

CHAPTER 1

Introduction to Transgenesis

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Over the past decade, a number of techniques have been developed that allow the introduction of defined, cloned DNA sequences into animal germ lines. Once inserted, these sequences, now called transgenes, are stably passed on from generation to generation. In other words, the transgene becomes a part of the genetic make-up of that particular line of animal. Every individual of a particular line will carry the transgene in every cell of its body. Of fundamental importance has been the observation that transgenes are often expressed—that is they are functional—and that this expression is subject to correct tissue-specific, developmental, and physiological regulation. It is therefore now possible to analyze the role and regulation of specific cloned genes within the whole organism. Such organisms are called transgenic organisms.

Of all technical achievements that have advanced biological sciences, few have opened up such possibilities as transgenic methodologies. The ability to change selectively the genetic make-up of a multicellular organism and thereby permanently alter the activity of particular proteins has important bearing on all areas of biological investigation. The extension of transgenic techniques to mammalian species has caught the imagination of both the scientific and general communities, providing

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cause for optimism and, at the same time, some concern when the implications for medicine and agriculture are considered.

Despite the notoriety of transgenic animals, the techniques for producing transgenics are not widely available in research laboratories, being found associated with the developmental biology laboratories where the techniques originated. The primary aim of the present volume is to facilitate the expansion of transgenic experimentation such that it becomes a commonly available approach. To this end, all of the techniques required for both the production and analysis of transgenic animals are described. Although a number of basic molecular cloning techniques are described herein, additional reference may be required, however, for some techniques used in the building of transgene constructs. The reader is referred to other volumes in this series (e.g., ref. 1).

The emphasis of the present volume is on transgenic mice, since this species has been the principal model in most transgenic studies. Comprehensive details for the production of transgenics in two other mammalian species, namely rats and sheep, are also included. Rats are included since they are the model of choice in many physiological and pharmacological studies, and sheep are included as an example of the genetic manipulation of livestock. In addition, techniques and applications of transgenesis in two nonmammalian species are also presented.

The superiority of a transgenic approach compared with the use of models such as cultured cells, for example, is clear to anyone investigating complex biological systems. However, the most appropriate design of transgene experiments is, in our experience, not always so clear to investigators with no experience of this approach. Thus, the results of a lengthy (the time-scale of transgenic experiments is in months and years rather than days) first-time study may simply inform the investigator that he or she should have spent more time thinking about the construction of the transgene or that a different strategy would have better answered the question. We feel that a knowledge of the transgenic literature is essential to the successful adoption of this technology into a novice laboratory. As an introduction to the field, we have therefore included short review chapters that consider the application of transgenesis to three selected complex systems, namely the brain, the immune system, and cancer. An additional intro-

ductory chapter considers the commercial applications of transgenesis, with particular emphasis on the genetic manipulation of sheep. It is hoped that with this additional insight, new investigators will be better equipped to use the techniques described here, and to apply transgenesis to its maximum potential.

Legal Obligations of Researchers and Animal Welfare

Many of the procedures described in the present volume are the subject of governmental regulation. Investigators should consult the appropriate local authorities before embarking on any study involving animal experimentation and/or genetic engineering. Researchers must always consider the fundamental principle of ethical research (2), which is that experimental animals must not be subjected to avoidable distress or discomfort. Research animals should be acquired and cared for in accordance with standards established by the National Institutes of Health (3).

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CHAPTER 2

Transgenic Rodents and the Study of the Central Nervous System

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1. Introduction

The following view of transgenic studies applied to the understanding of brain function is written as a guide for neuroscientists who may be considering transgenesis techniques in the pursuit of their research. Although most important studies are covered, the chapter is not a comprehensive review of the literature. Rather, it is intended to convey the possibilities of a particular technique, and thus provide an indication of both strategies and attainable goals. In addition to providing information relevant to experimental design, particular areas of neuroscience that have benefited from transgenic approaches will also be discussed. Developmental neurobiology is not specifically addressed; for a recent review of genomic manipulations in neuron/glia lineage analysis, *see ref. 1*. The first section of the present chapter describes the use of transgenics to localize *cis*-acting elements within neuronal genes, which act in the mediation of cell-specific and regulated expression. Transgenic mice have been chosen, to an extent, by default as models for neuronal gene analysis since suitable, permanent neuronal cell lines are not available for transfection studies. Analysis of enhancer/promoter regions is a daunting undertaking in transgenics and there is a strong argument for combining these studies with DNA-mediated transfection experiments in heter-

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ologous cell lines. Transgenic animals are, however, much more than model expression systems; they provide a unique opportunity to study both the regulation and role of neuronal genes in the context of integrated brain systems. The second section deals with approaches to neuronal gene function, concerning both gain-of-function and loss-of-function techniques. In the third section, the use of transgenics in the molecular analysis of neurological syndromes is discussed: Are transgenic rodents useful models for human brain diseases? Finally, the capacity to generate neuronal cell lines following targeted expression of oncogenes to specific neurons is assessed. The transgenic studies discussed here employ mice as the experimental animal, and, with one exception, use DNA microinjection techniques (*see* Chapters 18 and 19) in the generation of transgenics. At the time of writing, no studies have been published in which neuronal transgenes are expressed in rats. However, the techniques for producing transgenic rats are now available (*see* Chapters 26–31); given the ubiquitous use of rats by neuroscience investigators it is anticipated that the rat will be increasingly used to provide transgenic models for brain research.

2. Characterization of Regulatory Regions/Elements in Neuronal Genes

The primary aim, indeed the *sine qua non*, of transgenic studies is to direct expression of particular genes to specific groups of cells within heterogeneous cellular systems. Cell/tissue-specific expression is both an essential prerequisite to functional analysis of transgene expression and a requirement of studies in which the role of *cis*-acting regulatory elements are investigated in an appropriate cellular context; i.e., in the presence of corresponding *trans*-acting factors. The multiplicity of cell-types in the mammalian brain, each exhibiting unique patterns of gene expression, represents the extreme example in biology of a complex system in which precise genetic control is essential to permit the variety of phenotype. Although cell-specific transgene expression has been attained in a number of studies on the mouse central nervous system (CNS), it is not possible to make specific recommendations on the design of recombinant gene constructs for use in transgenic studies on the brain. On the contrary, comparison of different experiments has revealed considerable variation in the size and make-up of DNA constructs that appear to be required to direct

neuronal expression of particular genes. However, the literature is now of sufficient breadth to allow some useful generalizations to be drawn and questions may reasonably be posed regarding the size and composition of DNA constructs.

3. Design of DNA Constructs for Neuronal Transgene Expression

The quick, and probably most appropriate, answer to the question "How big a piece of DNA must be used to give neuron specific expression?" is "As big as possible"; in other words, use the largest available clone. Unlike other techniques, in which genetic information may be transmitted to experimental animals by viruses, for example, the microinjection technique offers no physical limit on the size of DNA construct used. Our current understanding of transcriptional control mechanisms, which suggests the presence of regulatory elements throughout the transcription unit, including elements >10 kb 5' to the transcriptional start site, certainly supports the "more is better" approach. Since neuron-specific regulatory elements have not been identified and, of course, complete sequence of the investigated gene may not be available, such an approach appears justified. It is also supported by experimental findings; for example studies on the SCG10 gene (2; *see also* Table 1) have revealed that while constructs containing either 3.5 or 4.2 kb of 5' flanking sequence direct neuron-specific expression of a reporter gene, use of only 0.55 kb of proximal promoter sequence results in a "relaxed" or deregulated pattern of expression, which includes nonneuronal tissues. Since reporter levels still remained highest in the brain, it is apparent that neuron-specific expression of SCG10 may be achieved through selective repression in other tissues mediated by silencer elements in distal 5' regions (2). The results of another study (3; *see also* Table 1) are consistent with a similar interpretation with respect to specific patterns of peripheral transgene expression. The latter study and others (e.g., 4, *see also* Table 1) are, furthermore, consistent with distal 5' elements regulating neuronal expression since, in both cases, appropriate expression is found in peripheral tissues but not in the brain. Although more studies are required, some general features do appear to be emerging, namely that neuronal expression is specified by regulatory sequences additional to those that permit nonneuronal expression. The location

Table 1
Expression of Neuronal Transgenes in Mice

Gene		Flanking DNA		Neuronal expn.			Peripheral expn.			Ref
		5'	3'	+/-	Sp.	Ec.	+/-	Sp.	Ec.	
SCG10/CAT	(r)	4.2	0	+	ND	ND	+	+	-	2
		3.5	0	+	ND	ND	+	+	-	2
		0.55	0	+	ND	ND	+	+	+	2
NSE/lacZ	(r)	1.8	0	+	+	-	+	-	+ ^a	5
NGF	(h)	8.0	7.5	+	+	-	ND	ND	ND	7
L7/lacZ	(m)	4.0	2.0	+	+	-IPN	-	-	-	8
GnRH	(m)	5.0	3.5	+	+	-PVN	+	+	+	9
VP/Tag	(b)	1.25	0	-	-	-	+	-	+	18
VP/CAT	(b)	1.25	0	+	-	+	+	-	+	20
OT	(b)	0.6	2.6	+	-	+	+	-	+	12
OT/VP	(r)	0.36/1.4	0	+	+	-	-	-	-	11
PNMT	(h)	2.0	4.0	-	-	-	+	+	+	3
PNMT	(h)	8.0	4.0	-	-	-	+	+	-	3
POMC/neo	(r)	0.77	0	-	-	-	+	+	-	4
GRF/Tag	(h)	1.6	0	-	-	-	+	-	+	19
GRF/NGF	(h)	1.6	0	+	ND	ND	-	-	-	19
GRF/cHras	(h)	1.6	0	+	ND	ND	-	-	-	19
DBH/lacZ	(h)	5.8	0	+	+	+ ^b	+	+	+	6

Abbreviations: CAT, chloramphenicol acetyltransferase, DBH, dopamine β -hydroxylase, Ec, ectopic expression; GnRH, gonadotrophin hormone-releasing hormone, GRF, growth hormone releasing factor, IPN, interpeduncular nucleus only, NSE, neuron-specific enolase, PNMT, phenylethanolamine *N*-methyltransferase, PVN, paraventricular nucleus only; Sp., cell-specific expression, Tag, SV 40 large-T antigen

ND Not determined/reported

Promoters are (b) bovine, (h) human, (m) mouse, (r) rat

^aTesticular expression (often observed as ectopic site of expression in transgenics with neuronal promoters)

^bSee ref. 6 for excellent discussion of mechanisms of ectopic expression

of such sequences may, however, be highly variable; whereas 8 kb of 5' flanking sequence was not sufficient to direct expression of the phenylethanolamine *N*-methyl-transferase gene (PNMT) to neurons in the brainstem (3), a construct containing only 1.8 kb of the neuron-specific enolase gene (NSE) resulted in panneuronal expression of a β -galactosidase marker, with peripheral expression in testis only (5). Furthermore, with respect to the PNMT transgene (3), a recent study (6) has shown that a construct containing only 5.8 kb of 5' sequence from the closely related dopamine β -hydroxylase (DBH)

gene was sufficient to direct specific expression to noradrenergic neurons in the brainstem. A more sophisticated approach to designing constructs for neuronal transgenesis studies would include a consideration of factors other than simply the length of 5' flanking sequence. First, the possible presence of regulatory elements within intronic, exonic, and 3' flanking regions should be borne in mind. It is noteworthy that some of the successful transgenic studies employing neuronal genes (7-9; *see also* Table 1), have used constructs that include all of these regions. For example, 8 kb of the mouse L7 gene was sufficient to direct a highly specific, endogenous pattern of transgene expression to the mouse brain with a low level of ectopic expression in a single brain nucleus (8). Second, regulatory elements may be located in adjacent, linked genes. This phenomenon, which has been described for globin genes (10), is apparent for the neuroendocrine peptide genes vasopressin (VP) and oxytocin (OT). Construction of a "mini-locus" transgene containing 5' flanking sequence of both VP (1.4 kb) and OT (0.36 kb) resulted in cell-specific expression of OT in the mouse hypothalamus; VP was not detected (11). Previously, an OT construct containing 0.6 kb of flanking sequence failed to produce an appropriate expression pattern (12; *see also* Table 1), indicating that correct expression of OT is dependent on sequences contained in the VP gene. Further studies are required to gain an understanding of the full extent of regulatory interactions between these linked genes. Recent transgenic studies have therefore described promoters that may be used to direct transgene expression in a brain-specific manner. Aside from the practical applications in potential functional investigations, these studies have provided a basis for understanding the mechanisms that underlie neuron-specific gene expression. Following the localization of regulatory elements to relatively large regions of DNA, it is anticipated that subsequent studies will lead to the precise delineation of these elements. For example, the pan-neuronal expression pattern obtained using the NSE gene (5) and the Thy-1 gene (13) may allow the identification of neuron-specific elements and *trans*-acting nuclear proteins. At the present time neuron-specific control elements have not been described, although some interest has been focused on the octameric sequence 5'-GCCAGCC-3', which is present in the proximal promoter region of several neuronally expressed peptide genes (14,15). Characterization of such elements

may provide powerful tools for future studies. Currently, promoters such as NSE and Thy-1 may be used to transform neurons in a non-specific manner. The L7 promoter (8), on the other hand, is an example of a promoter that may be used to manipulate selectively the phenotype of specific groups of brain cells.

4. Ectopic Neuronal Expression of Chimeric Transgenes

Ectopic expression of transgenes in cells that do not express the corresponding endogenous gene has been found in a number of transgenic studies in which chimeric "fusion" gene constructs have been employed. Analysis of this phenomenon has revealed that the unexpected pattern of expression results from a combinatorial action of *cis*-acting elements from both promoter and reporter regions of the transgene, which cannot be predicted from the expression patterns of the individual elements (16). For example, a construct containing the mouse metallothionein-I (MT-I) promoter linked to either the rat or human growth hormone gene produced a unique pattern of expression in several brain regions (17). This surprising result is clearly of interest with regard to the developmental relationships between different groups of neurons and may provide novel insights into developmental events. However, ectopic expression is not desirable in studies which are designed to target specific groups of neurons. First, the expression pattern obtained may be entirely inappropriate. Thus, transgenics produced with neuronal promoters linked to SV 40 large T-antigen sequence exhibited no transgene expression in brain, rather tumors and hyperplasia in ectopic peripheral sites (18,19; *see also* Table 1). Analysis of alternative constructs, in which different "reporters" were used, resulted in neuronal expression (19,20; *see also* Table 1) indicating that the ectopic pattern obtained with the initial constructs may result from a unique synergistic interaction between the two parts of the chimeric gene. Second, even in cases where the desired brain expression is obtained, the severe consequences of simultaneous ectopic expression may confound interpretation of the experiment as a result of either secondary effects or impaired health of the animal. It may be possible to prolong the experiment; for example, thymectomy of mice exhibiting thymic hyperplasia (19) increased the survival time of transgenics expressing a growth hormone releasing factor

(GRF) fusion gene. Clearly, however, ectopic expression associated with chimeric genes may seriously interfere with transgenic studies and care should be taken both in the design of constructs and in the interpretation of unexpected patterns of expression.

5. Transgenic Approaches to Neuronal Gene Function

Early transgenic studies in which overexpression of the growth hormone gene resulted in abnormally large mice provided a dramatic example of how transgenesis could be used to probe the function of proteins (21). Targeting of neural genes to specific brain tissues is now available as a technique to investigate the role of more enigmatic neural proteins. The gain-of-function approach may be applied to both normal mice and to genetic mutants; in the latter case it is possible, through gene therapy to correct the genetic error by replacing the impaired gene. In the former case, however, transgene expression may not be associated with measurable changes in functional activity. Thus, transgene expression may be controlled by homeostatic mechanisms, endogenous gene activity may be suppressed, or receptor pathways may be down-regulated. An alternative loss-of-function approach may therefore be more appropriate to investigate the functional role of neural genes. Recent advances in technique have provided a number of ways by which the activity of either specific cells or genes may be abolished.

5.1. Gain of Function Experiments

In an extension to the original growth hormone transgenic experiment (21), a more recent study (22) has utilized transgenic technology to demonstrate a direct role for GRF in the control of somatic growth. Overexpression of the hypothalamic hypophysiotrophic peptide GRF was associated with pituitary hyperplasia, increased plasma levels of growth hormone, and accelerated growth. As well as providing direct evidence of the role of GRF, these mice may also provide potentially useful experimental models for clinical growth disorders. The strategy in this study involved a chimeric transgene, the promoter region of the mouse MT-I gene linked to the coding region of the human GRF gene, which offers distinct advantages. GRF is expressed at high levels in ectopic sites such as the liver where it is not subject

to physiological feedback control, indeed expression can be induced and regulated by supplying heavy metals in the animals drinking water. Potential problems with this approach include the possibilities that sufficient product will not be delivered to the site of physiological response or that precursor peptides will not be subjected to appropriate processing to yield biologically active peptides. In other studies precise targeting to the tissue of interest is a requirement of the experiment. Selective overexpression of nerve growth factor (NGF) in pancreatic islets by use of the insulin promoter has elegantly demonstrated the role of NGF in regulating pancreatic innervation by sympathetic neurons (23). Genetic manipulation of a mutant mouse strain, the hypogonadal (hpg) mouse, has provided a classic example of how transgenesis can be used to modify neuronal phenotype (9). The hpg mouse exhibits a genomic deletion of 33.5 kb in the region of the gonadotrophin releasing hormone (GnRH) gene, which prevents the production of GnRH, a hypothalamic decapeptide that plays a pivotal role in reproductive development and function. Postnatal gonadal development is blocked in such mutants resulting in infertility. Since it has been possible to show that the GnRH neurons develop normally in hpg mice therefore indicating that the infertility may be solely as a result of the absence of GnRH expression, a gene therapy approach appeared to be possible. This idea was fully vindicated in a study in which a transgene containing the wild-type mouse GnRH gene (*see also* Table 1) was inserted into the germ line of wild-type mice; through a series of selective matings with heterozygous hpg mice, transgenic homozygous hpg mice were produced. The latter animals exhibit cell-specific GnRH mRNA expression in the hypothalamus, and both males and females are fully fertile. The successful outcome of this project, achieved despite the inherent difficulty of dealing with infertile animals, has provided an impetus for similar studies in the future.

5.2. Loss of Function Experiments

Techniques are now available by which specific cell types can be ablated in transgenic animals. This powerful tool has been developed through the use of cytotoxic genes such as the A subunit of diphtheria toxin (DT-A) or ricin, which may be linked in transgene constructs to cell-specific regulatory elements (24,25). Cells expressing such constructs in transgenics are killed. Although the possibilities of this tech-

nique are exciting, a number of problems may limit its application in studies of the brain. Embryo lethality resulting from toxin expression in cells essential for embryo survival is clearly a potential hazard; even where tissue-specific promoters are used, minimal ectopic expression may be sufficient to result in death caused by the potent nature of these toxins. The recent development of an attenuated DT-A gene (26), which requires a higher level of expression to produce a lethal effect, may prove to be a more versatile tool in genetic ablation experiments. Another problem with these studies regards the possibility that early ablation of one cell type may lead to abnormal development of other cells through the absence of cell interactions. Specificity of ablation may not be obtained and hence any loss of function would be uninterpretable. The use of an inducible toxic gene, such as tk from herpes simplex virus in transgene constructs (27), may circumvent the developmental side effects of cell ablation since transgene expression may be induced at later stages of development. However, since only dividing cells can be killed (27), this approach is of limited use in the brain. A third problem with the ablation technique is that a residual number of cells has generally been shown to escape ablation, thus confusing interpretation. Despite these drawbacks, further application of the cell ablation technique is anticipated. A transgenic mouse model of demyelinating disease has recently been produced (28) using a myelin basic protein (MBP)/DT-A construct (*see* Section 6.1.). Techniques for disrupting the expression of specific endogenous genes are also available. One promising technique involves the use of constructs designed to express antisense (m)RNA. In the only reported success to date (29), expression of MBP antisense (m)RNA in the brains of transgenic mice reduced levels of both MBP mRNA and MBP, resulting in the shiverer mutant phenotype. An alternative procedure in which specific genes are targeted and disrupted through a homologous recombination event is technically more difficult to achieve, but is potentially more reliable than the antisense construct approach. In the former technique, embryo-derived stem (ES) cells are transformed with a targeting vector containing a modified form of the targeted gene that is functionally impaired. A series of selection procedures are then undertaken to enrich for cells in which the rare event of homologous recombination has occurred. Following the identification of a suitable cell line, blastocysts are microinjected

with these cells and chimeric mice are generated. Through selective breeding procedures, with reference to coat color, it is possible to generate hetero- and homozygous animals that exhibit the mutated genotype. A recent experiment of this type has successfully disrupted the *int-1* gene, producing a homozygous mouse with a severe ataxic phenotype associated with major defects in midbrain and cerebellar development (30). Previous suggestions that *int-1* was involved in brain development (*see ref. 30*) have, therefore, been dramatically validated. Although the tools for performing these experiments are available for mice (*see Chapters 23 and 24*), the development of ES cell lines has yet to be achieved for rats.

6. Transgenesis Applied to Neurology

In the field of neuroscience an immediately apparent application of transgenic technology lies in the development of animal models “designed” to exhibit specific neurological syndromes and disease states. The current emphasis on neurodegenerative diseases, which reflects an increasingly evident incidence within aging populations, has provided a major impetus for such studies. In cases where characterized genes have been implicated in the etiology of brain disorders, it is possible to design transgene constructs to probe the gene’s role either through gain-of-function, or loss-of-function approaches. In addition to the scientific approach, however, a “chance approach” to the study of brain pathology has also proven of value since a number of transgenic insertional mutants that exhibit neurological symptoms have been generated. Here, transgene (identity irrelevant) insertion has disrupted an endogenous gene(s) (identity unknown) within a known locus that is associated with a particular pattern of pathology/behavioral abnormality. As described below, transgenic, insertional-mutant mice should facilitate cloning of genes that are affected by spontaneous mutations.

6.1. Transgenic Models of Neurological Diseases

A compelling use of transgenics as models for neurodegenerative diseases has been in the study of prion protein (PrP) genes, which are thought to act in the pathogenesis of scrapie and possibly other, similar diseases. Transgenic mice with hamster PrP gene constructs cre-

ated by Prusiner and colleagues have provided a role for the PrP gene in modulating scrapie infectivity and in the development of species-specific neuropathology (31,32). In a recent extension of this work a direct attempt to model the scrapie-related Gerstmann-Straussler-Scheinker syndrome (GSS) in transgenic mice proved to be extremely successful (33). GSS is a human neurodegenerative disease that has been genetically linked to an amino-acid substitution in the human PrP gene (*see ref. 33*). Mice expressing a mouse PrP gene construct with an identical mutation develop neuropathology characteristic of GSS, indicating PrP as a potential molecular basis for GSS. Further PrP transgenics are anticipated in the continuing elucidation of prion biology (*see ref. 32*). The pathology observed in the hamster PrP transgenic mice is particularly interesting with regard to Alzheimer's disease (AD) since characteristic amyloid plaques associated with the production of a protease-resistant PrP are found. Since there is no good animal model for AD, the PrP transgenics may be additionally useful in this respect. At the same time, studies are also in progress to develop transgenic Alzheimer's models using constructs based on the Alzheimer Amyloid Precursor Protein (APP) gene (34).

The preliminary results of the latter study have provided an indication of potential problems in the generation of neurological models since a number of neurological traits that have been identified in the offspring of transgenic mice are not necessarily linked with transgene expression, rather the features may be symptomatic of particular inbred lines. Considerable care, including the use of more than one line of transgenics, is therefore recommended in the evaluation of such models. The retraction (35) of a recent study (36) in which AD-like neuronal degeneration was initially observed in transgenic mice expressing a Thy-1/APP fragment transgene has also served to highlight the potential problems that may be encountered in the phenotypic analysis of transgenics.

6.2. Insertional Mutations

The production of insertional mutants whereby endogenous neurally expressed genes are disrupted by transgenes is not really surprising given the high percentage of genes that are expressed in the brain. Particularly fortunate, however, have been studies in which known neurological loci have been affected (37,38). For example,

production of transgenics using a SV40/dihydrofolate reductase gene construct resulted in a mutant mouse line that exhibited neuropathology identical to the purkinge cell degeneration (pcd) mutant mouse. The transgenic mutation was found to be allelic with the pcd locus (38). A procedure for cloning genes associated with the pcd locus is then apparent since the transgene sequence provides a probe to screen genomic libraries and hence obtain flanking sequence from the transgene insertion site. The continued proliferation of transgenic experimentation should generate many more insertional mutants that map to neurological loci and therefore provide systems to investigate the molecular basis of neuropathologies.

7. Generation of Neuronal Cell Lines Through Transgenesis

The ability to target specific groups of neurons in transgenic mice (*see* Section 2.) provides for the possibility of immortalizing these cells through targeted tumorigenesis using transgene constructs containing cell-specific promoter elements linked to oncogene sequences. Permanent neuronal cell lines derived from such tumors would be invaluable experimental tools for neurobiology, which has lacked this facility in the past owing to the difficulty in transforming mature neurons. Although there has been one notable success (39) in this area of transgenesis, the technique may not be generally applicable to all groups of neurons. The system in which targeted tumorigenesis has been successfully applied in the brain is the hypothalamic GnRH cell group. As described previously (*see* Section 5.1.) the mouse GnRH promoter gives cell-specific expression in transgenic mice (9); linking this promoter to the SV40 T-antigen oncogene resulted in the production of brain tumors (39). As may be anticipated from other studies (*see* Section 4.), tumors were found in numerous ectopic sites; however, in one transgenic founder an appropriately located tumor (anterior hypothalamus) was found. Since this tumor expressed GnRH, part of the tissue was taken for tissue culture, and, following a lengthy process of subculturing, a cell line was established. These immortalized cells are remarkable in that they exhibit features characteristic of differentiated neurons including GnRH expression and secretion. In addition to providing a model system for investigation of genetic elements regulating GnRH mRNA expression, a unique system for

investigating factors controlling secretion of this peptide has also been obtained. Considerable advances in our understanding of these neurosecretory neurons are anticipated. In contrast to the latter experiment, other studies using neuronal promoters linked to SV40 T-antigen have not resulted in neuronal tumors. Although this may result from an inappropriate expression pattern (3,19), one study has clearly shown that T-antigen is expressed in a targeted groups of neurons in the absence of tumorigenesis (40). Thus, most neurons may be refractory to the transforming actions of oncoproteins; the results of these studies are, in fact, consistent with the view that differentiated neurons are terminally postmitotic. GnRH neurons may represent the exception to this rule being (possibly) derived from the neural crest and exhibiting GnRH expression prior to the cessation of mitotic capability (41). Further studies are necessary to establish whether higher levels of T-antigen expression are required to transform other neuronal groups, or whether other oncogenes may be more appropriate tools for these studies. At the present time, alternative approaches such as somatic cell fusion (42) may be more profitable.

8. Conclusion

The development of transgenic techniques during the 1980s has significantly broadened the range of experimental approaches to biological problems. The techniques are of particular value to complex biological systems, such as the mammalian brain, which are intractable to comprehensive analysis by classical techniques. Thus far, transgenesis has been applied to specific neuronal systems that have presented as convenient model systems. The current challenge for neuroscientists is to adopt and refine these techniques such that they become widely applicable in all areas of neuroscience.

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CHAPTER 3

Transgenic Animals and the Study of Cancer

David Murphy

1. Introduction

This chapter seeks to outline the strategies being employed to exploit transgenic mice as a tool in the study of cancer. Rather than catalog the many different oncogene and oncogene-promoter combinations that have been introduced into transgenic mice to elicit tumors, I will concentrate on a single system that epitomizes the general approach. That system, pioneered by Philip Leder of Harvard University, is mammary carcinogenesis induced by oncogene expression directed by the Mouse Mammary Tumor Virus (MMTV) enhancer-promoter.

2. Multistep Carcinogenesis

Tumor development in the whole animal is thought to be a multi-step process (1–4). This process, which could be defined as a bad situation becoming worse, has been termed tumor progression and is crudely diagrammed in Fig. 1. A normal cell with the burden of its inherited genetic makeup, which may predispose it to cancer, is subject to successive environmental insults (5), causing a gradual accumulation of genetic changes that may result in cancer. Such mutant cells are influenced by many complex host responses and influences that can either encourage or retard tumor growth. Crucial to the development of malignancy is angiogenesis—the cancer obtains a blood supply and can thus expand in size, since it is not restricted to obtaining nutrients by diffusion (6). The tumor finally becomes a killer when it

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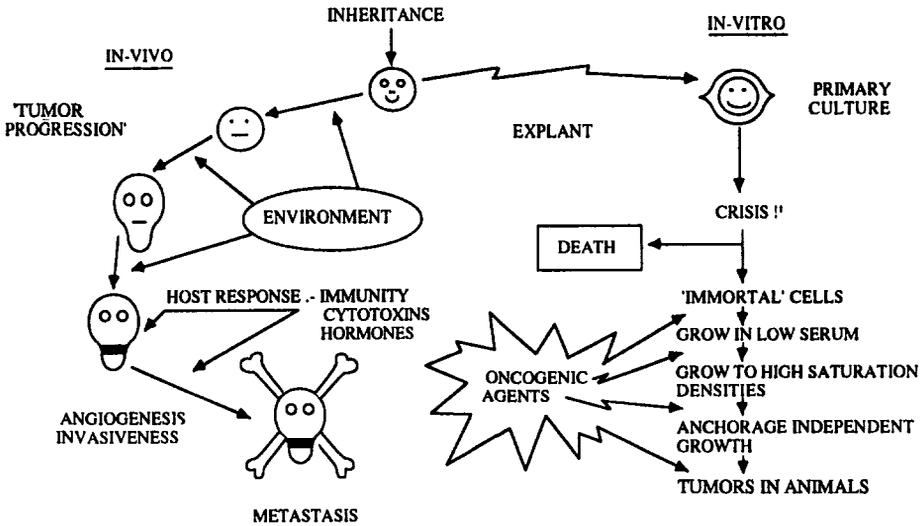


Fig. 1. Diagrammatic representation of tumor progression in vivo and its mimicry in culture.

becomes invasive—it eats into surrounding normal tissue, and when it metastasizes—that is, it spreads to other parts of the body (7).

3. Biology of Tumor Cells

Tumor biologists can mimic the progression process in vitro, but only to a very limited degree (8; Fig. 1). Normal cells can be explanted from an animal into culture, but they only have a limited life-span.

After a few rounds of cell division, the culture will undergo a “crisis” with most cells dying, but a few cells survive. These are cells that have undergone a genetic change and have become immortalized; that is, they can grow and divide indefinitely in culture. Such cells can then be further transformed by oncogenic agents. This term “transformation” can be defined as the acquisition of growth characteristics not exhibited by the parental cells.

Immortalization is itself a transformation step, and once a cell has been transformed once, it can be transformed again. There is therefore a hierarchy of transformed phenotypes (Fig. 1). The ability to grow in low serum (that is, the ability to grow in the absence of certain growth factors and nutrients) is a less stringent assay of transformation than growth to high saturation density (which is the ability to

grow without growth being inhibited by neighboring cell; that is, the phenomenon of contact inhibition is lost). This is a less stringent assay than the ability to grow without a substratum (or anchorage-independent growth), which is a less stringent assay than tumor production in animals. Thus progression to a pathologically more destructive phenotype can be mimicked *in vitro*, and we can hypothesize that each step in the progression of a tumor is caused by a gene, and such a gene could be called an oncogene.

4. Oncogenes and Tumor Progression Factors

An oncogene can be defined as a DNA sequence capable of disrupting normal growth controls resulting in pathological cellular proliferation. However, some of the genes involved in tumor development in the whole animal, for example, those activities involved in angiogenesis, invasiveness, and metastasis, would not fit into this classical notion of an oncogene. They are not directly involved in cellular proliferation, although they may indirectly promote proliferation by, for example, in the case of angiogenesis factors, providing transformed cells with additional nutrients. Thus, an oncogene is not necessarily a gene that causes cancer. Many other types of genes also contribute to the overall stepwise process that is tumorigenesis. Such genes encode what have been called "tumor progression factors." Although cellular proliferation and its pathologies can be studied *in vitro* in detail, it is difficult to envisage how the processes of invasiveness, metastasis, and angiogenesis, as well as the interactions between the host and the tumor, could be studied using simplistic *in vitro* systems. Complex systems are best studied intact, which means using transgenic animals if a "reverse genetic" approach is to be adopted (9,10).

Many oncogenes have been identified, mainly but not exclusively from *in vitro* studies. These genes can be fitted into two classes—recessive and dominant. When a function involved in the suppression of growth is lost through a mutation and transformation results, the oncogene is termed recessive (11). When a transforming function is gained through mutation, the oncogene is dominant.

Dominant, or gain-of-function, oncogenes were originally described in oncogenic DNA (8) or RNA (12,13) viruses of mice and other organisms. Such viruses were shown to be able to induce cancer in

infected organisms and/or transform cells cultured in vitro. Tumor viruses carry dominant oncogenes that they bring into cells, resulting in transformation. There is, however, little evidence for direct viral involvement in the vast majority of cancers, human or animal. Rather, many spontaneous animal and human tumors have been shown to be associated with dominant “cellular oncogenes.” These cellular oncogenes are derived by the mutation of normal cellular genes (called proto-oncogenes) that may have some role in the control of normal cellular growth. Four lines of evidence pointed to the notion that normal cells can be transformed by alteration of the activity of their own genes:

1. Certain RNA tumor viruses were shown to contain transduced derivatives of cellular genes (12–14)
2. Retroviruses that lack their own oncogenes were shown to transform by insertional mutagenesis. Integration of the provirus adjacent to a cellular proto-oncogene resulted in the activation of that gene and its conversion into an oncogene (15).
3. Tumors were demonstrated to contain endogenous cellular genes that were capable of inducing transformation when introduced by gene-transfer techniques into cultured cells (16–19).
4. Characteristic chromosomal abnormalities (amplifications, translocations) found in some tumors were shown to be associated with the derangement of the expression of specific proto-oncogenes (20,21)

The mutations that afflict proto-oncogenes can be divided into two broad classes: (1) mutations that alter the regulation of the expression of the gene—either the level of expression of the gene, the place of expression, or the developmental timing of expression or (2) mutations that alter the function of the protein.

Dominant oncogenes can be divided into two classes, based on their ability to effect different sorts of growth changes in cells in vitro: (1) immortalizing genes, which are able to convert primary cells to immortal cells (22,23), and (2) transforming genes, which convert immortal cells to fully transformed cells (22,24). Transforming genes and immortalizing genes have been shown to cooperate in vitro (25–27). The action of an immortalizing gene is needed before transformation can take place; that is, a transforming gene can only transform an immortalized cell—it has no effect on a primary cell. It became apparent from these experiments that the oncogenes that resulted in immortal-

ization and the oncogenes that progressed a cell toward a fully transformed phenotype could be fitted into two classes based upon their subcellular localization. Thus, immortalizing genes tend to have a nuclear localization, implicating them in transcriptional, posttranscriptional, or replicative processes (22,23). Often proto-oncogenes converted into immortalizing oncogenes by the subversion of their regulation. Transforming genes, on the other hand, tend to be localised in the cytoplasm or cell membrane, implying a role in the transmission of aberrant growth signals from the exterior to the nucleus (22,24). Such oncogenes tend to be the result of mutations in the protein coding sequences of the proto-oncogene. Thus, obtaining a fully transformed phenotype requires the cooperation of two different sorts of oncogene, resulting in the subversion of normal growth controls at two different levels. This is consistent with the concepts of multistep carcinogenesis and tumor progression.

An example of an immortalizing gene is the *myc* gene. The protein encoded by this gene is found in the cell nucleus, which implies that it might be involved in the control of transcription, or the control of DNA replication and cell division (23). Proto-oncogene *c-myc* becomes an oncogene when its normal regulation is subverted. When, through a DNA rearrangement or amplification, the *c-myc* gene is overexpressed or inappropriately expressed, the *c-myc* protein can lead to unregulated cell division.

An example of a transforming gene is the *ras* gene. The *ras* protein is located on the inner side of cell membrane and is related to the G-protein family of signal transduction molecules (23). The normal *ras* gene is not transforming, but the mutated one is. The only change is a single base pair mutation leading to a single amino acid substitution in the protein sequence. Thus, a single point mutation converts a normally innocuous gene into an oncogene, presumably because the altered protein transmits unregulated growth signals.

5. Oncogene Expression in Transgenic Mice

The relevance of in vitro studies on oncogenes to the ontogeny of cancer in whole animals has always been in doubt. However, very soon after the development of the technology of murine transgenesis, oncogenes were introduced into whole animals (9,10). Three basic

conclusions arose from these studies. First, it was shown that the expression of transgenic oncogenes results in the inevitable development of tumors in susceptible tissues. Second, it has become apparent that particular oncogenes have particular cell-type specificities; that is, an oncogene expressed in one tissue, say the mammary gland, may cause a malignancy, but in another tissue, it may be benign or have no effect whatsoever. Third, transgenic experiments have shown that although oncogenes are necessary, they are not sufficient for tumor development in transgenic mice. Other events are necessary, which is reminiscent of the concept of multistep carcinogenesis involving the participation of tumor progression factors. Transgenic mice allow the analysis of these events in the whole organism.

Note that, thus far, studies in transgenic mice made by the microinjection method have dealt only with dominant oncogenes. Microinjection adds new genes to the gene pool of the organism, leaving the endogenous genes unchanged, and to see any effect, the transgene must be dominant. With the new ability to mutate specific endogenous mouse genes using homologous recombination in ES cells (*see* Chapters 23 and 24), however, it will be possible to create recessive events to look at loss of function oncogenes.

6. Mammary Cancer in Transgenic Mice

Philip Leder's group at Harvard have used transgenic animals to study the involvement of oncogenes in mammary cancer, and this is a system that has now been adopted by many other groups. The system depends on the construction of hybrid oncogenes using the promoter and enhancer region from the MMTV. The promoter-enhancer region of MMTV is tissue specific in both virus-infected mice and transgenic animals (28,29), being reproducibly expressed in mammary gland, lungs, kidney, salivary glands, testis, prostate, seminal vesicles and lymphoid tissue. In virally infected animals, MMTV induces mammary tumors. Analysis of these tumors revealed that oncogenesis was a consequence of integration of the virus adjacent to one of at least three cellular genes. The expression of these genes (*int-1*, now called *Wnt-1*; 30; *int-2* and *int-3*; 15) is activated by the MMTV promoter-enhancer. The cellular *int* proto-oncogenes are thus converted into oncogenes. Leder and his group took advantage of the tissue specificity of the MMTV promoter-enhancer region. They asked if

Table 1
Expression of Oncogenes in Transgenic Mice Under the Control
of the Mouse Mammary Tumor Virus Enhancer-Promoter Region

Oncogene	Oncogene Type	Ref.
<i>c-myc</i>	Nuclear protein; immortalizing gene	31-33
<i>v-Ha-ras</i>	G-protein-like cell-membrane-associated GTP binding protein; transforming gene.	33,34
SV40 T-antigens	Large T is a nuclear protein (binds p53 and Rb tumor suppressor gene products); involved in transcriptional regulation and DNA replication; cytoplasmic/membrane component; has both immortalizing and transforming functions.	35
<i>int-1 (Wnt-1)</i>	Secreted glycoprotein growth factor	36,37
<i>int-2</i>	Epithelial cell growth factor; related to basic fibroblast growth factor	36,48
<i>ret</i>	Activated receptor-type tyrosine kinase	39
<i>c-neu</i>	Activated receptor-type tyrosine kinase. Epidermal Growth Factor receptor related	40,41
<i>N-ras</i>	Member of <i>ras</i> family	42
TGF α	Secreted epithelial growth factor; EGF related	43

tumors arose in transgenic animals bearing hybrid oncogenes made up of the MMTV promoter-enhancer region linked up to other oncogenes. The two oncogenes they initially chose to investigate were a cellular immortalizing gene, *c-myc*, and a virally derived transforming gene, *v-Ha-ras*. Many other oncogenes have now been linked to the MMTV control sequences and introduced into transgenic animals. These experiments are summarized in Table 1.

Female transgenic mice bearing the MMTV-*c-myc* transgene developed mammary tumors, as did all the transgenic offspring (31,32). In addition, expression of the transgene resulted in lymphoblastic lymphomas, testicular tumors, and mast cell tumors.

Similarly, the MMTV-*v-Ha-ras* induced mammary tumors in female transgenic mice, indicating that in an experimental situation, this gene can cause cancer (33). Also, male mice bearing the MMTV-*v-Ha-ras* gene developed mammary tumors—males do have mammary epithelial cells, just much fewer of them than females. Tumors were also iden-

tified in the salivary gland, and hyperplasia was observed in both harderian glands. Lymphoblastic lymphomas were also observed. Another group has also produced mice bearing an MMTV-*v-Ha-ras* transgene (34). They, too, observed mammary tumors and bilateral harderian gland hyperplasia. Additionally, these mice also developed splenomegaly and primary bronchio-alveolar lung adenocarcinomas.

Thus, when inappropriately overexpressed, the immortalizing *c-myc* gene and the transforming *v-ras* gene can cause tumors in whole animals. However, in a number of other tissues, expression of the transgene was found, but this did not have a pathological effect, nor did it perturb normal development (32). Therefore, inappropriate expression of an oncogene itself is not enough to result in cancer. A particularly striking example of this is the lung tumor phenotype observed by Tremblay et al. (34) in their MMTV-*v-Ha-ras* transgenic mice. Although expression of the transgene was detected in the lung tissue the MMTV-*v-Ha-ras* transgenic mice derived by Sinn et al. (33), no tumors were ever observed. Some genetic difference between the strains of mouse used to make the transgenic animals in the two groups was suggested as the reason for this difference (34). Further, different tissues or cell types apparently respond differently to oncogene expression. Although some tissues develop full-blown tumors, others, like the harderian gland, become hyperplastic, but never progress to a malignant phenotype. Some tissues are totally unresponsive to oncogene expression. Oncogene action is thus tissue specific.

Even in tissues that develop tumors, it was noted that expression of the oncogene is not in itself enough to elicit a tumor. The expression of an oncogene may predispose a tissue to tumorigenesis, but additional mutations are needed before a tumor actually develops. Three observations support this conclusion. First, tumor development only occurs following a long period of latency. In the MMTV-*c-myc* mice, the transgenic oncogene is expressed in all ten female mammary glands on maturation, but tumors do not arise until the mouse is 6 to 14 mo old, after two or three pregnancies. In the MMTV-*v-Ha-ras* mice, the latency period is shorter, but is still up to 6 mo. This implies that further genetic events (mutations) are needed and that these take time to accumulate. Second, despite the expression of the oncogenes in all ten female mammary glands, only one or two of

these glands develop tumors. Again this implies that secondary events are needed to bring about a tumor and that these accumulate at random. Third, cells in the MMTV-*c-myc* transgenics were shown to undergo a preneoplastic stage of cell division prior to the onset of a truly tumorigenic phenotype. Together, these data imply that oncogenes identified in culture are solely involved in disorders of cellular proliferation, and the development of true cancers requires the development of other, as yet unidentified, types of functions, such as angiogenesis.

Transgenic mice have validated the notion that oncogenes are important in tumors in whole animals as well as in tissue-culture cells. They have also shown that oncogenes have different effects in different cell types. Significantly, transgenic mice are now being used to demonstrate directly that cancer is indeed a multistep process involving the cooperation among different oncogenes and between oncogenes and other types of cellular genes ("tumor progression factors"). The different genetic events that result in cancer are therefore now being elucidated in transgenic mice. As described above, an overexpressed *myc* gene is able to cooperate with an activated *ras* gene in vitro to transform a normal primary cell fully. Similarly, Leder and his colleagues produced "double transgenics" bearing both the MMTV-*c-myc* transgene and the MMTV-*v-Ha-ras* transgene by mating mice bearing single transgenic oncogenic transgenes (33). Such mice show an accelerated rate of mammary carcinogenesis when compared to mice bearing only one of the two hybrid oncogenes. This experiment demonstrates that cancer is indeed a multistep process in vivo and validates the concept of oncogene cooperation in whole animals. This concept was proposed after work on tissue culture cells, and this experiment extends the notion to real tumors in real animals. However, it was again noted that only a few of the ten separate mammary glands develop into tumors. The rate of tumor formation is increased in "double transgenics," but the number of cancers is not changed, even though both transgenes are expressed in all ten glands. Thus, oncogene expression alone (even expression of multiple cooperating oncogenes) is unable to elicit tumorigenesis. Further genetic events are required, and transgenic mice will be an invaluable tool in the search for these tumor progression genes. In a similar experiment, Kwan et al. have

demonstrated cooperation between the *Wnt-1* and *int-2* proto-oncogenes (36).

7. Tumor Progression Factors and Transgenic Mice

It is to be expected that the role and identity of tumor progression factors will soon be analyzed using transgenic animals. The protocol will be analogous to that used by Leder to demonstrate cooperation between immortalizing and transforming genes in transgenic mammary cancer. Independently derived animals bearing transgenes consisting of a specific promoter driving the expression of oncogenes or potential tumor progression factor genes can be mated to produce mice carrying any number of different hybrid transgenes, all of which will be expressed in the same tissue. The pathological consequences of such coexpression can then be analyzed. If, as is thought (6), angiogenesis is crucial to the development of a malignancy, then the coexpression of an angiogenesis-inducing factor with an oncogene in a particular susceptible tissue may accelerate the development of a tumor. Alternatively, it may increase the number of independent tumors arising from that tissue type or the size of individual tumors. Similarly, it is possible that the acquisition of an invasive phenotype by a tumor is owing to the activation of particular protease genes, and tumor metastasis is perhaps a consequence of changes in the way that the tumor cell interacts with the extracellular matrix. With the identification of genes encoding such proteases and matrix attachment factors (receptors), their role in tumor development can be tested directly in transgenic animals.

8. Identification of Genes Cooperating with Oncogenes

Techniques have now been developed that allow for the identification and cloning of novel endogenous murine genes that cooperate with a transgenic oncogene to elicit tumorigenesis. These techniques, termed retrovirus-tagged mutagenesis, involve the infection of a transgenic animal with a reterovirus that lacks an oncogene, but that is able to promote tumorigenesis through the inadvertent insertion within or adjacent to a proto-oncogene, thereby increasing its expression or altering its structure. Viral infection may result in accelerated tumorigenesis in a transgenic animal as a consequence of the functional cooperation with the transgene of a mutated gene. The gene

can then be identified and cloned by virtue of its proviral "tag." Using the Moloney Murine Leukemia Virus as a tag in E μ -*myc* transgenic mice, a novel zinc finger gene has been identified as a collaborator with the *myc* gene in preB-cell lymphomagenesis (44,45). It is possible that MMTV may find similar utility in studies of mammary carcinogenesis.

9. Toxicity Testing and Tumor Therapies

Cancer in humans is thought to be caused principally by exposure to environmental carcinogens that result in mutations. Transgenic mice are now being used to screen for such carcinogens. The MMTV-c-*myc* mice developed by Leder are commercially available (marketed by Charles River, Wilmington, MA). The toxicity of particular substances can be tested by exposing the transgenic animals to it and asking if the pathology of the tumorigenesis is altered. Conversely, transgenic animals with reproducible and predictable tumor development will increasingly be used to test novel anticancer therapies.

10. Conclusion

The reproducibility and predictability of tumorigenicity in transgenic mice are allowing the different stages in tumorigenesis to be studied using the powerful tool of reverse genetics in the whole organism. By using transgenic animals, the role of specific oncogenes and tumor progression factors is being directly analyzed with a new precision. Transgenic models of particular cancers offer the promise of a better understanding of the process of tumor progression, with a consequent improvement in therapeutic approaches to the human disease.

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CHAPTER 4

Transgenic Animals and the Study of the Immune System

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1. Introduction

Transgenic technology, the introduction of genetic information into the germ line of an animal to alter its genetic constitution stably, provides a powerful tool for studying the development, functions, and malfunctions of complex biological systems (1–3). The main advantage has been that it is possible to study the gene functioning in its natural milieu. This is particularly informative when the genes have been manipulated such that the regulation and tissue specificity of expression are altered. Although transgenic animals bear the introduced gene in every tissue, expression of that gene may be widespread or directed to particular cell lineages, depending on the regulatory sequences chosen (1–3).

Using transgenic animals, one can start to assay the function of a particular gene in a great variety of diverse cell types for which tissue culture lines are often not available. One can therefore attempt to assess whether a particular gene has perturbed differentiation within particular lineages. The perturbations may help to delineate early maturation stages and to clarify how differentiation is controlled. The regulation of the gene could also be studied in organized, discrete structures or compartments that often cannot be recapitulated *in vitro*. In addition, the introduced genes are subjected to a network of hormonal and lymphokine stimuli in its natural environment. Therefore, one can begin to evaluate cooperativity between different genes of

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diverse cell types within the immune system. These conditions are usually too complex or impossible to reproduce in cultured cells. It is therefore not surprising that a great number of transgenic animals (mostly mice) have been generated to gain new insights into many of the key immunological questions (2–4). These include studies on the mouse and human Major Histocompatibility Complex (MHC) antigens; immunological self-tolerance; T-cell receptor and immunoglobulin genes; and lymphokines and their receptors. Many of these topics have been reviewed extensively (1–4). This chapter intends to provide a selective view on how molecular immunologists have exploited the production of transgenic mice to study genes encoding the MHC molecules.

2. Major Histocompatibility Complex (MHC)

2.1. Introduction

The MHCs of the mouse (the H-2 complex) and of humans (the HLA complex) encode highly polymorphic cell surface and secreted glycoproteins that are important for immune regulation and function (5,6). Transplantation antigens that are important in allograft rejection are encoded by class I genes that map to the H-2K/D/L and HLA-A, -B, -C subregions in the mouse and humans, respectively. In addition, there are class I genes that map to the murine Qa and Tla subregions. Mature cell-surface class I MHC molecules are found to be noncovalently associated with the β -2 microglobulin (β -2M). The level of expression of the class I MHC molecules is developmentally regulated. In adults, although the class I MHC molecules are expressed on the surface of many different cell types, the level of expression is highest in lymphoid cells. The level of expression of class I MHC genes could also be enhanced by interferon (IFN). The MHC molecules function as copresenter of foreign antigens to T-cells and are thus required for antigen recognition. Recognition by cytotoxic T-lymphocytes (CTL) of foreign/tumor antigen along with self-MHC class I molecules leads to the subsequent lysis of antigen-bearing target cells. The class II MHC genes, on the other hand, encode membrane-bound glycoproteins composed of two noncovalently associated polypeptide chains termed α and β (5). These are the I-A and I-E in the mouse, and DR, DQ, and DP molecules in humans. The class II molecules are essential for the presentation of antigenic peptides to T-helper cells to trigger a cascade of immunological responses that result in

killing of antigen-presenting cells and induction of an antibody response. Class II molecules are found primarily on B-lymphocytes, thymic epithelial cells, activated T-lymphocytes, and antigen-presenting cells of the monocyte/macrophage lineage. Within the MHC, in addition to the class I and class II loci, numerous other genes have also been described (5,6). There is immense interest in studying genes encoding the highly polymorphic class I and class II loci, since they are directly involved in genetic regulation of immune responsiveness. Recently, several of these MHC genes have been utilized to produce transgenic mice in the hope of addressing some of the fundamental immunological questions.

2.2. Production of Congenic and Mutant Mouse Strains

Transgenic mice constructed on an inbred background provide a novel approach to the study of class I gene expression and antigen function. In the transgenic system, a single well-defined gene can be placed on an inbred background in one generation. If the transgene is properly expressed and the locus carrying the transgene can be bred to homozygosity, the resulting mouse strains would be, in principle, analogous to congenic lines. These transgenic mice would be identical to the parental inbred mouse strain, except for the addition, at one histocompatibility locus, of a foreign allele introduced by microinjection. Yoshioka et al. introduced the H-2D^d class I gene into the C57BL/6 mice by microinjection (7). The H-2D^d transgene was found to be expressed in a tissue-specific manner that paralleled that of the endogenous H-2K^b gene. The H-2D^d transgene was inducible in response to interferon and suppressible by transformation with the human adenovirus 12. In addition, all the transgenic H-2D^d mice were found to be tolerant of the H-2D^d antigen. Therefore, spleen cells from the transgenic H-2D^d mice failed to generate an anti-H-2D^d CTL response *in vitro*, suggesting that the H-2D^d antigen is recognized as a self-molecule in these animals. Similar observations were made when transgenic mice were generated by microinjecting the human HLA genes into mouse embryos. The human HLA-B27 molecules could be used by the mouse T-cell as a restriction element against viral antigens in HLA-B27/human β -2M double-transgenic mice (8). Moreover, the introduction of HLA-B27 into C57BL/6 or (C57BL/6 \times SJL/J)F1 mice

in the absence of human β -2M gene could also result in the expression of the HLA-B27 molecules at a level comparable to the endogenous H-2^b and H-2^s class I molecules. Transgenic mice carrying the HLA-A2.1 gene were also generated (9). Spleen cells from these transgenic mice expressed the HLA-A2.1 molecules on their cell surface in association with mouse β -2M. The cells, however, contain more HLA mRNA than endogenous H-2 class I mRNA. In contrast, the amount of HLA: β -2M is low. There is also a large pool of non- β -2M-associated HLA heavy chain inside the cell. These results indicated that in these transgenic mice, HLA-A2.1 seems to compete poorly with the H-2 heavy chains for mouse β -2M.

In most of the transgenic mice produced, the introduced MHC molecules behave as self-antigens, and could function as transplantation antigens in skin graft reactions and as restriction elements in antiviral or antibacterial T-cell response. The introduction of MHC genes by microinjection of mouse embryos would therefore circumvent the painstaking task of developing congenic mouse strains by classical breeding method and through the isolation of spontaneous class I mutant mice.

2.3. Identification of Tissue-Specific Transcription Regulatory DNA Sequences

Many studies involving generation of transgenic mice are aimed at understanding normal developmental processes. By introducing genes that are normally expressed in a tissue-specific manner, one can assess whether *cis*-acting DNA elements involved in developmental programming of gene expression are present. Then, by producing transgenic mice with a series of mutant genes, one can define the precise sequence required for tissue-specific expression. In this respect, the class II MHC molecules provide a good model system for the identification of multiple-enhancer elements for expression and compartmentalization. In the thymus, the class II molecules occur on epithelial cells of the cortex, and more abundantly on interdigitating reticular cells and epithelial cells of the medulla. It is in these well-defined areas of the thymus that "education" of T-lymphocytes takes place, that is, the shaping of the T-cell receptor repertoire toward recognition of foreign antigens in association with self-MHC and away from reactivity against self-MHC alone. Widera et al. (10) microinjected

two fragments containing 1.4 kb of 5'-flanking and 0.5 kb of 3'-flanking region of the E α gene into (H-2^{bxs}) mice that do not express their endogenous E α gene. The transgene was expressed in thymic tissue and in adherent spleen cells, and was induced in peritoneal exudate cells by γ -interferon. However, in contrast to normal mice, there was no expression of E α in B-lymphocytes of the transgenics. Since transgenic mice made with constructs containing 3.2 and 2 kb of 5'-flanking sequences show the normal expression pattern of the E α gene, it appears that deletion of 5'-flanking sequences between -1.4 and -2 kb inactivated or eliminated regulatory sequences required for expression of E α specifically in B-cells. The presence of this B-cell control region at -2 to -1.4 kb was also confirmed by van Ewijk et al. (11), who analyzed the E α gene expression in a more detailed fashion by the creation of a set of transgenic mouse lines after introduction of E α genes carrying deletions in its promoter/enhancer elements. By this approach, the authors further demonstrated a dissociation of expression in thymic cortical vs medullary cells, in B-cells vs non-B cells, and in germinal center vs follicular B-cells. Besides allowing conclusions about the role of individual promoter elements in controlling gene expression in different tissues, the set of transgenic lines described by these authors offered the potential for additional studies on the role of class II MHC molecules in various compartments of the immune system. The transgenic mouse system also allows the detection of crucial transcriptional regulatory elements. In vitro studies demonstrated the importance of the conserved regulatory sequence motifs, the X and Y boxes, in the 5'-flanking region of the MHC class II genes. Dorn et al. (12) confirmed the importance of these sequences by investigating the effect of their deletion on E α RNA synthesis in transgenic mice. Although mutant genes that had either the X or Y box deleted could still be transcribed, the efficiency of transcription was drastically reduced, and furthermore, the RNA initiation was no longer accurate.

2.4. Studying Developmental Regulation of Gene Expression

The expression of the MHC class I genes in embryonic cells is developmentally regulated and differentiation-dependent. Although human chorionic villus trophoblast cells do not express MHC class I

antigens, maturation of embryonic cells is generally accompanied by an increase in expression of MHC class I genes. Oudejans et al. (13) produced transgenic mice containing both the human HLA-B27 and β -2M genes. By applying *in situ* hybridization with biotinylated single-stranded RNA probes complementary to HLA-B mRNA on tissue sections of embryonic and extraembryonic tissues from transgenic mice, these authors were able to study genetic regulation of MHC class I gene transcription in developing extraembryonic cells, including trophoblast cells. It was found that in contrast to extraembryonic stromal cells and embryonic tissues, which contain mRNA coding for HLA-B27, specific transcripts were not detected in labyrinthine, or spongiotrophoblast, nor in trophoblastic giant cells. These cells are devoid of the HLA-A and HLA-B locus class I transcripts. Cell determination (trophoectoderm determination), therefore, results in the lack of expression of HLA-A and HLA-B.

The molecular mechanisms that control MHC class I gene expression in extraembryonic trophoblasts, in particular in extravillous trophoblast, are difficult to study since purified cell suspensions of normal extravillous trophoblasts are difficult to obtain and to culture. As illustrated above, with the introduction of transgenic technology, these experiments are now becoming feasible.

2.5. Relating Structure of MHC Genes to Function

A number of lymphocyte surface proteins are anchored in the cell membrane by glycosphosphatidyl inositol (known as GPI) linkages instead of hydrophobic protein domains. Antibodies specific for Qa-2, a GPI-anchored MHC class I antigen, can activate mouse T-lymphocytes, whereas antibodies against H-2 are not normally mitogenic for T-lymphocytes. The transgenic mouse system has been utilized to provide direct evidence of a biological function associated with the GPI membrane anchor.

To study the importance of the GPI-anchor Qa-2 in the activation of T-lymphocytes, Robinson et al. (14) produced transgenic mice expressing either normal GPI-anchored Qa-2 or Qa-2 molecules with a membrane-spanning protein domain derived from H-2. They demonstrated that only lymphocytes derived from transgenic mice carrying GPI-anchored forms of Qa-2 could be activated *in vitro* by Qa-2-specific antibodies and showed the biological importance of

the phospholipid membrane anchors in the process of transmembrane signaling in this pathway of T-lymphocytes activation.

2.6. Models for Gene Therapy

Although DNA introduced into mammalian somatic cells or injected into mouse eggs recombines with coinjected molecules quite readily, the foreign DNA typically integrates randomly within the chromosomes (1-3). Achieving targeted insertion therefore requires effective selection or screening strategies, or a method for reducing the nonhomologous events. One of the unique features of the MHC genes that make them of particular interest for insertion into mice is that inbred mice can be used that lack some of the MHC genes. The "defective" MHC gene can then be replaced as a model for gene therapy.

There are several mouse strains that do not express surface MHC class II I-E molecules. Strains of H-2^b and H-2^s haplotypes do not express a surface I-E molecule because of a 630-bp deletion that removes the first exon and part of the promoter in the E α gene. The E α chain, however, is synthesized and can be found in the cytoplasm. Brinster et al. (15) corrected this genetic defect by homologous recombination using the normal, functional E α allele (from BALB/c) spanning the deletion. In the transgenic mice they produced, the corrected E α gene could be transmitted to progeny, and these mice were bred to homozygosity. mRNAs were produced from the corrected allele in a tissue-specific manner in these transgenic mice. Unfortunately, these mRNAs were of different sizes from the wild-type; many point mutations were introduced, and no E α protein could be detected. These pioneer experiments, nevertheless, demonstrated that it is feasible to target foreign DNA to a specific locus of the mouse genome in fertilized mouse eggs. Potentially, the targeting of foreign DNA to specific chromosomal sites by homologous recombination would provide an invaluable genetic model for studying gene function and correcting genetic defects.

2.7. Induction of Immunological Tolerance

The immune system of an organism normally does not react to its own cells and tissues. Tolerance to self-antigens is a critical feature of the immune system. The repertoire for antigen bound to self-MHC proteins of peripheral T-lymphocytes in a given animal is controlled

in several ways: by the collection of germ-line genes available in the animal, by removal of self-reactive cells during tolerance induction, and by positive selection of cells bearing receptors biased for recognition of antigen in association with self-MHC. Certain MHC molecules in combination with the products of other genes seem to bind very well to particular parts of T-cell receptors, thus leading to deletions of large portions of the T-cell repertoire (16). In addition, functional silencing of particular elements in the immunologic repertoire, whereby antigen interacts with the lymphoid system to impair its later capacity to respond to that antigen, is an important mechanism in immunologic tolerance for both T- and B-lymphocytes.

In some cases, the study of tolerance to self has been greatly aided by animal models of spontaneous or experimentally induced autoimmunity. With transgenic technology, it is possible to engineer antigens that are well defined in terms of three-dimensional structure and T-cell subset responses for microinjection. Furthermore, expression of the introduced gene can be targeted to tissues that are readily accessible for histological analysis, and in some cases, timing of expression can be controlled.

In the T-lymphocyte pool, it appears that tolerance induction primarily occurs during lymphocyte development in the thymus. Once the T-cell repertoire has been shaped in the thymus, it does not appear to be subjected to further mutation in the periphery. Potentially autoreactive T-cells are deleted presumably on exposure to the appropriate self-antigen presented by either bone-marrow-derived cells or thymic stromal cells. Strong support for this conclusion comes from the study of transgenic mice containing a high frequency of T-cells in the preimmune repertoire with receptors for the male (H-Y) antigen (17). Despite the fact that peripheral T-cells in transgenic animals of both sexes expressed the transgenes, tolerance to the H-Y antigen was observed only in male transgenic offsprings. This was shown to involve deletion of self-reactive T-cells early on in thymocyte differentiation at the stage when the cells expressed both CD4 and CD8, indicating a role for the two coreceptors for MHC molecules in tolerance induction.

Although this mechanism of intrathymic deletion is effective for ridding the T-cell repertoire of autoreactive lymphocytes, it may not be as effective in inducing tolerance to antigens expressed specifically in other nonlymphoid tissues. These tissue-specific antigens are

of great importance, since many of the major failures of tolerance to self (i.e., autoimmune disease) are owing to immune responses to these "paraenchymal self" antigens.

Mice expressing I-E with the normal tissue distribution (on B-lymphocytes, macrophages, dendritic cells, and thymic epithelium) induce tolerance to self-I-E by clonal deletion in the thymus. By targeting the expression of an MHC class II antigen I-E to the pancreatic β islet cells in I-E⁻ mice using the insulin promoter, transgenic mice produced were found to be tolerant to the I-E transgene encoded antigen despite the absence of detectable I-E expression in the thymus or spleen (18). In vitro studies with the isolated I-E⁺ islet cells suggested that class II antigens on nonlymphoid cells were in fact not stimulatory to T-lymphocytes. Instead, they induced a specific "T-cell paralysis," and reactive T-cells exposed to the islet cells were rendered anergic to subsequent rechallenge by normal lymphoid class II⁺ stimulators.

These observations were further confirmed by targeting transgene expression to the acinar cells of the exocrine pancreas using promoter sequences isolated from the elastase gene (19). It was found that tolerance in T-lymphocytes can be induced and maintained by expression of the class II antigen exclusively in peripheral nonlymphoid cells. Adoptive transfer studies indicated that T-cells from tolerant mice can mediate resistance to organ-specific immune attack, in mature nontolerant T-cells in vivo, but the resistance may not involve specific suppressor T-lymphocytes. These experiments demonstrated that T-cell tolerance by clonal paralysis does occur during normal T-cell development in vivo.

Immunoglobulin (Ig) genes encode antibody molecules expressed exclusively in the B-lymphocyte lineage. B-lymphocytes are derived from proliferating pre-B precursor cells and exit from the mitotic cycles that generate them as small, nondividing lymphocytes lacking membrane Ig (mIg). They then develop mIg, first IgM and later IgM plus IgD, during a nonmitotic maturation phase. It has been postulated that, for the B-lymphocytes, mIg is the vital signal transducer.

Nemazee and Burki have studied B-cell tolerance in transgenic mice using genes for IgM anti-H-2^k MHC class I antibody (20). In H-2^d transgenic mice, tissue-specific expression of the IgM anti-H-2^k protein was obtained. The idiotype encoded by the transgenes was found only on B-lymphocytes. It was found that about 25–50% of the splenic

B-lymphocytes bear mIg of this specificity, and a high anti-H-2^k cytotoxic antibody titer encoded by the transgenes is produced. In contrast, H-2^{d/k} transgenic mice lack B-lymphocytes bearing the anti-H-2^k idio-type and contain no detectable anti-H-2^k antiserum. B-lymphocytes with the predetermined transgene-encoded antiself-specificity were absent, suggesting that very large numbers of autospecific B-lymphocytes can be eliminated by clonal deletion. This deletion probably occurred at some time shortly after they first expressed their mIg receptors and as a consequence, most of them never developed into antibody-secreting cells. These results would therefore support the hypothesis that B-lymphocytes are made tolerant by antigen in the absence of antigen-reactive T-lymphocytes.

It can be seen that the use of transgenic mice to study self-recognition by the immune system provides us with important information about the properties of tolerance toward self and the mechanisms by which it is achieved, which in turn reflects on the nature and development of the immune system. This approach of introducing into the germ line of an animal of a known gene coding for a normally foreign antigen by means of a specific promoter to direct its expression to specific tissues would allow the synthesis of this particular antigen as an authentic self-molecule at a particular stage in development and in a particular site.

2.8. Production of Polymorphic Anti-HLA Antibodies

Antibodies employed for HLA tissue typing are routinely alloantisera obtained from multiparous women. In many cases, these antibodies have broad specificities and are crossreactive with many different serologically ill-defined determinants. The use of monoclonal antibodies has reduced significantly many of the problems associated with alloantisera. However, immunization of normal mice with HLA antigens results mostly in antibodies against framework determinants. In this regard, production of HLA transgenic mice allows the possibility of raising monoclonal antibodies against polymorphic HLA determinants. This is so because HLA transgenic mice are, in principle, tolerant to the framework determinants; antibodies produced by them should be mainly directed to the allele-specific determinants. This is indeed the case and was recently reported by Hammerling et al. (21). These authors succeeded in obtaining, at a high frequency, antibodies specific for the

HLA allelic determinants by immunization of various HLA class I transgenic mice with spleen cells from other HLA transgenics or with HLA-transfected mouse fibroblasts. The potential of this strategy for the production of antibodies for HLA serotyping is enormous.

2.9. Disease Models

Genetic factors are known to influence host resistance to certain tumors. Over the years, researchers have had to be contented with randomly found animal models of genetic disease. However, despite many years of looking, no suitable animal mutants have been found for many abundant human genetic diseases. With the transgenic technology, it has now become possible to deliberately produce animal models of human genetic disease.

In the mouse, natural resistance to transplantable lymphomas has been linked to the MHC locus on chromosome 17 (22). To investigate this H-2-linked phenomenon, Hoglund et al. (23) used the H-2D^d transgenic mouse strain D8 on B6 background (7) and reported that these transgenic mice have acquired "natural" resistance to T-cell lymphomas of B6 origin in parallel with expression of the transgene product. Therefore, the D8 mice were more resistant to sc challenge of RBL-5 lymphoma cells than B6 controls. The direct role of the H-2D^d antigen was further investigated by the use of (D8 × B6)F1 crosses and (D8 × B6)F1 × B6 backcrosses. The latter showed cosegregation with regard to D^d antigen expression and lymphoma resistance. The inherited pattern was consistent with control by a single dominant gene, and it is therefore most likely that the D^d gene is responsible for the resistance of the D8 mice. These results also provide direct evidence to support models that hypothesize that MHC class I genes play an important role in conventional F1 hybrid resistance.

To create a mouse model for human insulin-dependent diabetes, Allison et al. (24) utilized the rat insulin promoter to overexpress the H-2K^b gene product in the pancreatic β cells of transgenic mice. The transgenic mice, whether syngeneic or allogeneic to the transgene, developed insulin-dependent diabetes. However, no T-cell infiltration was detectable in these transgenic mice whether there was compatibility between the transgene and the H-2 type of the recipient mouse, suggesting a direct, nonimmune role for the transgenic class I molecules in the disease process.

There are over 40 human diseases that have been associated with various MHC haplotypes (25). Specific alleles have higher frequencies (presumed susceptibility alleles or markers for these alleles), whereas others have lower frequencies (presumed protective alleles or markers) compared with frequencies in ethnically matched control populations. In general, the reasons for these associations are unknown. HLA transgenic mice, therefore, offer the possibility of establishing mouse models of HLA-linked diseases. This possibility was fully exploited by Luthra et al. (26) to study the correlation between HLA-B27 and ankylosing spondylitis. These authors reported that HLA-B27 transgenic mice are more susceptible to infection with the bacteria *Yersinia enterocolotica* than normal litter mates. Therefore, infection of HLA-B27 transgenic mice with 10^4 live *Yersinia enterocolotica* bacterial cells would lead to paralysis in the hind legs in 30–40% of the treated animals.

3. Concluding Remarks

This chapter describes some of the scope with which transgenic mice can be applied to address complex problems in the immune system. As a tool, transgenic mice produce many useful reagents for studying the immune system. These include the production of congenic mice (7–9) and the production of allele-specific antibodies (21). Also, transgenic mice are providing insights into the properties of self-tolerance (19,20). When new genetic information is added to the genome of a mouse, that gene (or genes) and, as a consequence, its gene product, are genetically and phenotypically self. Thus, one can ask if such new products are recognized as self by the immune system and if so, what the characteristics of that nonresponsive condition are. This would, in turn, reflect upon the nature and development of the immune system.

One of the most important applications of transgenics is to provide a model system for answering questions about gene activity in in vivo animal systems. As described above, expression of any gene could be directed to a specific cell type (or types) by the construction of fusion genes that are regulated by heterologous promoters consisting of tissue-specific enhancer elements. This approach provides a means to define the characteristics of individual stages in development and differentiation of the immune system, as well as the underlying enhancers/promoters that elaborate regulation and compartmentalization of gene

expression (11). Furthermore, it would be possible to inhibit specific gene functions in the immune system by utilization antisense constructs. Antisense DNA/RNA have been utilized to inhibit the expression of eukaryotic genes in several biological systems (27). Transgenics provide a potential approach to creating mice deficient in the expression of any cloned genes. Recombinant constructs that have been designed to express at higher levels than corresponding endogenous genes by the inclusion of strong enhancer/promoter elements could be applied in order to achieve maximum inhibition *in vivo*.

Last, but not least, the transgenic mouse model offers a new tool for studying human genetic diseases. Gene products could be overproduced to create a disease state, as exemplified by the induction of diabetes with overproduction of MHC antigens in pancreatic islets (18), or, if one has cloned a gene for a dominant disorder resulting from an altered gene product, one could attempt to correct the disease gene by gene homologous recombination and targeting (15). The ultimate application of such studies obviously would be treatment of genetic diseases.

Nevertheless, I do not want to finish this chapter without mentioning some of the present limitations of transgenic technology. Certain areas of this powerful technology still need further refinement. There is at present no control on the number of copies of the introduced genes to be integrated into the host genome, nor can one control the site of integration except by the laborious manipulations of homologous recombination in embryonic stem cells followed by subsequent introduction into blastocysts (28). One might have to incorporate large distant upstream regulatory sequences in order to achieve maximum gene activity in the transgenics (29). It could be a long process to try to identify some of these regulatory elements. The expression of foreign gene products could lead to the disruption of programmed normal developmental processes and, in some cases, could result in abnormality of reproductive function (30). Furthermore, the biological effects of the expression of foreign genes are quite unexpected in some cases. For example, in the experiment described by Allison et al. (24), it was their intention to induce diabetes in mice as a consequence of the inappropriate MHC class I expression in pancreatic β -islet cells. Although diabetes could be generated, the onset of disease did not require incompatibility between self-MHC and the transgene-encoded

product. In addition, the authors found no evidence for autoimmunity in the transgenic mice that they created (24), and the disease state can be induced by targeting a variety of genes to the β -islet cells. Despite these cautious notes, the transgenic technology still provides one of the best, if not the only model at present to study many complex biological systems.

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CHAPTER 5

The Commercial and Agricultural Applications of Animal Transgenesis

Kevin A. Ward and Colin D. Nancarrow

1. Introduction

The commercial potential for transgenesis techniques is substantial, particularly in the fields of animal and plant agriculture. This results from productivity being a function of genetic potential and interaction with the environment, but environmental factors being only partially subject to influence by the farmer. Thus, concentrating on genotype improvement becomes an important goal if substantial cumulative gains in productivity are to be made. Historically, the genetic potential associated with important animal production traits, such as wool growth, milk yield, and body growth, has been improved by selective breeding, whereby phenotypically superior animals are used as parental stock for following generations. The high quality of the domestic animals in use in farming today compared with those of earlier centuries is witness to the success of the approach, but nevertheless, the method has significant limitations that have frustrated animal breeders for many years. The complex genetic interactions that combine to produce a particular animal phenotype result in slow genetic gain, averaging at best about 1–3% per year. In addition, separating a desired production trait from one or more undesirable traits is often very difficult. However, the most important of these limitations is the inability to transfer genetic information between species, because of the biological barrier that prevents interspecific breeding.

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All the above constraints arise from the mechanism of transfer of DNA between animals, whereby whole chromosomes constitute the unit of genetic exchange. Transgenesis offers a possible solution, although it should not be considered a universal cure. There can be little argument that the successful transfer of recombinant DNA to mouse embryos by the technique of pronuclear microinjection of single-cell embryos (1-5) and the subsequent demonstration that the technique could be used to alter the growth properties of mice (6,7) heralded one of the most significant technical advances in modern biology. Its application to agriculturally important animals is now well-documented (8-15), and the effort is now directed toward making the commercial promises a reality. This is by no means an easy matter, however. Although gene transfer may overcome some of the difficulties associated with conventional breeding techniques, it brings with it a new set of problems. Foremost among these is the need to understand the physiological processes of domestic animals at a molecular level. This is necessary to enable genes that influence important production traits to be identified and isolated. It has become apparent over the past few years that very few such traits actually fall into this category. Second, it becomes important for the appropriate molecular modifications to be made to the chosen gene so that its effect on the animal is as desired. Third, it is important to determine whether, in the process of gene insertion, an existing gene of importance has been damaged, which will require a detailed assessment of the general physiology of the animal.

The various production traits that are amenable to manipulation by transgenesis techniques are as follows:

1. The endocrine system;
2. Biochemical pathways;
3. The structural proteins of the textile fibers and milk; and
- 4 The immune system.

Although it is theoretically easier to manipulate the products of animals rather than actual body composition, nevertheless, to date, most efforts have been directed toward the latter target. This presumably reflects a paucity of knowledge of the physiology involved in the production of animal products. This chapter will concentrate on those areas that are currently undergoing active research in an attempt to increase the productivity of domestic animals.

2. The Modification of the Endocrine System of Domestic Animals

Manipulation of the endocrine status of domestic animals was an obvious early target for transgenesis following the pioneering research of Palmiter et al. (6,7) in laboratory mice and, as a consequence, has received considerable attention over the past few years. The results have been reviewed in detail several times in recent years (13–18), so in this section, only the overall conclusions from the work will be summarized, together with some evaluation of the commercial potential of the approach.

When the genes used in the initial mouse experiments were transferred to pigs, sheep, and rabbits (8), using the mouse metallothionein-I (MT-I) promoter and the human or the bovine growth hormone coding sequences, constitutive expression of the transgenes was obtained (13,14,16). This gave rise to a large rise in the level of circulating growth hormone but, in contrast to the mice, did not result in larger animals. Moreover, the animals were physiologically abnormal (13,14), and it became clear that the larger domestic animals did not respond to elevated growth hormone concentrations in the same way as laboratory mice (19,20).

The initial results in pigs and sheep were obtained with transgenes encoding heterologous growth hormone proteins (bovine or human), and therefore, it was suggested that the poor growth response may have been owing to poor recognition of the hormone, particularly in view of a report that a faster-growing transgenic pig had been produced that contained a transgene-encoding porcine growth hormone (15). However, such an explanation now appears unlikely, because transgenic sheep with elevated levels of the natural ovine growth hormone and transgenic pigs with elevated porcine growth hormone all exhibit the same poor growth and abnormal physiology, as do those animals with excess heterologous hormone (9,21). Rexroad et al. (19) showed that transgenic sheep with excess bovine growth hormone were diabetic, which suggests that the animals become acromegalic, diabetes being a typical symptom of this disease state. Nancarrow et al. (18,21) looked in detail at the physiology of transgenic sheep containing extremely high levels of the natural sheep growth hormone, and arrived at similar conclusions to those of Rexroad and his col-

leagues. The animals had an elevated basal metabolic rate and an associated high cardiac output, IGF-1 was elevated, and renal function was impaired. Bone growth was abnormal, particularly in the front limbs, and the internal organs of the transgenic sheep were significantly larger than controls. The animals showed obvious symptoms of diabetes, and all died within one year of birth.

For the commercial application of this line of research, the chronic high production of growth hormone must be avoided. Although a number of attempts at achieving this goal have been made, as yet none have been successful. One approach has been to control the actual release of the growth hormone by the action of other genes. Thus, the gene encoding the growth-hormone-releasing factor has been placed under metallothionein promoter control by Pursel et al. (13,14), and the resulting transgenic animals produced elevated levels of the releasing factor, but the concentration of growth hormone itself was not altered. As a result, the transgenic animals remained healthy and of normal appearance, and did not grow at any faster rate than controls. In a similar experiment (22), growth-hormone-releasing factor coding sequences were placed under the control of the human transferrin gene promoter, but similar results were obtained.

A different approach has been to use promoters other than that of the metallothionein gene. Wagner (12) placed the growth-hormone-coding sequence under the control of a promoter sequence isolated from the gene encoding the enzyme phosphoenolpyruvate carboxykinase, the expression of which can be regulated by the ratio of carbohydrate:protein in the diet, but again, no growth of the transgenic animals beyond that of controls was obtained. Rexroad et al. (22) have used the albumin promoter to direct growth-hormone synthesis to the liver in transgenic sheep, but this also failed to provide the appropriate regulation for enhanced growth in these animals.

It is apparent that animals with enhanced growth and improved feed efficiency are possible using the approach of modifying growth-hormone levels, but only if the production of the hormone is very tightly regulated so that its concentration remains within physiological limits and if the transgene can be rendered transcriptionally silent at will. With current technology, this is a formidable challenge, but achieving it is essential for commercial application of the results.

3. Modification of the Biochemistry of Domestic Animals

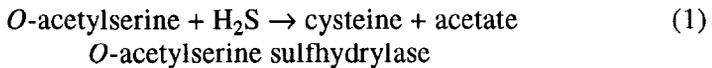
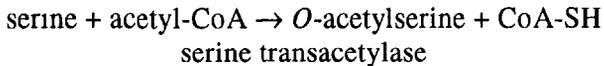
During the evolution of our major species of domestic animals, a significant number of metabolic pathways have been lost by irreparable damage to the genetic information that encodes them. The end products of these pathways must be supplied as essential nutrients in the diet, and there are instances where this source is insufficient for maximum productivity. Genetic engineering now provides the ability to transfer genes encoding these pathways from organisms where the pathways are functional, thus restoring functional biosynthetic capacity to the target species. In order for this to be useful, however, it is necessary to identify substrate or nutrient limitations to important production traits. This is not a simple task, because many production traits are influenced by a variety of different factors depending on the actual husbandry situation, and the identification of these factors requires a detailed knowledge of the physiology of the production trait. Such knowledge is often not available, because the detailed physiology of the particular production trait is only partly understood. There are, however, two current areas of research where this approach may succeed.

3.1. The Cysteine Biosynthetic Pathway

The Australian wool industry provides one useful area where this approach might succeed. When sheep are fed a diet that simulates that of the grazing animal, the amino acid cysteine is rate limiting for wool growth. Although cysteine itself is not an essential amino acid for mammals, it can only be synthesized from methionine. Thus, the supply of either methionine or cysteine is essential in the sheep diet. In order to increase the rate of wool growth, it is clearly necessary to increase the supply of one of these two amino acids. However, direct dietary supplementation of these amino acids is not effective as a method of increasing their supply, because the unique features of ruminant digestion ensure that added amino acid is degraded by the resident microflora. In the past, different approaches have been taken to overcome the degradation that occurs. For example, the ruminal degradability of proteins can be reduced by treatment with formaldehyde (23), which prevents ruminal degradation, but allows digestion

in the distal part of the digestive tract. Alternatively, methionine itself can be encapsulated in a medium that survives the rumen, but not the lower digestive tract (23). However, these methods have not been economical in the practical farm environment.

An alternative approach is to introduce into the sheep genome the genes that encode the cysteine biosynthetic pathway. These genes are functional in prokaryotes and the auxotrophic eukaryotes, and are thus available for modification for expression in the digestive tract of sheep. The actual pathway for the biosynthesis of cysteine in *E. coli* is complex. It consists of two discrete parts, a pathway for the reduction of a sulfur source to sulfide, and a simple two-step pathway to combine the sulfide with the amino acid serine to produce cysteine. The genes that encode the second part of the process are *cysE* and *cysK*, encoding the enzymes serine transacetylase (SAT) and *O*-acetylserine sulfhydrylase (OAS). These catalyze the following reactions:



The research involved in this type of genetic engineering project can be divided into four parts:

1. Isolation and characterization of the appropriate DNA coding and promoter sequences;
2. Construction and testing of various fusion genes in cell culture to determine the optimum configuration of the various components;
3. Testing of the preferred gene in transgenic mice to determine the efficiency and tissue-specificity of expression in vivo; and
4. Transfer of the preferred gene to transgenic sheep.

The *cysE* and *cysK* genes from *E. coli* have been isolated and fully characterized (24–26), thus providing the necessary coding sequences for the gene-transfer experiments. In order to modify the genes for expression in eukaryotic cells, the bacterial coding sequence has been inserted downstream from the sheep metallothionein-Ia promoter with the sheep growth-hormone gene used for stabilization of the mRNA transcribed from the fusion gene product (27,28). The general design of these genes is shown in Fig. 1. When mouse L-cells were transformed

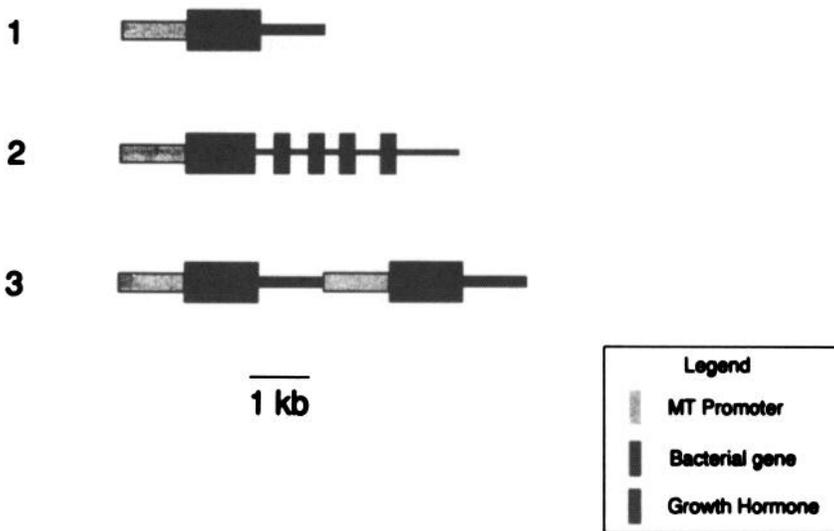


Fig. 1. Diagrammatic representation of the modifications made to bacterial coding sequences to provide expression in eukaryotic cells. Gene 1 contains exon 5 of the sheep growth-hormone gene 3' to the bacterial sequence. Gene 2 is similar, but contains the entire sheep growth-hormone gene. Gene 3 consists of a fusion of two gene 1 sequences, such that a single piece of DNA encodes the enzymes necessary for the cysteine synthesis or glyoxylate cycle biochemical pathways.

with these genes, mRNA transcripts of the predicted sizes were detected with probes encoding the appropriate *cysE* or *cysK* coding sequences (28). Extracts prepared from the transformed cells contained readily detectable levels of the enzymes SAT and/or OAS (Table 1).

The expression of the *cys*-encoding fusion genes was examined in transgenic mice. Only those genes containing exon 5 of the sheep growth-hormone gene located 3' to the bacterial coding sequence were expressed at detectable levels in zinc-induced transgenic mice. This information is of general relevance to the expression of genes in transgenic animals, since the genes containing only exon 5 do not possess any exon/intron structure. Introns therefore are not an obligatory requirement for the expression of foreign genes in transgenic mice (but *see* 29).

Having established the fact that both genes can be independently transcribed and translated in transgenic mice, the transfer of both genes was examined. The transcription and translation of both *E. coli*-derived sequences have been demonstrated in animals containing the combina-

Table 1
 Activity of Serine Transacetylase (SAT) and *O*-Acetylserine Sulphydrylase (OAS) in Extracts from Cells Transformed with Various Fusion Genes Containing Either the *cysE* (CE) or *cysK* (CK) Gene of *Escherichia coli*^a

Construct	Zn-induced		Uninduced	
	SAT	OAS	SAT	OAS
pMTCE10	2796	—	777	—
pMTCE11	255	—	68	—
pMTCK7	—	1350	—	—
pMTCK11	—	162	—	—
pMTCEK1	268	6960	86	1242

^aGenes were constructed as shown in Fig 1 pMTCE10, pMTCK7, and pMTCEK1 contain only exon 5 of the sheep growth-hormone gene pMTCE11 and pMTCK11 contain the complete sheep growth hormone gene Enzyme activity is expressed as μ moles substrate utilized (SAT) or product formed (OAS) /30 min /mg protein

tion gene MTCEK1, which encodes both the *cysE* and *cysK* sequences in a single piece of DNA (Fig. 1). Zinc-inducible SAT and OAS activities (Table 2) were measured in the intestinal epithelium, the kidney, and the liver. Clearly, the actual biosynthesis of cysteine in these animals requires the presence of the necessary substrates in the tissues that express the transgenes, and current experiments are directed toward establishing whether such biosynthetic activity can be demonstrated in the mice. Since earlier studies have already indicated that genes that are expressed in transgenic mice are also expressed at high level in transgenic sheep, we have also commenced the transfer of the MTCEK1 gene to sheep.

Rogers et al. (24,30,31) have used a similar approach to achieve the same goal of enabling sheep to synthesize cysteine from H₂S. In this case, the source of genes to provide the appropriate coding sequences is the bacterium, *S. typhimurium*. The two genes being used are the *cysE* gene, which is essentially the same as the *E. coli* gene, and the *cysM* gene, which encodes an OAS enzyme significantly different from that encoded by the *cysK* gene both in *E. coli* and *S. typhimurium*. After demonstrating expression of the genes in sheep cells in culture after being fused to the SV40 late promoter and SV40 polyadenylation signal sequences (30,31), they have been joined together in a single piece of DNA, and each coding sequence has been placed under the control of

Table 2
Activity of Serine Transacetylase (SAT) and *O*-Acetylserine Sulphydrylase (OAS) in Tissue Extracts Prepared from Transgenic Mice^a

Mouse line	Organ	SAT	OAS
CK7-26	Intestine	—	206
	Kidney	—	352
	Liver	—	13
CE10-29	Intestine	6546	—
	Kidney	0	—
	Liver	0	—
EK1-28	Intestine	15144	519
	Kidney	0	938
	Liver	0	156
	Bran	0	90

^aCK7-26 contains the gene pMTCK7, CE10-29 contains pMTCE10, and EK1-28 contains pMTCEK1. Specific activity is measured as nmoles substrate utilized (SAT) or product formed (OAS) / 30 min/mg protein

a promoter derived from the long terminal repeat of the Rous sarcoma virus. This DNA has provided constitutive expression in transgenic mice and transgenic sheep, but unfortunately, not in tissues where the necessary substrate for the reactions might be expected. The research now in progress aims at providing the genes with a promoter more suited to directing their expression in tissues where the appropriate substrates for cysteine biosynthesis might be available (31).

3.2. The Glyoxylate Cycle

A second project designed to improve the general efficiency of feed utilization in sheep, and to increase specifically nutrient supply to sheep wool follicles involves the introduction of the glyoxylate cycle to sheep. The rationale for this research stems from the fact that the microorganisms that populate the rumen in sheep consume essentially all available carbohydrate in the ingested feed and produce a range of fermentation products, the most important of which, from an energy viewpoint, are the volatile fatty acids. After absorption by the sheep, these are used directly for energy or, if gluconeogenic, are converted to glucose to provide the carbohydrate that is essential for the proper function of several key tissues. Included in these tissues are the wool follicles, which have a high demand for glucose (32).

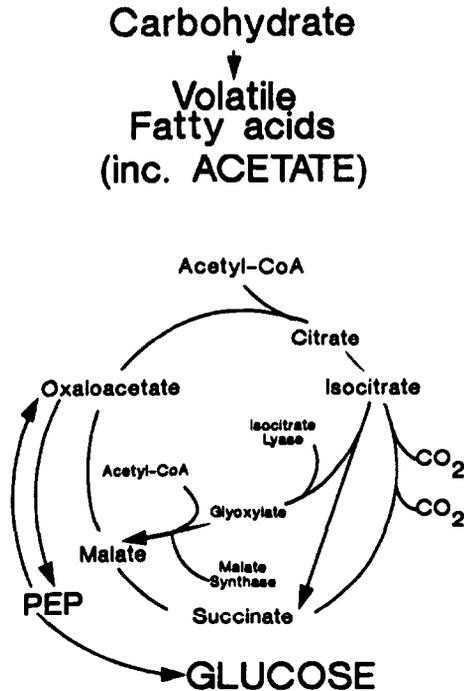


Fig 2 The biochemical reactions of the glyoxylate cycle.

On pastures where the nongluconeogenic volatile fatty acid acetate predominates, sheep are prone to ketonuria and a suboptimal growth of wool (33,34). It has been suggested that if the abundant supply of acetate in these animals could be utilized for glucose production, some of these problems might be overcome. Acetate can serve as a source of glucose in organisms that possess the enzymes necessary to catalyze the reactions of the glyoxylate cycle (35) (Fig. 2) where isocitric acid from the tricarboxylic acid cycle is cleaved to succinate and glyoxylate by the action of the enzyme isocitrate lyase. Succinate is a gluconeogenic substrate, whereas the glyoxylate produced in the reaction is combined with another molecule of acetate to produce malate, thus providing the necessary substrate for continuation of the cycle. This second reaction is catalyzed by the enzyme malate synthase.

In *E. coli*, the enzyme isocitrate lyase is encoded by the gene *aceA*, whereas malate synthase is encoded by the *aceB* gene. These genes

Table 3
Activity of Isocitrate Lyase and Malate Synthase in Extracts
of Zinc-Induced L-Cells Transformed with Fusion Genes
Encoding the Enzymes of the Glyoxylate Cycle^a

Construct	Isocitrate lyase	Malate synthase
pMTaceA1	76	—
pMTaceB1	—	1.7
pMTaceA2	68	—
pMTaceB2	—	34.3

^apMTaceA1 and pMTaceB1 contain the complete sheep growth-hormone sequence, whereas pMTaceA2 and pMTaceB2 contain only exon 5, as shown in Fig 1. Specific activities are expressed as nmoles of product formed /20 min /mg protein and are corrected for a low level of malate synthase activity in L-cell control extracts. No isocitrate lyase activity was detected in untransformed L-cells.

have both recently been isolated and sequenced (36–38), and their modification for transcription in eukaryotes has been carried out in similar fashion to that described for the cysteine genes, using the sheep MT-Ia promoter sequence and exon 5 of the sheep growth-hormone gene. When transferred to L-cells in culture, all three genes produced RNA transcripts of the predicted sizes (27,39), and these were translated into active enzyme as indicated by the activities of isocitrate lyase and malate synthase in extracts prepared from the transformed cells (Table 3). The genes were then transferred to mice, and their expression examined in liver, kidney, and intestinal tissues, where mRNAs were detected that hybridized with appropriate probes for the bacterial coding sequences and were of the predicted sizes (39). Cell-free extracts from the same tissues showed active isocitrate lyase and malate synthase activities, indicating that the animals have the enzymic potential for the operation of the cycle (39). The detailed physiology of these animals is currently under investigation.

3.3. Other Possible Biochemical Pathways

The commercial possibilities associated with the manipulation of domestic animal biochemistry are assuming wider acceptance in the field of genetic engineering, but there are a number of significant technical difficulties associated with the approach as it is currently being used. The most important of these is the requirement for simple

pathways, made necessary by the size limitations of current cloning and microinjection procedures. The size limit of the transgene at present, using bacterial coding sequences of the normal 1–2 kb, would appear to be about four or five genes.

The other important limitation to the methodology at present is controlling the expression of genes that might interfere with the metabolic flux of important cellular intermediates. This is addressed in a recent review (40) that looks at the possibility that the *E. coli* genes for threonine biosynthesis and lysine biosynthesis could be used to introduce the biosynthetic pathways for both amino acids into transgenic livestock (40). A computer-simulation study of the metabolite flux that might ensue if the appropriate enzymes were to be produced in animals is included in this work (40).

4. Structural Protein Modification

The commercial potential for the modification of structural proteins is considerable, because such modification would not be expected to affect the viability of the animal. The growing acceptance of the relevance of transgenic animals in domestic animal production has resulted in several approaches in this area receiving serious consideration. The most obvious proteins of commercial value are: (1) the proteins of milk and (2) the proteins of the textile fibers (wool, cashmere, and mohair).

4.1. Milk Proteins

The mammary gland is a useful target for the expression of transgenes that have been modified to produce foreign proteins of high value e.g., pharmaceutical and growth factors. The concept stems from the proposal of Palmiter et al. (6) that transgenic animals might be used for the manufacture of valuable proteins, and the use of the mammary gland itself for this purpose was pioneered by Clark and his colleagues, using the sheep β -lactoglobulin gene as a promoter source and the sheep as the target species (11,41–45). Because of the impracticality of using larger animals to test gene constructs, much of the early investigative work has been carried out in transgenic mice. The mouse is a useful test system, since it has been shown (46,47) that the sheep gene is expressed specifically in the mouse mammary gland and that this expression shares some developmental similarities with the temporal sequence in the sheep.

Foreign proteins have been produced in the mammary glands of mice and sheep in experiments that involved joining the β -lactoglobulin promoter to the coding sequences of two human proteins, α 1-antitrypsin and blood clotting factor IX. These genes were expressed in transgenic mice and in transgenic sheep, although the level of expression of the transgenes was substantially lower than that of the endogenous β -lactoglobulin gene (42). Nevertheless, concentrations of 25 μ g/mL of human factor IX protein and 5 mg/mL of human α 1-antitrypsin in these early experiments (42) clearly demonstrated the potential of the approach. Recently, this expression has been increased by the introduction of intronic sequences into the transgene, resulting in high levels production of human α 1-antitrypsin in mice (29,48).

Other promoter sequences derived from milk protein genes have now been used to produce human proteins of therapeutic value in the mammary gland. Thus, in transgenic mice, the mouse whey acid protein (WAP) gene promoter has been used to produce human tissue plasminogen activator (49,50) and human CD4 protein (51), and the bovine α S1-casein gene promoter has directed high level expression of human urokinase (52). Furthermore, in a highly significant series of experiments, the expression of the mouse WAP gene at very high levels in the mammary glands of transgenic pigs has recently been demonstrated (53). Thus, both the WAP and β -lactoglobulin promoters appear commercially attractive for high-value protein production.

Another commercially attractive approach to the manipulation of milk composition is to change some of the normal constituent proteins or lipids, although no transgenic animals have yet been reported where this has been achieved on a commercial scale. Considerable variation can occur naturally in the protein:lipid ratio of milk, with present consumer preference tending toward higher values, and therefore, it should be possible to alter these properties genetically. Thus, manipulation of the genes encoding the milk proteins to favor higher protein content would be of significant advantage, provided that the protein content was maintained at a level compatible with the general micellar structure of milk. It may be possible to achieve this goal either by altering the promoter sequences that control the expression of the milk protein genes in the mammary gland or by inhibiting the transcription of genes involved in lipid biosynthesis.

4.2. The Proteins of the Textile Fibers

The structural proteins of the important textile fibers (wool, cashmere, and angora) also represent a useful target for genetic manipulation, and some preliminary research in this area has been foreshadowed (31). The unique sulfur-rich keratin proteins that make up these fibers contribute the majority of the physical properties of the fibers, partly as a result of their unique amino acid composition and partly because of their arrangement within the fiber cells. Thus, it is probable that manipulation of unique keratins may alter the textile properties of the fiber. Since many of the keratin genes have now been characterized (54–57), this is a realistic goal.

Recently, a complete keratin gene encoding a type-II wool protein was expressed in transgenic mice (58). The gene was expressed at different levels in the various transgenic animals, and when this expression was high, the wool protein caused significant abnormalities in the structure of the mouse hair. This is probably the result of incompatible pairing of the wool protein with the mouse keratins and, hence, provides some guide to the limits for keratin manipulation. This important experiment demonstrates that a complete keratin gene can be expressed tissue-specifically in mice, thus opening the way for the use of this species for initial testing of keratin genes with minor alterations to coding or control sequences.

The modifications to wool proteins that might be considered include reduction of the number of cysteine residues per keratin molecule, changing the number of amino acid side chains in order to modify dyeing properties, and manipulating the proteins of the fiber cuticle to produce a “cashmere-like” wool fiber. Other modifications to be considered are the inclusion of proteins with specific toxicity to moths to prevent the destructive action of the keratinases secreted by these insects, and the modification of cuticle structure to reduce or eliminate the shrinkage that wool fabrics can undergo during normal washing.

5. Conclusions

The transition from laboratory experiments to transgenic domestic animals with significant improvements in productivity is a difficult problem, because the delicate physiological balance of the animals is easily disrupted leading to lowered overall performance. However,

such is the effort currently being placed in this area that commercial animals can be predicted with confidence within the next few years. The most likely animals will be those modified for the production of high-value proteins in the mammary gland, but animals with modified biochemistry will also be under trial within the next five years. The work on the manipulation of animal biochemistry that has been described here will serve as a useful guide to the extent to which animal metabolism can be altered in general. In addition, the next few years are likely to see more emphasis placed on the production of animals with altered structural protein production.

The fact that it is now six years since the first transgenic domestic animals were reported and yet no commercially viable animal has been produced is compelling evidence for the difficulties associated with development of genetic-engineering concepts from laboratory to field. However, nothing has yet been found that diminishes the potential usefulness of the approach, and there is little doubt that the industry can look forward to unique and productive animals during the current decade.

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CHAPTER 6

P Element-Mediated Germ-Line Transformation of *Drosophila*

Mark O'Connor and William Chia

1. Introduction

In contrast to many organisms, *Drosophila* embryos do not integrate injected DNA at an appreciable frequency. For this reason, the generation of germ-line transformants has relied on the utilization of transposable elements to effect the chromosomal integration of injected DNA (1,2). The success of this approach has depended largely on our understanding of the biology of P elements and the syncytial nature of the early *Drosophila* embryo. The first 13 embryonic divisions following fertilization are nuclear, resulting in the formation of a syncytium. Consequently, if microinjection into the posterior end of the embryo is carried out prior to cellularization, a proportion of the microinjected DNA will be present in the cytoplasm of the pole cells, the progenitor cells of the germ line.

In practice, the DNA to be injected is comprised of two components. The first consists of a helper plasmid containing a defective P element that, although capable of producing the P transposase, which can act in *trans* on another P transposon, is itself immobile. The second component consists of a transposon construct in which the sequence to be integrated as a transgene is situated between the 31-bp P element inverted terminal repeats along with a suitable marker. The transposase produced by the helper plasmid will act on the inverted repeats of the transposon construct and facilitate the integration of the transposon into essentially random chromosomal sites of the recipient's germ

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line. Both P element biology and the characteristics of P element-mediated transformation have been reviewed extensively (for example, ref. 3). In this chapter, we will deal primarily with the technical details necessary for obtaining germ-line transformants.

1.1. An Outline of Events Involved in the Generation of Germ-Line Transformants

1. Construct the desired plasmid containing the transgene, marker, and necessary P element sequences for transposition.
2. Coinject the transposon along with a defective helper plasmid supplying the P element transposase.
3. Mate the survivors (Go) to an appropriate strain that will allow for the scoring of the marker carried on the transposon construct.
4. Select for transformed progeny that have acquired the marker carried on the transposon, and balance the transformants.
5. Test the structure and copy number of the transgene(s) in the transformant lines.
6. Choose unrearranged single insert lines for phenotypic analysis.

1.2. Choice of Transposon and Marker Gene

A large number of vectors suitable for constructing transposons have been described. We will consider here three of the more widely used ones. The transformation vectors based on *rosy* (*ry*) as a scorable marker were the first to be used. One of the most versatile versions of the *ry*-based vectors is pDM30 (4), which can be obtained from the Rubin laboratory. The major advantage of using *ry*-based vectors is that, since 1% of wild-type *ry* expression is sufficient to yield *ry*⁺ eye color, insertions into positions that result in a low level of expression can still be recovered. However, the *ry* gene is large (usually a 7.2-kb *Hind*III fragment carrying *ry* is used), and this results in a less than optimal size of vector. For example, the large size of *ry*-based vectors can make the construction of transposons more difficult and can also contribute to a decreased transformation frequency.

Another popular series of transformation vectors uses the *white* (*w*) gene as a marker (5). In the most widely used *w* vectors, a mini-*white* gene (6) with a subthreshold of *w*⁺ activity is used. There are several advantages associated with these mini-*w*-based vectors. First, the gene is small, ~4 kb, compared to *ry*. Second, since mini-*w* has subthreshold activity, for most insertions, flies that are heterozygous for mini-*w* can be distinguished from flies that are homozygous on the

basis of eye color. Finally, *w* is easier to score than *ry* when large numbers of flies are involved. The latest versions of these vectors (the *Casper* series) can be obtained from Carl Thummel or Vince Pirrotta.

A third series of vectors are those based on G418 antibiotic selection (7). In these vectors, the bacterial neomycin-resistance gene is used as a selectable marker in place of visible markers, such as *ry* and *w*. The advantage of using such vectors is that transformants can be selected on *Drosophila* food containing G418 (usually 500–1000 mg/mL), eliminating the chore of screening a large number of flies for a visible marker. However, the major disadvantage is that the window of G418 concentration, which will allow true transformants to survive, but which will reduce the leakage of nonresistant animals to an acceptable level, is narrow. Consequently, transformants resulting from insertions into chromosomal sites that yield a low level of expression will not be recovered.

Other transformation vectors, such as those based on *Adh*, which allow for selection on media containing alcohol, have also been described. In addition, a transformation vector (pCaWc) in which both the transposon and the transposase are carried on the same plasmid molecule (with the transposase located outside of the P element 31-bp repeats) has been successfully employed for obtaining transformants (8). There are also “shuttle vectors” that now exist, which greatly facilitate the construction of complex transposons. These vectors (e.g., pHSX; 8) contain large polylinkers flanked by restriction enzyme sites, like *NotI* (which occurs only very rarely and enables several DNA fragments to be assembled and then excised as one contiguous piece). The construct can then be inserted into the single *NotI* site of transformation vectors, like pDM30, or the *Casper* series. Finally, transformation vectors designed for placing genes under the control of *HSP70* and actin promoters have been described (9), in addition to transformation vectors designed to facilitate the insertion of desired sequences upstream of a *LacZ* reporter gene to drive its expression (6,9).

1.3. The Choice of Transposase Source

There exist a number of plasmids that, when injected, can provide the P element transposase necessary to mobilize the coinjected transposon. Two of the most widely used sources are pp25.7wc (wings clipped; 10) and pUCHsΔ2-3 (11). The wings clipped transposase source contains a complete 2.9-kb P element, in which the last 22 bp

have been deleted so that the element is no longer mobile. The pUCHs Δ 2-3 transposase source comprises of the engineered transposase gene (Δ 2-3), in which the intron separating the second and third exons (normally only spliced in the germ line) has been removed (11,12). This modified transposase gene is placed under the control of the *HSP70* promoter, although the constitutive expression off this promoter is of a sufficiently high level such that heat shock is not really necessary. Injecting this construct will result in the transient expression of a functional transposase in both germ-line and somatic tissues. An alternative approach to coinjecting a plasmid that provides a transposase source is to inject embryos that possess a chromosomal source of the Δ 2-3 transposase (13,14).

2. Materials

2.1. The Microinjection System

The injection apparatus we use can be seen in Fig. 1. This system consists of:

1. A Leitz micromanipulator;
2. A Nikon inverted-phase contrast microscope;
3. A vibration-free table, on which the microscope is mounted;
4. The loaded needle, containing the DNA to be injected; and
5. A collar (Narishige, Tokyo) into which the needle is placed and that is, in turn, attached to the micromanipulator.

Although the micromanipulator is used to position the needle, injection is carried out by moving the microscope stage with the embryos on it. We use an air-filled system to deliver the DNA into the embryos. This consists of a 60-mL glass syringe attached to the collar by a piece of rubber tubing (although Narishige Teflon™ tubing may also be used).

This system may appear very basic, but we find that the syringe imparts adequate control of DNA delivery without producing the problems often encountered when using a fluid-filled transmission system, and also has the advantage of being much cheaper. Injection needles are prepared from borosilicate capillaries (for example, Clark Electromedical [Reading, UK] GC100TF-15 capillaries) using a pipet puller. A relatively inexpensive two-stage vertical needle puller can be used, such as the PB-7 model from Narishige.

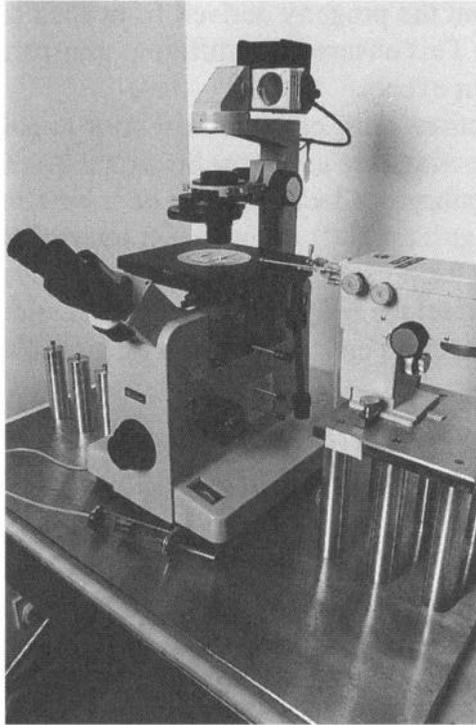


Fig. 1. The typical arrangement of the apparatus used for injection of *Drosophila* embryos.

2.2. Fly Requirements

In general, a large number of embryos (in the region of 500–1000) need to be injected for each construct in order to produce several independent transformants. In our hands, between 25 and 75% of injected embryos will hatch as larvae. Approximately 50% of the larvae will eclose as adults, and between 50 and 80% of the surviving adults will be fertile. Each surviving adult will be individually mated, and approx 200 progeny from each mating will be scored for the marker present on the transposon construct. Although the frequency with which germ-line transformants are produced varies depending on the construct injected (3), in general, on the order of 10% of the surviving adults will produce at least one germ-line transformant among its progeny. Therefore, it is reasonable to aim at obtaining about 100 adult survivors for any given construct injected. We usually collect only one

transformant from the progeny derived from each surviving adult to establish stocks. This ensures that different transformants originated from independent events.

Since the injections must be performed prior to pole-cell formation, 1-h embryo collections are used (*see* Section 3.3.). Therefore, the fly strain used for embryo collections must be robust enough to provide sufficient eggs (at least 100) during a 1-h interval. One further consideration is that the presence of defective P elements in the injection host strain can affect the frequency of transformation. Consequently, care should be taken to ensure that such elements are not present in the chosen host strain.

2.3. Miscellaneous Requirements

2.3.1. Materials Required for DNA Preparation

1. Qiagen anion-exchange columns.
2. Injection buffer: 5 mM KCl, 0.1 mM Na phosphate, pH 7.8.
3. 0.45- μ m Millipore filters.

2.3.2. Materials Required for Egg Collection and Egg Processing

1. Egg collection chamber. This can be made from open-ended plastic cylinders of any sort large enough to contain a few hundred flies. The chambers should have fine gauze placed over one end for ventilation, and once the flies have been placed into the chambers small Petri dishes containing yeast-glucose food, smeared with moist, live yeast are taped to the other end.
2. A glass or plastic tube with a Nitex gauze over one end.
3. 50% (v/v) Household bleach.
4. 0.02% (v/v) Triton X-100.
6. Black nitrocellulose filters.
7. A fine paint brush.
8. Coverslips (22 \times 40 mm).
9. A solution of Sellotape in n-heptane.
10. Voltalef oil.

3. Methods

3.1. DNA Preparation

Plasmid DNA for microinjection may be prepared either by the cesium chloride-ethidium bromide centrifugation method, or by the more convenient QIAGEN anion-exchange columns produced by

QIAGEN Inc. (Chatsworth, CA). The latter method produces clean DNA and is not only quicker, but also avoids the use of ethidium bromide and organic solvents, such as phenol and chloroform, which could potentially reduce embryonic survival rates.

The concentration of DNA for microinjection needs to be quite high (between 400 and 600 $\mu\text{g}/\text{mL}$) with “helper” plasmid, if used, at a concentration of 200 $\mu\text{g}/\text{mL}$. The DNA to be injected should be ethanol precipitated and given a 70% ethanol wash before being redissolved in injection buffer. Aliquots of 20 μL can then be stored at -20°C .

Prior to loading the DNA into injection needles, the aliquots should be heated to 65°C for 10 min to ensure that the DNA is fully dissolved and then spun through a 0.45- μm Millipore filter for a couple of minutes to remove any dust or particles that could potentially block the needle.

3.2. Needle Preparation

To obtain a needle that possesses the appropriate shape, the first-stage pull should generate a stretch with a length of about 8 mm and a diameter of approx 200 μm . The heating filament should then be moved to the center of this stretch so that the second pull produces a very fine tip of approx 2 mm in length with an end of between 1 and 5 μm in diameter. The heater settings for the first and second pull will need to be determined empirically in order to produce a good-quality needle.

Once a needle has been prepared, the simplest way to load it with the DNA solution is to add 1–2 μL of the injection DNA at the back of the capillary with a micropipet. The internal filament that runs along the length of the capillary draws the DNA solution to the front of the needle, which can now be placed into the collar of the microinjection system.

The survival of injected embryos is affected to a large extent by the sharpness of the needle. In order to obtain a sharp point, the needle can be broken at an angle against a cover slip mounted onto a glass slide. This process is visualized using the inverted-phase microscope, and is made easier by placing a drop of the Voltalef halocarbon oil on the junction between the slide and the cover slip where the needle is to be broken. When the needle breaks, a small amount of the oil can usually be seen to enter the tip. The flow of DNA can now be tested by applying a little pressure to the syringe. The needle is now ready to use for microinjection.

In between injecting embryos, the needle can be lowered into a small (5 cm) Petri dish lid containing Voltalef halocarbon oil. This helps prevent evaporation of the DNA solution and the concomitant clogging of the needle, which can otherwise occur.

3.3. Egg Collection

Synchronous and abundant batches of eggs are required for injections. In general, 300–600 adults will produce enough eggs for a few days of microinjections. The flies should be transferred into collection chambers. To optimize egg laying, the flies should be kept at 25°C for a further 2 d in the chambers before starting egg collections for injection, with the Petri dishes containing the food being changed every day. At the end of the second day, and every subsequent day, the flies should be transferred to 18°C overnight and then returned to 25°C on the morning of collection. The first hour's collection should be discarded, since female flies tend to retain eggs until fresh food is supplied. Thereafter, at 60-min intervals, the collection plates can be removed and replaced with new ones.

The eggs to be injected are washed off the collection plates with distilled water, and passed down a glass or plastic tube that contains a nitex gauze over one end to retain the embryos. The eggs are now ready for dechoriation.

3.4. Preparation of Embryos for Microinjection

1. The first step in preparing the eggs for microinjection requires the removal of the tough outer chorion (*see* Note 1). Chemical dechoriation involves placing the tube with nitex gauze and embryos into a beaker containing 10 mL of a 50% solution of household bleach. The beaker and tube are gently shaken and, after 2–2.5 min of dechoriation, the bleach is diluted by the addition of an equal vol of a 0.02% Triton X-100 solution. The tube is then removed from the beaker, and the eggs thoroughly washed with distilled water.
2. The embryos are then transferred onto a black nitrocellulose filter with a fine paint brush and then lined up along one of the ruled lines on the filter in such a way that the micropile is nearest to the operator. It is important to keep the filter damp to prevent the eggs from drying out.
3. When 50–60 embryos have been lined up, they are transferred to a 22 × 40 mm cover slip, which can be made adhesive by the prior application of a solution of Sellotape in n-heptane. The cover slip with attached embryos is then stuck onto a microscope slide using a small drop of Voltalef oil.

and a little pressure. The whole slide is then placed inside an air-tight box containing silica gel in order to desiccate the embryos (*see* Note 2).

4. At the end of the desiccation period, the eggs are taken out of the box containing the silica gel and covered with a layer of Voltalef oil. This oil, although being oxygen permeable, is water impermeable and therefore prevents any further desiccation of the embryos. The embryos are now ready to be injected.

3.5. Microinjection of *Drosophila* Embryos

1. Once the needle is lifted safely out of the way, the slide containing the embryos is placed on the microscope stage, so that the eggs have their posterior facing the needle. The micromanipulator is then used to bring the needle into the same plane as the line of eggs.
2. The tip of the needle should be brought level with the center of the first egg, and this is gaged by running the very end of the needle up and down the edge of the embryo. This ensures that the needle will not slide over the surface of the egg and will also help decrease the amount of damage caused to the embryo. The embryo is then moved toward the needle with a purposeful motion, so that the vitelline membrane is just penetrated. The needle should then be drawn back so that the tip is only just within the cytoplasm. Most of the embryos to be injected will be in the early cleavage stage (15 min to 1 h 20 min), and will have a space between the posterior pole and the vitelline membrane. It is important that the needle is inserted through the space, and that the DNA is deposited in the posterior pole of the embryo proper. It is here, at the posterior pole, that the germ line will be formed. The embryo is then injected with a quantity of DNA solution equivalent to approx 1% of the egg's total volume, and the needle removed. The procedure is then repeated until all the embryos have been injected (*see* Note 3).
3. Any embryos in which pole cell formation has already taken place are killed by being run through with the needle and are not counted among those eggs that have been successfully injected.
4. The cover slip containing the injected embryos is removed from the slide and placed onto a flat yeast-glucose-charcoal plate. A further thin layer of Voltalef oil is then applied to the line of embryos, and the plate placed into a box kept humid by damp tissues. The box is then placed on a level surface in an 18°C incubator for 48 h. If the plate is not kept level, the Voltalef oil will run off, and the embryos will overdesiccate and die.
5. After this time, hatched larvae are counted, then transferred into vials containing fly food, and returned to the 18°C incubator to develop. The

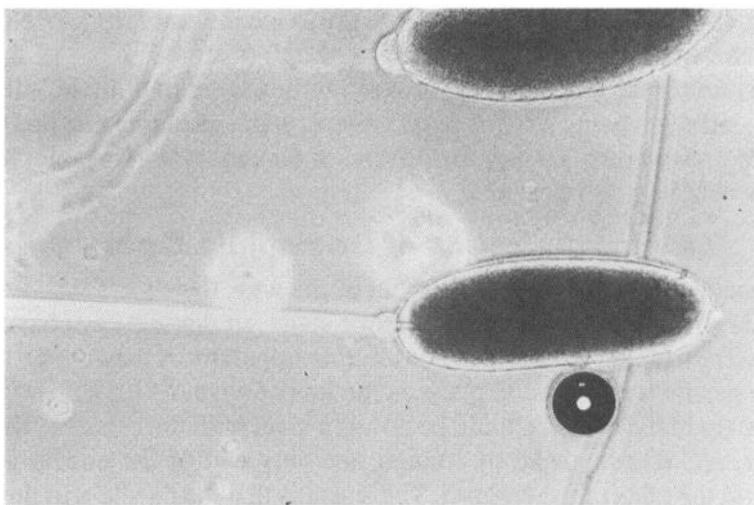


Fig. 2. The microinjection of *Drosophila* embryos illustrating the region of the embryo targeted for injection. Note also the "bubble" of cytoplasmic material leaking from the embryo, which should be removed.

percentage survival to first instar larvae can be determined by dividing the number of survivors by the number of successfully injected embryos.

4. Notes

1. There are two methods of dechoriation that can be employed: chemical and mechanical. However, we favor the chemical method, since it is far easier and less time-consuming.
2. This stage is of vital importance if the embryos are to withstand being punctured and accommodate the volume of DNA being introduced. Moreover, this step of the procedure is also probably the most crucial, in terms of survival rates, since there is only a narrow margin between a sufficient reduction of egg turgor and excessive drying, which kills the embryos. If possible, preparation of embryos should be carried out in an environment with constant temperature and humidity conditions, since this will facilitate the determination of the optimum desiccation time. However, if this is not possible, the experimenter will have to determine the desiccation time empirically, because this will tend to fluctuate depending on the climatic conditions. As a starting point, we have generally used desiccation times of between 10 and 15 min.

3. If the embryo has not been desiccated enough, or if too much DNA solution has been injected, then cytoplasm may leak out of the egg, reducing its chances of survival (see Fig. 2). We have found that increased survival rates can be achieved by removing the "bubbles" of cytoplasm. This is easily carried out by having a constant flow of DNA coming out of the needle, which is then brushed passed the line of embryos.

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CHAPTER 7

Gene Transfer by Microinjection in the Zebra Fish *Brachydanio rerio*

*Hong-Woo Khoo, Lay-Hong Ang,
and Hueh-Bin Lim*

1. Introduction

Although transgenesis studies of fish started relatively later compared to those of mammals, they have not been completely neglected, and in recent years, many publications have appeared in this field. Reviews of transgenesis in fish are given by Maclean et al. (1), Hew (2), Maclean and Penman (3), and Ozato et al. (4). Much attention had been focused on commercially important food species, especially in the attempt to insert beneficial genes, such as the antifreeze and growth-hormone genes, into salmonids, such as trout and salmon. Zhang et al. (5) have successfully shown transfer, expression, and inheritance of rainbow trout growth-hormone cDNA microinjected into the common carp.

The use of aquarium fish, such as zebra fish (*Brachydanio rerio*) for transgenic studies is advantageous in that many eggs can be produced daily on a year-round basis (6). In vitro fertilization of the zebra fish is easily conducted. Also, the rapid development time of the embryos, within 3–4 d into free-swimming fry and their short spawning cycle (maturing within 4 mo) allowed for faster experiments (7). The technique of microinjection in zebra fish will be described here.

Germ-line transformation of the zebra fish by microinjection of naked DNA was first conducted by Stuart et al. (6,8). DNA is micro-

injected into the cytoplasm, as used in other species by Chourrout et al. (9) and Guyomard et al. (10), of one-celled embryos instead of the more tedious methods of microinjection of naked DNA directly into the oocyte nuclei as practiced for the medaka *Oryzias latipes* (4) or through the micropyle of the egg (11). Sources of information on breeding and rearing conditions, egg production, spawning cycle, egg collection, and embryo development of zebra fish are described by Roosen-Runge (12), Hisaoka and Battle (13), Hisaoka and Firlit (14,15), Laale and McCallion (16), Schirone and Gross (17), Eaton and Farley (18), and Laale (19).

2. Materials

1. Suitable aquarium facilities (see refs. 12–19).
2. Wide-mouthed Pasteur pipets.
3. Injection chambers: These are made by punching holes into a layer of agarose contained in a 3.5-mm diameter disposable plastic Petri dish. Fill the plastic Petri dish with 1.5 mL of 2% (w/v) molten agarose, and let it harden. Puncture the solidified agarose gel with the tip of a glass Pasteur pipet with orifices of about 1.9–2.0 mm in external diameter, and remove the central cut-out gels by aspirating with a Pasteur pipet attached to a vacuum setup, or remove them by lifting them out with a pin. Flood the injection chamber with embryo fluid
4. Embryo fluid: 17 μM NaCl, 0.4 μM KCl, 0.48 μM CaCl₂, 0.66 μM MgSO₄, and 1 $\mu\text{g}/\text{mL}$ methylene blue.
5. DNA solution: 5 μL of the DNA to be microinjected as at concentration of 2 mg/mL is diluted with 95 μL of 0.1M Tris-HCl, pH 7.5, and 0.25% phenol red, to give a final DNA concentration of 100 $\mu\text{g}/\text{mL}$. The dye facilitates the estimation of the volume of DNA injected.
6. The microinjection system consists of a three-dimensional micromanipulator, a micropipet holder holding the micropipet, a binocular microscope, and an automatic microinjector (Fig. 1). More details on the use of micromanipulators and micromanipulation are given by El-Badry (20).
7. Microinjection pipets: Pull fine glass capillaries (Drummond Scientific Co., Broomall, PA), 100 μL vol, 1.4 mm od, and 0.9 mm id, with a micropipet puller (vertical micropipet puller—type PE-2, Narishige Scientific Instrument Lab, Japan, or any equivalent). The tip of the micropipet should be about 0.05 mm. If it is too fine, it will not penetrate the chorion. Just before microinjection, break the micropipet tip with a blunt forcep to produce a sharp pipet tip, which is required to penetrate the egg chorion.

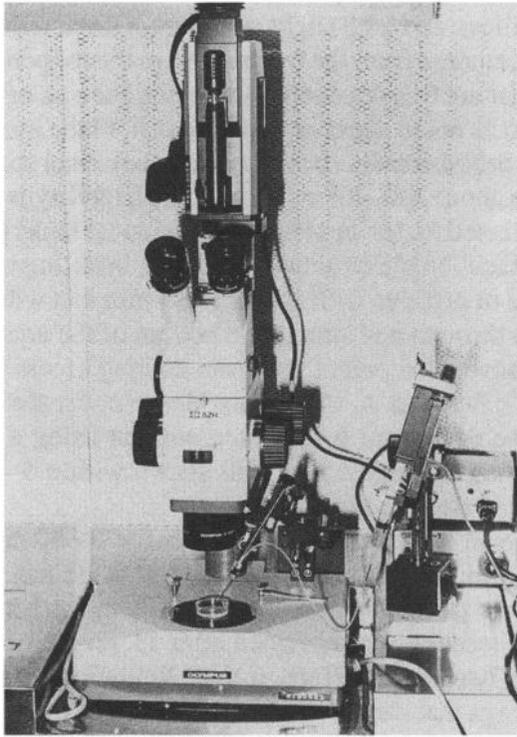


Fig. 1. Microinjection setup showing the three-dimensional micromanipulator, the micropipet holder, the microinjector, the binocular microscope, and the optional video camera attachment.

3. Methods

3.1. Preparation of Eggs

1. Maintain broodstock zebra fish in clear plastic aquariums (20 L) containing 10–15 adults aged about 3–4 mo in each tank. Use tap water that has been aerated and kept overnight to remove the chlorine.
2. Spontaneously spawn the zebra fish, and collect the fertilized eggs at the one-celled stage prior to the first cleavage, as follows. Prepare the broodstocks for spawning by feeding with frozen “bloodworms” and live tubifex for a week prior to spawning. The temperature of the water in the aquariums should be maintained between 25 and 32°C. The light regime is 12 h light and 12 h dark.
3. Select ripe females from the stock tanks the evening (4 to 5 PM) before microinjection is planned, and place them in a tank of fresh, standing

water with some aquatic plants. Select suitable gravid females (with enlarged bellies), and with slight pressure on the abdomen, examine the oocytes that emerge from the oviduct out to the ovipore. Select females with eggs that are translucent. Examination for ripe eggs must be swift and gentle so as not to stress or harm the fish. In the morning, more than 80% of the selected females should be ready for natural spawning. A female can produce about 200–400 eggs, and fertilizability is usually high.

4. Keep the selected males and females in separate tanks prior to fertilization. Place each female with two males in a breeding trap composed of an enclosure of nettings with apertures >1 mm that will allow fertilized eggs to pass through and sink to the bottom of the aquarium, but small enough to prevent the parent fish from gaining access to and eating the eggs (Fig. 2). Stuart et al. (6) use marbles to cover the floor of the tank to protect the eggs from being eaten without using a breeding enclosure. If the fish are ready, they will spawn within 5–10 min after the opposite sexes are placed together.
5. The eggs persist in the one-cell stage only for 10–30 min after fertilization. Hence, microinjection at the one-cell stage has to be conducted within this time period. Spawn the fish during the early morning between 7 and 9 AM, because eggs spawned after 11 AM are often overripe and not viable. There is, therefore, a time limitation that determines the number of eggs that can be microinjected per day by one operator.
6. Collect the recently fertilized and water-hardened eggs with wide-mouthed pipeters or droppers, and carefully arrange the eggs into the holes of a preferred injection chamber. Each egg is rolled into and pressed lightly into a hole, where it will be held firmly and will not roll around when it is injected.

3.2. Injection of Zebra Fish Eggs

1. Fill the microinjection pipet with DNA solution (*see* Section 2.). Place the pipet tip into about 50 μ L of DNA solution on wax paper, and suck up into the pipet using the suction of a 6-mL syringe.
2. Inject the DNA using a continuously flowing pipet maintained with air pressure by an electrically driven automatic microinjector (Fig. 3), which maintains the pressure while injection is conducted. Precalibrate the amount injected by counting the number of seconds that the needle stays in the egg before withdrawing it. Inject the eggs at the one-celled stage, 10–35 min after fertilization. Inject about 10 nL DNA (about 1 ng).
3. Injection can be done through the tough chorion and directly into the germinal disk. Quite often, the germinal disk tends to rotate away. Another

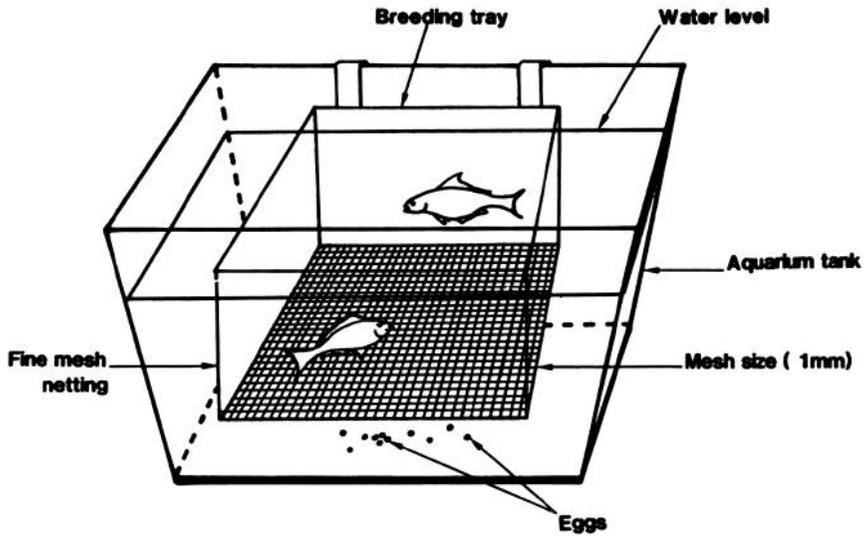


Fig. 2. Breeding setup for collection of spawned and fertilized eggs.

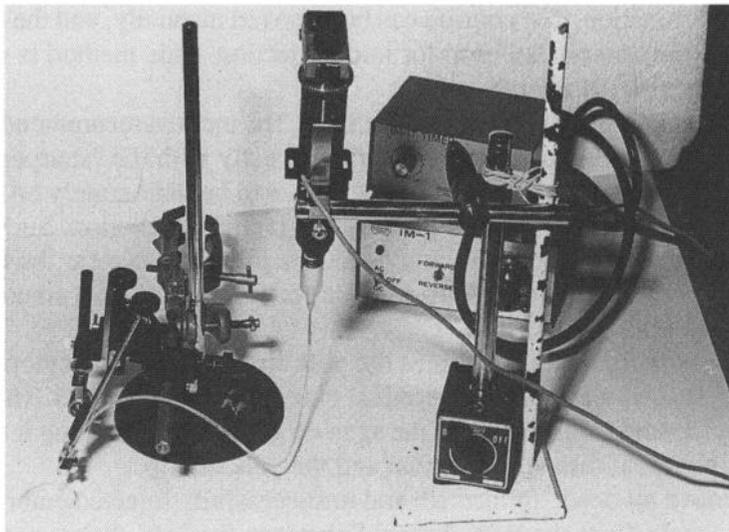


Fig. 3. Close-up of microinjection setup.

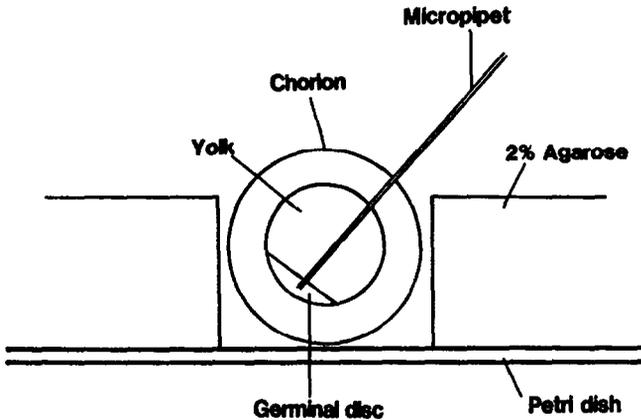


Fig. 4. Micromjection through chorion and yolk into the cytoplasm of germinal disc.

approach is to inject through the yolk and then into the cytoplasm of the germinal disc by placing the egg with the germinal disc diametrically opposite the point of entry of the micropipet into the yolk (Fig. 4). About 150 eggs could be injected in this manner in each session. One of the problems of direct injection through the chorion is that the micropipet tends to break because of the tough chorion, and this can be overcome by dechoriation. The chorion can be removed manually, and the embryo placed in embryo solution for microinjection. This method is used by Stuart et al. (6) for zebra fish.

Dechoriation is time-consuming. The method recommended here for zebra fish is to pierce the chorion directly with the "sharpened" tip of the micropipet. The micropipet tip has to be deliberately broken, so that a jagged sharp edge is produced to pierce the chorion. Such deliberate breakage is made to produce a sharp tip and to clear the clogged section of the micropipet tip when it occurs to maintain a smooth flow of DNA during injection.

The micropipet penetrates the yolk first and then the cytoplasm of the one-cell blastodisk or germinal disc from the yolky end. The egg is preprepared for injection in the agarose gel wells by rotating it to place the germinal disc at the bottom and the yolk on top.

- 4 Remove all dead, uninjected, and unsuccessfully injected embryos immediately from the Petri dishes. Submerge the Petri dishes containing the remainder of the injected embryos in a small aquarium containing dechlorinated tap water, and add methylene blue to inhibit bacterial and fungal growth. The surviving eggs hatch in 3 d.

5. Rearing of embryo to adult: Rear the embryos in small aquariums (14 × 14 × 25 cm) for the first month (*see* Note 1).

4. Notes

1. Injected embryos that develop to the neurula stage will normally complete their development to fry stage. During the first 2 wk after hatching, the fries can be fed with egg yolk or Liquifry (Intervet Ltd., UK). *Artemia nauplius* (brine shrimp) and large solid food should not be given during the first 2 wk after hatching, but can be given after that period for a month. When the fish are larger, feed them with *Moina sp.* and live tubifex worms to speed up their growth and maturation in 2–3 mo. Frys fed on fish flakes alone will take about 4–5 mo to reach maturity. Change tank water once every 2–3 wk to minimize infection.
2. Potentially transgenic fish can be biopsied at a number of developmental stages. For embryos, remove from the aquarium, rinse in PBS, and store in Eppendorf tubes at -70°C . Collect free swimming zebra fish fries, after the yolk sac has been absorbed and immediately after the fry has become free swimming (96 h after fertilization). Store them singly in Eppendorf tubes at -70°C after rinsing in PBS. For adult zebra fish, remove one of the pectoral fins by cutting near the base of the pectoral, rinse in PBS, and store in separate Eppendorf tubes at -70°C . DNA extraction and Southern analysis are as described in Chapters 34 and 37, respectively.

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CHAPTER 8

Transgenic Induction in *Salmonid* and *Tilapia* Fish

Norman Maclean

1. Introduction

Why produce transgenic fish? There are two chief reasons for introducing novel genes into animals. The first is as a means of increasing knowledge of gene regulation in that particular group of organisms. The second is that transgenic induction may involve some economic benefit from the modified organism in terms of its increased growth potential, disease resistance, or other desirable genetic trait. Both reasons are of importance in the context of transgenic fish. Fish are good candidates for transgenic induction for several reasons. They lay large numbers of eggs, and both fertilization and development are external to the body of the female (except in a few species of mouth-brooding fish and ovoviviparous species, such as the guppy [*Poecilia*]). Also, the eggs are usually quite large and may be readily pierced by a suitable glass needle.

A third reason is that the piscine genome is readily manipulated, since no mechanism of gender imprinting of genes, such as occurs in mammals, has been observed in fish. Thus, gynogenetic manipulation is fairly straightforward. A further plus for *Salmonid* fish is that, since they are intensively and extensively farmed, eggs and milt are widely available, at least for certain periods of the year. Additionally, some trout farms have brood stock that are maintained in conditions of artificially controlled day length, thereby shifting the onset of sexual maturity and allowing egg and milt production over periods of the

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year not normally covered by the breeding cycle. Thus, rainbow trout eggs are available from some farms for up to 9 mo of the year. *Tilapia* species are widely cultured as food fish in Asia, Africa, and Israel, and are easy to culture in the laboratory. They mature in 4–6 mo in farm conditions, and females lay eggs every 3–4 wk. Transgenic induction in fish is, however, not without some difficulties. Eggs of some fish species are enclosed within a very stout chorion, and this may prove hard to pierce with a small microneedle. A more major objection is the long generation time of many fish species. Under normal laboratory conditions, this is likely to be at least 2 yr in the rainbow trout and perhaps 9 mo in *Tilapia* species. Some small species, such as zebra fish (Chapter 7) and medaka, have shorter generation times, but offer no economic dimension to the experimental program.

1.1. Methods of DNA Introduction

The introduction of novel cloned DNA sequences into fertilized eggs can be achieved by a range of methods. They are as follows:

1. Introduction of the novel sequences by transfection into totipotent cells; Cell lines can then be isolated that are positive with respect to the novel sequence; that is, they have incorporated one or more copies into the chromosomal DNA. Nuclei from such transgenic cells can then be microinjected into fertilized eggs, following destruction or removal of the egg nucleus. This method has not been attempted with fish eggs.
2. Mixing of fertilized eggs with a solution containing many copies of the novel gene sequence, followed by electroporation to facilitate entry of some of the DNA molecules into the eggs: This method has been used with some success in fish by Inoue et al. (1)
3. Mixing of multiple copies of the chosen DNA sequence with sperm, followed by fertilization of eggs with such sperm, so using the sperm as a carrier system: Although the method has been employed with both sea urchins (2) and (controversially) mice (3), it has not to my knowledge been successfully used with fish eggs.
4. Microinjection of a concentrated solution of DNA into the fertilized eggs by glass microneedles: Most work with transgenic fish has involved this approach, and this is the method that will now be discussed in detail.

One of the problems with attempting the injection of DNA solutions into fish eggs is that it is not normally possible to visualize the egg nucleus. Since the chromosomes within the nucleus are the prime target for the DNA, this is clearly an unfortunate state of affairs. Although

one group of workers has successfully introduced DNA solution into the enlarged 4C nuclei of fish oocytes (4), all other groups, including my own, have chosen to inject into the egg cytoplasm in the vicinity of the nucleus. Presumably, some DNA may then enter the nucleus by passage through the nuclear envelope, or more probably, at the following mitotic divisions, when the nuclear membranes are temporarily dissolved, some of the injected copies will pass into the nucleoplasm and gain access to the chromosomes. The injection into cytoplasm seems to require the introduction of larger concentrations of DNA than is used, for example, for injection of mammalian eggs, where the nucleus can be directly targeted.

It is crucial to position the DNA as near to the nucleus as possible within the egg. This means that the correct end of the eggs must be identified, and that the needle must pierce the thick chorion (or pass down the micropyle), pass through the egg membrane, and stop in the superficial layer of cytoplasm overlying the abundant yolk. If the needle reaches the yolk, not only will injection be futile, but the needle will almost certainly be blocked and will have to be rejected.

Another important consideration is the age or developmental stage of the eggs. Fertilized eggs rapidly undergo cell division, and incorporation of DNA after a few rounds of cell division will result in transgene mosaicism, sometimes with the result that no copies of the transgene are in crucial tissues, such as gonad. Mosaicism of some degree, especially of multiple copies being integrated at distinct times after the initiation of cell division, is commonplace with fish eggs, especially with species where development is very rapid, e.g., zebra fish (5). We do not yet know how serious the mosaicism problem will be with *Tilapia*, but the work of Brem et al. (6) indicates that it is a likely outcome; a majority of the transgenic rainbow trout produced in our laboratory prove to be transgenic in all tissues, but also mosaic in some. For these reasons, egg injection should take place as soon after fertilization as possible, although in *Salmonids*, since delayed water hardening will also delay development, fertilized eggs can be stored for up to 3 d prior to injection.

1.2. Future Uses of Transgenic Fish

Fish are good candidates for transgenic studies. It now seems clear that our knowledge of fish genes and fish gene regulation will rapidly advance with the increased use of fish gene libraries and trans-

genic induction with sequences derived from them. It also seems probable that, in the near future, transgenic fish with improved disease resistance, delayed sexual maturity, or increased growth rate will make a significant and valuable contribution to commercial aquaculture.

2. Materials

1. Suitable aquariums.
2. Fish eggs.

Rainbow Trout: These fish naturally spawn in autumn and winter, and the strains kept in most fish farms will be sexually active in October and November. Sexual maturity is determined by the onset of breeding coloration, especially in cock fish, and by the fact that when fish are handled and gently squeezed along the sides of the belly, eggs or milt are released. The hen fish should not be allowed to become overripe, or egg viability will be low. Since, as will be discussed later, it is advantageous to avoid water hardening of *Salmonid* eggs, the fish should be netted, lightly anesthetized as necessary by a 5-min incubation in 0.04 mg/L ethyl-aminobenzoate, dried with a cloth, and then stripped of eggs or milt into a clean, dry receptacle. The container should be closed to prevent evaporation. The eggs can be fertilized directly by mixing the eggs and milt in the ovarian fluid, and the fertilized eggs may be kept for up to 3 d at 4°C. Milt should be checked for good sperm motility and may also be stored for up to 24 h at 4°C if well oxygenated, although its viability is less than that of the eggs.

Tilapia: *Tilapia* species used in our research laboratory is *Oreochromis niloticus*. Since this is a very aggressive species, mature males and females have to be kept separately. They should be maintained in glass aquariums in pairs, but with a transparent plastic divider to separate the pairs and prevent the males from attacking or even killing the females. Nest building will be evident if a 4-cm layer of pebbles is placed on the bottom of the aquarium. When both have built nests, the female should be watched closely for signs of egg laying. Whenever this occurs, both fish should be removed and stripped (no anesthesia is necessary). We normally fertilize and water harden the *Tilapia* eggs immediately.

3. Injection needles: Much of the efficiency of transgenic fish production depends on the production of good needles. Those used for trout egg injection are made from sealed capillary tubes (244-831) bought from Gallenkamp Ltd., Belton Park West, Loughborough LE11 0TR, UK. These are sterile inside the sealed tube, and are 10-cm long and 1.5-mm external diameter. Such needles are hand pulled in a low Bunsen burner flame to provide a parallel sided length of tubing with an inner diameter of

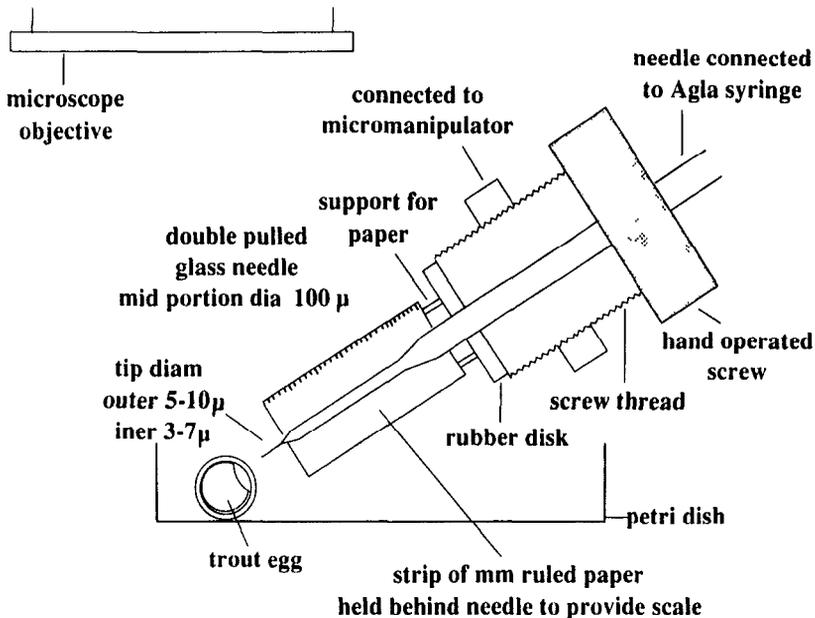


Fig. 1. Diagram of needle and microinjection apparatus used for injection of novel genes into *Salmonid* eggs.

about 100 mm. As seen in Fig. 1, this is the area of tubing used for volume calibration. Following this initial diameter reduction, the needles are then subjected to a second reduction of the tip section. This is achieved, essentially on a trial-and-error basis, by hanging each double-ended needle in a clamp, with a plasticine weight attached to the opposite end, and the narrowed 100- μ section passing through a 1-cm diameter heated metal coil. When the needle is in place, a current is passed through the coil via a rheostat, so that the metal coil glows red. This results in melting of the glass and the falling under weight of the terminal portion of the tubing. Although this might be thought to provide two usable needles from one length of capillary tube, in practice, only the lower needle is used. The tip diameter of this needle should be 5–10 μ on the outside (approx 3–7 μ id). The long tip of the needles can be usefully broken off to leave a sharp angle and projecting about 1 cm from the volume calibration area. Such needles should be sterilized on their outer surfaces by uv light and stored in a box on plasticine mounts prior to use.

Tilapia needles for *Tilapia* egg injection are derived from a different type of glass tubing than those used for trout egg injection. They are

microelectrode tubes, available from Plowden & Thompson Ltd., Dial Glass Works, Stowbridge, West Midlands, DY8 4YN, UK. They come as 10-cm long tubes with an outer diameter of 1 mm and an inner bore of 0.58-mm diameter. A long solid glass fiber is attached continuously to one part of the inner bore of the tubes, thereby facilitating loading of the needle with solution from the bottom end. They are subjected to a single pull in an automatic pipet puller (C. F. Palmer Ltd., London), to give a tip diameter of 2–3 μ od (1–2 μ id). Since no calibration area of the needle is available, the volume injected must be determined by measuring volumes of drops expressed by a needle under particular time/pressure constants or by arithmetic calculation given the geometry of the needle tip

4. 4.5 cm² Plastic weighing boat.
5. Small hair loop or a stout horse hair or a fine wire loop.
6. Stereomicroscope
7. Micromanipulator
8. Agla micrometer syringe (Wellcome Beckenham, UK).
9. Nylon tubing.
10. Liquid paraffin.
11. DNA for injection: The DNA for injection should be linear rather than circular, and it is best to cut out the cloning vector DNA and inject only copies of the inserted cloned gene. It now seems that the choice of regulatory elements is very important if appropriate transgene expression is to be achieved. In particular, in most laboratories, gene constructs in which structural genes are driven by mammalian promoters are expressed aberrantly or not at all in fish cells and tissues (7–9). To achieve satisfactory expression, either fish derived or viral promoters should be used. Maximal expression is likely to depend on the inclusion of appropriate enhancer sequences in the construct (10). Approximately 10⁶ copies of the DNA fragment are introduced into the fish egg in 18 μ L of a solution of NaCl-Tris buffer (88 mM NaCl, 10 mM Tris-HCl, pH 7.5).

3. Methods

3.1. Trout Eggs

1. When used for injection of trout eggs, each needle is calibrated by careful measurement of the internal diameter of the calibration chamber by means of a stage micrometer. When the needle is mounted in the chuck of the micromanipulator, a length of millimeter ruled graph paper is held behind it, so that the column of liquid can be visualized and the movement of its meniscus noted against the millimeter scale. Trout eggs are routinely injected with 10⁶ copies of the chosen DNA sequence, nor-

- mally in 18 nL of NaCl-Tris buffer (88 mM NaCl, 10 mM Tris-HCl, pH. 7.5). The DNA copy number in solution may be determined in $\mu\text{g}/\text{mL}$ by using Avogadro's number and the known size of the DNA construct.
2. In order to permit injection, a chosen precalibrated sterile needle is mounted in a chuck clamp attached to a Prior micromanipulator. The thick end of the needle is linked via a length of flexible nylon tubing to an Agla syringe. The nylon tubing is filled with sterile liquid paraffin to transmit hydraulic pressure from the syringe barrel to the DNA solution in the needle. An air space of 2–5 mm is left between the DNA solution and the liquid paraffin, but too long an air gap results in excessive backlash and imprecise injection. The DNA solution is loaded into the needle after the needle has been linked to the Agla syringe and mounted in the micromanipulator. To do this, the needle tip is introduced into a droplet of the DNA solution in a small sterile weighing boat, and the Agla syringe used to draw up about 100 nL into the needle, leaving the appropriate air gap between the DNA solution and the column of liquid paraffin.
 3. Fertilized and recently water-hardened trout eggs should now be available, held at approx 5°C . Water hardening some 5 min before injection enables the micropyle area of the egg to be more readily recognized by the gathering of the oil droplets. A small square plastic weighing boat of 4-1/2 cm square is used to hold the eggs. The weighing boat is fitted with an insert of filter paper cut to shape as shown in Fig. 2, the eggs being held in the corners of the boat. The boat and filter paper should be presterilized by UV, and the paper surface wetted with sterile water to prevent drying out of the egg. Four eggs can now be placed in the weighing boat. They are best lifted by use of a small hair loop, using a stout horse hair or even a fine wire loop of a diameter less than the diameter of the egg. Under a suitable illuminated stereomicroscope, the egg should then be manipulated by means of the loop and movements of the boat, so that the micropyle area of the egg (recognized by a shallow depression in the chorion surface) is facing up at an angle of approx 45° toward the needle. Although large trout eggs may have a visible micropyle (as do eggs of atlantic salmon, *Salmo salar*), in our experience, it is not usually possible to locate precisely the micropyle of rainbow trout eggs. This implies that the needle must simply pierce the chorion (in the area of gathering lipid droplets) in order to enter the cytoplasm below. Since the chorion of the *Salmonid* egg hardens in water, fertilization in ovarian fluid without water prevents hardening. Only when the egg is placed in water some 5 min prior to injection does hardening actually begin. However, the chorion will remain relatively plastic for some minutes, and during that time, injection can be effected

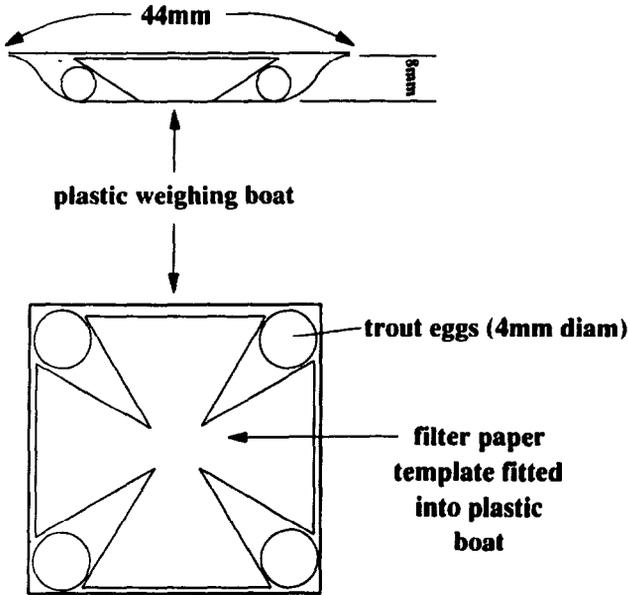


Fig. 2. Diagram of modified plastic weighing boat, filter paper, and *Salmonid* eggs, as used in egg injection procedure.

4. The needle tip, now loaded with the DNA solution, should be brought up to the chorion surface by micromanipulation, so that the end of the needle causes a dimple in the egg in the micropyle region. If the manipulator is then given a gentle tap, the needle should pierce the chorion and enter the cytoplasm below. Rotation of the micrometer screw on the Agla should now result in the injection of the DNA solution, the volume injected being determined by the movement of the meniscus in the calibration area. The needle is withdrawn following injection, and another egg lined up for the next injection. Four eggs can be water hardened as a batch, and, when these are all injected, another four can be water hardened in turn.

Some trial and error is needed with needles. If the needle point is too fine, it will simply bend rather than enter the chorion. If it is too wide, it will enter the egg under extreme pressure only, but yolk may leak out of the hole, and the viability of such damaged eggs is very poor. Needles must be sharp and fine, but resistant to bending, which means that the fine terminal section should not be too long and should taper gently up to the tip itself. A good needle may be used to inject up to 20 eggs before it becomes damaged or blocked, and must be dis-

carded. With experience, more than 20 trout eggs can be injected in an hour, and an experienced injector can do up to 100/d.

5. Within minutes of injection, eggs should be placed in clean well-oxygenated water and shielded from strong light. They are best left undisturbed for a period of at least 1 h since eggs are extremely sensitive to shaking for the first 30–60 min after introduction to water. Rainbow trout egg should be kept in running water at a temperature close to 12°C. If the temperature is significantly <12°C, development will be prolonged beyond 28 d to hatching, whereas if it is significantly higher, the viability of the eggs will be reduced. Eggs in my laboratory are maintained in dechlorinated mains water with aeration, but recirculation systems can also be employed. The eggs are rather susceptible to infection with *Saprolegnia* fungus, and should be exposed once per day to a dose of malachite green solution (zinc-free malachite green in a 1:500,000 dilution in water for 1 h). Over the first 2 wk of development, trout eggs should be carefully watched, and dead eggs removed. If injection is causing a reduction in viability, the problems are most likely to arise within the first 10 d. Hatching of fry at 12°C should occur in about 28 d. Fry will require feeding a few days after hatching as the yolk sac is gradually absorbed.

3.2. Tilapia

1. *Tilapia* eggs are substantially smaller than trout eggs, but have a conspicuous micropyle and can be readily injected by the needle passing down the micropyle. Since needles for *Tilapia* egg injection are not double-drawn, there is no volume calibration region, but the tip region is subject to the same considerations as mentioned in the context of trout egg injection; that is, it must be sharp, fine, and strong. Since *Tilapia* eggs are not injected directly through the chorion, there is no reason to avoid water hardening. Thus, the eggs should be available in sterile water immediately after fertilization. The needle is mounted as before in a micromanipulator and linked to an Agla syringe by a length of air-filled nylon tubing. However, the egg can usefully be held in a device consisting of a short piece of nylon tubing opened out into a short funnel at one end. This funnel should be large enough to accommodate part of the egg, but the tube diameter should be small enough to prevent movement of the egg into the bore of the tube. A short rubber tube with a mouth piece can then be fitted to this “egg restrainer,” and the restraining tube attached to a micromanipulator arm. The tube and funnel are placed under the water surface of a Petri dish, and the eggs are injected under water. This allows a single *Tilapia* egg to be placed

in the funnel and manipulated by a hair loop, so that the micropyle is offered up to the needle tip. Slight suction on the rubber attached to the funnel (by mouth) will ensure that the egg remains in place while being injected. It also ensures that the egg will be separated easily from the needle after injection has been completed.

2. In *Oreochromis niloticus*, eggs are naturally incubated in the buccal cavity of the female fish. Since injected eggs have to be kept in separate batches, it is necessary to find an alternative incubation method. A frequently used and successful method is to keep eggs in glass or plastic funnels, immersed in tanks of water, and through which a current of water is pumped from below. This current is regulated so that the eggs or embryos are constantly agitated, tumbling gently within the funnel. Antibiotic (Streptomycin sulfate 0.05 mg/mL and penicillin G, 50 IU/mL) should also be added to the water. Under these conditions, and if maintained at an optimal temperature of 25/26°C, the embryos can be removed from the funnels some 4–5 d after hatching, and feeding can commence when the yolk sac has been absorbed.

4. Notes

1. Since fish eggs have nuclei that cannot be readily visualized under a dissecting microscope, it is necessary to inject DNA into the cytoplasm. This fact probably accounts for the high number of copies of the transgene that must be injected in order to achieve a reasonable percentage of transgenic fish. In my laboratory, we achieve ratios of 15% of all fish hatching from injected eggs proving transgenic in blood, following injection with 10^6 copies of the transgene into trout eggs (11).
2. Fish tagging: Since some fish must be assayed for transgenism by DNA purified from biopsy material, it is necessary to identify individual fish and be able to select known transgenics for breeding purposes. In case of *Tilapia*, fish can be identified by fin clipping, and the shorter generation times mean that the tagging system need not last for so long. We have used two alternative tagging systems for rainbow trout. In the one, VI tags obtainable from Northwest Marine Technology Inc. are inserted with a special tool into the transparent cartilage above the eye socket. Since each plastic tag is separately numbered, each fish can be identified. These tags must be inserted while the fish is anesthetized with benzocaine (ethyl-amino benzoate). The tags are permanent, but often become partially illegible with older fish. A better, but more expensive system of tagging involves the use of PIT tags (Fisheagle Ltd, c/o J. Taylor, Little Faringdon Mill, Lechdale, Gloucestershire GL7 3QQ). These are glass-encased transponders that are placed in the body cavity

of each fish, under anesthesia, using a special applicator. The hand-held sensor can be used to locate and identify individual tagged fish within a range of approx 1 m. These tags work effectively and are virtually immortal. The fish tolerate them well, but they are somewhat expensive and require skill to insert. They may be reused if recovered from the carcass of a tagged fish.

3. In any set of transgenic experiments, an early goal is the acquisition of evidence that one or more copies of the transgene are incorporated into the chromosomal DNA of the fish. Appropriate assays can be carried out at an early stage of development following egg injection by sacrificing a proportion of the fish for DNA extraction. Alternatively, fish can be grown on, tagged, and DNA prepared from a biopsy sample.

If small fish are to be sacrificed, each individual fish can be dissected, its skin, eyes, and internal organs removed, and the remaining tissues homogenized in a Tris, CaCl₂, SDS solution (500 μ L of 1 mM Tris-HCl, pH 8, 10 mM EDTA, 250 mM NaCl, 1% sodium dodecyl sulfate, and 15 μ L of 10 mg/mL proteinase K), and incubated overnight at 37°C. This is followed by conventional phenol/chloroform and chloroform/isoamyl alcohol extraction and the DNA purified from each (see Chapter 34).

When older fish are to be biopsied, blood is the best source of DNA, and in the experience of my own laboratory, the transgenic status of blood cells is a good indicator of total transgenism. Blood can be withdrawn from the dorsal vein following anesthesia, and DNA prepared from the blood cells in the usual way. Alternatively, some adult fish can be killed, and different tissues used as sources of DNA in studies on transgenic mosaicism.

4. Germ-line transmission assays: When transgenic fish have been identified and tagged, they may be kept for breeding to provide evidence for germ-line transmission of the transgene. It is best to tag all fish prior to biopsy, and then to remove the small adipose fin of all trout proving positive in blood. This makes for easy identification if they are to be biopsied again. Rainbow trout will take 2 or even 3 yr in most aquarium systems to become sexually mature. It is convenient to examine a sperm sample for the transgene signal when stripping male transgenics. Eggs are more difficult because of the excessive volume of yolk compared with the small yield of DNA. Not all fish proving positive in sperm necessarily yield transgenic progeny, although the reasons for this are unclear. A great advantage of *Tilapia* is, of course, that they will become sexually mature at 8 or 9 mo only, so evidence of germ-line transmission can be obtained much more rapidly. Because of mosaic distribu-

tion of the transgene in the gametes, ratios of transgenics in F1 progeny are often <50%. However, if copies of transgenes are incorporated into more than one chromosome, as is often the case, then more than 50% of the F1 generation will prove to be transgenic, but a range of different Southern blot restriction fragment patterns will be obtained from different F1 fish (12).

5. Transgene expression: Assaying for transgene expression in fish is little different from similar assays in other classes of transgenic animals. It is important to ascertain whether a particular promoter will drive expression in fish cells. To this end, constructs in which the chosen promoter is spliced to reporter genes, such as CAT (chloramphenicol acetyl transferase), *LacZ* (β -galactosidase), or luciferase, may be either transfected into tissue cells of the chosen fish species or even injected into muscle of small fish, using the procedure successfully exploited in mammals (13). Alternatively, transient expression assays can be run in embryonic fish if embryos are sacrificed within a few days of injection.

Once a promoter has proven effective, it can be spliced onto the transgene of choice, and again there are arguments for choosing a structural gene of piscine origin. A considerable number of such sequences have now been cloned in various laboratories around the world. It may be necessary to include enhancer elements or other positive regulatory sequences in the construct, as well as polyadenylation sequences at the 3' terminus.

Most promoters are likely to have some tissue specificity of action, and thus, it may be necessary to dissect a transgenic fish and monitor extracts from different tissues for evidence of transgene expression. Secreted proteins, such as growth hormone, may be detected by radioimmunoassay of serum protein by blood biopsy. Interestingly, if the signal sequence for such a protein is absent through the use of cDNA transgene, the growth hormone may still be detected within cells (14).

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CHAPTER 9

An Overview of Transgenic Mouse Production

David Murphy and David A. Carter

1. Introduction

Three basic techniques have emerged for making transgenic mice: two of these, retroviral infection of preimplantation embryos and the manipulation of embryonal stem cells, have attributes which make them desirable in some experiments, but the third technique, microinjection, is by far the most efficient and popular. All but one of the transgenic species described in the present volume are produced by microinjection. The isolation and manipulation of murine Embryonal Stem Cells (ES cells) and the production of chimeric mice are described in Chapters 23 and 24.

An overview of the microinjection protocol is presented here. It is specifically relevant to mice, although the essential elements are similar for all mammalian species, particularly rats. In this context, this chapter is intended to link together the individual elements of the protocol (detailed in separate chapters) and thus provide new investigators with a summary of the various skills and facilities that are required.

2. Preliminary Requirements

There are two basic requirements for transgenic animal production, namely a healthy colony of animals and a cloned DNA sequence that will form the transgene. Both demand careful preparation if the transgenic experiment is to be successful. The establishment and care of suitable mouse and rat colonies are described in Chapters 12 and

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27. Techniques for making transgene constructs are beyond the scope of this volume, and the reader is referred to one of the many manuals available that describe recombinant DNA protocols. Particular care should be given to the final steps in the purification of the DNA construct (*see* Chapter 11). Attention to the design of the transgene construct is crucial and is discussed in Chapter 10.

3. Outline of Transgenic Mouse Production

The essential steps in the production of transgenic mice are shown in Table 1. The time taken to perform these procedures will depend on the skill and experience of the operator. With the exception of the day of microinjection, when prolonged attention is required, most of the procedures can be easily incorporated into a normal working day. At the same time, care should be taken to adhere strictly to the timing of injections and so on. Novice experimenters may prefer to delay the implantation of injected eggs until the day after microinjection. In addition to spreading the work load, this will also allow quantification of egg survival.

Following parturition, the number of pups should be counted, and this number marked on the cage together with the birth date. Any abnormalities should be checked at this stage. Expression of particular transgenes could result in severe abnormalities that are incompatible with maturation. In these cases, care must be taken to monitor the deterioration of the offspring and to kill them for analysis if necessary. Tissue samples taken will confirm identity as a transgenic and can be used for any of the analytical procedures described in this book. In other cases, transgene expression will be associated with embryonic lethality, clearly difficult to detect when occurring at early stages of development. Attenuated development prior to parturition will require careful sampling procedures if transgene expression is to be monitored.

As soon as transgenics have been identified by analysis of biopsied DNA, they should be mated so that a line is quickly established. Thus, sufficient animals for expression analysis/experimentation may be derived. Care must be taken to maintain the line, despite the pressing needs of an experiment. In some cases, the transgenic founders will be infertile—an *in vitro* fertilization (IVF) procedure may then be necessary (Chapter 22). Many investigators now routinely kill

Table 1
Summary of Transgenic Mouse Production (Micromjection)

Day	Procedure	Chapter
1	Start superovulating females (egg donors)	13
3	Continue superovulation protocol—mate females with stud males (fertilize eggs)	13
	Mate mature females (egg recipients/surrogate mothers) with sterile (vasectomized) males	13 and 15
4	Collect eggs (fertilized one-cell) into culture	16 and 17
	Micromject eggs with DNA fragment	18 and 19
	Implant surviving eggs into surrogates	14 and 20
20–22	Birth of pups	
	Caesarean section and fostering may be necessary	21
~40	Take DNA samples (tail biopsy) identification of transgenic animals	34
	Analyze DNA by slot-blot	35
	PCR	36
	or Southern blotting	37
	Breed transgenics, use IVF if necessary	22
>40	Determine expression of transgene by RNA analysis	38–44
	<i>In situ</i> hybridization	45
	Immunocytochemistry	46
	Reporter enzyme assay	49
	(Use nontransgenic litter mates as negative controls)	
	Identify pathologies by postmortem examination	47 and 48

founders in order to determine expression patterns of a particular transgene rapidly. Once a transgene has been shown to elicit the desired tissue-specific pattern of expression, mice bearing it can be rapidly rederived for further detailed analysis.

Analysis of the expression of transgenes will, of course, depend on the particular experimental goal, but some general points are worth noting. As a rule, analysis begins with a comprehensive sampling of tissues that will provide information on the specificity of transgene expression. The survey should include both tissues in which expression is anticipated and tissues in which the endogenous gene is not expressed. Design of the transgene should have taken into account strategies for differentiating between transgene and endogenous gene (*see* Chapter 10). Sensitivity of analyses should be considered. For

example, a simple Northern blot (Chapter 39) of total cellular RNA may not detect transgene mRNA, which is readily detected by the polymerase chain reaction (PCR; Chapter 41). An additional caveat that must be borne in mind is that transgenes may be transcribed efficiently, but, subsequently, translation may be blocked. We (Ang, H.-L., Carter, D. A., and Murphy, D., unpublished observations) have produced two transgenic mouse lines bearing a construct that acts as an efficient expression unit in transfected tissue culture cells. However, in mice, the same construct does not produce active CAT reporter enzyme, although Northern analysis reveals ubiquitous expression of CAT RNA.

Finally, an important point to remember during the analysis of transgenics is that the observed expression pattern may be quite different to that hoped for or predicted. Interaction between different elements of the transgene may result in expression that cannot be fully explained by our current understanding of gene regulation.

CHAPTER 10

Transgene Design

*David Murphy, David A. Carter,
and Duncan R. Smith*

1. Introduction

The exact structure of a microinjection transgene depends entirely on the aims of a particular experiment. In this context, reference to the chapters on the applications of transgenesis and technical details of transgenes described in references therein are highly recommended (*see* Chapters 2–5). However, certain general rules should be considered when designing a construct for eventual introduction into a transgenic organism. A transgene should operate like any other gene in the targeted cells of an organism, and hence, structural elements must be appropriately recognized by the transcriptional, posttranscriptional, and translational machinery of the host. A comprehensive review of the mechanisms of gene control is beyond the scope of the present volume, but the following points are indicative of the type of considerations that should contribute to the design of a transgene.

2. Transcriptional Control

1. What is known about the promoter and enhancer sequences of the gene of interest?
2. Have studies on the transfection of the gene into cultured cells identified any elements that should be included or eliminated from the transgene?
3. If little is known about the transcriptional regulatory elements, then one should aim to build as big a transgene as possible, even resorting to the use of a cosmid clone (*see* Note 1).

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4. If the transgene is from a different species than the host, are species differences in expression likely to complicate the analysis of expression?
5. Do not neglect introns (*see* Section 3.) or sequences beyond the 3' end of the last exon. Noncoding sequences can contain important regulatory elements, and regions many kilobases upstream or downstream of the start of transcription can contribute to the proper control of transcription initiation and termination.
6. Consider the position of regulatory elements within a transgene. Although "enhancers" are thought to be orientation and position independent, other signals, such as the TATA box, should be correctly positioned with respect to a functional transcription start site.

3. Posttranscriptional Processes

1. Introns have been shown to improve the efficiency of overall transgene activity (1). Include at least one intron in the transgene. This can be placed within the 3' untranslated region of the transgene so as not to disrupt any coding sequences.
2. The first and the last exon of the transgene can be any length. However, the size of internal exons is constrained, possibly by the machinery that recognizes exon/intron boundaries and engenders splicing. The construction of internal exons of greater than 300 bp by, for example, the insertion of a reporter element, may result in poor splicing efficiency and low expression of the transgene overall.
3. The proper location of signals for polyadenylation is important, not only to engender proper synthesis of the poly(A) tract, but also to facilitate appropriate transcription termination and message 3' end formation.

4. Translation

1. A reporter gene, which is best located in the first or last exon (*see* Section 3.) should possess a suitably located, functional translation initiation codon (AUG). Care should be taken to ensure that no functional initiation codons are present upstream of the one that should be utilized.
2. Alternatively, the reporter could be synthesized as an in-frame fusion with another coding sequence.

5. Posttranslational Processes

1. The protein product of the transgene may be destined for a particular subcellular location. Does the transgene contain the appropriate signals, such as those that mediate, for example, processing, secretion, nuclear localization, and so forth.
2. The transgene protein product may require posttranslational modifica-

tion for activity, for example, phosphorylation or glycosylation. Are the signals that engender these processes present and/or accessible?

The process of making transgenic animals is both lengthy and laborious. The experimenter should carefully consider whether further cloning procedures would give the experiment a greater chance of success.

The design of the transgene should take into account strategies for differentiating between the transgene and the endogenous gene, at the level of genomic and expression analysis. Sequence differences between species can be exploited or reporters can be included.

The presence of contiguous vector-derived DNA sequences in a fragment of injected DNA has been shown to inhibit the expression of mammalian transcription units in mice. For example, Townes et al. were able to increase the expression of a β -globin transgene by up to 1000-fold by removing plasmid sequences (2). Bacterial reporter genes, such as chloramphenicol acetyl transferase (CAT), have not been reported to inhibit the expression of transgenes, but this possibility ought to be considered. Similarly, cryptic regulatory elements in bacterial sequences contained within transgenes might alter the expression pattern. As a general rule, therefore, investigators should remove all vector sequences prior to injecting cloned eukaryotic genes in order to maximize the quality, quantity, and reproducibility of transgene expression. Appropriate restriction sites should be engineered at the ends of the transgene to facilitate the easy isolation of the microinjection fragment. The cleaved fragment is then gel purified (*see* Note 2 and Chapter 11). Finally, having designed and built a transgene, the experimenter might wish to test whether it is capable of acting as a functional expression unit in transfected culture cells (*see* Chapter 55) before proceeding to the transgenic animal.

6. Notes

1. Cloned DNA of any size can be introduced into mice by microinjection, including λ clones up to 50 kb in length (3).
2. Linear DNA has been shown to integrate fivefold more efficiently than supercoiled DNA (2), but the structure of the fragment ends created by different restriction enzymes has no effect (3,4).

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CHAPTER 11

Isolation of DNA Fragments for Microinjection

Duncan R. Smith and David Murphy

1. Introduction

The purification of a DNA fragment for microinjection is extremely important. This chapter describes a rapid and efficient technique for isolating specific DNA fragments from agarose gels run in Tris-acetate buffer, and was first described by Vogelstein and Gillespie (1). Agarose blocks containing the DNA fragment of interest are cut from gels and dissolved in NaI, a chaotropic salt that at concentrations of around 4M is able to solubilize agarose. Glass beads are then added, which, in this concentration of NaI, efficiently bind to the released DNA fragments. RNA, proteins, and other impurities fail to bind to the glass fragments. Following a few washing cycles, the purified DNA is eluted from the glass into a low-salt buffer. This method produces DNA of sufficient purity for most applications. No further purification is needed for the DNA fragment to be subcloned, labeled using standard methods, or released by restriction endonucleases. However, further purification is recommended if the DNA is to be microinjected into fertilized one-cell eggs. Passage through a Sephadex G-50 column previously equilibrated in MiTE removes all contaminating solutes and solvents (e.g., ethanol) that might be deleterious to the egg. Filtration through the 0.45- μ m filter removes particulate matter that might block the microinjection pipet.

Glass bead isolation fulfills all the criteria to be the method of choice for the isolation of DNA fragments from gels:

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1. It is rapid. A fragment can be isolated and ready for further processing within 2 h.
2. The method is efficient. Eighty percent recovery can be anticipated in most cases.
3. Its simplicity means that a large number of samples can be processed simultaneously.
4. The reagents used are relatively nonhazardous. Additionally the oxidation of the NaI (used to dissolve the agarose) to a purple-colored compound indicates the location any spillages!
5. The DNA fragment is extremely pure following processing. The quality is such that the DNA can be directly used in all manner of enzymatic reactions, and with little further purification, it is suitable for microinjection into fertilized one-cell eggs.

The biggest disadvantage of the technique is that the recovery of small (<500–800 bp) DNA fragments is not efficient. This can lead to substantial losses, although some will always be recovered.

Another possible disadvantage of the technique is that large (>15 kb) DNA fragments might potentially be broken during the wash cycles. If a large DNA fragment were to bind to two or more glass beads, then on washing and, hence, separation of the beads, the DNA strand might be broken. In practice, we have not found this to be a problem.

2. Materials (see Note 1)

1. Powdered glass flint, available from glass supply companies (e.g., Eagle Ceramics Inc., 12267 Wilkins Avenue, Rockville, MD 20852).
2. Autoclaved, filtered (0.2 μm) water.
3. Concentrated nitric acid.
4. 40X TAE buffer: 1.6M Tris-HCl, pH 8.0, 0.8M sodium acetate, and 0.04M EDTA.
5. Ethidium bromide (10 mg/mL) in sterile distilled water.
6. Long-wave (365 nm) UV light transilluminator.
7. 6M NaI: This is prepared by dissolving 90.8 g of NaI and 0.5 g of Na_2SO_4 in water to a final vol of 100 mL. Filter through a 0.45- μm Nalgene filter, and then add a few Na_2SO_4 crystals to the filtrate. The Na_2SO_4 crystals do not dissolve properly, but serve to prevent the oxidation of the NaI. Store at 4°C protected from light.
8. Ethanol wash solution: 50% (v/v) ethanol, 0.1M NaCl, 10 mM Tris-HCl, pH 7.5, and 1 mM EDTA. Store at -20°C.
9. Sterile (autoclaved), filtered (0.2 μm) MiTE (Microinjection TE): 10 mM Tris-HCl, pH 7.4, and 0.2 mM EDTA (see Note 2).

10. Sephadex G-50 slurry (*see* Note 3): This is prepared by swelling G-50 powder in water followed by autoclaving.
11. 1-mL Sterile disposable syringes.
12. Baked glass wool: Bake at 250°C for at least 3 h to destroy all contaminating nucleases.
13. Millipore (Bedford, MA) filter type HV (catalog number SJHV004NS, autoclaved).

3. Methods

3.1. Preparation of Glass Bead Slurry

1. Mix 250 g of the powdered glass flint with 500 mL of sterile water. Allow the heavier particles to settle over 1 h. Decant the fines into centrifuge tubes. Discard the heavy particles. Recover the fines by centrifugation (1000g, 5 min).
2. Resuspend the pelleted fines in 200 mL of sterile water. Add 200 mL of concentrated nitric acid, and bring to the boil on an electric hot plate. **Carry out this step in an efficient fume hood.**
3. Allow to cool, and then centrifuge (1000g, 5 min) to recover the glass beads.
4. Carry out repeated cycles of washing in sterile water and centrifugation until the pH of the suspension has reached neutral.
5. Store the glass bead slurry as a 50% (v/v) slurry. The slurry can be stored at -20°C, with working stocks being maintained at 4°C.

3.2. Recovery and Purification of DNA Fragments from Agarose Gels

1. Digest the DNA using appropriate restriction enzymes according to the recommendations of the manufacturer (*see* Chapter 50).
2. Fractionate the digested DNA in an agarose gel using 1X TAE buffer containing ethidium bromide (0.5 µg/mL). (*See* Chapter 51).
3. Visualize the DNA fragments using a long-wave UV light transilluminator, and carefully excise the fragment of interest, using a fresh, sterile scalpel blade, in as small a volume as possible. Dice the agarose block, and transfer to a 1.5-mL Eppendorf-type centrifuge tube.
4. Add as much 6M NaI as possible. The minimum ratio of 6M NaI to agarose should be 3:1. If this cannot be achieved in a single tube, then divide the agarose between a number of tubes.
5. Incubate at 65°C with occasional agitation (vortexing) until the agarose has completely dissolved.
6. Add 1 µL of glass bead slurry for every 2 µg of DNA. Mix well, and incubate on ice for 1 h with occasional agitation.

7. Recover the glass beads by centrifugation in an Eppendorf-type microcentrifuge. To ensue a loose pellet that can be readily resuspended, spin for only 1–2 s.
8. Discard the supernatant, and resuspend the pellet in 0.5 mL of 6M NaI. Repellet the glass beads (1–2 s, microcentrifuge), and repeat the NaI wash.
9. Resuspend the glass beads in 0.5 mL of ethanol wash. Centrifuge briefly to pellet the glass beads, and repeat the ethanol wash.
10. After removing the supernatant, vortex the pellet in the small amount of ethanol wash that remains in the tube, and then recentrifuge. Carefully remove as much of the supernatant as possible, but do not allow the pellet to dry out.
11. Add sterile water (>50 μ L) and incubate at 65°C for 30 min to elute the DNA. If the DNA fragment is to be used for microinjection, then elute in sterile, filtered MiTE.
12. Centrifuge the glass beads (this time pellet for 2 min at full speed), and transfer the supernatant containing the DNA to a fresh tube.
13. Prepare a spun Sephadex G-50 column. Block the end of a 1 mL disposable syringe barrel with baked glass wool, and fill with Sephadex G-50 slurry until all the excess liquid drains out and the beads are packed. Position the column in a disposable 15-mL plastic conical centrifuge tube, and centrifuge at 1000g for 3 min. Equilibrate the column by filling it with sterile filtered MiTE and then draining by centrifugation as before. Repeat this five times.
14. Load the DNA fragment (maximum vol 100 μ L), previously eluted from glass beads in MiTE, onto the column, and centrifuge at 1000g for 5 min. Collect the purified DNA in a fresh 15-mL centrifuge tube previously rinsed in sterile filtered MiTE.
15. Pass the DNA through a small 0.45- μ m Millipore filter (type HV) using a sterile disposable syringe previously washed with sterile filtered MiTE.
16. Assay the DNA by spectrophotometry or by comparing the ethidium bromide staining of an aliquot fractionated through an agarose gel with the fluorescence of DNA standards of known concentration (*see* Note 4).

4. Notes

1. Glass bead DNA isolation kits are commercially available from Bio 101 (La Jolla, CA). The GeneClean II kit is apparently able to recover DNA from Tris-borate as well as Tris-acetate agarose gels.
2. Microinjection DNA is dissolved in a buffer of 10 mM Tris-HCl, pH 7.4, 0.1–0.25 mM EDTA. Higher concentrations of EDTA and low concentrations of MgCl₂ are toxic to eggs.
3. Many manipulations of DNA and RNA leave the nucleic acid in a solution containing unwanted salts, nucleotides, or radioactive moieties. In the

majority of cases, ethanol precipitation will not entirely remove these or will add further salts. For this reason, Sephadex gel-exclusion chromatography is often employed to purify DNA fragments. Sephadex is a bead formed, crosslinked dextran. The crosslinking is carefully controlled, giving rise to pores of a particular size. Each bead therefore can be considered to consist of a network of holes, all having identical size and shape. A molecule passing down a Sephadex column will pass through a volume that is dependent on the size of the molecule. A molecule that is too large to enter a pore will only pass through the volume of the column not occupied by the beads. A small molecule (such as a solute molecule) will be small enough to enter the pores and, therefore, the molecule will pass through a much larger volume and so take a longer time to pass down the column. Since the size of the pore can be controlled, a range of gel-exclusion (or gel-filtration) matrices are produced. The size of a molecule that can just enter the pore is called the exclusion limit (i.e., larger molecules are excluded). Pharmacia (Uppsala Sweden), for example, makes eight Sephadex gel-filtration matrices, G-10, G-15, G-25, G-50, G-75, G-100, G-150 and G-200, in a range of grades (superfine, fine, medium, and coarse). Their exclusion limits are, respectively, 700, 1500, 5000, 10,000, 50,000, 100,000, 150,000, and 200,000 daltons. Note that these limits are calculated for Dextrans. Molecules of intermediate size will fractionate according to their mol wt. For example, G-50 will fractionate in the range 500–10,000 daltons. Therefore, a mixture of salts and DNA will fractionate cleanly. The DNA will be excluded from the pores and so will pass straight through the column. Salts and nucleotides will be small enough to enter the pores and so will be retained on the column. One further advantage of this method is that the sample does not become appreciably diluted.

4. DNA fragments are injected at a concentration of 1–5 $\mu\text{g}/\text{mL}$. Excessively high concentrations of DNA will kill eggs (2). It is estimated that around 1–2 pL of DNA solution are introduced into the pronucleus by microinjection. Thus, depending on the size, around 500 copies of the DNA fragment are introduced. Some workers make dilutions of the injection DNA stock (e.g., 1, 2.5, and 5 $\mu\text{g}/\text{mL}$) and rotate between these different solutions during a microinjection session as the pipets are changed.

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CHAPTER 12

Establishing a Colony for Efficient Production of Transgenic Mice

David A. Carter

1. Introduction

A specific and dedicated mouse colony is required if transgenic mice are to be efficiently produced. The structure of such a colony is described in the present chapter. Although the provision of these animal facilities will not present a problem to large research establishments, smaller laboratories must weigh the commitment of animal housing and maintenance against the extent of the proposed transgenic experiment and consider whether a collaboration with an established transgenic unit may best suit their purposes. In addition to the “production-line” mice, a transgenic facility must be able to accommodate large numbers of transgenics under analysis, possibly of multiple lines and generations. Time-consuming husbandry is required, which will involve both scientific and animal house staff. On deciding to establish a new transgenic unit, investigators must therefore ensure that junior scientific staff are prepared to take on extensive animal work—work that begins when the transgenics have been made. Staff with no animal experience must be trained by skilled animal-care operatives and should continue to seek advice at all times. There is no substitute for direct training, but novice workers may refer to manuals, for example (1). Prior to starting animal work, staff must consult appropriate government offices that regulate research animal use and obtain any necessary documentation. Even in countries where animal

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use is not monitored, it is essential that recognized standards of welfare and hygiene are maintained. With regard to animal health requirements (*see* Note 1), facilities need not conform to Specific Pathogen-Free (SPF) standards, but minimal precautions against infection should be taken. For example, entry to the facility should be restricted to essential staff only, who should wear fresh protective garments and gloves. The specific mouse population described here is commonly used in many transgenic units and should be adequate for three microinjection sessions per week. F2 zygotes, although less readily available, are chosen for microinjection, since the generation of transgenics and maintenance of lines is more efficient compared with inbred mice (2). Particular inbred lines must, of course, be used where a defined genetic background is a requirement of the experiment.

2. Methods

2.1. Groups of Mice Required for Production of Transgenics

Four groups of mice (*see* Note 2) are required:

1. Immature (12–14 g, 4–5 wk old) F1 hybrid females: These mice act as egg (zygote) donors for microinjection. The eggs are harvested following a superovulation protocol and fertilization (*see* Chapter 17). We use F1 animals generated from matings between C57BL/6J and CBA/J mice; other hybrids have been successfully used.

A typical microinjection session will employ 10 F1 hybrid females, giving approx 250 injectable eggs. To provide 30 F1 females/wk, it will be necessary to maintain 50 breeding cages, each containing a male (CBA/J) and a female (C57BL/6J). Since many more females than males are generally required, the number of female progeny can be maximized by placing two females with each male and culling male progeny at birth. The frequency of litter production can be maximized by taking advantage of the postpartum estrus in female mice; thus, males should not be removed from the cage following parturition. In general, the breeding colony should be carefully monitored to provide both accurate dates for weaning and superovulation, and fecundity data. Alternatively, it may be more convenient to dispense with a breeding colony and purchase a suitable supply of F1 hybrid females from an outside source.

2. Mature (2–12 mo) F1 hybrid males—“studs”: Sexually mature F1 hybrid males (C57BL/6J x CBA/J) are required for mating with the immature female F1s to provide fertilized F2 zygotes. A subcolony of at least 20

studs should be individually housed (for example, 33 × 15 × 13 cm cages) and mated on alternate days. Although these animals should act as good studs for up to 1 yr, a record of the plugging rate (*see* Chapter 13) will indicate studs that need to be replaced from time to time. Studs can be obtained either from the breeding colony or purchased. A stock of males may be caged together provided that they have been simultaneously weaned and not exposed to females. However, potential studs should be individually caged at least 1 wk prior to mating, since sperm production may be suppressed in subordinate males within a group.

3. Mature (>6 wk, >19 g), pseudopregnant females: These mice (0.5 d pc) act as recipients for the microinjected F2 eggs. The state of pseudopregnancy, in which the eggs will develop in an environment free from “competing” fertilized eggs, is achieved by mating mature estrous females with sterile (vasectomized, *see* Section 2.1., step 4) male mice. The females may be conveniently taken from the F1 colony or may be of another strain with equally good maternal characteristics. A subcolony of at least 30 mature females will be required to provide five pseudopregnant recipients/d, since in a randomly cycling colony, approx 25% of the mice will exhibit estrus on a particular day (*see below*). Ovulating females are chosen for mating with the vasectomized males following an examination of vaginal characteristics. A scheme to facilitate the identification of ovulating female mice is described in Chapter 13. As an alternative to vaginal examination and if there are sufficient vasectomized males, it is possible to establish a larger number of matings (20–30) to obtain sufficient pseudopregnant animals from a randomly cycling group of females. If sufficient recipients are not obtained on a particular day, injected eggs may be maintained in culture while a second series of matings is set up. Conversely, extra pseudopregnant mice should be saved and reused for matings 10–14 d later.
4. Sterile stud males (2–24 mo): Sterile males, usually vasectomized (*see* Chapter 15), are required for the production of pseudopregnant recipient mothers. Between 20 and 30 individually caged mice of any strain (Parkes or Swiss are excellent performers) are required. They should be mated on alternate days only. Animals that consistently fail to produce plugs should be replaced.

2.2. Transgenic Lines

Following the production of transgenic founders (mice that develop from injected eggs), these animals must be quickly mated in order to establish a transgenic line. F2 hybrid transgenics are generally mated with F1 hybrids. Analysis of the founder phenotype should not begin

until the transgene has been successfully passed to a subsequent generation. If the founders will not mate it may be necessary to perform an *in vitro* fertilization procedure (*see* Chapter 22). With normal fecundity, large numbers of potential transgenics for experimentation will be generated within a short space of time. It is essential that transgenic breeding is carefully monitored and recorded as soon as a line is established, so that confusion does not arise and valuable animals are not lost. In some cases, it may be necessary to establish a homozygous line, particularly where large numbers of mice are required for analysis. This may be achieved through heterozygous crosses and analysis of offspring. It should be noted, however, that a significant proportion of DNA integration events in transgenic mice have been shown to produce recessive lethal mutations, so homozygous animals cannot be generated.

3. Notes

1. Unless animals are maintained in an SPF unit, it is inevitable that sporadic microbial infections will occur in the colony. Careful monitoring of the colony for signs of infection will allow the removal of unhealthy mice and prevent a potentially disastrous widespread infection. Sick animals should be sent for veterinary analysis. A 6-mo check of the colony's health status by a veterinarian is advisable. Professional veterinary care is mandatory, although reference to texts (for example, 3) may be useful. The loss of a valuable transgenic line through infection may be prevented by maintaining the line in a collaborating laboratory or by freezing embryos. The health status of an infected, but surviving line can be restored by either Caesarean (*see* Chapter 21) or oviduct transfer (*see* Chapter 20) rederivation.
2. An additional group of animals that may be required are foster mothers. Such mice are necessary in cases where a very low number of embryos develop in the pseudopregnant recipient; the resultant fetuses may be too large for normal birth to occur, and a Caesarean section must be performed. These procedures are described in Chapter 21. Foster mothers can be obtained by setting up matings 1 d later than the pseudopregnant matings. Alternatively, if the colony is sufficiently large, suitable females should be available on most days.

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CHAPTER 13

Mating Mice

David Murphy

1. Introduction

In the process of creating and analyzing transgenic mice, matings between male and female animals are required for the following reasons:

1. To produce fertilized one-cell eggs for microinjection. Natural matings between mature females (over 6–7 wk of age) and stud males (over 7–8 wk of age) can be used to supply the one-cell eggs. Such matings can provide around 10 F2 eggs/mouse (from matings between CBA/J × C57Bl/6 or C57Bl/10 F1 male and female mice). However, it is preferable to mate the stud males with superovulated immature females. Such regimes can increase the yield of eggs per animal three- to fivefold, depending on the strain of mouse used.
2. To prepare 0.5-d postcoitum (pc) pseudopregnant recipient females to act as surrogate recipient mothers for microinjected one-cell eggs.
3. To maintain stocks of normal and transgenic animals. It is beyond the scope of this chapter to describe the animal husbandry techniques required to maintain a large breeding colony of normal or transgenic mice. Suffice to say that in most circumstances, colonies are expanded by the encouragement and meticulous monitoring of natural matings. However, it should be noted that an unusually large number of transgenic strains have reproductive defects. This can be a consequence of a variety of lesions, ranging from gross gonadal dysfunction to behavioral problems. It is sometimes necessary to intervene in order to maintain and expand a transgenic line. Such intervention might entail either superovulation of a female transgenic mouse or in vitro fertilization using either or both transgenic eggs and sperm (Chapter 22).

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A full description of mouse reproductive physiology is beyond the scope of this book. The reader is referred to specialist manuals (1–3).

2. Materials

1. Follicle-stimulating hormone (FSH): This can be obtained from Intervet Laboratories Ltd. (Trade name: Folligon). Alternatively, Pregnant Mares Serum Gonadotrophins (PMSG; Sigma) can be used. Make up to 50 IU/mL with sterile 0.9% (w/v) NaCl. Store frozen in 1-mL aliquots.
2. Human chorionic gonadotrophin (hCG): This can also be obtained from Intervet Laboratories Ltd. (Cambridge, UK) (Trade name: Chorulon) or from Sigma (St. Louis, MO). Make up to 50 IU/mL in sterile 0.9% (w/v) NaCl. Store frozen in 1-mL aliquots. *Note that as with any reagent derived from human material, preparations of hCG may carry infectious agents, and should be handled with care.*
3. Sterile 1-mL disposable syringes and 25-g needles
4. Sterile 0.9% (w/v) NaCl.
5. P200 Gilson pipet and sterile yellow tips.
6. Dissecting microscope (e.g., Nikon SMZ10-TD)

3. Methods

3.1. Natural Matings

1. All mice are maintained on a constant light–dark cycle. Sophisticated systems that vary the intensity of light with the time of day (0% light 1830–0630 h; 25% light 0630–0830 h; 75% light 0830–1130 h; 100% light 1130–1300 h; 75% light 1300–1600 h; 25% light 1600–1830 h) are preferable, but simple, on/off regimes (dark 1700–0600 h; light 0600–1700 h) are adequate.
2. Between 1600 and 1800 h, place a single mature female (1.5–4 mo old) in the cage of a single male. Oestrous females should be preselected on the basis of the gross physical appearance of their vaginas (Table 1) or the cytology of vaginal smears (Table 1). Smears are obtained by gently washing out the cellular contents of the vagina with 50- μ L sterile 0.9% NaCl using a P200 Gilson pipet fitted with a sterile yellow tip. The smear is placed on a microscope slide, and the cytology is examined under a dissection microscope. Females can also be selected at random, without preselection, but clearly the efficiency of plugging will be greatly reduced. Note that overenthusiastic smearing can induce pseudopregnancy in a female mouse!
3. On the following morning, examine the females for a copulatory plug. This is evidence that a successful coupling has taken place. The plug consists of a creamy-white mass of coagulated sperm and proteins block-

Table 1
Identification of the Estrus Stage of a Female Mouse

Stage in estrus cycle	Gross vaginal appearance	Cytology
Diestrus	Small opening Tissues blue Moist	Pencil dots
Proestrus	Gaping Reddish pink Moist	Early: Larger dots Late: Less-defined circular dots
Estrus	Striations on dorsal and ventral lips Gaping Lighter pink Less moist Pronounced striations	Squamous cells "zig zags"
Metestrus I	Pale Dry	
Metestrus II	Dorsal lip less edematous As in Metestrus I Dorsal lip receded Dorsal lip less edematous White cellular debris lines inner wall and may fill vagina	

ing the vagina. Often the plug is obvious—it is hard and blocks the vagina entirely. Sometimes the plug has a softer, more fluid consistency. Note that often the plug is not externally visible, lies deep within the vagina, and can only be observed by careful examination of the vagina using a smooth blunt probe. Care should be taken not to confuse the cellular debris often found in the vagina at metestrus with a copulatory plug.

4. Efficiency: Without preselection of estrus females, between 10 and 25% of the females presented to the stud males should mate as evidenced by a copulatory plug. With preselection for females actually in estrus, the plugging rate should be around 80%.

3.2. Superovulation

1. Between 1000 and 1200 h, inject 5–10 sexually immature female mice (4–5 wk old, 12–14 g) intraperitoneally (ip) with 100 μ L of 50 IU/mL FSH (i.e., 5 IU/mouse) using a sterile disposable syringe fitted with a 25-g needle.
2. Between 46 and 48 h later, inject the same mice with 100 μ L of 50 IU/

mL hCG (i.e. , 5 IU/mouse) ip using a sterile disposable syringe fitted with a 25-g needle. Immediately place each female with a stud male mouse of appropriate strain.

3. On the following morning, examine the females for the presence of a copulatory plug (*see* Section 3.1.). Some 80–100% of the females should have undergone a successful mating.

4. Notes

1. Female mice maintained on the light–dark cycle described here ought to ovulate every 4–5 d, 5 h after the onset of darkness at around midnight. Males will copulate with estrous females in the middle of the dark period, at around 0030 h. Fertilization will take place between 30 min and 2 h later. Eggs can be harvested and injected anytime between 10.30 h and midnight of the following day (0.5 d pc). After this time, the fertilized eggs will start to undergo the first cleavage division to give two-cell embryos
2. Pseudopregant females 0.5 d pc can be implanted with microinjected eggs anytime during the day that follows an infertile coupling.
3. Although virgin females can make suitable pseudopregnant recipients, experienced mothers are preferable. They are better able to cope with care of their litter (especially since the litter may be unusually small or large) and with the traumas associated with the experimental manipulations they undergo.

References

1. Tuffery, A. A. (ed.) (1987) *Laboratory Animals: An Introduction for New Experimenters*. Wiley, Chichester, UK.
2. Poole, T. B. (ed.) (1989) *The UAFW Handbook on the Care and Management of Laboratory Animals*. Longman, Harlow, UK.
3. Foster, H. L., Small, J. D. and Fox, G. F. (eds.) (1983) *The Mouse in Biomedical Research*. Academic, London, UK.

CHAPTER 14

Anesthetizing Mice

David A. Carter

1. Introduction

Operations, such as vasectomy (*see* Chapter 15) and oviduct transfer (*see* Chapter 20), are performed on anesthetized animals. It is essential that the experimenter is familiar with this procedure and that the anesthetic has been tested: A lethal dose of anesthetic administered prior to an oviduct transfer is an unfortunate end to a day spent collecting and microinjecting eggs. The anesthetic described here is Avertin (administered ip); other anesthetics, perhaps routinely used in the experimenter's animal house, may be used.

2. Materials

1. Avertin: A 100% stock is made by mixing 10 g of tribromoethyl alcohol with 10 mL of tertiary amyl alcohol. A working solution is 2.5%, diluted from stock in sterile water. Both stock and working anesthetic should be stored at 4°C, protected from light.
2. Sterile disposable 1-mL syringes.
3. Sterile disposable 0.5 × 16 mm needles.
4. Animal balance.

3. Methods

1. Weigh the mouse. Use between 15 and 17 μL of 2.5% Avertin/g of body wt. The dose varies between batches and should be redetermined when a fresh stock is prepared. The anesthetic should be taken up into a 1-mL syringe fitted with a 0.5 × 16 mm needle.
2. The mouse should be restrained by tightly gripping the dorsal skin between thumb and fingers, such that the skin is stretched tight over the

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ventral abdomen and the animal is immobilized. The technique should, ideally, be demonstrated by an experienced animal worker. With the other hand, introduce the needle directly into the abdominal cavity in a position level with the top of the hindlimb and laterally so as to avoid the bladder. Again, seek expert advice if you are unsure. Inject the anesthetic (ensure that the injection is ip) and wait momentarily before withdrawing the needle.

3. The animal should be unconscious within a few minutes and remain anesthetized for 30–60 min (*see* Note 1). An appropriate level of anesthesia is indicated by the maintenance of rapid breathing and the absence of a blinking reflex while blowing on the eyes.

4. Note

1. If an adequate level of anesthesia does not develop, inject additional anesthetic (25% of initial vol)

CHAPTER 15

Vasectomizing a Mouse

David Murphy

1. Introduction

Vasectomized males are needed to engender pseudopregnancy in mature female mice. Such pseudopregnant females act as hormonally and physiologically competent recipients for microinjected fertilized one-cell eggs (*see* Chapter 20). Vasectomized males retain their sexual potency and leave a copulatory plug after mating, but, of course, fail to transmit any sperm. Some laboratories use genetically sterile males for this purpose rather than mice rendered sterile by surgery.

2. Materials

1. Avertin anesthetic (2% [v/v]); *see* Chapter 14.
2. 70% (v/v) Swabbing ethanol in a squeeze bottle.
3. Sharp-pointed, fine dissection scissors.
4. Sharp watchmaker's forceps (size 5).
5. Blunt forceps.
6. Bunsen flame.
7. Curved surgical needle (size 10, triangular, pointed).
8. Surgical silk suture (size 5.0).
9. Wound clips (9 mm) and wound clip applicator (e.g., Clay Adams, available from Arthur M. Thomas Co., Philadelphia, PA).

3. Methods

1. Restraining the animal with one hand (Chapter 14) introduce the 2% (v/v) Avertin anesthetic (15–18 $\mu\text{L/g}$) intraperitoneally (*see* Note 1).
2. After ensuring that the animal is completely anesthetized (Chapter 14), lay the animal, abdomen side up, on the lid of a 9-cm Petri dish. Spray the lower half of the abdomen with swabbing ethanol.

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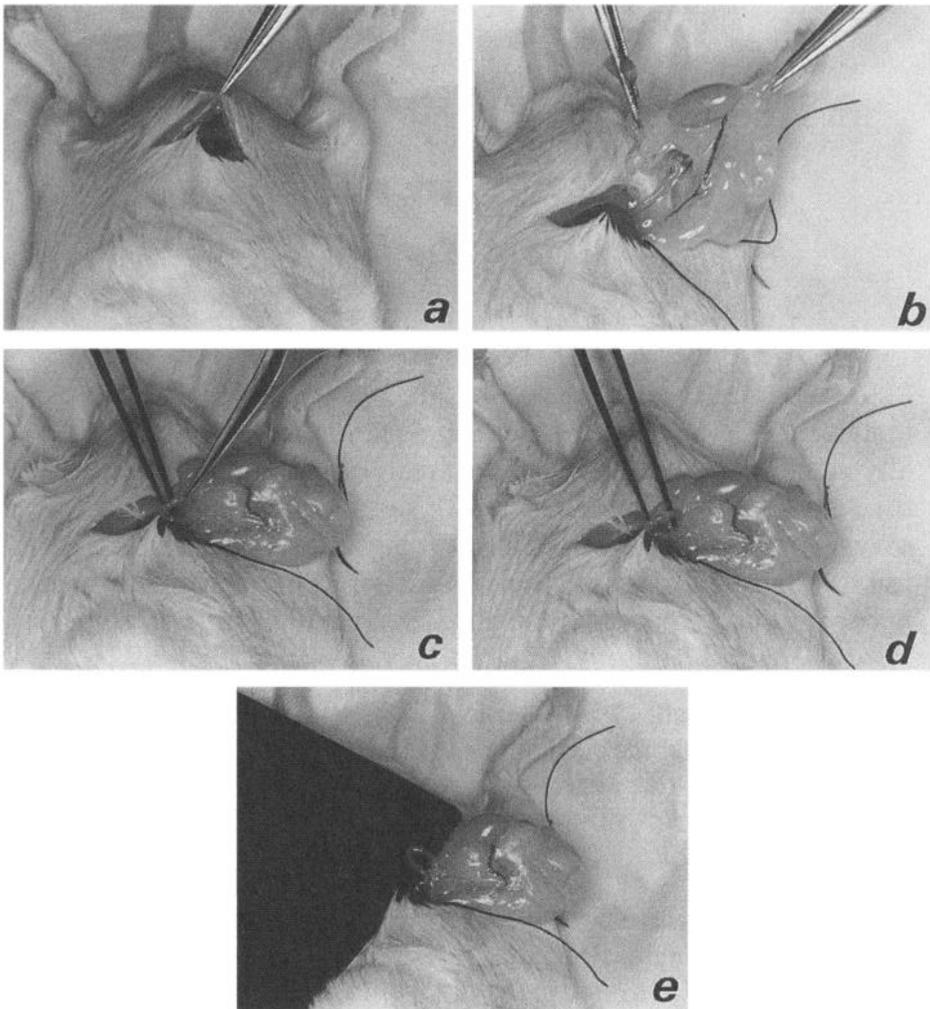


Fig. 1. Steps in the vasectomy of a male mouse. *See text* for details.

3. Comb the hair away from the proposed incision site with the points of a pair of fine forceps.
4. Lift the skin away from the body wall using a pair of fine, but blunt forceps, and make a 1-cm transverse incision at a point level with the hip joint (Fig. 1a). To reduce bleeding, stretch the incision to around 1.5 cm with the outer (blunt) edges of the blades of a pair of opened scissors.
5. Make a 1-cm incision through the body wall, parallel to the skin cut (Fig. 1a). Again stretch the incision.

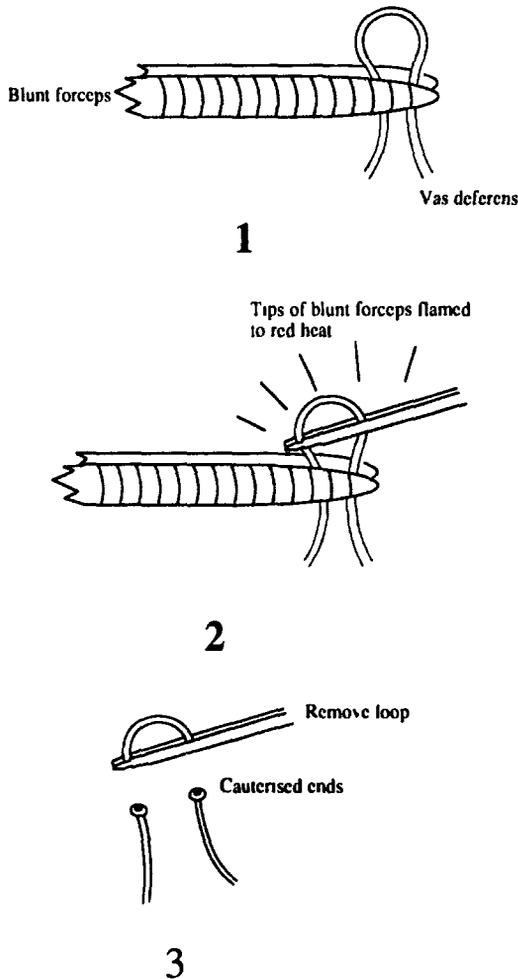


Fig. 2. Cauterization of the vas deferens. See discussion on next page.

6. Introduce a single stitch into the anterior side body-wall wound.
7. Pull out one side of the reproductive tract by grasping the large associated fat pad using a blunt pair of forceps (Fig. 1b).
8. Identify the vas deferens (Fig. 1b; held by left forceps). The vas deferens links the epididymis to the penis and associated glands. It is a rather rigid tract with a pronounced blood vessel running along its side. Remove the membranes attached to the vas deferens using fine forceps and scissors (Fig. 1c and d). The vas deferens is clearly seen against the black background (Fig. 1e).

9. Hold the vas deferens in a loop with a pair of forceps. Heat a pair of blunt forceps (tip width around 2 mm) until glowing red. Grip the vas deferens loop with the hot forceps for a few seconds both to burn away the tube and cauterize the exposed ends (Fig. 2).
10. Ensure that the cauterized ends are well separated and then return the reproductive tract to the inside of body wall using a blunt pair of forceps.
- 11 Repeat with the other side of the reproductive tract.
12. Sew up the body wall with at least two stitches.
13. Clip the skin with two wound clips (*see* Notes 2 and 3).

4. Notes

1. The vasectomy operation should be performed on young, healthy, sexually mature males of around 8 wk of age. Any strain with good sexual performance can be used. We have found that Swiss 3T3 mice perform well.
2. Following the operation, the mice should be caged individually and allowed to recuperate for at least 3 wk prior to the presentation of their first female.
3. Vasectomized males should be presented with females, at the most, every other day. The performance of each male should be monitored. Any male that fails to mate on three successive occasions, as indicated by its inability to deposit a copulatory plug, should be replaced.

CHAPTER 16

Preparation of Culture Media for Fertilized One-Cell Mouse Eggs

David A. Carter

1. Introduction

Two types of culture media (1) are required for the *in vitro* manipulations involved in the process of making transgenic mice. (1) M16: This is used for maintenance of eggs in microdrop cultures (*see* Chapter 17) in a 37°C incubator gassed with 5% CO₂. Since this medium is buffered with bicarbonate alone and the eggs are extremely susceptible to pH changes, it is not suitable for maintaining eggs outside the incubator. (2) M2: This is used for maintaining eggs outside the incubator during prolonged manipulations, such as collection or microinjection. M2 has essentially the same components as M16, although the bicarbonate is partially replaced with HEPES buffer to facilitate survival outside the CO₂ incubator. However, the eggs should not be maintained in M2 for periods longer than 30 mins.

2. Media Preparation

Preparation of M16 and M2 is described in Tables 1 and 2. The following three criteria should be strictly adhered to:

1. All solutions should be made up using sterile disposable plastic containers and pipets. The complete removal of contaminating detergents from glassware is essential for egg survival and difficult to achieve.
2. Water should be either double glass distilled or purified by filtration (Milli-Q) and stored in clean plastic containers.
3. All chemicals should be of tissue-culture grade (Sigma, St. Louis, MO) and stored under appropriate environmental conditions. Of all the com-

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Table 1
Preparation of Concentrated Component Stocks
for M2 and M16 Culture Media

10X A ^{a,b}	For 100 mL	5.534 g NaCl 0.356 g KCl 0.162 g KH PO ₄ 0.293 g MgSO ₄ · 7H ₂ O 2.61 g Na lactate 1.0 g glucose 0.06 g penicillin 0.05 g streptomycin
10X B ^{b,c}	For 100 mL	2.101 g NaHCO ₃ 0.01 g phenol red
100X C ^{b,c}	For 10 mL	0.036 g Na pyruvate
100X D ^{b,c}	For 10 mL	0.252 g CaCl ₂ · 2H ₂ O
10X E ^{b,d}	For 100 mL	5.958 g HEPES 0.01 g phenol red

^aWeigh out all components (except sodium lactate) into a 100-mL container. Weigh out the sodium lactate into a beaker, and add to the first container. Rinse the beaker with water, and use this wash to make up the A stock to 100 mL.

^bFilter all concentrated stocks through 0.45- μ m filters into sterile plastic tubes. Store frozen at -20°C. Working stocks can be kept at 4°C. At 4°C, A, D, and E will keep for 3 mo, but B and C should be changed at 2-wk intervals.

^cWeigh out the components into 10- or 100-mL containers, and make up with water.

^dWeigh out the components into a beaker, and dissolve in 50 mL of water. Adjust to pH 7.4 with 0.2M NaOH, and then make up to 100 mL with washings from the beaker.

Table 2
Preparation of M2 and M16 from Concentrated Stocks^a

Stock	M2	M16
10X A	10 mL	10 mL
10X B	1.6 mL	1.6 mL
100X C	1.0 mL	1.0 mL
100X D	1.0 mL	1.0 mL
10X E	8.4 mL	—
Water	78 mL	78 ml
BSA	400 mg	400 mg
Total	100 mL	100 mL

^aMeasure the water into a container. Aliquot the concentrated stocks into the water, and then carefully rinse the pipet by drawing the liquid up and down. Add the BSA, and mix gently until dissolved. Filter through a 0.45- μ m filter, using a large sterile disposable syringe, into sterile containers. Store at 4°C. Prepare fresh each week.

ponents, bovine serum albumin (BSA), which acts primarily to reduce the stickiness of the eggs, is the most likely source of contaminating toxins. Several different products, and possibly batches, may need to be tested before a safe supply is obtained (*see* Note 1).

3. Notes

1. In a recent experiment in which egg survival in culture was compared using media prepared with eight different BSA products (from three different suppliers), only one product permitted survival (64%) to the blastocyst stage (Ho, M. Y., Carter, D. A., and Murphy, D., unpublished). Although most products permitted 50% survival over 24 h in culture, a toxic effect was clearly evident at this early stage. The product giving best survival was: Sigma albumin, bovine (crystallized, lyophilized, essentially globulin free, cell culture tested, Product # A 4161).
2. Osmolarity of the medium does not have to be routinely checked, but, for reference, the values should be: M16, 288–292 mosmol/L; M2, 285–287 mosmol/L.

Reference

1. Hogan, B., Constantini, F., and Lacy, E. (1986) *Manipulating the Mouse Embryo—A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

CHAPTER 17

Collection of Fertilized One-Cell Mouse Eggs for Microinjection

David A. Carter

1. Introduction

Following superovulation and mating (*see* Chapter 13), the immature F1 females are killed, and the fertilized F2 eggs are dissected out from the upper part of the oviduct or ampulla. The procedure for egg collection (1) is described in the present chapter. Eggs may be recovered several hours before they are due to be injected. A suitable time for collection would be 11 AM to 12 noon for animals maintained on a light-dark cycle with the mid-dark-phase time at 12 midnight. The procedure is straightforward, and once the use of the mouth-operated pipet holder is mastered, the vast majority of eggs from 10 mice can be obtained in <1 h.

1.1. Preliminary Procedures

A number of techniques must be learned prior to beginning the egg collection procedure.

1. Rapid and humane killing of mice: It is essential that mice be killed quickly and humanely. The recommended method is to break the neck—cervical dislocation. Holding the animal by the tail on a wire cage top, allow it to run such that it becomes stretched out in the process of pulling on the cage bars with its forelimbs. Apply firm pressure at the base of the skull either with a spatula blade or by pinching with thumb and forefinger. A simultaneous pull on the tail will break the neck.
2. Microdrop cultures: These are 20–40 μL drops of M16 medium placed in an array (using a micropipet) on the bottom of a 35-mm culture dish. Light paraffin oil, which prevents evaporation, is carefully flooded over

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the drops until they are submerged (*see* Note 2). The microdrops are reasonably robust, but will be disrupted on shaking.

3. Egg-transfer pipets: Small pipets used for transferring eggs between culture dishes must be fashioned from hard glass capillaries. Using a small gas flame, the middle of a BDH hard glass capillary should be heated until soft. After withdrawing from the flame, the ends should be pulled apart rapidly until the two halves break apart. If the pipets are too long (the "pulled" region should be no more than 3 cm long) or too narrow (<200 μm), an appropriate mark should be made with a diamond pencil and the excess glass snapped off. The internal diameter of a transfer pipet should be around 300 μm , and the end should be flush. A 30-min practice session should enable the production of useful pipets. The pipets are assembled into a mouth-operated system made up of a mouthpiece, plastic tube (approx 60 cm), and a pipet holder. It is advisable to practice the operation of this system prior to the collection of a large numbers of eggs (*see* Note 1)

2. Materials

1. M2 and M16 media (*see* Chapter 16).
2. Hyaluronidase (Sigma, St. Louis, MO): 10 mg/mL in M2 stored at -20°C (stable for several months).
3. Light paraffin oil (Fluka [Buchs, Switzerland] or other; *see* Note 2).
4. 70% Ethanol in squeeze bottle.
5. 35-mm Sterile tissue-culture dishes.
6. Hard glass capillaries, 1.5 mm od.
7. Mouth pieces, which also double as pipet holders (Arnold R. Horwell Ltd., West Hampstead, London, UK).
8. Pipet holders.
9. Flexible connecting tubing for pipet system.
10. Diamond pencil.
11. Dissecting scissors, one regular, one fine.
12. Watchmaker's forceps. 2 \times #5.
13. Stereo microscope with understage illumination (e.g., Nikon [Tokyo] SMZ-10TD).
14. Fiberoptic light source (e.g., Nikon).
15. 37 $^{\circ}\text{C}$ Incubator gassed with 5% CO_2 .

3. Methods

1. At least 1 h before collecting the eggs, prepare eight culture dishes of media:
 - 2X M16 equilibrated in 37 $^{\circ}\text{C}$ incubator.
 - 2X M16 microdrop cultures equilibrated in 37 $^{\circ}\text{C}$ incubator.
 - 4X M2 left at room temperature.
2. Kill the plugged donor females as described above.

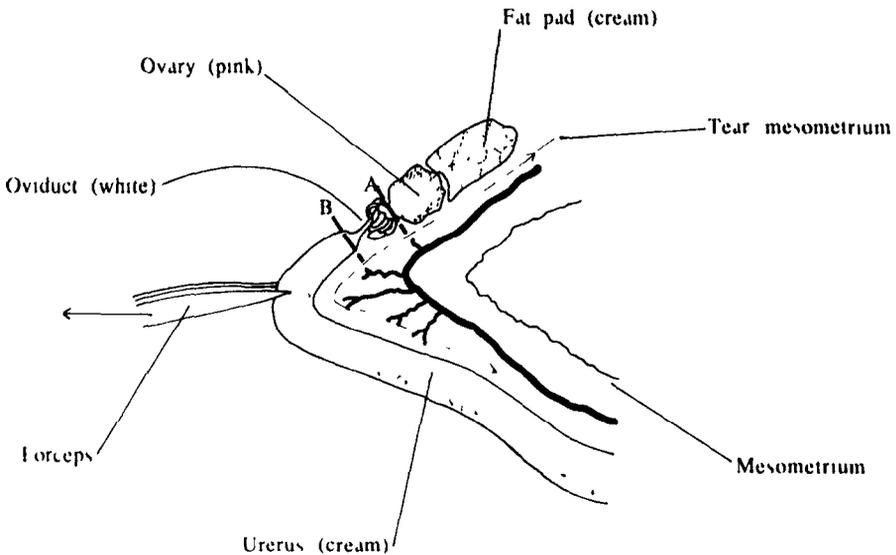


Fig. 1. Dissection of the female reproductive tract of a mouse showing the procedure for removal of the oviduct prior to egg collection.

3. Lay the animals out on absorbant paper, and soak the ventral surface of the abdomen with 70% ethanol.
4. Pinch up the skin with fingers, and make a small cut in the midline (exact position is not critical) using the regular scissors. It is then possible to skin the animal by pulling the skin away from the position of the cut toward the head and tail. The abdomen should be completely exposed. The body wall (peritoneum) should then be cut away to expose the abdominal organs. By pushing the coils of gut out of the way, the uterus and attached ovary (see Fig. 1) should be clearly visible on both sides of the abdominal cavity. The coiled oviduct is located between the ovary and uterus.
5. Gripping the uterus with watchmaker's forceps as indicated in Fig. 1, lift up the reproductive tract. This facilitates trimming of the membrane (mesometrium) that joins the reproductive tract to the body wall. The fine scissors should be used to puncture the membrane and trim it away from the oviduct.
6. Maintaining the same grip with the forceps, a cut should be made between the ovary and oviduct using the fine scissors (cut A, Fig. 1). The cut should be as close as possible to the oviduct.
7. Transferring the forceps to the oviduct itself (grip firmly but carefully), make a second cut between the uterus and oviduct (cut B, Fig. 1)

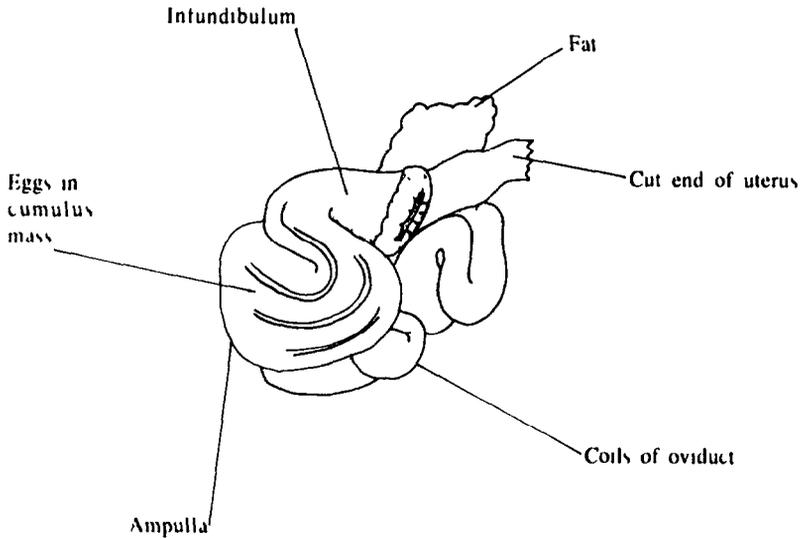


Fig. 2. The mouse oviduct as viewed under a dissecting microscope showing the position of eggs in the swollen ampulla.

8. Place the oviduct in one of the dishes of M2.
9. Dissect out the oviduct from the other side of the animal and then proceed with the rest of the female donors. All the oviducts may be collected in the same dish of M2.
10. When viewed under the dissecting microscope (10–20X mag.), the oviducts should appear as a mass of opaque coils with a single transparent, swollen region, termed the ampulla (*see* Fig. 2). The ampulla is the target for egg collection since, at this stage, it contains the cumulus mass (numerous fertilized eggs surrounded by cumulus cells). Eggs may be visible through the walls of the ampulla
11. Using one pair of forceps to hold the oviduct down, tear the ampulla with a second pair of watchmaker's forceps. Take care to orientate the tissue such that the cumulus mass is visible when it escapes from the ampulla. If the mass does not spill out immediately, it may be necessary to tease the eggs out with forceps. Discard the empty oviduct and repeat the procedure with the remaining tissues. Occasionally, an oviduct will not exhibit a prominent ampulla. In these cases, the forceps can be used to tear the oviduct apart to search for eggs. To facilitate rapid transport to a fresh dish of medium, take care to collect the cumulus masses in a single location within the first dish.

12. Using an egg transfer pipet (*see* Note 1), collect all the eggs and transfer to a second dish of M2.
13. Mix the cumulus masses with 50 μL of hyaluronidase delivered from a thawed tube of stock hyaluronidase using a transfer pipet. Enzymatic digestion is necessary to separate the eggs from cumulus cells. A few minutes treatment is sufficient and the eggs should not be in contact with the enzyme for a longer period. Pipetting the eggs up and down will speed the separation.
14. Transfer the eggs to a third dish of M2 using the transfer pipet. Wash the eggs by repeatedly collecting and transferring to a new location in the dish. Each time the eggs are transferred, the small cumulus cells should be abandoned such that virtually none are remaining in the final wash. Repeat with a fourth dish of M2.
15. Wash the eggs twice in one of the equilibrated dishes of M16.
16. Transfer the eggs to microdrop culture (approx 30 eggs/microdrop), and incubate at 37°C in 5% CO until required for microinjection.

4. Notes

1. A practice session with the mouth-operated pipet system is essential prior to dealing with a large number of eggs. Use media alone and then a small number of eggs to learn the technique. Experiment with potential mistakes, such as blowing bubbles into the media, a common problem that can cause a time-consuming dispersal of eggs. Ensure that the pipet does not become loose in the holder.
2. After BSA (*see* Chapter 16), paraffin oil is the second most likely source of toxins that will kill eggs. Experiment with noninjected eggs to test that development to the blastocyst stage (4 d in culture, by which time a ball of cells containing a blastocoel is formed) is permitted in more than 50% of the original one-cell eggs. It may be necessary to test several different products and possibly batches. In a recent experiment (Ho, M. Y., Carter, D. A., and Murphy, D., unpublished) in which different oils were compared, we have found that egg survival in culture was excellent using Fluka paraffin oil (Cat. No: 76235).

Reference

1. Hogan, B., Constantini, F., and Lacy, E. (1986) *Manipulating the Mouse Embryo—A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

CHAPTER 18

Microinjection of Cloned DNA Fragments into Fertilized One-Cell Mouse Eggs

I. Manual Injection

David Murphy

1. Introduction

Central to the process of making transgenic mice is the physical introduction of cloned DNA fragments into fertilized one-cell mouse eggs. First described 10 years ago by a number of investigators (1–5), microinjection remains the most popular and successful of the methods currently available for generating transgenic animals. Microinjection continues to be the method of choice, because the advantages of speed and reliability far outweigh the demands placed on the investigator for precise technical skill and expensive equipment.

This chapter describes the equipment requirements for microinjection, the assembly of these components into a microinjection system, and the process of microinjection itself. Described here is the manual execution of the injection process—the DNA is forced into the nucleus of the fertilized one-cell egg via an injection needle linked to a simple system consisting of an air-filled tube connected to a 50-mL syringe. This system has proved more than adequate over the years and, indeed, affords the skilled operator a certain degree of fine control over the amount of DNA introduced into the egg. However, the process is slow and the injection pipets must often be changed many times during a microinjection session. In the next chapter (Chapter 19), I describe

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one of the recently developed automatic injection systems. Here the injection pipet is linked to an accurate pumping system. The use of such systems can speed up the process of microinjection considerably.

2. Materials

- 1 Inverted microscope with the following features:
 - a. Image erected optics.
 - b. A fixed stage (the objective moves when focusing rather than the stage).
 - c. A condenser with a long working distance.
 - d. 10X eyepieces.
 - e. A 4X objective.
 - f. A 40X objective.
 - g. Suitable optics: Nomarski differential interference contrast (DIC available from Nikon, Tokyo) optics provide the optimal microscopic system for observing the structure of fertilized one-cell eggs so that they will be readily injected. DIC optics are expensive, and glass injection chambers are required. Plastic injection chambers are compatible with Hoffman (Greenville, NY) modulation contrast optics, but the resolution is inferior to that afforded by Nomarski microscopy. If neither Hoffman nor Nomarski optics are available, eggs can be viewed under bright field. Phase-contrast microscopy is not compatible with any microinjection system.

The Nikon Diaphot-TMD equipped with the diasopic DIC Nomarski attachment TMD-NT2 is an excellent and robust, but affordable microscope system widely used for microinjection.

2. Micromanipulators: Two micromanipulators are used—one (usually the left-hand) is used to manipulate the holding pipet, which holds the egg in place while it is injected; the other (usually the right-hand) is used to manipulate the injection pipet. The most commonly used micromanipulators are the M type supplied by Leitz. This system has convenient joy-stick control of fine horizontal movement in two planes. The micromanipulators and the microscope must be positioned on a purpose-built baseplate (Fig. 1), which must be custom engineered (a plan has been published; 6) Narishige (Tokyo) produces economically priced micromanipulation systems compatible with its Diaphot-TMD microscope. These systems are very flexible and have the advantage that they do not require a purpose-built baseplate.
3. Micropipet holders (Leitz [Wetzlar, Germany] single-instrument holders fitted with Leitz single-instrument tubes if Leitz Type-M micromanipulators are used).

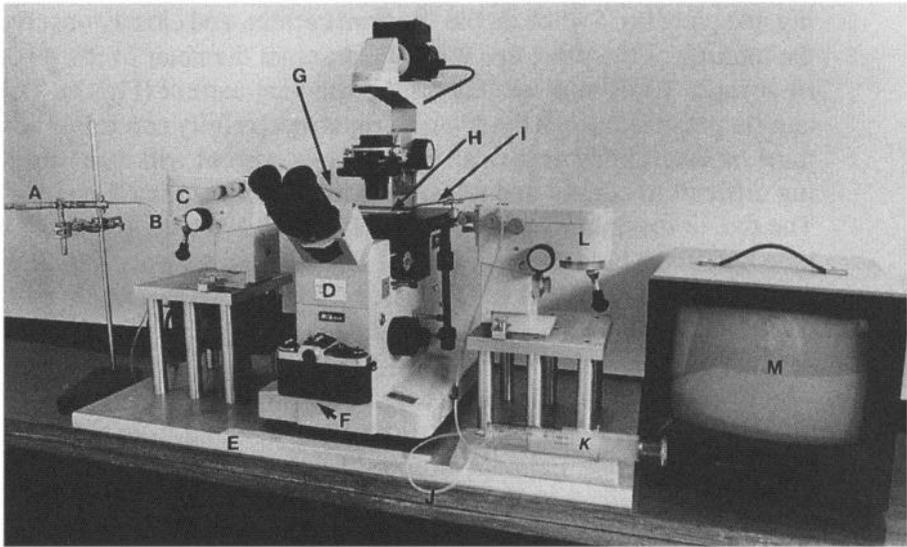


Fig. 1. Typical arrangement of the equipment needed for the microinjection of fertilized one-cell eggs. A. Agla micrometer syringe. B. Liquid-paraffin-filled tube. C. Left-hand micromanipulator. D. Inverted microscope. E. Base plate. F. Camera (optional). G. Left-hand instrument tube with holding pipet. H. Microinjection chamber (depression slide) sitting on fixed stage. I. Right-hand instrument tube with injection pipet. J. Air-filled tube. K. Glass 50-mL syringe. L. Right-hand micromanipulator. M. Video system (optional).

4. Holding pipets: A holding pipet holds the fertilized one-cell egg in place while it is injected. Holding pipets are prepared as follows:
 - a. Draw a 1-mm od Leitz hard-glass capillary (catalog number 520119) in a gas flame. Grip the ends between forefinger and thumb, and turn the middle of the capillary in the hottest part of the flame until the glass softens. Then withdraw the pipet from the flame, and simultaneously pull on both ends. Reject any capillaries that are not drawn straight.
 - b. Using a diamond pencil, score the drawn section 2 cm from the shoulder, and break the capillary at this point. Ensure that the capillary is suitable for forging. It should be perfectly straight and the broken end must be flush, with no jagged edges. The drawn section should have an external diameter of $100\ \mu\text{m}$ ($\pm 20\ \mu\text{m}$).
 - c. Vertically mount a suitable drawn capillary in a microforge (e.g., Narishige MF-9). Using a 4X objective lens, focus on the tip of the pipet. Bring the filament of the microforge close to, but not touch-

ing, the pipet tip. Switch on the filament current, and closely observe the melting of the pipet tip. When the internal diameter of the pipet tip reaches 10–15 mM , switch off the filament current (Fig. 2). The current passing through the filament must be carefully controlled and must be determined empirically. Too much current will make forging difficult to control and may result in the filament itself melting. The size of the holding pipet aperture is very important. Too small a hole will make holding pipet control difficult and may also result in the pipet becoming blocked. Too large a hole will also make control difficult and may allow eggs to be drawn into the pipet, resulting in damage or loss.

- d. Move the pipet to a horizontal position, and observe under low magnification. Position the microforge filament 1–2 mm from the tip of the pipet. Move the filament close to the pipet, but ensure that it is not touching. Switch on the filament current, and allow a bend to be introduced into the end of the pipet. Switch off the current when the end of the pipet is 15° to the horizontal (Fig. 2).

A single holding pipet will usually suffice for a single microinjection session. Holding pipets cannot be reused. Large numbers of holding pipets can be made in advance and stored in a Petri dish. The structure of a holding pipet is diagrammed in Fig. 2.

5. Microinjection pipets: A microinjection pipet is used to introduce the DNA fragment physically into the nucleus of the fertilized one-cell egg. Microinjection pipets are prepared from thin-walled glass capillaries with an external diameter of 1 mm. Capillaries with an internal filament (e.g., Clark Electromedical Instruments [Reading, UK], catalog number GC 100 TF-15) are useful, because they allow the pipet to be back-filled, by capillary action, from the end distal to the injection tip. Microinjection pipets are drawn using a commercially available pipet puller (e.g., David Kopf [Tujunga, CA] Verticle Pipette Puller Model 720, or the Narishige PB-7). Injection tips should have a $1\text{-}\mu M$ opening. A larger tip will burst the egg, whereas a smaller tip will easily block. Microinjection pipets should be prepared as needed.
6. The microinjection chamber: Glass depression slides are suitable microinjection chambers for inverted microscopes fitted with any optical system. Glass depression slides should be siliconized by rinsing in a solution of 3% (v/v) dichloromethyl silane in chloroform. The slide is then thoroughly rinsed with water and a standard household detergent. Prior to use, rinse the slide with ethanol, and dry carefully with tissue paper. Ensure that the slide is devoid of dust particles.
7. Agla micrometer syringe (Wellcome, Kent, UK) or equivalent

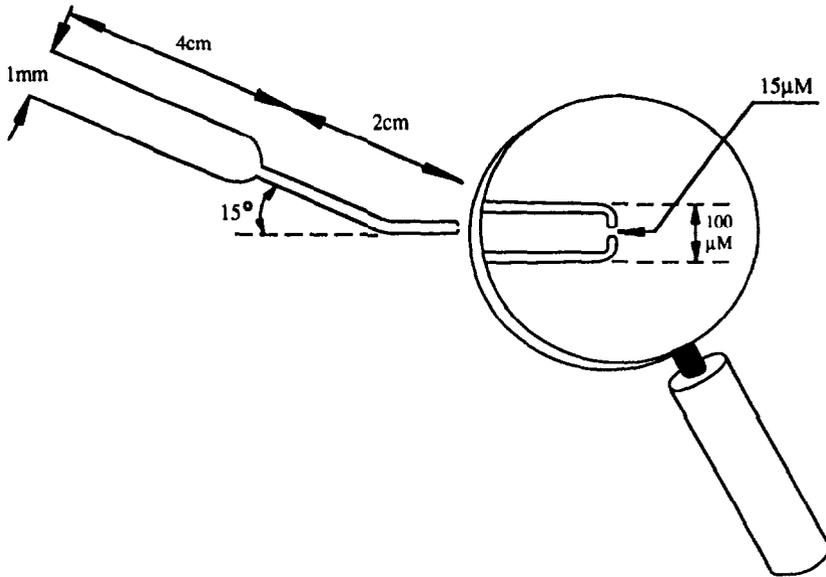


Fig. 2. Construction of a typical holding pipet.

8. Microinjection pump: This can be as simple as an air-filled 50-mL syringe with a ground-glass plunger, or as sophisticated as an automatic injection system (e.g., Narsihige Pico-Injector PLI-188).
9. M2 culture media (Chapter 16).
10. M16 culture medium (Chapter 16).
11. CO₂ tissue culture incubator (37°C, 5% [v/v]).
12. Light liquid paraffin.
13. 26-g needles (5-cm long).
14. Tygon (Akron, OH) tubing ($\frac{3}{32}$ in id, $\frac{5}{32}$ in od).
15. One clamp stand.
16. Diamond pencil.
17. Disposable 1-mL syringes.
18. Fluorinert electronic liquid (3M company [St. Paul, MN], FC77).
19. Dissecting microscope (e.g., Nikon SMX10TD).

3. Methods

3.1. Assembly of the Microinjection System

A typical microinjection system is shown in Fig. 1.

3.1.1. The Microinjection Chamber (Fig. 3)

1. Place a flat drop of M2 embryo culture medium (around 100 μL) in the well of an ethanol-washed, dried, siliconized glass depression slide.
2. Cover the M2 drop with light liquid paraffin oil to prevent evaporation.
3. Place the injection chamber on the stage of then inverted microscope, and using the 4X objective, focus on the bottom of the M2 drop

3.1.2. The Holding Pipet (Fig. 4)

1. Connect the Agla micrometer syringe to a Leitz instrument tube using 1 m of Tygon tubing. Fill the system with light paraffin oil, making sure that air bubbles are excluded. Position the Agla micrometer syringe on a clamp stand in a convenient position close to the left-hand micromanipulator
2. Fill a holding pipet with Fluorinert electronic liquid using a 5-cm long 26-g needle connected to a 1-mL disposable syringe. Insert the holding pipet into the paraffin-oil-filled instrument tube. Tighten the instrument tube captive nut to secure the holding pipet in position.
3. Clamp the instrument tube into the instrument tube holder located on the left-hand micromanipulator. Rapidly adjust the Agla micrometer syringe until liquid stops flowing out from the end of the holding pipet. Do not allow air to flow into the holding pipet.
4. Position the tip of the holding pipet around 2 cm above the center of the microinjection chamber. Manipulate the instrument tube, and adjust the instrument tube holder such that the tip of the holding pipet is horizontal and straight. Observe the holding pipet through the microscope using a 4X objective lens. The shadow of the pipet ought to be visible. If it is not, gently lower the pipet. Further lower the holding pipet into the microinjection chamber by adjusting the fine controls of the micromanipulator. Monitor the process by observation through the 4X objective lens. Adjust the manipulator such that the holding pipet is just above the bottom of the injection chamber. The pipet should move freely in the horizontal plane throughout the field of view when operated by the joy-stick control. Ensure that the pipet does not catch on the bottom of the injection chamber.
5. Adjust the Agla micrometer syringe until the meniscus between the M2 medium in the injection chamber and the Fluorinert in the holding pipet is located at the shoulder of the bend in the holding pipet.

3.1.3. The Microinjection Pipet (Fig. 3)

- 1 Connect a Leitz instrument tube holder via a 1-m length of Tygon tubing to either a 50-mL syringe with a ground-glass plunger (lubricated with liquid paraffin), or an automatic injection system (*see* Chapter 19).

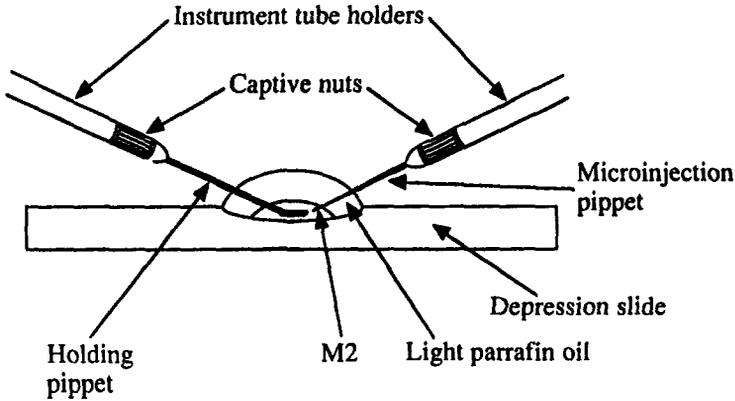


Fig. 3. Side view of the microinjection chamber.

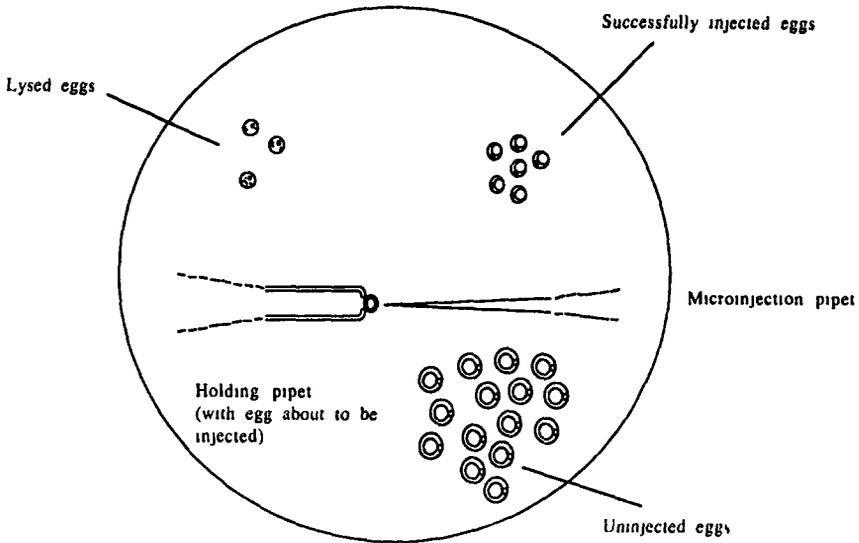


Fig. 4. Low magnification (40X) view of the microinjection chamber.

The system is air-filled.

2. Back-fill a freshly drawn microinjection pipet with DNA solution (*see* Notes 1-3). Place the end distal to the tip into the DNA solution, and allow the liquid to ride into the tip by capillary action via the internal filament. As soon as liquid can be seen in the tip, withdraw the pipet from the DNA solution.

3. Position the pipet into an instrument tube, and secure by tightening the captive nut. Place the instrument tube in the instrument tube holder of the right-hand micromanipulator. Using the instrument tube holder controls, move the tip of the injection pipet until it is 2 cm above the injection chamber. The tip should be at an angle of approx 15–20° to the horizontal. Using the fine vertical control of the micromanipulator, lower the tip into the injection chamber until it is just above the chamber floor. Observe this procedure through the microscope using a 4X objective. To avoid breaking the tip, ensure that the pipet does not contact the bottom of the injection chamber.
- 4 Using the horizontal controls of the micromanipulators, bring both the injection pipet and the holding pipet to the center of the field of view. Switch to the 40X Nomarski objective, and focus on the holding pipet. Using the fine vertical micromanipulator control, bring the tip of the injection pipet into the same focal plane as the holding pipet. Ensure now that the microinjection pipet can move freely in the horizontal plane when operated by the joy-stick control.

3.2. Microinjection of Fertilized One-Cell Eggs

1. Remove 20 fertilized one-cell eggs from microdrop storage in the 37°C, 5% CO₂ incubator (*see* Note 4). While observing the eggs using a dissecting microscope, transfer the eggs into M2 medium contained in a 35-mm tissue culture Petri dish. Rinse the eggs in M2. Discharge the eggs into the injection chamber using a mouth-operated general egg-transfer pipet. Observe this procedure under the microscope using the 4X objective. Attempt to keep the eggs grouped together as they are discharged. Locate them in the bottom half of the field of view, just below the holding and injection pipets (Fig. 4). When discharging the eggs into the injection chamber, be sure to avoid the release of air bubbles, which, at best, will obscure the view of the eggs, and, at worst, will result in the loss of the eggs and necessitate the reassembly of the microinjection system.
- 2 Using the fine vertical micromanipulator controls, readjust the vertical position of the holding and microinjection pipets such that they are located in the same focal plane as the eggs.
3. Using the horizontal joy-stick micromanipulator control, bring the tip of the holding pipet up to an egg. Gently rotate the Agla syringe control such that the egg is drawn onto the tip of the pipet. The egg should be firmly held on the end of the holding pipet, but care should be taken to avoid distorting and, hence, damaging the egg.
4. Move the egg to the center of the field of view, and then switch to the

40X Nomarski objective lens. Focus up and down to locate the two egg pronuclei. The larger of the pronuclei (the male pronucleus) is usually targeted. It may be necessary to move the egg in order to locate the targeted pronucleus in a convenient position for injection. The egg can be turned by gently expelling it from the end of the holding pipet. Then, using the holding pipet, the egg can be rolled into a suitable position before being sucked back onto the pipet tip.

5. Ensure that the egg is tightly held by the holding pipet by applying half a turn to the Agla syringe control. Ensure that the egg is not damaged by the application of too much suction, although distortion of the egg zona pellucida is not a problem.
6. Carefully focus on the targeted pronucleus. Using the right-hand horizontal micromanipulator joy-stick control, bring the microinjection pipet tip up to the zona pellucida. Adjust the fine vertical micromanipulator control to bring the microinjection pipet tip into the same focal plane as the pronucleus. Squeeze hard on the 50-mL injection syringe. It is sometimes possible to see the DNA solution being ejected from the pipet end either by the observation of the mixing with the M2 medium, or by observation of a slight movement of the egg or of contaminating particulate material in the medium. This is a useful indication that the microinjection pipet is not blocked.
7. With one rapid, but smooth movement, introduce the tip of the microinjection needle into the targeted pronucleus by penetrating the zona pellucida and the egg membrane (*see Note 5*). As soon as the pipet tip appears to be in the nucleus, squeeze hard on the 50-mL injection syringe (operated with the left hand). One of three things will now happen:
 - a. Successful injection is indicated by the swelling of the pronucleus (Fig. 5). Continue to introduce DNA into the nucleus by exerting pressure on the 50-mL syringe until the nucleus has reached approximately twice its normal volume. Then withdraw the injection pipet in a single, smooth, rapid movement.
 - b. Failure to penetrate the elastic cell egg membrane is indicated by the appearance at the microinjection pipet tip of a small, clear drop of liquid (Fig. 6). Such is the elasticity of the egg membrane that it can be pushed to form an invagination stretching from one side of the egg to the other. To penetrate the egg membrane, continue to push the microinjection pipet as far as the holding pipet (being careful not to damage the needle by knocking it against the holding pipet tip). Experienced egg microinjectors can “feel” the egg membrane give way. Pull the tip back to the nucleus, and then squeeze on the microinjection syringe again.

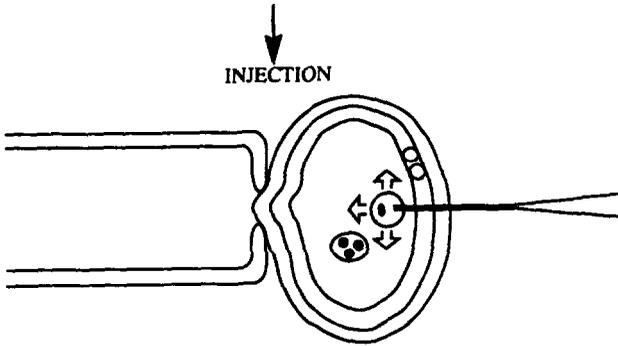
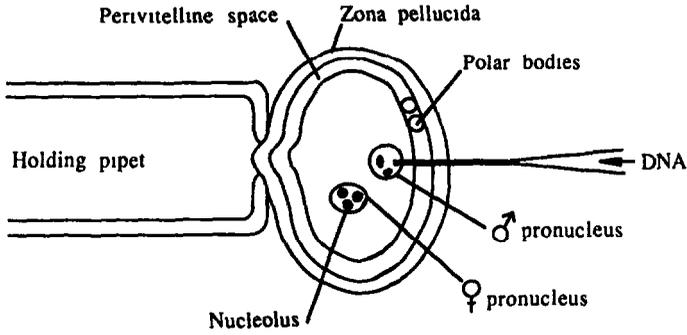


Fig. 5. Injection of fertilized one-cell eggs. High-magnification (400X) microscopic view.

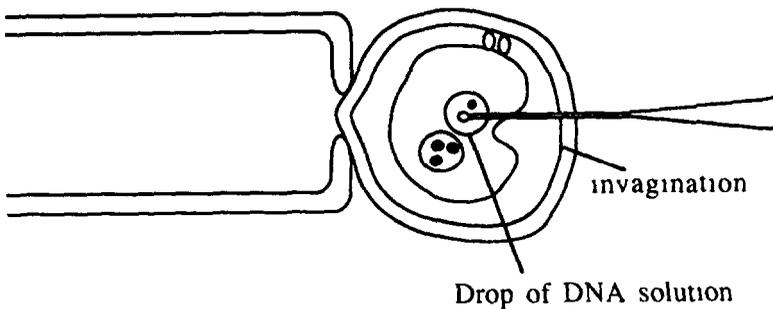


Fig. 6. Failed penetration of the fertilized one-cell egg owing to the elasticity of the egg membrane.

- c. A blockage of the microinjection pipet is indicated by the lack of ejection of any material from the pipet tip. The pipet is probably blocked and should be changed. Alternatively, the pipet puller may be producing microinjection pipets with sealed tips or tips with excessively small openings. Try adjusting the pipet puller such that the tip aperture is slightly larger. Finally, it may be necessary to replace the stock of DNA solution if it has become contaminated with particulate material that could block the pipet tip.

Some investigators are able to enlarge the aperture of a blocked or sealed tip by rubbing it gently against the holding pipet. However, the resulting tip may well damage the eggs.

8. Following the withdrawal of the microinjection pipet from the egg, cytoplasmic particles may be observed to flow out of the egg into the perivitelline space. This is indicative of egg lysis. If the eggs lyse following two or three successive attempts at injection, the microinjection pipet should be replaced.
9. Switch back to the 4X objective lens, and move the injected egg away from the microinjection pipet. Eject the egg from the holding pipet. Injected eggs should be sorted into two groups—those that have survived injection, and those that have not (Fig. 4).
10. Inject all the eggs in the batch, and then return those that have survived to M16 microdrop culture at 37°C in a 5% CO₂ tissue culture incubator via two washes in 5% CO₂ equilibrated M16
- 11 Eggs that have survived injection (*see* Note 6) are returned by oviduct-transfer surgery and returned to the natural environment afforded by a pseudopregnant recipient female (*see* Chapter 20). Eggs can be transferred into the surrogate recipient on the same day as injection, or they can be transferred following culture overnight when most will have developed to the two-cell stage.

4. Notes

- 1 The quality of the solution of DNA introduced into the egg is crucial to the success of the method. DNA fragments for microinjection should be purified as described in Chapter 11. The composition of the solvent in which the DNA is dissolved is extremely important. The presence of even small amounts of Mg²⁺ will kill an injected egg, as will an excessively high concentration of EDTA. It has been determined that the optimal buffer consists of 10 mM Tris-HCl, pH 7.4, containing EDTA to 0.1–0.25 mM (microinjection TE, or MITE; 7).
- 2 The DNA should be injected at a concentration of 1–5 mg/mL. Excessively high DNA concentrations can kill the injected egg (7), but gener-

ally there is no correlation between the number of DNA molecules introduced, and the structure or copy number of the resulting transgene.

3. The physical state of the injected DNA has little effect on the success of a transgenic experiment. Cosmid clones of up to 50 kb in length can be introduced with the same efficiency as smaller molecules (4). Linearized DNA fragments apparently are able to integrate at an efficiency fivefold greater than circular molecules (7). The structure of the DNA fragment ends created by different restriction endonucleases has no effect on the efficiency of generating transgenics, or on the organization of the resulting transgene (7,8).
4. Fertilized one-cell eggs should be maintained in M2 for as brief a period as possible (maximum 30 min to 1 h). Thus, eggs held in microdrop culture should be processed for injection in small, manageable batches. A skilled operator ought to be able to inject 20–40 eggs in 15 min.
5. When injecting the pronucleus, it is worth trying to avoid the sticky nucleolus. If this attaches to the injection pipet, it may be drawn out of the nucleus, thus killing the egg.
6. In the hands of a practiced investigator, 80% of eggs ought to survive the insult of microinjection.

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CHAPTER 19

Microinjection of Cloned DNA Fragments into Fertilized One-Cell Mouse Eggs

II. Automatic Injection

David Murphy

1. Introduction

In recent years, a number of automatic microinjection systems have appeared on the market. These systems replace the simple manual syringe system for forcing the DNA solution out of the microinjection pipet and into the pronucleus of a fertilized one-cell egg. The advantages of such automatic systems are twofold: (1) Because injection is triggered by a foot-operated peddle, the hands are left free to operate the joy-stick controls of the micromanipulators. Since the hands are not constantly moving from one piece of apparatus to another, the process of microinjection is speeded up considerably. (2) Through the application of a low, constant (balance) pressure, DNA solution is flowing out of the holding pipet throughout the injection session. This prevents back-flow of M2 medium into the injection pipet, which would otherwise considerably dilute the DNA solution, and it also prevents blockage of the pipet. Using an automatic injection system, it is found that pipets need not be changed as often as required when using a manually operated system. This chapter describes the operation of an economical injection system supplied by the Narishige company (Tokyo).

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2. Materials

1. Narishige Pico-Injector (PLI-188).
2. Compressed air supply: The input gas pressure must be 105–150 psi. The Hitachi (Tokico) Package Oilfree Bebicon PO-O 75PSB (maximum output 121 psi) is compatible with the Narishige injection system. Bottled gas can be used if a regulator is fitted.
3. Connecting tubings (refer to manufacturer's instructions). The assembly and testing of the system, particularly the high-pressure gas supply, should be carried out by a trained professional.
4. Injection DNA (*see* Chapters 10 and 11).

3. Methods

3.1. Assembly of the Narishige Injection System

Note: The initial assembly of the Narishige Pico-injection system should be carried out by a trained operative.

1. The air supply is connected to the injection system via the Pressure Input port on the back panel of the instrument (Fig. 1).
2. The injection system is connected to the instrument tube via the P_{OUT} outlet on the front panel of the instrument (Fig. 2).
3. Care must be taken to ensure that there are no air leaks in the system.
4. Plug in the injection foot pedal to the appropriate foot switch input on the back panel of the instrument.
5. The microinjection system is assembled exactly as described in Chapter 18, except that the 50-mL syringe used for manual operation of the injection is replaced by the Pico-Injector.

3.2. Operation of the Automatic Injection System

1. With the output tap closed, switch on the Hitachi Bebicon air compressor, and allow the air tank to charge (around 2 min).
2. Switch on the Pico-Injector. Turn down the balance ($P_{BALANCE}$) and injection (P_{INJECT}) pressure taps to zero (Fig. 1). The output pressure of these modes is on the PRESSURE digital display when the PRESSURE METER SOURCE control is switched to the appropriate position.
3. Open the tap of the compressor air tank to allow pressurized air into the Pico-Injector
4. Set the balance and injection pressures by manipulation of the appropriate valves. Initial balance pressure ($P_{BALANCE}$) should be set at between 2 and 5 psi.
5. Load a microinjection pipet with DNA (*see* Chapter 18, Manual Injection), secure it in the instrument tube, and position it in the injection chamber as described in Chapter 18.

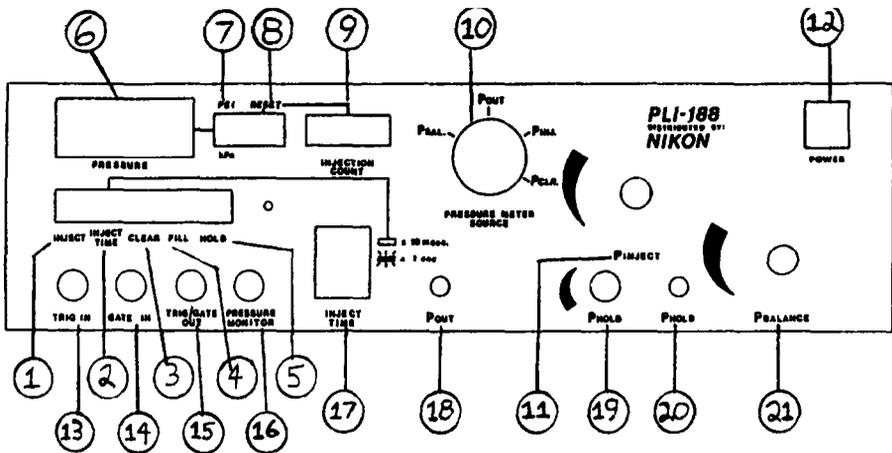


Fig. 1. The Pico-Injector PLI-188. Reproduced with kind permission of Nikon-Narishige. Front panel controls and connections. 1. INJECT push button. This is pushed to trigger the injection pressure manually for the time set by the internal timer. 2. INJECT TIME push button. This determines the time multipliers (10 ms or 1 s) for the internal injection timer. 3. CLEAR push button. Pushing this button activates a 0.5-s pressure surge (supply pressure) to clear a clogged pipet. The surge can be extended by pushing the button for longer than 0.5 s. 4. FILL push button. This button is pushed to apply suction to the delivery pipet in order to fill it from the tip. 5. HOLD push button. This button is pushed to apply a small suction to a holding pipet. A cell can be thus held in place for injection. Pushing the button a second time releases the suction. Our experience has been that sticky cells, like fertilized one-cell mouse eggs, fail to be released. 6. PRESSURE display. Displays the "gage" pressure selected by the PRESSURE METER SOURCE switch. 7. PRESSURE UNITS push button (select psi or kP). 8. RESET push button. Resets the injection count to zero. 9. INJECTION COUNT display. Displays total number of manual injections triggered. 10. PRESSURE METER SOURCE switch. Selects the various measured pressures engendered by the system. P_{INJ} is the pressure applied during injection. P_{BAL} is the constant applied pressure. P_{CLR} is the supply pressure and that delivered during operation in the CLEAR mode. P_{FILL} is the negative pressure applied in FILL mode. P_{OUT} is the pressure currently being delivered to the output port. 11. P_{INJ} control. Used to set the injection pressure (0.4–60 psi). 12. POWER push button. AC power on or off. 13. TRIG IN connector. This connector is for electrical initiating injection. 14. GATE IN connector. This connector is for external timing of the duration of injection. 15. TIG/GATE OUT connector. This output can be used to trigger or synchronize other electrical instruments. 16. PRESSURE MONITOR out. This connector delivers an electrical level whose amplitude gives the actual pressure applied to the meter. 17. INJECT TIME switch. This two-digit switch is used to set the duration of injection when timed internally. The units are set by the INJECT TIME switch. 18. POUT connector. This connector is attached to the injection pipet using the output hose supplied with the instrument. In use, the injection, balance, fill, and clear pressures are delivered through this port. 19. P_{HOLD} control. Sets the low suction (0–0.1 psi) applied to the P_{HOLD} connector. 20. P_{HOLD} connector. This connector is attached to the holding pipet and delivers the low suction pressure set by the P_{HOLD} control. 21. P_{BAL} control. This control sets the balance pressure (0.1–10 psi).

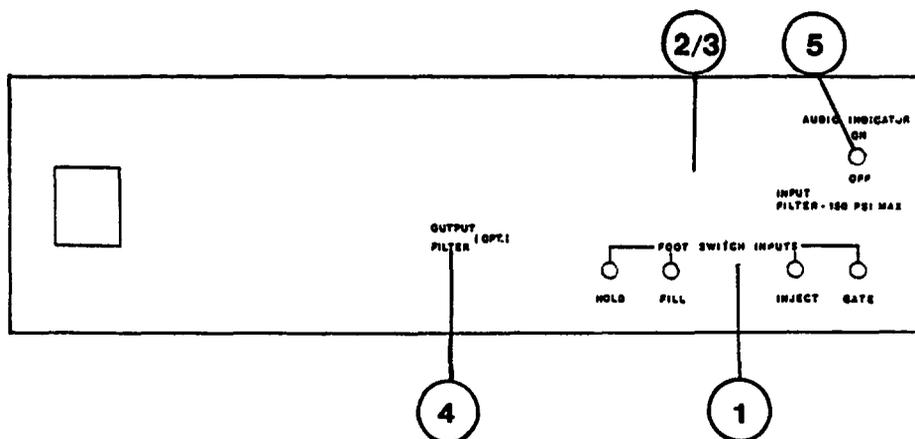


Fig. 2. The Pico-Injector PLI-188. Reproduced with kind permission of Nikon-Narishige. Rear panel controls and connections. 1 FOOT SWITCH INPUTS. These optional footswitch connectors can be used to enable remote, foot-operated control of the HOLD, INJECT, FILL, and GATE functions. 2. PRESSURE INPUT connector. This input delivers compressed gas to the instrument (105–150 psi). 3. INPUT FILTER. The input filter removes particles larger than $0.1\ \mu\text{m}$ as well as liquid from the input gases. 4. OUTPUT FILTER (optional). An output filter is recommended if the FILL function is being used. 5. AUDIO INDICATOR switch. A buzzer, which sounds during injection, is activated and inactivated by this switch.

6. The injection pressure (P_{INJ}) must be determined empirically. The optimum procedure would be to match the Pico-Injector parameters with a particular set of pipet puller settings that give good injection pipets. If these parameters are not changed between injection sessions, then only minor adjustments will be necessary when setting up the system at the beginning of each session.
7. Set the injection mode to GATE (i.e., manual) mode. Thus, length of the injection is controlled by foot pedal.
8. Proceed with the egg injection as described in Chapter 18, except that the introduction of DNA into the egg nucleus is triggered by foot pedal activation of the Pico-Injector inject mode.

4. Notes

1. The inject mode of the Pico-Injector can be ignored, and injection achieved by using the constant BALANCE outflow.
2. The Pico-Injector has a separate internal vacuum pump that can be connected to the holding pipet. Also foot-pedal operated, this system poten-

tially allows the holding pipet, now an air-filled system, to be remotely operated. Depression of the foot pedal activates the suction pump, which draws an egg onto the holding pipet. Further depression of the foot pedal switches off the vacuum pump, and the egg is released. However, in practice, the stickiness of the egg prevents release from the end of the pipet. The positive pressure exerted by the manual, oil-filled Agla micrometer syringe system is necessary to expel the egg.

3. The Pico-Injector is fitted with a CLEAR mode, activation of which momentarily forces air at 120 psi through the injection pipet, thus clearing any blockages.
4. By operation of the FILL push button, suction is applied to the delivery pipet, allowing microinjection pipets to be loaded with injection solution from the tip.
5. The injection count display records the number of times the injection mode is activated.
6. Every so often during operation, the air pressure in the Air Compressor tank will drop below threshold level, activating the air pump. The pump operates for 10–20s and, although rather noisy, should not affect the microinjection process.

CHAPTER 20

Delivery of Microinjected Eggs to Surrogate Mothers by Oviduct Transfer

David A. Carter

1. Introduction

Following the microinjection procedure (*see* Chapters 18 and 19), the eggs must be transferred to pseudopregnant (0.5 d pc) recipient mothers. Embryos from the one-cell through the morula stage (0.5–2.5 d pc) are transferred into the ampullae by a procedure called an oviduct transfer (1), described herein. The oviduct transfer is preferably performed on the same day as the microinjections or, alternatively, on the following day. The latter timing will allow the experimenter to observe whether injected eggs have developed to the two-cell stage, although it is preferable to minimize the time in culture. The number of eggs transferred to recipients should be targeted to produce a litter size of five to ten. In practice, the number of eggs required to achieve this number will depend on the competence of the experimenter and other factors, such as the quality of the DNA preparation (*see* Chapter 11). A suitable number of eggs delivered to each recipient mouse is 30. Oviduct transfer is not an easy technique, and novice experimenters are advised to practice first on cadavers, until they are happy with the procedure. A convenient dye may be used in place of eggs to visualize correct delivery of the pipet contents into the ampulla.

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1.1. Preliminary Procedure

In addition to practicing the oviduct-transfer technique itself, one other technique must also be learned before starting an experiment, namely, the preparation and loading of oviduct-transfer pipets. A similar, although finer, pipet to the egg-transfer pipet (*see* Chapter 17) is required to deliver eggs to the recipient's oviduct. The pipet is fashioned exactly as described for the transfer pipet (*see* Chapter 17), except that the "pulled" region should have an internal diameter of around 150 μm . The small diameter (just large enough to accommodate eggs) is essential for a successful oviduct transfer. The oviduct-transfer pipet is further modified compared with the simple transfer pipet by flame-polishing the tip. This is achieved by touching the end of the pipet momentarily in the gas flame. Generally, a sufficient degree of "rounding off" will occur if the pipet is withdrawn as soon as a yellow flame becomes visible at the tip. Correct loading of the oviduct-transfer pipet is also an essential feature of good transfer technique and should be practiced prior to an important experiment. The loading arrangement is shown in Fig. 1. The pipet is first filled with light paraffin oil to just past the shoulder. The viscosity of the oil provides greater control over the movement of eggs. A small bubble of air is then taken up, followed by some M2, and then another bubble. The eggs are then collected, preferably in a stacked rank with a minimal volume of medium. A third air bubble is taken up followed by a final column of M2. The total length of the eggs/bubbles/medium should not exceed 2 cm. The loaded pipet, attached to the mouth pipetting system, may be conveniently stored during the surgery by fixing it to a piece of plasticene on the surgical microscope.

2. Materials

1. M2 medium (*see* Chapter 16).
2. 70% Ethanol in squeeze bottle.
3. 2.5% Avertin (*see* Chapter 14) (or alternative anesthetic).
4. Light paraffin oil (Fluka [Buchs, Switzerland] or other; *see* Chapter 17).
5. 35-mm Sterile tissue-culture dishes
6. Hard glass capillaries, 1.5 mm od (BDH, Ltd., Poole, UK).
7. Mouth pieces, which also double as pipet holders (Arnold R. Horwell Ltd., West Hampstead, London, UK).
8. Pipet holders.
9. Flexible connecting tubing for pipet system.

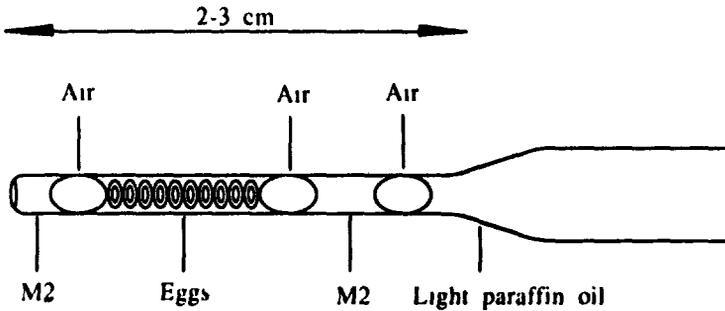


Fig. 1. The tip of an oviduct-transfer pipet enlarged to show the arrangement of eggs, air bubbles, and media.

10. Diamond pencil.
11. Dissecting scissors, one regular, one fine.
12. Watchmaker's forceps 2 × #5.
13. Fine blunt forceps, one pair.
14. Surgical silk suture (5.0).
15. Curved surgical needles (size 10, triangular, pointed).
16. Autoclips and applicator (9 mm, Clay Adams, Becton Dickinson, Parsippany, NJ).
17. Artery clip (1- $\frac{1}{2}$ in).
18. Sterile disposable 1-mL syringes.
19. Sterile disposable 0.5 × 16 mm needles.
20. Petri dish lid (9 cm).
21. Stereo microscope with understage illumination (e.g., Nikon [Tokyo] SMZ-10TD).
22. Surgical microscope with optional assistants viewing head (e.g., Carl Zeiss Jena [Oberkochen, Germany] OPM 212T with head model 050).
23. Fiber optic light source (e.g., Nikon).
24. Animal balance.
25. Alcohol burner.

3. Methods

1. Surgical instruments should first be sterilized by dipping in 100% ethanol and flaming with the alcohol burner.
2. Anesthetize a 0.5-d pc pseudopregnant recipient female as described (see Chapter 14).
3. Place the animal ventral side down on the 9-cm Petri dish lid. After soaking the mouse's back with 70% ethanol, a small transverse incision (<1 cm) is made with the regular dissecting scissors, about 1 cm to

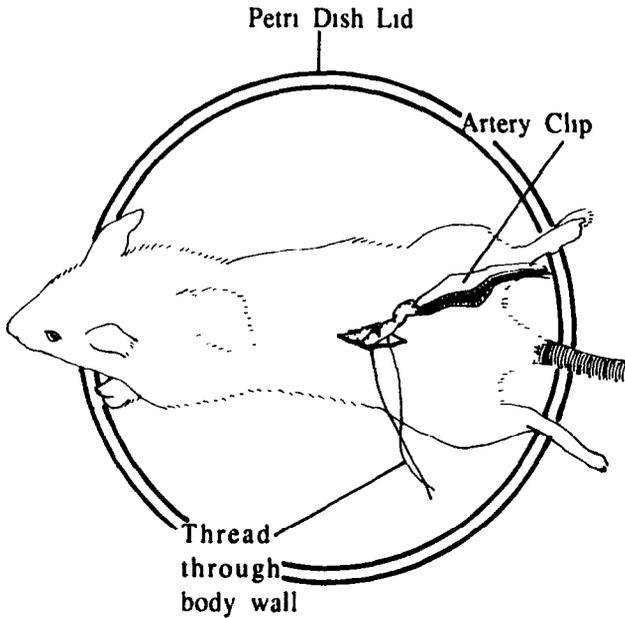


Fig. 2. Dissection of the mouse for oviduct transfer. Schematic diagram of an anesthetized mouse showing the exteriorized ovary and oviduct held with an artery clip that is attached to a fat pad. See Fig. 3 for fine details.

the left of the spinal cord, at the level of the last rib (Fig. 2). The incision should be stretched to prevent bleeding.

4. The orange-colored ovary (and possibly a white fat pad attached) should be visible beneath the body wall. A 3–5 mm cut should be made through the body wall at a point a few millimeters away from the ovary, using the fine scissors. Stretch the incision to prevent bleeding.
5. A single stitch should be introduced through the body wall on one side of the incision and left in place.
6. Using the fine, blunt forceps, pull out the fat pad joined to the ovary. The oviduct and uterus will be pulled out simultaneously. Attach the artery clip to the fat pad, taking care to avoid the ovary. The reproductive tract may then be held in position over the back of the animal by the artery clip. Ensure that the coils of the oviduct are uppermost (Fig. 2).
7. The preparation should then be moved (on the Petri dish) to the stage of the surgical microscope. Illumination may be provided by the microscope itself or from the fiberoptic light source. The experimenter may find it necessary to use both for the subsequent microsurgery. The oviduct should be viewed under 10–20X magnification.

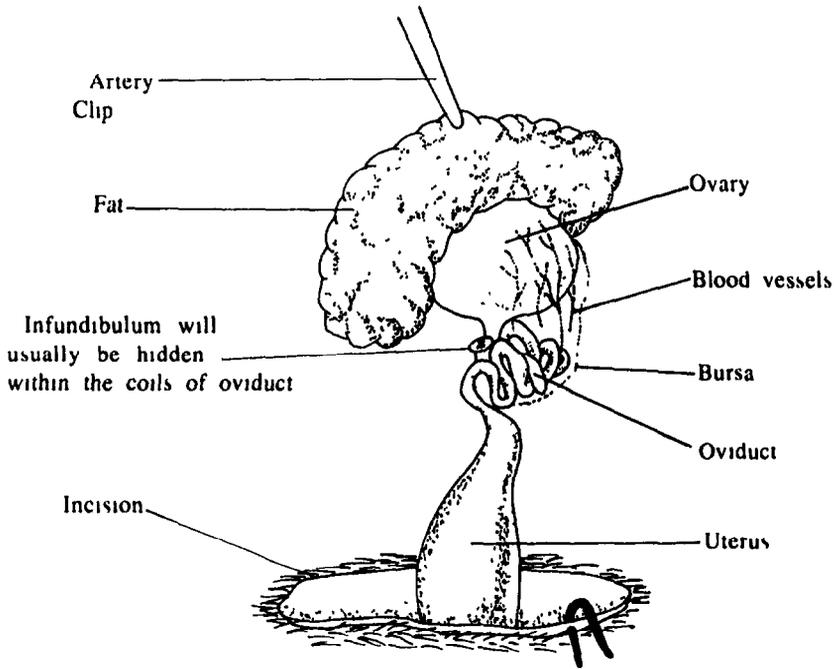


Fig. 3. Schematic diagram of the mouse ovary and oviduct prepared for oviduct transfer. The infundibulum is located within the coils of the oviduct and may be accessed by penetrating the transparent membrane (bursa) that covers the cavity, oviduct and ovary.

8. Gently orient the oviduct coils to reveal a cavity (*see* Fig. 3) that lies below the ovary and behind the coils of the oviduct. The opening of the oviduct or infundibulum (the target of the transfer procedure) is located within this cavity behind a transparent membrane, the bursa, which covers the cavity, oviduct and ovary (*see* Fig. 3). Part of the skill in performing a successful oviduct-transfer operation lies in ripping the bursa to the extent that the infundibulum is readily accessible while not causing unnecessary damage and bleeding. If excessive bleeding is frequently encountered, preventative measures may be taken (*see* Note 1). The bursa should be torn with watchmaker's forceps at a point above the infundibulum and away from capillaries. If the infundibulum is not visible through the bursa, simply rip the membrane at a convenient point, and continue the search. The infundibulum may be lifted out of the cavity when gently gripped with watchmaker's forceps.
9. The eggs should now be prepared for transfer as described above (*see* Section 1.1.)

10. Having carefully stored the transfer pipet, return to the mouse, and mop up any excess blood using small screws of tissue paper held in the watchmaker's forceps.
11. Pull out the infundibulum using watchmaker's forceps such that the opening can be accessed by the oviduct-transfer pipet. The experimenter must decide which hand offers the finest control to achieve this operation. Using the other hand, push the tip of the transfer pipet toward the top of the infundibulum. The opening is not visible until penetrated and may be located by gentle prodding with the tip of the pipet. The tissue will "give" at the opening, and the pipet will enter easily. Push the pipet into the infundibulum until it has entered the ampulla. Correct placement is important: The pipet tip must be far enough into the infundibulum so that it does not fall out when the eggs are expelled (a common error), but not so far in that the opening is against the wall of the ampulla, so restricting escape of the eggs.
12. Expel the contents of the pipet into the ampulla, and monitor delivery of the eggs (which are not visible) by the appearance of bubbles. The appearance of three bubbles will ensure that all the eggs have been expelled. If excessive pressure is required, it is possible that the tip of the pipet will be forced against the wall of the ampulla: Withdraw slightly. Alternatively, the tip of the transfer pipet may become blocked with clotted blood. If this occurs, it may be necessary to return to the stereomicroscope and reload the pipet.
13. Withdraw the oviduct-transfer pipet. After removing the artery clip, grip the fat pad with a pair of blunt forceps, and return the reproductive tract back inside the body wall. Sew up the body wall with a stitch, and then clip the skin together with an autoclip.
14. Repeat on the other side, provided that additional eggs are available and the mouse remains fully anesthetized.
15. Return the mouse to its cage, and leave it undisturbed in a warm area until it has recovered from the anesthetic (*see* Note 2). Label the cage with details of the DNA construct, number of eggs transferred, and the date.

4. Notes

- 1 Excess bleeding can be prevented by a prior application of epinephrine (Sigma [St. Louis, MO], 20–50 μL of 0.1% solution diluted in 0.01M HCl) made through the bursa at the top of the oviduct using a fine needle (30 g) and 1-mL disposable syringe.
- 2 Although laboratory rodents can survive operations with minimal precautions against infection, it has been reported that treatment with antibiotic will increase oviduct-transfer efficiency, presumably by

preventing minor infections of the oviduct. For rats, at least, a single postoperative injection of Binatol (50 mg, ip), an ampicillin-based product from Bayer, has been shown to be effective (Mullins, J. J., personal communication).

Reference

1. Hogan, B., Constantini, F., and Lacy, E. (1986) *Manipulating the Mouse Embryo—A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

CHAPTER 21

Caesarean Section and Fostering

David Murphy

1. Introduction

There is great variation in the survival rate of microinjected eggs introduced into pseudopregnant recipient female mice. If the survival rate is low, then the few eggs that do develop tend to be “overnourished” and grow into larger fetuses compared to the embryos of a normal-sized litter. Under such circumstances, the overgrown embryos can sometimes fail to be delivered, because their size prevents normal passage down the birth canal. If pregnancy proceeds for 2 or 3 d beyond the normal gestation period of 19–21 d, there is a danger that the pups will die *in utero*. Such pups can be rescued by caesarean section and fostering. Fostering may also be needed to save the valuable transgenic, but unweaned, pups born normally to a mother that unexpectedly dies.

2. Materials

1. 70% (v/v) Swabbing ethanol in a squeeze bottle.
2. Sharp, fine dissecting scissors.
3. Sharp watchmaker’s forceps (size 5).
4. Blunt forceps.

3. Methods

3.1. Caesarean Section

1. Kill the mother by cervical dislocation (Chapter 17).
2. Soak the abdomen in swabbing ethanol.
3. Skin the lower half of the animal, and cut open the body wall to reveal

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the pregnant uterus. Carefully remove the uterus by cutting at the oviducts and the cervix, and by tearing away the attached membranes.

4. Carefully cut the uterus into sections, each containing a single pup. Gently squeeze the pups out of the uterine tube using blunt forceps.
5. Carefully dissect away the membranes that surround the pups, and cut the umbilical cord.
6. Wipe away any fluid from the area of the nose and the mouth, and then gently and rhythmically squeeze the chest of the animal with blunt forceps in order to stimulate breathing.
7. Place the pups on a prewarmed damp tissue, and keep warm (around 30°C) until fostered.

3.2. Fostering

1. Remove the foster mother from her pups (*see* Notes 1 and 2).
2. Introduce the fostered pups to the natural pups, and mix them up.
3. Attempt to make the foster mother urinate on the mixed litter. Urination can often be stimulated simply by restraining the female with one hand as described in Chapter 14.
4. Release the mother into the cage containing the pups, and leave undisturbed for at least 3 h.
5. Remove as many of the natural pups to leave a litter of around 12. The foster mother will be unable to care for a larger litter.

4. Notes

1. The age of the natural pups of the foster mother should be approximately the same as the pups to be fostered, give or take 1 or 2 d.
2. The foster mother should be of a strain with good maternal characteristics. Preferably, the coat color of the natural pups should distinguish them from the fostered pups. Transgenic pups derived from (CBA/J)/(C57Bl) F2 eggs will be black or agouti, and can be readily distinguished from the albino pups of, for example, the Swiss 3T3 strain.

CHAPTER 22

In Vitro Fertilization of Mouse Eggs

David A. Carter

1. Introduction

Some transgenic animals may prove to be infertile through an inability to mate or rear a litter. It will therefore be necessary to perform an in vitro fertilization (IVF) procedure (1) in order to continue the line. The technique involves superovulating females and fertilizing the eggs with sperm taken from the dissected epididymis of males. IVF eggs are then transferred to a 0.5-d pc pseudopregnant recipient mother. The animals used will depend on the particular experiment; if C57BL/6J \times CBA/J F1 hybrids were used for the original transgenic production, these should also be chosen for the IVF procedure. Males should be at least 6 wk of age and preferably studs that were rested on the preceding night. Females should be at least 4 wk of age. The described protocol employs a single male and 10 females.

1.1. Preliminary Procedure

A specific medium (Whittingham's) is required for the IVF procedure. It should be made up according to Table 1.

2. Materials

1. Whittingham's medium (*see* Section 1.1.).
2. M16 medium (*see* Chapter 16).
3. 70% Ethanol in squeeze bottle.
4. Light paraffin oil (Fluka [Buchs, Switzerland] or other; *see* Chapter 17)
5. 35-mm Sterile tissue culture dishes.
6. Micropipet and sterile tips (e.g., Gilson [Villiers-le-Bel, France] Pipetman, P200).

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Table 1
Preparation of Whittingham's Medium for IVF of Mouse Eggs^a

Component	g/100 mL
NaCl	0.5803
NaHCO	0.2106
Glucose	0.1000
KCl	0.0201
Na HPO ₄ • 12H ₂ O	0.0056
Na pyruvate	0 0055
Penicillin	0.0063
Streptomycin	0.0050
CaCl ₂ • 2H ₂ O	0.0264
MgCl ₂ • 6H ₂ O	0.0102
Na lactate	0.35 mL of 60% syrup
Phenol red	0.1000

^aAll components are available from Sigma (St Louis, MO) and should be of tissue-culture grade. All containers should be sterile plastic, and water must be either double glass distilled or filtered (Milli-Q). The components should be weighed out, made up to 100 mL with water, filtered through a 0.45- μ m filter, aliquoted, and stored at -20°C. Prior to use, the medium must be supplemented with 30 mg/mL bovine serum albumin (Sigma, albumin, bovine [crystallized, lyophilized, essentially globulin free, cell-culture tested] # A 4161).

7. Egg transfer pipet and mouth-operated pipet system (*see* Chapter 17).
8. Dissecting scissors: 1 regular, 1 fine.
9. Watchmaker's forceps. 2 × #5.
10. Stereo microscope with understage illumination. (e.g., Nikon [Tokyo] SMZ-10TD).
11. Fiber optic light source (e.g., Nikon).
12. 37°C Incubator gassed with 5% CO₂.

3. Methods

1. Two days before the IVF procedure, start the superovulation of F1 females as described in Chapter 13.
2. On the day before the IVF procedure or at least several hours before (minimum 3 h), prepare drop cultures (*see* Chapter 17) of Whittingham's medium (supplemented with 30 mg/mL BSA) · 1 × 500 μ L drop and 1 × 1000 μ L drop in separate 35-mm dishes under oil. The dishes should be incubated at 37°C in 5% CO₂.
3. At least 1 h prior to the IVF procedure, prepare a 35-mm dish contain-

ing M16 for washing and another 35-mm dish containing M16 microdrop cultures (*see* Chapter 17). Incubate at 37°C in 5% CO₂.

4. Kill males by cervical dislocation (*see* Chapter 17) 12 h after the females have been injected with hCG. Place the animal ventral side up on a sheet of absorbant paper, and soak the lower abdomen with 70% ethanol. Locate the testes by palpating the lower abdomen. Make a small incision close to the testes with the regular scissors, and draw out the testes by gently gripping with watchmaker's forceps. The epididymus can be clearly observed as a white mass of coils at the base of the testes, which is colored gray/white by comparison. Gripping the epididymus carefully with the watchmaker's forceps, dissect out the epididymus using the fine scissors, taking care to remove any fat. The attached vas deferens (a fine white tube that leads away from the epididymus) should be cut as close as possible to the epididymus. Place the epididymus into the 500- μ L drop of pregassed Whittingham's medium under oil. Dissect out the other epididymus, and place it in the same drop of medium.
5. Gently squeeze out the sperm from the distal end of the epididymus using watchmaker's forceps. The sperm should emerge very easily in a continuous stream; if not, it may be necessary to puncture the end of the epididymus with the forceps (*see* Note 1). Speed is essential: The sperm should be maintained at room temperature for as short a time as possible.
6. Incubate the sperm for 1.5 h at 37°C under 5% CO₂ for capacitation to occur.
7. Kill the females 12.5 h post-hCG, and dissect out the oviducts as described (*see* Chapter 17). Place the oviducts into the 1000- μ L drop of pregassed Whittingham's medium (supplemented with 30 mg/mL BSA). Release the cumulus masses using an egg transfer pipet for manipulation as described (*see* Chapter 17). Incubate at 37°C in 5% CO₂.
8. At 13.5 h post-hCG, add 100 μ L of sperm suspension to the drop containing the eggs using a micropipet. Use sterile tips. The sperm concentration should be in the range $1-2 \times 10^6$ sperm/mL. It is not necessary to determine the concentration.
9. Incubate the IVF culture (egg/sperm mixture) for 4 h at 37°C in 5% CO₂. A minimum period of 3 h should be adequate if time is limited.
10. At the end of the fertilization period, the eggs should be transferred to a dish of warm, pregassed M16 and washed, using an egg transfer pipet (*see* Chapter 17), to remove as much of the sperm as possible. Next, transfer the eggs to M16 microdrop culture, and incubate at 37°C in 5% CO₂ overnight.
11. On the next day, transfer the eggs to 0.5-d pc pseudopregnant recipient

mothers as described (*see* Chapter 20). Fertilized eggs should be at the two-cell stage, although surviving one-cell eggs should not be discarded.

4. Note

1. Should sperm be difficult to obtain from the epididymus, it is advisable to quickly kill another male and repeat the dissection.

Reference

1. Hogan, B., Constantini, F., and Lacy, E. (1986) *Manipulating the Mouse Embryo—A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY

CHAPTER 23

The In Vitro Isolation of Murine Embryonic Stem Cells

Dave Wells

1. Introduction

Embryonic stem (ES) cells are derived directly from those progenitor cells of early mouse embryos that subsequently form all of the tissues of the fetus itself and that under appropriate culture conditions, can be maintained continuously in an undifferentiated state in vitro (1-3; see Note 1). When introduced into a mouse blastocyst or aggregated with morulae, however, the ES cells are capable of responding to in vivo developmental signals and participate in normal embryogenesis leading to the formation of chimeric offspring (4). Furthermore, ES cells are also capable of colonizing the trophoctodermal and primitive endodermal lineages of the extraembryonic membranes (5,6) and of supporting complete fetal development, with newborn pups resulting from the aggregation of stem cells with tetraploid cleavage-stage embryos being 100% ES cell-derived (7). The ES cells may also colonize the germ line in chimeras and form fully functional gametes (4). Hence, in combination with gene targeting, ES cells provide a powerful approach for introducing novel genetic change into the mouse genome (8).

This chapter details the materials and methods required for the isolation of murine ES cells; to date the mouse is the only species in which ES cells have been isolated and shown to be pluripotent in vivo. Embryonic cells that morphologically resemble murine ES cells have been isolated from the golden hamster (9), pig (10,11), sheep (12), and cow (10); however, no one has yet demonstrated that these

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cells are capable of participating in normal embryo development and contributing to a chimeric animal in these species.

The subsequent sections describe: the tissue-culture facility required; media and standard culture procedures used; the production of mouse embryos from different developmental stages; the procedures used for the isolation of ES cells from each of these different embryonic stages; and routine in vitro ES cell-culture techniques.

2. Materials

2.1. The Tissue-Culture Facility

The isolation and maintenance of stem cells should ideally be conducted in a laboratory established solely for sterile ES cell culture. Standard sterile tissue-culture procedures should be employed throughout. Personnel entry should be restricted, and no mice should be handled in the tissue-culture laboratory. The majority of tissue culture is performed within the vertical-flow cabinet. However, in the course of ES cell isolation, it is necessary to operate in the dissecting microscope. With care, microbial contamination can be kept to a minimum. Testing for mycoplasma-infected cells should be periodically undertaken.

The basic equipment in this laboratory should include:

1. A vertical-flow tissue-culture cabinet.
2. Humidified, 5% CO₂ (in air) incubators set at 37°C.
3. A bench-top centrifuge.
4. A water bath.
5. An inverted, phase-contrast microscope (magnification: 40, 100, and 200X).
6. A binocular, dissecting microscope with transmitted illumination (magnification: 12–100X) within a small still-air or laminar flow cabinet.
7. One gas Bunsen burner, with a pilot flame, within each culture cabinet.
8. Pipets, either glass or plastic disposables (e.g., Sterilin Ltd. [Hounslow, UK] or Costar [Cambridge, MA]; pipet sizes: 1, 5, 10, and 25 mL)
9. A hand-held pipet-aid.
10. A vacuum pump, with two waste traps to aspirate spent medium through a tube connected to disposable, heat-sterilized, long-form glass Pasteur pipets.
11. Glassware designated specifically for stem cell culture should be washed without any detergents. Glassware should be soaked for 1 to 2 d in a bucket of double-distilled water and rinsed several times in ultrapurified water (e.g., Milli-Q Water Purification System; Millipore, Bedford, MA). After draining, general glassware is heat-sterilized (at 180°C for 2 h) while bottles with screw-on tops are autoclaved (at 101 kPa for 20 min).

12. 1-mL cryopreservation tubes (Nunc, Roskilde, Denmark).
13. Tissue-culture plastics (*see* Section 3. for details).

2.2. Tissue-Culture Reagents and Solutions

1. All tissue-culture medium described here is based on a formulation of Dulbecco's Modified Eagle's Medium (DMEM) without sodium pyruvate, but high in glucose (4500 mg/mL). This can be purchased in either liquid form (1 or 10X; dilute appropriately) or powdered form from tissue-culture suppliers (e.g., Flow Laboratories Inc. [McLean, VA], Gibco Laboratories, Life Technologies Inc. [Grand Island, NY], and so on). An alternative medium formulation favored by some researchers is Glasgow's MEM (13).
2. Serum: Both fetal calf serum (FCS) and newborn calf serum (NCS) are required. The most suitable serum is selected after testing several samples provided by suppliers for their respective plating efficiencies with established ES or feeder-dependent embryonal carcinoma cell lines. A known number of ES cells are seeded onto replica plates containing 10 or 30% serum from each test batch. The plates are then incubated for 7 d, and the stem cell colonies stained and counted. After washing once with phosphate-buffered saline (PBS) (*see* item 12 *below*), the plates are stained with a 2% (w/v) solution of methylene blue for 2–5 min. The plates are then rinsed with water and allowed to dry. The average plating efficiency can then be calculated for each serum. The batch giving the highest plating efficiency, with no toxicity at 30% serum concentration, is then purchased in bulk order. It is generally not necessary to heat-inactivate bovine serum used for mouse ES cell work; however, with some batches used at high concentrations, serum toxicity may be observed owing to high levels of complement. Heat treatment at 56°C for 30 min to inactivate complement may markedly decrease toxicity.
3. 200 mM L-glutamine is added to medium to 1% (v/v), since this amino acid is unstable.
4. ES cell-culture media is supplemented with 1% (v/v) of a 100X stock solution of MEM nonessential amino acids (NEAA; e.g., Flow Laboratories). The final concentrations of the added NEAA are therefore: glycine, L-alanine, L-asparagine, L-aspartic acid, L-glutamic acid 0.1 mM each; L-proline, L-serine 0.2 mM each).
5. 100X β -mercaptoethanol stock (10 mM). 7 μ L of β -mercaptoethanol is dissolved in 10 mL of PBS (*see* item 12 *below*) and added freshly made to ES cell-culture medium to a final concentration of 0.1 mM to enhance stem cell attachment and growth (14).
6. Antibiotics are sometimes used to a final concentration of 50 IU/mL penicillin and 50 μ g/mL streptomycin (*see* Section 3.2. and Note 2).
7. HEPES buffer is used at a final concentration of 20 mM to maintain the pH

of the medium during manipulations outside the incubator for extended periods.

- 8 Buffalo rat liver (BRL) conditioned medium (optional; *see* Section 3.4.1. and Note 3).
9. DIA/LIF (*see* Section 3.4.1. and Note 3): Murine or human recombinant forms of DIA/LIF may be prepared by transfecting the appropriate expression plasmids into either COS cells (15), yeast cells, or *Escherichia coli* (16). The DIA/LIF factor may be purified from the supernatants of the transfected cells. The murine recombinant form of DIA/LIF is commercially available ("ESGRO," Gibco Laboratories, Life-Technologies).
10. Mitomycin C (Sigma, St. Louis, MO): Appropriate precautions must be taken when handling this potential carcinogen. The stock solution is prepared by dissolving a 2-mg vial of mitomycin C in 5 mL of PBS (i.e., 400 $\mu\text{g}/\text{mL}$), which is stored at 4°C for no longer than 1 mo. Mitomycin C is used at a final concentration of 10 $\mu\text{g}/\text{mL}$.
11. Trypsin/EGTA solution (TEG): To 1 L of sterile water, add:

7.0 g NaCl
 0.3 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$
 0.24 g KH_2PO_4
 0.37 g KCl
 1.0 g D-glucose
 3.0 g Tris-(2-amino-2-[hydroxymethyl] propane-1,3-diol)
 1.0 mL Phenol red

Dissolve ingredients, discard 100 mL of the salts solution, and then add:

100 ml 2.5% (10X) trypsin in modified Hank's balanced salts solution (Flow Laboratories)
 0.4 g EGTA (ethyleneglycol-tetraacetic acid)
 0.1 g PVA (polyvinyl alcohol)

TEG is adjusted to pH 7.6, filter-sterilized (0.22 μm), aliquoted into 20-mL sterile, plastic universals, and stored at -20°C. TEG is utilized for the subculture of both fibroblast and ES cell lines and for the disaggregation of embryonic outgrowths and putative stem cell colonies in the process of ES cell isolation. The powdered ingredients should be of tissue-culture purity and kept solely for ES cell culture.

12. PBS: Ca^{2+} and Mg^{2+} free PBS may be prepared by either dissolving preformulated tablets (e.g., Flow Laboratories) or by adding the following ingredients to 1 L of sterile water:
 - 10.0 g NaCl
 - 0.25 g KCl
 - 1.44 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$
 - 0.25 g KH_2PO_4

- PBS is adjusted to pH 7.2. Aliquot into 500-mL bottles, sterilize by autoclaving, and store PBS at room temperature.
13. Gelatin (from porcine skin; Sigma) is made as a 0.1% (w/v) solution in sterile water, and dissolved and sterilized by autoclaving
 14. Pancreatin-Trypsin solution is Ca^{2+} - and Mg^{2+} -free PBS containing 2.5% (w/v) pancreatin (porcine; Sigma), 0.5% (w/v) trypsin (porcine 1:250; Sigma), and 0.5% (w/v) polyvinylpyrrolidone (M_r 10,000), further supplemented with 0.1% (w/v) D-glucose, 1% (v/v) of a 100X stock solution of nonessential amino acids (Flow Laboratories), and 20 mM HEPES buffer (pH 7.4; 17).
 15. Mouth-controlled, hand-pulled, plugged Pasteur pipets are used to transfer embryos between culture dishes. These are constructed by rotating the pipet over a Bunsen burner, first to soften the glass. After withdrawal from the flame, the pipet is quickly pulled to produce a fine capillary, approx 6 cm long and with an internal diameter of 150–200 μm . The end of the capillary is broken cleanly to produce a square tip by scoring the glass with another finely pulled capillary. (Similar principles are used to produce finer pipets to manipulate ES cell colonies during isolation procedures outlined in Section 3.3.) A modified Pasteur pipet bulb may be utilized as an adaptor to connect the end of the pulled pipet to a length of rubber tubing with a fitted mouthpiece. Fine control over the movement of embryos within the pipet is aided by first aspirating several minute volumes of medium interspaced with air bubbles.

2.3. ES Cell-Culture Media

Two media formulations are used for ES cell culture. For ES cell isolation, medium contains 20% (v/v) serum (ES_{20}), whereas established ES cell lines may be maintained in medium with only 10% (v/v) serum (ES_{10}) and supplemented with recombinant DIA/LIF (*see* Section 3.4.1. and Note 3). In both instances, serum comprises a 50:50 mixture of selected batches of fetal calf serum (FCS) and newborn calf serum (NCS).

1. Medium for murine embryonic stem cell isolation (ES_{20} medium):
 - 64 mL Dulbecco's Modified Eagle's Medium (1X)
 - 10 mL Fetal calf serum
 - 10 mL Newborn calf serum
 - 1 mL 200 mM L-glutamine
 - 1 mL 100X nonessential amino acids stock
 - 1 mL 100X β -mercaptoethanol stock (final concentration: 0.1 mM)
 - 13 mL sterile water

Osmolality: ca. 290 mOsm/kg H_2O (*see* Note 4).

2. Medium for murine embryonic stem cell maintenance (ES₁₀ medium).

- 74 mL Dulbecco's Modified Eagle's Medium (1X)
- 5 mL Fetal calf serum
- 5 mL Newborn calf serum
- 1 mL 200 mM L-glutamine
- 1 mL 100X nonessential amino acids stock
- 1 mL 100X β-mercaptoethanol stock (final concentration: 0.1 mM)
- 13 mL sterile water

Osmolarity: ca. 290 mOsm/kgH₂O (*see* Note 4). To ES₁₀ medium, BRL-conditioned medium or recombinant DIA/LIF may be added (*see* Section 3.4.1. and Note 3).

All media are filter-sterilized after preparation. Because of the large volumes concerned, it is often convenient to utilize resterilizable, 47-mm filter holders (Millipore, Bedford, MA), housing a glass-fiber prefilter overlying a 0.22-μm nitrocellulose filter. The medium is pushed through the filter with a 60-mL syringe. Media are stored at 4°C and warmed to 37°C immediately before use. Medium that is older than 2 wk should be supplemented with 1% (v/v) of 200 mM L-glutamine. Note that no antibiotics are used for ES cell isolation or culture (*see* Note 2).

2.4. STO Cell-Culture Media

DMEM₁₀ medium is used for the routine culture of the STO fibroblast cell line, used to prepare feeder cells for coculture with ES cells.

Medium for STO fibroblast culture (DMEM₁₀ medium):

- 89 mL Dulbecco's Modified Eagle's Medium (1X)
- 5 mL Fetal calf serum
- 5 mL Newborn calf serum
- 1 mL 200 mM glutamine

Media are filter-sterilized and stored as described in Section 2.3.

2.5. Animals

Although ES cells have been isolated from a variety of inbred, outbred, and F₁ crosses, there does appear to be an effect of mouse genotype on the ease with which ES cells can be isolated (18), with embryos from the 129 strain yielding stem cells at significantly higher frequencies than other mouse genotypes (19).

In order to produce embryos, a supply of 6- to 8-wk-old virgin females are required for superovulation. Following hormone treatment, these

are mated with individually caged stud males. Mice are usually housed in a light cycle of 14 h light (04:00 h to 18:00 h) and 10 h dark, or a 12 h light (06:00 to 18:00) and 12 h dark cycle. Mice are fed and watered *ad libitum*.

2.5.1. Materials for Superovulation

The hormones used to superovulate mice are pregnant mare's serum gonadotrophin (PMS, Folligon; Intervet [Boxmeer, Netherlands]) to increase follicle production and human chorionic gonadotrophin (hCG, Chorulon; Intervet) to induce ovulation. Both PMS and hCG are prepared by dissolving the lyophilized powders in sterile 0.9% (v/v) NaCl, to give a final concentration of 50 IU/mL. The hormones are then aliquoted, stored at -20°C , and replaced after 2 mo.

2.5.2. Materials for Anesthesia

Anesthetic is prepared by mixing separately 1 mL of Hypnorm (Janssen Pharmaceuticals [Beerse, Belgium]; containing 0.315 mg fentanyl citrate and 10 mg fluanisone) and 1 mL of Hypnovel (Roche Products Ltd. [Basel, Switzerland]; containing 5 mg midazolam hydrochloride) each with 2 mL of distilled water, **before** combining the two solutions together. The anesthetic is stored at room temperature and replaced after 4 wk. An alternative anesthetic may be used (*see* Chapter 14).

2.5.3. Additional Materials and Reagents

1. Standard surgical equipment and dissecting tools.
2. Cotton suture (5/0 Mersilk, Ethicon Inc. [Somerville, NJ]).
3. Michel clips
4. Progesterone solution (10 mg/mL dissolved in absolute ethanol or corn oil).

2.6. Materials for Karyotype Analysis

1. 0.56% (w/v) KCl.
2. Fixative: 3 vol of absolute methanol to 1 vol of glacial acetic acid.
3. 2X standard saline citrate (SSC): 0.3M NaCl, 0.03M trisodium citrate.
4. Gurr's phosphate buffer, pH 6 (e.g., BDH Chemicals, Poole, UK).
5. 0.25% (w/v) Trypsin (porcine; Difco Laboratories [Detroit, MI]) dissolved in Gurr's phosphate buffer.
6. 5% (v/v) Giemsa Gurr's R-66 stain (e.g., BDH) in Gurr's phosphate buffer.

3. Methods

3.1. Preparation of Feeder Cells

Historically, ES cells have been isolated and maintained on layers of mitotically inactivated, embryonic fibroblast “feeder” cells. If cultured in the absence of feeders, the ES cells rapidly differentiate (1; see Note 5). The most commonly utilized fibroblast feeder layers have been those prepared from the continuous STO cell line (20). Other embryonic fibroblast cell lines also capable of maintaining stem cells include C3H 10T1/2 cells (21) and BALB-3T3/A31 cells (22). Alternatively, primary embryonic fibroblasts may be isolated from fetuses in the third trimester of pregnancy. Although such feeders have been favored by some workers (23), a major disadvantage in their use is the limited in vitro life-span of these primary cells (24). In order to prepare feeder cell layers for coculture with embryos or ES cells, the fibroblast cells must be mitotically inactivated. Typically, this is achieved by treatment with the drug mitomycin C (20) or by exposure to irradiation (25).

3.1.1. Routine Culture of STO Fibroblast Cells

1. The STO fibroblast line (20) is routinely cultured in DMEM₁₀ medium in tissue-culture flasks, commonly with a 25-cm² growth area surface (e.g., Costar, Nunclon).
2. Once confluent, the fibroblasts are passaged, or subcultured, into fresh flasks. The old medium is aspirated from the flask, and the cells washed once with 10 mL of PBS.
3. After the PBS is aspirated, 2 mL of TEG are added, and the flask incubated at 37°C for 3 min. After swirling the flask to obtain a single-cell suspension (checked in the inverted microscope), the trypsin solution is neutralized by adding 3 mL DMEM₁₀.
4. Typically, for routine culture, a 1/10 aliquot (i.e., 0.5 mL) of this suspension is added to 10 mL of fresh DMEM₁₀ in a new flask. Under such a subculture regime, flasks may reach confluency after 4–5 d. The subculture ratio and the number and growth area of the flasks seeded may be varied to suit the demand for STOs.
5. The flasks are then transferred into the CO₂ incubator with either their caps loosened or tightened after being gassed while in the flow cabinet via a sterile, plugged Pasteur pipet connected to a cylinder containing 5% CO₂, 20% O₂, and 75% N₂.

3.1.2. Preparation of STO Fibroblast Feeder Cell Layers

Mitotically inactivated STO feeder cell layers are prepared using the following protocol.

1. The old medium is aspirated from a subconfluent flask of STOs and, for a 25-cm² flask, replaced with 10 mL of DMEM₁₀ containing 10 µg/mL of mitomycin C (Sigma). The STO flasks are then incubated for 3 h.
2. After treatment, the mitomycin C medium is aspirated, and the STO cells are washed three times with 10 mL of PBS, before being trypsinized with 2 mL of TEG. After 3 min incubation at 37°C, the STOs are dissociated into single cells, and the TEG neutralized with 8 mL of DMEM₁₀.
3. The total 10-mL suspension is then transferred into a 20-mL conical, plastic universal. A minute volume of the suspension is drawn into a Pasteur pipet and transferred to a hemocytometer, in order to count the number of cells.
4. The STO cell suspension is centrifuged at 1000 rpm for 5 min, and the supernatant aspirated. The cell pellet is then disrupted by carefully flicking the tube, and the cells are resuspended in DMEM₁₀ medium, to one of three alternative densities (*see below*).
5. For convenience during the isolation of an ES cell line, feeder cell layers may be prepared on different sized surface areas, for example, either in microdrops under toxicity-tested, lightweight paraffin oil, in 1.75 cm² four-well plates (Nunclon), or in 25-cm² flasks. To enhance STO cell attachment, all culture surfaces are pretreated with gelatin. Microdrops may be prepared by placing two rows of five 10-µL drops of gelatin on 6 cm diameter tissue-culture dishes, overlaid with 5 mL of paraffin oil. For wells and flasks, just enough gelatin should be added so as to cover the culture surface. The gelatin is stored for about 1 h in the tissue-culture cabinet, before aspiration and replacement with the freshly prepared, inactivated STO cell suspension.
6. The STOs should be plated at a density to ensure a uniform, confluent monolayer of cells. For microdrops, 20 µL of a suspension containing 3×10^5 STO cells/mL are introduced; each (1.75 cm²) well of a four-well plate should receive 1 mL of a suspension containing 1.5×10^5 cells/mL; whereas 25 cm² flasks are seeded with 1×10^6 STO cells in 5 mL of DMEM₁₀ medium.
7. Feeders should generally be used within 5 d, after which time fresh feeder layers should be prepared. Before the feeder layers are used for coculture with embryos or ES cells, the old medium is aspirated and fresh ES medium introduced.

3.1.3. Preparation of Primary Embryonic Fibroblast Feeder Cell Layers

Primary embryonic fibroblasts are isolated from 13- or 14-d-old mouse fetuses.

1. Fetuses are dissected from the uteri and the placental tissue, and soft internal organs removed using fine scissors and watchmaker's forceps.
2. The fetal carcass is washed in PBS and transferred into a dish containing 2 mL of TEG solution. The carcass is finely minced, and the tissue incubated for 5 min at 37°C.
3. The trypsin is then neutralized with 5 mL of DMEM₁₀, and the contents of the dish transferred to a conical universal. The large pieces of cellular debris are allowed to settle over a few minutes, and the supernatant containing the embryonic cells is transferred to a 25-cm² flask and incubated.
4. After 24 h, the medium is changed to remove dead cells, and the primary embryonic fibroblasts are cultured as for STOs (Section 3.1.1.).
5. Since these primary cells will become senescent after 15–20 cell divisions, the isolation procedure must be made on a regular basis to ensure the feeder cells are continuously available. To ensure a confluent monolayer of mitotically inactivated embryonic fibroblasts, the cells should be seeded at twice the density as recommended for STOs in Section 3.1.2., step 6.

3.2. Production of Mouse Embryos for Embryonic Stem Cell Isolation

This section describes the methods for the production of morulae, delayed and nondelayed blastocysts, and primitive ectoderm from egg-cylinder-stage embryos (*see* Note 4).

3.2.1. Superovulation

1. Six- to 8-wk-old virgin females are injected ip with 5 IU (i.e., 0.1 mL) of PMS at approx 13:00 h of the light cycle.
2. Forty-seven hours later, the same females are injected ip with 5 IU (i.e., 0.1 mL) of hCG. Females are then paired with stud males (of the appropriate strain) and checked for copulation plugs the following morning (designated d 0.5 pc). It is accepted that ovulation occurs approx 12 h after the administration of hCG, that is, around the midpoint of the dark cycle.

3.2.2. Oviductal Recovery of 16–20 Cell Morulae

1. Female mice are killed by cervical dislocation on d 2.5 pc. They are laid out on their backs on absorbant tissues, and the abdominal surface sterilized with 70% ethanol.

2. The abdominal skin is pinched up with forceps and a small cut made across the midline. Grasping both sides of the cut firmly with forceps, the skin is pulled in opposing directions to expose the abdominal body wall, which is in turn cut open. The alimentary tract is displaced to one side to reveal the reproductive organs near the dorsal surface of the female.
3. With the reproductive organs located, the uterine horn is grasped with watchmaker's forceps and cut with fine scissors just below the uterotubal junction. Holding the cut end of the uterus, the oviduct is pulled taut, and a cut is made between the oviduct and the ovary. The oviduct is then transferred into a dish containing DMEM₁₀ + 20 mM HEPES + antibiotics (50 IU/mL penicillin and 50 µg/mL streptomycin) and placed in a dissecting microscope.
4. Using watchmaker's forceps and the tip of a blunt 30-g needle attached to a 1-mL syringe filled with medium the oviductal coils are manipulated to locate the opening of the oviduct (the infundibulum).
5. The tip of the blunt needle is carefully inserted into the infundibulum and grasped with the forceps. Approximately 0.1 mL of medium is slowly introduced through the oviduct to flush the embryos out of the tract and into the dish.
6. The dish containing the oviductal flushings is searched for embryos under low magnification in the dissecting microscope. Morulae are aspirated into a mouth-controlled, hand-pulled, plugged Pasteur pipet (*see* Section 2.2., item 15). The embryos are then collected from the flushings and pooled into a 25-µL drop of ES₂₀ medium under oil. This aids their subsequent location.

3.2.3. Uterine Recovery of Blastocysts

1. Females are killed by cervical dislocation on d 3.5 pc. The reproductive tract is exposed as described in Section 3.2.2.
2. The uterus, containing the blastocysts, is removed by cutting across the base of the cervix, but above the bladder, and cutting away the mesometrium membrane holding the uterus to the body wall. Both uterine horns are cut just below the junction with the oviduct, and the uterus is placed on clean paper tissue. The uterine horns are then cut from the cervix, and any remaining mesometrium or adipose tissue trimmed away.
3. A 25-g needle, attached to a syringe containing DMEM₁₀ + 20 mM HEPES + antibiotics (*see* Section 3.2.2.), is inserted 2–3 mm inside the tubal end of the uterine horn and grasped firmly with forceps. Approximately 1 mL of medium is flushed through each horn and collected in a 6-cm plastic dish. The embryos are then collected from the flushings (*see* Section 3.2.2.) and pooled into a 25-µL drop of ES₂₀ medium under oil

3.2.4. Production of Implantationally Delayed Blastocysts

1. An ovariectomy is performed on superovulated females 2.5 d pc (26) Mice are anesthetized with a 0.15- to 0.20-mL ip injection of a Hypnorm and Hypnovel mixture (*see* Section 2.4.2.).
2. Once unconscious, the back of the mouse is wiped with 70% alcohol. A pair of blunt forceps is used to pick up the skin, and dressing scissors are used to make a 10-mm lateral incision across the midline, posterior to the last rib. Paper tissues, moistened with 70% alcohol, are used to open the wound and remove any cut hair by wiping in a head-to-tail direction.
3. Sliding the skin from side to side, the position of the right-hand ovary is located beneath the body wall. The region directly overlying the ovary is grasped with pointed watchmaker's forceps and a 5-mm incision made in the body wall with sharp iris scissors. By exerting gentle pressure on both sides of the incision, the ovarian fat pad can be exteriorized and grasped with blunt forceps.
4. Very carefully, the associated ovary, oviduct, and the top of the uterine horn are also pulled out. The weight of the fat pad is generally sufficient to keep the ovary from slipping back inside the abdominal cavity, provided a small incision is made.
5. The mouse is then transferred, on absorbant tissues, to the stage of a binocular dissecting microscope, with a fiberoptic incident light source. Focusing on the ovary at low magnification (ca 10X), two watchmaker's forceps are utilized to tear open the ovarian bursa delicately, encapsulating the ovary and the oviduct
6. With the ovary released, a small, pretied loop (ca. 5 mm diameter) of fine suture cotton (5/0 Mersilk; Ethicon) is slipped between the ovary and the oviduct, and pulled tight with forceps to ligate the ovarian blood vessels. All of the ovarian tissue is then cut away, using iris scissors.
7. The fat pad and oviduct are then replaced inside the abdominal cavity by lifting up one edge of the incision and pushing in the fat pad with blunt forceps. The ovariectomy is then repeated on the other side. The incisions in the body wall are closed with single sutures (5/0 Mersilk; Ethicon), whereas the skin may be closed with either sutures or one or two Michel clips.
8. While the female is still unconscious, 0.1 mL of 10 mg/mL progesterone is injected sc into the flank region. To aid postoperative recovery, the mouse should be placed onto a heated blanket.
9. The delayed blastocysts are recovered from the uterine horns as outlined in Section 3.2.3. by flushing between d 6.5 and 8.5 pc (i.e., 4–6 d after ovariectomy or 3–5 d, respectively, after blastocyst formation).

3.2.5. Recovery of Egg-Cylinder-Stage Embryos 5.5 d pc

1. Females are sacrificed by cervical dislocation, and the belly region swabbed with 70% alcohol. The abdominal cavity is then opened to reveal the reproductive tract as in Section 3.2.2. In turn, each uterine horn is cut just below the utero-tubal junction.
2. Grasping the end firmly with forceps, the uterus is pulled taut, and scissors used to tear away the mesometrium. Next, the uterine horn is opened by sliding the tip of a pair of iris scissors down along the antimesometrial wall of the uterus, moving toward the cervix. Keeping the uterus pulled taut, forceps are used to “shell” the decidua (containing the embryos) out of the uterus.
3. The decidua are placed into a dish containing DMEM₁₀ + antibiotics (50 IU/mL penicillin and 50 µg/mL streptomycin) and 20 mM HEPES buffer, added to maintain the pH during subsequent manipulations outside the incubator.
4. In a dissecting microscope, each deciduum is delicately teased apart with flame-sterilized watchmaker’s forceps, to dissect out the early egg-cylinder-stage embryo, easily identified as a dark speck from the trophoblast tissue. A representative illustration of the dissected embryo is shown in Fig. 1. The trophoblast and parietal endoderm tissues are torn away from the implantation site during the dissection procedure and, hence, appear ragged. A proamniotic cavity has often been developed by this stage, and a clear division between the primitive ectoderm and the extraembryonic ectoderm (of trophectodermal origin) is often observed.
5. Isolation of pure primitive ectoderm tissue is accomplished by first, cutting away the extraembryonic regions of the egg cylinder utilizing a fine-glass microneedle constructed from a hand-pulled, Pasteur pipet (“cut” in Fig. 1). Then, the overlying layer of visceral endoderm is removed with an enzymatic pretreatment. After rinsing in PBS, the tissue is incubated for 5 min in a 250-µL drop of pancreatin-trypsin solution at 4°C.
6. The dish is then flooded with DMEM containing 10% bovine serum to neutralize the crude proteases, and the tissue transferred to a fresh droplet of ES₂₀ medium under paraffin oil and incubated for 1 h at 37°C.
7. Following this recovery period, the endoderm layer often cleanly separates from the primitive ectoderm by repeatedly aspirating the tissue gently up and down inside a fine, flame-polished, mouth-controlled Pasteur pipet.
8. This treatment generally yields relatively pure clumps of ectoderm, which may then be either explanted intact into tissue culture for ES cell isolation or first disaggregated into smaller pieces, comprising several

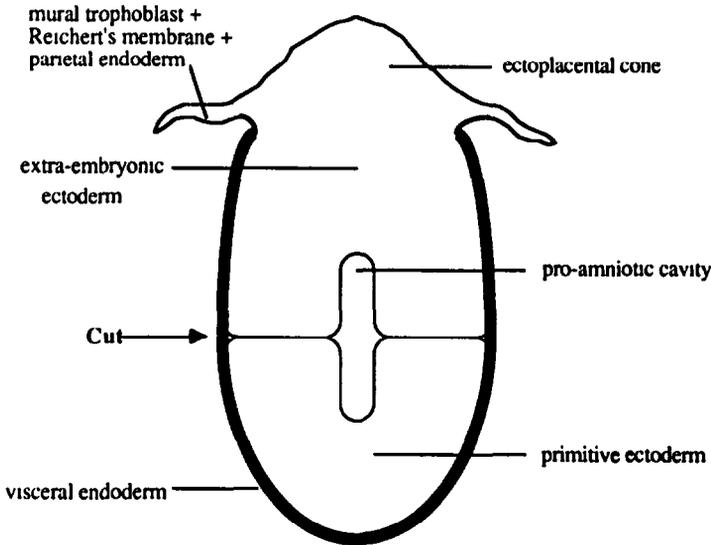


Fig. 1. Representative illustration of a d 5.5 pc egg cylinder-stage mouse embryo following dissection from the deciduum, showing the position of the lateral cut made in order to isolate the embryonic portion of the embryo

cells each. This is accomplished by incubating the ectoderm in TEG for 3–5 min at 37°C followed by mechanical dissociation, using a fine pipet.

3.3. Isolation of Murine Embryonic Stem Cells

This section describes the typical procedures utilized to isolate ES cells from three different embryonic stages: namely, from blastomeres of disaggregated morulae, from the ICM (inner cell mass) of delayed and nondelayed blastocyst-stage mouse embryos, and from d 5.5 pc primitive ectoderm. Emphasis is placed on the isolation of ES cells from blastocysts. However, apart from the timing of the disaggregation of the embryo outgrowth, the isolation procedures used are essentially identical for each of these developmental stages. ES cells are morphologically identified as small, rounded cells with a large nuclear to cytoplasmic ratio, containing one or more prominent nucleoli and no overtly specialized cellular structures; that is, characteristics of a typically undifferentiated cell phenotype.

After embryo recovery, the blastomeres, blastocysts, or primitive ectoderm tissue are cocultured on STO feeder layers prepared in

microdrops (Section 3.1.2.). ES₂₀ medium is routinely used for ES cell isolation (*see* Note 6). The medium is changed on these microdrops 2–3 h before the embryos are introduced. Using a sterile, hand-pulled Pasteur pipet, cells from one embryo are transferred to each microdrop. Microdrops allow the progress of each embryo to be monitored individually. The additional advantages of using microdrops are that any ES cell colonies arising from a single embryo can be pooled together, without the necessity for cloning as required for group cultures, and the spread of infection is minimized.

3.3.1. Isolation of Embryonic Stem Cells from Blastocyst-Stage Embryos

There are two general strategies used to divert the ICMs of blastocysts from their normal fate of differentiation and, instead, encourage their continued proliferation *in vitro* in order to isolate ES cells. The method described here is based on that originally reported by Evans and Kaufman (1) and subsequently detailed by Robertson (24). Briefly, the technique involves the culture of intact blastocysts for a limited time until they grow to resemble egg-cylinder-like structures. The embryonic portion of this outgrowth is then enzymatically and mechanically manipulated, and subsequently propagated in appropriate culture conditions. The second strategy described by Martin (2) involves the culture of ICMs following the immunosurgical destruction of the trophoctoderm from day 3.5 pc blastocysts (27). A proportion of such ICMs were reported to give rise directly to colonies of stem cells, apparently without the necessity of any further specific manipulation (2). Although it may be more efficient to isolate ES cells following immunosurgery than simply by explanting blastocysts intact into culture (12 vs 6%; 28), the latter procedure is more commonly adopted because of its greater ease and general convenience.

3.3.1.1. CULTURE OF INTACT BLASTOCYSTS

1. Intact d 3.5 pc blastocysts explanted into microdrops hatch from the zona pellucida and attach to the STO feeder cells after 24–36 h by the outgrowth of the trophoctodermal cells (Fig. 2a). These cells spread out and differentiate to form a monolayer of large, flat trophoblast cells. As a consequence of this disorganization, the ICM becomes exposed to the culture environment and appears initially as a small nest of cells in the center of the trophoblast outgrowth after 2–3 d (Fig. 2b)

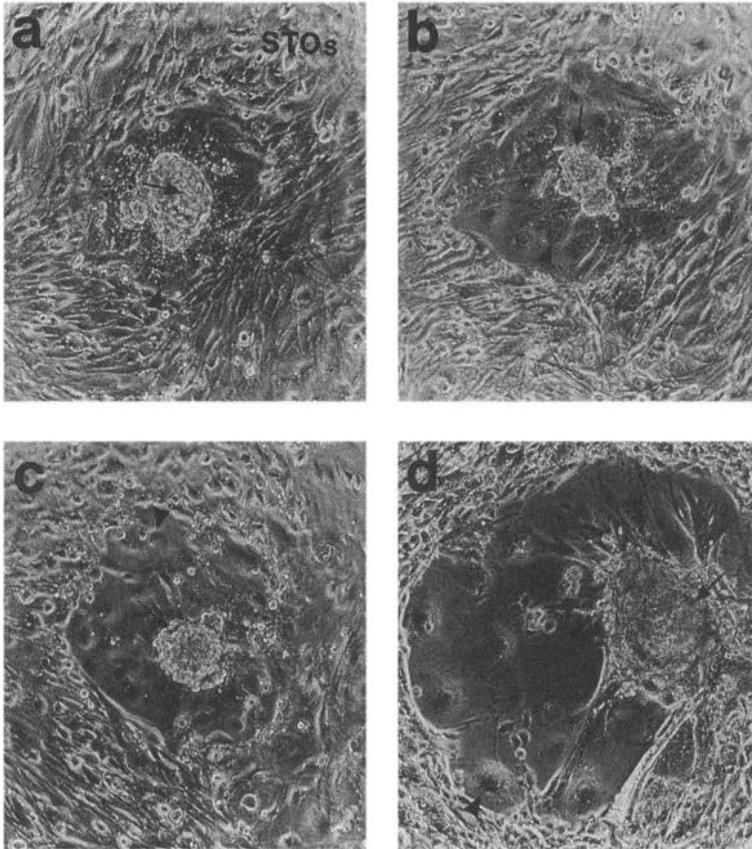


Fig. 2. Attachment and outgrowth of d 3.5 pc blastocyst-stage mouse embryos on feeder cell layers. In the above panels, the ICM is indicated by arrows and the trophoblast by arrowheads. Embryos hatch from the zona pellucida and attach to the STO feeder cells via the trophectoderm after 24–36 h of culture (a). In the process of attachment the blastocysts collapse, sequestering the ICM (a). Subsequently, the trophectoderm differentiates into giant trophoblast cells (a), which grow out radially to expose eventually the ICM after 2 or 3 d (b). The ICM outgrowth proliferates as a three-dimensional mass of cells and is disaggregated once it has reached a size similar to that in (c) after a total of 4 to 5 d *in vitro*. ICM outgrowths should be disaggregated before they begin to differentiate into endoderm (d). Photographs taken using phase-contrast microscopy.

2. After embryo attachment, the medium on the microdrops should be replaced daily. The ICM cells continue to proliferate, and the outgrowths are allowed to grow to a suitably large size (determined by experience; *see* Fig. 2c), but without signs of endoderm differentiation, before they are disaggregated. Such outgrowths are generally obtained after 4–5 d of

culture with d 3.5 pc blastocysts. Delayed blastocysts, however, generally require a 6-d culture interval before they reach a suitable size because of their slower rates of *in vitro* growth initially. Variability between embryos exists in the rate of ICM growth, and thus, each embryo must be monitored and assessed daily in the inverted microscope. If cultured without intervention, cells of the embryonic outgrowth differentiate (Fig. 2d) and have a much reduced capacity to yield ES cells.

3.3.1.2. DISAGGREGATION OF ICM OUTGROWTHS

1. Two to 3 h before ICM outgrowths are to be disaggregated, the cultures should be refed with fresh medium. The disaggregation procedure is performed by transferring the culture dish to the dissecting microscope, and with a blunt glass probe, prepared from a flame-polished, hand-pulled Pasteur pipet, the ICM is "picked off" from the surrounding layer of trophoblast.
2. With a mouth-controlled, drawn-out Pasteur pipet, the ICM is then transferred to a 20- μ L wash-drop of TEG on a 6-cm dish (without oil) before being placed into a fresh drop of TEG and incubated at 37°C for 5 min.
3. Returning the dish to the dissecting microscope, a finely pulled Pasteur pipet with a tip diameter approximately one-fifth the size of the ICM clump is used to break up mechanically the partially digested outgrowth into several small pieces, each comprising around 15 cells. All of the cellular pieces from one embryo are then transferred to a fresh STO microdrop containing ES₂₀ medium. This disaggregation is termed "passage one."
4. Approximately 6 h after disaggregation, the medium is changed on the culture to remove any dead cells and any residual traces of the trypsin solution.

3.3.1.3. IDENTIFICATION OF EMBRYONIC STEM CELLS

The majority of colonies arising from the disaggregated outgrowths of blastomere, ICM, or primitive ectoderm origin will be of a differentiated morphology. Trophoblast and endodermal colonies are generally the most common (Fig. 3a); however, patches of fibroblast-like, neuronal (and with extended culture), beating muscle may also be observed. Undifferentiated cells possessing an ES-like morphology (so called at this stage, since many of the colonies initially displaying this cellular morphology may differentiate by the second or third passage) are identified as small, rounded cells with a large nuclear-to-cytoplasmic ratio and containing one or more prominent nucleoli (Fig. 3b). On the STO feeders, the ES cells tend to grow in tightly-packed,

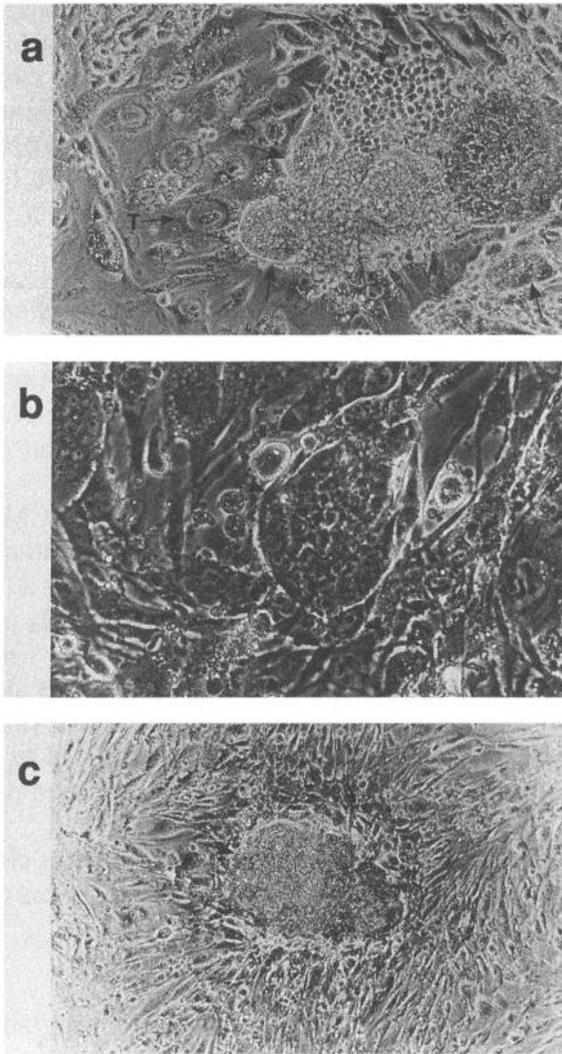


Fig. 3. Morphology of cell colonies following disaggregation of ICM-derived outgrowths. Giant trophoblast cells (T) and endodermal-like cells (E) are the most common cell colonies present in first-passage cultures of disaggregated ICMs (a). However, some small ES-like colonies are present among the assortment of differentiated cells (a) (ES-like colonies are arrowed in all panels). Higher magnification of a small ES-like colony 2 d after the first passage (b) shows the typical undifferentiated cell morphology. ES cells grow in close opposition with one another, with each cell having a high nuclear-to-cytoplasmic ratio and one or more prominent nucleoli (b). After 7 d of culture on the STO feeders, some cells of the disaggregated ICM may grow into tightly packed, three-dimensional ES-like colonies, where clear intercellular boundaries are difficult to distinguish (c). The colony shown in panel (c) was subsequently expanded and maintained as a permanent ES cell line. Photographs taken using phase-contrast microscopy.

three-dimensional colonies, where clear intercellular boundaries are difficult to distinguish (Fig. 3c). At this stage, ES cells have to be identified on the basis of their cell morphology. This skill only comes with experience. There are a number of stem-cell-specific (or pluripotent cell specific) markers that can be used to verify the ES cell phenotype and distinguish these cells from their differentiated derivatives. These cell markers include both immunohistological and biochemical types. For example, high-alkaline phosphatase enzyme activities are observed in ES cells (25). ES cells express the SSEA-1 antigen (29) and bind the monoclonal antibody ECMA-7 (30) on the cell surface. Pluripotent stem cells fail to express cytoskeletal markers indicative of differentiated cell types, such as identified by the monoclonal antibody TROMA-1 (30). Recent immunohistological studies have gone some way toward characterizing the carbohydrate antigens present specifically on the surface of ES cells or their differentiated cell derivatives (31,32).

The first passage in culture is used to screen out all of the differentiated colonies and select only those displaying a stable ES-like cell morphology. This is achieved by employing a relatively long culture interval of 5–8 d and feeding the cultures initially every other day, but more frequently whenever the media becomes acidic.

3.3.1.4. EXPANSION OF EMBRYONIC STEM CELLS INTO PERMANENT CELL LINES

The first passage cultures are monitored daily and disaggregated once ES-like cell colonies attain a “suitably large size,” without having commenced differentiation (e.g., Fig. 3c). Because not all of the colonies derived from each embryo will be of a similar size, the approach to disaggregation at the second passage depends on the number of stably growing colonies in each culture. If several ES-like colonies are present in the first-passage microdrop, then the entire microdrop may be trypsinized.

1. The old medium is aspirated, the cells washed *in situ* with PBS, and 20 μ L of TEG introduced.
2. Following 3–5 min at 37°C, the dish is transferred to the dissecting microscope and a fine, mouth-controlled, pipet used to dissociate further the colonies into single-cells. This is important, so as to prevent subsequent differentiation.
3. The cell suspension is then transferred into a STO feeder well of a four-

well plate, containing 1 mL of ES₂₀ medium. After 1 or 2 d, small colonies of ES-like cells may be visualized in some of these cultures.

If only one or two ES-like colonies in the first passage culture are ready for disaggregation, it is considered best to pick these out of the microdrop, whereas any remaining ES-like colonies, which are considered too small to survive readily the trypsinization procedure, are left to proliferate further. The larger ES-like colonies are picked off the STO feeder layer with a blunt glass probe, washed briefly in TEG, and then incubated at 37°C in a fresh drop of TEG for 3 min. After partial digestion, the colonies are disaggregated mechanically into single cells with a fine pipet and introduced into a fresh STO microdrop, before being eventually expanded into four-well plates, as described above.

From the four-well plates, the ES-like cells are subsequently expanded into 25-cm² flasks containing a layer of STO feeder cells.

1. The ES-like cells are cultured within the wells for 4–5 d, feeding as required, by which time the colonies may have merged to form a monolayer of cells.
2. Working in the flow cabinet, the medium within the well is aspirated, and the cells washed with 1 mL of PBS. Following a 3- to 5-min incubation in 0.25 mL of TEG, a hand-pulled Pasteur pipet (tip diameter ca 1 mm) and bulb are used first to introduce around 0.5 mL of ES₂₀ medium to neutralize the trypsin and then to pipet the suspension vigorously to disaggregate the cells.
3. The single-cell suspension is then transferred into a flask containing 10 mL of ES₂₀ medium. The flask is then cultured in the CO₂ incubator with either the cap loosened or tightened after being gassed with a 5% CO₂, 20% O₂, and 75% N₂ mixture.

3.3.2. Isolation of Embryonic Stem Cells from Blastomeres

The following is based on the method of Eistetter (33) for the establishment of ES cells from single blastomeres. (The author has attempted to repeat this work, but all initial ES-like colonies eventually differentiated into trophectoderm.)

1. To obtain single blastomeres from compacted 16- to 20-cell morulae, the zonae pellucidae must first be removed. This may be accomplished by treatment of embryos in either acid Tyrode's solution (pH 2.1) or with an enzymatic solution comprising 0.5% (w/v) pronase and 0.5% (w/v) polyvinylpyrrolidone (M_r 10,000) in HEPES buffered "M2" medium (17).

2. The zona-free morulae are then incubated at 37°C in Ca²⁺- and Mg²⁺-free phosphate-buffered saline containing 0.3% (w/v) EDTA for 3 min
3. Dissociation of the blastomeres is completed mechanically utilizing a finely pulled Pasteur pipet, with a flame-polished tip. All of the blastomeres from each embryo may be explanted together into one feeder microdrop after washing extensively in ES₂₀ medium.
4. Eistteter (33) reported that the blastomeres rapidly attach to the feeder cells; however, in the author's hands, the majority of blastomeres remain free floating and continue to divide forming miniblastocysts or trophoctodermal vesicles.
5. After 3 or 4 d of culture, colonies with an ES-like morphology may be visualized and, depending on their size, should be picked out and lightly disaggregated in TEG before being plated onto feeder layers away from the influence of differentiated colonies. Propagation of the blastomere-derived ES cells is the same as described previously for blastocysts.

3.3.3. Isolation of Embryonic Stem Cells from d 5.5 pc Primitive Ectoderm

Apart from the timing of the first passage, methods for the establishment of primitive ectoderm-derived ES cells are identical to those described previously for blastocyst-derived ES cell lines.

1. The isolation history of an ectoderm-derived ES cell line is illustrated in Fig. 4. The d 5.5 pc egg cylinder-stage embryo in Fig. 4a shows the position of the cut made to remove the extraembryonic region and the location of the visceral endoderm tissue overlying the primitive ectoderm.
2. The primitive ectoderm is isolated as described in Section 3.2.5. and generally results in relatively pure clumps of tissue (Fig. 4b), which are then explanted onto STO feeder layers prepared in microdrops.
3. After 2 or 3 d of culture, undifferentiated outgrowths are commonly observed (Fig. 4c), which are then lightly disaggregated with TEG before any obvious signs of differentiation into several small pieces each comprising around 10–15 cells. Despite this early first passage, extensive differentiation often results in the cultures, with many endodermal and flattened epithelial-like colonies developing. However, trophoblast colonies are rarely observed, except in instances of contamination with cells of extraembryonic origin, since d 5.5 pc primitive ectoderm no longer has the potential to develop trophoctoderm (34). After a 6- to 7-d culture interval, first-passage colonies that remain undifferentiated should be trypsinized into single cells and passaged.
4. From these cells, second-passage colonies of typical ES cell morphology may grow in some cultures, as in Fig. 4d. Although d 5.5 pc primitive

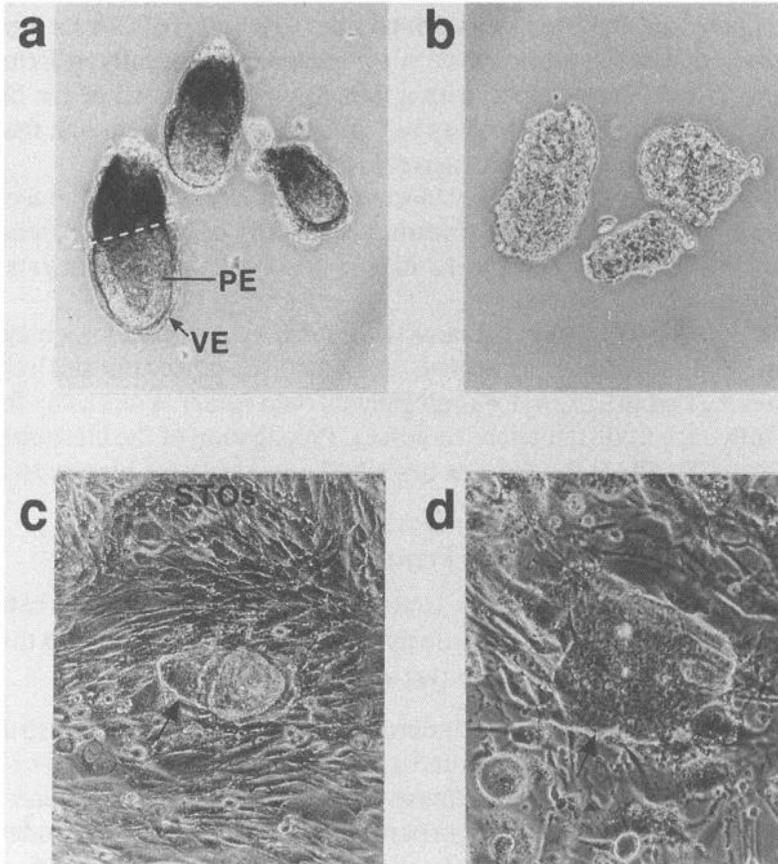


Fig. 4. Establishment of an ES cell line from d 5.5 pc primitive ectoderm. Egg-cylinder-stage embryos dissected from the decidua on d 5.5 pc (a). Primitive ectoderm (PE) tissue (b) is isolated from the embryos by first cutting away the extraembryonic region along the dotted line in (a) and then utilizing an enzymatic procedure to remove the overlying visceral endoderm (VE). Panel (c) shows an intact ectoderm explanted onto the STO feeder cells. From the undifferentiated outgrowth (arrowed) in (c), colonies of ES cell morphology were observed at the second passage (d) and subsequently expanded into a permanent stem cell line. Photographs taken using phase-contrast microscopy.

ectoderms may often give rise to outgrowths that appear remarkably like established ES cells from the outset (as has been reported for immunosurgically isolated ICM outgrowths; 2), in the author's experience, most of these differentiate extensively on disaggregation. Because of the relatively large number of cells in the d 5.5 pc primitive ecto-

derm, differentiation may be minimized by lightly disaggregating the isolated ectoderms prior to culture.

3.4. Culture of Established Murine Embryonic Stem Cells

This section describes the procedures used for the routine maintenance of ES cells, the freezing and thawing of cell lines, the induction of *in vitro* differentiation, and the karyotype analysis of stem cells.

3.4.1. Maintenance of Embryonic Stem Cell Lines

Cell lines maintaining a stable ES cell morphology may be routinely cultured in 25-cm² flasks. After the fifth passage, ES cells can be “weaned” from the STO feeder layer, and the serum concentration in the medium reduced by half. ES cells are subsequently maintained on gelatin-coated flasks in medium (ES₁₀) supplemented with DIA/LIF to prevent ES cell differentiation (*see* Note 6). This factor may be supplied from either BRL-conditioned medium or a recombinant source. The supplementation of DIA/LIF to medium provides a complete substitute for feeder cells preventing the differentiation of isolated stem cells and, hence, greatly simplifies the routine culture of ES cell lines. For BRL-conditioned medium, the optimal concentration minimizing spontaneous ES cell differentiation is a 60% (v/v) strength, diluted with fresh ES medium (35). ES cells are generally grown in media containing 10 ng/mL DIA/LIF (equivalent to 1000 U/mL) to maintain the undifferentiated stem cell morphology (15,16). In the situation where DIA/LIF is not purified from the supernatant, a titration assay is necessary to determine the volume of crude DIA/LIF supernatant to add to ES₁₀ medium in order to prevent ES cell differentiation. Established ES cells retain their full developmental potential and capacity to colonize the germ line in chimeras when maintained in the “crude” BRL-conditioned medium (36) and in the presence of purified recombinant DIA/LIF (37).

3.4.2. Subculture of Embryonic Stem Cells

Established ES cell lines are generally passaged every 3–4 d, changing the medium every other day or whenever it becomes acidic.

- 1 When confluent, the cells are refed 2–3 h before the passage to maximize subsequent cell survival. The medium is then aspirated, and the cells washed once in PBS.

2. After removal of the PBS, 2 mL of TEG are added, and the flask incubated at 37°C for 3 min. The extent of trypsinization is periodically monitored in the inverted microscope. To minimize spontaneous differentiation of ES cells, the aim is to produce a single-cell suspension. To achieve this, it may sometimes be necessary to knock the flask gently against the bench.
3. Once the cells have been dissociated, the trypsin is neutralized with 3 mL of ES₁₀ + DIA/LIF medium, and the suspension mixed thoroughly. Routinely, the cells can be subcultured by transferring a 1/10 aliquot of this suspension (i.e., 0.5 mL) into a pregelatinized flask (0.1% [w/v] gelatin in sterile water for around 1 h) containing 10 mL of fresh ES₁₀ + DIA/LIF medium. The volume of the aliquot dispensed can be varied depending on the requirement for ES cells; however, it should not be reduced below a 1:10 ratio.

3.4.3. Freezing and Thawing of Embryonic Stem Cells

Generally, it is wise to freeze several ampules of cells as soon as possible after isolating a new ES cell line. For long-term storage, the ampules are kept under liquid nitrogen. The cryoprotectant used in the medium is dimethyl sulfoxide (DMSO) at a final concentration of 10%. Although the procedures described here are specifically for ES cells, similar methods can be used for the freezing and thawing of STO and BRL cells.

3.4.3.1. FREEZING CELLS

The ES cells harvested from each confluent 25-cm² growth area (ca 1 × 10⁷ cells) are frozen in a 1.0-mL cryotube (Nunc/lon).

1. Cells are collected by trypsinization (Section 3.4.2) and are then pelleted in a conical, plastic universal by centrifugation at 1000 rpm for 5 min.
2. The supernatant is aspirated, and the cells resuspended in 0.5 mL of ES₁₀ + DIA/LIF medium.
3. The final volume (i.e., 1.0 mL) is made up with 0.5 mL of the freezing medium (20% [v/v] DMSO in ES₁₀ + DIA/LIF medium), which is added slowly, while gently flicking the universal.
4. The 1.0-mL suspension is dispensed into the cryotube, which is then wrapped in a few layers of paper tissue in a polystyrene box before being placed in a -70°C freezer overnight.
5. For long-term storage, the cryotubes are clipped onto freezing canes and plunged into liquid nitrogen.

3.4.3.2. THAWING CELLS

1. Once retrieved from the liquid nitrogen, the cryotube is thawed quickly in a 37°C water bath, until the ice crystals have all melted.

2. The cryotube is sterilized by wiping with 70% alcohol.
3. Working at room temperature within the flow cabinet, a sterile Pasteur pipet and bulb are used to transfer the 1-mL cell suspension in the cryotube into 9 mL of ES₁₀ + DIA/LIF medium in a conical universal. The cells are then pelleted at 1000 rpm for 5 min.
4. The supernatant is aspirated, and the cells resuspended in 10 mL of ES₁₀ + DIA/LIF medium, transferred to a pregelatinized, 25-cm² tissue-culture flask, and cultured in the CO₂ incubator.
5. The medium should be changed after approx 6 h to remove any cell debris.

By seeding the thawed cells at high density, the flasks should reach confluence within 1–2 d.

3.4.4. Induction of In Vitro Differentiation in Embryonic Stem Cells

By the suspension culture of cellular aggregates, ES cells can be induced to differentiate along pathways thought to be analogous to those of early embryonic development, leading to the formation of embryoid bodies. These may be simple structures comprising an outer layer of endodermal cells or may progress into fluid-filled, cystic embryoid bodies. These comprise an inner layer of ectodermal-like cells, with a Reichert's membrane separating a presumed outer layer of parietal endodermal cells. These cystic embryoid bodies contain alpha-fetoprotein and transferrin and are thus analogous to the visceral yolk sac of the postimplantation-stage mouse embryo (23).

1. Suspension culture is conducted in agarose-coated dishes. These are prepared by applying a base layer consisting of 2% (w/v) agarose (Type 1; Sigma) in PBS, which is dissolved and sterilized by autoclaving. Approximately 1.5 mL is added per 6-cm dish to give an even layer and is left to set at room temperature. A second, thin layer is then applied using 1% (w/v) agarose in PBS. Once this has set, 5 mL of DMEM₁₀ + 0.1 mM β -mercaptoethanol is added to each dish and incubated to allow equilibration. Before use, the medium should be replaced.
2. ES cells are lightly trypsinized with TEG for 1–2 min. By gently rocking the flask, large clumps of cells detach and the trypsin should then be immediately neutralized with DMEM₁₀ medium + 0.1 mM β -mercaptoethanol.
3. An approx 1/20 aliquot of this aggregate suspension is dispensed into a 6-cm agarose-coated dish. At higher seeding densities, the individual aggregates adhere to each other.
4. Cultures are fed regularly, aspirating the old medium by either transfer-

- ring the suspension into a conical universal to allow the embryoid bodies to settle, or by simply tilting the dish, before fresh medium is added.
5. Utilizing these procedures, simple embryoid bodies form within 2–4 d and become cystic after 7–10 d.
 6. If simple embryoid bodies are allowed to attach to a tissue-culture surface, the resulting differentiation is chaotic and a wide range of different cell types form, which may be identified utilizing standard histology or specific cell markers.

3.4.5. *Karyotype Analysis of Embryonic Stem Cells*

Karyotype analysis is used to determine the sex and chromosome complement of a cell line. In order to obtain germ-line transmission of the ES cell genotype in chimeras, it is vital that a high proportion of cells within the cell line have an euploid chromosome complement and a modal number of 40 chromosomes. It is important to check the karyotype of an ES cell line routinely with increasing time in culture, since there is the risk of selecting aneuploid cells, which exist within most ES cell lines. To regenerate a karyotypically normal cell line, the ES cells may be single-cell cloned, and diploid cultures identified and reexpanded.

This section describes the methods to prepare cells in metaphase in order to perform chromosome counts and G-banding analysis, which involves the denaturation of the chromosomes with hot saline and trypsin in order to identify individual chromosomes on the basis of their unique banding pattern, and to determine whether any abnormalities are present.

3.4.5.1. PREPARATION OF METAPHASE SPREADS

Metaphase spreads of ES cells may be prepared from cultures exposed to colcemid or, preferably, by utilizing cultures in an exponential phase of cellular growth, in order to maximize the number of cells in mitosis. The quality of the mitotic spreads is generally superior when colcemid is not used.

1. ES cells are trypsinized and pelleted in a 20-mL conical universal as described previously (Section 3.4.2.).
2. The medium is aspirated and the cell pellet disrupted, before a Pasteur pipet and bulb are used to introduce dropwise, around 0.5 mL of a 0.56% (w/v) KCl solution. Once mixed, excess hypotonic KCl solution is added to make 10 mL and left at room temperature for 15 min, to allow the cells to swell.

3. Following centrifugation (1000 rpm for 5 min) and aspiration of the supernatant, freshly prepared fixative (3 vol of absolute methanol to 1 vol of glacial acetic acid) is slowly added dropwise, while flicking the tube to prevent the cells from forming clumps.
4. Excess fixative is added to make 10 mL and the cells are left for 5 min, at room temperature, before pelleting (1000 rpm for 5 min) and aspiration of the supernatant. This cycle is repeated two further times, each time with freshly prepared fixative. The cells are finally suspended in around 0.5 mL of fixative and are then ready for producing mitotic spreads.
5. Metaphase spreads of the fixed, swollen cells are made on clean, wet, glass microscope slides that have been chilled on ice. A quantity of the fixed suspension is drawn into a hand-pulled Pasteur pipet (tip diameter ca 1 mm) with a bulb. Several drops of suspension are released onto the slide from a height of around 100 cm.
6. The undersurface of the slide is wiped dry, and evaporation of the fixative is aided by warming the slide briefly in the flame of a Bunsen burner, as well as blowing across the surface of the slide (taking care not to inhale the fixative vapor). The height from which the cells fall and the rate of fixative evaporation are both important variables in maximizing the rupture of the swollen cells and, hence, the spreading of the chromosomes.
7. For determining the modal chromosome number, the slides can be stained immediately in a 3% (v/v) solution of Gurr's Giemsa stain in PBS for 15 min. The slides are then rinsed in two changes of distilled water and allowed to air-dry before counting chromosomes. For the purposes of G-banding, the best staining results are obtained by first storing the slides in a dust-free place for 10–14 d after preparation of the mitotic spreads.

3.4.5.2. G-BANDING ANALYSIS

1. Slides are incubated in 2X SSC at 60°C for 1 h. The slides are then rinsed four to five times in distilled water and stored temporarily in a rack under water.
2. Each slide is individually immersed in a 0.25% (w/v) trypsin solution in Gurr's phosphate buffer (pH 6.8) for between 7 and 15 s at room temperature.
3. The trypsin remaining on the slide after this digestion is neutralized in Gurr's buffer containing 5% (v/v) NCS.
4. The slides are rinsed further in two changes of buffer before being stained in freshly prepared 5% (v/v) Giemsa Gurr's R-66 stain in Gurr's buffer (pH 6.8) for 8–10 min.

5. The slides are finally rinsed in two changes of buffer, followed by two changes of distilled water, and allowed to air-dry. The incubation times of the slides in trypsin and stain should be determined empirically in order to optimize the clarity of the banding patterns.
6. G-banded metaphase spreads are examined with a standard format, bright-field microscope utilizing oil immersion, objective lenses (maximum magnification around 1000X). Several suitable mitotic spreads with no or minimal overlapping of chromosomes should be photographed. G-banded chromosomes are identified according to the Standardized Genetic Nomenclature for mice (38), and karyograms constructed to determine the sex of the ES cell line and whether populations exist within the cell line that possess chromosomal abnormalities.

4. Notes

1. In the mouse, ES cells have been isolated from the blastomeres of 16- to 20-cell morulae (33), from the inner cell mass (ICM) of blastocyst-stage embryos (1,2), from the primitive ectoderm of the d 5.5 pc egg-cylinder-stage embryo (19) and recently from d 8.5 pc primordial germ cells (39). ES cell lines derived from the ICM have been obtained from fertilized d 3.5 pc or implantationally delayed blastocysts and from parthenogenetically (40,41) and androgenetically produced blastocyst embryos (42).

Historically, the majority of workers have isolated ES cell lines from the readily available d 3.5 pc blastocyst-stage embryo. ES cell cultures have been obtained from both intact blastocysts and immunosurgically isolated ICMs with equal success (ca. 10%; 28). From experience, blastocysts that have undergone a period of implantational delay are expected to generate, on average, a threefold increase in the efficiency of ES cell isolation compared to nondelayed, d 3.5 pc embryos (19,43). The increase in the potential of delayed embryos to yield ES cells may arise either as a consequence of the small, yet significant increase that occurs in the number of cells in the ICM (44), or may be a result of some epigenetic change in gene expression. Although primitive endoderm has formed in the implantationally delayed embryo, no further differentiation of the ICM occurs (45). Therefore, the normal pattern of expression of genes responsible for the differentiation of the ICM is halted, and this may be an essential feature in the establishment of ES cell lines *in vitro*.

Recent evidence indicates that there is a period of embryonic development, from d 2.5 to d 8.5 pc, from which pluripotent cells present within the murine embryo can be successfully isolated and maintained in culture as ES cells. Eistetter (33) has reportedly isolated ES cells from individual blastomeres of decompacted morulae with significantly greater effi-

ciency than from blastocysts cultured conventionally (37 vs 8%). Although possible, the establishment of ES cell lines from postimplantation-stage embryos is both technically more demanding and less successful than from preimplantation stages (19,39). Since detailed chimeric analyses have not been reported, it is not known whether these blastomere-, primitive ectoderm-, and primordial germ cell-derived cell lines are of the same lineage as ICM-derived ES cells, or if their *in vivo* developmental potentials are "frozen" at the embryonic stage from which they are isolated.

2. For both ES cell isolation and routine culture, **no** antibiotics are used. If a culture becomes infected (especially if by fungus or yeast), it is generally wise to discard it from the laboratory. If, however, a particularly valuable culture becomes infected with bacteria, it may be extensively washed in Ca^{2+} - and Mg^{2+} -free PBS before medium containing 50 IU/mL penicillin (sodium salt) and 50 $\mu\text{g}/\text{mL}$ streptomycin is added.
3. Smith and Hooper (35) found that medium conditioned by Buffalo rat liver (BRL) cells contained a factor that was a potent inhibitor of stem cell differentiation. This factor was termed stem cell differentiation inhibitory activity (DIA). Structural and functional comparisons have shown that DIA is identical to the murine myeloid leukemia inhibitory factor (LIF; 15,16). Furthermore, a third growth factor, human interleukin for DA cells (HILDA), has been shown to be essentially identical to the human LIF protein (46,47).
4. It has been found that the osmolarity of DMEM, as supplied by Gibco, is approx 345 mosM/kg H_2O , and is still considered very high after all of the media supplements and sera have been added (ES₂₀: ca. 335 mosM/kg H_2O and ES₁₀: ca. 340 mosM/kg H_2O ; 48). Although an initial study found no significant differences in the efficiency of ES cell isolation with media of varying osmolarities (Wells, unpublished data), the plating efficiency of an established ES cell line was observed to have been optimal with medium of 290 mosM/kg H_2O (J. McWhir, IAPGR, Edinburgh, Scotland, personal communication). Hence, it is recommended that the osmolarity of both ES₁₀ and ES₂₀ media be reduced to around 290 mosM/kg H_2O by the addition of 13% (v/v) sterile water (see Section 2.3.).
5. The function of the feeder cells, in addition to providing a more suitable attachment surface for direct coculture (20), is in the active suppression of stem cell differentiation. It has recently been shown that a factor known as DIA/LIF (see Note 3), which inhibits the differentiation of stem cells, is produced by these feeder cells as both a diffusible protein and in an immobilized form, associated with the extracellular matrix (21). Furthermore, it has been shown that in the coculture sys-

tem, ES cells secrete a heparin-binding growth factor responsible for the stimulation of DIA/LIF expression in the feeder cells (49). It has also been recently shown that recombinant DIA/LIF, added in the culture medium, can replace feeder cells in the isolation of ES cell lines, which may retain their capacity for germ-line transmission (50,51). However, it is recommended that feeder cell layers be used for the isolation of new ES cell lines (but not necessarily for their maintenance once established; see Section 3.4.1.), since the coculture system may aid in producing cell lines with an euploid chromosome complement.

6. ES₂₀ medium is used routinely for ES cell isolation. However, others have demonstrated the use of medium conditioned on PSA-1 embryonal carcinoma cells (2) or BRL cells (13) to facilitate the isolation of ES cells. However, Robertson and Bradley (43) have observed that although conditioned medium may enhance the growth of primary colonies, the majority tend to be of a "retrophoblast" lineage and ultimately differentiate. Furthermore, a comparative study has shown no significant effect of cell-conditioned medium on the efficiency of ES cell isolation (28).

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CHAPTER 24

Production of Chimeras Derived from Murine Embryonic Stem Cells

Dave Wells

1. Introduction

Embryonic stem (ES) cells are undifferentiated cells derived from early mouse embryos, which under appropriate culture conditions proliferate continuously *in vitro*. ES cells have been demonstrated to be pluripotent *in vivo* from their capacity to form teratocarcinomas (1,2) and germ-line chimeric mice (3), dependent on the environment into which the stem cells are introduced. When ES cells are introduced under the kidney capsule, *in vivo* differentiation is chaotic with the teratocarcinoma composed of a wide variety of different cell types. If, however, the stem cells are returned into a preimplantation mouse embryo, *in vivo* differentiation proceeds in a normal and organized manner, and the ES cells colonize the three primary cell lineages of the developing embryo: the primitive ectoderm, endoderm, and mesoderm. This leads to the formation of chimeric offspring composed of cells of two different genetic constitutions: the host embryonic cells and those derived from the ES cells. The ES cells are capable of contributing to every tissue in the fetus, including the primordial germ cells (3,4). Furthermore, the trophectodermal and primitive endodermal derivatives in the extraembryonic tissues of the conceptus may also be colonized by ES cells (5,6). Recent studies have shown that murine ES cells are capable of supporting complete fetal development, following the aggregation of stem cells with tetraploid-cleavage-stage embryos (7).

Although ES-cell-derived chimeras may be generated relatively easily (an average of 35% of pups born were chimeric in a study of 17

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independently derived ES cell lines; 4) achieving germ-line transmission is much more difficult and requires the use of euploid ES cells (*see* Note 1). Unlike embryonal carcinoma cells (isolated from teratocarcinomas), Evans et al. (4) found no significant fetal losses, nor any evidence of tumor formation in any of the ES-cell-derived chimeras produced in a large study. In fact, there has been only one report of an extragonadal teratocarcinoma derived from ES cells in a chimeric mouse, presumably resulting as a consequence of the “escape” of one or more stem cells from the regulative control of the inner cell mass (ICM) following injection into the host blastocyst (8).

ES cells are ideally suited to genetic modification and subsequent selection *in vitro*. In combination with their capacity to colonize the germ line of mice, ES cells are being increasingly utilized to introduce precise mutations into the mouse genome via the application of homologous recombination (gene targeting) technology, in order to study the developmental function of specific genes. The design of the targeting vectors used in these gene knock-out experiments, their introduction into ES cells, and the selection of recombinants are all beyond the scope of this volume, and the reader is referred the following references (9–17).

This chapter describes two methods for generating chimeric mice: either from the physical injection of ES cells into host embryos (morula and blastocyst stages) or the aggregation of stem cells with cleavage-stage embryos. The following sections consider the general mouse requirements, culture media, the embryo injection and aggregation procedures, embryo transfer to recipient females, and the analysis of chimeric offspring.

2. Materials

2.1. Mouse Requirements

Mice are required to act as donors to supply host embryos and pseudopregnant recipients to foster the manipulated embryos.

2.1.1. Donor Mice

The choice of mouse strain to use as donors depends on the genotype of the stem cells and the combination of cell markers necessary to distinguish the host embryonic cells from those cells in the chimera of stem cell origin (*see* Note 2). The procedure for superovulation of donor mice has been described previously (*see* Chapter 23, Section 3.2.1.; *see* Note

3). To produce embryos of the correct developmental stage at a convenient time of day for the experimenter, the light cycle and the timing of the gonadotrophin injections are important parameters. Mice are normally housed in a cycle of 12 h light and 12 h dark. With natural matings, estrus females ovulate 3–5 h after the onset of the dark period, and mating occurs around the midpoint of the dark cycle. With superovulated females, it is generally assumed that ovulation occurs 12 h after the administration of hCG, which should be timed to occur around the midpoint of the dark cycle to optimize fertilization. The approximate time of initiation of embryonic development is determined by checking females for copulation plugs the following morning. This is designated d 0.5 postcoitum (pc).

Eight- to 16-cell morula-stage embryos are found in the oviduct on d 2.5 pc. By d 3.5 pc in the mouse, the embryos have entered the uterus and developed to the expanded blastocyst stage. The methods for recovering these two embryo stages from the reproductive tract have been described previously in Chapter 23, Section 3.2. Following recovery, embryos are group-cultured in microdrops (25 μ L) of the appropriate medium (Section 2.2.) under oil, until they are required for manipulation.

2.1.2. Recipient Mice

Pseudopregnant recipient females are used as surrogate mothers for the manipulated embryos. Recipients can be of any genetic strain that is available; however, to optimize embryonic survival, it is best to use F₁ hybrid strains (e.g., C57BL \times CBA). This is because a higher proportion of F₁ recipients tend to maintain a pregnancy, and they also tend to be better mothers.

Pseudopregnant recipients are obtained from natural matings between females and vasectomized males (*see* Chapters 13, 15, and 20). The approximate time of ovulation is determined by checking for copulation plugs the following morning, which is designated d 0.5 pc. These matings must be set up so that the recipient females are 1 d less advanced developmentally than the donor embryos that they will receive.

2.2. Embryo Culture

Cleavage- and blastocyst-stage mouse embryos are cultured at 37°C in a humidified 5% CO₂ (in air) incubator. However, these two embryonic stages must be cultured in separate media because of differences in their energy substrate requirements.

2.2.1. Media for Cleavage-Stage Embryos

Cleavage-stage embryos do not have a glucose-based metabolism, and are cultured in Medium 16 (M16) with pyruvate as an energy source, supplemented with 4 mg/mL bovine serum albumin (BSA) immediately before use:

Medium 16 (18). To 100 mL of Analar water, add:

0.5534 g	NaCl
0.0356 g	KCl
0.0162 g	KH ₂ PO ₄
0.0294 g	MgSO ₄ ·7H ₂ O
0.0252 g	CaCl ₂ ·2H ₂ O
0.32 mL	Sodium lactate (60% syrup)
0.0036 g	Sodium pyruvate
0.1000 g	D-Glucose
0.0010 g	Phenol red
0.2106 g	NaHCO ₃

Filter-sterilize (0.22 μm), and store at 4°C for not more than 3 wk.

For manipulations conducted outside of the incubator, it is recommended that Medium 2 (M2) with 4 mg/mL BSA, supplemented with HEPES buffer, be used to maintain the pH:

Medium 2 (19). To 100 mL of Analar water, add:

0.5534 g	NaCl
0.0356 g	KCl
0.0162 g	KH ₂ PO ₄
0.0294 g	MgSO ₄ ·7H ₂ O
0.0252 g	CaCl ₂ ·2H ₂ O
0.32 mL	Sodium lactate (60% syrup)
0.0036 g	Sodium pyruvate
0.1000 g	D-Glucose
0.0010 g	Phenol red
0.0337 g	NaHCO ₃
0.5000 g	HEPES (free acid)

Filter-sterilize (0.22 μm), and store at 4°C for not more than 3 wk.

2.2.2. Media for Blastocysts

Blastocysts have a glucose-based metabolism and may be cultured in the medium used for ES cells (ES₁₀ medium; see Chapter 23, Section 2.3.). For manipulations conducted outside of the incubator, it is

recommended that medium supplemented with HEPES buffer be used to maintain the pH. For blastocysts, HEPES is added to ES₁₀ medium to give a final concentration of 20 mM.

2.2.3. Media for ES Cell Culture

These have been described in detail (*see* Chapter 23).

2.2.4. Media for Removing Zona Pellucida

1. Acidified Tyrode's solution. To 100 mL of Analar water, add:

- 0.800 g NaCl
- 0.020 g KCl
- 0.024 g CaCl₂·2H₂O
- 0.010 g MgCl₂·6H₂O
- 0.100 g D-Glucose
- 0.400 g Polyvinyl pyrrolidone (PVP 40)

Adjust to pH 2.1 with Analar HCl. Filter-sterilize (0.22 μm), and store aliquots at -20°C.

2. Pronase solution:

- a. Make 0.5% (w/v) pronase solution (Calbiochem) in Medium 2 (*see* Section 2.2.1.) with 4 mg/mL bovine serum albumin (M2 + BSA).
- b. Sprinkle 1.0% (w/v) polyvinyl pyrrolidone (PVP 40) on top of solution, and dissolve slowly.
- c. Incubate at 37°C for 2 h to digest contaminating nucleases.
- d. Pre-soak dialysis tubing in M2 + BSA and dialyze solution at 4°C for 18 h against excess M2 + BSA in a beaker.
- e. Filter-sterilize (0.22 μm), and store aliquots at -20°C.

2.2.5. Decompaction and Aggregation Media

1. Decompaction of morulae is assisted by incubation of embryos in Ca²⁺- and Mg²⁺-free phosphate-buffered saline (PBS) containing 0.3% (w/v) EDTA. PBS may be prepared by either dissolving preformulated tablets (e.g., Flow Laboratories) or by adding the following ingredients to 1 L of sterile water:

- 10.0 g NaCl
- 0.25 g KCl
- 1.44 g Na₂HPO₄·12H₂O
- 0.25 g KH₂PO₄

PBS is adjusted to pH 7.2. Aliquot into 500-mL bottles, sterilize by autoclaving, and store PBS at room temperature.

2. Aggregation of cleavage-stage embryos may be assisted by the inclusion of phytohemagglutinin (0.1% [w/v]) in media.

2.3. Equipment Requirements for ES Cell Injection into Embryos

The injection chamber used here is prepared simply by introducing a 250- μ L drop of medium into the center of a 90-mm plastic bacteriological dish and overlaying this with lightweight paraffin oil. The dish is then transferred to the microscope stage. The medium comprises ES₁₀ + DIA/LIF (*see* Chapter 23) with 20 mM HEPES buffer to maintain the pH with the manipulations conducted outside the incubator. Some workers have recommended the use of equipment to cool the injection chamber to 10°C (20). However, the procedure described here works very satisfactorily at room temperature.

2.3.1. Preparation of Micropipets

Two micropipets, constructed from glass capillary tubing, are utilized for the embryo manipulations. The embryo is immobilized by gentle suction with a holding pipet, and an injection pipet is used to introduce the ES cells either into the blastocoelic cavity of blastocysts (Fig. 1; Section 3.2.1.) or under the zona pellucida of morula-stage embryos (Section 3.2.2.).

2.3.1.1. INJECTION PIPETS

The injection pipets are prepared by pulling thin-walled, 1.0-mm diameter glass capillary tubing (e.g., GC100T-15; Clark Electromedical Instruments) on a horizontal or vertical pipet puller (e.g., Campden Instruments; Kopf Instruments). The settings are adjusted to produce a capillary with a gradual taper over a 15-mm length of glass, from the shoulder to the needle point. The tip of this pulled capillary is then broken at right angles at an external diameter of 18–20 μ m utilizing a microforge (e.g., Defonbrune; Narishige; Research Instruments). This diameter is generally just large enough to accommodate the stem cells. The capillary is positioned horizontally on the microforge with the small glass bead (0.2–0.5 mm in diameter) on the platinum wire, heating filament directly below the capillary at the required diameter (determined by a graticular eyepiece). With the glass bead heated slightly (the temperature has to be determined empirically), the equatorial plane of the capillary is slowly brought into contact with the glass bead, so that the two just barely fuse together. The filament is then switched off, and at the same instant

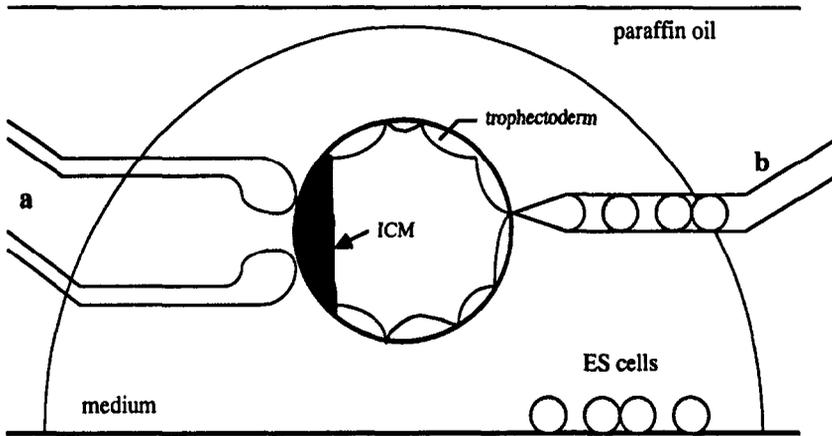


Fig. 1. Orientation of micropipets within the manipulation chamber. A holding pipet (a) is used to immobilize the blastocyst by gentle suction, while an injection pipet (b) introduces the ES cells into the blastocoelic cavity of the embryo. (Figure not to scale.)

the spring-loaded connecting rod, holding the filament, is depressed and so causes the capillary to break cleanly at the point of contact with the glass bead, which retracts as it cools.

To allow the injection pipet to enter the embryo cleanly, a sharp tip is required. With a good deal of patience and skill some workers are able to snap the glass capillary by hand in the dissecting microscope using a sharp scalpel blade and a spongy silicon rubber mat (20). Although it may be a little more time-consuming, using a microgrinder to bevel the pipets is certainly a more reproducible method to construct the desired injection tip. The tip of the pipet is bevelled to an angle of 45° . This is accomplished by grinding the pipets for 3–5 min each, on a slowly rotating (60 rpm) aluminum oxide disk (1- μm grade; 3M) lubricated with distilled water and with a stream of air flowing through the inside of the pipet, to keep the internal bore free of debris. With the aid of the microforge, a sharp point is then put onto the leading edge of the bevelled pipet. With the pipet positioned vertically, the filament is heated to a low temperature, at which the pipet tip just barely fuses to the glass bead. Once fused, the pipet is quickly raised away from the filament to draw out a short glass spike. It is important that the spike is not too long, since during the injection

procedure, the blastocyst may collapse before the pipet has entered the blastocoelic cavity.

To provide a "siliconizing" effect, the tips of the injection pipets are washed in a 1.25% (v/v) detergent solution of Tween-80 in distilled water using a syringe and a piece of plastic tubing to draw the solution inside the fine capillary repeatedly. The pipets are then allowed to dry at room temperature before use.

2.3.1.2. HOLDING PIPETS

The holding pipet is constructed from a 10-mm-long capillary tube (external diameter 1.0 mm; e.g., GC100-10, Clark Electromedical Instruments). The taper may be fashioned by either utilizing a pipet puller as described above or by hand. A small section of the capillary tubing may be uniformly heated over the pilot flame of a propane gas burner. Once softened, the capillary is withdrawn from the flame and pulled quickly by hand, to produce a gradual taper. It is desirable that the tip of the holding pipet have an external diameter of 90–100 μm , which is 1–2 cm from the shoulder of the capillary. This pulled capillary is then broken at right angles by positioning the capillary vertically on the microforge, at the required diameter, adjacent to the glass bead on the heating filament. The capillary is brought into contact with the glass bead in an equatorial position and delicately scored at this point with a fine diamond pencil. The bottom of the capillary (which is waste) is gently tapped, and the glass usually breaks cleanly at the desired diameter. Because of the relatively large diameter of the holding pipet, it is generally easier to break the pipet squarely using the above procedure than trying to use the microforge as described for the injection pipet, especially if the glass bead is small, as is necessary for constructing the finer injection pipets. The tip of the holding pipet is then heat-polished by placing the tip above, but just clear of, the hot glass bead on the microforge, causing the glass to melt and produce a concentric orifice of 20–25 μm in diameter.

2.3.1.3. SECONDARY STRUCTURE IN PIPETS

The appropriate bends need to be created in the pipets with the microforge, so they can enter the injection chamber and allow the tips of the pipets to remain at the same focal level when they are being moved in a horizontal plane. The exact angles depend on the type of chamber being used. To allow entry into the injection chamber constructed from a bac-

teriological dish, both the injection and holding pipets are bent at an angle of around 30°, approx 4 mm from the respective tips (Fig. 1). It is important to orientate the injection pipet correctly on the microforge, so that once the bend has been created, the bevel of the pipet will be in the desired orientation when assembled on the micromanipulation arm.

2.3.2. Micromanipulator Assembly

This section describes the micromanipulatory, optical, and suction systems, and the orientation of the pipets in the injection chamber.

2.3.2.1. MANIPULATORS AND OPTICS

Embryo injections are performed using hand-operated micromanipulators to control the three-dimensional movement of the two pipets in the injection chamber precisely. The two brands commonly used by most researchers are Leitz and Narishige. One micromanipulation arm is mounted on each side of the microscope. The injection and holding pipets can be mounted on either of these micromanipulators depending on comfort for the operator. For embryo injections, ideally an inverted, phase contrast microscope with a magnification range of 40X to 200X and with a fixed stage should be used (e.g., Nikon Diaphot; Leitz Diavert). Such microscopes provide a large working distance between the stage and the condenser to accommodate the injection chamber, and to facilitate simple entry of the pipets into the chamber. Phase-contrast optics are more than satisfactory; however, if Differential Interference Contrast objectives are used, then the injection chamber must be constructed of glass and not plastic. Normal upright microscopes may also be used; however, because of the shorter working distance, a hanging drop injection chamber must be used. This may be constructed from a normal glass microscope slide with two lengths of glass, 22-mm long and 2-mm square, adhered to the longitudinal edges of the slide with Vaseline to support a normal square glass coverslip, also held in place with Vaseline. A small volume of medium is introduced into the middle of the cavity, and each side is back-filled with mineral oil to prevent evaporation.

2.3.2.2. MICROSUCTION

The two instrument holders on the manipulation arms are each connected to micrometer spring-loaded syringes using thick-walled (Portex) tubing (1.0–2.5 mm id). The tubing is filled with lightweight inert min-

eral oil (Fluorinert 77 or paraffin oil). For ease of operating, each micro-syringe should be placed on the opposite side to the manipulator that controls the movement of the pipet. In this way, both the movement and suction of one pipet can be controlled simultaneously with both hands. For the holding pipet, a 0.5- to 1.0-mL ground glass syringe may be used. To allow finer control of the suction with the injection pipet, a 50- to 250- μ L Hamilton syringe is ideal. Before fixing the pipets into the respective instrument holders, the tubing must be purged of air bubbles using a 20-mL reservoir syringe filled with inert oil, connected into the hydraulic line via a three-way tap. The pipets are locked into the instrument holders, and using the reservoir syringes, each pipet is filled with inert oil.

2.3.2.3. ORIENTATION WITHIN THE INJECTION CHAMBER

The pipets are aligned in the injection chamber with the tips parallel to the bottom surface, so that they remain in the same focal level when moving in a horizontal plane (Fig. 1). The type of injection chamber used dictates the secondary structure of the pipets and the arrangement of the micromanipulation arms to allow entry into the chamber. The injection chamber recommended here is prepared by simply introducing a 250- μ L drop of medium into the center of a 90-mm plastic bacteriological dish, overlaid with lightweight paraffin oil. Thus, a 30° bend created at the tips of the pipets means that with the instrument holders angled at 60°, the tip of the pipets are parallel with the bottom of the chamber. With the hanging drop injection chamber, the pipets must enter the chamber horizontally, and thus, the secondary bends must allow for this different orientation.

Once the pipets are positioned within the chamber, medium from the chamber is drawn a short distance into each pipet using the reservoir syringes, and then the three-way taps are turned back to the micrometer-controlled syringes. Several hundred freshly trypsinized ES cells are then introduced into the chamber and allowed to settle to the bottom before commencing injections.

3. Methods

3.1. Preparation of Embryonic Stem Cells

For routine culture and maintenance of ES cells, refer to Chapter 23, Section 3.4. Briefly, the cells are cultured on a 3-d passaging regime, with ES cells being used for chimera production on d 1 or 2 of the

growth cycle. To maximize stem cell viability, the medium should be changed every day. (Important: *see* Note 4).

3.1.1. Preparation of ES cells for injection into embryos

For injection into host embryos, the ES cells are trypsinized into a single-cell suspension and a small volume, containing several hundred cells, is introduced into the injection chamber on the microscope stage. After 1–2 h of micromanipulation, these stem cells should be discarded, and a new injection chamber prepared with freshly trypsinized ES cells.

3.1.2. Preparation of ES Cells for Aggregation with Morulae

For aggregation with morulae, the ES cells are prepared by lightly trypsinizing the cells for 1–2 min to give a suspension consisting of small clumps of cells. A mouth-controlled pipet is then used to pick out healthy clumps individually comprising 10–15 cells each, which are transferred to microdrops of ES cell medium and incubated for 1–2 h. This allows the cells to recover and form rounded masses. The microdrops should be prepared in bacteriological dishes to prevent the cells from adhering to the plastic.

3.2. Injection of Embryonic Stem Cells into Mouse Embryos

The procedure described here is a simplified version of the technique detailed by Bradley (20).

3.2.1. Blastocyst Injection Procedure

1. Embryos to be injected are transferred from the incubator into the injection chamber only in small groups to prevent them from being exposed to lower temperatures for prolonged periods.
2. The injection pipet is lowered to the base of the chamber, and 10–15 healthy ES cells for one injection are individually selected and gently drawn into the pipet by microsuction, so that they are positioned one directly behind the other.
3. The expanded blastocysts are held by suction onto the holding pipet. By patiently blowing and sucking medium through the orifice of the holding pipet, the embryo can be rolled along the base of the chamber until it is eventually oriented in the desired position. It is preferable that the ICM region of the blastocyst be positioned either over the orifice of the pipet (as in Fig. 1), or that the ICM be at either the north or south pole positions.

4. Focus, at high magnification on a “junction” between where two trophoctodermal cells join together around the equatorial plane of the embryo. Then raise the injection pipet to this same focal level. The injection pipet is then brought to the point just adjacent to this cellular junction (Fig. 1).
5. With a controlled rapid forward movement on the manipulation joystick, the pipet is pushed into the blastocoelic cavity. This movement is critical to the success of the operation. If the pipet is moved too slowly, then the blastocyst may collapse. If, on the other hand, the injection pipet is moved too rapidly in an uncontrolled manner, it may either damage the embryo extensively or break the tip of the pipet if it makes contact with the holding pipet. It is generally easier to inject blastocysts when they are very expanded, with the trophoctoderm “stretching” the zona. If necessary, blastocysts may be cultured until they reach this stage.
6. With the injection pipet inside the blastocoelic cavity, the ES cells are gently blown out of the pipet and into the embryo. It is important to have a controlled microsuction system. Air or leaks in the hydraulic line often cause the stem cells to rush in and out of the pipet. It may be important not to introduce more than 20 ES cells into the blastocyst, since there is the potential for abnormal fetal development and tumor formation if the stem cells are not karyotypically normal (8).
7. The injection pipet is then carefully withdrawn from the blastocyst and the injected blastocyst released from the tip of the holding pipet. Shortly thereafter, the blastocysts generally collapse because of the punctured trophoctoderm.
8. Once the embryos in the chamber have all been used, the successfully injected blastocysts are transferred to the incubator in a droplet of ES₁₀ + DIA/LIF medium under oil, where they slowly begin to reexpand. After 30–60 min of culture, the ES cells may be seen adhering to the ICM of the host blastocyst. Injected embryos should not be cultured for more than 6 h before being transferred to recipients; otherwise many of them may begin to hatch from the zona and are therefore more difficult to handle. Injected blastocysts should be transferred regardless of whether or not they have fully reexpanded.

3.2.2. Morula Injection Procedure

Recent evidence suggests that by injecting ES cells into earlier cleavage-stage embryos, the stem cells contribute more extensively to the tissues of the chimera than from blastocyst injections (21). The procedure uses the same micromanipulatory setup as described for blastocyst injections. The procedure is technically straightforward,

simply requiring the injection pipet to enter through the zona pellucida and introduce around 10 ES cells. The stem cells may be deposited underneath the zona, since they tend to migrate into the center of the morula mass owing to the differences in the cell-surface properties between the ES cells and the cleavage stage blastomeres. It is because the ES cells tend to assume an internal position that they contribute more to the resultant ICM and, hence, to all of the tissues of the subsequent fetus. The procedure relies on injecting ES cells before the onset of compaction. If compacted 8- to 16-cell morulae are recovered from donor mice, they may be decompacted by briefly incubating embryos at 37°C in Ca²⁺- and Mg²⁺-free PBS containing 0.3% (w/v) EDTA.

3.3. Aggregation of Embryonic Stem Cells with Morulae

This procedure does not require the sophisticated and expensive micromanipulatory equipment necessary for embryo injections. However, it is not a method that is greatly favored by many researchers to produce chimeras, since it requires the use of a compromise medium because of the different nutrient requirements between ES cells and cleavage-stage embryos (*see* Note 5). As the zona is removed from the eight-cell embryos during the aggregation procedure, the ES-cell-embryo aggregates must be cultured to the blastocyst stage before they can be transferred to recipients. The culture medium must therefore contain pyruvate to support the cleavage-stage embryos and serum, rather than BSA, for the maintenance of the stem cells. In the method described here, a group of ES cells are aggregated with two eight-cell embryos. Although the resultant blastocyst is composed of more cells than normal, the fetus is not larger than usual, since size regulation occurs in the primitive ectoderm shortly after implantation (22).

3.3.1. Removal of Zonae Pellucidae

The zona may be digested from eight-cell embryos by treatment with either acidified Tyrode's solution or with a pronase enzymatic solution.

1. Using a finely pulled, mouth-controlled Pasteur pipet, a few embryos at a time are transferred into a 25- μ L drop of either Tyrode's or pronase solution (these drops do not have to be under oil).

2. While monitoring the progress in the dissecting microscope, the embryos are quickly removed from the drop just before the last remnants of the zona are about to dissolve. This usually takes between 20 and 30 s for the Tyrode's solution and 2 and 3 min for the pronase solution, with digestions performed at room temperature.
3. The zona-free embryos are then washed through several microdrops of M2 + BSA to remove traces of the digesting agents. The zona-free eight-cell embryos are then cultured in pairs at 37°C in microdrops of M16 + BSA for 1–2 h to increase the "stickiness" of the cell surfaces. It is important that the microdrops be set up in bacteriological dishes and not tissue-culture dishes to prevent the embryos from adhering to the plastic surface.

3.3.2. *Decompaction*

For the aggregation procedure to be successful, the embryos must not be compacted. If some compacted morulae are recovered from donor mice they may be decompacted by incubating them at 37°C in Ca²⁺- and Mg²⁺-free PBS with 0.3% (w/v) EDTA, until the cells have separated and individual blastomeres are distinguishable.

3.3.3. *Aggregation Procedure*

1. A small clump of 10–15 ES cells (prepared as described in Section 3.1.2.) and two zona-free eight-cell embryos are transferred to each 5- μ L drop of M16 with 10% (v/v) fetal calf serum under oil in a bacteriological dish.
2. The ES cells are sandwiched between the two embryos by blowing streams of medium through a fine, mouth-controlled Pasteur pipet. Since the cells are very sticky, it is important not to manipulate them with the pipet itself; otherwise they will adhere to the glass.
3. To ensure that the cell surfaces remain sticky, it is necessary that the manipulations be performed at, or near, 37°C and that the cells do not cool extensively. Cooling can be minimized by having only 3 or 4 microdrops/dish and having a heated microscope stage. The aggregates should be checked after 30 min to ensure that they are beginning to adhere together.
4. If there are problems in getting the aggregates to form, then some of the medium in the microdrop can be aspirated to bring the oil/medium meniscus down onto the embryos to force them together. Aggregation can also be enhanced by chemical agents, such as phytohemagglutinin (0.2 μ g/mL; 23). The aggregates are cultured overnight to the blastocyst stage in M16 with 10% fetal calf serum and transferred to the uteri of 2.5 pc recipients (Section 3.4.).

3.4. Embryo Transfer

Manipulated embryos are surgically transferred into the reproductive tracts of pseudopregnant female recipients to allow the embryos to develop *in vivo*. Pseudopregnant females are produced from matings with vasectomized male mice. Vasectomy is described in Chapter 15. Blastocyst-stage embryos are transferred to the uterus, whereas morulae are transferred to the oviducts. In the case of manipulated embryos, it is best to transfer the embryos to pseudopregnant recipients that are developmentally less advanced than the embryos themselves. This allows the transferred embryos time to resynchronize with the reproductive tract following the trauma of the microsurgery and *in vitro* culture. Thus, blastocysts are transferred to recipients that are 2.5 d pc, whereas injected morulae may be transferred to recipients 0.5–1.5 d pc.

The procedure for uterine embryo transfer is outlined in this section. Oviduct transfer is described in Chapter 20. Surgical operations may be performed on the laboratory bench. However, it is important to minimize animal stress and, hence, losses owing to embryonic resorptions.

1. Anesthetize the mouse.
2. Swab the back of the mouse with 70% (v/v) alcohol.
3. A pair of blunt forceps are used to pick up the skin and dressing scissors used to make a 10-mm lateral incision across the midline, posterior to the last rib. Paper tissues, moistened with 70% (v/v) alcohol, are used to open the wound and remove any cut hair, by wiping in a head-to-tail direction
4. Sliding the skin from side to side, the position of the ovaries can be visualized beneath the body wall, appearing quite pinkish with an associated cream-colored fat pad. The region of the body wall directly overlying the ovary is grasped with pointed watchmaker's forceps and a 5-mm incision made in the body wall with sharp iris scissors. By exerting gentle pressure on both sides of the incision, the ovarian fat pad is exteriorized and grasped with blunt forceps.
5. Very carefully, the associated ovary, oviduct, and the top of the uterine horn are also pulled out. The weight of the fat pad is generally sufficient to keep the ovary from slipping back inside the abdominal cavity.
6. The mouse is then transferred, on absorbent tissues, to the stage of a binocular dissecting microscope, with an associated fiberoptic incident light source.
7. In another dissecting microscope, with transmitted illumination, the embryos to be transferred are aspirated into a hand-pulled Pasteur pipet,

with an internal diameter just larger than the embryos themselves and with a square tip. The pipet should be preloaded with alternate media and air bubbles, which allows fine control over the movement of the embryos. Embryos are drawn up into the pipet one directly behind the other, so as to introduce the minimum of medium into the reproductive tract when the embryos are transferred. Typically, nine manipulated embryos are transferred unilaterally. If more embryos are to be transferred, it is best to transfer them bilaterally. However, no more than 12 embryos should be transferred per recipient, and no fewer than five embryos should be transferred to each side of the reproductive tract, since they should be crowded to their normal density to prevent large fetuses from developing, which may result in pre- or early postnatal death.

8. Focusing on the region of the uterotubal junction (magnification around 10X), a pair of watchmaker's forceps are used to grasp the top of the uterine horn, and gently lift it up slightly to allow a 25-g hypodermic needle to puncture the uterine wall and enter the lumen.
9. While still holding the top of the uterus and observing down the microscope, the tip of the transfer pipet containing the embryos is inserted 5 mm inside the uterine lumen, through the hole created with the needle.
10. The embryos are then gently blown into the uterine lumen using the air bubbles along the pipet as markers. After transfer, the pipet should be checked to ensure that all of the embryos were expelled into the reproductive tract.
11. The reproductive organs are returned to the abdominal cavity by lifting up one edge of the incision and gently pushing in the ovarian fat pad with blunt forceps. The incision in the body wall can be closed with a single suture (5/0 Mersilk; Ethicon), whereas sutures or Michel clips may be used to close the skin. To aid postoperative recovery, mice should be placed on a heated blanket before they are returned to a clean cage. If pregnant, the females will litter 17 d later.

4. Notes

1. In achieving germ-line chimerism, male ES cell lines have a greater potential, because the XY chromosome constitution is more stable *in vitro* (24), sex conversion produces some chimeric males that transmit only the ES cell genotype (3,4), and breeding from males is more rapid. There are very few reports of germ-line female chimeras from XX ES cells (25) or *in vitro* isolated XX embryonal carcinoma cells (26,27). Female ES cell lines often suffer either a complete or partial loss of one X chromosome, thought to be a compensatory mechanism for X-inac-

tivation (28). A normal, stable chromosome complement is vital for transmission through the gametes. This generally implies the use of ES cells with a short culture history, since the risk of selecting aneuploid cells increases the longer the cells remain *in vitro*. This has practical implications for obtaining germ-line transmission following gene-targeting experiments. However, germ-line transmission from ES cells following 260 cell generations *in vitro* has been demonstrated, but not without periodic subcloning to identify and reexpand euploid cells (29).

Because the Y chromosome is principally responsible for sex determination in mammals, the integration of XY stem cells with an XX host embryo, results in sex-conversion in a proportion of formerly female embryos. These sex-converted animals transmit only the XY stem-cell-derived genotype in their sperm (30). However, up to two-thirds of sexually converted phenotypic males may in fact be sterile hermaphrodites from a low contribution of ES-derived XY somatic cells in the female germinal ridge, which exert only a mild masculinizing influence (30). In the situation where XY ES cells are combined with an XY embryo, the phenotypic male chimeras produced may transmit the ES genotype in only a small fraction of their sperm (typically 0.3–3%; 30).

The simplest means of assessing germ-line transmission is through the use of appropriate coat color markers. For instance, if the ES cells are derived from a strain of mouse with homozygous-dominant pigmentation alleles and these stem cells are injected into an embryo from an albino mouse strain (homozygous recessive), then by backcrossing the chimera to an albino mouse, any transmission of the stem cell genotype through the germ line will be detected by the presence of pigmented progeny (which will be heterozygous at the pigmentation locus). If suitable coat color combinations are not available, the GPI isoenzymes may be used instead.

Factors that may increase the contribution of ES cells into the germ-line of chimeras include:

- a. Increasing the number of stem cells injected (from 3–5 to 10–15 cells; 24);
- b. The choice of mouse strain from which to obtain host embryos (e.g., stem cells isolated from the 129/Sv strain have entered the germ line at a greater frequency when injected into C57BL/6 host embryos compared to MF1 or CD-1 strain embryos. 11),
- c. Utilizing strains of mice carrying specific fertility mutations to produce host embryos (e.g., the *W* mutation [“dominant white spotting”] 24); and
- d. The developmental stage of the host embryo (21).

The injection of ES cells into morula-stage embryos has shown more extensive chimerism in midgestation fetuses compared to those from blastocyst injection (21). This may in turn increase the probability of obtaining germ-line chimeras and appears more efficient than earlier attempts at ES cell morula aggregation experiments (31).

It is worth mentioning that the vast majority of workers that have demonstrated germ-line transmission from ES chimeras, have utilized ES cells derived from embryos of the 129 mouse strain (including a number of substrains; 32). The only other mouse strain reported in the literature, from which ES cells have been derived and have produced germ-line chimeras, is the CD-1 strain (29). However, there is no biological reason to assume that euploid ES cells derived from the embryos of any mouse strain would not be capable of colonizing the germ line.

2. Depending on the nature of the study, pigmentation, biochemical, or histochemical markers (introduced into the ES cells) may be used:
 - Pigmentation markers: An appropriate combination of coat color markers is the simplest way to detect overt chimeras and to assay for germ-line contributions. Pigmentation markers are only useful post-natally; however, pigment can be detected in the eye of the fetus from d 10 pc onwards.
 - Biochemical markers: Biochemical markers are required for mid-gestational analyses and for analyzing the extent of chimerism in internal body organs. The most commonly utilized marker is glucose phosphate isomerase (GPI), since these isoenzymes (present in one of three forms in mice) are ubiquitously expressed and can be easily separated electrophoretically and stained. For the methods of electrophoresis and staining of the GPI isoenzymes, the reader is referred to Eicher and Washburn (33), McLaren and Buehr (34), and Bradley (20).
 - Histochemical markers: The fate of ES cells introduced into the pre-implantation embryo can be followed as they participate in embryogenesis and the formation of a chimeric offspring, if the stem cells are tagged by a genetic tracer. ES cells stably expressing β -galactosidase, under the control of an endogenous promoter following electroporation, have been used to generate chimeras, and the pattern of colonization by the stem cells followed by *in situ* histochemical staining of whole fetuses (6,21). An extension of this approach is to use β -galactosidase in so-called enhancer trap and promoter trap vectors to identify novel developmentally regulated genes on the basis of temporally and spatially restricted patterns of enzyme staining in the chimeric conceptus (35,36).
3. In producing embryos from donor mice, some workers have preferred

to use natural matings, since superovulation tends to yield some embryos that are morphologically abnormal or developmentally retarded. Although this may be true, these disadvantages are overridden by the advantages superovulation gives in terms of synchronizing females and maximizing the utilization of mice where mouse stocks are limited.

4. In order to obtain germ-line chimerism, a high proportion of the stem cells in a cell line must have a normal chromosomal constitution. Thus, the karyotype should be checked before beginning attempts to generate chimeras (*see* Chapter 23, Section 3.4.4.). The longer cells remain in tissue culture, the greater the chance that the proportion of aneuploid cells in the cell line will increase. By single-cell cloning, however, euploid cell cultures can be identified and reexpanded. ES cell cultures should also be regularly checked for mycoplasma contamination. Such infection causes fetal death and drastically reduces the success in obtaining chimeras (20). Infected cell lines should be discarded, and improved aseptic tissue-culture techniques adopted.
5. Despite the disadvantages, aggregation has been used successfully to produce ES-cell-embryo-derived chimeras (7,31). The aggregation of tetraploid four-cell embryos with ES cells has been used to produce newborn pups that were 100% stem-cell-derived (7). Tetraploid embryos were used in order to establish the placental connection. However, because of their ploidy, the tetraploid cells were excluded from the developing fetal tissues. The reason why these ES-derived newborn pups failed to survive postnatally is not yet clear (7).

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CHAPTER 25

Cryopreservation of Transgenic Mouse Lines

Kimball O. Pomeroy

1. Introduction

A transgenic animal represents an enormous investment in time and money. Animals can be destroyed through disease, fire, malfunctions in the control of the environment, negligence, sabotage, or accidental disposal. Researchers can protect valuable transgenic lines from accidental destruction by “banking” them in liquid nitrogen. Cryopreservation can also reduce animal costs by decreasing the number of live animals investigators must maintain. Often, when one is trying to produce a transgenic animal, some lines will be derived that may not initially appear interesting. These animals can be stored in liquid nitrogen for future recovery and study. The maintenance of just one line of mice, say 25 mice at 15 cents/d, can cost over \$1000 (US) in a single year.

Most cryopreservation research has focused on mice and cattle. The goals of such research have been to:

1. Better understand the principals of cryopreservation,
2. Develop methods for freezing that result in higher viability, and
3. Develop simpler methods for freezing embryos.

Methods that work for freezing embryos of one species are often inappropriate for the freezing of other species.

Cryopreservation involves a number of steps. These are:

1. Superovulation of donor females (*see* Chapter 13).
2. Surgical or nonsurgical embryo recovery (*see* Chapter 17 and Note 1).

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3. Exposure of embryos to freezing solution containing culture medium (such as PBS; *see* Section 2.), serum, or albumin and a cryoprotectant (*see* Note 2)
4. Freezing of embryos.
5. Thawing of embryos.
6. Removal of the cryoprotectant.
7. Transfer of the embryos to a suitable recipient.

Several variables can be modified to optimize the freezing protocol:

1. Type and concentration of the cryoprotectant.
2. Rate of addition of the cryoprotectant.
3. Duration and temperature of the exposure to the cryoprotectant prior to cooling.
4. Temperature of ice formation.
5. Cooling rates.
6. Temperature at which embryos are plunged into liquid nitrogen.
7. Warming rates.
8. Rate and temperature of dilution of the cryoprotectant.

Before attempting to freeze the embryos of a valuable transgenic line, one should first freeze embryos of nontransgenics in order to establish the freezing procedure in your laboratory. Not only should trials be conducted in which embryos are frozen, thawed, and cultured, but embryos should also be successfully transferred into recipients, resulting in viable offspring. Next, one should estimate the number of samples to freeze by taking into account the number of embryos needed to produce adequate numbers of male and female mice to rescue the line. This number can then be doubled, so that ideally the line can be stored in two separate liquid nitrogen containers. These containers should be equipped with liquid-nitrogen-level monitors. All freezing straws should be labeled and crossreferenced in a book that records details on the transgenic line, the number of embryos in the straw, the date the embryos were frozen, and the freezing protocol used. This last item is important, since freezing procedures may change and the method of freezing determines the thawing method.

There are probably more ways to freeze mouse embryos than any other species. Most of these methods have been determined empirically. Two methods are described here. The first involves a slow cooling protocol that demands a programmable freezing machine (1). The second is a fast cooling method developed at the Salk Institute (San

Diego, CA) and uses a -70°C freezer (1). Some mouse lines may be more sensitive to damage using the fast cooling protocol.

2. Materials

Equipment needed to freeze embryos is often already found in laboratories that produce transgenic animals.

1. Stereomicroscope.
2. Embryo-transfer pipets (*see* Chapter 17).
3. Tissue-culture dishes.
4. Carbon dioxide (5% [v/v]) incubator set at 37°C .
5. Culture media (*see below*).
6. Liquid-nitrogen storage tank.
7. A programmable freezing machine: Recent advances in the freezing of embryos of some species, including the mouse (2), allow for freezing without such expensive equipment. Instead, freezing is accomplished by the use of a -70°C freezer or by direct plunging into liquid nitrogen.
8. Phosphate-buffered saline (PBS): Add the following ingredients to 1 L water:
 - 10 g NaCl
 - 0.25 g KCl
 - 1.44 g $\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$
 - 0.25 g KH_2PO_4Adjust to pH 7.2, aliquot into 500-mL bottles, and filter-sterilize (0.2- μ pore).
9. Fetal calf serum (FCS).
10. Sucrose.
11. Glycerol.
12. Forceps.
13. Embryo culture medium (e.g., M2; *see* Chapter 16).
14. Freezing straws.

3. Methods

3.1. Slow Cooling

3.1.1. Freezing

1. Place eight-cell to early-blastocyst stage embryos (2.5–3 d post-hCG; *see* Chapters 13 and 17) into PBS containing FCS (10% [v/v]).
2. Rinse the embryos twice in 2 mL of PBS containing FCS (10% [v/v]).
3. Place the embryos into 2 mL of PBS containing FCS (10% [v/v]) and 5% (v/v) glycerol for 10 min.
4. Place the embryos into PBS containing FCS (10% [v/v]) and 10% (v/v) glycerol for 10 min, during which time label, load, and seal the straws.
5. Cool embryos at $-2^{\circ}\text{C}/\text{min}$ and hold at -6°C for 5 min.

6. Seed straws with forceps cooled in liquid nitrogen, and hold at -6°C for 5 min.
7. Cool straws to -30°C at $-0.5^{\circ}\text{C}/\text{min}$.
8. Plunge straws into liquid nitrogen for storage.

3.1.2. Thawing

1. Place straws into a 37°C water bath until the ice melts.
2. Remove the embryos from the straws, and place in PBS containing FCS (10% [v/v]), 10% (v/v) glycerol, and 0.6M sucrose for exactly 10 min
3. Transfer the embryos into culture medium, and rinse twice by transferring into fresh culture medium.
4. Culture the embryos until transferred into synchronized recipients (*see* Chapter 20).

3.2. Fast Cooling

3.2.1. Freezing

1. Place eight-cell to morula mouse embryos (2 5 d post-hCG) into PBS containing 10% (v/v) FCS.
2. Rinse the embryos twice in 2 mL PBS containing 10% (v/v) FCS.
3. Place the embryos into PBS containing 10% (v/v) FCS, 3.25M glycerol, and 0.5M sucrose (freezing solution).
4. Load the embryos into labeled straws.
5. Maintain the embryos at room temperature, and ensure that the total exposure to freezing solution is 20 min.
6. Place the straws horizontally onto the bottom of a -60° to -70°C freezer.
7. Cool the straws in the freezer for 5–15 min.
8. Place a small liquid-nitrogen container in the freezer. Rapidly plunge the straws into the liquid nitrogen.
9. Place the straws in canes and store.

3.2.2. Thawing

1. Place the straws into a 25°C water bath until the ice melts.
2. Expel the embryos into 2 mL of PBS containing 10% (v/v) FCS and 0.5M sucrose for exactly 12 min.
3. Transfer the embryos into culture medium, and rinse twice.
4. Culture the embryos, or transfer into synchronized recipients (*see* Chapter 20).

4. Notes

1. The number of embryos recovered can vary among individuals as a consequence of the environmental conditions, the superovulation regimen, or the age of the animal. It is also known that some transgenic lines have impaired fertility.

2. The most commonly used cryoprotectants are glycerol, dimethyl sulfoxide (DMSO), ethylene glycol, and propanediol (propylene glycol).

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CHAPTER 26

Introduction to the Physiology and Husbandry of the Rat

Chiew-Hun Phang

1. Introduction

Until recently, the mouse has been the animal of choice for transgenic studies. This is because the scientists who pioneered transgenic technologies emerged from the fields of mouse genetics and embryology. The mouse will continue to be the most convenient mammalian model for asking developmental, genetic, and immunological questions. Basic cancer studies will also continue to use the mouse as its principal animal model. However, in fields such as physiology and neurobiology, the mouse is a somewhat inconvenient system and, for scientists carrying out studies in these areas, it is the rat that has for many years provided the most appropriate model. The recent development of techniques that allow for the generation of transgenic rats will allow the application of reverse genetic techniques to physiological and neurobiological problems (1).

The rat is a hardy animal with a reproductive capacity as good as the mouse. Rats have a relatively short gestation and generation times and produce large litters (>10 pups per pregnancy). The major disadvantage of rats compared to mice is their size. Animal breeding and holding facilities need to be considerably larger for rats than they would be for the same number of mice. But it is the large size of the rat that has made it such an attractive model for physiologists. The rat is accessible for a whole range of physiological, surgical, and neu-

robiological manipulations that would be unthinkable on the mouse. Such manipulations range from the trivial—it is much easier to measure the blood pressure of a rat than a mouse—to the extremely complex, such as the introduction of probes into specific areas of the brain for electrophysiological readings, cellular ablation, drug delivery, and so forth. As such there is now a huge literature on the physiology and neurobiology of the rat that serves as a solid basis for today's molecular studies. The mouse is much less understood in these respects. In order to apply the benefits of transgenic technologies to the study of physiology and neurobiology, researchers are now turning to the rat. Further, it is likely that the advantages of the rat as a transgenic animal will have medical and commercial application. Transgenic rats will be used to produce accessible disease models. The medical sciences of neurology and neuropharmacology will greatly benefit from the use of transgenic rats. Transgenic rats may find a place in toxicity and drug testing laboratories and, finally, pharmaceuticals themselves might one day be made as foreign proteins expressed in transgenic rat milk.

2. Reproductive Physiology of the Rat

It is beyond the scope of this book to describe fully the reproductive physiology of the rat, and the interested reader is referred to the many excellent books on the subject (2–5). Just a few important points are represented here. Rats reach sexual maturity by the age of 8 wk. Sex in immature rats is differentiated by observation of the perineum. Male rats have a greater distance between the anus and the genital papilla compared to female rats. The gestation period of the rat is 22–23 d. The abdomen of a pregnant rat becomes obviously enlarged only during the final week of pregnancy. Under normal mating conditions, the litter size of the rat is about 12. From our laboratory observations, the rat normally gives birth during the day.

2.1. The Male Reproductive System

The testes of the male are enclosed in two separate scrotal sacs suspended outside the body. The structure of the male reproductive tract is diagrammed in Fig. 1. Within the testes are coiled loops of seminiferous tubules where spermatogenesis takes place. These tubules eventually drain into the epididymis, which is a storage and maturation area for spermatozoa. During copulation, the spermato-

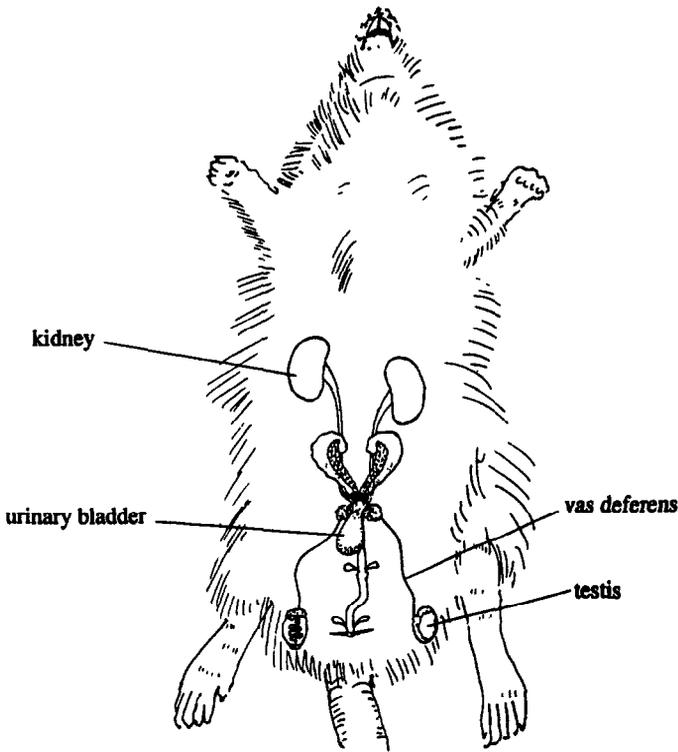


Fig. 1. The male reproductive system.

zoa are carried from the epididymis via the vas deferens and the ejaculatory duct and emitted through the penis.

2.2. The Female Reproductive System

The female reproductive tract is diagrammed in Fig. 2. The ovaries of the female lie close to the kidneys and are surrounded by fat. Each ovary appears as a mass of follicles and is red in color. Located below the ovary are the coiled tubes of the oviduct leading down to either the left or right uterine horn. The uterine horns open to the exterior via the vagina. Surrounding the ovary and oviduct is a transparent membrane, the bursa ovarii.

The female reproductive system goes through regular cyclical changes known as the estrous cycle. Each cycle lasts between 4 and 5 d (*see* Chapter 27).

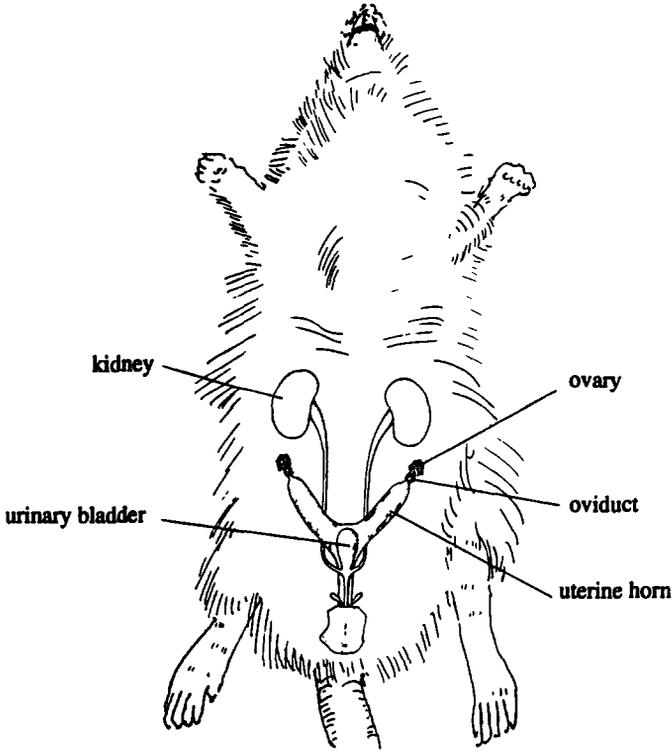


Fig. 2. The female reproductive system.

3. Animal Husbandry

The reader is referred to various excellent books and manuals that describe the establishment and maintenance of healthy rat breeding colony (6).

4. The Production of Transgenic Rats

The process of generating transgenic rats by microinjection of cloned DNA into fertilized one-cell eggs is essentially the same as for mice. Differences in the anatomy and size of the rat compared to the mouse, and particularly, differences in the reproductive endocrinology of the female rat compared to the female mouse, have required standard techniques developed on the mouse to be adapted to the rat. These adaptations are described in detail in the following four chapters. Chapter 27 refers to the reproductive endocrinology of the rat,

and describes protocols for mating and superovulating female animals. Chapter 28 describes the surgical procedure used to vasectomize the male rat. The harvesting of fertilized one-cell eggs from a mated, superovulated female rat is described in Chapter 29, and their reimplantation into a pseudopregnant recipient following microinjection is detailed Chapter 30.

The process of microinjection of fertilized one-cell rat eggs is the same as described for mice in Chapter 18 (manual) and 19 (automatic). Note that the rat egg membrane is considerably more elastic than the mouse egg membrane. This makes the penetration of the egg more difficult and demands particularly good injection pipets. The pronuclei of rat eggs are also smaller than mouse egg pronuclei, perhaps making targeting of the male pronucleus more difficult. The rat egg cytoplasm is very granular, sometimes obscuring the view of the pronuclei.

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CHAPTER 27

Mating of Rats

Chiew-Hun Phang

1. Introduction

For the production of transgenic rats, matings are carried out under controlled conditions in order to obtain a supply of one-cell fertilized eggs, and to obtain pseudopregnancy in females, which will act as surrogate mothers for the microinjected eggs.

2. Materials

1. Follicle stimulating hormone (FSH). This is supplied by Intervet Laboratories Ltd. (Cambridge, UK) under the trade name of Folligon. Pregnant Mares Serum Gonadotrophins from Sigma (St. Louis, MO) can be used if the above is not available. It is dissolved in sterile 0.9% (w/v) NaCl to a concentration of 50 IU/mL and stored frozen.
2. Human chorionic gonadotrophin (hCG). This is supplied by Intervet under the trade name of Chorulon or it can be obtained from Sigma. It is dissolved in sterile 0.9% (w/v) NaCl to a concentration of 50 IU/mL and stored frozen.
3. Sterile 1-mL disposable syringes.
4. Sterile 0.9% (w/v) NaCl or sterile PBS solution is prepared as follows: sodium chloride (8.0 g), potassium chloride (0.2 g), disodium hydrogen phosphate (1.15 g), and potassium dihydrogen phosphate (0.2 g). The aforementioned are dissolved in water and made up to a 1-L solution and autoclaved before use.
5. Dissecting microscope (e.g., Nikon [Tokyo] SMZ10-TD).

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3. Methods

3.1. Matings to Supply Fertilized Eggs

In our laboratory, young and immature Sprague Dawley female rats (*see* Note 1) of 5-wk-old are used for superovulation by the administration of gonadotrophins as follows:

1. At 1200 h, inject 5–10 sexually mature female rats intraperitoneally with 10 IU of FSH (*see* Note 2).
2. 56 h later, inject the females intraperitoneally with 10 IU of hCG (*see* Note 2) before placing each with a stud male (*see* Note 2)
3. In the morning, the females are subjected to a vaginal smear (*see* Section 3.3) to check for the presence of sperm, which is an indication of successful copulation. In rats, despite successful mating, the copulatory plug sometimes has been lost by the next morning. Hence, only females that have a positive sperm smear (*see* Section 3.3.) are used to supply fertilized eggs. Some 50–80% of superovulated females should mate

3.2. Matings to Obtain Pseudopregnancy in Females

It has been found that experienced mothers make better surrogate mothers compared to virgins. A colony of such rats are maintained and are smeared vaginally (*see* Section 3.3.) every morning to determine their estrous stage. Female rats maintained on a 12-h day and 12-h night cycle will ovulate roughly every 4 d (*see* Note 5). The cell characteristics of each of the stages of the estrous cycle are listed in Table 1.

1. On the morning of microinjection, separate female rats that are, by vaginal smearing, determined to be in the proestrous stage and place individually in the cage of vasectomized rats at 1800 h.
2. Alternatively, insertion of a smooth glass rod with a diameter of 7 mm into the vagina of the rat for 1 min will induce pseudopregnancy.
3. There will be no copulatory plug, neither will there be sperm in the vagina to indicate a successful mating since vasectomized rats are used. Hence, rats in the proestrous stage are usually prodded with the glass rod as a back-up procedure before placing them with vasectomized rats

3.3. Vaginal Smearing of Rat

The various stages of the estrous cycle of the rat are most accurately determined by vaginal smearing. Vaginal smearing is carried out at approximately the same time each morning. The rats are iden-

Table 1
Changes in Cellular Content of Vaginal Smears
Associated with an Ovulating Female Rat

Stage of estrous cycle	Cell characteristics
Estrous (Ovulating)	Mostly squamous/cornified cells
Metestrous	Mostly leucocytes
Diestrous	Epithelial cells and leucocytes
Proestrous	Mostly round, nucleated cells

tified either by ear-punches or tail-marks. Vaginal smearing of the rat is performed as follows:

1. Fill 1-mL syringe with about 0.1–0.2 mL of PBS or 0.9% saline solution.
2. Hold the female rat with one hand such that the opening to the vagina is exposed.
3. With the other hand, insert the syringe into the opening until resistance is felt. Expel the contents of the syringe and then immediately draw back the plunger. Cells from the vagina will be picked up in this manner.
4. Transfer the contents of the syringe to a microscope slide and examine the cells under a light microscope with a 40X magnification to determine the various stages of the estrous cycle (Fig. 1).
5. To determine the presence of sperm in the vagina, the contents on the microscopic slide are viewed under a much higher magnification (200X).

4. Notes

1. In our laboratory, we have used only Sprague Dawley strain rats for transgenic production. However, Mullins et al. (1) used fertilized eggs that were derived from matings between Sprague Dawley female and WKY male rats to make their transgenic rats.
2. The injection of hormones, FSH, and hCG can be varied by 1 or 2 h without much affecting the efficiency of ovulation or the quality of eggs.
3. For maintaining stocks of normal or transgenics strains, rats are mated with the least amount of intervention by controlled transfer of male and female rats between cages.
4. The superovulation regime employed by Mullins et al. differs from that of our laboratory. Their method (2) describes superovulation of rats using continuous infusion of FSH using a subcutaneous minipump. This method of superovulation produces about 60–85 oocytes per rat.
5. Some female rats do not go through the normal four phases of the estrous cycle. Instead, they always remain in a particular phase of the cycle. Such rats are in anestrus phase and they are not suitable for mating purposes.

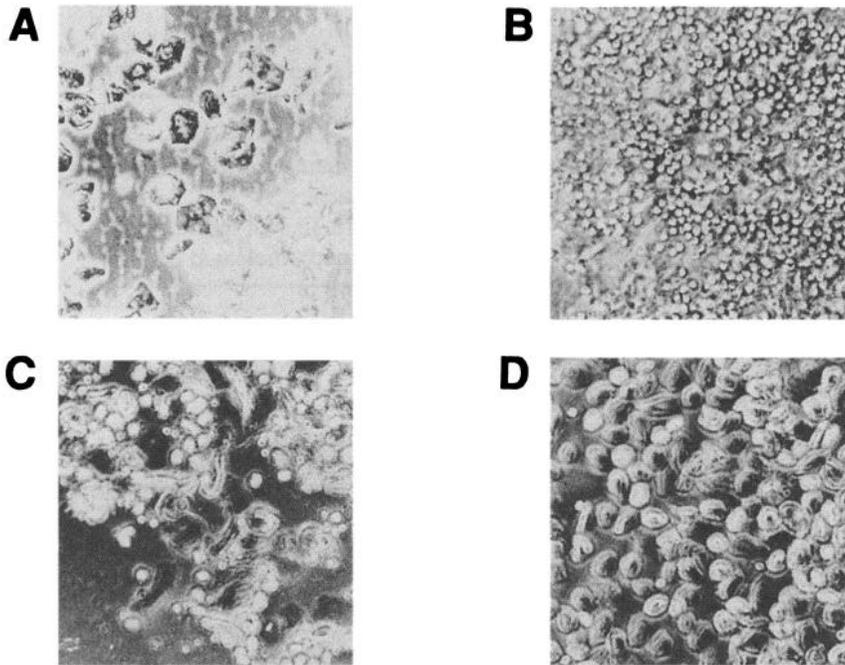


Fig. 1. The morphology of cells from vaginal smears of rats in various stages of the estrous cycle. (A) Estrous stage; (B) Metestrous stage; (C) Diestrous stage; (D) Proestrous stage.

Acknowledgments

The author would like to thank Zeng Qi (IMCB) for her contribution to the development of the superovulation regime. Victor Goh (National University Hospital, Singapore) is thanked for the pictures of the vaginal smears.

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CHAPTER 28

Vasectomy of the Male Rat

Chiew-Hun Phang

1. Introduction

Mature male rats that are sexually active are vasectomized and used to produce pseudopregnancy in sexually mature female rats. These pseudopregnant female rats are used as surrogate mothers. Microinjected fertilized one-cell eggs are implanted into the oviduct of such recipients. As no sperm is emitted from the vasectomized rat during mating and as vasectomized rats, unlike vasectomized mice, do not leave a copulatory plug after mating, there is no direct indication of a successful sterile mating. Hence, a proestrous female is usually chosen to mate with a vasectomized male rat because such a receptive female at this stage of its estrous cycle is more likely to copulate with the male rat.

2. Materials

1. CRC cocktail is made with: 1 part of Hypnorm™ from Janssen Pharmaceuticals; 1 part of Dormicum™ from Roche; and 2 parts of water.
2. 70% (v/v) swabbing ethanol in squeeze bottle.
3. Blunt forceps.
4. Sharp watchmaker's forceps.
5. Curved surgical needle.
6. Surgical silk suture
7. Cotton thread.
8. Wound clips (9 mm) and wound clip applicator.

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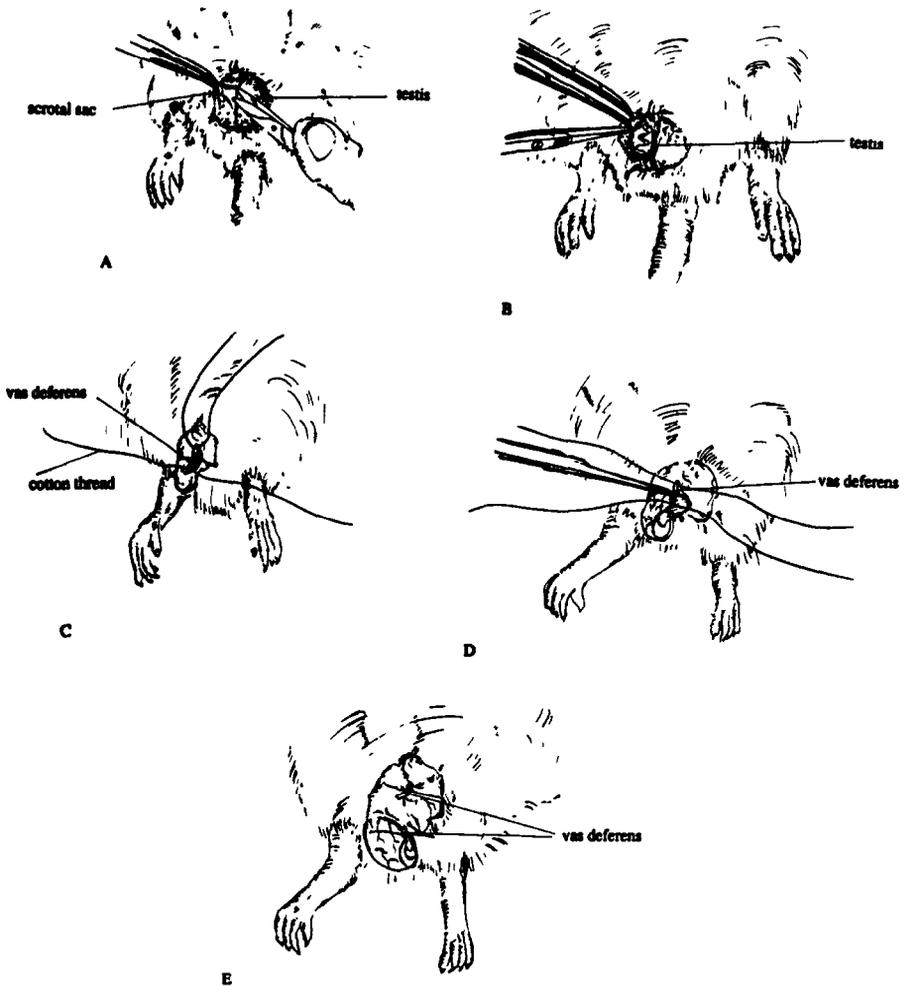


Fig. 1. Vasectomy of the male rat.

3. Methods

1. Restrain the animal (*see* Note 1) with one hand or with a rat restrainer and inject CRC cocktail (0.3 mL/100 g) intraperitoneally Swab the lower abdomen with 70% alcohol
2. Lift the scrotal skin covering the testes with a pair of fine forceps and make an incision of 1.5 cm (Fig. 1A and B).
3. Using a pair of blunt forceps, gently pull out the fat pad lying at the bottom of the testis to reveal the testis, epididymis, and vas deferens.

4. Free the vas deferens from the membrane that is attached to it. Tie tightly at two points using cotton thread (Fig. 1C). Then cut the vas deferens using a pair of scissors at the site between the two tightened ends (Fig. 1D and E).
5. Return the organs to the inside of the body using a pair of blunt forceps.
6. Close up the body wall and the skin with at least three stitches.
7. Repeat on the other testis.
8. Cage the vasectomized animal individually. After 3–4 wk, it can be used to produce pseudopregnant mothers (*see* Note 2).

4. Notes

1. Healthy, sexually mature male rats of about 5-wk-old are used for the vasectomy operation.
2. Vasectomized males tend to grow to a large size. They are replaced if they get too big and become incompatible in size with the presented females.

CHAPTER 29

Harvest of Fertilized One-Cell Rat Eggs

Chiew-Hun Phang

1. Introduction

The harvest of fertilized one-cell eggs of rats is very similar to mice (*see* Chapter 17). However, in the case of rats, collagenase is used instead of hyaluronidase for the removal of cumulus mass from around the eggs.

2. Materials

1. 70%(v/v) swabbing ethanol in squeeze bottle.
2. Fine pair of scissors.
3. Large pair of scissors.
4. Sharp watchmaker's forceps.
5. M16 medium (*see* Chapter 16).
6. M2 medium (*see* Chapter 16).
7. Liquid paraffin.
8. 35-mm tissue culture dishes.
9. Egg transfer pipet (*see* Chapter 17).
10. Stereo dissecting microscope.
11. Collagenase (Sigma, St. Louis, MO) is made to 10 mg/mL in M2 medium.

3. Methods

3.1. Harvest of One-Cell Fertilized Eggs

Collect fertilized one-cell eggs at 14.00 h on the day following mating with stud (*see* Note 1).

As in the case of culturing fertilized eggs from mice, rat eggs that are in the one- or two-cell stages are maintained in M16 microdrop cultures

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in a 37°C tissue culture incubator gassed with 5% (v/v) CO₂. However, when the eggs are manipulated outside the incubator, they are kept in M2 medium.

1. About 1 h before the harvest of the eggs, set up two 35-mm tissue culture dishes containing M16 medium and two 35-mm tissue culture dishes containing small drops of M16 medium covered with liquid paraffin. Allow to equilibrate in the 37°C incubator gassed with 5% (v/v) CO₂. In addition, prepare four 35-mm tissue culture dishes containing M2 medium and leave at room temperature.
2. Kill female rats (smear positive for sperm) by cervical dislocation: Hold the animal by its tail and stun it on a hard surface, e.g., a table top. Then apply a firm pressure at the neck with a large pair of scissors and pull on the tail. If the animal is too large to be killed in this manner, it should be decapitated using a very large pair of scissors or a rodent guillotine.
3. Lay the rat on its back and swab the abdomen with 70% (v/v) alcohol.
4. Make an incision through the skin and the body wall at the lower half of the abdomen. The coiled oviduct located between the ovary and the uterus can be seen once the intestines are moved to the side (Fig. 1A).
5. Using a pair of fine forceps, grip the uterus to stretch the reproductive tract and tear the mesometrium (a membrane that joins the reproductive tract to the body wall) with a pair of fine scissors.
6. Make a cut between the ovary and the oviduct and another between the uterus and the oviduct while holding the oviduct with a pair of fine forceps (Fig. 1B).
7. Place the oviduct in M2 medium.
8. Remove the oviduct from the other horn of the reproductive tract as described in steps 5–7. Then dissect out the oviducts from the rest of the female rats and place in the same dish of M2 medium.
9. View the oviducts under the 20X magnification of a stereo dissecting microscope. Swollen ampulla with the cumulus cells can be seen under this magnification.
10. Tear the ampulla using two pairs of fine watchmaker's forceps to release the fertilized cells surrounded by cumulus cells from the oviduct. Repeat with the rest of the ampullae.
11. Transfer the cumulus masses to a fresh dish of M2 medium using a general egg transfer pipet. Individual eggs are released from the cumulus cells by incubating the cumulus masses with 100 µL of collagenase solution for a few minutes. Usually, it is necessary to pipet the eggs up and down to remove cumulus cells that stick stubbornly to the eggs.

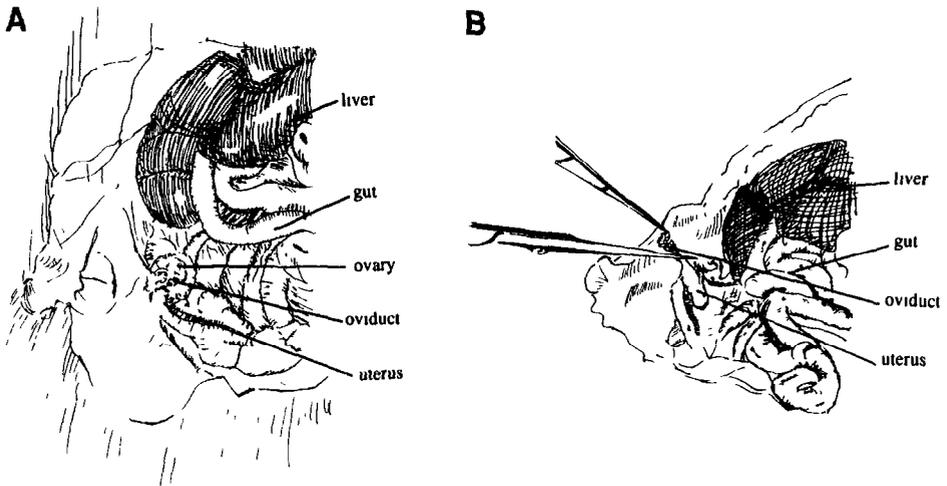


Fig. 1. Harvest of fertilized one-cell rat eggs.

12. Rinse individual eggs twice in fresh M2 medium to remove traces of enzyme and cell debris.
13. Finally, wash the eggs twice in the M16 medium before transferring to the M16 microdrop culture.
14. Incubate the eggs at 37°C in 5% (v/v) CO₂ prior to microinjection.

4. Note

1. Harvesting of fertilized one-cell eggs can be carried out earlier than 1400 h after mating with the stud male. In this laboratory we routinely harvest eggs as early as 1000 h. The one-cell eggs are then incubated until 1400 h to allow the pronuclei of the eggs to develop. The eggs are then ready for microinjection.

Acknowledgments

The author thanks Zeng Qi for her contribution to the work that optimized the timing of the harvesting of fertilized one-cell eggs.

CHAPTER 30

Implantation of Microinjected Eggs

Chiew-Hun Phang

1. Introduction

Microinjected eggs are normally incubated overnight in M16 micro-drop culture at 37°C in CO₂ until 1400 h. By this time the eggs will have developed as far as the two-cell stage. The eggs are then implanted into surrogate mothers. As the membrane covering the oviduct of the rat is much tougher than the mouse, we normally cut it with a very fine pair of scissors rather than tearing it. For a female rat that had been successfully induced into pseudopregnancy, the infundibulum will be swollen and the opening of the infundibulum can be clearly seen under the 10–20X magnification of the dissecting microscope.

2. Materials

1. 70% (v/v) swabbing ethanol in squeeze bottle.
2. Fine pair of scissors.
3. Large pair of scissors
4. Artery clip.
5. Sharp watchmaker's forceps.
6. Blunt forceps.
7. Stereo dissecting microscope.
8. Oviduct transfer pipet (*see* Chapter 20).
9. M16 medium (*see* Chapter 16).
10. M2 medium (*see* Chapter 16).
11. Paraffin oil
12. Curved surgical needle.

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13. Surgical silk suture.
14. Cotton thread.
15. Wound clips (9 mm) and wound clip applicator.
16. CRC cocktail (*see* Chapter 28).
17. Epinephrine (Sigma, St. Louis, MO) is made to 0.1% (w/v) in 0.1M HCl.
18. Ampicillin (e.g., Binatol, Bayer, Leverkusen, Germany).

3. Methods

1. Anesthetize the pseudopregnant recipient with CRC cocktail (0.3 mL/100 g).
2. Position the rat to lie on its front and swab the lower half of the animal with 70% alcohol.
3. Lift the skin beside the spine and about 1–2 cm above the hind leg and make an incision of 1–2 cm with a large pair of scissors (Fig. 1A).
4. Make a smaller incision of 0.5 cm through the body wall beneath the position of the opened skin with a fine pair of scissors.
5. Using a pair of forceps, locate the fat pad that is joined to the ovary, oviduct, and uterus. Pull out the fat pad and attach an artery clip, taking care not to touch or damage the ovary. Adjust the artery clip such that the reproductive tract with the coils of the oviduct is displayed in the uppermost position (Fig. 1B).
6. View the oviduct under the stereo dissecting microscope at 10–20X magnification with illumination from a fiber optic light source.
7. Carefully pick up the transparent membrane covering the ovary, oviduct, and the enclosed cavity with a fine pair of watchmaker's forceps. Introduce a drop of epinephrine (0.1%) into the cavity through a syringe fitted with a 26-g needle (1). The epinephrine prevents local bleeding when the membrane is cut. A lapse of 20 s should be allowed before attempting to cut the membrane (*see* Note 1).
8. The membrane is tough and it is easier to cut through it using a fine pair of scissors than to tear it with watchmaker's forceps.
9. The infundibulum—the swollen opening of the oviduct—is hidden behind the coils of the oviduct under the ovary. Gently move the oviduct coils aside to display the infundibulum.
10. At this point, the eggs are prepared for transfer. Remove about 20 micro-injected eggs from the M16 microdrop culture and wash in M2 medium before loading them into an oviduct transfer pipet. First, fill the pipet with paraffin oil to the shoulder. Follow this with a small air bubble, more M2 medium, then another air bubble. Next, draw up the eggs in a minimum volume of medium followed by another small air bubble, and finally a short column of M2 medium (*see* Chapter 20).

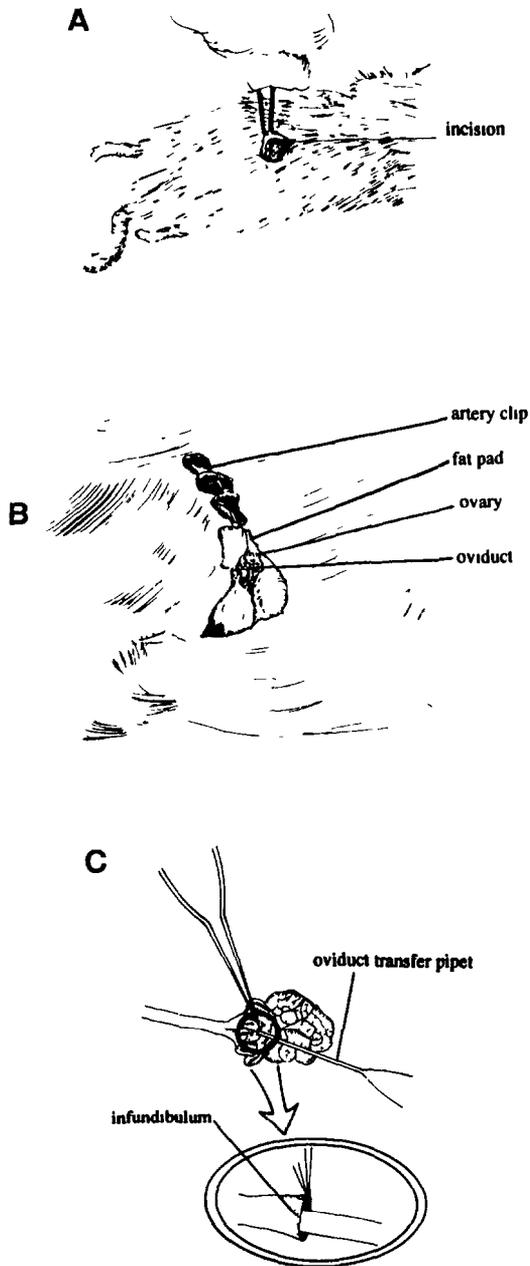


Fig 1 Implantation of microinjected eggs into the oviduct of a pseudopregnant recipient

- 11 Before introducing the eggs into the infundibulum, any body fluids, blood, or blood clots that have formed around the oviduct must be mopped up with a piece of rolled-up tissue.
12. Pick up the infundibulum very carefully at its side with a pair of watchmaker's forceps. Usually the opening of the infundibulum can be seen
13. Push the tip of the oviduct transfer pipet into the opening of the infundibulum and expel the contents of the pipet. (Fig 1C). The appearance of three air bubbles in the ampulla is an indication that the eggs have been successfully introduced into the ampulla.
14. Withdraw the oviduct transfer pipet, remove the artery clip, and return the reproductive tract to the body cavity without damaging the reproductive organs.
- 15 Close up the body wall with two or three stitches. Close up the skin with autoclips.
16. Repeat the implantation of microinjected eggs on the other oviduct if there are further eggs.
- 17 A single dose of ampicillin (50 mg) can be delivered intraperitoneally (see Note 2).
18. When the rat recovers from the effect of anesthesia, return it to the animal holding unit to await the arrival of pups after 21 d of gestation.

4. Notes

1. The transparent membrane covering the ovary of the rat is transversed with fairly large blood capillaries. The use of epinephrine allows the membrane to be cut without much blood loss and hence the infundibulum can easily be located.
- 2 A single dose of ampicillin has been noted to reduce low grade infections that can afflict the rat postsurgery. The antibiotic can improve the efficiency of pregnancy markedly (J. Mullins, Edinburgh; personal communication).

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CHAPTER 31

Cryopreservation of Transgenic Rat Lines

Kimball O. Pomeroy

1. Introduction

The following protocol is for freezing rat blastocysts by direct plunging into liquid nitrogen (1). Although this protocol is designed for freezing the embryos in polypropylene cryotubes, it could readily be adapted to 0.5-mL straws. For a general discussion of cryopreservation, the reader is referred to Chapter 25.

2. Materials

Equipment needed to freeze embryos is often already found in laboratories that produce transgenic animals.

1. Stereomicroscope.
2. Embryo-transfer pipets (*see* Chapter 17).
3. Tissue-culture dishes.
4. Carbon dioxide (5% [v/v]) incubator set at 37°C.
5. Liquid-nitrogen storage tank.
6. Phosphate-buffered saline (PBS): Add the following ingredients to about 800 mL water: NaCl (10 g), KCl (0.25 g), Na₂HPO₄·12H₂O (1.44 g), KH₂PO₄ (0.25 g). Adjust to pH 7.2, adjust the volume to 1 L, aliquot into 500-mL bottles, then sterilize by filter-sterilization (0.2-μ pore).
7. Bovine serum albumin (BSA).
8. Freezing medium VS1:
 - 20.5% (v/v) dimethyl sulfoxide (DMSO)
 - 15.5% (w/v) acetamide
 - 10% (v/v) propylene glycol
 - 6% (w/v) polyethylene glycol (PEG) 8000

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- Freezing solutions are made in PBS containing 0.4% (w/v) BSA.
9. Polypropylene cryotubes (12.5 × 48 mm).

3. Methods

3.1. Cooling

1. Expose rat blastocysts (5 d post-hCG) to PBS containing 0.4% (w/v) and 12.5% (v/v) VS1 for 5 min at room temperature.
2. Transfer the embryos to PBS containing 0.4% (w/v) BSA and 25% (v/v) VS1, and maintain at room temperature for 5 min.
3. Place embryos in VS1 at 4°C, and transfer 40 µL containing the embryos to a precooled (4°C) polypropylene tube for 15 min.
4. Plung into liquid nitrogen and store.

3.2. Thawing

1. Thaw the tubes on ice.
2. Add 200 µL of PBS containing 0.4% (w/v) BSA and 50% (v/v) VS1 to the thawed tube (at 4°C).
3. After 10 min, add 400 µL of PBS containing 0.4% (w/v) BSA and 25% (v/v) VS1, and leave at 4°C for 10 min.
4. Place the embryos in PBS containing 0.4% (w/v) BSA and 12.5% (v/v) VS1 at room temperature for 5 min.
5. Wash the embryos twice in PBS containing 0.4% (w/v) BSA.
6. Transfer the embryos into synchronized recipients.

Reference

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CHAPTER 32

Production of Transgenic Sheep

*Colin D. Nancarrow, James T. A. Marshall,
and Kevin A. Ward*

1. Introduction

The production of transgenic sheep has proven difficult compared to the mouse and lower animals. The work load is far greater and the rates of success far less by most criteria. However, the benefits to human and animal health and agricultural productivity are potentially enormous (Ward and Nancarrow, Chapter 5) and support for the continuation of the work is assured. Unfortunately, the low rate of transgenesis for sheep, at about 1% of injected, transferred embryos, means that investigation of the regulation of expression of the transgenes, their phenotypic effects, and optimization of the fusion gene constructs, all of utmost importance to the agricultural industry, can seldom be addressed. We know now that the mouse may not be a good model for the sheep, an example being the ovine metallothionein-ovine growth hormone fusion gene, GH9 (1-3), for which expression and phenotypic effects were quite different for sheep and mice. In sheep, pronuclear microinjection of several hundred copies of the foreign gene into embryos is the only published method used to regularly produce transgenics and it will be the standard by which future methods for incorporation of the transgene are judged.

Here, we are considering the production of transgenic sheep, from preparation of the animals through to the establishment of pregnancies in recipient ewes. Particulars relating to the preparation of the

fusion genes and the identification of transgenic progeny are common with other species and will be detailed in other chapters of this book. Readers are referred to an excellent review of the more general aspects of production of transgenic ruminants by Wilmut and Clark (4).

This chapter details methods that allow for the generation of transgenic sheep by the physical introduction of DNA into embryos. However, other methods for producing transgenic sheep are being developed and their potential is discussed below.

1.1. Sperm as Vector

The report of Lavitrano et al. (5) whereby transgenic mice were produced following in vitro fertilization with sperm that had been preincubated with DNA created great hope, particularly within those working with farm animals. Although Gandolfi et al. (6) have reported similar success in pigs, many other laboratories, including ours, have failed to repeat the phenomenon, particularly in mice. However, there is recent evidence that DNA can be carried into cattle eggs at fertilization and subsequently identified in blastocysts by the polymerase chain reaction (PCR) (7,8). The significance of these results is that treated spermatozoa may eventually be available frozen in straws for artificial insemination (AI), a far cry from microinjection. Perhaps with the appropriate genes, we will eventually see treated semen used in a "terminal" fashion, to produce a high proportion of transgenic animals that will be used for their immediate phenotypic effect and not for transmission of the gene through the germ line.

The problems with this approach have been the low rate of integration, the rearrangements that occur in the transgene, and the possibility of formation of nonintegrated, replicating episomes resulting from such sequences as the SV40 origin of replication. More research is needed to evaluate further this interesting method.

1.2. Retroviral Vectors

It is possible for recombinant, attenuated vectors to be integrated into chromosomes but the success in producing transgenics has been limited. Problems relate to size limitations on the transgene that can be carried, interference of transgene expression by viral sequences, the high chance of producing chimeric animals, and a slight but important possibility of recombination with disease viruses (4,9).

1.3. Embryo Stem Cells

Perhaps the most important of new approaches to the many problems of production of transgenic livestock is the use of cultured embryo stem cells. These cells can be prevented from differentiating in culture and when introduced into the blastocoele cavity, they can take part in formation of any or all parts of the conceptus. However, chimeras may still be obtained and germ-line transmission may not be possible in all offspring. Transgenes can be introduced into these pluripotent mouse cells by electroporation or chemical means, the integration can be site-directed if required and selection of only those cells transformed can be carried out (10). Despite encouraging results with porcine and, to a lesser extent, ovine lines (11), embryonic stem cells have not yet been established. These methods are so important to genetic engineering that it will only be a matter of time before ruminant stem cell lines are established and used for production of transgenics.

Microinjection remains as the method for production of transgenic sheep at present, despite large efforts being put into other areas. There has been little improvement for nearly a decade in this technique and like many groups we can only hope that our search for more efficient methods will be successful. The most encouraging appear to be the use of sperm as a vector and the establishment of readily accessible methods of producing embryo stem cells. These two methods can be seen to have differing uses in genetic engineering. Not until one of these is developed to the extent that the production of transgenic embryos becomes a matter of course will the scientific community be able to get on with the most important work of all, which is to investigate gene constructs and evaluate their phenotypic effects on animal health and production.

2. Materials

2.1. Animals

The breed of our choice has been, for practical reasons, either the Australian Merino or a Border Leicester \times Merino (BLM) cross. In the ideal situation, one should use the breed for which the transgenes have been tailored (*see* Note 1).

The major sheep breeds have similar reproductive physiology and so it is expected that the methods discussed here will suit all, with

little alteration being necessary. However, parameters such as the timing and efficiency of detection of pronuclear visibility appears to vary considerably from experiment to experiment and this aspect may need to be investigated when using new breeds and with different environmental conditions operating.

2.2. Hormones

The general principals that allow the manipulation of the reproductive physiology of the sheep are discussed in detail in Note 2.

2.2.1. Follicle Stimulation and Superovulation

2.2.1.1. PITUITARY FOLLICLE STIMULATING HORMONE (FSH)

Preparations of FSH derived from pituitary glands of pig or sheep or the urine of postmenopausal women are available. These preparations usually are costly, have little luteinizing hormone (LH) contamination, and, because of their short half-life, need to be injected twice daily over a period of 3–4 d. Although they are very successful in producing embryos, they may be less attractive to users considering the other work inputs needed to produce transgenic sheep. Lesser amounts can be used in a single dose combination with pregnant mares' serum gonadotropin (PMSG), however the final choice should be made in the light of personal experience. Preparations available include: F.S.H.-P. (Burns-Biotec Lab. Inc., Omaha, NE), Folltropin-V® (Vetrepharm Inc., Ontario, Canada), and Ovagen® (Immuno-chemical Products Ltd., Auckland, New Zealand).

2.2.1.2. PMSG

This hormone is produced by the endometrial cups of the pregnant mare and stimulates the secondary wave of ovulations around 40–120 d of gestation. It is extracted from blood serum for use as an FSH-like hormone and contains partially purified fractions of an FSH-like glycoprotein. The half-life of this material is long and care must be taken not to use excessive amounts, because follicular development and estradiol secretion will continue after ovulation. With excessive numbers of ovulations, this may lead to problems with sperm transport, fimbrial recovery of eggs, and egg transport through the oviduct, resulting in low yields of eggs and low fertilization rates. Properly used, however, PMSG preparations are a cheap and efficient means of increasing ovulation rate (OR) to satisfactory levels. Intrauterine

insemination must be used in conjunction with progestagen pessaries and PMSG to negate the problems of defective sperm transport, to give control over insemination time, and to ensure that fertilization takes place. Preparations used in this laboratory are: Folligon® (Intervet International B. V., Boxmeer, Holland) and Pregnecol® (Heriot Agvet Pty Ltd., Ferntree Gully, Victoria, Australia).

2.2.2. Estrous Cycle Regulation

The length of estrous cycles can be controlled and the time of ovulation synchronized by either prolonging or reducing the progestational phase. Generally, once the source of progesterone, the corpus luteum (CL) or a pessary, has been removed, onset of estrus and the preovulatory LH surge will occur in the first ewes close to 48 h later. Previous stimulation with FSH or PMSG will bring these events forward by some 24 h.

2.2.2.1. PROGESTAGENS

Intravaginal pessaries are of two types, including Repromap® sponges (Medroxyprogesterone Acetate, Upjohn Pty Ltd., Sydney, Australia), Chronogest® sponges (Flugestone Acetate, Intervet, Sydney, Australia), and CIDR, a silastic coated spring (Controlled Internal Drug Release, Carter Harvey Holt Plastic Products, Hamilton, New Zealand).

The sponges are impregnated with a synthetic gestagen and the CIDR with progesterone, all releasing progestagen at a relatively constant rate when inserted. Both are easy to use and progestagen secretion ceases immediately they are removed. To be effective, they must remain intact until the endogenous CL has undergone luteolysis. Fortunately, if they are applied during the first few days of the cycle, normal luteinization fails and further estrus and ovulation is inhibited by the device. Thus, they need only remain inserted for a minimum of 11–12 d to a recommended maximum of 14 d. Their main advantage over prostaglandins is this flexibility and the fact that they can be used outside the breeding season. They provide the progestational priming that is needed for estrus expression and proper endocrinological preparation of the uterus for acceptance of the embryo (12).

2.2.2.2. PROSTAGLANDINS

These drugs cause luteolysis and a lowering of plasma progesterone that is detectable some 8–12 h later. They are not effective during the first 5–6 d of the cycle so either excess sheep are treated, vasc-

tomized rams are used to detect the stage of the cycle accurately and ewes from d 6–16 injected with prostaglandin, or two injections are given about 10 d apart.

Effective products that have been used with sheep are Estrumate® (Cloprostenol; Pitman-Moore, Sydney, Australia) and Lutalyse® (prostaglandin F2 α , Upjohn Pty Ltd., Kalamazoo, MI).

2.2.3. Control of LH and Ovulation

Although good estrous cycle synchronization can be achieved with progestagen or prostaglandin treatment, some ewes may be slow to respond. The preovulatory LH peaks may occur up to 48 h later than those of most ewes about 24 h following pessary removal. As this event will dictate the time of ovulation, fertilization, and subsequent pronuclear development, there is good reason to control it as tightly as possible (Fig. 1). LH secretion can be finely controlled by treating the animals with gonadotropin releasing hormone (GnRH). This is now produced synthetically and can be obtained from several sources such as Novachem (Melbourne, Australia) and Sigma Chemical Co. (St Louis, MO).

All our laboratory manipulations with embryos are now timed relative to a GnRH injection that elicits an LH surge within the hour that is physiologically equivalent to the onset of estrus, or d 0 of the cycle.

2.3. Media

2.3.1. General Considerations

It is important that standards are maintained and variables reduced to a minimum. Water quality is of utmost importance and a good water purification system is required. Media and other additives should be prepared from either AR or tissue culture grade chemicals. Unless otherwise indicated, sterile techniques should be used for all procedures. All sera used are heat-inactivated (60°C for 30 min), filter sterilized, and stored at -18°C until thawed for use. Recommended materials are: Milli-Q filtration system (Millipore Corp., Bedford, MA) and Millex-GS sterilizing filters (0.22- μ m pore size, Millipore Corp.).

2.3.2. Sperm Collection and AI

A simple buffer, phosphate buffered saline (PBS) with 0.4% bovine serum albumin (BSA) (pH 7.1) is all that is required for this work.

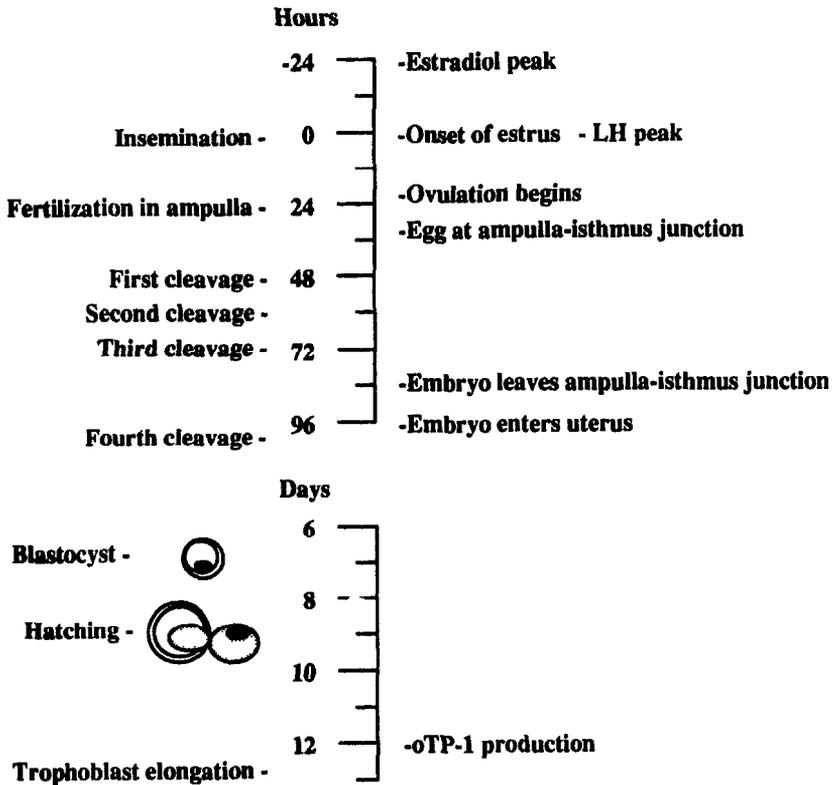


Fig. 1. Timing of early embryonic development in sheep.

2.3.2.1. DULBECCO'S PBS

Prepare stock solutions A and B as follows for five times concentrated PBS, without antibiotics (5X PBS⁻).

- Solution A: NaCl (40 g), KCl (1 g), Na₂HPO₂ (5.75 g), KH₂PO₄ (1 g). Make to 1 L with Milli-Q water and autoclave.
- Solution B: CaCl₂ (0.5 g), MgCl₂·6H₂O (0.5 g). Make to 50 mL with Milli-Q water and syringe filter (0.2 μm).

Solutions A and B can be stored for 3 mo at 4°C.

To make normal PBS⁺ (1X PBS), dilute 10 mL 5X PBS⁻ stock B with 400 mL autoclaved Milli-Q water. Slowly add 200 mL 5X PBS⁻ stock A. Make up to 1 L with autoclaved Milli-Q water. Store indefinitely at 4°C.

2.3.2.2. PBS⁺ + 0.4% BSA

To make PBS⁺ + 0.4% BSA (and this has to be fresh) add penicillin and streptomycin to the required amount of PBS⁺ to a concentration of 100 U/mL, add BSA (w/v) and stir to dissolve. Adjust pH to 7.1 with 1N HCl and sterilize by syringe filter. Antibiotics can be added as powder or as a stock solution of 100,000 U/mL in water.

2.3.3. *Embryo Flushing and Holding Media*

2.3.3.1. PBS⁺ + 5% NORMAL EWE SERUM

Add penicillin and streptomycin to the required amount of PBS⁺ (as in Section 2.3.2.2.); we use 10 mL/ewe. Add thawed ewe serum, adjust the pH to 7.4 with 1N HCl, and sterilize by filtration (0.2 μ m). Make fresh daily. Keep a small amount separate for holding and injection medium (see Section 2.3.4.2). Serum from several ewes is collected and pooled. It is not used if blood cell lysis has occurred.

2.3.3.2. SYNTHETIC OVIDUCT FLUID MEDIUM (SOFM) PLUS HEPES

This medium can be used to hold embryos whenever they are in an air atmosphere. SOFM (13) is made as in Section 2.3.5.2. but with the addition of 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES; pH 7.4) in place of Stock B.

2.3.4. *Media for Pronuclear Injection*

2.3.4.1. DNA INJECTION VEHICLE

Fusion gene DNA is added to TE buffer containing 10 mM Tris-HCl and 1 mM EDTA, pH 8.0, to the concentration required, usually between 1 and 5 μ g/mL.

2.3.4.2. INJECTION MEDIUM

Either medium described in Section 2.3.3. can be used. They are overlaid with paraffin oil (BDH Chemical Ltd., Poole, UK).

2.3.5. *Embryo Culture*

Successful culture of microinjected embryos can be achieved using any of three systems. The choice is one of convenience.

2.3.5.1. IN VIVO SYSTEMS

Either medium described in Section 2.3.3. can be used as a vehicle in which to transfer the embryos into the oviducts of rabbits or sheep and to flush the embryos out at the end of the culture period.

2.3.5.2. CELL-FREE SYSTEMS

Ovine embryo culture from the one-cell to the hatched blastocyst stage utilizes SOFM+20% heat-inactivated human serum (HIHS) (14). Blood is collected from fasted males, the serum removed, heat-inactivated, and stored frozen in aliquots.

SOFM is made from five stock solutions as follows:

- Stock S: NaCl (31.47 g), KCl (2.67 g), KH₂PO₃ (0.81 g), Na lactate ([60%] 3.10 mL), glucose (1.35 g), penicillin (0.30 g), and streptomycin (0.25 g). Make to 500 mL with water, sterilize, store 3 mo.
- Stock B: NaHCO₃ (2.101 g) and phenol red (0.05 g). Make to 100 mL with water, sterilize, store 2 wk.
- Stock M: MgCl₂·6H₂O (0.500 g). Make to 50 mL with water, sterilize, store 3 mo.
- Stock C: Na pyruvate (0.072 g). Make to 20 mL with water, sterilize, store 1 wk.
- Stock D: CaCl₂·2H₂O (1.260g). Make to 50 mL with water, sterilize, store 3 mo.

Mix the stocks in the amounts listed below depending on volume needed, add HIHS to 20% (v/v), adjust pH to 7.4 and osmolarity to 280–300 mosM/L, and filter-sterilize. Discard after use.

Stock S	5.0 mL	or	1.0 mL
Stock B	5.0 mL	or	1.0 mL
Stock M	0.5 mL	or	0.1 mL
Stock C	0.5 mL	or	0.1 mL
Stock D	0.5 mL	or	0.1 mL
Water	<u>38.5 mL</u>	or	<u>7.7 mL</u>
	50.0 mL		10.0 mL

It is also possible to use conditioned medium from oviduct cell cultures (*see* Section 2.3.5.3.).

2.3.5.3. OVIDUCT CELL COCULTURE

Secretions from oviduct cells are capable of supporting embryo development to the blastocyst stage (6,15,16). Although conditioned medium from oviduct cells can also be used, it is possibly easier to utilize the coculture technique. The media used contain standard items.

1. Earle's salts (Gibco Europe Ltd., Paisley, UK).
2. TCM 199 (Gibco).

3. Newborn calf serum (NCS, Sera Lab. Ltd., Crawley Down, UK).
4. Fetal calf serum (FCS; Sera Lab.).
5. Kanamycin (Sigma).
6. 4-well culture plates (Nunc; Gibco).

2.4. Equipment

The production of transgenic sheep is costly in time, labor, and assets. All the equipment listed below is readily available. Much effort is being spent on methods to relieve this burden and methods with promise are examined in Section 1.

2.4.1. General Equipment

The production of media, embryo recovery and handling, embryo culture, and transgenic production requires that the laboratory be equipped to tissue-culture standard. Items required include:

1. pH meter and combination electrode (BJ321 Activon, Sydney, Australia).
2. Analytical balance
3. General glassware (top quality Pyrex) and plasticware (cell culture grade).
4. Tissue culture grade detergent (7X, ICN Biomedicals, Sydney, Australia).
5. An inverted microscope with a range of phase-contrast objectives (Nikon TMS, Nikon, Tokyo, Japan).
6. Binocular microscopes with magnification 2- to 65-fold for embryo searching.
7. An incubator for maintaining media and equipment at 38.5°C.
8. A laminar flow hood (DFMG4, Gelman Sciences Pty Ltd., Sydney, Australia).
9. Laparotomy cradle for restraint and elevation of sheep.

All glassware requires a thorough prewash with acid and extensive rinsing with tissue-culture grade water. Plasticware should be tested for suitability for embryo culture; mouse embryos can be used for this.

2.4.2. Intrauterine Insemination

2.4.2.1. COLLECTION OF SEMEN

This equipment is needed for collection and examination of sperm motility.

1. Artificial vagina (AV) and rubber lining.
2. Glass sperm-collecting vessel.
3. Dissecting microscope (Zeiss 47 50 57, Carl Zeiss, Oberkochen, Germany)
4. Cold light source (Schott KL 1500, Fibre Optics, Wiesbaden, Germany).

2.4.2.2. INSEMINATION

For intrauterine insemination, the following items are needed:

1. Full laparoscopic equipment including 7-mm diameter trocar-cannulae, a 6.5-mm 30° Hopkins laparoscope, grasping forceps, and high intensity fiber optic light source 485 (Karl Storz, Stennings, Sydney, Australia).
2. Carbogen cylinder and valve.
3. Insemination pipets, 150-mm long flame pulled and sharpened Pyrex glass tubing (4 mm od, 1 mm id). These may be reused after resharpening.
4. Adjustable cradle for restraint and support of ewes.
5. Osti animal clippers (Sunbeam Corp., Sydney, Australia).
6. Local anesthetic (2% procaine, Orgamol Talfar, Evionnaz, Switzerland).
7. Antibiotic (e.g., Penstrep, Troy Labs, Sydney, Australia).
8. Syringes and needles.

2.4.3. Embryo Recovery and Transfer

2.4.3.1. SURGICAL EQUIPMENT

1. Scalpel handle and blades, hemostats, scissors, suture, needle holder, suture needles, forceps, swabs, and towel clips.
2. Fenestrated drapes, 10-mL syringe, 18-g and 25-g needles, sterile normal saline.
3. Polyvinyl catheters 100-mm length (1.0 mm id × 1.5 mm od and 1.5 mm id × 2.0 mm od).
4. Induction anesthetic (Pentothal, Boehringer Ingelheim, Sydney, Australia).
5. Antibiotic (Penstrep).
6. Analgesic (Finadyne, Heriot Agvet Pty Ltd., Melbourne, Australia)
7. Topical antiseptic (Betadine, Faulding Pharmaceuticals, Adelaide, Australia).
8. Laparoscopic equipment (as described in Section 2.4.2.2.).
9. Sterile surgical tray.

2.4.3.2. EMBRYO HANDLING

1. Sterile embryo blocks and cover glasses.
2. Embryo handling pipets (flame pulled from Pasteur pipets to 250 μm id).
3. Mouth tubes (Silastic tubing, 0.132 in. id × 0.183 in. od, Dow Corning, Midland, MI).
4. Depression slides for manipulation, thinnest ones selected for pronuclear visualization.
5. Drummond microdispenser (Drummond Scientific Co., Broomall, PA).
6. 3-1/2 French Tom-Cat catheter 5-1/2 in. long (Sherwood Medical, St. Louis, MO).
7. Jelco 16-g IV catheter placement unit (Johnson & Johnson, Tokyo, Japan).

2.4.4. Pronuclear Microinjection

2.4.4.1. MICROSCOPE AND OPTICS

A research microscope with Nomarski accessories giving differential interference contrast (DIC) optics is essential.

1. Nikon Inverse Microscope Diaphot-TMD (Nikon, Japan).
2. Diascopic DIC Nomarski Attachment TMD-NT20.

2.4.4.2. MICROMANIPULATORS AND MICROINJECTOR

For pronuclear microinjection, the following items are needed:

1. Two micromanipulators (e.g., Leitz micromanipulator, Ernst Leitz, Wetzlar, Germany).
2. Compressed air operated microinjector (Eppendorf 5242, Eppendorf, Carl Zeiss, Hamburg, Germany)
3. Micrometer syringe with luer fitting (Dixon Scientific Co., Beckenham, UK; Hamilton Co., Reno, NV).

The line connecting the syringe and the holding pipet is filled with fluorinert.

2.4.4.3. FINE NEEDLE PRODUCTION

1. Capillary tubing (1.2–1.5 mm id, Kimble, Toledo, OH).
2. Glass tubing (GC 100TF.15, Clark Electromedical Instruments, Reading, UK).
3. Vertical Pipet Puller (Model 700D, David Kopf Instruments, Tujunga, CA).
4. Flaming Brown Micropipet Puller (Model P.80/PC, Sutter Instrument Co., San Rafael, CA).

Holding pipets are made from Kimble tubing and pulled on the Vertical Pipet Puller to 20–40 μm id with the settings: heater 65 and solenoid 5. They are flame-polished before use. Microinjection needles are pulled from GC 100TF.15 glass tubing on the Flaming Brown using the settings: heat 400, pull 40, velocity 30, time 40. Settings on all pipet pullers will vary with environmental conditions.

2.4.5. Embryo Culture

1. IR 1500 Automatic CO₂ incubator (Flow Laboratories, Bioggio, Switzerland).
2. Sealed plastic container (e.g., a lunch box).

The box contains a stainless steel grid standing in, but extending above, a 1-cm layer of distilled water through which bubbles the gas mixture of 5% CO₂, 5% O₂, and 90% N₂ (Fig. 2).

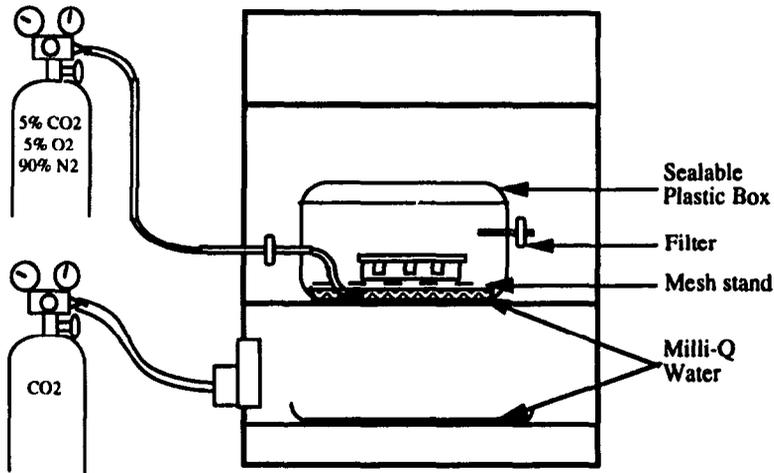


Fig. 2. Diagram illustrating in vitro cell-free culture system. See Section 3.5.1.2.

2.4.6. Pregnancy Diagnosis

1. Ultrasound scanner (Vetscan, Tokyo Keiki, Tokyo, Japan).
2. Intracorporeal probe 5MHz linear transducer (Vetscan).
3. 3.5 MHz external linear transducer (Vetscan).

Other scanners and sector probes are also adequate for this work.

3. Methods

3.1. Synchronization of Estrus and Superovulation

For the provision of pronuclear stage embryos for microinjection and subsequent transfer to oviducts or uterus, three groups of ewes are treated as Donor Group, Recipient Group A, and Recipient Group B as in Table 1. There can be variations on this theme.

The number of sheep entering this program depends on the yield of embryos, the experimental requirements, and the performance of the research team. Normally we would use 16 donor ewes and 14 recipients in the program described in Table 1 for Donors and Recipient Group A. This typically results in 100 embryos to examine and 30–70 embryos for transfer after microinjection (see Section 3.4.).

3.1.1. Donor Ewes (see Table 1 and Note 3)

- 1 Donors receive 1200 IU PMSG. This may be increased to 1500 or even 1800 IU with care if the response drops as the breeding season closes

Table 1
Protocol for the Provision of Embryos
for Pronuclear Microinjection and Embryo Transfer

Day	Time	Donor group	Recipient group	
			A	B
-15	Monday	Sponges in	Sponges in	Sponges in
-2	Sunday	0800 h PMSG 1200 IU	PMSG 400 IU	
-1	Monday	1800 h Sponges out	Sponges out	
0	Tuesday	0800 h —	—	Sponges out
0	Tuesday	0800 h —	—	PMSG 400 IU
0	Tuesday	1800 h GnRH 50 µg	GnRH 50 µg	—
1	Wednesday	0830 h Uterine AI	—	Detect marks ^a
2	Thursday	0800 h Embryo recovery	—	Detect marks
2	Thursday	0900 h Microinjection	—	—
2	Thursday	1200 h Culture embryos	Oviduct transfer	—
3	Friday	—	—	Detect marks
8	Wednesday	1000 h Assess blastocysts	Uterine transfers	Uterine transfers

^aContinue estrus detection twice per day for 3 d (d 3 Friday).

and the ewes enter a state of anestrus. Sponges are removed 34 h after PMSG was given. This time difference may be up to 48 h (17), but our experience has shown that there is no particular advantage in this.

2. An alternative method is to use FSH preparations, usually as a series of eight injections of 3 mg given twice per day (18). In this case the pessaries are withdrawn or prostaglandin injected at the time of the fifth treatment (19).
3. The beginning of ovulation is regulated by sponge removal. GnRH (50 µg) treatment 24 h after sponge removal induces and synchronizes ovulation in delayed sheep (see Section 2.2.3., Note 4, and Table 1).
4. Eggs are recovered after the cumulus cells are lost but before their initial division. Within this period, the pronuclear membranes form and the pronuclei become visible prior to fusion (see Note 2).

3.1.2. Recipients for Oviduct Transfers (Table 1)

1. In the recipients, a minimal dose of 400 IU PMSG aids estrus synchronization following sponge removal and aligns the group with the donors by bringing forward the onset of estrus to 24 h after pessary removal.
2. GnRH (50 µg; Table 1) prevents estrus in some animals, presumably by inhibiting estradiol synthesis through the premature LH surge in delayed ewes (see Note 5).

3.1.3. Recipients for Uterine Transfers (Table 1)

Recipient Group B is included if uterine transfers are to take place following embryo culture.

1. Sponge removal is delayed compared to donors and the 400 IU PMSG is given simultaneously.
2. Ewes must be examined both morning and evening for raddle marks from vasectomized rams so that appropriate synchrony between embryos and recipients can be obtained.

3.1.4. Cyclic Flock

We also keep a group of untreated ewes with teaser rams for general purposes and, as estrous cycle data are known for each ewe, they can be used at short notice for recipients, by treating them simultaneously with PMSG (400 IU) and prostaglandin (Section 2.2.2.2.).

3.2. Artificial Insemination (see Note 6)

Transcervical AI of the superovulated ewe is at best a very difficult procedure and therefore not practical. Standard methods for direct deposition of semen into the uterus of the ewe are those of laparotomy or laparoscopy. We recommend laparoscopy; it is faster, requires local anesthetic, and the smaller incisions reduce trauma, infection, tissue dehydration, and adhesions.

3.2.1. Collection of Semen

1. Three rams are kept trained to the AV and are not exposed to natural mating.
2. The temperature of the water jacket is kept at approx 45°C.
3. The rams are introduced singly to a restrained teaser ewe experiencing estrus, and semen is collected using the AV and glass collecting cup as the rams attempt to mount.
4. Semen is pooled and diluted 1:1 with PBS⁺ + 0.4% BSA, pH 7.1. Semen may be held at room temperature for a few hours. Frozen semen also can be used for intrauterine AI.

3.2.2. Laparoscopic Insemination

1. Feed and water are withheld from ewes for 24 h.
2. The animals are restrained on a laparoscopy cradle in dorsal recumbency and the hind quarters elevated.
3. A small area of the ventral abdomen is clipped free of wool. Local anes-

thetic (2% [w/v] procaine) is introduced subcutaneously and intramuscularly at two sites, one in each of the lower abdominal quadrants midway between flank and umbilicus and 2–3 cm off midline. A single intramuscular dose of 3–5 mL antibiotic (Penstrep) is given to reduce chances of bacterial infection.

4. A trocar-cannula, attached by a flexible gas hose to a regulator on a carbogen cylinder, is inserted intra-abdominally at the right-hand site, the trocar removed and replaced by the laparoscope attached to the light source. A trocar-cannula (without gas) is inserted in the left-hand site, the trocar is then removed and replaced with SEMM grasping forceps. Approximately 1–2 L of carbogen gas are passed into the abdominal cavity.
5. The uterus is located and manipulated minimally, using the forceps, to a position suitable for insemination. The forceps are removed and replaced by the insemination pipet into which was drawn 40–50 μ L of diluted semen using a 1-mL syringe attached to the nonsharpened end of the pipet.
- 6 Each uterine horn is pierced through its antimesometrial surface with the pipet and the diluted semen deposited into the lumen. This is repeated for the other horn.
7. The pipet, laparoscope, and cannulae are removed and the incisions closed with suture or Michel clips if necessary.

3.3. Collection of Embryos

It is estimated from this program that embryos are collected about 14 h after fertilization.

1. Ewes are taken off food and water for 24 h prior to surgery.
2. Premedication of 1 mL Finadyne and 3 mL Penstrep are given intramuscularly for analgesic and antibiotic reasons.
3. The abdomen and sides of the neck are closely clipped and the ewes seated in an adjustable cradle or placed on a surgical table. The surgical site is swabbed with Betadine and the ewe anesthetized with 15–20 mL (0.75–1.0 g) of 5% (w/v) Pentothal injected via the jugular vein. Full sterility of the operation site is maintained throughout.
- 4 The hindquarters of the ewe are elevated and a midline incision of approx 4 cm made in the abdominal wall anterior to the udder. The pendant uterus is located, exteriorized, and supported on swabs damped with warm saline.
5. A catheter is inserted for a distance of 1.5 cm in the ampulla and held in place by an assistant with the end opening into a prewarmed embryo block. The oviducts are flushed separately with 2–5 mL of flushing

medium using a 25-g needle placed in the isthmus proximal to the uterotubal junction.

6. The contents of the blocks are immediately searched for embryos. The embryos are then transferred to a second block containing 0.2 mL of holding medium. When half the donors are flushed, this group of embryos is transferred to the microinjection laboratory. The remainder follow as soon as possible.
7. The incisions are closed internally with Chromic 2 or Metric 4 Silk using single or blanket stitch and externally with silk using a blanket stitch. The sheep are laid in sternal recumbancy for recovery and are not used again for these purposes.

3.4. Microinjection

1. Embryos are transferred to a hollow ground cavity slide for examination and manipulation using the Nikon research microscope, at 200–320-fold magnification. Unsuitable embryos are removed in groups based on degeneracy, lack of fertilization, and absence of pronuclei. The accuracy of this selection may be checked by culture for 5 d in Walker's medium (20).
2. Centrifugation has been used to increase the visibility of pronuclei and the yield of injectable embryos (*see* Note 7). Embryos placed in an Eppendorf tube with holding medium are spun for 1–3 min at 11,000–13,000g in a microfuge prior to examination for pronuclei.
3. Embryos to be microinjected are contained in a drop of medium covered with paraffin or silicone oil on a depression slide chamber where they are examined under high power to assess visibility of the pronuclei (Fig. 3).
4. Individuals are immobilized using a holding pipet with gentle suction. They can be moved around by the injection pipet until one of the pronuclei can be seen in a suitable position for microinjection.
5. The injection pipet is filled with DNA solution (*see* Note 8) and clamped into the tube connected to the injector. Pressures are adjusted, the pipet aligned with the embryo and tested for its ability to squirt the injectant out. The gas input pressure is maintained at 400 kPa. The holding pressure of the injector is maintained at 6–10 kPa to prevent the DNA solution from withdrawing and the holding medium from entering into the capillary. Usually, the minimum pressure necessary for the solution to be streaming slowly from the pipet is adequate. Injections are carried out for 0.1–0.9 s at a pressure of approx 40 kPa (1), although up to 220 kPa has been used (14). The clearing pulse is set at 15 kPa. If the injection pipet blocks, it can be cleared with this pulse, chipped against the holding pipet or thrown away. Immediately following a clearing pulse, holding medium may enter the pipet if the holding pressure is insufficient. Injec-

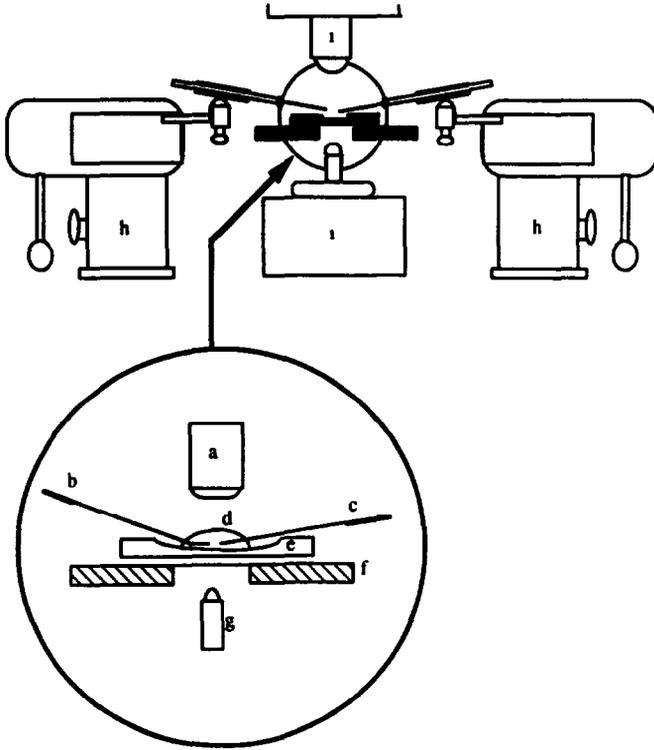


Fig. 3. Schematic representation of the micromanipulation procedure for the injection of the pronucleus of sheep embryos. (a) condenser; (b) holding pipet; (c) injection pipet; (d) drop of medium containing eggs and covered by liquid paraffin; (e) depression slide; (f) microscope stage; (g) objective; (h) micromanipulators; (i) microscope with Nomarski optics.

tion pipets should be changed when: (1) the pronuclei consistently fail to swell, (2) the pipet continues to drag material out of the perivitelline membrane, (3) the pipet fails to pierce the various membranes, (4) the pipet appears dirty or clogged, (5) the pipet breaks and the tip is $>1 \mu\text{m}$ in diameter.

6. At this stage, the egg should be repositioned so that the pronucleus is in the proximal half and, if possible, at a normal position with respect to the pipet, otherwise the pipet may not pierce the pronucleus but rather roll over it.
7. Using high power, refocus on the pronucleus and bring the injection pipet into the same focal plane. Keeping the pipet in focus, thrust it into the embryo with sufficient force to pierce the plasma and pronuclear

membranes. This can be difficult and it is likely that severe damage may result if the membranes are unduly damaged (21).

8. Once the tip of the needle appears within one pronucleus, the injector is activated and the pronucleus observed to swell. If this does not occur, either the object is not the pronucleus, or the membrane has not been pierced and a second attempt must be made. The needle is quickly withdrawn and the remainder injected. We have estimated that some 1–2 pL DNA solution enters the pronucleus.
9. Injected eggs are transferred back to an embryo block and a new group selected for injection.
10. Injected embryos are left at room temperature for 1 h before they are assessed for survival from the treatment. They are considered to have lysed if the perivitelline space, which disappears after injection, is not regained. Accuracy of this examination may also be checked by culture. Data in Table 2 demonstrate that a majority of pronuclear visible fertilized eggs develop to blastocysts and that 70% of those in which pronuclei were not observed were, in fact, fertilized although only 49% developed at suitable rates.

3.5. Embryo Culture and Transfer

Once injected, embryos may be either cultured, *in vivo* or *in vitro*, or transferred immediately back into recipient ewes. Culturing provides a means of assessing survival of the embryos and enables other manipulations to be performed if necessary. For example, examination of biopsy samples by PCR may permit transgenic blastocysts to be identified as well as their sex (22; *see* Note 9). The PCR multiplies exclusively any small sequences of DNA that are specified by synthetic flanking oligonucleotides. The great excess of this sequence can usually be detected following electrophoresis. The remaining portion of the transgenic blastocyst can then be transferred. Thus less recipients are needed as nonviable and nontransgenic embryos can be eliminated.

3.5.1. Embryo Culture

3.5.1.1. IN VIVO CULTURE

Culturing in rabbit or sheep oviducts for approx 3 d has proven successful (20,23) but is not a method favored in our laboratory. Any second transfer carries the risk of all attendant losses being repeated, in addition to the extra workload. If this technique is required, use the methods that are described in Section 3.5.2., with a ligature being applied at the uterotubal junction to prevent the embryos moving into the uterus.

Table 2
Development of Embryos in Culture Following Pronuclear Microinjection^a

Stage of development	Number of embryos and percentage		
	1 cell	2-32 cells	Morulae + Blastocyst
PN visible controls ^b	3 (11.5)	3 (11.5)	20 (76.9)
PN not visible ^c	46 (29.7)	33 (21.3)	76 (49.0)
Buffer injected ^d	27 (57.4)	5 (10.6)	15 (31.9)
DNA injected ^d	45 (50.0)	17 (18.9)	28 (31.1)

^aEmbryos were cultured in SOFM + HIHS (Section 3 5 1 2) for 5 d

^bThese represent the developmental potential of fertilized eggs

^cThese include any eggs that may not have been fertilized

^dAll were selected as pronuclear visible (PN)

3.5.1.2. CULTURE IN CELL-FREE MEDIA (SEE NOTE 10)

1. Ova are grouped according to assessment after microinjection and washed in SOFM+HIHS before culture in 1-mL vol in 24-well multi-well dishes at 38.5°C under humidified 5% CO₂: 5% O₂: 90% N₂ atmosphere for 5-7 d.
2. These dishes are placed in the plastic box (Fig. 2) inside an automatic CO₂ incubator and slowly gassed. Embryos should remain undisturbed, with handling, temperature changes, gas changes, and so forth kept to a minimum for the period of culture.
3. At the end of this period, embryos are examined for development. The d 5 embryos should be late morula to early blastocyst and d 7 should be blastocysts. It appears that development occurs somewhat faster than predicted *in vivo* (see Fig. 1) and a reassessment of the synchrony of recipients may be needed.
- 4 Blastocysts and morulae are washed in holding medium (Section 2.3.3.1.) before transfer.

3.5.1.3. OVIDUCT CELL COCULTURES (SEE NOTE 11)

Epithelial cells flushed from the oviducts of sheep quite readily culture in the medium TCM 199 and support embryo development (15,24,25). Usually these cultures are established as outgrowths for 3 d before embryos are cocultured with them (see Note 12).

- 1 Cells are flushed from oviducts of ewes on d 2 of the cycle with PBS containing 10% (v/v) NCS and allowed to sediment for 10 min.
2. The cells are washed and resuspended in 4 mL of TCM 199 containing Earle's salts, 10% FCS, 2.2 g/L NaHCO₃, and 75 mg/L kanamycin.

3. The resuspended cells are plated into wells and incubated in an atmosphere of 5% CO₂ in humidified air.
4. Medium is replaced after 3 d when the cells had colonized and the embryos were then incubated on these layers for up to 3 d.

3.5.2. Transfer to Oviducts

Preparation of sheep and surgical protocols are identical to those for embryo recovery (Section 3.3.).

1. Exposed ovaries of recipient sheep are examined and ovulation points noted. Transfers are made into the side ipsilateral to the ovulation. If ovulation has occurred in both ovaries, embryos may be transferred to either or both oviducts, although there does not appear to be any advantage in doing the latter.
2. Embryos for transfer are taken up in 2 µL of holding medium with a Drummond Microdispenser and deposited about 2 cm into the ampulla of the oviduct. We assume that about 80% of injected embryos fail to survive and transfer 3–6 embryos into the oviducts of each recipient. The reproductive tract is gently returned and the incisions closed as described previously (Section 3.3.).

3.5.3. Transfer to the Uterus

After 5–6 d of culture, blastocysts are transferred directly to the uterus. This may be done surgically via laparotomy or by laparoscopy. Usually one or two blastocysts would be transferred at this stage of development.

3.5.3.1. LAPAROTOMY

Preparation and surgical procedures for laparotomy transfers are as for embryo collection (Section 3.2.).

1. A perforation is made in the uterine wall midway along the horn ipsilateral to the CL using the blunt end of a suture needle.
2. Blastocysts are aspirated into a flame polished and washed Pasteur pipet with 10–30 µL of holding medium.
3. The tip of the pipet is passed through the perforation and the contents gently discharged into the uterine lumen.

3.5.3.2. LAPAROSCOPY

The initial procedure is that outlined under laparoscopic AI (Section 3.2.2.).

- 1 The uterus is located, the ovaries examined using laparoscopic holding forceps to manipulate them, and the CLs counted.

2. The horn of the uterus ipsilateral to the ovulation(s) is grasped with the forceps about 5–10 cm from the uterotubal junction and pulled up toward the body wall.
3. The embryos to be transferred, usually two, are drawn into a 3-1/2 French Tom-Cat catheter attached to a 1-mL syringe. An air space is located on either side of the medium containing the embryos.
4. The uterine lumen is cannulated by inserting a Jelco IV placement Catheter intra-abdominally at a point 1–2 cm from the left-hand cannula (containing forceps) and toward the midline. The catheter is slowly inserted into the elevated uterine horn at a point close to the holding forceps and toward the uterotubal junction until it is located in the lumen.
5. The stylus is removed and the Tom-Cat catheter inserted in the cannula until freely maneuverable within the uterine lumen. The embryos are slowly expelled.
6. The placement catheter, laparoscope, and cannulae are removed and the incisions are closed where necessary.

3.5.4. Postoperative Care

Ewes are removed from the cradles and placed in a sternal recumbancy position. Food and water are offered once they have recovered. They should remain in quiet surroundings for several days; we choose to keep them indoors until pregnancy diagnosis has been performed by ultrasound.

3.6. Diagnosis of Pregnancy

For managerial reasons, it is useful to determine the pregnancy rates as soon as possible. This evidence is valuable in determining the fate of the injected embryos. It has been found in mice that fetuses can be resorbed at higher rates for injected embryos than for control embryos. The reason for this is not obvious, although the transgene may affect some important developmental process. Abnormal fetal mortality has occasionally been observed in sheep (1,4) although the numbers are insufficient to draw conclusions. Perhaps synchrony between the blastocyst and recipient ewe is compromised by alterations in developmental rates and parameters of ovine trophoblast protein-1 (oTP-1) secretion. Nevertheless, it appears that the injection itself may influence survival of the embryo or fetus by some unknown mechanism.

Sheep are fasted for 24 h prior to ultrasound scanning using either intracorporeal or abdominal probes. Pregnancy is detected in recipi-

ent ewes 28–42 d after GnRH treatment, using the intracorporeal probe that gives virtually 100% accuracy at this stage. Several large spaces representing the expanded uterine lumen can be seen in pregnant sheep. Subsequent examination for multiple fetuses is made with external probes at 50–65 d of gestation and appropriate regimes initiated to increase feed intakes to these ewes after 125 d.

4. Notes

1. The breed of animal used is dependent on the aims of the experiment. If successful, the cysteine double fusion transgene, Cys EK (26), will ultimately be used in Merinos to promote wool growth; growth hormone genes that reduce fat content or increase growth rates will be used in any of the various meat-sheep and so on, although in the future some transgenes may be of general use, in animal health for instance.
2. The general principles that allow manipulation of the reproductive physiology of sheep for the purposes of transgenic sheep production will be discussed here.
 - a. Estrous cycle and ovulation. Regulation of the estrous cycle is necessary so that sufficient embryos may be obtained at appropriate times for subsequent laboratory work. This basically means that embryos recovered from superovulated ewes should have maximum pronuclear visibility for microinjection. Regulation, using the principles espoused above, is directed toward:
 1. Control of estrous cycle length with either progestagen implants or injections of prostaglandin F₂ α or analogs.
 2. Stimulation of follicle development with FSH preparations that are used in conjunction with estrous cycle regulating drugs to obtain multiple ovulations.
 3. Control of time of ovulation and fertilization by stimulating LH release with an injection of hypothalamic GnRH.
 4. Timing of intra-uterine AI.

Sheep are seasonal breeders that undergo spontaneous ovulation in cycles of around 17 d. Usually 1–2 eggs are shed, with some exceptions such as the Booroola Merino of Australia and the Finnish Landrace of the UK. These sheep may release around 6–10 eggs at each cycle, an OR suitable for an effective transgenic program and one produced in other sheep only by hormonal treatments. However, to our knowledge, these prolific breeds have not been used as embryo donors for microinjection. Ovulatory cycles begin in response to decreasing day length, they end as a result of increasing day length. The length of the breeding period will vary considerably with breed, environment, and latitude.

This does not necessarily present a problem for continuation of this work out of season, as stimulation of estrus and multiple ovulation is achieved by the use of hormonal treatments.

The reproductive cycle is characterized by a short follicular phase initiated by a decline in levels of blood plasma progesterone concentrations following luteolysis, the demise of the CL. LH pulses from the anterior pituitary increase in frequency and elicit a pulsatile secretion of estradiol, gradually increasing in amplitude. The primary oocyte is halted in development with its diploid pairs of chromosomes held separate at the equatorial plate (metaphase I) (27). At a critical stage, the secretion of estrogen from the dominant follicles increases rapidly and exerts a positive feedback effect resulting some 24 h later in the onset of estrus (d 0 of the cycle) and a surge of LH. This preovulatory surge of LH occurs via GnRH and has a threefold effect:

1. First, in concert with estrogen (28), further maturation of primary oocytes is initiated and they undergo the first reduction division when extrusion of the first polar body occurs. These secondary oocytes then progress to metaphase II. This process takes about 24 h and requires LH stimulus of the surrounding cumulus cells. If eggs are to be produced from *in vitro* matured oocytes harvested from slaughterhouse material, then granulosa and cumulus cells must be included in the incubation mixture with LH, FSH, and estradiol for 24 h. *In vitro* fertilization can then be attempted.
2. Second, the ovulatory process is initiated and ovulation begins some 21–27 h later, coinciding with oocyte maturation. Ovulation occurs on d 1 of the estrous cycle.
3. Third, estradiol secretion is terminated by inhibition of the enzymes of androgen synthesis and aromatization in thecal and granulosa cells respectively. This process of luteinization later results in the progesterone production from the CL that is necessary to inhibit further ovulation and to support pregnancy.

The mode of action of FSH and its interaction with LH has still not been adequately explained. For the purposes of genetic engineering, it is thought that it facilitates the maturation of follicles and inhibits the LH-induced atresia that prevents these follicles from entering the final stages of development and oocyte maturation. Stimulation of the ovaries with FSH preparations is essential if superovulation is used to provide embryos for transgenesis.

- b. Fertilization and embryo development. The ovulated egg is collected by the fimbria of the oviduct and moves rapidly to the junction of the ampulla and isthmus where it remains for some 72 h. In the presence

of rams, mating occurs from the onset of estrus, and capacitated spermatozoa accumulate in clumps at the uterotubal junction from where they are slowly released. Thus the egg may be fertilized quickly after ovulation and this act reinitiates development with extrusion of the second polar body finalizing the reduction division. Formation of the pronuclei, their fusion, and the first embryonic cleavage occur during the next 24-h period. Visualization of the pronuclei is essential for microinjection of the fusion gene. We have found this to be most difficult on a regular basis as it varies from day to day, all other conditions under our control being equal. During the next 3 d, up to 96 h after the onset of estrus and the LH surge, the embryo undergoes three cleavage divisions (Fig. 1). Finally, it moves through the muscular isthmus, the uterotubal junction, and into the ipsilateral uterine horn. Here the embryo develops into a morula, then a blastocyst, until it finally hatches from its zona pellucida at around d 9.

- c. **Pregnancy.** Rapid elongation of the trophoblast occurs from d 12–13 and this involves invasion of the lumen of the contralateral uterine horn. Evidence of implantation at the caruncles has been found from d 16. The trophoblast of 12- to 21-d-old blastocysts secretes a protein, called oTP-1, that is essential for the maintenance of CL function past this time (29). In the absence of embryos, or possibly when embryos are retarded or otherwise defective, oTP-1-mediated suppression of luteolysis does not occur. Under these conditions, luteolysis takes place with a rapid fall in progesterone secretion around d 14–15, pregnancy fails, and ovulation recurs. This is a finely tuned mechanism with perhaps 24 h maximum leeway for oTP-1 secretion before the luteolytic process becomes irreversible. oTP-1 acts locally on the uterus, which results in it being able to prevent luteolysis in only the ipsilateral ovary. Thus it is important to transfer embryos and blastocysts back into the oviducts or uterine horns on the same side as the ovary with the CL and to have close synchrony between the developmental stage of the embryo and the recipient ewe's cycle. Once pregnancy is established, secretion of progesterone by the CL continues throughout, although placental steroidogenesis is sufficient from d 50 of gestation to maintain pregnancy in its own right. Further development and maturation of the cotyledons of the syndesmochorial placenta takes place throughout a pregnancy lasting 149–151 d.
3. With Merino or BLM sheep, OR can range from an average of 8–18 for each experimental run. Most variation comes from ewe genotype, season, and batch of hormone. GnRH treatment 24 h after sponge removal induces and synchronizes ovulation in delayed sheep.

4. In our initial experiments to produce pronuclear zygotes, many were obtained too early and were covered with cumulus cells indicating that ovulation had been delayed in those ewes (17). When GnRH was used and collections were made 56 or 62 h after sponge withdrawal, 8 and 14 h after ovulation, 0 and 6% of fertilized eggs had cleaved. Collection at 64 h with no GnRH treatment resulted in 23% cleavage. Subsequently, Walker, Smith, and Seamark (19) showed that GnRH reduced the median time for all ovulations from 60 to 45 h and from 54 to 48 h for FSH and PMSG treatment, respectively. The majority of ovulations occurred between 42 and 51 h although the earliest were around 36 h after sponge withdrawal. Recovery rates also vary greatly from day to day, the limits being 30–75%. In general, very high ORs are accompanied by poor recovery rates.

Our results obtained for the year 1990 are presented in Table 3. These data are representative of several years' experiments; but as an example of variability, in 1991 we found ORs of 19 with recovery rates of approx 60%. Our rate of pronuclear visibility (47%) is lower than expected but the reasons for this are not evident.

5. Ovulation does occur in these recipients. The advantage is that no vasectomized teaser rams are needed. In one experiment, we showed that pregnancy rates following embryo transfer were the same for recipients that had been detected with vasectomized rams compared to those that did not experience estrus
6. AI is essential to these techniques and the use of GnRH to synchronize LH peaks enables AI to be carried out confidently on all donors some 10–24 h prior to ovulation. When AI was carried out at the time of GnRH treatment and compared to mating on three occasions during the estrous period, fertilization rates increased from 50–55% up to 80–98% in favor of AI (17).
7. Centrifugation of embryos is essential for the pig and cow (30,31) and has been used for sheep embryos. Some of our early work did indicate that centrifugation was useful with an additional 13% of total embryos being identified for injection (32), but there is little advantage gained over and above optimized microscopic technique (4,17,30). Cellular organelles are redistributed and the more opaque contents cleared away from the pronuclei. It may be worthwhile retesting this method if one is establishing the transgenic techniques.
8. The integrity of the fusion gene should be checked on a minigel before and after a series of injections to ensure that it has not been degraded. Each aliquot should be used once only. The concentration of DNA in the injection buffer is important. Transgenic sheep were only obtained

Table 3
Results of Embryo Production and Manipulation, 1990

Parameter	Number	%	Parameter	Number	%
Donors	454		Ovulations/donor	10.5	
Ovulations	4755		Eggs/donor	5.55	
Eggs recovered	2521	53.0	Recipients	143	
Eggs examined	2161	85.7	Transferred/recipient	4.72	
Degenerate	64	3.0	Pregnant at d 28	78	
Cleaved	34	1.6	Pregnant at d 60	64	44.8
Cumulus	24	1.1			
PN not visible	1059	49.0			
PN visible	988	46.6			
Eggs transferred	675	68.3	Lambs born	94	13.9

when the concentration of GH9 was 5 $\mu\text{g}/\text{mL}$. Higher concentrations can be toxic to mouse embryos (33), although integration frequencies tended to rise simultaneously. The optimum concentration for sheep should be investigated when establishing a system and will probably lie somewhere between 1 and 10 $\mu\text{g}/\text{mL}$. Our experiment showed that only 43% of embryos injected with buffer were capable of developing and there was no difference when DNA was used (Table 2)

9. Identification of transgenic blastocysts. DNA amplification (PCR) is used in conjunction with culturing of injected embryos to identify those that are transgenic for their transfer to recipients (Section 3.5.). Theoretically, one or a few cells is all that is necessary to remove for analysis, but this will depend on the sensitivity of the particular assay. There has to be a careful selection of oligonucleotides to specify the transgene alone. If, as with GH9, both the promoter and expressed gene are sourced from the subject species, then transgene-specific sequences will have to span both the promoter and the expressed gene. A program such as Oligo (version 3.4, National Biosciences, Hamel, MN) or GeneJockey (Biosoft, Ferguson, MO) may be used to select the most stringent oligonucleotides to reduce false sequences of DNA being synthesized. We have found false DNA band patterns to be a problem, however careful selection of the oligonucleotides can prevent this. By the use of DNA amplification, transgenic blastocysts can be identified (22) for transfer into recipient animals, thus increasing the efficiency of transgenic production.
10. In a detailed comparison, Walker et al. (20) found that there were no differences in percentages (32.6–50.0%) of microinjected zygotes developing to elongated conceptuses at d 14, irrespective of whether they had been

cultured *in vivo* in oviducts or *in vitro* for 1 or 3 d before transfer, or transferred to recipients within 4 h of microinjection. Likewise, development to lambs was similar and ranged from 20.0–26.4% of injected zygotes, indicating a significant loss of conceptuses between these two periods.

During 1990, we transferred 675 microinjected zygotes at a rate of between 2 and 6 per ewe (average 4.7, Table 3). There was no effect of number transferred to each ewe on the percentage surviving to lambs. More recently, we have been culturing embryos (20) to investigate their developmental rates and we have found that 31% form transferable blastocysts after 5 d (Table 2). Our data are also similar to Walker's in that 54% of zygotes failed to divide. A study by Rexroad and Wall (34) clearly showed that embryo survival following injection of buffer was reduced from 73 to 28%, data in accordance with ours and that confirm that the physical act of microinjection itself is a major impediment to the success of this work (21).

11. Rexroad and Powell (25) found that cells obtained at any stage of the cycle could be used. It is thought that an oviduct specific factor is needed for progression through the 8–16 cell block when the embryonic genome is activated. The success of Walker's method, however, shows that whatever these factors may be, they are not necessarily specific to the oviduct, or at least HHS provides a suitable substitute.
12. As an alternative, the embryos may be cocultured with either the initial epithelial cell suspensions or with conditioned medium from these cultures as has been successful for bovine material (35).
13. *In vitro* egg production and fertilization. Methods are available for the *in vitro* maturation of oocytes harvested from ovaries collected at the abattoir (36,37). Although the costs associated with animals are reduced, more labor is required in the laboratory and this method may not be so attractive in the final analysis. There is the possibility of better control of fertilization time and pronuclear visibility to suit one's needs, but we do not see sufficient advantage at this stage. This may not be the case for cattle because of the greater cost differentials. Although commercial yields of calves remain low, it shows that there is considerable room for improvement (4), and perhaps this area should be reassessed later.

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CHAPTER 33

Cryopreservation of Transgenic Sheep Lines

Kimball O. Pomeroy

1. Introduction

Although a method exists for freezing sheep embryos directly into liquid nitrogen without a programmable freezing machine (1), a more traditional slow cooling method is described here. Either dimethyl sulfoxide (DMSO) or ethylene glycol can be used as a cryoprotectant in this slow cooling method (2). Pregnancy rates for frozen embryos are similar to those of fresh embryos. For a general discussion of cryopreservation, the reader is referred to Chapter 25.

2. Materials

Equipment needed to freeze embryos is often already found in laboratories that produce transgenic animals.

1. Stereomicroscope.
2. Embryo-transfer pipets (*see* Chapter 17).
3. Tissue-culture dishes.
4. Carbon dioxide (5% [v/v]) incubator set at 37°C.
5. Liquid-nitrogen storage tank.
6. Programmable freezing machine.
7. Phosphate-buffered saline (PBS): Add the following ingredients to 1 L water:

10 g NaCl
0.25 g KCl
1.44 g Na₂HPO₄·12H₂O
0.25 g KH₂PO₄

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Adjust to pH 7.2, aliquot into 500-mL bottles and sterilize by filter-sterilization (0.2- μ pore).

8. Fetal calf serum (FCS).
9. Dimethyl sulfoxide (DMSO).
10. Forceps.
11. Sucrose.
12. Glass freezing ampules.

3. Methods

3.1. Cooling

1. Place morula or blastocysts in PBS containing 10% (v/v) FCS, and each of 0.5, 1.0, and 1.5M DMSO for 10, 10, and 20 min, respectively.
2. Place the embryos in glass ampules containing PBS containing 10% (v/v) FCS and 1.5M DMSO. Leave at room temperature for 20 min.
3. Cool the embryos at $-1^{\circ}\text{C}/\text{min}$ to -7°C , and seed the ampules by grasping them with forceps cooled in liquid nitrogen.
4. Cool at $-0.3^{\circ}\text{C}/\text{min}$ to -35°C , and then at $-0.1^{\circ}\text{C}/\text{min}$ to -38°C .
5. Plunge the ampules into liquid nitrogen and store

3.2. Thawing

1. Place the ampules in a 37°C water bath until the ice has melted.
2. Recover the embryos from the ampule.
3. Place the embryos in PBS containing 10% (v/v) FCS, 1.5M DMSO, and 0.5M sucrose for 10 min.
4. Transfer the embryos into PBS containing 10% (v/v) FCS and 0.5M sucrose for 10 min.
5. Transfer the embryos into PBS containing 10% (v/v) FCS and, hence, into synchronized recipients.

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CHAPTER 34

Isolation of Genomic DNA from Tail Tissue

David Murphy

1. Introduction

Transgenic animals are identified by the presence of transgene sequences in genomic DNA isolated from biopsy material. A suitable method of obtaining this genomic DNA must fulfill three criteria:

1. The method must be rapid and easily performed. Up to 100 genomic DNA preparations may have to be performed simultaneously. In an active transgenic laboratory, many thousands of transgenic mice may be analyzed annually.
2. The DNA should be pure enough to be cleaved by restriction enzymes for subsequent Southern blot analysis.
3. The acquisition of the biopsy must not cause undue distress to the animal. A large amount of genomic DNA is not required, it is therefore not necessary to perform major surgical operations, such as partial hepatectomy, to obtain tissue samples. Any techniques that require anesthesia or result in large losses of blood (e.g., tail bleeding or sampling blood from the eye) are distressing to an animal and put the life of a potentially valuable transgenic founder at risk.

The method described in this chapter fulfills all of these requirements: The genomic DNA is produced within 24 h of the acquisition of the biopsy material with a few easy manipulations. The biopsy material is obtained from the tail of the young mouse. This can be obtained without anesthesia and the wound rapidly heals. An external pain reliever and/or disinfectant can be applied to the wound.

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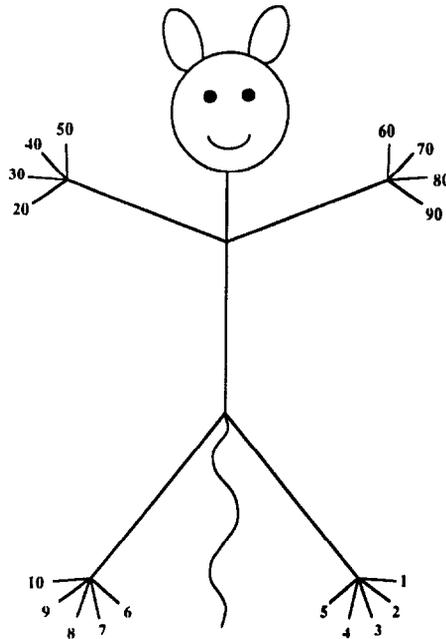
Some regulatory authorities have asked researchers to anesthetize mice for tail biopsy. This is not recommended: The administration of any anesthetic to mice will inevitably lead to death in a certain proportion of the animals. Death or infection following tail biopsy is unknown, at least in this laboratory.

2. Materials

1. Fine, sharp, clean dissection scissors Sterilization by autoclaving is recommended.
2. PK buffer: 50 mM Tris-HCl, pH 8.0, 100 mM EDTA, 100 mM NaCl, 1% (w/v) SDS, 100 mg/mL Proteinase K (PK; Sigma, St Louis, MO, or Gibco-BRL, Gaithersburg, MD).
3. 1 mg/mL RNase A. RNase is made up in water and incubated in a boiling water bath for 10 min to denature any contaminating DNase.
4. Tris buffered phenol: To prepare this, 100 g of molecular biology grade phenol (Gibco-BRL) is melted at 65°C and equilibrated overnight with 100 mL of Tris-HCl, pH 7.4, 24 mL of 0.5M EDTA, 7.5 mL of 10M NaOH, and 243.5 mL distilled, autoclaved water. Following the removal of most of the upper aqueous phase, 8-hydroxyquinoline (100 mg) and β -mercaptoethanol (200 μ L) are added and the phenol is stored frozen.
5. Tris buffered phenol/chloroform/isoamylalcohol (24:24:1).
6. 100% Ethanol
7. 70% Ethanol.
8. Sterile distilled water.

3. Methods

1. Restrain the animal with one hand (*see* Chapter 14) and use a sharp pair of fine scissors to cut off approx 1 cm of tail (*see* Note 1). Place this tissue in 700 μ L of PK buffer contained in an autoclaved 1.5-mL Eppendorf-type centrifuge tube. **At the same time, tag the animal** (*see* Note 2).
2. Mince the tail with a fine pair of scissors (this step is optional; *see* Note 3).
3. Incubate at 55°C overnight, preferably with gentle agitation
4. Add 5 μ L of 1 mg/mL RNase A. Incubate at 37°C for 1–2 h.
5. Add 700 μ L of Tris buffered phenol. Extract the digested tail by gentle mixing with the phenol until homogeneous.
6. Separate the phases by centrifugation at 10,000 rpm in a microcentrifuge for 5 min. Transfer the viscous upper aqueous phase and the interphase to a fresh tube.
7. Add 700 μ L of Tris buffered phenol/chloroform/isoamylalcohol (24:24:1).



Viewed from below

Fig. 1. A scheme for marking mice by toe clipping. The toe is clipped up to the first joint. Up to 100 animals can be so marked. See discussion on p. 312.

- Gently mix the phases until emulsified and separate by centrifugation at 10,000 rpm in a microcentrifuge for 5 min.
8. Carefully avoiding the interphase, transfer the aqueous phase to a fresh tube. Add approx 1 mL 100% ethanol. Gently mix. A stringy white precipitate of genomic DNA should be formed.
 9. Pellet the precipitate by centrifugation at 10,000 rpm in a microcentrifuge for 2 min.
 10. Discard the supernatant and rinse the pellet with 70% (v/v) ethanol. It is permissible to vortex gently at this stage. Repellet the DNA by centrifugation for 1 min at 10,000 rpm in a microcentrifuge.
 11. Remove as much of the supernatant as possible and dry the pellet under vacuum.
 12. Resuspend the pellet in 250 μ L of sterile distilled water. Incubate at 37°C for several hours to enable the DNA to dissolve.
 13. Assay the yield of DNA by UV spectrophotometry (260 nm) (see Note 4).
 14. Store genomic DNA at 4°C (see Note 5).

4. Notes

1. Mice should be weaned (at least 3 wk old) before they are biopsied.
2. At the same time as the tail tissue is obtained, the mouse should be tagged in one of four ways:
 - a. Ear punching;
 - b. Toe clipping (a toe clipping scheme is shown in Fig. 1);
 - c. Caging (weaned animals can be placed individually in marked cages);
 - d. Electronic tagging systems are now available (Lab Products Inc., Maywood, NJ).
3. If the DNA is to be used for polymerase chain reaction analysis (*see* Chapter 36), cross-contamination, which can be introduced by the scissors, should be avoided. The tail tissue will digest without mincing.
4. Approximately 100–200 mg of genomic DNA can be isolated from 1 cm of tail tissue, which is sufficient for a large number of Southern analyses. The quality of the DNA is such that it is cleaved by most common restriction endonucleases.
5. Vortexing and repeated freeze-thawing shears genomic DNA and should be avoided.

CHAPTER 35

Slot Blotting of Genomic Tail DNA

David Murphy

1. Introduction

Slot or dot blotting is a technique whereby nucleic acids can be applied to a solid matrix, unfractionated, using a vacuum manifold. Slot blotting is the quickest, easiest, and, apart from polymerase chain reaction, probably the most sensitive assay of transgenic animal genotype (1). However, it can only be applied to animals that bear a transgene that has hybridizable segments with little or no homology to the host genomic DNA—for example, a hybrid gene with a viral or procaryotic reporter element, or a gene from another species with sufficient sequence divergence to allow the transgene to be distinguished from the host gene. Genomic slot blotting should not be used for the initial identification of a transgenic founder animal. For this, Southern blotting is preferable as it can give more information about the number of integration sites and the presence of transgene rearrangements, deletions, and so forth. However, genomic slot blotting is useful for the rapid screening of subsequent generations of transgenics when detailed information about the structure of the transgene is not needed.

2. Materials

1. 3M NaOH.
2. 5M NH₄ acetate.
3. Nylon hybridization matrix (e.g., Amersham [UK] Hybond-N).
4. 20X SSPE: 3.6M NaCl, 200 mM Na phosphate buffer, pH 6.8, 20 mM EDTA.

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5. Slot-blot applicator (e.g., Schleicher and Schuell, Dassel, Germany) linked to a vacuum line.
6. UV light (312 nm) transilluminator.

3. Methods

1. Adjust 5–15 μg of genomic tail DNA (*see* Note 1) and copy number standards (*see* Note 2) to 0.3M NaOH in 200 μL . Include controls (*see* Note 3).
2. Incubate 10 min in a boiling water bath and quench on ice.
3. Add 400 μL of 5M NH_4 acetate. Apply to a nylon hybridization membrane (*see* Note 4), previously equilibrated with 2X SSPE, using a slot-blot applicator.
4. When the DNA solution has entirely passed through the filter, disassemble the slot-blot applicator. Rinse the filter in 2X SSPE. Air-dry, then bake at 80°C for 60 min.
5. Covalently crosslink the DNA to the matrix by exposure, DNA side down, on Saran wrap, to a UV light (312 nm) transilluminator for 2 min (*see* Note 5).
6. Proceed as described in Chapter 54 (Filter Hybridization).

4. Notes

1. Genomic DNA prepared using rapid techniques from tail tissue are severely contaminated and difficult to quantitate accurately by spectrophotometry. Semiquantitative slot-blot analyses should therefore be performed in duplicate with one filter being hybridized to a transgene probe, the other with a probe for a gene endogenous to the host animal. False negatives can thereby be avoided.
2. Transgene copy number can be determined by slot-blotting by comparing the level of hybridization to copy number standards (prepared by diluting a known quantity of the unlabeled transgene DNA) to the level of hybridization to dilutions of the transgenic mouse genomic DNAs. The latter figure should be corrected with respect to the hybridization of a probe to an endogenous standard host gene.
3. Slot blotting is notoriously prone to give false positive signals. This can best be avoided by: (1) rigorous testing of the probe using Southern blots to ensure that it is specific for the transgene; (2) inclusion in all assays of adequate negative (i.e., nontransgenic) controls; (3) assiduous preparation of all probes, solutions, DNAs, and so forth in order to avoid contamination.
4. Note that these methodologies have been developed for neutral nylon membranes (e.g., Amersham Hybond-N) and have not been tested on

positively charged membranes (e.g., Amersham Hybond-N+, Bio-Rad [Richmond, CA] Zeta-Probe, NEN-Du Pont [Boston, MA] Genescreen Plus).

5. The time needed to covalently UV crosslink nucleic acids to a nylon filter must be determined empirically. The energy output of UV transilluminators varies considerably. Too much exposure will damage the nucleic acids, and reduce the autoradiographic signal following hybridization. Too little exposure will not link the nucleic acids to the filter. During hybridization and washing the target nucleic acids will be lost, again resulting in a reduced autoradiographic signal. To determine the optimal exposure time, identical filters bearing a known nucleic acid are exposed to the UV transilluminator for differing lengths of time. Following hybridization to the same probe, the optimum time, corresponding to the strongest autoradiographic signal, can be determined. Note that the energy output of a transilluminator changes over a period of months and thus regular recalibration is required. Stratagene (La Jolla, CA) produces a system that emits a fixed, measured amount of UV energy.

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CHAPTER 36

Genomic Analysis of Transgenic Animals by the Polymerase Chain Reaction

Cathy Abbott

1. Introduction

Once transgenic animals have been made and founder animals identified, it is usually necessary to establish lines of animals carrying the transgene. Since the founder animal will be hemizygous with respect to the transgene, at most 50% of its offspring will be transgenic. If the original animal is a mosaic, the proportion of transgenic offspring will be much lower. In either case, it is desirable to have a rapid, easy, and preferably economical screening method.

Screening is carried out by detecting the presence of either the transgene itself or its protein product. The protein product can sometimes be detected conveniently by, for example, immunodiffusion using an antibody that is specific for the product of the transgene and not its endogenous counterpart. However, this approach requires not only the availability of a specific antibody, but also that the transgene be efficiently expressed and in a tissue (such as blood) that can be easily removed without sacrificing the animal. For these reasons, it is usually more convenient to screen for the presence of the transgene itself, since any tissue can be used (in principle at least) for the preparation of DNA. This can be accomplished by hybridizing Southern blots of DNA prepared from putative transgenics with a probe that distinguishes between the transgene and any equivalent endogenous gene. However, since the advent of polymerase chain reaction (PCR) technology, it is now possible to screen mice for the presence of a

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transgene quickly and easily, provided that the primers are carefully chosen. The advantages of PCR are the ease of preparation of samples for analysis, and its speed (tens of mice can easily be analyzed in a day). In addition, the sensitivity of the reaction means that only small amounts of material are needed.

The design of primers and the precise conditions for PCR depend on the nature of the transgene to be detected. Transgenes often fall into one of three categories as described below.

1. The transgene can be a gene cloned from a different species, often human, for which there is a homologous gene in the mouse. In such a case, where the transgene differs perhaps only slightly from its endogenous equivalent, primers obviously have to be designed so that they can amplify only the transgene. If sequence is available for both genes, then primers can be chosen in regions of least homology. However, if only the sequence of the transgene is available, primers chosen from intron sequence will usually give a species-specific PCR product. The cycling conditions may need to be adjusted to maximize this effect; for example, high annealing temperatures and fewer cycles than usual may be necessary. Sequence from the 3' untranslated region of a gene will also usually yield species-specific primers, although this is not always the case (1). The main problem with this approach is the possibility of contamination of "negative" tubes with PCR products; however, this can be avoided by careful handling (*see* Note 1).
2. The transgene will sometimes be cloned from the same species as the transgenic animal, e.g., a "minigene," so that there are only minor differences between the transgene and the endogenous counterpart. If a minigene construct is to be used, i.e., the promoter of a gene linked to its coding sequence from a cDNA clone, it should be possible to design primers that give PCR products of different lengths from the transgene and the endogenous gene by amplifying across an intron. If the transgene differs from the homolog by only a single nucleotide, then primers flanking the mutation could be used to amplify both genes, the products of which are then distinguished by either restriction enzyme digestion or hybridization to an oligonucleotide specific for the transgene.
3. The easiest screening can be accomplished where the transgene contains a reporter gene, such as bacterial CAT, which does not have a homolog in the transgenic animal. In this case, primers designed from the sequence of the reporter gene will be completely specific for the transgene. An advantage of this approach is that the same primers could be used for any transgenic experiments involving the same reporter.

There are many methods available for the rapid preparation of DNA from tissues suitable for PCR (however, *see* Note 2). I generally prefer to use the standard, but fairly quick, method of preparing DNA given below, which is adapted slightly from that of Hogan et al. (2). This preparation yields DNA of high enough quality for Southern blots if this should prove to be necessary (for accurate estimation of copy number, for example). However, if speed is of prime importance, an alternative quick preparation method is given that is suitable for preparation of DNA from tail tips. Other quick methods also exist for the preparation of DNA for PCR from small amounts of blood or hair.

2. Materials

- 1 STE: 50 mM Tris-HCl, pH 8, 100 mM NaCl, 100 mM EDTA, and 1% (w/v) SDS.
2. Proteinase K (20 mg/mL).
3. Phenol-chloroform-isoamylalcohol (25:24:1): Phenol should be equilibrated in TE (10 mM Tris-HCl, pH 7.5, 1 mM EDTA). An equal vol of TE is mixed with phenol melted at 65°C. The phases are allowed to separate, and then the buffer aqueous phase is removed. Repeat the procedure until the pH of the phenol is 7.5.
4. Chloroform.
5. 3M Sodium acetate.
6. Isopropanol.
7. 10X PCR buffer. 0.5M KCl, 15 mM MgCl₂, 0.1M Tris-HCl, pH 8.3, and 1 mg/mL gelatin.
8. 2 mM Stocks of dATP, dCTP, dGTP, and dTTP (Pharmacia, Uppsala, Sweden, ultrapure dNTP kit)
9. Forward and reverse PCR primers (*see* Note 3).
10. *Taq* DNA polymerase.
11. Thermal cycling system: Many such systems are commercially available.

3. Methods

3.1. DNA Preparation

3.1.1. Method 1

1. Incubate the tail tip overnight at 55°C in 700 µL of STE and 20 µL of 20 mg/mL proteinase K.
2. Extract twice with 700 µL of phenol/chloroform.
3. Extract once with 700 µL of chloroform.
4. Add 70 µL of 3M sodium acetate and 400 µL of isopropanol, invert to mix, and spool out the DNA.
5. Redissolve the DNA in 200–500 µL of water.

3.1.2. Method 2

1. Incubate tail tip as described in Section 3.1.1. in 200 μL of STE and proteinase K.
2. Take 80 μL of the digest, and add 50 μL of isopropanol.
3. Spool out the DNA, and then dissolve it in 50 μL of water.
4. Boil for 15 min, and then use 2 μL for each PCR reaction.

3.2. PCR—Basic Protocol

1. Mix the following in a 0.5-mL Eppendorf tube:
 - 10 μL of 10X PCR buffer
 - 10 μL each of 2 mM dATP, dCTP, dGTP, and dTTP (Pharmacia ultrapure dNTP set)
 - 50 pmol each primer
 - 1 μg DNA
 - Water to 100 μLLiquid paraffin can be layered over the mix to prevent evaporation.
2. Incubate the mixture at 95°C for 5 min. This will inactivate any contaminating nucleases and make the DNA single stranded.
3. Add 1 U *Taq* polymerase, and start the cycling reaction. One cycle would typically be 94°C 15 s, 55°C 30 s, and 72°C 1 min for 30 cycles; however, the choice of cycling conditions depends on the melting temperature of the oligonucleotides, the length of the expected product and the degree of specificity required (*see* Note 4).
4. Run a 10- μL aliquot of the PCR reaction on a 2% agarose minigel in 1X TAE stained with ethidium bromide (*see* Chapter 51) and viewed under UV light. Check that the band obtained is of the predicted size.

4. Notes

1. Contamination occurs when PCR products get into PCR reagents. This can be avoided by maintaining physically separate areas for the setting up of PCRs and the analysis of PCR products (4). If contamination does occur (so that, for example, all mice appear to be transgenic), there is a published method by which reagents can be treated with UV irradiation (5). My personal choice, where practicable, is to throw out all reagents and start again.
2. A problem that is sometimes encountered with PCR is nonamplifiable DNA. If consistently negative results are being obtained, the DNA should be amplified with primers that will amplify an endogenous gene and that should therefore give a positive result. If DNA is unamplifiable, it can be cleaned up (6), or fresh DNA can be prepared from the mouse. If this is necessary, it is best to spool out the DNA as quickly as possible

after the precipitation stage, since this seems to minimize nonamplification problems.

3. Choice of oligonucleotides: These are typically 20–25 mers with a roughly equal proportion of GC to AT. It is important that the GC content of each oligonucleotide is equal to that of its partner so that the annealing temperatures will be identical. I prefer to use 25 mers with about 60% GC content. The size of the fragment to be amplified is usually between 200 and 500 bp, although good results can be easily obtained for up to 1 kb. The fragment should not be <100 bp for good resolution. An extension time of 1–2 min should be ample for amplification products of up to 1 kb.

Most primers operate well in buffers containing 1.5 mM MgCl₂. However, certain pairs of primers have different optimum requirements for Mg²⁺ concentration, and it is sometimes necessary to carry out titrations using Mg²⁺ concentrations ranging from 1.1–2.5 mM MgCl₂ (3). There seems to be no way of predicting the Mg²⁺ optimum from sequence data; it must always be determined empirically.

4. In order to increase specificity, annealing should be at as high a temperature and for as short a time as possible for amplification to occur. A nontransgenic mouse DNA should always be included in the PCR as a negative control. If necessary, the number of cycles could also be adjusted to help control specificity.

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CHAPTER 37

Genomic Analysis of Transgenic Animals

Southern Blotting

Duncan R. Smith and David Murphy

1. Introduction

Although subsequent generations can be rapidly screened using slot blotting (Chapter 35) or the polymerase chain reaction (PCR; Chapter 36), founder transgenic animals should initially be analyzed by Southern blotting for two reasons. First, low copy number transgenics or mosaic transgenics (where only a small number of the cells in a biopsy may be transgenic, and hence the effective copy number of the transgene in the DNA preparation is less than one) may not be clearly identifiable using slot blots because of high background. Such animals can, however, be unambiguously identified by the presence of bands of predicted sizes on Southern blot filters (1,2). Note that PCR can also clearly detect low copy-number transgenes. Second, both slot blot and PCR are unable to give direct information on transgene copy number, transgene integrity (i.e., the presence or absence of gross deletions, insertions, and so forth), and the number of independent transgene integration sites. Careful choice of restriction enzymes used to cleave the DNA, followed by Southern blotting, can reveal information about all of these features of a transgenic animal.

The technique of Southern blotting is well known (2). DNA is cleaved with restriction enzymes (Chapter 50) to produce fragments that are fractionated according to size in an agarose gel (Chapter 51).

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The DNA is then partially cleaved by depurination (to facilitate the transfer of larger DNA fragments) and alkali denatured by sequential soaking of the gel in solutions containing HCl and NaOH respectively. The denatured DNA fragments are then transferred to a solid matrix or filter (usually a nylon membrane) for subsequent hybridization to a specific labeled probe (Chapter 37).

A transgene integrated into the genome of a host animal can be distinguished from homologous endogenous genes in two ways:

1. Differing restriction patterns. If the transgene is derived from another species, it is likely that some restriction enzyme sites will be species-specific, and endogenous and exogenous DNA can be distinguished on the basis of the size of restriction fragments. Even if the transgene is identical to the endogenous gene, the former will be flanked by different restriction sites compared to the latter, resulting in different sized restriction fragments.
2. Transgene specific probes. A transgene may have hybridizable segments with little or no homology to the host genomic DNA; for example, a hybrid gene with a viral or prokaryotic reporter element, or a gene from another species with sufficient sequence divergence to allow the transgene to be distinguished from the host gene.

2. Materials

1. Molecular biology grade agarose (Gibco-BRL, Gaithersburg, MD).
2. 10X TBE: 108 g Boric acid, 55 g Tris base, and 40 mL 0.5M EDTA, pH 8.0, to 1 L. Autoclave to sterilize.
3. 20 × 20 cm gel casting tray, running tank, and electrophoresis power pack.
4. 10 mg/mL ethidium bromide in water (**caution: extremely carcinogenic**).
5. 20X SSPE: 3.6M NaCl, 200 mM Na phosphate, pH 6.8, 20 mM EDTA.
6. 10X loading dye: 40% (v/v) glycerol, 10X TBE, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol.
- 7 Nylon hybridization membrane (e.g., Amersham [UK] Hybond-N; *see* Note 1).
8. Capillary transfer system (*see* Note 2).
9. Depurination buffer: 0.25M HCl.
10. Denaturation buffer: 1.5M NaCl, 0.5M NaOH.
11. Transfer buffer: 1.5M NaCl, 0.25M NaOH.
12. 312 nm UV light transilluminator.
- 13 Copy number standard (*see* Note 3).
14. Markers (size standards; *see* Note 4).
15. Genomic DNA (from tail biopsy; *see* Chapter 34).

16. Restriction enzymes and buffers (*see* Chapter 50; *see* Note 5).
17. Labeled probe DNA (*see* Chapter 53).

3. Methods

1. Cut 10–15 μg of genomic biopsy (usually tail) DNA (Chapter 34) with an appropriate restriction endonuclease as described (Chapter 50).
2. Make an agarose gel as described (Chapter 51).
3. To the digest, add 1/10 vol of loading dye, mix well, and load the samples into the gel slots. Include appropriate standards on the gel (*see* Notes 3 and 4).
4. Apply a current across the gel (negative to positive, 4 V/cm) and run until the fast (bromophenol) blue has reached the end of the gel.
5. After electrophoresis, illuminate the gel using a UV light transilluminator (312 nm). Photograph the gel with a ruler placed alongside the marker DNA lane.
6. Remove unused portions of the gel with a clean scalpel blade.
7. Incubate the gel in approx 3 gel vol of depurination buffer with gentle agitation at room temperature for 30 min, or until the bromophenol blue in the loading dye turns yellow.
8. Decant the depurination buffer and replace with 3 gel vol of denaturation buffer. Incubate with gentle agitation at room temperature for 30 min.
9. Decant the denaturation buffer and replace with 3 gel vol of transfer buffer. Equilibrate the gel with gentle agitation at room temperature for 30 min.
10. Place the gel on the platform of a capillary transfer system filled with transfer buffer. A diagram of a transfer system is shown in Fig. 1. The system is made up of a platform that sits in a reservoir containing transfer buffer. A wick, made up of four thicknesses of 3MM paper is placed over the platform, soaked in transfer buffer. All air bubbles must be removed from the wick. The width and length of the platform corresponds to the size of the gel and the wick is cut to the same width. The platform and reservoir are made to the same height.
11. Cut a piece of Hybond-N (Amersham) to the same size as the gel. Wet this by floating it on distilled water then rinse in transfer buffer. Place the filter on the gel and smooth out any air bubbles.
12. Cut four pieces of 3MM paper to the same size as the gel. Soak two in transfer buffer and place over the filter. Smooth out any bubbles. Place two dry filters onto the sandwich, then a stack of dry paper towels. Place a 1-kg weight on top and allow transfer to proceed at least 12 h.
13. Disassemble the transfer system. Prior to separating the gel from the filter, the position of the gel slots can be marked. If this is done with a

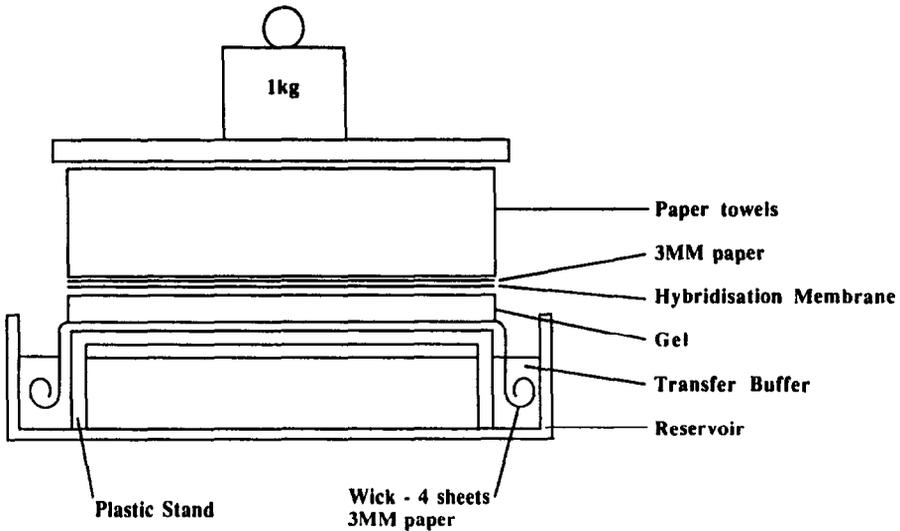


Fig. 1. A typical capillary action Southern transfer system.

pencil, the marks will appear on the resulting autoradiograph. Rinse the filter in 2X SSPE and bake the filter at 80°C for 20–60 min

14. Covalently crosslink the DNA to the matrix by exposure to a 312 nm UV light transilluminator. Place the filter, DNA side down, on a piece of Saran wrap and expose for 2–3 min (*see Note 6*).
15. Hybridize the filter as described in Chapter 54 (Filter Hybridization).

4. Notes

1. Note that these methodologies have been developed for neutral nylon membranes (e.g., Amersham Hybond-N) and have not been tested on positively charged membranes (e.g., Amersham Hybond-N+, Bio-Rad [Richmond, CA] Zetaprobe, NEN-Du Pont [Boston, MA] Genescreen Plus).
2. The capillary transfer system described here is efficient but time-consuming. A number of companies now market systems (vacuum blotting: Pharmacia LKB [Uppsala, Sweden], Hybaid [Twickenham, UK]; positive pressure blotting: Stratagene [La Jolla, CA]) that reduce the transfer process to as little as 1 h.
3. Transgene copy number can be determined by Southern blotting by comparing the level of hybridization to copy number standards (prepared by diluting a known quantity of the unlabeled transgene DNA fragments) to the level of hybridization to transgenic mouse genomic

- DNAs. The latter figure should be corrected with respect to the hybridization of a probe to an endogenous standard host gene.
4. The size of a hybridizing band is determined relative to DNA standards of a known size (e.g., bacteriophage lambda cut with *Eco* RI and *Hind* III, or the convenient 1-kb ladder marketed by Gibco-BRL). It is best to radioactively end-label (see Chapter 43) DNA standards such that an image of their position is produced on the final autoradiograph.
 5. The number of independent integration sites can be determined by cutting the genomic DNA with an enzyme that does not cleave the transgene. The number of bands produced will therefore correspond to the minimum number of integration sites. Note that as transgenes often integrate as large head-to-tail or head-to-head tandem arrays, the bands produced by this analysis might be particularly large, demanding the use of low percentage gels. Note that if, when using enzymes that cleave the transgene, junction fragments can be identified, they too can give an indication of the number of integration sites.
 6. Efficient crosslinking of DNA to nylon filters is achieved with an optimal amount of exposure to UV light. After a certain value the efficiency decreases with increasing exposure. For this reason it is best to calibrate a UV source before usage. This can be done by taking filters with an identical amount of DNA on each, exposing them to UV for different lengths of time, and then hybridizing them to the same probe. The strongest signal will establish the optimal time for exposure. Note that the energy output of a standard UV transilluminator varies with the age of the bulb, thus necessitating regular recalibration. Some manufacturers (e.g., Stratagene) now produce UV crosslinkers that automatically expose the filter to the radiation for the optimal time, delivering a fixed dose of energy.

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CHAPTER 38

The Preparation of RNA from the Tissues of Transgenic Animals

David Murphy

1. Introduction

The analysis of RNA is often the easiest and quickest method of determining the pattern of transgene expression in a transformed organism. Such analyses demand that the RNA extraction method be relatively rapid and painless. Some RNA preparation methods, for example, those that require a CsCl₂ ultracentrifugation step, do not fulfill such demands. The methods presented here, however, allow RNA to be isolated rapidly (within hours for the minipreparation method) from many transgenic animal tissues simultaneously. The methods are based on the use as a lytic agent of guanadinium thiocyanate, a strong denaturant that destroys cellular integrity as protein structure is lost. Simultaneously, RNases lose activity, so the method is particularly useful for the isolation of RNA from transgenic mouse tissues rich in this enzyme (for example, pancreas and lung). Following acid-organic extraction of the lysate and centrifugation, high-mol-wt genomic DNA partitions at the interphase, and denatured proteins partition into the organic phase and interphase, leaving the RNA in the aqueous fraction. This is further purified by ethanol precipitation. The resulting RNA can be analyzed by any of the techniques described in Chapters 39, 40, 41, 43, and 44.

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2. Materials (see Note 1)

1. Denaturing solution A (DSA): 4M guanadinium thiocyanate, 25 mM sodium citrate, pH 7, and 0.5% (w/v) sarkosyl. This solution is prepared by dissolving 472.8 g solid guanadinium thiocyanate in 500 mL DEPC-treated water (see Notes 2 and 3) followed by the addition of 100 mL of DEPC-treated 250-mM sodium citrate, pH 7, and 5 mL of 100% (w/v) sarkosyl. The solution is made up to 1 L with DEPC-treated water and purified by passage through a 0.45- μ m Nalgene (Rochester, NY) filter.
2. Denaturing solution B (DSB): 5M guanadinium thiocyanate, 10 mM EDTA, and 50 mM Tris-HCl, pH 7.5. This solution is prepared and filtered similarly to DSA (see step 1 above).
3. DEPC-treated 2M sodium acetate, pH 5.5 (see Notes 2 and 3).
4. RNA-grade phenol: Molecular-biology-grade phenol (Gibco-BRL, Gaithersburg, MD) is melted at 65°C and then equilibrated with an equal vol of DEPC-treated water. Following the addition of 8-hydroxyquinoline to 0.1% (w/v), much of the aqueous phase is removed, and the phenol is stored frozen until required.
5. Chloroform:isoamyl alcohol (24:1).
6. 4M LiCl: This solution is autoclaved and then purified by passage through a 0.45- μ m Nalgene filter.
7. 1-mL Sterile disposable syringes and sterile 25-g needles.
8. Homogenizer (e.g., Ultra-Turax T25, Janke and Kunke, Staufen, Germany).

3. Methods (see Note 4)

3.1. Minipreparation of RNA

1. To 50 mL of DSA, add 360 μ L of β -mercaptoethanol (to 0.1M). Mix well.
2. Collect tissues. Tissue samples can be collected directly into 500 μ L DSA/ β -mercaptoethanol on ice and processed immediately. Alternatively, tissues can be collected into empty DEPC-treated 1.5-mL Eppendorf-type tubes on dry-ice, frozen, and stored at -80°C until required. Frozen tissues should not be allowed to thaw prior to homogenization, but should be disrupted still frozen directly into DSA/ β -mercaptoethanol. Thawing releases RNAses from disrupted intracellular stores. Finely chopping the tissue while fresh may assist the subsequent homogenization.
3. Homogenize tissues in 500 μ L of a solution of DSA/ β -mercaptoethanol by suction, 5–10 times, using a sterile disposable syringe, through a 25-g needle. Tougher samples may require a larger needle.
4. Extract by the sequential addition of 50 μ L 2M sodium acetate, pH 5.5, 500 μ L of RNA-grade phenol, and 100 μ L of chloroform:isoamyl alco-

- hol (24:1). Following mixing, incubate on ice for 10 min, centrifuge (Eppendorf [Hamburg, Germany] 5415 Microfuge, 5 min, 14,000 rpm), and then transfer the upper aqueous phase to a fresh tube. The interface between the organic and aqueous phases should be carefully avoided. This can be difficult, since the interface contains genomic DNA and can be very viscous. Precipitate nucleic acids by incubation with 1 mL of 100% ethanol at -70° for 1 h.
5. Recover the nucleic acids by centrifugation, resuspend in 250 μ L of DSA/ β -mercaptoethanol, and reprecipitate with ethanol.
 6. Wash the pellet with 75% ethanol, dry under vacuum, and resuspend in 12 μ L of DEPC-treated water (*see* Note 5).
 7. Assay 2 μ L of the RNA in 500 μ L of DEPC-treated water by UV light spectrophotometry (260 nm). One absorbance (A) unit is equivalent to an RNA concentration of 40 μ g/mL (*see* Note 6).

3.2. Maxipreparation of RNA (see Note 7)

1. To 50 mL of DSA, add 360 μ L of β -mercaptoethanol (to 0.1M). Mix well.
2. Collect tissues. Tissue samples can be collected directly into 5–20 mL DSA/ β -mercaptoethanol on ice and processed immediately. Alternatively, tissues can be collected into empty sterile disposable 50-mL centrifuge tubes on dry-ice, frozen, and stored at -80°C until required. Frozen tissues should not be allowed to thaw prior to homogenization, but should be disrupted still frozen directly into DSA/ β -mercaptoethanol. Finely chopping the tissue while fresh may assist the subsequent homogenization.
3. Disrupt tissues in 5–20 mL of a solution of DSA/ β -mercaptoethanol using an Ultra-Turax homogenizer or equivalent. Use 5 mL of DSA/ β -mercaptoethanol for every 250 mg of tissue.
4. Following the sequential addition of 2M sodium acetate, pH 5.5 (1/10 vol of the homogenate), RNA-grade phenol (1 homogenate vol), and chloroform-isoamyl alcohol (24:1; 1/5 original homogenate vol), incubate the mixture on ice for 15 min.
5. Following centrifugation at 10,000g at 4°C for 20 min, transfer the nucleic acids in the upper aqueous phase to a fresh tube, carefully avoiding the interface, and precipitate with 2 vol of 100% ethanol.
6. Recover the nucleic acid pellet by centrifugation at 10,000g at 4°C for 30 min following incubation at -80° for 60 min.
7. To 50 mL of DSB, add 360 μ L of β -mercaptoethanol. Mix well. Resuspend the RNA pellet in 5 mL of DSB/ β -mercaptoethanol. Precipitate the RNA by the addition of 6 vol of 4M LiCl followed by incubation at 4°C overnight.
8. Recover the RNA by centrifugation at 10,000g at 4°C for 90 min. Resus-

pended the pellet in 5 mL of DSA/ β -mercaptoethanol, and reprecipitate with 2 vol of 100% ethanol followed by centrifugation.

9. Wash the RNA pellet with 70% (v/v) ethanol, dry, and resuspended in DEPC-treated water (*see* Note 5). Store frozen until required.
10. Assay 2 μ L of the RNA in 500 μ L of DEPC-treated water by UV light spectrophotometry (260 nm). One absorbance unit is equivalent to an RNA concentration of 40 μ g/mL (*see* Note 6).

4. Notes

1. All receptacles and equipment should be rendered RNase-free. The homogenizer probe and glass (Pyrex) centrifuge tubes can be baked at 250°C for 3 h. Plastic Eppendorf-type tubes and pipet tips should be washed in a solution of 0.1% DEPC, and then autoclaved. Sterile disposable plastic tubes, syringes, and needles are generally regarded as RNase-free.
2. The success of this method is dependent on the elimination of RNase molecules from all equipment and reagents used in the preparation of the RNA. Solutions should be treated with DEPC (diethylpyrocarbonate) where possible. DEPC is added to the solution to 0.1%, and following incubation at room temperature for 3 h, the solution is autoclaved. Autoclaving, as well as sterilizing the solution, also inactivates the DEPC, which will itself destroy RNA unless so treated. Solutions can be further purified by passage through 0.45- μ m Millipore-type filters. Note that Tris-buffers cannot be treated with DEPC. These can be made up with DEPC-treated water, autoclaved, and filtered.
3. **Caution:** DEPC is highly toxic. Use only in a fume hood.
4. The choice of method depends on the size of the tissue to be processed. If the amount of lysis solution used is too low, then the resulting RNA preparation will be contaminated with genomic DNA. The miniprep method can maximally accommodate tissue samples the size of a mouse thymus, two mouse ovaries, or a single rat hypothalamus. A mouse brain, heart, kidney, or spleen can readily be processed in 5 mL of lysis buffer using the maxiprep method.
5. Resuspending a dry RNA pellet in water can often prove difficult. Heating at 65°C for 15 min and persistent vortexing can encourage a sample to go into solution. Alternatively, samples can be sonicated using a fine, clean probe.
6. The RNA prepared using these methods is of sufficient quality to be used in any of the analytical procedures described in Chapters 39, 40, 41, 43, and 44. However, further purification of polyadenylated RNA (mRNA) may sometimes be necessary. Methods for this have been described (1,2). Note that kits for this procedure are now available from a number

of suppliers (e.g., Pharmacia [Uppsala, Sweden], Bio-Rad [Richmond, CA], and Promega [Madison, WI]).

7. The two methods differ only in the inclusion of a LiCl precipitation step in the maxiprep method. This is necessary in order to eliminate fragments of DNA that cofractionate with the RNA following the extraction with phenol. The DNA fragments are produced by the shearing of genomic DNA by the action of the homogenizer.

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CHAPTER 39

Northern Blotting

David Murphy

1. Introduction

Northern blotting is the technique whereby RNA molecules are denatured, fractionated through agarose on the basis of size, and then transferred to a solid matrix for subsequent hybridization to a specific labeled probe (1). The technique can be used to analyze the pattern of transgene expression in an organism, and changes in that pattern following physiological and developmental transitions. It is important to note that the technique depends on being able to distinguish the transgene RNA from the RNAs produced by the homologous endogenous gene. Thus, the transgene must be modified such that, although both transgene and endogenous RNAs are detected with the same probe, the former RNAs have a different mobility to the latter. Alternatively, the transgene RNAs should contain hybridizable segments with little or no homology to host RNAs expressed in the tissues being examined—for example, RNAs derived from a hybrid gene with a viral or prokaryotic reporter element or a gene from another species with sufficient sequence divergence to allow the transgene RNAs to be distinguished from the host RNAs using specific probes.

2. Materials

1. Molecular-biology-grade agarose (e.g., Gibco-BRL, Gaithersburg, MD).
2. Formaldehyde (Fluka, Buchs, Switzerland).
3. 20X MAE: 0.4M MOPS, pH 7, 0.1M sodium acetate, and 0.02M EDTA.
4. Gel casting tray (20 × 20 cm), running tank (e.g., Pharmacia [Uppsala, Sweden] GNA-200), and electrophoresis power pack.

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5. 20X SSPE: 3.6M NaCl, 200 mM sodium phosphate buffer, pH 6.8, and 20 mM EDTA.
6. Loading buffer (3:1): 5 mL formamide (Fluka; *see* Note 1), 0.5 mL 20X MAE, 1.7 mL formaldehyde (Fluka), and 0.3 mL DEPC-treated water
7. 10X Loading dye: 40% (v/v) glycerol, 10X MAE, 0.2% (w/v) bromophenol blue, 0.2% (w/v) xylene cyanol, and 10 mg/mL ethidium bromide.
8. Nylon hybridization membrane (e.g., Amersham [UK] Hybond-N; *see* Note 2).
9. Capillary transfer system.
10. 312 nm Ultraviolet light transilluminator.

3. Methods

1. Weigh out an appropriate volume of agarose (*see* Note 3) into a clean, autoclaved 500-mL flask.
2. Add 250 mL DEPC-treated water, and boil in a microwave oven until the agarose has completely melted. The preparation of DEPC water is described in Chapter 38.
3. Allow to cool to approx 65°C, and then add 18 mL of 20X MAE and 60 mL formaldehyde. Mix well, and then pour into the sealed gel casting tray containing a comb. Ensure that all bubbles are removed. Formaldehyde is harmful if inhaled: Perform these steps in a fume hood. When the gel has set, unseal the ends of the gel, remove the slot comb, and place the gel in a running tank containing sufficient 1X MAE just to cover the gel.
4. Add 3 vol of 3:1 loading buffer to 1 vol of RNA (maximum 50 µg) in water. Mix well, and then heat at 65°C for 15 min to denature the RNA. Add 1/10 vol loading dye, mix well, and then place the samples in the gel slots.
5. Apply a current across the gel (negative to positive; 4 V/cm), and run until the fast blue (bromophenol blue) has reached the end of the gel.
6. Place the gel on a capillary transfer system filled with 20X SSPE. The construction of this is described in Chapter 37. Cut a piece of Hybond-N (Amersham) to the same size as the gel. Wet this by floating it on DEPC-treated water, and then rinse in 20X SSPE. Place the filter on the gel, and smooth out any bubbles.
7. Cut four pieces of 3MM paper to the same size as the gel. Soak two in 20X SSPE, and place over the filter. Smooth out any bubbles. Place two dry filters onto the sandwich and then a stack of dry paper towels. Place a 1-kg weight on top, and allow transfer to proceed for at least 12 h.
8. Disassemble the transfer system. Prior to separating the gel from the filter, the position of the gel slots can be marked. If this is done with

pencil, the marks will appear on the resulting autoradiograph. Bake the filter at 80°C for 20–60 min.

9. Covalently crosslink the RNA to the matrix by exposure to a 312-nm UV light transilluminator. Place the filter RNA side down on a piece of Saran wrap™, and expose for 2–3 min (*see* Note 4). During this time, the fluorescent RNA can be observed because of interchelation of the ethidium bromide contained within the loading dye. The position of the 28S and 18S ribosomal RNA bands (*see* Note 5) can be marked, as well as the position of any RNA markers used (e.g., Gibco-BRL). The ethidium bromide stain can also indicate the integrity of the RNA samples used, the equality of loading of the different samples, and the efficiency of transfer. A photograph can be taken of the filter.
10. Proceed as described in Chapter 54.

4. Notes

1. Formamide should be deionized before use. To do this, formamide is mixed and stirred for 1 h with Dowex XG8 mixed bed resin, followed by filtration through 3MM paper. Fluka formamide (catalog number 47670) does not require deionization.
2. Note that these methodologies have been developed for neutral nylon membranes (e.g., Amersham Hybond-N) and have not been tested on positively charged membranes (e.g., Amersham Hybond-N+, Bio-Rad [Richmond, CA] Zeta-Probe, NEN-Du Pont [Boston, MA] Genescreen Plus).
3. The percentage of agarose used in the gel will determine the ability to resolve different sized RNAs. For examining RNAs >2 kb, use gels of 1% agarose. Smaller RNAs require progressively higher agarose contents up to a maximum of 1.8% for RNAs <500 bp.
4. The time needed to UV crosslink covalently nucleic acids to a nylon filter must be determined empirically. The energy output of UV transilluminators varies considerably. Too much exposure will damage the nucleic acids and reduce the autoradiographic signal following hybridization. Too little exposure will not link the nucleic acids to the filter. During hybridization and washing, the target nucleic acids will be lost, again resulting in a reduced autoradiographic signal. To determine the optimal exposure time, identical filters bearing a known nucleic acid are exposed to the UV transilluminator for differing lengths of time. Following hybridization to the same probe, the optimum time, corresponding to the strongest autoradiographic signal, can be determined. Note that the energy output of a transilluminator changes over a period of months, and thus regular recalibration is required. Stratagene (La Jolla, CA) pro-

duces a system that emits a fixed, measured amount of UV energy.

5. The size of an RNA species revealed by Northern analysis can be determined by comparison with either the 28S (4.7 kb) or 18S (1.9 kb) ribosomal RNA bands endogenous to the RNA preparation, or by using commercially available RNA mol-wt markers (Gibco-BRL, Boehringer Mannheim, Mannheim, Germany). Note that the majority of eukaryotic mRNAs bear polyadenylate tracts of varying length (30–400 nucleotides) at the 3' end. The size of the mRNA core can be determined by Northern analysis following the enzymatic removal of the poly(A) tail using RNase H in the presence of oligo(dT) (2).
6. The steady-state level of an RNA species and changes in the amount of an RNA species following a developmental or physiological change can be determined relative to specific RNA standards generated by *in vitro* transcription of cloned cDNAs by bacteriophage RNA polymerases (SP6 or T7;3). Such determinations should be compared to internal standards (i.e., an RNA species that does not change in level) assayed by reprobng the Northern filter in order to control for loading or transfer artifacts.

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CHAPTER 40

RNA Slot Blotting

David Murphy

1. Introduction

Using slot blots, RNA can be applied, unfractionated, to a solid matrix. Slot blotting can be used as a rapid method for analyzing changes in transgene RNA quantity following developmental or physiological changes. However, it can only be applied to animals that bear a transgene that has hybridizable segments with little or no homology to host RNAs expressed in the same tissues being examined—for example, RNAs derived from a hybrid gene with a viral or prokaryotic reporter element or a gene from another species with sufficient sequence divergence to allow the transgene RNAs to be distinguished from the host RNAs.

2. Materials

1. Loading buffer (3:1). 5 mL formamide (Fluka [Buchs, Switzerland]; *see* Note 1), 1.7 mL formaldehyde (Fluka), 0.3M DEPC-treated water, and 0.5 mL 20X MAE (20X MAE: 0.4M MOPS, pH 7, 0.1M Na acetate, and 0.02M EDTA).
2. DEPC-treated 20X SSPE: 3.6M NaCl, 200 mM Na phosphate buffer, pH 6.8, and 20 mM EDTA.
3. Slot-blot applicator linked to vacuum line (e.g., Schleicher and Schuell, Dassel, Germany).
4. Nylon hybridization matrix (e.g., Amersham [UK] Hybond-N); *see* Note 2.
5. 312 nm UV light transilluminator.
6. RNA (*see* Notes 3–5).

3. Methods (see Note 5)

1. Add 3 vol of 3:1 loading buffer to the RNA sample (up to 50 μg total cellular RNA). Incubate at 65°C for 15 min to denature.
2. Add SSPE to 2X to a final vol of up to 500 μL .
3. Apply to the nylon hybridization matrix using the slot-blot applicator.
4. Disassemble the slot-blot system, and rinse the filter in DEPC-treated 2X SSPE.
5. Bake the filter at 80°C for 1 h. This step dries the filter and assists with the fixing of the RNA to the matrix.
6. Covalently crosslink the RNA to the matrix by exposure to a 312-nm UV light transilluminator. Place the filter RNA side down on a piece of Saran wrapTM, and expose for 2–3 min (see Note 6).
7. Proceed with hybridization as described in Chapter 54.

4. Notes

1. Formamide should be deionized before use. To do this, formamide is mixed and stirred for 1 h with Dowex XG8 mixed bed resin, followed by filtration through 3MM paper. Fluka formamide (catalog number 47670) does not require deionization.
2. Note that these methodologies have been developed for neutral nylon membranes (e.g., Amersham Hybond-N) and have not been tested on positively charged membranes (e.g., Amersham Hybond-N+, Bio-Rad [Richmond, CA] Zeta-Probe, NEN-Du Pont [Boston, MA] Genescreen Plus).
3. Total cellular RNA prepared using rapid techniques can be contaminated with genomic DNA and therefore will be difficult to quantitate accurately by spectrophotometry. Semiquantitative slot-blot analyses of transgene RNA should therefore be performed in duplicate with one filter being hybridized to a transgene probe, and the other with a probe for a gene endogenous to the host animal that does not change in level with the physiological or developmental change being examined.
4. Slot blotting of RNA can be used to rapidly quantitate changes in the steady-state levels of specific RNA species following developmental or physiological change. The hybridization of the probe to dilutions of test RNAs is compared to the signal generated by hybridization of the same probe to dilutions of specific RNA standards generated by *in vitro* transcription of cloned cDNAs by bacteriophage RNA polymerases (SP6 or T7). Such determinations should be compared to internal standards (i.e., an RNA species that does not change in level) assayed by probing a duplicate filter or reprobing the same filter to control for loading artifacts.

5. Slot blotting is notoriously prone to giving false positive signals. This can best be avoided by:
 - a. Rigorous testing of the probe using Northern blots to ensure that it is specific for the transgene RNA;
 - b. Inclusion in all assays of adequate negative (i e., nontransgenic) controls; and
 - c. Assiduous preparation of all probes, solutions, RNAs, and so on, in order to avoid contamination.
6. The time needed to UV crosslink covalently nucleic acids to a nylon filter must be determined empirically. The energy output of UV transilluminators varies considerably. Too much exposure will damage the nucleic acids and reduce the autoradiographic signal following hybridization. Too little exposure will not link the nucleic acids to the filter. During hybridization and washing, the target nucleic acids will be lost, again resulting in a reduced autoradiographic signal. To determine the optimal exposure time, identical filters bearing a known nucleic acid are exposed to the UV transilluminator for differing lengths of time. Following hybridization to the same probe, the optimum time, corresponding to the strongest autoradiographic signal, can be determined. Note that the energy output of a transilluminator changes over a period of months, and thus, regular recalibration is required. Stratagene (La Jolla, CA) produces a system that emits a fixed, measured amount of UV energy.

CHAPTER 41

Analysis of Gene Expression by PCR

Peter Koopman

1. Introduction

Methods used to study gene expression rely on two basic conditions. First, sufficient tissue must be available for analysis, and second, the gene must be expressed at a level high enough to be detectable by the method used. Where the gene of interest is expressed in small tissues or groups of cells, for example, in mouse embryos, or where transcripts are present in vanishingly low amounts, it is often difficult to perform Northern blotting, RNase protection assays, or *in situ* hybridization. In these cases, the exquisite sensitivity of the polymerase chain reaction (PCR) can provide a solution. PCR has been used to detect transcripts present at down to 1 copy/1000 cells (1), and in samples as small as a mouse blastocyst (2–4) or even a single cell (5).

In addition to this sensitivity, the specificity of PCR can often be an important consideration. In the case of a gene that is a member of a closely related multigene family or a heterologous transgene that differs only slightly in its coding sequence from the corresponding endogenous gene, it can be difficult to obtain a suitably specific probe for expression studies. PCR can distinguish transcripts that differ in as little as one nucleotide.

PCR is quick (from tissue to result in as little as 1 d), inexpensive, does not require special skills or radioactivity, and can be used to assay several types of transcripts simultaneously. This chapter emphasizes the study of gene expression in transgenic animals, but the techniques described can be used in any situation where the advantages of PCR are called for.

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2. Materials

2.1. RNA Preparation

1. Solution D: 25 g guanidinium thiocyanate (Sigma, St. Louis, MO), 29.3 mL water, 1.76 mL 0.75M sodium citrate, pH 7, 2.64 mL 10% sarkosyl, and 38 μ L β -mercaptoethanol. Store aliquots at -70°C . Thawed aliquots can be kept in the dark at room temperature for up to 1 mo
2. 2M Sodium acetate, pH 4 and pH 5.8.
3. Water-saturated phenol.
4. Chloroform:isoamylalcohol (49:1).
5. RNase-free glycogen (e.g., Boehringer, Mannheim, Germany) 20 mg/mL.
6. Diethylpyrocarbonate (DEPC)-treated water: Add 100 μ L DEPC (Sigma) to 1 L distilled water, shake vigorously, and autoclave.
7. 5X DNase buffer: 200 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 30 mM MgCl_2 .
8. DNase I (e.g., Boehringer).
9. RNase inhibitor (RNasin, e.g., Promega, Madison, WI).

2.2. Reverse Transcription

1. 5X RT buffer: 0.25M Tris-HCl, pH 8.3, 375 mM KCl, and 15 mM MgCl_2 (supplied by Gibco-BRL, Gaithersburg, MD).
2. 3.75 mM Nucleotides: Mix equal vol of 15 mM dATP, dGTP, dCTP, and dTTP to yield 3.75 mM of each.
3. Primer (*see* Note 1).
4. Dithiothreitol (DTT): 100 mM.
5. Moloney Murine Leukemia Virus (MoMuLV) reverse transcriptase (BRL).

2.3. PCR

1. 5X PCR buffer. 250 mM Tris-HCl, pH 9.0, 75 mM ammonium sulfate, 35 mM MgCl_2 , 0.85 mg/mL BSA (molecular biology grade), and 0.25% (v/v) NP40. This buffer requires a high nucleotide concentration (1.5 mM) to compensate for the high magnesium ion concentration.
2. Nucleotides as in RT reaction.
3. PCR primers.
4. *Taq* polymerase.
5. Mineral oil.
6. Thermal cycling machine.

3. Methods

3.1. RNA Preparation

An extremely versatile method of RNA extraction is the acid guanidinium phenol chloroform (AGPC) method (6), which can be scaled to accommodate any amount of tissue (*see also* Chapter 38).

Minimum handling leads to high RNA yields, although DNA contamination seems to be inevitable with this method. The following adaptation is suitable for a single cell or up to 10 mg of tissue.

1. Add 100 μL solution D to tissue in Eppendorf tube. Vortex and/or flick with needle until tissue disintegrates.
2. Add 10 μL 2M sodium acetate, pH 4, and mix.
3. Add 100 μL unbuffered, water-saturated phenol and mix.
4. Add 20 μL of chloroform:isoamyl alcohol (IAA) 49:1, and then mix very well.
5. Chill on ice for 15 min, and then spin fast for 15 min at 4°C (e.g., maximum microfuge speed).
6. Take the top phase into a new tube. If dealing with $<10^5$ cells or 1 mg tissue, add 1 μL of RNase-free glycogen at 20 mg/mL.
7. Add 240 μL ethanol. Freeze on dry ice or at -70°C .
8. Thaw and spin fast for 15 min at 4°C.
9. Wash the pellet with 80% EtOH, drain, and dry.
10. Resuspend in 5–100 μL DEPC-treated H_2O . Heat to 65°C for 10 min to ensure dissolution and inhibit secondary structure; then snap chill. Store at -70°C .

If DNase treatment is necessary (*see* Note 2), perform steps 1–9 above, and then proceed as follows:

- 10a. Resuspend in 50 μL of DNase buffer containing 2 U RNase-free DNase I and 2 U RNase inhibitor. Incubate for 2 h at 37°C.
11. Extract once with phenol:chloroform:IAA 25:24:1, spin for 5 min, and remove the aqueous phase to a new tube. The phenol used here should be buffered with 1M Tris-HCl, pH 7–8.5.
12. Precipitate with 6 μL of 2M sodium acetate, pH 5.8, and 150 μL ethanol, freeze, and then spin for 15 min in a microfuge.
13. Wash the pellet twice with 80% ethanol, drain, and dry.
14. Resuspend in 5–100 μL DEPC-treated H_2O . Heat to 65°C for 10 min to ensure dissolution and to inhibit secondary structure formation; then snap-chill before reverse transcription.

3.2. Reverse Transcription (*see* Note 1)

3.2.1. Large-Scale RT

1. Set up the following reaction:

DEPC- H_2O	To 30 μL
5X RT buffer	6 μL
Nucleotides, 3.75 mM each	1 μL
Primer, 500 ng/ μL	1 μL

DTT, 100 mM	1 μ L
RNA, 1 μ g	1 μ L
Reverse transcriptase 200 U	1 μ L MoMuLV, BRL

2. Incubate at 42°C for 30 min (ample to reverse transcribe 2.5 kb).

3.2.2. Small-Scale RT

1. Resuspend the RNA in 5 μ L of DEPC-treated H₂O. Transfer to a PCR tube.
2. Add 2.5 μ L of RT mix. (This mix is made exactly as above, omitting water and RNA.)
3. Overlay with 30 μ L of mineral oil.
4. Incubate at 42°C for 30 min. PCR reagents can be added directly to this tube.

3.3. PCR

1. Set up the following reaction:

H ₂ O	To 50 μ L
Nucleotides	20 μ L as in RT reactions
5X PCR buffer	10 μ L
Primers, 500 ng each	x μ L
<i>Taq</i> polymerase	y μ L 0.5–2.5 U; should be titrated for optimal results
RT reaction	z μ L (<i>see</i> Note 3)

x , y , and z are variables to be determined for each individual experiment.

2. Ensure that mixture is covered with mineral oil.
3. Denature for 2 min at 94°C, and then perform 30 cycles as follows.

For plate temperature-driven machines:

95°C	30 s
T° C	1 min; <i>see</i> Note 4
72°C	1 min; <i>see</i> Note 5

For tube thermocouple-driven machines:

94°C	5 s
T° C	30 s
72°C	30 s

4. Cool slowly to room temperature. Analyze 5–10 μ L on a 2% agarose/TBE gel (*see* Note 6).

Refinements of this basic protocol are described in Notes 7–10.

4. Notes

1. Common priming strategies for RT reactions include using gene-specific primers, random hexamers, or oligo-dT 12–18 mers. I have had best results with oligo-dT, and this has the further advantage of allow-

ing the RT product to be assayed for several transcripts, either simultaneously or sequentially.

The RNA yield from 10 mg tissue should be at least 1 μg . This should allow a 30- μL "large-scale" RT reaction; if less tissue is used or if the gene of interest is expressed at very low levels, a 7.5- μL "small-scale" reaction can be used. If PCR is to be performed using primers from within one exon, duplicate reactions should be set up with and without reverse transcriptase.

2. Ideally, primers to be used for PCR will correspond to two different exons, so that genomic DNA will give a larger product than cDNAs from the transcript being assayed (or no product at all where large introns are involved). Where both primers are of necessity within one exon, the RNA preparation must be treated with DNase I, so that PCR bands will indicate mRNA expression and not contaminating DNA.
3. The products of RT reactions can be used directly in PCR reactions with no further purification. Usually one-sixth of a large-scale RT reaction is amplified or the whole of a small-scale RT reaction; the amounts can be adjusted according to requirements.
4. T is an annealing temperature suitable for the primers used. It should be determined empirically, but is usually between $T_m - 10$ and $T_m + 10$ ($^{\circ}\text{C}$), where:

$$T_m = 2 \times \#(\text{A} + \text{T}) + 4 \times \#(\text{G} + \text{C}) \quad (1)$$

5. These extension times at 72°C are suitable for products up to 1 kb. They should be increased 30 s for each additional kilobase.
6. A suitable 5X loading dye for agarose gels that does not obscure PCR products is. 10 mM Tris-HCl, pH 7–8.5, 1 mM EDTA, 20% Ficoll, and Orange G to taste.
7. Improving sensitivity: A number of simple strategies can be used to manipulate the sensitivity of detection. These include:
 - a. The number of cycles can be increased. Some workers have used up to 60 cycles, but depending on conditions and reagents used, it is doubtful that much enzyme activity would survive this punishment. Also, the amount of specific product can plateau beyond a certain number of cycles, favoring the amplification of undesired products.
 - b. Enzyme can be supplemented after a suitable number of cycles (say 20). The amount of work to be done by the polymerase doubles with each cycle, yet enzyme activity decreases with each denaturation step
 - c. The agarose gel can be blotted onto a nylon filter that can then be hybridized to a radioactive probe for the gene of interest. The probe

can include both primer sequences, since the short length of probe/primer homology will not permit probe binding to undesired "ghost" PCR bands at high stringency.

- d. A second round of PCR can be undertaken using a second pair of primers nested within the first pair. For example, after 20 cycles in the first round, 1 μL is transferred into a second reaction containing the nested primers, and 20 further cycles executed.

The paradox of all these strategies is that the greater the sensitivity, the greater the risk of revealing a false positive caused by contamination, thus invalidating the experiment. Great care needs to be taken to avoid this problem (*see* Note 10).

8. Quantitation: Applied to studies of gene expression, quantitative PCR is an attempt to measure the abundance of transcripts in a tissue using the amount of final PCR product as a guide. A number of properties of PCR mean that quantitation is full of pitfalls. For example, because PCR proceeds exponentially, a small difference in amplification efficiency between two otherwise identical samples will yield vastly different amounts of product. Many regard PCR as semiquantitative at best.

One factor required for quantitation is an exact exponential relationship between the number of cycles and the amount of product. It is generally held that this relationship breaks down beyond 15–20 cycles, depending on conditions. Beyond this, the amount of abundant product begins to plateau, whereas less abundant products may continue to be amplified exponentially.

Another problem is tube-to-tube variability. This can only be overcome by multiple independent assays of the same starting tissue. Clearly, this will magnify the work load of the experiment severalfold, but without this extra effort, attempts at quantitation are meaningless.

The most effective way to measure product is to use a radioactive label. Radioactivity can be measured after removal of unincorporated label from the product or after excision of the product from a gel. This should always be related back to a standard curve made using different dilutions of a control RNA sample.

9. Using PCR to distinguish closely related genes. To distinguish between the gene of interest and any closely related genes (for example, a heterologous transgene vs its endogenous counterpart), at least one nucleotide must differ between the two transcripts. Normally, there will be many more differences, for example, in the 5'- or 3'-untranslated regions of the transcripts, that will allow the design of primers that will specifically recognize only the cDNA of interest

Where the number of nucleotide differences is limited, these should be incorporated into the 3' end of the oligonucleotides, to reduce the possibility of false priming. The specificity enhancer Perfect Match (Stratagene, La Jolla, Ca) may be useful in these situations, but this reagent must be carefully titrated so as not to inhibit amplification.

Often, differences between two genes will create or abolish a restriction enzyme recognition site. If this is the case, the primers can be designed to amplify both gene products, which can then be distinguished on an agarose gel after the appropriate restriction digest. If all else fails, sequencing of PCR products can be used to identify which of two closely related genes is expressed.

10. Safeguards and controls: The exquisite sensitivity of PCR is at once its greatest asset and its greatest problem. Unless painstaking care is used, contamination is almost certain to arise, causing false positives and ruining experiments.

The specific DNA molecule being amplified poses the greatest threat. Such vast quantities of these molecules are being produced that it is easy for some of them to find their way into PCR reagents. Other dangers include plasmid subclones of the gene of interest, which are likely to be wafting around the lab and lurking on key pieces of equipment.

Safeguards against contamination are described in a recent *Nature* article (7). The importance of extreme care from the outset cannot be overemphasized. The highest priority is physical separation of the PCR setup area from the normal lab area where PCRs are run and analyzed. Preferably dedicated equipment—in particular, pipets, centrifuges, and freezers—should be used. It is helpful to UV-irradiate pipets between experiments. Reagents should be made up from stock powders and solutions especially reserved for PCR. Buffers and primers should be aliquoted when made up, and aliquots changed frequently. Gloves should be changed often, particularly after touching anything other than your PCR equipment.

Each assay should, of course, include a positive control that may be a known expressing tissue or some purified RNA from such tissue. It is also essential to include a nonexpressing (e.g., nontransgenic) tissue or perform a mock analysis of water, as a negative control.

It is useful to include primers for an irrelevant, but ubiquitously expressed gene, in addition to the primers for the gene of interest, as a control for the quality and quantity of RNA in each sample. I use the following primers for mouse *hypoxanthine phosphoribosyltransferase* (*Hprt*):

Hprt.1a: 5'-CCTGC TGGAT TACAT TAAAG CACTG-3'

Hprt.1b: 5'-GTCAA GGGCA TATCC AACAA CAAAC-3'

These primers have been used at annealing temperatures from 45 to 65°C, and so are compatible with almost any other primer set (8–10). They give a product of 354 bp, and span exons 3–8 of the *Hprt* cDNA (11). No PCR product is obtained from genomic DNA. To ensure that they do not obscure the signal from the gene of interest, the *Hprt* primers can be used down to 25 ng/reaction.

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CHAPTER 42

Nuclear Run-On Analysis of Transcription

David Murphy

1. Introduction

The methods described in Chapters 39, 40, 43, and 44 can only provide information about steady-state levels of transgene RNA. Any differences in specific RNA levels observed in different tissues of a transgenic organism, or any changes in RNA level as a consequence of a physiological or developmental change, cannot, using these techniques, be ascribed to transcriptional controls. Such differences could equally be a consequence of posttranscriptional mechanisms that govern RNA stability. The nuclear run-on assay is, however, a direct, accurate measure of the level of transcription of a particular gene, and can be used to quantitatively measure differences in transgene transcription as a consequence of tissue-specific, developmental or physiological regulation. The method depends on the *in vitro* incorporation of radioactive ribonucleotides into RNA by RNA polymerase II complexes associated with nascent RNA chains within intact, isolated nuclei. The number of RNA polymerase complexes associated with a particular gene is a measure of the rate of transcription of that gene. Thus, the incorporation of radioactive ribonucleotides into a particular RNA is a measure of the rate of transcription. The level of transcription of a particular gene is assayed by isolating the labeled RNA from nuclei following *in vitro* incubation with radioactive precursors and using this RNA to probe specific cloned DNAs fixed to a matrix. The level of hybridization is directly related to the level of transcription of the

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gene of interest, and can be measured by scintillation counting or densitometric scanning following autoradiography. The procedures described here (1,2) have been developed in this laboratory by adapting previously published protocols (3–5).

2. Materials (see Note 1)

1. Lysis buffer: 10 mM HEPES, pH 7.9, 10 mM NaCl, 3 mM MgCl₂, 0.5% NP40, and 50 U/mL human placental RNase inhibitor (RNasin, Amersham, UK).
2. Storage buffer: 50 mM HEPES, pH 7.9, 40% (v/v) glycerol, 5 mM MgCl₂, 0.25 mM EDTA, and 500 U/mL RNasin.
3. 10X Run-on buffer. 1.5M NaCl, 25 mM MgCl₂, 50 mM Mg acetate, 10 mM MnCl₂, 20 mM DTT, 1.25 mM EDTA, 5 mM ATP (Pharmacia, Uppsala, Sweden), 5 mM GTP (Pharmacia), 5 mM CTP (Pharmacia), 20 mM creatine phosphate, 30 U/mL creatine phosphokinase (Boehringer Mannheim, Mannheim, Germany), and 5 mg/mL heparin.
4. Nucleoside 5' diphosphatekinase (Boehringer Mannheim).
5. α -³²P-labeled UTP (3000 Ci/mmol; Amersham) (see Note 2).
6. DEPC (diethylpyrocarbonate)-treated water (see Note 1). This is prepared by adding 0.1% (v/v) DEPC to distilled, deionized, or reverse-osmosis purified water. Following incubation at room temperature for 3 h, the mixture is autoclaved.
7. Vanadylribonucleoside complex (VRC; Gibco-BRL, Gaithersburg, MD)
8. RNase-free pancreatic DNase I
9. 10X Proteinase K (PK) buffer: 10 mM Tris-HCl, pH 7.4, 50 mM EDTA, 10% (w/v) SDS, and 1.6 mg/mL PK (Sigma, St. Louis, MO, or BRL).
10. Tris-buffered phenol/chloroform/isoamyl alcohol (24:24:1): To prepare Tris-buffered phenol, 100 g of molecular-biology-grade phenol (BRL) is melted at 65°C and equilibrated overnight with 100 mL Tris-HCl, pH 7.4, 24 mL 0.5M EDTA, 7.5 mL 4M NaOH, and 243.5 mL DEPC-treated water. Following the removal of most of the upper aqueous phase, 8-hydroxyquinoline (100 mg) and β -mercaptoethanol (200 μ L) are then added, and the phenol is stored frozen.
11. DEPC-treated 0.3M Na acetate, pH 5.5 (see Note 1): DEPC is added to the solution to 0.1% (v/v). Following incubation at room temperature for 3 h, the solution is autoclaved.
12. DEPC-treated 0.2M NaOH (see Note 1) DEPC is added to the solution to 0.1% (v/v). Following incubation at room temperature for 3 h, the solution is autoclaved.
13. 2.4M HEPES.

14. Sephadex G-50 slurry: This is prepared by swelling G-50 powder in water followed by autoclaving.
15. Disposable 1-mL syringes.
16. Autoclaved glass wool.
17. Wash buffer A: 50 mM Na phosphate buffer, pH 6.8, and 0.1% (w/v) SDS.
18. Wash buffer B: 50 mM Na phosphate buffer, pH 6.8, and 1% (w/v) SDS.
19. 2X SSPE: 360 mM NaCl, 20 mM Na phosphate buffer, pH 6.8, and 1 mM EDTA.
20. DNase-free RNase A (10 mg/mL): This is made by resuspending the RNase A dry powder in water. Following incubation in a boiling water bath for 10 min and quenching on ice, the solution is aliquoted and frozen at -20°C until required.
21. 0.3M NaOH.
22. 5M NH_4 acetate.
23. Hybond-N (Amersham) nylon hybridization membrane (*see* Note 3).
24. Slot-blot apparatus linked to a vacuum line.
25. Ultraviolet light (312 nm) transilluminator.
26. Hybridization buffer: 500 mM Na phosphate buffer, pH 6.8, 7% (w/v) SDS, 1 mM EDTA, and 15% (v/v) formamide (Fluka, Buchs, Switzerland) (*see* Note 4).

3. Methods

3.1. Preparation of Nuclei

1. Collect finely chopped tissue in 5–20 mL ice-cold lysis buffer. For every 100 mg of tissue, use at least 5 mL of lysis buffer.
2. Using a Dounce homogenizer, subject the tissue to 15 strokes. Incubate on ice for 3 min, and then apply 10 further strokes. Allow large particulate material to settle before transferring the supernatant to a sterile plastic disposable centrifuge tube.
3. Spin the homogenate at 500g for 10 min. The supernatant, which contains the cytoplasmic RNA, can be stored frozen if required.
4. Resuspend the pellet in 100 μL of storage buffer, and snap freeze in liquid nitrogen. Store the nuclei at -80°C until required.

3.2. Nuclear Run-On Reaction

1. To 100 μL of nuclei suspension, add 20 μL 10X run-on buffer, nucleoside 5' diphosphatekinase to 12 U/mL, RNasin to 500 $\mu\text{g}/\text{mL}$, ^{32}P UTP (100–500 μCi), and DEPC water to final vol of 200 μL .
2. Incubate at room temperature for 60 min.
3. Add VRC to 2 mM (2 μL of 200 mM stock) and 10 μL of 60,000 U/ μL

- pancreatic DNase I (RNase-free) and incubate at room temperature for 30 min.
4. Add 1/10 vol (25 μL) of 10X PK buffer. Incubate at 37°C for 1 h.
 - 5 Extract once with 250 μL of Tris-buffered phenol/chloroform/isoamyl alcohol.
 6. Remove the aqueous phase to a fresh tube, and reextract the phenol phase with 100 μL 0.3M Na acetate.
 - 7 Precipitate the pooled aqueous phases with 2 vol of 100% (v/v) ethanol. Incubate at -80°C for 1 h, and centrifuge for 5 min in an Eppendorf microcentrifuge to recover the RNA.
 8. Resuspend the pellet in 45 μL of ice-cold 0.2M NaOH. Incubate on ice 10 min, and then neutralize with 5 μL of 2.4M HEPES.
 9. Prepare a spun Sephadex G-50 column. Block the end of a 1-mL disposable syringe barrel with autoclaved glass wool, and fill with Sephadex G-50 slurry until all the excess liquid drains out and the beads are packed. Position the column in a disposable 15-mL plastic conical centrifuge tube, and centrifuge at 1000g for 3 min. Equilibrate the column with DEPC water by filling the column with DEPC water and then draining it by centrifugation as before. Repeat this five times.
 10. Load the labeled RNA onto the column, and centrifuge at 1000g for 5 min. Collect the purified RNA in a fresh 15-mL centrifuge tube.
 11. Determine the incorporation of labeled nucleotide into RNA by scintillation counting of 1 μL of purified product. Spot 1 μL onto the center of each of two 2.4-cm Whatman DE81 disks. Wash *one* of the disks six times, 5 min each wash, in 0.5M Na_2HPO_4 Wash the same disk in water twice, 1 min/wash, and then twice in 95% (v/v) ethanol, 1 min/wash. Dry both filters, and then place in an aqueous scintillation fluid and count in a liquid scintillation counter. The unwashed filter measures the total radioactivity in the sample, whereas the washed filter measures the radioactivity incorporated into RNA.
 12. Hybridize to cloned DNAs fixed to a nylon matrix (prepared as described in Section 3.3.) using the procedures described in Section 3.4.

3.3. Preparation of Slot-Blot Filter

1. Add 200 μL 0.3M NaOH to 5 μg of plasmids containing genomic or cDNA clones of interest (*see* Note 5).
2. Boil for 10 min, and quench on ice.
3. Add 400 μL 5M NH_4 acetate. Apply to a nylon hybridization membrane (e.g., Amersham Hybond-N), previously equilibrated with 2X SSPE, using a slot-blot applicator.
4. Rinse the filter in 2X SSPE. Air-dry and then bake at 80°C for 60 min.

5. Covalently crosslink the DNA to the matrix by exposure, DNA side down on Saran™ wrap, to a UV light (312 nm) transilluminator for 2 min (*see* Note 6).
6. Rinse the filter in wash buffer A.
7. Prehybridize in hybridization buffer at 65°C for at least 5 min.

3.4. Hybridization and Washing (*see* Note 7)

1. Identical filters are hybridized with equal counts corresponding to equal amounts of RNA synthesized in nuclei isolated from different tissues from the same animal or from equivalent animals, or from the same tissue taken from different animals at different developmental stages or subjected to different physiological conditions.
2. Filters are incubated for at least 60 h at 65°C with labeled RNA in as small a vol of hybridization buffer as possible (1–2 mL depending on the size of the filter).
3. Following hybridization, the filters are washed twice in wash buffer A for 10 min at room temperature and then twice in wash buffer A for 10 min at 65°C.
4. The filters are rinsed five times in 2X SSPE at room temperature, and then incubated for 60 min at room temperature in 2X SSPE containing 10 µg/mL RNase A.
5. Following two final 15-min washes at 65° in wash buffer B, the filters are subjected to autoradiography.

4. Notes

1. Diethylpyrocarbonate (DEPC) is a strong inactivator of all RNases. All solutions (*except those that contain Tris*) should be treated with DEPC (0.1% [v/v]) for 3 h, followed by autoclaving. Solutions should then be filtered through 0.22-µm sterile filtration units (Millipore, Bedford, MA).
2. The nuclear run-on techniques depend on the manipulation of large amounts of ³²P-labeled material. Due care should be exercised throughout.
3. Note that these methodologies have been developed for neutral nylon membranes (e.g., Amersham Hybond-N) and have not been tested on positively charged membranes (e.g., Amersham Hybond-N+, Bio-Rad [Richmond, CA] Zeta-Probe, NEN-Du Pont [Boston, MA] Genescreen Plus).
4. Formamide should be deionized before use. To do this, formamide is mixed and stirred for 1 h with Dowex XG8 mixed bed resin, followed by filtration through 3MM paper. Fluka formamide (catalog number 47670) does not require deionization.
5. The cloned DNAs applied to the slot-blot filter can either be cDNAs or genomic clones. Given that intron sequences are represented in the

- labeled pre-mRNAs generated in this assay, the use of genomic clones can often be more sensitive (4), although care must be taken to ensure that no repetitive sequences are present in the target DNA.
6. The time needed to UV crosslink nucleic acids covalently to a nylon matrix must be determined empirically. The energy output of UV transilluminators varies considerably. Too much exposure will damage the nucleic acids and reduce the autoradiographic signal following hybridization. Too little exposure will not link the nucleic acids to the filter. During hybridization and washing, the target nucleic acids will be lost, again resulting in a reduced autoradiographic signal. To determine the optimal exposure time, identical filters bearing a known nucleic acid are exposed to the UV transilluminator for differing lengths of time. Following hybridization to the same probe, the optimum time, corresponding to the strongest autoradiographic signal, can be determined. Note that the energy output of a transilluminator changes over a period of months, and thus, regular recalibration is required. Stratagene produces a system that emits a fixed, measured amount of UV energy.
 7. Hybridization should be performed in a tightly sealed vessel using as little probe as possible. Usually, this has meant the use of strong plastic bags, sealed with a bag sealer and incubated in a water-filled plastic box in a shaking water bath. Such containment systems are prone to leaks and contamination, and can be extremely messy, both when applying and removing the hybridization solution.
 8. In choosing cloned DNAs to hybridize with the labeled RNA, it should be noted that RNA polymerase III is also active in *in vitro* run-on reactions. Thus, clones should be used that do not contain transcribed middle repetitive sequences (e.g., the human Alu family or the rodent B1 and B2 series). Such repeats are very common in genomic clones and are often to be found in the 3'-untranslated regions of mRNAs.
 9. All assays should incorporate negative and positive controls. Negative controls should correspond to the vector sequences contained within the plasmids that carry the cloned genes assayed. Hybridization to vector sequences should be negligible. A positive control could be a ubiquitously expressed gene that is not subject to tissue-specific, developmental, or physiological regulation. Such a gene is difficult to find! The choice of such a control depends on the nature of the experiment. For example, in experiments comparing levels of expression in the rat brain following different physiological stimuli, we have found the genes encoding either the intermediate filament Gh14 Fibrillary Acidic Protein (6) or the cell-surface marker Thy-1 (7) extremely useful (2).

10. By isolating and analyzing RNA from the cytoplasmic fraction derived in Section 3.1, it is possible to compare directly transcription and steady-state RNA levels. RNA is prepared from the cytoplasmic fraction by the addition of 1/10 vol of 10X PK buffer. Following incubation at 37°C for 60 min, the nucleic acids are isolated by two rounds of extraction with Tris-buffered phenol/chloroform/isoamyl alcohol, followed by precipitation by 2 vol of ethanol. After incubation at -80°C for at least 1 h and centrifugation, the nucleic acid pellet is washed once with 70% ethanol, dried, and resuspended in DEPC-treated water.

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CHAPTER 43

S1 Nuclease Protection Mapping

Duncan R. Smith

1. Introduction

The use of S1 nuclease to map the start site of a transcription unit is a well-established technique. Based on the method of Berk and Sharp (1), it has undergone many refinements over the years. S1 nuclease mapping requires a relatively detailed knowledge of the gene structure and sequence data (or a *very* good restriction map) of the first exon and several hundred bases of upstream sequence. Although S1 nuclease mapping is mainly used to map transcription start sites accurately (to be described in this chapter), this method can also be used to map intron–exon junctions (2). Note that in order to identify a transcriptional start site unambiguously, S1 nuclease mapping should be used in conjunction with primer extension (Chapter 44).

S1 nuclease protection and primer extension can be used in transgenic analysis to ask if the transcriptional activity of a transgene is directed from the appropriate start site. Such an analysis demands that the transgene transcript can be distinguished from the RNA of the corresponding endogenous gene. A transgene may have hybridizable segments with little or no homology to the host genomic DNA—for example, a hybrid gene with a viral or prokaryotic reporter element, or a gene from another species with sufficient sequence divergence to allow the transgene to be distinguished from the host gene.

The basic S1 nuclease mapping technique consists of hybridizing a labeled DNA probe that partially overlaps a RNA species of interest to that RNA species and using the single-strand-specific S1 nuclease

to digest away the nonhybridizing regions. When using S1 nuclease to map a transcription initiation site, the DNA probe normally consists of a genomic DNA fragment that starts within the first exon and extends upstream past the initiation site. The DNA will hybridize to the RNA up to the first base transcribed, and the upstream sequences will remain unpaired. S1 nuclease will digest away the unpaired DNA and RNA, giving a protected fragment that corresponds in size to the length of the RNA from the transcriptional start site to the restriction site within the first exon. The product can then be analyzed on a denaturing acrylamide gel (or agarose gel, depending on the size of the protected fragment), usually in conjunction with a sequencing ladder.

S1 nuclease mapping is a sensitive technique, and as such, variations in experimental conditions can have a great effect on the analyzed product. It is usually necessary to perform experiments designed to optimize the conditions used for hybridization. Once the optimal conditions have been defined, the S1 nuclease mapping is highly reproducible and can be used in quantitative analysis (*see* Note 1). One of the main determinants of the conditions to be used is the probe itself. Probes can be of two types, single-stranded and double-stranded. Both types of probes have a number of advantages and disadvantages, and the experimenter must decide which is better for each experiment. Both types of probes will be considered here.

1.1. S1 Nuclease Mapping with Double-Stranded Probes

Double-stranded probes have the advantage of being relatively simple to produce, but the disadvantage is that the reaction conditions for the hybridization must be such that they drive the formation of DNA/RNA duplexes, rather than DNA/DNA duplexes. Fortunately, in the presence of high concentrations of formamide, RNA/DNA hybrids are more stable than DNA/DNA hybrids (3). Hence, by hybridizing in the presence of 80% formamide and 0.4M NaCl, it is possible to choose a temperature that is above the T_m of the DNA/DNA duplex, but below that of the DNA/RNA duplex. By using such conditions of hybridization, DNA/RNA hybrids are formed in the absence of a DNA/DNA duplex (*see* Note 2).

It is preferable to use probes end labeled at only one point on the strand complementary to the RNA to be analyzed. The easiest way to

achieve this is by the selection of a probe generated by cleavage with two different restriction endonucleases, one enzyme site located within the putative first exon and the second site within the putative 5'-flanking region of the gene. The label will be attached to the restriction site within the first exon. This is normally done by digesting the DNA from which the probe will be produced with the restriction enzyme that cleaves within the first exon. The DNA is then dephosphorylated with calf intestinal alkaline phosphatase, and the 5' ends are labeled with polynucleotide kinase. The kinase is inactivated. The labeled DNA is purified and then cleaved with a second restriction endonuclease that cuts upstream of the region in which transcription initiation is thought to occur. The resulting DNA fragment, which can be gel purified (*see* Chapter 11), is labeled at a single point within the putative first exon.

1.2. S1 Nuclease Mapping with Single-Stranded DNA Probes

With single-stranded DNA probes, the hybridization conditions are less difficult to define, and hybridization can be performed either in aqueous solution or in formamide buffer. The main difficulty lies in producing the single-stranded probe. Several methods exist. One of the most commonly used is to end label a double-stranded probe (which will label both strands) and then separate the two strands on a nondenaturing acrylamide gel (4). Alternative methods involve the *de novo* production of a single-stranded probe by the use of an M13 clone. Obviously, this requires the possession of a suitable genomic fragment spanning the putative transcription initiation site, which is cloned in M13 in the appropriate orientation. A primer is then hybridized to the M13 template and Klenow fragment polymerase used in the presence of labeled dNTP to produce a labeled, synthesized strand that is complementary to and partially overlaps the RNA of interest (5). A restriction cut is made upstream, past the site at which the gene is thought to initiate transcription in order to facilitate the isolation of a probe of defined length. The advantages of this method are two-fold. First, the two strands of DNA (M13 template strand and probe strand) are of very different sizes, so the labeled, synthesized fragment of defined length can easily be separated on an alkaline agarose denaturing gel and purified. Second, the probe can be uniformly labeled

to extremely high specific activities by the inclusion of a radioactively labeled nucleotide in the extension buffer. High specific activity probes are useful for mapping rare transcripts. However, such probes must be used within 24 h for best results, since a considerable amount of radiation-induced damage (cleavage) occurs. Apart from the overnight hybridization temperature and the hybridization buffer, single-stranded probes are treated in an identical manner to double-stranded probes (*see* Note 3).

2. Materials (*see* Note 4)

2.1. Dephosphorylation

1. DNA: either a purified DNA fragment in water, 1X TE, or a restriction digest mixture after incubation with the appropriate restriction enzyme (*see* Chapter 50).
2. 2X CIP buffer: 2 mM ZnCl₂, 2 mM MgCl₂, and 20 mM Tris-HCl, pH 8.3
3. Calf intestinal alkaline phosphatase (or bacterial alkaline phosphatase). CIP and BAP are often supplied as ammonium suspensions. The ammonium ions must be removed by dialysis with 1X TE before use.
4. Phenol saturated in 1X TE.
5. 5M NaCl.
6. 1X TE: 10 mM Tris-HCl, pH 8.0, and 1 mM EDTA.
7. Ice-cold absolute ethanol (-20°C).

2.2. Kinase Reaction

(5' Overhang or Oligonucleotide)

1. 10X Polynucleotide kinase buffer: 500 mM Tris-HCl, pH 7.6, 100 mM MgCl₂, 50 mM DTT, and 100 mM spermidine (pH 7.6).
2. γ -[³²P]-ATP at 3000 Ci/mM, 10 μ Ci/ μ L.
3. 1 pmol Dephosphorylated ends (either DNA fragment or oligonucleotide)
4. 1 U Polynucleotide kinase.
5. Sterile distilled water.
6. Incubating oven or water bath.

2.3. Kinase Reaction (3' Overhang or Blunt End)

1. 10X Buffer A: 100 mM Tris-HCl, pH 9.5, 10 mM spermidine, and 1 mM EDTA.
2. 10X Buffer B: 500 mM Tris-HCl, pH 9.5, 100 mM MgCl₂, and 50 mM DTT.
3. 2–5 U Polynucleotide kinase.

4. γ -[³²P]-ATP 3000 Ci/mM, 10 μ Ci/ μ L.
5. 1 pmol dephosphorylated ends in water or 1X TE.
6. Sterile distilled water.
7. Boiling water bath
8. Incubating oven or water bath at 37°C.

2.4. S1 Nuclease Mapping

1. 10X hybridization buffer: 4M NaCl, 400 mM PIPES, pH 6.4, and 10 mM EDTA.
2. 10X S1 nuclease buffer: 500 mM NaAc, 45 mM ZnSO₄, and 2.8M NaCl, pH 4.5.
3. DNA probe in water: either single-stranded or double-stranded and labeled at either a single point or uniformly labeled (*see* Notes 1, 5, and 6).
4. Deionized formamide.
5. RNA to be analyzed, usually between 5 and 500 μ g of total RNA (*see* Notes 6 and 7).
6. Absolute ethanol at -20°C
7. S1 nuclease (*see* Note 8).
8. Phenol/chloroform/isoamyl alcohol (25:25:1). Phenol saturated with 100 mM Tris-HCl (pH 8.0) is added to an equal vol of chloroform and 1/25 vol isoamyl alcohol.
9. 8–12% Denaturing polyacrylamide sequencing gel, if expected product is in the range 20–800 bp; otherwise, a denaturing agarose gel.
10. tRNA at 20 mg/mL in water as negative control (molecular biology grade; *see* Note 6)
11. Formamide dye mix: 96% deionized formamide, 10 mM EDTA, pH 8.0, 0.125% (w/v) xylene cyanol, and 0.125% (w/v) bromophenol blue.
12. 80% (v/v) Ethanol at -20°C.
13. Sterile DEPC-treated water (*see* Note 4).

3. Methods

3.1. Removal of Terminal Phosphate Groups

1. If starting with a restriction digest, heat-inactivate the restriction endonuclease (10 min, 70°C), and then dilute 1:1 with 2X CIP buffer. If starting with purified DNA in water or 1X TE, then also dilute 1:1 with 2X CIP buffer.
2. Add 1–2 U of CIP.
3. Incubate at 37°C 30 min to 1 h.
4. After incubation, phosphatases cannot be heat-inactivated, so add 80 μ L of 1X TE, and then 100 μ L of saturated phenol. Vortex well (1 min).

Following centrifugation, remove the upper, aqueous layer, and repeat the extraction twice more.

5. Either directly apply the aqueous layer to a preparative gel (*see* Chapters 51 and 11 and Note 9), or add 6.5 μL 5M NaCl and 250 μL ice-cold absolute ethanol, incubate at -20°C for 30 min to 1 h, and recover DNA by centrifugation.

3.2. 5'-End Labeling **(5' Overhang or Oligonucleotide) (see Note 10)**

1. Mix together in a tube 2 μL 10X polynucleotide kinase buffer, 1 pmol of dephosphorylated DNA ends (Note 11), 25–30 μCi γ -[^{32}P]-ATP, 1 U polynucleotide kinase, and sterile distilled water to give 20 μL final vol.
2. Incubate at 37°C for 30 min.
3. Proceed to the hybridization step (Section 3.4.; *see* Note 12).

3.3. 5'-End Labeling **(3' Overhang or Blunt End) (see Note 10)**

1. Prepare dephosphorylated DNA solution (at 0.1 pmol/ μL ; *see* Note 11) in 10 mM Tris-HCl, pH 9.5, 1 mM spermidine, and 0.1 mM EDTA (1X buffer A).
2. Denature the DNA by placing tube in a boiling water bath for 3 min.
3. Briefly spin tube in an Eppendorf centrifuge (full speed, 2 s), then quickly chill on ice, and rapidly add 2 μL of buffer B.
4. Add 2–5 U of polynucleotide kinase.
5. Add 3 μL of γ -[^{32}P]-ATP, and sterile distilled water to a 20- μL final vol.
6. Incubate at 37°C for 30 min.
7. Proceed to the hybridization step (Section 3.4.; *see* Note 12).

3.4. Hybridization

1. The labeled probe is dissolved in water to give approx 20,000 cpm/ μL . Ideally, this should be between 0.05 and 0.005 pmol of probe.
2. Between 5 and 500 μg of RNA is taken from storage, dried down (*see* Notes 7 and 13), and resuspended in 24 μL deionized formamide. Include controls (*see* Note 6).
3. In a sterile Eppendorf, mix together the 24 μL of RNA, 3 μL of probe in water, and 3 μL of 10X hybridization buffer (*see* Note 3).
4. The tube is capped firmly, and the tube placed in a boiling water bath for 2 min.
5. After denaturation, the samples are incubated at the chosen incubation temperature (*see* Notes 3 and 14) overnight. It is important not to let the temperature of the tubes fall below the overnight incubation temperature (*see* Note 15).

3.5. S1 Nuclease Mapping

1. After incubation, 270 μ L 1X S1 nuclease buffer, containing 200 U (*see* Note 8) S1 nuclease, are rapidly added to each tube containing the hybridization reactions. The samples are briefly vortexed and placed on ice (*see* Note 16). After a short centrifugation to bring the liquid to the bottom of the tube, the samples are incubated at $\sim 37^{\circ}\text{C}$ for 1 h.
2. The S1 nuclease reactions are stopped by placing on ice. Each sample is extracted once with phenol/chloroform, ethanol precipitated, washed once with 80% (v/v) ethanol (ice cold), dried and dissolved in 4 μ L of formamide loading dye, boiled for 2 min, and loaded onto a denaturing polyacrylamide sequencing gel of appropriate concentration (*see* Note 17).

4. Notes

1. When S1 nuclease mapping is intended for quantitative analysis, it is important to ensure that the probe is present in excess over the mRNA species of interest. Hence the amount of both DNA and RNA in the mapping must be known.
2. Invariably, when double-stranded probes are used in S1 nuclease mapping reactions, a small amount of the probe reanneals to itself. This reannealed probe will be protected from S1 nuclease digestion and will appear on the gel band corresponding in size to the full-length probe.
3. With single-stranded probes, hybridization can take place either in aqueous solution or in the presence of formamide buffer. Hybridization temperatures are 30°C below the duplex T_m ; for example, if a long (>50 bp) single-stranded probe is used, 65°C incubation would be appropriate. In practice, RNA/DNA hybridizations are more commonly carried out at lower temperatures, e.g., 40°C for a long single-stranded probe, in the presence of 50% formamide.
4. It is important that all reagents, solutions, and tubes are RNase free. This can be achieved by treatment with Diethyl pyrocarbonate (DEPC). Plastic and glassware are soaked in a 0.1% (v/v) solution of DEPC for 2 h, followed by autoclaving. Solutions should be made up with sterile distilled water in DEPC-treated plastic and glassware, and can then have DEPC added to 0.1% (v/v) followed by autoclaving. Solutions containing Tris-buffers cannot be treated in this manner, so normally such solutions are made up without Tris, treated with DEPC, and autoclaved. Then sterile (autoclaved) Tris-buffer is added just prior to use.

Disposable plastic gloves should be worn at all times when handling glass and plasticware and while making solutions. Many laboratories keep separate chemical stocks and electrophoresis equipment only for RNA work, and solutions should not be used for both RNA and

DNA work. This is especially important when the large amount of RNase A used in most DNA preparation techniques is considered.

5. This technique has to be optimized for each probe. Hybridization conditions vary with each probe. However, once a set of optimal conditions have been defined, then results are exceptionally reproducible.
6. Some probes can give rise to artifactual bands. The reasons for this are unclear. However, the presence of probe-derived artifacts makes it important to include adequate controls when undertaking S1 nuclease mapping. Three negative controls and one positive control are commonly employed. Negative controls include a reaction tube that contains all of the reagents except the RNA undergoing mapping. In its place is either distilled water, an equal amount of tRNA (such as yeast tRNA), or an equal amount of RNA from a tissue that is known (from Northern blot analysis) not to have the message of interest. This reaction tube is then treated in an identical manner to other mapping reactions. Any bands appearing in the negative control must therefore be probe-derived artifacts. Often (but by no means always) these artifact bands can be avoided by changing the conditions (such as the overnight hybridization conditions or the temperature of the S1 mapping reaction) or in some circumstances, using a slightly different probe.

One positive control that is commonly employed is to produce an *in vitro* transcribed RNA from a cDNA clone. This *in vitro* RNA is then mapped as for *in vivo* RNA samples. An *in vitro* transcribed RNA will produce a single mapped band on a gel, of a known size. This control provides confirmation that the mapping conditions are at least approximately right. Many vectors on the market today allow the production of *in vitro* transcripts, and they are normally supplied with excellent protocols for producing *in vitro* RNA transcripts.

7. The amount of RNA to be mapped in each reaction depends on the abundance of the message. Usually this information is available from prior Northern blot analysis (*see* Chapter 39). For relatively abundant messages (the signal after Northern analysis is visible after 2–20 h), between 10 and 20 μg of total RNA give a good signal. For rarer messages (a few days to weeks for a signal on a Northern blot), RNA should be increased 20- to 50-fold (up to about 500 μg of total RNA). For very rare messages, it is often better to use poly (A+) mRNA (5 μg of poly [A+] mRNA are roughly equivalent to 500 μg of total RNA).
8. S1 nuclease from different manufacturers comes in vastly different concentrations. The amount of S1 nuclease to add to a reaction must be derived somewhat empirically. There are also batch variations with lots of enzyme, and practically speaking, it can be noted that when chang-

ing from one batch of the enzyme to another (from the same manufacturer), the reaction conditions (either amount of enzyme or time of digestion) must be slightly modified. A reasonable starting digestion would consist of approx 200 U of S1 nuclease and a reaction of about 1 h at 37°C. In this laboratory, good results have been obtained with enzymes purchased from Amersham (Amersham, UK) or Pharmacia (Uppsala, Sweden), although other manufacturers may retail enzymes of similar quality.

9. Even if the DNA is to be purified by preparative gel electrophoresis, it is advisable to phenol-extract the DNA first. Reasons for this are twofold. First, at this stage, there is a large amount of protein present (in most cases, inactivated restriction endonuclease as well as the kinase), which can cause poor preparative runs. Second, phosphatases are very resistant to both denaturation and removal from the DNA. Problems in subsequent labelings could be encountered if the phosphatase is not completely removed.
10. These methods will label a double-stranded DNA molecule on both strands (i.e., at both 5' termini) and an oligonucleotide at one point.
11. Polynucleotide kinase is sensitive to certain contaminants, which can severely reduce the efficiency of labeling. The most commonly encountered of these are found after agarose gel electrophoresis. Certain contaminants are copurified with the DNA from the gel slice. These contaminants are batch variable. Some batches present no problems to subsequent labeling of DNA; others can completely eliminate labeling activity. If low labeling activity is encountered, the DNA can be cleaned up by G-50 spin column chromatography (as described in Chapter 11). Rephenol extraction or repeated precipitation of the DNA will *not* clean up the DNA.
12. End-labeled DNA molecules should be used as soon as possible after production. Although the sample may still seem hot after storage, degradation of the DNA will occur by radiation-induced cleavage (damage) of the DNA. The longer the storage of the sample, the more damage that will occur, irrespective of how the sample is stored. If possible, use the probe within 2 d of synthesis.
13. Do not overdry the RNA, since this can lead to problems with resuspension. If problems are encountered resuspending the RNA, then warming to 65°C for a few minutes followed by vortexing can help. This cycle of warming and vortexing can be continued until the RNA is fully redissolved.
14. Optimally, the incubation temperature is below the RNA/DNA T_m and above the DNA/DNA T_m . This should be calculated for the RNA/probe pair. In most cases, it is better to experiment with a range of temperatures, spaced at 2–3°C intervals around the optimal temperature.
15. It is important not to allow the temperature to drop when taking the

hybridization mixture from the denaturing water bath to the overnight incubation oven or water bath. If the temperature does drop below the DNA/DNA duplex T_m , the probe will rapidly reanneal. In this case, there will be no probe available for DNA/RNA duplex formation and the mapping will fail. Practically, it is often convenient to perform the denaturing step in a boiling water bath (such as a beaker filled with water on a hot plate) and then remove the whole beaker to an air incubator set at the overnight hybridization temperature.

16. It is important to undertake this step as rapidly as possible to prevent the reformation of a DNA/DNA duplex. Once the hybridization has been diluted with ice-cold S1 nuclease buffer (containing S1 nuclease), the DNA/RNA duplexes are "locked" into place. The samples are placed on ice to prevent the S1 nuclease from working, so that all the samples can be placed into a water bath at the same time, and so have the same amount of digestion.
17. Markers for polyacrylamide gels can be simply produced from commercially available DNA markers. Approximately 10 μg of DNA gel marker (such as Phi X 174 /*Hae* III, or the 123-bp marker from Gibco-BRL, Gaithersburg, MD) are dephosphorylated as in Section 3.1., phenol-extracted, and precipitated with Na acetate and ethanol. This DNA is then resuspended in 20–50 μL 1X TE and stored at -20°C until required. An aliquot of approx 1 μL is then labeled by kinase treatment (*see* Section 3.2.) in approx 10 μL . After kinsing between 200 and 400 μL of formamide, loading dye is added directly to the reaction. Normally between 1 and 4 μL are needed to produce a good signal after overnight exposure at -70°C with an intensifying screen. The labeled marker can be stored at -20°C , and is usable for between 4 and 6 wk. The marker must be denatured by boiling for 2–3 min prior to loading.

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CHAPTER 44

Primer Extension

Duncan R. Smith

1. Introduction

Primer extension can be viewed as a technique complementary to S1 nuclease mapping (*see* Chapter 43). In S1 nuclease mapping analysis, a DNA:RNA hybrid is resected back to the point of divergence between the RNA and the DNA (1). In primer extension, a DNA probe is annealed to an RNA template and then extended in a 3'–5' direction to the start of the RNA molecule (2). The most common usages for this technique are in determining the size of full-length RNA transcripts and mapping transcription initiation sites.

In most cases, a short oligonucleotide of length 25–35 nucleotides is made complementary to the RNA species of interest, such that the primer oligonucleotide will lie some 30–150 nucleotides downstream from the suspected end of the transcript. The oligonucleotide is normally 5'-end labeled (*see* Chapter 43) and then hybridized in aqueous solution to the RNA transcript. Reverse transcriptase is then used in the presence of deoxynucleotides to extend the primer to the 5' terminus of the RNA molecule. The extended product is then denatured from the RNA template and analyzed on a denaturing polyacrylamide sequencing gel. The length of the expected product can be analyzed by comparing it to standard radioactively labeled markers run on the same gel. Alternatively, if a definitive assignment of the start site of transcription is required, then the product of the primer extension should be run alongside a sequencing ladder.

Several important variations on the basic method exist. First, for rare messages, it is often difficult to detect a signal with an end-labeled

oligonucleotide. For this reason, some workers include a radioactive nucleotide in the reaction mix. If, for example, ^{32}P -dCTP is included in the reaction mix in the place of cold dCTP, then radioactive dCTP will be incorporated along the length of the product. This method increases by several orders of magnitude the detectable signal. However, this method also increases the background noise. Care must be taken with this approach to ensure that the final *molarity* of nucleotide in the reaction mix is not so low that it provides insufficient nucleotide for the enzyme to function. Second, many RNA molecules have very strong secondary structure formations at their 5' ends. In these cases, it can often prove impossible for the reverse transcriptase to elongate through the hairpin or loop structure. It is often necessary, therefore, to use a DNA restriction fragment as a primer, with the fragment being some 50–200 nucleotides long. In this case, slightly different reaction conditions must be used. The hybridization conditions are those used for S1 nuclease mapping (*see* Chapter 43), and for the same reason, a double-stranded DNA fragment has to be denatured and a DNA:RNA hybrid produced. This hybrid can be achieved in the presence of high formamide (3) and at temperatures above the T_m of the DNA:DNA hybrid, but below that of the DNA:RNA hybrid. If this approach is used, it is important to remember that the final product detected will be the sum of the DNA probe and the extended sequences. Although the expected extension might be only about 100 bp, if an 800 bp restriction fragment is used as a primer, then the product will be about 900 bp. For this reason, the practical upper limit on a DNA restriction-fragment primer is around about 200 bp (especially where assignments of size to a single base pair must be made). In this method, again, the DNA primer is either end labeled, or a radioactive nucleotide can be included in the reaction mix.

Note that when analyzing transgene transcripts, some means must be devised to distinguish the foreign RNA from the product of the endogenous gene. A transgene may have hybridizable segments with little or no homology to the host genomic DNA, for example, a hybrid gene with a viral or prokaryotic reporter element or a gene from another species with sufficient sequence divergence to allow the transgene to be distinguished from the host gene. Also note that the unambiguous description of a start of transcription should employ both primer extension and S1 mapping (Chapter 43).

2. Materials (see Note 1)

1. A short oligonucleotide primer (25–35 nucleotides long), 5'-end labeled with ^{32}P (see Chapter 43) and complementary to the RNA species of interest.
2. RNA (5–50 μg total RNA) in 5 μL water (for oligonucleotide-primed extension) or in 24 μL deionized formamide (for restriction-fragment-primed extension).
3. Hybridization buffer:
 - A (for oligonucleotide primed extension): 2M NaCl and 50 mM PIPES, pH 6.4.
 - B (for restriction-fragment-primed extension): 4M NaCl, 400 mM PIPES, and 10 mM EDTA, pH 6.4.
4. Reverse transcriptase (see Note 2).
5. 0.02M DTT.
6. 1M Tris-HCl, pH 8.0.
7. Sterile distilled water (RNase free; see Note 1).
8. 0.1M MgCl_2 .
9. 1 mg/mL Actinomycin D in water (store in the dark at -20°C , since actinomycin is light sensitive). (See Note 3).
10. dNTP mix. 10 mM dCTP, 10 mM dATP, 10 mM dGTP, and 10 mM dTTP (see Note 4).
11. Phenol/chloroform/isoamyl alcohol (25:25:1): Phenol saturated with 100 mM Tris-HCl (pH 8.0) is added to an equal vol of chloroform and 1/25 vol isoamyl alcohol.
12. 1X TE: 10 mM Tris-HCl and 1 mM EDTA, pH 8.3.
13. Absolute ethanol at -20°C .
14. Formamide loading buffer: 96% deionized formamide, 10 mM EDTA, pH 8.0, 0.125% (w/v) xylene cyanol, and 0.125% (w/v) bromophenol blue.
15. An 8–12% denaturing polyacrylamide sequencing gel.
16. Radioactively labeled size markers (see Note 5).
17. α - ^{32}P -dCTP if using radioactive incorporation method (see Section 1.).
18. A boiling water bath.
19. Water bath or incubating oven.

3. Methods

3.1. Oligonucleotide-Primed Primer Extension

1. Combine the following in an Eppendorf tube: 5 μL RNA in water, 3 μL radioactively labeled oligonucleotide, and 2 μL 5X hybridization buffer A.
2. Place the tube for 2 min in a boiling water bath and then incubate for between 2 and 4 h at 30 – 40°C .
3. Prepare the following mix just prior to the end of the hybridization

period: 2 μL dNTP mix, 1 μL 1M Tris-HCl, pH 8.0, 2 μL 0.1M MgCl_2 , 1 μL 1 mg/mL actinomycin D, 1 μL 0.02M DTT, 2 μL DEPC-treated sterile water, and 1 μL reverse transcriptase.

4. When the hybridization period is over, add the dNTP/Tris/ MgCl_2 /DTT/reverse transcriptase/actinomycin stock to the hybridization solution and mix well. Incubate the reaction tube at 37°C for 2 h.
5. After incubation, add 80 μL 1X TE and 100 μL phenol/chloroform. Mix well by vortexing and spin 5 min in an Eppendorf centrifuge at full speed. Remove the aqueous phase to a fresh tube, and add 10 μL of 3M sodium acetate, pH 5.2, and 250 μL of absolute ethanol at -20°C. Incubate at -20°C for more than 1 h. Centrifuge the sample in a refrigerated Eppendorf centrifuge for 15 min at full speed at 4°C. Pour off the ethanol and air-dry the tube (*see* Note 6). Redissolve the pellet in 4 μL of formamide loading buffer, denature by boiling for 3 min, and then load onto a denaturing polyacrylamide sequencing gel.

3.2. Restriction-Fragment-Primed Primer Extension

1. Combine together 24 μL of RNA in formamide, 3 μL of the DNA restriction fragment primer (at 20,000 cpm/ μL), and 3 μL of hybridization buffer B.
2. Place in a boiling water bath for 3 min to denature DNA. Incubate overnight at a temperature above the T_m of the DNA:DNA duplex, but below that of the DNA:RNA duplex (*see* Note 7).
3. Because formamide inhibits reverse transcriptase, it is necessary to purify the nucleic acids by precipitation. Add 170 μL of DEPC-treated water and 500 μL of cold (-20°C) absolute ethanol. Incubate at -20°C for more than 1 h. Recover the nucleic acids by centrifugation in an Eppendorf centrifuge at top speed for 15 min at 4°C. Wash the pellet with 80% (v/v) ethanol (ice cold), and air-dry the pellet (*see* Note 7).
4. Redissolve the pellet in 12 μL DEPC-treated sterile water, and then add 2 μL dNTP mix, 1 μL 1M Tris-HCl, pH 8.0, 2 μL 0.1M MgCl_2 , 1 μL 1 mg/mL actinomycin D, 1 μL 0.02M DTT, and 1 μL reverse transcriptase. Incubate the tube at 37°C for 2 h.
5. After incubation, add 80 μL of 1x TE and 100 μL of phenol/chloroform/isoamyl alcohol. Mix well and spin for 5 min in an Eppendorf centrifuge at full speed. Remove the aqueous phase to a fresh tube, and add 10 μL 3M Na acetate, pH 5.2, and 250 μL absolute ethanol at -20°C. Incubate at -20°C for 1 h or longer. Centrifuge the sample in a refrigerated Eppendorf centrifuge for 15 min at full speed and 4°C. Pour off the ethanol and air-dry the tube (*see* Note 6). Redissolve the pellet in 4 μL

formamide loading buffer, denature by boiling for 3 min, and load onto a denaturing polyacrylamide sequencing gel.

4. Notes

- 1 It is important that all reagents, solutions, and tubes be RNAase free. This can be achieved by treatment with diethyl pyrocarbonate (DEPC). Plastic and glassware are soaked in a 0.1% (v/v) solution of DEPC for 2 h, followed by autoclaving. Solutions should be made up with sterile distilled water in DEPC-treated plastic and glassware, and can then have DEPC added to 0.1% (v/v) followed by autoclaving. Solutions containing Tris-buffers cannot be treated in this manner, so normally such solutions are made up without Tris, treated with DEPC, and autoclaved. Then sterile (autoclaved) Tris-buffer is added just prior to use.

Disposable plastic gloves should be worn at all times when handling glass and plasticware, and while making solutions. Many laboratories keep separate chemical stocks and electrophoresis equipment only for RNA work, and solutions should not be used for RNA and DNA work. This is especially important when the large amount of RNase A used in most DNA preparation techniques is considered.

2. Reverse transcriptase is now available from many manufacturers. In my hands, Gibco-BRL (Gaithersburg, MD) Moloney Murine Leukemia Virus RNase H-Reverse transcriptase (M-MLV H-RT[Superscript]) works well.
3. Actinomycin D is both carcinogenic and mutagenic, so it should be handled with extreme care. Solutions of actinomycin D are light sensitive and should be stored in the dark.
4. Each dNTP is made initially as a 100-mM stock at pH 7.0. dNTPs are initially dissolved in water at a slightly higher concentration and then the pH adjusted with NaOH to 7.0. The concentration is then adjusted to 100 mM.
5. Markers for polyacrylamide gels can be simply produced from commercially available DNA markers. Approximately 10 μg of DNA gel marker (such as Phi X 174 /Hae III, or the 123-bp marker from Gibco-BRL) are diphosphorylated as in Chapter 43, phenol extracted, and precipitated with Na acetate and ethanol. This DNA is then resuspended in 20–50 μL 1X TE and stored at -20°C until required. An aliquot of approx 1 μL is then labeled by kinase treatment (*see* Chapter 43) in approx 10 μL . After kinasing, between 200 and 400 μL of formamide loading dye are added directly to the reaction. Normally between 1 and 4 μL are needed to produce a good signal after overnight exposure at -70°C with

- an intensifying screen. The labeled marker can be stored at -20°C , and is usable for between 4 and 6 wk. The marker must be denatured by boiling for 2–3 min prior to loading.
6. Do not overdry RNA, since this can lead to problems with resuspension. It is best to either air-dry RNA samples or, if using a vacuum drier, make sure that the pellet is *just* dried (or even slightly “damp” looking). If overdrying does occur, then resuspension can be aided by repeated cycles of heating to 65°C , followed by vigorous vortexing.
 7. Optimally, the incubation temperature is below the RNA:DNA T_m , and above the DNA:DNA T_m . This should be calculated for the RNA/probe pair. In most cases, it is better to experiment with a range of temperatures, spaced at $2\text{--}3^{\circ}\text{C}$ intervals around the optimal temperature.

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CHAPTER 45

***In Situ* Hybridization Analysis of Transgenic Tissues**

Judith McNiff Funkhouser

1. Introduction

In situ hybridization (ISH), also called *in situ* hybridohistochemistry (ISHH), has been used since the 1970s to localize mRNA within a tissue. This technique is now routine in many laboratories, and some standard procedures are emerging.

Probes used for ISH can be identical to those used for Northern analyses. The use of cDNA (1,2) or RNA (3) probes has been reported. Shorter probe fragments (<300 bp) often give a greater signal, because their smaller size allows penetration through the tissue to the location of the RNA. With even shorter oligonucleotide probes (4,5), penetration is easier, and they can be designed to distinguish between closely related mRNAs.

For routine screening, frozen tissue provides the shortest preparation time. Therefore, in this laboratory, we routinely use fresh frozen tissue, cryosections with minimal storage, and minimal pretreatment. The optimal hybridization stringency for high signal-to-noise ratio is determined mathematically (*see* Note 1) and tested experimentally.

Use of a radioactive labeled probe is a proven approach for detection of a tissue mRNA and is a good initial choice to assure accuracy. Following hybridization, tissue sections on slides are washed, dried, and applied to X-ray film, which is exposed for a few days and developed. The sections can be stained and compared to the film. Alternatively, for more precise localization, the slides can be emulsion dipped,

exposed for a longer time (a few days to 1 mo), and then developed. The grains (signal from the probe) are then in a layer just above the section that is stained through the emulsion. Both layers can be seen through the light microscope with bright field, whereas dark field can be used to visualize only the grains giving a higher magnification image similar to film autoradiography. When the signal can be seen to come from a particular cell or cells, they can be identified by their morphology, location, and staining characteristics. Immunohistochemistry can be combined with ISH on the same or adjacent sections to identify a particular cell type within the relatively thick frozen section or to locate the protein product of a gene (6).

Alternate approaches designed to increase the ISH sensitivity and precision are available, including the use of nonradioactive probes and fixing the tissue by immersion or perfusion, which can improve the morphology and make cell identification easier. The fixed tissue can be handled subsequently as frozen sections. These can be cut 6- to 60- μm thick. The thicker sections give a "louder" signal from a cluster of cells. Alternatively, tissue can be embedded in paraffin or plastic for a thinner section allowing visualization of details. The paraffin yields a 4- to 6- μm section, which ensures there are no multiple layers of cells. The plastic yields a 0.5- to 1- μm section, which ensures no overlay of cells. Also, since one cell can be seen and identified in several adjacent sections, immunohistochemistry and ISH can be carried out on separate sections, and colocalization of an mRNA and its protein determined without the compromises necessary for performing two techniques on the same section. Some plastics can also be cut for electron microscopy, allowing subcellular visualization of mRNA within a cell or identification of signal source when cell processes are intertwined.

2. Materials

2.1. Tissue and Slide Preparation

1. Cryostat (a rotary microtome set in a freezer).
2. Isopentane: Place in a stainless-steel beaker set in an ice bucket filled with a dry ice/acetone mixture
3. Black boxes:
 - a. Plain plastic boxes for 25 slides: These protect against condensed moisture for freezer storage of slides.
 - b. Black boxes with 25-slide polyacetyl rack and dessicant compartment: These are used for exposure of slides to the emulsion.

4. Tissue culture standard "Milli Q" purified water (i.e., filtered through activated charcoal, deionizing resins, and an ultrafilter).
5. All solutions used on the tissue prior to hybridization should be made with diethyl pyrocarbonate (DEPC; Sigma, St Louis, MO) treated water. Add 1 mL of DEPC to 1 L of "Milli Q" H₂O, and shake vigorously. Allow to stand several hours or overnight, and then autoclave.
6. Fixatives:
 - a. 4% (w/v) Paraformaldehyde in 0.1M phosphate buffer pH 7.4: Heat 4 g paraformaldehyde in 50 mL deionized water to 60–70°C and titrate until clear with 10N NaOH (a few drops). Cool to 40°C, and then mix with 50 mL of 0.2M phosphate buffer.
 - b. Picric acid-paraformaldehyde (PAF) in 0.1M phosphate buffer pH 7.4: Heat 4 g paraformaldehyde in 30 mL of saturated picric acid to 60–70°C, and titrate until clear with 10N NaOH. Cool to 40°C. Mix with H₂O to a volume of 100 mL, and then add an equal volume of 0.2M phosphate buffer.
7. 10N NaOH (20 g/50 mL H₂O).
8. 0.2M Phosphate buffer (pH 7.3):
Na₂HPO₄ (28.2 g/l)
NaH₂PO₄ (6.9 g/250 mL)
Titrate the above two solutions to pH 7.3. Treat with DEPC (0.1% [v/v]), and then autoclave.
9. PBS containing heparin is the initial wash for the perfusion start. To 100 mL of 20 mM phosphate buffer diluted from the above stock with DEPC-treated water, add 8 g sodium chloride and 100 U of heparin.
10. 30% (w/v) sucrose in 0.1M phosphate buffer.
11. Perfusion fixation setup (optional; see Note 2):
 - a. Two 1-L bottles with hose connections at the bottom.
 - b. Silicone tubing (³/₈ in.) to fit the bottle hose connections.
 - c. Three-way stopcock, which fits the above tubing.
 - d. "Venoset" from a medical supply distributor provides a bubble trap, tube, and tubing clamp, and a lower slip end, which fits any syringe needle. The top will fit the ³/₈-in. tubing connected to the stopcock.
 - e. Syringe needle, for rats, 18 g; for mice, 25 g.
 - f. "Intramedic" polyethylene tubing (the largest size that will fit into the aortic artery of the animal—for rats 7430, for mice 7405). Take a piece about 1.5-in. long, hold it up to a match flame, and flare one end. Fit the other end onto a tight-fitting needle.

Place the bottles 75 cm above the animal. This provides adequate pressure to perfuse rats and mice. Fill one bottle with fixative, and let it flow through to the stopcock. Fill the other bottle with an isotonic buffered saline (e.g., PBS), and fill the tubing with the bubble trap upside

down until the trap is half full. Turn the trap leaving it half full, fill the remainder of the tubing, and close the clamp. The catheter is inserted according to the directions given below with the clamp slightly open allowing a slow flow. There must be a positive pressure on the catheter tip as it is inserted to avoid any air bubbles entering the blood vessels. Air will block the flow. Once the tip is in place increase the flow.

12. Microslides, precleaned Superfrost (Dynalab, Rochester, NY or Fisher Scientific, Pittsburgh, PA). These do not have to be washed prior to use. Many other brands first have to be washed with detergent.
13. Acid cleaning solution (for cleaning slides and glassware):
 - Potassium dichromate, 20 g.
 - Water, 200 mL.
 - Sulfuric acid, 20 mL
14. 0.25M Ammonium acetate.
15. 50 $\mu\text{g/mL}$ Poly-L-lysine (Sigma #P1274) in 10 mM Tris-HCl buffer (pH 8.0).
 - Stock solutions:
 - 10 mM Tris-HCl buffer pH 8.0.
 - Poly-L-lysine 10 mg/mL in 10 mM Tris-buffer. pH 8.0. Store at -20°C in 1.5-mL aliquots.
 - Dilute 200X to make the working solution (1.5 mL in 300 mL 10 mM Tris-HCl).
16. Glass staining jars, acid cleaned.
17. Slide racks to fit staining jars (may be glass or metal).
18. OCT (a "Tissue Tek" product, Miles Inc., Elkhart, IN) for mounting small frozen tissue pieces to microtome chuck. Sterile deionized water can also be used and is preferable with fresh frozen tissue, since the OCT does not wash away after fixation in paraformaldehyde and the probes have a tendency to stick to it, thereby increasing the background.

2.2. End Labeling with Terminal Transferase

1. Oligonucleotide in sterile "Milli Q" water.
2. New England Nuclear (Boston, MA) 3'-end labeling kit: The terminal transferase reaction adds a string of labeled nucleotides to the 3' end of the oligonucleotide. The kit contains the enzyme, the reaction buffer, cobalt chloride, and standard DNA.
3. New England Nuclear $\alpha\text{-}^{35}\text{S}$ dATP: This brand contains a small amount of DTT and tricine, which stabilizes the labeled nucleotide, but does not interfere with the terminal transferase reaction.
4. Sephadex G50 swollen in Tris-EDTA.
5. TEN: 50 mM Tris-HCl, pH 7.5, 10 mM EDTA, and 150 mM NaCl.

2.3. Hybridization

All solutions made should be sterile and put into sterile containers, either autoclaved glass or sterile disposable plastic. Try to keep things as sterile as possible while working. Fingerprints contain RNase. Gloves should be worn while working to reduce the chance of fingerprint contamination of any item used. Slides can be handled with forceps. Make sure the forceps are clean. If in doubt, wash in detergent, soak in 1*N* NaOH, rinse in water, dip in 95% (v/v) EtOH, and let dry.

1. 1*N* NaOH (can be used to inactivate RNase that is present on any surface that needs to be used).
2. Aluminum foil: Fresh off the roll, aluminum foil provides a clean RNase-free surface on which your slides can be placed.
3. Square polypropylene slide mailers (A. H. Thomas, Swedesboro, NJ): Treat with deionized water containing DEPC (0.1% [v/v]) for several hours or overnight and then autoclave.
4. Phosphate-buffered saline (PBS: 10 mM phosphate buffer: To 0.2*M* stock diluted 20X with DEPC treated water, add 0.9% [w/v] NaCl).
5. 0.25% Acetic anhydride (v/v) in triethanolamine buffer: Make stock solutions of 0.1*M* triethanolamine adjusted to pH 8 with HCl, DEPC treat with 0.1% (v/v) for several hours, and autoclave. Immediately before use, add 0.25 mL acetic anhydride/100 mL.
6. Absolute ethanol.
7. 95% (v/v) and 70% (v/v) Ethanol: Dilute absolute ethanol with DEPC water.
8. Chloroform.
9. Parafilm.
10. Moisture tight plastic container with a flat bottom: Make perspex or Plexiglas™ (5–8 mm thick) racks to fit the interior. Use small squares of plastic glued to the bottom surface to space the racks apart when stacked one above the other. With the slides placed flat on these racks, this system allows you to place a large number of slides in the same box. The interior is kept moist by placing a wet filter paper in the bottom under the first plastic rack.

2.4. Hybridization Buffer

1. Stock solutions.
 - a. 20X SSC: 3*M* NaCl, and 0.3*M* sodium citrate, pH 7.0. Treat with DEPC (0.1% [v/v]) and autoclave.
 - b. Denhardt's solution (50X stock): Dissolve 100 mg each of Ficoll, polyvinylpyrrolidone, and BSA in 10 mL of DEPC-treated water,

filter through a 0.2- μm filter into autoclaved microfuge tubes, and store at -20°C in 400- μl aliquots.

- c. 1M DTT in 0.1M sodium acetate: Filter through 0.2- μm filter into autoclaved microfuge tubes, and store at -20°C in 100- μL aliquots.
 - d. Yeast tRNA (Sigma) in DEPC-treated water: Filter through 0.2- μm filter. Store at -20°C in 100- μL aliquots.
 - e. Sheared salmon sperm (ss) DNA (Sigma): Dissolve at 10 mg/mL in DEPC-treated water. Heat in a boiling water bath for 10 min.
 - f. 50% (v/v) deionized formamide (Fluka, Buchs, Switzerland).
 - g. Dextran sulfate (500,000 mol wt).
2. Hybridization buffer (20 mL). Mix the following:

	Amount	Final conc.
Deionized formamide	10 mL	50% (v/v)
20X SSC	4 mL	4X
Sheared ssDNA 10 mg/mL	1 mL	500 $\mu\text{g/mL}$
Yeast tRNA	0.1 mL	5 mg/mL
50X Denhardt's sol'n	0.4 mL	1X
Dextran sulfate*	2 g/4.5 mL DEPC H ₂ O	10% (w/v)

*Vortex to dissolve

This solution can be stored at -20°C for at least 2 mo. Store in single-use aliquot (e.g., 1 mL) in autoclaved microfuge tubes. To use with a ^{35}S -labeled probe, add 10 μL 1M DTT/mL of hybridization buffer prior to use.

3. Oligonucleotide probe diluted to a concentration of 100 ng/mL.

2.5. Visualization

1. X-Ray film.
2. Emulsion (NTB-2) for optical autoradiography: Melt the emulsion in 45°C water bath, and dilute 1:1 with 2% (v/v) glycerol in H₂O.
3. Dark room with safe lights equipped with filters to match the film or emulsion—**caution:** The film specifications presume a 15-w bulb in the safe light kept at least 4 ft away from your work or a 25-w bulb used indirectly with the safe light directed at the ceiling or a wall. Kodak NTB-2 needs a Kodak #2 red filter. X-Ray films use a Kodak GBX filter.
4. Dipping jar: Electron Microscopy Sciences (Fort Washington, PA) markets a well-designed one that allows 25 slides to be dipped in 14 mL of 1:1 diluted emulsion and is well supported to avoid spilling.
5. Develop film in an X-ray film processor.
6. Develop emulsion-dipped slides in staining jars.

Kodak D19 developer for 2 min 30 s.
Rinse in water for 30 s.
Fix in 5% sodium thiosulfate for 6 min.
Rinse in running tap water for 15 min.

3. Methods (see Table 1)

It is an absolute necessity to use controls together with the transgenic sections. The most obvious negative control is to apply the probe specific for the transgene to sections from nontransgenic animals. Numerous artifacts may be apparent. When initial criteria for nonartifactual signals have been met, it can be further confirmed with some of the following:

1. The signal is not present if the tissue is digested with RNase A prior to hybridization.
2. The signal can be decreased or eliminated by competing the hot label with a large (10–20-fold) excess of cold label.
3. The signal is changed in intensity by known physiological changes
4. The use of a random oligonucleotide of the same length does not produce a similar signal.

If possible, a positive control should also be run. Use sections from a tissue known to contain the mRNA of interest.

3.1. Tissue Preparation

The first screening by ISH for localizing transgene expression is done on cryosections of fresh frozen tissues that have indicated expression by RNA isolation and Northern analysis (see Note 1). The procedure used in this laboratory is that of S. L. Lightman and W. S. Young (7,8).

1. Quick and careful freezing of the tissue is necessary to avoid damage by autolysis. Tissue should be removed from one animal at a time and frozen without delay. Work quickly, or damage by autolysis will occur. "Freeze artifact" caused by ice crystal formation in the tissue will leave holes in the tissue and will make it difficult to section. Some tissues can be frozen on dry ice, but many require faster freezing to improve the morphology. Use a liquid intermediate, isopentane, in a stainless-steel beaker cooled in a dry ice/acetone mixture. The tissue is wrapped loosely with several openings in aluminum foil and quickly immersed in isopentane. It is kept moving in the isopentane to prevent the buildup of an insulating film at the tissue isopentane interface. When frozen, remove to dry ice for transport to the freezer, or place directly into the freezer. The tissue blocks keep well stored at -70°C .

Table 1
Hybridization Protocol

PAF	15 min
PBS	5 min
0.25% (v/v) Acetic anhydride in triethanolamine	10 min
70,80,95,100% (v/v) EtOH	.1 min each
Chloroform	5 min
100,95% (v/v) EtOH	1 min each
Air-dry	
Apply 50 μ L hybridization buffer with labeled probe	
Cover with parafilm cover slip	
Hybridize	Overnight
Wash, 1X SSC 55°C four times	20 min each
Wash, 1X SSC rt two times	1 h each
Rinse, dH ₂ O two times	2X 1 dip
Air-dry	
Dip in 1:1 diluted emulsion	2X 1 dip
Air-dry in dark	
Pack into black slide boxes with dessicant	
Seal with plastic tape	
Leave in cold 4°C	1-30 d
Develop D19	2 min 30s
Acid stop	30 s
Fix	5 min
Rinse water twice	5 min each
Counterstain, H + E	
Dehydrate 70, 95, 100% (v/v) ETOH	1 min each
Cover slip with xylene-based mounting media	

- Before sectioning, the slides must be prepared. The more costly precleaned slides ("Super-frost") can be used directly from the box. Many other slides require washing in soap or detergent followed by a water rinse. All slides should be acid cleaned and rinsed thoroughly in copious amounts of running water. Finally, rinse in double-distilled or "Milli Q" H₂O. To keep the sections on the slides during extensive washings, the slides must be "subbed." Subbed slides are coated with either chrom alum gelatin or poly-L-lysine. For poly-L-lysine (9), dip in 0.25M ammonium acetate solution, oven-dry, soak in poly-L-lysine for 30 min, and dry protected from dust. They can be oven-dried. The slides should be used within 2 wk.

- 3 Mount the frozen tissue onto the specimen holder or chuck of the cryostat. This can be done within the microtome freezer which is set at -23°C or colder. Use sterile ice water and freeze a layer onto the surface of a cold dry chuck. Add a second layer of water and, before it freezes completely, place the frozen tissue in the cold water and hold with cold (-23°C) forceps while it freezes. Work fast so the tissue does not melt. Very small tissues (e.g., the mouse pituitary) will have to be frozen in OCT in order to facilitate handling and to prevent freeze-drying during storage. It can then be mounted onto the chuck with OCT. When the section is fixed in paraformaldehyde, the OCT will also be fixed and will not wash off the slide. The probe has some tendency to stick to this, and it will increase the background staining.
4. Sectioning is done on a cryostat. A novice should not attempt to use the cryostat without guidance from an experienced operator. The section thickness can vary between 6 and 50 μm . Cut $\approx 10\text{-}\mu\text{m}$ sections at -20°C for routine screening. Sections of 30–50 μm might be used if you are trying to scan, say, a whole brain in serial sections. Sections of 10–20 μm are good for routine work. Thinner sections will give greater detail. Sections are cut using an even motion on the handle. They are picked up off the cold knife by melt mounting onto the coated slide. If microscopic inspection shows small cracks in the tissue parallel to the knife edge, the temperature is set to low. If pressing a gloved thumb onto the block face immediately prior to the cutting stroke relieves this problem, then turn up the temperature. Too warm a temperature will yield a compressed section. Compression can also be caused by an improperly positioned antiroll plate or a dull knife. The correct temperature with a sharp, clean knife edge and a properly adjusted antiroll plate will yield a section that lies flat on the cold knife surface and can be picked up by melting onto a warm slide. Dry the section at 40°C on a warm plate for 1–2 min, then refreeze in the cryostat, place in black slide boxes with tight-fitting covers, and store at -70°C until use. Do not store too long. Some tissues keep, but some do not. Section storage can be improved for some tissues by fixing the section immediately after cutting and drying at 40°C . Fix for 15 min to 1 h, rinse in PBS, dip in 70% (v/v) ethanol, and dry. Refreeze and store. Many authors agree that a cross-linking fixative, like paraformaldehyde, provides good mRNA retention within the tissue (10). The addition of picric acid (11) has improved the results in this laboratory with better morphology and better detection of mRNA. When ready to use, the slides should be removed from the freezer. If unfixed, then they should be placed immediately in fixative for 10–15 min and then rinsed in an isotonic buffer, e.g., PBS. If

the tissue is already fixed, the slides can be placed directly into PBS.

5. Tissue pretreatment prior to hybridization should be kept to the minimum necessary to allow probe access to the RNA within the tissue and to minimize nonspecific sticking of the probe. Following Lightman and Young's (7) procedures, our slides are dipped into 0.25% (v/v) acetic anhydride in triethanolamine for 10 min to minimize nonspecific sticking. The sections are then dehydrated for 1 min each in 70, 80, 95, and 100% (v/v) ethanol, and then left in chloroform for 5 min to damage the lipid layer of the cell membrane enough to allow probe access. Following a rinse in 100% (v/v) and 95% (v/v) ethanol for 1 min each, the slides are allowed to air-dry in a 37°C warm room.

3.2. Probe Preparation

1. Probes are 3'-end labeled with terminal transferase (12) using a New England Nuclear kit according to the manufacturer's instructions and are prepared immediately prior to use. To a sterile microcentrifuge tube, add your probe plus the following solutions found in the kit.

2 μL Probe DNA (200 ng in H_2O)

6 μL Water

12.5 μL Reaction buffer

5 μL α - ^{35}S dATP

2.5 μL CoCl_2 .

Mix and centrifuge to the bottom of the tube.

2. Add 2 μL of terminal transferase. Mix gently, and place in a water bath at 37°C for 1.5–2 h. The temperature should not drop to 36°C, since this will give a much reduced labeling. Stop the reaction by placing the tube in an ice bucket.
3. Transfer the mixture to a Sephadex G50 column, and rinse the tube with 30 μL TEN. Centrifuge for 3 min at 672 g, and recover 50 μL containing the probe. Use immediately. If storage is desired for a short period, then add DTT to a 10-mM concentration.

3.3. Hybridization and Washing

1. To 1 mL of hybridization buffer, add 10 μL of 1M DTT, mix, and add 10 μL (\approx 35 ng) of labeled probe and vortex. Ten microliters of this mix should count \approx 100,000 cpm or above. If it is lower, the amount of DNA can be increased up to three times. Beyond this, there is too much background. Compare your probe labeling with the standard provided in the NEN kit.
2. To hybridize with a minimum of hybridization buffer, the slides need to be placed flat in a moist chamber. A Petri dish with moistened filter

paper in the bottom will work for a few slides. Plastic refrigerator storage boxes work very well for larger numbers. Put moistened filter paper in the bottom, and place the slides flat. Pipet 50 μL onto each slide (this volume should be adjusted to the cover sections easily). A parafilm "cover slip" cut to fit over the sections can be used to spread the buffer over the sections and hold it there without excessive evaporation. There should be no air bubbles under the cover slip. Hybridize overnight at 37°C (see Note 1).

3. Wash sections in 1X SSC. A typical schedule would be to wash at 55°C, with four changes of 20 min each, followed by two room temperature washes of 1 h each. The wash time and temperature will have to be adjusted for each probe. With an unknown probe, try several wash schedules aiming for a clear signal readily detected above background. The stringency of the wash can be varied low to high by increasing the temperature, lengthening the time, or reducing the salt concentration. Finally, dip the slides in distilled water, and let them dry.

3.4. Visualization

1. Film autoradiography: In an X-ray cassette, the slides must be held firmly against the film with even pressure on all slides. This can be accomplished by gluing the back of the slides to a piece of stiff paper or poster board. The choice depends on the depth available in the cassette. The film is inserted and exposed for a few days at -70°C, and then developed. It can be compared to the same slide stained and cover slipped after exposure, or to adjacent sections stained and cover slipped.
2. Emulsion autoradiography: The NTB-2 is diluted 1:1 before use. In the dark room, use a clean spatula to transfer the room temperature emulsion to a sterile screw-cap centrifuge tube. Warm in a water bath to 45°C until the emulsion melts. Approximately 15 mL of gelled emulsion melts to 7 mL. Mix with 7 mL of deionized H₂O using a clean slide, then dip a trial slide, and check for an even coating and freedom from bubbles, indicating the emulsion is melted and well mixed. Dip each slide, wipe the reverse side, and then stand the slides on end. Leave them to dry in the dark (13). Turn off even the safe light. After they are dry, 1–2 h later, pack them into black boxes with silica gel and seal them shut with plastic tape. Expose at 4°C for a few days to 1 mo.
3. Develop the slides in Kodak D19 for 2 1/2 min. Rinse for 30 s in an acid stop bath, and fix in 5% (v/v) Na thiosulfate. This can be followed by staining the section in many of the usual histological stains. Hematoxylin eosin is the most common (see Chapter 48). Follow by dehydration in ethanol and cover slip with a xylene-based mounting media. Brain

sections can also be stained in neutral red nissal stain for 4 min, rapidly dehydrated by incubation in each alcohol for 15 s, cleared in xylene, and cover slipped. The light-red stain provides excellent contrast to the black grains of the autoradiographic signal.

4. Autoradiographs can be examined with bright-field microscopy or dark field. The bright field allows you to see the stained section and the grains in the emulsion in the same field. The dark field allows visualization of the autoradiographic grains only, allowing a quick scan for label above background and facilitating a grain density comparison over tissues of different staining densities.

4. Notes

1. Positive controls are useful in finding the correct hybridization and wash conditions for particular probes. A calculation of the melt temperature (T_m) can be used as a place to start. Using the formula given in reference (14), calculate the T_m for DNA–DNA hybrids:

$$T_m = 81.5^\circ\text{C} - 16.6[\log_{10}(\text{Na}^+)] + 0.41(\%G + C) - 0.63(\%F) - (600/l) \quad (1)$$

$[\text{Na}^+]$ is the concentration of sodium ions in the hybridization mix, %G-C is the percentage of G and C residues in the probe, %F is the concentration of formamide (% v/v) in the hybridization mix, and l is the length of the probe.

Because of the greater stability of DNA–RNA hybrids, their T_m will be 10–20°C higher than this figure. Use a temperature 30–40°C below the T_m for hybridization, and vary the wash temperature low to high to vary the stringency. At some point below the T_m , you should find a hybridization and a wash temperature that leave a clear signal against a relatively clean background. Remember that too high a temperature will destroy the section leaving you with nothing to label. Increase the percentage of formamide to lower the T_m . Lowering the salt concentration and lengthening the time are alternative ways to increase wash stringency. For long washes, sterile conditions are necessary to avoid digestion of the tissue by enzymes from external sources.

2. Once the expression is localized, there may be some need to pinpoint further its location. The improved morphology provided by tissue fixed prior to freezing can often be useful. The tissue can be fixed by immersing in fixative immediately on removal from the animal. The fixative volume should equal 20 times tissue volume or more. To reduce further the possibility of autolysis, the fixative can be perfused through the vasculature prior to removal of the tissue. For a rat, prepare 400–500

mL of fixative (PAF). Anesthetize the rat with an overdose of Nembutal. Working quickly, open the abdomen, puncture the diaphragm to collapse the lung, and cut through the ribs on both sides of the sternum. Lift the sternum with a hemostat, and cut the pericardium. Rest the hemostat above the animal's head. Using a curved forceps or a curved mosquito hemostat to hold the thread, pass thick (#1) suture thread under the aorta, and push up above the heart so that it can be loosely tied around the large blood vessels exiting the heart. With sharp scissors, cut the left ventricle and insert a cannula through the left auricle into the aorta. Tighten the tie around the cannula. Increase the flow rate. The right auricle should swell, indicating that the perfusion is working, and cut it immediately to "create a drain." Allow 150–250 mL of fixative to flow through in 15–30 min for a mouse; increase the volume to 300–400 mL for a rat. Stop the flow, remove the required tissue, and immerse it into fixative, at least 20 times the tissue volume. Cut into small pieces to ease infiltration. After a total fixation time of 1–2 h, transfer to 30% (w/v) sucrose until it sinks. For good cryoprotection, the sucrose must penetrate the tissue fully, allowing it to sink in these solutions. Since it is important for ISH not to leave the tissue in the sucrose very long (no more than overnight), it may be necessary to perfuse a second solution of the fixative with 10% (w/v) sucrose solution and cut into small enough pieces to allow penetration of 30% (w/v) sucrose within a few hours. Freeze the tissue. The blocks can be stored at -70°C . For most tissues, the blocks store well. The cryosections do not store as well. Some tissues can be stored; others will suffer a severe loss of signal.

Taking advantage of the improved morphology of fixed tissue, it can be embedded in paraffin or plastic for thinner sections and greater resolution. For paraffin-embedded tissue, the ISH is done on the sections (15). There may be sensitivity loss owing to extensive processing. Hydrochloric acid, pronase, or proteinase K digestion will probably be necessary to access the mRNA. The concentration and time needed for your tissue will have to be determined experimentally. For plastic sections, the ISH can be done prior to embedding in plastic (16) or subsequent to plastic embedding (17).

Fixation with glutaraldehyde (2–4% [v/v]) can be used alone or with the paraformaldehyde (add 0.5–1% [v/v] glutaraldehyde) to retain the mRNA during processing. It will then be necessary to incorporate enzyme pretreatment into your protocol.

Since autoradiography yields a signal on a separate film of emulsion layers, a second approach to more closely locating mRNA is to use a non-radioactive labeling technique (18,19). These make use of the

methods developed for immunohistochemistry (see Chapter 46). Graid et al. (20) have compared several of the techniques using a biotin-labeled DNA probe and found that visualization with an antibiotin antibody gave a signal with the least background. This approach can also be used with a deoxygenin tag (21,22) or a 5 Bromo 2' deoxyuridine tag (23). The latter two may be more versatile, since neither one occurs naturally in animal tissues.

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CHAPTER 46

Immunohistochemical Analysis of Transgene Expression

Judith McNiff Funkhouser

1. Introduction

Immunohistochemistry can be used to localize the peptide or protein product of the transgene, or to identify particular cell types within tissue sections by labeling a specific protein. There are many ways to do this. Today many involve a primary antibody to the protein of interest, and a secondary antibody plus a fluorescent or enzyme tag to visualize the reaction (1; see Note 1). The fluorescent tag is observed by viewing it through a microscope equipped with a light source of the correct wavelength to excite the molecules of the tag, which then emit light of a specific wavelength, visible through filters. Because the fluorescent material contains a limited number of molecules, the label is temporary and needs to be photographed to save the result obtained. The second protocol given here uses a fluorescent tag. In contrast, the enzymes alkaline phosphatase and horseradish peroxidase can be visualized with chromogens that create a permanent slide, which can be examined immediately in a simple bright-field microscope and reviewed later. Additionally, it allows a clear bright-field or Nomarski view of the tissue, together with the label. These approaches give a one-enzyme molecule for one antibody-antigen reaction. For proteins in low abundance, the reaction may be enhanced by Sternberger's peroxidase antiperoxidase (PAP) method (2), which attaches a three-enzyme complex to the secondary antibody. Alternatively, the enhancement is accomplished by using a biotin-labeled secondary antibody

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that binds a streptavidin molecule complexed to enzyme molecules (3). The latter approach is the first method described here. It has yielded good results in a variety of tissues, and, with the correct dilution of the antibodies and streptavidin complex, results in a clear signal (*see* Note 2).

Slides stained for a protein by immunohistochemistry can also be stained for mRNA using *in situ* hybridization (*see* Note 3). Compromises will be necessary in both protocols, and it may not be possible in all cases.

2. Materials

1. The glassware should be acid cleaned (*see* Chapter 45).
2. Coplin jars work well for washing a few slides. Staining dishes and racks (glass or metal) facilitate the operation when more than 10 slides are being processed together.
3. Superfrost slides (Dynalab, Rochester, NY) can be used directly out of the box and coated with poly-L-lysine (*see* Chapter 45) prior to sectioning.
4. Sectioned tissue: In general, the best access to most antigens is provided by cryosectioned frozen tissue. Cryosections of tissues are best cut from animals perfused with PAF (*see* Chapter 45). The freezing permeabilizes the membrane so that little pretreatment is required. The easiest preparation is to use fresh frozen tissue. Work quickly when removing the tissue, and freeze immediately (*see* Chapter 45) or all will be lost to autolysis. The frozen tissue can be stored for some time prior to cryosectioning, but plan on cutting, fixing, and immediately reacting the sections. This will give the greatest access of antibody to antigen. Storage of the sections risks the breakdown of the antigen. Perfusion or immersion fixation prior to freezing the tissue will better preserve many antigens, and will better preserve the morphology and localization. Perfusion-fixed sections can sometimes be stored frozen for a short period of time, allowing more flexibility into your schedule. The length of storage time possible depends very much on the particular tissue and the particular antigen. It can vary from none to 6 mo. The protocol given above is done with perfusion-fixed, cryosectioned tissue, and works on brain sections stored as long as 6 mo and in pituitary sections stored a maximum of 1–2 wk. Immunostaining can be done on paraffin or plastic (4,5) embedded tissue. The thinner sections give greater resolution, but require more pretreatment steps. The paraffin must be removed, and in some cases, the plastic etched or permeabilized. Enzyme pre-

treatment may be necessary. Some antigens can be degraded by the embedding process. In these cases, the immunostaining can be done on frozen or vibratome sections, which are subsequently embedded in plastic and sectioned thinner, 0.5–2 μ , for greater resolution. The vibratome section will yield better morphology.

2.1. Immunohistochemistry—Peroxidase, Diaminobenzidine (DAB) Reacted

1. Primary antibody: The selection of the primary antibody is the most important choice. Aim for one with high affinity and specificity for the antigen of choice. The specificity is most necessary because the tissue section provides an abundance of other proteins, and it is important that the antibody does not stick to them. It is best to use antibodies tested for immunohistochemistry or immunocytochemistry by the manufacturer.
2. Secondary antibody: The secondary antibodies are anti-IgG to the host animal of the primary antibody. It should be whole molecule or Fab₂ fragment. Purchase only those tested for immunohistochemistry.
3. Blocking serum: This is serum from the host animal in which the secondary antibody was produced. This serum is applied to the sections prior to antibody application to block the nonspecific sticking of the antibody to the tissue. Keep frozen in small aliquot.
Use 2% blocking serum in phosphate-buffered saline (PBS) with 0.1% (w/v) saponin + 1% (w/v) bovine serum albumin (BSA) for 10 min prior to antibody application.
4. Streptavidin peroxidase (Sigma, St. Louis, MO): The stock solution is 1 mg/mL in sterile PBS. Store at 4°C. To use, dilute 1:200 with PBS containing 0.1% (w/v) saponin.
5. All buffers should be freshly made with deionized double-distilled water or with water purified by filtration through a "Milli Q" system. PBS can be autoclaved and stored unopened until needed
Stock solution of 0.2M sodium phosphate buffer, pH 7.4.
 - a. Na₂HPO₄: 28.2 g/1000 mL dH₂O
 - b. NaH₂PO₄: 6.9 g/250 mL dH₂OTitrate the first solution with the second to pH 7.4.
6. Phosphate-buffered saline (PBS): 0.2M Sodium phosphate buffer stock (*above*) is diluted 20X to 10 mM. Then add 0.9 g NaCl/100 mL. Add 0.1% (w/v) saponin to all PBS solutions used prior to streptavidin applications. Add 1% (w/v) BSA and 0.1% (w/v) saponin to PBS used to dilute serum or an antibody.
7. Saponin (Merck, Darmstadt, Germany), 0.1% (w/v), is added to all anti-

body- or serum-containing solutions and in all wash buffers used prior to the streptavidin application. Saponin, which is added to all serum- and antibody-containing solutions, replaces some of the lipid in the membrane and coats the antibodies allowing them to pass through (6). It results in less damage than Triton -X-100, which is used in many protocols for the same purpose.

8. Diaminobenzidene (Sigma) 0.5 g/5 mL in PBS: Store in small aliquots. Defrost once and dilute 1:200 to use. Wear disposable gloves when weighing out. Store frozen 50-mg aliquot so as to reduce the amount of handling necessary on a routine basis.
9. Hydrogen peroxide (H₂O₂) 30% (v/v) solution (Merck, stabilized): Store at 4°C.
10. Glutaraldehyde. 1% (v/v) in 0.1M sodium phosphate buffer. Mix 2 mL of 50% (v/v) glutaraldehyde with 48 mL of H₂O and 48 mL of 0.2M phosphate buffer.
11. Neutral red: 1.25% (w/v) in water: Filter through Whatman #1 paper just before use.
12. Ethanol.
13. Mounting media for cover slips. Use a xylene-based mounting media.

2.2. Enhanced Peroxidase Histochemistry: (Glucose Oxidase-DAB-Nickel)

1. 0.2M Acetate buffer: Dissolve 27 g of sodium acetate (anhydrous) in 1 L of water and adjust to pH 6.0 with a dilute acetic acid (3 mL/250 mL).
2. Glucose oxidase DAB nickel solution (GDN):
 - Stock solution A: 2.5 g of nickel ammonium sulfate to 50 mL of 0.2M acetate buffer (pH 6).
 - Stock solution B: 50 mg of 3,3'-diaminobenzidine dihydrochloride in 50 mL of water.Mix solutions A and B just before use, and add 200 mg of β-D-glucose (Sigma), 40 mg of ammonium chloride, and 0.5–1 mg of glucose oxidase (Sigma, type VII).

2.3. Immunohistochemistry—Fluorescent Label

1. Primary antibody: Use monoclonal or affinity-purified polyclonal antibody.
2. Secondary antibody: Use whole antibody or Fab₂ fragment made against the IgG of the host animal in which the primary antibody was produced
3. Blocking serum: Use serum from the host animal in which the secondary antibody was produced.
4. Permafluor (Shandon, Lipshaw). This is an aqueous mounting media.

2.4. Commercial Kits

Commercial kits are available to do immunostaining. To use them, follow the manufacturer's instructions. They are best for doing only a few slides. Ordering the components separately allows the system to be tailored to any number of slides and allows the flexibility to change the concentrations used to obtain optimal results for your tissue.

3. Methods

All steps are performed at room temperature except the primary antibody incubation, which is done at 4°C overnight. The washes are carried out in acid-cleaned glassware, either coplin jars for 1–10 slides or staining dishes with rack for larger numbers. The antibody incubations are performed with the slides laid flat in a moist chamber (*see* Chapter 45). Use several slides as negative controls, that is, put through all the solutions, but omit the primary antibody. These control sections are essential to the interpretation of your results (*see* Note 4).

3.1. Immunohistochemistry—Peroxidase, DAB Reacted

The pretreatment of the slides consists of washing in isotonic buffer, blocking the endogenous peroxidase activity with H₂O₂, and blocking nonspecific binding with BSA and serum. The primary antibody is incubated overnight at 4°C.

1. Wash in PBS, pH 7.4, for 5 min.
2. Treat with 3% (v/v) H₂O₂ in PBS containing 0.1% (w/v) Na Azide for 3 min.
3. Wash in PBS containing 0.1% (w/v) saponin, 1% (w/v) BSA, and 2% (v/v) blocking serum for 10 min.
4. Drain slides and pipet on 50–100 µL of first (primary) antibody appropriately diluted (*see* Note 2) in PBS containing 0.1% (w/v) saponin and 1% (w/v) BSA. Incubate overnight at 4°C in a moist chamber.

After a thorough wash, apply the secondary antibody and incubate 1–2 h.

5. Wash twice in PBS containing 0.1% (w/v) saponin for 5 min each.
6. Wash in PBS containing 0.1% (w/v) saponin, 1% (w/v) BSA, and 2% (v/v) serum for 10 min.
7. Drain slide and pipet on 50–100 mL of secondary antibody appropriately diluted (*see* Note 2) in PBS containing 0.1% (w/v) saponin and 1% (w/v) BSA. Incubate in a moist chamber for 1–2 h.

Following a second thorough washing, apply the streptavidin–peroxidase complex to the sections and incubate up to 1 h.

8. Wash twice in PBS containing 0.1% (w/v) saponin for 5 min each.
9. Wash in PBS for 5 min.
10. Drain the slide and pipet on 50–100 mL of streptavidin peroxidase complex diluted 1:200 in PBS. Incubate in a moist chamber for 45–60 min.

Following a thorough wash, apply the chromogen DAB (*see* Note 1) to the sections to react with the bound peroxidase enzyme, which produces an insoluble dark red-brown reaction product. The background and negative control sections should be light tan.

11. Wash three times in PBS for 5 min each.
12. React with 0.05% (w/v) DAB containing 0.01% (v/v) H_2O_2 in PBS. The solutions used should be mixed just before use and protected from bright light. Incubate for 5 min.
13. Stop the reaction by rinsing in PBS for 5 min.
14. Post fix in 1% (v/v) glutaraldehyde (*see* Note 5) for 10 min.
15. Rinse in PBS for 5 min.

Lightly counterstain (*see* Note 6) with neutral red (especially good for brain or spinal cord sections), or use Hematoxylin or Hematoxylin eosin (*see* Chapter 45). Some slides should be kept with no counterstain.

16. Stain in neutral red for 2–4 min., dehydrate in each of ethanol 70, 95, 100, and 100% (v/v) for 15 s each. Clear in two changes of fresh xylene, 2 min for each wash.
17. Cover slip using a xylene-based mounting media.

3.2. Enhanced DAB Reaction

An enhanced DAB reaction (7), which will detect a greater amount of peroxidase in the tissue, uses the glucose oxidase reaction as the source of H_2O_2 and coats the peroxidase–DAB reaction product with nickel. The combination of these steps results in a black reaction product against a cleaner light gray background. The procedure replaces the DAB incubation (steps 12 and 13, Section 3.1.) with the following three steps:

1. Rinse in 0.1M acetate buffer, pH 6.
2. Incubate in freshly prepared GDN solution for about 20 min. Protect from bright light.
3. Rinse in 0.1M acetate buffer, pH 6.

3.3. Immunohistochemistry—Fluorescent Labeled

The simpler procedure, which uses a single tagged secondary antibody, can often be used (8). Animals are best perfusion fixed with PAF (9; *see* Chapter 45).

The pretreatment includes washing only with isotonic buffer and permeabilizing the cell membranes with Triton-X-100 to allow the antibody access to the antigen, and to block nonspecific sticking of the antigen with BSA and serum. Incubate with the primary antibody overnight at 4°C.

1. Wash in PBS, pH 7.4, for 10 min.
2. Wash in PBS, pH 7.4, with 0.1% (v/v) Triton-X-100 for 10 min.
3. Treat with PBS containing 0.1% (v/v) Triton-X-100 and 10% blocking serum for 10 min.
4. Drain the slides and apply 50–100 μL of the primary antibody diluted (*see* Note 2) in PBS containing 1% (v/v) Triton-X-100. Incubate at 4°C overnight in a moist chamber.

After a thorough wash and a second application of blocking serum, apply the secondary antibody.

5. Wash twice for 10 min each in PBS containing 0.1% (v/v) Triton-X-100.
6. Treat with PBS containing 0.1% (v/v) Triton-X-100 for 10 min.
7. Incubate for 45–60 min in secondary antibody diluted (*see* Note 2) in PBS with Triton-X-100.

After a thorough wash, cover slip the sections with an aqueous mounting media.

8. Wash in PBS, three changes for 5 min each.
9. Mount the cover slip with permafluor.

Examine the sections with a fluorescent microscope equipped with narrow band width, blue excitation wavelength filters (*see* Note 1).

4. Notes

1. The peroxidase enzyme is used here to visualize the antigen. Its reaction with diaminobenzidine (DAB) quickly yields a reliable and easily seen brown reaction product that is insoluble in alcohol and other solvents, allowing a permanent cover slip to be mounted. DAB is carcinogenic and should be handled with care. The reaction product for DAB is also electron dense, so similar protocols can be used for electron and light microscopy. The enhanced DAB system referred to in the above protocol produces excellent sensitivity to detect small amounts of antigen.

If the secondary antibody is tagged with a fluorescent molecule, such as fluorescein (FITC) or rhodamine, then following the application of this antibody, the slide can be rinsed and a cover slip mounted with an aqueous mounting media made for use with fluorescent sections and viewed with a fluorescent microscope. The fluorescent scope should be equipped with filters that pass a blue excitation light (450–490 nm wavelength) to your FITC-stained specimens and a green excitation light (510–560 nm wavelength) for the rhodamine-stained specimen. Additional filters will cut out the background of emitted light from the tissue limiting the visualization to the wavelength of interest. There is less background fluorescence from aldehyde-fixed tissues at the wavelengths used for rhodamine. The results must be photographed, since the fluorescence emitted will quench with exposure to the excitation light and the slide is not permanent. Stained slides can be stored at 4°C until ready to be examined. Warm to room temperature, and cover slip before viewing on the microscope. The aqueous mounting media should contain an anti-quench agent (for example, *p*-phenalenediamine) to slow the quenching or fading of the fluorescence. The commercial aqueous mountant suggested here contains an anti-quench agent and holds the cover slip permanently in place. The slides should be stored in the dark.

Colloidal gold is a third chromogen that yields a permanent slide and can be used for light and electron microscopy. Choose a 1 or 5 nm diameter gold particle to minimize mechanical obstruction to antibody attachment. A streptavidin–gold complex can be substituted in the above protocol in place of the streptavidin–peroxidase complex. It is then visualized for light microscopy with the noncarcinogenic silver enhancement. Kits for silver enhancing gold are available from a number of companies (Janssen Pharmaceutical [Beerse, Belgium], Sigma, and Vector Stain [Burlingame, CA] to name a few). The gold needs no enhancement for use in electron microscopy.

2. Each primary and secondary antibody employed will require empirical determination of the appropriate dilution to use. Plan on trying different concentrations of primary antibodies. Start with a concentration recommended by the manufacturer or one used for that antibody in the literature, and use dilutions above and below this. Adjust the concentrations according to the results. The secondary antibody and the streptavidin concentrations can also be changed. They should be diluted if the background staining is too dark. The negative controls and background staining should have a light tan color with the DAB reaction product and a light gray background with a nickel-enhanced reaction.

The timing used in the above protocols (overnight at 4°C) allows the use of lower concentrations of antibody. Incubations of 1 h at room temperature with higher concentrations can be used for some antigens if you are in a hurry or for less stable antigens.

3. Staining the same sections for immunohistochemistry and *in situ* hybridization (ISH) is possible, and yields information on colocalization of mRNA and its protein product (10,11). The same fixative is used for both. Perfusion fixation with Zamboni's picric acid paraformaldehyde fixation has worked well for both immunohistochemistry and ISH staining in our laboratory. Optimize the staining for the immunohistochemistry and ISH separately. Combining them will then require some adjustments. Because of the long incubation times necessary, keep the solutions and conditions as sterile as possible. Growth inhibitors can be used, but with caution. Na azide (0.1% [w/v]) is useful, but is not compatible with peroxidase staining. Thimerisol (also called thiomersal) has been used successfully (12). If the immunostaining is being done first, then an RNase inhibitor can be added to all the solutions used for immunostaining. When glutaraldehyde is used as a primary fixative or a postfixative, the amount should be kept low, 0.1–0.5% (v/v), and one should expect to need enzyme pretreatment for either immunohistochemistry or ISH done following its application. If the antigen is unusually stable, the ISH can be done first, followed by the immunohistochemistry. The chromogen used for the first reaction must be stable to all solvents used subsequently. DAB or gold provides a stable marker. The second chromogen need not be stable, but must be a contrasting color. For example, a DAB reaction with its red-brown product can be used for one, whereas a radioactive label (black grains) or silver-enhanced gold (again a black label) is used for the other. Each of the steps involved in these protocols affects the others, and in working them out, a balance must be found that allows the whole to function.
4. When doing immunohistochemistry, it is essential to run negative controls along with the experimental tissue. The negative control can be done most easily by omitting the primary antibody from some slides and leaving everything else the same. Any reaction product produced by endogenous enzyme or nonspecific sticking will be seen on this slide, and will not be considered a positive signal. A second way to produce a negative control is to preabsorb the primary antibody with an excess amount of purified antigen and then to use this as a primary antibody on some slides.
5. Glutaraldehyde (1% [v/v]) is applied to the sections after immuno-

staining. This immobilizes and stabilizes the antigen/antibody complex and the enzyme used to detect it. It is an optional step useful for some antigens. In some cases, paraformaldehyde is sufficient for stabilization. These can be applied prior to DAB staining.

6. Counterstaining is often desirable to see the tissue location of the immunostaining better. Some sections should be left without counterstain to maximize the clarity of the immunostaining, since the counterstain can mask some areas of immunostaining. A hematoxylin eosin (*see* Chapter 48) counterstain yields a familiar picture of most tissues. For central nervous system sections, the black nickel or silver enhanced reaction products will stand out well against a neutral red nissal-stained section.

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CHAPTER 47

Postmortem Examination of Transgenic Mice

Kum Fai Chooi

1. Introduction

Postmortem examination of transgenic animals should ideally be performed by or with the help of a trained pathologist. This is because although the steps in the examination can be manually performed by anyone, the interpretation of the findings can only be done with the knowledge of veterinary medicine. This chapter describes the steps in a postmortem examination (necropsy), and does not attempt to describe pathological lesions and their significance. This method is a systematic approach to detecting gross abnormalities. These abnormalities can then be further examined at the microscopic level or be submitted to other laboratory tests (*see* Note 1). Prior knowledge of the anatomy of the animal species examined is necessary (1). Always perform necropsies on freshly killed animals or as soon as they are discovered dead. Necropsies performed on animals that have been dead for more than 24 h generally do not yield much useful information or material for further study.

The postmortem examination should be performed with a complete history of the case foremost in the investigator's mind. The information should include:

1. Strain of animal (Balb/c, CBA, and so forth);
2. Age;
3. Sex;

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These data quickly alert the investigator to the kinds of conditions that may or may not be present. For example, female C3H-A mice, older than 7 mo, are prone to mammary tumors. Tumors of the lymphoid tissue may occur in older C57Bl and SJL mice.

4. The transgene introduced;
5. Recent or relevant manipulations performed (drug, surgical, or other treatments) and observations on the state of the animal (alert, quiet, incoordinated, other abnormal functions, or gross abnormalities); and
6. Results of previous necropsies, if animals of the same or a similar line have already been examined.

A complete necropsy should be performed when a new transgene is being studied. The transgene may cause changes in organs where it is not expected to or no changes in organs where it is expected to.

2. Materials

- 1 Dissecting board and pins.
2. Scalpel, scissors, forceps, and other dissecting instruments.
3. Fixatives of choice, e.g., 70% (v/v) alcohol, 10% (v/v) buffered formalin, or Bouin's solution (*see* Notes 2–5).
4. 10% Buffered formalin (keeps for months at room temperature):
 - Formalin (40% formaldehyde) (100 mL)
 - Sodium dihydrogen phosphate monohydrate ($\text{NaH}_2\text{PO}_4 \cdot \text{H}_2\text{O}$) (4 g)
 - Anhydrous disodium hydrogen phosphate (Na_2HPO_4) (6.5 g)
 - Water to 1000 mL
5. Bouin's solution (make up fresh):
 - Picric acid, saturated aqueous solution (75 mL)
 - Formalin (40% formaldehyde) (25 mL)
 - Glacial acetic acid (5 mL)

Fix tissues for 24 h. Store tissues in 70% (v/v) ethanol.
6. Containers with labels (for organs to be fixed in, e.g., Sterilin containers)
7. Anesthetic (e.g., Avertin, *see* Chapter 14).
8. Gloves (**never work without gloves**; beware of zoonotic diseases).
9. Biohazard bags (or similar for disposal of organs).

3. Methods

1. Observe the animal first in its cage and then on the table for signs of abnormality. Sick animals are usually listless, huddle in a corner, will tend not to move even when removed from the cage. Careful examination of the animal while still alive will alert the investigator to concentrate on any abnormal organ/structure during the postmortem examination.

2. Euthanize the animal (for mice, *see* Chapter 17).
3. Examine the animal when it is dead. Note any abnormalities. This is to derive as much information as possible in preparation for subsequent steps in the investigation. For example, palpate any lumps. Palpation indicates if a lump is an abscess, cyst, or excessive tissue growth. An abscess would need to be excised under sterile conditions if the pus is to be collected for microbiological examination.
4. Lay the animal on its back. Stretch it out, and pin each of the four legs down through the footpad.
5. Wet the skin with 70% (v/v) alcohol. Make an incision in the skin from the lower jaw to the genital area.
6. Retract the skin, and pin it down with pins.
7. Use forceps to pick up the midline of the body wall, incise with scissors, and cut anterior and posterior.
8. Break the mandible at the point in between the incisors. Hold onto the tongue with forceps, and dissect out the tongue and attached organs: esophagus, trachea, lungs, and heart. Sever the esophagus at the diaphragm with care not to spill stomach contents. In the event that the stomach is engorged with food, tie the distal esophagus with a short piece of thread before severing.
9. Examine the thoracic organs before the abdominal organs to avoid contamination with abdominal contents. With a pair of scissors, cut into the esophagus from anterior to posterior. Examine the exposed surface. Palpate the pulmonary lobes. With scissors, cut into the trachea, starting from the larynx all the way into the bronchi. Examine the exposed surfaces. The heart is normally examined attached to the lungs. Make incisions in both the right and left side of the heart to examine the auricles and ventricles (*see* Note 6).
10. Examine and dissect the liver at its hilus from the alimentary tract. Check that the bile duct is patent by gently squeezing the gallbladder and watching for the flow of bile. Incise into the liver parenchyma.
11. Remove and examine the spleen. Make random incisions into the parenchyma.
12. Remove the entire gastrointestinal tract (stomach to rectum) and lay aside (*see* Note 7). Examine the pancreas. Cut along the greater curvature of the stomach, and representative lengths of duodenum, jejunum, ileum, large intestine, and rectum.
13. Examine the adrenal glands before the urogenital organs. For the latter, start by removing the kidneys from their attachment to the body wall, leaving the ureters attached at the hilus. Gently dissect out the ureters until they connect with the bladder. Dissect out the bladder and the urethra to the external orifice. In females, remove and examine the ova-

- ries, oviduct, and uterus together with the urinary tract. In males, remove and examine the testis, epididymis, and vas deferens together with the urinary tract. Make a longitudinal cut in the kidney to separate it into two equal halves. This is done to expose the cortex, medulla, and pelvis. Peel away the renal capsule, and examine the cortical surface. Cut into the bladder and urethra to examine the internal surfaces.
14. Examination of the head and brain is normally done at the end of routine necropsies. Sever the head from the neck at the junction of the occipital condyles and the atlas (first cervical vertebra). Do this from the ventral aspect. Examine the entire head region if this has not been done. Remove the skin. Using scissors, cut away the cranium. Start at the base of the skull, and cut upwards. Lift the cranium, and free it from the underlying meninges. Remove the brain starting from anterior to posterior, cutting at the cranial nerves at the base to free the brain. If the brain is to be examined histologically, place entire organ intact in 10% (v/v) buffered formalin (*see* Notes 2 and 3). If brain is not to be kept, cut in 0.5-cm transverse sections for inspection.
 15. Remove the eye from the socket. Start by cutting from the lateral canthus both dorsally and ventrally to meet at the medial canthus. If eye is to be examined histologically, place entire organ intact in Bouin's solution (*see* Notes 3 and 4). If it is not to be kept, make a longitudinal cut through the midline, including the optic nerve to separate the organ into two. Examine the internal structures.
 16. To remove the Harderian glands, first remove the eyes. Reach back into the socket with forceps, and gently pull out the horseshoe-shaped gland in the back of the socket.
 17. Record all abnormal findings. A brief description of the significant gross abnormalities detected should be recorded for every animal that is necropsied. The necropsy record should also indicate the type of organs and tissues collected for further examination. For example, brain and mammary gland may be collected for histopathological examination, but the liver may well be retained for microbiological analysis. It takes time for histopathological and other laboratory examinations. Thus, proper records make it easier to keep track of a case until all the tests are finalized.

Analysis of lesions in transgenic animals can be complicated if there is concurrent disease in the experimental animals. It cannot be stressed enough that the examination should be done with the aid of people knowledgeable in veterinary medicine and pathology.

4. Notes

1. Although a systematic examination is recommended, the investigator should collect samples for histological examination as soon as they are detected to minimize postmortem changes. Take representative area(s) of the tissue of interest/lesion, and place in fixative as soon as possible. Always include adjacent normal/unaffected tissue. Tissues should be dissected with sharp blades and with clean cuts in the plane of interest. Do not assume abnormalities seen in the same organ are identical. For example, differential diagnoses for nodules in the ventral abdominal area could include mammary tumors, skin tumors, or granulomas. When in doubt, take samples from all abnormal areas.
2. Organs must occupy no more than 10% of the total volume of the fixative that they are kept in. For all organs, 10% (v/v) buffered formalin can be used. However, eyes and testis are better preserved in Bouin's solution.
3. Avoid fixing entire organs (except for the brain and eye, which must be "hardened" before they can be trimmed properly for histological examination). If it is necessary to have the entire organ, make incisions at intervals to allow penetration of fixative. The thickness of organs should not exceed 5 mm for best penetration of fixative.
4. When Bouin's solution is used, remember to change to alcohol after 24 h to minimize irreversible yellow staining of tissues.
5. Place small organs into individually labeled containers.
6. The heart can be examined in more detail if necessary. This involves examination starting from the right auricle, right ventricle, left auricle, and left ventricle in that order. The semilunar valves and atrio-ventricular valves are also examined (2).
7. The digestive tract is best examined separately from the rest of the organs to minimize contamination with intestinal contents. This can be done at the end of the necropsy or with separate sets of instruments in a separate area of the examination table.

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CHAPTER 48

Histological Examination of Transgenic Mice

Kum Fai Chooi

1. Introduction

Histological examination is important to detect and differentiate among different types of tissue changes at the microscopic level. An enlarged organ can be hyperplastic, hypertrophied, or neoplastic. It can also be merely inflamed or infected. Only a histological examination can differentiate among the various growth disturbances and pathological changes at the microscopic level. Histopathology is indispensable when neoplasia induced by transgenes is the subject of study (1–4).

Tissues removed from the animal need to be processed before they can be examined under the microscope. The steps involved are fixation, dehydration, clearing, impregnation, embedding, sectioning, and staining. The aim of fixation is to prevent tissue decay, and maintain tissue and cellular structure for study. Tissue should be treated as soon as possible after removal. The amount of fluid used should be at least ten times the volume of the tissue. The most common fixative used is 10% (v/v) buffered formalin. The aim of dehydration is to remove water from the tissue for the later step of paraffin embedding. Clearing involves replacing alcohol with a wax solvent, since alcohol and wax are not miscible. Toluene is a suitable reagent for this purpose. Impregnation of the tissues supports them when they are sectioned. Tissue is finally embedded with molten paraffin wax and sectioned.

The hematoxylin and eosin (H and E) stain is routinely used for preliminary examinations. This is usually sufficient to give initial

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information on the nature of the lesion. For further analysis of tissue sections, various types of special staining can be employed ranging from staining for tissue components and microorganisms to immunohistochemical staining.

2. Materials

1. Automatic tissue processor (a series of glass jars, if this is not available).
2. Microtome.
3. Tissue-embedding center (Reichert-Jung, Cambridge Instruments, Buffalo, NY).
4. Staining dishes and racks (various sizes).
5. Circular water bath (Shandon/Lipshaw, Pittsburgh, PA).
6. Warming plate (for slides).
7. Harris's hematoxylin. The following components are needed:
 - Hematoxylin (2.5 g)
 - 100% (v/v) ethanol (50 mL)
 - Ammonium alum (aluminum ammonium sulfate) (50 g)
 - Distilled water (*see* Note 1; 500 mL)
 - Mercuric oxide (1.5 g)
 - Glacial acetic acid (20 mL)Prepare as follows:
 - Dissolve the alum in hot distilled water.
 - Dissolve the hematoxylin in 100% (v/v) ethanol.
 - Mix the two solutions. Heat to boiling. Remove from heat.
 - Add mercuric oxide (beware of excessive bubbling). Reheat briefly.
 - Cool flask in running water.
 - Add glacial acetic acid. Filter if too much precipitate is present.
8. Eosin.
9. Glass slides and cover slips (clean and grease-free).
10. Slide boxes and slide racks (Thomas, Swedesboro, NJ).
11. Ethanol.
12. Toluene.
13. Gelatin.
14. Paraffin wax/Paraplast (Thomas).
15. Diamond marker for slides.
16. Flat forceps.

3. Methods

3.1. Tissue Preparation

1. Fix the tissues in 10% (v/v) buffered formalin (*see* Chapter 47) for at least 24 h (*see* Notes 2 and 3).
2. Trim the tissues to the required size and to the region of interest (5).

- Place the tissues in the automatic tissue processor. Tissues can be manually taken through the same series of solutions, with constant agitation throughout. The following regime should be employed:

Reagent	Routine (total 18 h, overnight)
50% (v/v) alcohol	2 h
70% (v/v) alcohol	2 h
95% (v/v) alcohol	2 h
100% (v/v) alcohol	2 h
100% (v/v) alcohol	2 h
Toluene	2 h
Toluene	2 h
Paraplast	2 h
Paraplast	2 h

In most machines, the beakers are static and hold the processing fluids, whereas the tissues are held in individual containers or cassettes in a suspended basket, and mechanically moved from one beaker to another. Agitation is provided by vertical or rotating movements of the basket. Tissues can be routinely processed overnight so that they are ready for embedding the next morning.

3.2. Embedding (for Centers with Cassettes)

Techniques vary with different models. It is best to follow the manuals provided. The following general steps apply (*see* Note 4).

- Warm the base mold. Fill the mold with paraffin wax until half full.
- With hot forceps, place the specimen at the bottom of the mold with the surface to be sectioned face downwards.
- Place the mold on the chiller plate for a few seconds to fix the specimen.
- Place the cassette bottom on top of the mold. Fill with paraffin.
- Place on the cold plate for final cooling. The specimen is now ready for sectioning.

3.3. Microtomy

- Cut embedded tissues at 4–5 μm thickness. (Good-quality sections require an understanding of the equipment used and practice with guidance. *Also see* Note 5.)
- Transfer the ribbon of sections cut from the microtome to the circular water bath (45–50°C with 1% [w/v] gelatin added). Place the free end of the ribbon on the water first and spread the rest over the water surface.
- Separate individual sections by touching the junction between sections with the end of a pair of flat forceps.

4. Pick up the sections by passing a slide under them and gently lifting out of the water. Drain off excess water carefully. Label the slides with a diamond pencil on the same side as the section to prevent the section being wiped off during later examination or staining.
5. Place the slides directly on a slide warmer (55–60°C) for 15 min. After the water has evaporated, transfer the slides to an upright position in a slide rack to prevent dust settling. Leave overnight.

3.4. Staining Procedure (see Notes 4–10)

Routine hematoxylin and eosin:

1. Remove paraffin wax by submerging the slide in xylene, 2 × 10 min.
2. Dip in absolute alcohol, followed by 95% alcohol (2 min) and 70% alcohol (2 min).
3. Wash in distilled water (1 min).
4. Stain in hematoxylin (1 min).
5. Wash in running tap water (not distilled or Milli-Q) until the section is blue (approx 1 min).
6. Dip in acid /alcohol one to four times (a few seconds each time).
7. Repeat step 5.
8. Treat with 1% (w/v) aqueous eosin for 2 min.
9. Wash in running tap water (approx 2 min).
10. Dip in 70% (v/v) followed by 90% (v/v) alcohol (1 min each).
11. Wash in absolute alcohol, two changes (2 min each).
12. Clear in two changes of xylene (2 min each).
13. Mount in a suitable medium (Entellen, Merck). Allow sufficient time for the mounting medium to dry.
14. Examine under light microscope (nuclei are blue, cytoplasm are pink). See references 5, 6, and 7, for descriptions of normal tissues.

4. Notes

1. Use distilled water (neutral pH) to make up stains, unless tap water is specified.
2. Place the issue in fixative immediately after removal from the animal. This is to minimize postmortem changes and to maintain cellular structure.
3. Bone and other calcified tissue cannot be sectioned unless the calcium salts are removed. Refer to decalcification procedure in (8)
4. During embedding, do not allow the specimen to dry or harden.
5. Ensure that the knife is sharp and set at the correct angle in the microtome.
6. Constituents of stains should be dissolved in the order given in the formula.
7. Glassware used in staining should be thoroughly cleaned and rinsed in distilled water, and dried.

8. The staining procedure is ideally carried out by placing slides in a staining rack and submerging in a staining dish containing the solutions as stated.
9. A control section of tissue should be tested with each new batch of stain as a routine.
10. Special stains: Different components of a tissue can be differentiated more clearly by the use of special staining procedures. For example, Periodic acid-Schiff stains basement membranes and allows them to be differentiated from the surrounding connective tissue. For more information on staining of the different tissue components, refer to reference 8.

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CHAPTER 49

Reporter Enzyme Assays

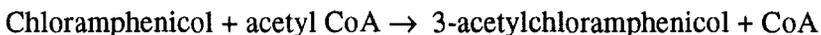
Karen Pardy

1. Introduction

Reporter genes code for proteins that have a unique enzymatic activity and are used to assess the transcriptional properties of DNA elements. The use of reporter genes in transgenic animals provides a rapid method for the detection of transgene expression, which is easily distinguishable from expression of the corresponding endogenous gene of the animal. The regulatory sequences of a chosen gene are fused to a readily assayable protein coding region, examples of which are chloramphenicol acetyl transferase (CAT), β -galactosidase, and luciferase. Sensitive assays are available for each of these proteins that facilitate detection and quantitation of transgene expression. The use of these reporter enzymes allows a more rapid and sensitive method of detection than the analysis of specific transgene transcripts within the transgenic animals. The reporter enzymes described in this chapter are CAT, β -galactosidase, and luciferase.

1.1. Chloramphenicol Acetyl Transferase

CAT is a bacterial enzyme and has the advantage that it has no mammalian counterpart. The enzyme catalyzes the following reaction:



The CAT assay was originally described by Gorman et al. in 1982 (1) and is based on the extent of acetylation of ^{14}C chloramphenicol using acetyl CoA as the acetyl donor group. The acetylated products of this reaction are then separated from the nonacetylated substrate

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by thin-layer chromatography and analyzed by autoradiography. This process of separation is time-consuming and has led to the development of nonchromatographic extraction methods. Sleight (2) has developed a nonchromatographic assay for CAT activity that uses an organic solvent to extract both the acetylated and nonacetylated forms of chloramphenicol from labeled acetyl CoA. This assay is both sensitive and rapid, and is the method described in this chapter.

1.2. β -Galactosidase

The enzyme β -galactosidase, which catalyzes the hydrolysis of β -galactosides, including lactose, is encoded by the *E. coli* *LacZ* gene (3). The enzyme activity is measured by a simple photometric assay that measures the hydrolysis of the substrate *o*-nitrophenyl β -D-galactopyranoside (ONPG) by β -galactosidase in cell-free extracts. β -galactosidase can also be monitored histochemically using the substrate X-Gal (5-bromo-4-chloro-3-indoyl β -D-galactoside) (4). A potential disadvantage of using β -galactosidase as a reporter enzyme is the presence of endogenous β -galactosidase activity in some mammalian tissues, including brain. However, the pH optimum for this enzyme is low (pH 3.5), whereas that of the *E. coli* enzyme is pH 7.3 (5). The presence of false positives can be minimized by performing the assay at pH 7.5 and by including normal tissue extract as a control.

1.3. Luciferase

The reporter gene for luciferase was cloned from the firefly (*Photinus pyralis*), a bioluminescent organism (6). Firefly luciferase catalyzes the following reaction (7):



The expression of firefly luciferase is measured in the presence of ATP and luciferin by the emission of light, which allows very rapid detection of transgene expression. The emission of light is measured using a luminometer, which detects both the peak of the light flash and the integrated light output over a period of time. The assay is very sensitive and may be used in the analysis of the transcriptional activity of weak promoters. The luciferase assay also has the advantage of using a nonradioactive substrate, and there is no similar enzyme activity in mammalian cells.

2. Materials

2.1. Chloramphenicol Acetyl Transferase Assay

1. 0.25M Tris-HCl, pH 7.8.
2. 0.25M Tris-HCl, pH 7.8, and 5mM EDTA.
3. 8 mM Chloramphenicol.
4. 0.5 mM Acetyl CoA (Sigma, St. Louis, MO).
5. ¹⁴C acetyl CoA (Amersham, Amersham, UK).
6. Ethyl acetate (4°C).
7. Liquid scintillation vials.
8. Scintillation fluid.
9. Liquid scintillation counter.

2.2. β -Galactosidase Assay

1. 0.1M Sodium phosphate, pH 7.5.
2. ONPG: 4 mg/mL in 0.1M sodium phosphate, pH 7.5.
3. 0.1M MgCl₂ and 4.5M β -mercaptoethanol.
4. 1M Na₂CO₃.
5. Spectrophotometer.
6. Cuvets: 0.5–1.0 mL.

2.3. Luciferase Assay

1. Incubation buffer: 25 mM glycylglycine, pH 7.8, 15 mM MgSO₄, 4 mM EGTA, 15 mM potassium phosphate, pH 7.8, 1 mM DTT, and 2 mM ATP.
2. Luciferin solution: 0.2 mM luciferin (Sigma) dissolved in 25 mM glycylglycine, pH 7.8, 15 mM MgSO₄, 4 mM EGTA, and 2 mM DTT.
3. Luminometer with auto-injection system (Lumac Biocounter M2500, Landgraaf, The Netherlands).

3. Methods

3.1. Chloramphenicol Acetyl Transferase Assay

1. Tissue extracts are prepared by homogenization in 0.2 mL of 0.25M Tris-HCl, pH 7.8, followed by three rounds of freeze-thawing by immersion in liquid nitrogen (3 min) and in a 37°C water bath (3 min). Vortex the suspension thoroughly after the final thawing step, and then spin at 14,000 rpm in a microcentrifuge at 4°C for 10 min. Retain the supernatant for the analysis of CAT activity. Prior to assay, heat the extract to 65°C for 10 min to destroy endogenous CAT inhibitors (*see* Note 1).
2. Set up the incubation mixture as follows:

- 20 μL 8 mM chloramphenicol
- 30 μL tissue extract
- 20 μL diluted acetyl CoA solution
- 30 μL 0.25M Tris-HCl, pH 7.8, and 5 mM EDTA.

The diluted acetyl CoA solution is prepared by diluting a 0.02-mL aliquot of ^{14}C acetyl CoA containing 1 mCi with 0.5 mM unlabeled acetyl CoA to a concentration of 5 mCi/mL.

3. Incubate at 37°C for 1 h, and then transfer the samples to an ice bath.
4. Extract the labeled reaction products twice into 100- μL aliquots of cold ethyl acetate. After addition of ethyl acetate to each sample, mix the layers by vortexing, and then separate by centrifugation at 14,000 rpm for 3 min at 4°C. After the first extraction, remove 80 μL of the organic phase; after the second extraction, remove 100 μL of the organic phase and combine the two.
5. Add the combined organic extracts to 2 mL of scintillation fluid, and determine radioactivity by liquid scintillation spectroscopy.

3.2. β -Galactosidase Assay

1. Prepare tissue extracts as for the CAT assay, **but do not heat** to 65°C prior to assay (see Note 2).
2. Set up the incubation mixture as follows:
 - 3 μL 0.1M MgCl_2 and 4.5M β -mercaptoethanol
 - 66 μL ONPG
 - 30 μL tissue extract
 - 201 μL 0.1M sodium phosphate, pH 7.5.
3. Incubate the reactions at 37°C for 30 min or until a faint yellow color develops.
4. Stop the reactions by the addition of 500 μL of 1M Na_2CO_3 to each tube. Then read the absorbance of each reaction at a wavelength of 420 nm.

3.3. Luciferase Assay

- 1 Homogenize tissue in 100 mM potassium phosphate, pH 7.8, and 1 mM DTT. Then prepare tissue extracts as for CAT, **but do not heat** to 65°C prior to assay (Notes 3 and 4).
2. Set up the incubation mixture as follows:
 - 0.10 mL tissue extract
 - 0.36 mL incubation buffer.
3. To initiate the reaction, inject 0.2 mL of luciferin solution into each sample using an automatic injection system. Measure integrated light output for 5 s after injection of luciferin using the free program mode of the luminometer.

4. Notes

1. Quantification of CAT activity in tissue extracts can be achieved by including a standard curve constructed using dilutions of purified CAT enzyme (Pharmacia-LKB), in each assay.
2. Positive and negative control samples should be included in each β -galactosidase assay. A 30- μ L aliquot of normal tissue extract may be used as a negative control, and purified *E. coli* β -galactosidase is used as a positive control sample. Purified *E. coli* β -galactosidase may be used to construct a standard curve and, therefore, allow quantification of the enzyme activity of the samples.
3. Freeze-thaw lysis of tissue extracts can cause some denaturation of luciferase (7). In order to avoid any such loss of activity, the enzyme can be extracted by detergent lysis (homogenize in 1% Triton X-100, 25 mM glycylglycine, pH 7.8, 15 mM MgSO₄, 4 mM EGTa, and 1 mM DTT). The lysates are spun at 14,000 rpm for 5 min at 4°C, and the supernatants transferred to fresh tubes and vortexed briefly before the assay.
4. Luciferase activity can be visualized in intact cells and can therefore be used to study tissue-specific gene expression in transgenic animals in a noninvasive manner. It is also possible to measure luciferase activity using scintillation counters (8). However, this method is less sensitive than assay of tissue extracts.

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CHAPTER 50

Restriction Endonuclease Digestion of DNA

Duncan R. Smith

1. Introduction

The ability to cleave DNA at specific sites is one of the cornerstones of today's methods of DNA manipulation. Restriction endonucleases are bacterial enzymes that cleave duplex DNA at specific target sequences with the production of defined fragments. These enzymes can be purchased from the many manufacturers of biotechnology products. The nomenclature of enzymes is based on a simple system, proposed by Smith and Nathans (1). The name of the enzyme (such as *Bam* HI, *Eco* RI, and so forth) tells us about the origin of the enzyme, but does not give us any information about the specificity of cleavage (see Note 1). This has to be determined for each individual enzyme. The recognition site for most of the commonly used enzymes is a short palindromic sequence, usually of either 4, 5, or 6 bp in length, such as AGCT (for *Alu* I), GAATTC (for *Eco* RI), and so on. Each enzyme cuts the palindrome at a particular site, and two different enzymes may have the same recognition sequence, but cleave the DNA at different points within that sequence. The cleavage sites fall into three different categories, either flush (or blunt) in which the recognition site is cut in the middle, or either with 5' or 3' overhangs, in which case unpaired bases will be produced on both ends of the fragment. For a comprehensive review of restriction endonucleases, see Fuchs and Blakesley (2).

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2. Materials

1. A 10X stock of the appropriate restriction buffer (*see* Note 2).
2. DNA to be digested (*see* Notes 3 and 4) in either water or 1X TE (10 mM Tris-HCl, pH 8.3, and 1 mM EDTA)
3. Bovine serum albumin (BSA) at a concentration of 1 mg/mL (*see* Note 5).
4. Sterile distilled water (*see* Note 6).
5. The correct enzyme for the digest (*see* Note 7).
6. 5X Loading buffer: 50% (v/v) glycerol, 100 mM Na₂EDTA, pH 8, 0.125% (w/v) bromophenol blue (Bpb), and 0.125% (w/v) xylene cyanol.
7. Spermidine at 100 mM (*see* Note 8).

3. Methods

1. With the exception of the enzyme, all solutions are thawed and then placed on ice.
2. A final vol for the digest is decided on, usually between 10 and 50 μ L (*see* Note 9), and then to a sterile Eppendorf tube are added 1/10 vol reaction buffer, 1/10 vol BSA, between 0.5 and 1 μ g of the DNA to be digested (*see* Note 3), and sterile distilled water to make up to final vol.
3. The enzyme is taken directly from the -20°C freezer, and the desired units of enzyme (*see* Notes 7 and 10) removed from the stock pot with a clean sterile pipet tip and added to the reaction mix. The constituents are mixed (*see* Note 11).
4. The tube is then incubated at the correct temperature (*see* Note 12) for approx 1 h. Genomic DNA can be digested overnight.
5. The reaction is then normally analyzed by running an aliquot on a suitable agarose gel (*see* Chapter 51).
6. The aliquot to be analyzed (usually 1–2 μ L) is mixed with 2 μ L 5X concentrated loading buffer and sterile distilled water to give 10 μ L final vol, and then loaded onto an agarose gel for analysis (*see* Chapter 51).

4. Notes

1. Enzymes are named according to the system proposed by Smith and Nathans (*1*) in which enzymes are named according to the bacteria from which they are first purified. So, for example, a restriction enzyme purified from *Providencia stuartii* would be identified by the first letter of the genus name (in this case *Providencia* and hence *P*) and the first two letters of the specific epithet (in this case *stuartii* and hence *st*) joined together to form a three-letter abbreviation—*Pst*. The first restriction isolated from this source bacteria would therefore be called *Pst* I (with the number in roman numerals), the second *Pst* II, and so on. Note, however, that the name of the enzyme gives no information about the

specificity of cleavage, and this must be determined from one of the numerous lists of enzymes and cleavage specificities. (The catalog of most suppliers of restriction enzymes will provide extensive information about restriction enzymes, such as specificity of cleavage, optimal reaction conditions, number of cleavage sites in common DNA templates, and so on, and these catalogs should be treated as valuable sources of information.)

2. Each enzyme has an optimal reaction buffer. The recommended reaction conditions are normally to be found on the manufacturer's assay sheet. In practice, many enzymes share common conditions, and it is possible to make up reaction buffers that are suitable for a number of enzymes. The vast majority of enzymes will work in one of three buffers, either a high-, low-, or medium-salt buffer, recipes for which are given below. These buffers are normally made as a 10X stock and then 1/10 final vol is added to each digest. Great care must be taken in matching the buffer to the enzyme, since the wrong buffer can give either a dramatically reduced activity, altered specificity, or no activity at all. Several manufacturers of restriction enzymes now provide the correct buffer with their enzymes as an added benefit, and it is recommended that where these buffers are provided, they are used.
 - High-salt buffer (1X): 100 mM NaCl, 50 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, and 1 mM DTT.
 - Medium-salt buffer (1X): 50 mM NaCl, 10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, and 1 mM DTT.
 - Low-salt buffer (1X): 10 mM Tris-HCl, pH 7.5, 10 mM MgCl₂, and 1 mM DTT.

In addition, two "universal buffers" are occasionally used, which are buffers in which all restriction enzymes have activity, although in some cases, activity can be reduced to only 20% of optimal activity. These are the potassium-glutamate buffer (3) and the potassium-acetate (4) buffers. These buffers can be particularly useful when a piece of DNA must be digested by two enzymes having very different optimal buffers.

3. The amount of DNA to be digested depends on subsequent steps. A reasonable amount for a plasmid digestion to confirm the presence of an insertion would be 500 ng to 1 μ g, depending on the size of the insert. The smaller the insert, the more DNA that should be digested to enable visualization of the insert after agarose gel analysis.
4. The DNA to be digested should be relatively pure and free from reagents, such as phenol, chloroform, alcohols, salts, detergents, and chelating agents. Any trace amounts of these chemicals will inhibit or inactivate the restriction endonuclease activity.

5. Bovine serum albumin is routinely included in restriction digests to stabilize low protein concentrations and to protect against factors that cause denaturation.
6. Good-quality sterile distilled water should be used in restriction digests. Water should be free of ions and organic compounds, and must be detergent free.
7. An enzyme unit is defined as the amount of enzyme required to digest 1 μg of a standard DNA in 1 h under optimal temperature and buffer conditions. The standard DNA used is normally λ DNA. Hence, for *Eco* RI (for example), there are five sites for this enzyme in λ . If one is digesting pBR322 which has one site with 1 U of enzyme for 1 h, this is actually a fivefold overdigestion.
8. Digests of genomic DNA are dramatically improved by the inclusion of spermidine in the digest mixture to a final concentration of 1 mM, since the polycationic spermidine binds negatively charged contaminants. Note that spermidine can cause the precipitation of DNA at low temperatures and so should not be added while the reaction is kept on ice.
9. The smallest practical volume in which to undertake a restriction digest is 10 μL . Below this, pipeting errors can introduce significant errors in the reaction conditions. This volume also allows the entire digest to be loaded onto a small agarose gel after the addition of the stop/loading buffer. If the stock DNA concentration is too dilute to give 0.5–1 μg in 5–6 μL , then the reaction can be scaled up to 20–50 μL . If double-digestion is to be undertaken (i.e., digestion with two different enzymes), then the recommended minimum vol is 20 μL and then 1 μL of each enzyme can be added and the glycerol concentration kept low (see Note 10).
10. Many enzymes are susceptible to the presence of glycerol. The majority of stock enzymes are provided in approx 50% (v/v) glycerol. A restriction digest in which more than approx 10% (v/v) glycerol is present can give cleavage at different sites from the normal (the so-called “star” activity). For this reason, it is advisable to keep the enzyme volume:reaction volume at 1:10 or lower. Similar “star” activity can result from incorrect salt concentrations.
11. Stock restriction enzymes are very heat labile and so should be removed from -20° storage for as short a time as possible.
12. Note that the incubation temperature for the vast majority of restriction endonucleases is 37°C , but that this is not true for all enzymes. Other enzymes, such as *Taq* I and *Sma* I, require different optimal temperatures (in this case, 65° and 25°C , respectively). It is wise therefore to check new or unfamiliar enzymes before use.

13. If large-scale preparative digests are to be undertaken (100–500 μL reaction mixes), then the reaction is scaled up accordingly. However, care must be taken to ensure that the reaction components are fully mixed, especially with regard to the viscous constituents, such as DNA solutions and stock restriction enzymes. For all volume digests, vortexing should be avoided, since this can significantly reduce the activity of the enzyme. For small volumes, mixing can be achieved by “tapping” or gently flicking the tube with a finger (often followed by a brief 1–5-s spin in an Eppendorf centrifuge to deposit the reaction at the bottom of the tube). For larger volumes, mixing can be achieved by gentle pipeting, taking liquid from the bottom of the reaction volume and mixing at the top of the reaction volume until a homogenous solution is achieved.

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CHAPTER 51

Agarose Gel Electrophoresis

Duncan R. Smith

1. Introduction

After digestion of DNA with a restriction enzyme (Chapter 50), it is usually necessary, for both preparative and analytical purposes, to separate and visualize the products. In most cases, where the products are between 200 and 20,000 bp long, this is achieved by agarose gel electrophoresis. Agarose is a linear polymer that is extracted from seaweed and sold as a white powder. The powder is melted in buffer and allowed to cool, whereby the agarose forms a gel by hydrogen bonding. The hardened matrix contains pores, the size of which depends on the concentration of agarose. The concentration of agarose is referred to as a percentage of agarose to volume of buffer (w/v), and agarose gels are normally in the range of 0.3 to 3%. Many different apparatus arrangements have been devised to run agarose gels; for example, they can be run horizontally or vertically, and the current can be conducted by wicks or the buffer solution. However, today, the “submarine” gel system is almost universally used. In this method, the agarose gel is formed on a supporting plate, and then the plate is submerged into a tank containing a suitable electrophoresis buffer. Wells are preformed in the agarose gel with the aid of a “comb” that is inserted into the cooling agarose before the agarose has gelled. Into these wells are loaded the sample to be analyzed, which has been mixed with a dense solution (a loading buffer) to ensure that the sample sinks into the wells.

Apparatus for electrophoresis consists of four main parts. A power supply (capable of at least 100 V and currents of up to 100 mA), an electrophoresis tank, a casting plate, and a well-forming comb. Apparatus for electrophoresis is available from many commercial suppliers and tends to be fairly expensive. However, electrophoresis apparatus is arguably one of the most vital pieces of equipment in the laboratory. Alternatively, apparatus can be "homemade" with access to a few sheets of perspex and minor electrical fittings. The construction of such apparatus is outside the scope of this chapter, but can be found in Sambrook et al. (1), Sealey and Southern (2), or Boffey (3).

The migration of DNA in an electric field depends on a large number of factors, such as conformation of the DNA (i.e., supercoiled, linear, or nicked circle), composition of the buffer used (type of buffer and ionic strength), and percentage of agarose. However, the essence of electrophoresis is that when DNA molecules in an agarose gel matrix are subjected to a steady electric field, they first orient in an end on position (4,5) and then migrate through the gel at rates that are inversely proportional to the \log_{10} of the number of base pairs (6). Larger molecules migrate more slowly because of greater frictional drag and because they worm their way through the pores of the gel less efficiently than smaller molecules (1). Note, however, that this only applies to linear molecules, and circular molecules (such as plasmids) migrate much more quickly than their mol wt would imply because of their smaller apparent size with respect to the gel matrix. An excellent treatment of the theory of gel electrophoresis can be found in Sambrook et al. (1).

2. Materials

1. Molecular-biology-grade agarose (high melting point; *see* Table 1).
2. Running buffer at 1X and 10X concentrate (Table 2).
3. Sterile distilled water.
4. A heating plate or microwave oven.
5. Suitable gel apparatus and power pack (*see* Section 1.).
6. Ethidium bromide in water at 10 mg/mL (*see* Note 1).
7. An ultraviolet (UV) light transilluminator (long wave, 365 nm).
8. DNA to be analyzed (*see* Note 2).
9. 5X Loading buffer (*see* Note 3): Many variations exist, but this one is fairly standard: 50% (v/v) glycerol, 50 mM EDTA, pH 8.0, 0.125% (w/v) bromophenol blue, and 0.125% (w/v) xylene cyanol.

Table 1
Resolution of Agarose Gels

Agarose %	Mol-wt range	Comments
0.2%	5–40 kb	Gel <i>very</i> weak; Separation in 20–40 kb range improved by increase in ionic strength of running buffer (i.e., Loenings E; <i>see</i> Table 2); only use HMP agarose
0.4%	5–30 kb	With care can use LMP agarose.
0.6%	3–10 kb	Essentially as above, but with greater mechanical strength
0.8%	1–7 kb	General-purpose gel separation not greatly affected by choice of running buffer; bromophenol blue runs at about 1 kb
1%	0.5–5 kb	As for 0.8%
1.5%	0.3–3 kb	As for 0.8% Bpb runs at about 500 bp
2%	0.2–1.5 kb	Pour while hot, and do not allow to cool to 50°C
3%	0.1–1 kb	Can separate small fragments differing from each other by a small amount; must be poured rapidly onto a prewarmed glass plate.

3. Methods

1. An appropriate amount of powdered agarose (Table 1) is weighed carefully into a conical flask.
2. One-tenth final volume of 10X concentrated running buffer is added (Table 2).
3. Distilled water to the final vol is added, and the contents of the flask mixed by swirling.
4. The flask is placed on a hot plate or in a microwave until the contents just start to boil (and all the powdered agarose is melted).
5. The contents are cooled to approx 50°C, and ethidium bromide solution added to give a final concentration of 0.5 µg/mL. The gel mixture can then be poured into the gel apparatus.
6. A comb to provide wells is inserted into the apparatus, and the gel is left until solid.
7. When the gel has set, the comb can be carefully removed and the solidified gel (still on its gel plate support) placed into the running apparatus, which is filled with 1X running buffer, above the level of the wells.
8. The DNA samples are then loaded into the wells of the gel. All samples are loaded at the same time. It is usual to include a marker in one of the lanes (i.e., a sample that has been predigested and for which the prod-

Table 2
Commonly Used Agarose Gel Electrophoresis Running Buffers

Loenings E buffer

High-ionic strength and not recommended for preparative gels

For 5 L of 10X

218 g Tris base

234 g $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$

18.6 g $\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$

Glycine buffer

Low-ionic strength, very good for preparative gels, but can also be used for analytical gels.

For 2 L 10X

300 g glycine

300 mL 1M NaOH (or 12 g pellets)

80 mL 0.5M EDTA, pH 8.0

Tris-borate EDTA (TBE buffer)

Low-ionic strength can be used for both preparative and analytical gels.

For 5 L 10X

545 g Tris

278 g Boric acid

46.5 g EDTA

TAE buffer (Tris-acetate buffer)

For 1 L 50X

242 g Tris base

57.1 mL glacial acetic acid

100 mL 0.5M EDTA (pH 8.0)

uct band sizes are well known). Many such markers are commercially available.

9. The lid to the apparatus is closed and the current is turned on (*see* Note 4).
10. The gel is usually run between 1 and 3 h, depending on the percentage of the gel and its size.
11. After electrophoresis, the sample is removed from the apparatus, and the products of the digestion can be viewed on a UV transilluminator (*see* Note 1)

4. Notes

1. Many workers do not like to include ethidium bromide in their gels and their running buffers, preferring instead to stain their gels after electrophoresis. It must be noted that ethidium bromide promotes the damage of DNA when viewed under UV light (by photoniccking). For this reason, if the gel is run with ethidium bromide in the gel and the running

buffer, it is best to limit the amount of viewing of the gel to prevent damage to the DNA molecule and subsequent smearing on reelectrophoresis. However, with this limitation in mind, there is no noticeable difference between running the gel with and without ethidium bromide, providing that one system is solely used (i.e., either always run your gels with ethidium in the gel and running buffer, or never). The reason for this is that ethidium intercalation can affect the mobility of the DNA, especially where circular plasmids (either supercoiled or nicked circle) are concerned. However, if the presence or absence of ethidium is kept constant, then no difficulty is encountered. Note that ethidium bromide is both carcinogenic and mutagenic, and therefore must be handled with extreme caution.

2. The amount of DNA that can be visualized in a single band with ethidium bromide staining can, in ideal circumstances, be as low as 10 ng. In general circumstances, a fragment consisting of approx 100 ng of DNA will provide a well-visualized band. Note that these figures are for single bands. If a digestion produces a large number of bands, then relatively more DNA will have to be loaded to ensure that all bands are seen.
3. Loading buffer is a dense solution (usually either glycerol or Ficoll), which when mixed with a DNA solution (or restriction digest) gives the sample sufficient density to fall to the bottom of the sample well, which is already filled with running buffer. Loading buffers normally contain either one or two marker dyes, which migrate in the electric field in the same direction as the DNA. Two commonly used dyes are bromophenol blue and xylene cyanol. These dyes migrate at different rates from each other. In a 0.8% (w/v) agarose gel, bromophenol blue migrates with DNA of approx 1 kb. Xylene cyanol in the same gel migrates at approx 4 kb. These dyes are useful for monitoring the progress of an electrophoretic run and, thus, ensuring that the DNA does not pass out of the bottom of the gel.
4. When running an analytical gel, the optimal resolution is obtained at about 10 V/cm of gel. When fragments of 5 kb and above are to be analyzed, better resolution is obtained at about 5 V/cm. Fragments smaller than 1 kb are normally resolved better at higher V/cm.

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CHAPTER 52

Working with Radioactivity in the Transgenic Animal Laboratory

Duncan R. Smith

1. Introduction

In recent years there has been a large increase in the number of non-radioactive methods of detection of nucleic acids (*see* Chapter 45). Despite this, the vast majority of laboratories still utilize radioactive labeling. Although it is possible to foresee that in 10–15 years radioactive nucleotides and X-ray film will be unheard of in the laboratory, for now they remain the system of choice.

In general, radioactive labeling of nucleic acids, using the methods such as random-primer labeling (Chapter 53), gives a greater degree of simplicity, sensitivity, and flexibility. Coupled with this is an increase in the use of automated imaging systems, which can detect the pattern of radioactivity in a gel or sample and provide a quantitative computer image. These systems offer advantages in the manipulation of the image, with regards to contrast and manipulating the data to provide statistical data and publishable figures. Despite this, the vast majority of laboratories still rely on the use of autoradiography to detect the position and intensity of the radioactivity.

Many manufacturers produce radioactive products for molecular biology (such as Amersham, Amersham, UK and NEN-DuPont, Boston, MA). It is possible to buy nucleosides and nucleotides labeled with a wide variety of isotopes such as ^{35}S , ^{32}P , ^{14}C , and ^3H . These

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four isotopes represent the most commonly used isotopes in the laboratory. All four of them are beta emitters, although the maximum energy of them varies greatly. This means that while common precautions must be taken as regards to handling them, their shielding requirements differ.

2. Safety

Ionizing radiation is both mutagenic and carcinogenic. Hence, care must be taken when handling radioactive isotopes, and in disposing of contaminated reagents and tubes. Each worker has a moral (and in many countries a legal) responsibility to ensure not only his or her own safety, *but also the safety of his or her colleagues*. In most countries, a system of licensing of radioactive workers exists, in which workers must have a basic familiarity with handling radioactivity, or work under a license holder.

In general, one handles radioactivity as one would handle any toxic chemical or pathogen, and so all the usual laboratory rules apply. Thus, one should always wear gloves and a lab coat and not consume food or drink in the laboratory. The major difference with radioactivity is that, when handling energetic emitters (such as ^{32}P), precautions must be taken to limit the exposure to ionizing radiation.

The other radioactive isotopes commonly encountered in the molecular biology laboratory, such as ^{35}S , ^3H , and ^{14}C , have such weak beta particles that all ionizing radiation is stopped by millimeters of air. Thus, providing they are external sources, they present no hazard to the worker. With weak beta emitters (^3H , ^{14}C , ^{35}S), radiochemical hazards are only encountered when the radioactivity enters the body (either orally or via wounds and so forth). With ^{32}P , the beta particles are very energetic (a range of 720 cm in air) and so exposure to ionizing particles from external sources can present a real hazard.

Three factors are involved in determining the exposure from a source of ionizing radiation. These are *time*, *distance*, and *shielding*. Thus, the less time that you handle the radioactivity the lower the exposure. The further you are away from a source of ionizing radiations, the lower the exposure. The more shielding between you and the radioactive source, the lower the exposure. In the laboratory, the most important of these factors is shielding. When handling ^{32}P , work is always undertaken behind a perspex screen about 1-cm thick (*see below*). Perspex of this thickness has the advantage of stopping all

beta particles (without producing harmful secondary radiation) as well as being transparent, thus allowing the worker to observe the experiment. In the laboratory the worker must always be aware of the three principles of time, shielding, and distance. Experiments must be planned and undertaken as quickly as possible and always behind a shield of perspex. In most laboratories an area (or ideally a separate room) is clearly designated for the handling of radioactive materials. This enables people not actively involved in radioactive work to be as far away as possible from a source of ionizing radiation. It is also strongly recommended that all equipment used for radioactive work (centrifuges, vacuum pumps, and the like) be clearly marked as such. This equipment should not be used for nonradioactive work. Similarly, radioactive materials should not contact equipment designated solely for nonradioactive use.

The radiation worker must remember that, when working with ^{32}P , it is impossible not to receive a measurable dose of radioactivity. However this should *only* be on the hands and lower forearms, and the use of proper shielding should prevent any whole body dose. The use of personal dosimeters is absolutely recommended (where this is not a legal requirement). Where large amounts of radioactivity (hundreds of microcuries to millicuries) are to be handled, the use of finger dosimeters is also recommended.

Molecular biologists face a particular hazard when handling radioactivity in that they are often working with relatively large amounts of radioactivity in a small volume of liquid. A small droplet can deposit enough radioactivity to contaminate a whole laboratory. This is usually caused by people relatively new to handling radioactivity. However, one of the main advantages of working with strong beta emitters (such as ^{32}P) is that a hand-held monitor (a Geiger-Mueller tube) can easily detect spillages, which can quickly be cleaned up. Every worker should therefore check the work area before, during, and after an experiment. The liberal usage of Geiger counters is to be strongly encouraged.

Weak beta emitters (^3H , ^{35}S , and ^{14}C) require no special shielding as the reaction vessels themselves provide enough protection. However, spillages of radioactivity are much harder to detect. Each worker should check for spillages by taking regular bench swabs, which are assayed in a scintillation counter.

3. Perspex

As discussed earlier, perspex is the ideal shielding material for ^{32}P . When lead is used as a shielding material, the beta particles are decelerated so quickly by the heavy lead nuclei that secondary X-rays are produced (“Bremsstrahlung”). Therefore an additional thickness of lead must be used to stop the secondary radiation. For this reason quite thick lead shielding must be used for ^{32}P . In contrast, perspex slows the beta particles less abruptly, and so no secondary radiation is produced. A 1-cm thickness of perspex is sufficient to shield from ^{32}P . Perspex has the added advantage of being transparent so the operator can easily see the experiment and manipulate them when required. Perspex shields, racks, and storage boxes can easily be made and several commercial manufacturers (such as Stratagene) now make purpose-designed perspex equipment for use in the molecular biology laboratory.

4. Solution

Radioactive nucleotides are normally shipped from the manufacturer in either Tricine (*N*-tris [hydroxymethyl]methylglycine) or water: ethanol (1:1).

In the case of the former, the nucleotide may be used directly in almost all reactions (Nick translation, random prime, sequencing, and so forth). In the latter the nucleotide *must* be desiccated under vacuum and redissolved in water before usage.

5. Specific Activity

Radioactive nucleotides are available with a wide range of specific activities. The researcher must be aware of this and select the appropriate activity for the experiment. Specific activity is an indication of the amount of label to the amount of carrier and is normally described as the number of curies per millimole. Common specific activities are in the range 400–3000 Ci/mM. The correct specific activity must be used for the correct experiment. For example a Sanger dideoxy sequencing reaction requires 1 μL (10 μCi) of ATP at 400 Ci/mM. If ATP at a specific activity of 3000 Ci/mM is used then 75 μCi of radioactivity is required to give the same molarity of ATP (of course the 3000 Ci/mM ATP could be diluted down with cold ATP to give a specific activity of 400 Ci/mM). In most cases, where the radioactive nucleotide is incorporated (primer extension, nick translation, ran-

dom prime labeling, sequencing, and so forth) the specific activity of the label is an important parameter.

6. Iodine

Iodine is not commonly encountered in the transgenic animal laboratory. However, there are occasions when its use is required, especially when protein analysis is undertaken. Most of the above recommendations must be modified when handling iodine. Iodine can represent a considerable hazard to the worker and those around. Firstly, in the molecular biology laboratory the isotope most commonly encountered is ^{125}I . This has a half-life of 60 d and is a gamma emitter. Iodine will most often be encountered initially as free iodine, which is highly volatile. Therefore this must be handled in a powerful fume hood, preferably in a specially designed room. Once the iodine has been conjugated to the molecule of interest the hazard from volatility is removed. As a gamma emitter, iodine is not shielded by perspex. Lead must therefore be used. Thick lead bricks must be used to shield successfully from the gamma rays. Waste products must be disposed of separately from other wastes. It is important when regularly handling iodine to have frequent thyroid checks, as this organ selectively takes up this element.

7. Spillages and Decontamination

Any activity in the wrong place is a spill and must be dealt with immediately. The spilt material should be wiped up with a swab of tissues, which are placed in the appropriate waste receptacles. The area is then decontaminated. Try the following decontamination materials in the following order: (a) distilled water, (b) water and detergent (decon 90), (c) a solution of EDTA, and (d) a+b+c+ion-exchange medium (fullers earth).

Decontamination of ^3H , ^{14}C , and ^{32}P should not be considered complete until a swab or tissue wiped over 100 cm^2 of the contaminated area gives $<1500\text{ dpm}$ when added to 10 cm^3 of scintillation fluid in a vial.

When contamination occurs on clothing, the clothing should be removed as quickly as possible and washed by normal laundry procedures. A solution of EDTA is useful in difficult cases.

Contaminated skin should be brushed for a long time with soap and warm water using a *soft* brush. If this is unsuccessful try titanium paste or EDTA soap. Skin decontamination should *never* be continued to the extent of damaging the skin.

CHAPTER 53

Random Primed Labeling of DNA

Duncan R. Smith

1. Introduction

Random primed labeling of DNA has now almost superseded the method of nick translation of DNA. Random primed labeling, based on the method of Feinberg and Vogelstein (1) is a method of incorporating radioactive nucleotides along the length of a fragment of DNA. Random primed labeling can give specific activities of between 2×10^9 and 5×10^9 dpm/ μ g (*see Note 1*). The following method is essentially that described by Feinberg and Vogelstein (2) in which a DNA fragment is denatured by heating in a boiling water bath. Then, random sequence oligonucleotides are annealed to both strands. Klenow fragment polymerase is then used to extend the oligonucleotides, using three cold nucleotides and one radioactively labeled nucleotide provided in the reaction mixture, to produce a uniformly labeled double-stranded probe. Each batch of random oligonucleotides contains all possible sequences (for hexamers, which are most commonly employed, this would be 4096 different oligonucleotides) and so any DNA template can be used with this method.

2. Materials (*see Note 2*)

- 1 DNA fragment to be labeled in water or 1X TE (*see Note 3*).
2. OLB buffer. Make up the following solutions :
 - Solution O: 1.25M Tris-HCl, pH 8.0, 0.125M MgCl₂ (store at 4°C).
 - Solution A: 1 mL Solution O, 18 μ L β -mercaptoethanol, 5 μ L dATP*, 5 μ L dTTP*, 5 μ L dGTP*. *Where each nucleotide has been previously dissolved in 3 mM Tris-HCl, 0.2 mM EDTA, pH 7.0 at a concentration of 0.1M.

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- Solution B: 2M HEPES, titrated to pH 6.6 with 4M NaOH. (Store at 4°C.)
 - Solution C: Hexadeoxyribonucleotides evenly suspended (this does not completely dissolve) in TE at 90 OD A₂₆₀ U/mL. (Store at -20°C.)
- To make OLB buffer, mix solutions A:B:C in a ratio of 100:250:150. Store OLB at -20°C.
4. A nucleotide labeled at the alpha position with phosphorous-32 (i.e., ³²P-dCTP; specific activity 3000 Ci/mM).
 5. Klenow fragment polymerase at 1 U/μL.
 6. Bovine serum albumin (BSA) at 10 mg/mL in water.
 7. A boiling water bath.

3. Methods (see Note 4)

1. Take about 30 ng of DNA to be labeled (the probe) and increase the volume to 31 μL with sterile distilled water
2. Boil the probe for approx 3 min.
3. Place tube immediately on ice.
4. In the following order add: 10 μL of OLB buffer, 2 μL of 10 mg/mL BSA, 5 μL of labeled nucleotide, and 2 μL of Klenow fragment. Mix all together by gentle pipetting.
5. Incubate at room temperature for 4–16 h (see Note 5).
6. Purify the probe if necessary (see Note 6).
7. Check incorporation of the radioactive moiety if necessary (see Note 7).
8. Use the probe for hybridization analysis at approx 25 ng/mL (see Note 8). Remember to denature before use.

4. Notes

1. Specific activity of the probe can be increased by using more than one nucleotide. It is possible to use all four nucleotides as labeled nucleotides, but with the already high specific activities obtainable by this method there are very few circumstances where this could be justified
2. Many manufacturers now produce kits (Amersham, Amersham, UK, NEN-Dupont, Boston, MA) for use in random primed labeling of DNA. These kits are simple and efficient.
3. This protocol is for purified DNA. In most cases the DNA can be used without purifying the DNA after preparative gel electrophoresis. In this case the gel slice is diluted with sterile distilled water at a ratio of 3 mL of water per gram of gel slice, the DNA denatured and the gel melted by boiling for 7 min, and then aliquoted into several tubes for either storage at -20°C (in which case the sample is boiled for 3 min before using) or immediate use.

4. In some cases (such as clone/clone Southern analysis) the random primed reaction can be scaled down to half the amounts given above.
5. Incubation times are optimal after about 4 h, whereby greater than 70% of the radioactivity has been incorporated, although it is often convenient to leave the reaction overnight (12–16 h).
6. It is usually not necessary to purify the probe. If the reaction has proceeded correctly, approx 70% of the label will be incorporated. The unincorporated label does not interfere with subsequent usage, although probes can be purified by spin columns (Chapter 11).
7. Incorporation of the activity can be checked by diluting down an aliquot of the multiprime reaction to give something in the order of 10^4 to 10^5 dpm in 1–10 μ l (50 μ Ci = 1.1×10^8 dpm). An aliquot of the diluted radioactivity is then spotted onto two Whatman DE81 disks. One of these is washed five times in 0.5M Na_2HPO_4 followed by two washings in water and one in 95% ethanol. Both filters are then dried and counted in a liquid scintillation counter in an aqueous scintillation fluid. The unwashed filter gives the total radioactivity in the sample (and so can be used to correct for counting efficiency), whereas the washed filter measures the radioactivity incorporated into the nucleic acid.
8. Note that with this method there is a net synthesis of DNA.

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CHAPTER 54

Filter Hybridization

David Murphy

1. Introduction

This chapter describes a standard method for the hybridization of labeled DNA probes to nucleic acids bound to a nylon matrix (1). Filters bearing bound nucleic acids produced by Northern blotting of RNA (Chapter 39), Southern blotting of DNA (Chapter 37), and slot blotting of DNA (Chapters 35) or RNA (Chapter 40) are hybridized to labeled probes using the method described below. The advantages of this method are, first, that the use of a high concentration of SDS in the hybridization buffer ensures a low background level of non-specific probe adherence to the membrane and, second, an extended period of filter prehybridization is not required. The inclusion of a large amount of SDS does, however, necessitate that the nucleic acids are covalently bonded to the matrix by UV light crosslinking. The inclusion of formamide (15% [v/v]) is also recommended in order to reduce the viscosity of the hybridization buffer. Formamide also has the effect of reducing the temperature of the hybridization reaction.

2. Materials

1. Hybridization buffer: 0.5M Na phosphate buffer, pH 6.8, 7% (w/v) SDS, 1 mM EDTA, 15% (v/v) formamide (Fluka; see Note 1).
2. Wash buffer: 50 mM Na phosphate buffer, pH 6.8, 0.1% (w/v) SDS.
3. Stripping solution. 0.1% (w/v) SDS in autoclaved water.
4. Heat sealer.
5. Shaking heated water bath.

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3. Methods

3.1. Hybridization (see Note 2)

1. Following UV light covalent crosslinking of the Northern, Southern, or slot-blot filter (see Chapters 35, 37, 39, and 40), wet the filter by floating it on wash buffer.
2. Prehybridize the filter by incubation in hybridization buffer at hybridization temperature for 5 min (see Note 3).
3. Hybridize by incubation at hybridization temperature (see Note 3) in hybridization buffer containing probe (see Note 4; usually <25 ng/mL) for at least 12 h with constant agitation in a shaking water bath (see Note 5).
4. Carefully store the probe (most probes can be used a number of times; see Note 6) and wash the filter twice in 200 mL of wash buffer at room temperature and four times in 200 mL of wash buffer at hybridization temperature (see Note 3).
5. Wrap the filter in thin plastic (e.g., Saran wrap) and expose to X-ray film. Do not allow the filter to dry.

3.2. Rehybridization of Filters

1. Filters can be stored (wrapped in plastic) at -20°C indefinitely.
2. To strip a filter of its old probe, rinse for 5×2 min in 250 mL of freshly boiled stripping solution.
3. Rinse once in wash buffer then hybridize directly. No prehybridization step is required.

4. Notes

1. Formamide should be deionized before use. To do this, formamide is mixed and stirred for 1 h with Dowex XG8 mixed bed resin, followed by filtration through 3MM paper. Fluka (Buchs, Switzerland) formamide (catalog number 47670) does not require deionization.
2. Note that these methodologies have been developed for neutral nylon membranes (e.g., Amersham Hybond-N) and have not been tested on positively charged membranes (e.g., Amersham [UK]Hybond-N+, Bio-Rad [Richmond, CA] Zeta-Probe, NEN-Du Pont [Boston, MA] Genescreen Plus).
3. The hybridization temperature (T_{hyb}) is usually 15°C lower than the melting temperature of the probe (T_{m}), i.e.:

$$T_{\text{hyb}} = T_{\text{m}} - 15^{\circ}\text{C}.$$

The T_{m} of the probe is dependent on its length (L ; in bases), its G/C content (%GC), the molar concentration of Na^+ (M) in the hybridiza-

tion buffer, the percentage of mismatch between probe and target (1% mismatch will alter the T_m by 1°C; %M), and the formamide content (percentage) of the hybridization buffer (%F). The following formula approximately applies to synthetic (oligonucleotide) probes up to 100 bases long:

$$T_m = 16.6 \log_n(M) + 0.41(\%GC) + 81.5 - (\%M) - 675/L - 0.65(\%F)$$

Oligonucleotide probes over 40 bases in length with perfect match to the target should be hybridized and washed at 65°C using the conditions described here. Random-primed (Chapter 53) or nick-translated DNA probes (both of which will have an average length of 100–200 bases) with perfect match to the target should also be hybridized and washed at 65°C. The hybridization temperature of shorter or mismatched probes should be calculated using the above formula, then tested empirically.

4. Probes are usually radioactively labeled nucleic acids prepared as described in Chapters 43 and 53. A variety of nonradioactive detection methods are now becoming commercially available. If using these systems, refer to manufacturer's instructions. The digoxigenin system marketed by Boehringer Mannheim (Mannheim, Germany) has been shown to be compatible with the methods described here (Murphy, unpublished observations).
5. Hybridization should be performed in a tightly sealed vessel using as little probe as possible. Usually this has meant the use of strong plastic bags, sealed with a bag sealer, and incubated in a water-filled plastic box in a shaking water bath. Such containment systems are prone to leaks and contamination, and can be extremely messy, both when applying and removing the hybridization solution. However, in recent years a number of companies have produced simple and safe hybridization and washing systems (e.g., Hybaid, Twickenham, UK), which when used properly can also improve the quality of the results obtained.
6. Radioactive probes in hybridization buffer can be used several times. Used single-stranded probes require no processing, but double-stranded probes need to be denatured. Following incubation in a boiling water bath for 10 min, double stranded are quenched on ice prior to reuse.

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CHAPTER 55

DNA Transfection

Karen Pardy

1. Introduction

Prior to the introduction of transgene constructs into animals, it is useful first to determine the presence of a functional promoter and transcription unit. This can be achieved by transient transfection of the construct into heterologous cell lines and subsequent measurement of reporter enzyme activity in cell extracts. Following ligation of the chosen promoter sequence to the reporter enzyme coding region in a suitable plasmid vector, DNA is purified and transfected into an appropriate cell line. Popular methods of transfection are the calcium phosphate method, electroporation, and lipofection. The latter method, although relatively expensive, provides a quick and reliable method of transfection and is the method described in this chapter. This method of transfection relies on a positively charged lipid, DOTMA (*N* [1-(2,3-dioleyloxy)propyl]-*N,N,N*-trimethylammonium chloride), which forms liposomes that interact with DNA and RNA and carries them into mammalian cells in culture (1). DOTMA is available commercially and has been shown to be suitable for a variety of cell lines.

2. Materials

1. Appropriate cell line and growth medium, e.g., CV1 cells in DMEM (Gibco-BRL, Gaithersburg, MD), 10% fetal calf serum.
2. 37°C incubator (humidified).
3. 95% O₂: 5% CO₂
4. OptiMEM I reduced serum medium (Gibco-BRL).
5. Lipofectin reagent (Gibco-BRL).
6. Purified plasmid DNA: 1–2 mg/mL in H₂O (*see* Note 1).

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7. Phosphate buffered saline (PBS).
8. Polystyrene tubes (e.g., Falcon 2064, Becton Dickinson, Parsippany, NJ) (*see* Note 2).

3. Methods

1. Plate cells in a 35-mm tissue culture dish in 2 mL of appropriate culture medium. Incubate cells at 37°C until they are 90–95% confluent.
2. For each 35-mm dish, dilute separately 0.6–12 µg plasmid DNA to 50 µL in OptiMEM I (reduced serum medium) and 18–30 µg lipofectin reagent to 50 µL in OptiMEM. The DNA and lipofectin reagent must be diluted separately to avoid precipitation.
3. Combine 50 µL of diluted DNA and 50 µL of diluted Lipofectin Reagent in a *polystyrene* tube (e.g., Falcon 2024, *see* Note 2) to obtain 100 µL complex solution per tissue culture dish. Mix gently (do not vortex) and let stand for 5 min at room temperature.
4. Wash the cells twice with OptiMEM I to remove serum that may inhibit transfection (*see* Note 3).
5. Add 2 mL of OptiMEM I to the cells and swirl plates gently to ensure all cells are covered.
6. Add 100 µL of lipofectin reagent DNA complex to the cells dropwise as uniformly as possible. Swirl plates gently to mix.
7. Incubate the cells for 5–24 h at 37°C in a humidified 5–10% CO₂ environment.
8. Remove medium.
9. Add 2 mL of appropriate culture medium to the cells and incubate at 37°C in a humidified 5–10% CO₂ environment for 48–72 h.
10. Harvest the cells by aspirating the medium and wash the monolayers twice with PBS.
11. Add 0.5 mL of PBS and harvest cells by scraping with rubber plunger. Transfer the cell suspension to an Eppendorf tube. Harvest the remaining cells with a second 0.5-mL aliquot of PBS.
12. Pellet cells by centrifugation for 5 min, 5000 rpm in a microcentrifuge at room temperature. Discard the supernatant (cell pellet may now be frozen if desired).
13. Resuspend the pellet in appropriate buffer for reporter enzyme assay

4. Notes

1. Plasmid DNA is prepared by alkaline lysis followed by purification using a CsCl gradient (2).
2. Do not use polypropylene tubes to mix DNA and lipofectin reagent, as the complex tends to adhere to these tubes.
3. Prior to harvest, cells should be washed thoroughly as albumin and other

serum components may interfere with reporter enzyme assays, e.g., chloramphenicol acetyl transferase.

4. In vitro expression of the reporter enzyme is not always a good indicator of expression in vivo.

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