Perspectives Series: Molecular Medicine in Genetically Engineered Animals

Recent Advances in Gene Mutagenesis by Site-directed Recombination

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Transgenic experimentation yields insights that could not be perceived otherwise among populations of mammalian organisms (reviewed in references 1 and 2). However, information gained may, on occasion, be somewhat limited should germline gene dysfunction be deleterious in embryonic development, thereby precluding analyses in various somatic compartments of the adult organism. The production of chimeric mice bearing genetic mutations specifically in various somatic cells allows the study of gene function in physiologic contexts that would otherwise be unavailable or lethal. One elegant cell type-restricted chimeric approach has been the use of the RAG 2-null embryos as recipients in gene-targeted embryonic stem (ES)[1] cell–derived lymphoid development (3). While a lack of cell or tissue development may result from ES cell clonal variation, use of multiple ES clones and complementation by gene transfer allow for controlled studies. Some considerations remain the inability to direct genetic variation to multiple, experimentally defined cell lineages and the potentially unique nature of each chimeric mouse generated.

Since inception, gene transfer experimentation has been generally limited to irreversible modifications of chromosomal DNA. Isolation of cells having undergone site-specific exchange or deletion of DNA sequence has only recently been developed using novel screening strategies for often low frequency events. To develop methods allowing high frequency site-specific chromosomal DNA modification, several laboratories have been investigating the activities of DNA recombinase enzymes to provide for additional experimental control over gene transfer and resulting mutagenesis among cell populations. Among the integrase family of recombinases, one of current relevance is termed Cre and exists as a 30-kD enzyme encoded within the genome of bacteriophage P1 (4). Recombination by Cre excises DNA residing between direct repeats of a 34-bp DNA substrate termed *loxP*. Cre recombination does not require ATP or topoisomerase activity (5) and has been found to function in heterologous eukaryotic genomes, includ-

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1. *Abbreviations used in this paper:* CMV, cytomegalovirus; ES, embryonic stem; tet, tetracycline.

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ing mammalian cell lines (6, 7). Another member of the integrase family is termed Flp and is encoded within the 2 μM circle of yeast (8). Flp recombinase acts identically to Cre and recognizes a similar DNA sequence substrate termed frt. Both use a transient high energy covalent peptide-DNA intermediate in DNA strand cleavage and ligation reactions. Flp has also been found to function in the genomes of *Drosophila melanogaster* and some mammalian cell lines after gene transfer (9, 10), although for reasons thus far uncertain, Cre recombinase appears to function at significantly higher efficacy in mammalian cells.

Transgenic studies of Cre recombinase activity previously reported that Cre could act in an efficient, heritable, tissue-, and site-specific manner to excise DNA specifically flanked by direct repeats of *loxP* at distinct chromosomal locations (11, 12). After recombination, excised DNA was degraded as it was not maintained extrachromosomally or found integrated elsewhere in the genome. Recombination and deletion of DNA residing between two direct repeats of *loxP* resulted in the elimination and activation of gene function, in the latter case by excision of a "stop" element that otherwise blocked expression of 3′ sequences. The assessed efficacy of Cre recombination was quite high, providing the expectation of using Cre-mediated recombination effectively for conditional gene mutagenesis techniques in embryonic stem cells and gene-targeted mice. That potential was initially achieved with the incorporation of functional recombinase target sites into the endogenous mouse immunoglobulin locus using homologous recombination in ES cells (13, 14). Subsequently, a novel targeting strategy was devised in which three *loxP* sites are used during homologous recombination to acquire ES cell clones ultimately lacking the selectable markers and yet bearing either systemic or conditional gene mutations upon mouse generation (15, and see below). These studies have developed a method enabling tissue-specific gene mutagenesis in vivo. Below, relevant experiences using this technique are described as well as expected advances in the near future.

Beginning with a cloning vector bearing three *loxP* sites, two of which flank the selectable marker genes *Neo* and *HSV-TK*, a targeting construct is generated that allows for the production of systemic and conditional gene mutagenesis models after homologous recombination (Fig. 1). *loxP*-containing isogenic gene targeting constructs thus designed and with several kilobases of genomic DNA have yielded homologous recombination frequencies of $\sim$ 0.025 among G418-resistant clones in experiments with nine different gene loci (unpublished data). While inclusion of *loxP* sites may not affect the frequency of homologous recombination, use of a *loxP*-specific probe in genomic Southern blotting reveals that $\sim$ 50% of gene-targeted ES clones lack the *loxP* site most distal from the *Neo* and *HSV-TK* cassette apparently as a result of crossover events specifically within the *loxP*-flanked genomic sequence (16, and unpublished data). ES cell clones that have undergone homologous recombination and retain all three *loxP* sites are electroporated with $\sim$ 1 μg of Cre expression vector in supercoiled plasmid form. ES cell sub-clones are then isolated after a 5–7 d selection in the presence of 0.5–2 μM gancyclovir begun 48–72 h after electroporation. In experience, virtually all resulting gancyclovir-resistant clones isolated have undergone either a Type I or Type II recombination yielding ES cells required for producing the systemic and conditional mutations in vivo, respectively (Fig. 1), often with the majority exhibiting the Type I deletion. However, gancyclovir resistant clones may also arise as a result of wild-type ES cell contaminants and from those cells bearing deleterious *HSV-TK* mutations. Several ways to increase the frequency of Type II recombination events are possible: one can reduce the amount of Cre vector used in the electroporation, use less efficient promoters to express Cre, and use Cre DNA sequence that lacks nuclear localization and eukaryotic translational consensus signals (see below).

After the production of chimeric and heterozygous mice from ES cell clones containing the Type II recombination, a breeding strategy to achieve conditional gene mutation in vivo

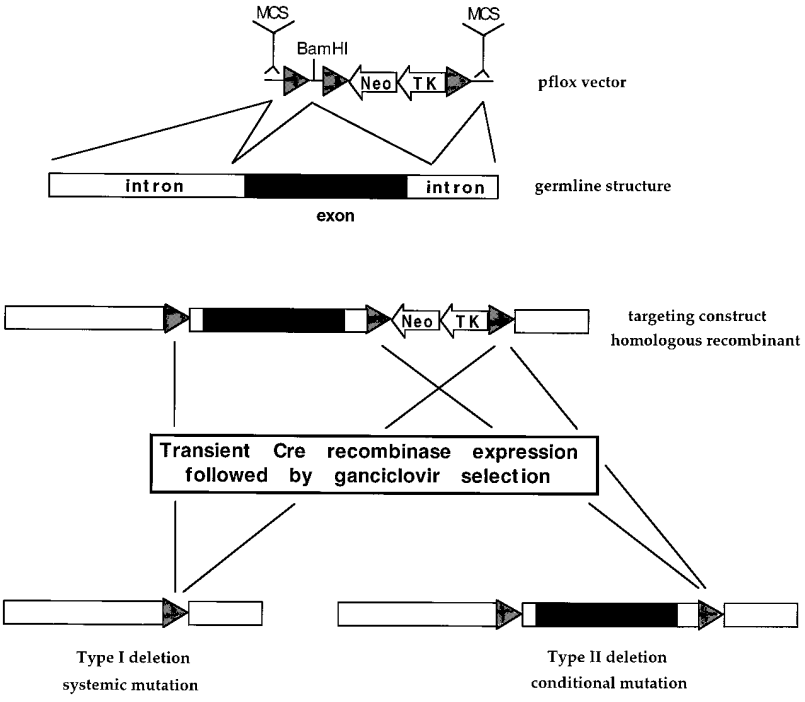

Figure 1. Systemic and conditional mutagenesis in embryonic stem cells as generated after homologous recombination and transient Cre recombinase expression. The frequency of Type I deletions is ten times higher than that for Type II deletions (see text). 34-bp *loxP* sites are depicted as shaded arrowheads (not to scale).

can be used (cover picture and legend). After two generations, mice bearing the Cre transgene and homozygous for the allele flanked by *loxP* sites (floxed) will theoretically represent ~20% of offspring (for unlinked loci) and can be used with littermates in controlled studies. Tissue and cell types undergoing recombination and mutagenesis are thus determined by those regulatory elements chosen to control Cre transgene expression. From conditional and somatic mutagenesis studies thus far, in vivo recombination has been obtained in cells comprising the liver, spleen, duodenum, heart, lung, uterus, kidney, eye lens, and muscle and in thymocytes and T cells (11, 12, 15–17), suggesting that perhaps all cell types will support Cre recombination. Importantly, the high efficiency of recombination induced in cells of the liver indicates that Cre recombinase can access chromatin and function effectively in postmitotic cells (17). Moreover, during the course of these studies, a higher efficacy of Cre recombination was obtained as a result of using Cre DNA sequence modifications (13) that incorporate both Kozak translational consensus and nuclear localization signals. Since Cre does not require cellular cofactors or DNA replication, a limiting step in efficient Cre recombination likely involves nuclear access in mitotic and postmitotic cells. Recombination efficiency may also vary due to chromatin structure in the region of the integrated *loxP* sites, as previously suggested (18). Presently, results with Cre transgenic mice and identical or distinct *loxP*-flanked alleles have not precluded alterations in nuclear Cre dosage as the key factor contributing to observed variations in recombination frequency.

An ability to temporally induce Cre recombination in vivo by addition of an exogenous stimulus has been described recently. In a transgenic study, Cre expression was placed under control of the interferon-inducible *Mx1* promoter and bred with animals bearing the *loxP*-flanked DNA polymerase-beta allelic structure. High efficiency recombination occurred among multiple cell types of adult animals after administration of the interferon agonist polyinosinic-polycytidylic acid (17). Two other inducible systems have been generated using chimeric recombinases (Flp or Cre) fused with estrogen-responsive elements (19, 20). In these studies, addition of estradiol or 4-hydroxytamoxifen induced site-specific recombinase activity on substrate DNA in vitro. Reports of inducible recombination herald a significant step forward in the ultimate ability to specifically alter chromosomal DNA structure at will in vivo and in a spatially restricted manner. In this regard, a system involving the tetracycline (tet)-resistance operon appears promising and may lack any extraneous physiologic impact intrinsic to mammalian hormonal and cytokine based induction systems. A mutated tet repressor fused with the VP16 activation domain (rtTA) has been developed that binds tet operator DNA in the presence of various tetracyclines and thereby activates transcription of the tetO-containing cytomegalovirus (CMV) minimal promoter (21). With low or no basal expression, over 1,000-fold inductions in enzyme activity have been reported to occur in mammalian cells and in transgenic mice (22, 23). To achieve inducible gene mutagenesis in a tissue-specific manner, the rtTA gene can be placed under the transcriptional control of a tissue- or cell-type specific regulatory element. Additionally, use of a single transgene construct harboring both functional cistrons (tissue-specific rtTA expression and tetO-CMV IE1-regulated Cre expression) would not increase breeding complexity in obtaining the mouse genotypes desired.

Of continuing relevance remains the diversity and availability of Cre recombinase transgenic mice. Studies that include their production and characterization are continually being reported, and it seems reasonable to deliver members of this increasingly large repertoire to an acceptable repository. Three Cre transgenic lines have been accepted by or deposited to Jackson Laboratories at present (*Mx1*-Cre, CMV-Cre, and *lck*-Cre) for dissemination as requested by the research community. Any one transgenic line may be used for numerous distinct studies, so unlike most other transgenic reagents, Cre recombinase transgenic mice do not themselves provide biological relevance. As implied, at present there is not any evidence for recombination in the unmodified mammalian genome after Cre expression in multiple cell types, as one would expect from the high statistical improbability of finding even one 34-bp *loxP* sequence within even the largest vertebrate genome.

The amount of DNA between *loxP* sites that Cre can recombine appears almost limitless, providing other unique experimental opportunities. The ability to irreversibly activate marker gene expression in early development is expected to enable key experiments in mammalian cell fate determination. Furthermore, it has been demonstrated that Cre recombinase can generate site-specific chromosome translocations in eukaryotic cells bearing *loxP* sites on heterologous chromosomes (24), allowing for the generation of specific karyotypic abnormalities comprising various novel disease models and the isolation of novel tumor suppressor loci (25, 26, and unpublished data). However, recombination over these distances appears rather inefficient, and screening or selection for site-specific translocation seems necessary. Moreover, recombination with endogenous cryptic *loxP* sites may also occur at low frequency (27).

The ability to manipulate large chromosomal DNA segments will likely be invaluable since the relatively long generation time of mammalian organisms otherwise precludes the ability to effectively screen for recessive and suppressor mutations. Such activity may however render problematic those studies that would require gene inactivation at multiple loci in the same cell. For these latter experiments, altering the 8 base spacer sequence of the *loxP* site may negate such events as Cre recombination, by way of the Holliday intermediate structure, depends upon precise basepairing during synapse formation (28). Synapse resolution by conservative recombination results in a remaining and functional *loxP* site, a fact that has been used to advantage to direct insertions of *loxP*-containing exogenous DNA into a chromosomal *loxP* site by Cre expression (29). It would appear that the modification of living genomes by site-directed recombination may become an integral approach in experimentation necessary to acquire a more complete understanding of mammalian development and physiology.

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