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TECHNOLOGY REPORT

Transgenic Mice Engineered to Target Cre/LoxP-Mediated DNA Recombination Into Catecholaminergic Neurons

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Summary: To introduce restricted DNA recombination events into catecholaminergic neurons using the *Cre/loxP* technology, we generated transgenic mice carrying the Cre recombinase gene driven by a 9 kb rat tyrosine hydroxylase (TH) promoter. Immunohistochemistry performed on transgenic mouse brain sections revealed a high number of cells expressing Cre in areas where TH is normally expressed, including the olfactory bulb, hypothalamic and midbrain dopaminergic neurons, and the locus coeruleus. Double immunohistochemistry and immunofluorescence indicated that colocalization of TH and Cre is greater than 80%. Cre expression was also found in TH-positive amacrine neurons of the retina, chromaffin cells of the adrenal medulla, and sympathetic ganglia. We intercrossed TH-Cre mice with the floxed reporter strain Z/AP and observed efficient Cre-mediated recombination in all areas expressing TH, indicating that transgenic Cre is functional. Therefore, we have generated a valuable transgenic mouse strain to induce specific mutations of "floxed" genes in catecholaminergic neurons. genesis 36:196–202, 2003.

Key words: tyrosine hydroxylase; Cre recombinase; transgenic mouse; catecholamine; dopamine; norepinephrine; loxP

INTRODUCTION

Dopamine, norepinephrine, and epinephrine are three bioactive catecholamines that play fundamental roles as neurotransmitters in the central and peripheral nervous system. Almost four decades ago Dahlstrom and Fuxe identified and mapped the major central catecholaminergic pathways in the mammalian central nervous system using neuroanatomical and histochemical techniques (Hillarp *et al.*, 1966). A common hallmark of these neurons is the expression of tyrosine hydroxylase (TH), the rate-limiting enzyme in catecholamine biosynthesis that converts the phenolic amino acid tyrosine into the catechol 3,4-dihydroxyphenyalanine (L-DOPA). A myriad of brain, neuroendocrine, and peripheral catecholaminergic neurons orchestrate diverse responses to environmental and internal stimuli. Norepinephrine participates in arousal and vigilance through projections that originate in the locus coeruleus. It is also the final neurotransmitter in the sympathetic autonomic system and, together with the epinephrine released from the adrenal medulla, provide the most rapid and effective general response to acute stress. Central dopaminergic pathways are involved in complex behaviors such as the control of locomotion, cognitive and emotional functions, the rewarding properties of natural reinforcers and drugs of abuse, and the neuroendocrine control of synthesis and release of prolactin and α-MSH from the pituitary gland. Dopamine also participates in the visual and olfactory systems through the amacrine and periglomerular cells present in the retina and olfactory bulb, respectively.

Midbrain dopaminergic neurons are particularly susceptible to premature degeneration, as observed in Parkinson's disease and some forms of dementia. The causes for this earlier cell death are still under intense investigation but they seem to involve a higher vulnerability to oxidative stress damage (Mouradian, 2002). Recently,

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mutations in the genes coding for α-synuclein and parkin have been shown to be responsible for some familiar forms of premature dopaminergic brain neuronal death (Gwinn-Hardy, 2002). Although these genes are expressed throughout the central nervous system, it is still unclear why DAergic neurons are more vulnerable to the effects of the mutant alleles. Development and maintenance of a neuronal dopaminergic phenotype depend on genes such as brain-derived neurotrophic factor and the transcription factor NR4A2 (nurr-1) that are also expressed in many other cell types (Guillin *et al.*, 2001; Zetterström *et al.*, 1997). To unravel the properties that ubiquitously expressed genes have in catecholaminergic neurons during development, in normal brain physiology, and in disease it is essential to induce gene mutations specifically in this population of neurons. Recent developments in mouse molecular genetics allow the targeting of DNA recombination events to a limited subset of somatic cells that share a common expression phenotype. This strategy is based on the ability of the P1 bacteriophage recombinase Cre to promote the specific excision of a genomic sequence flanked by two *loxP* sites (*floxed* gene; Rossant and McMahon, 1999; Nagy, 2000). Expressing Cre recombinase under the transcriptional control of a cell-specific promoter restricts the gene mutation to a particular cell type.

To introduce gene mutations specifically in catecholaminergic neurons, we have generated transgenic mice that carry a 9 kb rat TH promoter (Min *et al.*, 1994) driving the expression of Cre recombinase. Here we show that these transgenic mice express functionally active Cre recombinase in catecholaminergic neurons and could be used to promote mutations of *floxed* genes to this particular type of neurons.

RESULTS AND DISCUSSION

Pronuclear microinjection of a TH-Cre expression construct into B6CBF2 mouse zygotes generated two transgenic mice identified by PCR and confirmed by dot blot hybridization of tail genomic DNA. A transgenic founder (line #12) was propagated to subsequent generations, whereas the female founder #6 was infertile. Evaluation of the expression pattern of Cre recombinase in line #12 was performed by an immunohistochemical analysis on alternate coronal and sagittal fixed brain sections from F_1 and F_2 transgenic mice. Using a polyclonal anti-Cre antibody we observed that Cre immunoreactivity paralleled that of TH in all brain areas rich in catecholaminergic cell bodies. Figure 1 shows alternate Vibratome sections collected 50 μm apart from different brain areas that were incubated either with a polyclonal TH antibody (left) or a polyclonal Cre antibody (right). A similar pattern of TH and Cre immunoreactivity was observed in the locus coeruleus (A6 cells; Fig. 1a,b); substantia nigra pars compacta (A9 cells), and ventral tegmental area (A10 cells; Fig. 1c,d); arcuate (A12 cells) and periventricular (A14 cells) nuclei of the hypothalamus (Fig. 1e,f); zona incerta (A11 and A13, data not shown); and in the periglomerular layer of the olfactory bulb (A16 cells; Fig. 1g,h). The antibody against Cre did not show any labeling in brain sections collected from non transgenic mice (data not shown).

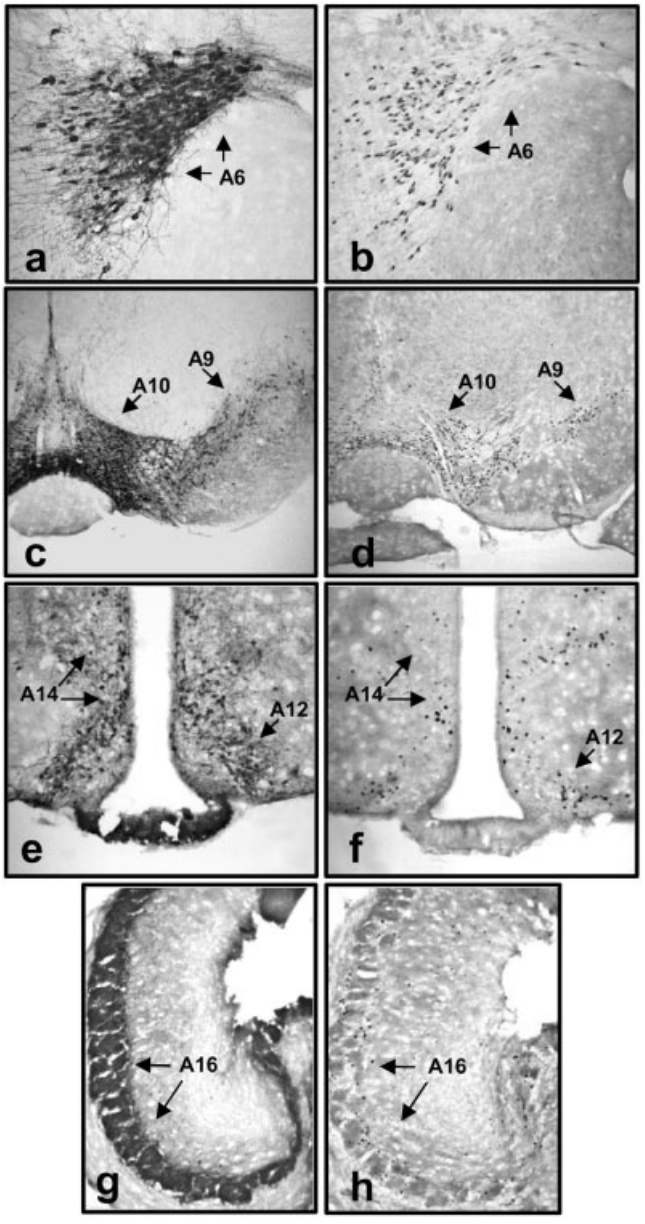

FIG. 1. Localization of endogenous TH and transgenic Cre recombinase in different brain areas revealed by immunohistochemistry. A polyclonal anti-TH antibody (left panels) or a polyclonal anti-Cre antibody (right panels) were used to incubate alternate 50 μm brain sections of TH-Cre transgenic mice collected at the level of the locus coeruleus (A6 cells, **a,b**), substantia nigra pars compacta, and ventral tegmental area (A9 and A10 cells, **c,d**), arcuate and periventricular nucleus of the hypothalamus (A12 and A14 cells, **e,f**) and the periglomerular layer of the olfactory bulb (A16 cells, **g,h**). All sections follow a coronal orientation except **a** and **b**, which are sagital.

To determine whether Cre and TH were expressed within the same neurons we performed double-staining

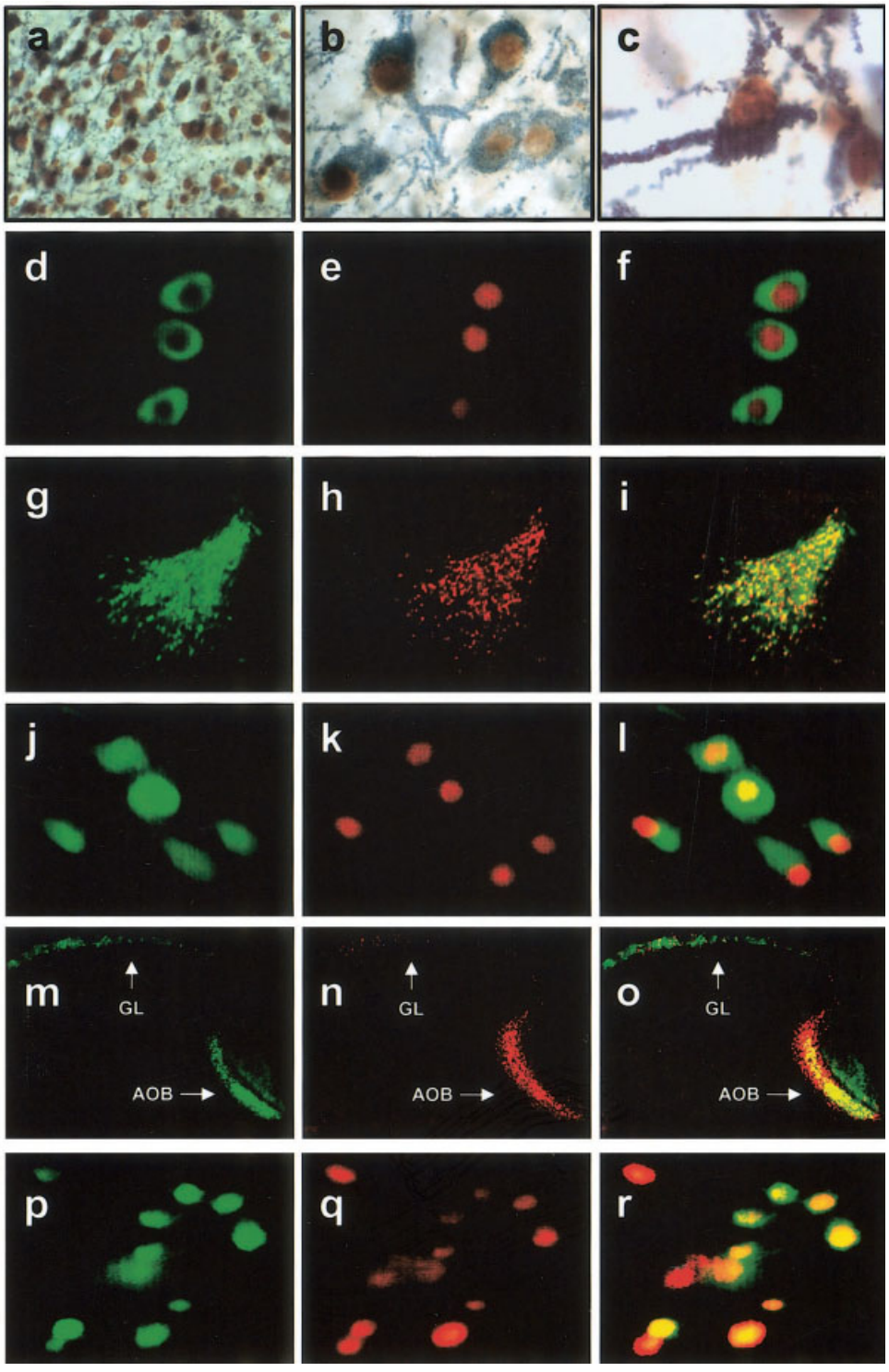

FIG. 2. Double-staining immunohistochemistry (**a–c**) and double immunofluorescence (**d–r**) for endogenous TH and Cre recombinase in different brain areas. Fifty-μm sections at the level of A9 (**a,b**) or A12 cells (**c**) of TH-Cre transgenic mice were incubated with a polyclonal anti-Cre antibody and then reincubated with a polyclonal anti-TH antibody. Cre immunoreactivity is evidenced by brown nuclei while blue cytoplasms indicate TH immunoreactivity. Simultaneous immunofluorescence was obtained using a monoclonal anti-TH antibody (**d,g,j,m,p**) and a polyclonal anti-Cre antibody (**e,h,k,n,q**) on 50 μm sections of TH-Cre transgenic mice at the level of the substantia nigra pars compact a (**d–f**), locus coeruleus (**g–l**), and olfactory bulb (**m–r**). Superimposed microphotographs obtained with both antibodies are shown (**f,i,l,o,r**). In the olfactory bulb neurons in both the periglomerular layer (GL) and the accessory olfactory bulb (AOB) were detected.

immunocytochemistry or double immunofluorescence on coronal and sagittal 50 μm sections obtained from fixed brains of transgenic mice. Because the Cre gene carries a nuclear localization signal and TH is a cytoplasmic enzyme, we were able to distinctly label Cre-expressing nuclei with a brown pigment (diaminobenzidine) and TH-positive cytoplasms with blue (benzidine dihydrochloride). Examples are shown in Figure 2, where Cre-expressing nuclei colocalize with TH-positive neurons in the pars compacta of the substantia nigra

(Fig. 2a,b) and in a group of neurons of the arcuate nucleus of the hypothalamus (Fig. 2c). Colocalization was also studied by double immunofluorescence using simultaneously a mouse monoclonal anti-TH antibody and a rabbit polyclonal anti-Cre antiserum followed by a fluorescein-coupled antimouse IgG antibody and a rhodamine-coupled antirabbit IgG antibody. Figure 2 shows examples of these results in the substantia nigra (Fig. 2d–f), locus coeruleus (Fig. 2g–l) and olfactory bulb (Fig. 2m–r). Using both double-labeling techniques we determined that transgenic Cre colocalized within more than 80% of TH neurons in all regions analyzed including the substantia nigra, ventral tegmental area, locus coeruleus, arcuate nucleus of the hypothalamus, zona incerta, and olfactory bulb. A small percentage of TH-positive neurons within these nuclei did not evidence Cre expression. We also detected Cre immunoreactivity in other areas of the brain such as the lateral septal nucleus, bed nucleus of the stria terminalis, the amygdaloid complex, and piriform cortex, where TH appears to be expressed at earlier developmental stages (Mezey, 1989; Asmus *et al.*, 1992; Asmus and Newman, 1993; Min *et al.*, 1994). In addition, we detected ectopic expression in the ventrolateral thalamus, superior colliculus, and a few cells in the striatum and lateral hypothalamus.

To analyze whether TH-Cre mice expressed a functional recombinase, we bred them with Z/AP transgenic mice (Lobe *et al.*, 1999). Z/AP mice carry a double-reporter transgene that provides a reliable readout of Cre recombinase activity. In Z/AP mice the ubiquitously active CMV enhancer/chicken β-actin promoter drives the expression of a *lacZ* gene flanked by *loxP* sites followed by the human placental alkaline phosphatase (hPLAP) coding sequences. Because the *lacZ* gene has been built with a triple polyadenylation signal, hPLAP expression is only expected to occur after Cre-mediated excision of the *floxed lacZ*. Therefore, a positive hPLAP signal will originate only in neurons expressing functional transgenic Cre. TH-Cre.Z/AP double transgenic mice showed intense hPLAP activity in all brain areas where Cre and TH are expressed. Figure 3b shows hPLAP activity in the olfactory bulb, olfactory tubercle, mesencephalic dopamine neurons, and the locus coeruleus. In addition, hPLAP is also clearly observed in the striatum and nucleus accumbens (Fig. 3b) because it is transported anterogradely to the nerve terminals, as is endogenous TH (Fig. 3a).

Cre expression has also been detected in other regions rich in catecholaminergic neurons such as in amacrine cells of the retina, adrenal medulla, and sympathetic ganglia. Nuclear expression of Cre was observed along the inner nuclear layer of the retina. Figure 4a,b shows two retinal sections obtained from a TH-Cre transgenic mouse that were incubated with a polyclonal TH antibody (Fig. 4a) or a Cre antibody (Fig. 4b) and developed with diaminobenzidine. Figure 4c shows a retinal section taken from a TH-Cre.Z/AP double transgenic mouse. A positive hPLAP reaction was observed in neuronal cell bodies along the inner nuclear layer and their dendritic

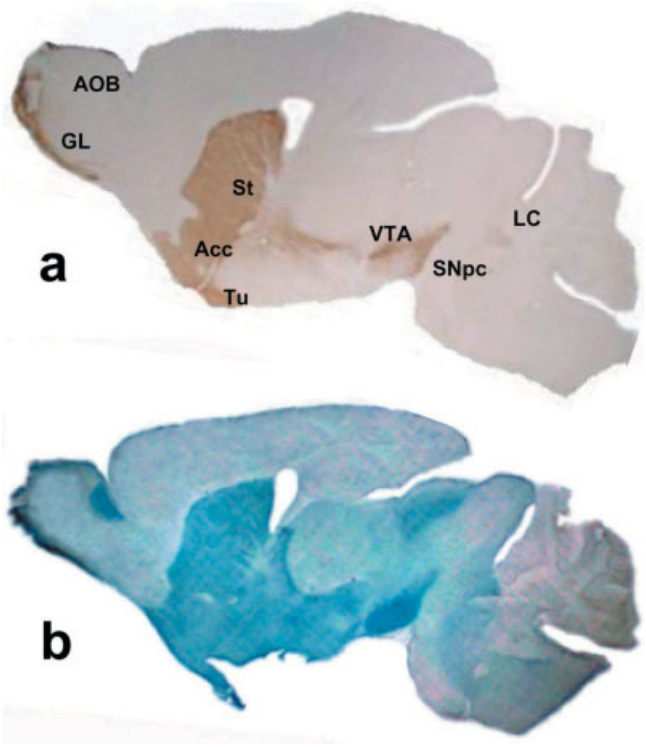

FIG. 3. Human placental alkaline phosphatase (hPLAP) activity in a representative sagital brain section of a double transgenic TH-Cre.Z/AP mouse revealed by in situ histochemistry. hPLAP activity (**b**) follows that of endogenous TH immunoreactivity (**a**) and is evident in the locus coeruleus (LC), midbrain dopaminergic neurons of the ventral tegmental area (VTA) or substantia nigra pars compacta (SNpc), olfactory tubercle (Tu) and neurons of the glomerular layer (GL), and the accessory olfactory bulb (AOB). Both hPLAP and TH undergo anterograde transport and are detected in the striatum (St) and the nucleus accumbens (Acc). hPLAP staining in the ventrolateral thalamus and superior colliculus is probably originated from ectopically expressed Cre in these areas.

projections in the inner plexiform layer of the retina (Fig. 4c). Colocalization of Cre and TH was confirmed by double immunofluorescence. A monoclonal anti-TH antibody labeled amacrine neurons (Fig. 4d) that also evidenced Cre-immunoreactive nuclei (Fig. 4e,f).

In microtome sections of the adrenal medulla of TH-Cre mice, expression of the transgene was detected more clearly in catecholaminergic chromaffin cells located in the most external layers of the medulla, as observed by immunohistochemistry using a Cre antibody (Fig. 5b) or histochemical detection of hPLAP in a TH-Cre.Z/AP double transgenic mouse (Fig. 5c).

The developmental expression of Cre paralleled that of TH in E13.5 transgenic embryos, as shown in the midbrain (Fig. 6a–c) and the trigeminal ganglion (Fig. 6d–f). TH and Cre expression were also evident in developing dorsal root ganglia (Fig. 6g–i) and the spinal cord, as described previously (Price and Mudge, 1983; Jonakait *et al.*, 1984; Schimmel *et al.*, 1999). TH and Cre were also detected in sympathetic ganglia of the para-

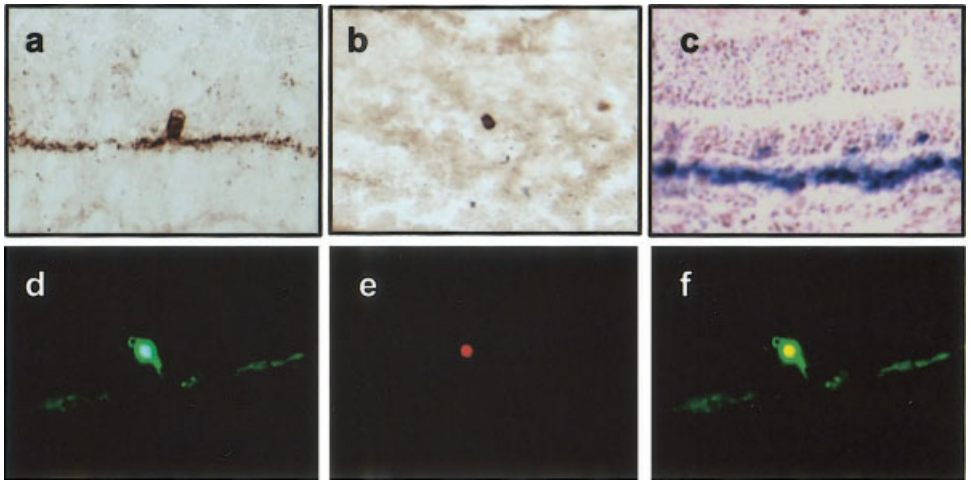

FIG. 4. Localization of endogenous TH and transgenic Cre recombinase in the inner nuclear layer of the retina of a TH-Cre mouse. Immunohistochemistry using an anti-TH antibody showing a representative amacrine neuron forming a dendritic plexus (**a**) or an anti-Cre antibody showing nuclear staining in the inner nuclear layer (**b**). hPLAP activity is evident in cells and dendritic plexus of a double TH-Cre.Z/AP mouse retina (**c**). Colocalization of endogenous TH (**d**) and transgenic Cre (**e**) are depicted by immunofluorescence in a representative amacrine neuron. The superimposed image is shown (**f**).

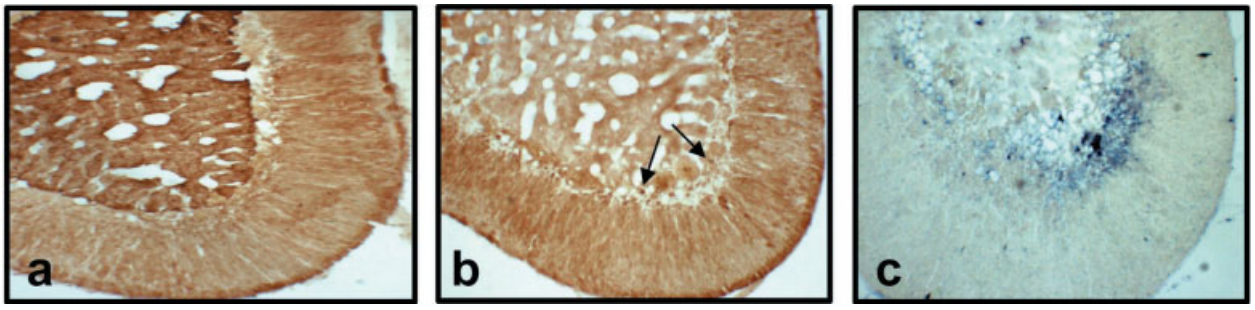

FIG. 5. Localization of endogenous TH and transgenic Cre recombinase in the adrenal medulla of a TH-Cre.Z/AP double transgenic mouse. Immunohistochemistry using an anti-TH antibody showing a representative adrenal section (**a**) or an anti-Cre antibody showing nuclear staining in the periphery of the medulla (**b**, arrows). hPLAP activity is evident in the peripheral layer of the adrenal medulla confirming the immunohistochemical results (**c**).

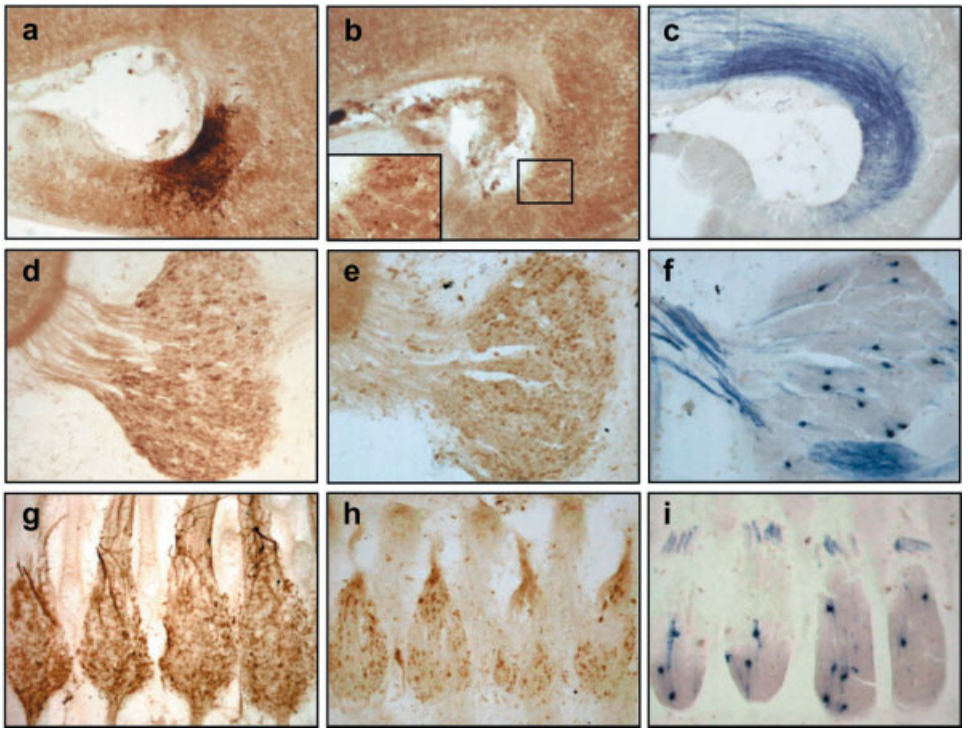

FIG. 6. Localization of endogenous TH and transgenic Cre recombinase in an E13.5 mouse embryo at the level of the developing midbrain (**a–c**), trigeminal ganglion (**d–f**), and dorsal root ganglia (**g–i**). Immunohistochemistry using an anti-TH antibody (**a,d,g**), an anti-Cre antibody (**b,e,h**) and hPLAP activity (**c,f,i**) performed on serial sections from a TH-Cre.Z/AP double transgenic mouse. The inset (**b**) shows intense nuclear Cre immunoreactivity in several developing midbrain neurons. Note that at this early developmental stage, hPLAP-positive cells are fewer than those detected with the Cre antibody, suggesting that *loxP*-mediated DNA recombination is not an immediate process.

vertebral column and in a small number of neurons of the dorsal root ganglia of adult transgenic mice (data not shown). Similar results were obtained in transgenic mice carrying a 9 kb or a 4.5 kb of the rat TH promoter (Min *et al.*, 1994; Schimmel *et al.*, 1999). This expression pattern is consistent with the interpretation that these promoters have all necessary *cis*-acting elements to direct authentic expression of reporter genes in transgenic mice while lacking other regulatory regions required to switch off the TH gene in neurons that express TH only during development. In the particular case of TH-Cre mice, the absence of postnatal TH silencing elements would not be critical because even a minimal active transcriptional window for Cre during development could be sufficient to mediate DNA recombination in the limited number of *loxP* sites present in each neuronal nucleus.

MATERIALS AND METHODS

Animals and Gene Construct

Animals were housed and maintained in accordance with the *Guide for the Care and Use of Laboratory Animals* of the Public Health Service (USA). They were exposed to a 12 h light–dark cycle and food and water were provided ad libitum. Transgenic mice were produced by microinjecting one of the pronuclei of fertilized B6.CBF$_2$ oocytes with ~500 molecules of the linearized 10.5 kb TH-Cre transgene fragment dissolved in 1 pl of a sterile solution containing 5 mM Tris-HCl, pH 7.4, and 0.1 mM EDTA. Microinjected eggs were transferred into the oviducts of pseudopregnant CF-1 foster mothers. The offspring were analyzed by PCR using the primers Cre1 (5′-GGA CAT GTT CAG GGA TCG CCA GGC G-3′) and Cre2 (5′-GCA TAA CCA GTG AAA CAG CAT TGC TG-3′) and confirmed by dot blot hybridization of tail genomic DNA using a random primed [^{32}P] radiolabeled probe generated with a 760 bp EcoRI/EcoRV fragment of Cre coding sequences. The TH-Cre transgene was constructed using Bluescript SK+/- (Stratagene, La Jolla, CA) as the plasmid backbone. A 9 kb Hind III/Eco RI fragment containing the rat TH promoter was excised from plasmid pTH9000 (generously provided by Drs. Tong Joh and Jin Son; Min *et al.*, 1994) and fused to a 1.2 kb Mlu I/Mlu I fragment containing coding sequences for a nuclear localization signal followed by Cre recombinase from bacteriophage P1 (GenBank accession number X03453). The polyadenylation site of the SV40 late T antigen gene was inserted 3′ to the Cre sequences. The 10.5 kb TH-Cre transgene was excised from the final plasmid as a Sal I/Not I fragment, separated by agarose gel electrophoresis, collected by electroelution, and purified through an ion-exchange Elutip-D column (Schleicher & Schuell, Keene, NH).

Z/AP reporter transgenic mice (Lobe *et al.*, 1999) were obtained from Dr. C. Lobe (Sunnybrook Health Science Center, Toronto, Ont) and genotyped by staining ear punch biopsies. Briefly, the tissue samples were collected into PBS in 96-well dishes, then fixed for 5–30 min in 0.2% glutaraldehyde in PBS, and finally rinsed three times in PBS for 5 min. Tissues were immersed in X-gal colorimetric reaction solution (2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% Nonidet-P40, 100 mM sodium PB pH 7.3, 1 mg/ml X-gal, 6 mM potassium ferrocyanide, and 5 mM potassium ferricyanide). Blue staining was usually evident after 15 min. TH-Cre and Z/AP transgenic lines were intercrossed to obtain F_1 mice expressing hPLAP only in catecholaminergic neurons.

Tissue Preparation

Mice were anesthetized with Avertin (2,2,2 tribromoethanol, Aldrich, Milwaukee, WI) 300 mg/kg, i.p., and perfused through the left cardiac ventricle with 10 ml of 0.9% NaCl followed by 50 ml of ice-cold 4% paraformaldehyde in PBS, pH 7.4. Brains were removed and postfixed in the same formaldehyde solution overnight at 4°C and sectioned at 50-μm intervals with a Vibratome 1000 (Ted Pella, Redding, CA). For frozen sectioning, cryoprotection was performed by subsequent overnight incubations in PBS containing 10, 20, and 30% sucrose over 3 days. Samples were then included in 30% sucrose in PBS and frozen at –20°C for at least 1 h. Blocks were cryosectioned at 30 μm using a sliding microtome (Leica SM2000R, Germany), collected in cold potassium phosphate buffer (KPBS: 140 mM NaCl, 20 mM K_2HPO_4, pH 7.4), and used for immunohistochemistry, immunofluorescence, or enzymatic histochemical staining.

Immunohistochemistry

Sections were incubated 1 h in 1% H_2O_2 in KPBS to inactivate endogenous peroxidases and then rinsed twice for 20 min with KPBS. A rabbit polyclonal anti-Cre antiserum was used at 1:2,000–3,000 dilutions (Covance/Babco, Princeton, NJ) and a rabbit polyclonal anti-TH antiserum was used at a 1:500 dilution (Chemicon International, Temecula, CA). Sections were incubated with primary antibody over night at 4°C in a solution containing KPBS, 0.3% Triton X-100, and 2% normal goat serum (NGS). After washing twice for 20 min with KPBS, sections were incubated with a solution containing biotinylated goat antirabbit IgG 1:200 (Vector Laboratories, Burlingame, CA) diluted in KPBS/0.3% Triton X-100 for 2 h at RT followed by two washes with KPBS for 20 min. Sections were then incubated with avidin/biotin complex ABC Vectastain Elite Kit (Vector) for 1 h at RT. After washing once for 20 min in KPBS, sections were rinsed for 20 min in TBS (150 mM NaCl; 50 mM Tris-HCl; pH 7.2–7.6). Finally, sections were exposed to a solution of 0.025% diaminobenzidine, 0.05% H_2O_2 in TBS, monitored under a stereomicroscope for color development, and then washed in TBS to stop the reaction.

For double-labeling immunohistochemistry, sections were first incubated with an anti-Cre antibody as described above. After stopping the diaminobenzidine reaction in TBS, sections were rinsed in KPBS and incubated with the anti-TH antibody and the procedure followed as explained above until the 20-min wash in

TBS. Then, sections were washed twice with 0.01 M sodium PB (pH 6.0) for 10 min, fixed with 0.1% glutaraldehyde in 0.01 M sodium PB (pH 6.0) for 10 min, and rinsed with the previous solution twice for 10 min followed by a 10-min incubation in a solution of 0.01% benzidine dihydrochloride (Sigma, St. Louis, MO) and 0.025% sodium nitroprusside (Sigma) in 0.01 M sodium PB (pH 6.0). Sections were then transferred to a similar solution with the addition of 0.005% H_2O_2 and incubated until the signal developed. Reactions were stopped by transferring the sections to 0.01 M sodium PB (pH 6.0). For single or double immunohistochemistry, sections were mounted on microscope slides by floating them on a prewarmed 1% gelatin solution containing 0.1% chromium potassium sulfate, air-dried for 10–12 h, dehydrated in 70, 95, 100% ethanol solutions and xylene for 5 min, and coverslipped under Permount (Fisher, Pittsburgh, PA).

Immunofluorescence

Sections were coincubated overnight at 4°C with a rabbit polyclonal anti-Cre (1:3,000) antibody and a mouse monoclonal anti-TH antibody at 1:500 (Diasorin, Stillwater, MN) both in KPBS/0.3% Triton X-100, 2% NGS. After washing twice with KPBS for 20 min at RT, sections were incubated in KPBS/0.3% Triton X-100 containing goat antimouse IgG coupled to fluorescein at 1:200 (Boehringer Mannheim, Indianapolis, IN), and goat antirabbit IgG coupled to rhodamine at 1:200 (Boehringer Mannheim). Finally, sections were washed twice in KPBS for 20 min at RT, mounted on microscope slides by floating them on PBS, and coverslipped under FluorSave reagent (Calbiochem, La Jolla, CA).

hPLAP Staining

Free-floating sections were refixed in cold PBS containing 0.2% glutaraldehyde for 10 min and then washed three times in PBS for 5 min. Activity of endogenous alkaline phosphatases was inactivated by incubating sections in PBS at 70–75°C for 30 min. Sections were then rinsed with PBS, washed in a buffer containing 100 mM Tris-HCl, pH 9.5; 100 mM NaCl, and 10 mM $MgCl_2$ for 10 min and transferred to a staining solution containing 100 mM Tris-HCl, pH 9.5; 100 mM NaCl; 50 mM $MgCl_2$; 0.01% sodium deoxycholate; 0.02% NP-40; 337 μg/ml nitroblue tetrazolium salt (Sigma), and 175 μg/ml 5-bromo-4-chloro-3-indolyl phosphate toluidinium salt (Sigma). The staining reaction was allowed to proceed for 10–30 min at RT in a dark chamber. Sections were then washed in PBS, mounted on slides, dried for 3 h at RT, dehydrated through an ethanol series and xylene, and mounted with coverslips using Permount (Fisher Scientific).

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