Transgenic Mice: An Irreplaceable Tool for the Study of Mammalian Development and Biology

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Abstract. Stable integration into the mouse genome of exogenous genetic information, i.e., the creation of transgenic mice, has become a privileged way of analyzing gene function in normal development and pathology. Both gene addition and gene replacement may be performed. This has allowed, in particular, the creation of mice in which precise mutations are introduced into a given gene. Furthermore, in recent years, strategies that induce the expression of a mutation in a given type of cell and/or at a given time in development have been developed. Thus, the transgenic methodology affords a unique and irreplaceable tool for the study of mammalian development and biology and for the creation of animal models for human genetic diseases.

The ability to introduce new genetic information into the germline of complex organisms, i.e., the creation of transgenic organisms, has completely revolutionized the study of all aspects of their biologic processes. This is particularly true in the case of mice, which represent a favored model for the study of mammalian development and physiologic processes. Here, I briefly review some aspects of the generation and use of transgenic mice, with special emphasis on targeted mutagenesis, which results in the creation of transgenic animals bearing mutations deliberately introduced in a particular gene or genomic region of interest. Many reviews of various aspects of transgenic technology have been published, and only a few of them (among the most recent, whenever possible) are quoted here; readers are referred to the first comprehensive review (1) and to a recent book and references cited therein (2).

Transgenesis by Addition of Cloned DNA Sequences: Pronuclear Microinjection

One route for the creation of transgenic mice, which was designed in the early 1980s, involves direct microinjection of cloned DNA into the pronuclei of fertilized eggs. This procedure produces mice in which the transgene is stably integrated into the mouse genome (generally as tandem head-to-tail arrays of variable length). This situation allows monitoring of transgene expression in all cell types and at any desired time in the developing embryo and postnatal animal. Indeed, it was soon demonstrated that a given transgene may be expressed specifically in time and space. However, a disadvantage is that transgene integration is apparently random; therefore, neighboring sequences might interfere, both qualitatively and quantitatively, with proper regulation of the transgene under study. Such an effect is referred to as a “position effect” (3) and might obscure analyses of transgene regulation. Interestingly, specific DNA sequences, termed locus control regions, have been discovered in the vicinity of some genes that render transgene expression copy number dependent and position independent (4). Furthermore, particular sequences in the locus control regions were recently demonstrated to act as boundaries between active and inactive chromatin (5). Despite the position effect, it has become possible, by creating sufficient numbers of transgenic mice carrying different regions of a gene, to delineate the cis-acting regulatory sequences implicated in correct temporal and spatial gene expression.

Fusion Genes and Targeting of Gene Expression

The availability of characterized regulatory sequences responsible for tissue-specific expression allowed the creation of transgenic mice in which the expression of a given gene is deregulated. This is achieved by the use of fusion genes as transgenes. Depending on the promoter/regulatory elements used and the coding sequences fused to them, enhanced or ectopic expression of either the normal gene product or a modified gene product may be obtained. Therefore, the transgenic approach permitted gain-of-function genetic modifications, the effect of which could yield insights into the biologic and/or pathophysiologic role of the gene product under study. Other uses of fusion transgenes include the use of a reporter gene such as Escherichia coli β-galactosidase to define expression patterns (6) and the use of a toxic agent to ablate a particular population of cells, to obtain insights into their biologic function (7).

Transgenes as Insertional Mutagens

When any exogenous DNA is introduced into the mouse germline, it may be integrated into the genome in such a way that it perturbs the expression of an endogenous gene, causing a mutation, which may result in a phenotype when transgene insertion is rendered homozygous. Systematic intercrossing between transgenic mice thus becomes a way of screening for
insertional mutations. The main advantage of this approach is that the transgene can serve as a molecular tag to recover the affected gene (for review, see reference (8)). Indeed, despite the fact that transgene insertion frequently entails genomic rearrangements at the site of insertion, several studies have illustrated the attractiveness of this approach, and several new genes of interest could be isolated in this way (see, for example, references (9 and 10) and references cited therein).

Embryonic Stem Cells and Generation of Programmed Modifications of the Mouse Genome

Knockout

Despite their widespread use in genetic studies of mouse biologic processes, gene-addition transgenic mice have important limitations; neither the copy number nor the site of integration is controlled, resulting in difficulty obtaining reliable specific expression of the transgene. Furthermore, with the exception of rare mutations obtained via serendipitous integration of a transgene into an endogenous gene, only gain-of-function mutations can be obtained in these transgenic mice. These limitations were overcome in the late 1980s with the combination of two methodologies, one that resulted in the generation of embryonic stem (ES) cells and the other that allowed investigators to perform and identify homologous recombination between incoming DNA and the homologous endogenous genomic sequence. ES cells were derived from preimplantation embryos and were shown to retain, in culture, the remarkable ability to colonize a host embryo, including its germline (11,12). The availability of ES cells, which can be cultured in large numbers, yields the possibility of selecting various genetic modifications in culture and obtaining corresponding mice via the generation of germline chimeras. In particular, methods that permit the replacement of an endogenous allele by a disrupted allele via homologous recombination in ES cells have been developed (13,14). This is generally achieved by the use of targeting vectors containing sequences of the gene of interest and a selection cassette inserted in an exon, interrupting the unit of transcription (Figure 1A). After introduction of the vector into ES cells, selection is performed and the resistant clones are screened for the presence of homologous recombinants, which contain the disrupted allele of the gene under study. Mice carrying the disrupted allele are subsequently obtained via the generation of germline chimeras. This approach has yielded enormous possibilities for the study of gene function. Indeed, >1000 genes (see the Transgenic/Targeted Mutation Database; http://tbase.jax.org/) have been targeted in this way, and the study of the phenotypes of the mutant embryos or mice thus created has clarified the function of genes in the development and physiologic processes of mice.

Knockin

An interesting extension of the knockout approach is the so-called knockin approach, in which the targeting vector contains, in addition to the selection cassette, a cDNA of interest inserted in-frame in an exon of the gene under study (Figure 1B). The cDNA is thus expressed in place of the endogenous gene product. The main advantage of this approach is that it allows tight control of the expression of the cDNA of interest, because the cDNA is placed in the context of the complete set of cis-acting regulatory elements that normally control the expression of the endogenous genes; furthermore, this approach avoids the position effect encountered in gene-addition transgenesis (see above). A common use of this approach is the targeting of the LacZ reporter gene, which

![Figure 1](https://example.com/figure1.png)

**Figure 1.** Knockout and knockin strategies. (A) Knockout. The targeting vector contains a selection cassette inserted into an exon and surrounded by sequences homologous to the endogenous allele. After homologous recombination between the targeting vector and the endogenous homologous sequences, a null allele is created. (B) Knockin. The method is as depicted in A, except that a cDNA of interest is inserted in-frame in an exon of the gene to be targeted. In the mutated allele, the gene of interest is expressed in place of the endogenous gene product.
permits accurate definition of the expression pattern of endogenous genes (see, for example, references (15 and 16). This approach has also proven to be a powerful tool for studying the functional relationships among members of a gene family and their potential abilities to functionally compensate for each other (see, for example, reference (17).

Other Types of Mutations

In the strategies described above, the modification of the target locus governed by the design of targeting vectors is mediated by the endogenous recombination machinery of the ES cells. In recent years, an important breakthrough has been made by taking advantage of site-specific recombinases, which were discovered in lower organisms but were demonstrated to work efficiently in mammalian cells (for review, see reference (18). Because of space limitations, I focus only on the most widely used, i.e., the Cre recombinase of P1 bacteriophage. This enzyme promotes recombination via recognition of a 34-bp, asymmetric, nucleotide sequence (termed \( \text{loxP} \)) (Figure 2A). Depending on the relative orientation of the \( \text{loxP} \) sites, Cre may catalyze excision (same orientation) or inversion (opposite orientation) of the DNA segments lying between these sites (Figure 2, B and C).

The use of the Cre/\( \text{loxP} \) system has considerably widened the scope of targeted mutagenesis in ES cells. First, it has been instrumental in the creation of new types of mutations, in addition to null mutations. “Clean” (i.e., devoid of a selection cassette that might interfere with gene regulation) and subtle (e.g., point mutations) mutations can be created. To this end, a targeting vector that bears the desired mutation in the gene of interest and the selectable marker in an intron, flanked by two \( \text{loxP} \) sites in the same orientation (“floxed” sequence), is constructed. ES cells targeted in the endogenous gene are then identified and transfected with a Cre-expressing vector, which promotes the excision of the selectable marker; this results in ES cells bearing the desired mutation in the gene of interest, with the presence of only one \( \text{loxP} \) site as a foreign sequence (Figure 3, C and D) (19) (for review, see references 20 and 21). Alternatively, the floxed selection cassette may be excised \textit{in vivo} by crossing male chimeras bearing the targeted allele with mice expressing Cre during early development (Figure 4, lower) (22,23); it should be noted that there exists an alternative method to create clean mutations by homologous recombination in ES cells, which relies on a hypoxanthine phosphoribosyl transferase-based selection system (Figure 3, A and B) (24) (for review, see references 20 and 21). The ability to create subtle mutations allows refinement of the analysis of gene function; furthermore, it allows the creation of murine

![Figure 2. Cre/\( \text{loxP} \) system of recombination. (A) The \( \text{loxP} \) sequences recognized by Cre are shown. The small arrows indicate the site of cleavage by Cre in the \( \text{loxP} \) sequence, which initiates the process of recombination. (B and C) The DNA segment is either deleted (B, same orientation of the \( \text{loxP} \) sites) or inverted (C, opposite orientation of the \( \text{loxP} \) sites).](image-url)
models of human genetic diseases, which are frequently attributable to subtle genetic modifications (e.g., point mutations).

Another important use of the Cre/loxP system is the creation of deletions, which are valuable tools for functional analysis of the mammalian genome (for review, see reference (25). First, two loxP sites are introduced in the chosen loci, in the suitable orientation, via two steps of homologous recombination; subsequent excision of the chromosomal DNA between the two loxP sites is induced by transfection of a Cre-expressing construct in ES cells. An elegant in vivo extension of this approach was recently described. Transgenic mice carrying an Sycp-1 (encoding synaptonemal complex protein-1)/Cre fusion gene were shown to express Cre specifically during the prophase of the male first meiotic division (26); crossing such transgenic mice with mice carrying loxP sites on homologous chromosomes yielded double-transgenic male animals, which produced sperm with deletions or duplications of the genomic region between the two loxP sites. Progeny of these male animals were demonstrated to carry these chromosomal rearrangements. This approach should provide a powerful means for functional analysis of complex genomic regions (27).

Cre/loxP System and Conditional Mutagenesis

Finally, and most importantly, the Cre/loxP system has provided the possibility of conditional mutagenesis, i.e., the ability to induce disruption (or expression) of any gene in any cell type and/or at any time of development (for review, see references (20 and 21). This is important for at least two reasons. First, when a mutation entails embryonic lethality, the study of gene function later in development is precluded. Second, when a gene exhibits a complex pattern of expression, the study of its function may be obscured when all cells of an individual express the mutation, as is the case in knockout mice; therefore, it may be valuable to induce the mutation in one cell type or tissue at a time. The approach to achieve inducible gene targeting is basically simple and involves two types of transgenic mouse lines; the first bears the target gene (or gene segment) flanked by two loxP sites in the same orientation and positioned in such a way that it does not prevent normal gene activity (floxed gene); the second line contains a fusion transgene expressing Cre recombinase. When these two mouse lines are crossed, depending on the promoter/regulatory sequences present in the fusion transgene, the floxed gene is deleted and a null mutation is created in particular cells.

Figure 3. Generation of clean mutations. (A and B) Double replacement. hpri− embryonic stem cells are used. In the first step (A), a targeting vector, containing an hpri expression cassette, is used. hpri− clones [hypoxanthine/aminopterin/thymidine (HAT)-resistant] are selected and screened for the presence of homologous recombinants. In the second step (B), the targeting vector is composed of sequences homologous to the endogenous gene of interest with a point mutation (*). After homologous recombination, the hpri selection cassette is removed, and the corresponding hpri− clones (with the mutation) can thus be selected using 6-thioguanine. Note that the second targeting event may be repeated with different types of mutations. (C and D) Use of the Cre/LoxP system. The targeting vector contains a floxed selection cassette and a point mutation (*). The ES clones containing the corresponding modified allele are then transfected with a Cre expressing vector that permits the excision of the selection cassette.
Figure 4. Use of the Cre/loxP system in vivo. (Upper) Conditional knockout. In the A × B mice, Cre is specifically expressed in the central nervous system (NCS) (using a Cre fusion gene with the appropriate regulatory sequences); consequently, the floxed allele is deleted in the brain but not in the other tissues of the animal. (Lower) Deletion of a floxed sequence in all cells of the A × C mice. This approach is used, for example, to remove a selection cassette (Figure 3) and entails the expression of Cre in female germline fusion genes, e.g., phosphoglycerate kinase/Cre or Zona Pellucida protein 3/Cre.

Figure 5. Use of a Cre fusion protein (Cre-ind) to control the time of induced gene targeting. (A) The agonist acts on the inactive ligand-binding domain/Cre fusion protein and activates it. (B) In A × B double-transgenic mice (containing the Cre/ind fusion gene and the floxed allele), the floxed allele is deleted only after injection of the agonist.
or tissues (Figure 4, upper). It is important to note that two requirements must be fulfilled for conditional gene targeting with the Cre/loxP system, i.e., (1) the floxed allele must be created in such a way that it is still functional and (2) targeting of Cre expression must be tightly controlled. One valuable way to address the latter issue could be to knockin the Cre coding sequences in genes whose specificity of expression is well known and appropriate for the particular question being addressed, as illustrated in a recent study (28). Conditional mutagenesis can be refined to induce a mutation not only in a given type of cell but also at a given time in development. To that end, two approaches are being developed. The first relies on the production of fusion proteins containing Cre and the ligand-binding domain of a steroid receptor; in such chimeric proteins, the activity of Cre becomes hormone dependent (Figure 5). Therefore, recombination between loxP sites could be controlled in cells carrying the chimeric Cre by the addition of ligand. Such an approach was demonstrated to be operational controlled in cells carrying the chimeric Cre by the addition of DNA sequences, thus promoting the activity of Cre in the presence of tetracycline (tetracycline-controlled transactivator) could bind only to the tetracycline repressor sequences linked to the herpes VP16 activating protein composed of a mutated version of the tetracycline receptor sequences in transgenic mice (36). The second system was devised by Gosden et al. (33). Those authors demonstrated that a fusion protein composed of a mutated version of the tetracycline repressor linked to the herpes VP16 activating sequences (reverse tetracycline-controlled transactivator) could bind only in the presence of tetracycline to the tetracycline operator (tetO) DNA sequences, thus promoting the activity of a LacZ or luciferase reporter gene. This result, which was originally observed in cultured cells (33), was extended to transgenic mice (34,35). It should be possible to adapt this strategy to the control of Cre expression. Therefore, transgenic mice that contain two transgenes, one expressing the reverse tetracycline-controlled transactivator and the other consisting of tetO sequences and a minimal promoter linked to the Cre gene, could be generated. Depending on the specificity of the promoter used to express the fusion protein, such transgenic mice should express Cre, after tetracycline treatment, in a given cell type at the time of treatment. Indeed, the feasibility of such a strategy was elegantly demonstrated in a recent study (36).

Concluding Remarks

Almost 20 years ago, the first transgenic mice were born from zygotes microinjected with cloned DNA. Many studies have since addressed almost all aspects of mouse biologic features using this transgenic approach, thus illustrating its unique versatility. However, the development of homologous recombination in ES cells has considerably widened the scope of transgenic technology. Indeed, the repertoire of genetic modifications that can be introduced via ES cells in the germ-line of mice is almost unlimited. Not only null mutations but also discrete and clean mutations (such as point mutations or micro-deletions or -insertions) as well as chromosomal rearrangements, including large deletions, inversions, or translocations, may be created. Lastly, strategies have been developed that allow the induction of mutations restricted to a given cell type or tissue, permitting conditional mutagenesis. Taken together, the variety of tools designed to modify the mouse genome have provided unprecedented opportunities for the study of gene function in the development and physiologic and pathophysiologic processes of this complex organism. There is little doubt that these tools will continue to be refined and will thus increasingly contribute to our knowledge of mammalian biologic processes.

Acknowledgments

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References

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