Endocrine, *vol. 19, no. 3, 229–238, December 2002* 0969–711X/02/19:229–238/$20.00 © 2002 by Humana Press Inc. All rights of any nature whatsoever reserved.

Gene Targeting in the Mouse

Advances in Introduction of Transgenes into the Genome by Homologous Recombination

Ravi P. Misra and Stephen A. Duncan[1]

Departments of Biochemistry and [1]Cell Biology, Neurobiology and Anatomy, Medical College of Wisconsin, 8701 Watertown Plank Road, Milwaukee, WI

The ability to stably introduce genes into the germline of animals provides a powerful means to address the genetic basis of physiology. Introduction of genes to generate transgenic animals has facilitated the development of complex genetic models of disease, as well as the in vivo study of gene function. However, one drawback of traditional transgenic technologies in which genes are microinjected into early-stage embryos is that there is little control over where and in how many copies genes are introduced into the genome. The development of animal transgenic technologies, which take advantage of homologous recombination mechanisms and the manipulation of embryonic stem (ES) cells, allows investigators to target and alter specific loci. In mouse transgenic systems, a plethora of sophisticated gene-targeting strategies now permit investigators to manipulate the genome in ways that essentially allow one to introduce virtually any desired change into the genome. Fur-thermore, when coupled with systems that allow for conditional gene expression, these gene-targeting strategies allow both temporal and tissue specific control of alterations to the genome. In the present review we briefly discuss some of the more recent gene-targeting strategies that have been developed to address the limitations of traditional animal transgenesis.

Key Words: Gene targeting; homologous recombination.

Transgenic Approaches to Studying Gene Function In Vivo

Techniques for introducing genes stably into the germline of experimental mammals provide a powerful means to investigate complex biological phenomena and to generate animal models of disease. In the most common approaches, genes are introduced or altered either by genetic manipulation of embryonic stem (ES) cells or by microinjection of DNA into the male pronucleus of fertilized eggs. Microinjection-based approaches are well suited to examining the physiological effects of overexpression or misexpression of gene products of interest. Such approaches also allow analysis of transcriptional regulatory elements in vivo. While a large number of studies have used a pronuclear injection approach to address gene expression and physiological function,there exist limitations on its usefulness. Notably, pronuclear injection results in an unpredictable number of transgene copies being incorporated at random locations in the recipient genome. This can result in ectopic expression of the transgene because both copy number and the site of integration can influence transgene expression. This can be a particular disadvantage in gene expression studies, which rely on independent analysis of multiple *cis*-acting regulatory regions. To account for these effects, multiple independent lines of transgenic mice need to be established for each experiment. Another limitation is presented by expression of transgenes that confer developmental lethal phenotypes. In such cases no founder line can be established, and, therefore, it is impossible to repeat an analysis with a specific transgenic mouse embryo. In part, to address these limitations various strategies have been developed that typically rely on targeting transgenes to a specific genomic locus by homologous recombination in pluripotent ES cells, thereby allowing precise modification of the gene of interest *(1)*. These ES cell lines can then be used to generate transgenic animals (Fig. 1) *(2)*. Using these approaches, it is possible to create mice of virtually any genotype. In addition, such gene-targeting methodologies can be coupled with bacterially derived site-specific recombination systems that allow introduction of loss-of-function mutations in the context of both developing and intact animals. Gene targeting strategies that create such "knock-out" mice having a null genotype often provide definitive experimental evidence regarding the functions of encoded proteins, and strategies that knockout genes "conditionally" can address developmental lethal phenotypes.

The principles of gene targeting were first established in experiments with *Saccharomyces cerevisiae*. During this time many of the basic tools that facilitated gene targeting in eukar-

Received October 31, 2002; Accepted November 15, 2002.

Author to whom all correspondence and reprint requests should be addressed: Dr. Ravi P. Misra, Department of Biochemistry, Medical College of Wisconsin, 8701 Watertown Plank Rd, Milwaukee, WI 53226. E-mail: rmisra@mcw.edu

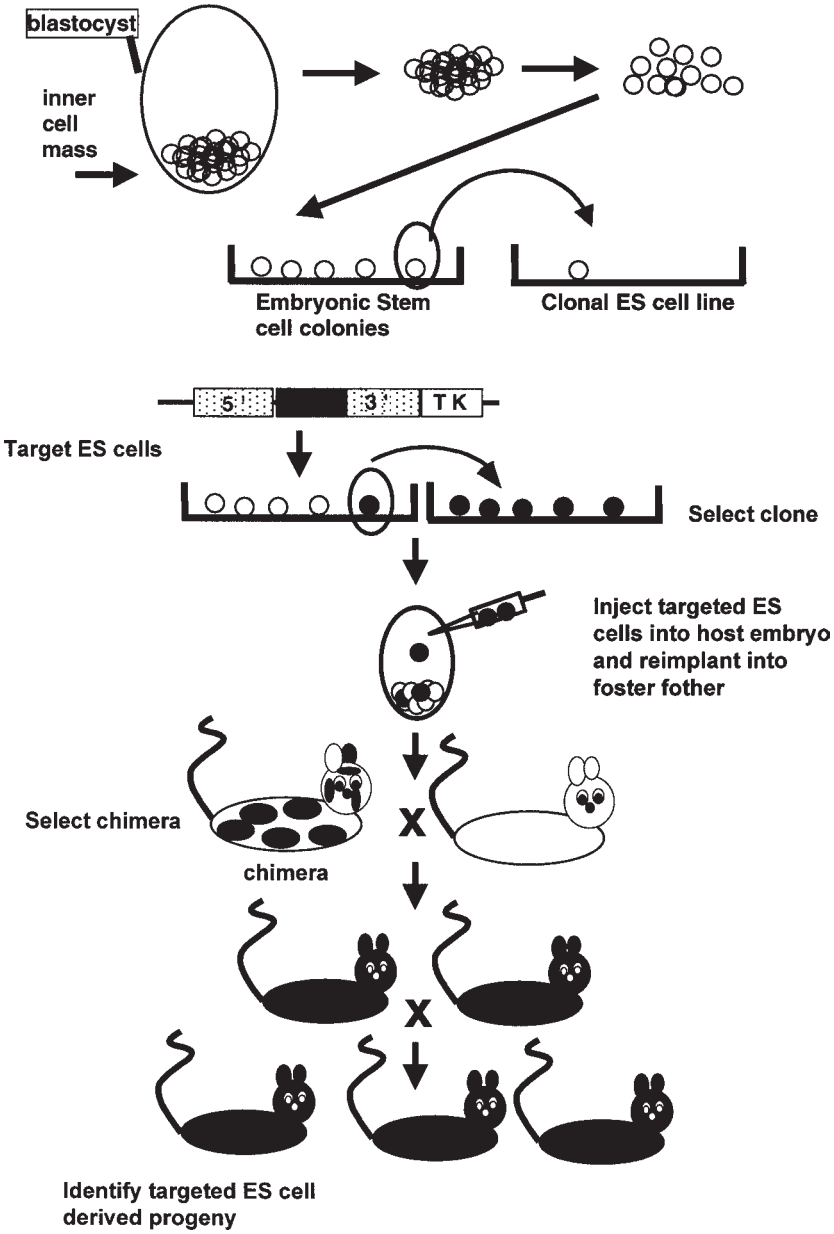

Fig. 1. Embryonic stem cells are isolated from the inner cell mass of blastocysts and ES cell lines are isolated. Gene targeting is carried out during the cell culture stage using targeting constructs and selectable markers to identify cells that are correctly targeted. Targeted cells are then injected into blastocysts derived from different strains, and injected embryos are reimplanted into pseudopregnant foster mothers. Embryos go on to develop chimeric animals that are test bred to identify germline transmission, where chimeras result from ES-cell-derived gametes. Chimerism and transmission of ES-cell-derived gametes can be monitored using coat color markers.

yotic cells by homologous recombination were established *(3,4)*. Lin et al. first demonstrated that homologous recombination in mammalian cells was feasible by introducing DNA into an artificial locus *(5,6)*. Smithies and co-workers subsequently demonstrated that homologous recombination could occur at an endogenous locus, the β-globin gene, in erythroleukemic cells *(7)*. In these experiments they used a targeting vector containing β-globin homologous sequences and a gene encoding neomycin resistance. After selecting for neomycin-resistant colonies, they found that the endogenous β-globin gene had successfully been modified. Application of gene targeting to the generation of transgenic animals was furthered by the demonstration that homologous recombination could be carried out in embryonic stem (ES) cells *(8)*. ES cells are derived from the inner cell mass (ICM) of blastocysts, and like ICM cells they can contribute to all embryonic tissues, including the germ cells, in developing mice. This means that genetic alterations made in cultured ES cells can be relatively easily transmitted through the mouse germline *(9,10)*.

While gene-targeting methods have proven to be extremely powerful means to address gene function, a number of technical issues limit the facility of the original approaches. One problem is that the generation of targeting vectors often utilizes large regions of genomic DNA, and their construction can be labor-intensive and complex. Also, alterations to the genome that globally affect somatic cell function often result in a developmental arrest that prevents any analysis of gene function in adult tissues or cells. This can be particularly frustrating when the hope is to obtain physiological data from the altered alleles. To address these issues a variety of modifications to the original gene-targeting strategies have been developed. In the remaining sections of this review we will focus on discussing some recent advances in gene-targeting procedures that address traditional limitations.

Site-Specific Recombination and Conditional Gene Expression

The study of alleles in the adult that normally affect embryonic development can be achieved through the use of site-specific recombination systems *(11,12)*. Recent advances using bacterial and eukaryotic recombinases that act on specific nucleotide sequences have allowed researchers to inactivate or activate gene function in a conditional manner. The basic procedure combines the incorporation of small *cis*-acting elements into given genomic sites by homologous recombination using controlled recombinase expression. Two of the most well-developed and widely used recombinase systems are the Cre/*loxP* system from bacteriophage P1 and the Flp/FRT system from *S. cerevisiae (13)*. Both the Cre and Flp recombinases are members of the lambda integrase family of site-specific recombinases. These recombinases mediate recombination between two target elements thereby excising the intervening DNA. The reaction occurs with high fidelity and requires only the recombinase and the target DNA sequence. Because the Cre/*loxP* system is more widely used, the following discussion will focus on the Cre/*loxP* system.

*The Cre/*loxP *Site-Specific Recombination System*

The Cre/*loxP* system consists of Cre protein, a site specific approx 38 kd DNA recombinase isolated from bacteriophage P1, and target *loxP* (locus of crossover in P1) elements. The *loxP* elements are 34 bp nucleotide sequences (Fig. 2D), originally found in the P1 genome, consisting of two 13 bp inverted repeats flanking a 8 bp nonpalindromic core *(14–16)*. Typically, *loxP* sites are introduced flanking the DNA sequences to be excised (Fig. 2). Depending on the orientation of the *loxP* sites relative to each other, recombination can result in irreversible excision of the intervening DNA, if the sites are head-to-tail, or inversion of the intervening sequences, if the sites are oriented head-to-head (Fig. 2D). Recombination can occur independent of the topology of the DNA, and in terminally differentiated postmitotic cells as well as dividing cells.

*Generation of Null Alleles Using Cre/*loxP*-Mediated Recombination*

In conventional gene-targeting approaches, the most common strategy is to inactivate the target gene by introducing a positive selectable marker, typically a drug-resistance gene that replaces or disrupts the target gene coding sequence. A significant drawback to this approach is that incorporation of the selectable marker can affect the target locus in unexpected ways. For example, its presence can unpredictably affect regulation and processing of the target gene, or the promoters driving expression of the selectable marker may interfere with expression of neighboring endogenous genes. In some cases endogenous genes lying over 100 kb from a targeted gene have been affected *(17)*. It is therefore beneficial to remove the selectable marker and generate a "clean" mutation in which all aspects of the target gene remain wild type except for the region encoding the desired mutation. An additional advantage to generating a clean mutation is that it allows a second site in the genome to be targeted using a similar selection strategy as the first site. The Cre/*loxP* system can be effectively used to generate a "clean" mutation *(13)*. One way this can be accomplished is outlined in Fig. 2A. Here the selectable marker genes are flanked with *loxP* recombination sites (floxed). Following homologous recombination and inactivation of the target locus, Cre-recombinase-mediated excision of the selection cassette can be performed either in vitro or in vivo, leaving a clean excision.

loxP recombination sites are asymmetric, and as a result, depending on the orientation of the two *loxP* sites relative to each other, there are different outcomes for the recombination event (Fig. 2D). When two *loxP* sites are oriented head-to-tail, the resulting recombination products include a circular molecule containing a single *loxP* site and a continuous product from which the circle was excised also containing a single *loxP* site. This type of reaction is largely irreversible, because the excised circle is lost as an episomal product, and therefore this reaction results in an excision or deletion reaction. In contrast, when *loxP* sites are oriented head-to-head, the resulting recombination event yields an integrative event in which there is an intrachromosomal recombination and a resulting inversion of the sequences between the *loxP* recombination sites (Fig. 2D). In this case, the *loxP* recombination sites are regenerated and the inverted product can continue to be a substrate for recombination as long as Cre recombinase is present. To address this issue, two strategies have been employed. In one case recombinase expression can be transient. In practice in mammalian cells, limiting Cre recombinase is generally significantly more challenging than in lower eukaryotes and bacteria. Another approach is to utilize asymmetric mutant *loxP* sites *(18–20)*. In this approach, mutant *loxP* sites are introduced into the genome at the desired genomic regions. These sites are designed such that after Cre-mediated recombination one wild type site and one mutant *loxP*

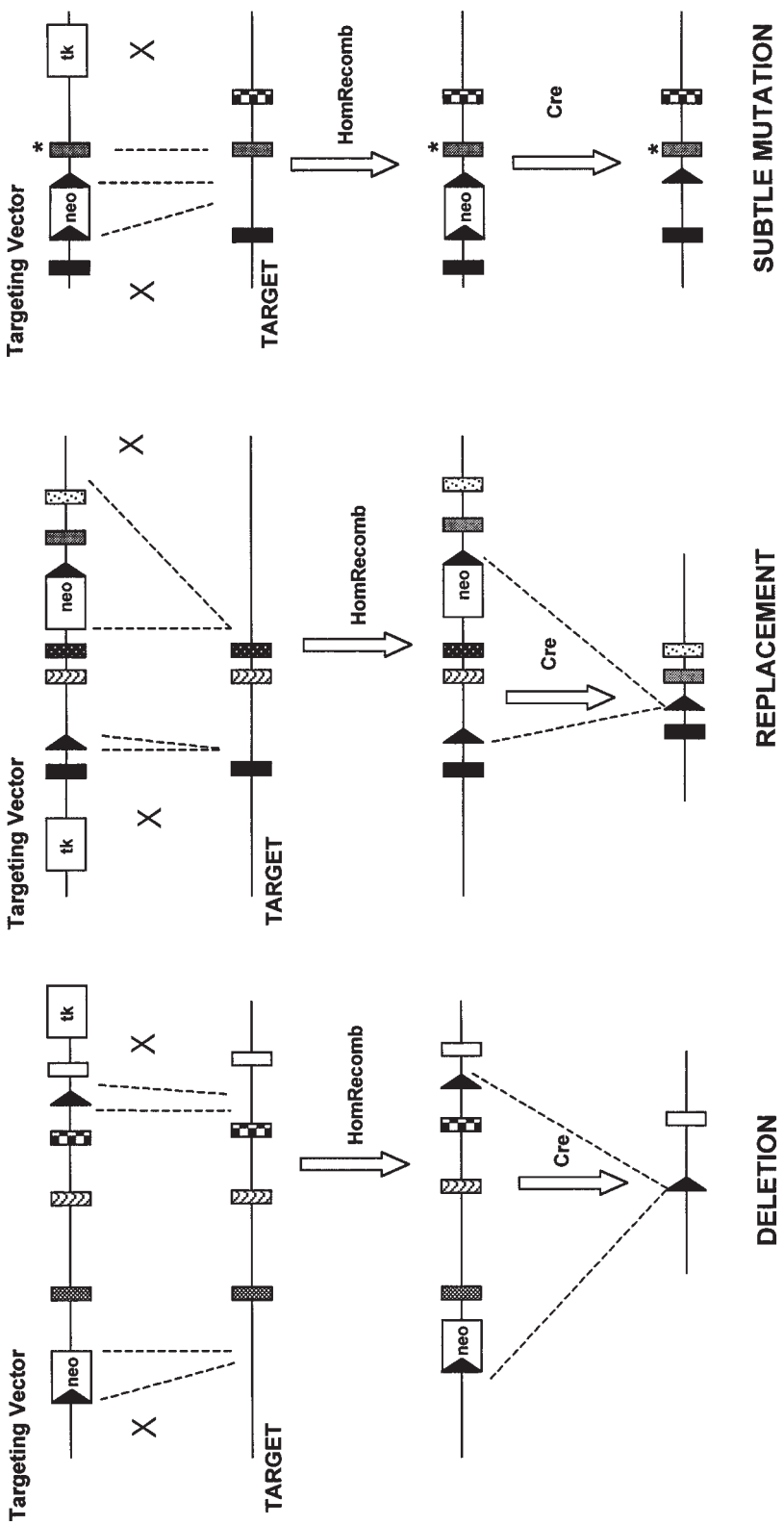

Fig. 2. Cre-*lox*-mediated targeting. (**A**) Cre-*lox*-mediated generation of a deletion. Two *loxP* sites are introduced by homologous recombination (HomRecomb) into the target locus such that they flank the genomic regions of interest, stippled, hatched, and checkered exons of target genes, and a neomycin-selection gene, which is used to select for recombinant ES cell clones. Transient expression in targeted ES cells with Cre recombinase leads to excision of the floxed DNA segment along with the neomycin gene. (**B**) Gene replacement. *loxP* sites are introduced at the 3' end of the neomycin gene and between the hatched and dark stippled exons. Heterologous sequences containing the light stippled and gray exons are inserted next to the *loxP*-flanked region that is replaced upon Cre-mediated recombination. (**C**) Cre-*lox*-mediated introduction of subtle mutations into a target gene. A subtle mutation in the gray exon (asterisk) and the *loxP*-flanked neomycin gene are introduced in between the black and checkered exons replacing the wild-type gray exon. The *loxP*-flanked neomycin gene is deleted upon Cre expression. In **A**, **B**, and **C**, exons are shown as rectangles and *loxP* sites are triangles. Crossover points are indicated with X's and dashed lines show insertion sites of selectable markers.

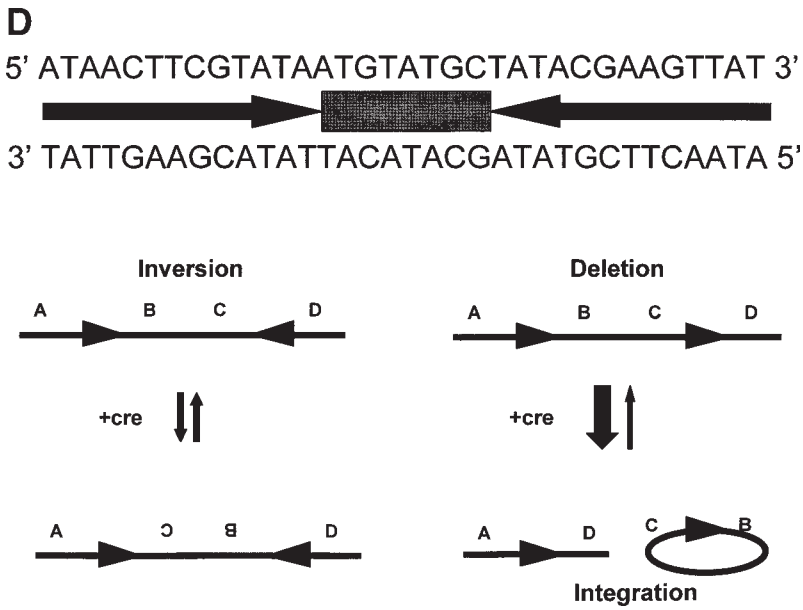

Fig. 2. (**D**) Top: *loxP* Cre recombinase target site. A single *loxP* site is depicted that contains inverted 13 bp symmetry elements flanking an 8 bp A:T rich nonpalindromic core, which imparts directionality to the element (gray rectangle). Bottom: Cre recombinase catalyzed reactions. Triangles indicate *loxP* sites. Cre will invert the DNA between two inverted *loxP* sites, or excise a circular molecule from between two directly repeated target *loxP* sites separated by at least 82 bp. **A**, **B**, and **C** are adapted from ref. *11*, and **D** is adapted from ref. *13*.

site are generated. The mutant site is designed to have a poor affinity for Cre recombinase. Therefore, the forward reaction is heavily favored, and the reaction is essentially irreversible.

Gene Replacement Using the Cre/loxP Recombination System

The Cre/*loxP* system can also be used to swap endogenous sequences with heterologous sequences in a "knock-in" approach that results in replacing the endogenous sequences with desired sequences (Fig. 2B). This type of approach is advantageous for a variety of purposes and is frequently used to swap exonic sequences to study function or to generate chimeric proteins. Knock-in strategies can also be used to introduce reporters into specific genomic loci allowing gene expression patterns using endogenous regulatory sequences to be studied (*see* below). In a replacement strategy the targeting vector contains heterologous sequences that are directly linked to a selection gene, heterologous sequences that will replace the endogenous sequences, and homologous targeting sequences. *loxP* sites flank the selection gene and the homologous targeting sequences. Upon integration, selection, and recombinase-mediated deletion of the selection marker, the endogenous locus is replaced with heterologous replacement sequences (Figs. 2B and 2C). The knock-in approach can be used to swap in relatively large (kb) regions, or sequences containing smaller point mutations.

*Conditional Gene Inactivation Using the Cre/*loxP *System*

Often an initial targeting event is designed to disrupt the function of the target gene, remove a selectable marker, and generate a "clean" mutation that upon production of a transgenic animal will be manifested in all tissues. However, the Cre/*loxP* system can also be used to generate alleles in which disruption of the gene is conditionally dependent on the presence of Cre. When *loxP* sites are introduced into nonfunctional regions of the gene (e.g., intronic regions) that flank essential coding sequences, the allele is said to be "floxed." In the absence of Cre the "floxed" allele expresses a wild-type product. However, in the presence of Cre-recombinase, recombination between *loxP* sites results in a deletion of flanked exons and a loss-of-gene function. This general strategy has been employed to disrupt gene function in both a temporal and tissue-specific manner. Usually Cre is supplied by mating mice harboring the "floxed" allele with a second line of transgenic mice expressing Cre in specific cell types or tissues. More recently, strategies that rely on Cre recombinases whose own expression is under the control of drug-inducible promoters have been developed that allow for more precise temporal control over recombinase expression (for review see refs. *13,21–23*). Although it is most common to provide Cre by mating with a Cre-expressing transgenic mouse, it is also feasible to provide Cre by adenovirus infection. This has been particularly suitable for deletion of alleles in the liver *(24)*.

Expression of Genes by Targeted Knock-In

An important application of transgenic technologies is to express gene products in a controlled manner. Typically, there is interest to express a transgene in a tissue-specific or temporal-specific fashion. To accomplish this, expression of the transgene is mediated by appropriate transcrip-

tional regulatory elements. This requires the availability of well-characterized transcriptional regulatory elements that direct transgene expression in specific cell types. The availability of such elements is surprisingly limited. However, utilizing endogenous regulatory elements can circumvent this limitation. The use of endogenous regulatory elements is achieved by introducing the transgene into a defined genomic locus by homologous recombination in ES cells as discussed above. While this approach requires knowledge of the expression pattern of the endogenous gene, it does not require any analysis of gene regulatory elements. Such a knock-in approach would, in effect, significantly increase the repertoire of regulatory elements available to drive transgene expression, because nearly any gene's regulatory sequences would potentially be available to drive transgene expression. Moreover, this type of approach ensures that a single copy of the transgene is introduced into a defined genomic location, which largely avoids the negative influences of random transgene integration. The downside, however, is that every individual transgene an investigator wishes to express must be independently targeted to the chosen genomic locus. This can be tedious, especially if the rate of recombination at the chosen locus is low. Recently, a number of methods have been described that allow transgenes to be successively targeted to marked genomic loci. One approach, discussed in detail below, is particularly simple because it relies on reconstitution of neomycin resistance as a positive selectable marker, and so correctly targeted ES cell clones can be efficiently identified by growth in G418.

Successive Introduction of Transgenes into a Given Genomic Locus

Figure 3A illustrates the overall strategy used to introduce single-copy transgenes into a defined genomic locus. For convenience the procedure can be considered in three distinct steps. First a specific genomic locus is chosen based on known gene expression data. In the example presented here, the Hnf3α locus was used because Hnf3α is expressed throughout the embryonic gut endoderm of the mouse. Once the locus is chosen, a targeting vector is designed to introduce a *phosphoglycerate kinase* (pgk) promoter/*loxP*-Neo cassette that confers resistance to G418 in mammalian cells. Importantly, the pgk promoter of this cassette is flanked by *loxP* elements so that the promoter can easily be removed using Cre recombinase. In step 2 a Cre-expression plasmid is transiently introduced into the pgk/*loxP*-Neo targeted ES cells and Cre-mediated deletion of the pgk promoter results in the cells reverting to G418 sensitivity while retaining the Neo gene. The generation of these G418-sensitive ES cells containing Neo integrated into the Hnf3α locus forms the basis for targeting transgenes to this locus. In step 3, a general targeting vector is constructed that allows the introduction of transgenes into the Hnf3α/Neo locus (Fig. 3B). Two arms of homology mediate targeting of transgenes to the Hnf3α/Neo locus. The 3' arm consists of nucleotides 29 to 626 of the Neo coding sequence with an accompanying pgk promoter flanked by *loxP* elements. This truncated Neo cassette is inactive and activity is dependent on reconstitution of sequences encoding the carboxyl end following homologous recombination *(25)*. The 5' region of homology consists of, in this case, Hnf3α genomic sequence. Although this is a multiple-step procedure once the chosen locus has been targeted by homologous recombination, subsequent manipulations are extremely efficient. Indeed in the final step, where the transgene of interest is targeted to the desired locus, it was found that 100% of G418-resistant ES cell colonies were correctly targeted *(26)*. Transgenic mice and embryos can then be generated from these ES cells by standard injection into blastocysts and subsequent breeding of the resulting chimeric mice.

Sauer and co-workers and Hardouin et al. have also described alternative Cre-mediated approaches to target specific loci. In the approach of Sauer and co-workers single-copy transgenes are targeted to defined genomic sites that have been marked by *loxP* elements using a double *loxP* targeting strategy *(27,28)*. This approach relies on co-transfection of a Cre expression plasmid along with a *loxP*-targeting vector that carries the transgene. Hardouin et al. have recently described a method to introduce transgenes to loci identified by gene trapping. In this case integration of the transgene at the gene-trap locus is mediated by Cre recombinase and translation of the transgene is under control of an internal ribosomal entry site (IRES) *(29)*.

Chosen Site Integration—Targeting to the HPRT Locus

The problem of random transgene integration can be also be overcome by targeting transgenes to the *hypoxanthine phosphoribosyl transferase* (Hprt) locus *(27,30,31)*. The *Hprt* locus is a particularly suitable site for the integration of transgenes because it exists as an X-linked gene present as a single copy in male ES cells. Moreover, the *Hprt* gene is ubiquitously expressed and so provides a favorable chromatin environment for transgene expression. Indeed, it has been demonstrated that the level of expression of transgenes inserted into the *Hprt* locus is directed solely by exogenous transcriptional regulatory elements *(30)*. A method that allows the introduction of single-copy transgenes into this locus has been described by Bronson et al. *(30)*. Using this approach single-copy transgenes are introduced 5' of a defective *Hprt* locus by homologous recombination in ES cells to reconstitute HPRT activity (Fig. 4A). This is an extremely efficient procedure because targeted clones that reconstitute the *Hprt* gene can be selected on the basis of growth in HAT medium. Following this method, upward of 90% of HAT-resistant ES cell clones contain correctly targeted transgenes *(30)*. A major advantage of this targeted transgenic approach compared to traditional transgenics is that transgene expression is predictable, so expression at ectopic sites is largely avoided. Mice derived from independent ES cells clones containing a transgene expressed from the same pro-

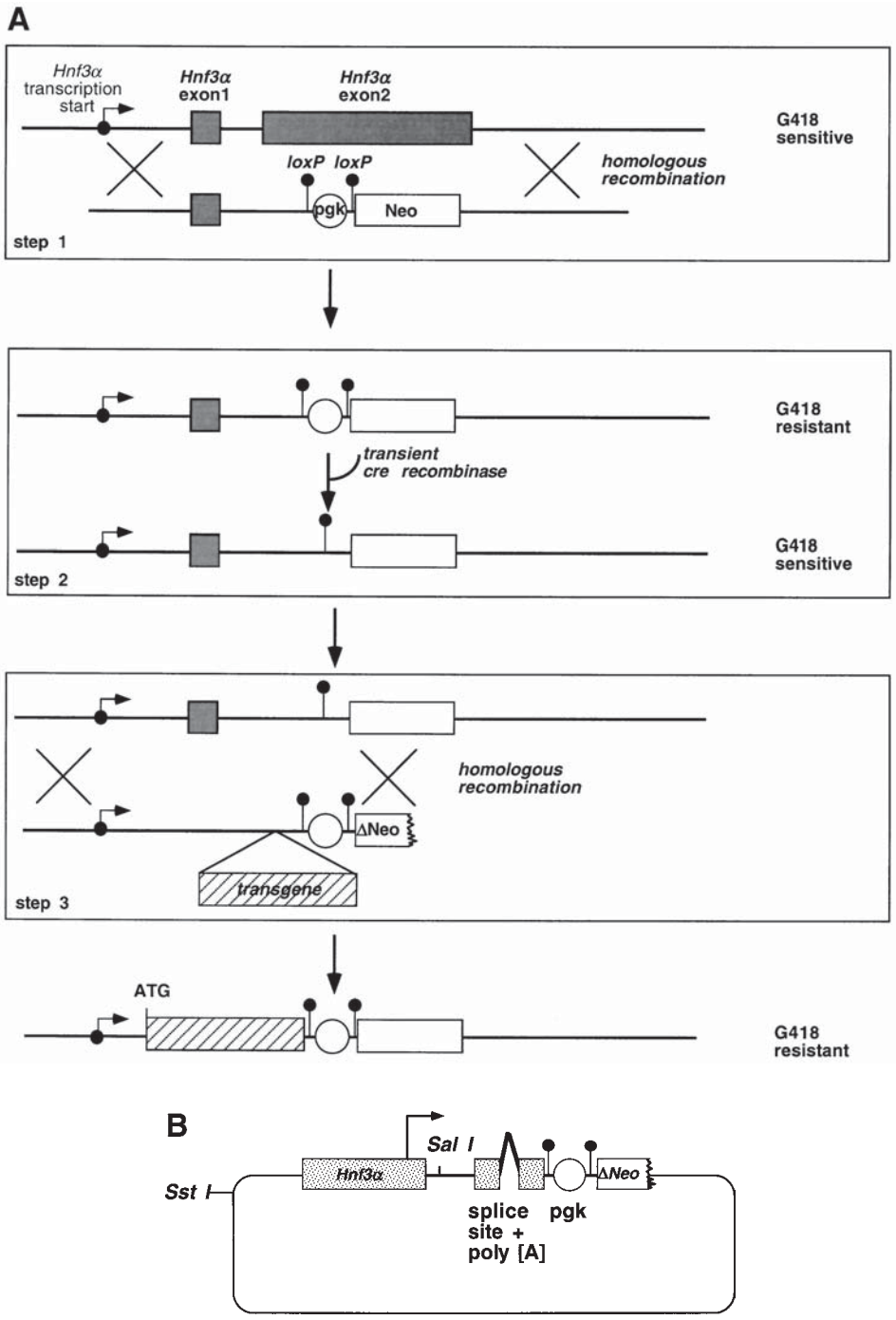

Fig. 3. (**A**) Strategy used to introduce transgenes into a defined genomic locus in ES cells. For convenience the approach can be considered in three steps (boxes). In step1, a cassette containing *neomycin phosphotransferase* coding sequence (Neo) that is expressed from a *phosphoglycerate kinase-1* (pgk) promoter (open circle) flanked by *loxP* elements (stems) is targeted to a chosen locus, in this case *Hnf3α*, by homologous recombination. Cells containing the Neo cassette are resistant to the pharmacological inhibitor G418. In step 2, sensitivity of the targeted ES cells to G418 is restored by removing the *pgk* promoter by transiently introducing a plasmid that expresses Cre recombinase. In step 3, transgenes (hatched box) are introduced to the locus through homologous recombination. The short arm of homology contains a truncated Neo gene (ΔNeo) that lacks phosphotransferase activity that can be expressed from a *pgk* promoter. The long arm of homology consists of genomic DNA sequences lying 5' to the intended site of integration (not drawn to scale). Homologous recombination reconstitutes expression of Neo and generates G418-resistant ES cells. The site of transcriptional initiation of *Hnf3α* is indicted as a black dot with an arrow. (**B**) Schematic of a general targeting vector used to introduce transgenes into the *Hnf3α* locus by homologous recombination. The 5' arm of homology contains *Hnf3α* genomic DNA that extends 3' of the *Hnf3α* transcriptional start site (arrow). Coding sequences can be introduced into a unique *Sal1* site that lies 5' to sequences containing an intron and polyadenylation signal for efficient RNA processing. A truncated Neo gene (ΔNeo) that lacks phosphotransferase activity provides the short arm of homology. Expression of ΔNeo is regulated by a *pgk* promoter flanked by *loxP* elements. For electroporation into ES cells, the targeting vector can be made linear by digesting with *Sst1*.

moter all exhibit comparable and cell-type-specific levels of expression *(30–33)*.

Production of Embryos by Tetraploid Aggregation—A Powerful Tool to Study Embryonic Lethal Phenotypes and Developmental Gene Expression

Analysis of dominantly acting alleles that cause embryonic lethality can often uncover fundamental mechanisms that control diverse cellular processes. However, by definition, it is not feasible to establish founder lines expressing such alleles. To overcome this problem investigators have historically relied upon transient transgenic procedures *(34)*. In this approach, fertilized eggs are injected with a transgene construct and are allowed to develop to a predetermined embryonic stage *in utero* before being collected for phenotypic analysis. Unfortunately, because of the randomness of transgene integration, transgene expression in each embryo can differ, which can ultimately confound any phenotypic analysis. This means that statistically significant numbers of transgenic embryos must be generated and analyzed for each transgene, a process that can be prohibitive if the phenotype is complex.

Recently, many of these limitations have been overcome by combining genetic manipulation of ES cells with the ability to generate embryos directly from ES cells by tetraploid embryo complementation (see Fig. 4B) *(35–37)*. Tetraploid embryos, produced by the fusion of two-cell-stage (diploid) CD-1 embryos, are able to implant but do not complete development when transplanted into recipient surrogate mother. However, normal embryogenesis will occur if the tetraploid embryos are aggregated with embryonic stem cells prior to transplantation. Lineage tracing studies within the resulting chimeric embryos have demonstrated that tetraploid cells contribute exclusively to extraembryonic structures, i.e., yolk sac endoderm, placenta, chorion, and trophectoderm lineages, while the embryo proper is derived solely from the ES cells. This allows the relatively rapid analysis of dominant embryonic lethal phenotypes. Recently, HPRT-negative ES cells have been generated that allow one to couple the power of introducing single-copy transgenes to the *Hprt* locus with efficient generation of embryos by tetraploid complementation *(30,38)*. This is particularly appealing because each embryo produced in this manner is, in effect, clonal because it is generated from a single clonal ES cell line. This allows reproducible comparison of the phenotype generated by a given transgene at different developmental stages. Moreover, because the transgene is present as a single copy at a known site of integration, it circumvents the need to generate many different transgenic lines from a given construct.

This approach is not only useful for phenotypic analyses but can also be used to rapidly identify transcriptional regulatory elements that control developmental and cell-specific expression of genes in vivo as has been reported for the αMHC locus *(38)*. There are two major advantages of the *Hprt*/tetraploid approach over traditional transgenic methods. The first is that it is extremely rapid—once the targeting construct is in hand it takes approx 4–5 wk to generate transgenic mouse embryos. The second is due to the presence of the transgene at a known and constant integration site, which allows reproducible comparison of expression from specific regulatory elements at different developmental stages. In addition, because of the reproducibility of expression from embryo to embryo, this technique could potentially allow comparative quantitative analyses of reporter gene expression in vivo.

Gene Targeting By Recombineering

As discussed above, the introduction of modifications into genes via homologous recombination in ES cells relies on targeting constructs that contain large segments of DNA homologous to the gene to be targeted. Typically, these sequences are isolated from lambda phage libraries or bacterial artificial chromosomes (BACs) that contain large regions of genomic sequences. Manipulating these large DNA fragments requires complex multiple-step cloning strategies and, in many cases, the complexity of the cloning increases when additional elements, such as *lacZ* or *GFP* reporter genes, *loxP* sites, or point mutations, are required. One of the major limitations in using standard cloning practices is the need for suitable restriction endonuclease cut sites that allow appropriate positioning of cloned DNA fragments. As a consequence, the generation of targeting constructs is often the rate-limiting step in a given gene-targeting project. Recently, however, bacteriophage-based homologous recombination systems that work efficiently in *E. coli* have greatly simplified the process by which complex gene targeting constructs are manufactured (recently reviewed in refs. *39* and *40*). These approaches initially based on the RecET recombination system of phage Rac, allow alteration of DNA molecules by recombination with linear DNA fragments and promise to revolutionize our ability to generate transgenic animals. In the original ET system, Stewart and colleagues demonstrated that linear dsDNA generated by PCR and flanked by short (42-bp) regions of homology to a target plasmid could be efficiently integrated into the plasmid by introducing the linear DNA fragment into *recBC sbcA* strains of *E. coli (41)*. Although initial experiments were conducted in *sbcA* strains of *E. coli*, it was later shown that any *E. coli* strain could support homologous recombination if the appropriate phage genes are expressed from plasmids or are incorporated into the bacterial genome.

The term "recombineering" has been coined to describe the use of homologous recombination in *E. coli* to manipulate genomic sequences. Recombineering makes it possible to introduce virtually any type of mutation into a BAC, or other large target DNA, using PCR-amplified, linear,

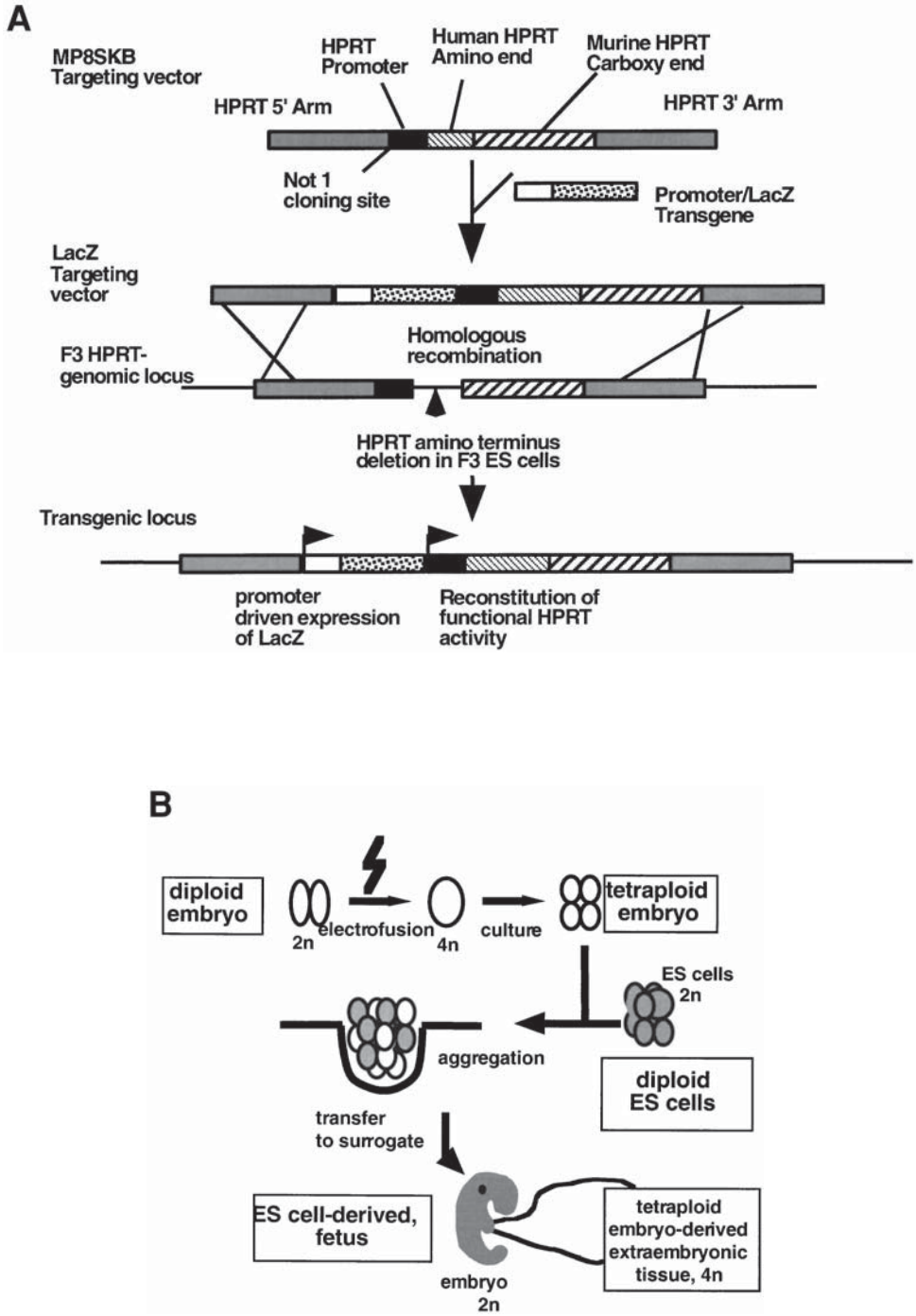

Fig. 4. (**A**) Strategy for targeting promoter/*LacZ* transgenes to the *Hprt* locus. The F3 ES cells are HPRT negative due to a deletion of the 5' end of the *Hprt* gene. However, HPRT function is reconstituted after recombination with the targeting vector mp8SKB containing the transgene of interest. This allows an efficient selection strategy to detect integration of transgenes at the *Hprt* locus. (**B**) Diagram illustrating tetraploid aggregation: fetuses produced by this method are derived entirely from ES cells, while tetraploid cells contribute to the extraembryonic tissue.

double-stranded DNA targeting cassettes that have short regions of homology with the target sequences at their ends. Recombineering promises to greatly decrease the time it takes to create transgenic mouse models by conventional means, and to facilitate the ability to carry out complex targeting strategies. Many of the strategies utilizing recombineering approaches allow targeting constructs to be generated within 1 wk—far faster than average conventional cloning strategies.

Over the past decade ever more sophisticated application of homologous recombination techniques and ES cell culture approaches have made it possible to conceive of

generating virtually any desired mutation in the germline of mice. With these technologies in hand, it is now conceivable to genetically dissect the function of every gene in the mammalian genome.

Acknowledgments

This work was supported by grants from the National Heart Lung and Blood Institute (HL67272), and the American Heart Association-Northland Division to R.P.M.; and from the National Institute of Diabetes and Digestive and Kidney Disease (DK55743 and DK60064) to S.A.D.

References

1. Thomas, K. R. and Capecchi, M. R. (1987). *Cell* **51,** 503–512.
2. Thompson, S., Clarke, A. R., Pow, A. M., Hooper, M. L., and Melton, D. W. (1989). *Cell* **56,** 313–321.
3. Hinnen, A., Hicks, J. B., and Fink, G. R. (1978). *Proc. Natl. Acad. Sci. USA* **75,** 1929–1933.
4. Orr-Weaver, T. L., Szostak, J. W., and Rothstein, R. J. (1981). *Proc. Natl. Acad. Sci. USA* **78,** 6354–6358.
5. Lin, F. L., Sperle, K., and Sternberg, N. (1984). *Mol. Cell. Biol.* **4,** 1020–1034.
6. Lin, F. L., Sperle, K., and Sternberg, N. (1985). *Proc. Natl. Acad. Sci. USA* **82,** 1391–1395.
7. Smithies, O., Gregg, R. G., Boggs, S. S., Koralewski, M. A., and Kucherlapati, R. S. (1985). *Nature* **317,** 230–234.
8. Hasty, P., Ramirez-Solis, R., Krumlauf, R., and Bradley, A. (1991). *Nature* **350,** 243–246.
9. Bradley, A. (1991). *Curr. Opin. Biotechnol.* **2,** 823–829.
10. Capecchi, M. R. (1989). *Trends Genet.* **5,** 70–76.
11. Kuhn, R. and Schwenk, F. (1997). *Curr. Opin. Immunol.* **9,** 183–188.
12. Hasty, P., Abuin, A., and Bradley, A. (2000). In: *Gene targeting*, 2nd ed. Joyner, A. L. (ed.). Oxford University Press, Oxford, pp. 1–35.
13. Dymecki, S. M. (2000). In: *Gene targeting*, 2nd ed. Joyner, A. L. (ed.). Oxford University Press, Oxford, pp. 37–99.
14. Sternberg, N. and Hamilton, D. (1981). *J. Mol. Biol.* **150,** 467–486.
15. Sternberg, N., Hamilton, D., and Hoess, R. (1981). *J. Mol. Biol.* **150,** 487–507.
16. Le, Y. and Sauer, B. (2000). *Meth. Mol. Biol.* **136,** 477–485.
17. Pham, C. T., MacIvor, D. M., Hug, B. A., Heusel, J. W., and Ley, T. J. (1996).*Proc. Natl. Acad. Sci. USA* **93,** 13090–13095.
18. Zhang, Z. and Lutz, B. (2002). *Nucleic Acids Res.* **30,** E90, 1–5.
19. Araki, K., Araki, M., and Yamamura, K. I. (2002). *Nucleic Acids Res.* **30,** E103–E103.
20. Araki, K., Araki, M., and Yamamura, K. (1997). *Nucleic Acids Res.* **25,** 868–872.
21. DeMayo, F. J. and Tsai, S. Y. (2001). *Trends Endocrinol. Metab.* **12,** 348–353.
22. Gao, X., Kemper, A., and Popko, B. (1999). *Neurochem. Res.* **24,** 1181–1188.
23. Sauer, B. (1998) *Methods* **14,** 381–392.
24. Piston, D. W., Knobel, S. M., Postic, C., Shelton, K. D., and Magnuson, M. A. (1999). *J. Biol. Chem.* **274,** 1000–1004.
25. Beck, E., Ludwig, G., Auerswald, E. A., Reiss, B., and Schaller, H. (1982). *Gene* **19,** 327–336.
26. Zhao, R., Fahs, S. A., Weiler, H., and Duncan, S. A. (2001). *BMC Dev. Biol.* **1,** 10.
27. Soukharev, S., Miller, J. L., and Sauer, B. (1999). *Nucleic Acids Res.* **27,** e21, i–viii.
28. Fukushige, S. and Sauer, B. (1992). *Proc. Natl. Acad. Sci. USA* **89,** 7905–7909.
29. Hardouin, N. and Nagy, A. (2000). *Genesis* **26,** 245–252.
30. Bronson, S. K., Plaehn, E. G., Kluckman, K. D., Hagaman, J. R., Maeda, N., and Smithies, O. (1996). *Proc. Natl. Acad. Sci. USA* **93,** 9067–9072.
31. Evans, V., Hatzopoulos, A., Aird, W. C., Rayburn, H. B., Rosenberg, R. D., and Kuivenhoven, J. A. (2000). *Physiol. Genomics* **2,** 67–75.
32. Cvetkovic, B., Yang, B., Williamson, R. A., and Sigmund, C. D. (2000). *J. Biol. Chem.* **275,** 1073–1078.
33. Guillot, P. V., Liu, L., Kuivenhoven, J. A., Guan, J., Rosenberg, R. D., and Aird, W. C. (2000). *Physiol. Genomics* **2,** 77–83.
34. Sasaki, H. and Hogan, B. L. (1994). *Cell* **76,** 103–115.
35. Nagy, A., Gocza, E., Diaz, E. M., et al. (1990). *Development* **110,** 815–821.
36. Nagy, A. and Rossant, J. (1993). In: *Gene targeting: a practical approach.* Joyner, A. (ed.). Oxford University Press, Oxford, pp. 147–149.
37. Nagy, A., Rossant, J., Nagy, R., Abramow-Newerly, W., and Roder, J. C. (1993). *Proc. Natl. Acad. Sci. USA* **90,** 8424–8428.
38. Misra, R. P., Bronson, S. K., Xiao, Q., et al. (2001). *BMC Biotechnol.* **1,** 12.
39. Copeland, N. G., Jenkins, N. A., and Court, D. L. (2001). *Nat. Rev. Genet.* **2,** 769–779.
40. Zhang, Y., Muyrers, J. P., Testa, G., and Stewart, A. F. (2000). *Nat. Biotechnol.* **18,** 1314–1317.
41. Zhang, Y., Buchholz, F., Muyrers, J. P., and Stewart, A. F. (1998). *Nat. Genet.* **20,** 123–128.