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Functional expression of Cre recombinase in sub-regions of mouse CNS and retina

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Abstract We describe a strategy for generating CNS and retina sub-region-specific mutations using the Cre/loxP system. Transgenic mice expressing Cre recombinase under the control of the c-kit promoter were established. Functional Cre expression was predominantly found to be restricted to the CA1, CA2 and CA3 regions of the hippocampus, the anterior region of the dentate gyrus, and to the ganglion cell layer of the retina.

Key words: Embryonic development; Transgenic mouse; Hippocampus; Ganglion

1. Introduction

The possibility of generating defined mutations in embryonic stem cells by gene targeting has contributed significantly to the understanding of gene function [1]. Several problems associated with the conventional knockout technique have, however, become apparent. Thus, the frequently observed embryonic lethality in mice harboring two copies of targeted non-functional alleles precludes functional analysis of such a gene at later stages of development [2]. Another potential problem is derived from the fact that germ line incorporation of the mutation results in the presence of the targeted locus in all cells. This may lead to secondary, non-cell autonomous phenotypes that may be difficult to distinguish from cell autonomous phenotypes. Moreover, major phenotypic changes due to a targeted mutation may obscure more subtle phenotypes.

One approach to overcome these limitations is based on the utilization of the Cre/loxP recombination system [3] to introduce the mutations in a tissue-specific [4] or inducible fashion [5]. The Cre recombinase originates from the phage P1 and mediates site-specific recombination between 34 bp repeats, termed loxP sites. This system has proven to be a powerful tool in several genetic systems, such as yeast [6], plants [7] and mice [8]. In mice, the Cre/loxP system has been used to accomplish both cell-type restricted activation of transgenes (TG) [8] and generation of cell type restricted null alleles by the deletion of LoxP flanked (floxed) gene segments [9].

Here we report the establishment and characterization of a transgenic mouse strain, c-kit(151)Cre, carrying a construct which expresses Cre recombinase under the control of the c-kit promoter. The c-kit promoter has been reported to be active in a variety of tissues including hematopoietic cells [10], melanocytes, testis, ovary [11], areas of the CNS [12] and during early embryogenesis [13]. The herein described c-kit(151)Cre mice functionally express Cre recombinase in the CA1, CA2 and CA3 regions of the hippocampus, in the anterior region of the dentate gyrus and in the ganglion cell layer in the retina.

2. Materials and methods

2.1. Generation of Cre transgenic mice

The c-kit promoter [14] was amplified by PCR of genomic DNA from a C57Bl/6 mouse using the following primers, forward: 5′ CCC AAG CTT AGG GAG AGG TGC TAG 3′; reverse: 5′ CCG CTC GAG TGC GCT AGA CTC TGA 3′.

The 300 bp fragment containing the promoter was digested with *Hin*dIII and *Xho*I, and inserted into a vector comprising the Cre gene and the 3′ UTR containing the A(n) from the MT-I gene (kindly obtained from Dr. F. Alt) [15]. The promoter was sequenced and verified against the published sequence [14]. The construct was gel-purified and injected into pronuclei of fertilized oocytes of F1(C57Bl/6×CBA) mice. The injected zygotes were transferred to pseudo-pregnant female mice by standard procedures. Transgenic mice were detected by Southern blot hybridization of *Bam*HI-digested tail-derived DNA using a 0.4 kb probe complementary to the Cre gene. Six founder mice were obtained and used to establish transgenic lines by backcrossing to C57Bl/6 mice. In this study, the characterization of mice from the c-kit(151)Cre line is reported.

2.2. Detection and quantification of functional Cre recombinase expression

Transgenic mice were crossed to a mouse strain harboring a reporter gene (neomycin resistance gene) flanked by loxP sites [16] (Fig. 1A). Organs from mice carrying the Cre transgene and a floxed neomycin resistance (Neo) reporter gene segment were dissected out. The brains were dissected so that the hippocampal region was present in the midbrain fractions. Genomic DNA was prepared using a Nuclon™ 'st for soft tissue' kit (Amersham int., Buckinghamshire, UK). 10 μg of DNA was run on a 0.85% agarose gel and blotted onto a Zeta-Probe® GT membrane (Bio-Rad Laboratories, CA, USA). The filters were hybridized and washed according to the manufacturer's recommendations. The fragment used as a probe to detect Cre-mediated recombination was a 0.6 kb *Xba*I–*Hpa*I fragment hybridizing to a region 3 kb 3′ of the Neo gene (Fig. 1A). The intensity of the three bands representing wild type (10.1 kb), floxed (6.2 kb) and loxed (13.0 kb) alleles (Fig. 1A,C) was determined using a phosphorimager (Molecular Dynamics, Amersham Pharmacia Biotech AB, Uppsala, Sweden). The deletion index was calculated as follows: deletion index in percent = intensity of loxed allele/(intensity of loxed allele+intensity of floxed allele)×100 [16,17].

2.3. Histochemical analysis of functional Cre expression

Transgenic mice from the c-kit(151)Cre line were crossed to cAct-XstopXlacZ transgenic mouse strain [18] (kindly provided by Dr. D.J. Anderson). Offspring was genotyped by Southern blot hybridization of genomic DNA from tail tips. Probes used to detect double transgenic mice were the 0.4 kb Cre probe described above and a 3 kb probe covering most of the lacZ gene. Dissected organs were rapidly

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frozen in mounting media (Tissue-Tek® O.C.T. 4583 Compound; Sakura Finetek, The Netherlands). Cryosections (20 μm) were prepared and fixed 2 min in 99.5% ethanol. The fixed sections were subjected to X-gal staining overnight at 37°C. The X-gal staining contained 1 mg/ml X-gal (4-chloro-5-bromo-3-indolyl-β galactosidase; Saveen Biotech AB, Malmö, Sweden), 4 mM $K_4Fe(CN)_6$, 4 mM $K_3Fe(CN)_6$ and 2 mM $MgCl_2$ (Sigma Chemicals, MO, USA) in PBS. The X-gal-stained slides were washed twice in PBS and were counterstained with hematoxylin (Histolab Products AB, Sweden) before microscopy.

3. Results

3.1. Generation and characterization of Cre transgenic mice

Transgenic mice expressing Cre recombinase under the control of the c-kit promoter were generated as described in Section 2. Mice of the c-kit(151)Cre transgenic strain were crossed to a reporter mouse strain carrying a neomycin resistance gene flanked by loxP sites (Fig. 1A). The offspring from this cross were genotyped for presence of the Cre transgene and the floxed allele of the reporter gene by Southern blot analysis of tail-derived DNA. Among the mice positive for both the transgene and the reporter locus, approximately 50% were found to functionally express Cre specifically in brain and to some extent in lymphoid tissue but expression was absent in all other organs and tissues tested (Fig. 1C). To quantify the extent of Cre-mediated recombination in different organs and tissues, Southern blot hybridization analysis was performed as described in Section 2. The probe in Fig. 1A was used for identifying Cre-mediated recombination events in the reporter locus. The probe detects the 10 kb wild type allele and the 6.2 kb floxed allele as well as the 13 kb band representing the locus after Cre-mediated recombination (loxed allele) in a *Bam*HI-digested genomic Southern blot hybridization (Fig. 1C). The relative intensity of the signals representing the different alleles was quantified using a phosphorimager. In the brain, cortex, midbrain and cerebellum were analyzed separately. Interestingly, while no Cre-mediated recombination events were detected in cerebellum, a reproducible level of the loxed reporter allele was observed in DNA samples from midbrain and cortex (Fig. 1D).

In the remaining 50% of the offspring, both the 6.2 kb band representing the floxed allele and the 13 kb band representing the loxed allele were detected in tail-derived DNA. These mice were found to have undergone Cre-mediated deletion in all organs and tissues tested, thus establishing a mosaic pattern unique to each individual mouse. The observed mosaic is most likely to be the result of functional Cre expression early in embryogenesis. Similar observations have been reported for other Cre-transgenic mice [16,17]. This group of mice was not studied further.

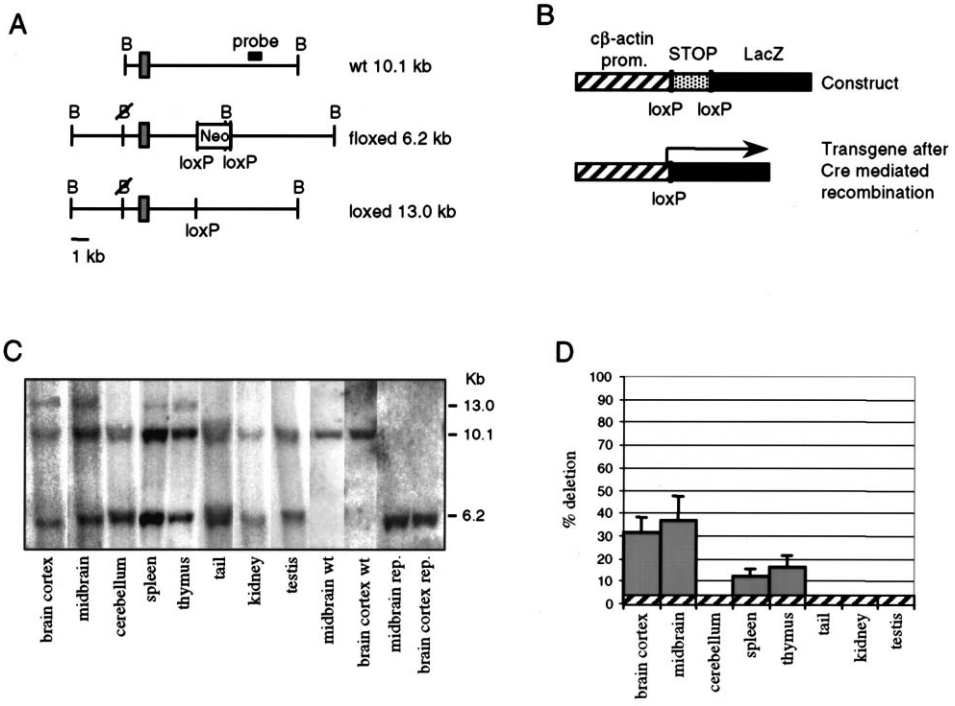

Fig. 1. A: Schematic representation of wild type locus, targeted locus and locus after Cre-mediated recombination of the Neo reporter mice. Sizes of the detected Southern blot hybridization fragment are given for each allele. B = *Bam*HI, struck-out B = destroyed *Bam*HI site. B: A schematic representation of the construct used to generate the cAct-XstopXlacZ transgenic mice (adopted from Tsien et al. [4]) and the transgene after Cre-mediated recombination. Hatched box represents the chicken β-actin promoter. Shaded box represents the transcription inhibitory (stop) sequence and the filled box the lacZ coding sequence. LoxP sites are represented by vertical lines. Arrow denotes transcription. C: Representative Southern blot hybridization analysis of Cre-mediated recombination in eight organs from a mouse carrying the c-kit(151)Cre transgene together with a loxP-flanked Neo allele (Fig. 1A). Lanes 9–12 represent organs from Cre^- control mice. *Bam*HI-digested genomic DNA was hybridized with the probe indicated in B. The three bands represent: loxed (13 kb) allele, wild type (10.1 kb) allele and floxed (6.2 kb) allele. Lane 1: cortex, lane 2: midbrain, lane 3: cerebellum, lane 4: spleen, lane 5: thymus, lane 6: tail, lane 7: kidney, lane 8: testis, lane 9: midbrain wild type, lane 10: cortex wild type, lane 11: midbrain $Neo^{+/+}$ reporter, lane 12: cortex $Neo^{+/+}$ reporter. D: Histogram showing the extent of deletion in eight organs analyzed in five mice carrying the c-kit(151)Cre transgene together with a loxP flanked Neo allele. Hatched bar represents lower detection level. Gray bar represents mean measured deletion. Error bar denotes standard error of the mean.

3.2. Distribution of loxed cells in CNS

To further assess the functional expression of the Cre transgene on a cellular level in the CNS, the c-kit(151)Cre mouse strain was crossed to the cAct-XstopXLacZ TG mouse strain [18]. The cAct-XstopXLacZ mice carry a transgene comprising a chicken β-actin promoter driving expression of the lacZ gene encoding the β-galactosidase enzyme (Fig. 1B). A floxed 'stop' sequence [8] separates the promoter and the lacZ coding sequence, making this transgene transcriptionally inactive unless subjected to Cre-mediated deletion. This feature of the cAct-XstopXLacZ TG mouse facilitates the detection of tissues and individual cells that have undergone Cre-mediated recombination by the fact that the β-galactosidase enzyme is expressed. To determine the functional expression pattern of Cre recombinase, coronal sections of brains from c-kit(151)-Cre/cAct-XstopXLacZ double transgenic mice were analyzed for β-galactosidase activity by X-gal staining. Microscopic examination of the stained sections revealed a distinct pattern of β-galactosidase activity restricted in hippocampus to CA1, CA2 and CA3 as well as part of the dentate gyrus (Fig. 2A–C). The CA1, CA2 and CA3 area cells showed a homogeneous staining from the most anterior to the most posterior part of the region, respectively. The dentate gyrus, however, exhibited a more restricted staining pattern. Towards the anterior end of the dentate gyrus, X-gal staining was defined to a single cell layer in the periphery of the structure (Fig. 2E), while the equivalent layer of the posterior end displayed no β-galactosidase activity (Fig. 2F). Less distinct, but reproducible X-gal staining was also observed in a region corresponding to the limbic cortex (Fig. 2D) [19]. Analysis of the whole brain by serial sections revealed no functional Cre expression in any other part of the brain, including cerebellum which was negative for X-gal staining, confirming the observations made by Southern blot hybridization (Fig. 1C). No β-galactosidase activity could be detected in brains of Cre^{-}/cAct-XstopXlacZ mice (Fig. 2G). In addition to the observed expression of Cre in CNS, analysis also revealed a specific X-gal staining in the ganglion cell layer of the retina (Fig. 3A).

3.3. Functional Cre expression in CNS and retina is detected at least from 18.5 days of embryonic development

To establish at what point in development the observed

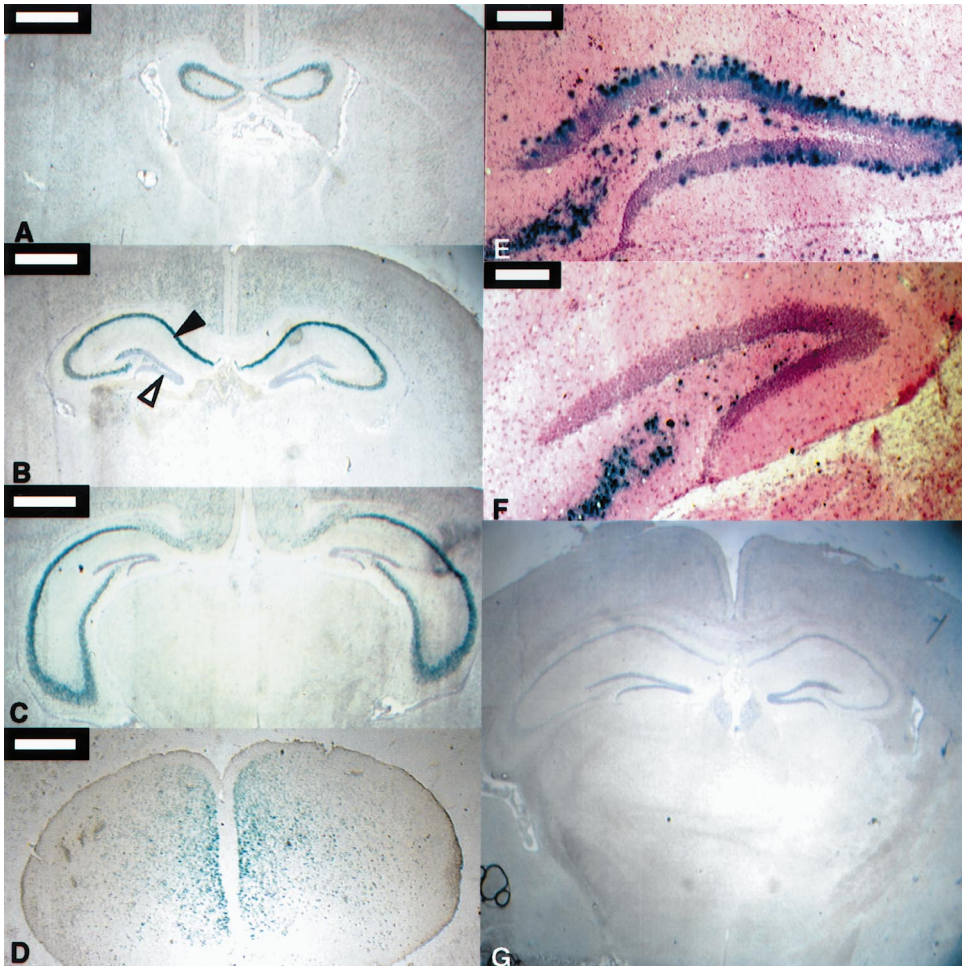

Fig. 2. Cre-mediated recombination in the brains of c-kit(151)Cre/cAct-XstopXlacZ double transgenic mice as visualized by X-gal staining. Coronal sections of brains from 12-week-old mice were stained with X-gal and counterstained with hematoxylin. In sections containing the anterior (A), medial (B), and posterior (C) parts of hippocampus, the X-gal staining is restricted to hippocampus. In the anterior part of cerebral cortex (D) the X-gal staining coincides with limbic cortex. Arrowheads in (B) indicate hippocampus (filled) and dentate gyrus (open). Scale bar = 1 mm. Cre-mediated recombination in the dentate gyrus was restricted to the peripheral cell layer in the anterior parts of the structure (E), while in the more posterior part (F), no staining was detected. Scale bar = 0.1 mm. In coronal sections of brain from Cre^{-}/cAct-XstopX-lacZ transgenic mice, only the hematoxylin counterstain is seen.

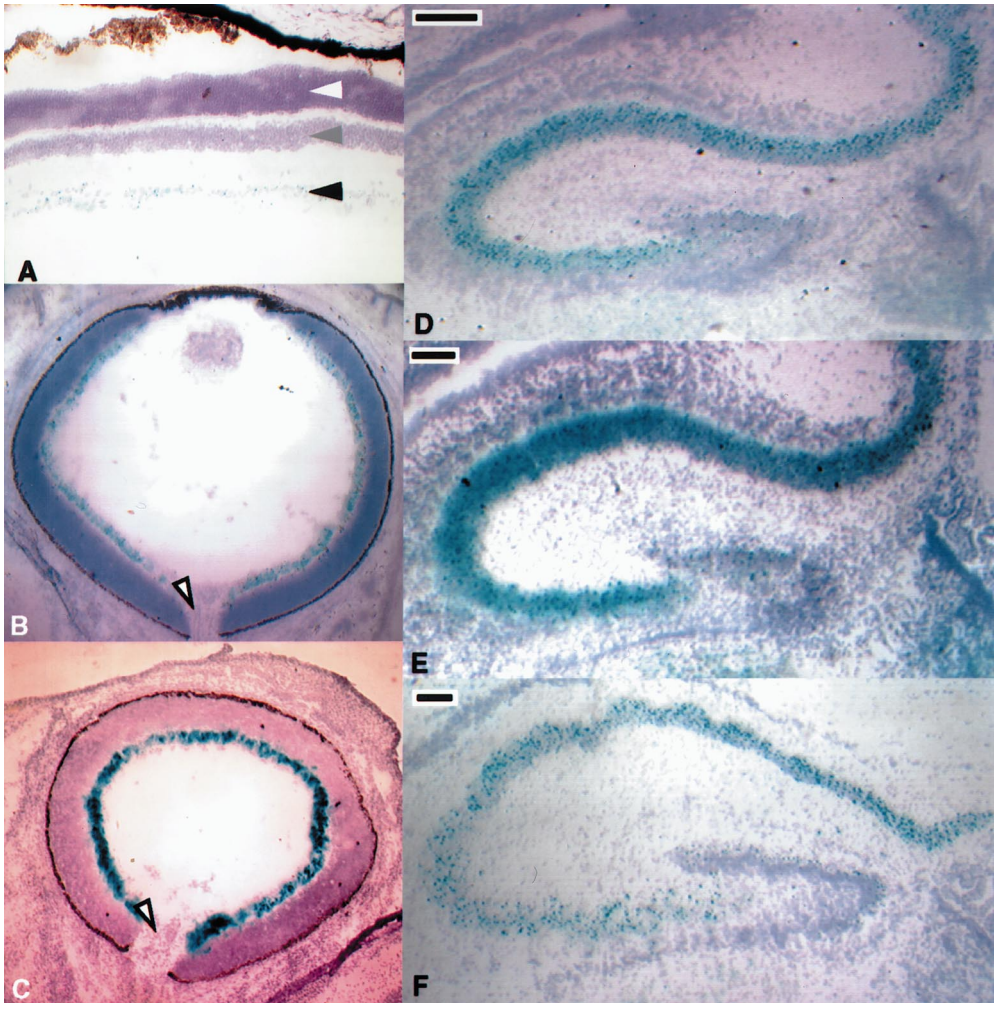

Fig. 3. A–C: Sections of eyes from c-kit(151)Cre/cAct-XstopXlacZ double transgenic mice stained with X-gal and counterstained with hematoxylin. In the retina of 12-week-old mice, only the ganglion cell layer showed X-gal staining (A). Arrowheads indicate photoreceptor cells (white), bipolar nerve layer (gray), ganglion cell layer (black). In sections from the eye of E19.5 (B) and E18.5 (C) embryos corresponding staining was observed. Arrowheads indicate optic nerve. D–F: Coronal sections of brains showing one hemisphere of the hippocampal region from E18.5 (D), newborn (E) and P5 (F) double transgenic mice. Scale bar = 0.1 mm.

recombination events mediated by Cre occurred, embryos of E12.5 to E19.5 as well as newborn and P5 mice were obtained from c-kit(151)Cre/cAct-XstopXLacZ mice and analyzed for β-galactosidase activity. The X-gal-stained ganglion cell layer observed in adult mice was visible in the developing retina from E18.5 to adult (Fig. 3A–C). In sections of the hippocampal regions of double transgenic embryos the X-gal-stained hippocampal cells observed in adult mice (Fig. 2A–C) could be traced back to 18.5 (Fig. 3D–F).

4. Discussion

We have established a mouse line that specifically and functionally expresses Cre recombinase in defined sub-populations of the brain and retina. This represents a unique model for introducing conditional genome alterations in these regions. Similar mice have previously been reported by Tsien et al. [4], who generated transgenic mouse lines expressing Cre recombinase under control of the α-calcium-calmodulin-dependent kinase II promoter. The c-kit(151)Cre mouse, however, differs significantly from these particularly concerning the embryonic Cre recombinase expression. In the c-kit(151)Cre line functional Cre expression is detected from E18.5, while in the mice studied by Tsien and colleagues [4] Cre-mediated recombination was reported to occur during the third postnatal week. Another unique feature of the c-kit(151)Cre line is Cre-mediated recombination in the ganglion cell layer of the retina.

The observed retina specific Cre expression in the c-kit(151)Cre mice differs from the reported endogenous amacrine cell specific c-kit expression [20]. Additionally, no functional Cre expression was detected in cerebellum of the transgenic mice analyzed here despite the described high expression of c-kit in this region [21]. The reported endogenous expression of c-kit in hippocampal regions [22], however, overlaps with the expression of Cre recombinase observed in the c-kit(151)Cre mice. The discrepancies of the endogenous expression of c-kit and the Cre expression controlled by the c-kit promoter in the transgene could have at least two non-mutually exclusive explanations. First, position effects dependent on integration site are known to influence transgene expression [23]. Alternatively, the promoter segment used in the transgenic construct could lack regulatory sequences necessary to mimic the endogenous expression pattern of c-kit.

Irrespective of the gene regulatory mechanism controlling Cre expression, it is clear that the functional expression pattern of Cre recombinase observed in these mice provides an excellent tool for studying gene function in the corresponding tissues. The specific expression of Cre recombinase in the CA1, CA2 and CA3 regions of hippocampus and dentate gyrus provides a tool to study genes controlling development and physiological functions of these CNS regions. Additionally, the Cre expression described in retina in these transgenic mice facilitates exploration of retinal axon guidance and topographic mapping in the mammalian visual system [24,25].

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