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Genetic engineering of embryonic stem cells via site-directed DNA recombination

Anna Norman[1], Dr. Mark MacInnes[2]

This article reviews recent advances in genetic engineering of mammals utilizing DNA recombination techniques to produce targeted genome modifications. The general objective of these technologies is to discover novel gene functions via manipulation of gene expression, regulation, or encoded protein sequences. The advantage of gene site-directed DNA recombination is that the engineered variant remains in the normal context of its chromosomal locus. This feature is especially important for studying gene function in the context of its regulation in animal development. The target gene can be subject to both gain of function or loss of function mutations. In addition to precisely crafted modifications of single genes, alternatively, site-directed DNA recombination can also produce chromosomal changes including segment deletion, or inversion, or loss of heterozygosity between a homologous chromosome pair. Site-directed recombination is accomplished by certain mechanisms of DNA exchanges that were first discovered in bacteria and their viruses (bacteriophages). We will illustrate how these systems permit specific modification of the mammalian genome. Recombination enzymes of the integrase family such as the Cre protein (cyclization recombinase) have a well-characterized site-specific recombination mechanism. Cre recombinase catalyzes DNA strand exchanges in palindromic DNA target sequences called the locus of crossover (lox site). The biochemical flexibility of Cre interactions with lox sites permits a novel approach to mammalian gene targeting. For example, lox site orientation and-or change of sequences can modify the specificity of DNA exchange in fascinating ways.

This approach has been most successfully adapted for site-directed recombination in mouse embryonic stem cells (ES cells). For in vivo analyses, ES cells can be implanted into embryos to contribute in utero to the germline tissues. In progeny chimerical mice, the novel genetic trait may be transmitted via sperm or egg to offspring. ES cells also differentiate in vitro into numerous cell types allowing direct assessment of cell lineage phenotypes. Some of the differentiation properties of mouse ES cells in vitro have been confirmed in both human and primate ES cells. An understanding of ES cell genetic engineering and its potential applications is therefore of critical medical and ethical importance. Despite the successes of these approaches in murine ES cells, site-directed recombination technology has unresolved questions about its utility for human genetic and tissue therapy.

DNA Recombination in Embryonic Stem Cells

In the present review, we will focus on technologies for targeted DNA recombination in mouse embryonic stem cells (ES cells). These primordial stem cells are derived from pre-implantation embryos of the blastocyst stage (Evans and Kaufman, 1981; Martin, 1981). ES cells have the unique property of *pleuripotency*, that is the ability of the cells to differentiate *in vivo* into all subsequent tissues that arise from within the embryo (reviewed in Smith, 2001). Upon injection of ES cells into host blastocyst stage embryos, the ES cells contribute *in utero* to embryonic development of all of the somatic and germinal tissue lineages. A novel line of mice is established when a novel genetic trait is transmitted in the germline of the founding progeny animals . To date, there is a literature of *several thousand* genetically novel lines of mice created from ES cells.

ES cells also exhibit remarkable proficiency to differentiate *in vitro*, providing researchers with many derivative cell types for direct genotype to phenotype

[1] College of St. Benedict/
St. John's University

[2] Biosciences Division
Los Alamos National Laboratory

analyses (O'Shea, 1999). Other mammalian cell systems that are permissive for targeted DNA recombination include chicken DT40 cells (Dhar et al., 2001; Winding and Berchtold, 2001) and Chinese Hamster ovary (CHO) cells (Thompson and Schild, 2001). Recently, some of the characteristics of pleuripotency found mouse ES cells *in vitro* have been replicated with both human and monkey ES cell lines (Cibelli et al., 2002; Reubinoff et al., 2001; Reubinoff et al., 2000). These findings highlight the crucial medical relevance and ethical implications of ES cell genetic research regarding the genetic manipulation of human embryonic stem cell lines.

Complementary Strategies for Targeted DNA Recombination in ES Cells

Altering target genes within ES cells employs two complementary strategies: homologous recombination and integrase mechanisms. Homologous recombination (HR) entails double reciprocal exchanges between DNA molecules dependent largely on overall sequence identity. HR has long been used to introduce exogenous DNA sequence into a similar target sequence within mouse ES cells (Thomas and Cappechi, 1986). In ES cells, HR coupled with appropriate selection using antibiotics for the desired recombinant cells provides an efficient method to produce gene-targeted mutations. Selected cells (clones) derived from single progenitor cells must be screened for HR by molecular methods (primarily polymerase chain reaction - PCR) to identify those with the desired genetic modification. The mechanism of HR within mammals has a complex biochemistry (Thompson and Schild, 2001). In contrast, prokaryotic DNA recombinases provide a complementary and less complex avenue towards site-directed genetic recombination. These mechanisms require much less sequence identity than HR, decreasing the amount of foreign DNA that is incorporated. In addition, while the best characterized DNA recombinase enzymes are of bacterial and yeast origin, they efficiently catalyze recombination in mammalian cells.

The detailed biochemical understanding of prokaryotic DNA recombinases and their substrate target sequences has allowed researchers to efficiently create specific cell type, or developmental control of the timing of genetic alterations in mice. This feat is accomplished by placing Cre recombinase under the control of a cell-type-specific gene promoter with the desired pattern of expression (reviewed in Sauer, 1998). Another advantage of site-directed DNA recombination is that DNA exchange is targeted by certain short oligonucleotide DNA sequences (e.g. *lox* sites) pre-positioned via HR in the mouse chromosome. In the specific case of DNA insertion, recombination sites in the plasmid vector identical to those in the genome provide the substrate for plasmid to chromosome recombination-mediated insertion of sequence. Variations in *lox site* targeting allow repeated exchanges of genetic material at the same mammalian genome site, or alternatively, into separate sites in the same genome.

A caveat to this enthusiastic introduction to site directed DNA recombination is the recent discovery of Cre recombinase-mediated genotoxicity in the form of chromosome aberrations (Loonstra et al., 2001; Thyagarajan et al., 2000). Characterization of this untoward consequence of recombinase activity has led to successful efforts to minimize its confounding effects on site-directed recombination studies in cells, *in vitro* and in tissues, *in vivo*. We will briefly survey potential applications of these technologies for human gene therapy in the context of therapeutic cell type rep*lac*ement derived from human ES cells. In a related topic not covered here, others have recently reviewed advances in molecular methods of assembling gene targeting vectors by HR in *E. Coli* bacterial artificial chromosome vectors (BACs) (Copeland et al., 2001).

The CRE/*lox* Site Recombination Mechanism

The integrase family of DNA recombinases shares the biochemical feature of bimolecular reaction kinetics whereby the enzyme recognizes a specific DNA recombination sequence. Members of this family include Cre recombinase from bacteriophage P1, bacteriophage lambda integrase, the yeast Flp recombinase, and the bacterial XerCD recombinases. These enzymes accomplish DNA strand exchange in a two step process between DNA substrates. One pair of strands is exchanged to form a recombination junction intermediate that does not move, while the second pair of strands is then exchanged during resolution of the junction. Van Duyne (2001) reviewed the structural biology of these recombinases with emphasis on the crystal structures of Cre with its DNA substrate (van Duyne, 2001). Cre recombinase and to a limited extent Flp recombinase (Seibler et al., 1998) have been used for enzyme mediated site-directed DNA recombination. We will describe in some detail how Cre recombinase interaction with its substrate DNA can be altered in ways that produce diverse genetic outcomes in mammals.

The Cre protein (38 kDa) is encoded by the *E. Coli* phage P1. P1 is maintained inside *E. Coli* cells as a single copy, circular DNA plasmid molecule. The role of Cre protein is to exchange and separate copies of P1 that arise after its replication in order to allow partitioning of the two P1 molecules at each cell division (Hoess and Abremski, 1984; Sternberg and Hamilton, 1981). The target site of Cre is the *loxP* sequence of 34 base-pairs (bp), containing two 13 bp inverted repeats flanking an 8 bp core sequence (Figure 1). Two Cre molecules bind to each *loxP* site, one on each half of the palindrome (van Duyne, 2001). Cre molecules bound to DNA then form a tetrameric complex bringing two *lox* sites into proximity. The Cre-mediated strand cleavage and exchange between *lox* sites occurs following the first bases and before the last base of the 8 bp cores. (The reader is referred to van Duyne (2001) and references therein for beautiful crystal structure representations of this complex). The DNA strand asymmetry of the 8 bp core also confers directionality on the *loxP* site (Hoess and Abremski, 1984). *loxP* orientation determines the type of recombination that will occur between *loxP* sites. Cre recombinase catalyzes both inter-molecular DNA exchanges and intra-molecular excision or inversion (Figure 2). If two *loxP* sites in the same molecule are co-aligned, Cre recombination will preferentially produce excision and circularization of the DNA between the sites (Figure 2A) (Baubonis and Sauer, 1993; Sauer and Henderson, 1989). Cre also catalyzes the reverse reaction, the integration of DNA into a single *loxP* (Figure 2A). However, integration is quite inefficient since the inserted DNA is immediately flanked by duplicated *loxP* sites, which permit re-excision (Araki et al., 1997). When two *loxP* sites are inverted in orientation intra-molecular recombination will produce an orientation switch of the insert (an inversion) with a 50:50 probability (Figure 2B) (Hoess et al, 1986, Feng et al, 1999).

The Cre/*lox* system as outlined above can be used to introduce certain kinds of gene mutations as well as chromosomal inversions, truncations, or deletions (Zheng et al., 2000; Feng et al, 1999). Further, Cre-induced mitotic

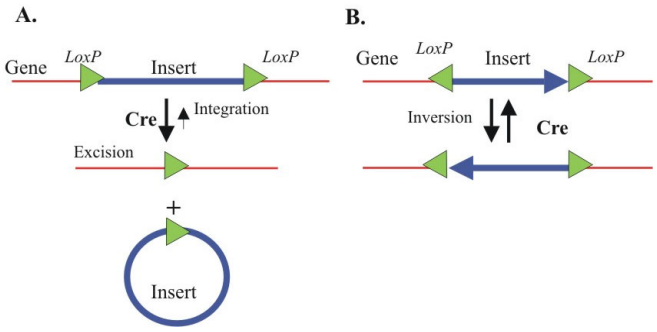

Figure 2. Cre mediated *loxP* recombination reactions at single *loxP* sites

(A) Homologous *loxP* sites flanking an insert recombine circularize the insert. B) Recombination between inverted *loxP* sites leads to a 50:50 probability of segment inversion.

chromosome recombination between single *loxP* sites on each member of a homologue pair has also been used to create genetic mosaics in mouse ES cells (Liu et al., 2002). This experiment simulates chromosome loss of heterozygosity (LOH) that is seen in many types of tumors. Cre-induced mitotic recombination in a tissue lineage *in vivo* would permit studies of LOH effects on development or in adult mouse tissues.

The recombination properties of Cre at a single *loxP* site select against the insertion of precise DNA segments into the target chromosome. Upon targeted integration of DNA, the *loxP* site is duplicated, leading to the highly favored intra-molecular excision (Fig. 2A). When an integration event does occur, not only is the DNA of interest integrated into the genome, DNA from the targeting plasmid vector is integrated as well. Alternative strategies have been devised using the Cre/*lox* system in order to create higher frequency and stability of insertion events, *in vitro*, and ultimately to eliminate plasmid DNA introduction into the genome.

Left hand palindrome *Right hand palindrome*

8 bp core

loxP ATAACTTCGTATA GCATACAT TATACGAAGTTAT

Figure 1. DNA sequence of wild type *loxP* site

The 13 bp inverted repeats (palindromes) flank an 8 bp asymmetric core sequence where the recombination exchange takes p*l*ace. One Cre recombinase molecule binds to each palindrome sequence (not shown). Strand cleavage positions are after the first, and before the last base of the 8-bp core.

CRE Recombination with Mutant *lox* Sites: Strategies for DNA to Chromosome Insertion

Albert et al. (1995) found that mutations in *loxP* permit integration of DNA at a plant target site in the plant genome while avoiding its immediate re-excision. This strategy was also successful for integrating foreign gene DNA into a mouse chromosome (Araki et al., 1997). A single mutant *lox* site in which nucleotides were altered in the right hand palindrome was pre-positioned by HR in the chromosome target (Figure 3). In the targeting vector, a distinct *lox* mutation was incorporated into the left hand palindromic element. The two mutant *lox* sites were in co-alignment and still enabled Cre to catalyze inter-molecular recombination in ES cells. However, the integration resulted in the creation of two *de novo lox* sites, one containing both left-end and right-end mutations, while normal *lox* P was generated at the other site. Cre poorly recognizes the LE+RE *lox* site, which inhibited re-excision between it and the *loxP*. This approach facilitated efficient insertion of a targeting cassette.

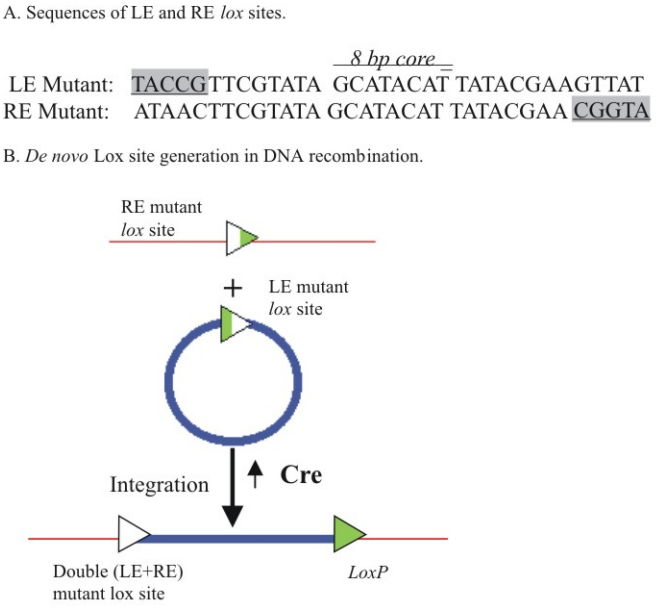

Figure 3. The LE/RE mutant *lox* site strategy for segment integration

Cre-mediated recombination between the mutant right end (RE) and left end (LE) *lox* sites produces a trapped product double mutant (LE+RE) *lox* site and a WT *loxP* site that are less susceptible to intra-molecular excision.

The idea that different *lox* sites may not recombine efficiently but that identical *lox* site recombination remains proficient led to an in-depth study of these interactions. Lee and Saito (1998) identified many mutant *lox* sites that recombine efficiently with an identical partner complex but not with *loxP* (Figure 4A). For example, *lox 2272* and *lox 2372* sequences contain two nucleotide changes in the core 8-bp sequence (Lee and Saito, 1998). The *lox* FAS site occurs naturally in *Saccharomyces cerevisiae* (Sauer, 1996). *Lox FAS* has a completely different consensus core sequence from *loxP* while remaining an efficient substrate for Cre. This fact illustrates the plasticity of *lox* sites. The *lox 511* site contains a single nucleotide mutation in the core sequence (Hoess et al., 1986). The recombination efficiency between homologous and heterologous pairs of *lox* sites has been studied in *E. Coli* (Siegel et al., 2001) (Table 1). Their results show that these heterologous pairs of *lox* sites undergo recombination at a much lower frequency than homologous pairs.

Siegel et al. (2001) used a green fluorescent protein (GFP) gene flanked by heterologous *lox* sites in the *lac* Z reporter gene (responsible for production of β-galactosidase) of a plasmid DNA. Before recombination, the bacterial colonies expressed GFP and emitted green fluorescence. Correct recombination events resulted in excision of the GFP gene and permitted the *lac* Z gene to be translated, producing a loss of GFP fluorescence and concomitant expression of functional *lac* Z gene. Such *E. Coli* recombinant colonies were non-fluorescent and blue dye colored on X-gal medium. The assay quantified the accumulated recombination events over many generations of colony growth (~18h) and therefore it was very sensitive to low levels of correct recombination. The assay also distinguished aborted or aberrant recombination repair products from true GFP excision via absence of correct *lac*Z gene activation. These findings indicated that recombination between homologous pairs of *lox* sites (whether similar or dissimilar to *loxP*) can occur efficiently *in vivo* while recombination between heterologous pairs occurs much less efficiently. Lee and Saito (1998) also noted the occurrence in some combinations of arrested intermediate recombination structures in their *in vitro* plasmid assay system. In these situations, recombination proceeded to exchange one DNA strand but, due to the heterozygosity of the *lox* sites, the intermediate wasn't able to resolve into the final recombination product. The potential persistence of arrested intermediates between heterologous *lox* sites may have implications for the use of the Cre/*lox* system in mammals (see below).

Given the low level of Cre-mediated recombination between several heterologous pairs of *lox* sites, new gene targeting techniques were developed that exploit this selectivity. The DNA to be inserted into the genome is constructed so that

Table 1. Recombination frequencies in *E. coli* among three mutant lox sites and loxP. Reprinted with permission of the authors. Note, Siegel et al (2001) reported recombination results for a sequence originally thought to be *lox2272*. However, upon inspection the published sequence it was not *lox2272* but rather *lox2372* (from Lee and Saito, 1998). (A. Bradbury, personal communication)

	WT	2272	FAS	511
WT	99.6			
2272	0.5	99.7		
FAS	0.2	1.7	99.4	
511	10.3	1.6	0.0	99.8

it is flanked by heterologous *lox* sites. The genomic target contains the matching *lox* sites by HR pre-placement. In the presence of Cre, during a double-reciprocal recombination event, there occurs a 50:50 probability of replacement of the *lox*-flanked chromosomal DNA by the targeting allele (Figure 4B). This exchange is referred to as recombinase-mediated cassette exchange, or RMCE. The RMCE system permits efficient insertion of *lox*-flanked DNA into the mammalian genome (Feng et al., 1999; Kolb, 2001; Trinh and Morrison, 2000). It is used to swap wild-type functional gene segments with knockout or otherwise mutated gene segments without incorporation of extraneous DNA.

Kolb used HR and site-specific RMCE to successfully insert a reporter gene into the mouse β-casein locus (Kolb, 2001). Kolb created a targeting construct consisting of *lox* 2272 and *loxP* sites flanking a selection marker that was integrated into the ES cells genome via HR. Recombination with a targeting construct containing a luciferase reporter gene flanked by *lox*2272 and *loxP* sites resulted in the efficient switching of the *lox*-flanked cassettes (Figure 4B). Typically, the HR step involves using selection markers such as geneticin (G418) or hygromycin resistance for positive selection placement of the *lox*-flanked gene segment. In RMCE, the selection marker is removed to avoid dysregulation of the modified allele. The loss of the selection marker by site directed recombination is tested by replica plating of cell clones in the appropriate selection medium.

CRE Recombinase Expression: Regulation in Mammalian Development

The Cre/*lox* system for genetic recombination also permits *lox*-flanked target gene alteration via stage- or tissue-specific control dependent upon the regulation of Cre gene expression *in vivo*. The majority of mammalian genes are thought to have developmentally regulated expression or they may express only in specific tissues. Because *lox* sites are quite short, their presence in the genome does not generally impair expression of their 'host' gene (Silver and Livingston, 2001; Trinh and Morrison, 2000). Often,

A. WT *loxP* and Mutant *lox* site sequences:

	8 bp core
loxP	ATAACTTCGTATA GCATACAT TATACGAAGTTAT
lox FAS	ATAACTTCGTATA TACC TTTC TATACGAAGTTAT
lox 2272	ATAACTTCGTATA GGATAC TT TATACGAAGTTAT
lox 2372	ATAACTTCGTATA GGATACC T TATACGAAGTTAT
lox 511	ATAACTTCGTATA GTATACAT TATACGAAGTTAT

B. Insertion of *floxed* sequences via double reciprocal recombination.

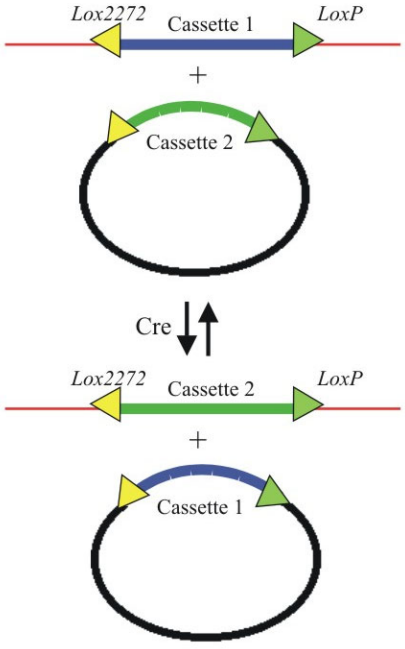

Figure 4. Recombinase Mediated Cassette Exchange (RMCE)

A) Sequence differences in mutant *lox* sites. B) Heterologous *lox* sites (in this example *loxP* and *lox* 2272) sites flanking an insert can be used to swap pre-placed genome cassette (Cassette 1) for a targeting insert (Cassette 2) that is flanked by the same *lox* sites. Recombination events that result in successful cassette exchange are assessed by molecular analysis or by antibiotic growth selection.

expression of a mutation in the whole animal rather than at a specific time or tissue location would prove lethal, thereby preventing the study of phenotypes of the gene (Schipani, et al., 2001). The targeted gene is flanked by *loxP* sites and then integrated into the genome via HR. The altered ES cells are then developed into mice with the *lox*-flanked gene intact. The Cre gene is engineered to express under the control of a cell-type-specific promoter, whichever suits the purpose of the study (Metzger and Chambon, 2001; Metzger and Feil, 1999; Schipani et al., 2001). A line of Cre tissue-specific expression transgenic mice is created separately and evaluated for cell-type specific Cre-expression. When the two transgenic mouse lines are mated, the progeny of doubly transgenic genotype will enable activation of Cre expression in the appropriate cell type or time of development. The mutation (usually an excision recombination) arises by Cre expression in most of the affected cells. Typically at low levels of Cre expression, some cells are mutated while others are not, creating genetic mosaicism in the tissue. Mosaicism may be useful for phenotype interpretation by providing modified and unmodified cells side by side.

In addition, it is important to assess whether Cre may cause untoward effects such as cell death arising from Cre expression in the transgenic parental and non-targeted littermate mice. Many transgenic mouse lines thought to have tissue-specific Cre-transgene expression appear normal outwardly and by histology (Lewandoski, 2001; Nagy and Mar, 2001). We shall see next why careful examination is warranted of tissue specific Cre-transgene expression.

CRE Recombinase Genotoxicity

While site-directed recombination is a useful tool for genetic manipulation, Cre recombinase is also inherently toxic to many mammalian cells lines. This toxicity is the result of the recombinase activity of Cre (Loonstra et al., 2001; Silver and Livingston, 2001). These researchers have reported total cessation of cell replication, cell death, and an abundance of chromosomal aberrations and aneuploidy following *high level* Cre recombinase expression. These events could be the result of illegitimate DNA recombination or strand breaks induced in the mammalian genome. Another observation consistent with this notion is that cells cultured in the presence of high levels of Cre showed an increase in the number of cells in the G2/M phase of the cell cycle (the period just before mitosis begins and mitosis itself). This result indicates that the DNA damage is severe enough to trigger marked G2/M cell cycle checkpoint arrest (for a RUR review of cell cycle check points, see the article by Renthal in this issue).

A corroborating discovery was that of the existence of pseudo-*lox* sites in the mammalian genome (Thyagarajan et al., 2000). Illegitimate mammalian genomic *lox* sites elicited Cre-mediated recombination. Indeed, Cre also induces recombination at secondary recombination sites that occur naturally in *E. Coli* and in yeast (called *lox*B sites) (Sauer, 1996; Sternberg and Hamilton, 1981). As Cre catalyzes apparent interaction between pseudo-*lox* sites in mammalian cells, these events could therefore result in deletions or other chromosome alterations. Consequently, Cre induced breaks at endogenous chromosomal sites may possibly complicate the interpretation of Cre/*lox* experiments.

The studies that elucidated this problem also offered possible technical solutions. Loonstra et al. used a hormone-regulated Cre gene that was expressed at negligible levels without induction (Loonstra et al., 2001). When cells were subjected to supra-basal but not saturating levels of the hormone, Cre expression was elevated sufficiently to catalyze excision of a *lox*-flanked reporter gene without inhibiting cell growth and without production of visible chromosome aberrations. In their work cited in Loonstra et al (2001) a moderate level of the hormone elicited complete excision of another *lox* flanked genomic target without apparent genotoxicity. Others have employed variations on 'hit and run' strategies utilizing a negative feedback loop to circumvent overt Cre genotoxicity (Pfeifer et al., 2001; Silver and Livingston, 2001). Cre expression vectors were engineered to produce low levels of Cre coupled with a genetic negative feedback loop to limit the amount of Cre in the cells. Silver and Livingston used a retroviral vector containing a Cre expression gene with a single *lox* 511 site in its LTR. This retroviral vector was engineered so that following its reverse transcription and genome integration, the Cre-expression vector contained duplicated LTRs with co-aligned *lox* 511 sites. When Cre was expressed at a level high enough to cause recombination between the *lox* 511 sites, the entire Cre gene was auto-excised removing further synthesis of Cre after a few cell generations. This strategy resulted in cells capable of targeted excision of *lox*-flanked sequences in an unlinked target gene. As Cre expression was limited, there were no observed genotoxic effects.

It is also likely that the amount of Cre expressed in ES cells in culture can be controlled simply during the gene transfer process. Linear DNA introduced into ES cells by the technique of electroporation is efficiently integrated

into the genome, either randomly or by HR, in ES cells. In contrast, circular plasmid DNA has approximately 8 fold lower probability of chromosome integration (Taniguchi et al., 1998). This difference can be exploited to control magnitude and duration of Cre-recombinase expression simply via the transient presence of Cre expression plasmid. In addition, a fluorescence reporter plasmid called 'Cre-Stoplight' has been developed recently to bioassay Cre recombinase activity in live cells by epifluorescence microscopy or flow cytometry (Yang and Hughes, 2001). The plasmid incorporates dual reporter gene cassettes containing a *lox*-flanked Discosoma coral fluorescent protein, DsRed, and a transcriptionally inactive green fluorescent protein (EGFP). When sufficient Cre is expressed in cells containing Cre-Stoplight the DsRed gene is excised and rendered inactive by virtue of its *flanking lox* sites. Then the upstream promoter is brought into apposition to the EGFP gene. Therefore, mouse ES cells taking up DNA after 72 hours show considerable fractions of cells (> 20 %) with both red and green epifluorescence caused by the switch of DsRed to GFP production (K. Nowak and M. MacInnes, unpublished observations). We are now investigating the utility of monitoring levels of Cre recombinase by transient expression of Cre Stoplight to obtain efficient GFP activation, and presumably recombination at specific genomic target sites. As indicated above, for *in vivo* experiments similar engineering of tissue-specific autoexcision, or autoregulation, of Cre transgene may help avoid the possibility of confounding non-specific genotoxicity in the developmental stage or tissue of interest.

Site-directed Recombination in Mammalian Functional Genomics and Human Gene Therapy

The Cre/lox recombination system and HR have given researchers powerful tools for investigating novel mammalian genes. Conversely, they can also be used to create controlled gross deletions, inversions, and chromosome mitotic recombination in order to characterize certain genetic disease processes. The rich applications of Cre/lox hold promise for elucidating thousands of novel gene functions, an essential integrative genetics component of the functional genomics / systems biology era. The recent completion of the draft sequences of both mouse and human genomes will greatly facilitate building HR and Cre/lox recombination vectors in both mouse and human cells. The production of gene targeting vectors for HR and Cre/lox strategies must be automated into a high-throughput enterprise in order to realize the full potential of these approaches (Copeland et al., 2001). Characterization of gene function in ES cells and their derivatives in vitro would facilitate preliminary genetic analyses without necessitating very costly and ethically questionable production of tens of thousands of new mutant mouse lines.

It is of great interest whether HR gene targeting is possible in human ES cells given that few or no diploid human cell lines have yet proved useful for HR and site-directed recombination. Recent provocative research produced successful isolation of stem-like cells from human and monkey embryos (Reubinoff et al., 2001; Reubinoff et al., 2000; Thomson and Marshall, 1998) and from parthenogenetically activated Macac eggs (Cibelli et al., 2002). Similarly, adult stem cells have been isolated from mammalian bone marrow, liver, pancreas and brain (for a review see Clarke and Frisen, 2001). These milestones raise controversial ethical possibilities that human cell therapy (and huES cell genetic engineering) may become a reality for numerous diseases with a genetic component. In theory, human stem cells could be 'corrected' through retroviral vector incorporation or via HR, and this approach complemented by Cre/lox genetics. Two major technical concerns in cell replacement therapy are, first, the possibility of implanted cell/tissue rejection. Ideally, this difficulty is circumvented by use of the patient's own (autologous) stem cells. The second technical problem concerns a significant possibility of a carcinoma arising from implanted stem-like cells. As illustrated in this brief review, we have shown how activation or reversal of targeted genetic modifications can be engineered using Cre/lox. This approach may offer opportunities to provide additional safeguards against neoplasia in therapeutic strategies involving cell replacement with human stem cells.

About the Author

Anna Norman is currently a junior at the College of St. Benedict in St. Joseph, Minnesota where she is majoring in Biology. After graduation, she hopes to attend either medical school or to enroll in a joint MD / PhD program. During the summer of 2001, she was accepted to a National Science Foundation sponsored Research Experience for Undergraduate students (REU) Program hosted by the Los Alamos National Laboratory. Under the mentorship of Dr. Mark A. MacInnes, a geneticist in Biosciences Division, she tested recombination efficiency of a homologous and heterologous pair of *lox* sites flanking

a marker gene introduced into mouse ES cells. Using recombinant DNA techniques, Ms. Norman assembled the plasmid vectors containing pairs of *lox* sites flanking a drug resistance marker gene. These vectors were then linearized and introduced into mouse ES via electroporation. Recombination efficiency was analyzed based on the number of colonies that grew, and cell growth rate, in culture medium containing antibiotics. Using this method she found resistance to intra-molecular recombination between *lox* 2372 and *lox* FAS but proficient as recombination as expected between *loxP* sites. During the experiment, quantifying colony yield and cell regrowth confirmed that certain toxic effects of Cre recombinase occurred in mouse ES cells leading in part to the discussion of Cre genotoxicity in this review. Improvement of methods outlined in the review is a major emphasis of the MacInnes laboratory at this time.

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