Site-specific recombination of a transgene in fertilized eggs by transient expression of Cre recombinase

(microinjection/chicken β-actin promoter/β-galactosidase)

KIMI ARAKI*†, MASATAKE ARAKI*†, JUN-ICHI MIYAZAKI‡, AND PIERRE VASSALLI*§

*Department of Pathology, Centre Médical Universitaire, University of Geneva, CH 1211, Geneva 4, Switzerland; and †Department of Disease-Related Gene Regulation Research (Sandoz), University of Tokyo, Faculty of Medicine, Hongo 7-3-1, Bunkyo-ku Tokyo 113, Japan

Communicated by K. Rajewsky, Institut für Genetik der Universität zu Köln, Cologne, Germany, September 26, 1994

ABSTRACT An efficient method of transgene modulation in fertilized eggs has been developed that uses the Cre/loxP recombination system. Twelve transgenic mouse lines carrying a chicken β-actin promoter-loxP-chloramphenicol acetyltransferase (CAT) gene-loxP-β-galactosidase gene construct were produced. After selection of the line showing the highest expression of the CAT gene in a variety of tissues, eggs of this line were injected in the male or female pronucleus with a Cre expression vector placed under the control of the chicken β-actin promoter and kept in a circular form to avoid genomic integration. This resulted in a transient expression of Cre, leading to recombination of the transgene as detected by galactosidase expression and DNA analysis. Recombination was completed before the morula stage with both types of pronuclear injections and occurred with a very high frequency; no mosaicism, no incomplete recombination, and no integration of the Cre sequence were observed in 18 mice born with this modified transgene. The β-galactosidase gene was expressed in various tissues at levels comparable to those found for the CAT gene in the founder line. This Cre transient expression system should be useful for breeding transgenic lines in which transgene expression leads to sterility or lethality—in particular, for selecting transgenic lines with high expression of a potentially lethal transgene whose full activity is difficult to explore in a conventional transgenic system because of the risk of selecting for transgenic lines carrying only poorly expressed transgenes.

Development of the technology for gene transfer in the germ line (1), leading to the production of transgenic animals, has had an enormous impact in biology. When the transgene results from random integration of a DNA fragment injected into a fertilized egg’s pronucleus, its level of expression in the resulting transgenic animal varies and depends upon factors which cannot be controlled, such as the integration site and the transgene copy number. Usually, several transgenic mouse lines are created to ensure that the phenotype resulting from the presence of a transgene is reproducible and/or to select lines with high expression of the transgene. However, special difficulties arise when expression of the transgene results in sterility or is lethal before the age of reproduction. This situation requires the repeated production of new transgenic mice, each with a different transgene integration site and copy number, resulting in unwanted variations in experimental conditions. An additional complication may arise when high expression of a transgene is lethal, since transgenic founder embryos with weak expression of the transgene will be selected. Thus, observation of a partial phenotype may result in erroneous interpretations concerning the exact functions of the explored gene.

To overcome these difficulties, expression of a potentially toxic transgene should be suppressed. This would allow the use of a single founder line stably transmitting the same transgene, provided reexpression of the transgene can be achieved under conditions controlled by the investigator. This could be obtained by a DNA recombination event mediated by the bacteriophage P1 cre gene, which encodes a recombinase, Cre, cleaving DNA at loxP sequences. The transgene may be introduced into a mouse in a "silenced" form by placing a loxP-flanked DNA sequence between its promoter and its coding sequence, thus preventing transcription. The transgene may be "reactivated" by mating mice of this line with mice of another transgenic line, expressing a cre transgene. Application of the Cre/loxP system to transgenic mice has already been reported, indicating efficient excision of the DNA located between the two loxP sequences in doubly transgenic mice, at least in tissues expressing the cre gene (2, 3). The aim of the present study was to develop a technique which allows the rapid and reproducible removal of the transgene silencing sequence in all cells from a mouse, without introducing any other permanent genetic modification. This was achieved by microinjecting a cre gene into fertilized eggs, where it is only transiently expressed. In addition, we have used, as a reporter/silencer sequence, a loxP-flanked chloramphenicol acetyltransferase (CAT) gene; expression of this reporter gene can thus be used for the selection of transgenic founder lines with the highest expression level.

MATERIALS AND METHODS

Plasmid Constructions and Production of Transgenic Mice. The Cre expression vector pCAGGS-Cre was constructed by inserting the cre gene, obtained as a 1.1-kb Xho I-Mlu I fragment from pBS185 (ref. 4; gift from B. Sauer), into the EcoRI site of pCAGGS (5). The transgene vector pCAG-CAT-Z, containing a chicken β-actin gene (CAG) promoter-loxP-CAT gene-loxP-lacZ region, was constructed from pCAG-lacZ (5) by inserting a CAT gene [derived from pBLCAT3 (6)] flanked by directly repeated loxP sequences [derived from pBS64 (7), gift from B. Sauer] into the HindIII site located between the CAG promoter and lacZ. This 6.9-kb construct (Fig. 1) was purified by electrophoresis and elution from NACS PREPAC (BRL) and used for microinjection. The plasmid pCAGGS-Cre was purified by NACS PREPAC, suspended in 1 mM Tris-HCl, pH 7.5/0.1 mM EDTA at 5 ng/µl, and used for microinjection. Transgenic mice were produced as described (8).

Abbreviations: CAG, chicken β-actin promoter; CAT, chloramphenicol acetyltransferase; X-Gal, 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside.

†Present address: Institute of Molecular Embryology and Genetics, Kumamoto University School of Medicine, Kuhonji 4-24-1, Kumamoto 862, Japan.

§To whom reprint requests should be addressed.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. §1734 solely to indicate this fact.
Southern Blot Analysis and PCR. Tails and tissues were lysed with NaDodSO₄/proteinase K (Boehringer Mannheim) and then treated with phenol/chloroform, 1:1 (vol/vol), precipitated with ethanol, and dissolved in 10 mM Tris-HCl, pH 7.5/1 mM EDTA. DNAs (5 µg) were subjected to electrophoresis in a 1% agarose gel. After transfer to nylon membranes (Boehringer Mannheim), Southern hybridizations were performed with probes ³²P labeled by random priming: (9). For PCR analysis, DNAs (0.5–1 µg) were submitted to 27 or 32 cycles of amplification (with each cycle consisting of 1 min at 94°C, 2 min at 55°C (or 65°C for cre primers), and 2 min at 72°C) on a thermal cycler. The 5' primers and 3' primers were CAT2 (5'-CAGTCATGGTGCCTAATGCACC-3') and CAT3 (5'-ACTGGTGAAACATCCCA-3'), respectively, for the CAT gene; Z1 (5'-GGTTACCCAACTTAATCG-3') and Z2 (5'-GTGAGCAATTAAACCTC-3'), respectively, for the lacZ gene (2); and Cre1 (5'-GGACATGTTTACAGG ATCGCCAGGC-3') and Cre2 (5'-GCATAACACGG TAAACAGCATTGC-3'), respectively, for the cre gene. The 5' primer and 3' primer for exploring the junction between CAG promoter and lacZ were AG-2 (5'-CTGCTAACCATTGTTCATGCC-3') and Z3 (5'-GGGCTCTTGCATTAGG-3'), respectively. Half of the reaction volume was analyzed by electrophoresis in a 1.4% agarose gel.

Detection of CAT and β-Galactosidase Activities. Organs were homogenized in extraction buffer (15 mM Tris-HCl, pH 8.0/60 mM KCl/15 mM NaCl/2 mM EDTA/1 mM dithiothreitol/0.4 mM phenylmethylsulfonyl fluoride) (10) with a glass homogenizer. After centrifugation at 13,000 × g for 15 min at 4°C, the protein concentration of the supernatant was determined with a kit from Bio-Rad (based on the Bradford method), and the amount of CAT in the samples was determined with a CAT-ELISA kit from Boehringer Mannheim. β-Galactosidase activity in the sample was measured by incubation with o-nitrophenyl β-D-galactopyranoside (11). For detection of β-galactosidase activity in early-stage (two- or four-cell) embryos, embryos were fixed in 2% glutaraldehyde solution for 5 min, treated with 0.1% Triton X-100 for 5 min, washed in phosphate-buffered saline, and incubated in stain solution [8.4 mM KCl/84 mM phosphate buffer, pH 7.5/1 mM MgCl₂/3 mM KFe(CN)₆/3 mM K₃Fe(CN)₆/3% 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-Gal)] overnight at room temperature. Whole embryos (13th day of gestation) were stained for β-galactosidase activity with X-Gal according to the method of Allen et al. (12).

RESULTS

Experimental Design. The experimental design is outlined in Fig. 1. The first step is the production of transgenic lines in which the coding region of the transgene of interest is preceded by a reporter/silencer transgene surrounded by loxP sequences. The line(s) are then screened to determine which one(s) shows the strongest expression of the reporter/silencer. In this study, in order to easily monitor the expression of the two transgenes, we used the CAT and lacZ genes, and the injected construct was thus CAG promoter-loxp-CAT-loxp-lacZ (CAG-CAT-Z). In the next step, eggs are collected from mice of the transgenic line(s) and submitted to pronuclear injection of the plasmid pCAGGS-Cre in a circular form; this results in transient Cre expression and recombination at the loxp sites. In embryos carrying the “recombined” transgene, β-galactosidase activity is now detectable; the pCAGGS-Cre plasmids disappear rapidly by dilution during embryonic development.

The Cre Transient Expression System. To confirm that the CAG promoter works efficiently in fertilized eggs, we first injected circular pCAG-lacZ into fertilized eggs and stained them by X-Gal at the two-cell stage. All cells were stained very strongly (data not shown), showing that the CAG promoter has strong activity in eggs. We compared injection into the female or male pronucleus and found that at the two-cell stage the β-galactosidase staining was stronger with those injected in the female pronucleus, which may reflect the difference in the starting time of expression. The important point was that both pronuclear injections led to constant expression of the injected plasmid, which probably indicates that the CAG promoter is especially strong; indeed, other investigators, who injected lacZ plasmids placed under the control of different promoters into mouse eggs, have reported β-galactosidase expression in only 50% (12) or less (13) of the injected eggs.

Production and Selection of Transgenic Lines. The CAG-CAT-Z construct was injected into fertilized eggs from B6D2F1 × B6D2F1 mice, and 12 transgenic founders were obtained. F₁ mice were then obtained from each founder, and CAT activity present in the hearts of these F₁ mice was determined. Three lines were found to display a high, comparable level of CAT activity (data not shown). For further experiments, we chose one of these lines, CAG-CAT-Z 34, which carries about four copies of the transgene in head-to-tail arrangement (see Fig. 3c), since it gave offspring more rapidly.
whereas noninjected were which percentage at cleus. Injected Tlwo-cell efficiently shows that the embryos were injected (d) Embryos analyzed by PCR using four kinds of primer sets. As shown in Fig. 3a, the Z1 and Z2 primers were designed to explore whether the lacZ gene had been transmitted or not, the Z3 and AG2 primers to detect the recombination event, and the CAT2 and CAT3 primers to explore whether the CAT gene had been deleted completely. In the case of incomplete recombination and/or of mosaicism, the DNA samples should show PCR-amplified DNA sequences with the use of CAT2/CAT3 primers and Z3/AG2 primers. Primers Cre1 and Cre2 were used to detect cre gene integration.

As shown in Table 2, 18 of these 47 mice carried the transgene. Irrespective of the site of pronucleus injection, in all these 18 transgenic mice (which expressed lacZ; see below), DNA amplification using Z3 and AG2 primers gave a DNA segment of the expected size, whereas no amplified DNA segment was seen when the CAT2/CAT3 or Cre1/Cre2 primer pairs were used (Fig. 3b). Liver and kidney DNAs from some of these recombinant-transgene-carrying mice gave the same pattern as tail DNAs (data not shown). This indicates that the expression of Cre in the injected transgenic eggs led to the excision of all CAT genes present in head-to-tail arrangement, that the cre gene was not integrated in the genome, and that the recombination event probably occurred very early in the embryos, since no mosaicism was detected.

Southern blot analysis of tail DNAs confirmed the existence of the expected recombination. As shown in Fig. 3a, recombination should reduce the four copies of the CAG-CAT-Z transgene array of transgenic line 34 to a recombined allele.

difference was observed at the four-cell stage after either type of injection, this probably indicates that injection into the male pronucleus results in a later start of Cre expression. In one experiment, the pCAGGS-Cre plasmid was injected at 1 ng/μl instead of 5 ng/μl; about half of the eggs were stained, indicating that the plasmid concentration used in all other experiments was in excess. With both types of injection, however, in some embryos only half of the cells were stained. This might reflect a difference in the level of expression of β-galactosidase or may suggest that recombination is a late event occurring in only one cell of the two-cell-stage embryo. To explore the second possibility, injected eggs were transferred into foster mothers, and the resulting embryos were stained at 13 days of gestation; three embryos out of five were stained (Fig. 2), with a ubiquitous and strong pattern of staining, suggesting that the recombination event had in fact occurred at the single-cell stage or eventually did occur in all cells; exploration of the placenta of these embryos by PCR showed that only the placentas of the stained embryo contained a lacZ gene, in the absence of the CAT gene (see below and Fig. 3). These experiments indicated that Cre works efficiently in eggs and probably leads to transgene recombination in all eggs injected with the Cre plasmid, since the frequency of eggs expressing β-galactosidase activity corresponds to the expected frequency of eggs carrying the transgene.

Mice Born with a Transgene Recombination Show Absence of Mosaicism, Complete Recombination, and Lack of Cre Integration. We transferred into pseudopregnant mice 60 and 146 eggs injected with the Cre plasmid in the female or male pronucleus and obtained 16 and 31 mice, respectively, which were studied at 4–6 weeks of age. First, tail DNAs were examined by PCR using four kinds of primer sets. As shown in Fig. 3a, the Z1 and Z2 primers were designed to explore whether the lacZ gene had been transmitted or not, the Z3 and AG2 primers to detect the recombination event, and the CAT2 and CAT3 primers to explore whether the CAT gene had been deleted completely. In the case of incomplete recombination and/or of mosaicism, the DNA samples should show PCR-amplified DNA sequences with the use of CAT2/CAT3 primers and Z3/AG2 primers. Primers Cre1 and Cre2 were used to detect cre gene integration.

As shown in Table 2, 18 of these 47 mice carried the transgene. Irrespective of the site of pronucleus injection, in all these 18 transgenic mice (which expressed lacZ; see below), DNA amplification using Z3 and AG2 primers gave a DNA segment of the expected size, whereas no amplified DNA segment was seen when the CAT2/CAT3 or Cre1/Cre2 primer pairs were used (Fig. 3b). Liver and kidney DNAs from some of these recombinant-transgene-carrying mice gave the same pattern as tail DNAs (data not shown). This indicates that the expression of Cre in the injected transgenic eggs led to the excision of all CAT genes present in head-to-tail arrangement, that the cre gene was not integrated in the genome, and that the recombination event probably occurred very early in the embryos, since no mosaicism was detected.

Southern blot analysis of tail DNAs confirmed the existence of the expected recombination. As shown in Fig. 3a, recombination should reduce the four copies of the CAG-CAT-Z transgene array of transgenic line 34 to a recombined allele.

Table 1. Number of stained eggs after pCAGGS-Cre injection

<table>
<thead>
<tr>
<th>Parental origin of injected pronucleus</th>
<th>Two-cell stage</th>
<th>Four-cell stage</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Injected eggs</td>
<td>Stained</td>
</tr>
<tr>
<td>Female</td>
<td>15</td>
<td>7 (47%)</td>
</tr>
<tr>
<td>Male</td>
<td>17</td>
<td>4 (24%)</td>
</tr>
</tbody>
</table>

The eggs were obtained as described in Fig. 2, and pCAGG-Cre was injected into the male or female pronucleus. After staining with X-Gal, stained eggs were counted.
consisting of a single copy of the CAG promoter, the residual loxP site, and the lacZ gene. In agreement with this expectation, the recombined transgenic DNAs did not show the 4.4-kb band observed with the intact transgene (Fig. 3c Left) but instead showed the 3.1-kb band expected from the recombined DNA, with an intensity compatible with a single copy (Fig. 3c Right).

The lacZ Transgene in the Recombined Mice Shows a Tissue Distribution and Expression Level Comparable to Those of the Reporter/Silencer CAT Gene in the Parent Mice. Expression of the intact or recombined transgene was determined in the liver, kidney, spleen, heart, lung, and muscle (Table 3). In mice bearing the nonrecombined transgene, the CAT gene was expressed at very high levels in heart and muscle and only at low levels in other tissues. In these mice, the transgene had no detectable β-galactosidase activity. In mice bearing a recombined transgene, β-galactosidase activity was very high in muscle and heart; in the other tissues, it was not above the background level. This probably reflects the difference in sensitivity of the assays used, the CAT assay being more sensitive because of the lack of endogenous activity. Transmission of the recombined transgene to progeny showed no evidence of instability.

**DISCUSSION**

These results establish a highly efficient method of genomic recombination whose product is subsequently expressed in all tissues and cells of the resulting animals. This method is based on the injection of a circular pCAGGS-Cre plasmid, which allows transient expression of Cre, into the pronucleus of eggs obtained from transgenic mice bearing a transgene containing two loxP sequences. The method presents the following features. (i) It is rapid: many eggs can be obtained from a single transgenic mouse, avoiding the requirement of prolonged breeding and mating of two transgenic lines otherwise necessary for the expression of the selected transgene and of the cre gene within the same mouse. (ii) It is very efficient, since it appears that, provided the injection of pCAGGS-Cre into a pronucleus is successful, recombination takes place in all cases. (iii) The recombination takes place shortly after injection: since the recombination event occurs at a very early stage of the embryo, the risk of mosaicism appears to be very low. (iv) The method does not further modify the genome, since the circular plasmid is not integrated.

This system has a number of useful applications. (a) Breeding transgenic lines in which transgene expression leads to lethality, sterility, or any source of inability to have progeny. In utero lethality should, however, occur later than the 10th day, since earlier lethality might not be easily recognized from egg damage resulting from microinjection. (b) Selecting transgenic lines with high expression (as done in the present work), an especially useful procedure when there is a chance that high transgene expression might induce in utero lethality. However, several transgenic lines should be selected, because transgene expression level may be related, in some integration sites, to copy number of the transgene; since the recombination event reduces the copy number to 1, the expression level may be, in such cases, reduced after recombination. (c) Gene targeting introducing loxP sequences. A variety of strategies can be considered for the selective removal of fragment of genes (instead of complete “knockouts”), using loxP sequences surrounding the fragment to be removed. In an experimental design based on this principle, a targeted neomycin-resistance gene was deleted by Cre transient expression in embryonic stem cells (14, 15). Instead of screening embryonic stem cells for successful recombination after transient expression of cre introduced by electroporation, nonrecombined cells can be used to prepare mice (provided the nondeleted gene alteration is not toxic) in whose eggs recombination could be achieved.

**Table 2.** PCR analysis of transgene status

<table>
<thead>
<tr>
<th>Parental origin of injected pronucleus</th>
<th>No. of injected eggs</th>
<th>No. of newborns</th>
<th>lacZ+ (Z1/Z2 PCR)</th>
<th>Recombined (AG2/Z3 PCR)</th>
<th>CAT (CAT2/CAT3 PCR)</th>
<th>Cre (Cre1/Cre2 PCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female</td>
<td>60</td>
<td>16</td>
<td>7</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Male</td>
<td>146</td>
<td>31</td>
<td>11</td>
<td>11</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

The injected eggs were transferred into foster mothers, and mice born as the result of this procedure were used when 3–4 weeks old. Tail DNAs were examined by PCR and the results with various primers (see Fig. 3 and Materials and Methods) are tabulated. Positive control for PCR detection of the cre sequence consisted of 1 pg of the cre plasmid and 1 μg of DNA from the tail of a nontransgenic mouse.
We thank Dr. Brian Sauer for the gift of the cre-containing plasmid, Jean-Dominique Vassali and Dominique Belin for comments on the manuscript, Mr. Jean-Claude Rumbeli and Mr. Etienne Denkinger for photographic work, and Ms. Jacqueline Ntah for typing the manuscript. This research was supported by the Swiss National Foundation (31-37516.93). K.A. was supported by Glaxo Institute of Molecular Biology (Geneva).


Table 3. Detection of CAT protein and β-galactosidase activity from various tissues of transgenic (Tg) mice with and without transgene recombination

<table>
<thead>
<tr>
<th>Assay</th>
<th>Mice</th>
<th>Liver</th>
<th>Kidney</th>
<th>Spleen</th>
<th>Heart</th>
<th>Lung</th>
<th>Muscle</th>
</tr>
</thead>
<tbody>
<tr>
<td>CAT</td>
<td>CAG-CAT-Z Tg</td>
<td>1.3</td>
<td>1.4</td>
<td>1.4</td>
<td>200</td>
<td>8.0</td>
<td>960</td>
</tr>
<tr>
<td></td>
<td>Non-Tg littermates</td>
<td>0.2</td>
<td>0.2</td>
<td>0.3</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>β-Galactosidase</td>
<td>CAG-CAT-Z Tg</td>
<td>0</td>
<td>0.01</td>
<td>0</td>
<td>0.11</td>
<td>0</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Recombined Tg</td>
<td>0</td>
<td>0.29</td>
<td>0</td>
<td>25.4</td>
<td>0.13</td>
<td>14.9</td>
</tr>
<tr>
<td></td>
<td>Non-Tg littermates</td>
<td>0</td>
<td>0.07</td>
<td>0</td>
<td>0.04</td>
<td>0</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Homogenates were prepared and CAT activity was measured by a CAT-ELISA kit (Boehringer Mannheim), and β-galactosidase activity by the o-nitrophenyl β-D-galactopyranoside method (1 unit of β-galactosidase is defined as the amount hydrolyzing 1 nmol of substrate per minute at 28°C). Values are averages for three mice.