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Cell-type and stage-specific gene knockouts in mice: An achievement of a long-sought goal

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The importance of gene action in regulating the biology of higher eukaryotes is hardly a matter of dispute. But until recently we have been unable to say whether a given gene was essential for the functioning of a particular developmental pathway or not. The reason was that there was no way to ensure that a gene was inactivated in just one cell type, and not in others. This situation has now changed.

Technical advances in handling and manipulating mouse embryos have made a great difference to the study of mammalian biology. It was the ability to create uniparental embryos by nuclear transplantation that pinpointed the essential features behind the phenomenon of genomic imprinting[1,2]. Studies with transgenic mice led to an improved understanding of the various aspects of tissue-specific gene expression and gene imprinting. Adding yet another dimension to the problem of tissue-specific gene expression is the control of developmental time sequences in higher eukaryotes. Developmental biologists long cherished the hope that they would one day be able to 'knock out' endogenous genes at will and so learn more about *when* a gene is essential (in addition to where it is essential).

That hope was realized by two groups, those of Capecchi in USA and Gruss in Germany, when they succeeded in using homologous recombination to create knockouts[3,4]. Capecchi and co-workers used embryonic stem cells (ES cells) and a phenotypic selection method to screen for homologous recombination involving the gene of interest, whereas Zimmer and Gruss designed a PCR approach to fish out ES cells that had undergone homologous recombination (Figure 1). In both these approaches the selected ES cells were used to create chimaeric mice. Subsequently, a transgenic line was established by selection of second-generation mice that carried the transgene. The next challenge was of achieving selected gene knockouts in a specific tissue type at a specific stage in development.

What looked improbable until the other day has now been achieved by Gu *et al.*[5]. A bacteriophage system provided the key. Phage P_1, a bacteriophage of *E. coli*, has a recombinase of the integrase family which helps in circularizing phage DNA during both the lytic and the lysogenic cycles. The system, called Cre-lox, comprises a 34 kDa protein (Cre, Cyclization

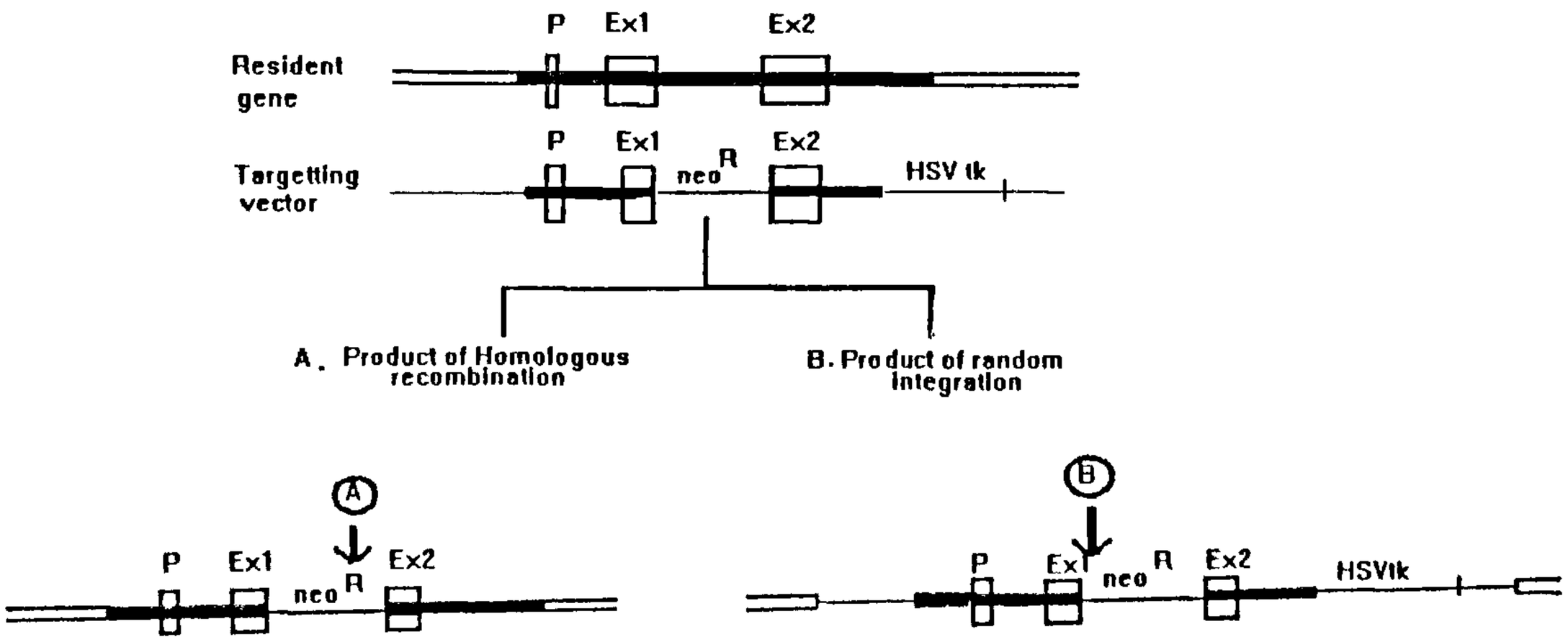

Figure 1. Strategy for creating knockout mice by homologous recombination. P is a promoter sequence, Ex1 and Ex2 are two exons of the gene to be disrupted, neo^R is the bacterial neomycin gene. HSVtk is the thymidine kinase gene from herpes simplex virus. After transformation, embryonic stem cells are selected for homologous recombinants on a medium containing G418 and gancyclovir; only type-A transformants survive. Selection can also be carried out by PCR with appropriate oligonucleotides.

*re*combination protein) that is necessary and sufficient to bring about recombination in regions flanked by a 34-base-pair sequence lox P1 (*lo*cus of crossing-over (*X*) in P1) that contains a 13-base-pair inverted repeat sandwiching an 8-base-pair sequence (Figure 2). When the lox P1 sequences are in the same orientation, Cre recombinase causes the excision of the DNA present between them. It was previously shown that the Cre-lox system can work in mammalian cells in culture and also in transgenic animals[6–8]. Orban *et al.*[8] could demonstrate tissue-specific deletion of a stop sequence from the bacterial β-galactosidase gene in T-cells. This led to the expression of β-galactosidase in transgenic mice that made Cre recombinase under the control of the lck promoter (a promoter specifically expressed in T-lymphocytes). They could show that the excision of the stop sequence within the β-galactosidase gene, and therefore the expression of β-galactosidase, occurred only in T-cells and occurred at a stage coinciding with T-cell emigration to peripheral compartments Thus they had achieved both tissue-specific as well as stage-specific excision.

Marth and Orban, together with Gu, Mossmann and Rajewsky[5] have combined the strategy for tissue-specific and developmental-stage-specific excision with the knockout scheme of Mansour *et al.*[3] The question that Gu *et al.*[5] have tried to address concerns the possible role of DNA polymerase β (pol β) in T-cell development. Polymerase β is known to be involved in the DNA repair needed for somatic gene rearrangements and hypermutation at antigen receptor gene loci. The strategy they used is shown in Figure 3. It involves three steps. The first was to create a transgenic line expressing Cre recombinase under the lck promoter. A second transgenic line contained a pol β promoter and at least one exon flanked by lox P1 sequences (a 'floxed' pol β gene). A third transgenic mouse had the DNA polymerase β gene knocked out. All the transgenic mice were created by making use of ES cells that were appropriately transformed. The transgenic mice with a floxed pol β gene contained one wild-type pol β gene. Even otherwise, the floxed DNA polymerase β could express itself in spite of having lox P1 sequences flanking the first exon. In order to create a homozygous tissue-specific knockout for the gene, a double transgenic containing two floxed pol β genes (homozygous pol β floxed) and Cre recombinase under an lck promoter could have been used. Instead, the authors obtained a transgenic mouse which contained Cre recombinase cloned under lck promoter, one homologue of pol β gene partially deleted and the other pol β homologue floxed. (Cre^+ pol β^Δ/pol β floxed). In this mouse, after the expression of Cre recombinase, only the T-cells are homozygous for a pol β deletion. During embryogenesis and in the adult, all tissues except the T-lymphocytes will have one functional copy of DNA polymerase β (that copy is the floxed pol β).

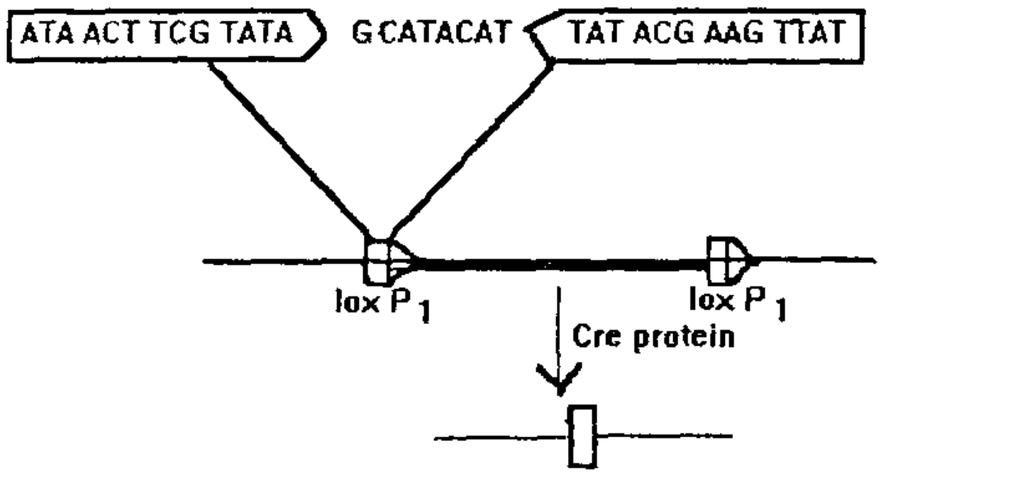

Figure 2. Cre-lox system of phage P1

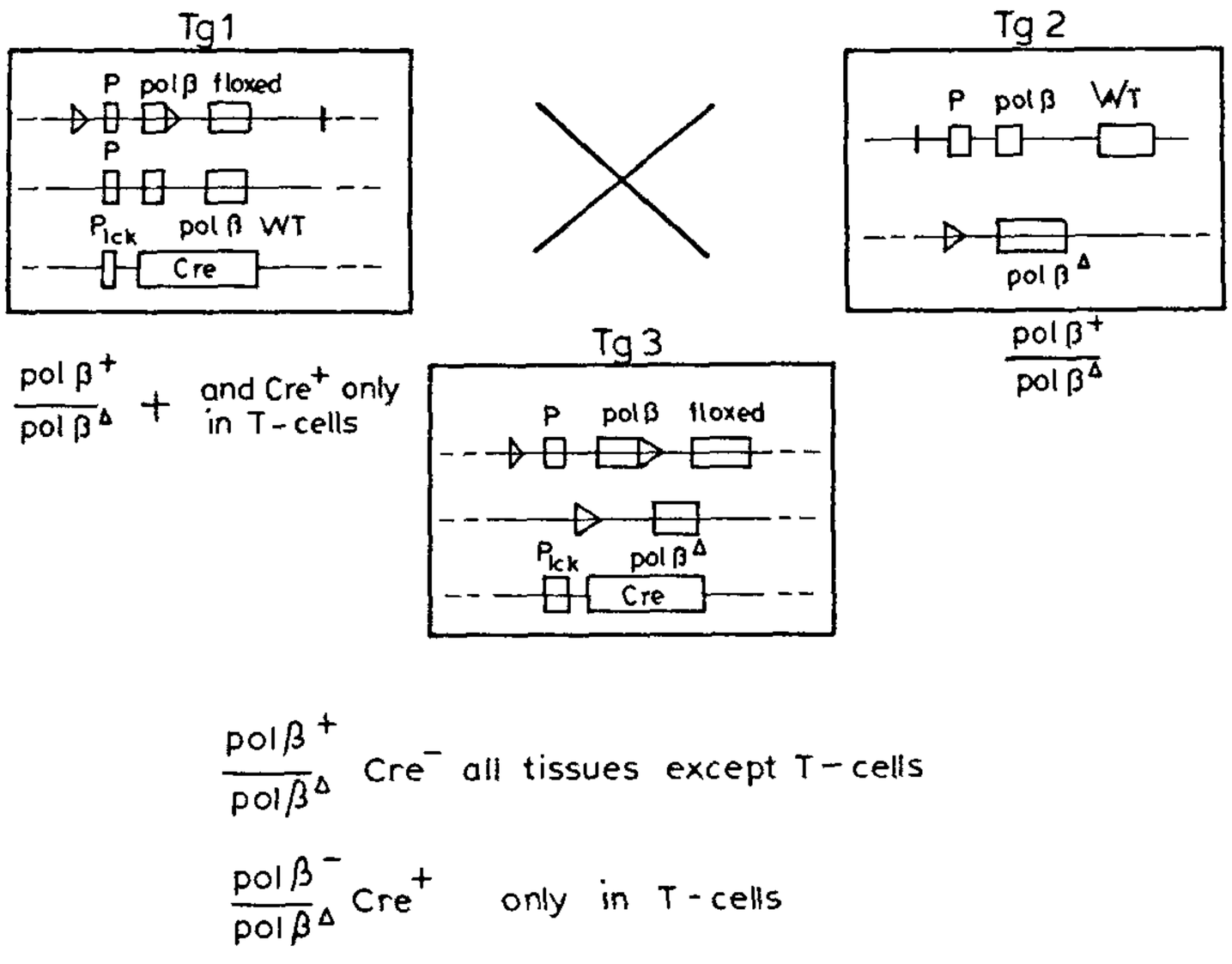

Figure 3. Outline of the strategy used to create conditional knockout. Tg is transgenic mouse, WT is wild type. Floxed means flanked by lox P1 sequences, pol β^Δ is partial deletion of the pol β gene, P is a promoter sequence. P_{lck} is the T-cell-specific lck promoter.

The targeting construct used to bring about homologous recombination in ES cells has the HSVtk gene within the region of homology unlike the construct used by Mansour *et al.*[3] The targeting constructs made by Capecchi and coworkers had a neomycin-resistant (neo^R) gene within the regions of homology of the endogenous gene that is targeted for knockout and the HSVtk gene was distal to this region. Thus, the HSVtk gene is eliminated whenever a homologous recombination occurs, whereas it is retained if there is a random integrand

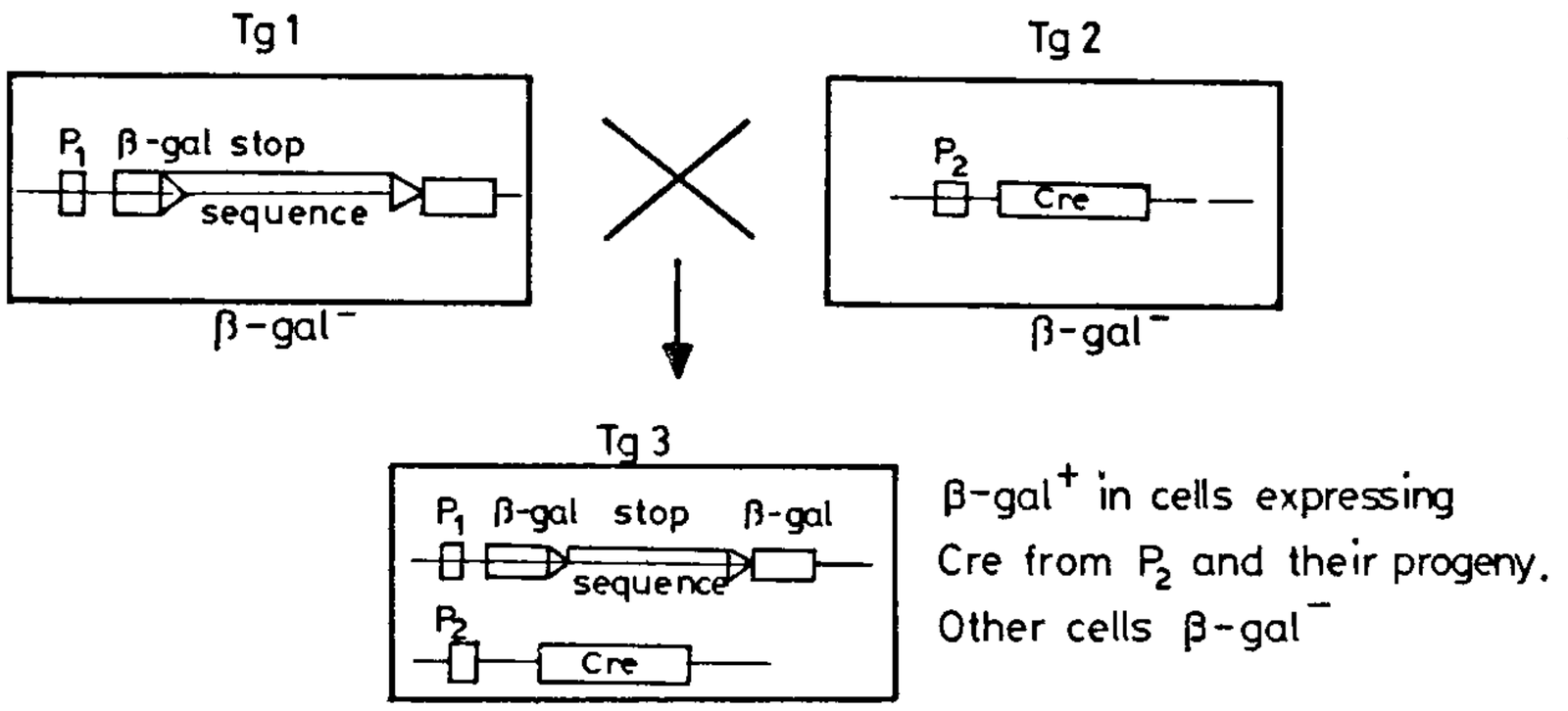

Figure 4. Strategy to bring about conditional activation Tg is transgenic mouse, P_1 and P_2 are promoter sequences.

(Figure 1). Random integration can be selected against by growing transformants in the presence of gancyclovir, a drug which eliminates cells expressing the HSVtk gene. A phenotypic selection for homologous recombinants among the ES cells is not possible in the experiments of Gu *et al.*[5] because the HSVtk gene is within the region of homology.

In this strategy the selection for various transformants used for creating chimaeras is based on Southern analysis, meaning that it is rather labour-intensive. Future approaches will probably simplify the selection procedures. It appears possible to retain the positive–negative selection for homologous recombination based on selection for G418 resistance (neo^R gene) and sensitivity to gancyclovir by cloning the HSVtk gene distal to the region of homology.

The effect of a conditional knockout of DNA polymerase β in T-lymphocytes was assessed by various criteria. The mice appeared normal in development and were fertile. Analysis of T-lineage cells in thymus in terms of total number and the expression of surface markers like CD4 and CD8 indicated a similar pattern in mutants and the wild-type mice. The data do not provide any evidence of impairment of T-lymphocyte differentiation due to the knockout of DNA polymerase β.

What are the likely offshoots of this? The technique of creating tissue-specific and developmental-stage-specific knockouts, or of conditional gene inactivation, is a powerful tool that can be exploited for different ends. While conditional inactivation has been demonstrated in this model system, conditional activation can also be contemplated (Figure 4). There are several other questions that developmental biologists will want to address with the new approach. One has to do with fate-mapping or tracing cell lineages. A strategy for tracing cell lineages would be to have a transgenic mouse having a reporter gene (e.g., for β-galactosidase) under a strong promoter and containing a stop sequence flanked by lox P1 sequences. By expressing Cre recombinase from a tissue-specific promoter active at the desired stage in development, one can have a situation wherein β-galactosidase is silent in all the tissues except those where the Cre recombinase is expressed and the stop sequence from the β-galactosidase gene is excised. Once this event happens in a progenitor cell, all its progeny cells will also express β-galactosidase. How does this approach compare with that of expressing a reporter gene under a tissue-specific promoter and tracing the expression of the reporter gene? Conditional activation approach requires that the fidelity of expression of tissue-specific promoter be maintained only in the progenitor cells for expressing Cre recombinase. The latter route would need tissue-specific expression patterns to be maintained in every progenitor as well as in progeny cell.

Conditional inactivation offers a clear advantage if the aim is to create point mutations at desired sites[9]. This could be accomplished by introducing a fragment of the gene of interest, containing a point mutation and a floxed neomycin-resistant gene (neo^R). After selection of transformants for neo^R, the neomycin gene can be excised by expressing Cre recombinase transiently. Further steps can be carried out to create homozygous transgenic mice. In such situations, selection will have to distinguish between the transgene and the endogenous gene at the level of a single-nucleotide difference.

Conditional inactivation is particularly attractive for creating models for genetic disorders. One can design transgenic mice such that gene(s) implicated in a specific disorder is(are) knocked out specifically in a tissue which is known, or suspected, to be affected by the disorder. For instance, one could knock out the protein kinase gene implicated in myotonic dystrophy in neuromotor tissues. In dominant disorders like Huntington's chorea, an effect ought to be seen even in the heterozygous state. To sum up, a significant amount of information is awaited over the next few years from studies using conditional inactivation or activation strategies in the mouse.

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