Conditional Gene Knockout Using Cre Recombinase

*Yunzheng Le and Brian Sauer**

Abstract

Cre recombinase has become an important instrument for achieving precise genetic manipulation in mice. Many of these desired genetic manipulations rely on Cre's ability to direct spatially and temporally specified excision of a predesignated DNA sequence that has been flanked by directly repeated copies of the *loxP* recombination site. Success in achieving such conditional mutagenesis in mice depends both on the careful design of conditional alleles and on reliable detection of *cre* gene expression. These procedures include PCR, immunohistochemistry and the use of a recombination-proficient GFP-tagged Cre protein.

Index Entries: Cre; site-specific recombination; knockout; FACS; ES cells; GFP; STOP cassette; fusion proteins; transgenic mice; *loxP*.

1. Introduction

The directed introduction of null mutations into defined genes has proven invaluable in elucidating gene function in a variety of experimental organisms. In the last decade or so this approach has been extended to mice ***(1)*** by the combined use of homologous recombination in murine embryonic stem (ES) cells to precisely target a mutation to a desired gene and subsequent derivation of mice carrying the targeted gene alteration from the genetically manipulated ES cells (e.g., by injection of gene-modified ES cells into blastocysts with subsequent germline transmission). In most instances null, or knockout (KO), mutations have been generated in mice by either simple insertion of a *neo* selectable marker in the target gene or *neo* insertion coupled with deletion of a critical region of the target gene. Targeted null mutations in a gene of interest, however, can lead to embryonic lethality in mice, thus obscuring the particular role of that gene in a target tissue or in the adult.

Site-specific recombination strategies allow circumvention of the problem of embryonic lethality by directing gene ablation in a spatially and temporally controlled manner. Because the Cre recombinase of phage P1 catalyzes efficient excisive recombination in mammalian cells ***(2)***, it has been become a useful tool for generating a conditional KO ***(3)***. In addition, Cre-mediated excision has been useful both for targeted activation of genes in transgenic mice and for elimination of the selectable drug marker that is necessarily left in the genome after homologous gene targeting in ES cells ***(4–6)*** (*see* **Note 1**).

The 38 kDa Cre recombinase catalyzes DNA recombination between specific 34-bp sequences called *loxP* ***(7)***. This site exhibits two inverted 13-bp repeats and a central asymmetric 8-bp core region that confers an overall directionality to the site (**Fig. 1**, *see* **Note 2**). Cre-mediated recombination between two directly repeated *loxP* sites on a DNA molecule results in excision of DNA between the two *loxP* sites. Mutant sites, such as

*Author to whom all correspondence and reprint requests should be addressed: Dr. Brian Sauer, Developmental Biology Program, Oklahoma Medical Research Foundation, Oklahoma City, OK 73104. E-mail: sauerb@omrf.ouhsc.edu

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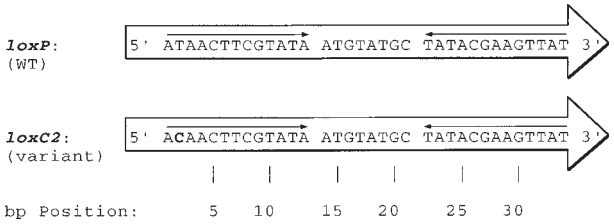

Fig. 1. Recombination sites for Cre recombinase. The wild-type *loxP* and variant *loxC2* sites are shown, with bp positions indicated below. The 13-bp inverted repeats are indicated by the thin arrows above the sequence. The *loxC2* site differs from the wt site by replacing the T at position 2 with a C, and is named accordingly. By convention, the sense of the overall directionality of the *lox* site is as indicated by the large arrow. Note that *loxG33* would be functionally equivalent to *loxC2*, because of symmetry.

loxC2, having base changes in the outer 4 bp of one of the inverted repeats (positions 1–4 or 10–13) are also recognized by Cre recombinase ***(8)***. The *loxC2* site is a useful alternative to *loxP* when the site will be placed into transcribed sequences because it decreases stability of the 13-bp inverted repeat-derived hairpin in RNA. Such hairpins in the 5' untranslated RNA region can diminish translation.

1.1. Strategies for Genomic Manipulation

Conditional gene deletion allows assessment of a gene's function in a target tissue without disturbing expression of that gene in nontarget tissues. Two components are required: a target mouse carrying a gene-modified allele of the gene to be ablated and a *cre* transgenic mouse that expresses Cre under the control of a promoter with the desired spatial and temporal pattern of expression. Exact placement of *lox* sites in the target gene will depend both on the type of deletion event desired and on constraints imposed by the structure of the target gene. An example of a target-gene modification strategy in ES cells for the generation of a conditional KO is shown in **Fig. 2A** (*see* **Note 3**). The *neo* selectable marker, flanked by two directly repeated *lox* sites (a lox^2 *neo* cassette), is placed at one of the deletion endpoints (shown here in the first intron) and a third *lox* site is placed at the other deletion endpoint (shown here in the 5' leader before the first ATG in exon I) (*see* **Note 4**). Standard homologous recombination in ES cells is used to modify the target-gene locus (shown here by a double crossover event).

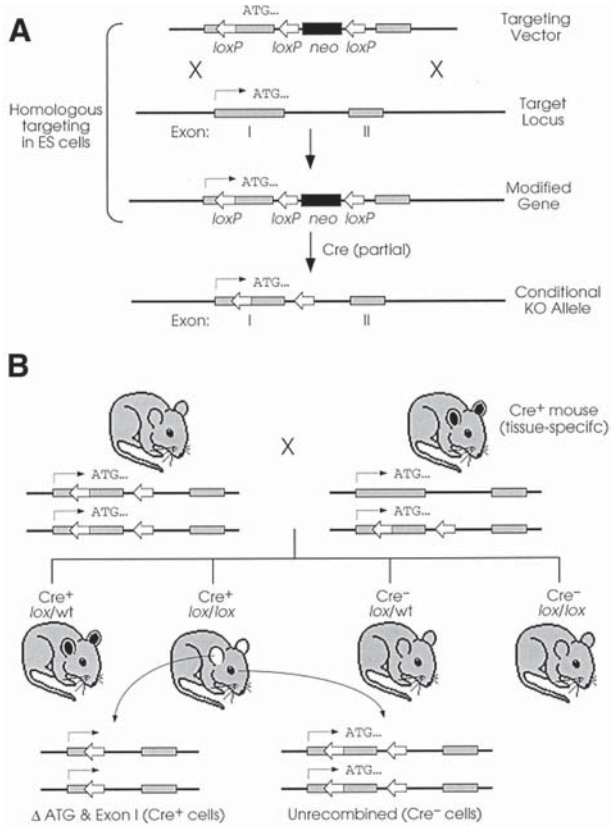

Fig. 2. A conditional KO strategy. **(A)** Targeting of the genomic locus by homologous recombination generates a modified gene that carries the correctly placed *lox* sites (indicated by white arrows) and the *neo* selectable marker (black rectangle). A second transfection step with a *cre* expression plasmid can be used to give partial excision. ES cells with the desired genomic structure are injected into blastocysts to give chimeric founders. **(B)** Mating strategy for a conditional KO. The desired double "transgenic" inherits a conditional allele from one parent and the *cre* transgene and the second copy of the conditional allele from the other. Note that the *cre* transgenic parent used in this cross is the product of a previous cross of a *cre* transgenic with the conditional KO mouse so that it is both heterozygous at the target KO locus and positive for the *cre* transgene. Tissue-specific Cre expression is shown here by the black ears. In the Cre^+ mouse homozygous for the conditional allele, productive recombination in the ear is represented by the white ears.

Because *neo* could interfere with the correct expression of the target gene (depending on its placement in a particular strategy), it may be prudent to remove it by a limited Cre-mediated recombination event that removes the *neo* interval but leaves intact the genomic interval that is to be later targeted for deletion. Removal of *neo* can be effected in ES cells by transient transfection with a Cre-expressing construct.

As shown in **Fig. 2B**, conditional gene KO is accomplished by the mating of two animals, one having the *lox*-modified target locus (most conveniently a homozygote) and the other carrying the *cre* transgene and, in addition, one copy of either a null allele in the target locus or a copy of the *lox*-modified target locus. Cre-mediated recombination then excises the target gene from the genome in a manner that reflects the (tissue-specific) pattern of expression of the *cre* gene. Penetrance of expression of the *cre* transgene will govern the ratio of deletion vs nondeletion on a per cell basis in the target tissue (*see* **Note 3**).

A similar mating strategy is used for targeted activation of a transgene or endogenous gene in a tissue-specific developmental or inducible fashion (**Fig. 3**). A "STOP" cassette flanked by two directly repeated *loxP* sites (a lox^2 STOP cassette) is placed between the promoter and the gene to be activated ***(4)***. STOP is designed to block gene expression and consists of a stuffer region (from the yeast *HIS3* gene), the SV40 polyadenylation region, and errant optimized ATG translational start and splice donor signals. Cre-mediated excision removes STOP thus permitting target-gene expression under the control of the adjacent promoter. Two animals are required: one expressing Cre with the desired spatial and temporal pattern and the other either carrying a lox^2 STOP-equipped transgene or, alternatively, an animal modified by homologous gene-targeting in ES cells to carry the lox^2 STOP cassette in the desired endogenous gene. Mating of these two animals results in activation of the target gene in those cells that both express (or have expressed in a progenitor cell) Cre recombinase and are capable of expressing the target gene, as determined by the specificity of the promoter used in the *lox* target strain.

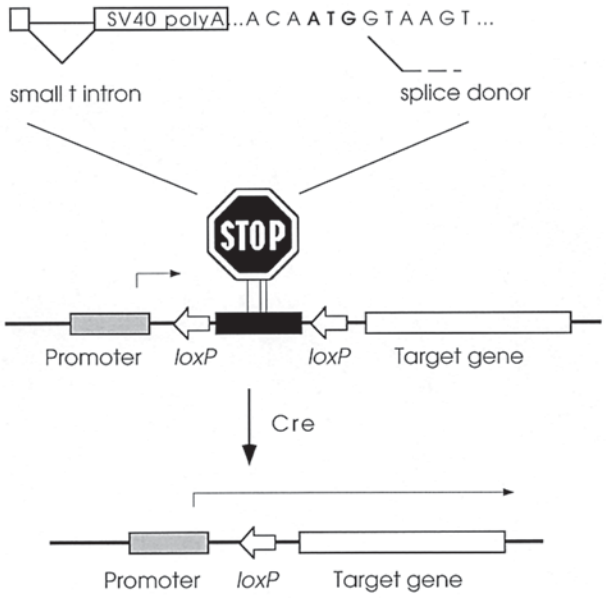

Fig. 3. Recombinational activation of gene expression. A synthetic STOP sequence (GenBank Accession No. U51223), is flanked by two directly repeated *lox* sites (white arrows) and is placed between a promoter and the target gene to be activated. Cre expression removes the STOP signal to allow target gene expression, leaving behind a single 34-bp *lox* site.

To target recombination to a particular tissue, Cre should be specifically expressed in the target tissue. This can be achieved by making a transgenic mouse with *cre* under the control of a promoter with the desired expression characteristics. Alternatively, the *cre* gene can be targeted to be under the control of an endogenous promoter in the genome by using homologous recombination in ES cells (a "knock-in"). The pattern of Cre mouse expression should be checked using a Cre-specific antibody ***(2,9)***, rtPCR, or *in situ* RNA hybridization. This is important because some promoters are susceptible to position effects, and resulting founder lines may express the transgene in a mosaic fashion in the target tissue or misexpress the transgene in an unwanted tissue. Gene ablation (or gene activation) will be difficult to achieve in the majority of cells in a target tissue if Cre is expressed in a variable and mosaic fashion. *cre* knock-ins may thus be preferred unless the promoter used in a transgenic strategy has already

been established to behave with the desired expression pattern in a transgenic (e.g., by monitoring expression of a *lacZ* reporter gene).

2. Materials

2.1. Detection of the cre Transgene by PCR

1. DNA oligonucleotide primers are stored at –20°C at a stock concentration of 3 µ*M*.
   Sense: 5' AGG TGT AGA GAA GGC ACT TAG C 3'
   Antisense: 5' CTA ATC GCC ATC TTC CAG CAG G 3'
2. 10X PCR buffer: 500 m*M* KCl, 100 m*M* Tris-HCl pH 8.3, 20 m*M* $MgCl_2$, stored at –20°C.
3. dNTP's: 12.5X stock solution, 2.5 m*M* of each in H_2O, stored at –20°C.
4. AmpliTaq polymersase (Perkin-Elmer, Norwalk, CT) at 250 U/50 µL, stored at –20°C.
5. Perkin Elmer 9600 thermocycler, or equivalent, and 0.2 mL PCR reaction tubes.

2.2. Antibody Detection of Cre Protein in Cells

1. Anti-Cre mAb 7.23 (Berkeley Antibody Co., Richmond, CA), 1 mg/mL.
2. Formaldehyde (Electron Microscopy Sciences, Ft. Washington, PA), 16% solution.
3. Normal goat serum (Life Technologies, Inc., Gaitherburg, MD).
4. FITC-conjugated goat anti-mouse IgG1 polyclonal serum (Southern Biotech., Birmingham, AL).
5. Phosphate-buffered saline (PBS); PBS/B (PBS + 0.5% BSA + 0.01% NaN_3); PBS/B + S (PBS/B + 0.5% saponin). Filter with 0.2-µm filter.
6. Epifluorescence microscope for FITC detection.

2.3. Gene Popout in ES Cells by GFPcre

All cell culture reagents are from Life Technologies, Inc., unless otherwise indicated.

1. Culture dishes: Falcon 3002 (6 cm).
2. PBS without calcium and magnesium.
3. 0.2% Gelatin (Sigma) in PBS, autoclaved.
4. Irradiated mouse embryonic fibroblasts (EFB, made in the laboratory).
5. ES medium: Dulbecco's modified Eagle's medium (DMEM) suplemented with 20% fetal calf serum (FCS, Hyclone, Logan, UT), 1000 U/mL ESGRO, 2 m*M* L-glutamine, 0.1 m*M* of nonessential amino acids and 0.1 m*M* of β-mercaptoethanol, with 50 U/mL of penicillin, and 50 µg/mL of streptomycin.
6. 0.1% trypsin in PBS with 0.05 m*M* ethylene diamine tetraacetic acid (EDTA).
7. Plasmid pBS500, EF1α-GFP*cre* ***(10)*** or pBS598 ***(11)***.
8. 2.5 *M* $CaCl_2$.
9. 50 m*M* BES solution: 50 m*M*, *N,N-bis*[2-hydroxyethyl]-2-aminoethanesulfonic acid, 280 m*M* NaCl, and 1.5 m*M* Na_2HPO_4, adjust pH to 6.96 with HCl.
10. Epifluorescence microscope for FITC (GFP) detection.

3. Methods

3.1. Detection of the cre Transgene by PCR

After identification of *cre* transgenic animals by Southern blotting, it is convenient to monitor *cre* transgene transmission by PCR, using standard genomic "tail DNA" ***(12)***. PCR amplification yields a 411 bp product diagnostic for *cre* (GenBank Accession No. X03453).

1. In a 0.2-mL reaction tube add 50–500 ng of genomic DNA, 5 µL of 10X PCR buffer, 4 µL of 10X dNTPs, 5 µL of each sense, and antisense primers, and H_2O to 50 µL total volume.
2. Denature 5 min at 94°C.
3. Add 0.5 µL of AmpliTaq polymerase.
4. Perform 25 or 30 cycles of amplification: 30 s at 94°C, 30 s at 63°C, 1 min at 72°C.

3.2. Antibody Detection of Cre Protein in Cells

Indirect immunofluorescence with anti-Cre specific antibody is used to assess tissue-specific expression and also the level of mosaicism of expression in a population of cells. Detection by antibody quickly identifies Cre-expressing animals that may be suitable for further analysis ***(9)***. Alternatively, or in addition, rtPCR can be performed using the *cre* oligos described in **Subheading 3.1.**

1. Cre-expressing cells are washed once with PBS and fixed with PBS + 2% formaldehyde for 20 min at room temperature. All steps are at RT unless otherwise indicated.
2. Wash twice with PBS and once with PBS/B.

3. Permeabilize with PBS/B + S for 6 min.
4. Block with PBS/B + 5% normal goat serum for 30 min.
5. Incubate with anti-Cre mAb 7.23 (diluted 1/100 in PBS/B + S) for 15 min. Incubate longer if necessary.
6. Wash three times with PBS/B + S.
7. Incubate with FITC-conjugated goat antimouse IgG1 polyclonal serum (10 µg/mL in PBS/B + S containing 7% normal goat serum) for 20 min.
8. Wash three times with PBS/B + S.
9. Examine by epifluorescent microscopy.

3.3. Gene Popout in ES cells by GFPcre

After targeted modification of a locus in ES cells by homologous recombination, removal of the selectable marker (for example *neo*) may be desired in order to preclude interference by the marker gene on correct gene expression at the target locus. Incorporation of a *lox*^{2} *neo* cassette into the homologous targeting vector allows subsequent Cre-mediated removal of the *neo* gene from the targeted locus. This step is facilitated by use of a functional fusion between Cre and an enhanced fluorescent derivative of the GFP of *Aequorea victoria* ***(13)***. Transient transfection with either the GFP*cre* fusion construct pBS500 ***(10)*** or the EGFP*cre* fusion construct pBS598 ***(11)*** results in cells that are simultaneously GFP$^+$ (easily identified because they are fluorescent) and Cre$^+$ (and, hence, committed to excision of *neo*). Because the transfection efficiency of ES cells is often low, it is convenient to enrich for productively transfected ES cells by using fluorescence (**Fig. 4**). We have found that gene transfer by calcium phosphate coprecipitation with DNA ***(14)*** to give somewhat more routinely efficient transient transfection than electroporation. Alternatively, standard electroporation of ES cells ***(15)*** with pBS500 (EF1α-GFP*cre*) or pBS598 (EF1α–EGFP*cre*) can be used with good results.

1. Plate 2×10^6 ES cells on a gelatinized 6-cm dish seeded with 2×10^6 irradiated EFB two days before the transfection. Change medium next day and also on the second day, 3 h before transfection.
2. Combine 9 µg pBS500 or pBS598 DNA with H_2O (total volume for DNA and H_2O is 165 µL), add 18.3 µL 2.5 *M* $CaCl_2$ and mix. Add the DNA solution drop-wise into 183 µL BES solution, mixing gently and continuously. Incubate 20 min at RT.
3. Wash the dish of ES cells with 5 mL PBS, add 2 mL 0.1% trypsin solution and incubate 2 min at RT. Remove the trypsin solution and incubate the dish at 37°C for 5 min. Add 4 mL medium to inactivate the trypsin. Count the cells and dilute the ES cells in the medium to 3×10^5 cell/mL. This ES cell suspension will be mixed with the DNA/$CaPO_4$ coprecipitate.
4. Mix the DNA/$CaPO_4$ coprecipitate solution with 5 mL of the ES cell suspension (1.5×10^6 ES cells total). Plate the mixture on a 6-cm gelatinized dish and incubate overnight at 37°C.
5. Wash the transfected ES cells with DMEM and replace with fresh medium 16 h after transfection.
6. Fluorescence should be observed in about 2–7% of the GFP*cre*-transfected cells 2 d after transfection. After trypsinization, fluorescent (Cre$^+$) ES cells can be either sorted manually at this time with a micromanipulator, or by FACS.

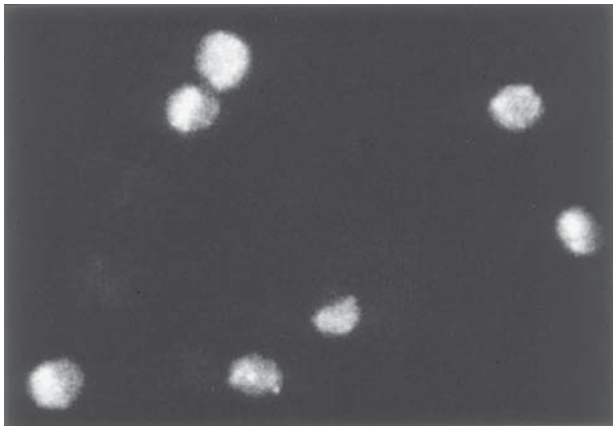

Fig. 4. GFPCre expression in ES cells. ES cells were transiently transfected with pBS500 (EF1α-GFP*cre*) by $CaPO_4$ coprecipitation. Two days later cells were trypsinized and examined for fluorescence.

4. Notes

1. Genomic manipulation strategies with the distantly-related DNA recombinase FLP (from yeast 2-µ circle) are conceptually similar to those for Cre recombinase. Somewhat greater variability in efficiency of recombination in mammalian cells has been reported for FLP ***(16,17)***.

2. There are two "ATG's" in the conventional orientation of *loxP* as shown in **Fig. 1**. However, in the reverse orientation there are no ATGs, hence the reversed orientation is preferred when inserting the *lox* sequence into the 5' RNA leader region of a gene.
3. In the conditional KO strategy, the *cre* transgenic parent in the "knockout activation" cross is heterozygous at the target KO locus for the conditional allele. Alternatively, the *cre* parent can be heterozyous at the target locus for a wt and a complete null. In this case, it should be verified that animals heterozygous for the null allele at this locus are phenotypically wt.
4. To evaluate the recombination potential of plasmids containing two *loxP* sites, it is convenient to transform the plasmid into the Cre-expressing *E. coli* strain BS591 [F^- *recA1 endA1 hsdR17* δ*lac*(*lacZYA-argF*)*U169 supE44 thi-1 gyrA96* (λ *imm434 nin5* X1-*cre*)]. Plasmid DNA isolated from this strain will have undergone Cre-mediated recombination and can be checked by a simple restriction digest ***(18)***. Alternatively, and more rigorously, *lox* regions should be sequenced in the final plasmid construction.
5. PBS + 0.1% Triton X-100 for 6 min also gives permeabilization of cells for indirect immunofluorescent detection of Cre.
6. Cre protein can be detected in target tissues by standard Western blotting or immunoprecipitation by using the anti-Cre mAb 7.23 at dilutions of 1/1000 and 1/150, respectively.
7. The pattern of Cre expression from a *cre* transgenic or knock-in animal may also be determined using the GFP*cre* gene. This potentially would allow direct detection of Cre but most likely would only be useful in situations in which the promoter used is known to give relatively strong expression (to allow direct detection of fluorescence) in the target tissue. Since only a single catalytic event is required for productive recombination in a cell, the actual amount of Cre required per cell is not high, and antibody detection is potentially more sensitive than reliance on fluorescence from GFPCre.
8. As an alternative to excision of the *loxP*-flanked *neo* gene in ES cells by transient transfection with a *cre* plasmid, ES cells can be injected directly into blastocysts to give a chimeric founder that is then mated with the EIIa-*cre* mouse ***(19)***. The EIIa promoter directs Cre expression only in fertilized zygotes and early embryos, and is not expressed postimplantation, thus allowing *neo* removal from the germline. In multi-*lox* transgene arrays, both partial and complete deletion events can be obtained that are then transmitted through the germline.
9. GFPCre localizes to the cell nucleus ***(10)*** since Cre carries an endogenous nuclear localization signal ***(20)***.
10. ES cells should be trypsinized completely to facilitate FACS sorting and to ensure elimination of mosaic colonies. Following sorting of ES cells transiently transfected with GFP*cre*, resulting colonies should be confirmed for excisive recombination by Southern or a suitable PCR assay. Most (≥80%) fluorescent cells will give rise to colonies carrying the desired *lox*-delimited deletion. DNA analysis will also confirm that the colony is not mosaic for both recombinant and nonrecombinant cells.

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