JCI The Journal of Clinical Investigation

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J Clin Invest. 1996;98(3):600-603. https://doi.org/10.1172/JCI118828.

Perspective

Perspectives Series: Molecular Medicine in Genetically Engineered Animals

Conditional Gene Targeting

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Gene targeting: The classical approach

Central to an understanding of the in vivo function of genes is their analysis by mutation, that is, inactivation or modification of a gene by mutation and the study of the consequences of the mutation in the mutant organism. In mammals, before gene targeting, this approach was limited to the rare spontaneous mutations reflected in obvious phenotypes, as in the case of inheritable diseases in the human. The targeted mutagenesis of the mouse germline was thus a fundamental breakthrough in this area of research. In its original form, gene targeting involves the inactivation of a given gene in the genome of embryonic stem (ES) cells by homologous recombination (1–3). ES cells derive from an early stage of mouse development and have retained their totipotency, thus they can participate in the generation of cell lineages of the mouse (including germ cells) if transferred into an early mouse embryo. Transfer of mutant ES cells into mouse embryos thus allows the transmission of the mutation in question into the mouse germline.

In the classical experiment of Thomas and Capecchi (3) gene inactivation was achieved by replacing a predetermined gene segment with a mutant version of this segment, through homologous recombination. Since the latter is infrequent in mammalian cells, the isolation of the mutant ES cells requires stringent selection. This was achieved by placing a selectable gene (in that case the neomycin resistance gene) into the targeted locus in a manner that allows its expression (and hence cellular selection) while inactivating the target gene.

Refining classical gene targeting by the Cre-loxP recombination system

A limitation of classical gene targeting comes from the presence of a selection marker gene in the targeted locus. Since this gene must be active in order to allow ES cell selection, it must always be considered that through its expression it might affect the mutant phenotype in an unpredictable way. A par-

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Received for publication 19 June 1996.

1. *Abbreviation used in this paper:* ES, embryonic stem.

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J. Clin. Invest.

0021-9738/96/08/0600/04 $2.00
Volume 98, Number 3, August 1996, 600–603

ticularly serious problem arises if gene targeting is used for the modification or elimination of regulatory DNA sequences, as the selectable gene brings in its own promoter and enhancer. For this reason, attempts have been made to eliminate the selection marker genes from the targeted locus (4–7).

We have used the bacteriophage-derived Cre-loxP recombination system (8, 9) for this purpose which had been shown to perform well in mouse cells in vitro (10) and in vivo (11, 12) and which appeared to offer a broad range of applications in gene targeting. The Cre enzyme, a member of a large family of recombinases (13), recognizes a sequence motif of 34 bp, called loxP (9). If a DNA segment is flanked by two loxP sites in the same orientation, Cre excises that segment from the DNA, leaving a single loxP site behind. (A piece of DNA flanked by loxP sites in opposite orientations will be inverted by the enzyme, a reaction that waits to be exploited in the gene targeting field [14, 15].) Considering this site as innocent, "clean" deletions can thus be produced. If in a gene targeting experiment the selection marker gene on the targeting vector (subsequently inserted into the target locus by homologous recombination) is flanked by loxP sites, it can later be removed from the ES cell genome via transient transfection of a Cre-expression vector into these cells. By appropriately positioning the two loxP sites, one can use this system as a general system of mutagenesis, producing deletions (16–18), gene replacements, insertions (often called "knock-ins") (19, 20), point mutations, (21) etc. However, we shall focus here on yet another and perhaps the most important aspect of Cre-loxP–mediated targeted mutagenesis, namely conditional (rather than general) gene targeting.

Conditional gene targeting

Why do we want to achieve conditional, i.e., cell-type–specific and/or inducible, gene targeting beyond targeted mutagenesis of the mouse germline? There are several reasons. First, germline mutations may be lethal, in which case there is no mouse to study gene function. Second, genes may exert their function at several stages of ontogeny and in different cell types. In the latter case, complex phenotypes may result, where it is difficult to distinguish cell-autonomous from more complex lesions. In the former case, classical gene targeting will allow one to identify the initial stage at which the target gene plays a critical role, but not necessarily later stages. Finally, in medical research, conditional gene targeting could allow one to generate models of somatically acquired genetic diseases (such as most forms of cancer) rather than of inherited ones.

The strategy of conditional targeting of endogenous genes that we have developed (16) consists of flanking a target gene or gene segment with loxP sites in ES cells by classical gene targeting and deleting the selection marker gene by transient transfection with a Cre-encoding plasmid. This protocol yields ES cell mutants in which the gene segment of interest is either flanked by loxP sites or deleted. Either mutation can be transmitted into the germline. In the former case, the mutant mice carry a functional, but loxP-flanked gene in their genome (Fig. 1 *A*). In the latter the gene is deleted in all cells of the body,

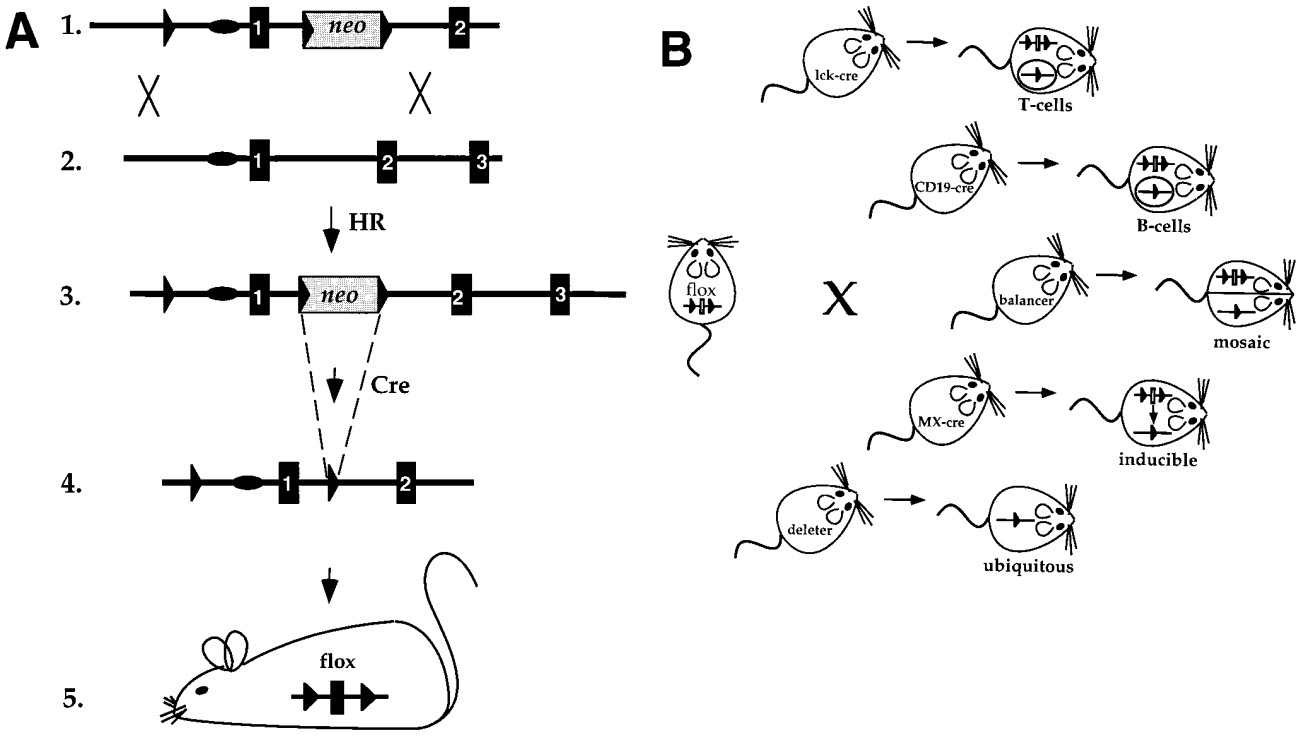

Figure 1. (*A*) Generation of mice with loxP-flanked target genes (flox). (*1*) Gene targeting vector containing three loxP sites (*filled triangles*), two of them flanking the Neomycin resistance (neo) gene. The HSV-tk gene, often used for negative selection, is not shown. (*2*) Target gene in the genome of ES cells (exons drawn as filled boxes). (*3*) Genomic locus modified by homologous recombination (*HR*) between vector and target gene. (*4*) Deletion of the loxP-flanked neo gene by transient Cre expression in ES cells. Two loxP sites remain in the target gene. (*5*) Generation of a loxP-containing mouse line from modified ES cells. (*B*) Utilization of Cre transgenic mice for conditional gene targeting. The mouse strain harboring two loxP sites in the target gene (see *A*) can be crossed to various strains expressing Cre recombinase. The target gene becomes inactivated only in cells expressing Cre but remains active in all other cells of the body. The Cre-expressing strains are described in the text.

generating the situation of a classical knockout experiment, except that no selection marker gene remains in the mutant locus.

Conditional targeting of loxP-flanked genes or gene segments can be achieved by crossing into the mutant animal a Cre-transgene from which Cre recombinase is expressed in a cell-type–specific or inducible manner (Fig. 1 *B*). The initial experiment, done in collaboration with J. Marth's group, established that in principle, cell-type–specific gene targeting can be successfully performed in this manner (lck-cre; 16). We subsequently generated a Cre transgene under the control of an interferon α/β (IFNα/β) inducible promoter and showed that in this system 100% deletion of a loxP-flanked gene segment could be achieved in the liver upon injection of IFNα/β and up to 98% in lymphocytes, whereas lower degrees of deletion were seen in other tissues (MX-cre; 22). Thus, both cell-type–specific and inducible gene targeting are feasible using the Cre-loxP recombination system, the efficiency in the system can reach 100% (as also recently shown by J. Marth and colleagues for T cell–specific gene targeting [23]) and Cre recombinase can do its job in resting cells such as the liver. Note also that conditional as well as classical gene targeting can be used not only for gene inactivation, but also, by appropriately constructing targeting vectors, for the introduction of subtle mutations (21) and also the activation of a gene (see reference 12).

The extent to which conditional gene targeting can be applied in biological and medical research depends at this point, at a technical level, on the availability of Cre-transgenic strains in which Cre is expressed at sufficiently high levels, but under strict cell-type–specific and/or inducible control. Indeed, if Cre expression could be induced specifically in any given cell type, that would for many purposes represent an ideal system of conditional gene targeting. While this goal has not yet been reached, fusion proteins in which Cre is fused to the ligand binding domain of a steroid receptor and which can be activated inside the cell by a steroid antagonist may allow for a successful approach to this problem (24–26). Alternatively, one may consider using systems of inducible, cell-type–specific transcriptional control like that developed by Bujard and colleagues, with tetracycline as the inducer (27, 28).

At present, a limited number of Cre transgenic animals is available which allows gene targeting in T cells (23), B cells (CD19-cre; 29 and R. Rickert, J. Roes, and K. Rajewsky, unpublished data), and, inducible by IFNα/β, in a variety of tissues but predominantly liver and lymphocytes (22). Many other such strains are presently being generated worldwide, so that one can expect that a large set of Cre transgenics will soon be at the disposition of the scientific community, making conditional gene targeting a routine approach in the analysis of mammalian gene function in vivo.

We have recently generated two Cre transgenic mouse lines which behaved unexpectedly, but still turn out to be particularly useful in this context. The first is a strain, designated balancer, which allows partial gene inactivation in essentially all tissues of the body, generating a mosaic situation in which competition between wild-type and mutant cells can be studied in situ. In a situation in which Cre-mediated deletion results in homozygous mutant cells, a simple Southern analysis of the extent of deletion in various tissues of the mosaic mutant animal allows one to pinpoint the sites at which the gene in question plays a functionally critical role (Betz, U.A.K., C. Voßhenrich, K. Rajewsky, and W. Müller, manuscript submitted for publication). The second Cre-transgenic strain, called deleter, mediates deletion of loxP-flanked gene segments in essentially all cells of the body, including germ cells (30). Crossing this strain to a strain in which a target gene is flanked by loxP sites on one or both homologous chromosomes, one obtains ubiquitous deletion which can also be transmitted into the germline, producing a classical knockout, again without a selection marker gene in the mutant locus.

Given these options of targeted mutagenesis through transgenes encoding Cre under various kinds of genetic control, we now mostly perform gene targeting experiments such that only a single mutation of the target gene is transmitted into the germline, namely a loxP-flanked, functional version of the wild-type locus. The resulting animals are healthy and indistinguishable from the wild-type. However, by crossing them to the various Cre-transgenics, any type of conditional mutagenesis as well as a classical (general) knockout can be obtained. Since the production of a strain carrying a loxP-flanked allele represents only little more effort than that of a classical knockout, we recommend that this protocol be generally considered when gene targeting experiments are being planned.

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