Transfer of Cloned Genes into Mouse Embryos

Development of techniques to introduce cloned foreign genes (called **transgenes**) into mouse embryos has permitted immunologists to study the effects of immune-system genes in vivo. If the introduced gene integrates stably into the germ-line cells, it will be transmitted to the offspring. Two techniques for producing transgenic mice are described in this section; one of these has been used to produce **knockout mice,** which cannot express a particular gene product (Table 23-7).

Transgenic Mice

The first step in producing transgenic mice is injection of foreign cloned DNA into a fertilized egg. In this technically demanding process, fertilized mouse eggs are held under suction at the end of a pipet and the transgene is microinjected into one of the pronuclei with a fine needle. The transgene integrates into the chromosomal DNA of the pronucleus and is passed on to the daughter cells of eggs that survive the process. The eggs then are implanted in the oviduct of "pseudo-pregnant" females, and transgenic pups are born after 19 or 20 days of gestation (Figure 23-17). In general the

TABLE 23-7 COMPARISON OF TRANSGENIC AND KNOCKOUT MICE

Characteristic	Transgenic mice	Knockout mice
Cells receiving DNA	Zygote	Embryonic stem (ES) cells
DNA constructs used	Natural gene or cDNA	Mutated gene
Means of delivery	Microinjection into zygote and implantation into foster mother	Transfer of ES cells to blastocyst and implantation into foster mother
Outcome	Gain of a gene	Loss of gene function

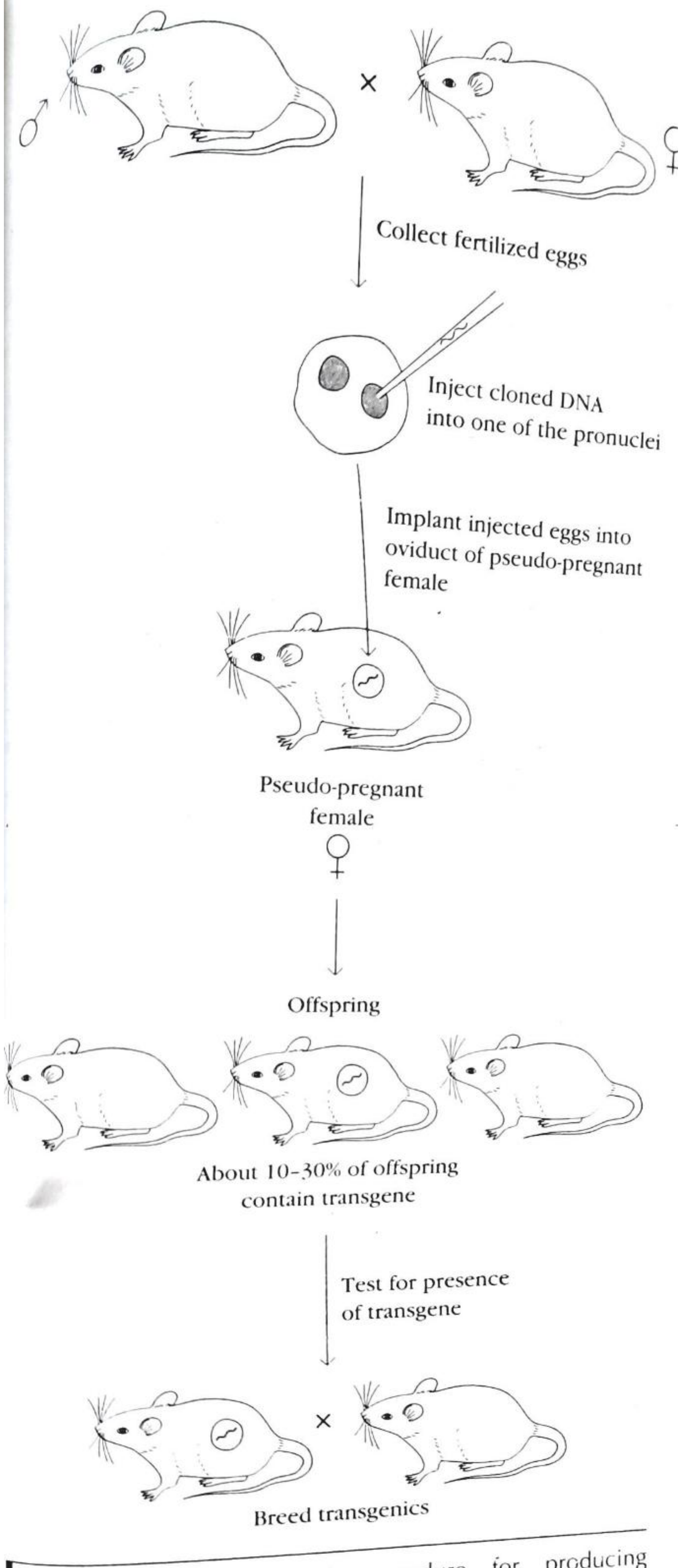


FIGURE 23-17 General procedure for producing transgenic mice. Fertilized eggs are collected from a pregnant female mouse. Cloned DNA (referred to as the transgene) is microinjected into one of the pronuclei of a fertilized egg. The eggs are then implanted into the oviduct of pseudopregnant foster mothers (obtained by mating normal females with a sterile male). The transgene will be incorporated into the chromosomal DNA of about 10%–30% of the offspring and will be expressed in all of their somatic cells. If a tissue-specific promoter is linked to a transgene, then tissue-specific expression of the transgene will result.

efficiency of this procedure is low, with only one or two transgenic mice produced for every 100 fertilized egg collected.

With transgenic mice, immunologists have been able to study the expression of a given gene in a living animal. Although all the cells in a transgenic animal contain the transgene, differences in the expression of the transgene in different tissues has shed light on mechanisms of tissue-specific gene expression. By constructing a transgene with a particular promoter, researchers can control the expression of a given transgene. For example, the metallothionein promoter is activated by zinc. Transgenic mice carrying a transgene linked to a metallothionein promoter express the transgene only if zinc is added to their water supply. Other promoters are functional only in certain tissues; the insulin promoter, for instance, promotes transcription only in pancreatic cells. Transgenic mice carrying a transgene linked to the insulin promoter, therefore, will express the transgene in the pancreas but not in other tissues.

Because a transgene is integrated into the chromosomal DNA within the one-celled mouse embryo, it will be integrated into both somatic cells and germ-line cells. The resulting transgenic mice thus can transmit the transgene to their offspring as a Mendelian trait. In this way, it has been possible to produce lines of transgenic mice in which every member of a line contains the same transgene. A variety of such transgenic lines are currently available and are widely used in immunologic research. Included among these are lines carrying transgenes that encode immunoglobulin, T-cell receptor, class I and class II MHC molecules, various foreign antigens, and a number of cytokines. Several lines carrying oncogenes as transgenes also have been produced.

Gene-Targeted Knockout Mice

One of the limitations with transgenic mice is that the transgene is integrated randomly within the genome. This means that some transgenes insert in regions of DNA that are not transcriptionally active, and hence the gene is not expressed. To circumvent this limitation, researchers have developed a technique in which a desired gene is targeted to specific sites within the germ line of a mouse. The primary use of this technique has been to replace a normal gene with a mutant allele or a disrupted form of the gene, thus knocking out the gene's function. Transgenic mice that carry such a disrupted gene, called knockout mice, have been extremely helpful to immunologists trying to understand how the removal of a particular gene product affects the immune system. A variety of knockout mice are being used in immunologic research, including mice that lack particular cytokines or MHC molecules.

Production of gene-targeted knockout mice involves the following steps:

- Isolation and culturing of embryonic stem (**ES**) **cells** from the inner cell mass of a mouse blastocyst
- Introduction of a mutant or disrupted gene into the

cultured ES cells and selection of homologous recombinant cells in which the gene of interest has been knocked out (i.e., replaced by a nonfunctional form of the gene)

- Injection of homologous recombinant ES cells into a recipient mouse blastocyst and surgical implantation of the blastocyst into a pseudo-pregnant mouse
- Mating of chimeric offspring heterozygous for the disrupted gene to produce homozygous knockout mice

The ES cells used in this procedure are obtained by culturing the inner cell mass of a mouse blastocyst on a feeder layer of fibroblasts or in the presence of leukemia inhibitory factor. Under these conditions, the stem cells grow but remain pluripotent and capable of later differentiating in a variety of directions, generating distinct cellular lineages (e.g., germ cells, myocardium, blood vessels, myoblasts, nerve cells). One of the advantages of ES cells is the ease with which they can be genetically manipulated. Cloned DNA containing a desired gene can be introduced into ES cells in culture by various transfection techniques. The introduced DNA will be inserted by recombination into the chromosomal DNA of a small number of ES cells.

The insertion constructs introduced into ES cells contain three genes: the target gene of interest and two selection genes, such as neo^R, which confers neomycin resistance, and the thymidine kinase gene from herpes simplex virus (tk^{HSV}), which confers sensitivity to gancyclovir, a cytotoxic nucleotide analog (Figure 23-18a). The construct often is engineered with the target-gene sequence disrupted by the neo^R gene and with the tk^{HSV} gene at one end, beyond the sequence of the target gene. Most constructs will insert at random by nonhomologous recombination rather than by gene-targeted insertion through homologous recombination. As illustrated in Figure 23-18b, a two-step selection scheme is used to obtain those ES cells that have undergone homologous recombination, whereby the disrupted gene replaces the target gene.

The ES cells obtained by this procedure are heterozygous for the knockout mutation in the target gene. These cells are

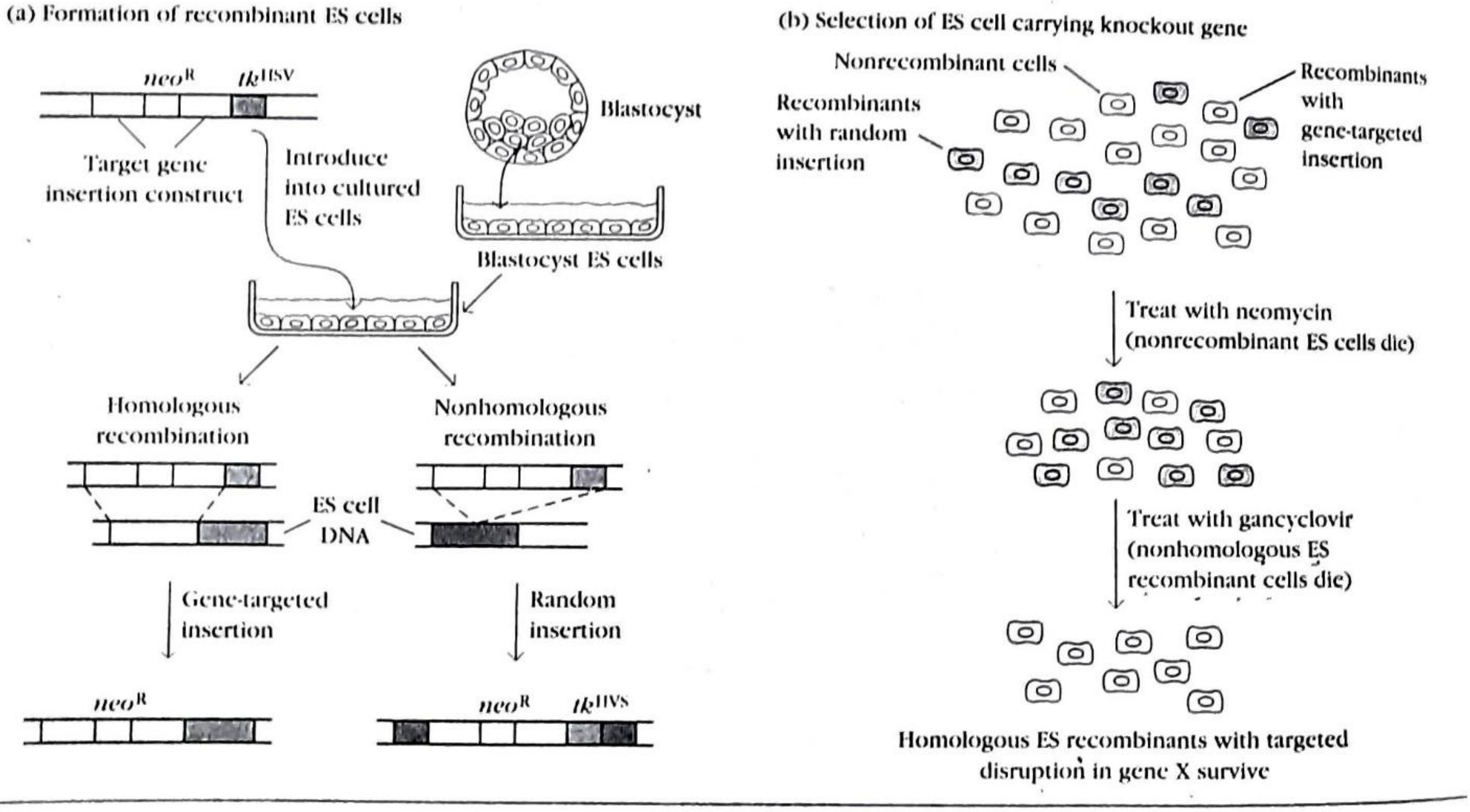


FIGURE 23-18 Formation and selection of mouse recombinant ES cells in which a particular target gene is disrupted. (a) In the engineered insertion construct, the target gene is disrupted with the neo^R gene, and the thymidine kinase tk^{HSV} gene is located outside the target gene. The construct is transfected into cultured ES cells. If homologous recombination occurs, only the target gene and the neo^R gene will be inserted into the chromosomal DNA of the ES cells. If nonhomologous recombination occurs, all three genes will be inserted. Recombination occurs in only about 1% of the cells, with nonhomologous recombination much more frequent than homologous recombination. (b) Selection with the neomycin-like drug G418 will kill any nonrecombinant ES cells because they lack the neo^R gene. Selection with gancyclovir will kill the nonhomologous recombinants carrying the tk^{HSV} gene, which confers sensitivity to gancyclovir. Only the homologous ES recombinants will survive this selection scheme. [Adapted from H Lodish et al, 1995, *Molecular Cell Biology*, 3rd ed., Scientific American Books.]

clonally expanded in cell culture and then injected into a mouse blastocyst, which subsequently is implanted into a pseudo-pregnant female. The transgenic offspring that develop are chimeric, composed of cells derived from the genetically altered ES cells and cells derived from normal cells of the host blastocyst. When the germ-line cells are derived from the genetically altered ES cells, the genetic alteration can be passed on to the offspring. If the recombinant ES cells are homozygous for black coat color (or other visible marker) and they are injected into a blastocyst homozygous for white coat color, then the chimeric progeny that carry the heterozygous knockout mutation in their germ line can be easily identified (Figure 23-19). When these are mated with each other, some of the offspring will be homozygous for the knockout mutation.

The "Knock-In" Technology

In addition to deleting a gene of choice, it also is possible to replace the endogenous gene with a mutated form of that gene. As in the strategy for knocking out a gene, DNA constructs that carry mutations in a particular gene can be exchanged for the endogenous gene. It also is possible to replace all of an endogenous gene with a DNA sequence of choice. In a recent report, for example, the CD4 gene was replaced with β-galactosidase. In these experiments, the CD4 promoter was left intact to drive the expression of β-galactosidase, which catalyzes the color change of certain reporter chemicals to blue. Using the blue color as an indicator, the experimenters could visualize cells in the developing thymus that normally transcribe CD4. Because

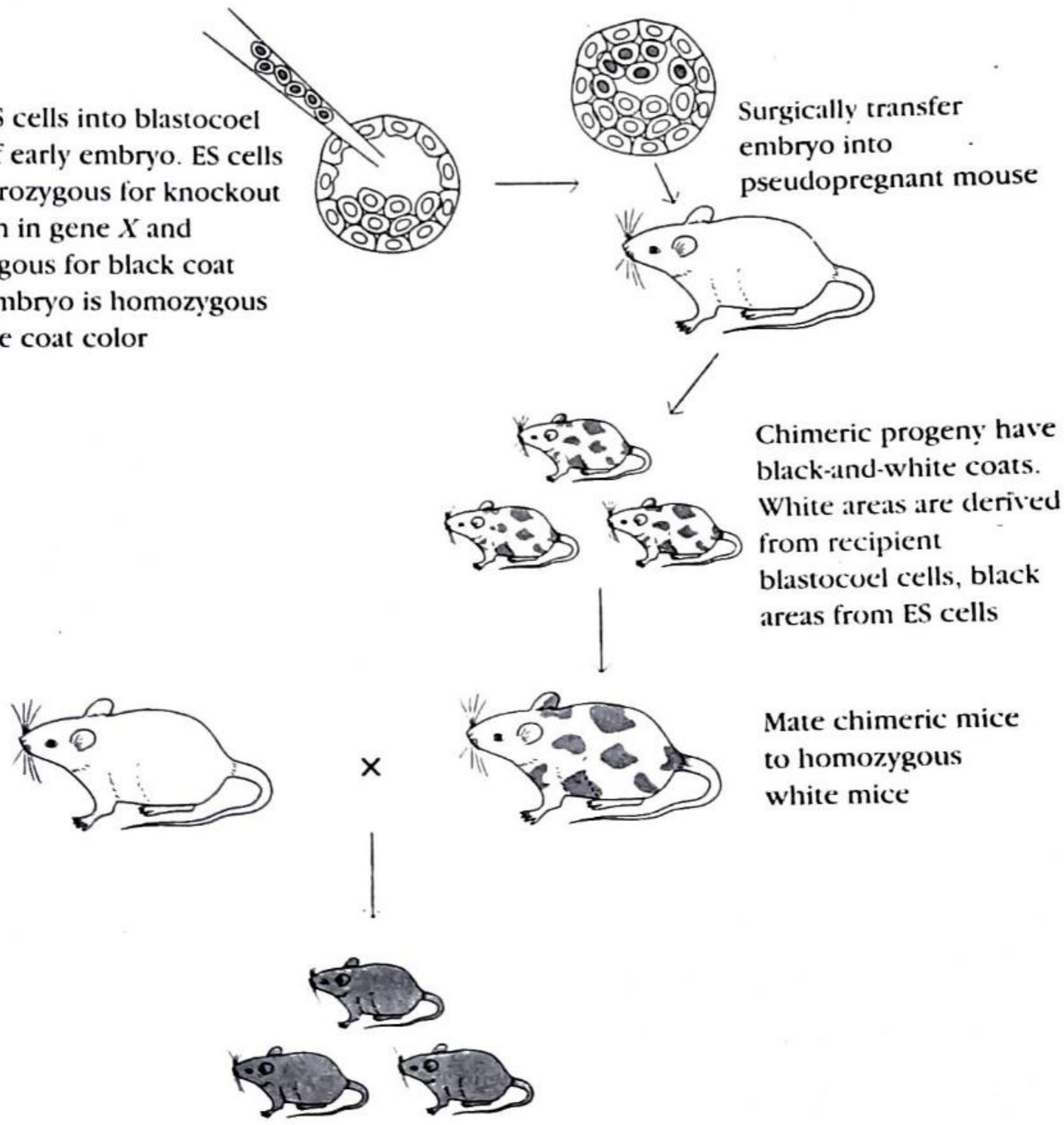
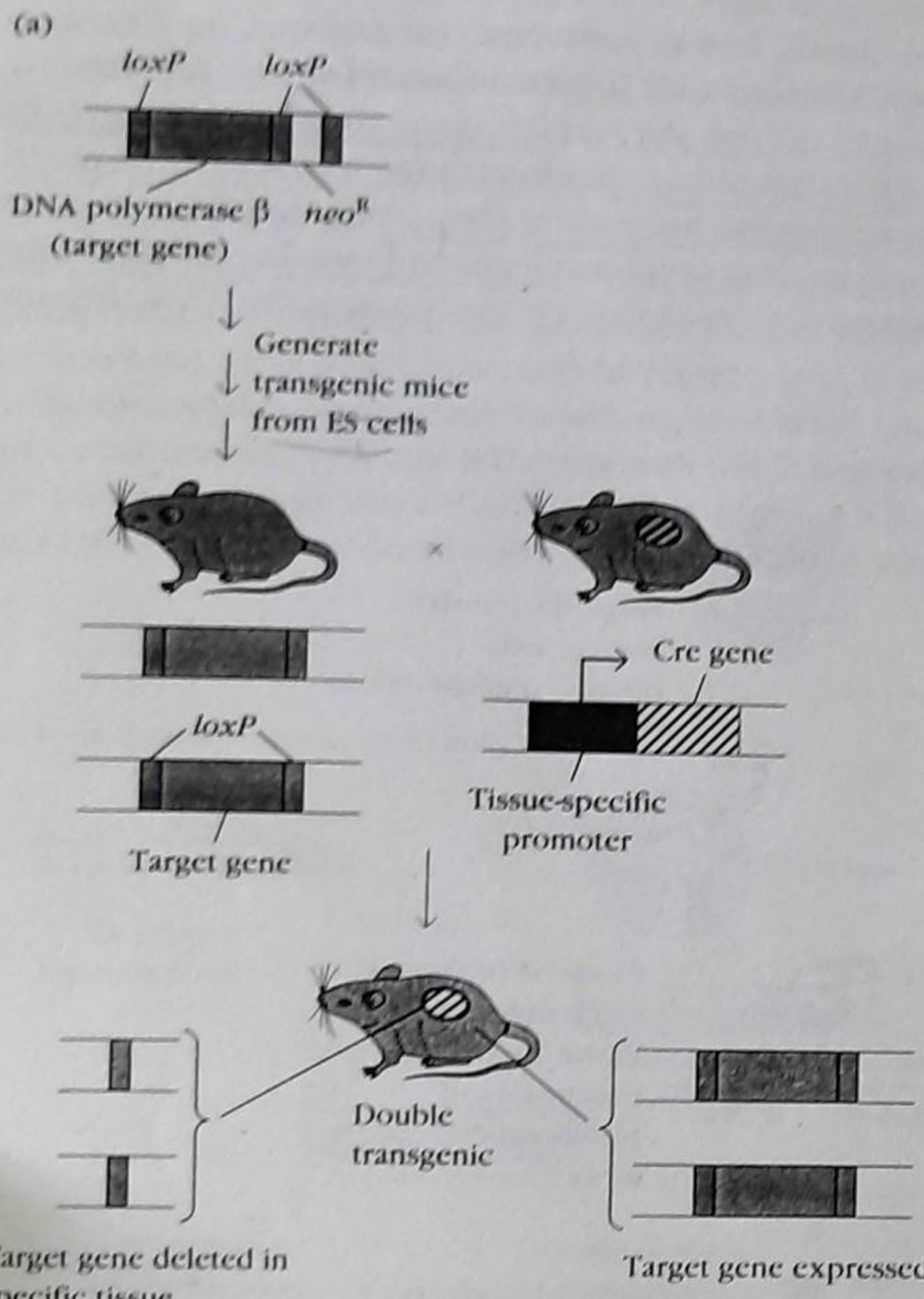
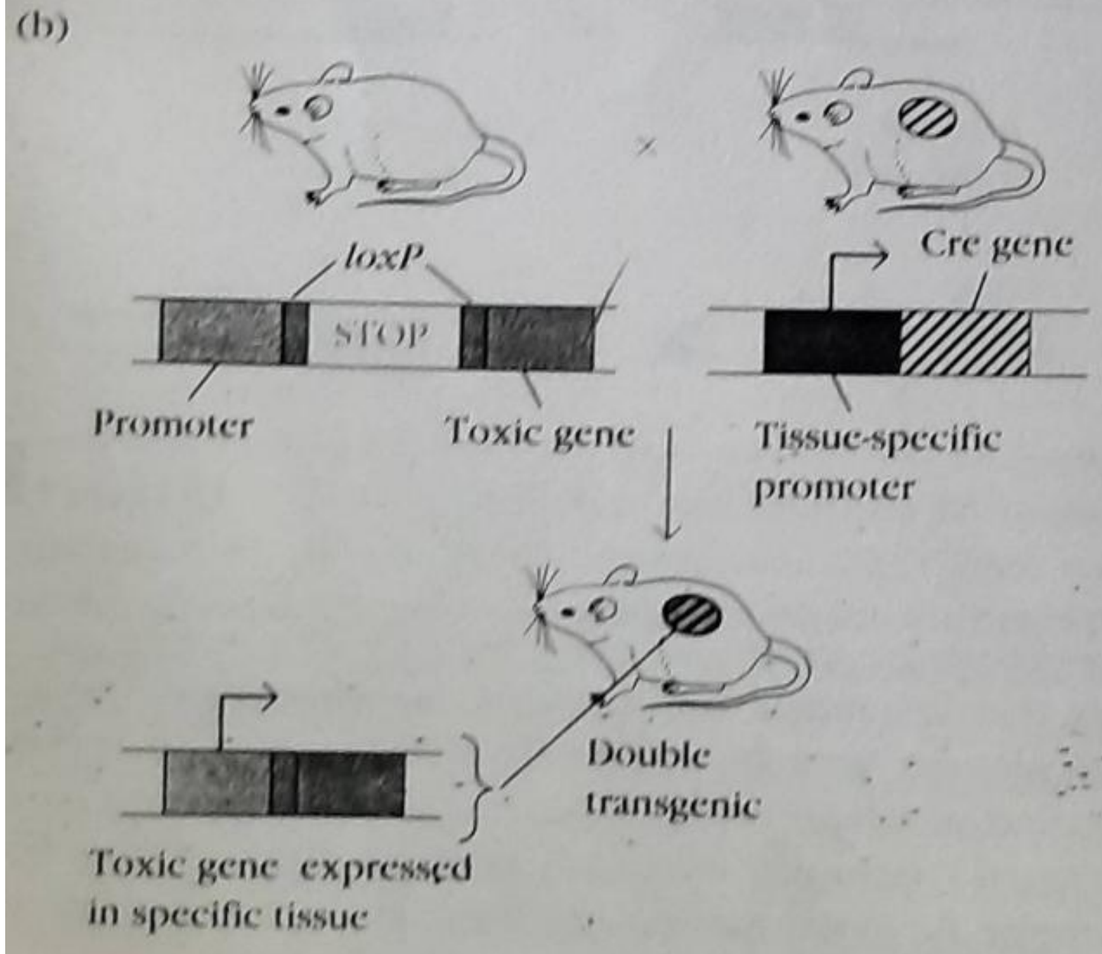


FIGURE 23-19 General procedure for producing homozygous knockout mice. ES cells homozygous for a marker gene (e.g., black coat color) and heterozygous for a disrupted target gene (see Figure 23-18) are injected into an early embryo homozygous for an alternate marker (e.g., white coat color). The chimeric transgenic offspring, which have black- and-white coats, then are mated with homozygous white mice. The all-black progeny from this mating have ES-derived cells in their germ line, which are heterozygous for the disrupted target gene. Mating of these mice with each other produces animals homozygous for the disrupted target gene, that is, knockout mice. [Adapted from MR Capecchi, 1989, *Trends Genet.* **5**:70.]

the CD4 promoter drove the expression of β-galactosidase, only those cells destined to express CD4 turned blue in the presence of the reporter chemicals. Data from these experiments were useful in tracing CD4/CD8 lineage commitment in developing T cells.





Inducible Gene Targeting—The Cre/lox System

In addition to the deletion of genes by gene targeting, recent experimental strategies have been developed that allow the specific deletion of a gene of interest in precisely the tissue of choice. These technologies rely on the use of site-specific recombinases from bacteria or yeast. The most commonly used recombinase is Cre, isolated from bacteriophage P1. Cre recognizes a specific 34-bp site in DNA known as *loxP* and, upon recognition, catalyzes a recombination. Therefore, DNA sequences that are flanked by *loxP* are recognized by Cre and the recombinational event results in the deletion of the intervening DNA sequences. In other words, animals that ubiquitously express Cre recombinase will delete all *loxP*-flanked sequences. The real innovation of this technique is that expression of the Cre recombinase gene can be controlled by the use of a tissue specific promoter. This allows tissue-specific expression of the recombinase protein and thus tissue-specific deletion of DNA flanked by *loxP*. For example, one could express Cre in B cells using the immunoglobulin promoter, and this would result in the targeted deletion of *loxP*-flanked DNA sequences only in B cells.

This technology is particularly useful when the targeted deletion of a particular gene is lethal. This is true in many circumstances. For example the DNA polymerase β gene is required for embryonic development. In experiments designed to test the Cre/*lox* system, scientists flanked the mouse DNA polymerase β gene with *loxP* and mated these

FIGURE 23-20 Gene targeting with Cre/*loxP* (a) Conditional deletion by Cre recombinase. The targeted DNA polymerase β gene is modified by flanking the gene with *loxP* sites (for simplicity, only one allele is shown). Mice are generated from ES cells by standard procedures. Mating of the *loxP*-modified-mice with a Cre transgenic will generate double transgenic mice in which the *loxP*-flanked DNA polymerase β gene will be deleted in the tissue where Cre is expressed. In this example, Cre is expressed in thymus tissue (striped) so that deletion of the *loxP*-flanked gene occurs only in the thymus (white) of the double transgenic. Other tissues and organs still express the *loxP*-flanked gene (orange). (b) Activation of gene expression using Cre/*lox*. A *loxP*-flanked translational STOP cassette is inserted between the promoter and the potentially toxic gene, and mice are generated from ES cells using standard procedures. These mice are mated to a transgenic line carrying the Cre gene driven by a tissue-specific promoter. In this example, Cre is expressed in the thymus, so that mating results in expression of the toxic gene (blue) solely in the thymus. Using this strategy, it is possible to determine the effects of expression of the potentially toxic gene in a tissue-specific fashion. [Adapted from B Sauer, 1998, *Methods* **14**:381.]

mice with mice carrying a Cre transgene under the control of a T-cell promoter (Figure 23-20a). The results of this mating are offspring that express the Cre recombinase specifically in T cells. Using such mice, the scientists were able to examine the effects of deleting the enzyme DNA polymerase β specifically in T cells. The effects of the deletion of this gene could not be examined in a conventional gene-targeting experiment, because deletion of DNA polymerase β throughout the animal would be lethal. However, with the Cre/*lox* system, it now is possible to examine the effects of the deletion of this gene in a specific tissue of the immune system.

The Cre/*lox* system also can be used to turn on gene expression in a particular tissue. Just as the lack of a particular gene may be lethal during embryonic development, the expression of a gene can be toxic. To examine tissue-specific expression of such a gene, it is possible to insert a translational stop sequence flanked by *loxP* into an intron at the beginning of the gene (Figure 23-20b). Using a tissue-specific promoter driving Cre expression, the stop sequence may be deleted in the tissue of choice and the expression of the potentially toxic gene examined in this tissue. These modifications of gene-targeting technology have been very useful in determining the effects of particular genes in cells and tissues of the immune system.