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Specificity and Efficiency of Cre-Mediated Recombination in Emx1–cre Knock-in Mice

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Emx1 is a mouse homologue of the Drosophila homeobox gene *empty spiracles* and its expression is restricted to the neurons in the developing and adult cerebral cortex and hippocampus. We reported previously the creation of a line of transgenic mice in which the cre gene was placed directly downstream of the putative Emx1 promoter using ES cell technology. We showed that Cre protein was present in the cerebral cortex of the transgenic mice and was able to mediate loxP-specific recombination *in vitro*. In the present study, the specificity and efficiency of the cre-mediated recombination were determined using three independent lines of reporter mice and a combination of histochemical staining, neuronal culture, and Southern detection of the genomic DNA. Our results showed that the recombination was highly efficient in all three lines of reporter mice tested and confirmed that the deletion was restricted to the neurons in the cerebral cortex and hippocampus. Furthermore, we have determined that the recombination efficiency in the cerebral cortex was 91%. Our results suggest that Emx1 is not expressed in every neuron in the developing and adult cerebral cortex. This line of cre mice should contribute to the studies of cortical development and plasticity. © 2000 Academic Press

Key Words: **cerebral cortex; hippocampus; Cre/loxP; transgenic mice; Emx1; knock-in; reporter mice; neuronal culture; recombination efficiency.**

Gene targeting in mice using ES cell technology has revolutionized the field of mammalian biology (1). It allows the production of mutant mice containing a desired mutation in any gene of interest. Conventional gene targeting techniques produce animals with mutations in all cell types. This approach could result in embryonic or neonatal lethality and could make it impossible to analyze the functions of the genes in adult animals. Recent development in conditional knockout technology made it feasible to create gene modifications in a tissue and/or temporal restricted manner (2, 3).

A recent strategy takes advantage of a site-specific recombination system derived from bacteriophage P1 (2; reviewed in 4). Cre (causes recombination) is a member of the Int family of recombinases and could perform efficient recombination at loxP (locus of X-ingover, P1) sites. The Cre recombinase can excise, from the chromosome, DNA fragment that is flanked by loxP. By restricting the cre expression to certain tissues, restricted mutation or deletion of the loxP flanked genes could be achieved. Therefore, the key to the tissue-specific gene inactivation using the Cre/loxP system is the creation of the cre transgenic mice. We initiated a study recently to characterize the putative promoter derived from a homeobox gene, the Emx1 gene, which specifically expresses in the developing brain.

Emx1 is a mouse homologue of the Drosophila homeobox gene *empty spiracles* (*ems*). Its expression starts around embryonic day 9.5 (E9.5) and is exclusively confined to the dorsal telencephalon (5). The Emx1 gene is expressed in virtually every neuron of the developing embryonic and postnatal cerebral cortex. No expression of the Emx1 gene has been detected in glial cells (6).

We reported previously the successful expression of the cre and lacZ genes in an Emx1-specific manner by directly inserting these genes into the exon 1 of the Emx1 gene. We have also demonstrated that Cre protein was present in the cerebral cortex of the transgenic mice and was able to mediate loxP-specific recombination *in vitro* (7). However, the effectiveness of deletion and the tissue or cell types that underwent cre-mediated recombination *in vivo* were not determined. Here, we report the deletion efficiency and spec-

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ificity of the Emx1-cre knock-in mice as tested with three different lines of reporter mice.

MATERIALS AND METHODS

Mice. The creation of the Emx1-cre knock-in mice has been described elsewhere (7). Z/AP mice (8) and loxPlacZ mice (9) were gifts from Corrine Lobe and Andrew Nagy of the University of Toronto, and David Anderson of California Institute of Technology, respectively. Rosa 26 cre reporter mice (R26R; 10) were purchased from Jackson laboratory. Heterozygous reporter mice (Z/AP, loxPlacZ, or R26R) were crossed with Emx1-cre heterozygotes or homozygotes to generate the double transgenic mice for the determination of the deletion efficiency.

Genotyping. The genotyping for the Emx1-cre and loxPlacZ mice was done as published previously (7, 11). Z/AP mice were genotyped according to the original procedure (8) using β-galactosidase staining of the tail biopsies from the mouse pups. Rosa mice were genotyped using the PCR methods as detailed in the original paper (10).

Southern analysis of brain genomic DNA. Animals were sacrificed by an overdose of pentobarbital and their brains were removed. A coronal cut was made through the tectum and the posterior part was used to extract control DNA from the cerebellum and brainstem. The cortices were peeled off from the anterior part of the cut and were used to isolate the DNA from the cerebral cortex. The isolation of genomic DNA was done essentially according to the protocol for extracting DNA from tail biopsies (12). After hybridization with a 2-kb fragment derived from the lacZ coding region, the band intensity was quantified using a Fuji phosphor imager BS1000.

Histochemistry. X-gal staining of the whole mount and brain sections was done as described (11). For differential lacZ staining of the sections from R26R and R26R/Emx1-cre mice, the floating sections were stained for 30 min using the same procedure outlined previously (11). Staining for the alkaline phosphatase was done according to the standard procedure (8).

Culture of cortical neurons. Cortical cell culture was done based on the protocol as described (13). Briefly, cultures of cerebral cortical neurons were derived from newborn transgenic mice positive for either Z/AP or Emx1-cre/Z/AP. Cortices were dissected out then rinsed several times with ice-cold dissociation media (DM: 1× HBSS, Life Technologies, 14180061, supplemented with 0.5% glucose, 0.7% sucrose, 0.35% $NaHCO_3$, and pH 7.2). Cortices were cut into small pieces using sterile razor blades and then treated with trypsin for 10 min. at 37°C. The released cells were then dissociated mechanically and filtered with a cell strainer (Fisher Scientific, 70 μm). The cells were counted and cultured using poly-lysine coated culture plates at $0.5–4.0 \times 10^5$ cells/cm^2 in basal medium eagle (BME; Sigma) containing 25 mM KCl and 10% fetal bovine serum (FBS; HyClone). After being cultured for 18–24 h, β-D-arabinofuranoside (Ara-C) was added to a final concentration of 10 μM. At days 2 and 4, one-third of the culture medium was replaced with fresh BME containing 25 mM KCl, 10% FBS, and 10 μM Ara-C. On day 5, cells were washed with PBS three times and fixed in 0.4% glutaraldehyde in PBS for 10 min. The cells were then treated with a rinse solution, and stained with X-gal solution overnight as described for the brain sections (11).

RESULTS

To determine whether the cre transgene could mediate recombination *in vivo*, we have used three lines of indicator mice including the chicken-β-actin-promoter-loxP-stop-loxP-lacZ indicator mouse (loxPlacZ mice; 9), the Z/AP mouse (8), and the Rosa26 cre reporter mice (R26R mice; 10). Experiments conducted using any one of these lines indicated an efficient cerebral cortex-specific gene deletion mediated by Emx1-cre knock-in mice.

LoxPlacZ indicator mice. In the progeny carrying both Emx1-cre and loxP-stop-loxP-lacZ transgenes, when the cre is active in a given cell, the stop sequence between the two loxP sequences will be removed, leading to activation of the lacZ expression; thus the cell will be stained blue using X-gal as a substrate. However, since Emx1-cre knock-in mice already have lacZ activity in the cerebral cortex, it is difficult to determine the effectiveness of recombination using lacZ staining. We used Southern blot analysis instead to assess the extent of deletion *in vivo*.

Genomic DNA was isolated from the cerebral cortex of the mice containing both the Emx1-cre and loxPlacZ loci. We also isolated DNA from the cerebellum and brainstem as controls for regional specificity. The isolated DNA was digested with XbaI, which cuts only once inside the 8.2-kb DNA fragment that was used to create the lacZ indicator mice. The digested DNA was separated, blotted, and probed with radioactive probes covering the 5′ part of the lacZ gene, which is present both in Emx1-cre and loxPlacZ loci (Fig. 1A).

Since in most of the cases, the injected transgene is integrated in a head-to-tail array, *Xba*I digestion of transgenic loxPlacZ DNA should yield an 8.2-kb repetitive band and two "junctional fragments" of novel lengths from the two ends of the array. The lacZ probe we used should detect only one of these two "junctional fragments." Therefore, the loxPlacZ locus should produce an 8.2-kb band and a 12-kb 3′-junctional band containing a 1.4-kb stop sequence flanked by two loxP sequences. The 12-kb band would be reduced to 10.6-kb in cells that undergo Cre-mediated recombination. The Emx1-cre knock-in locus should give rise to a 9-kb band.

As shown in Fig. 1B, three bands, representing the Emx1-cre and loxPlacZ loci, could be detected by Southern hybridization of DNA prepared from the brainstem and cerebellum. However, an additional band (10.6 kb) was found in DNA isolated from the cerebral cortex. We determined that the ratio of the 10.6-kb band (recombined) to the 12-kb band (native) was 1.24 ± 0.20 ($n = 3$). In other words, about $55 \pm 8\%$ of the cells in the cerebral cortex of the double transgenic mice underwent Cre-mediated recombination. It should be noted that the Emx1-cre gene is expressed only in the neurons (6). According to a quantitative study of the visual cortex of the adult albino rat, the mean cortical volume numerical density of neurons was $60{,}020 \pm 3840/\text{mm}^3$ and $49{,}040 \pm 2610/\text{mm}^3$ for the combined glial cell types (14). Hence, about 55% of the cells in the visual cortex of the adult albino rat are neurons. Although we do not know the glia-to-neuron ratio for the entire mouse cerebral cortex, our results

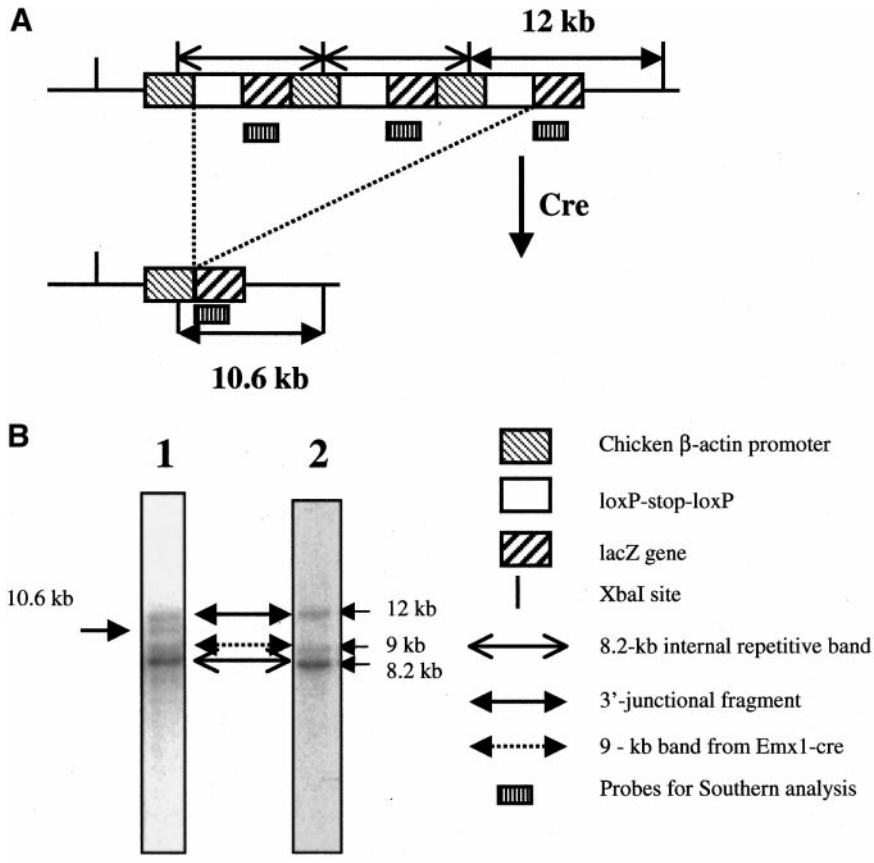

FIG. 1. (A) Genomic organization of the loxP-stop-loxP locus. For simplicity, only three copies of the transgene are shown. In the presence of Cre protein, the stop sequences (open boxes) and the majority of the transgenes are deleted in the cerebral cortex and hippocampus. The size of the 3′ junctional fragment (arrows) is reduced from 12 to 10.6 kb. Filled boxes denote transgenes inserted into the mouse genome. Each copy of the transgene consists of a chicken β-actin promoter, a loxP-stop-loxP cassette, and a lacZ coding region followed by a poly(A) sequence. Horizontal thin lines indicate the genomic DNA sequences near the insertion site. (B) Southern blot analysis of Cre-mediated recombination in the mice containing both the Emx1-cre and loxP-stop-loxP transgenes. Arrows in B indicate the fragments detected in the genomic Southern hybridization. The DNA was digested with XbaI. The location of the probes used was indicated in A. Brainstem and cerebellum were collected by cutting through the tectum coronally. The cerebral cortexes were then peeled from the midbrain and were extracted for genomic DNA. The recombined band is only present in the cerebral cortex (lane 1) and is absent in the brainstem and cerebellum (lane 2).

suggest that the majority of the cortical neurons in the double transgenic mice underwent cre-mediated recombination.

R26R indicator mice. Although the Emx1-cre mice contain their own lacZ gene coding for a nuclear-localized β-galactosidase, we have utilized the differential strength of the Rosa26 and the Emx1 promoters to show that Emx1-specific deletion could be achieved in R26R mice. Postnatal brains were sectioned and stained for a shorter period of time. As shown in Fig. 2F, after a 30-min incubation in X-gal staining solution, the sections derived from double transgenic mice (R26R/Emx1-cre) showed blue staining that was restricted to the cerebral cortex and hippocampus, while in sections derived from control mice (Emx1-cre) no staining could be seen (Fig. 2G), suggesting Emx1-cre could remove the stop sequence in the Rosa26 locus efficiently during development in a region-specific manner.

Deletion studies using Z/AP mice. The experiments conducted above used indirect measurements to estimate the efficiency of the deletion. It would be desirable to use another line of indicator mice such as Z/AP (8) in which Cre-mediated recombination leads to the activation of a reporter gene other than the lacZ gene that we have put next to the cre gene. Z/AP mice express the lacZ gene before Cre-mediated recombination throughout all embryonic and adult stages using a strong CMV enhancer/chicken β-actin promoter. Cre-mediated excision, however, removes the lacZ gene as well as a stop sequence, leading to the expression of a second reporter gene, the human alkaline phosphatase (AP) gene.

As shown in Fig. 2C, at E12.5, AP staining of a Emx1-cre/Z/AP embryo showed a similar pattern as the lacZ staining of a Emx1-cre embryo (Fig. 2A), while a wild-type embryo showed no AP staining at all (Fig. 2B). It should be noted that the embryo in Fig. 2A was

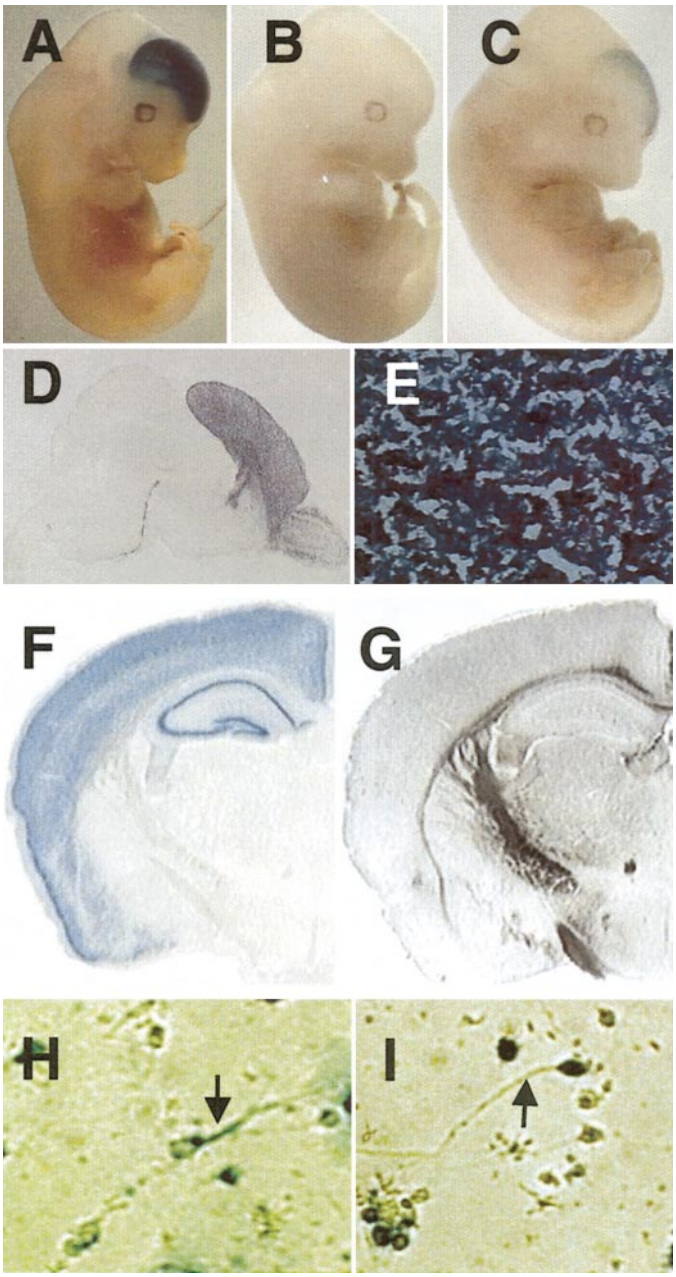

FIG. 2. E12.5 embryos stained for β-galactosidase (Emx1-cre, A) and AP activity (wild type, B; Emx1-cre/Z/AP, C). The staining is restricted to developing cerebral cortex. Sagittal section (D) of a P3 Emx1-cre/Z/AP mouse brain stained for AP. Anterior is right. High magnification (400×) view of barrel cortex stained with AP (P3, E). Dorsal is up. X-gal staining of coronal brain sections from Emx1-cre/R26R mice (F) and Emx1-cre mice (G). Blue staining could be detected in the cerebral cortex and hippocampus of Emx1-cre/R26R mice (F). Dorsal is up and lateral is left. X-gal-stained cortical neuronal cultures from Z/AP (H) and Emx1-cre/Z/AP (I) mice. Arrow in (H) indicates a neuron with blue process while the arrow in (I) points to a neuron without the blue staining in the process, suggesting a deletion of the lacZ gene in the Z/AP locus. Note the soma (including nucleus) of the neuron is blue.

stained for lacZ, which is more sensitive than the staining for AP. Therefore, the weaker staining pattern in Fig. 2C should not be construed as partial deletion. At P3, the staining was restricted to the cerebral cortex and the axon tracts formed by cortical efferents (Fig. 2D), since the AP product is not localized in the nucleus and could distribute in cytoplasm and neuronal processes. Upon close examination at high magnification (400×, Fig. 1E), virtually every cell stained purple in the barrel cortex. When we used a Stereo Investigator to quantify the percentage of the cells stained positive for the alkaline phosphatase in the barrel cortex, we could not find a single cell unstained. However, it is difficult to distinguish between glial cells and neurons using AP staining at this age.

Culture studies using Z/AP mice. Since the lacZ gene in the Z/AP mice codes for a β-galactosidase that is not nuclear-localized as in the Emx1-cre mice, we were able to develop a deletion assay based on the differences of the cellular localization. Cortical neurons from the Z/AP and Emx1-cre/Z/AP newborn mice (n = 2 each) were isolated, cultured, and stained for lacZ. The number of neurons having blue staining either in dendritic or axonal processes was counted. In cultures derived from the Z/AP mice, 77 ± 4% of the neurons contained blue processes (Fig. 2H), while only 6.8 ± 0.5% showed blue staining in their processes when both Emx1-cre and Z/AP loci were present (Fig. 2I). No blue staining was detected in the processes of neurons derived from Emx1-cre mice. These results suggest that a CMV enhancer/chicken β-actin promoter could only direct the lacZ/alkaline phosphatase expression to about 77% of the neurons. Therefore, taking the uniformities of lacZ expression into consideration, about 8.8% of the cortical neurons failed to undergo cre-mediated recombination *in vivo* and the rest (about 91%) achieved recombination successfully.

DISCUSSION

Previously, we created a line of transgenic mice that express the cre gene in an Emx1-specific manner using knock-in strategy and demonstrated that the Cre was working at least *in vitro* using our biochemical assays (7). In the present study, we have used three different lines of reporter mice to show that, regionally, the deletion was restricted to the cerebral cortex and hippocampus, and occurred in 91% of the neurons in the cerebral cortex.

The first conclusion from this study is that the Emx1-cre knock-in mice could delete loxP-flanked sequences completely independent of their chromosomal locations and their DNA sequence environment. DNA analysis using loxPlacZ indicator mice showed an efficient excision of target sequences (Fig. 1). Histochemical staining of sections and cultured neurons prepared from the Z/AP/Emx1-cre or R26R/Emx1-cre double transgenic mice also demonstrated that an effective deletion occurred in the cerebral cortex and hippocam-

pus. Furthermore, we have used Emx1-cre mice recently to delete the NMDAR1 gene, which codes for an essential subunit of the NMDAR receptors, a subtype of glutamate receptors in the mammalian central nervous system (reviewed in 18). Western blot analysis of the NMDAR1 proteins in the hippocampus showed a complete deletion of the NMDAR1 gene (S.H., X.L.J., and Y.L., unpublished). Taken together, the Emx1-cre mice we created could mediate loxP-specific recombination in an extremely effective manner independent of chromosomal loci.

The second conclusion draw from the current study is that Emx1 is not ubiquitously expressed in every cortical neuron as the published study implied (6). Our culture study indicated a 91% deletion efficiency using the Emx1-cre knock-in mice. Past studies have established that the level of Cre proteins in the cell is an important factor in mediating loxP-specific recombination (15). We could not rule out that the 8.8% of neurons that failed to undergo cre-mediated recombination could be due to their low level of Cre proteins. However, Chan and his colleagues showed independently that 85% of the cortical neurons are Emx1 positive using *in situ* hybridization (16). While our number is slightly higher than theirs, the difference could be attributed to a small percentage of the cortical neurons that express the Emx1 gene transiently during earlier embryonic development. Recombination-based systems have been used successfully in lineage studies during development (9, 17). Our experimental design would enable us to capture this group of neurons in our counting and it would be extremely difficult to find all the Emx1-positive neurons using *in situ* hybridization. Furthermore, we have recently quantified the percentage of the Emx1-positive neurons in juvenile mice using β-galactosidase immunohistochemistry. Our results indicated that about 91% of the cortical neurons were Emx1-positive (S.H. and Y.L., unpublished). Taken together, these results suggest that Emx1 is not expressed ubiquitously in every cortical neuron as previously reported (6). Future studies should be directed at characterizing the neurochemical phenotypes of the 8.8% neurons that failed to undergo cre-mediated recombination and are presumably Emx1-negative.

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