Targeted oncogene activation by site-specific recombination in transgenic mice

(cre/lox lens development/simian virus 40 large tumor antigen)


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ABSTRACT An efficient and accurate method for controlled in vivo transgene modulation by site-directed recombination is described. Seven transgenic mouse founder lines were produced carrying the murine lens-specific αA-crystallin promoter and the simian virus 40 large tumor-antigen gene sequence, separated by a 1.3-kilobase-pair Stop sequence that contains elements preventing expression of the large tumor-antigen gene and Cre recombinase recognition sites. Progeny from two of these lines were mated with transgenic mice expressing the Cre recombinase under control of either the murine αA-crystallin promoter or the human cytomegalovirus promoter. All double-transgenic offspring developed lens tumors. Subsequent analysis confirmed that tumor formation resulted from large tumor-antigen activation via site-specific, Cre-mediated deletion of Stop sequences.

A desired goal of transgene technology is efficient and accurate manipulation of DNA sequences after their integration in the germ line. DNA recombinases that mediate integration or excision of sequences at specific recognition sites in both prokaryotic (1–5) and eukaryotic (6–10) systems are well suited for this purpose. The bacteriophage P1 recombinase Cre catalyzes reciprocal recombination at a specific locus of crossing over (lox) (11–16). The lox sequence is composed of two 13-base-pair (bp) inverted repeats separated by an 8-bp spacer region. Upon binding to the inverted repeats, Cre synapses with a second lox site and then cleaves the DNA in the spacer region to initiate strand exchange with the synapsed lox partner. No additional factors are required in the recombination.

In this study, we examine the potential of the cre/lox system to activate a dormant transgene in the mouse. The simian virus 40 (SV40) large tumor antigens (TAg) directed to the lens by a murine αA-crystallin promoter (mAα) cause malignant lens tumors (17). We inserted between mAα and TAg a specially designed Stop sequence that prevents gene expression and is flanked by lox sequences. By crossing the dormant TAg transgenic mouse lines with Cre-expressing transgenic lines, we report here that the Cre protein recognizes the lox sites of the mAα–Stop–TAg transgene and recombines the two lox sequences, thereby removing Stop and activating TAg. Our studies show that targeted transgene modification in the mouse can be performed efficiently and accurately with a prokaryotic recombinase.

MATERIALS AND METHODS

Recombinant DNA Constructs and Transgenic Mice. The mAα–Stop–TAg transgene was constructed from the previously described plasmid pOA366a-T (17) by inserting to the BamHI site between mAα and TAg a 1.3-kbp Stop fragment flanked by directly repeated lox sequences (5'-ATAACT-TGTAATAGCATACATTATCGAAGTTT-3') (Fig. 1). The Stop sequence was composed of the 550-bp C-terminal sequence of yeast His3 gene, 825 bp of the SV40 polyadenylation signal region, and a synthetic oligonucleotide (5'-GATCTGACAATGGIAA(TAAGCTT-3'), where ATG is a false translation initiation signal and GTAAGT is a 5' splice donor site). The human cytomegalovirus (hCMV)–cre construct was obtained by fusing the 1.2-kbp cre gene to the hCMV promoter as described (16) and the mAα–cre construct was derived from the hCMV–cre construct by changing the promoter sequence and by a T to G substitution at the –3 position. Transgenic mice were produced as described (18).

Screening of Transgenic Mice. The genotypes of all offspring were analyzed both by PCR and by Southern blots. For PCR analysis, mouse tail DNAs (2 μg) were amplified by 35 cycles (1 min 20 sec, 92°C; 1 min, 65°C; 1 min 30 sec, 72°C) on a thermal cycler. The 5' primer for cre was 5'-GGACATGTTCAGGGATCGCCAGGCG-3' and the 3' primer was 5'-GCATAACCCATGAAACAGCATGGCT-3'. The 5' and 3' primers for TAg were 5'-GGTCTTGAAGAGGTGTCCTGGGGGA-3' and 5'-CCTCAAGTCTCACCAGAAGCTCC-3', respectively. Twenty percent of the reaction volume was analyzed on a 1.75% agarose gel. For Southern blot analysis, tail genomic DNAs (5 μg) were digested with BamHI and separated on a 0.75% agarose gel. Transfer to GeneScreen filters (DuPont) and Southern hybridization were performed according to Maniatis et al. (19). A 2.2-kbp Tag I/BamHI fragment of SV40 viral DNA (Bethesda Research Laboratories) and a 0.4-kbp BamHI fragment of the 5' portion of cre were used as hybridization probes. The expression levels of the cre transgenes were determined either by a functional test in fibroblasts and kidney cells derived from hCMV–cre transgenic mice (20) or by a PCR amplification of cDNAs synthesized from total RNAs from mAα–cre lenses. Lens RNAs (2 μg) were converted to single-strand cDNA by Moloney murine leukemia virus reverse transcriptase and were amplified by 35 cycles of PCR as described above.

Phenotype Analysis of Mouse Lenses. Mouse eyes were fixed in 4% paraformaldehyde or 4% glutaraldehyde and 36.8% formaldehyde, embedded in methacrylate, sectioned, and stained with hematoxylin and eosin as described (21). Immunoperoxidase staining with an antibody specific to TAg was performed on frozen tissue sections (17) under the conditions previously reported (22), except that nonspecific

Abbreviations: SV40, simian virus 40; TAg, large tumor antigen; mAα, murine αA-crystallin promoter; hCMV, human cytomegalovirus promoter/enhancer.

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Fig. 1. DNA constructs used for generating transgenic mice. The Nco I fragment microinjected into fertilized mouse eggs is shown. maA-Stop-TAg contains the maA promoter separated from the SV40 TAg sequences by the 1.3-kbp regulatory stop fragment, which was flanked by directly repeated lox sites and inserted into the BamHI site between the maA promoter and the TAg gene of the previously described plasmid paeA366a-T (17). The 3.3-kbp HindIII fragment of pBS187 plasmid (16) contained the cre gene under the control of the hCMV promoter/enhancer (hCMV-cre). The 3.0-kbp SauI/HindIII fragment containing maA promoter and cre gene (maA-cre) was derived from pBS187 by changing the promoter sequence and by T to G substitution at the -3 position for translation enhancement (16). (A)n, polyadenylation signal; 5'SD, 5' splice donor site.

binding was blocked with normal goat serum from the Vectastain detection kit (Vector Laboratories).

Molecular Analysis of Double-Transgenic Mouse Genotypes. Total genomic DNAs (1 μg) of eyes and/or tails from each mouse were amplified with a 5' primer (5'-GGCCCTGGTCTGACTCAGTACCAGCAGG-3') identical to the maA promoter sequence and a 3' primer (5'-CCCCCGAGGACCTCTTTCTCAAAGACC-3') complementary to the TAg first exon sequence. Amplification occurred in 100 μl of PCR buffer with 1.6 mg of bovine serum albumin per ml by 35 cycles of PCR (1 min 20 sec, 96°C; 3 min 30 sec, 72°C with 5-sec automatic cycle extension). One-third volume of the PCR amplification products was loaded on 1.75% agarose gel for analysis. The 220-bp PCR amplification products were then isolated from the agarose gel by electroelution and cloned into pGEM4Z plasmid (Promega). Double-stranded DNAs were sequenced as described (23).

RESULTS AND DISCUSSION

The cre/lox Binary System. The design of our experiment calls for accumulation of chromosomal lox sites and active Cre recombinase in specific target cells of transgenic mice. In this constellation, the recombinase would excise a Stop signal that prevents expression of SV40 tumor antigens and would thereby initiate oncogenesis in the target tissue. The removal of even a single Stop sequence at an integration site that may contain more than one transgene copy would activate the oncogene.

Production of Transgenic Mice Carrying Either the Dormant TAg Oncogene or the Cre Recombinase Gene. The maA-Stop-TAg dorminant oncogene construct was designed to contain the 1.3-kbp Stop fragment inserted between maA and TAg (Fig. 1). The Stop sequence contained a 550-bp spacer derived from C-terminal sequences of the yeast HIS3 gene to enhance the efficiency of the downstream 825-bp SV40 polyadenylation signal in terminating transcripts initiated by the maA promoter. An additional safeguard to prevent TAg expression was a synthetic oligonucleotide, including a false translation initiation signal (ATG), and a 5' splice donor site (GTAAGT). The Stop fragment was flanked by directly repeated lox sequences. Cre expression constructs were obtained by fusing the 1.2-kbp cre gene to the maA promoter (maA-cre) or to the hCMV promoter (hCMV-cre). In the maA-cre construct, a T to G nucleotide substitution was introduced to the -3 position relative to the AUG codon to enhance translation (16).

Transgenic mice were produced by injecting separately all three gene constructs as linear fragments into the pronuclei of fertilized mouse eggs that were then transferred to oviducts of pseudopregnant foster mothers (18). Seven independent transgenic mouse founder lines harboring the maA-Stop-TAg construct exhibited normal translucent lenses, indicating that the Stop sequences effectively prevented TAg expression. Seven founder animals with the maA-cre transgene and three founders with the hCMV-cre transgene were also obtained. No abnormalities among founders or their offspring were observed. High cre expression lines were selected based (i) on the presence of functional Cre enzyme in fibroblasts and kidney cells from hCMV-cre transgenic lines or (ii) on a PCR analysis of cDNAs synthesized from total RNAs of maA-cre lenses as described in Materials and Methods. F1 progeny of two maA-Stop-TAg founder lines, maA-Stop-TAg1 with >50 copies and maA-Stop-TAg2 with ~50 copies of the transgene per haploid genome, were mated with those maA-cre and hCMV-cre transgenic lines that showed high cre expression (Table 1).

Table 1. Transgenic mouse lines and occurrence of tumors

<table>
<thead>
<tr>
<th>Genotype</th>
<th>No. of animals</th>
<th>No. of animals with lens tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1*</td>
<td></td>
</tr>
<tr>
<td>maA-Stop-TAg1</td>
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</tr>
<tr>
<td>maA-Stop-TAg2</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>maA-cre</td>
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<td>0</td>
</tr>
<tr>
<td>hCMV-cre</td>
<td>&gt;100</td>
<td>0</td>
</tr>
<tr>
<td>maA-Stop-TAg1/maA-cre</td>
<td>17</td>
<td>17</td>
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<td>10</td>
</tr>
<tr>
<td>maA-Stop-TAg1/maA-cre</td>
<td>4</td>
<td>4</td>
</tr>
<tr>
<td>maA-Stop-TAg2/hCMV-cre</td>
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<td>5</td>
</tr>
<tr>
<td></td>
<td>Experiment 2†</td>
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<td>10</td>
</tr>
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<td>WT</td>
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<tr>
<td></td>
<td>Experiment 3‡</td>
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<tr>
<td>WT</td>
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</tr>
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</table>

*The genotypes of the animals were determined by PCR and Southern blot analysis. From the seven transgenic mouse founder lines carrying the dormant maA-Stop-TAg gene, two were selected for mating. The A9874 founder line of the maA-cre lines was selected for its high cre expression based on lens RNA analysis by PCR. The hCMV-cre founder line DP769 was also chosen because of its high cre expression based on Cre activity as described. All 36 double-transgenic animals had cataracts and all single transgens had normal eyes.

†Analysis of four successive litters of F1 generation single transgenic maA-cre female and F1 generation single-transgenic maA-Stop-TAg1 male. Genotypes of all offspring (n = 40) were confirmed by PCR and Southern analysis. WT, wild type.

‡Analysis of offspring from a cross between maA-Stop-TAg1/maA-cre double-transgenic female and male (produced by matings as described in †), which were heterozygous for each transgene. Genotypes of all offspring (n = 32) were analyzed by PCR. WT, wild type.
All Double-Transgenic Offspring Exhibit Lens Cataracts. The double-transgenic offspring harboring both the maA-Stop-TAg and maA-cro or hCMV-cre transgenes were easily identified on the basis of lens cataracts upon eye opening 10 days after birth (Fig. 2B). The genotypes of all offspring were confirmed by PCR (Fig. 3A) and Southern blot (Fig. 3C) analysis of DNA obtained from tail biopsies at weaning age. Two separate matings between maA-Stop-TAg1 and maA-cro progeny generated 17 double transgenics, each with cataracts in both lenses, from a total of 65 pups. Likewise, double transgenics resulting from crosses between maA-Stop-TAg2 and maA-cro or between maA-Stop-TAg1 or -2 and hCMV-cre all had cataractous lenses at expected Mendelian frequencies (Table 1). More recently, crossing both cre transgenic lines with a third maA-Stop-TAg transgenic founder line with fewer copies than the first two founder lines has also resulted in offspring that exhibited cataractous eyes at expected Mendelian frequencies (data not shown).

Malignant Transformation Results from TAg Expression. Histological analysis of lens sections obtained from double-transgenic animals consistently revealed morphological changes characteristic of proliferating lens tumors (Fig. 2C). Instead of the single mitotically active layer of anterior epithelial cells seen in normal lenses of maA-Stop-TAg transgenic mice (data not shown), a multilayered heterogeneous epithelium consisting of anaplastic cells was observed in the anterior part of the double-transgenic lenses. Tongues of invasive growth were extending into the rest of the disorganized lens mass. The lens mass was punctuated with large cysts that most likely result from improper elongation of fiber cells. Immunostaining of frozen lens sections with a polyclonal antibody to TAg showed a strong signal in the anaplastic cell mass (Fig. 2D). TAg could not be detected in lenses of maA-Stop-TAg littermates (data not shown). Taken together, these results strongly suggest that the malignant transformation was due to TAg expression in the lenses of double-transgenic animals.

TAg Activation Is Due to Site-Specific Recombination in the Embryonic Mouse Genome. The activation of the dormant TAg by Cre was assessed by PCR analysis of transgene sequences in affected and control lenses. A 24-bp 3' primer complementary to the first exon sequence of TAg and a 23-bp 5' primer identical to the maA promoter sequence were synthesized. Amplifications with these primers predictably generate a 220-bp fragment from maA-TAg, representing the product of Cre-mediated Stop excision at the maA-Stop-TAg locus. The expected 220-bp fragment was indeed obtained with genomic DNA from double-transgenic mice lenses, but not with genomic DNA from control aA-Stop-TAg mouse lenses (Fig. 4A). Sequence analysis of amplified 220-bp cDNA fragments revealed one single lox site flanked by 5' maA promoter sequences and 3' TAg (Fig. 4B). This confirms that TAg activation had occurred, as predicted, via precise site-specific deletion of the Stop sequences in the genome of differentiating lens cells. While Stop excision

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**Fig. 2.** Phenotype of double-transgenic mice bearing both maA-Stop-TAg and maA-cro genes. (A) Eye of a normal maA-Stop-TAg1 single-transgenic mouse at 10 weeks of age. The albino mouse eye appears red. (B) Eye of a maA-Stop-TAg1/maA-cro double-transgenic sibling. (C) Lens pathology of a double-transgenic mouse. Hematoxylin and eosin staining of section from a 16-day-old maA-Stop-TAg1/maA-cro mouse. Aqueous humor (a), vitreous humor (v), cyst (c), and anaplastic cells (arrowhead) are shown. (×8.) (D) Indirect immunostaining with an antibody specific to TAg (22) of a lens section from the same animal as in C. Anterior epithelial cells (arrowhead) are strongly stained. The antibody did not stain lenses from single-transgenic littermates. (×6.)

**Fig. 3.** Genotypes of a maA-Stop-TAg1 × hCMV-cre litter. (A) Identification of maA-Stop-TAg and hCMV-cre transgenes by PCR amplification of tail DNA. Primers for TAg generated a 195-bp fragment and primers for cre generated a 269-bp fragment. PCR products were separated on a 1.75% agarose gel for analysis. Lanes: 1, offspring 1; 2, offspring 2; 3, offspring 3; 4, offspring 4. Offspring 1, 3, and 4 are double transgenic. (B) Identification of the Stop deletion from tail genomic DNAs from the same animals. The PCR strategy selected is shown in Fig. 4A. Amplification yielded the expected 220-bp fragment from the three double-transgenic offspring 1, 3, and 4. (C) Southern hybridization analysis of BamHI-digested tail DNAs from the same animals. The band of offspring 1 represents a single-copy BamHI fragment of the TAg, indicating that only one maA-TAg transgene is left in the genome, and Stop sequences (1.3 kbp) have been excised. Offspring 3 and 4 retain multiple copies of maA-Stop-TAg transgenes in their genomes.
to allow lox synopsis and excision of Stop. Cells activating TAg at slightly different stages of embryonic lens development may be expected to be immortalized by TAg and to give rise to foci of transformed cells that reflect the differentiated state of the initial target cell. Thus, a careful analysis of cell types found in the developing double-transgenic lens may enable us to describe more precisely the effects of tissue differentiation on the process of oncogenesis.

The amount of Cre recombinase activity produced in cre mice is likely to govern the efficiency with which recombination takes place. It is encouraging that the Cre recombinase in hCMV-cre mice is quite proficient for recombination despite the fact that it is derived from a transgene that lacks an optimal eukaryotic translation initiation signal (31, 32). In cultured cells, the change to an efficient Cre translation initiation signal for the cre gene results in a substantial increase in recombination ability (16). We anticipate that this enhancement would also occur in transgenic animals, thus allowing promoters of even moderate strength to be useful for driving Cre expression in binary systems.

The utility of the cre/lox system for transgenic technology is likely to go far beyond the application we chose for our study. Based on the findings we report here, a great many schemes can be designed aimed at modulating transgene activity precisely and efficiently in vivo while simultaneously reducing the transgene copy number at any given integration site, down to one. A potentially even more powerful tool for gene modulation in vivo is the known propensity of Cre to mediate not only excision but also integration events. Cre will insert DNA sequences at preestablished chromosomal lox sites in the mammalian genome (16). Such integration events are infrequent and are therefore likely to require selection in embryonic stem cells before they can be established in the mouse germ line.

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Fig. 4. PCR and sequence analysis of the lox regions in transgenes. (A) Identification of the Stop deletions from tail and eye DNAs of transgenic animals. The PCR strategy is shown below. Amplification yielded the expected 220-bp fragment from eye DNAs of all double-transgenic mice and from tail DNAs of maA-Stop-TAg/hCMV-cre mice. PCR analysis of maA-Stop-TAg tail transgenic DNA (lane 1) and eye DNA (lane 2), maA-Stop-TAg/maA-cre double-transgenic tail DNA (lane 3) and eye DNA (lane 4), maA-Stop-TAg/hCMV-cre double-transgenic tail DNA (lane 5) and eye DNA (lane 6). (B) Sequence analysis of the lox region from the 220-bp amplification product (10). The lox sequences are underlined with asterisks and the maA promoter sequences and the TAg sequences are boxed. The Xho I and BamHI sites flanking the lox sites are indicated.

occurred selectively in the eye lens in mice carrying the maA-Stop-TAg and the maA-cre transgenes, the constellation of maA-Stop-TAg plus hCMV-cre transgenes resulted in Stop excision in other Cre-expressing tissues as well (Figs. 3B and 4A). However, the phenotypes of the two types of the double-transgenic mice did not visibly differ because TAg is driven by the maA promoter and is therefore synthesized almost exclusively in the lens (17). Southern analysis of tail DNAs obtained from a double-transgenic litter demonstrated that the Cre expressed from the hCMV promoter is highly efficient in deleting not only the Stop sequences but the multiple copies of the maA-Stop-TAg transgene as well, until only a single maA-TAg with a single lox site is left in the genome (Fig. 3C).

Conclusions. Our studies have shown that the cre/lox system is a very powerful tool to activate a transgene by incorporating two different transgenes in one genome. In this respect, it may be compared with other binary systems that have been developed for a similar purpose (24-26). Because the Cre recombinase is a member of the Int family of recombinases (27), it is likely that other recombinases will be useful in directing precise site-specific DNA rearrangements in transgenic animals. For example, the FLP recombinase of Saccharomyces cerevisiae has been shown to be proficient for recombination in both Drosophila (28) and in cultured mammalian cells (29). The pSR1 recombinase of Zygosaccharomyces rouxii appears to be quite similar to the Cre and FLP recombinases and has been shown to function efficiently in S. cerevisiae (30).

The activation of TAg in the developing lens is likely to occur in any lens cell that produces Cre at a level sufficient.