METHODS: A Companion to Methods in Enzymology **14,** 381–392 (1998)
Article No. ME980593

Inducible Gene Targeting in Mice Using the Cre/*lox* System

Brian Sauer

Laboratory of Biochemistry and Metabolism, National Institute of Diabetes, Digestive and Kidney Disease, National Institutes of Health, Building 10, Room 9N119, Bethesda, Maryland 20892-1800

E-mail: sauerb@helix.nih.gov

Molecular techniques now allow the design of precise genetic modifications in the mouse. Not only can defined nucleotide changes be engineered into the genome of the mouse, but genetic switches can be designed to target expression or ablation of any gene (for which basic molecular information is available) to any tissue at any defined time. These strategies promise to contribute substantially to an increased understanding of individual gene function in development and pathogenesis. A powerful tool, both for the design of such genetic switches and for speeding the creation of gene-modified animals, is the Cre site-specific DNA recombinase of bacteriophage P1. Precise DNA rearrangements and genetic switches can be efficiently generated in a straightforward manner using Cre recombinase. In conjunction with inducible systems for controlling Cre expression and function, these recombination-based strategies are likely to have a profound impact on developmental biology and the generation of useful animal models of human disease.

For nearly a century the mouse has been used for genetic analysis, with this work leading to key insights in immunology, cancer biology, and development. Over the past 20 years the adoption of molecular biological techniques for the genetic manipulation of the mouse has resulted in a vast surge of interest in using the mouse as a model system for the investigation of almost all facets of mammalian biology. Remarkably, it has now become possible to genetically alter the mouse genome with nucleotide precision. Not only does this allow the direct assessment of gene function in intact animals, it also allows the design of increasingly useful animal models of human disease.

Incorporation of exogenous DNA into the mouse genome to produce a transgenic animal can be achieved by pronuclear injection of DNA in the fertilized zygote. Transgenic animals produced by this method are generally gain-of-function mutants since the transgene is designed either to express a novel gene product or to misexpress a normal gene. Precise alteration of endogenous genes (gene targeting) is accomplished by homologous recombination in embryonic stem (ES) cells and has been used extensively to generate null or "knockout" mutations. The derivation from preimplantation embryos of murine ES cells that retain totipotency even after gene targeting has allowed the generation of intact animals harboring the desired genetic alteration. Gene-targeted ES cells are injected into blastocysts and upon colonization of the germ line allow generation of intact animals with the desired genetic alteration. Detailed descriptions of these procedures are readily available (1, 2).

More recently, strategies exploiting site-specific DNA recombination have been incorporated into transgenic and gene-targeting procedures to allow *in vivo* manipulation of DNA in ES cells or living animals. A large number of site-specific DNA recombinases have been described from bacteria and yeast, and the recombination reactions that they catalyze

1046-2023/98 $25.00

span a wide range of complexity (3). Most catalyze efficient DNA recombination at sequences of from 25 to 150 bp in length, a size sufficiently large that these recognition sequences are not expected to naturally occur in the mammalian genome. The relative simplicity and efficiency of Cre recombinase from temperate phage P1 has made it particularly useful for this purpose. Placement of recombination sites into the genome and subsequent targeted expression of recombinase have allowed the development of genetic switches that can either ablate or turn on any desired gene in transgenic or gene-modified mice.

Cre-MEDIATED RECOMBINATION

Cre is the 38-kDa product of the *cre* (cyclization recombination) gene of bacteriophage P1 (4, 5) and is a site-specific DNA recombinase of the Int family (6). Because Cre is of prokaryotic origin, gene and protein designations follow standard bacterial genetic nomenclature (7). This convention also helps to avoid potential confusion with the similarly named *CRE* (cAMP response element). Cre plays two critical roles in the life of P1: it provides a backup mechanism for cyclizing P1 DNA after infection (8, 9), and it enhances P1 plasmid stability in bacterial lysogens by resolving dimeric plasmids for increased partition fidelity at bacterial division (10).

Cre recognizes a 34-bp site on the P1 genome called *loxP* (locus of X-over of P1) and efficiently catalyzes reciprocal conservative DNA recombination between pairs of *loxP* sites (11). The *loxP* site consists of two 13-bp inverted repeats flanking an 8-bp nonpalindromic core region that gives the *loxP* site an overall directionality that, by convention, is as depicted in Fig. 1. Cre-mediated recombination between two directly repeated *loxP* sites results in excision of the DNA between them as a covalently closed circle. Cre-mediated recombination between pairs of *loxP* sites in inverted orientation will result in inversion of the intervening DNA rather than excision. Breaking and joining of DNA is confined to discrete positions within the core region and proceeds one strand at a time by way of a transient phosphotyrosine DNA–protein linkage with the enzyme. Unlike many recombinases of the Int family, no accessory host factor or DNA topological requirements are required for efficient Cre-mediated DNA recombination. These two characteristics are key features that prompted the initial determination of the suitability of Cre for genomic manipulation in eukaryotic cells (12).

Not all 34 bp are essential for efficient recombination: the first 4 bp (from left to right in Fig. 1 for the left-hand repeat) of either of the 13-bp inverted repeats can tolerate some modification with little if any loss of recombinational proficiency or fidelity (13, 14). Modified *lox* sites are helpful in the design of genetic switches (see below).

Because each of the 13-bp inverted repeats of the *loxP* site binds a single Cre monomer and because Cre acts virtually stoichiometrically, the number of functional Cre molecules required for DNA synapsis and recombination is most likely 4 per recombination event (15). This is approximately what has been observed both *in vitro* and in *Escherichia coli* (unpublished results), although higher concentrations of Cre may be required in the environment of the eukaryotic cell (16). Consideration that the prokaryotic Cre protein might not enter the eukaryotic nucleus efficiently prompted the construction of Cre fusions carrying the nuclear localization signal (NLS) of SV40 T-antigen (17). Since Cre itself carries a signal(s) that directs localization exclusively to the cell nucleus in cultured mammalian cells (18), it is unclear to what degree addition of an exogenous NLS might contribute to enhanced site-specific recombination.

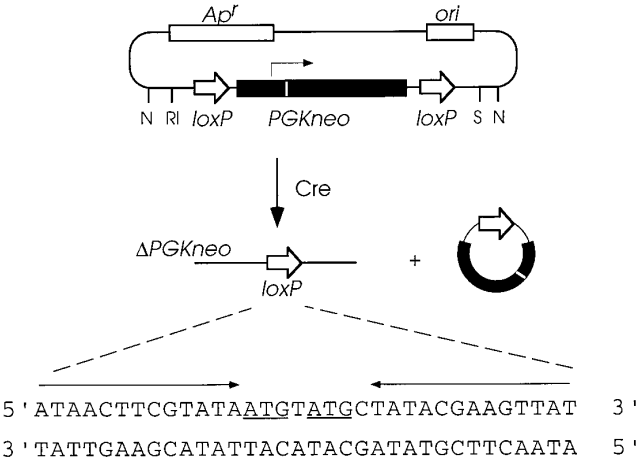

FIG. 1. Cre-mediated excisive recombination. Shown is the *lox*[2] *neo* plasmid pBS479 in which the *PGKneo* gene from pPNT (66) was placed between the two directly repeated *loxP* sites (indicated by open arrows) of pBS246 (20). In cells carrying an integrated copy of the *lox*[2] *neo* cassette, Cre-mediated recombination results in excision and subsequent loss of the *neo* gene. The inverted repeats of the *loxP* sequence are indicated by thin arrows, and potential ATG starts present in the orientation shown here are underlined. Restriction sites: N, *Not*I; RI, *Eco*RI; S, *Sfi*I.

TURNING GENES ON

There often arise instances where introduction of a transgene into the mouse results in either morbidity or such reduced viability that it is difficult or impossible to maintain the transgenic mouse line by breeding. In such circumstances it is advantageous to design a dormant transgene that can be activated after establishment of the transgenic line. One way of doing this is by inserting a *lox*^{2} STOP cassette (19, 20) between the (potentially toxic) transgene and its promoter (Fig. 2). After establishment of a transgenic line, the STOP signal can be removed by Cre-mediated excision, for example, by intercrossing with a second mouse expressing Cre, to activate the transgene as desired.

Lakso *et al.* (19) showed that the SV40 tumor antigen gene could be rendered completely quiescent by interposing a synthetic STOP sequence (consisting of the SV40 early polyadenylation signal, a false translational start, and a splice donor signal) between it and a lens-specific promoter. Resulting transgenic lines exhibited no incidence of tumor formation. On mating with a Cre mouse to generate doubly transgenic mice, recombinational activation of the tumor antigen gene occurred and resulted in the development of lens tumors in the offspring.

A critical issue in using the *lox*^{2} STOP strategy is that after recombination the remaining *loxP* site must not interfere with expression of the target transgene. Note that *loxP* contains two ATG translational start signals in one orientation (underlined in Fig. 1), but not in the other. Hence, it is important

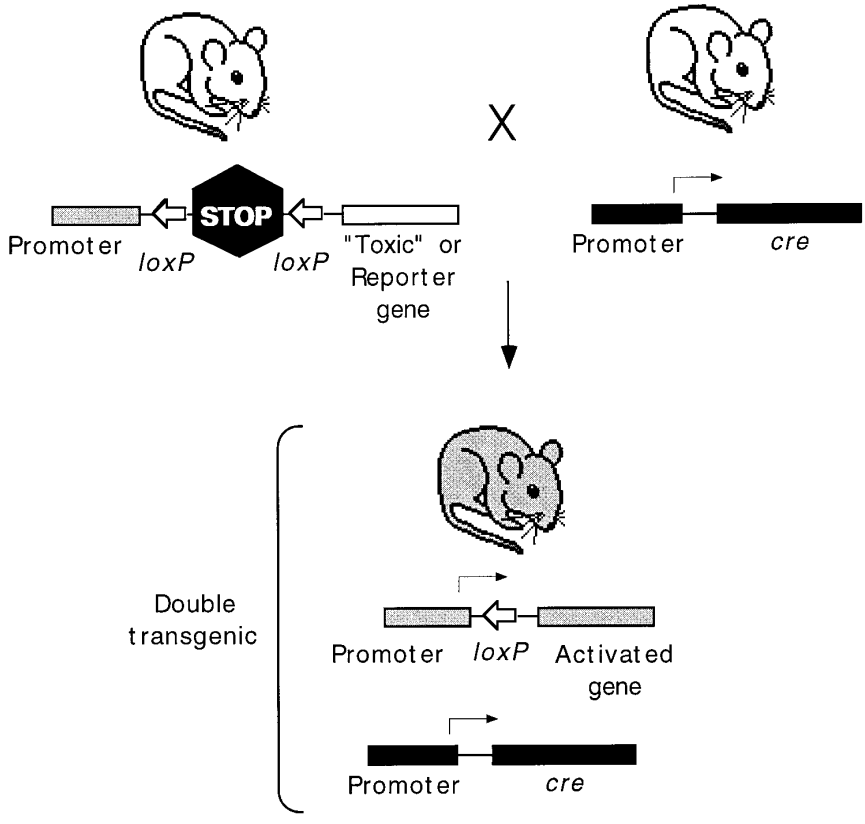

FIG. 2. Recombinational activation of gene expression. The *lox*^{2} STOP cassette from plasmid pBS302 (GenBank Accession No. U51223) is placed between the desired promoter and the "toxic" or reporter gene of interest. Mice carrying this dormant transgene are mated with a *cre* transgenic to generate a doubly transgenic mouse in which Cre has evicted the STOP sequence to activate the dormant transgene. In the example shown, the reporter transgene is capable of universal expression. Recombination by Cre occurred either ubiquitously or early after fertilization to give activation of the dormant transgene in all cells. Restriction of expression of the transgene to a particular tissue can be achieved by a suitable choice of promoters for both the reporter and the *cre* transgenes. The open arrows indicate the *loxP* site; thin arrows indicate transcription.

that the *lox*^{2} STOP cassette be oriented as shown in Fig. 2 so that there remains no false ATG start that could interfere with downstream transgene expression after excisive recombination. A second concern is that because of its palindromic structure, the *loxP* sequence will form a hairpin structure in transcribed RNA that can, if present in the 5′ leader, decrease downstream gene expression (21, 22). As discussed above, to minimize translational effects of *lox* that could affect gene expression in the design of a genetic switch, the *loxP* hairpin can be destabilized by introducing a recombinationally neutral alteration of one or more of the outer 4 bp into one of the site's inverted repeats (23).

The tissue specificity of expression for the recombinationally activated dormant transgene is a function both of the promoter specificity of the target transgene and of the promoter specificity of the Cre transgene. For example, use of a Cre transgenic mouse that expresses the recombinase ubiquitously, or that expresses Cre in the zygote, will allow activation of the dormant transgene so that the resulting expression pattern will be dictated solely by the promoter of the activated transgene. On the other hand, mating with a Cre mouse that expresses recombinase in a spatially or developmentally limited fashion will confine activation of the dormant transgene to the intersection of the two expression patterns.

Cre expression and ensuing recombination will evict the STOP sequence to allow target gene expression not only in the cell in which recombination takes place but also in its progeny. Thus the *lox*^{2} STOP strategy is a generally useful strategy both for characterizing the expression pattern of a *cre* transgene and for conducting lineage analysis in the mouse. A step in this direction is the development of a chicken β-actin promoter/*lox*^{2} STOP/*lacZ* reporter mouse that gives β-galactosidase expression in the nervous system after Cre-mediated transgene activation (24). Mating with a α-CaMKII-*cre* mouse activated expression of β-galactosidase in the CA1 cells of the cortex and hippocampus 3 weeks after birth in the doubly transgenic offspring. Note that cells that have become blue show either that Cre is being expressed in those cells or that Cre had been expressed in the lineage leading to it.

A complicating factor in these strategies is position effect variegation of the transgene (25). Position effects can lead to mosaicism of transgene expression in the target tissue and also to unexpected expression in other tissues, depending on the site of integration of the transgene. Clearly, concomitant mosaicism in expression of both the *cre* transgene and the *lox*^{2} STOP-tagged transgene could seriously hamper the attempt to activate gene expression in a majority of cells in the target tissue. For example, random mosaic expression in 60% of the target cell population for both the *cre* and the reporter transgenes would limit final reporter expression to only 36%. Acceptibility of such limitations will depend on the dictates of the particular experiment being planned. In the case of generating conditional knockout mutations (see below), mosaic *cre* expression may be a serious problem. Thus it would be prudent to screen transgenic lines for high penetrance of expression or, alternatively, to "knock-in" (26) the *cre* or reporter genes by homologous recombination in ES cells to a specific chromosomal locus displaying the desired expression pattern.

CONDITIONAL KNOCKOUTS AND MARKER EVICTION

Homologous recombination in pluripotent ES cells allows the targeted generation of a null or knockout mutation in mice. After gene targeting in ES cells, the desired mice are generated by blastocyst injection of the altered ES cells, subsequent germ line transmission of the engineered mutation, and appropriate matings to render the mutation homozygous. Because a null mutation can result in embryonic lethality that is noninformative for a gene's role either later in development or in the adult, it would be useful to devise conditional mutations of the target gene. Moreover, conditional mutations that could be confined to a particular cell lineage would greatly aid in the determination of a gene's function in that cell lineage or tissue.

Both dominant negative mutants and antisense RNA have been used to block activity of a target gene and thus can, in principle, be used to generate a null phenotype. In those situations where such strategies are likely to be effective, conditional expression of the dominant negative mutant can be attained by using the *lox*^{2} STOP strategy discussed above. After activation by mating with a *cre* transgenic with the desired tissue specificity, the dominant negative will be expressed in a manner dependent both on the pattern of Cre-mediated activation and on the promoter specificity of the activated transgene. Although this strategy may be useful for those genes for which there already exists

sufficient information to allow the design of a dominant negative mutant or a potent antisense construct, it is unlikely to be easily applicable to all genes.

An attractive alternative strategy for generating a spatially or temporally controlled conditional mutation is to modify the target gene by homologous recombination in ES cells so that it is flanked by *loxP* sites (Fig. 3). Mice containing such a modified gene are then crossed with mice expressing Cre in the desired target tissue, and Cre-mediated excision results in tissue-specific gene ablation. This strategy was first demonstrated by Gu *et al.* (27) using a mouse in which the promoter and first exon of the DNA polymerase β gene (*pol*β) were flanked by *loxP* sites. When mated to a transgenic mouse that specifically expressed Cre in T-cells, the *pol*β gene was inactivated in 40% of the T-cell population, but not in any other tissue. Incomplete elimination of the *pol*β gene from the targeted cell population in this instance may have been due to use of a wild-type *cre* transgene and most likely could be remedied by using a *cre* gene carrying a "Kozak" modification (17, 28, 29) that allows efficient translation in mammalian cells. Recombinational eradication of an endogenous gene can thus be targeted to a particular tissue or time by simply controlling expression of *cre*. Such conditional knockouts will be invaluable in obtaining a more complete understanding of gene function for genes that both play an essential role in the embryo (hence null mutations would be embryonic lethal) and provide a vital function in a particular adult tissue.

A particularly powerful feature of a conditional gene inactivation strategy using Cre is that the same *loxP*-tagged mouse can be used for gene ablation independently in a large number of different tissues, or at different developmental times, by simply mating it with a corresponding *cre* transgenic that displays the desired tissue or temporal specificity of expression. Thus, the same genetically modified animal can be used to answer a variety of different questions relating to the expression and function of the target gene. The use of the *loxP*-tagged conditional mutation strategy is doubly appealing because of the potential savings in effort and time that is necessarily associated with gene modification in ES cells and subsequent generation of an animal carrying the engineered mutation.

Conditional *loxP*-tagged mutations will also be of great value in distinguishing the relative roles of genes with apparently overlapping functions. A specific gene may be expressed in a particular adult tissue, for instance, in which there is also expressed a related gene that appears to perform a similar role. Construction of the appropriate double-gene-modified animals carrying conditional knockout mutations in these genes should allow a more accurate assessment of the relative contributions of the two genes to normal cell function in these tissues. In addition to Cre-mediated conditional loss-of-function mutations, conditional gain-of-function mutations can be designed to elucidate gene function. For example, an endogenous gene of interest can be disrupted or knocked out with the *lox*^{2} STOP cassette and then reactivated in a tissue-specific or temporally specific manner by mating with an appropriate Cre-expressing mouse.

Crucial to the success of these procedures, however, is the prior careful evaluation of the pattern of expression of the *cre* transgenic animal. Clearly, mosaic expression of recombinase in the desired tissue (or at the desired time) would defeat the intent to effect complete gene ablation in the target cell population. Conversely, unexpected expression of Cre in extraneous tissues would lead to gene ablation outside of the tissue of interest and thus complicate interpretation of the experiment. Mosaic gene expression is not uncommon in transgenic animals and is likely to be a function both of the site of integation and of the susceptibility to position effects of the promoter element. Judicious choice of a promoter displaying both position-independent gene expression and the correct combination of spatial and temporal specificity would, of course, be ideal, and some promoter/gene cassettes appear to manifest these properties. Alternatively, the use of chromatin insulator elements (30) may help in achieving these goals by ameliorating the effects of adjacent chromosomal sequences on expression of the *cre* transgene. In some cases, though, it may be preferable to obtain the desired specificity of expression by knocking-in the *cre* gene at a chromosomal locus displaying the desired pattern of expression.

The existence of both polyclonal (12) and monoclonal (31) antibodies to Cre permits Western and immunohistochemical analyses of candidate *cre* founder transgenic lines. Immunological techniques can thus be used to rapidly obtain a rough idea of the tissue-specificity of expression and to detect possible mosaicism in expression. Functional analysis of Cre activity, however, is the acid test for suitability of a particular transgenic or knock-in *cre* mouse for use in a particular conditional knockout or gene activa-

tion strategy. Because Cre-catalyzed excision is such a dramatic alteration in the genome, candidate *cre* transgenics can be mated with a reporter mouse carrying a *lox*^{2} gene cassette, and excision can be monitored simply and quickly by performing PCR analysis of target tissues with appropriately designed

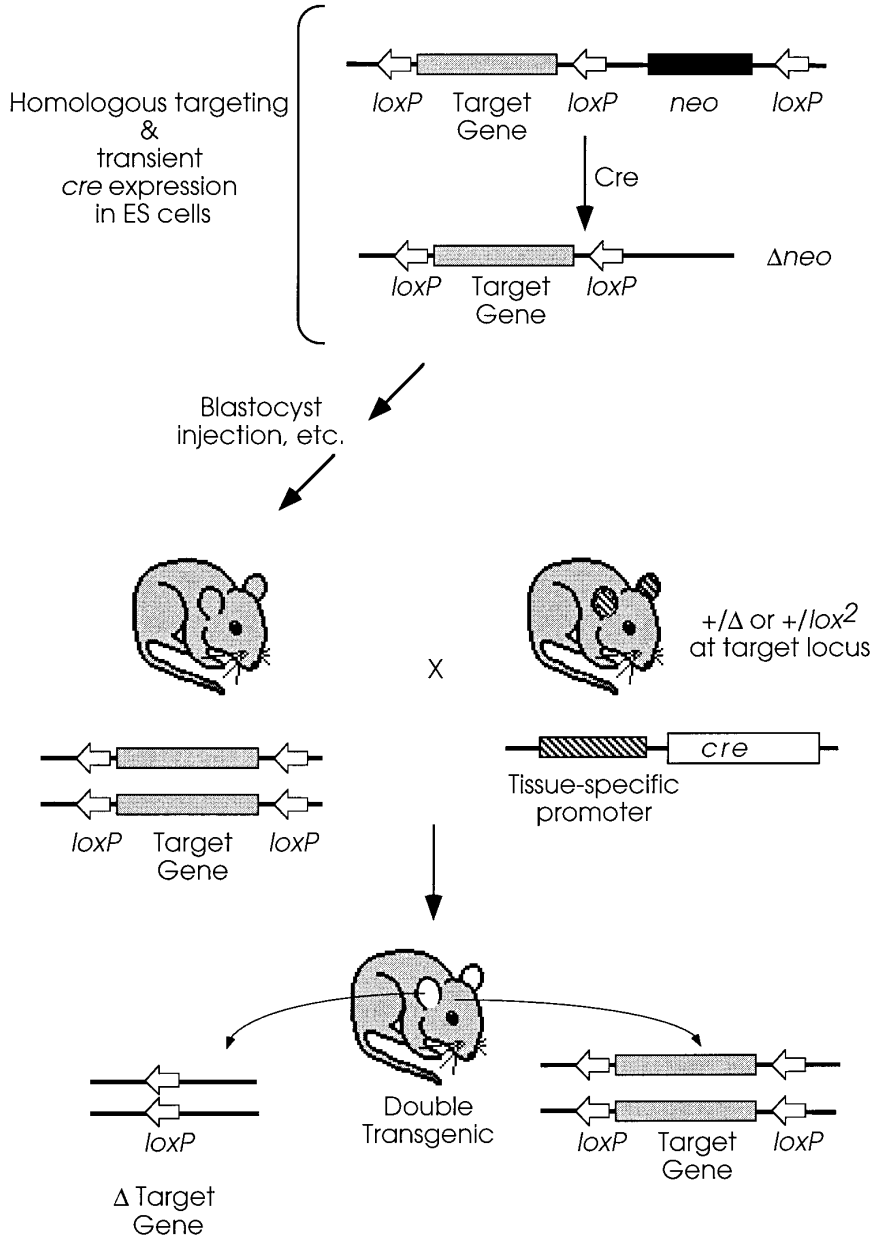

FIG. 3. Conditional gene ablation by Cre recombinase. The target endogenous gene is modified by homologous targeting in ES cells so that it is flanked by two directly repeated *loxP* sites (for simplicity, only one allele is shown). In those situations that require removal of the *neo* gene, a third *loxP* site is positioned so that *neo* can be excised by transient expression of *cre* in ES cells. The *HSVtk* gene can be incorporated into the *neo* interval to permit direct selection for Δ*neo* (27). After identification of Δ*neo* clones that retain the *loxP*-flanked target gene, mice are generated from ES cells by standard procedures. Mating of the *loxP*-modified mouse with a *cre* transgenic will generate a double-transgenic mouse in which the *loxP*-modified gene has been deleted in those tissues in which the *cre* transgene has been expressed. In the example shown, *cre* is expressed in the ears (striped) of the *cre* transgenic so that deletion of the *loxP*-modified target gene is confined solely to the ears (white) of the double transgenic.

primers. Still, the most satisfying analysis would allow a simple determination of the recombinational competence of each individual cell in the target tissue. As mentioned above, a reporter mouse carrying a *lox*^{2}STOP/*lacZ* transgene, that when activated gave good expression of *lacZ* in the nervous system, served as a powerful tool for identifying a *cre* transgenic with the desired pattern of expression in CA1 cells (24). This *cre* animal was then chosen to effect the specific ablation of a *loxP*-modified NMDA receptor gene in CA1 cells and thereby demonstrate a function for this receptor in spatial memory in the mouse (32). Reporter mice capable of expressing *lacZ* in all cells after Cre-mediated activation would be of great benefit in speeding the careful evaluation of the specificity of *cre* transgene expression, and a number of laboratories are currently developing such mice (33).

In knockout strategies, the endogenous locus in the mouse genome is targeted for *loxP* modification by homologous recombination in ES cells using a selectable marker, usually *neo*. Homologous targeting is achieved by standard procedures, with the targeting construct having a region of DNA homology that is 5′ to the target locus and another DNA segment that is homologous to the 3′ region. Sandwiched in between for conditional knockout strategies are the *loxP*-flanked region of the target gene destined for Cre-mediated excision and also the *neo* selectable marker. Optionally, the thymidine kinase gene (*tk*) of herpes simplex virus (HSV) can be appended to one of the homology arms to enhance the recovery of homologously targeted integrants using positive–negative selection (34).

Because the *neo* gene can itself adversely affect gene expression of neighboring genes (35–37), it may be important, depending on the positioning of the *neo* gene in the final targeted chromosomal locus, to be able to remove the *neo* gene from the targeted locus in a second step. Figure 3 shows that one way of achieving *neo* removal is to include a third directly repeated *loxP* site in the original targeting construct. Thus, one *loxP*-flanked interval is the gene designed for conditional inactivation, and the other *loxP*-flanked interval is *neo* itself. Complete Cre-mediated excision would, of course, remove both the *neo* gene and the targeted *loxP*-flanked DNA segment. However, transient expression of Cre in ES cells can lead to partial DNA excision, removing only the *neo* interval (27). To allow selection for the *neo* deletion, the HSV*tk* gene is included in the *neo* interval. After transient transfection with a Cre expression vector, colonies resistant to gancyclovir (TK$^-$) are screened for the desired deletion. The ratio of complete excision (target gene + *neo*) to deletion only of the *neo* interval is probably a function of the amount Cre activity introduced into the cell and thus is dependent both on the promoter used for *cre* expression and on the amount of DNA electroporated into cells. In addition, the frequency of *loxP* × *loxP* recombination may be sensitive to the distance between the sites (see below), allowing preferential excision of the *neo* interval in most cases.

Efficient eviction of the selectable marker by Cre can also simplify other genome modification procedures in ES cells. Introduction of point mutations into a gene of interest can be achieved by the strategy shown in Fig. 4, using a *lox*^{2} *neo* cassette (such as that shown in Fig. 1) for the selectable marker. After identification of those G418^R transformants in which homologous recombination has replaced one copy of the endogenous gene with the mutant variant on the targeting construct, the *neo* gene can be removed by Cre-mediated recombination, leaving behind only the 34-bp *loxP* site. Unless placed in a coding exon or another critical region of the gene, the *loxP* site is unlikely by itself to interfere with gene expression. Marker eviction by Cre also means that the selectable marker can be "recycled" for use in a second round of gene targeting (12, 38). This strategy has been used to sequentially disrupt both alleles of a target gene in ES cells with the same selectable marker (39). Marker recycling is useful because there are only a few different selectable markers that have been shown to work well in ES cells and also to allow use of a single line of drug-resistant feeder cells, since the feeder cells on which the ES cells are grown should also be resistant to the drug used for selection.

Cre catalyzes excisive recombination at chromosomal *lox* sites in 80–100% of cells into which a transient *cre* expression vector is introduced (18, 20). Hence, the major impediment to efficient *lox*^{2} marker eviction is the efficiency of DNA transfection, which can be fairly low ($\leq$5%) in ES cells using electroporation. As mentioned above, one solution is to simply select for excision with gancyclovir by including HSV*tk* on the DNA segment to be excised. A slight drawback to this strategy is that integration of the *tk* marker into the genome precludes its use for positive–negative selection in the original targeting construct. A promising alternative strategy, which does not impose an additional round of selective stress on the cells at the marker excision step, is to

use an expression vector carrying a gene fusion of green fluorescent protein (GFP) with *cre* (Fig. 5). The GFP*cre* fusion gene readily catalyzes excisive recombination when transfected into ES cells and also results in bright green cellular fluorescence (18). By using fluorescence-activated cell sorting (FACS) of GFP*cre* transiently transfected ES cells, an enriched population can easily be obtained in which 80% or more of the cells have excised the *lox*^{2} marker or are destined to do so. Sorted cells can then be plated for colony formation and Δ*neo* clones quickly identified for subsequent blastocyst injection. Optionally, recombinationally committed, fluorescent cells can be micromanipulated using a microscope equipped for epifluorescence (unpublished results). Parenthetically it should be noted that since knowledge of the tissue-specific pattern of Cre recombinase expression is critical for conditional gene knockout strategies, the GFP*cre* gene should also prove useful for tracing tissue-specific recombinase expression in transgenic mice.

A nagging worry in all excision strategies *in vitro* is that multiple rounds of ES cell manipulation may result in a population of cells with reduced totipotence, thus jeopardizing chances that the gene-modified locus will be transmitted through the germline. For simple marker removal, the second round of *cre* expression vector transfection of ES cells can be avoided by using instead a Cre-expressing mouse to remove the selectable marker. ES cells carrying the desired gene modification with a *lox*^{2} *neo* gene are injected directly into blastocysts to generate a chimera, as would be done in a standard knockout strategy. The chimera is then mated with a Cre-expressing mouse to excise the *neo* gene. Both CMV-*cre* (19, 40) and EIIa-*cre* (41) mice have been found useful for giving germline excision of *neo*. Alternatively, the *neo* gene can be excised by directly injecting zygotes

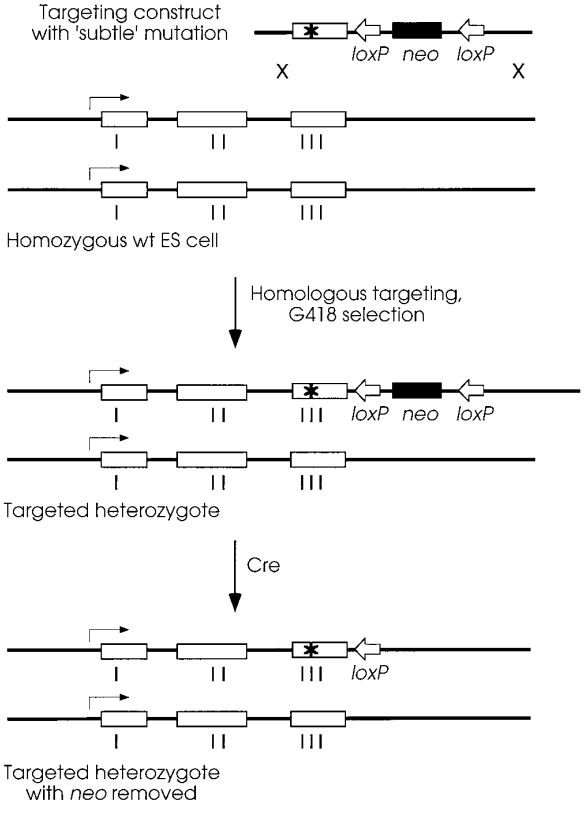

FIG. 4. Cre-mediated marker removal after introduction of a site-specific mutation. A point or other "subtle" mutation (indicated by an asterisk) is introduced into a target gene in ES cells by homologous targeting. In the hypothetical example shown here, the target gene has three exons, and the point mutation is placed into the third exon. Included on the targeting gene is a *lox*^{2} *neo* cassette for selection, the point mutation, and flanking homology. After homologous targeting, the *neo* gene is removed by transient expression of *cre* in ES cells. Alternatively, the *lox*^{2} *neo* ES cells can be used directly for production of mice, and the resulting chimera is mated with a Cre-expressing mouse (see text) to remove the *neo* gene. After Cre-mediated excision, only the small 34-bp *loxP* site remains in the genome.

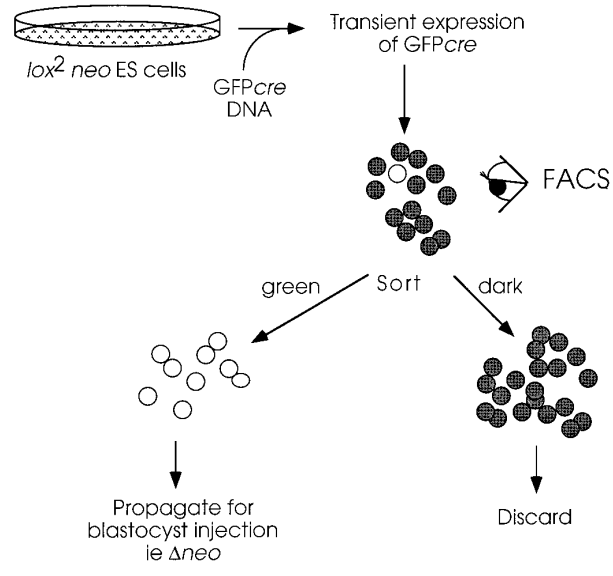

FIG. 5. Removal of *loxP*-flanked DNA in ES cells with a GFP*cre* fusion gene. ES cells carrying a *loxP*-flanked *neo* gene are transiently transfected with a vector carrying the GFP*cre* fusion gene and are FAC sorted 2 days later. Cells sorted as being (transiently) fluorescence-positive are highly enriched for cells that have undergone, or are destined to undergo, Cre-mediated marker excision.

with a DNA construct capable of expressing Cre in zygotes or early embryos (42, 43).

In the case of the EIIa-*cre* mouse, the EIIa promoter is active early in development, with high-level expression in the one-cell zygote and cessation of expression after implantation (44). The extent of excisive recombination is thereby frozen early in development and, by extension, also in the germline. Transgenic animals can thus be generated having different numbers of copies, and correspondingly different levels of expression, of a particular transgene at the same integration site. This strategy was used to demonstrate that oncogenicity in the embryonic lens from SV40 T-Ag expression could be significantly delayed by reducing the copy number of the T-Ag transgene to 1 (41).

INTERMOLECULAR RECOMBINATION AND CHROMOSOME TRANSLOCATIONS

Cre catalyzes not only intramolecular DNA recombination (excision and inversion) but also intermolecular recombination (integration and translocation). In cultured mammalian cells, this has been exploited to direct site-specific insertion of an introduced *loxP*-containing plasmid to a *loxP* target in the chromosome by cotransforming with a transient expression *cre* vector (29). Stable insertions are obtained from trapping the inserted DNA in the chromosome after a short burst of Cre activity. Although site-specific integration occurs more frequently than homologous targeting, identification of targeted integration events still requires the use of a selectable marker. Because the *loxP* target must first be placed into the genome (for example, by homologous recombination or by a more random method) it is convenient to incorporate the *loxP* site as part of a fusion gene, half of which is placed at the chromosomal target and the other half of which is on the targeting plasmid vector. Upon Cre-mediated recombination, a selectable marker gene is reconstructed, thereby permitting direct selection for the targeted chromosome insertion. A variety of *loxP* fusion markers have been generated for this purpose, including *tk* (29), *neo* (23), *hisD* (45), and, in ES cells, *Hprt* (46).

In a similar fashion, Cre can be used to orchestrate the production of precise chromosome translocations, deletions, and inversions (46–49). This is accomplished by first positioning *loxP* sites by homologous recombination in ES cells at the desired rearrangement endpoints. Each homologous targeting event for placement of the *loxP* sites consumes a selectable marker, and *neo, hph* (hygomycin resistance), and *pac* (puromycin resistance) have been used successfully in ES cells for this purpose. To facilitate subsequent selection for the desired recombination product, $Hprt^-$ ES cells are used for the two rounds of homologous targeting, and complementary halves of a *loxP–Hprt* fusion gene are placed at each rearrangement endpoint. Doubly targeted ES cells are then transiently transfected with a *cre* expression vector to catalyze recombination. This strategy has been used to directly select both a t(12;15) translocation between the *IgH* and the c-*myc* loci (46) and deletions and inversions at the *HoxB–wnt3* interval on chromosome 11 (48).

The Cre-mediated t(12;15) translocation was obtained at a frequency of about 5×10^{-8}. Inversions and deletions of 90 kb or 1 Mb at the *HoxB–wnt3* interval arose either at a similar frequency of about 1×10^{-7} or at a frequency of $1–5 \times 10^{-5}$, depending on the particular double-targeted ES clone. Clones giving the higher recombination frequency are those in which *loxP* sites have been targeted to the same chromosome homolog. Interestingly, the frequency of recombination in clones having *loxP* sites on different homologs was substantially lower and approached the frequency seen with translocation between chromosome heterologs. Cre also catalyzed DNA recombination between *loxP* sites located relatively far from each other in the *HoxB–wnt3* interval to generate inversions and deletions of 3–4 cM, albeit at a frequency somewhat lower than that seen for the shorter distances of 90–1000 kb.

For Cre-mediated inversions and deletions, design of the targeting *Hprt–loxP* constructs to permit $Hprt^+$ selection is straightforward if the relative orientation of the target loci is known. Correct orientation of the *loxP* sites is important to avoid the production of dicentric and acentric chromosomes after recombination. Such aberrant chromosomes would likely result in either cell inviability or loss of a normal karyotype and failure to contribute to the germline. Often, however, the relative orientation of the target loci either to each other or to the centromere is unknown. In these circumstances, it may be necessary to target each locus with both possible *Hprt–loxP* orientations (48) to ensure that a suitable double-targeted configuration is obtained.

A slightly different strategy that did not require the use of $Hprt^-$ ES cells was used to generate a 200-kb deletion of the amyloid precursor protein

(APP) locus (49). Two rounds of homologous recombination were used to target *loxP* sites to the desired deletion endpoints and also to position the HSV*tk* gene between the two directly oriented *loxP* sites. After transient transfection of the doubly-targeted ES cells with a *cre* expression vector, selection for resistance to FIAU resulted directly in the desired APP deletion. An intriguing alternative procedure that combined the second homologous targeting step with the Cre-mediated excision step was also presented. After placement of a *loxP* site at the first deletion endpoint along with the correctly positioned HSV*tk* gene, cells were cotransfected with the second *loxP* targeting vector and also with a *cre* expression vector. Simultaneous selection for FIAU resistance and for the dominant selectable marker on the second targeting construct (*hph*) directly gave the desired APP deletion. Although the frequency of recovery for the desired deletion was low, the substantial savings in time and effort achieved by this method is extremely attractive.

The ability to design precise inversions, deletions, and chromosome translocations in the mouse genome will have a profound impact on genetic mapping and strain construction in mice (20). In particular, many of the procedures worked out in *Drosophila* with novel chromosomes should be applicable to the mouse. Because Cre-mediated recombination is reciprocal, recombination between similarly oriented (with respect to the centromere) *loxP* sites placed at distinct loci on different chromosome homologs will result in both a deletion on one chromosome homolog and a duplication of the target interval on the other. Such balanced Cre-mediated recombination events have indeed been obtained in ES cells (48). The generation of defined segmental aneuploidies in mice will be useful not only in facilitating saturation mutagenesis of a predesignated chromosome interval, but also in evaluating the effect of increased or reduced ploidy of defined chromosome regions. This approach may be particularly valuable in identifying important chromosome regions that contribute to the many different features of trisomy 21 by allowing a systematic evaluation of increased copy number of defined chromosome 21 syntenic regions in the mouse (50; B. Sauer and M. Brennan, unpublished results).

SPECIFYING DNA RECOMBINATION IN TIME AND SPACE

Although position effects have in some instances influenced the efficiency of Cre-mediated recombination at *loxP* sites placed into the genome (51), for the most part, recombination appears to be primarily dependent only on the availability in the cell of Cre recombinase for recombination. Hence, in principle, the spatial and temporal occurrence of recombination can be completely specified by placing *cre* under the control of a promoter having the desired spatial and temporal pattern of expression. In actuality, however, some tissue-specific promoters may also be expressed at low levels in unwanted tissues or at inopportune times during development. Moreover, transgene position effects can also contribute to unexpected expression. Because even low levels of Cre expression may lead to recombination in some fraction of a cell population, use of a tightly regulated and well-characterized promoter is important in directing recombination to the correct target tissue at the correct time without unwanted recombination in nontarget tissues.

An attractive alternative to native promoters is to use synthetic inducible systems to control *cre* expression. The elegant tetracycline-regulated transcriptional systems (52, 53), presented in detail elsewhere in this issue, offer the possibility of inducing *cre* expression at a desired time either by simply dosing an animal with tetracycline or by withdrawing animals from tetracycline administration. In this strategy the *cre* gene is placed under the control of a minimal promoter (that by itself is transcriptionally silent) carrying a reiterated tet operator sequence. Transcriptional activation is achieved in cells that express a tet repressor–VP16 transcriptional activator fusion gene (tTA). Addition of tetracycline (or a tetracycline analog) prevents binding of tTA at the operator sequences to terminate gene expression. A variation is to use a mutant tet repressor–VP16 fusion that binds to operator DNA and thus activates gene expression only in the presence of tetracycline. Although mosaicism of transgene expression has limited the success of initial attempts at tetracycline control of Cre-mediated recombination in transgenic animals (54), it is likely that a judicious choice of promoter elements, or the use of knock-in strategies, will allow a robust implementation of this methodology. In a similar manner, inducible transcription of *cre* by a variety of other drugs or steroids, such as ecdysone (55, 56), RU486 (57), and dimerization-inducing synthetic ligands (58), may prove useful in mice. An intriguing second type of strategy is to control Cre function in the cell by fusing Cre to the ligand-binding domain of a steroid receptor (59–61). In cultured cells, Cre-mediated recombination by the

fusion protein is activated upon addition of ligand. The use both of steroid agonists that do not appreciably target endogenous steroid receptors and of ligand-binding domains responsive only to exogenously added ligands should allow adoption of this strategy in mice.

A complicating factor in all of these strategies is that they require multiple time-consuming mouse crosses to obtain within a single mouse the desired constellation of transgenes (for example, the regulatable *cre* gene, the transactivator gene, and the *lox*-modified target). For some purposes, an acceptable and much more rapid approach may be possible by viral-mediated gene transfer of Cre recombinase (62). Cre-expressing adenovirus vectors can be administered by intravenous injection to *loxP*-modified mice and cause recombination in the primary target tissues of liver and spleen (63, 64). Interestingly, topical application of the Cre vector to discrete regions of the brain resulted in localized recombination at the site of application (63). This is potentially a very powerful approach to achieving Cre-mediated gene ablation in a localized fashion, although it will be important to be able to distinguish the Cre-mediated gene ablation phenotype from effects due to viral infection or an associated immune response to viral infection.

CONCLUDING REMARKS

Cre DNA recombinase has become a powerful tool for the analysis of gene function in transgenic mice. Recombinational strategies in transgenic mice to turn genes on, ablate endogenous genes, and even build novel chromosomes in a tissue-specific and temporally defined manner now permit a level of genetic analysis hardly imaginable only a decade or two ago.

It is likely that further refinements and more sophisticated strategies will be developed using site-specific DNA recombination. The related recombinase FLP from *Saccharomyces cerevisiae* has also been used in transgenic mice (65), and its use in conjunction with Cre will undoubtedly permit genome manipulations that would be difficult to achieve with either recombinase alone. Moreover, the refinement of existing inducible gene expression systems and the development of new systems will allow even more precise control of recombinase expression. The combined use of these molecular tools will clearly be of considerable assistance in unraveling the complexity of mammalian development and in generating more sophisticated models of human disease.

ACKNOWLEDGMENT

I am grateful to Dr. U. Hochgeschwender for thoughtful comments on the manuscript.

REFERENCES

1. Wasserman, P. M., and DePamphilis, M. L. (1993) Guide to Techniques in Mouse Development, Academic Press, San Diego.
2. Hogan, B., Beddington, R., Constantini, F., and Lacy, E. (1994) Manipulating the Mouse Embryo: A Laboratory Annual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
3. Craig, N. L. (1988) *Annu. Rev. Genet.* **22,** 77–105.
4. Sternberg, N. (1978) *Cold Spring Harbor Symp. Quant. Biol.* **43,** 1143–1146.
5. Sternberg, N., Sauer, B., Hoess, R., and Abremski, K. (1986) *J. Mol. Biol.* **187,** 197–212.
6. Argos, P., Landy, A., Abremski, K., Egan, J. B., Ljungquist, E. H., Hoess, R. H., Kahn, M. L., Kalionis, B., Narayana, S. V. L., Pierson, L. S., Sternberg, N., and Leong, J. M. (1986) *EMBO J.* **5,** 433–440.
7. Demerec, M., Adelberg, E. A., Clark, A. J., and Hartman, P. E. (1966) *Genetics* **54,** 61–76.
8. Segev, N., and Cohen, G. (1981) *Virology* **114,** 333–342.
9. Hochman, L., Segev, N., Sternberg, N., and Cohen, G. (1983) *Virology* **131,** 11–17.
10. Austin, S., Ziese, M., and Sternberg, N. (1981) *Cell* **25,** 729–736.
11. Hoess, R. H., and Abremski, K. (1990) *in* Nucleic Acids and Molecular Biology (Eckstein, F., and Lilley, D. M. J., Eds.), Vol. 4, pp. 99–109, Springer-Verlag, Berlin/Heidelberg.
12. Sauer, B. (1987) *Mol. Cell. Biol.* **7,** 2087–2096.
13. Sauer, B., Whealy, M., Robbins, A., and Enquist, L. (1987) *Proc. Natl. Acad. Sci. USA* **84,** 9108–9112.
14. Sauer, B. (1996) *Nucleic Acids Res.* **24,** 4608–4613.
15. Mack, A., Sauer, B., Abremski, K., and Hoess, R. (1992) *Nucleic Acids Res.* **20,** 4451–4455.
16. Sauer, B., and Henderson, N. (1988) *Proc. Natl. Acad. Sci. USA* **85,** 5166–5170.
17. Gu, H., Zou, Y. R., and Rajewsky, K. (1993) *Cell* **73,** 1155–1164.
18. Gagneten, S., Le, Y., Miller, J., and Sauer, B. (1997) *Nucleic Acids Res.* **25,** 3326–3331.
19. Lakso, M., Sauer, B., Mosinger, J. B., Lee, E. J., Manning,

R. W., Yu, S.-H., Mulder, K. L., and Westphal, H. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6232–6236.

20. Sauer, B. (1993) *Methods Enzymol.* **225,** 890–900.
21. Kozak, M. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 2850–2854.
22. Sauer, B., and Henderson, N. (1989) *Nucleic Acids Res.* **17,** 147–161.
23. Fukushige, S., and Sauer, B. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 7905–7909.
24. Tsien, J. Z., Chen, D. F., Gerber, D., Tom, C., Mercer, E. H., Anderson, D. J., Mayford, M., Kandel, E. R., and Tonegawa, S. (1996) *Cell* **87,** 1317–1326.
25. Lacy, E., Roberts, S., Evans, E. P., Burtenshaw, M. D., and Constantini, F. D. (1983) *Cell* **34,** 343–358.
26. Hanks, M., Wurst, W., Anson-Cartwright, L., Auerbach, A. B., and Joyner, A. L. (1995) *Science* **269,** 679–682.
27. Gu, H., Marth, J. D., Orban, P. C., Mossmann, H., and Rajewsky, K. (1994) *Science* **265,** 103–106.
28. Kozak, M. (1986) *Cell* **44,** 283–292.
29. Sauer, B., and Henderson, N. (1990) *New Biologist* **2,** 441–449.
30. Chung, J. H., Whiteley, M., and Felsenfeld, G. (1993) *Cell* **74,** 505–514.
31. Schwenk, F., Sauer, B., Kukoc, N., Hoess, R., Müller, W., Kocks, C., Kühn, R., and Rajewsky, K. (1997) *J. Immunol. Methods* **207,** 203–212.
32. Tsien, J. Z., Huerta, P. T., and Tonegawa, S. (1996) *Cell* **87,** 1327–1338.
33. Akagi, K., Sandig, V., Vooijs, M., Van der Valk, M., Giovannini, M., Strauss, M., and Berns, A. (1997) *Nucleic Acids Res.* **25,** 1766–1773.
34. Mansour, S. L., Thomas, K. R., and Capecchi, M. R. (1988) *Nature* **336,** 348–352.
35. Artelt, P., Grannemann, R., Stocking, C., Friel, J., Bartsch, J., and Hauser, H. (1991) *Gene* **99,** 249–254.
36. Rijli, F. M., Dollé, P., Fraulob, V., Lemour, M., and Chambon, P. (1994) *Dev. Dyn.* **201,** 336–377.
37. Feiring, S., Kim, C. G., Epner, E. M., and Groudine, M. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8469–8473.
38. Sauer, B. (1994) *BioTechniques* **16,** 1086–1088.
39. Abuin, A., and Bradley, A. (1996) *Mol. Cell. Biol.* **16,** 1851–1856.
40. Schwenk, F., Baron, U., and Rajewsky, K. (1995) *Nucleic Acids Res.* **23,** 5080–5081.
41. Lakso, M., Pichel, J. G., Gorman, J. R., Sauer, B., Okamoto, Y., Lee, E., Alt, F. W., and Westphal, H. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 5860–5865.
42. Araki, K., Araki, M., Miyazaki, J.-I., and Vassalli, P. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 160–164.
43. Sunaga, S., Maki, K., Komagata, Y., Ikuta, K., and Miyazaki, J.-I. (1997) *Mol. Reprod. Dev.* **46,** 109–113.
44. Dooley, T. P., Miranda, M., Jones, N. C., and DePamphilis, M. L. (1989) *Development* **107,** 945–956.
45. Wang, P., Anton, M., Graham, F. L., and Bacchetti, S. (1995) *Somat. Cell Mol. Genet.* **21,** 429–441.
46. Smith, A. J. H., De Sousa, M. A., Kwabi-Addo, B., Heppell-Parton, A., Imprey, H., and Rabbitts, P. (1995) *Nat. Genet.* **9,** 376–385.
47. van Deursen, J., Fornerod, M., van Rees, B., and Grosveld, G. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 7376–7380.
48. Ramirez-Solis, R., Liu, P., and Bradley, A. (1995) *Nature* **378,** 720–724.
49. Li, Z.-W., Stark, G., Götz, J., Rülicke, T., Müller, U., and Weissmann, C. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 6158–6162.
50. Sauer, B. (1996) *Pharmacol. Tech.* **20,** 42–49.
51. Baubonis, W., and Sauer, B. (1993) *Nucleic Acids Res.* **21,** 2025–2029.
52. Gossen, M., and Bujard, H. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 5547–5551.
53. Gossen, M., Freundlieb, S., Bender, G., Muller, G., Hillen, W., and Bujard, H. (1995) *Science* **268,** 1766–1769.
54. St-Onge, L., Furth, P. A., and Gruss, P. (1996) *Nucleic Acids Res.* **24,** 3875–3877.
55. Christopherson, K. S., Mark, M. R., Bajaj, V., and Godowski, P. J. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 6314–6318.
56. No, D., Yao, T. P., and Evans, R. M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 3346–3351.
57. Wang, Y., DeMayo, F., Tsai, S., and O'Malley, B. (1997) *Nature Biotechnol.* **15,** 239–243.
58. Ho, S. N., Biggar, S. R., Spencer, D. M., Schreiber, S. L., and Crabtree, G. R. (1996) *Nature* **382,** 822–826.
59. Metzger, D., Clifford, J., Chiba, H., and Chambon, P. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 6991–6995.
60. Kellendonk, C., Tronche, F., Monaghan, A.-P., Angrand, P.-O., and Stewart, F. (1996) *Nucleic Acids Res.* **24,** 1404–1411.
61. Zhang, Y., Riesterer, C., Ayrall, A. M., Sablitzky, F., Littlewood, T. D., and Reth, M. (1996) *Nucleic Acids Res.* **24,** 543–548.
62. Anton, M., and Graham, F. L. (1995) *J. Virol.* **69,** 4600–4606.
63. Wang, Y., Krushel, L. A., and Edelman, G. M. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 3932–3936.
64. Rohlmann, A., Gotthardt, M., Willnow, T. E., Hammer, R. E., and Herz, J. (1996) *Nature Biotechnol.* **14,** 1562–1565.
65. Dymecki, S. (1996) *Proc. Natl. Acad. Sci. USA* **87,** 4712–4716.
66. Tybulewicz, V. L., Crawford, C. E., Jackson, P. K., Bronson, R. T., and Mulligan, R. C. (1991) *Cell* **65,** 1153–1163.