

An inbred 129SvEv GFP-Cre transgenic mouse that deletes *loxP*-flanked genes in all tissues

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ABSTRACT

A common method for generating mice with subtle genetic manipulations uses homologous recombination (HR) in embryonic stem (ES) cells to replace a wild-type gene with a slightly modified one. Generally, a drug resistance gene is inserted with the modified gene to select correctly targeted clones. Often, however, the presence of this drug resistance gene interferes with the normal locus and creates a null or hypomorphic allele. Flanking of the selectable marker by *loxP* sites followed by Cre-mediated deletion after drug selection can overcome this problem. The simplest method used to remove a *loxP*-flanked selectable marker is to breed an animal carrying a *loxP*-flanked drug resistance gene to an animal that expresses Cre recombinase in the germline. To date only outbred transgenic mice are available for this purpose. This can be problematic for phenotypic analysis in many organ systems, including the brain, and for the analysis of behavior. While attempting to make 129S6/SvEvTac inbred background (isogenic to our ES cells) mice that express Cre under the control of several tissue-specific promoters, we serendipitously generated a line that excises *loxP*-flanked drug resistance genes in all tissues, including the germline. This reagent allows deletion of *loxP*-flanked sequences while maintaining the mutation on an inbred background.

INTRODUCTION

A common method for generating mice with subtle genetic manipulations, for example inserting point mutations rather than creating a null allele, uses homologous recombination (HR) in embryonic stem (ES) cells to replace a wild-type gene

with a modified one. In most cases a neomycin resistance gene (*NeoR*) or other drug resistance gene is inserted into an intron or in the 3'-untranslated region (UTR) near the desired alteration to allow for selection of correctly targeted clones. The goal of this use of HR and insertion of *NeoR* is to make precise changes upstream of the *NeoR* insertion rather than to generate a null allele. However, in many cases the presence of the drug resistance gene interferes with the normal locus, resulting in a null or hypomorphic allele (1,2). To overcome this problem, the drug resistance gene, flanked by *loxP* sites, can be removed by exposure to Cre recombinase (Cre).

Cre can be introduced by several methods. First, transfection of a Cre-expressing plasmid into ES cells (1). Second, injection of a Cre-expressing plasmid into fertilized eggs (3,4). Finally, mating of the mouse mutant created using ES cell technology to a transgenic mouse that expresses Cre in the germline (1,5). The latter method is the simplest. To date only outbred mice are available at Jackson Laboratories for this purpose. The use of outbred mice can be problematic for phenotypic analysis in many organ systems, including the brain, and for the analysis of behavior (6). Another option is to use a recently described inbred mouse line in which the Cre recombinase expression cassette was inserted into the X-linked hypoxanthine guanine phosphoribosyltransferase gene (*Hprt*). The advantage of this mouse is that the researcher knows where the Cre is inserted in the genome (7). However, this insertion creates a null allele at the locus and produces a complete knockout of *Hprt* in the homozygous animal. Although this knockout does not produce a phenotype, it may lead to a synthetic phenotype in its offspring when it is bred to another transgenic mouse with a floxed allele. In order to maintain the mutation in an inbred background, transfection into ES cells or pronuclear injection of a Cre-expressing plasmid is required. Both of these latter methods are labor intensive. We generated a transgenic mouse line in an inbred 129S6/SvEvTac (129) strain that expresses Cre and demonstrate that deletion of a *loxP*-flanked *NeoR* gene (*loxP-NeoR-loxP*) occurs in all tissues, including the germline. This

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reagent can be used to delete *loxP*-flanked sequences in all tissues while maintaining a background isogenic to that of commonly used 129 ES cells.

MATERIALS AND METHODS

Transgene construct

The green fluorescent protein (GFP) gene was removed from pEGFP-C1 (Clontech) using *Eco47111/XhoI*. The GFP sequence was isolated by gel purification and inserted into *EcoRV/XhoI*-digested pBSKS (Stratagene) to generate pBSKSGFP. The 1.1 kb Cre recombinase gene was amplified using the plasmid pCre306C as a template (courtesy of Steve O'Gorman) using the 5' primer 5'-GCT AGC TCC GGA GAG CAA AAG CTG-3' and the 3' primer 5'-CTC TAG CTC GAG CTA ATC GCC ATC-3', which included a 5' *BspEI* site and a 3' *XhoI* site for use in generating an in-frame fusion of Cre to GFP. The product was gel purified and inserted into pBSKSGFP at the *BspEI/XhoI* site, to generate pBSKSGFPCre. The final transgenic vector was constructed by inserting the purified 1.86 kb *NotI* blunted GFPCre fusion cDNA into blunted *KpnI* and *NotI* sites in the plasmid hgPrnp (8,9) downstream of a modified prion promoter. The Prion.GFPCre plasmid (Fig. 1A) was purified using CsCl ultracentrifugation twice according to published methods (10). The purified plasmid was digested using *MluI* and *SalI* to release the backbone. The 5.7 kb Prion.GFPCre fragment was isolated after separation on a 1% agarose gel overnight at 4°C using Gene Clean (Qbiogene, Carlsbad, CA).

Production of transgenic 129 mice

All animal procedures were performed according to protocols approved by both The Salk Institute for Biological Studies and the National Institutes of Health Animal Care and Use Committees. The procedures for superovulation, embryo recovery, microinjection and transfer were similar to those described (10). Female 129 mice at 4 weeks of age were used as embryo donors after superovulation with 2.5 U pregnant mare's serum (PMS) or gonadotropin-PMS (catalog no. 367222; Calbiochem) and 2.5 U chorionic gonadotropin (catalog no. 0052-0315-10; Organon) 46 h apart. Stud males were 129 mice. Recipient females (CBy6F1, 8 weeks or older) were placed with CB6F1 vasectomized males. Microinjections were performed at 200× magnification using a Zeiss Axiovert 135M, Eppendorf micromanipulator 5171 and automatic Eppendorf microinjector 5242. Embryos were injected in M2 (catalog no. MR-015-D; Speciality Media) and then incubated in BMO2 (catalog no. MR-013-D; Speciality Media) at 37°C in 5% CO₂ overnight. The two-cell embryos were separated from the one-cell embryos the following morning and surgically transferred to primed recipients.

Determining where Cre-mediated excision occurs

Genotypes were determined by PCR using DNA prepared from tail biopsies or several brain regions, kidney, testes and liver using Phase-Lock Gel reagent (Eppendorf) after overnight lysis (11). Cre(+) mice were identified using the 5' Cre-specific primer 5'-CCG GGC TGC CAC GAC CAA-3' and 3' Cre-specific primer 5'-GGC GCG GCA ACA CCA TTT TT-3'. To determine if Cre-mediated excision occurred, PCR was

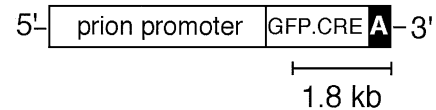


Figure 1. Transgene construct. A 5.5 kb minimal promoter and the first intron of the prion gene were ligated to a fusion protein of GFP (Met1–Lys239) and the Cre protein. A rat growth hormone poly(A)⁺ tail was used to ensure appropriate processing.

performed on DNA using primers to detect either a wild-type allele (Fig. 2A, upper panel, primers 1 and 2), the *loxP-NeoR-loxP* allele (Fig. 2A, middle panel, primers 1 and 3) or an allele where the *NeoR* gene was deleted leaving a single intact *loxP* site (Fig. 2A, lower panel, *loxP* primers 1 and 2). PCRs were run on 0.8% agarose-TBE gels.

Immunofluorescence

Mice were perfused with cold 4% paraformaldehyde transcardially. The kidney, testes, brain, and liver were dissected and submerged in 30% sucrose for 2 days before microtome sectioning (40 μm). Sections were stained with DAPI, analyzed and photographed using confocal microscopy (Bio-Rad).

Generating homozygous Cre(+) mice

A heterozygous 129 Cre(+) male was mated to a heterozygous 129 Cre(+) female. Cre(+) offspring derived from the mating were mated to wild-type animals and the genotypes of the offspring were determined by PCR. The probability of the mice being homozygous was calculated by using the Rule of Multiplication on a standard Punnett Square of a heterozygote mated to a wild type.

RESULTS AND DISCUSSION

Production of transgenic 129 mice

Of 40 superovulated female 129 mice, approximately 200–250 embryos were harvested and 97 embryos at the two-cell stage were recovered after microinjection. These embryos were implanted and 11 pups were born. Screening by PCR identified two Cre(+) founder animals. Both founders were bred to 129 wild-type mice to generate inbred F₁ offspring. Only one founder (98030B) produced Cre(+) transgenic offspring.

Cre(+) transgenic line deletes a *loxP*-flanked gene

The 98030B Prion.GFPCre transgenic founder line was studied to assess where Cre-mediated excision occurred. To generate appropriate mice, the founder male was bred to a Cre(-) female 129 mouse carrying a wild-type allele (Fig. 2A, WT) and a *NeoR* gene flanked by *loxP* sites (Fig. 2A, *loxP-NeoR-loxP*). DNA isolated from tails of Cre(+) offspring was screened for evidence of Cre-mediated deletion of the *loxP*-flanked *NeoR* gene. The 187 bp wild-type allele was amplified in both the breeder female (Fig. 2B, lane A, lower band) and the offspring (Fig. 2B, lane B, lower band), as would be expected. An additional 221 bp fragment was amplified in Cre(+) offspring (Fig. 2B, lane B, upper band), consistent with excision of the *NeoR* gene and retention of a single *loxP* site.

To determine the extent of Cre-mediated excision, PCR was used to detect any intact *loxP-NeoR-loxP* DNA. The breeder female had an intact *loxP-NeoR-loxP* allele (Fig. 2C, lane A'), whereas her Cre(+) offspring showed no amplification of the *loxP-NeoR-loxP* (Fig. 2C, lane B'), consistent with complete deletion of the *NeoR* gene in the Cre(+) offspring.

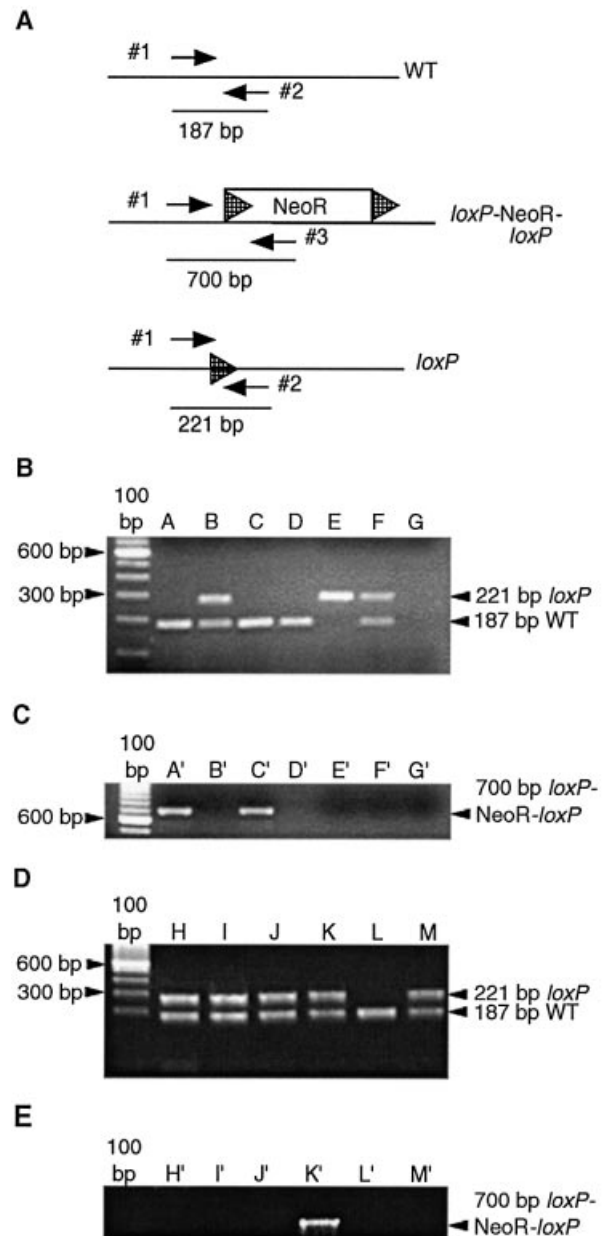
To test if the deletion occurred in the germline of the Cre(+) offspring whose tail DNA showed deletion of the *NeoR* gene, a Cre(+) male offspring (Fig. 2B and C, lanes B and B') was mated to a Cre(-) female carrying one wild-type allele (Fig. 2B, lane C) and one *loxP-NeoR-loxP* allele (Fig. 2C, lane C') and the genotype of three resulting offspring from the mating determined (Fig. 2B and C, lanes D-F and D'-F'). One offspring was Cre(+), but was homozygous for the wild-type allele (Fig. 2B, lane D), and therefore no *loxP-NeoR-loxP* products were amplified (Fig. 2C, lane D'). One of the offspring was Cre(+) and only showed amplification of the deleted allele (Fig. 2B, lanes E), consistent with inheriting the deleted allele from the male parent and also exhibiting Cre-mediated deletion of the *loxP-NeoR-loxP* allele (Fig. 2C, lane E') inherited from the female parent. Additionally, the offspring that was Cre(-) had one copy of the wild-type allele and one copy of the deleted allele (Fig. 2B, lane F) with no amplification of the *loxP-NeoR-loxP* allele (Fig. 2C, lane F'), demonstrating that paternal germline transmission of the deleted allele occurred. Lanes G and G' are included as negative controls (Fig. 2B and C).

Excision is complete in virtually all tissues

In order to determine in what other tissues *loxP-NeoR-loxP* excision occurred in the Cre(+) mice, PCR was performed on DNA isolated from the liver (Fig. 2D and E, lanes H and H'), testes (Fig. 2D and E, lanes I and I'), tail (Fig. 2D and E, lanes

J and J'), kidney (Fig. 2D and E, lanes K and K') and brain (data not shown) of the Cre(+) male animal that showed germline transmission of the deleted allele and no detectable *NeoR* gene in the tail DNA (Fig. 2C, lane B'). DNA from all tissues amplified both the deleted *loxP* and the wild-type alleles (Fig. 2D, lanes H-K). Lanes L and M are included as controls (Fig. 2D). A faint intact *loxP-NeoR-loxP* allele was amplified in the kidney (arrow indicating 700 bp fragment in Fig. 2E, lane K'), indicating that excision of the *NeoR* gene was incomplete in the kidney, but complete excision occurred in all other tissues studied (Fig. 2E, lanes H'-J'). However, because the intact *loxP-NeoR-loxP* allele was not passed on to any subsequent offspring, germline excision was complete (data not shown). Similar results were found for multiple other animals (data not shown). Lanes L' and M' are included as controls (Fig. 2E).

Figure 2. Location of the PCR primers and detection of various alleles. (A) Primers used to detect the wild-type allele (WT) and the deleted *loxP*-flanked *NeoR* allele are labeled 1 and 2. These primers amplify a 187 bp wild-type fragment. Primers 1 and 2 will also amplify the deleted *NeoR* allele (*loxP*), which has a residual 34 bp *loxP* site remaining after Cre-mediated excision of the *NeoR* gene has occurred. In this case, a 221 bp fragment is amplified. This PCR was designed so that the undelimited *loxP*-flanked *NeoR* allele would not amplify if present. Primers 1 and 3 amplify a 700 bp fragment when the *loxP*-flanked *NeoR* gene (*loxP-NeoR-loxP*) is present. NeoR indicates neomycin resistance gene and hatched arrowheads indicate *loxP* sites. (B) PCR results demonstrating amplification of the 187 bp wild-type allele and the 221 bp deleted *loxP* allele in tail DNA. (C) PCR amplification of the intact 700 bp *loxP-NeoR-loxP* allele in tail DNA from the same animals shown in (B). Lanes A and A' represent the female breeder. Lanes B and B' represent a Cre(+) male offspring of a mating between the animal in lane A and the Cre(+) founder. Lanes C and C' represent a female carrying a wild-type allele and a *loxP-NeoR-loxP* allele. The animal in lanes B and B' was mated to the animal in lanes C and C' and the genotypes of the offspring are shown in lanes D-F and D'-F'. Lane D represents a homozygous wild-type mouse, lane E represents a mouse carrying two *loxP* alleles and lane F represents a mouse with both a wild-type and a *loxP* allele. Lanes G and G' represent the negative control PCR in the absence of genomic DNA. (D) PCR results demonstrating amplification of the 187 bp wild-type allele and the 221 bp deleted *loxP* allele in DNA isolated from the liver (lane H), testes (lane I), tail (lane J) and kidney (lane K) of the F₁ mouse shown in (B), lane B. (E) PCR amplification of DNA isolated from the liver (lane H'), testes (lane I'), tail (lane J') and kidneys (lane K') of the F₁ mouse in (B), lane B. Lanes L, L', M and M' represent control amplifications of tail DNA isolated from a wild-type mouse and a mouse carrying both the wild-type and deleted *loxP* alleles, respectively.



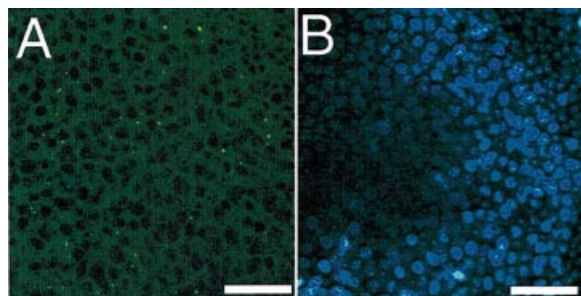


Figure 3. GFP expression is absent in the adult male germline. (A) GFP is not detected in the testes of an adult Cre(+) transgenic mouse. (B) DAPI staining of the testes section serves as a control. Scale bars indicate 5 μ m.

Cre is not detected in adult tissues

To determine if Cre was expressed in the germline of adult animals, testes from a Cre(+) animal were sectioned and visualized using the GFP marker. No GFP was detected in the testes (Fig. 3A). Therefore, the lack of Cre expression in the adult animal germline suggests that Cre must be expressed during early germ cell or embryo development. However, in spite of attempts to detect Cre in early embryos, we were unable to determine at which stage Cre expression and deletion occurred.

Homozygous Cre(+) line is normal and fertile

In order to generate homozygous Cre(+) mice, a heterozygous Cre(+) 129 male was mated to a heterozygous Cre(+) 129 female. To identify homozygous Cre(+) animals derived from the mating, the Cre(+) offspring were mated to wild-type animals and the numbers of Cre(+) mice determined. One female Cre(+) mouse had two litters resulting in five and seven mice, respectively. All of the mice were determined to be Cre(+). Therefore the probability is extremely low (0.00024) that this animal is not homozygous. A similar analysis of a Cre(+) male was performed. The 11 offspring from three litters resulting from mating to a wild-type female (four offspring from the first litter, five from the second and two from the third) were all Cre(+). Therefore, the probability of the male mouse not being homozygous is extremely low (0.000488).

In summary, we generated inbred homozygous Cre-expressing male and female mice that reliably and consistently delete a *loxP*-flanked gene in the germline. Deletion of the *loxP*-flanked allele occurs whether the Cre transgene is inherited from the mother or the father. The homozygous

mice are both fertile and phenotypically normal. This mouse has been accepted by the Jackson Laboratories for distribution under the name 129S6-TgN(Prnp-GFP/Cre)1Blw (Stock JR3960).

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