A Myelin Proteolipid Protein–LacZ Fusion Protein Is Developmentally Regulated and Targeted to the Myelin Membrane in Transgenic Mice

Patricia A. Wight, Cynthia S. Duchala, Carol Readhead,* and Wendy B. Macklin

Mental Retardation Research Center, UCLA Medial Center, Los Angeles, California 90024; and
*Department of Biology, California Institute of Technology, Pasadena, California 91125

Abstract. Transgenic mice were generated with a fusion gene carrying a portion of the murine myelin proteolipid protein (PLP) gene, including the first intron, fused to the *E. coli* LacZ gene. Three transgenic lines were derived and all lines expressed the transgene in central nervous system white matter as measured by a histochemical assay for the detection of β-galactosidase activity. PLP–LacZ transgene expression was regulated in both a spatial and temporal manner, consistent with endogenous PLP expression. Moreover, the transgene was expressed specifically in oligodendrocytes from primary mixed glial cultures prepared from transgenic mouse brains and appeared to be developmentally regulated in vitro as well. Transgene expression occurred in embryos, presumably in pre- or nonmyelinating cells, rather extensively throughout the peripheral nervous system and within very discrete regions of the central nervous system. Surprisingly, β-galactosidase activity was localized predominantly in the myelin in these transgenic animals, suggesting that the NH_2-terminal 13 amino acids of PLP, which were present in the PLP–LacZ gene product, were sufficient to target the protein to the myelin membrane. Thus, the first half of the PLP gene contains sequences sufficient to direct both spatial and temporal gene regulation and to encode amino acids important in targeting the protein to the myelin membrane.

MYELIN is a highly specialized extension of the plasma membrane elaborated by oligodendrocytes in the central nervous system (CNS)[1] and by Schwann cells in the peripheral nervous system (PNS). Myelin ensheathes a segment of an axon, forming a tightly compacted multilamellar structure (Raine, 1984), which functions to promote saltatory conduction of the nerve impulse (Huxley and Staempfli, 1949). Myelination is essential for nervous system function, with dysmyelination resulting in devastating forms of mental retardation. In addition, demyelination in the adult, such as in multiple sclerosis in humans, results in serious motor and sensory disorders. Myelination involves the coordinate expression of a small set of myelin-specific genes (for review see Campagnoni, 1988; Campagnoni and Macklin, 1988; Ikenaka et al., 1991; Mikoshiba et al., 1991). One of these genes, the proteolipid protein (PLP) gene provides an excellent model to study gene regulation for many reasons. (*a*) The PLP gene is abundantly expressed. Its gene products constitute roughly 50% of the total protein found in adult CNS myelin (Eng et al., 1968; Norton and Poduslo, 1973). (*b*) Its expression is cell-type specific, primarily restricted to oligodendrocytes. (*c*) The gene is regulated developmentally with peak expression occurring during the early postnatal period in rodents. (*d*) PLP gene expression can be studied in vitro in primary cultured oligodendrocytes in the absence of any neural signals (Dubois-Dalcq et al., 1986). (*e*) Mutations in this gene are devastating, and animals such as the *jimpy* mouse or the myelin-deficient rat are severely compromised, dying within the first two months after birth. (*f*) The PLP gene products are targeted specifically to the myelin membrane.

The PLP gene is present as a single copy in the genome (Milner et al., 1985; Diehl et al., 1986; Gardinier et al., 1986) located on the X chromosome (Willard and Riordan, 1985; Mattei et al., 1986). The general gene structure in the mouse (Macklin et al., 1987, 1991; Ikenaka, 1988) and human (Diehl et al., 1986) is quite similar; roughly 17 kb of DNA encompass seven exons with a large first intron (7–8 kb). The coding sequence of the PLP gene is highly conserved with 100% identity among the mouse, rat, and human sequences at the amino acid level and >95% identity at the

Patricia A. Wight's present address is Department of Physiology, University of Arkansas for Medical Sciences, Little Rock, AR 72205.

Carol Readhead's present address is Cedars Sinai Medical Center, D-3067, Los Angeles, CA 90048.

1. *Abbreviations used in this paper*: CNS, central nervous system; GC, galactosylceramide; ONPG, o-nitrophenyl-β-D-galactopyranoside; PLP, proteolipid protein; PNS, peripheral nervous system.

nucleotide level. As well, there exists a high degree of conservation for the 5'-and 3'-untranslated regions of the gene, even extending into the 5'-flanking DNA (Macklin et al., 1987).

Two protein products are generated from the PLP gene, by use of an alternative splice site within exon 3; PLP which contains 276 amino acids and a related protein, DM-20, which does not contain PLP residues 116–150 (Macklin et al., 1987; Nave et al., 1987; Simons et al., 1987). DM-20 protein is expressed prior to PLP during development but as development proceeds, PLP becomes the predominant protein (Kronquist et al., 1987; Gardinier and Macklin, 1988; LeVine et al., 1990; Schindler et al., 1990). Likewise, the transcripts encoding both proteins are temporally regulated (LeVine et al., 1990) with the expression of DM-20 mRNA preceding that of PLP mRNA and occurring embryonically in the mouse (Pham-Dinh et al., 1991; Ikenaka et al., 1992; Timsit et al., 1992*a*). Although PLP expression has been demonstrated in Schwann cells, it may not be incorporated into myelin sheaths (Puckett et al., 1987; Kamholz et al., 1992). Unlike the developmental switch observed in oligodendrocytes, both DM-20 mRNA and protein expression are higher than PLP mRNA and protein in rat sciatic nerve at postnatal day 14 (Pham-Dinh et al., 1991).

The transcriptional regulatory elements controlling PLP gene expression have been difficult to map in vitro, primarily due to a paucity of PLP-expressing cell lines. Several recent studies have used deletion–transfection analysis to localize transcriptional regulatory elements within the PLP gene. While these studies identified elements that modulate PLP gene expression in both a positive and negative fashion, none of these elements were sufficient to direct tissue-specific expression of the PLP gene entirely. In these experiments, 0.3–5.0 kb of 5'-flanking DNA of the PLP gene was used to drive reporter gene expression. The results indicate that the PLP promoter can be transcribed in a number of cell types, including many cells that do not express the PLP gene normally. Mouse PLP-luciferase chimeric genes had significant transcriptional activity in either hamster glial cells or mouse hepatoma cells (Cook et al., 1992). Likewise, fusion genes containing rat 5'-flanking PLP DNA promoted chloramphenicol acetyl transferase (CAT) expression in both glial and nonglial cells (Nave and Lemke, 1991). In contrast, human PLP sequences directed high level CAT expression in human astroglial cells but not in nonglial cells (Berndt et al., 1992), suggesting that this construct might contain some tissue-specific elements, although it is unclear why astroglial cells should express the reporter gene, since they do not express PLP. Taken together, these studies suggest that the 5'-flanking DNA of the PLP promoter can direct gene expression in many cells, while sequences important to restricting tissue-specific expression of the PLP gene may reside elsewhere in the gene. Since the structure of the PLP gene is highly conserved and the first intron accounts for almost half of the gene, we hypothesized that a tissue specific element(s) might reside within intron 1. To test this, transgenic animals were generated using a PLP–LacZ fusion gene which contained intron 1 and coded for the first 13 amino acids of PLP. Three transgenic lines were derived and all lines expressed the transgene (β-galactosidase) both spatially and temporally consistent with endogenous PLP expression. Transgene expression was maintained in primary cultured oligodendrocytes thus effectively tagging these cells for future use in transplantation studies. Surprisingly, a greater level of transgene expression was observed in the embryonic PNS as opposed to the CNS. In adult animals, β-galactosidase activity was present in the myelin, suggesting that the first 13 amino acids were sufficient to target the protein appropriately. Thus, these transgenic animals provide an extremely potent model to study gene regulation in the developing nervous system.

Materials and Methods

Transgenic Plasmid Construction

A KpnI–ApaI fragment encompassing the latter two thirds of PLP intron 1 and the first part of exon 2 was isolated from genomic lambda clone 9a (Macklin et al., 1987). The ApaI site was converted to a SphI site by removal of the 3'-overhang with T4 DNA polymerase and the subsequent ligation of a SphI synthetic linker (5'-GGCATGCC-3'; Promega, Madison, WI). The KpnI–SphI fragment was subcloned into the corresponding sites in pGEM-7Zf(−) (Promega) and the resulting plasmid was designated KS-5.5. A ClaI–KpnI fragment from lambda KK1 (Macklin et al., 1991) containing PLP 5'-flanking DNA, all of exon 1 and the first third of intron 1 was inserted upstream of PLP sequences in KS-5.5 at the ClaI and KpnI sites, thus regenerating PLP intron 1. This plasmid was termed CS(+). A LacZ expression cassette containing the HindIII–BamHI fragment from plasmid pCH110 (Pharmacia Biotech Inc., Piscataway, NJ) was subcloned into pBluescript KS(+) (Stratagene, La Jolla, CA) and designated pGAL5 (gift of N. Mori and D. Anderson, California Institute of Technology, Pasadena, CA). The HindIII site was converted to a SphI site by a fill-in reaction with DNA polymerase I large fragment and four dNTPs followed by the addition of a SphI linker, generating plasmid pGALSph. The ApaI–SphI PLP fragment from CS(+) was ligated into the ApaI and SphI sites of pGALSph generating plasmid AS(+)GAL. To remove an in-frame stop codon downstream from the PLP initiating methionine, pGAL5 was digested with KpnI to delete a 239-bp fragment and the remaining KpnI site was converted to a SphI site as described above, yielding plasmid pGALΔK. The final construct was designated PLP(+)Z and it was generated by replacing the LacZ expression cassette (SphI–NotI fragment) in AS(+)GAL with the analogous SphI–NotI fragment from pGALΔK which essentially removed the in-frame stop codon. Thus PLP(+)Z contains 2.4 kb of 5'-flanking PLP DNA, all of exon 1 and intron 1, and the first 37 bp of PLP exon 2 ligated to a trpS–LacZ fusion gene and SV-40 polyadenylation signals (nucleotides 209–3736 of pCH110).

Production of Transgenic Mice

PLP(+)Z was digested with ApaI and NotI and fractionated on a 0.7% agarose gel. A 14-kb band of DNA was excised from the gel and electroeluted. The DNA was subjected to several extractions with phenol/chloroform and then precipitated twice with 3 M sodium acetate and ethanol. The precipitate was redissolved in TE (10 mM Tris, 1 mM EDTA) at a concentration of ~200 copies/pl. Transgenic animals were generated by the injection of DNA into pronuclei of BDF2-fertilized mouse eggs (Hogan et al., 1986; Readhead et al., 1987). Fertilized eggs were incubated overnight in M16 media (Hogan et al., 1986) in a 5% CO_2 incubator at 37°C, and the next day, 15 to 20 two-cell stage embryos were transferred into the left oviduct of BDF1 pseudopregnant mouse. The mice were allowed to develop to term. At 3 wk of age, the animals were weaned and screened for transgene integration.

Screening for Potential Transgenic Founders

A piece of tail was biopsied from potential founder mice under anesthesia. DNA was isolated from tail biopsies by incubation overnight with proteinase K (100 μg/ml) in 50 mM Tris, 100 mM EDTA, at 50°C followed by one extraction with phenol, two extractions with phenol/chloroform (1:1) and a final extraction with chloroform alone. The DNA was precipitated with 3 M sodium acetate and ethanol. The DNA was resuspended with TE. The transgene was identified by Southern blot analysis. Genomic DNA (10 μg) was digested with BamHI, electrophoresed on 0.7% agarose gels, and transferred to nitrocellulose. The blots were hybridized to random primed LacZ sequences (SphI–NotI fragment from pGALΔK).

β-Galactosidase Histochemistry

β-galactosidase–expressing cells were identified in mice by staining with X-gal (5-bromo-4-chloro-3-indolyl-β-galactopyranoside; Promega) as described by Sanes et al. (1986) or with Bluo-gal (halogenated indolyl-β-D-galactoside; GIBCO BRL Life Technologies, Inc., Gaithersburg, MD) as described by Loewy et al. (1991). Briefly, mice were anesthetized with halothane and perfused intracardially with PBS followed by 0.5% paraformaldehyde-1.0% glutaraldehyde in PBS, pH 7.3, and finally in the same fixative containing 10% sucrose. Perfused animals were left undisturbed for 1 h after which tissues were dissected out and immersed in the fixative containing 10% sucrose. Upon dropping, tissue sank in the same fixative containing 25% sucrose (usually overnight at 4°C), and was stored at −80°C until cryostat sectioning (20–30 μm). The X-gal stain consisted of 1 mg/ml X-gal, 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, 0.02% NP-40, and 0.01% sodium deoxycholate in PBS. The Bluo-gal stain consisted of 2 mM Bluo-gal, 16 mM potassium ferricyanide, 16 mM potassium ferrocyanide, and 2 mM $MgCl_2$ in PBS. Stains were filtered with 0.45 μm membranes and incubated with tissue sections for 14–18 h at 37°C. Sections were rinsed twice for 15 min sequentially with 3% DMSO in PBS followed by PBS alone, refixed twice with 2% paraformaldehyde–0.2% glutaraldehyde for 15 min, dehydrated in ethanol (70, 80, 95, and 100%) cleared in xylene, and mounted with Permount (Fisher Scientific, Pittsburgh, PA).

Embryos were recovered on day 14.5 of gestation (0.5 corresponds to the day the vaginal plug was observed) and fixed overnight at 4°C in 2% paraformaldehyde–0.2% glutaraldehyde in PBS. Embryos were stained with X-gal, processed (to post fixation step) as described above, and photographed.

Subcellular Fractionation

Organs were rapidly dissected from mice and frozen on dry ice. Transgenic animals were identified by incubating a small section of cervical spinal cord in X-gal stain described above. Tissues were homogenized in 0.32 M sucrose (10% wt/vol), and membrane fractions were obtained by a modification of the methods of Whittaker and Barker (1972) and Norton and Poduslo (1973). Briefly, homogenates were centrifuged for 11 min at 1,000 *g* to generate P1 and S1. S1 was centrifuged at 17,000 *g* for 60 min to generate S2 and P2. S2 was centrifuged for 60 min at 100,000 *g* to generate the microsomal fraction and a soluble fraction. P2 was resuspended in 0.32 M sucrose and layered over a discontinuous sucrose gradient containing 12 ml 1.2 M sucrose and 12 ml 0.88 M sucrose, and centrifuged for 2 h at 53,000 *g* to generate P2A and P2B at the two interfaces and a pellet, P2C, which was resuspended as a mitochondrial fraction. P2A and P2B were diluted with water and recentrifuged at 75,000 *g* for 30 min. The resulting P2B pellet was the synaptosomal fraction, and P2A was the myelin fraction. Typically prepared crude nuclear preparations (P1 fraction) contained significant myelin contamination (data not shown). Nuclei for these studies were therefore prepared by centrifugation of the original 0.32 M sucrose brain homogenate through sucrose gradients containing 0.88, 1.2, and 1.8 M sucrose for 5 h. The resulting pellet was the nuclear fraction.

Samples were assayed for β-galactosidase activity by the method of Lim and Chae (1989). Samples were incubated with 3.5 mM *o*-nitrophenyl-β-D-galactopyranoside (ONPG; Sigma Immunochemicals, St. Louis, MO), in PBS containing 0.5% NP-40 for 30 min at 37°C. Enzyme activity was measured as absorbance at 420 nm.

Northern Blot Analysis

Total RNA was isolated according to the procedure of Chomczynski and Sacchi (1987) of which 20 μg was fractionated on 0.68 M formaldehyde–1.0% agarose gels and transferred to nitrocellulose filters in 20× SSC. Random primed probes were used to detect sequentially: LacZ (SphI–NotI fragment from pGALΔK described above), PLP (EcoRI fragment from pKK1.38; Macklin et al., 1991), and pCHOB (BamHI fragment from pCHOB; Harpold et al., 1979) transcripts.

Cell Culture and Immunohistochemistry

Primary mixed glial cultures (line 26H) were established according to Edmond et al. (1987). Briefly, cerebral hemispheres were dissected from neonatal mice (3 d old), freed of meninges, and mechanically dissociated in Ham's F-12/DME 1:1 (Irvine Scientific, Santa Ana, CA) containing 6 g/liter glucose and 10% FBS. The dissociated cells were plated onto 12-mm poly-L-lysine–coated glass coverslips in 24-well plates or directly in poly-L-lysine coated 6-well plates. Growth medium was changed every 3 d. After the indicated days in culture, cells were rinsed twice in PBS without Ca^{2+} and Mg^{2+}, and fixed for 1 h at room temperature with 4% paraformaldehyde in PBS, pH 7.3. Cells were washed twice with PBS and stored at 4°C in PBS until use. The cells were permeabilized with 0.1% Triton X-100 in TBS (50 mM Tris, 150 mM NaCl, pH 7.4) for 10 min, rinsed with PBS and incubated in 5% normal goat serum in TBS for 1–2 h at room temperature.

Galactosylceramide mAb (GC; Ranscht et al., 1982) was a kind gift from Joyce Benjamins (Wayne State University, Detroit, MI) and polyclonal β-galactosidase antibody was purchased from Organon Teknika-Cappel (Durham, NC). Appropriate antibody dilutions were made in 5% goat serum in TBS, added to the fixed, permeabilized cells and incubated for 1 h at room temperature. After 4 rinses with PBS, cells were incubated for 1 h at room temperature with fluorescein-isothiocyanate- or rhodamine-conjugated, species-specific secondary antibodies (Boehringer-Mannheim Biochemicals, Indianapolis, IN). After staining, coverslips were mounted with vinol mounting medium.

Results

PLP-LacZ Transgenic Mice

We developed a transgenic mouse model to study the spatial and temporal regulation of PLP gene expression in particular, and to serve as a useful marker of oligodendrocytes in general. The transgene was generated from two overlapping genomic lambda clones, KK1 and 9a (Fig. 1 *A*). An ApaI site in exon 2 was converted to a SphI site with synthetic linkers, thereby transforming the 5′ ApaI site to a unique site. The transgene contained 2.4 kb of 5′-flanking PLP DNA, all of exon 1 and intron 1, and the first 37 bp of exon 2 fused in frame to the LacZ gene. Therefore, the 5′- and 3′-splice sites of intron 1 were maintained for correct splicing, and the fusion protein contained the initiating methionine and the first 13 amino acids of PLP in addition to β-galactosidase sequences. The transgene (ApaI–NotI fragment) was purified away from vector sequences and microinjected into fertilized mouse eggs; 41 pups were born, and 34 survived to be screened. These mice were screened for the presence of LacZ sequences by Southern blot analysis of genomic DNA obtained from tail biopsies. Three founder mice bearing multiple copies of the PLP–LacZ construct were identified, and lines 20, 26, and 27 were established from these animals (Fig. 1 *B*). One of the lines, line 26, was characterized in greatest detail. Founder 26 was chimeric in her germline, and her transgenic progeny contained either a high copy number (Fig. 1 *B*, lane *26H*) or low copy number (lane *26L*) of the transgene (Fig. 1 *B*). The diversity in line 26 probably arose from a partial internal detection of multiple head to tail insertions of the transgene at a single chromosomal locus in the founder (data not shown). The two sublines have been maintained stably for over a year without any detectable change in transgene copy number suggesting that the insertion site was not at a recombinational "hot" spot. All three lines expressed the transgene based upon X-gal staining. The expression of the transgene in either subline of 26 or in line 27 was generally at a consistently high level, as determined by histological assay. Thus, lines 26H, 26L, and 27 were characterized further. Since line 20 expressed the transgene to a lower extent, it was not characterized in detail, although line 20 did express the transgene in a tissue-specific manner (see below).

PLP–LacZ Transgene Expression Is Tissue Specific

The tissue specificity of transgene expression was studied by

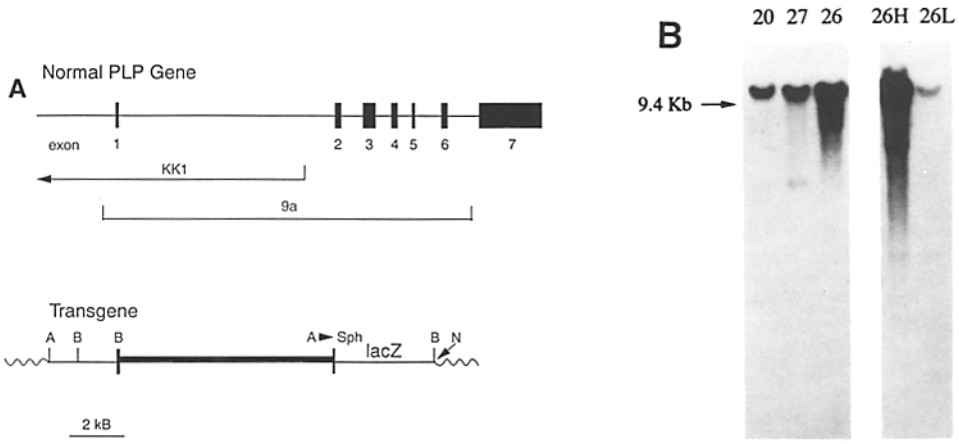

Figure 1. Generation of transgenic mice. (*A*) Structure of the mouse PLP gene and the PLP–LacZ transgene. The relevant structure of the mouse PLP gene including 2.4 kb of the 5′ upstream region and the entire exon-intron area is illustrated at the top. The gene structure is presented in a 5′ to 3′ orientation, with exons represented by black boxes. Overlapping lambda genomic clones, KK1 and 9a, were used to reconstruct PLP intron 1 and generate the transgene shown at the bottom of the figure. The transgene encompassed PLP sequences starting with a 5′ end 2.4 kb upstream from the cap site and proceeding downstream to the ApaI site in exon 2. The ApaI site in exon 2 was mutated to a SphI site, thus making the 5′ ApaI site unique in the transgene. The PLP sequences were fused in frame to a LacZ expression cassette (see Materials and Methods section for details). The ApaI–NotI fragment was purified away from vector sequences (indicated by wavy lines) and microinjected into fertilized mouse eggs to produce transgenic mice. Restriction sites are indicated as follows: ApaI, *A*; BamHI, *B*; mutated ApaI site, *A→Sph*; NotI, *N*. (*B*) Southern blot analysis of genomic DNA isolated from PLP–LacZ transgenic lines. Genomic DNA (10 μg) was digested with BamHI, fractionated on 0.7% agarose gels, transferred to nitrocellulose, hybridized to ^{32}P randomly primed fragments prepared from the LacZ region (SphI–NotI) shown in (*A*). The autoradiogram shown on the left compares results obtained from lines 20, 27, and 26H while the autoradiogram on the right contrasts the two sublines originating from founder animal 26.

analyzing LacZ mRNA levels and β-galactosidase enzyme activity. Total RNA was isolated from the liver, lung, heart, kidney, spleen, spinal cord, and brain of a 21-d-old mouse from line 26H. PLP–LacZ and PLP transcripts were detected only in the spinal cord and brain (Fig. 2). Similar results were obtained for enzyme activity, using tissue homogenates made from transgenic (26L and 27) and nontransgenic mice. β-galactosidase activity was present at detectable levels only in the brains and spinal cords of transgenic mice (Fig. 3). Enzyme activity was below the limits of detection in sciatic nerve, heart, liver, lung, spleen, kidney, or testes. However, when intact sciatic nerve from all three transgenic lines was incubated in X-gal buffer, β-galactosidase activity could be detected (data not shown). The length of time required for development of the blue reaction product was consistent with the known levels of transgene expression in these animals, i.e., sciatic nerve from lines 26 and 27 generated blue reaction product within a few hours, while sciatic nerve from line 20 required overnight incubation and nontransgenic sciatic nerve remained white. When sciatic nerve homogenates were analyzed by Western blot, sciatic nerve samples from lines 26 and 27 had detectable

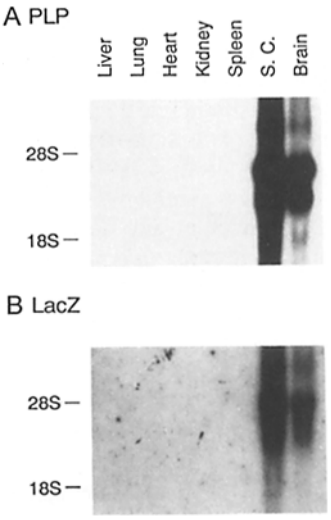

Figure 2. Northern blot analysis of mRNA expression in tissues. Total RNA was isolated from the indicated tissues of a 21-d-old transgenic mouse (line 26H). 20 μg was fractionated by electrophoresis through a denaturing formaldehyde agarose gel (1%) and transferred to a nitrocellulose filter. The blot was probed sequentially for LacZ, PLP, and CHOB transcripts. RNA loading was consistent among lanes, as judged by CHOB mRNA, which is encoded by a constitutively expressed gene (data not shown). Shown are the resulting autoradiograms for (*A*) PLP mRNA; 19-h exposure and (*B*) PLP–LacZ mRNA; 9-d exposure. The PLP–LacZ mRNA is slightly longer than the 3.2-kb mRNA for PLP. *S.C.*, spinal cord.

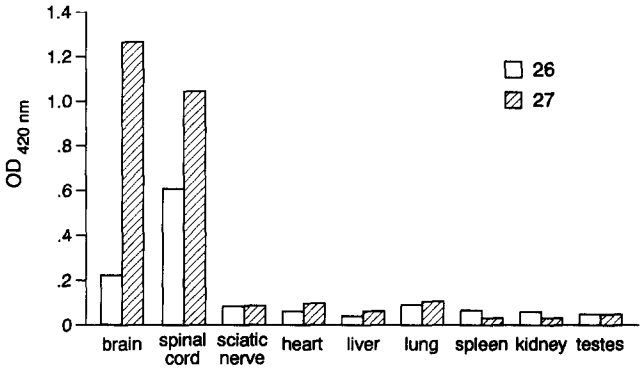

Figure 3. Spatial pattern of β-galactosidase activity. Tissues were isolated from adult transgenic mice (Line 26L and 27). Homogenates were prepared in PBS, and samples were incubated with 3.5 mM ONPG, 0.5% NP-40 in PBS (Lim and Chae, 1989). Samples were 20 μg of brain and spinal cord protein from line 26H, 5 μg of brain and spinal cord protein from line 27 and 100 μg protein from other tissues. Samples were incubated for 2 h and optical density was measured at 420 nm.

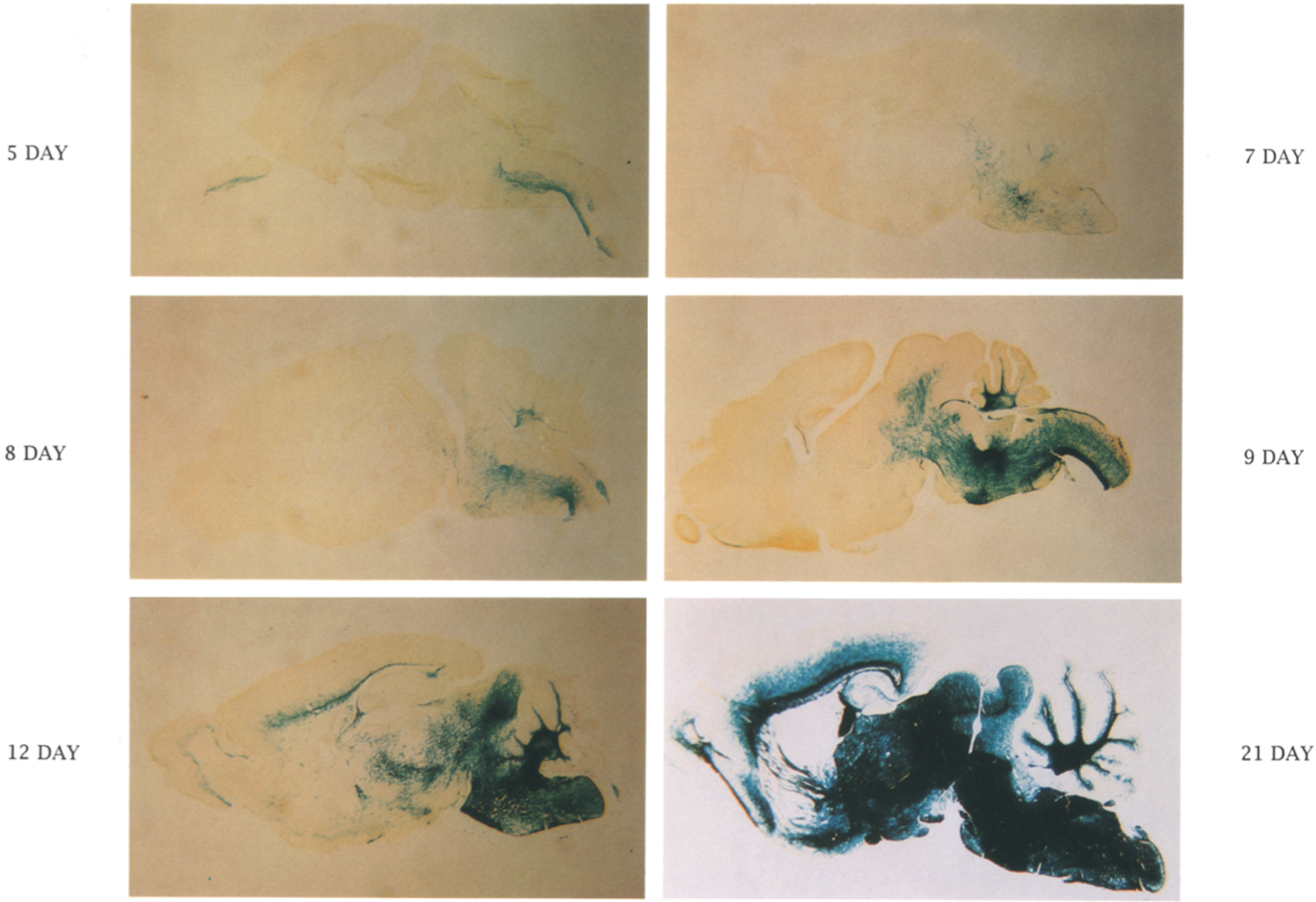

Figure 4. Temporal pattern of β-galactosidase activity in brain. Photomicrographs demonstrating X-gal staining from sagittal sections through transgenic mouse brains (line 26H) at the indicated ages. β-galactosidase activity was evident in the brain at postnatal day 5 and increased significantly up to day 21 generally following a caudal to rostral gradient.

β-galactosidase bands, which were present at significantly lower levels than those from brain or spinal cord; heart samples had no detectable β-galactosidase band (data not shown). No immunoreactive β-galactosidase band was seen in sciatic nerve from line 20, but this was not unexpected since the amount of immunoreactive β-galactosidase in the brain and spinal cord of line 20 mice was extremely low. The inability to detect β-galactosidase activity in sciatic nerve from any transgenic line in our enzyme assay or on Western blots of sciatic nerve from line 20 presumably reflects the extremely low level of the protein that is expressed in this tissue. The selective expression of the transgene in brain, spinal cord, and at low levels in sciatic nerve indicates that the transgene contained the PLP genomic sequences necessary to direct tissue specific expression.

Expression of the PLP-LacZ Transgene Is Developmentally Regulated

Examination of 20–30-μm brain sections stained with X-gal revealed that transgene expression (as measured by β-galactosidase activity) increased with age and that staining appeared to localize to white matter areas within the brain (Fig. 4). Similar developmental patterns were seen for line 27 and both sublines of line 26. While abundant blue staining was apparent in the transgenic animals, no staining was observed in the brains and spinal cords of nontransgenic littermates (data not shown). β-galactosidase activity was evident in the brains of mice (line 26H) at postnatal day 5 and increased significantly up to day 21 generally following a caudal to rostral gradient (Fig. 4). By day 5, transgene expression was detected in the regions of the olfactory tract, hindbrain, and faintly in the midbrain. Stain was first observed in the white matter tracts of the cerebellum at day 7 and rapidly increased, reaching saturating levels by day 21. Enzyme activity was detected in the diencephalon by day 8 and in the corpus callosum by day 12. Thus, transgene expression was regulated temporally and followed a caudal to rostral pattern, consistent with normal myelination. β-galactosidase activity in line 26L, which contained substantially lower copies of the transgene, showed temporal regulation as well, albeit to a slightly lower level than line 26H in younger animals (data not shown).

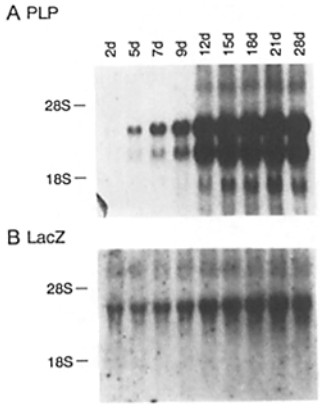

Figure 5. Northern blot analysis of developmental mRNA expression in brain. Total RNA was isolated from brains of transgenic mice (line 26L) at the indicated ages (postnatal days 2 through 28) and processed as described in Fig. 2. The blot was probed sequentially for LacZ, PLP, and CHOB transcripts. RNA loading was consistent among lanes as determined by hybridization with the CHOB probe (data not shown). Shown are the resulting autoradiograms for (*A*) PLP mRNA; 19-h exposure and (*B*) PLP-LacZ mRNA; 4-d exposure.

Northern blot analysis was performed to evaluate the temporal expression of transgenic PLP-LacZ and endogenous PLP mRNAs in the brains of mice from line 26L. Total RNA isolated from the brains of mice ages 2–28 d exhibited a developmental increase in PLP-LacZ mRNA (Fig. 5 *B*). The largest change in PLP-LacZ mRNA levels occurred between 9 and 12 d. Correspondingly, the three major size classes of mRNAs generated from the mouse PLP gene (3.2, 2.4, and 1.6 kb) are regulated developmentally and showed the greatest increase between 9 and 12 d (Fig. 5 *A*; the autoradiogram was overexposed in order to detect the 3.2-kb transcript in 2-d-old brain). The levels of PLP-LacZ mRNA were comparatively less than the PLP mRNAs at all times examined, except perhaps in the 2-d brain sample. Interestingly, the slope of the increase in PLP-LacZ mRNA levels was not as great as that for endogenous PLP transcripts.

The Transgene Is Expressed in Cultured Oligodendrocytes

To assess the cell type responsible for transgene expression, blue-stained regions from the brain were examined under high magnification (100×). Cells demonstrating β-galac-

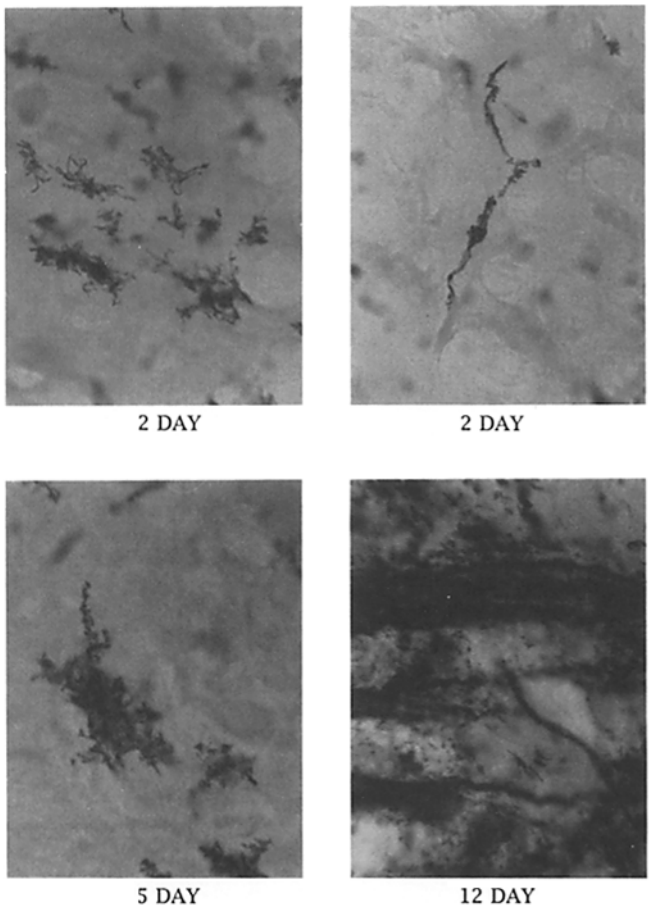

Figure 6. Morphology of transgene expressing cells. High magnification (100 × objective) of Bluo-gal-stained cells from brains of transgenic mice at the indicated ages. Staining showed cells with bipolar and stellate morphologies at the younger ages with a progression to highly arborized cells and white matter tracts staining by postnatal day 12.

Table I. Immunocytochemical Quantification of β-galactosidase (β-Gal) and GC Expressing Cells in Primary Brain Cultures

Days in culture	β-Gal–positive cells	GC-positive cells	β-Gal/GC
			%
9	8	11	72
14	28	34	82
22	66	73	90
28	30	33	90

Mixed primary glial cultures were prepared from neonatal transgenic brains (line 26H). Cells were fixed and double stained for β-galactosidase (β-Gal) and GC immunoreactivity. Three fields (200×) were examined at each time point and quantified for β-Gal and GC immunoreactivity. Shown are the total number of immunoreactive cells from the three fields as well as the percentage of GC-positive cells that were also positive for β-Gal. β-Gal positive cells were always positive for GC immunoreactivity.

tosidase activity were morphologically consistent with oligodendroglial development. Bipolar and stellate cells were observed at the younger ages with a progression to highly arborized cells and white matter tracts staining at the older ages (Fig. 6). To identify these cells positively as oligodendrocytes, primary mixed glial cultures were prepared from neonatal transgenic mouse brains and immunocytochemically evaluated for the oligodendroglial-specific marker, galactosylceramide (GC). When quantified for the number of GC and β-galactosidase immunoreactive cells, it was clear that at all ages studied, β-galactosidase was present only in cells that were also GC positive (Table I). Thus, the transgene was not expressed in astrocytes or microglia in these cultures. In addition, as cultures matured, an increasing percentage of GC-positive cells became β-galactosidase positive, until by 22–28 d in culture, 90% of the GC-positive cells were β-galactosidase positive.

The Transgene Is Expressed in Embryos

To evaluate PLP–LacZ expression during embryonic development, a male heterozygous for the transgene (line 26H) was bred to a nontransgenic female, and 14.5-d embryos were analyzed for β-galactosidase activity. X-gal staining was observed throughout the dorsal root ganglia and in the olfactory bulb in transgenic embryos at embryonic day 14.5 (Fig. 7). Significant staining of most PNS tracts was observed also. In fact, more PNS staining was observed than CNS staining. No staining was evident in nontransgenic littermates.

The Fusion Protein Is Targeted to the Myelin

The subcellular localization of the transgene product was studied by EM and subcellular fractionation. Electron micrographs of Bluo-gal–stained tissue suggested that the enzyme activity was localized in the compact myelin (Macklin et al., 1993). To confirm this, brain homogenates were fractionated and assayed for β-galactosidase activity. Dramatically higher levels of enzyme activity were detected in the myelin fraction prepared from transgenic brains than other tissues (Fig. 8). Thus, the nuclear, synaptosomal, mitochondrial, microsomal, and supernatant fractions from transgenic brains showed very little β-galactosidase activity. The small amount of activity in the synaptosomal fraction

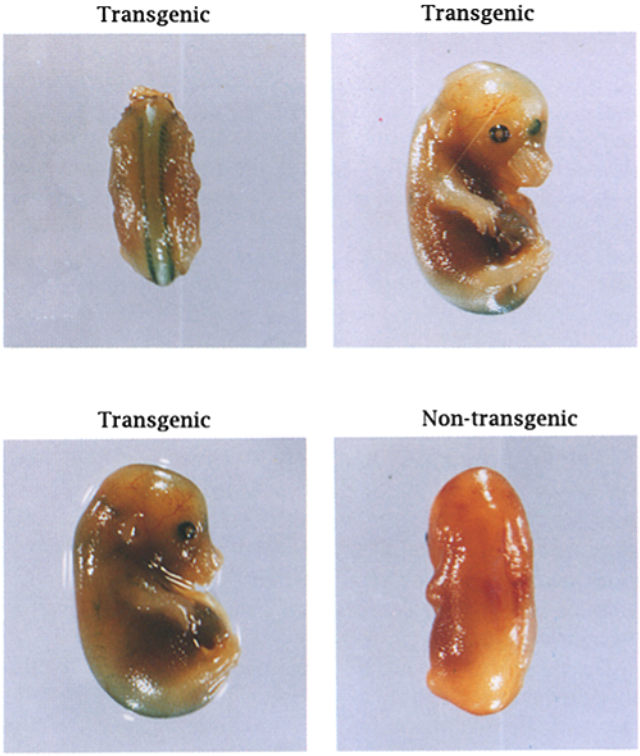

Figure 7. Transgene expression in 14.5-d embryos. Whole fetuses were stained for β-galactosidase activity with X-gal. Staining is evident in the dorsal root ganglia and the olfactory bulb of transgenic embryos but not in nontransgenic littermates.

was probably from myelin contamination, since this fraction contained PLP, as determined by Western blots (data not shown). Essentially no activity was found in fractions obtained from nontransgenic brains. The enrichment of activity in myelin was found for all three transgenic lines, although the level of activity in myelin from line 20 was quite low. These data demonstrate that the first 13 amino acids of PLP were sufficient to target the fusion protein to the myelin, and generally to no other subcellular fraction, including cytosol.

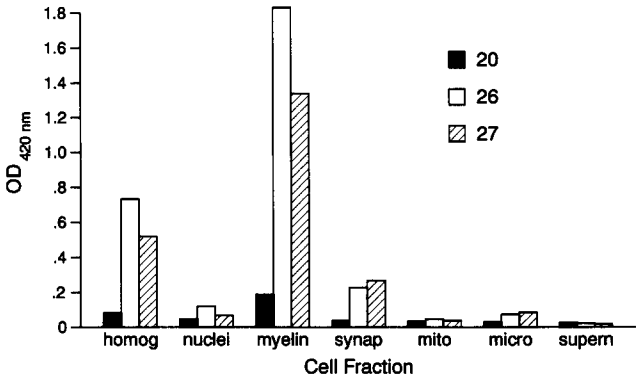

Figure 8. β-galactosidase activity in subcellular brain fractions. β-galactosidase activity was measured by incubating samples in 3.5 mM ONPG for 30 min and measuring absorbance at 420 nm. Samples, lines 26H and 27, 25 μg; line 20, 200 μg.

Discussion

The mechanisms that control oligodendrocyte differentiation in the developing nervous system have a major impact on many aspects of brain development. Oligodendrocytes are among the most abundant cells in the nervous system, and if their differentiation program is disrupted, many areas of the CNS are permanently impaired. These cells have a very well defined differentiation program, which can be investigated extensively in situ and in culture. Because they represent a relatively homogeneous cell population, which expresses a small set of myelin-specific genes, and because their differentiation program can proceed in culture in the absence of other cells and trophic factors, these cells are ideally suited for studies designed to understand lineage and the control of differentiation in the CNS. In fact, they have become an important model for investigating many of these questions. The current studies focus on one of these oligodendrocyte-specific genes; they investigate what regions of the PLP gene are important for tissue-specific expression, and how its expression is controlled in the developing nervous system.

The presence of positive or negative tissue-specific regulatory elements within introns has been demonstrated for a variety of genes including immunoglobulin heavy chain (Banerji et al., 1983; Gillies et al., 1983) and light chain genes (Queen and Baltimore, 1983; Picard and Schaffner, 1984; Pierce et al., 1991), collagen genes (Bornstein et al., 1987; Horton et al., 1987; Burbelo et al., 1991), and genes encoding adenosine deaminase (Aronow et al., 1989), β-globin (Magram et al., 1989), Thy-1 (Vidal et al., 1990), apolipoprotein B (Brooks et al., 1991), glial fibrillary acidic protein (Sarker and Cowan, 1991), p53 (Lozano and Levin, 1991), and PDGF-B (Franklin et al., 1991). We hypothesized that the mouse PLP gene might contain tissue-specific regulatory element(s) within the first intron since (*a*) the transfection results analyzing PLP promoter elements did not define unambiguously a tissue-specific element within the 5′-flanking DNA (Ikenaka et al., 1990; Nave and Lemke, 1991; Berndt et al., 1992; Cook et al., 1992); (*b*) the topology of the PLP gene is highly conserved evolutionarily; and (*c*) almost half of the PLP gene is contained within the first intron. A transgenic approach was used to test this hypothesis. In these studies, the transgene consisted of PLP sequences encompassing 2.4 kb of 5′-flanking DNA, all of exon 1 and intron 1, and the first 37 bp of exon 2 fused in frame to a LacZ expression cassette (Fig. 1 *A*). It should be noted that the presence of introns has been shown to increase the expression of transgenes in mice (Brinster et al., 1988; Choi et al., 1991; Palmiter et al., 1991). Thus, the inclusion of the first intron in our transgene may have been important from a nonspecific and/or technical perspective for transgene expression, and not because of specific enhancers within the intron. While our PLP–LacZ fusion gene did not specifically map tissue-specific regulatory element(s) of the PLP gene, the transgene appears to contain such elements, and we are generating additional transgenic lines with deletions in the first intron to test our hypothesis that such elements reside there.

The fusion gene contained sequence(s) sufficient to permit spatial and temporal regulation of the transgene in a manner similar to endogenous CNS PLP gene expression. PLP–LacZ and endogenous PLP/DM-20 transcripts were detected in the spinal cord and brain of a 21-d-old mouse (line 26H), and not in liver, lung, heart, kidney, nor spleen (Fig. 2). Parallel results were obtained when tissue homogenates were assayed for β-galactosidase activity (Fig. 3). One potential difference exists between expression of the transgene and the normal PLP gene. Recently, DM-20 mRNA has been found in the heart (Campagnoni et al., 1992). We were unable to detect any β-galactosidase activity in heart homogenates prepared from adult transgenic mice (Fig. 3). Perhaps the enzymatic activity was diluted to undetectable levels by nonexpressing constituents in these tissues, or possibly expression is reduced developmentally in the adult. Campagnoni et al. (1992) estimated that the level of PLP/DM-20 mRNA in the heart was ~0.1–0.2% of that in the brain. Conceivably our Northern blot analysis was below detection limits, since total RNA instead of poly(A)$^+$ RNA was used in our studies. However, since we were also unable to detect enzymatic activity or β-galactosidase protein in Western blots of heart samples, it is possible that the PLP genomic sequences necessary for mature myocardial expression are absent from the fusion gene and reside elsewhere in the PLP gene.

The PLP gene is expressed at a low level in Schwann cells (Puckett et al., 1987; Griffiths et al., 1989; Stahl et al., 1990; Agrawal and Agrawal, 1991; Gupta et al., 1991; Pham-Dinh et al., 1991; Ikeneka et al., 1992; Kamholz et al., 1992; Timsit et al., 1992*a*). β-galactosidase was present in sciatic nerves prepared from adult mice in all three transgenic lines. The enzyme activity was below the levels of detection with our enzyme assay, but other techniques demonstrated its presence. It is possible that the protein was undetectable in the enzyme assay because of its extremely low level. The Western blots suggest that the level of the protein in sciatic nerve is 2–5% that in brain. On the other hand, it is also possible that the inability to detect it in the enzyme assays results from lower enzyme stability in sciatic nerve than in brain. This possibility is suggested by the fact that intact sciatic nerve stained well with X-gal, and the length of time needed for the staining was not dramatically longer than that needed for intact spinal cord from the same transgenic line. While X-gal staining is not an accurate quantitative measure of enzyme activity, it is dependent upon the amount of enzyme present, and both spinal cords and sciatic nerves from lines 26 and 27 reacted histochemically much more rapidly than did spinal cords and sciatic nerves from line 20. Thus, it was somewhat surprising that no enzyme activity could be detected in sciatic nerve homogenates in the ONPG enzyme assay, since spinal cord and brain homogenates had measureable enzyme activities in this assay. Perhaps the β-galactosidase enzyme in sciatic nerve is more labile than the CNS β-galactosidase, which could be protected from denaturation by localization to the myelin membrane. It is unclear at present where the β-galactosidase protein is localized in sciatic nerve, but it has been reported that the PLP/DM-20 protein in Schwann cells is not present in PNS myelin, but is in the cell body (Puckett et al., 1987). Thus, the β-galactosidase activity could be more stable in myelin than in the soma.

Transgene expression was quite extensive in embryonic PNS, as well as in discrete regions of the embryonic brain, which received direct PNS input. As shown in Fig. 7, the

dorsal root ganglia and olfactory bulb stained intensively with X-gal in embryos at gestational day 14.5. As well, blue tracts were evident in the limbs, even extending out into the digits of some embryos. This may be correlated to endogenous DM-20 expression since DM-20 mRNA expression precedes that of PLP and appears to predominate in the embryo (Pham-Dinh et al., 1991; Ikenaka et al., 1992; Timsit et al., 1992*a*). What cell type in either the embryonic CNS or PNS is expressing the transgene and what the function of a PLP gene product is in these embryos are intriguing and important questions. Does DM-20 act in embryonic cells of the glial lineage to control some aspect of their differentiation? How does this protein, which appears to function in myelin as a hydrophobic structural protein in a multilamellar membrane, function in embryos, which have no such multilamellar membranes? These questions are being investigated with our transgenic lines.

The PLP–LacZ fusion gene also contained the necessary PLP genomic sequences to direct temporal expression of the transgene. Developmental regulation of transgene expression exists for LacZ mRNA as shown by Northern analysis (Fig. 5). A substantial increase occurred between 9 and 12 d for both PLP–LacZ and endogenous PLP transcripts. The levels of transgene mRNA were less than those for PLP/DM-20 mRNAs at all ages studied. Similar results were obtained with a MBP–LacZ transgene (Gow et al., 1992). Interestingly, the slope of the increase of PLP–LacZ transgene mRNA levels during development was quite different from that of the endogenous PLP mRNA. The PLP–LacZ mRNA increase appeared to be relatively consistent throughout development, while the PLP mRNA increase was more dramatic between 9 and 12 d. In other studies, we have demonstrated that the transcription rate of the PLP gene increases during development by several fold (Macklin et al., 1991). Since PLP mRNA levels rise by orders of magnitude during development, the transcription rate cannot be solely responsible for the increase in mRNA levels, which suggests that another level of PLP gene regulation must exist postranscriptionally. Other studies investigating the mechanisms controlling PLP gene expression have suggested that protein kinase C activity may control some aspect of PLP mRNA stability in differentiating oligodendrocytes (Asotra and Macklin, 1993). This mRNA stability could be controlled by secondary structure within the >2,000 bases of the 3′-noncoding segment of the PLP/DM-20 mRNAs. Since this segment is not present in the PLP–LacZ fusion transcript, transgene mRNAs would not be controlled in the same manner. On the other hand, that region of the PLP mRNA was included in the transgene expressed by Gow et al. (1992), and it did not appear to stabilize LacZ transcripts to any great extent.

The transgene was expressed in primary mixed glial cultures derived from neonatal brains. Immunocytochemical analysis showed the β-galactosidase activity was found only in putative oligodendrocytes, i.e., all β-galactosidase immunoreactive cells were immunoreactive also for GC, a marker for immature as well as mature oligodendrocytes (Table I). These data imply that oligodendrocyte progenitors do not express the transgene, since GC appears only in immature and mature oligodendrocytes, not in progenitors, which express A_2B_5 prior to GC (Raff et al., 1983). However, low levels of transgene expression probably would be below the levels of detection by immunocytochemistry. As cells matured in the mixed culture, and presumably begin to express more PLP, the number of β-galactosidase positive cells increased, as did the percentage of GC positive cells that were β-galactosidase positive. The subpopulation of oligodendrocytes that did not express the PLP–LacZ fusion gene at detectable levels was reduced with time in culture, and in older cells only 10% of GC positive cells did not appear to express β-galactosidase.

Since the immunoreactivity for β-galactosidase colocalized with that for PLP, subcellular localization of the fusion protein was investigated. Intriguingly, the transgene product was localized in the myelin membrane itself as determined by EM and subcellular fractionation of brain homogenates (Fig. 8). Thus it appears that the first 13 amino acids of PLP are sufficient to target the protein to myelin. Whether targeting to the myelin sheath is the result of an active process or a passive event, such as association of the short PLP peptide with the endogenous PLP/DM-20 proteins remains to be determined. It is noteworthy that, although PLP/DM-20 transcripts are translated on bound ribosomes (Colman et al., 1982), the microsomal fraction did not contain any β-galactosidase activity, suggesting that this chimeric protein is localized primarily in the sheath and is not retained in the endoplasmic reticulum. This is intriguing in that when DM-20 cDNA is transfected into HeLa cells, the protein accumulates predominantly in vesicles, presumably ER and Golgi, although some DM-20 protein moves to the plasma membrane (Timsit et al., 1992*b*). Thus, a more complete movement to the myelin membrane is effected with our PLP–LacZ fusion protein in situ, which does not accumulate in vesicles. This suggests that some cell-specific or developmental factors are important for the movement of these proteins within oligodendrocytes.

These data on the localization of the PLP–LacZ fusion protein are extremely important since they imply that a very small segment of this protein is sufficient to target it appropriately. Related data have been presented for cytochrome b5, where the carboxy-terminal 10 amino acids appear sufficient to target the protein to the ER (Mitoma and Ito, 1992), and for growth-associated protein (GAP-43) where the NH_2-terminal 10 amino acids have been reported to target reporter proteins to the growth cone in transfected cells (Zuber et al., 1989), although other reports about transfection of GAP-43 reporter constructs do not agree with the initial observation (Liu et al., 1991). Similarly, the first 12 amino acids (less than half of the presequence) of an imported mitochondrial protein could direct mouse cytosolic dihydrofolate reductase into the yeast mitochondrial matrix (Hurt et al., 1985). It should be noted that not all transgenic products in oligodendrocytes will target to the myelin membrane. For example, Gow et al. (1992) did not find β-galactosidase in the myelin membrane in their MBP–LacZ mice.

A search of the current data bases for sequence homology to the NH_2-terminal 13 amino acids of PLP did not reveal any significant identity to other proteins. However, identification of targeting sequences solely by sequence analysis is often times insufficient. For instance, many random sequences can functionally replace the secretion signal sequence of yeast invertase for targeting to the plasma membrane (Kaiser et al., 1987). Nonetheless, several of the amino acids in the NH_2-terminal portion of PLP warrant further discussion. Myristoylation of NH_2-terminal glycine

residues such as in Rous sarcoma virus pp60src has been shown to be important in targeting proteins (Pellman et al., 1985). Although PLP is acylated and it contains an NH_2-terminal glycine, the NH_2 terminus is not blocked and the predominant fatty acid is palmitic acid (Lees and Bizzozero, 1992). Recently, it has been shown that PLP cysteine residues at positions 5, 6, 9, 108, 138, and 140 are modified by long-chain fatty acids (Weimb and Stoffel, 1992). Thus, acylation of the first three cysteine residues may be important in targeting our fusion protein. On the other hand, PLP acylation has been shown to occur within the myelin membrane itself (Lees and Bizzozero, 1992), suggesting that covalent lipid modifications may not be important for targeting this protein. A di-leucine motif has been implicated in lysosomal targeting of T cell antigen receptor chains (Letourneur and Klausner, 1992), but our results did not indicate targeting of the fusion protein to the lysosome even though leucine residues are found in positions two and three of PLP.

The observation that the NH_2-terminal 13 amino acids of PLP were sufficient to target the fusion protein to the myelin membrane does not preclude the possibility that multiple recognition determinants may exist within the protein. The human low density lipoprotein receptor was recently shown to contain two different determinants that independently mediated sorting to the basolateral plasma membrane in MDCK cells (Matter et al., 1992). Additionally, targeting of multimeric proteins may require specific interactions of the subunits, and some multimeric proteins that fail to assemble correctly are not transported to their proper location. The NH_2-terminal domains of acetylcholine receptor subunits have been shown to contain recognition signals for the initial steps of receptor assembly (Verrall and Hall, 1992). It has been postulated that the PLP aggregates to form channels within the myelin membrane (Lees and Bizzozero, 1992). Thus, targeting of our fusion protein to the myelin membrane could be explained if the NH_2-terminal region of PLP was important in PLP aggregation.

The presence of β-galactosidase, which is a relatively large protein ($\sim$120 kD), in the myelin membrane has had no discernible effect on the function or stability of the myelin in these animals. Heterozygous adult mice (6–9 mo) from several lines and homozygous adult mice from line 26 have no obvious clinical signs of dysmyelination, and electron micrographs of adult tissue indicate no dramatic alteration in the appearance of compact myelin. Nevertheless, the presence of the enzyme in the myelin membrane alters it at some level, since staining of the membrane for enzyme activity disrupts compact myelin extensively (data not shown). Studies are under way to localize the enzyme more discretely within the myelin sheath.

The current studies have demonstrated that PLP–LacZ transgene expression is an excellent measure of PLP gene expression in embryos and postnatal animals, as well as in primary cultured oligodendrocytes. These cells are now tagged with LacZ with no apparent detrimental effect, and they should be ideal for transplantation studies. Further investigations into the consequences of targeting a new protein into the myelin membrane itself may provide important new insights into myelination and dysmyelination.

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