© 2002 Wiley-Liss, Inc.
genesis 35:63–72 (2003)

TECHNOLOGY REPORT

Inducible Site-Specific Recombination in Myelinating Cells

Nathalie H. Doerflinger,[1] Wendy B. Macklin,[2] and Brian Popko[3]*

[1]Institut de Génétique et de Biologie Moleculaire et Cellulaire (IGBMC), CNRS, Université Louis Pasteur, Illkirch, France
[2]Department of Neurosciences, Lerner Research Institute, Cleveland Clinic Foundation, Cleveland, Ohio
[3]Center for Peripheral Neuropathy, Department of Neurology, University of Chicago, Chicago, Illinois

Received 21 June 2002; Accepted 6 August 2002

Summary: To explore the function of genes expressed by myelinating cells we have developed a model system that allows for the inducible ablation of predetermined genes in oligodendrocytes and Schwann cells. The Cre/loxP recombination system provides the opportunity to generate tissue-specific somatic mutations in mice. We have used a fusion protein between the Cre recombinase and a mutated ligand-binding domain of the human estrogen receptor (CreER^T) to obtain inducible, site-specific recombination. CreER^T expression was placed under the transcriptional control of the regulatory sequences of the myelin proteolipid protein (PLP) gene, which is abundantly expressed in oligodendrocytes and to a lesser extent in Schwann cells. The CreER^T fusion protein translocated to the nucleus and mediated the recombination of a LacZ reporter transgene in myelinating cells of PLP/CreER^T mice injected with the synthetic steroid tamoxifen. In untreated animals CreER^T remained cytoplasmic, and there was no evidence of recombination. The PLP/ CreER^T animals should be very useful in elucidating and distinguishing a particular gene's function in the formation and maintenance of the myelin sheath and in analyzing mature oligodendrocyte function in pathological conditions. genesis 35:63–72, 2003. © 2002 Wiley-Liss, Inc.

Key words: myelinating cells; CreER^T; recombination

INTRODUCTION

The myelin sheath is the multilayered membrane structure that surrounds most nerve axons and facilitates rapid nerve conduction velocities by promoting salutatory conduction. Oligodendrocytes are responsible for myelinating the central nervous system (CNS), and Schwann cells myelinate the peripheral nervous system (PNS). Myelinating cells express a number of molecules critical to their differentiated function (Morell and Quarles, 1999; Baumann *et al.*, 2001; Garbay *et al.*, 2000). A better understanding of the molecular mechanisms by which these cells form and maintain the myelin sheath will likely advance our understanding of this critical structure, as well as facilitate the development of strategies for the repair of the injured sheath in demyelinating diseases such as multiple sclerosis and chronic inflammatory polyneuropathy (Scherer, 1997; Duncan *et al.*, 1997).

The examination of mouse mutants, spontaneous and experimentally engineered, that are specifically deficient in a particular myelin component has been an invaluable tool in the analysis of the myelination process (Werner *et al.*, 1998; Suter *et al.*, 1999; Martini, 2000; Yool *et al.*, 2000). To date, these mutations have been present in all cells at all stages of development in the animals examined. Such models are satisfactory for molecules that are "myelin specific," but to determine the function of molecules more widely expressed, the analysis of myelinating cell-specific mutants is preferable.

Fortunately, new tools have been developed that allow for the generation of conditional genome alterations by controlling the tissue or cellular specificity of the introduced mutations (Gao *et al.*, 1999). Conditional somatic mutations can be obtained using a recombination system based on the bacteriophage P1 site-specific Cre recombinase (Kuhn *et al.*, 1995; Nagy, 2000) under the control of either a tissue- or cell-specific promoter. The Cre recombinase efficiently catalyzes molecular recombination at specific 34-bp sequences termed loxP sites. Any DNA segment flanked by loxP sites, in the same orientation, will be deleted in cells expressing the Cre recombinase but will remain intact in nonexpressing cells. Numerous studies have demonstrated that Cre-mediated recombination can occur in a variety of cell types (Sauer *et al.*, 1989; Kuhn *et al.*, 1995; Kellendonk *et al.*, 1999; Feltri *et al.*, 1999; Nagy *et al.*, 2000). Furthermore, several recent studies have described mice expressing Cre recombinase under the control of the

* Correspondence to: Brian Popko, Jack Miller Center for Peripheral Neuropathy, Department of Neurology, University of Chicago, 5841 South Maryland Ave, MC2030, Chicago, IL 60637-1470.
E-mail: bpopko@neurology.bsd.uchicago.edu

Grant sponsors: Institut National de la Santé et de la Recherché Médicale (INSERM), Vaincre les Maladies Lysosomales (VML), and the Multiple Sclerosis Society.
Grant sponsor: National Institutes of Health, Grant numbers: NS027336 and NS034939.

DOI: 10.1002/gene.10154

myelin basic protein (MBP) promoter that allow for the introduction of oligodendroglial-specific alterations (Niwa-Kawakita *et al.*, 2000; Hisahara *et al.*, 2000; Sato *et al.*, 2001).

In addition to spatial specificity, the control of temporal specificity is also a desirable feature of an in vivo recombination system. For example, specific molecules may play distinct functions in the formation versus the maintenance of the myelin sheath. Steroid hormone receptors have been exploited to confer temporal control to the Cre recombinase (Metzger *et al.*, 1995; Kellendonk *et al.*, 1996). For example, Feil *et al.* (1996) developed a system for regulating the temporal specificity of recombination by generating a Cre recombinase fused to a mutated ligand binding domain of the human estrogen receptor (ER). The mutant ER (ER^T) is activated by the synthetic steroid tamoxifen but not by endogenous estradiol (Feil *et al.*, 1996). The $CreER^T$ fusion protein translocates from the cytoplasm of the cell, where it is functionally inactive, to the nucleus when bound to tamoxifen, thus allowing temporally (tamoxifen) controlled somatic mutagenesis of floxed target genes.

We set out to generate a mouse model system that would allow for the spatial and temporal control of gene inactivation in myelinating cells. Toward this goal, transgenic mice were generated in which $CreER^T$ expression was driven to myelinating cells using the regulatory sequences of the murine proteolipid protein (PLP) gene (Wight *et al.*, 1993; Griffiths *et al.*, 1998). The expression of transgenes driven by this PLP transcriptional control region is cell-type specific, primarily restricted to myelinating oligodendrocytes in the CNS (Wight *et al.*, 1993; Fuss *et al.*, 2000, 2001) and Schwann cells in the PNS (Mallon *et al.*, 2002). Furthermore, PLP is expressed earlier than MBP during development, beginning during embryonic life and at early developmental stages of the oligodendroglia lineage (Wight *et al.*, 1993; Belachew *et al.*, 2001; Mallon *et al.*, 2002). PLP-promoter-driven $CreER^T$ transgenic mice should allow for the molecular assessment of myelinating cell development and function in vivo from the progenitor stage to mature myelinating cells (Spassky *et al.*, 1998; Belachew *et al.*, 2001).

Previous studies have shown that the first half of the mouse PLP gene, containing 2.4 kb of 5′ flanking DNA, exon 1 and intron 1, is sufficient to direct both spatial and temporal transgene transcription that accurately reflects the expression of the endogenous PLP gene (Wight *et al.*, 1993; Fuss *et al.*, 2000, 2001). Three independent lines were generated carrying the PLP/$CreER^T$ transgene (Fig. 1A). Mice of all PLP/$CreER^T$ lines were fertile, transmitted the transgene in a Mendelian fashion, and showed no signs of disease. RT-PCR data, generated using a primer set that amplifies a region that spans the intron in the transgene, demonstrates the presence of transgene-derived mRNA primarily in CNS (brain and spinal cord), but also weakly in heart and testis in animals of each line (Fig. 1B). PLP/DM20 mRNA expression has previously been shown to be present in heart at levels at about 0.1% of that present in the brain (Campagnoni *et al.*, 1992), and PLP-driven mRNA was detected in the testis of transgenic animals (Fuss *et al.*, 2000).

To examine the expression of the $CreER^T$ protein in the three transgenic lines, we performed Western blot analyses using polyclonal antibody against the Cre protein (Kellendonk *et al.*, 1999) (Fig. 1C). In brain samples from transgenic mice of the three different lines, a protein with an apparent molecular weight of 67 kDa was detected. This corresponds to the expected size of recombinant $CreER^T$ fusion protein. No expression of $CreER^T$ protein was detectable in wild-type animal (Fig. 1C).

We further characterized the expression of the $CreER^T$ protein by immunohistochemistry in adult sections of brain, optic, and sciatic nerves using the same antiserum as used for immunoblotting. Expression of the $CreER^T$ protein is localized predominantly in white matter areas, with a scattered distribution within corpus callosum, anterior commissure, cerebellum, spinal cord, optic, and sciatic nerves (Fig. 2), and is consistent with the endogenous expression of the PLP gene (Wight *et al.*, 1993). The expression profile is essentially the same for all three PLP/$CreER^T$ transgenic lines. At the same time, no expression of $CreER^T$ protein is detectable in wild-type animals (data not shown).

To assess the identity of the $CreER^T$-positive cells in the CNS we performed several double-labeling immunohistochemistry experiments on frozen brain sagittal sections and examined areas with relatively simple morphology, such as cerebellum (Fig. 3A–C). Analysis of $CreER^T$ labeling using confocal imaging techniques revealed $CreER^T$-positive cell bodies in all white matter tracts (red labeling, Fig. 3A, D). For a more accurate localization of oligodendrocytes and their myelin sheath, monoclonal antibodies specific for MBP and PLP were used. As expected, cells positive for $CreER^T$ could be identified in areas that were positive for MBP or PLP (green labeling, Fig. 3B and 3C, respectively). $CreER^T$-derived fluorescence was confined to cell bodies. Nevertheless, a precise colocalization of cell bodies double positive for $CreER^T$ and MBP or PLP in myelin-rich CNS areas was not easily obtained since MBP and PLP are myelin-associated and do not accumulate in the cell bodies. Occasionally single cells could be distinguished at the edges of the white matter tracts where the overall MBP or PLP stain was less, with clear $CreER^T$-positive cell bodies and MBP- or PLP-positive cytoplasm and processes (Fig. 3A–C). To assess the identity of $CreER^T$-positive cells more accurately, we used antibodies that specifically label oligodendrocyte cell bodies (clone CC1 of adenomatous polyposis coli protein and gluthathione-s-transferase Pi [GST-Pi]), processes and somata of astrocytes (anti-glial fibrillary acidic protein [GFAP]), and neuronal cell bodies and dendrites (clone SMI32 of neurofilament H) for double-labeling experiments (Fig. 3D–I). As representative CNS regions, we analyzed corpus callosum, spinal cord and cerebellum. These studies clearly demonstrated that neither astrocytes nor neurons were labeled by $CreER^T$ (Fig. 3H, I). In general, a major-

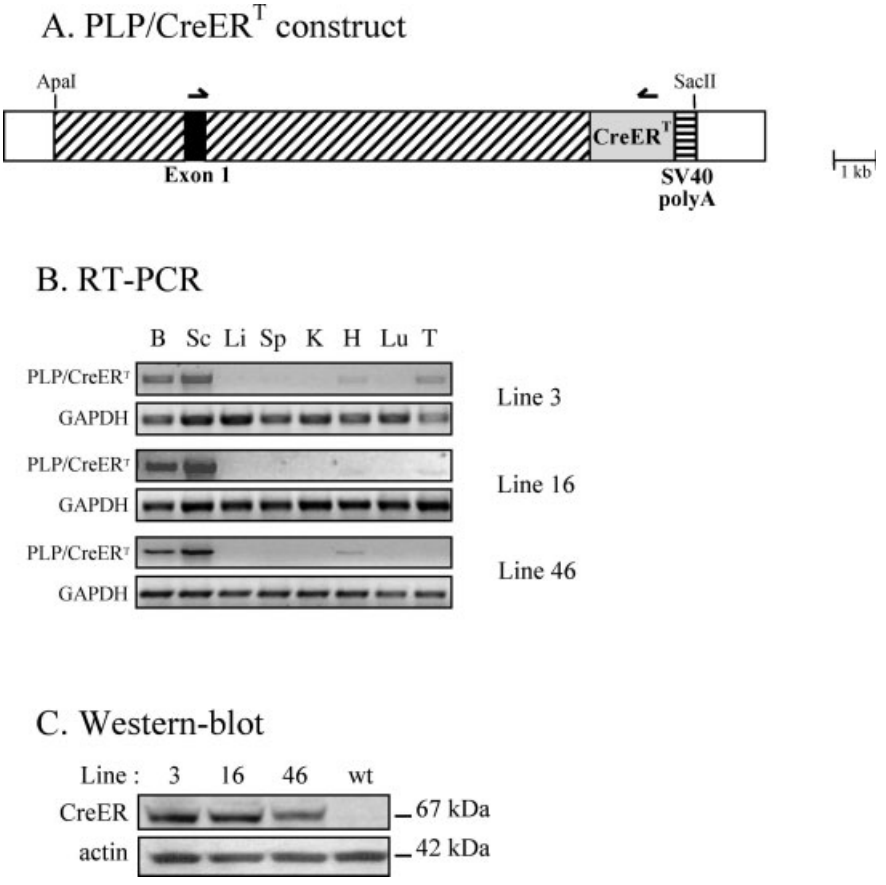

FIG. 1. Transgene construct and PLP/CreER^T expression in transgenic mice. (**A**) Structure of the PLP/CreER^T transgene and strategy to the detection of CreER^T mRNA by RT-PCR. To generate transgenic PLP/CreER^T mice we used the PLP cassette containing 2.4 kb of the 5′-flanking DNA, exon 1 and intron 1 of the PLP gene (black-and-white diagonally striped boxes), which have been shown to drive abundant oligodendrocyte expression (Fuss *et al.*, 2000). For transcription termination, a simian virus (SV) 40 poly(A) signal sequence (horizontally striped box) is present 3′ of the cDNA sequence encoding CreER^T (CreER^T box). White boxes represent vector sequences. (**B**) RT-PCR analysis of CreER^T mRNA expression. RNA was isolated from brain (B), spinal cord (SC), liver (L), spleen (Sp), kidney (K), heart (H), lung (Lu), and testis (T) of 3-week-old PLP/CreER^T mice. RT-PCR products corresponding to PLP/CreER^T mRNA (fusion between PLP exon 1 and CreER^T coding sequence) and GAPDH mRNA used as an internal control are indicated. (**C**) Western blot analysis of CreER^T expression. Twenty μg of total protein of brain from 3-week-old PLP/CreER^T and littermate control mice were analyzed. Membrane was incubated with polyclonal anti-Cre recombinase and monoclonal anti-actin antibodies. All three expressing transgenic lines and wild-type (wt) sample are showed. The expected specific band sizes are 67 kDa and 42 kDa for recombinant CreER^T fusion protein and actin, respectively.

ity of the CreER^T-positive cells were also CC1 (Fig. 3D, E) or GST-Pi (Fig. 3F, G) positive. The above data confirmed a restricted expression pattern of CreER^T in myelinating oligodendrocytes in the CNS of PLP/CreER^T mice.

To examine the steroid response of CreER^T-expressing oligodendrocytes, we compared the intracellular localization of the chimeric protein upon treatment of transgenic mice with tamoxifen or oil carrier alone. Immunohistochemistry analyses were performed on frozen spinal cord sections using polyclonal anti-Cre antibody and DAPI to stain cell nuclei. In the absence of tamoxifen treatment, all CreER^T protein was excluded from the nucleus (Fig. 4A, B). Three days of 1 mg/day tamoxifen treatment was sufficient to induce nuclear translocation of CreER^T (Fig. 4C, D).

The specificity of Cre-mediated recombination was tested functionally by crossing the PLP/CreER^T-expressing mice to animals harboring a floxed β-galactosidase (LacZ) reporter transgene (Araki *et al.*, 1995). In the reporter mice, LacZ expression is only activated in cells that express functional Cre recombinase, which removes a stop signal from the floxed transgene. Double-transgenic animals were treated for 8 consecutive days with a single daily injection of oil carrier or 1 mg/day of tamoxifen. The recombination event was analyzed by PCR in various tissues of the PLP/CreER^T:floxed LacZ reporter mice. Recombination of the floxed LacZ transgene was scored by amplification of a 384-bp PCR product using primers described by Niwa-Kawakita (2000), AG10 and Z6 (Fig. 5). These primers also allowed amplification of a 1972-bp product, corresponding to the nonrecombined transgene, which was observed in all samples (Fig. 5). In tamoxifen-treated floxed LacZ reporter mice carrying the PLP/CreER^T transgene, the

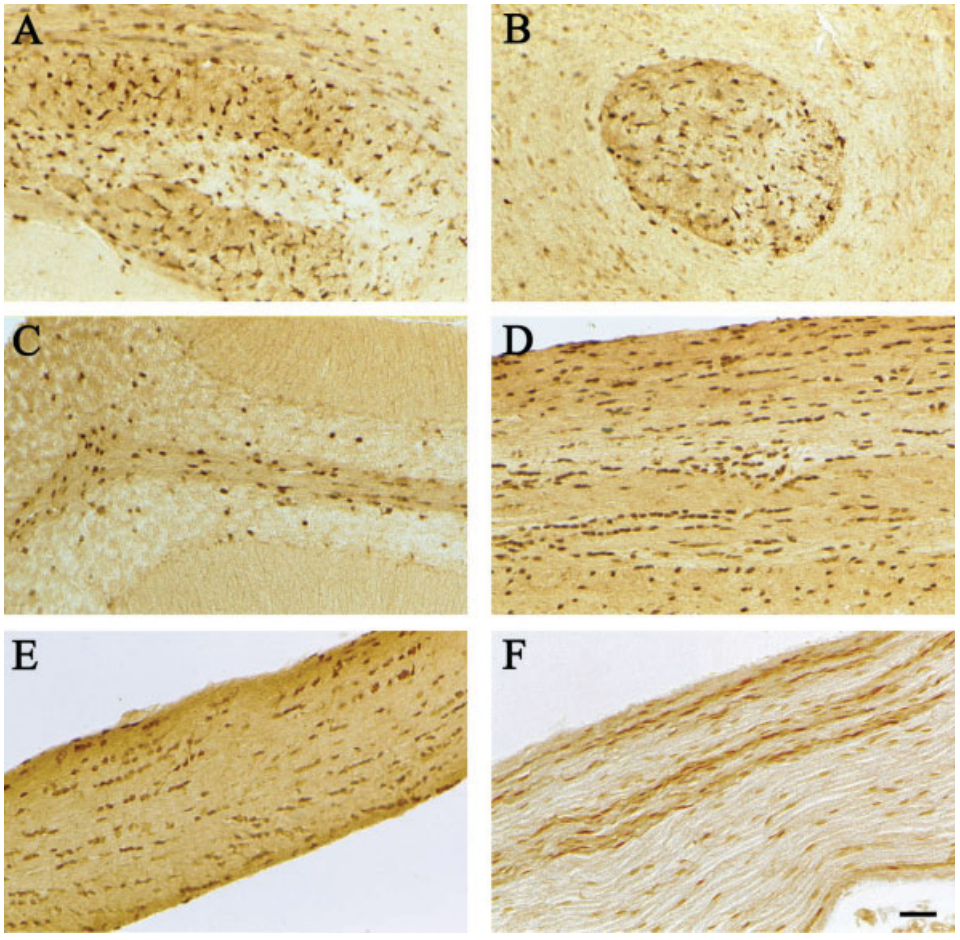

FIG. 2. PLP/CreER^T protein expression pattern in central nervous system and peripheral nervous system. Immunohistochemical staining of CreER^T recombinase (brown cell bodies) was performed on sections of 5-week-old PLP/CreER^T line 3 mice brain (**A–D**), optic (**E**), and sciatic (**F**) nerves using the same polyclonal anti-Cre antibodies as for Western blots. Expression of the transgene is localized predominantly in white matter areas and was consistent with the endogenous expression of the PLP gene. Details of this staining is showed in corpus callosum (A), anterior commissure (B), cerebellum (C), spinal cord (D), optic (E), and sciatic (F) nerves. Scale bars = 50 μm.

384-bp fragment specific for the deleted transgene was amplified principally in the CNS samples analyzed, thus confirming the spatial and temporal specificity of the PLP transgene for the CNS. Nevertheless, consistent with the PLP/CreER^T expression pattern described above, recombination was also weakly observed in heart and testis. At the same time, no deleted transgene was amplified in oil carrier treated animals (Fig. 5).

To determine the cellular localization of Cre-mediated recombination in the CNS and PNS, various tissue samples from PLP/CreER^T:floxed LacZ reporter mice, treated by oil carrier or tamoxifen, were analyzed for expression of β-galactosidase by X-gal staining (Fig. 6). In reporter mice carrying the PLP/CreER^T transgene, specific staining was observed in various regions of the CNS, only after tamoxifen treatment (Fig. 6A, B). Blue-stained cells were found primarily in white matter tracts of brain (Fig. 6C–F) and optic nerve (Fig. 6G). To a far lesser extent X-gal conversion was also detected in the sciatic nerves (Fig. 6H), confirming that the PLP promoter cassette used includes elements that stimulate transcription in Schwann cells (Puckett *et al.*, 1987; Mellon *et al.*, 2002). Immunofluorescence staining for both LacZ and Cre recombinase protein detected LacZ reporter expression in a significant proportion of Cre positive cells (Fig. 6A). Finally, dual label immunocytochemistry for both LacZ and CC1 primarily, although not exclusively, detected recombination in oligodendroglial cells, as assessed by LacZ reporter expression, after Cre-mediated recombination (Fig. 7B and C). It is unclear whether the lack of uniform labeling of LacZ-positive cells by the oligodendroglial marker reflects a population of oligodendrocytes that do not express CC1, as the work of Fuss *et al.* (2000) would suggest, or whether the PLP/CreER^T transgene is expressed in nonoligodendroglial cells, or some combination of these two possibilities.

The combination of the PLP promoter and the induction of the CreER^T recombinase by tamoxifen yields a highly selective conditional mutagenesis system for myelinating cells. The PLP/CreER^T transgene is expressed primarily in white matter tracts of the CNS, with less expression detected in Schwann cells, and even less in the heart and testis. In the CNS of these mice, cells labeled by their CreER^T expression could be clearly identified as mature oligodendrocytes by double immunocytochemical studies with oligodendrocyte-specific markers.

Expression of the PLP gene was initially thought to occur exclusively in the CNS, but PLP is clearly also expressed in Schwann cells (Puckett *et al.*, 1987). The

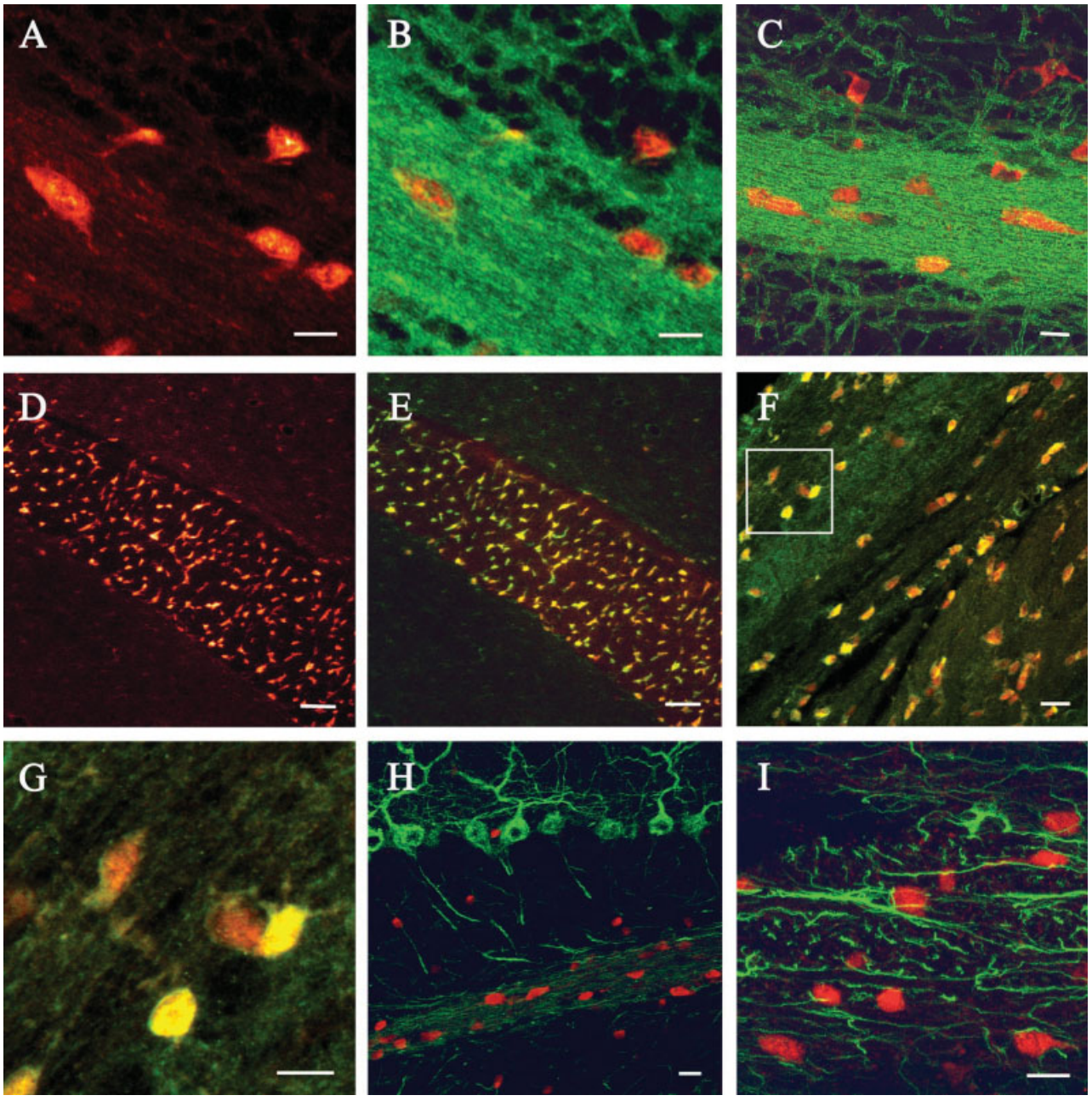

FIG. 3. Oligodendroglial CreER^T expression examined by confocal imaging. To assess the identity of the Cre-positive cells (red) in brain we performed double labeling (green) on 5-week-old frozen brain sagittal sections with antibodies specific for myelin sheath: MBP (**A, B**) and PLP (**C**); oligodendrocyte cell bodies such a CC1 (**D, E**) and GST-Pi (**F, G**); neurons: neurofilament H (**H**); and astrocytes: GFAP (**I**). Panels A and B show sections from line 3, panels C, D, E, F, G, and I display sections from line 46, and panel H is from line 16. Pictures show double labeling, with single (A/D) or double laser channel, localized in cerebellum (A/B/C), corpus callosum (D/E), or cerebellum (F/G/H/I). Scale bars = 10 μm, except for D and E, which equal 25 μm.

PLP expression cassette used in these studies contains transcriptional control elements that drive expression also in the PNS (Wight *et al.*, 1993; Jiang *et al.*, 2000; Mallon *et al.*, 2002), where it is expressed in both myelinating and nonmyelinating Schwann cells (Jiang *et al.*, 2000). The PLP promoter remains active after sciatic nerve transection (Jiang *et al.*, 2000), which suggests that the inducible system described here could be used to induce recombination in experimental models of peripheral nerve injury.

The availability of the PLP/CreER^T mice should allow for the detailed characterization of the function of proteins expressed by oligodendrocytes. The targeted expression of the Cre recombinase to these cells provides

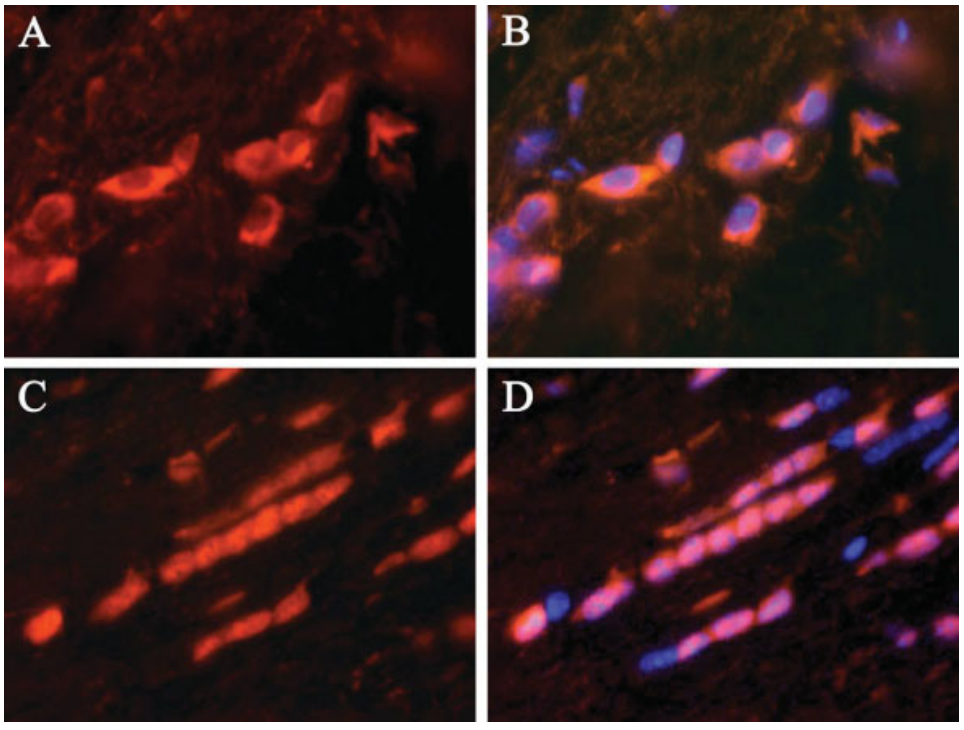

FIG. 4. Tamoxifen-induced nuclear translocation of CreER^T recombinase. Four-week-old line 3 PLP/CreER^T:floxLacZ double transgenic mice were injected for 3 consecutive days with tamoxifen (1 mg/day) (C/D) or with the sunflower oil carrier alone (A/B). Immunohistochemistry against Cre was performed on spinal cord cryosections (A/B/CD). The red label corresponds to Cre staining (A/B/C/D) and the cyan label to DAPI staining (B/D) of the nuclei.

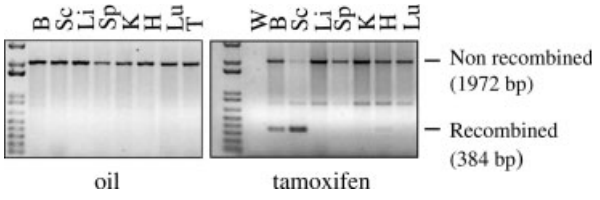

FIG. 5. PCR detection of CreER^T-mediated recombination in mice. Four-week-old line 3 PLP/CreER^T:floxLacZ transgenic mice were injected with vehicle (oil) or tamoxifen (1 mg/day) for 8 consecutive days. Excision of the floxed CAT cassette in various organs of oil-treated mice (left panel) or tamoxifen-treated mice (right panel) was analyzed by PCR of the junction between the β-actin promoter and the LacZ reporter gene. Recombination was scored by amplification of a 384-bp PCR product. The same primer set also allowed amplification of a 1972-bp PCR product corresponding to the non-recombined transgene. Presented samples are water (W), as a negative control of the PCR; brain (B), spinal cord (Sc), liver (L), spleen (Sp), kidney (K), heart (H), lung (Lu), and testis (T).

the means to tease out the oligodendroglial role played by genes expressed in cell types in addition to oligodendroglia. The inducible feature of the CreER^T fusion protein provides the opportunity to characterize the function of oligodendroglial proteins during the myelination process, as well as in mature animals. Furthermore, as demonstrated by the floxed LacZ reporter gene, the PLP/CreER^T animals can be used to temporally activate gene expression specifically in oligodendrocytes. A major problem with transgenic studies using a developmentally regulated promoter has been the expression of transgenes throughout development as well as in mature cells. Thus, investigating the impact of a protein in the mature cell cannot be separated from its potential to alter the development of the cell. The ability to activate cre-mediated recombination selectively in oligodendrocytes provides the opportunity to investigate the function of proteins in mature oligodendrocytes that have developed normally. These animals offer another tool in our efforts to comprehensively characterize the myelination process and at the same time investigate the function of mature oligodendrocytes in normal and pathological environments.

MATERIALS AND METHODS

Generation of the PLP/CreER^T Transgenic Line

For cell type-specific expression of the Cre recombinase, a 2-kb CreER^T fragment, containing the Cre open reading frame fused to a mutated form of the estrogen receptor ligand binding domain, was first isolated from pCreER^T plasmid (Feil, 1996), which was kindly provided by Dr. Pierre Chambon. This T4 DNA polymerase-treated EcoRI DNA fragment was inserted into the SmaI site of a modified version of the vector pNEB193 (Fuss *et al.*, 2000). The pPLP/CreER^T plasmid was constructed by cloning the 2.1-kb AscI-PacI restriction fragment containing the CreER^T sequence into the 14.5-kb PLP promoter cassette containing 2.4 kb of the 5′ flanking DNA, exon 1, intron 1 of the mouse PLP gene and a SV40 poly(A) signal sequence (Fuss *et al.*, 2000). The final 16.5-kb pPLP/CreER^T construct was cut with ApaI and SacII to release a 13-kb PLP/CreER^T fragment containing the transgene. For detection of the transgene by PCR analysis the CreER^T primers sense 5′-GAT GTA GCC AGC AGC ATG TC-3′

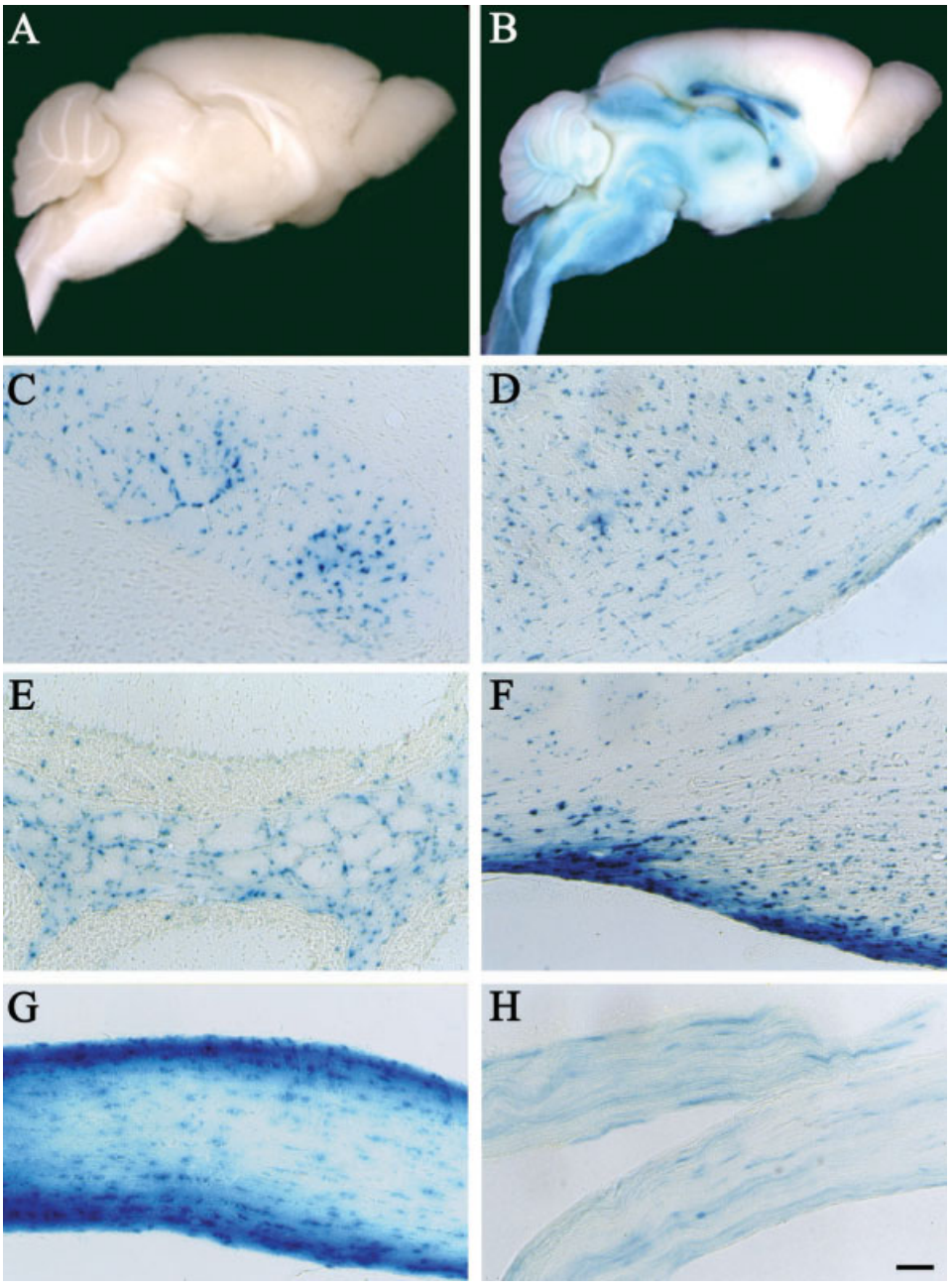

FIG. 6. X-Gal staining analysis of Cre-mediated recombination. To determine the localization of Cre-ER^T-mediated recombination after injection of tamoxifen (B–H) or oil carrier (A), various samples of line 3 PLP/CreER^T:floxLacZ mice [sagittal half-brain (A/B) and 30-μm frozen sections of sagittal brain (C–F), longitudinal optic (G) and sciatic (H) nerves] were analyzed for expression of β-galactosidase by X-gal staining. In the central nervous system, only after induction by tamoxifen, intense blue-stained cells were found in many heavily myelinated white matter tracts (B) such as corpus callosum (C), ventral spinal cord (D), cerebellum (E), brain stem (F), and optic nerve (G). Some scattered blue-stained cells were also detected in sciatic nerve (H). Scale bars = 50 μm (C–H).

and reverse 5′-ACT ATA TCC GTA ACC TGG AT-3′ were used to amplify a 532-bp PCR product.

Detection of CreER^T mRNA Synthesis in PLP/CreER^T Mice

CreER^T mRNA was detected by reverse transcriptase (RT)-PCR. Total RNA was extracted from brain, spinal cord, liver, kidney, heart, lung, and testis tissues of 3-week-old PLP/CreER^T mice with the TRIzol Reagent method (Life Technologies). cDNA was synthesized for 10 min at 37°C using 200 units of Moloney murine leukemia virus RT and 2 μg of RNA and was amplified by 30 cycles of PCR at 55°C using primers sense (5′-TCA GAG TCG CAA ACC TCG AG-3′) and reverse (5′-GAT GTA GCC AGC AGC ATG TC-3′) that amplify a 1.5-kb fragment of the PLP/CreER^T cDNA. As an internal control, a 400-bp cDNA fragment of the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was amplified in the same condition using the sense and reverse primers 5′-CCA CTC ACG GCA AAT TCA ACG GCA-3′ and 5′-TCC AGG CGG CAC GTC AGA TCC ACG-3′, respectively.

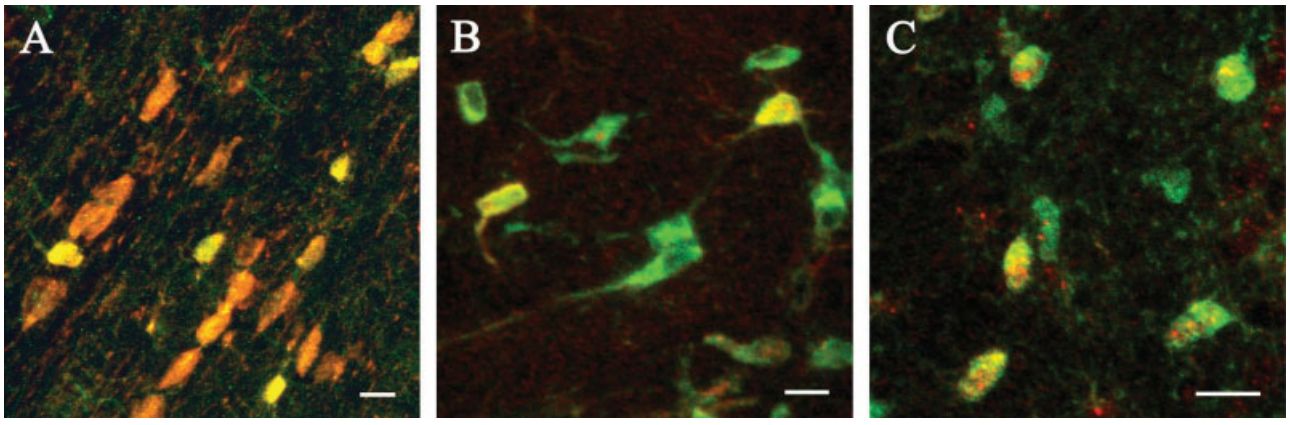

FIG. 7. Analyses of β-galactosidase expression pattern by confocal imaging. To assess the localization of β-galactosidase-expressing cells after Cre-mediated recombination, we performed double immunofluorescence labeling of β- galactosidase (red) and Cre recombinase (green) (**A**) or β-galactosidase (green) and CC1 (red) (**B, C**), on frozen tissue sections from 5-week-old line 3 PLP/CreER^T:floxLacZ mice treated for 8 consecutive days with tamoxifen. Panel shows staining of spinal cord (A), anterior commisure (B), and cerebellum (C). Yellow cells correspond to colocalization of both labeling. Scale bars = 10 μm.

Western Blot Analysis of CreER^T Expression

Total protein extracts were prepared from brain of 3-week-old transgenic PLP/CreER^T mice. Tissues were homogenized in 1% sodium dodecyl-sulfate (SDS), PBS (pH 7.3) for 30 s using a manual potter, and boiled for 10 min. Insoluble material was removed by centrifugation at 5000 × *g* for 10 min. Protein concentrations were determined using a modified Lowryl assay (Bio-Rad, Inc.). Twenty micrograms of each protein sample was resolved on 10% Bis-Tris gel (Novex), transferred to PVDF membranes (Millipore Corp.) and probed overnight at 4°C with polyclonal anti-Cre antibody (Covance BabCo) or 1 h room temperature with monoclonal anti-actin (clone AC-40; Sigma) at dilution of 1:5000 and 1:500, respectively. Antibodies were detected by enhanced chemiluminescence detection system following the instructions of manufacturers (ECL Plus; Amersham).

Immunohistochemical Analysis

For immunohistochemistry, 5-week-old transgenic PLP/CreER^T mice and nontransgenic littermate negative controls were used. Anaesthetized mice were perfused intracardially with PBS followed by 4% paraformaldehyde in 0.1 M phosphate buffer (PB), pH 7.3. Isolated tissues were postfixed for 1 h in the same fixative, cryopreserved in 30% sucrose in PB for 48 h at 4°C, embedded in OCT and frozen before cryostat sectioning (10–30 μm). Sections were permeabilized in −20°C acetone for 10 min, washed in PB, and blocked for 2 h at room temperature (RT) in PB containing 10% (v/v) normal goat serum and 0.1% (v/v) Triton X100 and incubated overnight at 4°C in athe primary polyclonal antibody (1:5000 dilution) generated against the Cre protein (Covance/BabCo) (Kellendonk *et al.*, 1999), diluted in the same blocking solution. After sections were rinsed in PB containing 0.1% (v/v) Triton X100, bound primary antibodies were detected by incubation with an anti-rabbit Cy3-coupled for 2 h RT. Sections were mounted in Vectashield mounting medium (Vector Laboratories Inc.). For immunoperoxidase staining Cre immunoreactivity was also detected, following the primary antibody, by the avidin-biotin complex method (Vectastain ABC-Elite kit; Vector Laboratories) and visualized with diaminobenzidine (DAB) chromogen (Fig. 2). For double labeling, monoclonal primary antibodies used with polyclonal Cre antibody were directed against MBP (SMI99 clone; Sternberger Monoclonals Inc.), PLP (clone plpc1; Serotec), *Adenomatous polyposis coli* protein (APC) (clone CC1; Calbiochem-Novabiochem Corp.), gluthatione-S-transferase Pi form (GSTPi) (Biotrin Int Ltd.), neurofilament H (SMI32 clone; Sternberger Monoclonals Inc.), and glial fibrillary acidic protein (GFAP) (SMI22 clone; Sternberger Monoclonals Inc.) The above procedure was repeated using anti-rabbit Cy3-coupled and anti-mouse conjugated to biotin secondary antibodies followed by streptavidin fluorescein (Jackson Immuno Research) to detect Cre polyclonal and mouse monoclonal antibodies, respectively. Images were generated on a Leica TCS-NT laser scanning microscope.

Tamoxifen-Induced Nuclear Translocation of CreER^T Recombination

To address the CreER^T-mediated recombination, the PLP/CreER^T lines were mated with a floxed LacZ (chicken β-actin gene promoter-loxP-CAT gene-loxP-LacZ region) reporter line (Araki 1995), which was kindly provided by Dr. Anton Berns, to generate double heterozygous transgenic mice PLP/CreER^T:floxLacZ (Araki *et al.*, 1995). A 10-mg/ml tamoxifen (ICN) stock suspension was prepared for the induction experiment by mixing 10 mg tamoxifen with 100 μl of ethanol and 900 μl of sunflower oil, followed by 30 min sonication. Four-week-old PLP/CreER^T:floxLacZ double-transgenic mice were injected for 3 to 8 consecutive days intraperi-

toneally with 100 μl of suspension (1 mg tamoxifen) or with the sunflower oil carrier alone. Immunofluorescence labeling against Cre was performed on spinal cord cryosections as previously described and nuclei were visualized with DAPI staining by adding 0.01% DAPI (4′,6-diamidino-2-phenylindole dihydrochloride; Sigma) in Vectashield mounting medium.

Recombination Analysis

PCR was also used to analyze CreER^T-mediated excision of the floxed CAT cassette from the floxLacZ target allele in various tissues of PLP/CreER^T:floxLacZ double heterozygotes after 8 days of treatment with tamoxifen or oil carrier. To explore the junction between the β-actin promoter and LacZ, the primers AG10 and Z6 described in Niwa-Kawakita (2000) were used. Recombination was scored by amplification of a 384-bp PCR product. The same primer set also allowed amplification of a 1972-bp fragment corresponding to the nonrecombined transgene.

Beta-Galatosidase Histochemistry

Recombination of the floxed LacZ transgene was analyzed by identification of β-galactosidase-expressing cells after staining with 5-bromo-4-chloro-3-indolyl-β-galactopyranoside (X-gal) as described by Wight *et al.* (1993). Briefly, anaesthetized mice were perfused intracardially with PBS followed by 1% paraformaldehyde, 0.5% glutaraldehyde in PBS, pH 7.3, and finally in the same fixative containing 10% sucrose. Tissues were immersed in the fixative solution containing 10% sucrose. Half-brain (hand-cut sagittally along the interhemispheric fissure), sciatic, and optic nerves were directly whole stained. Upon dropping, other half-brain sank in the same solution containing 25% sucrose (usually overnight at 4°C), and was embedded in OCT and frozen before cryostat sectioning (30 μm). The X-gal stain consisted of incubation at 37°C overnight in 1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM MgCl2, 0.02% NP-40, and 0.01% sodium deoxycholate in PBS. Whole organs or sections were rinsed twice with 3% DMSO in PBS followed by PBS alone, and refixed with 2% paraformaldehyde-0.2% glutaraldehyde. Whole organs were photographed and embedded in OCT before cryostat sectioning (30 μm). Then sections were dehydrated in ethanol (70, 80, 95, 100%), cleared in xylene, and mounted with Permount (Fisher Scientific). Recombination of the floxLacZ transgene was also analyzed by immunocytochemical detection on cryostat sections of β-galactosidase, with a polyclonal antibody (Cappel Research Reagents; ICN), and double labeling with the monoclonal antibody against APS protein, as described previously, or the polyclonal anti-Cre antibody using the tyramide signal amplification system (APS, NEN Life Science Products) according to the manufacturer's instructions. Streptavidin-Alexa Fluor 488 conjugate (Molecular Probes) was used to visualized biotin-labeled tyramide. The second antigen (β-galactosidase) was detected by a Cy3-conjugated goat anti-rabbit IgG serum.

ACKNOWLEDGMENTS

We thank Dr. Pierre Chambon for kindly providing the CreER^T cDNA and Dr. Anton Berns for providing the floxed LacZ reporter mice. We also thank April Kemper for technical assistance in the generation of the transgenic mice described and Dr. Jeffrey Dupree for advice with the morphological analyses. NHD was supported by a postdoctoral fellowship from the Institut National de la Santé et de la Recherché Médicale (INSERM), the French association Vaincre les Maladies Lysosomales (VML), and the U.S. National Multiple Sclerosis Society. This work was also supported by grants from the NIH (NS027336 and NS034939) to BP.

LITERATURE CITED

Araki K, Araki M, Miyazaki J, Vassalli P. 1995. Site-specific recombination of a transgene in fertilized eggs by transient expression of Cre recombinase. Proc Natl Acad Sci USA 92:160–164.

Baumann N, Pham-Dinh D. 2001. Biology of oligodendrocyte and myelin in the mammalian central nervous system. Physiol Rev 81:871–927.

Belachew S, Yuan X, Gallo V. 2001. Unraveling oligodendrocyte origin and function by cell-specific transgenesis. Dev Neurosci 23:287–298.

Campagnoni CW, Garbay B, Micevych P, Pribyl T, Kampf K, Handley VW, Campagnoni AT. 1992. DM20 mRNA splice product of the myelin proteolipid protein gene is expressed in the murine heart. J Neurosci Res 33:148–155.

Duncan ID, Grever WE, Zhang SC. 1997. Repair of myelin disease: strategies and progress in animal models. Mol Med Today 3:554–561.

Feil R, Brocard J, Mascrez B, LeMeur M, Metzger D, Chambon P. 1996. Ligand-activated site-specific recombination in mice. Proc Natl Acad Sci USA 93:10887–10890.

Feltri ML, D'Antonio M, Previtali S, Fasolini M, Messing A, Wrabetz L. 1999. P0-Cre transgenic mice for inactivation of adhesion molecules in Schwann cells. Ann N Y Acad Sci 883:116–123.

Fuss B, Mallon B, Phan T, Ohlemeyer C, Kirchhoff F, Nishiyama A, Macklin WB. 2000. Purification and analysis of in vivo-differentiated oligodendrocytes expressing the green fluorescent protein. Dev Biol 218:259–274.

Fuss B, Afshari FS, Colello RJ, Macklin WB. 2001. Normal CNS myelination in transgenic mice overexpressing MHC class I H-2L(d) in oligodendrocytes. Mol Cell Neurosci 18:221–234.

Gao X, Kemper A, Popko B. 1999. Advanced transgenic and gene-targeting approaches. Neurochem Res 24:1183–1190.

Garbay B, Heape AM, Sargueil F, Cassagne C. 2000. Myelin synthesis in the peripheral nervous system. Prog Neurobiol 61:267–304.

Griffiths I, Klugmann M, Anderson T, Thomson C, Vouyiouklis D, Nave KA. 1998. Current concepts of PLP and its role in the nervous system. Microsc Res Tech 41:344–358.

Hisahara S, Araki T, Sugiyama F, Yagami K, Suzuki M, Abe K, Yamamura K, Miyazaki J, Momoi T, Saruta T, Bernard CC, Okano H, Miura M. 2000. Targeted expression of baculovirus p35 caspase inhibitor in oligodendrocytes protects mice against autoimmune-mediated demyelination. EMBO J 19:341–348.

Jiang H, Duchala CS, Awatramani R, Shumas S, Carlock L, Kamholz J, Garbern J, Scherer SS, Shy ME, Macklin WB. 2000. Proteolipid protein mRNA stability is regulated by axonal contact in the rodent peripheral nervous system. J Neurobiol 44:7–19.

Kellendonk C, Tronche F, Monaghan AP, Angrand PO, Stewart F,

Schutz G. 1996. Regulation of Cre recombinase activity by the synthetic steroid RU 486. Nucleic Acids Res 24:1404–1411.

Kellendonk C, Tronche F, Casanova E, Anlag K, Opherk C, Schutz G. 1999. Inducible site-specific recombination in the brain. J Mol Biol 285:175–182.

Kuhn R, Schwenk F, Aguet M, Rajewsky K. 1995. Inducible gene targeting in mice. Science 269:1427–1429.

Mallon BS, Shick HE, Kidd GJ, Macklin WB. 2002. Proteolipid promoter activity distinguishes two populations of NG2-positive cells throughout neonatal cortical development. J Neurosci 22:876–885

Martini R. 2000. Animal models for inherited peripheral neuropathies: chances to find treatment strategies? J Neurosci Res 61:244–250.

Metzger D, Clifford J, Chiba H, Chambon P. 1995. Conditional site-specific recombination in mammalian cells using a ligand-dependent chimeric Cre recombinase. Proc Natl Acad Sci USA 92:6991–6995.

Morell P, Quarles RH, 1999. Myelin formation, structure, and biochemistry. In: Siegel GJ, Agranoff BW, Albers RW, Fisher SK, Uhler MD, editors. Basic neurochemistry: molecular, cellular, and medical aspects. Philadelphia: Lippincott-Raven Press. p 69–93.

Nagy A. 2000. Cre recombinase: the universal reagent for genome tailoring. genesis 26:99–109.

Niwa-Kawakita M, Abramowski V, Kalamarides M, Thomas G, Giovannini M. 2000. Targeted expression of Cre recombinase to myelinating cells of the central nervous system in transgenic mice. genesis 26:127–129

Puckett C, Hudson L, Ono K, Friedrich V, Benecke J, Dubois-Dalcq M, Lazzarini RA. 1987. Myelin-specific proteolipid protein is expressed in myelinating Schwann cells but is not incorporated into myelin sheaths. J Neurosci Res 18:511–518.

Sato M, Watanabe T, Oshida A, Nagashima A, Miyazaki JI, Kimura M. 2001. Usefulness of double gene construct for rapid identification of transgenic mice exhibiting tissue-specific gene expression. Mol Reprod Dev 60:446–456.

Sauer B, Henderson N. 1989. Cre-stimulated recombination at loxP-containing DNA sequences placed into the mammalian genome. Nucleic Acids Res 17:147–161.

Scherer SS. 1997. Molecular genetics of demyelination: new wrinkles on an old membrane. Neuron 18:13–16.

Spassky N, Goujet-Zalc C, Parmantier E, Olivier C, Martinez S, Ivanova A, Ikenaka K, Macklin W, Cerruti I, Zalc B, Thomas JL. 1998. Multiple restricted origin of oligodendrocytes. J Neurosci 18:8331–8343.

Suter U, Nave KA. 1999. Transgenic mouse models of CMT1A and HNPP. Ann N Y Acad Sci 883:247–253.

Yool DA, Edgar JM, Montague P, Malcolm S. 2000. The proteolipid protein gene and myelin disorders in man and animal models. Hum Mol Genet 9:987–992.

Werner H, Jung M, Klugmann M, Sereda M, Griffiths IR, Nave KA. 1998. Mouse models of myelin diseases. Brain Pathol 8:771–793.

Wight PA, Duchala CS, Readhead C, Macklin WB. 1993. A myelin proteolipid protein-LacZ fusion protein is developmentally regulated and targeted to the myelin membrane in transgenic mice. J Cell Biol 123:443–454.