Myelin Basic Protein Gene Contains Separate Enhancers for Oligodendrocyte and Schwann Cell Expression

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Abstract. The DNA sequence between position +36 and −1907 of the murine myelin basic protein gene contains the enhancer and promoter elements necessary for abundant and cell specific expression in transgenic mice. Surprisingly, the pattern of expression promoted by this DNA fragment is a subset of that exhibited by the endogenous myelin basic protein (MBP) gene. Fusion genes prepared with this promoter/enhancer and a Lac Z reporter gene are expressed only in oligodendrocytes and not in Schwann cells, whereas the endogenous MBP gene is expressed in both cell types. The level of transgene expression measured by nuclear run-on experiments is very substantial and rivals that of the endogenous MBP gene. Furthermore, this 1.9-kb DNA fragment directs transcription on the same (or very similar) developmental schedule as the endogenous gene. These results indicate that the MBP promoter/enhancer sequences are at least tripartite: a core promoter, the oligodendrocyte enhancer elements, and a third component that either expands the specificity of the oligodendrocyte enhancer to include Schwann cells or acts independently to specifically stimulate transcription in Schwann cells.

THE myelin ensheathments of the major axons in the central nervous system (CNS)[1] and peripheral nervous system (PNS) are remarkably similar in architecture and composition (26, 40) despite the fact that CNS myelin is elaborated by oligodendrocytes, a cell of neural tube origin (36), while PNS myelin is synthesized by Schwann cells which are of neural crest origin (29). Although some myelin proteins are found only in one or the other myelin, several proteins like myelin basic protein (MBP), proteolipid protein (PLP), and myelin-associated glycoprotein (MAG) are common to both types of cells (1, 31, 37, 54). These myelin proteins are expressed only in the two types of myelinating cells and in no other cell of the body. A common expectation is that each of the genes encoding these proteins is controlled by a set of *cis*-acting elements and *trans*-acting proteins that stimulate transcription in both cell types.

Early studies (24, 25, 43) focused attention on sequences extending 1.3-kb upstream of the MBP transcription start site as being important in MBP gene regulation. The results of Roach et al. (43, 55) showing that the transcription of the truncated MBP gene of the *shiverer* mutant mouse proceeds with the same efficiency and cell-type specificity as that of the wild type gene indicates that these *cis*-acting elements must reside 5′ to the middle of intron 2, the 5′ boundary of the *shiverer* deletion. The gene transfer experiments of Kimura et al. (25) further define the location of the tissue-specific transcription elements by showing that the MBP intronic sequences were not necessary for brain-specific expression. Taken together, these two studies show that the most likely locations of the binding sites for tissue- and cell-specific *trans*-acting factors are exon 1 of the MBP gene and the 1.3-kb sequence lying upstream of the cap site.

Subsequent studies of the sites within this 1.3-kb sequence that are able to bind *trans*-acting proteins have revealed a complex of elements in the 5′ promoter region of the MBP gene that are important in general transcription (3, 11, 34, 48, 49, 50).

A critical evaluation of the specificity, efficiency, and the completeness of the genetic elements lying within the 1.3-kb fragment has not been undertaken as yet and it is not known whether these elements promote expression only in oligodendrocytes and Schwann cells and how efficiently. Indeed, several recent experiments have raised concern that this DNA fragment may not contain the full complement of genetic elements necessary for efficient, cell specific expression. Friedrich et al. (15) created transgenic mice which bore a fusion gene fashioned from the MBP promoter, the vesicular stomatitis virus glycoprotein (VSV G) gene coding region and SV_{40} splice and polyadenylation signals. The animals expressed the VSV G protein in oligodendrocytes but only weakly. Furthermore, the mice exhibited source neuronal dystrophy suggesting that neurons were affected by

This manuscript is communication #113 from the Brookdale Center for Molecular Biology at the Mount Sinai Medical Center.

1. *Abbreviations used in this paper*: CNS, central nervous system; MAG, myelin-associated glycoprotein; MBP, myelin basic protein; MHC-1, class 1 major histocompatibility complex; PLP, proteolipid; PNS, peripheral nervous system; VSV-G, vesicular stomatitis virus glycoprotein.

low level expression of the transgene. It is unclear whether this apparent breakdown in specificity of expression was an inherent property of the MBP promoter/enhancer sequences used, or whether the SV_{40} sequences modified the specificity, a phenomenon previously observed by others (7, 45). In separate studies Yoshioka et al. (56) and Turnley et al. (53) used 1.3 kb of 5′ MBP sequence to promote expression of an appended class 1 major histocompatibility complex antigen (MHC-1). While both studies reported expression of the transgene in oligodendrocytes, they also reported significant expression in testes, seminiferous tubules, intestinal villi, liver, and lung.

In the present communication, we show that a 1.9-kb fragment of the MBP promoter/enhancer sequence lying just 5′ of position +36 contains the necessary *cis*-acting genetic elements for the abundant and cell-specific expression of sequences appended to it. The level of gene expression for the transgene, measured by nuclear run-on assays, rivals that of endogenous myelin basic protein gene. Furthermore, expression of the protein encoded by the transgene follows the same developmental schedule as the endogenous MBP. However, unlike the endogenous gene, the transgene is expressed only in oligodendrocytes and not in Schwann cells. These results indicate that the MBP promoter/enhancer sequences are at least tripartite: the core promoter, the oligodendrocyte enhancer element(s), and a third component that either expands the specificity of the oligodendrocyte enhancer to include Schwann cells or acts independently of the oligodendrocyte enhancer and specifically stimulates transcription in Schwann cells.

Materials and Methods

Creation and Identification of Transgenic Mice

DNA used to generate transgenic mice was prepared from the plasmids illustrated in Fig. 1 by digestion with Not-1 and separation of the resultant fragments by gel electrophoresis. DNA was purified from agarose gels by 2 rounds of adsorption to and elution from Geneclean (Bio 101, La Jolla, CA). The DNA was dissolved in microinjection buffer (10 mM Tris-HCl, pH 7.4, 0.2 mM EDTA), filtered twice through 0.2 μm filters and its concentration adjusted to 5 ng/μl (estimated by ethidium bromide staining).

Transgenic mice were produced in the Mount Sinai School of Medicine Transgenic Core Facility (New York). Microinjection and manipulation procedures were as described by Hogan et al. (21) with only minor modifications. C57Bl/6J × DBA/2J F_1 hybrid mice were used as the source of fertilized eggs which were re-implanted into CD-1 pseudopregnant females. Transgenic founders were identified by polymerase chain reaction (PCR) using synthetic oligonucleotide primers specific for the Lac Z transgene.

Histochemistry and Immunofluorescence

Mice were anesthetized and fixed by vascular perfusion with buffered 4% formaldehyde. For cryotomy, tissue pieces were infiltrated with 20% buffered sucrose, frozen, and stored at −80°C. Sections, 4–30 μm thick, were thaw mounted on gelatin-coated slides and stored at −20°C. Tissue fixed but not frozen was sectioned serially at 50-μm thickness with a Vibratome (Lancer, St. Louis, MO).

For β-galactosidase histochemical stain, cryostat or vibratome sections were incubated at 20° or 37°C for 30 min to 5 d in a solution containing 35 mM $K_3Fe(CN)_6$, 35 mM $K_4Fe(CN)_6$, 2 mM $MgCl_2$, 0.01% sodium deoxycholate, 0.02% NP-40, 0.15 M NaCl, 10 mM Na_2HPO_4, and 0.03–0.1% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) final pH 7.0.

For immunofluorescence, cryostat sections were stained with the following primary antibodies, used singly or in combination: anti-β-galactosidase (rabbit; Cappel Laboratories, Malvern, PA) (mouse; Promega Biotec, Madison, WI), anti-myelin basic protein (rabbit, anti-peptide amino acids 1–20; mouse, monoclonal) (16), anti-2′,3′-cyclic nucleotide phosphohydrolase (rabbit, gift of D. Colman, Columbia University, New York), anti-myelin-associated glycoprotein (rabbit, gift of R. Quarles), anti-P0 (rabbit, gift of D. Colman), anti-glial fibrillary acidic protein (GFAP) (rat, gift of V. Lee, Pennsylvania School of Medicine, Philadelphia, PA), anti-neurofilament (mouse, monoclonal, gift of V. Lee; rabbit, Chemicon, Temecula, CA). These were visualized with fluorescein- or biotin-conjugated, species-specific secondary antibodies (Amersham Corp., Arlington Heights, IL) and rhodamine-conjugated streptavidin (Molecular Probes Inc., Junction City, OR). Preparations were counterstained where appropriate with the nuclear dye DAPI (4′,6′-diamidino-2′-phenylindole) or with *m*-phenylenediamine (39). After staining, coverslips were mounted with buffered glycerol; for preparations using fluorescein, the glycerol contained 2% 1,4-diazabicyclo-(2,2,2)-octane to reduce fading (23).

Determination of Transgene Copy Number

The 3.1-kb coding region of Lac Z labeled with α-[^{32}P]-dCTP (3,000 Ci/mmol) by random-priming (Klenow large fragment, Promega Biotec) was hybridized to duplicate, 5-μg samples of transgenic mouse genomic DNA adsorbed onto nitrocellulose membranes (slot blots) for 24 h at 42°C in 6× SSC (1× SSC = 0.15 M NaCl in 15 mM sodium citrate), containing 50% formamide, 5× Denhardt's solution, 0.6% SDS, 50 mM phosphate (pH 6.9), and 0.2 g/l sonicated salmon sperm DNA. The blots were washed with solutions of increasing stringency up to 0.1× SSC containing 0.5% SDS at 55°C, and then exposed to X-ray film. Known amounts of the Lac Z coding region were included on the membranes to determine the number of copies of the transgene by visual comparison of the autoradiogram film darkenings.

β-Galactosidase Assays

Organs were rapidly dissected from mice and frozen separately on dry ice. Partially thawed samples were homogenized (Tekmar tissuemizer, Tekmar Co., Cincinnati, OH) at 0°C in 1–2 ml of 50 mM Tris buffer, pH 7.3, containing 250 mM sucrose, 150 mM NaCl, 5 mM EDTA, 1 mM DTT, and 0.1 mM leupeptin. Aliquots of each homogenate were stored at −80°C. The protein content of the homogenates was determined in duplicate assays (30). Enzyme activity was measured in duplicate assays using *o*-nitrophenol galactopyranoside (Sigma Chemical Co., St. Louis, MO) as the substrate, in an adaption of the β-galactosidase assay protocol recommended by the vendor (Sigma Chemical Co.). Two or three aliquots from each assay were removed (typically at 5–30-min intervals) and added to tubes containing Na_2CO_3 (0.5 M final) to terminate the reaction; each time-point was rendered optically clear using SDS (0.5% final) and centrifugation. Enzyme specific activity was determined from the average absorbance change at 420 nm per minute over the entire time course, using an extinction co-efficient for *o*-nitrophenol of 4,800 $M^{-1} \cdot cm^{-1}$ (12). Comparison of the enzyme activity in homogenates with that of purified β-galactosidase diluted in brain homogenate enabled determination of the absolute amounts of enzyme in organs from transgenic mice.

Western Blots

Aliquots of brain and spinal cord homogenates were diluted to a protein concentration of 2 mg/ml in 125 mM Tris, pH 6.8, containing 5% sucrose, 5% SDS, 0.5% (vol/vol) β-mercaptoethanol, and a trace of bromophenol blue then heated to 50°C for 10 min. This sample preparation procedure is a slight modification of that of Laemmli (28), which avoids aggregation of the myelin proteolipid and Wolfgram proteins. Purified β-galactosidase, diluted to 1 μg/ml in 5 mg/ml BSA (BSA, fraction V; Boehringer-Mannheim Biochemicals, Indianapolis, IN), was used as a standard; aliquots of this solution were added to wild-type brain homogenates for electrophoresis to ensure the β-galactosidase behaved, in subsequent steps, similarly to that present in transgenic tissue. The samples were electrophoresed in 6.5% SDS-polyacrylamide gels (28) then electroblotted onto Immobilon-P (Millipore, Bedford, MA) for 2 h at 0°C using a 0.25 M Tris–1.92 M glycine transfer buffer (51). The membrane was blocked overnight at 4°C in TBS (25 mM Tris buffer, pH 7.4, 136 mM NaCl, and 36 mM KCl) containing 0.5% (vol/vol) NP-40, 3% BSA, and 10% (vol/vol) normal goat serum (Sigma). Unless otherwise specified, subsequent processing of the western blots was carried out at 20°C in wash buffer (TBS, 0.5% [vol/vol] NP-40, 1% BSA and 1% [vol/vol] normal goat serum). Fractionated rabbit anti-β-galactosidase antiserum (Rockland, Gilbertsville, PA) and affinity-purified goat anti-rabbit IgG antibodies conjugated to alkaline phosphatase (Promega Biotec) were freshly diluted (1:10,000 and 1:7,500, respectively) in

wash buffer and pre-adsorbed to a mixture of non-transgenic mouse brain and spinal core homogenates (each 15 μg/ml) for 30 min then passed through 0.45 μm Millex-HV filters (Millipore Continental Water Systems, Bedford, MA). The blots were incubated in primary antiserum for 60 min, incubated for 45 min in several changes of wash buffer then incubated for 30 min in secondary antibody. After a further 45-min wash period using 0.5% NP-40 in TBS, alkaline phosphatase-antibody complexes on the blots were visualized using the Protoblot detection system (Promega Biotec).

Nuclear Run-on Assays

Nuclei were prepared using the method of Wiktorowicz and Roach (55). Briefly, brains were rapidly removed from decapitated transgenic mice, rinsed in ice-cold PBS and homogenized in 20 mM Tris buffer, pH 6.9, containing 0.75 M sucrose, 0.1% (vol/vol) Triton X-100 (Boehringer-Mannheim Biochemicals), 5 mM β-mercaptoethanol and 1 mM $MgCl_2$ using a Dounce homogenizer (Wheaton, Millville, NJ). 10 strokes of the loose-fitting pestle and 20 strokes of the tight-fitting pestle were used to dissociate the tissue which was then mixed with sucrose cushion buffer (20 mM Tris buffer, pH 6.9, 2.1 M sucrose, 5 mM β-mercaptoethanol and 1 mM $MgCl_2$) and layered over 10 ml of sucrose cushion buffer in a polypropylene ultracentrifuge tube (SW28; Beckman Instruments, Palo Alto, CA). Nuclei were pelleted at 15,000 rpm for 60 min at 4°C, gently resuspended in 3 ml of wash buffer (20 mM Tris buffer, pH 6.9, 20% [vol/vol] glycerol, 140 mM KCl, 10 mM $MgCl_2$, 1 mM $MnCl_2$, and 5 mM β-mercaptoethanol), and then centrifuged for 5 min at 3,000 rpm in a Beckman JS-5.2 rotor.

Transcription reactions were performed according to the method of Chen-Kiang and Lavery (8) with several modifications. Fresh nuclei pellets were resuspended in 1 ml of transcription buffer (wash buffer, pH 7.9, containing 14 mM β-mercaptoethanol, 1 mM of each of ATP, CTP, GTP, and S-adenosylmethionine, and 20 mg/ml tRNA), centrifuged for 3 min at 3,000 rpm and then resuspended in 190 μl of transcription buffer. After adding 0.5 mCi of α-[^{32}P]-UTP (800 Ci/mmol; Du Pont, Wilmington, DE) dissolved in 10 μl of transcription buffer, the nuclei were incubated at 0°C for 10 min. The transcription assay was carried out for 15 min at 30°C. The reaction mixture was centrifuged for 10 s to separate the nuclei and the supernatant fluid removed. The nuclei were then lysed by the addition of 300 μl of 10 mM Tris buffer, pH 7.4, containing 0.5 M NaCl, 50 mM $MgCl_2$, 2 mM $CaCl_2$, 300 U of RNase-free DNase I (Boehringer-Mannheim Biochemicals) and 40 U of RNAsin (Promega Biotec). Proteinase K (Sigma Chemical Co.), sodium sarcosinate (SLS), and EDTA (final 0.3 mg/ml, 0.5% and 5 mM, respectively) were added to the lysate and the mixture incubated for 30 min at 37°C. An equal volume of 10 mM Tris buffer, pH 7.0, containing 6 M guanidinium isothiocyanate, 1% SDS, and 0.3 M β-mercaptoethanol was added, the solution was then layered over a 1.2-ml cushion of 5.7 M CsCl and centrifuged overnight at 45,000 rpm using a Beckman TLS-55 rotor (9). The RNA pellet was dissolved in 10 mM Tris buffer, pH 7.4, containing 1 mM EDTA, and precipitated in ethanol.

Hybridizations

Deoxyribonucleic acid probes used for detecting ^{32}P-labeled nascent transcripts from nuclear run-on assays were as follows: MBP, 4 kb of mouse genomic clone (10) encompassing exon 2 and the 5′ end of intron 2 (KpnI to BamHI fragment); Lac Z, the entire 3.1-kb coding region (20); non-specific binding, pBluescript II SK+. The appropriate portions of the MBP and Lac Z gene were sub-cloned into pBluescript II SK+ (Stratagene, La Jolla, CA). Bacteria (XL-1 blue; Stratagene) transformed with these phagemids were grown in the presence of VCSM13 helper phage (Stratagene) and antisense single-stranded DNA was purified from the culture supernates by polyethylene glycol-ammonium acetate precipitation and phenol-chloroform extractions as recommended by the vendor. Alkali-denatured (2 M NaOH–3 M NaCl incubated at 25°C for 25 min) single-stranded probes were slot-blotted onto nitrocellulose filters (0.2 μg DNA/slot). In some experiments linearized, heat denatured double-stranded DNA was used instead of single-stranded antisense DNA. Blots were pre-hybridized for 2 h in 6× SSC, 5× Denhardt's solution, 0.6% SDS, 50 mM phosphate, and 0.2 g/L sonicated salmon sperm DNA at 65°C. Hybridization solutions containing the run-on transcripts were hybridized successively to three slot-blots of the target DNA sequences; each cycle of hybridization was 48 h. Blots were washed and then incubated for 20 min at 20°C in 2× SSC containing 0.2% SDS and 10 μg/ml of RNase A followed by an incubation of 20 min at 20°C in 2× SSC containing 0.2% SDS and 100 μg/ml of proteinase K. Finally, the blots were washed for 10 min at 20°C in 2× SSC containing 0.2% SDS and exposed to X-ray film.

Ribonuclease Protection Assays

Samples of total RNA purified from brain, spinal cord, or peripheral nerve (9) were hybridized for at least 12 h at 45°C in 0.2 M Pipes buffer, pH 6.4, containing 80% formamide, 2 M NaCl, and 5 mM EDTA with labeled antisense RNA transcripts specific for either the transgene or the endogenous MBP gene (33). Single-stranded RNA was digested at 30°C for 60 min with ribonucleases A and T1 (final concentrations: 40 μg/ml and 10,000 U/ml, respectively). After proteinase K digestion (30°C for 60 min) samples were electrophoresed on nondenaturing, 6% acrylamide gels, dried, and exposed to X-ray film.

Quantification of Autoradiograms and Western Blots

Autoradiograms from nuclear run-on and ribonuclease protection assays were digitized using a Datacopy GS plus flat-bed scanner (Xerox) and analyzed on an Apple MacIntosh computer using the software program, NIH Image, version 1.43 (written by Wayne Rasband). Several exposures of each autoradiogram were used to ensure signal intensities lay within the linear response ranges of the film. Photographs of western blots were treated similarly.

Results

The MBP Gene Promoter/Enhancer Elements and the Fusion Genes, MβP, and MβG

Earlier work by us (15) and others (3, 11, 34, 48, 49, 50) indicate that important *cis*-acting genetic elements lie upstream of the first exon of the murine MBP gene. This region of DNA was shown to be capable of promoting transcription both in vitro and in vivo in transgenic mice. Although the results obtained with transgenic mice showed that the fusion gene was capable of expression in oligodendrocytes, the cellular specificity was incompletely documented and little information is available about expression in other cells of the nervous system or elsewhere in the body. To study the cell specificity of expression promoted by the 5′ upstream region of the murine MBP gene (10), we constructed two expression vectors, each using different sequences for splice sites and polyadenylation signals. The MBP portion of these vectors is a DNA fragment that extends from position +36 to position −1907 of the MBP gene. This sequence was ligated to a polylinker containing HindIII, EcoR1, and BamH1 sites. The splice and polyadenylation signals were supplied by either a portion of the PLP gene (exon 6, intron 6, and exon 7) or the human β-globin genes (exon 2, intron 2, and exon 3). These splice and polyadenylation signals were cloned into a plasmid containing the MBP promoter/enhancer sequences leaving a unique BamH1 site into which cDNAs could be inserted. For convenience, synthetic oligonucleotides containing Not-1 sites were inserted at both sides of the entire assembly (Fig. 1). For the purpose of this study, we chose the truncated *Escherichia coli* Lac Z gene (20) as a reporter gene because β-galactosidase is functionally neutral in many eukaryocytes (14) and the assay for its activity is very sensitive.

The fusion genes were excised from the plasmid, purified and used to prepare transgenic mice. Five founder mice were created with MβP and two with MβG. Sections of brain, spinal cord, and peripheral nerve from all lines were examined histochemically with the chromogenic substrate, X-Gal. Of the MβP lines, four exhibited a pattern of β-galactosidase staining that was consistent with oligodendrocyte-specific expression. One of these showed, in addition, a small amount of punctate staining of the CNS. The fifth MβP line

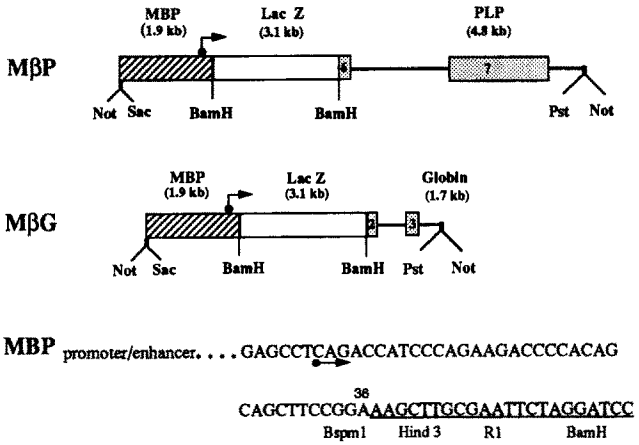

Figure 1. Diagram of MβP and MβG transgenes. A restriction fragment extending from a Sac-1 site (−1907) to a BspM1 site (+36) of the MBP promoter/enhancer was ligated to a polycloning site (underlined sequence) and subcloned into a derivative of pUC19 containing splice and polyadenylation signals supplied by either a Pst-1 fragment of PLP gene containing part of exon 6, intron 6, and exon 7, or a BamH1–Pst-1 fragment of murine β-globin gene. The Lac Z gene used as a reporter is a truncated version described by (20). Not-1 sites were inserted at either end of the constructs for easy excision of the transgene. The sequence shows the position of transcription initiation with a horizontal arrow; the superscript 36 indicates position +36 of the MBP gene and the end of the MBP promoter/enhancer sequence used here.

did not express detectable amounts of the Lac Z gene. Of the two MβG lines, one expressed the transgene in an oligodendrocyte-specific manner that was indistinguishable from the MβP mice. The second did not express the transgene at levels detectable by histochemistry.

Cell and Tissue Specificity of β-Galactosidase Accumulated in MβP3 Transgenic Mice

Preliminary experiments in which organs and tissues of heterozygous MβP transgenic mice were incubated in solutions of X-Gal revealed a striking specificity of transgene expression. Only neural tissue and, specifically, the CNS component, exhibited appreciable β-galactosidase activity. Notably, the PNS was devoid of detectable transgene expression. This specificity is illustrated in Fig. 3 *A* which shows intense staining of the section of spinal cord but no detectable staining in the attached peripheral nerve roots. The specificity in expression of β-galactosidase was further explored and quantified by spectrophotometric assays of β-galactosidase activity of cell free extracts prepared from various tissues of transgenic and non-transgenic litter-mate mice (Table I). As shown in Table I, only the CNS tissue of the MβP 3 and MβP 5 mice contained β-galactosidase activity that was demonstrably higher than the comparable tissue of the non-transgenic litter mates. Consistent with the results of the gross staining of pieces of spinal cord and dorsal root ganglia, only the CNS tissue expressed the transgenic β-galactosidase gene. Results qualitatively identical to these were obtained with MβG mice, showing that the PLP gene sequences used for splice/polyadenylation signals of the MβP mice did not appreciably contribute to the specificity of expression. Thus this specificity is conferred by the MBP promoter/enhancer sequences alone.

Table I. β-Galactosidase Levels in Transgenic and Wild-Type Tissues

	MβP3	MβP5	Wt
	(μg/gm wet weight tissue)		
Brain	2.78	3.66	0.03
Spinal cord	0.29	0.51	0.00
Sciatic nerve	0.00	0.01	0.00
Thymus	0.00	0.01	0.01
Lung	0.00	0.02	0.01
Heart	0.00	0.01	0.01
Liver	0.11	0.06	0.06
Spleen	0.02	0.05	0.01
Adrenals	0.00	–	0.00
Kidneys	0.08	0.13	0.11
Gonads	–	0.01	–
Skeletal muscle	0.00	0.00	0.01
Adipose	0.01	0.03	–

To map the regional distribution and cellular localization of β-galactosidase activity, we prepared sets of serial sections through complete sagittal half brains stained alternately for myelin and for β-galactosidase. The β-galactosidase histochemical reaction yielded very strong staining of MβP3 specimens, with reactivity concentrated in white matter throughout the brain and the major white matter tracts such as the callosal radiations, anterior commissure, fornix, and internal capsule clearly delineated (Fig. 2 *A*). This pattern was nearly identical to that obtained with immunoperoxidase staining for myelin basic protein (Fig. 2 *B*). Sections from control nontransgenic mice, stained for β-galactosidase, showed no reaction product and were nearly invisible (Fig. 2 *C*).

Strong β-galactosidase staining was also found in white matter throughout the transgenic spinal cord. By contrast, PNS, including spinal roots and trigeminal nerve and ganglion, was consistently negative for β-galactosidase activity in MβP3 mice (Fig. 3 *A*). High magnification revealed two components of β-galactosidase staining in MβP3 mice. First, both major white matter tracts and small bundles of myelinated axons were filled with and clearly delineated by diffuse staining of moderate intensity (Fig. 3 *B*). Where myelinated axons are sparse, as in the cerebellar granular layer or in parts of the cerebral cortex, individual stained axons could be distinguished (Fig. 3 *D*). Second, small cell bodies were strongly stained. These cells, 8–12 μm in diameter and filled to opacity with reaction product, were found throughout the CNS, at high density in white matter and at lower density in gray matter. In favorable sections one could follow stained processes connecting those stained cell bodies with bundles of myelinated axons (Fig. 3 *B*), a pattern characteristic of Golgi-impregnated oligodendrocytes (42).

Immunofluorescence with antibodies against the β-galactosidase yielded images nearly identical in pattern to those obtained with the histochemical reaction. White matter tracts exhibited uniform, weak immunofluorescence and contained intensely labeled small cell bodies whose perikarya appeared filled with antigen (Fig. 3 *C*). Simultaneous staining with the nuclear dye DAPI revealed many cells in white matter, and a majority in gray matter, which were not immunostained. Gray matter in different regions contained

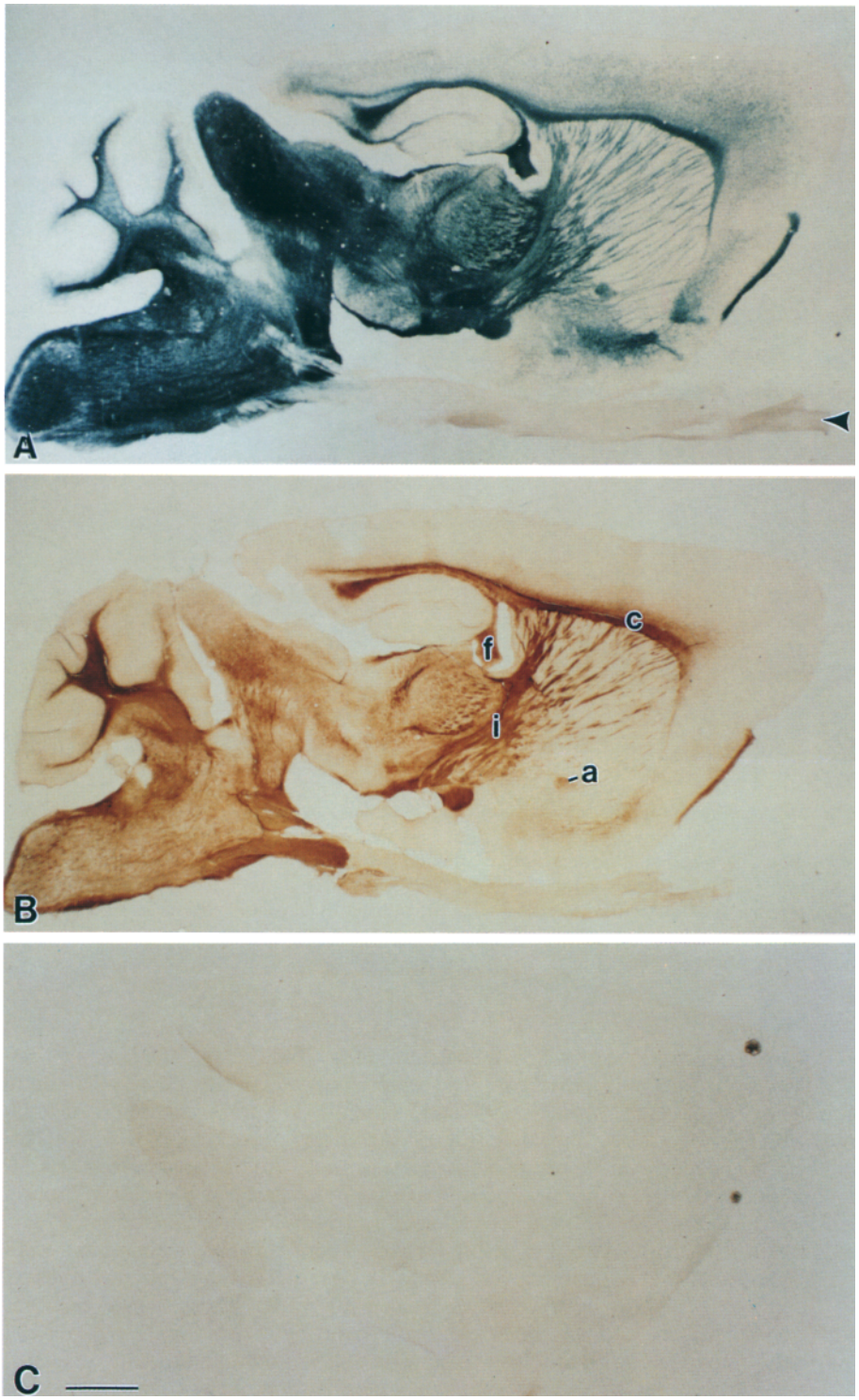

Figure 2. Bacterial β-galactosidase activity is concentrated in white matter of MβP3 transgenic mice. (*A*) Sagittal vibratome section from brain of transgenic MβP3 mouse, stained with X-gal for bacterial β-galactosidase activity. Major white matter structures are clearly delineated by the histochemical reaction. The trigeminal nerve and ganglion (*arrowhead* in *A*) are not stained. (*B*) Nearby section from the same specimen stained by immunoperoxidase for myelin basic protein, for comparison with *A*. (*a*) anterior commissure; (*c*) callosal radiations; (*f*) fornix; (*i*) internal capsule. (*C*) Vibratome section from control (nontransgenic) mouse brain incubated with X-gal, showing lack of staining throughout the brain. Bar, 1 mm.

immunofluorescent cell bodies in proportion to the local density of myelinated axons.

To positively identify the cells expressing the transgenic protein, we prepared cryostat sections of spinal cord and brain for simultaneous two color immunofluorescence, recording β-galactosidase in one channel and one of several cell-specific marker proteins in the second channel. The β-galactosidase immunoreactive cells and their processes showed no immunoreactivity for neurofilament protein (a neuron-specific marker) or for glial fibrillary acidic protein (GFAP; an astrocyte marker). CNP (2′,3′-cyclic nucleotide phosphohydrolase) is a myelin-specific protein; in CNS, it is found only in oligodendrocytes, where it is concentrated at the plasmalemma (6, 18, 52). In cryostat sections CNP immunostaining yielded bright rings of fluorescence at the plasmalemmas of oligodendrocytes, a pattern we previously

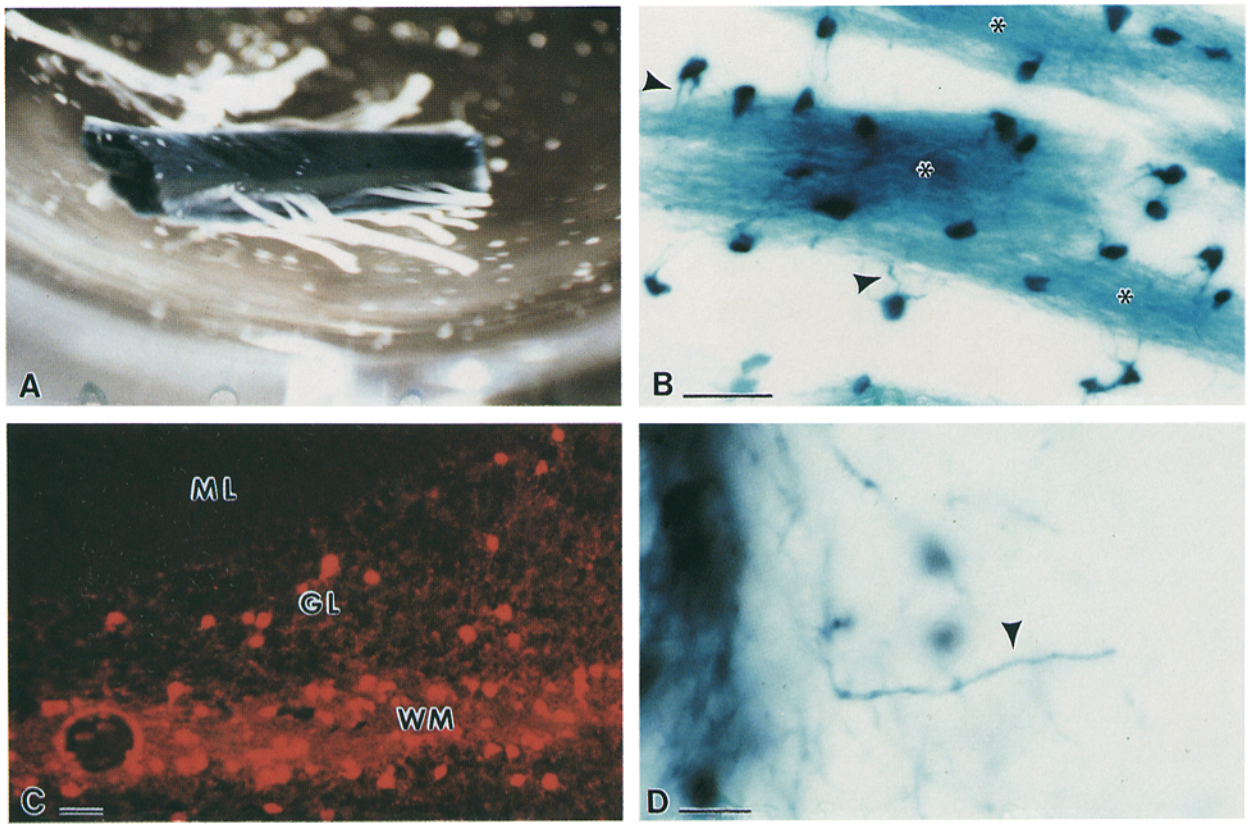

Figure 3. Localization of transgenic protein in MβP3 mice. (*A*) Spinal cord with attached spinal roots and nerves, incubated in X-gal. Spinal cord is stained but the peripheral nerves are not. (*B*) Vibratome section from caudoputamen, incubated with X-gal. Broad stained bands (*asterisks*) are bundles of myelinated axons. Oligodendrocyte cell bodies are stained, as are fine processes (*arrowheads*), connecting the oligodendrocyte cell bodies with myelin sheaths. (*C*) Cerebellar cortex, immunostained with polyclonal rabbit anti-β-galactosidase. Oligodendrocyte cell bodies, found in granular layer (*GL*) and subcortical white matter (*WM*), are strongly immunoreactive. In addition, the subcortical white matter shows diffuse fluorescence. The molecular layer (*ML*), which is virtually devoid of myelin in mouse, shows no immunofluorescence. (*D*) Granular layer of cerebellar cortex, stained with X-gal. A single stained myelinated axon (*arrowhead*) can be followed as it courses through the granular layer. Abbreviations as in *C*. Bars: (*B* and *C*) 40 μm; (*D*) 20 μm.

reported in semithin frozen sections (18). By contrast, β-galactosidase immunofluorescence filled the perikaryal cytoplasm of the same cells (Fig. 4). More than 95% of cell bodies positive for β-galactosidase, were also clearly positive for CNP and were therefore identified as oligodendrocytes; the remainder presented equivocal images. In similar preparations, immunofluorescence for neurofilament or GFAP antibodies never showed coincidence with that for β-galactosidase, indicating that the enzyme is not present in neurons or astrocytes of MβP3 mice.

These data show clearly that the MβP transgene is expressed strongly in oligodendrocytes of MβP3 mice. The perikarya and processes of these cells are diffusely filled with β-galactosidase, detected both histochemically and by immunofluorescence with two different antibodies. By contrast, expression in neurons and astrocytes occurs at levels below detection with our techniques.

Temporal Control of Transgene Expression During CNS Development

Myelin is almost completely absent from newborn mouse brain; its formation begins shortly after birth in a tract-specific sequence and reaches a peak of intensity at the end of the third postnatal week. To determine the temporal pattern of transgene expression, we stained sections of forebrain from 7-, 14-, and 21-d old MβP3 transgenic mice for β-galactosidase (Fig. 5). At 7 days postnatal (dpn) (Fig. 5 *A*), little reaction product was produced. Sections were nearly invisible at low magnification, although high magnification showed some stained cell bodies concentrated in areas destined to become white matter. A marked increase in overall staining was seen at 14 dpn, and major white matter tracts were clearly delineated (Fig. 5 *B*). These showed a further increase in staining intensity at 21 dpn (Fig. 5 *C*); staining at this age was however still weaker than in adult specimens. This pattern is consistent with the known accumulation of myelin in developing mouse brain (22, 47).

Typically, myelination is preceded by intense gliogenesis (44), and early formed oligodendrocytes differentiate and become immunoreactive for MBP even before myelin sheaths appear (46). In spinal cord and medulla of newborn MβP3 mice we found little myelin, but many cells were marked by X-gal staining for β-galactosidase. Differentiating oligodendrocytes exhibit a regular temporal progression in gene activation; among myelin specific proteins CNP is expressed early and MBP later. In two-color immunofluorescence preparations, we found CNP in all cells which were

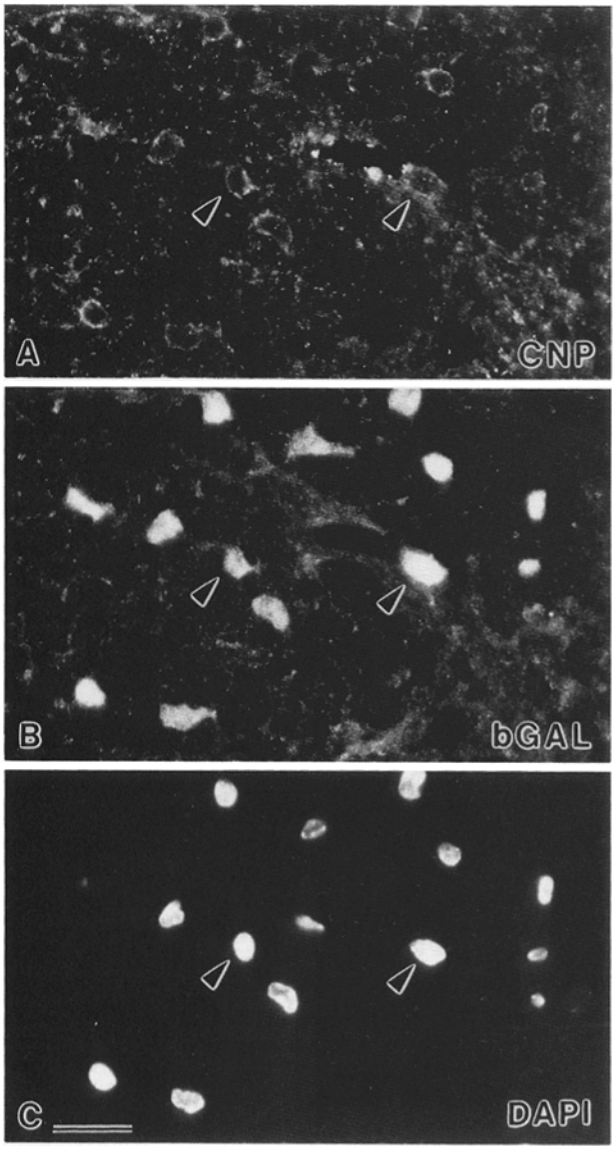

Figure 4. β-galactosidase is expressed in cells containing the oligodendrocyte-specific protein 2',3'-cyclic nucleotide phosphohydrolase (*CNP*). A cryostat section from spinal cord of an MβP3 transgenic mouse was stained by two-color double immunofluorescence for CNP (antiserum raised in rabbit) and for β-galactosidase (mAb) and counterstained with the nuclear dye DAPI. The identical field is shown in the three panels, viewed with fluorescein (*A*), rhodamine (*B*), and DAPI (*C*) fluorescence. 13 doubly immunofluorescent perikarya are illustrated; two are marked with arrowheads. Bar, 20 μm.

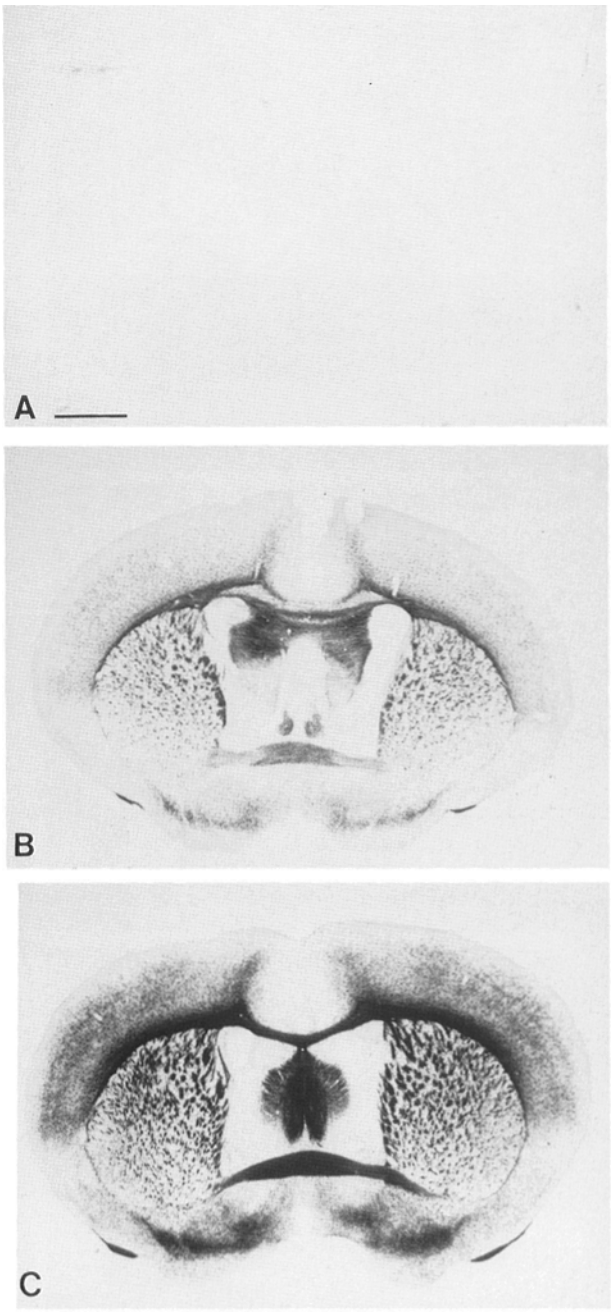

Figure 5. Expression of MβP transgene during postnatal development. Coronal vibratome sections were cut at the level of the anterior commissure of brains from MβP3 transgenic mice 7 (*A*), 14 (*B*), and 21 (*C*) days after birth and incubated with X-gal. Staining was negligible at 7 d and increased progressively thereafter. Bar, 1 mm.

immunofluorescent for β-galactosidase, indicating that early expression of the transgene is limited to oligodendrocytes. However, many CNP-positive perikarya lacked detectable β-galactosidase, suggesting that the MβP transgene, like the endogenous MBP gene, is activated after the CNP gene. Comparison of MBP and β-galactosidase immunofluorescence yielded a different pattern: almost all cells positive for either antigen were also positive for the other (Fig. 6). Thus, the endogenous MBP gene and the MβP transgene may be activated simultaneously within each maturing cell.

Variation of Expression

The localization of β-galactosidase was examined by histochemistry in all five MβP lines produced and both MβG lines. All five of the lines which expressed detectable enzyme showed striking localization of reaction product to CNS white matter (Fig. 7), and all showed strongly stained oligodendrocyte cell bodies and processes and weakly stained myelin internodes when examined at high magnifica-

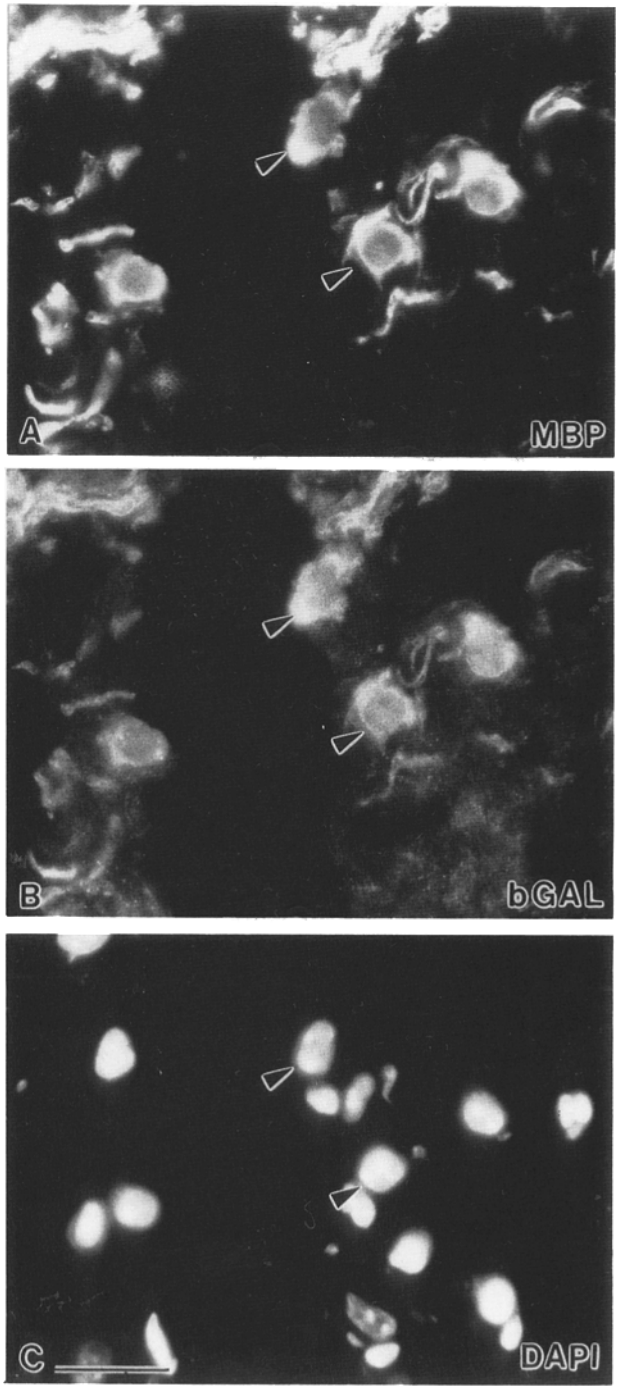

Figure 6. Coincidence of myelin basic protein (*MBP*) and β-galactosidase (*βGAL*) immunoreactivity in immature oligodendrocytes. A cryostat section from the spinal cord of a postnatal day 1, MβP3 transgenic mouse was stained by two-color double immunofluorescence and counterstained with the nuclear dye DAPI. The identical field is shown in the three panels, viewed with rhodamine (*A*), fluorescein (*B*), and DAPI (*C*) fluorescence. Immunoreactive cell bodies are indicated by arrowheads. Bar, 20 μm.

tion. However, the different lines showed some variation on this theme. Variation in the expression of transgenes in different lines of mice carrying the same transgene are frequently observed (5) and thought to arise because the transgenes of each line are integrated into a different site on the host chromosome and thus differently influenced by the surrounding endogenous sequences. Alternatively, the transgene of some lines may have undergone rearrangement and modification before insertion into the host chromosome.

In addition to diffuse, strong staining of oligodendrocytes, MβP5 also showed fine granular staining in some areas of CNS, including cerebral cortex and hippocampus. A second variation is exhibited by the MβP30 line: while expression is limited to oligodendrocytes, the histochemical reaction colored white matter unevenly (Fig. 7), suggesting that expression is strong in some oligodendrocytes and weak or absent in others. The MβG46 line showed strong expression of the transgene in oligodendrocytes, with a very small amount of granular staining of the type seen in MβP5.

Of the five β-galactosidase expressing lines examined, four showed absolute suppression of transgene expression in peripheral nerve. The fifth, MβP30, followed this pattern but did exhibit very weak and irregular staining of trigeminal nerve, in regions close to the CNS–PNS boundary.

The MβP Transgene Is Efficiently Transcribed

The steady state level of MBP mRNA in brain suggests that the MBP promoter may be one of the strongest in the CNS. It is not known whether this strength is an intrinsic property of the core promoter or whether distal elements, like domain control regions, or chromosomal position also play important roles. The observation that the transgenic MβP promoter contain the genetic elements conferring strict oligodendrocyte specificity and the appropriate temporal control of expression prompted us to compare the relative strengths of the transgene and endogenous gene promoters by a variety of techniques.

The levels of β-galactosidase activity found in transgenic brain and spinal cord (3 and 0.5 μg/gm wet weight tissue; Table I) are very small when compared to the abundance of MBP in these tissues (~1 mg/gm wet weight). This discrepancy suggested that much of the transgene product may turn over rapidly or be otherwise enzymatically inactive. However, Western blot analyses of transgenic tissue did not support this possibility as shown in Fig. 8. Only a single protein band of 116 kD was revealed by immuno probing of the Western blot. Comparison of the intensity of staining of these bands to those obtained with the graded amounts of highly purified β-galactosidase verified the levels determined by direct enzyme assay shown in Table I. Comparison of these numbers to the level of MBP in brain (1 mg of MBP/gm wet weight) indicates that β-galactosidase accumulation is $\sim 10^{-3}$ that of MBP in transgenic brain. However, when transgene expression was estimated by Northern blot rather than Western blot a much higher level relative to that of MBP was indicated. Visual comparison of Northern blot autoradiograms suggested that the steady-state ratio of β-galactosidase to MBP mRNA was about 10^{-2} (data not shown). This ratio was corroborated by RNase protection assays shown in Fig. 9. In that figure the MBP signal obtained with 0.3 μg of total brain RNA is comparable to the β-galactosidase signal obtained with 30 μg of brain RNA after allowing for differences in probe size, composition, and specific activity. Thus the ratio of transgene expression to endogenous MBP gene expression was either 10^{-3} or 10^{-2} depending on whether accumulated protein or mRNA was used as the basis for estimating the level of gene activity. This 10-fold difference might be traced to the differences in

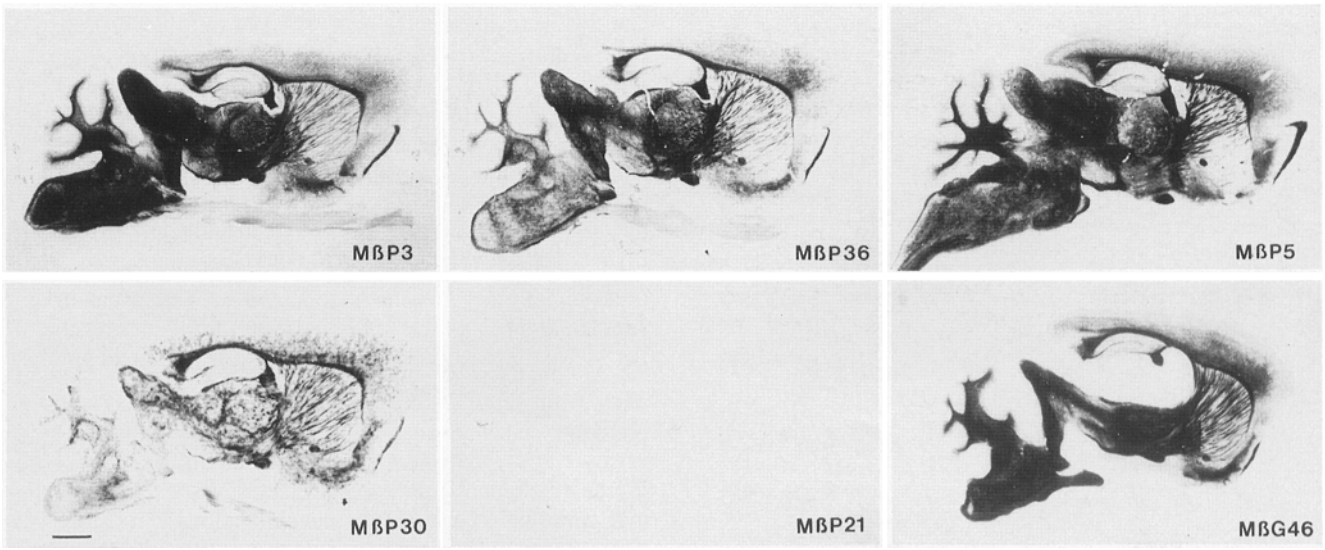

Figure 7. Comparison of β-galactosidase activity in brain of different MβP and MβG transgenic lines. Sagittal, 50-μm thick vibratome sections from brains of adult mice were reacted with X-gal. Bar, 1 mm.

preferred codon usage between eukaryotes and prokaryotes. Alternatively the Lac Z gene used in the transgene is a truncated form (20) that initiates protein synthesis at the second ATG of the wild type mRNA and that ATG is not in the preferred context (27). Thus the level of protein synthesis sustained by the β-galactosidase mRNA may be disproportionately low.

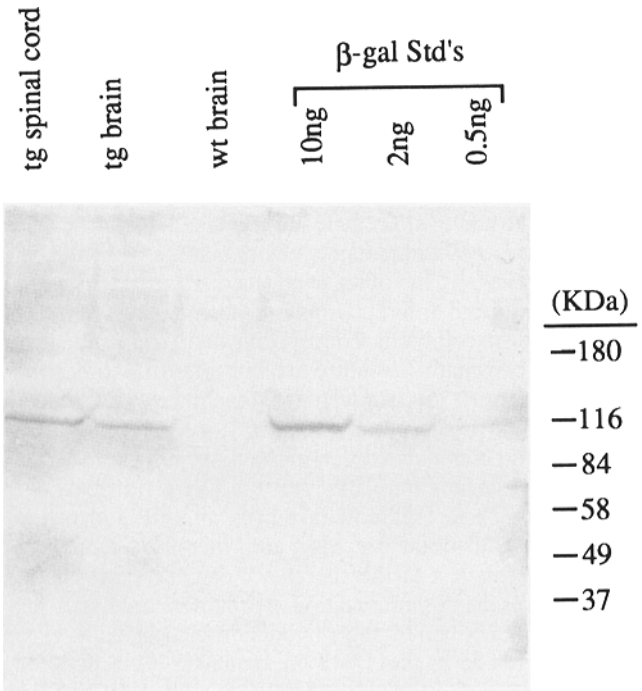

Figure 8. Western blot analysis of brain and spinal cord homogenates of MβP3. Samples of MβP3 brain and spinal cord homogenates and known amounts of purified β-galactosidase (added to wild-type mouse brain homogenate) were electrophoresed in 6.5% SDS–polyacrylamide gels. The separated protein bands were transferred to PVDF membranes and probed with polyclonal rabbit anti-β-galactosidase as described in Materials and Methods. Lane *1*, 120 μg protein from MβP3 spinal cord; lane *2*, 80 μg protein from MβP3 brain; lanes *3–6*, 80 μg protein from wild-type mouse brain; lanes *4–6*, *10*, 2, and 0.5 ng purified β-galactosidase, respectively.

The relative abundance of the mature mRNA or protein encoded by it are poor gauges of the relative promoter efficiency since these levels can be modulated by posttranscriptional and posttranslational events. Thus, although the ratio of β-galactosidase to MBP is $\sim 10^{-3}$, and the ratio of their mRNA is $\sim 10^{-2}$, the ratio of the promoter strengths may be much larger. To better estimate the relative frequency of transcription initiation of the genes we have performed nuclear run-on assays which directly compare the relative amounts of nascent transcripts of the two genes in the same transcription reaction mixture. The results of such experiments are shown in Fig. 10. Quantitative estimates of the relative amounts of nascent transcripts were obtained by adjusting densimetric measurements for the differences in base composition and the lengths of the unlabeled hybridization probes. These results indicate that the levels of transgene transcription in MβP3 and MβG 46 are 20–30% and 30–40%, respectively, of the endogenous MBP gene. These numbers are approximations since they do not take into account differences in gene dosage. On the one hand, the transgenic mice are heterozygous for the transgene but homozygous for the MBP gene. On the other hand, there are approximately 5–10 copies of the transgene present in the chromosome and we do not know how many of them are transcriptionally active.

MβP3 Transgene Transcripts Are Not Detected in the PNS

The surprising absence of β-galactosidase in peripheral nerve of MβP3 transgenic mice suggests that either the protein is not synthesized in this tissue, is inactive, or is rapidly degraded. We examined whether the peripheral nerve had detectable levels of β-galactosidase mRNA and, therefore, was capable of synthesizing the enzyme. In preliminary experiments, we could not detect β-galactosidase mRNA in Northern blot analyses of RNA isolated from peripheral

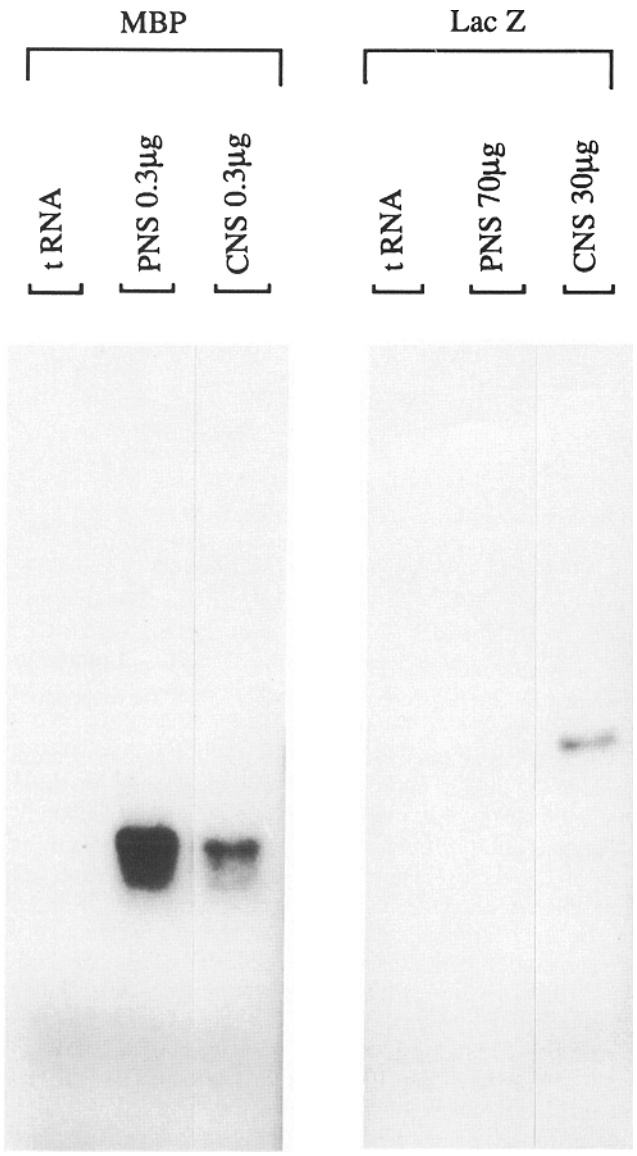

Figure 9. Analyses of PNS and CNS RNA by ribonuclease protection assays. The indicated amount of CNS RNA, PNS RNA or tRNA were hybridized to ^{32}P-labeled antisense probes specific for MBP or Lac Z, treated with ribonuclease, and electrophoresed in a non-denaturing 6% polyacrylamide gel. An autoradiogram of relevant portions of the dried gel are shown.

nerve under conditions that easily detected it in brain RNA (data not shown). We pursued these studies with RNase protection analyses since these have a much greater sensitivity. These analyses (Fig. 9) showed that we were unable to detect any β-galactosidase mRNA in 70 μg of total RNA prepared from sciatic and trigeminal nerves under conditions that easily detected MBP transcripts in 0.3 μg RNA. On the other hand, β-galactosidase mRNA was easily detected in 30 μg CNS RNA. Two conclusions can be drawn from these RNase protection experiments. First, the steady state level of β-galactosidase mRNA in the CNS is ~1% that of MBP mRNA, and second, the level of β-galactosidase mRNA in PNS is not detectable by our methods.

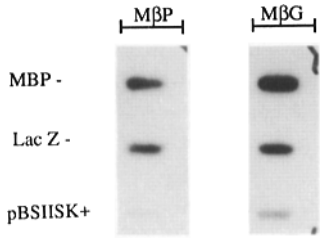

Figure 10. Estimation of in vitro run-on transcripts from nuclei from brains of MβP3 and MβG46 transgenic mice. Nuclear run-on experiments were performed as indicated in Materials and Methods. Autoradiograms of the membranes from the first of three successive hybridizations are shown.

Discussion

The transcription of eukaryotic genes is frequently thought of as being simultaneously controlled by two broad types of regulatory mechanisms. The first involves proteins that bind to target DNA sequences close to the initiation site (the core promoter elements) and are intimately associated with the mechanics of RNA initiation and elongation. The second set of regulators involves proteins that interact with DNA sites sometimes at considerable distances from the initiation site and restrict or stimulate transcription of the gene in certain cell types (enhancer and silencer elements). Recent investigations of the core promoter of the MBP gene have identified a number of target sites for transacting proteins within a few hundred bp of the initiation sites for transcription. These sites have been shown to be important in the initiation of in vitro transcription by extracts of HeLa cells, mouse brain, and mouse liver, or in vivo in cultured cells transfected with MBP promoter fusion genes. While it is clear that these sequences play a role in transcription, their participation in the events that restrict expression of the MBP gene to oligodendrocytes and Schwann cells is obscure since the experimental systems used to identify and define them (HeLa, hamster glial cell lines, NG 108-15 cells) normally do not express the MBP gene. An important difference between transfected genes and the endogenous gene may be that the former were amplified in *E. coli* immediately before testing, while the endogenous genes, on the other hand, have traversed all of the developmental and differentiation processes witnessed by the eukaryotic test cell. It is conceivable that post-replicative events and chromatin structure are important in conferring cell specificity to the transcription machinery of the cell. The transgenic mouse paradigm allows for a definitive study of the genetic elements conferring cell specificity for transcription, since not only has the test DNA traversed the developmental and differentiation programs, it is also presented in all cells of the organism and, therefore, represents a very stringent test of the specificity of gene expression.

In the present communication we demonstrate that the genetic elements conferring oligodendrocyte specific gene expression reside in the 1,943-bp sequence lying between position +36 and −1907 of the MBP gene. The MBP exons, introns and 3′ sequences are not required for strict specificity. The 1,943-bp promoter/enhancer sequences also confer the correct temporal expression of reporter genes, indicating that the genetic elements responsible for these changes are also present there. Surprisingly, the DNA elements conferring this exquisite control of expression in the oligodendrocytes were not sufficient to specify expression in the other cell which normally expresses the MBP gene, the Schwann cell. Expression of MβP transgene could not be detected in peripheral nerve by either of the very sensitive assays used,

β-galactosidase or RNase protection assays. Nevertheless, the absence of expression in the Schwann cell may not be absolute and some expression below that detectable by our methods may be suggested by other experiments. In experiments to be reported elsewhere (Peterson, A., S. Chang, H. Arnheiter, R. Lazzarini, A. Bernstein, and M. Brieitman, personal communication) we created transgenic mice carrying an attenuated diphtheria toxin gene controlled by a 6.5-kb DNA fragment containing the MBP promoter/enhancer. The transgenic mice had a *shiverer* phenotype as anticipated, and suffered from a striking deficiency in oligodendrocytes. Most of the myelin fibers in the PNS exhibited unremarkable features. However, some perturbations of the PNS were noted, in particular, some large axons were ensheathed but not myelinated and occasional phagocytosing cells were encountered, suggesting myelin and Schwann cell losses. These results suggest very limited transgene expression in the PNS. These results, together with those reported herein, suggest that discrete additional *cis*-acting elements are required for copious Schwann cell expression. The very low level of Schwann cell expression in the absence of the Schwann cell–specific elements might be adventitious.

The notion that oligodendrocyte-specific promoter elements might adventitiously stimulate low level expression in Schwann cells predicts that endogenous genes which are putatively oligodendrocyte-specific will exhibit some expression in Schwann cells. The recent demonstration that the PLP gene, previously thought to be expressed only in oligodendrocytes, is also expressed at low levels in Schwann cells may provide an example of low level, adventitious expression in the PNS by a "CNS-specific" gene (1, 37). Unlike the other structural myelin proteins (MBP and MAG) that are expressed in both Schwann cells and oligodendrocytes, the PLP is not preferentially incorporated into compact PNS myelin, perhaps because it is not acylated in Schwann cells as it is in oligodendrocytes (1). Thus it is without known function in the Schwann cell. In this regard it would be interesting to ascertain whether *shiverer* mice, which carry a deletion in the MBP gene removing sequences 3′ to the middle of intron 2 (35, 43), express any MBP-derived transcripts in the PNS. The absence of *shiverer* MBP transcripts in the PNS would signal that sequences in the deleted portion of the gene are important for expression in Schwann cells.

The clear specificity in expression that we observe with the MBP-Lac Z transgene is in sharp contrast to the broad pattern of expression reported for MBP-MHC-1 antigen transgenes (53, 56). The latter transgenes were expressed in lung, liver, testes, and intestinal villi, in addition to oligodendrocytes. It seems unlikely that this difference is caused by the amount of MBP gene used as the promoter/enhancer (1.9 kb in our transgene, 1.3 kb in the MHC-1 transgenes) since other transgenes employing the 1.3-kb MBP sequence were not expressed in liver (25). However, a major difference between the MHC-1 and the Lac Z transgenes is the presence of intronic sequences in the former which may contain enhancer elements as in immunoglobulin (17, 19, 38), β-globin (2, 4, 32) or PDGF (13). Conceivably these elements, if present, might contribute significantly to the pattern of transgene expression. On the other hand, we show in the present communication that two different transgenes, MβP and MβG, with introns, splice and polyadenylation signals derived from two different genes (PLP and β-globin genes, respectively) are expressed only in oligodendrocytes. These results argue strongly that the specificity we observe is conferred by the MBP promoter/enhancer and any PLP or β-globin derived enhancer elements contribute minimally to the pattern of expression.

Another feature of the MBP promoter sequence used in the present study that bears comment is its high efficiency of transcription. Although the steady state level of transgene mRNA was of the order of 1% that of the mRNA, nuclear run-on experiments showed that nascent transcripts of the transgene were synthesized at ~20–40% the rate of MBP transcripts. The apparent discrepancy between the two types of measurements suggests that the transgene mRNA has a shorter half life than most MBP mRNAs in this cell. Presently, we do not have any information on the origin of this more rapid degradation or how it compares to the rates of turnover of other prokaryotic mRNA sequences in eukaryotic cells. Nevertheless, the expectation is that the MBP promoter/enhancer described here has the potential to express genes fused to it at ~20–40% of the rate of MBP gene expression. This rate of gene expression is close to that estimated by Kimura et al. (25) and Readhead et al. (41) for their transgenic lines which employed the 5′ MBP promoter/enhancer sequences. Of the six transgenic lines tested by these two groups, four expressed in the 10 to 40% range. While there may be several explanations for the differences in transcription rate between the natural MBP gene and the transgenes, two seem particularly noteworthy. First, the site of insertion of the transgene may have a profound effect on the transgene expression. The higher transcription rate of the natural MBP gene therefore might arise because it resides at a site on chromosome 18 that is particularly conducive to transcription. Alternatively, the difference in the rates might result from the omission of an important part of the promoter/enhancer sequences in the transgenes. In this regard, the fact that the transgene promoter/enhancer is less efficient than the endogenous MBP gene is not totally unexpected since a binding site for a transcription factor was recently identified between position +25 and +45 of the MBP gene [48]. Since our promoter/enhancer fragment only extends to position +36, this site is incomplete in our transgenes. We are currently investigating this possibility and have generated mice (M′βP) bearing transgenes that include MBP sequences to position +48, so that we might compare the efficiencies of transgene transcription in these new lines.

We thank Beatrice Cubitt, Shaoli Sun, Xiaoqiao Jiang, and Xue Li, for their excellent technical assistance.

This work was supported by grant NS29056-02 from the National Institutes of Health and grant RG2238-A-1 from the National Multiple Sclerosis Society to R. A. Lazzarini.

Received for publication 14 May 1992 and in revised form 23 July 1992.

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