Gene targeting restricted to mouse striated muscle lineage

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ABSTRACT

Spatially and temporally regulated somatic mutations can be achieved by using the Cre/LoxP recombination system of bacteriophage P1. In order to develop gene knockouts restricted to striated muscle, we generated a transgenic mouse line expressing Cre recombinase under the control of the human α-skeletal actin promoter. Specific excision of a loxP-flanked gene was demonstrated in striated muscle, heart and skeletal muscle, in a pattern very similar to the expression of the endogenous α-skeletal actin gene. Therefore, the reported transgenic line can be used to target inactivation or activation of a given gene to the skeletal muscle lineage.

INTRODUCTION

Gene targeting is widely used to direct a variety of specific modifications to endogenous genes in mice (1). Gene knockout experiments generated inherited mutations in all cells, which may lead to embryonic lethality and therefore hamper the ability to analyze gene function or dysfunction during the post-natal period.

Spatially and temporally regulated somatic mutations can be achieved by using the Cre/LoxP recombination system of bacteriophage P1 (2,3). Cre recombinase efficiently promotes recombination between two 34 bp LoxP recognition sequences. The LoxP sequences can be inserted at targeted sites into the genome of embryonic stem cells through homologous recombination. Two LoxP sequences in the same orientation and flanking the region of interest allow the loxP-flanked gene region to be deleted in cells expressing Cre recombinase. Therefore, spatial and/or temporal excision will depend on the promoter activity driving Cre recombinase expression.

In order to develop gene targeting restricted to skeletal muscle fibers, we used a promoter region derived from the human α-skeletal actin (HSA) gene which is specifically expressed in striated muscles, heart and skeletal muscle (4,5). We report here a transgenic mouse strain that targets Cre recombinase expression to striated muscle fibers in a spatial and temporal pattern very similar to that of the endogenous α-skeletal actin gene.

MATERIALS AND METHODS

Construction of transgene

The promoter and the first exon of the HSA gene (from –2000 to +239, relative to the transcription initiation site) was isolated from pHSA2000CAT plasmid (kindly provided by Edna Hardeman) (4,5). PGS5 plasmid contains the rabbit β-globin intron, Cre recombinase transgene and SV40 polyadenylation signal (kindly provided by Daniel Metzger). The addition of a heterologous β-globin intron and polyadenylation site was introduced to ensure appropriate splicing and polyadenylation of the Cre RNA. A *Sal*I–*Hin*dIII–*Sal*I linker was inserted into the SalI restriction site of PGS5. The 2239 bp *Hin*dIII restriction-fragment of pHSA2000CAT plasmid was cloned into the *Hin*dIII site of PGS5. This plasmid was designated as pGS-HSA-Cre. Plasmid DNA was prepared from cleared bacterial lysates by banding on two successive ethidium bromide–caesium chloride gradients. The construct was then digested using *Not*I restriction enzyme and purified by sucrose gradient before injection.

Generation of transgenic lines and screening of transgenic mice

The purified *Not*I HSA-Cre gene construct was injected in zygote pronucleus of (C57BL/6J×SJL) F1 mice (6). Embryos were reimplanted into pseudopregnant CD1 foster mothers. DNA was extracted from tail biopsy in lysis buffer (100 mM Tris–HCl pH 8, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 200 µg/ml proteinase K) overnight at 55°C followed by phenol extraction, ethanol precipitation and resuspension in TE buffer. A 790 bp fragment of the Cre recombinase gene sequence was amplified using the following primers: Cre1, 5'-CCG GTC GAT GCA ACG AGT GAT-3'; Cre2, 5'-ACC AGA GTC ATC CTT AGC GCC-3'. A 340 bp fragment of the LacZ gene sequence was amplified using the following primers: Lac1, 5'-TCG TGC GGT GGT TGA ACT GCA CAC-3'; Lac2, 5'-CGA TTC ATT GGC ACC ATG CCG TGG-3'. Two microliters (200 ng) of the above preparation were used for PCR amplification reaction in buffer containing 200 µM each dNTP, 1 µM of each primer, 1.5 mM MgCl$_2$, 10 mM Tris–HCl pH 8.3 and 50 mM KCl. For genotyping embryos, yolk sacs were digested in 200 µl lysis buffer (50 mM KCl, 10 mM Tris–HCl pH 8.3, 1.5 mM MgCl$_2$, 0.45% Nonidet P-40, 0.45% Tween-20 and 100 µg/ml proteinase K) overnight at 55°C. Two microliters of

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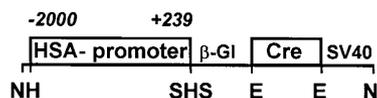
Figure 1. Schematic representation of the construct. The 2239 bp *Hin*dIII restriction fragment of pHSA2000CAT plasmid containing the HSA promoter from –2000 to +239 was linked to the Cre recombinase gene (Cre). The purified *Not*I HSA-Cre gene construct was injected in zygote pronucleus of (C57BL/6J×SJL) F1 mice. β-Gl, β-globin intron; SV40, SV40 polyadenylation signal; H, *Hin*dIII; N, *Not*I; S, *Sal*I; E, *Eco*RI.

this preparation were used for DNA PCR amplification after denaturation of proteinase K. For Southern blot analysis, 8–10 µg of total genomic DNA was digested using *Eco*RI restriction enzyme and transferred to a Hybond N+ membrane (Amersham) following standard procedures. The PCR amplification product of the Cre recombinase gene sequence as described above was used as probe.

Analysis of the transgene expression

To test the efficiency of Cre-mediated site-specific recombination, we used LacZ reporter mice carrying the chicken β-actin promoter and β-galactosidase coding sequence separated from each other by the CAT gene flanked by loxP sites (7). β-Galactosidase expression was assayed in embryos and older mice from crosses between transgenic mice and LacZ reporter mice. Double transgenic embryos and older mice were identified using PCR amplification analysis of Cre and LacZ transgenes. Noon of the day of the plug was considered 0.5 day post-coitum (d.p.c.).

Embryos were dissected in 1× PBS and fixed in 0.2% glutaraldehyde, 5 mM EGTA, 2 mM MgCl$_2$ in 1× PBS for 10 min at 4°C. Fixed embryos were washed three times in 2 mM MgCl$_2$, 1% Nonidet P-40 in 1× PBS and incubated for 3–4 h at 37°C in 1 mg/ml X-Gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide in the washing solution as described above. After staining, embryos were rinsed twice in the washing solution. Tissues from adult mice were embedded in OCT compound (Miles Laboratory, Incorporated) and frozen at –80°C in isopentane cooled on liquid nitrogen. Frozen sections of 10–12 µm were fixed in 2% formaldehyde and 0.2% glutaraldehyde in 1× PBS for 10 min at 4°C. Fixed tissues were washed twice in 1× PBS and incubated overnight at 37°C in 1 mg/ml X-Gal, 5 mM potassium ferrocyanide, 5 mM potassium ferricyanide and 2 mM MgCl$_2$ in 1× PBS. After staining, tissues were washed twice in 1× PBS and counterstained with safranine.

RESULTS

The construct contains the Cre recombinase transgene including an ATG separated from the HSA promoter by a β-globin intron and followed by the SV40 polyadenylation signal (Fig. 1). Two transgenic lines carrying the HSA-Cre transgene were selected based on the detection of the Cre recombinase transgene by using both PCR amplification and Southern blot analyses and were expanded (data not shown). Mice carrying the Cre recombinase transgene are phenotypically normal and were used for further characterization. HSA-Cre transgenic lines were crossed with LacZ reporter mice to facilitate the analysis of the Cre recombinase transgene activity. In the progeny that inherits both LacZ and Cre transgenes, Cre-mediated recombination of the CAT gene will restore LacZ gene transcription, thereby

allowing production of β-galactosidase protein in target cells which results in X-Gal blue staining (7). Of the two lines, only one (HSA-Cre79) gave a detectable β-galactosidase activity reflecting Cre recombinase activity and was studied further.

In adult tissues from double transgenic mice, gastrocnemius, triceps and biceps muscles stained uniformly blue with standard β-galactosidase incubation times, indicating a specific and strong Cre recombinase activity in skeletal muscle fibers except in some muscle fibers (Fig. 2A, f–h). In the heart, the vast majority of cardiomyocytes did not show β-galactosidase activity except some clusters of cells which are not obviously associated with particular cardiac structures (Fig. 2A, e). No β-galactosidase activity was detected in other tissues including liver, kidney, cerebellum, thymus, spinal cord, brain and spleen (Fig. 2 and data not shown). These data indicate specific Cre-mediated recombination activity in striated muscles.

In order to characterize Cre recombinase activity during development, embryos that inherit both Cre and LacZ transgenes were analyzed. At 9 d.p.c., somites showed detectable β-galactosidase activity, with an appearance of elongated cells in a rostro-caudal orientation corresponding to the initial formation of myotomal muscles (Fig. 2B, i and k). In the heart, clusters of cells or patches showed a β-galactosidase activity starting from 9 d.p.c. which did not correspond to any particular structures (Fig. 2B, j). By 9 but not at 12 d.p.c., the expressing line showed β-galactosidase activity in the trigeminal ganglion (Fig. 2B, i). At 12 d.p.c., segmental blocks of myotomal muscles showed strong LacZ staining from vertebrae and ribs, with fibers running predominantly in a dorsal to ventral direction (Fig. 2B, l). These data show that from 9 d.p.c., elongated cells of the somites accumulated LacZ gene activity indicating the presence of Cre recombinase expression in the myotome region of the somites. In neonates, skeletal muscles stained uniformly blue indicating an efficient Cre recombinase activity restricted to skeletal muscle fibers (Fig. 2B, m and n). Therefore, the α-skeletal actin promoter is able to drive Cre recombinase expression not only in mature striated muscle fibers but also in striated muscle cells of the somites and heart.

To test for proper function of the Cre recombinase, the transgenic line was crossed with a loxP-flanked allele generated by homologous recombination in ES cells (unpublished data) (Fig. 3A). Tissue-specific deletion of the reporter gene segment was demonstrated by PCR analysis using genomic DNA isolated from a variety of adult tissues in the offspring of these crossings. The PCR product is only detectable following excision of the LoxP flanked test gene segment by the Cre recombinase. As shown in Figure 3, a 450 bp fragment was successfully amplified in skeletal muscle tissue and more weakly in heart of double heterozygous mice carrying both HSA-Cre79 transgene and the loxP-flanked gene. Thus, targeted expression of the LacZ gene in skeletal and cardiac muscle tissues correlated with the deletion of the reporter loxP flanked gene segment in striated muscle. In addition, the absence of a deleted gene segment in the other tissues confirms the striated muscle-specificity of Cre recombinase activity.

DISCUSSION

Here, we report the creation of a system for genetic manipulation of cells of the striated muscle lineage in mice by the expression of Cre recombinase under the transcriptional control of the
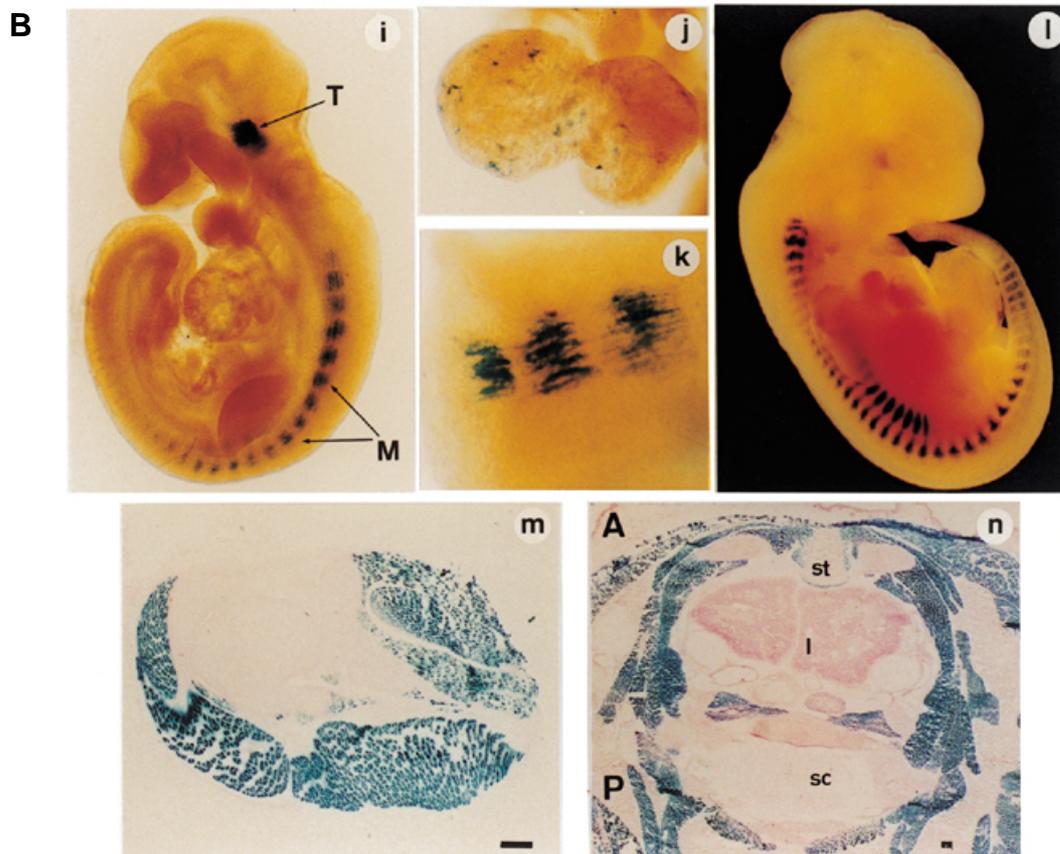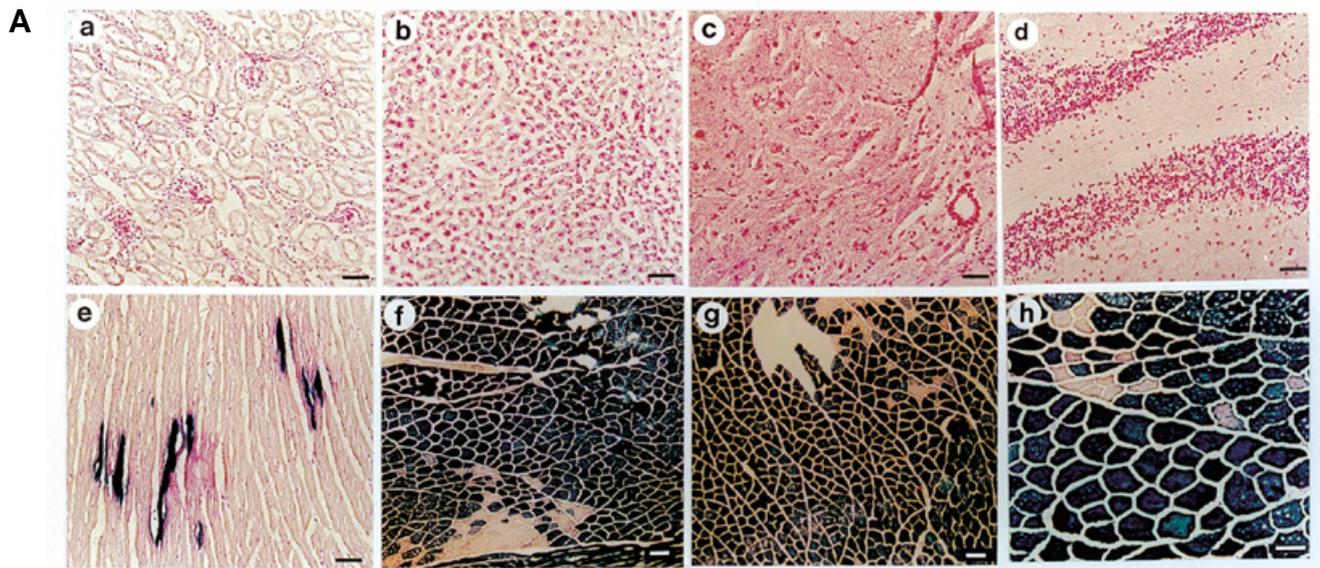
Figure 2. β-Galactosidase activity in tissues of mice carrying both HSA-Cre79 and LacZ transgenes. (**A**) In adult, no β-galactosidase activity was observed in kidney (a), liver (b), spinal cord (c) and cerebellum (d). Uniform and strong β-galactosidase staining was seen in gastrocnemius (f), biceps (g) and triceps (h) except in some muscle fibers. In heart (e), some isolated cardiomyocytes showed β-galactosidase staining. (**B**) (i) A 9.5 d.p.c. embryo shows β-galactosidase staining in the myotomal region of somites (M) and an ectopic expression of β-galactosidase in trigeminal ganglion (T). Higher magnification revealed the aspect of elongated blue cells in the somites with a rostro-caudal orientation (k) and a positive staining in clusters of cells in heart which did not correspond to any particular structures (j). At 12.5 d.p.c., segmental blocks of myotomal muscles showed strong LacZ staining with fibers running in a dorsal–ventral direction (l). (m) Cross-section of forelimb from neonate shows a uniform and strong β-galactosidase expression in all skeletal muscles. (n) Transverse section of thoraco-abdominal region from neonate reveals a strong LacZ staining in intercostal and paravertebral muscles. A, anterior; P, posterior; sc, spinal cord; l, liver; st, sternum. Scale bars 50 μm (a–e and h) and 100 μm (f, g, m and n).
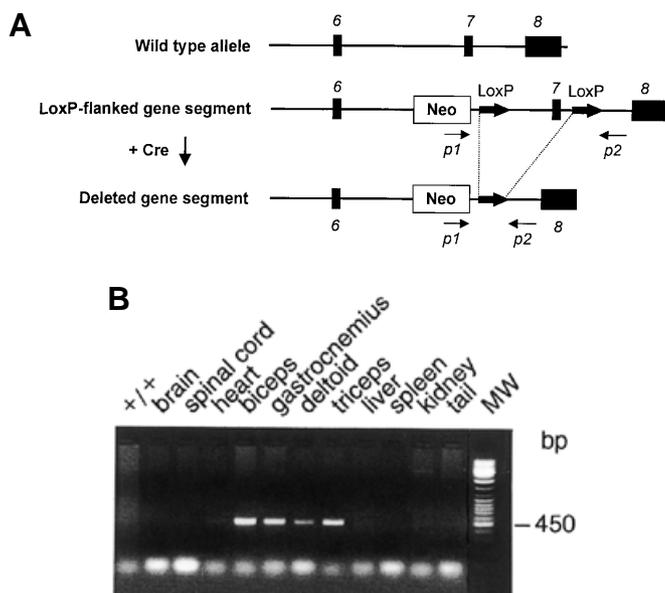
Figure 3. Deletion testing of Cre recombinase on a loxP-flanked gene region. (**A**) Schematic representation of the loxP-flanked gene segment generated by homologous recombination and used to detect Cre recombinase activity. The positions of the primers (p1 and p2) are indicated. The expected fragments before and after site-specific recombination are 2230 and 450 bp respectively. The HSA-Cre79 transgenic mice were crossed with this test strain. Mice carrying both the HSA-Cre79 and the loxP-flanked gene were selected. (**B**) Tissue-specific deletion of the reporter gene segment was demonstrated by PCR analysis using primers p1 and p2 from genomic DNA isolated from a variety of tissues. A 450 bp fragment was successfully amplified in skeletal muscle tissues and more weakly in heart indicating the presence of an efficient Cre recombinase activity in striated muscle. No ectopic expression of Cre recombinase was detected in the other tissues.

HSA promoter. The HSA promoter was chosen since it is transcribed specifically in striated muscles, heart and skeletal muscle (8). Moreover, HSA gene is expressed at the earliest stages of skeletal muscle development and differentiation (8).

The specificity of Cre mediated deletion was determined by using both LacZ reporter transgenic mice and mice carrying loxP-flanked gene segment generated by homologous recombination. The present study shows that the HSA-Cre79 transgenic line carrying the HSA gene promoter from –2000 to +239 is capable of specific expression of Cre recombinase in striated muscle, heart and skeletal muscle. In skeletal muscle, Cre recombinase shows a uniform and strong activity in skeletal muscle throughout the life of animal including skeletal muscle cells of the somites. In heart, Cre recombinase is expressed in some clusters of cardiomyocytes which did not correspond to any particular structures in the heart. These data are consistent with previous *in situ* hybridization experiments of α-skeletal actin transcripts showing a expression pattern during development similar to that observed in the present study (8). Ectopic expression of Cre recombinase in non-striated muscle is absent except in trigeminal ganglion at 9.5 d.p.c. Interestingly, transgenic experiments using different fragments of the promoter region of the mouse cardiac actin gene inserted upstream of a LacZ reporter gene showed a transgene expression in the trigeminal and facial acoustic ganglia at 10.5 d.p.c. (9). Yet, the endogenous α-skeletal or cardiac actin transcripts have not

been detected suggesting that either the promoters of both cardiac and skeletal actin are subject to similar position effects or low level of transcripts of both genes may have been missed while detection of β-galactosidase activity is extremely sensitive. Therefore, the present study shows an expression pattern of Cre recombinase similar to that of the endogenous gene in adult skeletal muscles. In addition, the Cre recombinase transgene is regulated correctly during embryogenesis having the same qualitative pattern of expression as the α-skeletal actin endogenous gene both in skeletal and cardiac muscles (8).

The *in vivo* definition of such specific and strong skeletal muscle expression of Cre recombinase as reported here is of potential importance for gene targeting experiments to skeletal muscle lineage. The targeted expression of the Cre recombinase in skeletal muscle tissue will facilitate the *in vivo* analysis of the function of a variety of genes in skeletal muscle. The Cre recombinase transgenic line can indeed be used to target inactivation or activation of a given gene to skeletal muscle fibers. In addition, this approach may avoid early embryonic lethality such as observed by knocking out the gene encoding dystroglycan, a central component of the dystrophin–glycoprotein complex, a protein assembly that plays a critical role in a variety of muscular dystrophies (10). Finally, since programmed motor neuron cell death is dependent on signals from their target tissue, skeletal muscle [reviewed by Oppenheim (11)], gene targeting restricted to skeletal muscle should enable testing of the role of candidate trophic signals on motor neuron survival *in vivo*.

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