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Cre/*lox*: One More Step in the Taming of the Genome

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Cre recombinase has become an important tool in the precise manipulation of the genome, and its adoption has led to the development of increasingly accurate mouse models for the understanding of gene function. Although much of current work exploits the alacrity and precision with which Cre catalyzes excisive DNA recombination, Cre also is adept at the insertion of heterologous DNA into the genome. The precision and efficiency with which Cre can target DNA to a predesignated locus in the genome promises to facilitate understanding of mutant genes and allelic variants in their natural chromosomal context.

Key Words: Cre recombinase; *loxP*; genome.

Introduction

At the molecular level, the mammalian genome can be a wild and unruly place. This, in turn, has profoundly complicated the facility and reliability with which genetically modified animal models can be made, particularly so in the generation of transgenic mice by pronuclear microinjection of DNA into fertilized zygotes. Such transgenes are stably incorporated into the genome, but in a haphazard, ill-defined manner. Although incorporation is often at a single site, thus facilitating subsequent genetics, the region of the genome into which the DNA is inserted is more or less random. Moreover, the injected DNA is commonly rearranged into tandem arrays with both inverted and directly repeated copies, and the number of copies incorporated into the genome can be either high or low. There are consequences: transgene expression in the target tissue (specified by the promoter used in the transgene construct) may be either high or low, or even mosaic, depending only on the particular founder animal isolate, and expression may occur in unexpected tissues. Thus, both the level and the tissue-specificity of transgene expression are often subject to chromosomal position effects. Occasionally, the transgene inserts into an endogenous gene, inactivating it. Although such mutants can be useful, more targeted approaches have generally proven more powerful in generating mutations in endogenous mouse genes.

Of course, gene targeting in totipotent murine embryonic stem (ES) cells using homologous recombination has mitigated many of these problems *(1)*. Alteration of a predetermined target gene, be it deletion, insertional inactivation, or replacement with a specific mutant allele, can readily be accomplished by homologous recombination in ES cells, with subsequent generation of the desired mutant mouse by injection of mouse blastocysts with the genetically altered ES cells. The use of site-specific DNA recombination systems brings yet another level of sophistication to the kind of genetic manipulation possible in designing highly precise mouse models for the understanding of gene function. The Cre (*c*yclization *re*combination) site-specific DNA recombinase of bacteriophage P1 *(2)* has been particularly important in this regard, both because it was the first such recombinase to be used for this purpose in mammalian cells and mice and because Cre is a particularly potent recombinase in eukaryotes.

Because Cre-mediated recombination at *loxP* (*l*ocus *o*f *cross*over, *p*hage) sites is both extremely efficient and also exquisitely dependent on the presence of the recombinase, excision from the genome of any DNA fragment flanked by the *lox* recombination sites can be effected by simply expressing Cre in a target tissue and at the desired time, for example, by choosing a promoter to drive recombinase expression having the desired spatial and temporal pattern of expression. This thus has allowed a general strategy applicable to any gene for the design of both conditional gain-of-function and loss-of-function mutations in the mouse. Moreover, quite a variety of additional applications have been developed for Cre recombinase expression in eukaryotes. Much of this has generally been reviewed previously *(3–5)*, and a number of these topics are explored in far greater depth in this issue of *Endocrine*. Here I will give a brief historical background and then discuss in more detail the use of Cre, not to remove DNA from the genome, but instead to deliver it to a predefined locus.

Background

Cre recombinase is the 38 kDa product of the P1 *cre* gene *(2)* and is a member of the Int family of tyrosine site-specific DNA recombinases *(6)*. Cre catalyzes conservative site-specific DNA recombination at a 34 bp site called *loxP* *(7,8)*. The *loxP* site is relatively small, consisting of an asymmetric 8 bp core region flanked by two 13 bp inverted repeats to which Cre binds. Recombination occurs by sequential

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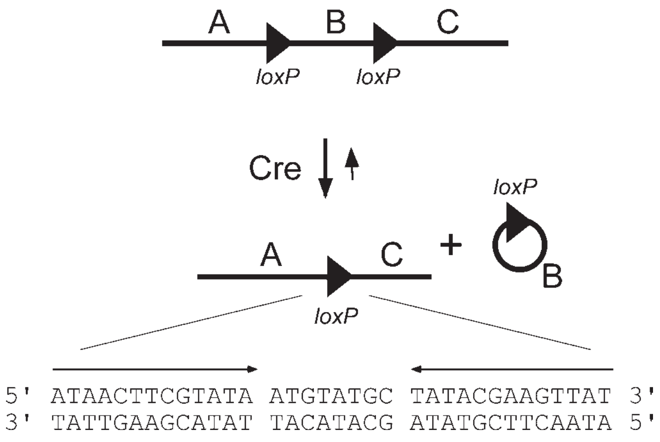

Fig. 1. Cre-mediated DNA recombination. The *loxP* site, represented by black triangles, is 34 bp in size and is composed of two 13 bp inverted repeat elements, to which Cre binds, surrounding a central 8 bp core or spacer region where actual breakage and rejoining of DNA takes place. A DNA segment flanked by two directly repeated *loxP* sites is excised by Cre as a covalently closed circular molecule. DNA recombination is conservative so no gain or loss of nucleotides occur during the recombination reaction.

breaking and rejoining of DNA strands within the central core region of *loxP* by way of a transient phosphotyrosine protein–DNA linkage with the enzyme. Recombination requires no accessory host protein or energy co-factors and, unlike most members of the Int family, proceeds efficiently in vitro with both linear and supercoiled DNA substrates. Cre-mediated recombination at two directly repeated *loxP* sites on the same DNA molecule, a *loxP*^{2} configuration *(9)*, precisely excises the intervening DNA segment as a covalently closed circle (Fig. 1). Recombination also proceeds efficiently at two sites in inverted orientation and in this case an inversion event results. Intermolecular recombination of *loxP* sites on separate DNA molecules gives a reciprocal translocation if both DNAs are linear and an integration event if at least one is circular.

Initial experiments to assess the feasibility of using site-specific recombination for eukaryotic genome manipulation showed that Cre's prokaryotic origin posed no hindrance to its ability to catalyze efficient excisive DNA recombination both in yeast *(10)* and in mammalian cells *(11,12)*. Thus, *loxP* sites placed onto the eukaryotic chromosome were readily accessible to recognition by Cre recombinase. Second, Cre could readily enter the eukaryotic nucleus. Entry into the nucleus had not been unexpected: Cre's small size (38 kDa) is less than the size limitation of 50–60 kDa imposed on passive diffusion into the nucleus by the nuclear pore. Nevertheless, subsequent work showed that Cre is actively transported into the eukaryotic nucleus due to the presence of endogenous sequences naturally present within the Cre protein *(13)*. In addition to excisive recombination to remove an unwanted gene from the genome, Cre also proved to be adept in vivo at intermolecular recombination to direct specific integration of an exogenous DNA to a target *loxP* site previously placed at a chromosomal site *(14)*. The stage was thus set for the development of Cre as a tool in the genetic and genomic engineering of mice.

In transgenic mice Cre-mediated recombination was first exploited to trigger the tissue-specific activation of an oncogene in a developmentally regulated manner *(15)*. Using a recombination-based gene activation strategy, two types of transgenic mice were generated. In the first the transgene consisted of the lens-specific αA-crystallin promoter driving expression of the SV40 large T-antigen (T-Ag), but with a *loxP*-flanked synthetic STOP sequence (*lox*^{2} STOP) interposed between the promoter and structural gene. This was designed so that T-antigen could not be expressed until Cre-mediated recombination removed the intervening DNA, but would then be expressed under the control of the αA-crystallin promoter. Mice generated carrying this construct did not express T-Ag and showed no evidence of tumor formation in the lens. The second type of transgenic mouse generated was a Cre-expressing mouse designed to activate the quiescent transgene in the first transgenic mouse. Doubly transgenic mice were obtained by crossing the *lox*^{2} STOP T-Ag mice with Cre mice carrying the *cre* gene under the control of either the lens-specific αA-crystallin promoter or the generally expressed CMV major immediate early promoter. All doubly transgenic progeny mice developed lens-specific tumors, with onset of T-Ag expression and subsequent tumor formation occurring just after the normal time at which αA-crystallin is normally expressed. A second set of experiments demonstrated the efficiency of Cre-mediated excision in transgenic mice *(16)*. Again, two types of transgenic mice were generated. One carried the *cre* gene under the control of the thymocyte-specific *lck* promoter. The other carried a *loxP*-flanked transgene consisting of the *lck* promoter driving *lacZ*. Intercrossing of the two transgenic lines generated doubly transgenic progeny. Molecular analysis showed that Cre had precisely removed the *lacZ* transgene in the target tissue by recombination at the flanking *loxP* sites. Thus, Cre could be used in transgenic mice to either eliminate or activate a transgene in a tissue-specific manner.

Coupling of site-specific DNA recombination strategies with homologous targeting in ES cells led to the ability to control similarly the expression of endogenous genes in the mouse. A convincing demonstration of the use of Cre to do this, and thereby generate a conditional mutation of an endogenous gene, was provided by a set of experiments in which the endogenous gene for DNA polymerase β (*polβ*) was modified by homologous targeting in ES cells so that the promoter and first exon were flanked by directly repeated *loxP* sites *(17)*. Resulting mice were phenotypically normal. However, mating of these mice with *lck*-*cre* transgenic mice produced progeny that carried both the *cre* transgene and also the *loxP*-modified *polβ* gene, and in these mice DNA polymerase β was ablated specifically in 40% of thymocytes due to Cre-mediated recombination. In principle, this binary Cre/*lox* strategy allows the tissue-specific knockout of any gene in the mouse genome. Not only can gene ablation be targeted spatially to a particular tissue, but gene ablation can also be targeted temporally by simply specifying the time at which Cre is active in the target tissue(s), for example, by using a developmentally regulated or inducible promoter to drive *cre* expression.

Such tissue-specific and temporally specified gene knockout strategies have become an invaluable tool for understanding gene function in the mouse. This is significant because in a developing multicellular organism a particular gene may play one role in one cell type and a somewhat different role in another cell type or tissue. Furthermore, knockout of a gene of interest may give rise to an embryonic lethal phenotype, thereby preventing understanding of its function in an adult tissue. Thus, postponement of the knockout phenotype until later in development or in the adult would allow a more complete understanding of the role of that gene. Conditional gene ablation does just that. It permits imposition of the knockout phenotype at the time and place desired by the experimenter, and thus it provides a generally applicable strategy for precise dissection of gene function in the mouse. A dramatic example of conditional mutagenesis is that used to confirm the intimate involvement of the *N*-methyl-D-aspartate receptor NMDAR1, complete knockout of which is neonatal lethal, in the acquisition of spatial memory in the mouse *(18,19)*. Conditional knockout of NMDAR1 was targeted to CA1 hippocampal neurons by mating mice carrying a *loxP*-flanked NMDAR1 gene with transgenic mice in which the *cre* gene had been placed under the control of an α-calcium-calmodulin dependent kinase II promoter expressing *cre* specifically in CA1 neurons. This generated progeny mice deficient in NMDAR1 only in CA1 neurons. Strikingly, these mice exhibited a pronounced deficit in spatial memory, but were otherwise normal.

To date, a large number of mouse lines have been generated carrying conditional alleles of many different endogenous mouse genes. In addition, a large number of mouse lines have been constructed that deliver tissue-specific or temporally specific *cre* expression. Several of these are discussed in detail elsewhere in this volume. A powerful feature, though, of the binary nature of the Cre/*lox* system is that a particular *loxP*-tagged conditional gene knockout can be mated with a battery of preexisting Cre-expressing mice, each with its own distinct pattern tissue-specific pattern of expression. Thus, the role of a conditionally tagged gene can be evaluated independently in a large number of tissues.

Cre-mediated recombination in mice and ES cells has been enlisted in the generation of chromosomal translocations, inversions, large nested chromosomal deletions, as well as simply removing the selectable marker and other unwanted DNA sequences that remain after homologous DNA targeting in ES cells. For this latter use, a GFP-*cre* fusion gene has been used in conjunction with FACS so that transfected cells that are transiently Cre$^+$ can be quickly sorted, thereby enriching for cells destined for recombination *(20)*. The GFP-*cre* fusion gene may also prove useful for identifying highly expressing cells or tissues in transgenic mice *(21)*.

Targeted Gene Placement

As noted above, Cre can target integration of an exogenous circular DNA carrying a *loxP* site to a *loxP* site resident in the genome of a eukaryotic cell. A frustrating limitation in molecular manipulation of mammalian cells is that stable integration of exogenous DNA into the mammalian genome generally occurs in a random fashion and rarely targets a specific locus by homologous recombination. The resulting variability in copy number and genome position between different transformants results in variable gene expression between different cell line isolates. Indeed, in transgenic mice this not uncommonly results in unexpected and variable patterns of gene expression from an injected transgene. Cre's ability to catalyze specific DNA recombination between chromosomal and extrachromosomal DNAs can help to minimize such variability in gene placement and do so at a frequency higher than that achievable by homologous targeting.

Because integration of a *loxP*-containing plasmid by recombination with a genomic *loxP* site results in the inserted DNA being flanked by two directly repeated *loxP* sites, and thus a substrate for Cre-mediated excisive recombination, transient Cre expression, for example, from a co-transformed *cre* expression plasmid *(14)* or from co-transfected purified Cre protein *(22)*, is necessary for stable DNA integration. Still, *loxP*-targeted integration occurred at a frequency of only about 10–25% of random, illegitimate DNA integration in those experiments. A promoter trap methodology using a promoterless *loxP* selectable marker on the targeting plasmid allowed direct selection for site-specific targeting by eliminating random plasmid integrants *(23)*. In initial experiments, greater than 90% of selected transformants were single-copy integrants targeted to the chromosomal *loxP* target, with only occasional recovery of integrants having two tandem copies of the targeting vector *(14)*. Complete elimination of random plasmid integrants was obtained by using an ATG-less *loxP-neo* fusion gene as the chromosomal target. With the *loxP* targeting vector providing both the promoter and the ATG start, all of the selected G418^R colonies were single copy integrants of the targeting vector, with nearly all showing identical levels of *lacZ* reporter gene expression in independent integrants *(23)*. The same strategy was used to construct isogenic cell lines expressing different alleles or isoforms of an angiotensin II AT_I receptor transgene to facilitate pharmacological evaluation *(24)*. Site-specific targeting of DNA to a chromosomal target does not itself abolish position effects on gene expression, instead site-specific targeting only guarantees that transgenes targeted to a particular *loxP*-tagged chromosomal locus site will experience the same chromosomal environment and will be single-copy. Thus, the variability of expression that comes from transgenes being integrated at different chromosomal positions can be eliminated by site-specific targeting. Such strategies do not negate the effect a particular chromosomal environment might have in influencing expression of the integrated transgene, but rather impose a uniform environment on transgenes targeted to that locus. An ideal *loxP*-tagged locus, then, would be one that is completely neutral with respect to transgene expression, so that the transgene would provide all signals necessary for governing transgene expression.

Both to permit generation of site-specific placement of a transgene in a mouse and to ensure that a Cre-targeted transgene experiences a known chromosomal environment, a single *loxP* site can be installed to a preselected locus by homologous targeting in murine ES cells. Placement of the *loxP* target at the mammary gland-specific whey acidic protein (WAP) locus allowed subsequent Cre-mediated targeting of a *loxP*-containing plasmid to that locus at a remarkable frequency of 23% compared to random DNA integration *(25)*. In a second ES cell example, and using a gene trap strategy to enrich for site-specific integrants, Cre-mediated targeting of a transgene to a *loxP* site placed at the genomic locus for β-casein gave a somewhat lower frequency (approx 1%) of site-specific to random integration *(26)*. However, in this latter case a more-sophisticated strategy was used to eliminate integration of nontransgene plasmid backbone sequences. The targeting plasmid was designed to have the transgene/selection cassette flanked by directly repeated *loxP* sites so that Cre first excises the cassette from the transfected plasmid and then integrates the excised *loxP*-containing circle into the genome in a second site-specific DNA recombination step *(14,27)*. A powerful feature of Cre-mediated targeting is that very large DNA inserts can be efficiently integrated at a chromosomal *loxP* target. Integration both of a 230 kb *loxP*-equipped BAC into a chromosomal *loxP* target in plants *(28)* and of circularized YACs into various chromosomal *loxP* targets in ES cells *(29)* has recently been achieved. Because ES cells can be injected into murine blastocysts to generate genetically modified mice, Cre-mediated integrative targeting in ES cells should prove to be a useful method for generating transgenic animals with predictable patterns of expression.

Two distinct strategies using mutant *lox* sites have been investigated to increase the frequency of Cre-mediated targeting of DNA to a chromosomal target. Because Cre-mediated recombination is normally reversible, one strategy to bias Cre-mediated recombination toward integration is to design the recombination reaction so that after integrative recombination one or both of the resulting *lox* sites flanking the inserted DNA becomes a poor recognition site for Cre. This strategy is based on the observation that *lox* sites mutant in the outermost 4–8 bp of a single “arm” or inverted repeat element (Fig. 1) do not recombine with each other, although they are still proficient in DNA recombination with an unmutated *loxP* site *(30,31)*. Presumably, cooperative binding of Cre to the inverted repeat elements of *loxP* site compensates for impaired binding at the mutated site during synapsis. Sites that are symmetrically mutated on both inverted repeats, however, become less competent for recombination with an unmutated *loxP* site because they bind Cre quite poorly. Since such doubly mutated sites would be generated by recombination between a left-arm mutated site and a right-arm mutated site, integration might be favored when using a chromosomal target *lox* site mutated in one arm and a targeting vector mutated in the other. After integrative recombination, the insert would be flanked by one doubly mutated site and one wild-type (wt) site so that excisive recombination would be inefficient. This strategy has had some success, both in plants *(32)* and in murine ES cells *(33)*. In ES cells with such mutated *lox* targets randomly placed into the genome, the frequency of site-specific integration of the reciprocally mutated *lox* site plasmid at the chromosomal target was generally between 1 and 8% of all drug-resistant colonies, with one ES line showing targeting as high as 16% of all selected colonies *(33)*.

All of the foregoing Cre-mediated integration strategies rely on a chromosomal target with only a single *lox* site. For convenience they can be referred to as single-*lox* targeting strategies. In contrast are a second type of integration strategies called double-*lox* integration strategies. These employ a chromosomal target having two heterospecific *lox* sites. Because the spacer region of the *lox* site acts as a homology sensor *(34)*, *lox* sites having the same spacer (homospecific *lox* sites) generally can recombine with each other, whereas recombination between heterospecific *lox* sites having nonidentical spacers is not favored. With this strategy the chromosomal target consists of two heterospecific *lox* sites, *loxP* and *lox511*, in an inverted configuration (Fig. 2) and can be designed such that they flank either a reporter gene or a negative selection marker. The same two heterospecific *lox* sites are present on the targeting vector but flank the gene to be inserted and/or a positive selection marker gene. Cre-mediated recombination between matching sites on the plasmid and on the chromosome results in what is effectively a double crossover event. This replaces the *lox*-flanked chromosomal interval precisely with the corresponding DNA segment on the plasmid. Because plasmid and other prokaryotic sequences can interfere with transgene expression *(35–38)*, one attractive aspect of this double-*lox* strategy for segmental genomic replacement is that no vector backbone sequences are incorporated into the target site by site-specific targeting.

Using a *lox-neo* fusion gene that permitted only site-specific integrants to become $G418^R$, direct comparison of single-*lox* and double-*lox* targeting to exactly the same chromosomal site in 3T3 cells indicated that double-*lox* target-

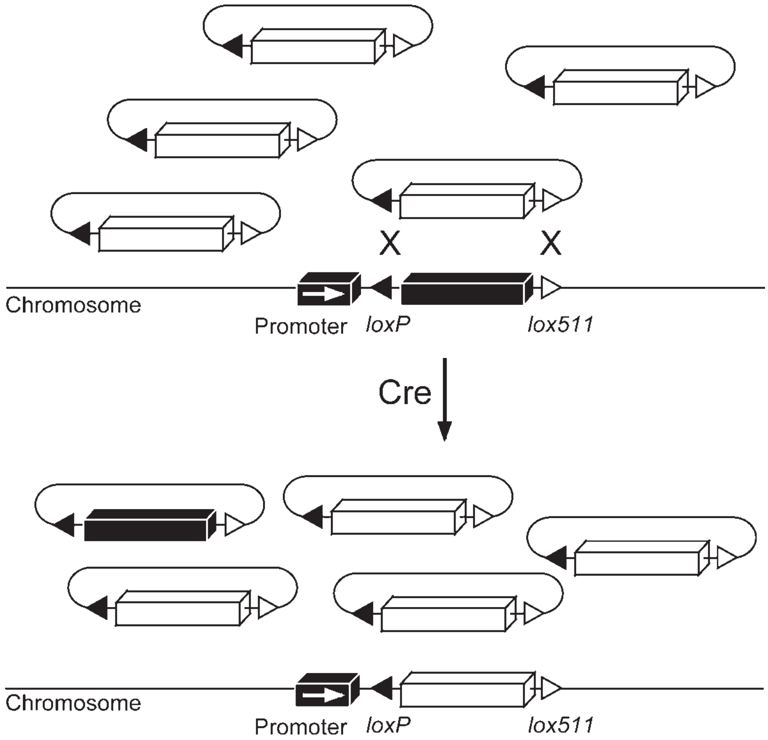

Fig. 2. Allele replacement by double-*lox* recombination. Heterospecific *lox* sites, represented by triangles (black, *loxP*; white, *lox511*), are shown flanking a particular allele (black box) of an endogenous gene on the chromosome and also a replacement allele (white box) on transfected circular plasmid DNAs. Alternatively, the *lox*-flanked gene (black box) can be a negative selectable marker placed there by homologous targeting. Recombination by transiently expressed Cre brings about what is formally a double crossover event between "like" *lox* sites, resulting in eviction of the endogenous black allele and replacement by the plasmid-borne white allele. Although additional recombination events between the new chromosomal allele with plasmid-borne alleles is possible, this will only regenerate the same white allele at the chromosomal locus. In the cartoon shown here, the *lox* sites are so positioned that allele replacement places the new white allele under the control of the endogenous promoter at this locus (black box with white arrow) to give a precise knock-in event.

ing was 40-fold more efficient than single-*lox* targeting and also required far less recombinase for optimal targeting efficiency *(38)*. In fact, random DNA integration was only a third as frequent as site-specific double *lox* targeting, with the overall frequency of site-specific targeting being 1% of all surviving cells or approx 6% of all cells taking up DNA. In similar experiments double-*lox* targeting to an arbitrarily chosen locus in mouse erythroleukemia MEL cells gave a frequency of site-specific targeting of between 0.2 and 0.5% of surviving cells using two sites designated L1 and L2 *(39)*. In these latter experiments the selection marker was flanked by directly repeated FRT sites so that it could be removed by Flp recombinase in a subsequent round of DNA transfection.

In ES cells, gene knock-in's by homologous recombination allow placement of a desired transgene or allelic variant at a desired locus. Such precise gene placement minimizes or eliminates position effects on gene expression by providing a natural chromosomal context with its attendant regulatory elements. However, the low frequency of targeting by homologous recombination and the requirement for large amounts of sequence homology to the target locus can make this a nontrivial endeavor. In contrast, double-*lox* targeting allows allele replacement at high frequency and does not require inclusion of genomic homology on the targeting vector. For example, after placement by homologous recombination of a double-*lox* target at the asialoglycoprotein receptor gene locus (*ASGR1*) in mouse ES cells, subsequent knock-in of any gene to that locus by double-*lox* targeting was readily obtained, occurring at a frequency of between 56 and 87%, far greater than that achievable by homologous recombination *(40)*. Efficient recovery of knock-in's placing a reporter gene under the control of the endogenous *ASGR1* promoter was achieved even without a positive selectable marker on the targeting construct by using a negative selection strategy to select for targeted replacement of a herpes TK gene placed between the two chromosomal heterospecific *lox* sites (Fig. 2). Dual selection (positive marker on the targeting plasmid, negative marker on the chromosome) gave 100% site-specific integration. Because the frequency of site-specific targeting was high, occurring in several percent of all cells taking up DNA, knock-in's could be identified in the absence of selection for drug resistance by simply screening colonies obtained after FACS enrichment from cells co-transfected with a combination of the targeting vector, the Cre-expressing vector, and a GFP-expressing construct that allowed identification of cells that had taken up DNA. One important observation from this work was that the frequency of site-specific targeting was insensitive to the transcriptional status of the locus; that is, the targeting frequency was the same whether or not an actively expressed gene was present at the chromosomal target. This suggests that double-*lox* targeting of silent loci in ES cells should be relatively efficient.

In a variation on the *loxP-lox511* double-*lox* strategy a pair of *loxP* sites in an inverted configuration was randomly placed into the genome of ES cells for use as a target for Cre-mediated integration *(41)*. Although inversion can occur by recombination between the inverted *loxP* sites, double crossover recombination can also proceed with a transfected targeting plasmid having similarly inverted *loxP* sites. Integrative targeting did in fact occur at high frequency, with inserts obtained in both possible orientations due to Cre-mediated inversional recombination either before or after recombination with the incoming plasmid. One potential concern in using *loxP* sites in an inverted configuration, besides the slight inconvenience that half of the targeted integrants will be an incorrect configuration for a precise gene knock-in, is that unequal sister chromatid exchange can lead to the production of dicentric and acentric chromosomes *(42)*. However, evaluation of an alternative to the *loxP-lox511* configuration was prompted by the observation that a low level of excisive recombination can occur between directly oriented *loxP* and *lox511* sites *(34,43)*.

Another variation is to replace the *lox511* site with an alternative heterospecific *lox* site called *lox2722* to reduce recombination with *loxP (44)*. The *lox2722* site differs from *loxP* at two positions in the spacer instead of the single alteration present in *lox511*. In these experiments, using a directly repeated configuration of heterospecific *lox* sites in ES cells, the *loxP-lox2722* set gave a higher frequency of correctly targeted positively selected integrants (50%) than did the *loxP-lox511* configuration (0–47%). This was even more dramatic using a dual positive–negative selection: 100% correct targeting with *loxP-lox2722* compared to 17–69% for the *loxP-lox511* direct repeat configuration. The relative increase in frequency was attributable to aberrant excisive recombination between the *loxP* and *lox511* sites that thus deleted the targeting interval and any gene that may have been integrated there.

Several technical variables probably contribute to the differences in targeting frequencies observed between these studies. One undoubtedly important variable is the relative orientation of *loxP* with respect to the heterospecific *lox* site. The results described above indicate that the "inverted" configuration with either *loxP* or *lox511* is far less susceptible to undesired excisive recombination with *loxP* than is the "direct" orientation, and that result has also been observed in *E. coli (45)*. The story may be somewhat more complicated, however. At first blush it would then seem that the inverted configuration of *loxP-lox511* should result in an appreciable number of targeted inserts in an inverted configuration, yet this was not observed *(40)* and so the incidence of such events may be infrequent. Other variables include the chromosomal locus being targeted, the particular Cre construct used, and the level of expression of recombinase in cells. This last point may be relevant because the amount of Cre required for double-*lox* targeting is considerably less than that required for single-*lox* targeting *(38)* and because in *E. coli* directly repeated *loxP* sites can be forced to undergo excision rather the expected inversion by high-level expression of Cre *(46)*. More work will shed light on these issues. Nevertheless, several quite workable strategies for targeted gene placement are clearly already available.

Similar gene placement strategies using heterospecific recombination sites have been described using FLP recombinase *(47,48)*. The term RMCE (*r*ecombinase-*m*ediated *c*assette *e*xchange) has been applied to all of this class of gene replacement strategies using heterospecific recombination sites *(49)*. As additional site-specific recombinases become available for use in mammalian cells, it is likely that the same kinds of heterospecific strategies will be developed for them. This naturally leads to the happy anticipation that multiple loci in the same cell can be targeted for allele replacement by site-specific targeting through use of different combinations of recombination sites and recombinases.

Closing Comments

Cre displays a very high degree of specificity in its ability to recognize and recombine DNA. Nevertheless, the mammalian genome is large and there do exist endogenous sequences that can serve as substrates at an appreciable efficiency for Cre-mediated DNA recombination *(50)*. In yeast expressing Cre recombinase such cryptic *lox* sequences behave as hotspots for nonsister chromatid exchange during G2 *(51)*, and it is likely that such mitotic sister and nonsister exchanges also occur in mammalian cells, although by themselves such exchanges should not generate any chromosomal anomalies. Recently, several labs have reported the occurrence of DNA rearrangements or chromosome aberrations associated with high-level Cre expression in mice or mammalian cells *(52,53)*. Because it is high-level expression, both with NLS-Cre or with a ligand-regulatable Cre-ER(T), that led to these chromosomal aberrations, one possibility is that overexpression may have contributed to recognition and recombination of endogenous DNA sequences. In support of this notion, high-level expression of Cre in *E. coli* can also lead to unexpected recombination between *lox* sites *(46)*, and high-level expression of Cre in yeast has been shown to cause chromosomal aberrations by inducing recombination with endogenous *lox*-like DNA sequences *(54)*. It may thus be prudent in mice to use the minimal amount of Cre expression required to catalyze the desired recombination event and not to use excessive amounts, i.e., more may not be better. The existence of high- and low-level *cre* alleles

(14), based on the presence or absence of optimal translation initiation signals, should allow the precise tailoring of the right amount of Cre expression with a chosen promoter. Conversely, because certain tissue-specific or developmentally regulated promoters may be quite weak, the availability of a codon-improved *cre (55)* should prove useful in those special circumstances. On a technical note, the urge to use very strong promoters to drive *cre* expression largely is prompted by an attempt to minimize the mosaicism or variability that often attends transgene expression in general. A superior strategy may be to minimize possible position effects imposed on *cre* transgene expression, for example, by using a knock-in strategy.

Site-specific DNA recombinases such as Cre have ushered in a large number of new strategies for manipulating the mammalian genome. Already conditional mutations of any gene in the genome are feasible to construct, and large-scale DNA rearrangements can be orchestrated at will. It is not unlikely that new and more remarkable strategies are close at hand in the further taming of the genome.

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