Overview of Gene Targeting by Homologous Recombination

The analysis of mutant organisms and cell lines has been important in determining the function of specific proteins. Gene targeting by homologous recombination in mammalian systems enables the production of mutants in any desired gene (Mansour, 1990; Robertson, 1991; Zimmer, 1992). Over the past two decades, gene targeting by homologous recombination has become a mainstay in gene-function investigation. This technology can be used to produce mutant mouse strains and mutant cell lines. Because most mammalian cells are diploid, they contain two copies, or alleles, of each gene encoded on a pair of autosomal (nonsex) chromosomes. In most cases, both alleles must be inactivated to produce a discernible phenotypic change in a mutant. The conversion from heterozygosity to homozygosity is accomplished by breeding in the case of mouse strains and by direct selection in cell lines.

More recent technological advances that have greatly increased the power to investigate gene function include spatial and temporal control of knockouts, mutation analysis, heterologous expression of proteins by "knock in" of coding regions into another gene, and recombineering for targeting construct production. Bacteriophage recombinase systems such as Cre with its recognition sequence, *loxP*, have played key roles in these technologies. Another recombinase system, the Flp/FRT system, can also be used (Fiering et al., 1993, 1999; Branda and Dymecki, 2004). The control can function along actual spatial coordinates when a viral gene transfer system is used, or in a cell- or tissue-specific fashion when restricted promoters are employed. Adding temporal regulation of Cre, as with the tetracycline-regulatable system (*UNIT 16.14*) or the modified Cre-ER fusion, allows temporal control as well.

To produce a mutant mouse strain by homologous recombination, two major elements are needed. An embryonic stem (ES) cell line capable of contributing to the germ line, and a targeting construct containing target-gene sequences with the desired mutation. ES cell lines are derived from the inner cell mass of a blastocyst-stage embryo. Maintaining ES cells in their undifferentiated state is a major task during gene targeting (*UNIT 23.3*). This usually is accomplished by growing cells on a layer of feeder cells (*UNIT 23.2*), which supply factors, most notably leukemia inhibitory factor (LIF), to keep cells undifferentiated. The targeting construct is then transfected into cultured ES cells (see *UNIT 23.5*). Homologous recombination occurs in a small number of the transfected cells, resulting in introduction of the mutation in the targeting construct into the target gene. Once identified, mutant ES cell clones can be microinjected into a normal blastocyst in order to produce a chimeric mouse. Because many ES cell lines retain the ability to differentiate into every cell type present in the mouse, the chimera can have tissues, including the germ line, with contribution from both the normal blastocyst and the mutant ES cells. Breeding germ-line chimeras yields animals that are heterozygous for the mutation introduced into the ES cell, and these offspring can be interbred to produce homozygous mutant mice.

Homologous recombination can also be used to produce homozygous mutant cell lines (see *UNIT 23.6*). Previously, inactivation of both alleles of a gene required two rounds of homologous recombination and selection (te Riele et al., 1990; Cruz et al., 1991; Mortensen et al., 1991). Now, however, inactivation of both alleles of many genes requires only a single round of homologous recombination using a single targeting construct (Mortensen et al., 1992). The homozygous mutant cells can then be analyzed for phenotypic changes to determine the function of the gene.

SOURCE OF DNA

Although nonisogenic DNA was used for constructs early in the history of homologous recombination, isogenic DNA (from the same mouse strain) gives higher recombination rates because homologous recombination requires stretches of exact base pair matches. Bacterial artificial chromosomes (BACs) from C57BL/6 and 129 strains are now available from genome repositories, making it more convenient to obtain DNA for constructs. Manipulations of large constructs is also possible using Red/ET recombineering methodology (Copeland et al., 2001).

Contributed by Richard Mortensen
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ANATOMY OF TARGETING CONSTRUCTS

Two basic configurations of constructs are used for homologous recombination—insertion constructs and replacement constructs (Fig. 23.1.1). Each can be used for different purposes in specific situations, as discussed below. The insertion construct is rarely used. It contains a region of homology to the target gene cloned as a single continuous sequence, and is linearized by cleavage of a unique restriction site within the region of homology. Homologous recombination introduces the insertion construct sequences into the homologous site of the target gene, interrupting normal target-gene structure by adding sequences. As a result, the normal gene can be regenerated from the mutated target gene by an intrachromosomal recombination event.

The replacement construct is the more commonly used construct. It contains two regions of homology to the target gene located on either side of a mutation (usually a positive selectable marker; see below). Homologous recombination proceeds by a double cross-over event that replaces the target-gene sequences with the replacement-construct sequences. Because no duplication of sequences occurs, the normal gene cannot be regenerated.

METHODS OF ENRICHMENT FOR HOMOLOGOUS RECOMBINANTS

Positive Selection by Drug-Resistance Gene

Nearly all constructs used for homologous recombination rely on the positive selection of a drug-resistance gene (e.g., neomycin or *neo*) that is also used to interrupt and mutate the target gene. When either insertion or replacement constructs are linearized, the drug-resistance gene is flanked by two regions of homology to the target gene. Selection of the cells using drugs (e.g., G418) eliminates the great majority of cells that have not stably incorporated the construct (see *UNIT 9.5*). However, in many of the surviving clones the construct has incorporated into the genome not by homologous recombination but rather through random integration. Therefore, methods to enrich for homologous recombinant clones have been developed.

Positive-Negative Selection

The most commonly used method for eliminating cells in which the construct integrated into the genome randomly, thus further

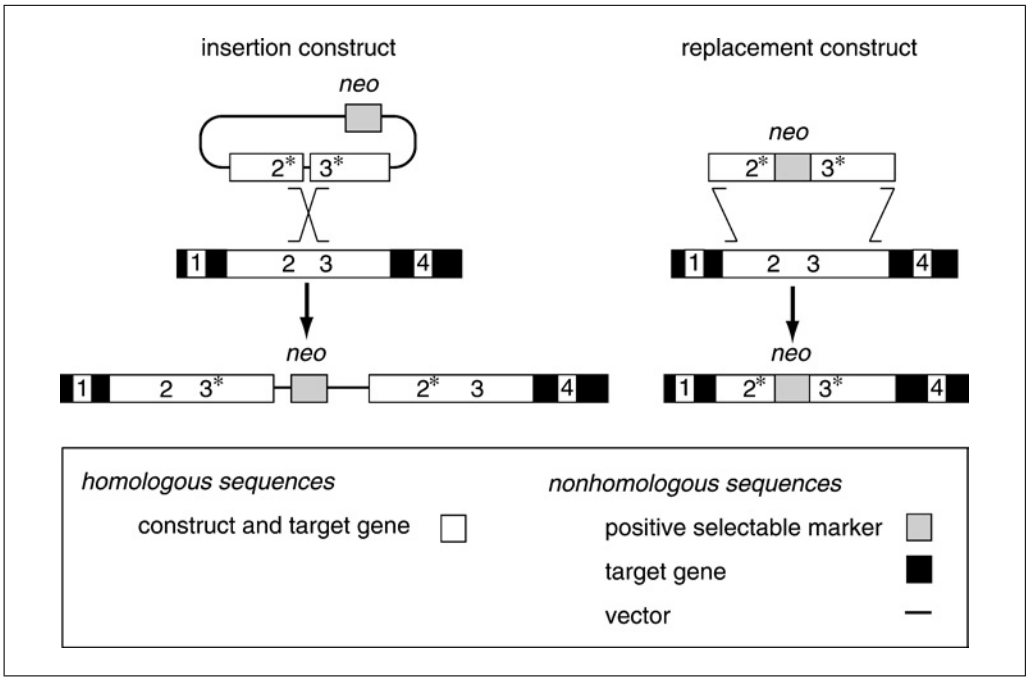

Figure 23.1.1 Two configurations of constructs used for homologous recombination. Numbers indicate target-gene sequences in the genome. An asterisk indicates homologous target-gene sequences in the construct. Replacement constructs substitute their sequences (2*, *neo*, and 3*) for the endogenous target-gene sequences (2 and 3). Insertion constructs add their sequences (2*, *neo*, and 3*) to the endogenous target gene, resulting in tandem duplication and disruption of the normal gene structure.

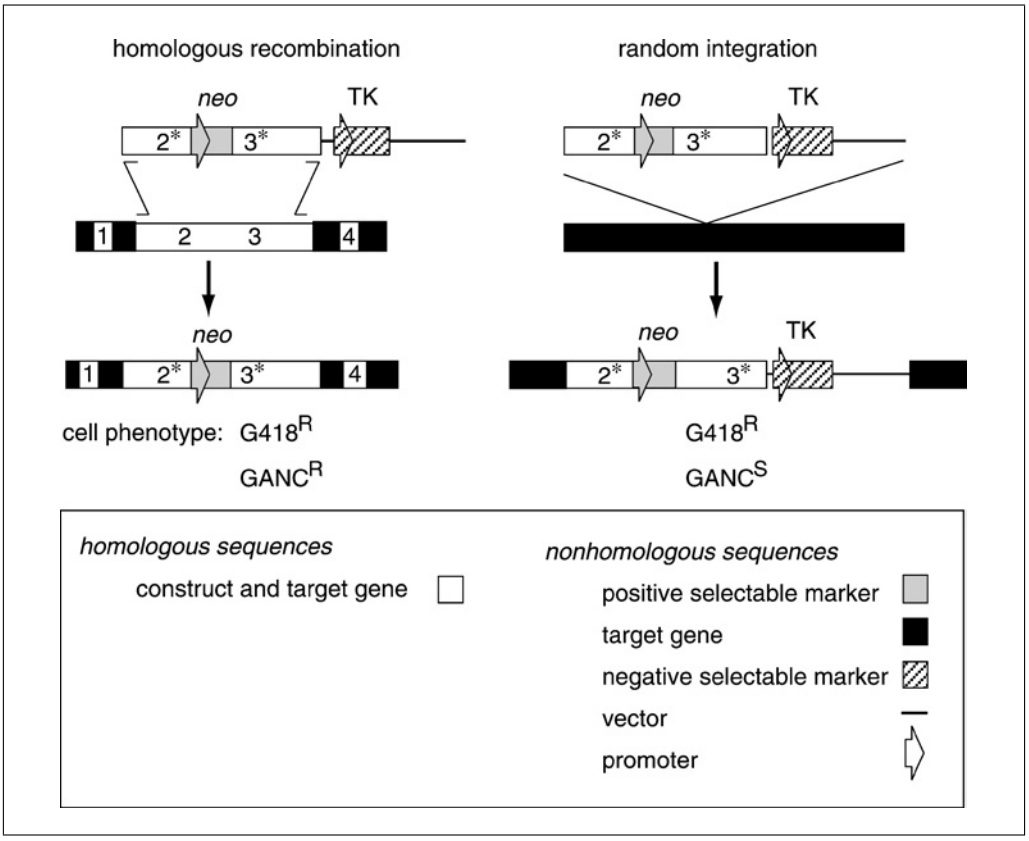

Figure 23.1.2 Enrichment for homologous recombinants by positive-negative selection using the TK gene. Homologous recombination involving cross-overs on either side of the *neo* gene results in loss of the TK gene. Random integration tends to preserve the TK gene. The presence of TK can be selected against because any cell expressing the gene will be killed by gancyclovir (GANC). Although both homologous recombinants and clones in which the construct integrated randomly are G418-resistant, only homologous recombinants are gancyclovir-resistant. The construct is shown linearized so that the plasmid vector sequences remain attached to the TK gene. This configuration helps preserve the integrity of the TK gene.

enriching for homologous recombinants, is known as positive-negative selection. It is only applicable to replacement constructs (Fig. 23.1.2; Mansour et al., 1988). In these constructs, a negative selectable marker (e.g., herpes simplex virus thymidine kinase, HSV-TK) is included outside the region of homology to the target gene. In the presence of the TK gene, the cells are sensitive to acyclovir and its analogs (e.g., gancyclovir, GANC). The HSV-TK enzyme activates these drugs, resulting in their incorporation into growing DNA, causing chain termination and cell death. During homologous recombination, sequences outside the regions of homology to the target gene are lost due to crossing over. In contrast, during random integration all sequences in the construct tend to be retained because recombination usually occurs at the ends of the construct. The presence of the TK gene can be selected against by growing the cells in gancyclovir; the homologous recombinants will be G418-resistant and gancyclovir-resistant, whereas clones in which the construct integrated randomly will be G418-resistant and gancyclovir-sensitive. In some cases, TK is inactivated without homologous recombination; thus, the gancyclovir-resistant clones must be screened to identify the true homologous recombinants. Other markers that are lethal to cells have also been used (e.g., diphtheria toxin; Yagi et al., 1990).

Endogenous Promoters

Constructs that rely on an endogenous promoter to express the positive selectable marker can also give enrichment of homologous recombinants (Fig. 23.1.3), but can only be used if the gene of interest is expressed in the cell line. They contain the coding region of a selectable marker (e.g., *neo*) but lack a promoter for the marker. The coding sequence for the marker usually interrupts, and is in frame with, an exon of the target gene. Thus, when homologous recombination occurs, a fusion protein is produced driven by the endogenous

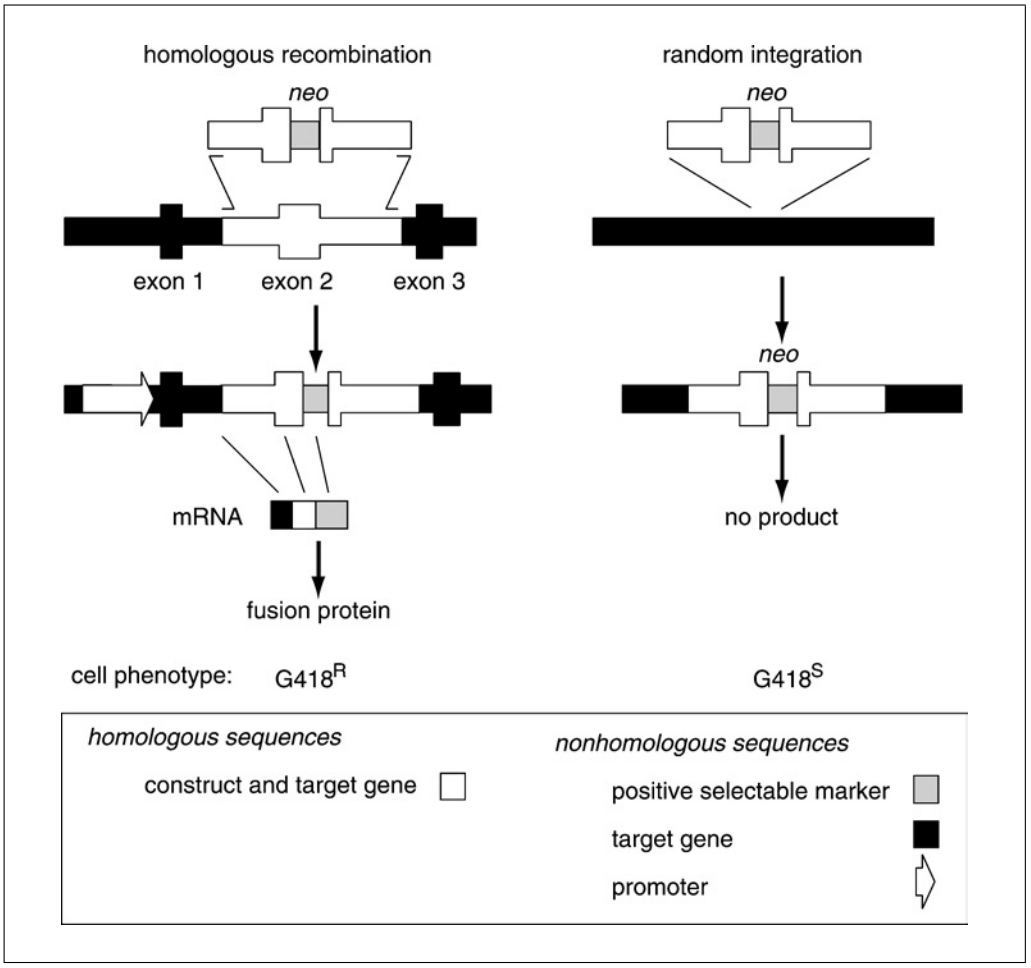

Figure 23.1.3 Enrichment for homologous recombinants using a positive selectable marker *(neo)* lacking a promoter. Clones in which integration of the construct provides an endogenous promoter to drive *neo* expression will be G418-resistant. The construct is designed so that homologous recombination will provide a promoter leading to *neo* expression, whereas random integration will most likely not provide a promoter, thus precluding *neo* expression.

target-gene promoter. In contrast, when random integration occurs, the selectable-marker protein is not usually produced. Therefore, homologous recombinants are G418-resistant, whereas cells in which the construct integrated randomly are G418-sensitive. Constructs containing a promoterless selectable marker can be generated in either replacement or insertion structure and can result in dramatic enrichment for homologous recombinants.

TYPES OF MUTATIONS

Gene Inactivation

Homologous recombination has most often been used to completely inactivate a gene (commonly termed "knockout"). Usually, an exon encoding an important region of the protein (or an exon 5′ to that region) is interrupted by a positive selectable marker (e.g., *neo*), preventing the production of normal mRNA from the target gene and resulting in inactivation of the gene.

A gene may also be inactivated by creating a deletion in part of a gene, or by deleting the entire gene. By using a construct with two regions of homology to the target gene that are far apart in the genome, the sequences intervening the two regions can be deleted. Up to 15 kb have been deleted in this way; thus, many genes could be completely eliminated (Mombaerts et al., 1991). Gene inactivations may also be controlled using the Cre/*loxP* recombinase system either spatially, through cell- or tissue-specific knockout, or temporally, through control of the activity or expression of the recombinase (see Cre/*loxP* System, below).

Mutations can be introduced that have multiple purposes. Homologous recombination has been used to introduce a replacement construct containing the coding sequence of β-galactosidase in frame with the 5′ end of the target gene. Downstream of the *lacZ* gene is a positive selectable marker driven by a

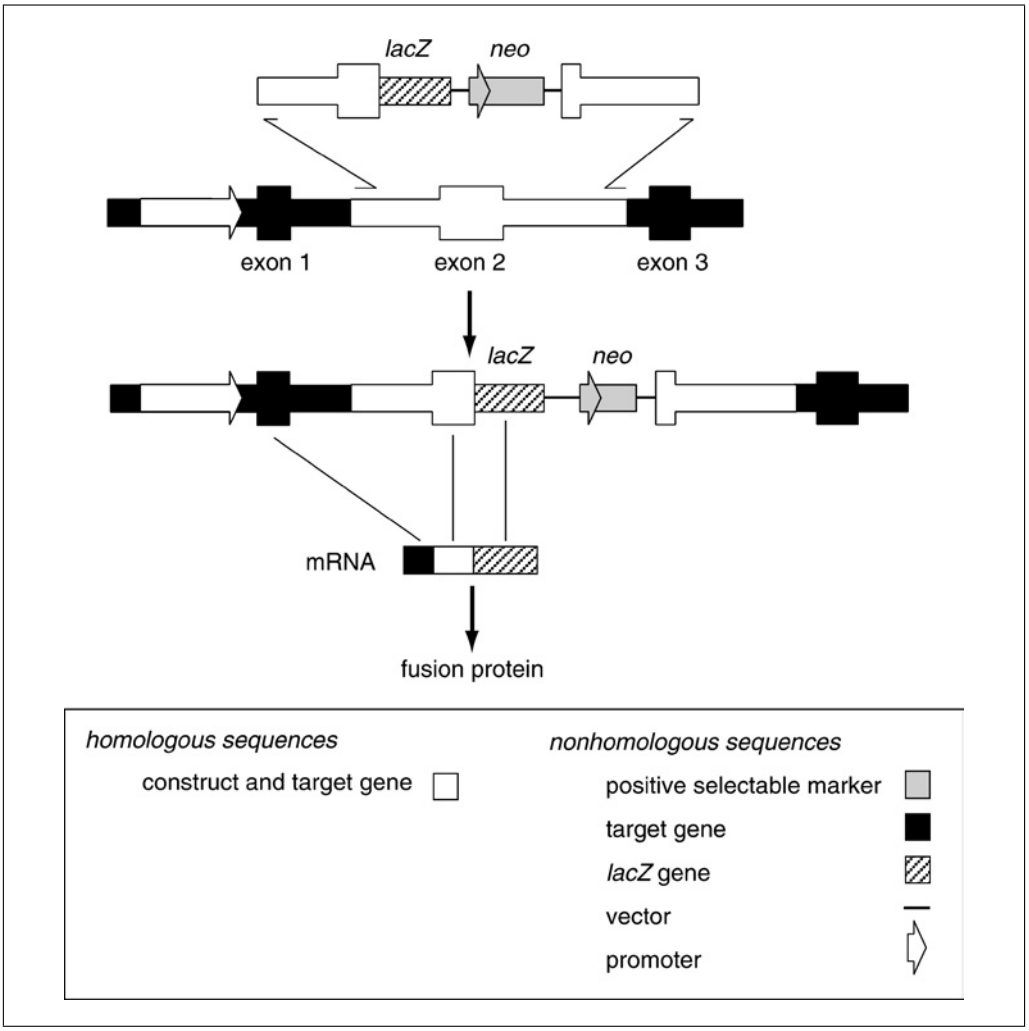

Figure 23.1.4 *LacZ* reporter construct for gene targeting. This construct has two purposes: to disrupt the target gene and to express the *lacZ* gene as a marker to monitor activity of the endogenous target gene's promoter.

heterologous promoter (Fig. 23.1.4). This construct not only disrupts target-gene function but also expresses a fusion protein with β-galactosidase activity, and thus can be used to monitor the activity of the endogenous gene's promoter in various tissues during development (Mansour et al., 1990). Targeting vectors can also be made that express β-gal without the fusion protein. Similarly, other coding sequences can be expressed from the endogenous promoter to achieve selective heterologous expression. Since this results in disruption of the gene, this approach is typically used only if the heterozygous knockout does not have a phenotype.

Subtle Gene Mutations

Homologous recombination can also be used to introduce subtle mutations in a gene. One method is analogous to a method in yeast called transplacement or allele replacement (*UNIT 13.10*). It is called "hit and run" (Hasty et al., 1991) because duplications are introduced into the target gene and then removed. An insertion construct containing both positive and negative selectable markers (e.g., *neo* and TK) is used to introduce a duplication that contains a subtle mutation, such as a point mutation, into the target gene sequence (Fig. 23.1.5). After selection for integration of the construct using the positive selectable marker (e.g., G418), homologous recombinants are identified by screening. A homologous recombinant clone is cultured and then the presence of the negative selectable marker is selected against (e.g., selection against TK using gancyclovir). This selects for an intrachromosomal recombination that eliminates the target-gene duplications and the selectable markers but leaves the mutant target-gene sequences substituting for the normal target-gene sequences. Surviving clones are screened for the correct intrachromosonal rearrangements, leaving the desired mutation. A second

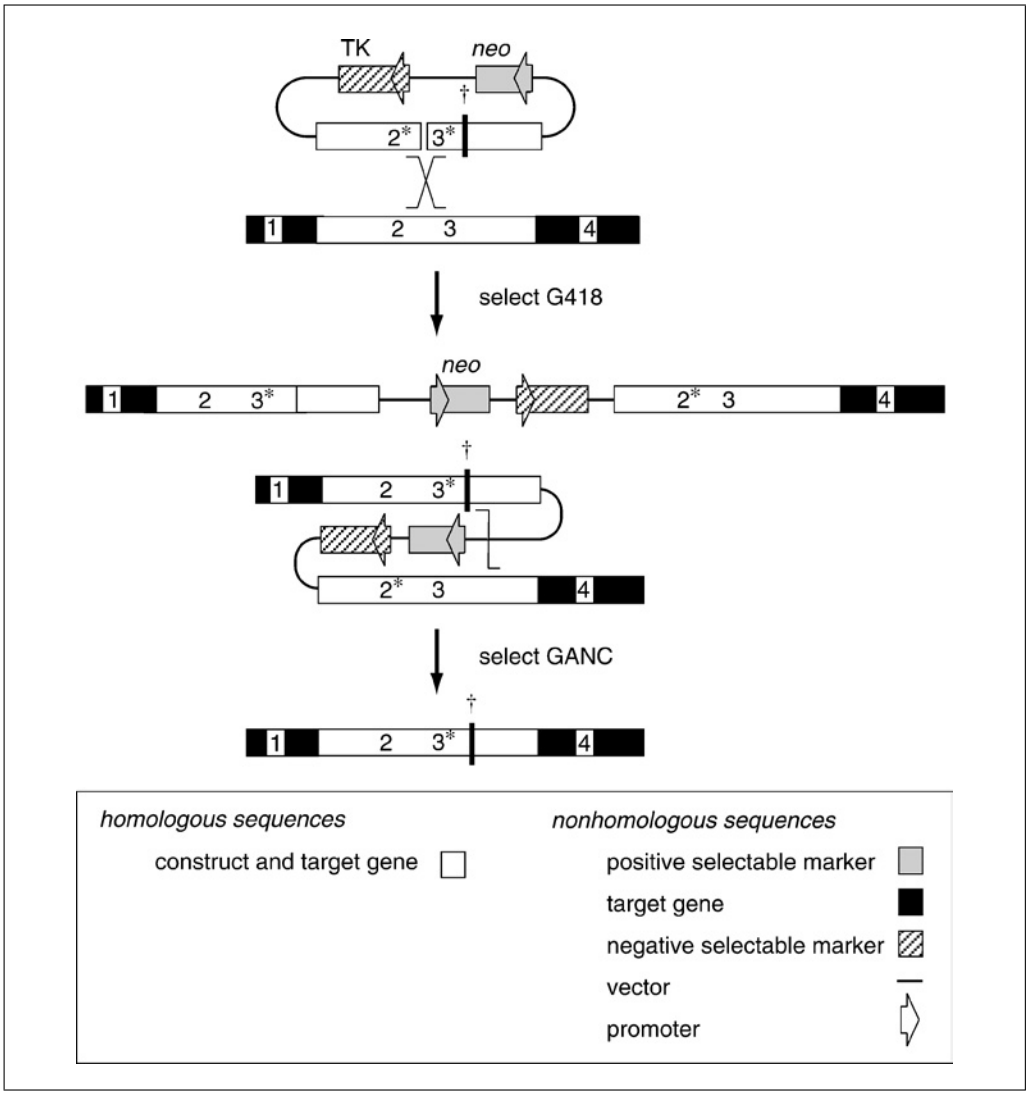

Figure 23.1.5 Allele replacement "hit and run." Cells are cultured in G418 to select for the integration of *neo*, then homologous recombinants are identified by screening and are cultured in gancyclovir (GANC) to select against the presence of the TK gene. This strategy may yield a reconstituted gene containing the subtle mutation present in the construct (indicated by the dark bar and †). Because intrachromosomal recombination may result in the loss of the subtle mutation, its presence must be verified (e.g., by a change in restriction site).

method of introducing subtle mutations into a gene is to insert the mutation by homologous recombination and then use the Cre/*loxP* system to remove the selectable marker.

Cre/*loxP* SYSTEM

The Cre/*loxP* system is derived from the bacteriophage P1. The recombinase Cre acts on the DNA site *loxP*. If there are two *loxP* sites in the same orientation near each other, Cre can act to loop out the sequence between the two sites, leaving a single *loxP* site in the original DNA and a second *loxP* in a circular piece of DNA containing the intervening sequence. Therefore, a properly designed targeting construct containing *loxP* sites can be used for introducing subtle mutations or for a temporally or spatially controlled knockout (for a review of the control of transgenes, see Sauer, 1993). Other recombinase systems, such as the Flp/FRT system, can be similarly useful (Fiering et al., 1995; Vooijs et al., 1998).

Removing the Positive Selectable Marker

Although many gene-inactivation approaches involving homologous recombination still use constructs that leave the positive selectable marker in the genomic DNA, it has become increasingly clear that this can cause a number of unanticipated effects. For example, the presence of the *neo* gene, often with its own promoter, can alter the expression of neighboring loci (Olson et al., 1996; Pham

et al., 1996). This can be a particular problem in gene clusters where neighboring genes are in the same family, since the genes affected may have similar or identical functions. As a result, slight differences in targeting constructs have led to marked differences in phenotype.

If the targeting construct includes *loxP* sites flanking the *neo* gene, then *neo* can be removed after targeting by transient expression of the Cre recombinase (as discussed in UNIT 9.5 and Fig. 23.1.3). This will leave the small *loxP* site in the genomic DNA, but the construct can be engineered so that this is in an innocuous location, such as an intron. Although theoretically even a *loxP* site could cause alterations in the expression of neighboring genes, no such cases have yet been reported. The efficiency of Cre recombination from transient expression reported in the literature varies widely, from ~2% to ~15% (Sauer and Henderson, 1989; Abuin and Bradley, 1996). This rate should be distinguished from the efficiency of Cre recombination in vivo, where the expression of Cre is derived from sequences integrated into the genome and therefore will show longer-lasting expression in nearly all cases.

Introduction of Subtle Mutations Using Cre/*loxP*

The strategy described in the previous section involves introducing subtle mutations by first duplicating sequences and then screening for intrachromosomal recombination that removes the redundant sequences and leaves the mutation. A limitation to this approach is that the second homologous recombination event occurs only infrequently. A more efficient method is to use a replacement construct containing the subtle mutation and then remove the positive selectable marker, which is flanked by *loxP* sites, using the Cre recombinase system (Fig. 23.1.6). This is

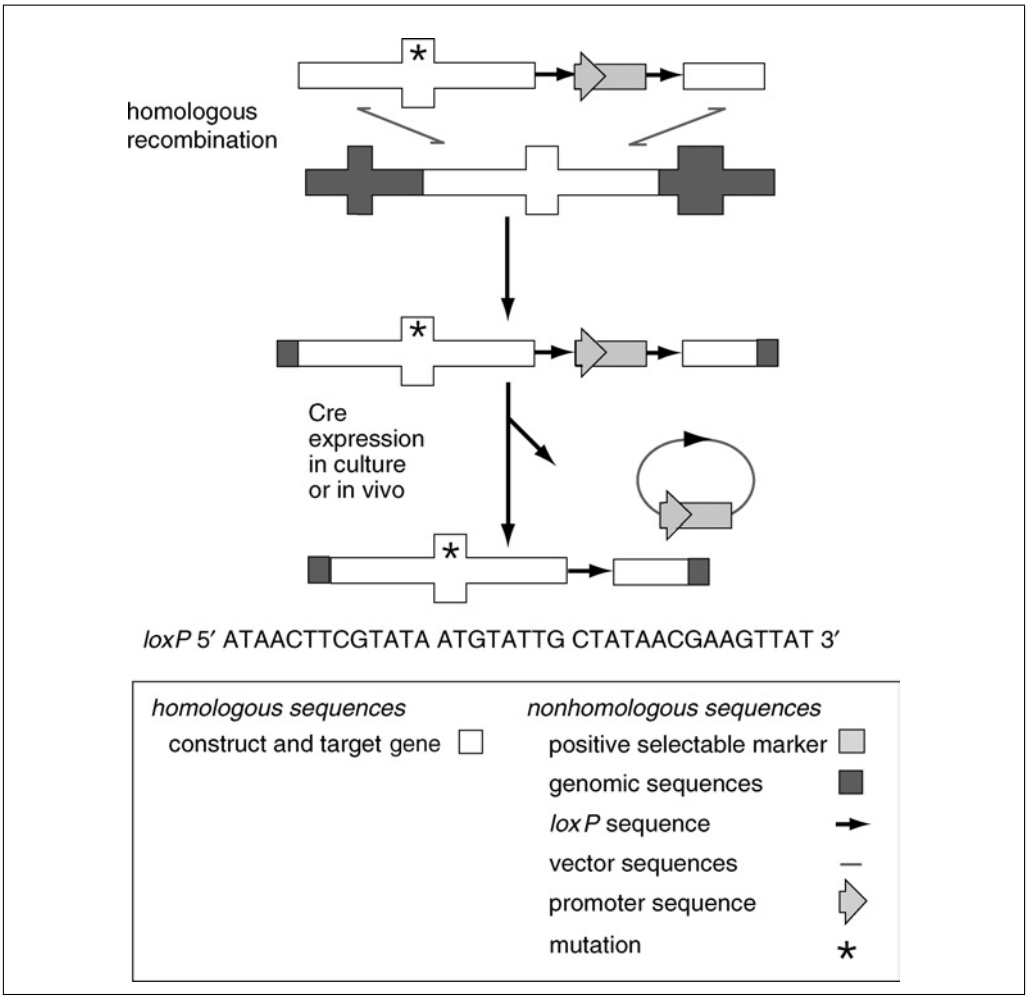

Figure 23.1.6 Using the Cre/*loxP* system to introduce subtle mutations. The subtle mutation is introduced along with the selectable marker in the targeting vector. The selectable marker is then removed by transient expression of Cre, which leaves only the subtle mutation and the small *loxP* site in a silent location.

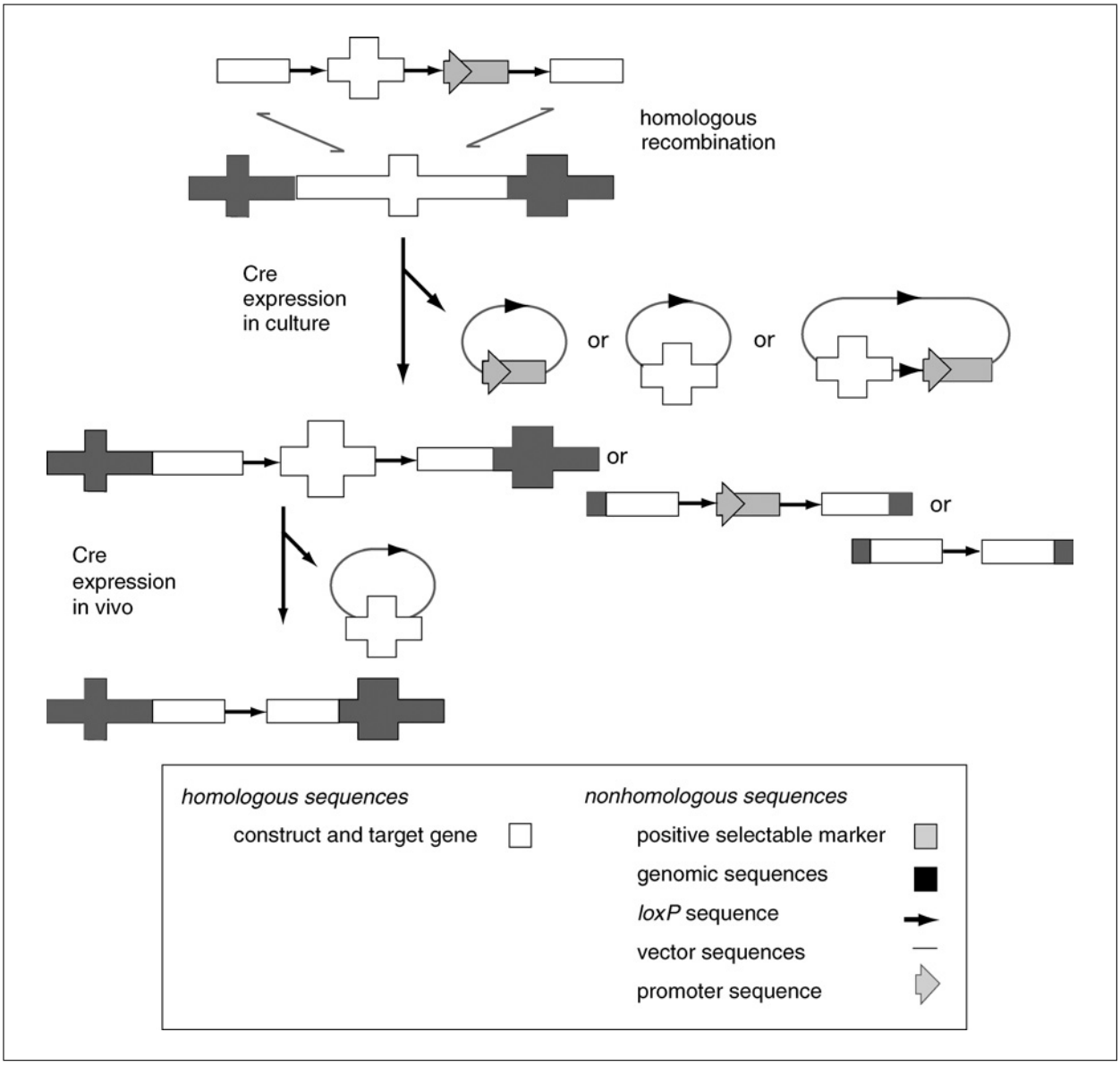

Figure 23.1.7 Conditional gene targeting using the Cre/*loxP* system. The targeting vector contains three *loxP* sites that flank the regions of the gene to be removed and the positive selectable marker *neo*. After homologous recombination is obtained, the selectable marker is excised from the gene by transient expression of Cre. The correct recombination is identified by screening by Southern analysis or PCR. The mutant ES cells are then used to produce a transgenic animal, which is finally bred to an animal line expressing Cre under either temporal or spatial control. Cre can also be ubiquitously expressed to obtain a knockout in all tissues, including the germ line.

identical in effect to removing the *neo* locus after gene inactivation, except that instead of an inactive gene, the replaced sequences contain a subtly mutated version. The selectable marker sequences can be removed by transient Cre expression in culture or by breeding animals to a Cre-deleter line (Williams-Simons and Westphal, 1999), provided that the marker does not cause lethality.

Spatial Control of Knockout

Spatially controlled targeted gene inactivations can be performed in two ways. The most common makes use of cell-specific promoters (sometimes called tissue-specific promoters, even though tissues are actually made of a number of different cell types). This approach begins with the creation of a transgenic animal that expresses Cre in only some cells using a cell-restricted promoter. A second transgenic animal line is then created by homologous recombination that contains *loxP* sites flanking a portion of the gene that is critical for activity, typically important exons (Fig. 23.1.7). Initially there are three *loxP* sites flanking this important gene region and the selectable

marker. After homologous recombination has been verified, Cre is transiently expressed and loops out regions of DNA between pairs of *loxP* sites. The resultant colonies are screened for the desired recombination (loss of the selectable marker but retention of all regions of the gene). Depending on the frequency of recombination at the site, it may be useful to use a construct that contains a negative selectable marker (such as cytosine deaminase in the example shown in Fig. 23.5.2) between the *loxP* sites along with the positive selectable marker (also see *UNIT 9.5*). In this way cells that have lost the markers can be selected.

While this approach was successful for many genes, the requirement for correct partial Cre recombination led to difficulties with other genes. Although recombination in vitro could go to completion, in other instances, and for unknown reasons, the desired recombination would not take place. Fortunately, use of another recombinase with its own sequence recognition sequences has simplified this technology (Fig. 23.1.8). A construct is created that uses Cre for the conditional mutation and Flp to remove the selectable marker. The removal of the selectable marker can be accomplished by Flp expression in vitro, but also by using a

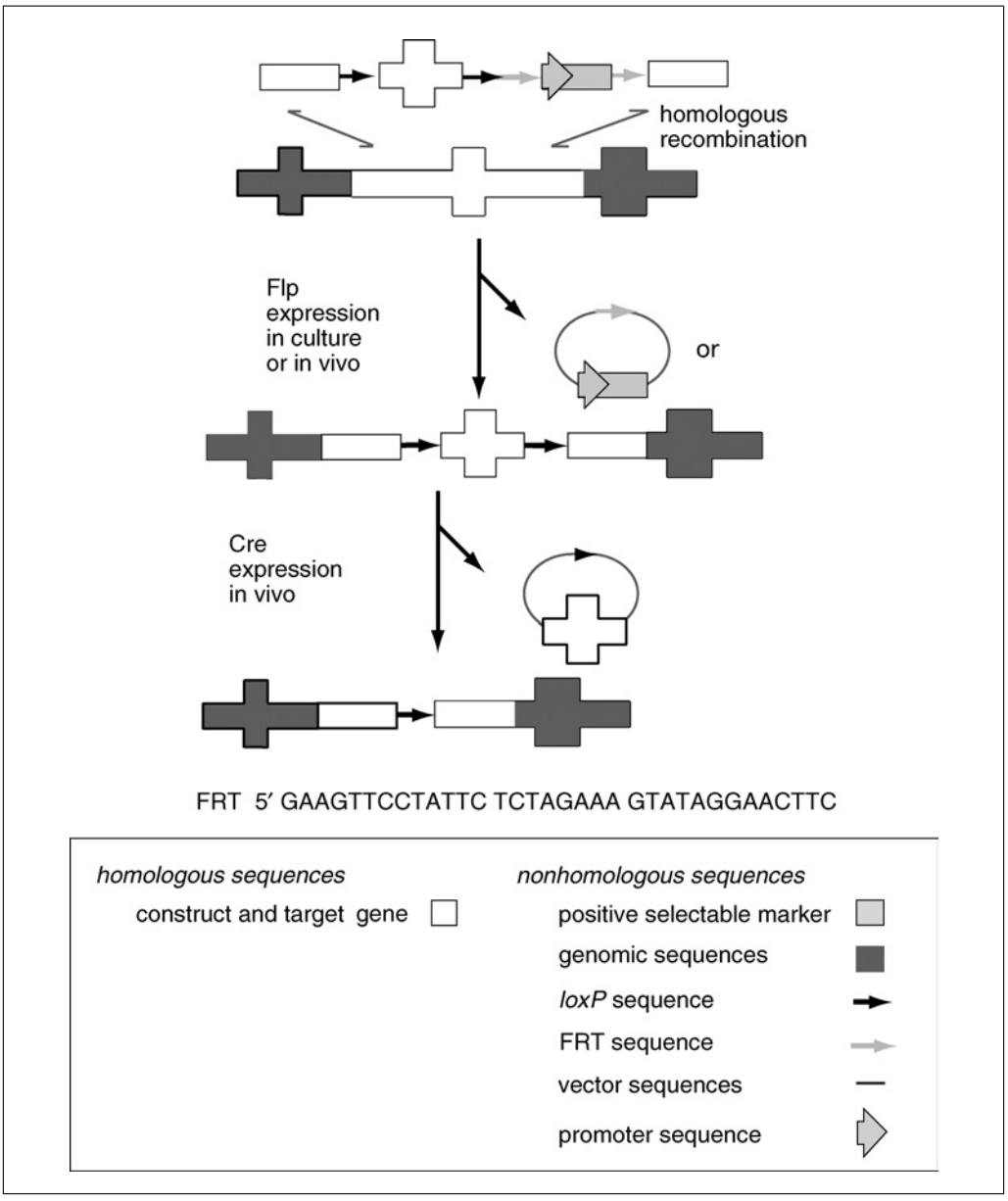

Figure 23.1.8 Alternative strategy for removal of selectable marker using Flp recombinase. The selectable marker is flanked by FRT sites for recognition and removal by the Flp recombinase allowing generation of a gene with only the *loxP* sites remaining. Otherwise the strategy is similar to that in Figure 23.1.7.

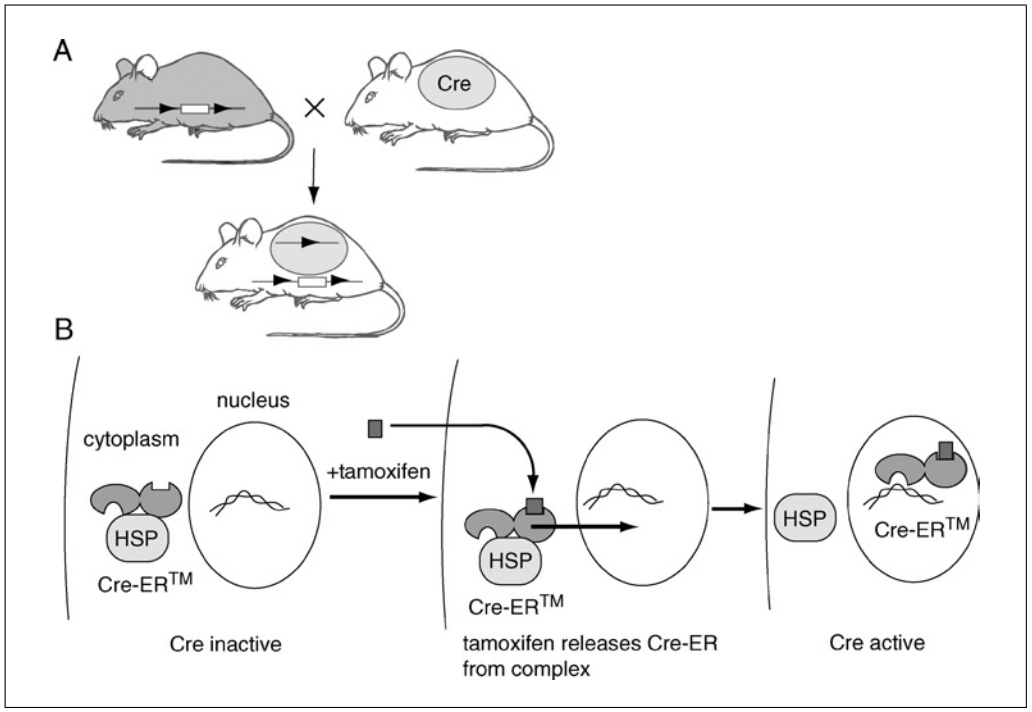

Figure 23.1.9 (**A**) Spatial control of Cre activity. Cre-mediated cell type–specific knockout relies on the production of a transgenic mouse expressing Cre only in the cell type of interest. By breeding these animals to an animal containing the construct with *loxP* sites flanking a critical region of the gene (called a "floxed" gene, for "flanking *lox*"), recombination occurs only in the cell types expressing Cre. The remainder of cells continue to express the target gene since the *loxP* sites typically do not alter expression. (**B**) Temporal control of Cre activity. In the absence of tamoxifen, the fusion protein is sequestered in the cytoplasm bound to heat-shock proteins (HSP). When tamoxifen is added, the fusion protein is released and translocates to the nucleus, where it is active for recombination. Abbreviations: ER, estrogen receptor; TM, tamoxifen.

Flp-deleter line (Rodriguez et al., 2000) after germline transmission. In vivo deletion has the advantage of minimizing the manipulation of the ES cell line, which can decrease germline transmission.

The targeted line will have normal expression of the targeted gene, since its only modification is the presence of *loxP* sites in innocuous sites (e.g., introns). When the two lines are bred together, the Cre recombinase will loop out the DNA—inactivating the gene—only in those cells where it is expressed (Fig. 23.1.9A). In this way, tissue-specific knockouts of a number of genes have been generated (Gu et al., 1994; Agah et al., 1997). The method also has the advantage that, once a transgenic line is generated with the desired restricted expression of Cre, the approach can be applied to a number of targeted lines. In addition, it is not necessary to make separate constructs for a restricted and a complete knockout, since Cre-expressing lines (called deleter lines) have been made that will produce rearrangement in all tissues when bred to the targeted line (Schwenk et al., 1995).

Another way of spatially controlling knockout is to use an expression system for Cre that can be applied to absolute location. In some cases, no restricted expression pattern is known for a gene that matches the desired spatial alteration; in others, the site may be particularly amenable to viral manipulation (as with an epithelial or endothelial surface) or accessible by direct injection (such as sterotactic injection of the central nervous system). By using a viral vector to express the Cre protein, it is possible to obtain knockouts that are spatially limited by the viral infection. This strategy has been applied to a number of tissues including the brain, liver, colon, and heart (Rohlmann et al., 1996; Wang et al., 1996; Agah et al., 1997; Shibata et al., 1997; van der Neut, 1997).

More refined spatial control has been achieved by using a combination of Cre and Flp in overlapping expression patterns. Effective recombination is achieved only where both recombinases are expressed (Awatramani et al., 2003).

Temporal Control of Knockout

In many cases, the phenotype of interest is in the adult animal but, because the gene is necessary for development, no adult animals are obtained. Delaying the expression of Cre activity until the animal is an adult would allow normal development, and then the knockout could be created in the adult (Rajewsky et al., 1996). This can be accomplished by using a conditional expression system (e.g., the tet-on, tet-off, or ecdysone systems; see *UNIT 16.14* and St-Onge et al., 1996) or other inducible system (such as an interferon-inducible promoter; Kuhn et al., 1995) to express Cre at the proper time. This would, however, require the construction of animals containing three transgenes. Another approach that has been used is the creation of a fusion protein with either a modified estrogen receptor (Feil et al., 1996, 1997; Zhang et al., 1996; Brocard et al., 1997) or a modified glucocorticoid receptor (Brocard et al., 1998). The estrogen receptor (ER) has been mutated so that it no longer responds to endogenous estrogens, but is activated by the administration of the partial estrogen agonist tamoxifen (Fig. 23.1.9B). Temporal control using Flp/FRT has also been achieved using a fusion of Flp with a modified estrogen receptor (Hunter et al., 2005).

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