CHAPTER

Genetic Engineering and Molecular Technology

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Overview: Use of Genetic Methods in Vertebrate Biology

Scientists have been performing genetic manipulations and analyses since Mendel first reported the heritable nature of physical characteristics in pea plants in 1865. With respect to rats, experimental biologists who crossbred rat strains for a variety of traits (Chapter 1) were occasionally rewarded by the advent of an unexpected functional or structural phenotype. Such serendipitously acquired mutant lines, most of which result from a spontaneous mutation to a single gene, were often expanded and characterized to serve as animal models of human diseases. However, a large-scale random breeding program to produce new lines with novel mutations would be very costly in rodents, even if combined with treatments that accelerate the mutation rate (e.g. Kasarkis *et al.*, 1998). Furthermore, random breeding schemes and mutagenic agents cannot be employed effectively to produce defects in specific genes of interest.

Fortunately, the revolution in molecular biology of the past few decades has made possible the intentional engineering of rat models for many human diseases (Heideman, 1991; Paul *et al.*, 1994;

Charreau *et al.*, 1996; Mullins and Mullins, 1996). The introduction of specific genes (and thereby new physiological properties) into the well-characterized biological backgrounds of common laboratory animals, including the rat, provides an *in vivo* means of examining the mechanisms by which the genes of interest control, and are regulated by, other molecules. The combined use of animals in which a gene has been either overexpressed, suppressed or deleted offers unique opportunities for basic researchers to dissect gene function at a fine level of detail. In addition, genetically engineered rats provide applied scientists with a new means of evaluating innovative therapeutic agents for potential efficacy, either by using rats in which human-like disease models have been engineered (e.g. hypertension (Paul *et al.*, 1994); myelinopathy (Sereda *et al.*, 1996)) or by creating rats in which one or more functional human genes have been introduced (arthritis (Hammer *et al.*, 1990); gene therapy (Jaffe *et al.*, 1992); lipoprotein metabolism (Swanson *et al.*, 1992)). The trend in academia and industry is to establish research teams in which scientists with divergent fields of expertise collaborate in the production and analyses of such models using many different tools.

Regardless of the ultimate application, genetically manipulated animals of any species are engineered and analyzed using comparable molecular techniques. A detailed technical consideration of these methods is beyond the scope of the current discussion. Therefore, we have chosen to focus on considerations that may affect the design, construction and interpretation of experiments in which genetically engineered rat models are employed. Where certain techniques have not yet been achieved in rats (e.g. 'knockouts'), we have extrapolated likely strategies from protocols used currently in mouse genetic engineering to provide background material for understanding their eventual application in rats.

Tools for Exploring Interesting Genes

Genes of biological interest that may be evaluated using rat models can be classed into several categories. First, a gene and its product may have an endogenous and rat-specific function, but this normal rat expression may confound efforts to understand certain aspects of human biology. An example of such a gene is α_2-microglobulin, a hepatic protein which collects in rat (but not human) renal epithelium following exposures to certain rat-specific chemical carcinogens, but only in male rats of strains that express the gene (Dietrich and Swenberg, 1991). Gene targeting ('**knockout**') technology can be employed to investigate the nature of such rat genes by deleting the gene and examining the effects (if any) resulting from its absence. Second, genes and their products may be highly conserved across taxonomic groups so that characterization in rats will provide relevant data that can be extrapolated across many species. Many vertebrate genes have such properties, including growth hormone (Matsumoto *et al.*, 1993), peripheral myelin protein 22 (Sereda *et al.*, 1996) and renin (Mullins *et al.*, 1990). Proteins derived from some conserved genes may exhibit biological activity in several species, while in other instances activity exists only in the species of origin (Ganten *et al.*, 1992). Finally, genes may be inactivated or missing in rats but have an important function in another species. These latter two classes may be examined by transgenic technology through 'overexpression' of a foreign DNA fragment that has been incorporated into the rat genome.

The design of genetic engineering experiments in rats will benefit from thorough background information, which may be obtained from many sources. Genotypic (including data regarding the DNA sequence and/or the distribution and extent of gene expression at the mRNA and protein level) as well as phenotypic data (such as *in vitro* or *in vivo* functional assays) is determined within the home laboratory or is acquired through collaboration or from commercial sources. Additional information often may be located through citation databases (e.g. Medline [www.ncbi.nlm.nih.gov/PubMed/])[1] by searching with keywords relevant to the gene of interest. In our experience, categories of keywords for which citation data may exist include the common and abbreviated gene names, the gene or protein sequences, tissues in which the gene is expressed, and diseases (animal or human) in which the gene is postulated to have a role. Comparable searches performed on the Internet using a commercial

[1] For brevity,the prefix 'http://' has been omitted from all Internet addresses (URLs) in the text. This prefix should be added when the URL is used to access the World Wide Web.

biology-based search engine (e.g. BioMedNet™ [http:/biomednet.com], Yahoo [www.yahoo.com/Science/Biology]) also may yield relevant information. However, in our experience random searches of the World Wide Web do not provide sufficient information to warrant their routine use. An exception would be participation in certain discussion groups (e.g. Embryo Mail [EmbryoMail@Ipsi.bare.usda.gov]; Transgenic List [www.med.ic.ac.uk/db/dbbm/tglist.htm]) that allow ongoing, real-time exchanges of text and pictorial data between researchers working with rats.

Bioinformatics melds data acquired from such diverse fields as biochemistry, experimental biology (animal, cellular and molecular), crystallography, and mathematics – to name a few – to find broad patterns within and among species that identify genes of interest. Specific branches of bioinformatic inquiry include **genomics** and **proteomics**, or the study of sequence and functional data for genes and proteins, respectively. While some questions in bioinformatics require formal training and very powerful (and expensive) computer platforms, a growing number of commercial software packages are available which can provide assistance to the general scientist. Examples of such applications include programs to compare a gene sequence with other gene sequences (e.g. BLAST and dbEST [both at www.ncbi.nlm.nih.gov]; GeneQuest™ from DNASTAR, Madison, WI, USA), software to select molecular probes for genetic analyses (e.g. Oligo™, National Biosciences, Plymouth, MN, USA), and compilations describing protein structure and function (e.g. Swiss-PROT [www.genebio.com/sprot.html]). Large collections of molecular data representing the pooled efforts of many laboratories are increasingly available for comparison of gene and protein sequences both within a species (e.g. Rat Genetic Database [www.nih.gov/niams/scientific/ratgbase]; 'RatMap' Rat Genome Database [http:ratmap.gen.gu.se/]) and across several species (e.g. Whole Mouse Catalog [www.rodentia.com/wmc/] GenBank [www.ncbi.nlm.nih.gov/PubMed]; Mouse Genome Informatics [www.informatics.jax.org]; The Genome Database [www.gdb.org]). These resources provide cross-links to rat genetic data and provide an invaluable resource to researchers engaged in studies of the normal and genetically engineered rat genome. Finally, several databases are being compiled (chiefly for mice at present) that describe the traits of genetically engineered animals (e.g. Induced Mutant Resource Database [www.jax.org/resources/documents/imr/]; Mouse Knock-Out and Mutation Database [http://biomednet.com/db/mkmd]; Transgenic and Targeted Mutation Database [www.jax.org/tbase]). As more genetically manipulated rat models are produced, we anticipate that relevant genetic and phenotypic data will be incorporated into these or comparable databases.

The Rat as a Species of Choice for Genetic Engineering

The mouse remains a preferred species for many transgenic applications. However, the rat is more suitable for many research questions. Desirable features of rat biology that would warrant their selection rather than a mouse model include more human-like physiological responses for some disease processes (e.g. arthritis (Greenwald and Diamond, 1988); cancer (Dycaico *et al.*, 1994); hypertension (Paul *et al.*, 1994; Charreau *et al.*, 1996)), an extensive behavioral database, and larger size (better suited to surgical manipulation and repeated blood sampling; Gill *et al.*, 1989). Even so, the relatively small size of rats removes many technical and financial drawbacks associated with breeding larger transgenic mammals, such as rabbits (Mullins and Mullins, 1996). Rats also share many useful traits with mice that are lacking in other laboratory animals of intermediate size (gerbil, guinea-pig, hamster, rabbit). These qualities include the availability of many normal and mutant strains, the presence of relatively homogeneous genetic backgrounds, and high fecundity (short gestation periods and good response to superovulation protocols; Robl and Heideman, 1994).

The successful production of transgenic rats was first described in the last decade (Hammer *et al.*, 1990; Mullins *et al.*, 1990). Since this time, new lines have been introduced rarely because few facilities routinely perform this technique in rats. Nevertheless, transgenic rat models provide essential mechanistic information that often assists in the biochemical dissection of human disease (e.g. chronic hypertension, autoimmune spondyloarthritides). In fact, rat models have often proven to have a

closer relationship to the human disease than do transgenic mice in which similar genetic material was inserted (reviewed in Charreau *et al.*, 1996). Genetic manipulation of rats will probably increase in the future where patent provisions for engineered animals (Lesser, 1995) promote such intellectually and economically profitable activities.

Several strains have been used successfully in the production of transgenic rats. Ideally, the use of genetically homogeneous inbred rat strains is preferable since variations in transgene expression or physiology arising from differences in genetic background can complicate the subsequent phenotypic analysis. Certain inbred strains, notably Fischer 344 (F-344) rats (Dycaico *et al.*, 1994; Veniant *et al.*, 1996; Lefevre *et al.*, 1997), have been used with success for genetic engineering. However, in practice most transgenic rats have been derived from outbred strains (e.g. Sprague-Dawley, Wistar) due to the ready availability of efficient superovulation protocols (Chapter 10; Mukumoto *et al.*, 1995) and the better reproductive performance of outbred rats (reviewed in Charreau *et al.*, 1996).

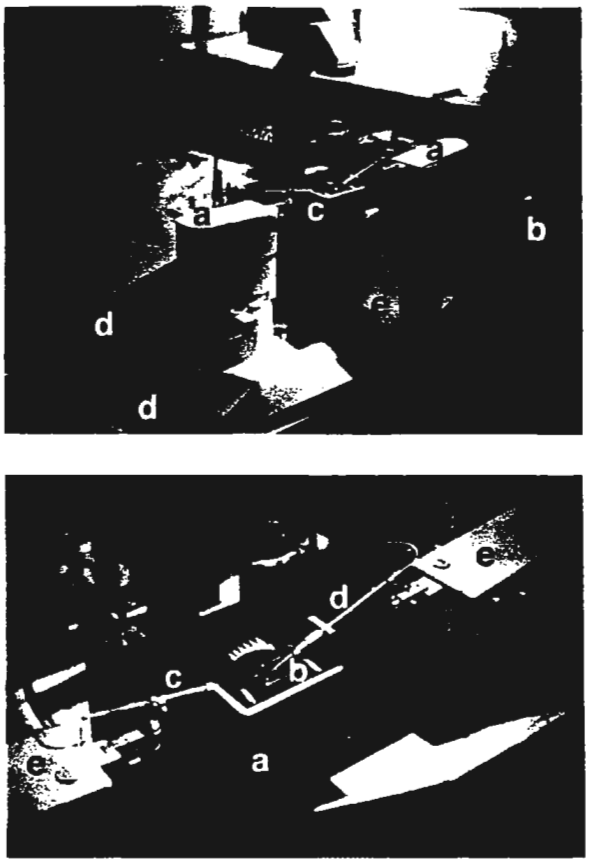


Figure 29.1 *Apparatus for pronuclear microinjection.* Introduction of transgenic DNA into rat zygotes (single-celled embryos) requires a suitable microscope (top panel) with servo-controlled pipettes for manipulating the cells (bottom panel). The layout of the injection station shown in panel A includes the micromanipulator controls for positioning the zygote (a), microliter pipette controls for introducing the DNA solution into a zygote (b), microscope stage (c), injector unit with keypad for controlling the delivery rate of the DNA solution (d), and microscope stand (e). In panel B, the microscope stage (a) supports a fluid-filled chamber (b) that contains suspended zygotes. The cells are immobilized with a holding pipette (c) and injected using a microinjection pipette (d). The placement of each pipette in three dimensions is controlled using the micromanipulator controls (e).

Genetic Manipulation of Rats

Equipment

A well-equipped molecular genetics facility contains dedicated laboratory areas for a variety of functions. The tasks to be performed in the production and analysis of genetically engineered rats will include recombinant DNA techniques (for generation of the transgene or targeting sequence as well as for analysis of the integration and expression of the inserted gene), cell culture, in-life and post-mortem assays of a transgene's effects on function and structure at the whole-animal and tissue levels, and photographic documentation. All instruments, reagents, and work areas for the molecular manipulations and cell culture activities must be separate from those used for in-life studies and post-mortem tissue processing. The specialized injection equipment used to transfer genetic material into single-celled (zygotes) or multicelled (blastocysts) embryos (Figure 29.1) can be housed in any room with good lighting. However, for the practical purposes of proximity to the animal rooms and cleanliness, this equipment is often placed in a surgical suite located within the transgenic animal facility. Tissue harvests are typically performed in a room that is outside the barrier that protects the animal colonies. Deliberate physical isolation of these *in vitro* and *in vivo* activities – modeled after features of facilities designed to perform the sensitive DNA amplification technique, polymerase chain reaction

(Dieffenbach and Devksler, 1993) – will help to prevent spurious analytical results in molecular assays that result from contamination of samples with extraneous animal, microbial or recombinant DNA.

Transgenic Technology

Transgenic technology is the science (and art!) whereby foreign genetic material is introduced directly into the genome of an animal. In general, the procedures used to generate transgenic rats (Robl and Heidemann, 1994; Charreau *et al.*, 1996) are comparable to those used in making transgenic mice (Hogan *et al.*, 1994; Wassarman and DePamphilis, 1993). Two methods, pronuclear microinjection and systemic gene delivery, have been employed in the production of rransgenic rat models.

Theory

Native rat genes contain alternating amino acid-encoding (exon) and noncoding (intron) regions controlled by various regulatory sequences (Figure 29.2). A typical transgene construct (Figure 29.3) consists of a gene's exons – ofren derived as a complimentary DNA (cDNA) sequence – flanked by a promoter (to control gene transcription) and a polyadenylation sequence (to enhance the stability of transgene messenger RNA). These three molecular elements need not be of rat origin, particularly where the structure and activity of the encoded protein products are conserved across species. Depending upon the nature of the scientific question, promoters are chosen based on their capacity to drive gene expression at high levels (Schmidt *et al.*, 1990) or their ability to regulate gene expression in many tissues (e.g. actin; Qin and Gunning, 1997) or at specific sites (e.g. casein or lactalbumin in mammary gland; Hirabayashi *et al.*, 1997).

During microinjection (see below), a few picoliters of solution containing about 100–200 copies of the transgene are introduced into a zygote (single-celled embryo, i.e. a fertilized ovum). Generally, integration of the transgenic DNA occurs randomly at a single site in the genome. Integration of the DNA may occur prior to DNA replication so that all cells of the rat, including the germline cells, will contain copies of the transgene. Alternatively, integration may occur after DNA replication is completed, resulting in the presence of the transgene in some but not all of the rat's cells (yielding a mosaic pattern of gene expression). Because each integration site is different, the resulting genetic background of each founder rat is unique. Transgene copies often aggregate into repeating linear arrays known as **concatemers** (Brinster *et al.*, 1985) either prior to injection of the foreign DNA or inside the rat pronucleus. Neither the number of copies in these concatemers nor their orientation during insertion can be controlled, and the number of copies often does not correlate with the degree of transgene expression. Instead, the extent of expression depends upon regulatory elements within the transgene construct; the location of transgene insertion (e.g. Clark *et al.*, 1994) – particularly with respect to enhancer or repressor sequences in the genome (Blackwood and Kadonga, 1998; Ogbourne and Antalis, 1998); and the proper orientation of the open reading frame for transcription. Levels of expression will vary greatly between different lines that contain the same transgenic construct.

Additional factors have been described that may affect the presentation and complicate the analysis of phenotypes in transgenic rats. First, transgene integration into a critical locus of the genome can cause an insertional mutation that disrupts the normal function of one or more essential genes (e.g. Woychik *et al.*, 1985). In such cases, the resulting effects of the induced mutation are superimposed on any changes associated with the expression of the transgene, and additional experiments will be required to fully separate the aspects of the genetic events. These unintended insertional mutations may have value as a guide to the genetic locus of an essential endogenous gene and, in some instances, as a novel model of a genetic disease. Insertional mutations are unique and occur in only a single animal line, while true transgenic phenotypes are comparable between all lines of transgenic mice established from different founders. Therefore, genesis of multiple lines derived from several different founder animals is a common research practice in order to confirm the presence of a true transgene-induced phenotype. Second, care must be given to controlling a variety of potential confounding conditions that may affect the severity of transgene-induced lesions. For example, the extent of arthritis and inflammatory bowel disease in rats transgenic for the human major histocompatibility complex antigen HLA-B27 is exacerbated by infection with intestinal or genitourinary tract bacteria but is ameliorated by preservation of a germ-free habitat

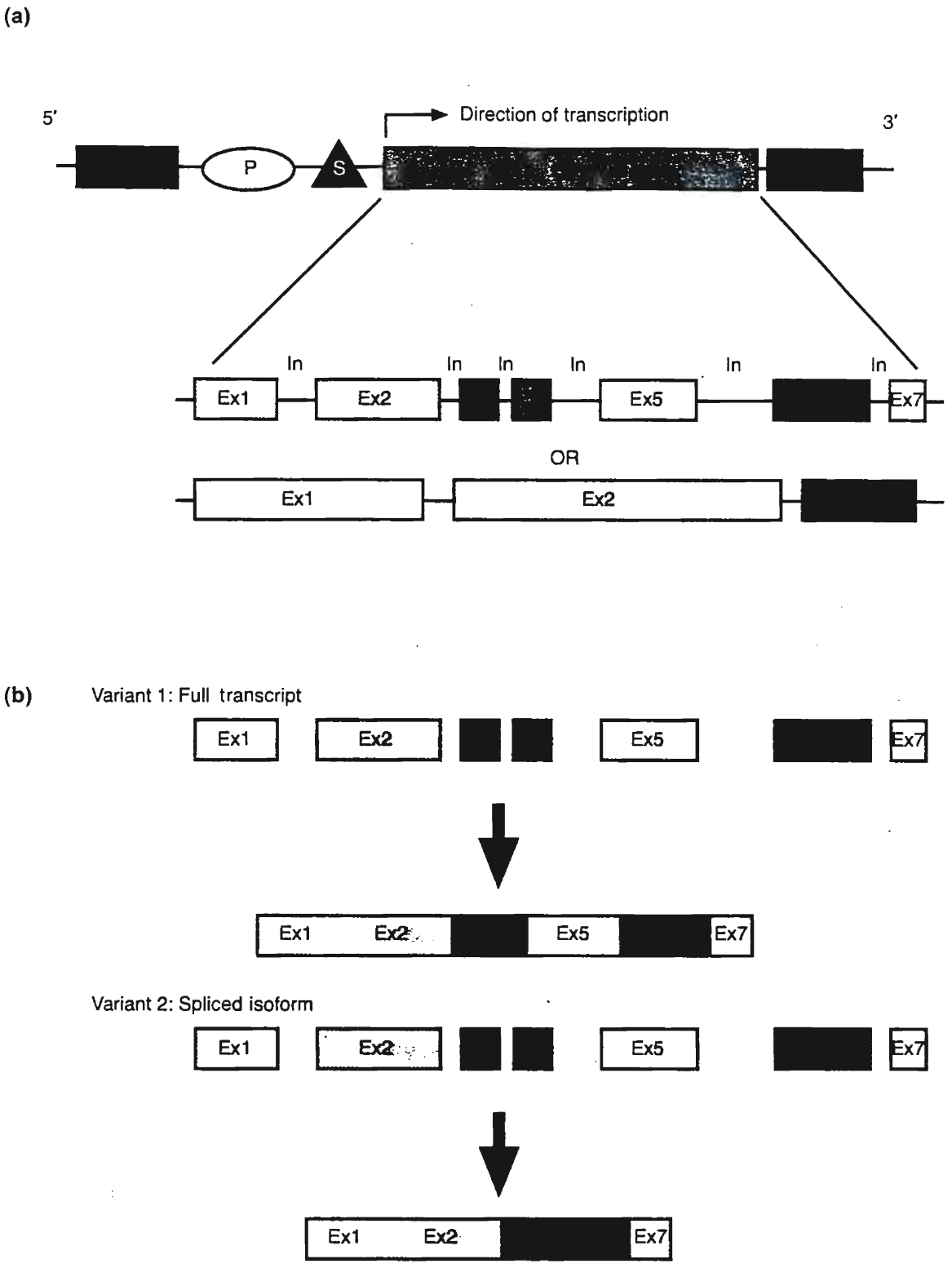


Figure 29.2 *Composition of a eukaryotic gene.* Mammalian genes often contain several regions including regulatory (R), promoter (P), signal (S), gene (G), and mRNA-stabilizing (A) sequences. Regulatory and promoter elements control gene transcription, the signal sequence indicates where transcription should be initiated, and the stabilizing sequence encodes the addition of a poly-adenosine (polyA) tail. (a) Most genes are divided into alternating amino acid-encoding regions: exon (Ex) sequences which are included in the mature RNA product, and intron (In) sequences that are transcribed but removed from the transcript as the exons are spliced together. (b) In some instances, alternative splicing during transcription of the exons yields different proteins. The lengths of the various genetic elements denote their relative number of nucleotides to indicate that not all exons are of the same size.

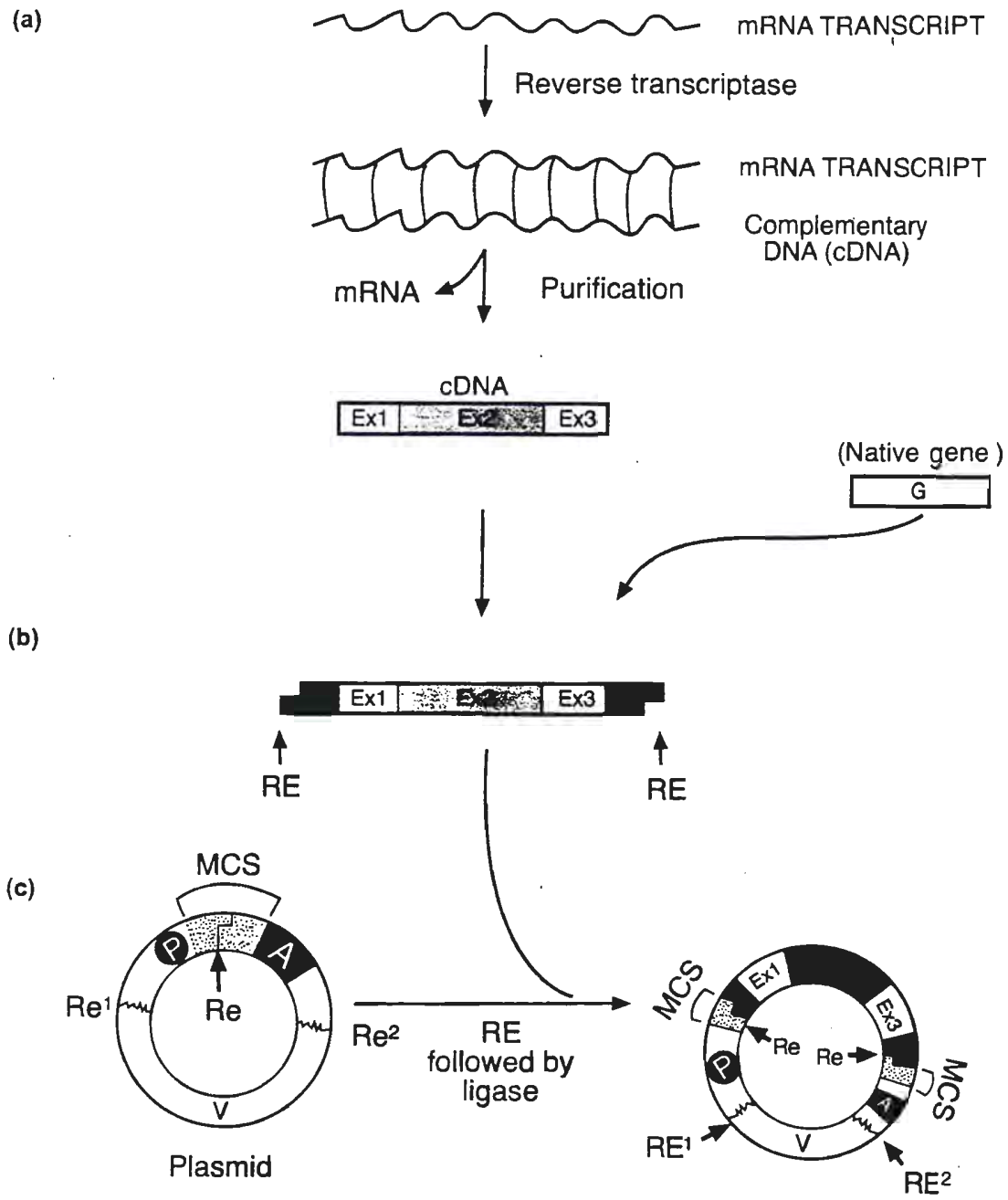
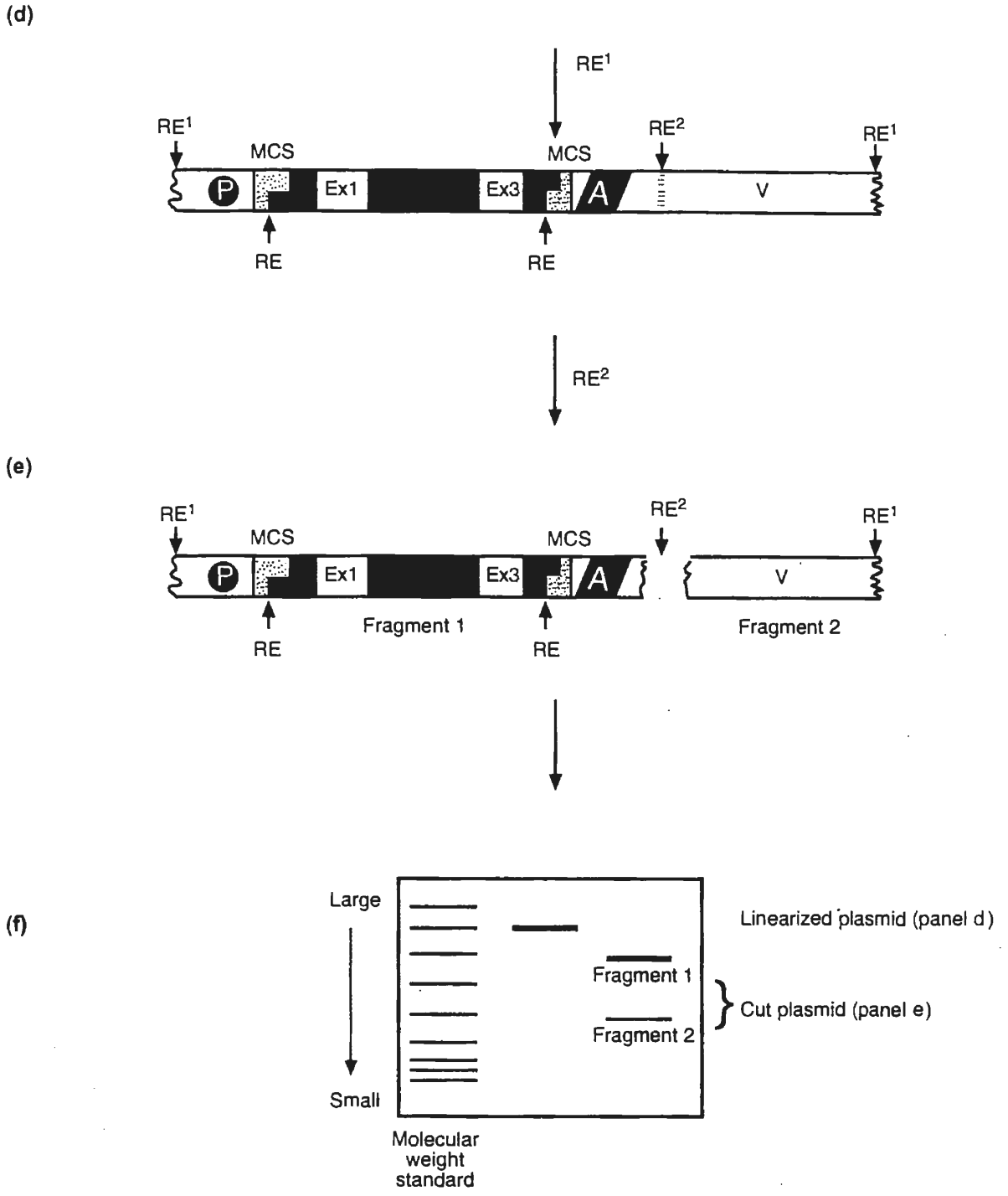


Figure 29.3 *Creating a transgenic construct.* (a) The DNA sequence of the gene of interest, or the complementary DNA (cDNA) representing the spliced exons (Ex) of the gene cloned from cellular mRNA, is the backbone on which the transgene is designed. In a series of steps, short sequences of linker nucleotides (L) containing sites for the action of a restriction enzyme (RE) are added to the ends of the DNA (b); different RE sites may be added to each end. When the DNA and the RE are mixed with a circular bacterial plasmid (the vector, V) containing a multiple cloning site (MCS; a region with cutting sites for several RE), the plasmid is cut and the DNA is inserted into the gap (c). Application of a ligase seals the DNA into the plasmid. Promoter (P) and mRNA stabilizing (A) sequences to direct the strength and location of transgene expression are added to (or already present in) the vector. The circular plasmid containing the transgene is introduced into bacteria to allow exponential amplification of the transgenic DNA. The bacteria are collected and treated with detergents to disrupt their membranes, and the plasmids are isolated by centrifugation. The plasmid is linearized using a different RE (d), and the vector (V) sequences are pruned with an additional RE to prevent possible deleterious effects of the vector sequences on expression of the transgene (e). The linearized construct is purified (f) prior to use.



(Taurog *et al.*, 1994). These confounding effects may require the investigation of apparent transgenic phenotypes under a variety of conditions.

Pronuclear microinjection

Most transgenic animals are created using pronuclear microinjection (Figure 29.4), a technique in which foreign DNA contained in a vector is injected into the pronucleus of a zygote isolated shortly after fertilization. The injected embryos are reimplanted in the oviduct of a recipient rat to yield potentially transgenic offspring (founders). For technical details of the superovulation and embryo transfer procedures in rats, see Chapter 10 or other reference materials (Pinkert, 1994).

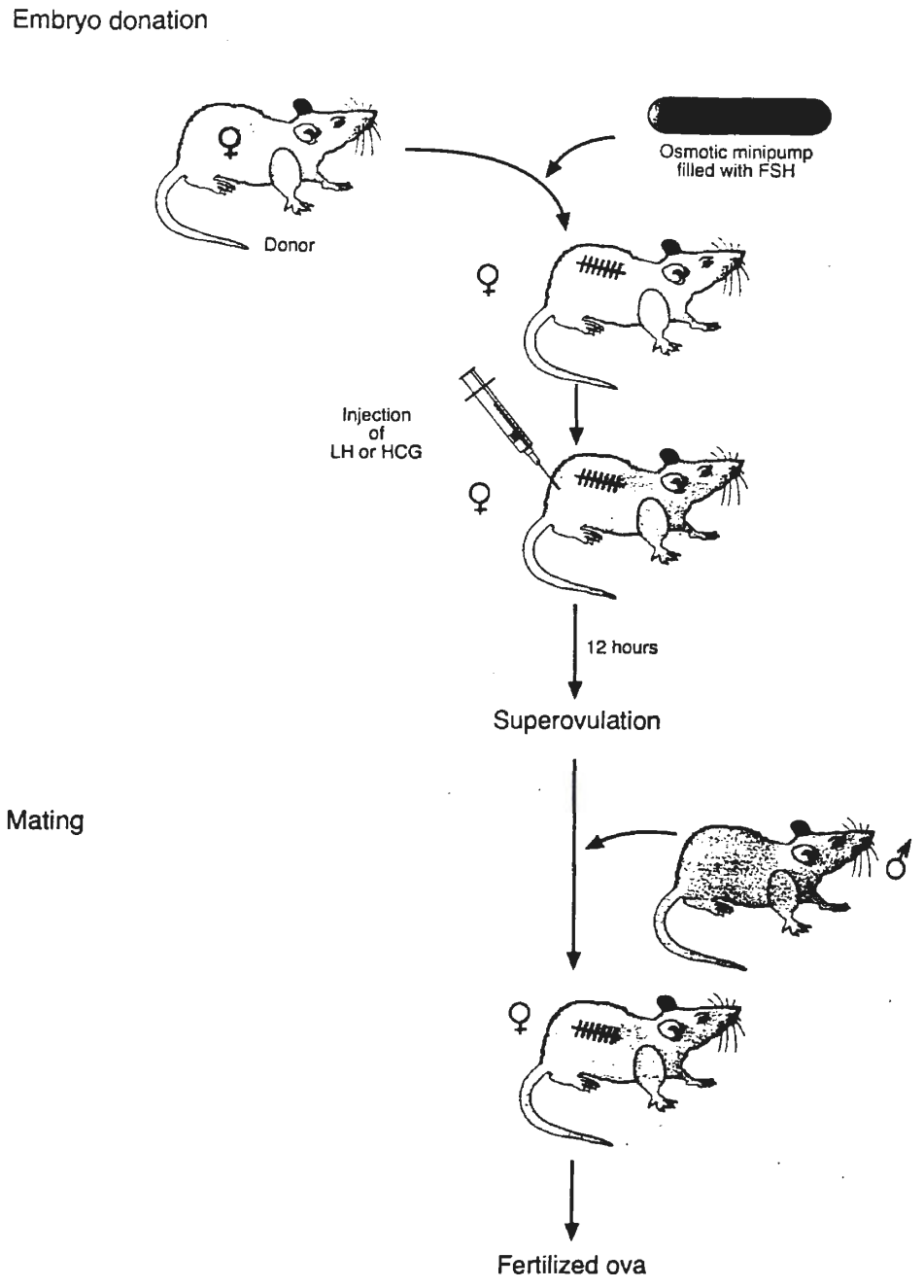
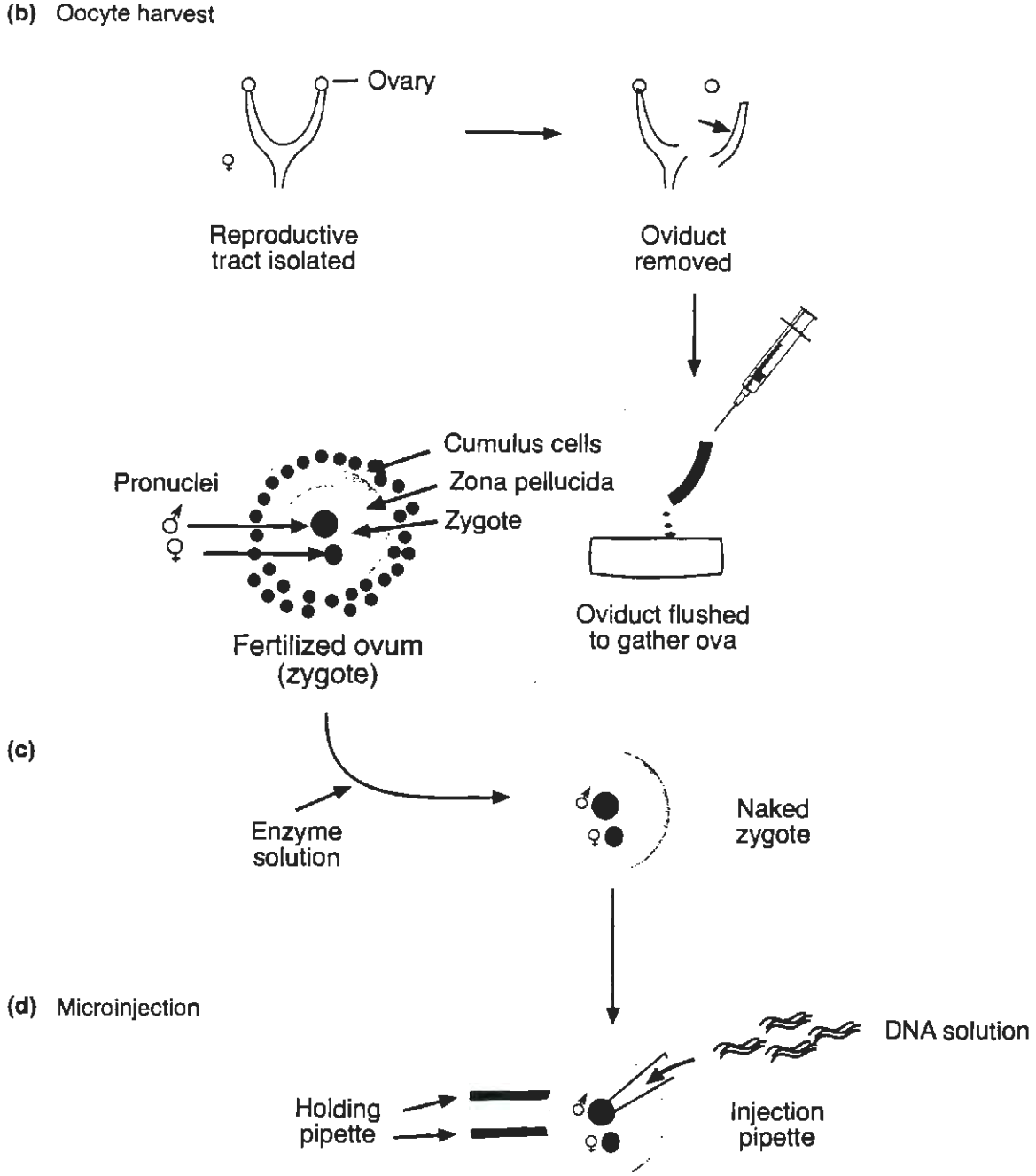
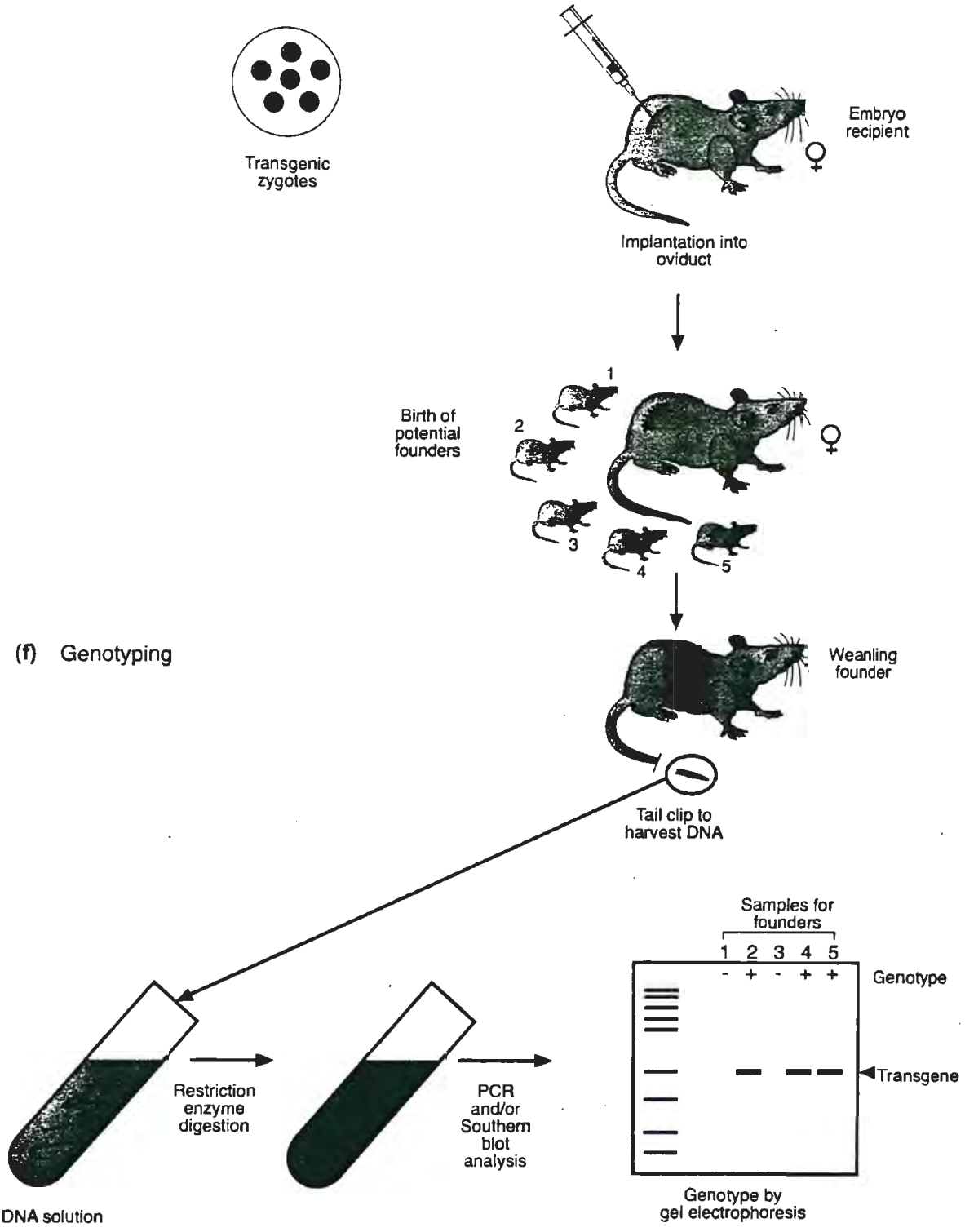


Figure 29.4 *Pronuclear microinjection technology.* The steps employed in microinjection protocols are superovulation, zygote isolation, microinjection, reimplantation and genotypic analysis of founders. First, embryo-donor females are treated with gonadotropic hormones to induce superovulation and then mated overnight with fertile males to produce fertilized ova (a). The oviducts are removed and flushed to gather the zygotes (single-celled embryos) (b). The outer cumulus layer is removed from each zygote by incubation in a protease solution (c). Zygotes with paired pronuclei are restrained with a holding pipette and then pierced with a microinjection pipette that contains the DNA solution (d). A few picoliters of DNA solution is introduced into one pronucleus, where one or more copies of the transgenic construct may be inserted at random into a break in the rat's genome. The embryos are reimplanted in the oviduct of a recipient female (e) to yield potentially transgenic offspring (founders). The genotype of each rat is determined by dissolving a tissue sample to isolate total genomic DNA, cutting the DNA with restriction enzymes, and then separating the DNA by electrophoresis (f). The probe detects a DNA sequence that is unique to the transgene. Once founder animals are established, PCR is often used to rapidly identify transgenic offspring.



Briefly, the sequential steps used in microinjection are superovulation, isolation of fertilized ova, microinjection and reimplantation. First, large numbers of rat zygotes are obtained by timed administration of pituitary- or placenta-derived gonadotropic hormones to young adult, embryo-donor rats. Common inducing agents include mixtures of follicle-stimulating hormone (FSH) and luteinizing hormone (LH), human chorionic gonadotropin (HCG), and pregnant mare serum gonadotropin (PMSG; a single molecule with both FSH and LH activities). In rats, the usual protocol is continuous infusion of FSH (using a subcutaneously implanted minipump) followed by injection of LH or HCG (Chapter 10; James McCabe, personal communication). Ovulation will occur about 12 hours after the LH or HCG bolus. Treated donor females are mated overnight with fertile males to produce zygotes (fertilized ova). More zygotes per rat may be obtained if older animals (greater than 10 weeks of age) are used as donors (Mukumoto *et al.*, 1995). In addition, the efficiency of fertile pairings may be increased by selecting female



rats in estrus based on the presence of cornified epithelial cells in vaginal swabs (Waynforth and Flecknell, 1992) or by measuring electrical conductivity from the vaginal mucosa using an impedance probe (e.g., Rat Estrus Cycle Monitor; Fine Science Tools, Foster City, CA, USA). Zygotes are gathered by flushing both oviducts (removed terminally under general anesthesia from copulation plug- or sperm-positive donor females) about 24 hours after the hormone injection. In outbred rats, typical superovulation protocols release from 20 to 80 ova per animal (Chapter 10; Robl and Heideman,

1994; Charreau *et al.*, 1996). Enough donor females are treated (usually 8–12) so that batches of at least 200 ova are available for microinjection. Batch processing increases the efficiency of the laborious microinjection step.

Next, zygotes are prepared for microinjection. These steps are conducted in a sterile environment. Zygotes are incubated in a protease solution to remove the associated cumulus cell layer and are then washed in culture medium. Zygotes with paired pronuclei are selected for microinjection, restrained with a holding pipette and then pierced with a microinjection pipette that contains the DNA solution (Figures 29.4 and 29.5). The perforations will reseal themselves after the pipette is withdrawn. A good operator can inject 80–150 rat zygotes per hour. The proportion of rat zygotes that disintegrate soon after injection varies greatly between batches of ova, with ranges of between 10% and 50% reported for the Sprague-Dawley (SD) strain (Charreau *et al.*, 1996).

After transgene injection, zygotes are reimplanted into the oviducts of female rats. Typically, females from outbred strains are chosen for their good maternal care and ready acceptance of cross-fostered pups. Embryo recipient females are often pseudopregnant animals obtained by mating with a vasectomized male (Chapter 10). As an alternative, pregnant females from a strain with pigmented hair (e.g. hooded rat) may be used as recipients for microinjected embryos from an albino strain (e.g. SD or F-344); at birth, the nontransgenic (colored) pups are culled (James McCabe, personal communication). Some investigators culture the microinjected zygotes overnight at 37°C to allow more accurate selection of viable (two-celled) embryos for reimplantation. Almost 80% of viable rat zygotes are reported to achieve the two-cell stage of embryonic development after this overnight incubation (Charreau *et al.*, 1996). However, other workers have attained up to a three-fold higher rate of pregnancy when embryo transfer is completed immediately after microinjection (Charreau *et al.*, 1996; James McCabe, personal communication).

Zygotes are reimplanted in batches of 20–25 into one oviduct of each pseudopregnant female, necessitating about 8–10 recipient females for each day of transfer. Again, some researchers (Charreau *et al.*, 1996) describe a three-fold higher pregnancy rate for bilateral transfer procedures (using 10–12 zygotes per side), presumably because the embryos do not have to migrate from one uterine horn to the other. The transfer of uninjected ('carrier') zygotes along with microinjected ones, while a common practice in mice to ensure larger litter sizes and sustainable pregnancies, may result in large litters with fewer transgenic pups in rats (Canseco *et al.*, 1994). The transfer of only microinjected zygotes routinely produces litters of normal size (e.g. 8–15 pups; Charreau *et al.*, 1996).

The typical survival rate for microinjected rat zygotes in culture (as reviewed by Charreau *et al.*, 1996) ranges between 30% and 40%, although higher rates of about 60% have been reported (Dycaico *et al.*, 1994). This efficiency is about half of that (70–90%) reported for comparably derived mouse zygotes (Brinster *et al.*, 1985; Taketo *et al.*, 1991; Canseco *et al.*, 1994). However, the proportion of microinjected rat zygotes that, upon return to a recipient rat's uterus, undergo implantation and yield live pups ranges between 15% and 30% for outbred donors and is about 4% in inbred strains (reviewed in Charreau *et al.*, 1996). This outcome in rats is higher than the reimplantation efficiencies recorded for most inbred and outbred mice (5–8%; Brinster *et al.*, 1985; Canseco *et al.*, 1994) except for the favored FVB/N strain (23%; Taketo *et al.*, 1991).

In special instances, *in vitro* fertilization and/or cryogenic storage may be used to continue or retain important rat lines (Chapter 10). The advantage of these two techniques is that the useful 'lifespan' of aging transgenic rats from poorly reproducing lines can be extended. This ability is particularly useful if the transgenic animal is male, due to the large number of ova that can be fertilized *in vitro* using sperm from a single individual. Typically the technique is employed only for critical lines because of the technical difficulties. Briefly, rats are treated with hormones to induce superovulation, and the unfertilized ova are collected. Ova are mixed with epididymal (mature) sperm and cultured for a variable length of time before being grouped in cryopreservation straws and frozen rapidly in liquid nitrogen. After thawing, fertilized ova are transferred into pseudopregnant recipient females. About 10% of the ova will yield living pups (Nakagata, 1993; Anzai *et al.*, 1994). Another recent advance, intracytoplasmic injection of freeze-dried and water-reconstituted sperm nuclei (Wakayama and Yanagimachi, 1998), may provide a much less expensive means of retaining important lines as it avoids the need for liquid nitrogen and low-temperature storage facilities.

Any or all of these genetic engineering procedures may be obtained as services from commercial vendors of transgenic rats.

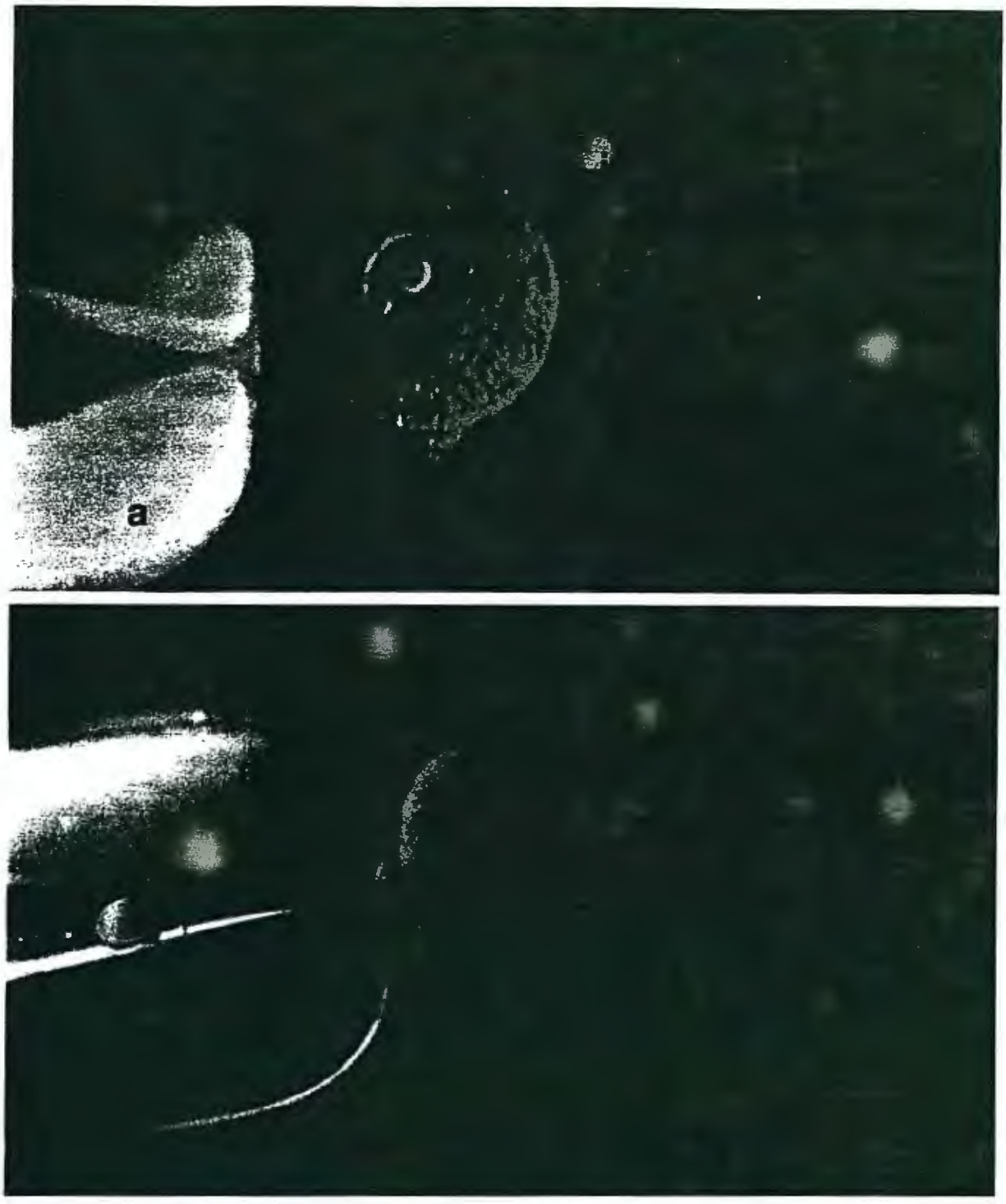


Figure 29.5 *Anatomy of rodent embryos during microinjection.* Zygotes (single-celled embryos) of the rat (upper panel) and mouse (lower panel) are immobilized by a holding pipette (a) while a microinjection pipette (b) is introduced into the large male pronucleus (c). The chief difference between the zygotes of these two species is that the rat cell has more flexible nuclear and plasma membranes, which renders penetration with the microinjection pipette more difficult. Nucleoli (d), cytoplasm (e), plasma membrane (f), perivitelline membrane (g), zona pellucida (h), polar body (i), and cumulus cells (j). Magnification, 490x.

Systemic gene delivery

The first genetically engineered animals were created by conveyance of transgenes using modified viral vectors. For this method, the vectors were engineered so that the host cells could not manufacture intact, infectious virus particles. The advantage of using viral vectors to deliver transgenes is that only a single transgene copy is integrated into the genome without the occurrence of transgene rearrangements or deletions that often arise during microinjection. The major disadvantage is that systemic gene delivery to multicelled organisms (adults or older embryos) typically does not lead to transgene integration in all cells, resulting in mosaic expression of the new gene. One of the most critical factors resulting from mosaic expression is the potential loss of a unique phenotype if the transgene is not transmitted to the germline cells of the transgenic animal. The lack of control over these essential factors is responsible for the shift to microinjection technology to produce transgenic animals in most facilities.

Nevertheless, systemic delivery systems may still have relevance in the exploration of those questions in which transgene expression in all cells is undesirable. For example, pronuclear microinjection with foreign DNA can induce a lethal phenotype in which the transgenic animal dies at some early stage of development, commonly before or shortly after birth. While such lethal events are interesting in and of themselves, the resulting inability to reproduce an adult disease model or establish a breeding colony may limit the utility of microinjection for such 'embryonic lethal' genes. In these cases, an alternative method is to introduce the transgene into the adult animal using a chemical (Simoes *et al.*, 1998) or, more commonly, a viral vector (Robbins *et al.*, 1998). In this manner, deleterious effects of the transgene to the developing embryo may be avoided. To our knowledge, this procedure has been reported only in mice (e.g. Sanes *et al.*, 1986; Holzinger *et al.*, 1995; Tsukui *et al.*, 1996; Baldwin *et al.*, 1997); however, implementation in the rat is feasible. Viral vector technology is described in more detail below in the section on Gene therapy.

Special methods in transgenic research

Modifications in the design of transgenic constructs have engendered several innovative ways to produce new and more finely controlled transgenic models. While many of these techniques have been validated in mice, there is no theoretical barrier to their use in rats.

One exciting approach is the advent of conditional gene expression in transgenics (Figure 29.6). This tool regulates the onset of expression, providing another means of avoiding the adverse effects associated with constitutive transgene overexpression during early development. In addition to the usual elements (promoter, transgene, polyadenylation sequence), the construct for a conditional transgene includes a ligand-inducible control system (Kühn *et al.*, 1995; Fishman, 1995). In this manner, gene expression will be essentially absent until activated by either the introduction (Passman and Fishman, 1994; Wang *et al.*, 1997) or withdrawal (Gossen *et al.*, 1995; Kistner *et al.*, 1996) of an exogenous ligand. For example, in one system, the metallothionein (MT) promoter directs basal expression of certain endogenous genes at low levels in many tissues. If the MT promoter is joined to a transgene, addition of heavy metals to the animal's diet induces the MT promoter and drives an increase in hepatic expression of the transgene of up to 100-fold (Palmiter *et al.*, 1983).

The main characteristics of an ideal conditional transgenic system would be low basal (no 'leakiness') and high peak activities, rapid induction upon administration of the ligand, and a single control protein (i.e. a simple signal transduction pathway). Some conditional gene expression systems require the engineering of two lines of transgenic animals: one carrying the gene of interest under the control of an inducible promoter and another expressing a -acting control protein (Byrne and Ruddles, 1989; Gardner *et al.*, 1996). Mating of the two lines results in progeny that contain both constructs, allowing the -acting control protein to act as the inducing ligand for the promoter that controls the transgene of interest. An alternative is to create double transgenic animals in which both the transgene and control protein have been introduced in the same construct (Schultze *et al.*, 1996). The best conditional gene expression systems have been engineered using nonmammalian control elements (e.g. Gossen *et al.*, 1993) since promoters of mammalian origin (e.g. MT) can react in a physiological manner and, therefore, are both leaky and subject to endogenous regulatory systems (Yarranton, 1992; Fishman, 1995). Ligands that have been validated in transgenic mice produced by microinjection include tetracycline (Furth *et al.*, 1994; Kistner *et al.*, 1996; Schultze *et al.*, 1996), the insect hormone

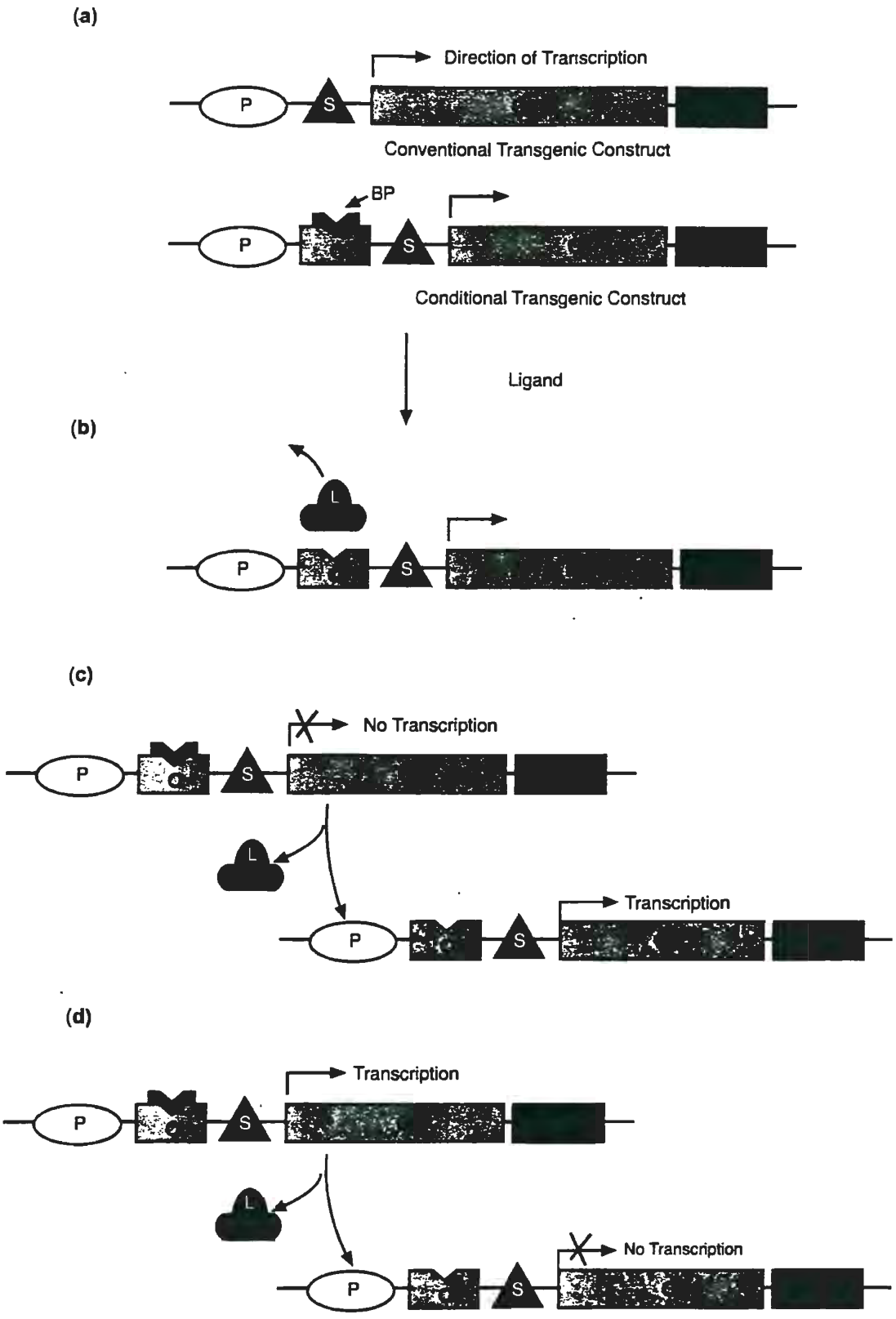


Figure 29.6 *Conditional gene expression.* Transgene expression may be controlled by the timed administration of an exogenous ligand. (a) In addition to the conventional elements in a transgenic construct (Figure 29.3), a conditional transgene includes an operator (O; a regulatory domain) with which the ligand-binding protein (BP) may interact. (b) Presence of the ligand (L) results in a conformational change in the ligand-binding protein, resulting in dissociation of the protein from the transgene. (c, d) Removal of this protein results in either initiation or cessation of the transgene's transcription. Notations for gene nomenclature are identical to those provided for Figure 29.2.

ecdysone (No *et al.*, 1996), and the progesterone analog mifepristone (RU486; Wang *et al.*, 1997). Conditional transgenic rats have been reported in which expression of marker genes injected directly into a specific organ of an adult was regulated using systemic administration of mifepristone (Oligino *et al.*, 1998) or tetracycline (Fishman *et al.*, 1994). To our knowledge, conditional transgenic rats have yet to be created by introduction of genetic material into embryos.

Another interesting possibility is the production of animals in which functional deficiencies have been imposed through the reduction of an endogenous gene's activity. Three methods have been described. First, the transgene product may act as a 'dominant negative' element with respect to the endogenous protein. In other words, the introduction of a single transgene allele can regulate a strong ('negative') function that reduces or cancels the normal ('positive') activity of an endogenous protein. An alternative possibility is that the transgene product binds and inactivates an endogenous protein. This latter paradigm has been employed *in vitro* and *in vivo* by the expression of transgenes that encode single-chain antibodies (scFv; Beerli *et al.*, 1994; Deshane *et al.*, 1997). Such transgenes are designed so that the protein product consists of the antigen-binding variable portion (Fv) of an antibody, directed against an intracellular antigen, but lacks a secretory tag. Expression of the scFv transgene results in manufacture and cytoplasmic retention of the scFv protein, leading to binding and inactivation of the antigen. Finally, a genetic ablation technique may be used to eliminate specific cell types by introducing a transgene that encodes a cytotoxic protein under the control of a tissue-specific promoter (Palmiter *et al.*, 1987; Borrelli *et al.*, 1988; Mintz and Kleiń-Szanto, 1992). Thus, all three schemes may yield functional phenotypes with features similar to those resulting from genotypic 'knockouts' produced by gene targeting. The main advantages of these three approaches are that this transgenic technology is usually simpler, less expensive, and more rapid than gene targeting.

Gene Targeting

A breakthrough approach in the manipulation of the mammalian genome was the isolation and culture of mouse embryonic stem (ES) cells, each of which is capable of contributing to the formation of all adult tissues. These cells were derived initially from the inner cell mass (representing the nascent embryo) of preimplantation blastocysts (Evans and Kaufman, 1981; Martin, 1981; Bronson and Smithies, 1994). At present, reliable ES cell lines have been obtained from only a few mouse strains. Development studies with ES cell cultures soon expanded to include successful germline transmission of genetic mutations introduced into mice by incorporation of recombinant DNA into cultured ES cells followed by introduction of the modified ES cells into the inner cell mass of blastocysts (Bradley *et al.*, 1984; Gossler *et al.*, 1986; Robertson *et al.*, 1986). While mouse ES cells injected into rat morulae also yield viable interspecific chimeric finders, pluripotent rat ES cells have yet to be isolated (Iannacconne *et al.*, 1994, with published erratum, 1997).

Recently, precise manipulation of the mouse genome has been accomplished through the gene targeting approach (Figure 29.7). Recent progress in rat ES cell biology (Charreau *et al.*, 1996; Takahama *et al.*, 1998) as well as the many similarities between mouse and rat reproduction suggest that gene targeting in rats will become feasible in the near future. Therefore, we believe that a discussion of this technology is appropriate here. First, recombinant DNA methods are used to alter the cloned sequence of a selected chromosome locus. This gene alteration is then transmitted into the genome of the ES cells through the process of homologous recombination (Smithies *et al.*, 1985; Doetschman *et al.*, 1987; Thomas and Capecchi, 1987; for reviews, see Capecchi, 1989, or Koller and Smithies, 1992). During homologous recombination (Figure 29.7), the introduced DNA will pair with the locus of endogenous DNA that possesses the complimentary nucleotide sequence. When the ends of the regions of nucleotide homology are cut, the DNA pieces are exchanged, and the transgene is incorporated into the genome (Folger *et al.*, 1982). Thus, the engineered gene is switched with the endogenous copy and is automatically integrated at the specific and proper location in the genome. The orientation of the homologous regions in vector and chromosomal DNA will determine whether the engineered gene replaces the endogenous one or whether the entire vector is inserted into the genome (Hasty and Bradley, 1993).

The properties of the engineered gene determine what its functional significance to the animal might be. For example, one common scenario is to disrupt the normal coding sequence of the gene, thereby disturbing normal gene expression and creating a null mutation (i.e. a 'knockout'). A second paradigm

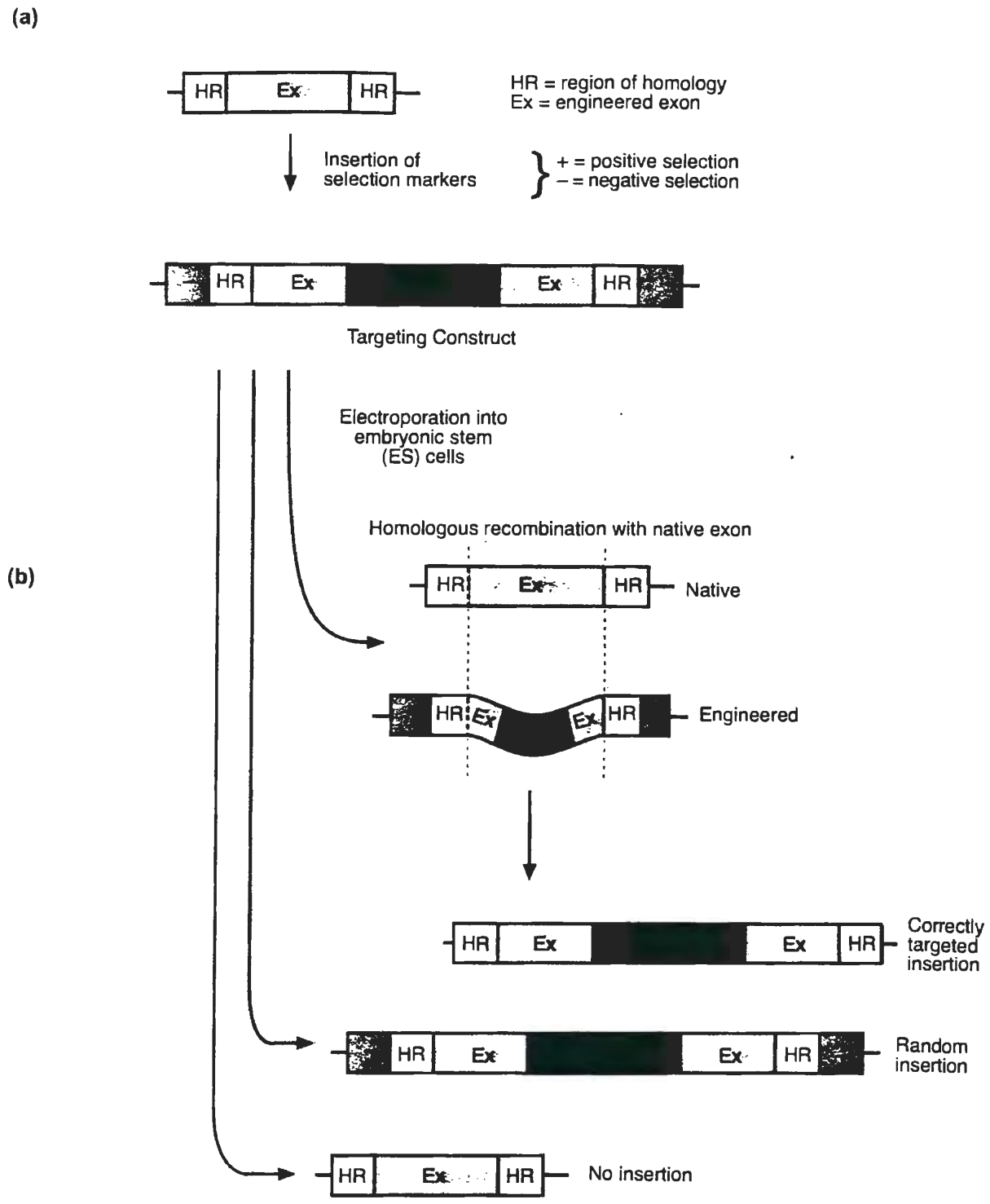


Figure 29.7 *Gene targeting technology.* The steps involved in targeting an endogenous gene with an engineered gene include design of the construct, selection of viable stem cell clones, injection of blastocysts, and reimplantation. (a) Commonly, the targeting construct consists of a plasmid containing the engineered gene linked to one or more additional genes that encode for xenobiotic selection markers. After amplification in culture, linearized plasmids are introduced into the embryonic stem (ES) cells. (b) The complimentary sequences of the endogenous gene and the plasmid-borne engineered gene align and are exchanged by homologous recombination. The ES cells are tested for the proper introduction of the engineered gene by addition of selection agents.

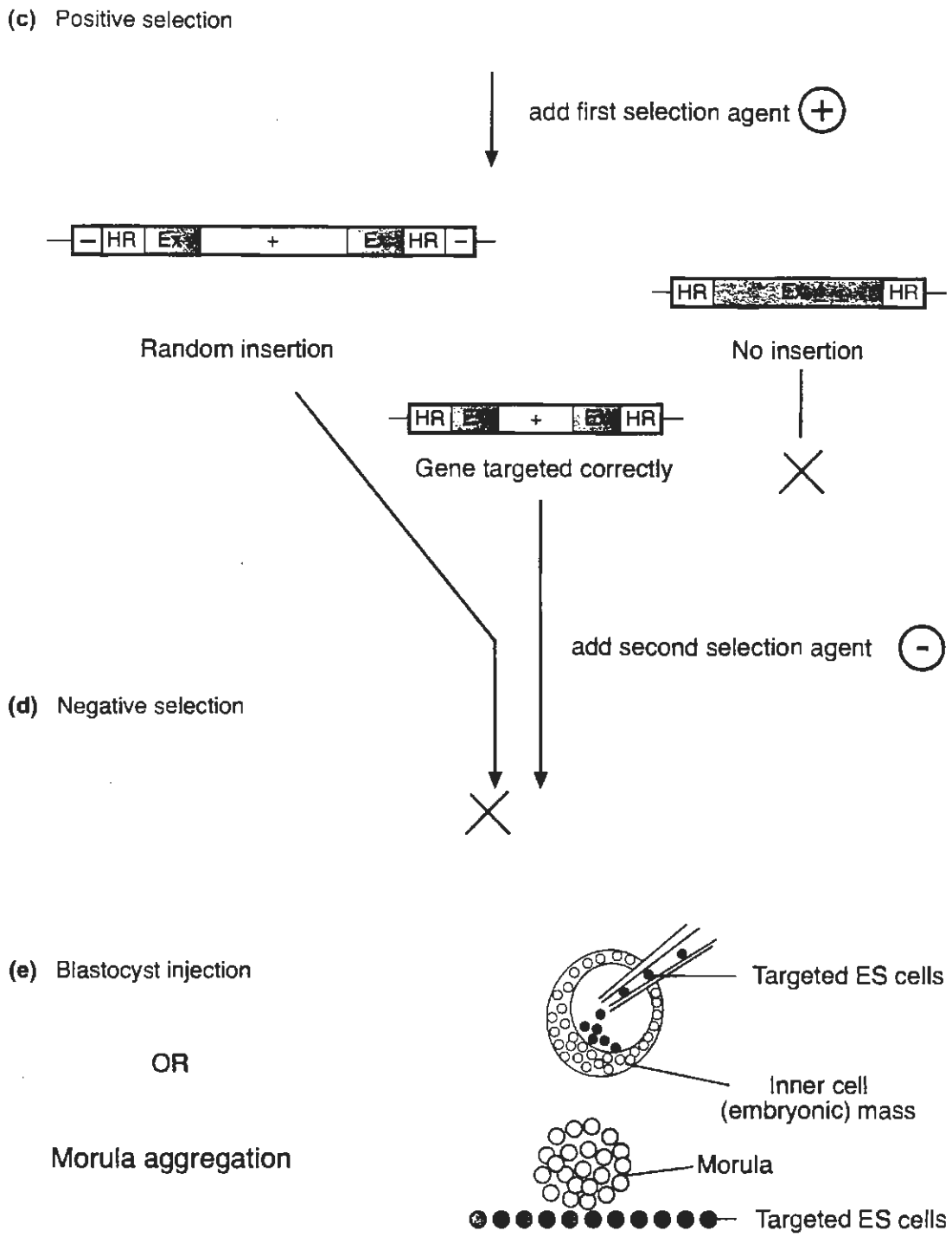


Figure 29.7 *(continued)* (c) For positive selection, ES cell clones survive in the presence of a toxic agent if they have incorporated correctly a targeting construct that includes an appropriate and functional copy of the toxin resistance gene. (d) Negative selection chooses ES cell clones with the appropriate integration of the targeting vector based on the loss of a toxin sensitivity gene and resistance of the ES cells to a toxic agent. When integration of the targeting vector occurs outside the region of homology, the sensitivity gene is retained, allowing the ES cells to die. (e) Cells from clones with the correctly targeted gene are expanded and then injected into the inner cell mass of a blastocyst or aggregated into a morula. The embryos are reimplanted in the oviduct of a recipient female. The resulting progeny are chimeric to variable degrees, depending upon the contribution of the ES cells to embryonic development. Note: In mice, the ES cells, the blastocyst or aggregation cells, and the recipient female are all derived from mouse lines with different coat colors, allowing for selection of chimeric progeny with the highest amounts of the targeted gene based on the greatest degree of coat color consistent with that of the animals from which the ES cells were established.

is to modify the targeting sequence into a constitutively activated form and insert it in place of the endogenous gene (a 'knockin'; Hanks *et al.*, 1995). A loss or gain of gene function may result in functional or structural deficits of varying degrees. Alternatively, the introduced mutation may have no apparent effect, suggesting that proteins derived from endogenous genes are compensating for the engineered DNA defect.

An engineered gene is added to a bacterial plasmid to produce the targeting vector (Hasty and Bradley, 1993) (Figure 29.7). The targeting construct is introduced into ES cells by using chemicals or electrical current (electroporation) to create transient pores in the cellular membranes. The efficiency of gene entry is low, and only a few of the cells which pick up the vector will actually undergo homologous recombination to replace the targeted endogenous gene with the engineered DNA in an appropriate orientation. The majority of the targeting constructs insert randomly within the genome, or do not integrate at all. Therefore, to identify ES cells that contain the properly targeted gene, many targeting protocols include positive and/or negative selection steps (Wurst and Joyner, 1993). A positive selection (Figure 29.7) identifies ES cells that have incorporated a drug-resistance gene that was retained by being designed internal to the regions of homology on the targeting vector. Culture of the targeted ES cells in the presence of the drug results in death of all ES cells in which the proper genetic recombination did not occur. In contrast, negative selection (Figure 29.7) is carried out by incorporating a drug-sensitivity gene external to the regions of homology such that correct integration of the targeting vector results in loss of the sensitivity gene and resistance of the ES cells to the selection agent. Individual cells are cultured, and colonies are screened for proper orientation and integrity of the targeted mutant gene. Once correctly targeted ES cells are identified, they are expanded into large cultures, isolated by trypsinization, and added to cells of the inner cell mass of the preimplantation blastocyst by microinjection or coaggregation (Wood *et al.*, 1993). At birth, the tissues of the progeny will be chimeras derived from both endogenous and engineered ES cells; extensive characterization then is required to select founders in which the engineered gene is transmitted through the germ line (i.e. in which the gene-targeted ES cells contributed to gonadal development) (Papaioannou and Johnson, 1993). However, a recent technical modification is the production of animals (mice) in which the embryonic component is formed entirely by gene-targeted ES cells (www.mshri.on.ca/nagy/Tetraploid/Tetra.htm). This technique will greatly reduce the time required to obtain a gene-targeted line. In addition, the ability to intermingle embryonic and extraembryonic (placental) components between wild-type and gene-targeted embryos will aid greatly in differentiating between embryonic and placental mechanisms by which targeted genes may lead to embryonic lethality (Rossant *et al.*, 1998).

Thorough screening of all ES cell cultures avoids the high labor, monetary and time costs associated with use of incorrectly targeted genes. In addition, ES cells should be surveyed for microorganisms to prevent introduction of pathogens into the breeding colony. In our experience, fewer than 3% of ES clones contain the engineered gene both in the correct orientation and with intact sequence. In mice, about 30% of injected embryos contain tissues that are derived from ES cells, and an even smaller fraction of these chimeric founders contain the engineered gene in their germ cells.

Special methods in gene targeting research

Two additional means of producing gene-targeted animals have been developed in recent years for use in research questions in which gene inactivation must be limited to selected times or tissues. The first procedure, administration of **antisense oligonucleotides**, is simple, relatively inexpensive, and can be used in rats at this time. In this method, large numbers of oligonucleotides are administered by intravenous injection (intra-amniotic microinjection for embryos; Chen and Hales, 1995). These agents are taken into cells, where they bind to mRNA molecules with complementary (sense) sequences. These duplexes are stable, so the protein cannot be made and the gene is effectively inactivated. However, the disadvantages of this technique include the variable extent of cellular uptake and the leakiness of the system resulting from incomplete mRNA binding.

An alternative protocol is the use of site-specific recombinases, including the Cre/*loxP* gene targeting strategy (Figure 29.8). The Cre recombinase, first identified in the bacteriophage P1, is a protein which excises any DNA sequence located between two *loxP* sites (a 34-bp (base pair) nucleotide sequence that has not been found in vertebrate

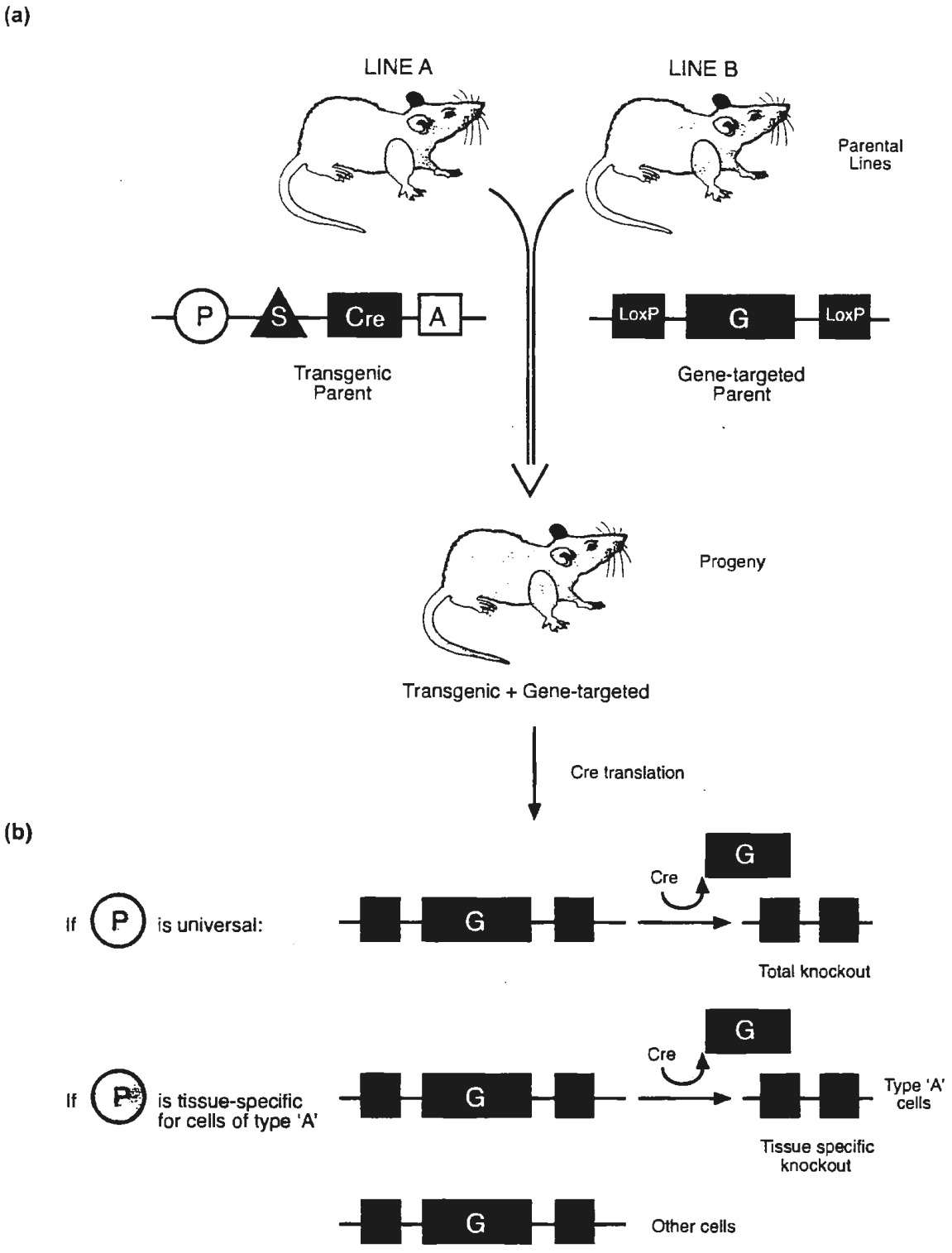


Figure 29.8 *Site-specific recombination: an alternative gene targeting strategy.* Microbial recombinase enzymes mediate the excision of genetic material located between adjacent loci of certain novel nucleotide sequences. This paradigm requires the creation of two genetically altered animal lines (a): a transgenic line incorporating a recombinase gene (e.g. the Cre protein) and a gene-targeted line in which the targeting construct contains a functional form of the endogenous gene of interest flanked by two recombinase recognition sites (e.g. the *loxP* locus, a unique 34-bp sequence recognized by Cre). The two parent lines of animals are normal, but crossing them results in progeny in which the *loxP*-flanked gene has been excised in cells that express the transgenic Cre protein (b).

genomes) (Sauer, 1993; Marth, 1996; Akagi *et al.*, 1997). This paradigm requires the creation of two genetically altered animal lines: a gene-targeted line in which the engineered construct contains a functional form of the gene of interest flanked by two *loxP* sites (Gu *et al.*, 1994; Kühn *et al.*, 1995), and a second transgenic line incorporating the Cre gene under the control of a suitable promoter. The two parent lines of animals are normal, but crossing them results in progeny in which the *loxP*-flanked gene has been excised in cells that express the transgenic Cre protein. Gene inactivation can be limited to a single tissue by placing the Cre transgene under the control of a tissue-specific promoter (Orban *et al.*, 1992; Gu *et al.*, 1994; Rajewsky *et al.*, 1996; Rohlmann *et al.*, 1996; Tsien *et al.*, 1996). In addition, Cre activity can be regulated by using a ligand-dependent conditional gene expression system to control Cre expression (Metzger *et al.*, 1995). The novel yeast-derived Flp recombinase system has been employed in a comparable fashion (Dymecki, 1996). The recombinase approach should prove to be especially useful for studying the loss of developmentally essential genes in specific tissues of adult animals, particularly where a global gene knockout approach would result in embryonic lethality (Copp, 1995).

Gene Therapy

We have discussed above the production of transgenic rats by administration of foreign genetic material in a viral vector. While this method has been used successfully to engineer rat models in order to answer basic research questions, an even more important consideration may be the preclinical application of such technology in rats to assess risk-to-benefit ratios for potential human gene therapies, including such uses as tissue-specific transfer of a gene to replace a defective protein (perhaps to cure a lysosomal storage disease) or to deliver a therapeutic agent (such as a cytotoxic protein to kill cancer cells). Chemical (Simoes *et al.*, 1998) and viral (Robbins *et al.*, 1998) vehicles are now being tested as therapeutic paradigms in many acquired and inherited human diseases. Potential rate-limiting steps in achieving successful gene transfer are the low efficiency of gene integration into the patient's genome in the proper target tissue and the short duration of activity that may result as the body's defense system degrades the transgene or the protein product. Such issues may be addressed using rat models of gene therapy.

Two basic approaches are used for gene therapy experiments. In the *ex vivo* method, host cells are removed from the animal, transfected while being cultured, and then reinserted into the same animal. The alternative *in vivo* technique requires the parenteral delivery of the genetic material, usually by intravenous (for systemic exposure) or targeted (for local action) injection, or by inhalation (for airway expression). Delivery may be made to the entire adult (Jaffe *et al.*, 1992), to a specific region (La Salle *et al.*, 1993), or to the conceptus (Baldwin *et al.*, 1997). In general, genes are delivered in viral vectors or in nonviral constructs; the latter systems may include the passive carriage of unmodified DNA (e.g. liposomes) or may involve the covalent modification of the transgene (e.g. chemical conjugates). The ideal system for gene therapy will yield efficient gene delivery, minimal pathogenicity (particularly immunogenicity), and stable and permanent integration of the transgene into the desired target tissue(s) and cells. Typically, viral vectors provide more effective gene incorporation but have greater liabilities with respect to immunogenicity and their innate pathogenicity toward the host. While several viral vectors are used commonly in animal studies and in human clinical trials, the most common are adenoviruses (with linear double-stranded DNA genomes) and retroviruses (with linear single-stranded RNA genomes).

Regardless of the viral type, certain aspects of gene therapy technology are universal. First, one or more viral genes are deleted (often by replacing them with the transgene DNA). Ideally, these truncated viral genomes can neither initiate viral reproduction in host cells (yielding a 'replication-defective' or 'replication-incompetent' vector) nor direct the production of viral proteins (thereby reducing its immunogenicity). However, compromises between the efficiency of gene transfer and the degree of viral pathogenicity must be balanced against the experimental question. For example, advantages of adenoviral vectors include their ability to infect many different cells (including nondividing ones), their high expression level in the host animal, and their high titer. However, the primary disadvantage is the transient expression of the transgene, which may result from two factors. One reason is the episomal (away from the chromosome) location of the transfected material, resulting in presence of the transgene for a short period in the cytoplasm rather than

permanent integration in the animal's genome. The second factor is the host's immune response against cells that express these viral proteins. In contrast, retroviral vectors are inserted permanently into the genome without expressing any immunogenic proteins. Nevertheless, stable infection with retroviral-borne transgenes occurs only in dividing cells, and levels of the transgene product may be low. Rats are important animal models for the preclinical biology and toxicology phases of gene therapy trials, although mice may be preferable for some applications since their smaller body size would allow for use of smaller amounts of the vector.

Cytogenetics

Cytogenetic technology has made large contributions to understanding the physical structure of the rat (and human) normal genome as well as in characterizing the genetic alterations that occur in certain diseases, most notably neoplasia (Kerler and Rabes, 1994). Karyotypic analysis (Figure 29.9), the assessment of chromosome structure, has been used for years to define gross abnormalities such as nondisjunctions and translocations (Mitelman, 1983; Ichikawa *et al.*, 1990; Montaudon *et al.*, 1990). This evaluation is made on metaphase chromosomes (termed 'spreads') that have been obtained by treatment of cells with colchicine (to arrest mitosis) and hypotonic chemical solutions (to rupture the cell and nuclear membranes, thereby releasing the condensed chromosomes).

Dye banding techniques (e.g. Giemsa or acridine orange) have revealed fine defects in chromosomal structure by demonstrating alterations in the location or width of various bands associated with more subtle genetic defects (Kerler and Rabes, 1994; Sargent *et al.*, 1996). Several standard dye-specific banding patterns have been described for the 21 pairs of rat chromosomes (Levan, 1974; Rønne *et al.*, 1987; Satoh *et al.*, 1989). These older, powerful methods of purely physical chromosomal analysis have been significantly enhanced by the recent advent of molecular techniques (e.g. fluorescent *in situ* hybridization (FISH), which assess the location of a specific DNA sequence (i.e. for a gene of interest) in conjunction with its chromosomal address. For example, FISH analysis performed with probes for at least two single-copy genes can determine the chromosomal location as well as the genetic distance between genes in metaphase spreads, in interphase (intact) nuclei, and in stretched DNA (Trask *et al.*, 1989). The resolution of chromosome mapping ranges from approximately 3 megabases (Mb) in metaphase spreads to about 100 kb in interphase nuclei, depending on the local chromatin structure (Lichter *et al.*, 1990). Molecular techniques even allow site-specific engineering of chromosomes to assess the impact of gene heterozygosity and chromosomal abnormalities in animals (Ramirez-Solis *et al.*, 1995).

Recently, the application of fluorescent cytogenetics technology has been extended from the mapping of single-copy genes with FISH to scans of the entire genome either by comparative genomic hybridization (CGH) or spectral karyotyping (Chang and Mark, 1997). In CGH (Parra and Windle, 1993), a global analysis of recurrent chromosomal defects (gains or losses of genetic material) is performed, usually by competitive hybridization of probes between differentially labeled normal (e.g. a green fluorochrome) and tumor-derived (e.g. red fluorochrome) DNA. The labeled probes are hybridized to metaphase spreads, and a comparison of the intensities of green and red fluorescence along the chromosomes reflects the relative abundance of normal gene sequences in the tumor. Regions of the DNA that are overexpressed in the tumor are seen as regions of high red intensity on the target chromosome. The difference can be quantified by digital imaging analysis, with current software (available for the rat, mouse and human) capable of detecting a change of 10 Mb (Kallioniem[illegible] *et al.*, 1992). DNA from formalin-fixed, [illegible]fin-embedded archival tissues may be as[illegible]d by CGH. For example, a small locus can be [illegible]ected from the tissue block or a single section [illegible]none *et al.*, 1998), and the DNA can then be amplified to examine genetic changes within a defined tissue or cell population (Speicher *et al.*, 1996). The microdissection approach is a particularly useful method for examining the early, subclinical stages of chronic diseases such as neoplasia.

Spectral karyotyping (SKY) surveys all chromosomes in a cell population simultaneously for many major rearrangement events, a screen not possible with other cytogenetic techniques. All chromosomes are labeled with oligonucleotide probes that have been conjugated to different fluorochromes (Telenius *et al.*, 1992; Liyanage *et al.*, 1996); for example, all 23 human chromosomes can be uniquely labeled using a combination of only five fluorochromes. To date, this method has

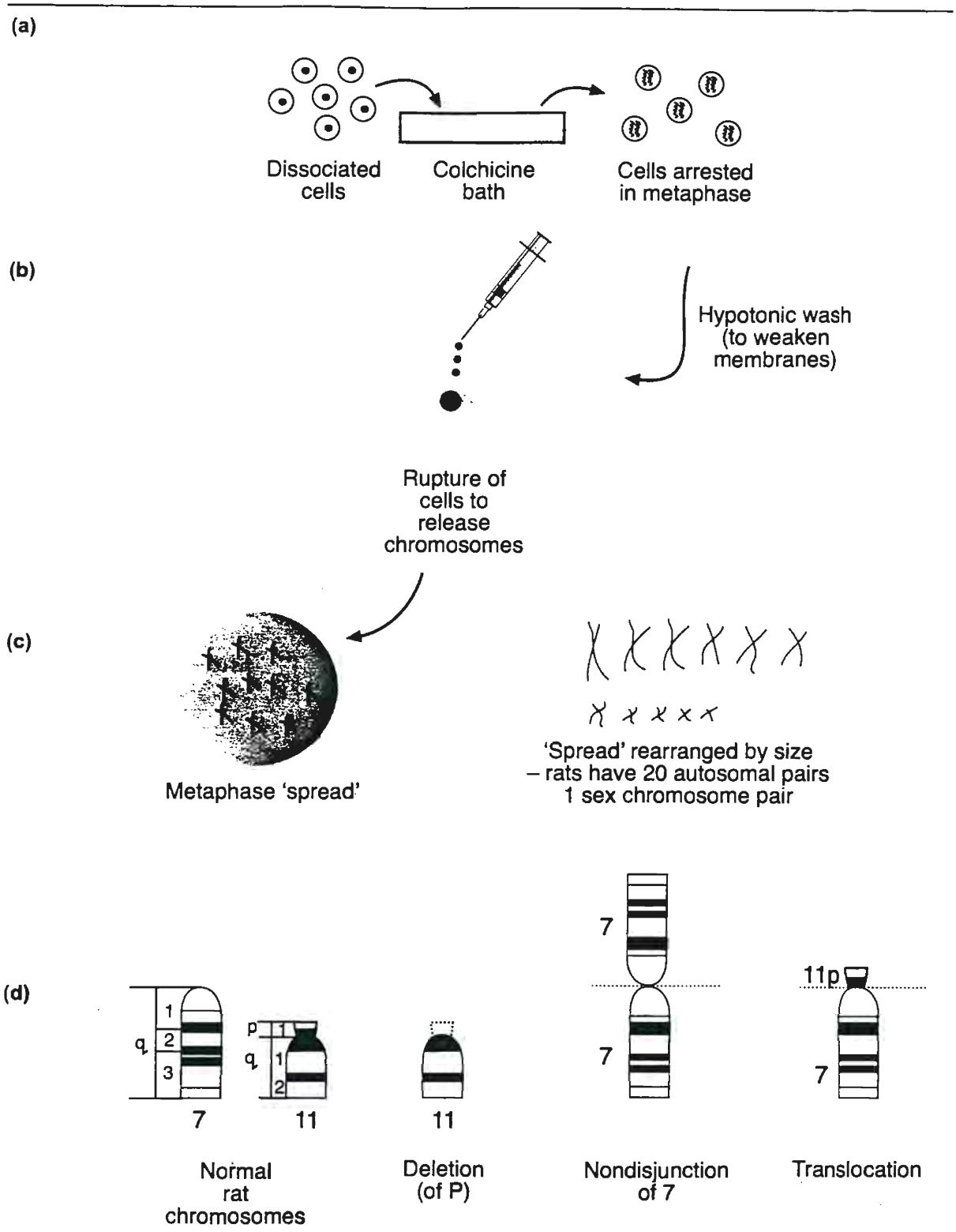
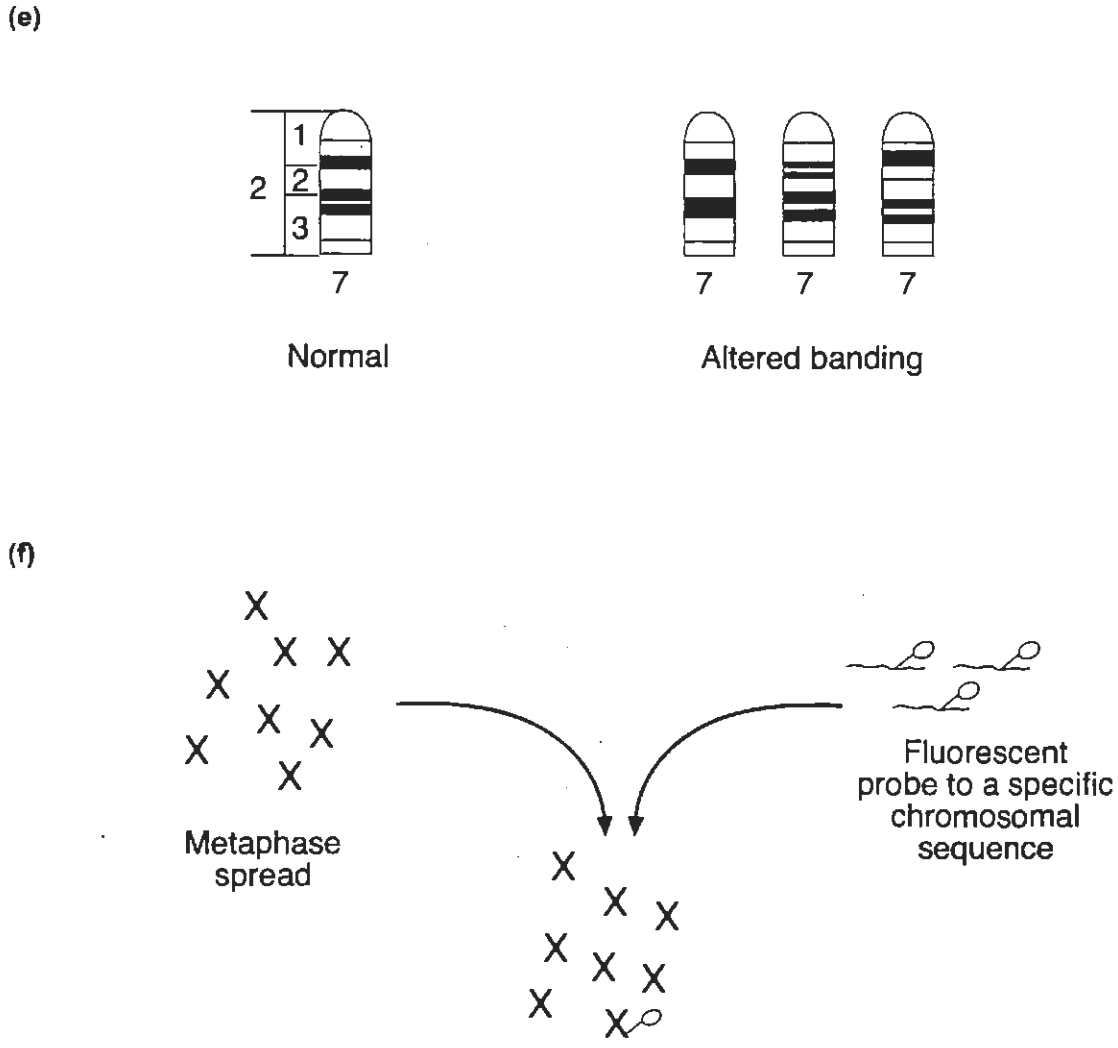


Figure 29.9 *Cytogenetic technology: karyotypic analysis.* Certain gross alterations in genomic DNA can be assessed by examining the structure of metaphase (condensed) chromosomes. (a) Dissociated cells are treated with colchicine to arrest mitosis at the metaphase stage. (b,c) Cells then are washed in hypotonic solutions to weaken the cellular membranes, and drops of the suspension are expelled onto glass slides to physically break the cells and release the condensed chromosomes. The chromosomes are stained and examined microscopically for major defects (d), such as deletions or nondisjunctions or translocations, as well as minor changes in the banding pattern (e). (f) These physical methods can be supplemented with fluorescent *in situ* hybridization (FISH), a molecular technique in which the chromosomal location of a specific DNA sequence can be determined by the use of an oligonucleotide probe conjugated to a fluorochrome. Banding pattern adapted from Kerler and Rabes (1994).



been performed only for human and mouse cells, but further work with genetically engineered rats will likely result in the adaptation of this technology for use in this species.

Cloning

The ready availability of large numbers of genetically identical rats would facilitate research by greatly reducing the variation in physiological response that exists between subjects with different genetic backgrounds. In the past, the production of highly inbred rat strains removed much, but not all, of the genetic heterogeneity between individual animals (Chapter 1). Recently, cloning methods that allow the generation of genetically identical individuals have proven feasible in mammals (Campbell *et al.*, 1996; Wilmut *et al.*, 1997; Wakayama *et al.*, 1998). A cloned embryo is constructed by introduction of an intact diploid nucleus (in the quiescent (G_0) stage of the cell cycle) into an enucleated oocyte (Figure 29.10). The isolated nucleus is joined to the oocyte either by electric fusion (Wilmut *et al.*, 1997) or microinjection (Wakayama *et al.*, 1998), and the genome is activated (to allow re-entry into the cell cycle) by electric pulses (Wilmut *et al.*, 1997) or chemical media (Bos-Mikich *et al.*, 1997). The cloned embryos are then implanted into the oviducts of recipient females. Primary cultures of embryonic, fetal or adult somatic cells have all been proven to work as sources for donor nuclei (Campbell *et al.*, 1996; Wilmut *et al.*, 1997), indicating that both partially and terminally differentiated genomes are capable of supporting the entire spectrum of development processes. In addition, adult cells have

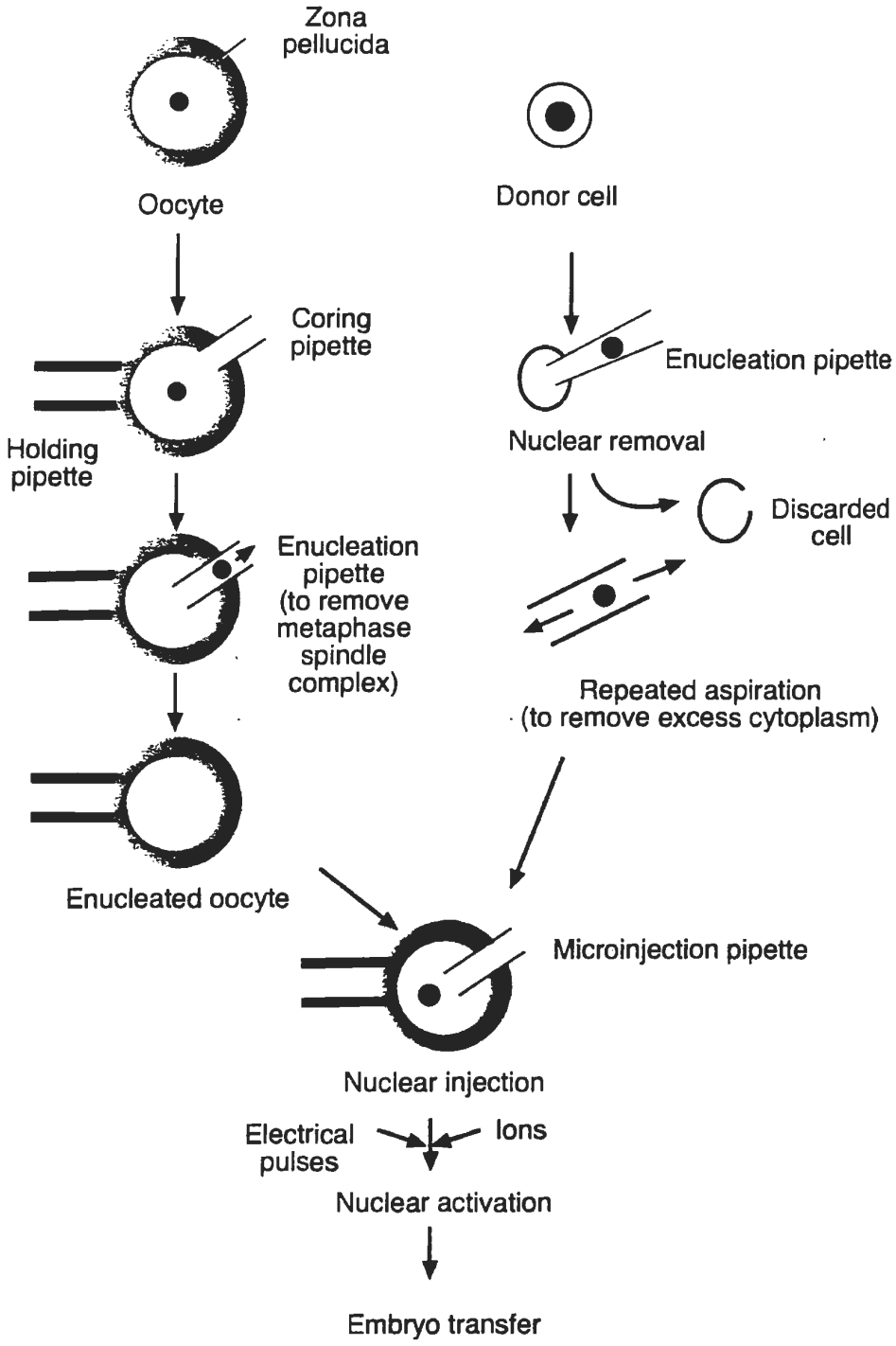


Figure 29.10 *Cloning.* A cloned embryo is constructed by introduction of an intact diploid nucleus (in the quiescent (G_0) stage of the cell cycle) into an enucleated oocyte. The isolated nucleus is joined to the oocyte by either electric fusion or microinjection, and the genome is activated (to allow re-entry into the cell cycle) by electric pulses or chemical media. The cloned embryos then are implanted into the oviducts of recipient females. Primary cultures of embryonic, fetal or adult somatic cells all have been proven to work as sources for donor nuclei. Adapted from Wakayama *et al.* (1998).

been shown to work without a passage in culture (Wakayama *et al.*, 1998). Cloning of rats has not yet been reported. However, the recently described 'Honolulu technique' (Wakayama *et al.*, 1998) has been used to clone several generations of identical female mice at a relatively high efficiency (2–3% survival to term of implanted embryos). We believe that rat cloning will be described in the near future.

Genotypic Analysis of Engineered Rats – Detection of Gene Insertion

Techniques used to produce and characterize genetically engineered rats require a considerable degree of technical expertise. Fortunately, the widespread acceptance of these molecular procedures has resulted in the collection of many 'recipes' into manageable volumes of material (e.g. Sambrook *et al.*, 1989; Brown, 1991; Ausubel *et al.*, 1998).

The presence of foreign DNA in a genetically engineered animal may be checked in both qualitative and quantifiable manners using one or more molecular assays. Briefly, cells are harvested from the rat. Commonly, the tail tips of weanlings are removed for this purpose at the same time that the mice are given individual identification marks (by a coded sequence of clipped toes and/or ear punches, a metal ear tag, or an implanted microchip). However, tail clips or ear punch remnants may be taken at an earlier age, or oral washes may be gathered from neonates (Irwin *et al.*, 1996), in order to expedite the genotyping process. The tissue specimens are dissolved in a buffered enzyme solution to release the total genomic DNA (with incorporated transgene). Next, DNA is purified using either a series of solvent extractions or by chromatographic separation. In many laboratories, each rat's genotype is checked first using the polymerase chain reaction (PCR). The exponential amplification offered by this technique can quickly differentiate between transgene-positive and non-transgenic animals. Typically, selecting several oligonucleotide primers that span both ends of the transgene increases the power of PCR. In this fashion, transcripts from transgenic and wild-type rats can be differentiated readily by sizing their distinct band migration patterns on an agarose gel. However, nonspecific annealing of oligonucleotide probes to DNA sites distant from the transgene may lead to false positive readings. For this reason, most laboratories confirm the PCR data by Southern analysis, in which unamplified DNA is separated on a gel. The omission of the enzymatic amplification step in this latter procedure prevents most erroneous results, but the trade-off is that the small quantity of unamplified transgenic DNA in the tissue sample may be difficult to detect. Interpretation of such faint bands is aided significantly by the use of restriction enzymes (specific for sites within the transgene). Digestion of transgenic and wild-type DNA strands results in restriction fragments of differing lengths, allowing detection of the two bands by their distinct gel migration patterns. Additional data obtained from the Southern blot are (1) confirmation that the founder rats have acquired the full-length transgene sequence without undergoing gene rearrangements or deletions, (2) information regarding the number of sites at which the transgene has been integrated, and (3) an estimate of the number of transgene copies that have been incorporated into the rat's genome. The multiplicity of integration sites is addressed by comparing the inherited bands of the founder with its progeny, and the bands of the offspring to each other; all rats are demonstrated to possess the same integration site if the DNA bands resulting from restriction enzyme mapping of the transgenic DNA are identical.

An assessment of transgene copy number is performed by comparing the signal strength of the transgenic bands with a control lane containing a known amount (usually a single copy) of an endogenous gene. A comparable analysis may be used to determine whether the offspring of a heterozygous mating are heterozygous (one copy) or homozygous (two copies) for the transgene. Finally, fluorescent *in situ* hybridization (FISH) analysis using transgene-specific probes may be used in cytological preparations of interphase (intact) cells to determine whether or not nuclei contain one or two copies of transgenic DNA (Dinchuk *et al.*, 1994; Nishino *et al.*, 1995).

Phenotypic Analysis of Engineered Rats

Incorporation of foreign DNA does not necessarily lead to useful gene expression. Often, inserted DNA is not transcribed into stable full-length mRNA, or mRNA is not translated into a structurally or functionally active protein. Similarly, transgenic expression of a non-rat protein may not be associated with any physiological effect in the rat (Ganten *et al.*, 1992). Ideally, a series of molecular biological and morphological assays may

be performed to characterize both the presence and the effect of the transgene's product (e.g. Sereda *et al.*, 1996). Again, protocols and application of these procedures within transgenic studies have been collected into reference manuals (Wassarman and DePamphilis, 1993; Hogan *et al.*, 1994; Pinkert, 1994; Ausubel *et al.*, 1998).

In general, gene expression is assessed by measuring either the DNA-derived message (mRNA) or, where possible, quantification of the presence or function of the ultimate transgene product (protein). Detection of the protein is usually preferable as high levels of mRNA often do not correlate with the quantity (or even capacity) for translation into protein. Furthermore, for some proteins the site of mRNA translation does not correlate with the ultimate site of protein activity. This circumstance is characteristic of tissues (e.g. brain) in which cell bodies contain RNA while the protein is transported to distant cellular processes, and of sites where the protein binds to receptors located on other cells or the extracellular matrix.

Quantitative Analysis of Gene Expression

Quantitative analysis is typically performed in homogenized tissues (for adult rats) or with pulverized conceptuses. Aliquots of homogenate are then purified to obtain the molecule to be assessed, either total RNA, mRNA or protein. The molecule is detected by hybridization with a single- or double-stranded nucleotide probe (for RNA) or binding of an antibody (for protein). These detection reagents are labeled with a radioisotope, a fluorochrome or an enzyme.

The analytical techniques of choice for RNA are the northern assay, reverse transcription polymerase chain reaction (RT-PCR), and the ribonuclease protection assay (RPA). Multiple probes may be readily prepared to interact with unique sequences that span both the gene-coding region, the promoter, and the flanking vector; the use of several probes may greatly increase the assay specificity. In methods employing electrophoresis, the identity of the molecule is verified by comparison to a standard (to evaluate size and migration characteristics) and, if necessary, by excising the band and sequencing the genetic material. Positive internal controls should be included in all assays, such as ubiquitously expressed **'housekeeping' genes** (e.g. actin or glyceraldehyde-3-phosphate dehydrogenase, GADPH; Goldsworthy *et al.*, 1993). The sensitivity and specificity of RPA and RT-PCR is increased over that of the northern assay because binding of the sense mRNA with a complementary probe occurs in the reaction buffer rather than on a solid membrane. In RPA, the generation of the double-stranded molecule is detected by the addition of a RNase with specificity for single-stranded nucleotides (RNA or DNA); the enzyme destroys all but the protected, hybridized message. The product of RT-PCR can be measured through detection of an appropriately sized molecule on a gel, or by real-time quantification of the hybridization reaction in solution using a luminescent detection system.

The quantifiable assays for the transgene-derived protein in tissues are the western analysis and enzyme biochemical procedures. The former technique measures the physical presence of the molecule, while the latter detects the presence and activity of the functional protein. In addition, secreted proteins encoded by transgenes may be detected in body fluids (e.g. serum) using enzyme-linked immunosorbent assays (ELISA) or enzyme kinetic tests to detect the physical or functional presence, respectively, of the protein. Multiple antibodies that interact with unique protein epitopes may be available. If a wild-type allele is replaced with a mutant allele or a marker gene, the use of probes with specificity for either the wild-type protein or the introduced molecule will increase the discriminating capacity of the assay.

Qualitative Localization of Gene Distribution

Once expression of the transgene has been confirmed in a genetically engineered line, a qualitative analysis to localize the RNA and/or protein is performed in tissues. These methods may detect molecules either in entire organs or embryos ('whole mount' procedures) or in tissue sections (slide-based procedures). If feasible, organs are preserved by fixation (usually by immersion in a dilute solution of buffered aldehyde) to preserve the overall tissue structure for subsequent analysis. However, in certain instances (e.g. proteins with delicate antigenic sites) the fresh tissues are embedded in mounting medium (e.g. O.C.T.; Miles Laboratories, Elkhart,

IN, USA) and flash-frozen in supercooled isopentane (2-methyl butane) to prevent degradation of the molecule while retaining acceptable tissue morphology. The analytical techniques used to localize sites of transgene expression are comparable to those described above for homogenized tissues. The principal method for demonstrating sense mRNA is *in situ* hybridization. Tissues are treated with a solution containing a complementary nucleotide probe labeled with an isotope, fluorochrome or enzyme. Next, multiple highly stringent washing steps (e.g. elevated temperature, low salinity) and a RNase bath are applied to remove nonspecifically bound probe. At present, isotopic methods are considered more sensitive, but it may be impossible to determine the exact cells in which transgenes are expressed. In contrast, probes conjugated to fluorochromes or enzymes often may be used to define the cellular and even subcellular distributions of mRNAs of interest. Protein products are defined by immunohistochemistry (Larsson, 1993; Osborn and Isenberg, 1994) and enzyme histochemistry (Chayen and Bitensky, 1994; Mercer, 1999). Again, the former method measures the physical presence of the transgene product, while the latter detects the presence and activity of the functional protein.

Antibodies are the main reagents used for immunohistochemistry. Anti-rat reagents may be available commercially for more common antigens (Weimer, 1996; Linscott, 1998); in addition, empirical testing may demonstrate the ability of antibodies directed against non-rat antigens to cross-react with rat molecules, thereby providing for their use in rat tissues (e.g. Smith, 1990). In many instances, however, antibodies must be made for the gene product of interest. Double-staining procedures to localize mRNA and protein simultaneously are possible by performing *in situ* hybridization and immunohistochemistry on the same sample. The mRNA method is commonly performed first because the nucleic acids are more vulnerable to the experimental conditions during this extended procedure.

Phenotypic Analysis

The two reasons for performing genetic engineering experiments are (1) to produce a rat model with specific biological features – often to generate an animal model of human disease – or (2) to introduce a gene sequence and to investigate the impact of altered gene expression *in vivo*. Regardless of the reason, engineered animals must be characterized fully to be of maximal use. Many different endpoints may be assessed, and the choice of which parameters to select often is dictated by the researcher's interests even more than the nature of any extraordinary findings. Two general categories to be evaluated are function and morphology of the rat. These features may be examined either in the intact animal *in vivo* or in relevant target tissues *in vitro*. Heterozygous animals should be included in the assessment of homozygous and control animals since partial penetrance (diluted intensity) of the transgene-induced phenotype may result from the reduced gene dosage (Lee *et al.*, 1995; Langheinrich *et al.*, 1996; Sereda *et al.*, 1996). In addition, the generation of chimeras with different ratios of transfected ES cells will assist in the study of gene dosage in developing and adult animals, particularly where a homozygous mutation results in an embryonic lethal phenotype. Finally, sexual dimorphism with respect to gene expression and/or the severity of the phenotype may occur (e.g. Veniant *et al.*, 1996; Cranston *et al.*, 1997), so rats of both sexes should be assessed.

Function represents an important aspect of any evaluation. The battery of tests to be performed will depend upon the prior or predicted knowledge regarding a novel transgene's phenotype. For example, engineered genes with sequences that are homologous to members of a gene family or which are components of a specific biochemical pathway may be anticipated to have phenotypes comparable to other members of the group, so the battery of endpoints might concentrate upon confirming a known pattern of defects (e.g. Cacalano *et al.*, 1998). In contrast, transgenes for which phenotypes have not been defined must be investigated using a more extensive regimen. Initial tests *in vivo* are made during the in-life portion of the experiment using behavioral and clinical endpoints. The data may be acquired repeatedly, are relatively noninvasive, and provide a dynamic assessment of a global physiological state. Transgene-induced behavioral alterations might only be manifested during a limited phase of the animal's life (e.g. the neonatal period, adulthood, senescence), or the effects may occur throughout life. For practical considerations (cost and time), many functional analyses are limited to neonatal and young adult animals. Conventional clinical pathology assays are performed on body fluids such as blood or urine and often include serum chemistry to assess organ function (chiefly

for the kidney, liver and pancreas) as well as hematologic parameters to assess blood cell populations. Flow cytometric analysis (Camplejohn, 1994) of cell populations (e.g. blood cells, dissociated lymphoid organs and tumors) may also provide useful information regarding transgene-induced alterations. In addition, *in vitro* functional assays might evaluate cell culture (Sereda *et al.*, 1996) or tissue slice (e.g. Alger *et al.*, 1984) models using endpoints such as cytokine production (Shikishima *et al.*, 1997), electrophysiological properties (Sereda *et al.*, 1996), or metabolism (Yamaguchi *et al.*, 1992). Advantages of functional assays include the speed with which data may be gathered; the non-invasive nature of certain tests, which allows repeated measurements in the same animal; and the quantifiable nature of the results. The chief disadvantages will probably be the wide degree of biological variation between responses of individual rats, and between animals of different strains (Crawley *et al.*, 1997). The shifting nature of functional endpoints *in vivo* will often necessitate special experimental designs with larger numbers of animals to control for this variation. In all cases, data should be compared to the findings for normal rats, ideally using age-matched wild-type littermates as controls.

Morphology represents another element of the characterization process, and it often is used as the 'gold standard' for defining the effects of genetic manipulation. Initial clinical observations are gathered for the living rat, with subsequent macroscopic (gross) assessments of organ systems at necropsy. Specific endpoints that might be noted include alterations in the size (larger, smaller, absent), shape, color or location of organs, or the presence of aberrant elements (e.g. extra organs, tumors). Tissues then are fixed and processed for microscopic evaluation. If no obvious abnormalities are noted, and if an expected target organ is not known, a battery of organ systems is screened using a routine tissue stain (such as hematoxylin and eosin). Next, organs with confirmed transgene-induced lesions are subjected to more extensive analysis to define the biological mechanism. Techniques of value for such research might include electron microscopy, immunohistochemistry, *in situ* hybridization, magnetic resonance imaging (Lukkarinen *et al.*, 1997) and morphometry. The choice of methods is usually selected on a case-by-case basis.

The increasing use of genetic engineering technology has led to a rapidly growing list of genes that have been shown to have a critical but previously unsuspected role in embryonic development. In many instances, alteration of these pathways during development announces its significance through the crude, but definitive, phenotype of embryonic lethality. Often, defining when and where the altered genes and their products exert their effects, as well as the mechanistic pathway involved, is extremely difficult. Initial attempts will probably include a sequential analysis of both gross and microscopic structure in the conceptus (and often the placenta). The goals at this stage are (1) to discover the developmental stage at which the lethal event is occurring and then (2) to find the target organ(s) in an earlier embryonic stage. The recent publication of several atlases describing prenatal development of rodents (Theiler, 1989; Kaufman, 1992; Altman and Bayer, 1995; Paxinos *et al.*, 1995) has greatly aided such investigations. Where feasible, insertion of a marker gene (Alam and Cook, 1990) in tandem with the transgene may provide a means of defining site(s) of early gene expression. Particular attention must be paid to ensure that any phenotype is the result of the desired genetic event and not an insertional mutation in an endogenous gene (Woychik *et al.*, 1985).

Disclaimer

The reference texts, software packages, and products mentioned in this chapter are included only for illustrative purposes. While these items have been used successfully by the authors, other products may serve the same purposes.

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References

Akagi, K., Sandig, V., Vooijs, M. *et al.* (1997) *Nucleic Acids Res.* **25**, 1766–1773.

Alam, J. and Cook, J.L. (1990) *Anal. Biochem.* **188**, 245–254.

Alger, B.E., Dhanjal, S.S., Dingledine, R. *et al.* (1984) In: Dingledine, R. (ed.) *Brain Slices*, pp. 381–437. New York: Plenum Press.

Altman, J. and Bayer, S.A. (1995) *Atlas of Prenatal Rat Brain Development*. Boca Raton: CRC Press.

Anzai, M., Nakagata, N., Matsumoto, K., Takahashi, Y. and Miyata, K. (1994) *Exp. Anim.* **43**, 247–250.

Ausubel, F.M., Brent, R., Kingston, R.E. *et al.* (1998) *Current Protocols in Molecular Biology*, Vols 1–3. New York: John Wiley.

Baldwin, H.S., Mickanin, C. and Buck, C. (1997) *Gene Ther.* **4**, 1142–1149.

Beerli, R.R., Wels, W. and Hynes, N.E. (1994) *J. Biol. Chem.* **269**, 23931–23936.

Blackwood, E.M. and Kadonga, J.T. (1998) *Science* **281**, 60–63.

Borrelli, E., Heyman, R., His, M. and Evans, R.M. (1988) *Proc. Natl Acad. Sci. USA* **85**, 7572–7576.

Bos-Mikich, A., Whittingham, D.G. and Jones, K.T. (1997) *Dev. Biol.* **182**, 172–179.

Bradley, A., Evans, M., Kaufman, M.H. and Robertson, E. (1984) *Nature* **309**, 255–256.

Brinster, R.L., Chen, H.Y., Trumbauer, M.E., Yagle, M. and Palmiter, R.D. (1985) *Proc. Natl Acad. Sci. USA* **85**, 4438–4442.

Bronson, S.K. and Smithies, O. (1994) *J Biol. Chem.* **269**, 27155–27158.

Brown, T.A. (1991) *Essential Molecular Biology: A Practical Approach*, Vols 1 and 2. Oxford: IRL Press.

Byrne, R.W. and Ruddle, F.H. (1989) *Proc. Natl. Acad. Sci. USA* **86**, 5473–5477.

Cacalano, G., Fariñas, I., Wang, L.-C. *et al.* (1998) *Neuron* **21**, 53–62.

Campbell, K.H.S., McWhir, J., Ritchie, W.A. and Wilmut, I. (1996) *Nature* **380**, 64–66.

Camplejohn, R.S. (1994) *J. Microsc.* **176**, 1–7.

Canseco, R.S., Sparks, A.E.T., Page, R.L. *et al.* (1994) *Transgenic Res.* **3**, 20–25.

Capecchi, M.R. (1989) *Science* **244**, 1288–1292.

Chang, S.S. and Mark, H.F. (1997) *Cytobios* **90**, 7–22.

Charreau, B., Tesson, L., Soulillou, J.P., Pourcel, C. and Anegon, I. (1996) *Transgenic Res.* **5**, 223–234.

Chayen, J. and Bitensky, L. (1994) In: Celis, J.E. (ed.) *Cell Biology: A Laboratory Handbook*, Vol. 2, pp. 245–252. San Diego: Academic Press.

Chen, B. and Hales, B.F. (1995) *Biol. Reprod.* **53**, 1229–1238.

Clark, A.J., Bissinger, P., Bullock, D.W. *et al.* (1994) *Reprod. Fertil. Dev.* **6**, 589–598.

Copp, A.J. (1995) *Trends Genet.* **11**, 87–93.

Cranston, A., Bocker, T., Reitmar, A. *et al.* (1997) *Nature Genet.* **17**, 114–118.

Crawley, J.N., Belknap, J.K., Collins, A. *et al.* (1997) *Psychopharmacology* **132**, 107–124.

Deshane, J., Siegal, G.P., Wang, M. *et al.* (1997) *Gynecol. Oncol.* **64**, 378–385.

Dieffenbach, C.W. and Devksler, G.S. (1993) *PCR Methods Appl.* **3**, S2–S7.

Dietrich, D.R. and Swenberg, J.A. (1991) *Fundam. Appl. Toxicol.* **97**, 749–762.

Dinchuk, J.E., Kelley, K. and Boyle, A.L. (1994) *Bio Techniques* **17**, 954–959.

Doetschman, T., Gregg, R.G., Maeda, N. *et al.* (1987) *Nature* **330**, 576–578.

Dycaico, M.J., Provost, G.S., Kretz, P.L. Ransom, S.L., Moores, J.C. and Short, J.M. (1994) *Mutat. Res.* **307**, 461–478.

Dymecki, S.M. (1996) *Proc. Natl Acad. Sci. USA* **93**, 6191–6196.

Evans, M.J. and Kaufman, M.H. (1981) *Nature* **292**, 154–156.

Fishman, G.I. (1995) *Trends Cardiovasc. Med.* **5**, 211–217.

Fishman, G.I., Kaplan, M.L. and Buttrick, P.M. (1994) *J. Clin. Invest.* **93**, 1864–1868.

Folger, K.R., Wong, E.A., Wahl, G. and Capecchi, M.R. (1982) *Mol. Cell. Biol.* **2**, 1372–1387.

Furth, P., St. Onge, L., Boger, H. *et al.* (1994) *Proc. Natl Acad. Sci. USA* **91**, 9302–9306.

Ganten, D., Wagner, J., Zeh, K. *et al.* (1992) *Proc. Natl Acad. Sci. USA* **89**, 7806–7810.

Gardner, D.P., Byrne, G.W., Ruddle, F.H. and Kappen, C. (1996) *Transgenic Res.* **5**, 37–48.

Gill, T., Smith, G.J., Wissler, R.W. and Kuntz, H.W. (1989) *Science* **245**, 269–276.

Goldsworthy, S.M., Goldsworthy, T.L., Sprankle, C.S. and Butterworth, B.E. (1993) *Cell Prolif.* **26**, 511–517.

Gossen, M., Bonin, A.L. and Bujard, H. (1993) *Trends Biol. Sci.* **18**, 471–475.

Gossen, M., Freundlieb, S., Bender, G., Müller, G., Hillen, W. and Bujard, H. (1995) *Science* **268**, 1766–1768.

Gossler, A., Doetschman, T., Korn, R., Serfling, E. and Kemler, R. (1986) *Proc. Natl Acad. Sci. USA* **83**, 9065–9069.

Greenwald, R.A. and Diamond, H.S. (1988) *Handbook of Animal Models for Rheumatic Diseases*, Vol. 1. Boca Raton: CRC Press.

Gu, H., Marth, J.D., Orban, P.C., Mossmann, H. and Rajewsky, K. (1994) *Science* **265**, 103–106.

Hammer, R.E., Maika, S.D., Richardson, J.A., Tang, J. and Taurog, J. (1990) *Cell* **3**, 1099–1112.

Hanks, M., Wurst, W., Anson-Cartwright, L., Auerback, A.B. and Joyner, A.L. (1995) *Science* **269**, 679–682.

Hasty, P. and Bradley, A. (1993) In: Joyner, A.L. (ed.) *Gene Targeting: A Practical Approach*, pp. 1–31. Oxford: IRL Press.

Heideman, J. (1991) *Bio/Technology* **16**, 325–332.

Hirabayashi, M., Takahashi, R., Sekiguchi, J. and Ueda, M. (1997) *Exp. Anim.* **46**, 111–115.

Hogan, B., Beddington, R., Costantini, F. and Lacy, E. (1994) *Manipulating the Mouse Embryo: A Laboratory Manual*, 2nd edn. Cold Spring Harbor Press: Plainview, New York.

Holzinger, A., Trapnell, B.C., Weaver, T.E., Whitsett, J.A. and Iwamoto, H.S. (1995) *Pediatr. Res.* **38**, 844–850.

Iannacconne, P.M., Taborn, G.U., Garton, R.L., Caplice, M.D. and Brenin, D.R. (1994) *Dev. Biol.* **163**, 288–292. [Published erratum appears in *Dev. Biol.* **185**, 124–125 (1997)].

Ichikawa, T., Kyprianou, N. and Isaacs, J.T. (1990) *Cancer Res.* **50**, 6349–6357.

Irwin, M.H., Moffatt, R.J. and Pinkert, C.A. (1996) *Nature Biotechnol.* **14**, 1146–1148.

Jaffe, H.A., Daniel, C., Longenecker, G. *et al.* (1992) *Nature Genet.* **1**, 372–378.

Kallioniemi, A., Kallioniemi, O.-P., Sudar, D. *et al.* (1992) *Science* **258**, 818–821.

Kasarkis, A., Manova, K. and Anderson, K.V. (1998) *Proc. Natl Acad. Sci. USA* **95**, 7485–7490.

Kaufman, M.H. (1992) *The Atlas of Mouse Development*, 2nd edn. San Diego: Academic Press.

Kerler, R. and Rabes, H.M. (1994) *Crit. Rev. Oncogenesis* **5**, 271–295.

Kistner, A., Gossen, M., Zimmermann, F. *et al.* (1996) *Proc. Natl Acad. Sci. USA* **93**, 10933–10938.

Koller, B.H. and Smithies, O. (1992) *Annu. Rev. Immunol.* **10**, 705–730.

Kühn, R., Schwenk, F., Aguet, M. and Rajewsky, K. (1995) *Science* **269**, 1427–1429.

Langheinrich, M., Lee, M.A., Boehm, M., Pinto, Y.M., Ganten, D. and Paul, M. (1996) *Am. J. Hypertension* **9**, 506–512.

Larsson, L.-I. (1993) *Appl. Immunohistochem.* **1**, 2–16.

La Salle, G.L.G., Robert, J.J., Berrard, S. *et al.* (1993) *Science* **259**, 988–990.

Lee, M.A., Boehm, M., Kim, S. *et al.* (1995) *Hypertension* **25**, 570–580.

Lefevre, P.A., Tinwell, H. and Ashby, J. (1997) *Mutagenesis* **12**, 45–47.

Lesser, W. (1995) In: Meyers, R.A. (ed.) *Molecular Biology and Biotechnology. A Comprehensive Desk Reference*, pp. 907–910. New York: VCH Publishers.

Levan, G. (1974) *Hereditas* **77**, 37–52.

Lichter, P., Chang, C.-J., Call, K. *et al.* (1990) *Science* **247**, 64–69.

Linscott, W.D. (1998) *Linscott's Directory of Immunological and Biological Reagents*, 10th edn. 4877 Grange Rd., Santa Rosa, CA.

Liyanage, M., Coleman, A., du Manoir, S. *et al.* (1996) *Nature Genet.* **14**, 312–315.

Lukkarinen, J., Groehn, O.H., Sinervirta, R. *et al.* (1997) *Stroke* **28**, 639–645.

Marth, J.D. (1996) *J. Clin. Invest.* **97**, 1999–2002.

Martin, G.R. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 7634–7638.

Matsumoto, K., Kakidani, H., Takahashi, T. *et al.* (1993) *Mol. Reprod. Dev.* **36**, 53–58.

Mercer, E. (1999) http://www.rodentia.com/wmc/; Laboratory Section, Technical Guides and Protocols' sub-section/

Metzger, D., Clifford, J., Chiba, H. and Chambon, P. (1995) *Proc. Natl. Acad. Sci. USA* **92**, 6991–6995.

Mintz, B. and Klein-Szanto, A.J. (1992) *Proc. Natl Acad. Sci. USA* **89**, 11421–11425.

Mittelman, F. (1983) *Cytogenet. Cell Genet.* **36**, 1–515.

Montaudon, D., Benchekroun, M.N., Londos-Gagliardi, D. and Robert, J. (1990) *Anticancer Res.* **10**, 1667–1676.

Mukumoto, S., Mori, K. and Ishikawa, H. (1995) *Exp. Anim.* **44**, 111–118.

Mullins, L.J. and Mullins, J.J. (1996) *J. Clin. Invest.* **97**, 1557–1560.

Mullins, J.J., Peters, J. and Ganten, D. (1990) *Nature* **344**, 541–544.

Nakagata, N. (1993) *J. Reprod. Fertil. Steril.* **87**, 479–483.

Nishino, H., Herath, J.F., Jenkins, R.B. and Sommer, S.S. (1995) *BioTechniques* **19**, 587–588, 590, 592.

No, D., Yao, T.-P. and Evans, R.M. (1996) *Proc. Natl Acad. Sci. USA* **93**, 3346–3351.

Oligino, T., Poliani, P.L., Wang, Y. *et al.* (1998) *Gene Ther.* **5**, 491–496.

Orban, P.C., Chui, D. and Marth, J.D. (1992) *Proc. Natl Acad. Sci. USA* **89**, 6861–6865.

Ogbourne, S. and Antalis, T.M. (1998) *Biochem. J.* **331**, 1–14.

Osborn, M. and Isenberg, S. (1994) In: Celis, J.E. (ed.) *Cell Biology: A Laboratory Handbook*, Vol. 2, pp. 361–367. San Diego: Academic Press.

Palmiter, R.D., Norstedt, G., Gelinas, R.E., Hammer, R.E. and Brinster, R.L. (1983) *Science* **222**, 809–814.

Palmiter, R.D., Behringer, R.R., Quaife, C.J., Maxell, F., Maxwell, I.H. and Brinster, R.L. (1987) *Cell* **50**, 435–443.

Papaioannou, V. and Johnson, R. (1993) In: Joyner, A.L. (ed.) *Gene Targeting: A Practical Approach*, pp. 107–146. Oxford: IRL Press.

Parra, I. and Windle, B. (1993) *Nature Genet.* **5**, 17–21.

Passman, R. and Fishman, G.I. (1994) *J. Clin. Invest.* **94**, 2421–2425.

Paul, M., Wagner, J., Hoffman, S., Urata, H. and Ganten, D. (1994) *Annu. Rev. Physiol.* **56**, 811–829.

Paxinos, G., Ashwell, K.W.S. and Törk, I. (1995) *Atlas of the Developing Rat Nervous System*, 2nd edn. San Diego: Academic Press.

Pinkert, C.A. (1994) *Transgenic Animal Technology: A Laboratory Handbook.* San Diego: Academic Press.

Qin, H. and Gunning, P. (1997) *J. Biochem. Biophys. Methods* **36**, 63–72.

Rajewsky, K., Gu, H., Kühn, R. *et al.* (1996) *J. Clin. Invest.* **98**, 600–603.

Ramirez-Solis, R., Liu, P. and Bradley, A. (1995) *Nature* **378**, 720–724.

Robbins, P.D., Tahara, H. and Ghivizzani, S.C. (1998) *Trends Biotechnol.* **16**, 35–40.

Robertson, E., Bradley, A., Kuehn, M. and Evans, M. (1986) *Nature* **323**, 445–448.

Robl, J.M. and Heideman, J.K. (1994) In: Pinkert, C.A. (ed.) *Transgenic Animal Technology: A Laboratory Handbook*, pp. 265–278. San Diego: Academic Press.

Rohlmann, A., Gotthardt, M., Willnow, T.E., Hammer, R.E. and Herz, J. (1996) *Nature Biotechnol.* **14**, 1562–1565.

Rønne, M., Shibasaki, Y., Poulsen, B.S. and Andersen, O. (1987) *Cytogenet. Cell Genet.* **45**, 113–117.

Rossant, J., Spence, A. and Rossant, J. (1998) *Trends Genet.* **14**, 358–363.

Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, 2nd edn. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press.

Sanes, J.R., Rubenstein, J.L.R. and Nicolas, J.-F. (1986) *EMBO J.* **5**, 3133–3142.

Sargent, L., Dragan, Y., Xu, Y.H., Sattler, G., Wiley, J. and Pitot, H.C. (1996) *Cancer Res.* **56**, 2985–2991.

Satoh, H., Yoshida, M.C. and Sasaki, M. (1989) *Cytogenet. Cell Genet.* **50**, 151–154.

Sauer, B. (1993) *Methods Enzymol.* **225**, 890–900.

Schmidt, E.V., Christoph, G., Zeller, R. and Leder, P. (1990) *Mol. Cell. Biol.* **10**, 4406–4411.

Schultze, N., Burki, Y., Lang, Y., Certa, U. and Bluethmann, H. (1996) *Nature Biotechnol.* **14**, 499–503.

Sereda, M., Griffiths, I., Pülhofer, A. *et al.* (1996) *Neuron* **16**, 1049–1060.

Shikishima, H., Ikeda, H., Yamada, S. *et al.* (1997) *Leukemia* **11** (suppl. 3), 70–72.

Simoes, S., Slepuskin, V., Gaspar, R., deLima, M.C.P. and Duzgunes, N. (1998) *Gene Ther.* **5**, 955–964.

Simone, N.L., Bonner, R.F., Gillespie, J.W., Emmert-Buck, M.R. and Liotta, L.A. (1998) *Trends Genet.* **14**, 272–276.

Smith, R.A. (1990) *J. Histotechnol.* **13**, 255–269.

Smithies, O., Gregg, R.G., Boggs, S.S., Kordewski, M.A. and Kucherlapati, R.S. (1985) *Nature* **317**, 230–234.

Speicher, M.R., Gwyn-Ballard, S. and Ward, D.C. (1996) *Nature Genet.* **2**, 368–375.

Swanson, M.E., Hughes, T.E., Denny, I.S. *et al.* (1992) *Transgenic Res.* **1**, 142–147.

Takahama, Y., Ochiya, T., Sasaki, H. *et al.* (1998) *Oncogene* **16**, 3189–3196.

Taketo, M., Schroeder, A.C., Mobraaten, L.E. *et al.* (1991) *Proc. Natl Acad. Sci. USA* **88**, 2065–2069.

Taurog, J.D., Richardson, J.A., Croft, J.T. *et al.* (1994) *J. Exp. Med.* **180**, 2359–2364.

Telenius, H., Pelmear, A.H., Tunnacliffe, A. *et al.* (1992) *Genes, Chromosomes Cancer* **4**, 263–267.

Theiler, K. (1989) *The House Mouse: Atlas of Embryonic Development.* New York: Springer-Verlag.

Thomas, K.R. and Capecchi, M.R. (1987) *Cell* **51**, 503–512.

Trask, B., Pinkel, D. and van den Engh, G. (1989) *Genomics* **5**, 710–717.

Tsien, J.Z., Chen, D.F., Gerber, D. *et al.* (1996) *Cell* **87**, 1317–1326.

Tsukui, T., Kanagae, Y., Saito, I. and Toyoda, Y. (1996) *Nature Biotechnol.* **1**, 982–985.

Veniant, M., Menard, J., Bruneval, P., Morley, S., Gonzales, M.F. and Mullins, J. (1996) *J. Clin. Invest.* **98**, 1966–1970.

Wakayama, T. and Yanagimachi, R. (1998) *Nature Biotechnol.* **16**, 639–641.

Wakayama, T., Perry, A.C.F., Zuccotti, M., Johnson, K.R. and Yanagimachi, R. (1998) *Nature* **394**, 369–374.

Wang, Y., DeMayo, F.J., Tsai, S.Y. and O'Malley, B.W. (1997) *Nature Biotechnol.* **15**, 239–243.

Wassarman, P.M. and DePamphilis, M.L. (1993) *Guide to Techniques in Mouse Development.* San Diego: Academic Press.

Waynforth, H.B. and Flecknell, P.A. (1992) *Experimental and Surgical Techniques in the Rat*, 2nd edn. San Diego: Academic Press.

Weimer, R.V. (1996) *MSRS Catalog of Primary Antibodies*, 3rd edn. Birmingham, MI: Aerie [http:www.antibodies-probes.com/].

Wilmut, I., Schnieke, A.E., McWhir, J., Kind, A.J. and Campbell, K.H.S. (1997) *Nature* **385**, 810–813.

Wood, S.A., Allen, N.D., Rossant, J., Auerbach, A. and Nagy, A. (1993) *Nature* **365**, 87–89.

Woychik, R.P., Stewart, T.A., Davis, L.G., D'Eustachio, P. and Leder, P. (1985) *Nature* **318**, 36–40.

Wurst, W. and Joyner, A.L. (1993) In: Joyner, A.L. (ed.) *Gene Targeting: A Practical Approach*, pp. 33–61. Oxford: IRL Press.

Yamaguchi, T., Tokita, Y., Franco-Saenz, R., Mulrow, P.J., Peters, J. and Ganten, D. (1992) *Endocrinology* **131**, 1955–1962.

Yarranton, G.T. (1992) *Curr. Opin. Biotechnol.* **3**, 506–511.