**Z/AP, a Double Reporter for Cre-Mediated Recombination**

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The Cre/loxP site-specific recombination system combined with embryonic stem cell-mediated technologies has greatly expanded our capability to address normal and disease development in mammals using genetic approaches. The success of this emerging technology hinges on the production of Cre-expressing transgenic lines that provide cell type-, tissue-, or developmental stage-specific recombination between loxP sites placed in the genome. Here we describe and characterize the production of a double-reporter mouse line that provides a convenient and reliable readout of Cre recombinase activity. Throughout all embryonic and adult stages, the transgenic animal expresses the lacZ reporter gene before Cre-mediated excision occurs. Cre excision, however, removes the lacZ gene, allowing expression of the second reporter, the human alkaline phosphatase gene. This double-reporter transgenic line is able to indicate the occurrence of Cre excision in an extremely widespread manner from early embryonic to adult lineages. It will be a valuable reagent for the increasing number of investigators taking advantage of the powerful tools provided by the Cre/loxP site-specific recombinase system. © 1999 Academic Press

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**INTRODUCTION**

The availability of mouse embryonic stem (ES) cell-mediated gene targeting has provided a tremendous amount of information about the biological function of many genes in normal and disease processes (Melton, 1994; Shastry, 1998; St-Jacques and McMahon, 1996). The general approach has been to create null mutations in a gene of interest and study the consequence of the gene deficiency. Similarly, transgenic misexpression has revealed the biological functions of many genes (Balling et al., 1989; Brunkow and Tilghman, 1991; DeRocco et al., 1997; Jegalian and De Robertis, 1992; Johnson et al., 1994; Lufkin et al., 1992; Zhang et al., 1994). These powerful approaches, however, have been limited by the fact that the alterations are present in the germline and are thus manifest in all cells at all times. If the genetic change has an early lethal phenotype, later phenotypes cannot be accessed (Nagy et al., 1998). Likewise, a genetic change that produces a complex, multicomponent phenotype could be more easily analyzed if the alteration were restricted to one tissue or cell type.

By combining the Cre/loxP site-specific recombination system with transgenic technologies, it has become possible to introduce conditional genome alterations that are spatially and temporally restricted (Sauer, 1998). The Cre recombinase of the P1 bacteriophage catalyzes recombination between two specific 34-bp consensus sequences (loxP sites) with high specificity and efficiency (Sternberg and Hamilton, 1981). The loxP sites are palindromic except for an 8-bp asymmetric core sequence that provides each loxP site with an orientation (Hoess et al., 1986). If two loxP sites lie in the same orientation in the same DNA strand, introduction of Cre enzyme will result in recombination

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between the loxP sites and excision of the intervening DNA sequence.

Cre recombinase is effective not only in bacteria but also in eukaryotic cells. Use of the Cre/loxP system in eukaryotic cells or in the mouse requires only that the components (Cre enzyme and loxP sites) be introduced (Sauer and Henderson, 1988). loxP sites can be introduced into the genome via different means of transgenesis. When a Cre recombinase transgene is also introduced, the Cre-mediated recombination between the loxP sites can lead to a specific deletion, translocation between chromosomes, or inversion of a DNA segment, depending on the location and orientation of the loxP sites. In the mouse the occurrence of the genome-altering excision event is dependent on the expression pattern of the Cre transgene. Overall, non-specific or early embryonic expression of Cre can result in a global excision (Nagy et al., 1998; Schwenk et al., 1995), while lineage/cell type-specific Cre expression can lead to a much more restricted pattern of excision (DiSanto et al., 1995; Gu et al., 1994; Sauer, 1998; Tarutani et al., 1997).

The spectrum of conditional genome alterations depends on a battery of Cre transgenic mice that possess different tissue and temporal specificity. The establishment of these lines is an ongoing effort in many laboratories. To achieve spatial restriction, the Cre recombinase coding sequence in these transgenic mice is under the regulation of a variety of tissue-specific promoters, or has been “knocked-in” to a gene (see URL: http://www.mshri.on.ca/develop/nagy/Cre.htm). Several approaches to provide inducible Cre activity in transgenic mice by administration of exogenous compounds are also now emerging, opening the possibility of restricting site-specific recombination to later stages in development (et al., 1996; Furth et al., 1994; Kellendonk et al., 1996; Rivera et al., 1996).

While these Cre transgenic mice are being developed, there has been no sufficient means to test the actual excision activity of the Cre recombinase at the individual cell level. We have designed a transgenic mouse, designated the Z/AP reporter (lacZ/human placental alkaline phosphatase) that uses a double-reporter system to provide a precise and accurate assay for Cre excision at the cellular level. Before Cre excision, cells express lacZ, while after excision lacZ’ expression is replaced with human placental alkaline phosphatase (hPLAP) expression. We illustrate a Cre-mediated, excision-dependent expression switch of the reporters at critical stages of development and in adult tissues.

MATERIALS AND METHODS

DNA Constructs

The Z/AP expression vector was constructed as follows. A triple repeat of the SV40 polyadenylation signal (3xP; provided by E. Mercer) was inserted into pBluescript (Stratagene). The βgeo (lacZ/neo fusion; Friedrich and Soriano, 1991) coding sequence was placed upstream of the poly(A) sequence, and the βgeo/3xP insert was then excised and inserted between two tandem loxP sites, also in pBluescript. The loxP-flanked βgeo/3xP insert was excised and placed into the XhoI site of the pCAGG vector (Niwa et al., 1991), which contains the CMV enhancer/chicken β-actin promoter and a rabbit β-globin pA. The insertion destroyed the XhoI site on the 5’ side of the loxP-flanked βgeo/3xP but conserved the XhoI site on the 3’ side. This vector is referred to as pCALL. The hPLAP coding sequence (provided by Thomas Lufkin) was inserted into the intact XhoI site, downstream of the loxP-flanked βgeo/3xP, to produce the Z/AP expression vector (see Fig. 1A).

ES Cell Growth and Electroporation

The R1 ES cell line (Nagy et al., 1993) was maintained essentially as described (Wurst and Joyner, 1993). The cells were grown in Dulbecco’s modified Eagle’s medium/high glucose (Gibco) supplemented with 15% preselected fetal calf serum (Hyclone), 1 mM sodium pyruvate (Gibco), 1 mM nonessential amino acids (Gibco), 2 mM L-glutamine, 10−6 M β-mercaptoethanol, and 1000 U/mL leukemia inhibitory factor (Mereau et al., 1993). For experiments in which drug selection was required, gentamicin (G418; Gibco) was added at an active concentration of 200 μg/mL.

For electroporation, approximately 6 × 107 cells were mixed with 20 μg of linearized DNA and electroporated at 250 V, 500 μF in a Bio-Rad Gene Pulser. After selection with G418 for 7 to 8 days, colonies were picked and grown in duplicate 96-well plates. One replicate was used for lacZ staining. Cells were fixed with 0.2% glutaraldehyde in PBS for 5 min. After three rinses in wash buffer (2 mM MgCl2, 0.01% deoxycholate, 0.02% Nonidet-P40, and 100 mM sodium pyruvate (Gibco), 1 mM nonessential amino acids (Gibco), 2 mM L-glutamine, 10−6 M β-mercaptoethanol, and 100 U/mL leukemia inhibitory factor (Mereau et al., 1993)). For experiments in which drug selection was required, Gentamicin (G418; Gibco) was added at an active concentration of 200 μg/mL.

Tetraploid and Diploid Embryo Aggregation

Three selected ES cell lines carrying the Z/AP vector were used for tetraploid embryo aggregation following our standard protocol (Nagy and Rossant, 1993). In brief, two-cell-stage embryos (1.5 dpc, days postcoitum) were flushed from superovulated ICR outbred females and placed in groups of 50 between two platinum electrodes (250 μm apart) overlaid with a large drop of 0.3 M mannitol solution, pH 7.4. The embryos were oriented by a high-frequency AC electric field provided by a CF-150 fusion instrument (BLS, Inc., Hungary) in such a way that the field of the subsequently applied DC pulse (40 V, 30 μs) was perpendicular to the plane between the two blastomeres of the embryos. The fused tetraploid embryos were cultured overnight and used for sandwich-type aggregation with ES cells (Nagy and Rossant, 1993) the next day at their four-cell stage.

ES cell ↔ diploid embryo aggregation chimeras were produced to obtain germline transmission of the Z/AP transgene. The procedure was similar to the tetraploid embryo aggregation except that the embryos were recovered at the eight-cell stage (2.5 dpc), no electroporation was performed, and only a single embryo was aggregated with each clump of ES cells. The aggregates were cultured overnight and transferred to ICR pseudopregnant recipients. The pregnancies with the tetraploid embryo aggregations were terminated at 9.5 and 12.5 dpc and the recovered completely ES cell-derived embryos were stained for lacZ expression as published (Nagy and
Rossant, 1993). Chimeras made with diploid embryos were tested for germline transmission by mating with ICR females.

**Genotyping**

To establish the genotype and transgene copy number in the Z/AP lines initially, genomic DNA was prepared from ear punch or tail biopsy samples, digested with EcoRV, and used in Southern blot analysis (Southern, 1975). The $^{32}$P-labeled probe was a 464-bp BglII-HindIII fragment, including the rabbit $\beta$-globin polyadenylation sequence of the Z/AP vector. Subsequently, the Z/AP reporter expression allowed us to use whole-mount in situ staining for both lacZ and hPLAP on tissue samples, such as ear punch or yolk sac, for genotyping. In this case, the tissue samples were put into PBS in 96-well dishes, then fixed for 5 to 30 min in 0.2% glutaraldehyde in PBS. After washing with PBS, tissues were stained in X-gal (see above). Staining was usually evident after 5 min.

**lacZ and hPLAP Staining**

For whole-mount lacZ staining of embryos and tissues, samples were rinsed in 100 mM sodium phosphate (pH 7.3) or PBS before fixing. Small embryos (E9.5) and tissues were fixed in lacZ fix (0.2% glutaraldehyde, 50 mM EGTA, pH 7.3, 100 mM MgCl$_2$ in 100 mM sodium phosphate, pH 7.3, or PBS) for 30 min on ice with shaking. Larger embryos were fixed in 2% paraformaldehyde and 0.2% glutaraldehyde in PBS for 30 min on ice with shaking and then bisected and fixed for an additional 30 to 60 min on ice in lacZ fix. Large tissues were fixed in lacZ fix for 4 h on ice with shaking: the tissue was bisected after the first hour to allow penetration of the fix solution. Following fix, samples were washed three times for 15 to 30 min in lacZ wash buffer (2 mM MgCl$_2$, 0.01% sodium deoxycholate, 0.02% Nonidet-P40 (NP-40) in 100 mM sodium phosphate, pH 7.3, or PBS). Staining was carried out in 0.5 mg/ml X-gal, 5 mM potassium ferrocyanide, and 5 mM potassium ferricyanide in lacZ wash buffer at 37°C or room temperature for 30 min to overnight, with shaking and protection from light. After

![Diagram](image-url)
completion of staining, samples were washed three times for 10 min in PBS and stored in lacZ wash buffer at 4°C. For alkaline phosphatase staining, embryos and tissues were fixed as for the lacZ whole-mount protocol above. For better penetration of the alkaline phosphatase (AP) substrates, 0.02% NP-40 and 0.01% sodium deoxycholate were added to the fix solution. Samples were rinsed in PBS and then endogenous alkaline phosphatases were heat inactivated by incubation in PBS at 70 to 75°C for 30 min. Samples were rinsed in PBS, washed in AP buffer (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 10 mM MgCl₂) for 10 min, and stained with BM Purple AP substrate (Boehringer Mannheim) at 4°C for 0.5 to 36 h. When staining was complete, the samples were washed extensively in PBS containing 0.1% Tween 20 and 2 mM MgCl₂.

To prepare sectioned embryos and tissues, samples were washed in PBS three times for 15 to 30 min after fixation and cryoprotected in 15% sucrose in PBS for 1 h at 4°C and then in 30% sucrose in PBS overnight at 4°C. They were then incubated in Tissue-Tek OCT (Sakura) at 4°C for at least 1 h prior to embedment in OCT over dry ice. Blocks were cryosectioned at 10 μm, placed onto polylysine-coated slides (Fisher Scientific), and dried for 1 to 4 h at room temperature before storage at −20°C.

Prior to staining, slides were refixed in cold PBS containing 0.2% glutaraldehyde for 10 min. For lacZ staining, slides were washed three times for 5 min in lacZ wash buffer and then stained in lacZ stain solution for 4 to 6 h at 37°C, protected from light. When the staining was complete, slides were rinsed in PBS before dehydration through a graded ethanol series and mounting with coverslips. For alkaline phosphatase staining, after fixation slides were washed three times in PBS for 5 min and endogenous alkaline phosphatase was inactivated by incubating slides in PBS at 70 to 75°C for 30 min. Slides were then rinsed with PBS, washed in AP buffer (see above) for 10 min, and overlayed with NBT/BCIP stain (100 mM Tris–HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl₂, 0.01% sodium deoxycholate, 0.02% NP-40, 337 μg/ml NBT (nitroblue tetrazolium salt; Boehringer Mannheim), and 175 μg/ml BCIP (5-bromo-4-chloro-3-indolyl phosphate, toluidinium salt; Boehringer Mannheim). The staining reaction was allowed to proceed for 10 to 30 min at room temperature. Slides were then washed in PBS, dehydrated through an ethanol series, and mounted with coverslips. For lacZ/alkaline phosphatase double staining, slides were first stained for lacZ activity and washed, endogenous alkaline phosphatase was heat inactivated, and the slides were stained for alkaline phosphatase activity.

**RESULTS**

**Z/AP Reporter Vector**

Three fundamental features were envisioned for an ideal transgene reporting Cre recombinase activity: (1) strong widespread expression, (2) a convenient reporter gene to be expressed only before Cre-mediated recombination, and (3) a different reporter gene to be expressed only after Cre-mediated recombination. A transgene was designed in such a way that Cre activity resulted in excision of the first reporter gene, which consequently permitted expression of the second reporter gene (Fig. 1A). A lacZ/neomycin fusion gene (βgeo; Friedrich and Soriano, 1991) coding sequence was used as the first reporter. Use of the βgeo provided not only a visual reporter but also a drug selection marker for ES cells carrying the expression construct. The βgeo was
followed by a triple repeat of the SV40 polyadenylation signal (Zinyk et al., 1998) to ensure a transcriptional stop and flanked by loxP sites. The hPLAP coding sequence (DePrimo et al., 1996; Millan, 1986) was used as our second reporter, following the loxP-flanked region. The hPLAP was not expected to be expressed until after Cre excision of the βgeo (Fig. 1A).

Several different promoters, including the CMV (Thomsen et al., 1984), human β-actin (DePrimo et al., 1996), pII-3-hydroxy-3-methylglutaryl coenzyme A reductase (pIIHMG-CoA) (Luskey, 1987), and a CMV enhancer/chicken β-actin hybrid gene promoter (pCAGG; Niwa et al., 1991), were tested in preliminary experiments for their potential to drive ubiquitous expression. This was accomplished by fusing the promoters to the lacZ gene, coelectroporating them with a PGK-neo<sup>R</sup> expression vector (Tubulewicz et al., 1991) into ES cells, and selecting G418-resistant colonies. The G418-resistant clones were then evaluated for uniformity and strength of lacZ expression. The pCAGG promoter provided the most consistent and strongest expression of the lacZ reporter, thus it was chosen for further use in the Cre-activity reporter transgene described above.

From our studies of the expression reliability of the promoters, we recognized that only a few percent of the transgene-positive ES cell clones displayed an overall expression of the lacZ gene, whereas most of the clones expressed lacZ in a mosaic manner. Each of these ES cell clones represented an individual integration site. We hypothesized that those integration events that provided overall lacZ expression within an ES cell clone could lead to a more reliable and widespread expression in corresponding transgenic embryos or animals. Therefore in our final strategy (depicted in Fig. 1B) we implemented an in vitro screen of the ES cell clones for overall and strong expression. Following these criteria, 3 of the total of 96 clones were selected for further analysis.

The selected ES cell clones were tested for in vivo transgene expression by ES cell tetraploid embryo aggregation to generate completely ES cell-derived embryos (Nagy and Rossant, 1993). The embryos were stained at E9.5 and E12.5 for lacZ expression and the results supported our expectation of achieving widespread expression in the embryo (not shown). Subsequently, ES cell diploid embryo aggregation was carried out (Wood et al., 1993) and one of these three cell lines provided germ-line transmission. Southern analysis of the F1 animals revealed that the transmitted line carried three independent sites of integration of the Z/AP transgene. One of the integration sites (H9) contained a single copy of the transgene. The second site (H18) was a tandem double-copy integration and the third site (H2) contained a multiple copy integration. In order to avoid any possible complications due to recombination between multiple loxP sites, these transgenes were segregated from each other by further out-crossing. After segregation, the lacZ expression was redetermined. H18 gave widespread mosaic expression. H2 displayed strong widespread expression but no double transgenic animals were obtained when it was crossed with a Cre transgenic line. The H9 integration retained strong lacZ expression and there was no problem in obtaining double transgenic animals when it was crossed with Cre lines. Therefore the H9

**FIG. 3.** Early embryonic expression of the Z/AP reporter transgene. (A) Blastocysts fathered by a hemizygous Z/AP transgenic male mouse, stained for lacZ. The strong staining of 50% of the embryos (8/14) indicates that the paternally inherited Z/AP transgene is already strongly expressed during preimplantation stages. (B–F) Sagittal sections of E9.5 embryos stained for lacZ (B, C) or hPLAP (D, E). Before Cre excision, embryos express lacZ (B) but not hPLAP (D), whereas after Cre excision, embryos do not express lacZ (C) but do express hPLAP (E). A higher magnification (F) shows the overall expression of the hPLAP reporter at the cell level.
line became the subject of all further detailed characterization. We refer to this mouse line as the Z/AP reporter line.

Z/AP Reporter Expression at Early Embryonic Stages

Single transgenic Z/AP mice and offspring of Z/AP hemizygous × CMV–Cre homozygous mice were analyzed for lacZ and hPLAP reporter gene expression to characterize the extent of expression and the lacZ/hPLAP reporter switch due to Cre-mediated excision. The CMV–Cre transgenic line was established in a previous study (Nagy et al., 1998), in which we found that the Cre recombinase was expressed at early embryonic stages and resulted in an overall excision of loxP-flanked sequences in a large proportion of double-transgenic embryos. As the generation number increased, the Cre-mediated excision became variable and we observed an increasing number of mosaic embryos or animals (A.N., unpublished). Some double-transgenic embryos, however, underwent complete excision of the βgeo and demonstrated the principle of this double reporter system. Single-transgenic Z/AP embryos were positive for lacZ activity and negative for hPLAP activity (Figs. 2A and 2B). Double-transgenic embryos that received both the Z/AP and the CMV–Cre transgene and underwent complete excision of lacZ were lacZ negative and instead stained for hPLAP (Fig. 2C). Thus, Cre excision resulted in the replacement of the lacZ expression with hPLAP expression. In some double-transgenic embryos the Cre-mediated excision was not complete and the embryos remained complementary mosaic for lacZ and hPLAP (data not shown).

Transgenic Z/AP embryos were tested as early as the blastocyst stage for transgene expression. To ensure that the source of expression was embryonic, the Z/AP transgene was transmitted paternally. The E3.5 preimplantation-stage blastocysts expressed the paternally inherited Z/AP transgene at a high level (Fig. 3A).

To characterize the expression at the cellular level, sagittal sections were performed on E9.5 Z/AP and Cre-excised Z/AP embryos (Figs. 3B–3F). We were not able to pinpoint any cell type other than erythrocytes in these embryos which did not express the reporters, regardless of whether it was intact Z/AP stained for lacZ (Fig. 3B) or excised Z/AP stained for hPLAP (Figs. 3E and 3F). We also noted that there was no leaky hPLAP expression from the intact Z/AP transgene (Fig. 3D) and that lacZ expression was eliminated upon Cre excision (Fig. 3C).

Z/AP Reporter Expression at the Newborn Stage

The successful switch in reporter gene expression persisted at the newborn stage. The whole-mount staining of a Z/AP transgenic newborn showed very intensive and extensive transgene activity (Fig. 4A). Various tissues, including brain, intestine, kidney, chondrocytes, lungs, liver, muscle, testis, heart, esophagus, pancreas, spleen, and adrenal gland, were sectioned and stained for either lacZ (not shown) or hPLAP (Figs. 4B–4Q). In the Z/AP single-transgenic mice, lacZ was expressed in all tissues tested with the exception of the nucleated erythrocytes, chondrocytes, and adipocytes (not shown). As observed for the Z/AP, CMV–Cre double-positive transgenic embryos, expression of Cre resulted in loss of lacZ activity and gain of hPLAP activity in all previously lacZ-positive tissues (Figs. 4B–4Q). Interestingly, we observed alkaline phosphatase activity in adipocytes and chondrocytes, which were lacZ negative (Fig. 4H), but not in erythrocytes (Figs. 4M, 4P, and 4Q).

Z/AP Reporter Expression in an Adult Mosaic for Cre Excision

To demonstrate an additional advantage of the Z/AP reporter line, adult Z/AP, CMV–Cre double-transgenic tissues with mosaic Cre excision of the βgeo reporter are shown in Fig. 5. The lacZ- and alkaline phosphatase-positive cells indicate the preexcision and postexcision status, respectively. These cells were arranged in a complementary manner that was clearly demonstrated by staining adjacent sections for the two reporters separately and double-staining for the two reporters on a single section (Fig. 5, left, middle, and right columns, respectively).

Transgene Expression with Sporadic and Tissue-Specific Deleters

Z/AP, CMV–Cre double-transgenic embryos occasionally showed a low-level sporadic excision of βgeo. In this case, only a few clustered hPLAP-positive cells were observed, indicating their clonal origin (Fig. 6A).

We recently generated a mouse line with a Cre recombinase "knock-in" into the tissue nonspecific alkaline phosphatase (TNAP) gene (Lomeli and Nagy, in preparation). The TNAP gene is expressed in primordial germ cells and, at a lower level, in the neural tube after gastrulation (Hahnel et al., 1990; MacGregor et al., 1995). Crossing the TNAP-Cre line with the Z/AP reporter mice provided a test

![FIG. 4. Z/AP reporter expression in newborn tissues. (A) A 5-day-old Z/AP transgenic pup was bisected sagittally and stained for lacZ expression. (B–E) Brain sections of Z/AP newborn mice before (B, C) or after (D, E) Cre excision stained for lacZ (B, D) or alkaline phosphatase activity (C, E). (F–Q) Tissue sections from double-transgenic (Z/AP, CMV–Cre) newborn mice with complete Cre-mediated excision of lacZ stained for alkaline phosphatase activity. (F) Intestine, (G) kidney, (H) trachea, (I) lung, (J) liver, (K) muscle, (L) testis, (M) heart, (N) esophagus, (O) pancreas, (P) spleen, and (Q) adrenal gland (m, muscle; c, chondrocytes; e, erythrocytes). All cells express the transgene except for erythrocytes.](image)
for its cell specificity of Cre excision. Sections of Z/AP, TNAP–Cre double-transgenic E13.5 embryos confirmed that hPLAP staining was confined to cells within the ventral neural tube (Fig. 6B) and gonads (Fig. 6C), pinpointing the sites of Cre activity.

**DISCUSSION**

An international collaboration is presently under way to develop a battery of transgenic mice expressing Cre recombinase with different tissue and temporal specificities (URL: http://www.mshri.on.ca/develop/nagy/Cre.htm). These mice will provide the possibility to carry out specific genome alterations, such as knockouts, induced chromosome aberrations, and conditional transgene activations, in a way that alterations can be restricted to specific tissues and time frames.

As this database of Cre transgenic mice has grown, three problem areas have become evident. First, many of the promoters being used to drive Cre expression have not been characterized for their activity during early embryonic development. Using such promoters to drive Cre can result in broad, nonspecific excision arising from early expression of the Cre enzyme. Second, most of the promoters are integration site-dependent as far as their specificity is concerned. For these, a large number of transgenic lines must be produced in order to find one that is satisfactory. Finally, an insufficient level of Cre expression may lead to only partial (mosaic) activity in the lineage of specificity. In this case, supplying the Cre recombinase with a nuclear localization signal might be a solution, as long as the level of background (unwanted) activity of the recombinase remains low. Because of these issues, a thorough characterization of the sites of Cre recombinase activity has to precede the use of a transgenic line in real experiments aimed at specific genome alterations. At this point, however, it is important to note that we still have to explore whether different floxed regions of the genome with comparable size are equivalent with regard to their ability to undergo Cre-mediated recombination. It is possible that certain chromosomal regions are more readily available for the recombinase than others. Therefore, we are eager to see the development of other reliable reporter lines with obviously different integration sites to reveal the extent of such variation.

The most suitable assay for Cre activity at the cellular level would be a transgenic mouse line that expresses a reporter gene in response to Cre recombination. In two previous Cre reporter mice, gain of lacZ activity was used to indicate Cre excision at the cellular level (Akagi et al., 1997; Tsien et al., 1996). In the first mouse, a cross to CMV–Cre transgenic mice showed that the lacZ reporter expression is limited to the nervous system and muscle (Tsien et al., 1996; Zinyk et al., 1998). A detailed characterization of the potential for lacZ reporter expression in all tissues was not carried out for the second reporter mouse (ACZL reporter line) (Akagi et al., 1997). Therefore, it was not clear with this ACZL reporter line whether an absence of lacZ expression was due to a lack of Cre excision or to a lack of potential for lacZ expression.

The difficulty of establishing satisfactory Cre activity reporter lines stems from two problems. First, conventional transgenesis via pronuclear injection frequently results in multiple-copy integration of a transgene. Multiple-copy integration can create an unwanted number of loxP sites and possible chromosomal instability (Lewandoski and Martin, 1997) or an unpredictable outcome of Cre-mediated excision. ES cell-mediated transgenesis provided a higher frequency of single-copy transgene integration. Although our final Z/AP line was derived from an ES line containing three separate integration sites, one of the three had only a single copy of the transgene, which was relatively easy to segregate and establish the desired Z/AP transgene. Second, it is difficult to obtain widespread expression from randomly integrated transgenes. The most reliable lacZ reporter lines so far are gene trap lines such as Rosa 26 (Friedrich and Soriano, 1991). One obvious way to reach a similar level of reliability for the Cre activity reporter mouse is to reintroduce a conditional lacZ construct into the Rosa-26 integration site, as has been recently reported (P. Soriano, in press). In the production of the Z/AP reporter line, our approach of using ES cell-mediated transgenesis and an in vitro selection from a large number of integration sites for overall strong expression shared similarities with the gene/enhancer trap approaches (Friedrich and Soriano, 1991; Gossler et al., 1989). This procedure likely selects for permissive integration sites or the presence of nearby general enhancers.

Although Z/AP is widely expressed at all stages, we knew that no transgenic or gene trap lines with proven ubiquitous expression have so far been made. Therefore, we carefully searched for nonexpressing cells in Z/AP embryos, since this information will be essential for those planning to use the line for evaluation of Cre activity, and found that the Z/AP transgene does not express lacZ in erythrocytes, chondrocytes, and adipocytes. Interestingly, after Cre excision only erythrocytes remained hPLAP negative. The simplest explanation for this discrepancy is the possible sensitivity difference between the lacZ and the hPLAP reporters.

**FIG. 5.** Expression in adult tissues. Tissues from adult double transgenic (Z/AP, CMV–Cre) mice with mosaic excision were sectioned and assayed for lacZ expression (left column), hPLAP expression (middle column), or both (right column). Expression of the reporters indicates the mosaic pattern of Cre excision in this mouse and their complementarity.
We envision several advantages of the Z/AP reporter mouse. First, the reporter transgene is widely expressed at all stages during the lifetime of the animal. In addition both reporters are easily visualized by an enzymatic staining reaction. Finally, it utilizes a binary reporter system in which the lacZ reporter is expressed before Cre excision and the human placental alkaline phosphatase reporter is expressed after Cre excision.

FIG. 6. Use of the Z/AP reporter to mark tissue-specific Cre activity. (A) A double-transgenic (Z/AP, CMV–Cre) E9.5 embryo stained for alkaline phosphatase activity. In this particular embryo the clusters of stained cells indicate a limited sporadic excision by CMV–Cre. (B, C) Transverse sections of an E13.5 double-transgenic (Z/AP, TNAP–Cre) embryo were positive for hPLAP staining in certain cells in the ventral neural tube (B) and in the gonads (C).
The binary reporter system discriminates between a lack of reporter expression and a lack of Cre excision. Cre-mediated excision is indicated by the loss of lacZ expression and a gain of hPLAP expression. The usefulness of the binary reporter system was demonstrated in the double-transgenic Z/AP, CMV–Cre adult mouse, in which the two reporters revealed the mosaic pattern of Cre excision within each tissue. The pattern of complementarity was strikingly different from organ to organ. Heart, pancreas, and testis, for example, contained large patches of lacZ-positive/hPLAP-negative cells and of hPLAP-positive/lacZ-negative cells. This pattern may reflect some common clonal organization of cells within these tissues. The liver, brain, and kidney, on the other hand, showed a very different picture: a fine mixing of lacZ-positive and hPLAP-positive cells.

We recently inserted the wild-type Cre gene into the tissue-nonspecific alkaline phosphatase gene by homologous recombination. To evaluate the site of Cre activity of this Cre transgenic line, it was crossed with the Z/AP reporter mice. The double-transgenic embryos stained for lacZ expression and a gain of hPLAP expression. The double-transgenic embryos convincingly demonstrated the expected sites (MacGregor et al., 1995) of Cre activity and specificity in the developing gonads and ventral neural tube.

The Z/AP reporter line will complement the ongoing efforts to develop the Cre/loxP system. This system opens up the possibility of making conditional alterations of the mouse genome, which is a critical advance in our ability to make suitable mouse models of human disease.

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