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Cre-mediated somatic site-specific recombination in mice

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ABSTRACT

Conditional mutant mice equipped with heterologous recombination systems (Cre/lox or Flp/frt) are promising for studying tissue-specific gene function and for designing better models of human diseases. The utility of these mice depends on the cell target specificity, on the efficiency and on the control over timing of gene (in)activation. We have explored the utility of adenoviral vectors and transgenic mice expressing Cre under the control of tissue-specific promoters to achieve Cre/lox-mediated somatic recombination of the *LacZ* reporter gene, using a newly generated flox *LacZ* mouse strain. When adeno Cre viruses were administered via different routes, recombination and expression of *LacZ* was detected in a wide range of tissues. Whereas in liver β-galactosidase activity was quickly lost by turnover of expressing cells, even though the recombined allele was retained, β-galactosidase in other tissues persisted for many months. Our data indicate that the flox *LacZ* transgenic line can be utilized effectively to monitor the level and functionality of Cre protein produced upon infection with adeno Cre virus or upon crossbreeding with different Cre transgenic lines.

INTRODUCTION

Recent advances in mouse embryology and molecular genetics have made it possible to specifically mutate defined genes in the germline of mice. Protocols using transgenesis (1) and homologous recombination in embryonic stem (ES) cells (2,3) permit overexpression, inactivation and modification of genes more or less at will. Mutations, identical to those observed in inherited or somatically acquired diseases such as in cancer, can now be mimicked in a mouse model system. These technologies are invaluable in assessing the role of genes in complex processes such as tumorigenesis, embryonic development and functioning of the immune system. However, there are a number of limitations as well, e.g. nullizygosity appears to be lethal in many instances or causes complex pleiotropic effects and, therefore, does not permit the development of an *in vivo* model system in which gene inactivation is restricted to a defined subset of cells (4).

To overcome these limitations, strategies for conditional, cell type-specific gene targeting (5) and inducible gene disruptions (6) have recently been developed. These systems take advantage of site-specific recombinases such as the Cre/loxP recombination system of bacteriophage P1, which requires only two well-characterized components: the 38 kDa recombinase protein Cre and the 34 bp long Cre-specific recognition sequence, loxP (7–9). The utility of this system has been shown both by the generation of conditional transgenic mice and the production of conditional gene knockouts. In the latter case, a target construct flanked by two loxP sites (flox) was used to modify the cognate gene by homologous recombination in ES cells. The expression level of the floxed allele is expected to be the same as that of the wild-type and should, therefore, not lead to phenotypic changes. Crossing of the floxed mice with transgenic mice carrying the Cre recombinase gene under the control of a cell type-specific promoter or an inducible promoter then leads to excision of the intervening sequences. This strategy has been shown to work in a number of settings (6,10).

However, the current systems do not provide optimal flexibility. Three issues are relevant in this respect: (i) recombination in other cells than the desired target cell might occur due to inappropriate or unexpected expression of the *Cre* transgene; (ii) lack of control over the developmental timing of recombination as a result of the nature of the promoter used or leakiness of the inducible promoter chosen; (iii) complexity of the breeding when (multiple) conditional alleles have to be combined with specific transgenes.

We have started to address these questions by: (i) generating a flox *LacZ* indicator mouse that permits monitoring of the activity and timing of the Cre recombinase *in vivo*; (ii) exploring the utility of vectors designed for somatic gene transfer to introduce the Cre recombinase at a desired time into somatic cells.

First, we have explored the utility of a Cre adenovirus (11,12) to mediate Cre/lox recombination *in vivo*. Our results confirm and further extend studies using adenoviral vectors to mediate recombination in mice (13). Earlier, the use of adenoviral vectors has been explored for their application in gene therapy protocols.

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From these studies it has become clear that adenoviruses can infect a variety of cell types *in vivo* irrespective of whether the cells are actively dividing or not (14–20). It has become apparent that in immunocompetent hosts expression of genes transduced by adenoviral vectors lasts, in general, <3 or 4 weeks, because of elimination of the infected cells through a cell-mediated immune response (21,22).

Here we show that, using the flox *LacZ* indicator mouse strain, adeno Cre virus can mediate recombination at high frequency in a range of somatic tissues. We also demonstrate that the switch-on of *LacZ*, rather than expression of *Cre* or viral structural genes, leads to elimination of cells that have undergone recombination of the chromosomally localized *LacZ* reporter gene. Finally, the system provides the potential for efficient and simple lineage marking.

MATERIALS AND METHODS

Generation of transgenic mice

The pCAG promoter–loxP–*CAT*–loxP–*LacZ* DNA for flox *LacZ* indicator mice was generously provided by Dr Kimi Araki (23). For cell type-specific expression of the Cre recombinase, a 1.4 kb fragment containing the *Cre* open reading frame with an N-terminal nuclear localization signal (nlsCre) (5) and synthetic intron was isolated from pOG231 (a gift of Dr O'Gorman). This fragment was inserted between 706 bp of the rat pro-opiomelanocortin (POMC) promoter 5′ flanking sequence (24) and SV40 splice and polyadenylation signal or the 1.1 kb segment of 5′ flanking sequence from the rat P0 gene (25) and rabbit β-globin 3′ splicing and polyadenylation signal. nlsCre was inserted between 1.3 kb of 5′ flanking sequence of human interphotoreceptor retinoid binding protein (IRBP) (26) and rabbit β-globin 3′ splicing and polyadenylation signal. The inserts were cut out, freed from vector sequences by agarose gel electrophoresis, purified and injected into fertilized FVB/N mouse (27) oocytes essentially as described (28). The founder mice were bred to FVB mice to maintain the line. The presence of the transgenes was determined by Southern analysis of tail tip DNA.

DNA analysis

DNA was isolated from tissues using standard procedures. Briefly, tissues were minced and digested overnight in SDS, proteinase K buffer. These were followed by two phenol extractions, one phenol/chloroform extraction, chloroform/isoamyl alcohol extraction, ethanol precipitation and resuspension in TE buffer. An aliquot of 10 µg DNA was digested with *Eco*RI and *Xba*I and subjected to Southern analysis. The 1.0 kb of *Sac*I–*Eco*RI *LacZ* fragment and Cre recombinase coding sequence amplified by PCR were used as probes to estimate recombination of the flox *LacZ* transgene and the quantity of adeno Cre viral genome. The band intensities were quantified with a Bioimaging analyzer (Fuji Bas 1000). Percentage recombination was calculated by subtraction of the background.

Construction of adenoviral vector

The Cre adenovirus vector was constructed and propagated essentially as described (11). Briefly, the *Hin*dIII fragment of pBS185 containing the *Cre* ORF under control of the hCMV immediate early promoter and the metallothionein I polyadenylation signal (29) was inserted into the *Hin*dIII site of pdE1spIA and co-transfected with pJM17 into 293 cells. The viruses were plaque purified and re-analyzed prior to preparation of large scale stocks. The recombinant adenoviral vector was propagated in 293 cells and purified by cesium chloride density centrifugation.

PCR analysis

For PCR analysis, 0.5 µg DNA were submitted with Perfect Match DNA Polymerase Enhancer (Stratagene) to 38 cycles of amplification (each cycle consisting of 1 min at 94°C,1 min at 57°C and 2 min at 72°C). To explore the junction between actin promoter and *LacZ*, AG2 (5′-CTGCTAACCATGTTCATGCC-3′) and Z3 (5′-GGCCTCTTCGCTATTACG-3′) primers were used.

Animal studies

All animals were housed in SPF facilities. Mice were moved and maintained in an isolator after administration of adeno Cre viruses. Aliquots of 0.2 ml viral solution (10^9 p.f.u.) were injected into the tail vein or 10^7 p.f.u. virus were injected into muscle, lung or under the skin.

Histochemistry

Mice were sacrificed and organs were fixed in 0.2% glutaraldehyde solution or mounted in OCT compound. After a rinse in detergent, organs were incubated in 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal) staining solution at 37°C essentially as described (28). A post-fixation was performed for 24–48 h in 10% formalin. Organs were dehydrated and embedded in paraffin for sectioning.

Frozen sections were fixed in 0.2% glutaraldehyde, 0.1 M phosphate buffer, pH 7.3, 5 mM EGTA, 2 mM $MgCl_2$ on ice for 5 min, rinsed three times in phosphate-buffered saline (PBS) with 2 mM $MgCl_2$ on ice, placed in detergent rinse on ice for 10 min, incubated in staining solution (5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40 in 0.1 M phosphate buffer, pH 7.3) at 37°C overnight. Then they were washed twice in PBS with 2 mM $MgCl_2$ at room temperature for 5 min each, rinsed in distilled water and counterstained in neutral red for 1 min. After staining, samples were rinsed in distilled water three times, dehydrated with ethanol (5 min each in 70 and 100%), cleared twice in xylene and mounted.

RESULTS

flox *LacZ* indicator mouse

To assess the efficiency of Cre-mediated site-specific recombination, we have generated flox *LacZ* reporter mice that carry the chicken β-actin promoter (30) and β-galactosidase (β-gal) transcription unit separated by the *CAT* gene flanked by loxP sites (Fig. 1). The *CAT* transcription unit prevents β-gal expression. However, when the *CAT* gene is excised, β-gal expression will ensue (23). Fourteen independent transgenic founders were produced and two of them carried one and two copies of the transgene. Expression of the *CAT* gene was estimated by Northern blot analysis of RNA isolated from the various tissues. Both strains showed high mRNA levels in heart and muscle, moderate levels in kidney, lung, thymus, spleen, stomach, intestine, brain, retina and peripheral nerves and low amounts of mRNA in liver (data not shown). The single copy transgenic line was selected for the experiments described below. No *LacZ* expression can be detected in this mouse line by X-gal staining without exposure to

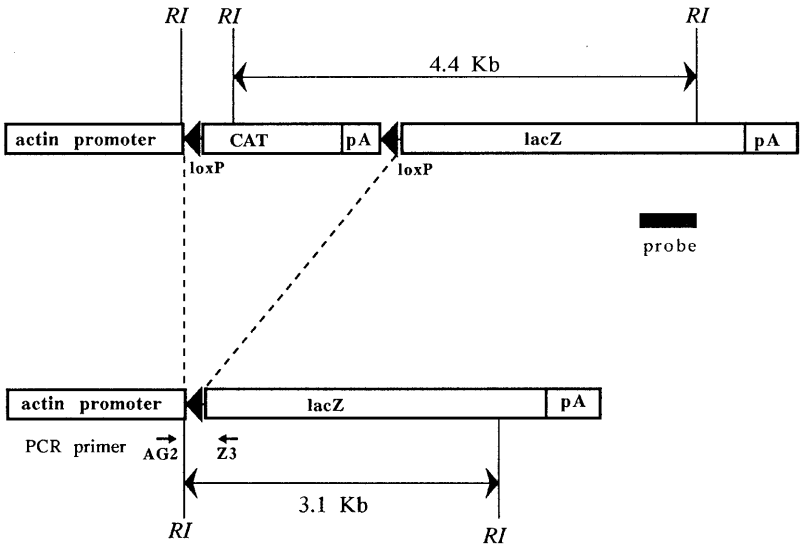

Figure 1. Cre-mediated activation of the *LacZ* gene. The widely active chicken β-actin (CAG) promoter is separated from the *LacZ* transcription unit by the *CAT* gene. pA and RI in the maps designate the SV40 polyadenylation signal and the position of the *Eco*RI site respectively. Cre-mediated recombination leads to juxtaposition of the *LacZ* gene to the promoter. The recombined and non-recombined transgenes can be discerned by *Eco*RI digestion as 3.1 and 4.4 kb fragments respectively. The 1.0 kb *Sac*I–*Eco*RI fragment (shown as a bar) was used as hybridization probe.

the Cre recombinase. Therefore, this transgenic mouse line allows evaluation of the recombination frequency not only by *LacZ* staining *in situ*, but also by Southern blot analysis on tissue DNA. Upon *Eco*RI digestion, the single copy transgene gives rise to fragments of 4.4 and 3.1 kb when unrearranged and recombined respectively (Figs 1 and 2A).

Somatic gene switching in flox *LacZ* reporter mice using adeno Cre virus

Recombinant adeno Cre virus was constructed as described (11). Aliquots of 10^9 p.f.u. vector or the same volume of physiological saline (220 µl) were infused into the tail vein of 6-week-old flox *LacZ* reporter mice and non-transgenic control mice; 5, 15, 30 and 60 days later the mice were sacrificed and frozen tissue sections were stained with X-gal and counterstained with neutral red or hematoxylin/eosin. Strong *LacZ* expression was observed in a wide range of tissues: liver, heart, pancreas, intestine, lung, muscle, kidney and adipose tissue. Furthermore, recombination could be detected by PCR in most tissues except brain (see Fig. 5).

The liver appeared the preferred target site after i.v. inoculation of the adeno Cre virus. It has been shown previously that nearly 100% of hepatocytes can be transduced *in vivo* when a large number of adenoviral particles are infused i.v. (31). Inoculation of 10^9 p.f.u. vector into the tail vein of flox *LacZ* indicator mice resulted in massive staining of the liver. About 65% of hepatocytes were stained by X-gal 5 days after injection (Fig. 3b and g), whereas no staining was observed in control livers (flox *LacZ* mice injected with saline; Fig. 3a and f). However, the fraction of β-gal-positive hepatocytes was reduced to 40, 4 and finally <1% of hepatocytes on days 15, 30 and 60 post-injection respectively (Fig. 3c and h, d and i, e and j and Table 1), probably as the result of a cellular immune response directed against the transduced cells and/or *LacZ*-expressing cells (see below). Evidence for a self immune response against hepatocytes was evident from perivascular infiltration of lymphocytes, extensive degeneration and necrosis of the liver and hypertrophy of cells. Whereas only few abnormalities were seen on day 5, extensive degeneration of hepatocytes and large numbers of aberrant giant nuclei were found at later stages. Lymphocyte infiltration increased especially in the portal triad. Liver regeneration started around day 15 and was mostly completed by day 30. Only few lymphocytic infiltrates were observed perivascularly on day 60 (Fig. 3l–o).

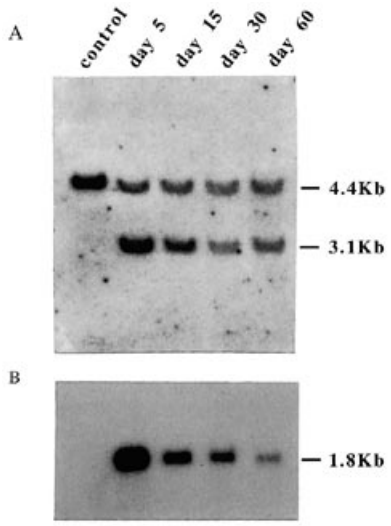

Figure 2. Quantification of adenoviral gene transfer and recombination frequency. Southern blot analysis of liver DNA from recipient mice was used to estimate the frequency of recombination (**A**) and the number of adenoviral vector copies (**B**). Total cellular DNA (10 µg) was prepared from liver of control (lane 1) and recipient flox *LacZ* indicator mice at 5, 15, 30 and 60 days after adeno Cre virus injection (lane 2–5 respectively) and digested with *Eco*RI and *Xba*I. Upon *Xba*I digestion, adeno Cre virus yields a 1.8 kb fragment harboring the Cre recombinase gene. 12, 24, 48 and 96 pg *Xba*I-digested plasmid DNA (pBS185) was applied to the same gel to serve as a copy number control. These amounts of DNA correspond to 1, 2, 4 and 8 copies/cell respectively when 10 µg cellular DNA was loaded per lane. *LacZ* (Fig. 1a) and the Cre ORF probes were used for hybridization to Southern blots. Band intensities were quantified with a Bioimaging analyzer. PBS185 plasmid served as a copy number control.

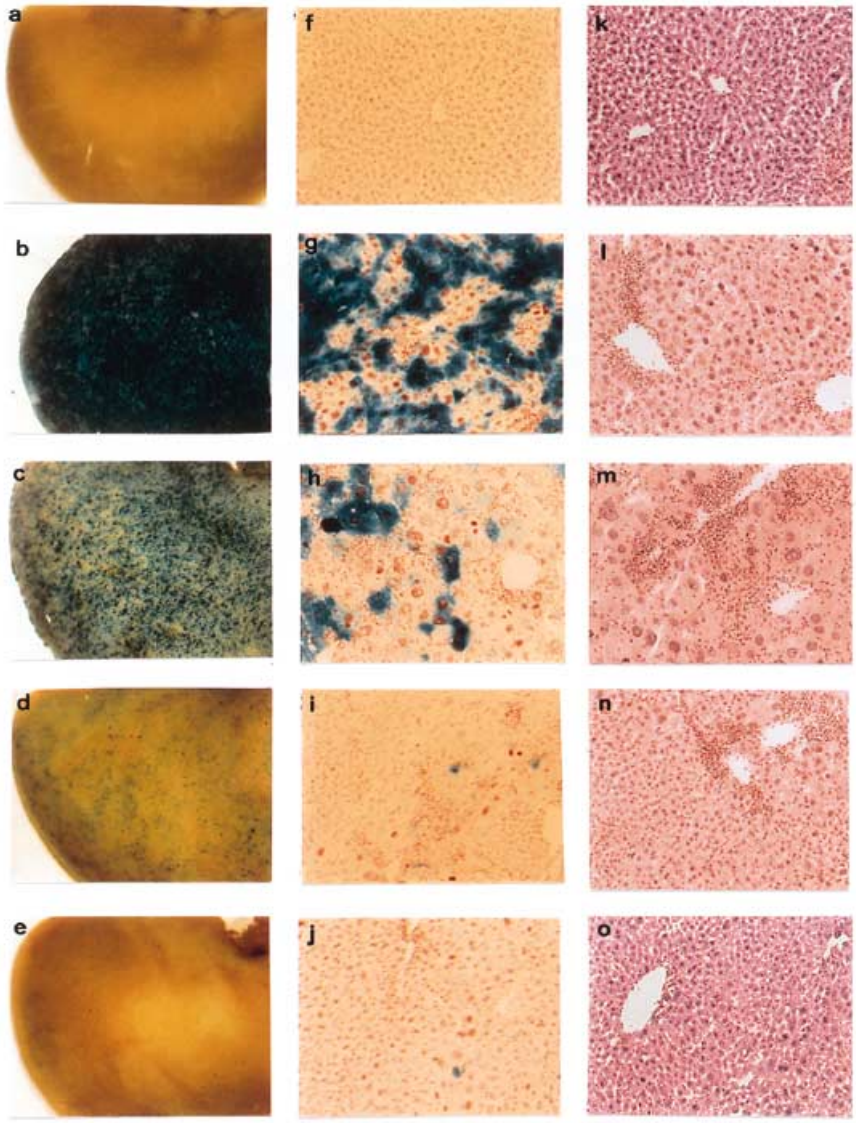

Figure 3. *LacZ* expression and histopathology in liver. 10^9 p.f.u. adeno Cre virus were injected into the tail vein. Livers were collected on days 5 (**b**, **g** and **l**), 15 (**c**, **h** and **m**), 30 (**d**, **i** and **n**) and 60 (**e**, **j** and **o**); **a**, **f** and **k** are controls (flox *LacZ* mice injected with saline). A lobe of liver (a–e) and frozen sections (f–j) were fixed and stained in 5-bromo-4-chloro-3-indolyl β-D-galactoside (X-gal). These sections were counterstained with neutral red. Frozen sections (k–o) were stained with hematoxylin/eosin for histopathology. Magnification: a–e, 8×; f–o, 20×.

Table 1. Summary of parameters measured in adeno Cre virus-infected livers of flox *LacZ* transgenic mice

	Day 5	Day 15	Day 30	Day 60
Recombination frequency by DNA analysis[a]	78%	69%	55%	54%
β-Gal-positive hepatocytes[b]	65%	40%	4%	<1%
Adeno Cre viral copies/cell[a]	5.5	1.5	1.0	0.5
Inflammation	+/–	+++	+	–
Regeneration	–	+/–	+	–

[a]Recombination frequency and adeno Cre viral copy number were calculated by quantifying band intensities of the 3.1 and 4.4 kbp bands (Figs 1 and 2).
[b]β-Gal-positive hepatocytes were counted in H&E stained sections (Fig. 3).

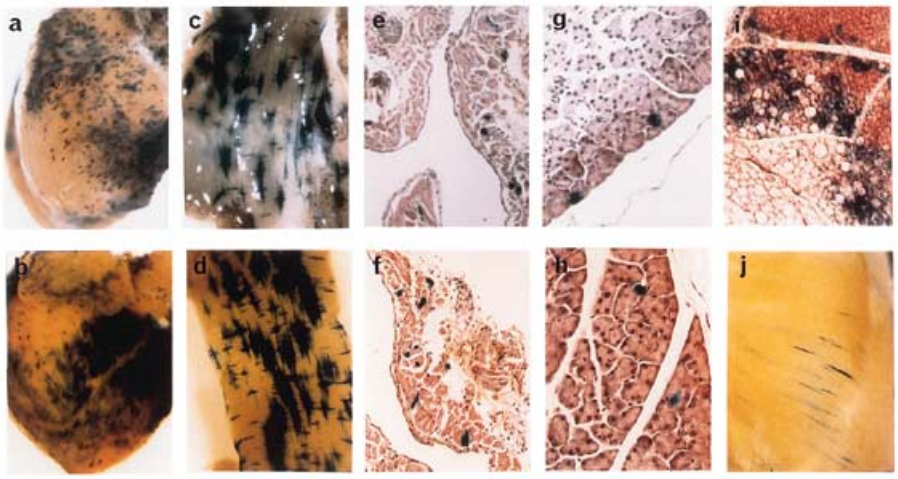

Figure 4. Adeno Cre virus-mediated recombination in transgenic tissues. β-Gal-positive cells in the flox *LacZ* indicator mice injected with adeno Cre viruses in the tail vein (**a**–**h**), s.c. (**i**) and intramuscularly (**j**). Heart (a and b), intestine (c and d), lung (e and f), pancreas (g and h), adipose tissue (i) and muscle (j). Tissues were harvested at 5 (a, c, e and g) and 30 days post-injection (b, d, f and h). Original magnification: a–d and j, 4×; e–i, 20×).

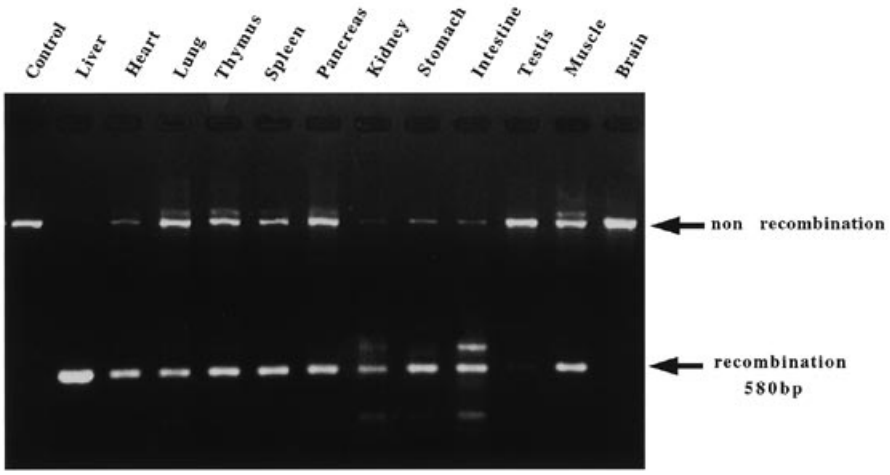

Figure 5. PCR analysis of cre-mediated recombination on the flox *LacZ* transgene in recipient flox *LacZ* mice. Tissues were harvested 5 days after adeno Cre virus injection for DNA isolation. Amplification from the recombined transgene by primers AG-2 and Z-3 yields a 580 bp fragment. The upper band corresponds to the non-recombined transgene. Liver DNA from an untreated flox *LacZ* mouse was used as a control.

Since infected hepatocytes can be efficiently eliminated by an immune response of the host (22,32,33), the amount of viral DNA retained in the liver would be expected to diminish during regeneration. Total DNA from liver of these mice was isolated and the amount of adenoviral DNA was quantified by Southern blot analysis. Viral DNA was still present at the latest time point tested (day 60). The amount of viral DNA detected at the different time points corresponded to 5.5, 1.5, 1.0 and 0.5 copies/cell on days 5, 15, 30 and 60 respectively (Fig. 2B and Table 1). Therefore, >90% of the viral DNA was lost by 60 days. Elimination of infected hepatocytes and re-population of the liver by non-infected hepatocytes seem to have caused the loss of *LacZ*-expressing hepatocytes. Surprisingly, Southern blot analysis showed that 60 days after adeno Cre virus injection >50% of hepatocytes still carried a recombined transgene (Fig. 2 and Table 1). Since regeneration of the liver is mostly completed by day 30, the ratio of recombined and non-recombined transgene in the liver remains unchanged after day 30. The large discrepancy between X-gal staining and Southern blot analysis is likely due to selective survival of hepatocytes that do not express the transgene (see Discussion).

When 10^6 p.f.u. adeno Cre virus were infused into the tail vein of flox *LacZ* mice a much lower frequency of recombination was observed, as illustrated by the *LacZ* staining 5 days after adeno Cre virus inoculation (Fig. 6).

Site-specific recombination in other tissues

After adeno Cre virus infusion into the tail vein of flox *LacZ* mice, β-gal-positive cells were observed at all the time points tested (up to 60 days post-injection) in cardiac muscle (Fig. 4a and b), the muscular layer of the main vessel walls in lung (Fig. 4e and f), the acinar cells of the pancreas (Fig. 4g and h), the longitudinal and circular muscular layer of the intestine (Fig. 4c and d), skeletal muscle and the endothelium of glomerular capillaries and tubular cells of the kidney. Southern blot analysis did not detect

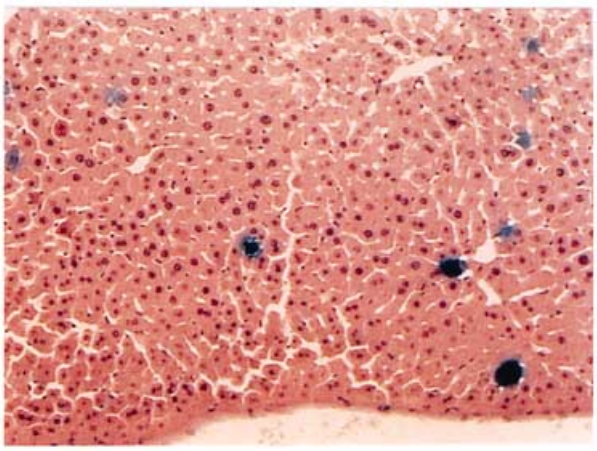

Figure 6. Reduced frequency of recombination in liver using lower titers of adeno Cre virus. 10^6 p.f.u. adeno Cre virus were injected into the tail vain of a flox *LacZ* mouse and the liver was harvested 5 days post-injection and stained for *LacZ*. β-Gal-positive hepatocytes surrounded by normal hepatocytes are observed.

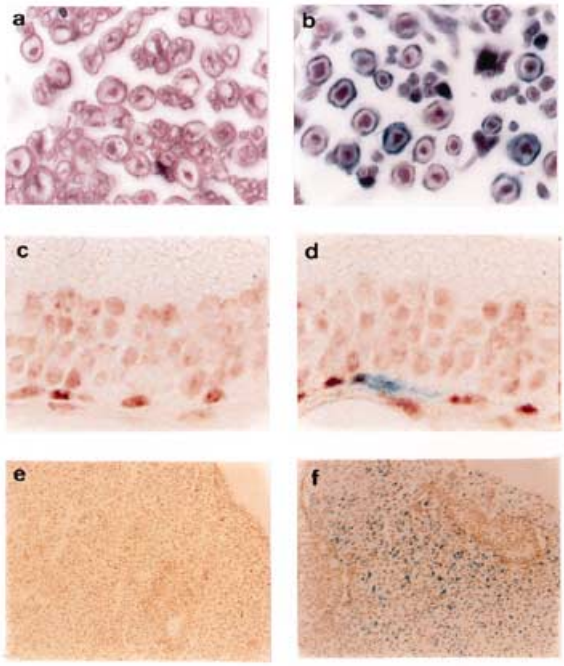

Figure 7. Cell type-specific Cre-mediated recombination in mice. flox *LacZ* indicator mice were crossbred with *P0 Cre*, *IRBP Cre* and *POMC Cre* transgenic mice. Sciatic nerve, eyes and pituitary gland were isolated from single (**a**, **c** and **e**) and double (**b**, **d** and **f**) transgenic mice and stained with X-gal. a and b, counterstained with H&E; c–f, counterstained with neutral red. Magnification: a–f, 100×; e and f, 10×.

recombination in these tissues but PCR analysis confirmed that recombination had occurred (Fig. 5). This indicates that the recombination frequency in these tissues was relatively low. Interestingly, in contrast to what we observed in liver, the number of β-gal-positive cells in these tissues did not diminish significantly over time.

Local administration of 10^7 p.f.u. adeno Cre virus into muscle (Fig. 4e), adipose tissue (Fig. 4f) and lung (not shown) resulted in *LacZ* staining in close proximity to the site of injection.

Tissue-specific expression of a transgenic Cre recombinase in flox *LacZ* indicator mouse

Three different Cre transgenic mice were produced.

P0 Cre transgenic mouse. To selectively direct expression of Cre recombinase to Schwann cells, we utilized the P0 promoter (25). P0 is the major structural protein of peripheral myelin and is expressed in myelinating Schwann cells in the later stages of myelination. The region of 1.1 kb of 5′ flanking DNA from the rat *P0* gene is effective in directing expression of heterologous genes to myelinating Schwann cells in transgenic mice. Five *P0 Cre* transgenic mice were obtained and crossbred with flox *LacZ* indicator mice. The sciatic nerve was isolated from double and single transgenic mice and stained for *LacZ* expression. Extensive staining of the sciatic nerve was observed in double (Fig. 7b), but not in single, transgenic mice (Fig. 7a). Histochemical analysis showed that *LacZ* was expressed in many, but not all, Schwann cells.

POMC Cre transgenic mouse. For pituitary-specific expression of the Cre recombinase, the POMC promoter was chosen (24). Six *POMC Cre* transgenic mice were obtained and crossbred with flox *LacZ* reporter mice. Pituitary glands were isolated from 8-week-old single and double transgenic mice. Large numbers of β-gal-positive cells were observed in the intermediate lobe (Fig. 7f) of the pituitary gland, while lesser staining was observed in the anterior lobe of double transgenic mice. No staining was seen in single transgenic mice (Fig. 7e).

IRBP Cre transgenic mouse. The *IRBP* promoter was used (26) to express the Cre recombinase in the retina. *IRBP Cre* transgenic mice were obtained and crossbred with the flox *LacZ* indicator mice. β-Gal expression was found to be restricted to a few scattered photoreceptor cells located in the outer nuclear layer directly opposite the retinal pigment epithelium in double transgenic mice (Fig. 7d). It was not possible to determine the extent of recombination, as the FVB/N mouse strain which was used for transgenesis carries the recessive *rd* (retinal degeneration) mutation (34) and, as a consequence of this mutation, a progressive degeneration of the photoreceptor cells, starting at day 8 after birth, is observed. Other tissues in the eye were negative for β-gal staining. None of the controls showed staining (Fig. 7c). Infrequently, tracks of staining cells were observed, indicating that a precursor cell had switched, resulting in marking of its descendants.

DISCUSSION

Recently, transgenic mouse systems have been described that permit inducible expression of the Cre recombinase which, in turn, can mediate recombination of chromosomally located recombination targets (6). While this work marks a milestone in the field of mouse reverse genetics, full exploitation of this system requires further improvements. The control of Cre expression appears critical in this respect: tissue specificity of expression, background activity, level of induction, control over fraction of cells in which expression can be induced and the timing of expression. These features are difficult to incorporate in *Cre* transgenic lines. Even when *Cre* transgenic mice meet some of

these requirements, time consuming and costly breeding will have to be performed to combine the different alleles (especially when multiple conditional null alleles are being analyzed). Somatic gene transfer to introduce the recombinase into cells *in vivo* is, therefore, an appealing option. We have generated a flox *LacZ* reporter mouse that permits evaluation of the performance of Cre-expressing systems based upon transgenic mice or somatic gene transfer vectors.

We have used an adeno Cre virus to monitor recombination *in vivo* in these *LacZ* reporter mice. Replication-deficient adenoviral vectors have been developed for gene therapy purposes and were shown to efficiently transduce genes into many different cell types *in vivo* in animal model systems. In general, gene expression is transient and extinction of gene expression is thought to be the result of the dilution of episomal viral DNA upon cell division as well as the consequence of the elimination of infected cells by the immune system (22,32,33).

Infusion of 10^9 p.f.u. adeno Cre virus into flox *LacZ* reporter mice caused extensive recombination of the flox–*LacZ* gene construct in hepatocytes. Close to 80% of the hepatocytes showed recombination at the DNA level. While it is possible that the percentage of cells infected by the adeno Cre virus was slightly higher, our data clearly show that adenoviral vectors can be used effectively to achieve efficient Cre/lox recombination.

Under conditions in which the vast majority of hepatocytes were infected by the adeno Cre virus, >90% of the viral genomes were cleared from the liver by day 60. Over this time period *LacZ* expression was reduced from 65 to <1%, although still >50% of the hepatocytes carried a recombined *LacZ* allele. A possible explanation for these observations is that the floxed transgene is not expressed in all cells. The lower frequency of *LacZ* staining (65%) as compared with the recombination frequency (78%) observed 5 days after adeno Cre virus inoculation, a time point at which an immune response has not yet been mounted, is in accordance with this. If we assume that β-gal acts as a potent antigen, as has recently been shown (35), *LacZ*-expressing cells might be an efficient target for immune destruction. Consequently, *LacZ*-expressing cells will be eliminated and hepatocytes not expressing *LacZ* will divide to substitute for the lost cells. The observation that at day 60 50% of the hepatocytes still carried a recombined allele, while not expressing *LacZ*, suggests that β-gal, rather than proteins synthesized from the viral template, elicited this immune response. The loss of adenoviral genomes from the regenerated liver would then primarily be the consequence of cell division. This raises an interesting issue with respect to the role of the vector-encoded proteins, including Cre, in the immune response, as 50% of the hepatocytes that survive have a history of expression of vector-encoded proteins. Either under the circumstances of our experiments β-gal is immunodominant and the immune system 'ignores' other viral antigens or expression of adenoviral vector-encoded genes and the *LacZ* transgene is extinguished after a short burst of transcription. Such silencing could be triggered by local cytokine production, which occurs as a consequence of a massive inflammatory response (36). Moreover, since liver macrophages (Kupffer cells) are infected by the adeno Cre virus, this may also contribute to a cytokine-mediated silencing of foreign genes in the liver, as has recently been described for inactivation of HBV transgene expression by LCMV-infected Kupffer cells (37). However, we would have expected a much further reduction in expression of the unrearranged *CAT* transgene (we still observed 20% of wild-type levels at 60 days after adeno Cre virus inoculation) if general transgene silencing was the predominant mechanism. Further analyses are in progress to address this question.

The observation that the sparsely observed *LacZ* expression in other tissues is not extinguished argues against a cellular immune response towards cells at these other sites. It is possible that the lower local antigen load fails to recruit immune cells to these sites and, therefore, permits survival of the *LacZ*-expressing cells. Alternatively, these different cell types might be less well equipped to present the antigens to the immune system.

A few points are worth emphasizing. Firstly, the immune response we observe in the liver calls for immune suppressive or tolerizing measures if an appreciable level of a foreign protein has to be produced in liver for a long time. These actions might be needed irrespective of the vector system used to transduce the gene. Secondly, the discrepancy between *LacZ* expression and the recombination frequency after adeno Cre virus infection points in this case to a mosaic expression pattern of the reporter transgene. Since this behavior of transgenes might be the rule rather than the exception, this could hamper widespread gene recombination in a particular tissue using transgenic Cre mice. Thirdly, somatic gene transfer, while probably not suitable at present to confer massive switching in all tissues, provides excellent control over the timing of recombination, while cell target specificity can be achieved both by the route of virus administration (20) and by the use of tissue-specific promoters within the viral vectors. These features are paired to a high transient expression level, no background and simple breeding requirements, making this approach particularly attractive if one wants to study the biological consequences of gene (in)activation in a range of different tissues, e.g. in assessing the oncogenic potential of loss of tumor suppressor gene function.

Studies with immunodeficient mice have demonstrated that prolonged gene expression from adenoviral vectors is possible (22,38). Therefore, suppression of the immune response permits expression of somatically introduced genes for long periods of time. Several studies have addressed ways to mitigate the immune response in immunocompetent animals. The temperature-sensitive mutant E2A recombinant adenovirus shows improved transgene persistence and a decreased inflammatory response in mouse liver (39). The administration of immunosuppressive agents such as cyclosporin A and cyclophosphamide resulted in persistent expression of somatically transferred transgenes (40). Moreover, the administration of CTLA4-Ig, which is known to block co-stimulatory signals between T cells and antigen-presenting cells, permitted persistent adenoviral-mediated gene expression in mice without the requirement for long-term immunosuppression (41). These strategies might also facilitate the persistence of cells that have undergone somatic recombination through infection by adeno Cre virus.

We have also demonstrated the utility of Cre recombinase transgenic mice to mediate tissue-specific recombination. Our *LacZ* reporter mouse will be of specific value to estimate the expression characteristics of distinct promoters, as it leaves an imprint that otherwise might escape detection. The utility of *Cre* transgenic mice could be further increased by the use of regulatable promoters for expression of the recombinases. The interferon-inducible promoter has been successfully used for conditional mutation of endogenous alleles. Other inducible systems have still to prove their suitability. The use of binary drug-inducible transcription (42–45) or direct fusion of the

recombinase to hormone binding domains (46) will, hopefully, permit further improvement of these conditional systems.

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