The Ectopic Expression of Myelin Basic Protein Isoforms in *Shiverer* Oligodendrocytes: Implications for Myelinogenesis

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Abstract. The myelin basic proteins (MBPs) are a set of peripheral membrane polypeptides that are required for the compaction of the major dense line of central nervous system myelin. We have used primary cultures of oligodendrocytes from MBP-deficient *shiverer* mice as host cells for the expression by cDNA transfection of each of the four major MBP isoforms. The distributions of the encoded polypeptides were studied by immunofluorescence and confocal microscopy and compared with patterns of MBP expression in normal mouse oligodendrocytes in situ and in culture. The exon II–containing 21.5- or 17-kD MBPs were distributed diffusely in the cytoplasm and in the nucleus of the transfectants, closely resembling the patterns obtained in myelinating oligodendrocytes in 9-d-old normal mouse brains. By contrast, the distribution of the 14- and 18.5-kD MBPs in the transfectants was confined to the plasma membrane and mimicked the distribution of MBP in cultures of normal adult oligodendrocytes. Our results strongly suggest that the exon II–containing MBPs are expressed first and exclusively during oligodendrocyte maturation, where they may play a role in the early phase of implementation of the myelination program. In contrast, the 14- and 18.5-kD MBPs that possess strong affinity for the plasma membrane are likely to be the principle inducers of myelin compaction at the major dense line.

DURING myelinogenesis, central nervous system oligodendrocytes extend processes that enwrap selected axons and generate a compacted multilamellar membrane spiral that by EM may be resolved into alternating major dense and intraperiod lines (Raine, 1984). The major dense line results from the close apposition of the cytoplasmic membrane leaflets that is brought about and stabilized by members of a set of peripheral membrane proteins termed the myelin basic proteins (MBPs)[1] (Dupouey et al., 1979; Privat et al., 1979; Readhead et al., 1987). The MBPs are the products of at least six mRNAs that are obtained by alternative splicing of a single primary transcript of the MBP gene, which contains seven exons (for review see Campagnoni, 1988). In mice and rats, four mRNAs are most abundant and encode polypeptides of 21.5, 18.5, 17, and 14 kD (de Ferra et al., 1985). All of these MBP isoforms contain exons I, III, IV, V, and VII; exon II is present in the 21.5- and 17-kD MBPs and exon VI is found in the 21.5- and 18.5-kD MBPs.

Physiological roles for each MBP polypeptide in myelin formation cannot be assigned with precision as yet. Transfection experiments in which the four major MBPs were individually expressed in nonglial (HeLa) cells yielded two distinct patterns of intracellular polypeptide distribution that were correlated with the presence or absence of the peptide sequence encoded by exon II (Staugaitis et al., 1990). These experiments showed that the 14- and 18.5-kD MBPs, which lack exon II (de Ferra et al., 1985) and are the predominant forms in purified compact myelin (Barbarese et al., 1977), associated with intracellular membranes in transfected HeLa cells. These results were expected, based upon the strong, nonspecific membrane-binding properties displayed by the 18.5-kD isoform in vitro (Smith, 1977; Boggs et al., 1982) and by the demonstration that the 14-kD MBP expressed transgenically can by itself induce major dense line formation (Kimura et al., 1989). Surprisingly, however, and in striking contrast, the exon II–containing 17- and 21-kD MBPs that are expressed in high relative proportion early in brain development (Barbarese et al., 1978; Staugaitis et al., 1990) demonstrated very limited membrane associations and in fact were found diffusely distributed in the cytoplasm and nucleoplasm.

These studies revealed that despite extensive homology, the individual MBPs display markedly different properties when expressed in HeLa cells and suggested that in myelinating cells, the isoforms may not be functionally equivalent in the elaboration of myelin (Staugaitis et al., 1990). In the

1. *Abbreviations used in this paper*: CNP, 2',3'-cyclic nucleotide 3'-phosphodiesterase; GalC, galactocerebroside; MAG, myelin-associated glycoprotein; MBPs, myelin basic proteins; PLP, proteolipid protein.

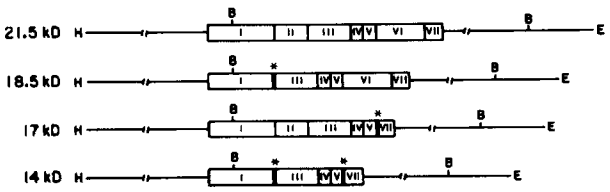


Figure 1. Schematic diagram of DNA inserts encoding the four MBPs expressed by transfection in *shiverer* oligodendrocytes. The bars depict the exon composition of each MBP. Asterisks denote the positions of exons that are spliced out of each isoform. Horizontal lines represent the 5' and 3' noncoding segments of the constructs. The 5' nontranslated region contains a portion (1.3 kb) of the MBP promoter. The 3' noncoding region is 1.5 kb in length, and is encoded by exon VII. (*H*) HindIII; (*B*) BalI; (*E*) EcoRI restriction sites.

present study, we have used the mouse mutant *shiverer*, in which the MBP gene is nonfunctional (Roach et al., 1983) but all other major myelin proteins are produced, as a source of oligodendrocytes into which cDNA "minigenes" encoding each MBP have been introduced by transfection. The intracellular distributions of the expressed products were mapped by high-resolution confocal microscopy. We have also compared the patterns obtained in the transfected *shiverer* oligodendrocytes with MBP distributions in normal mouse oligodendrocytes in vitro and in myelinating oligodendrocytes in tissue sections.

Materials and Methods

Culture of Mouse Oligodendrocytes

The preparation of mouse oligodendrocytes was modified from established procedures (Hirayama et al., 1983; Lubetzki et al., 1986). 3–4-wk-old normal mice (C57/B6J) and *shiverer* mice of the same genetic background were killed, and their brains removed and placed in HBSS (Ca^{2+} and Mg^{2+}-free; Gibco Laboratories, Grand Island, NY), containing 10 mM Hepes (pH 7.4), 25 mM Na bicarbonate, 50 μm 2-mercaptoethanol, 100 U/ml penicillin, and 100 μg/ml streptomycin. Tissue was kept in this buffer throughout the procedure. The cerebellum, brainstem, and olfactory bulbs were discarded. The meninges were removed and the tissue was finely minced. Tissue from normal mice was treated with 0.06% trypsin (30 min at 37°C), after which FCS (20%) was added. Trypsinization was not necessary to disrupt *shiverer* tissue. The cellular suspensions were then passed sequentially through 500 μm and 63 μm Nytex filters (Tetko, Inc., Briarcliff Manor, NY). A 45% Percoll (Pharmacia Fine Chemicals, Piscataway, NJ) gradient (20 ml) was formed by centrifugation (23,000 *g* for 45 min of 4°C). The filtrate (10 mls) was loaded onto this gradient and centrifuged (23,000 *g* for 15 min at 4°C) in a rotor (model SS 34; DuPont Instruments-Sorvall Biomedical Div., DuPont Co., Wilmington, DE). Cells were collected from the zone between the myelin and red blood cell layers. After three washings, cells were resuspended in Opti-MEM (Gibco Laboratories), supplemented with 1% dialyzed FCS, 100 U/ml penicillin, 100 μg/ml streptomycin, and 50 μm 2-mercaptoethanol), counted, and plated onto coverslips precoated with 10 μg/ml poly-L-Lysine (P2636; Sigma Chemical Co., St. Louis, MO). Cells were maintained at 37°C with 5% CO_2.

Plasmid Constructions

The mouse genomic clone HECC201 and the cDNAs pM44, pM72, pM23-900 encoding the mouse 21.5-, 17-, and 14-kD MBPs, respectively, were gifts from Dr. A. T. Campagnoni (University of California, Los Angeles, CA). The cDNA pHF43 encoding the 18.5-kD MBP was a gift of Dr. R. A. Lazzarini (Mount Sinai School of Medicine, New York, NY).

Genomic clone HECC201 is a 2,656 bp HindIII–EcoRI fragment, which contains a 5' flanking region (1,300 bp), exon I (218 bp), and a portion of intron I (1,138 bp). Previous work by others has shown that the 5' flanking region was sufficient to promote transcription in oligodendrocytes in vivo (Katsuki et al., 1988), and so we used this fragment to construct "minigenes" encoding the individual mouse MBP cDNAs. This was achieved by ligating the genomic "promoter region" to each individual cDNA at the BalI site located in exon I (Fig. 1). Because a second BalI site was present in exon VII of each mouse cDNA, the constructions shown in Fig. 1 were obtained as follows: HECC201 was digested with HindIII and BalI, and the fragment containing the promoter region was purified. A three-way ligation was performed using this fragment, the BalI–EcoRI fragment corresponding to the 3' end of exon VII, and the pGEM™-4 (Promega Biotec, Madison, WI) vector, which had been digested with HindIII and EcoRI. This plasmid was amplified in bacteria and purified, linearized with BalI and dephosphorylated. In parallel, each cDNA was digested with BalI and the internal fragment of each cDNA insert was purified and ligated to the BalI-digested plasmid described above to yield the minigenes shown in Fig. 1.

The 660-bp cDNA encoding the 14-kD rat MBP was described previously (Staugaitis et al., 1990). This was subcloned into the HindIII site of the pRSV vector (Forman et al., 1988). Ligation mixtures were used to transform Escherichia coli HB101 cells. Large scale plasmid preparations were purified by two cycles of CsCl equilibrium density gradient centrifugation followed by dialysis and precipitation (Maniatis et al., 1982).

Transfections

Shiverer oligodendrocytes at 2 d in vitro were transfected using the technique of Chen and Okayama (1987). Just before transfection, cells were fed with medium containing 3% dialyzed FCS. 2.5 μg of plasmid DNA in a calcium phosphate solution were added to each coverslip. A calcium phosphate-DNA complex was gradually formed on the cells over the course of 12 h at 35°C and 3% CO_2. For each experiment, a control transfection without DNA was always performed. The cells were then rinsed twice, fed with medium containing 1% dialyzed FCS, and immunostained 48 h later. For the cells transfected with the pRSV-MBP plasmid, sodium butyrate (5 mM) was added to the medium for 12 h before immunostaining to enhance expression (Gorman et al., 1983). Optimal transfection efficiencies were obtained using cells that were plated at high density (500,000 per coverslip) and cultured for 2 d. 10% of the plated cells survived the transfection procedure, and optimally, 30 cells per coverslip expressed the transfected cDNA. No positive cells were obtained when the transfection procedure was performed without cDNA.

Immunofluorescence

The rabbit polyclonal antisera to MBP and to 2', 3'-cyclic nucleotide 3'-phosphodiesterase (CNP) have been described (Colman et al., 1982; Bernier et al., 1987). The MBP antiserum recognizes each of the four major MBP isoforms (Staugaitis et al., 1990). Polyclonal antisera to the myelin-associated glycoprotein (MAG) and to the myelin proteolipid protein (PLP) were prepared in our laboratory and were tested on Western blots. All polyclonal antisera were affinity purified. The mouse mAb that recognizes galactocerebroside (Gal C), a major determinant on the oligodendrocyte surface, was from a hybridoma cell line produced by Dr. B. Ranscht (Cancer Research Institute, La Jolla, CA) (Ranscht et al., 1982).

After all transfections, Gal C hybridoma supernatant was diluted 1/5 in culture medium and added to live cells (15 min at 22°C). The cells were fixed (4% paraformaldehyde in PBS for 10 min) and permeabilized (0.05% Triton-X100 in PBS for 5 min). Coverslips were treated with 0.2% gelatin (in 100 mM Tris-HCl, pH 7.4 for 15 min), and MBP antiserum (diluted 1/100 in 0.2% Tris-gelatin) was added (30 min). After several washings, coverslips were incubated (25 min) in a mixture of FITC-conjugated goat anti-rabbit IgG and TRITC-conjugated goat anti-mouse IgG (Kirkegaard and Perry Laboratories, Inc., Gaithersburg, MD), diluted in Tris-gelatin, and after washing, were mounted onto slides using Citifluor (Citifluor LTD, Northampton Square, London, England) containing the nuclear stain, Hoechst 32258 (10^{-5} M).

Preparation of Tissue Sections from Normal Mouse Brain

Brains from 2, 6, and 9-d-old mice were removed and fixed by immersion in cold (4°C) 4% paraformaldehyde in PBS for 24 h, after which they were

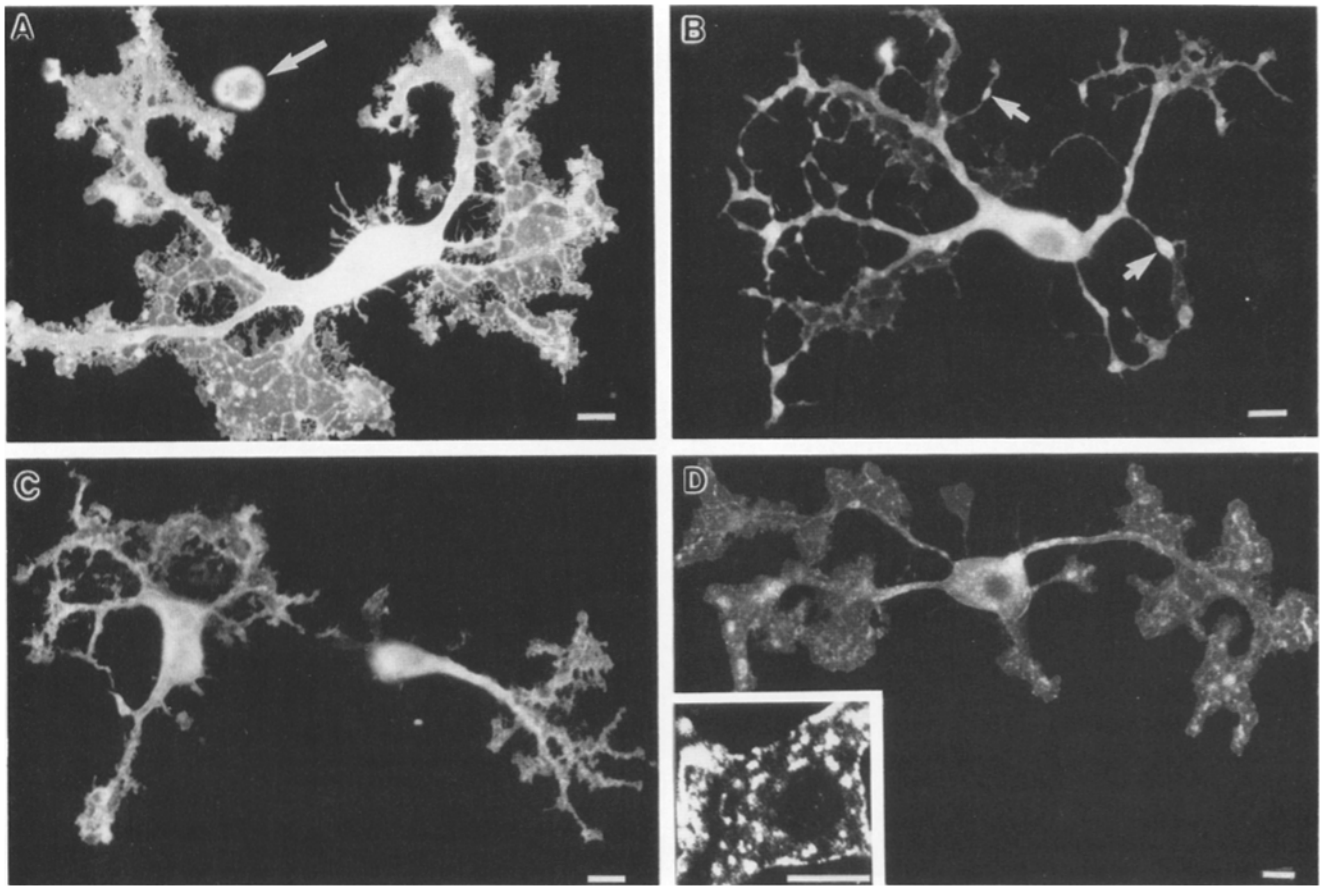


Figure 2. Distribution of myelin markers in *shiverer* oligodendrocytes at 2-d in vitro. (*A*) Gal C; (*B*) CNP; (*C*) MAG; (*D*) PLP. (*A*) Gal C distributes over the surface of the cell including the membrane expansions. Note that some oligodendrocytes have not yet elaborated processes but nevertheless express Gal C (*arrow*). (*B*) CNP is distributed within the cytoplasm of the cell body and processes in this cell. Areas of high fluorescence are commonly observed within thin processes (*arrows*). (*C*) and (*D*) MAG (*C*) and PLP (*D*) are transmembrane proteins that distribute in the cytoplasm and at the plasma membrane. PLP is associated with discrete membranous structures inside the cell body (*D, inset*) and the major processes. Bar, 20 μm.

infiltrated with fixative to which sucrose (40% wt/vol) was added. After 48 h, the brains were divided in the sagittal plane and parasagittal sections (10–20 μm) were cut on a cryostat (Hacker Instruments, Inc., Fairfield, NJ). After extensive washing in PBS, the tissue sections were preincubated in a solution containing PBS, 0.2% gelatin, and 0.1% Triton X-100 (PBSGT) for 1 h, 22°C. Antiserum to MBP was then diluted (1/200) in PBSGT and added to the sections (1 h at 37°C), followed by washing with PBSGT (4 × 15 min). The sections were incubated with FITC–conjugated secondary antibody diluted in PBSGT (1 h at 37°C). After washing (4 × 15 min), the tissue was mounted onto slides and coverslipped. Confocal analysis of stained myelin sheaths in these sections demonstrated that under these conditions the antibody penetrated the entire section.

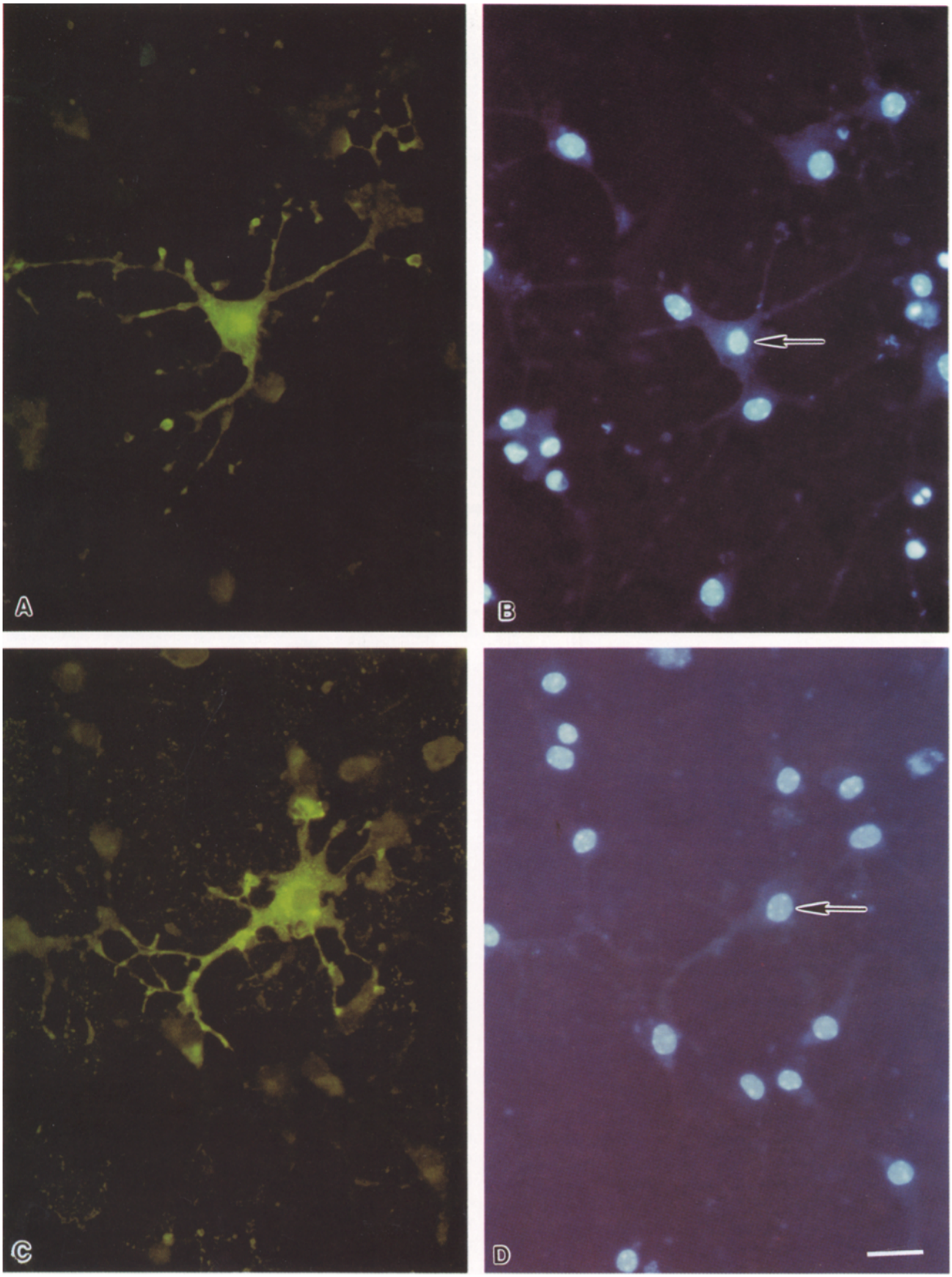
Confocal Microscopy

PHOIBOS-1000 confocal microscopy and image processing have been described (Staugaitis et al., 1990). Cells were scanned using a 63 × /1.4 Planapochromat or a 100 × /1.3 Neofluor objective (Carl Zeiss, Inc., Thornwood, NY). The initial scans were taken at a level close to the coverslip on which the cell was grown and then at 1-μm increments until several sections through the cell nucleus were obtained. Normally, 512 × 512 pixel images were obtained in which every other pixel on every other line was sampled (pixel spacing = 2). To obtain a maximum magnification and resolution of optical sections through the nucleus, every pixel was sampled (pixel spacing = 1). In these images, the pixel size equals 0.1 μm^2.

Results

Characterization of Adult Shiverer Oligodendrocytes In Vitro

The rates and the patterns of growth were similar for both normal and *shiverer* oligodendrocytes. At 2-d in vitro, most cells had started to grow cytoplasmic extensions that rapidly lengthened and branched, giving rise to an intricate network of thinner processes that apparently lay down flattened membrane sheets. Some cells grew more slowly (*arrow* in Fig. 2 *A*) and did not extend processes until 4- or 5-d in vitro. Regardless of morphology, at 2 d more than 95% of the cells expressed Gal C, CNP, MAG, and PLP. Gal C, a plasma membrane glycolipid, was expressed uniformly over the entire cell surface (Fig. 2 *A*), while CNP was localized throughout the cytoplasm of the cell body and the major processes (Fig. 2 *B*). MAG, a transmembrane glycoprotein, was detected primarily at the plasma membrane of the cell body and along the processes (Fig. 2 *C*). PLP, the major integral membrane protein of myelin, also distributed at the plasma mem-


A
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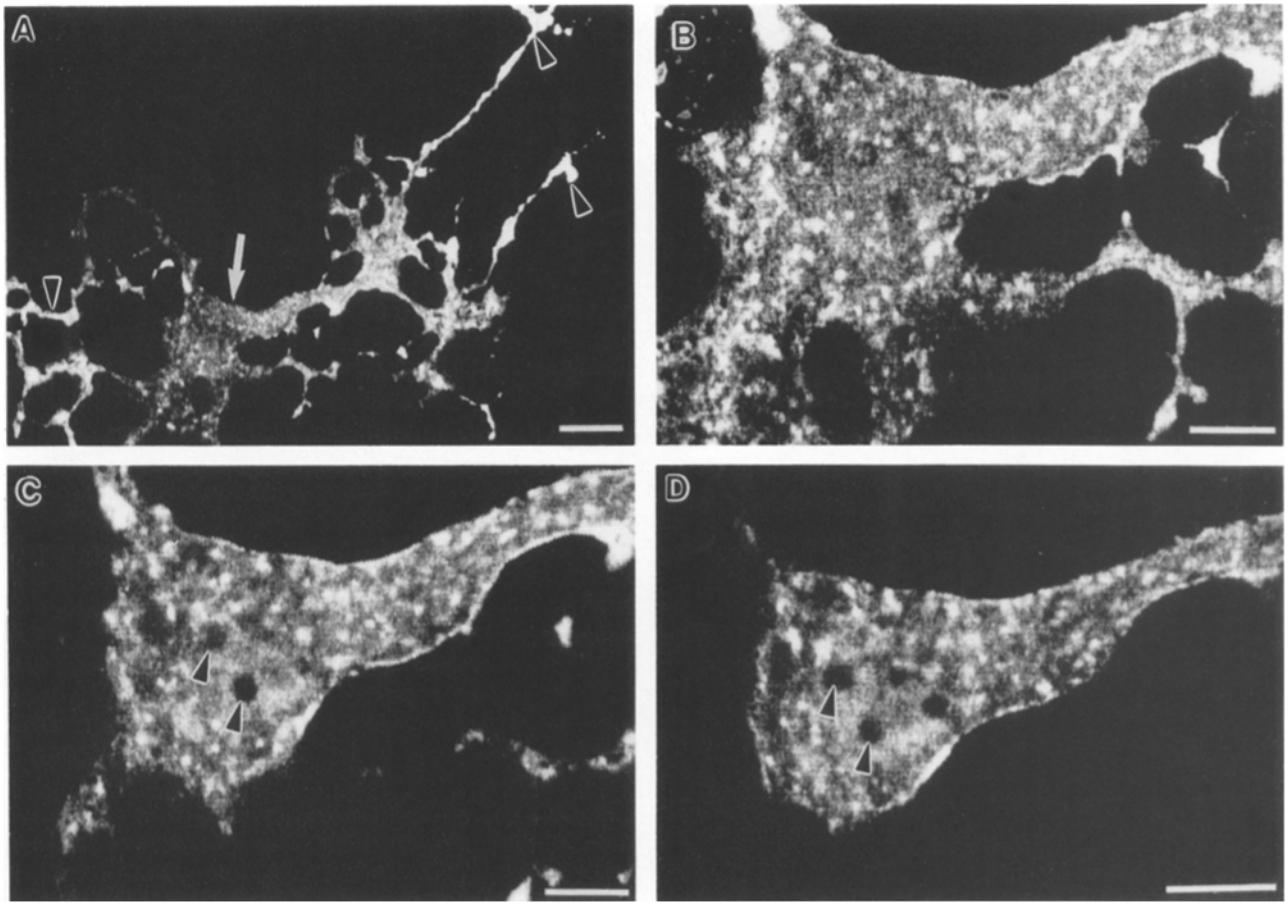

Figure 4. Optical sections through a *shiverer* transfectant expressing the 21.5-kD MBP. (*A*) The image was recorded at a level near the coverslip permitting the visualization of flattened membranes. Intense fluorescence is observed at the ends of the processes (*arrowheads*). (*B–D*) The cell body (4 *A*, *arrow*) was scanned at high magnification at consecutive 1-μm increments towards the top of the cell. Note the finely granular pattern throughout the cytoplasm and the nucleus, and the absence of nucleolar labeling. (*C* and *D*, *arrowheads*). Pixel spacing: (*A*) 2; (*B–D*) 1. Bars: (*A*) 20 μm, (*B–D*) 10 μm.

brane but in addition highly fluorescent intracytoplasmic clumps were observed (Fig. 2 *D*). By confocal microscopy (Fig. 2 *D*, *inset*), these clumps were resolved into discrete structures that probably represent membranous elements involved in the synthesis and transport of PLP (rough ER, Golgi apparatus, and associated membranes). As expected, MBP immunofluorescence was absent in *shiverer* oligodendrocytes (data not shown).

Our data therefore demonstrate that soon after plating in culture, both *shiverer* and normal adult oligodendrocytes synthesize the membrane and protein constituents characteristic of the myelinating state in vivo (Szuchet et al., 1986; Monge et al., 1986). In fact, the distributions and intensities of these markers in *shiverer* oligodendrocytes were virtually identical both to the normal adult oligodendrocytes in our cultures (Allinquant et al., 1989) as well as to those cultured by others (Dubois-Dalcq et al., 1986; Knapp et al., 1987, 1988; Dyer and Benjamins, 1989). These observations reinforced our expectation that *shiverer* oligodendrocytes would serve as physiologically meaningful host cells in which to study the properties of ectopically expressed MBPs.

Distribution of Individual MBP Isoforms in Transfected Shiverer Oligodendrocytes

For each MBP cDNA, expression was first detectable at 24 h and was maximal between 48–72 h. These cells displayed a variety of morphologies. Both small cells with short processes and large cells with elaborate processes and well developed membrane expansions were highly fluorescent. Based

Figure 3. Conventional epifluorescence microscopy of *shiverer* oligodendrocytes expressing the 21.5- (*A* and *B*) or the 14-kD MBPs (*C* and *D*). The cDNAs encoding the 21.5-kD MBP (*A* and *B*) or the 14-kD MBP (*C* and *D*) were transfected into *shiverer* oligodendrocytes and processed for microscopy 48 h later. In both cells, MBP fluorescence was strong in the processes and in the cell body. Staining of the transfected cells with Hoechst 32258 clearly confirms that in the cell body, the 21.5-kD MBP is contained within the nucleus (*B*, *arrow*), while the 14-kD MBP is not (*D*, *arrow*). Bar, 15 μm.

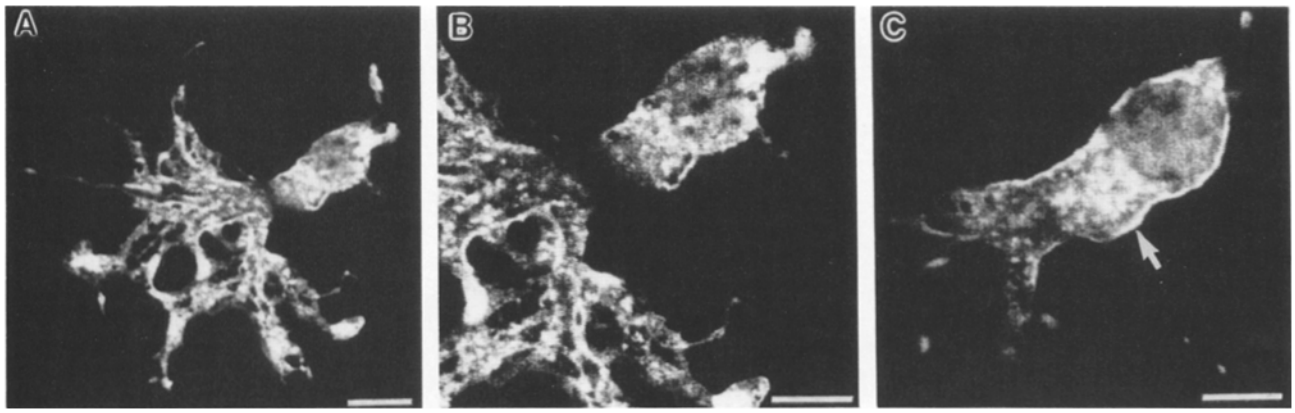

Figure 5. Optical sections of a transfected *shiverer* oligodendrocyte expressing the 17-kD MBP. (*A*) The image was recorded at a level close to the coverslip. MBP immunofluorescence is strong throughout the cell body cytoplasm and flattened membrane sheets; *B* is a magnification of the cell body at the same focal level as *A*; *C* is taken 1 μm above *B*. The MBP fluorescence is less intense in the nucleus than in the cytoplasm, and some may be detected at the plasma membrane (*arrow*). Pixel spacing: (*A*) 2; (*B* and *C*) 1. Bars: (*A*) 20 μm; (*B* and *C*) 10 μm.

upon fluorescence intensity, all MBP cDNAs were expressed at similar levels in the processes, membrane expansions and cell bodies (Fig. 3, *A* and *C*). The intracellular distribution of the individual MBPs in cell bodies, however, differed according to the particular isoform expressed. For the 21.5-kD MBP expressors, cell bodies typically appeared to be filled with immunofluorescence, and a defined, nuclear outline was rarely observed. In fact, in many cells, fluorescence appeared most intense in the region of the soma presumably occupied by the nucleus (Fig. 3 *A*), and by labeling with Hoechst 32258, this localization was confirmed (Fig. 3 *B*). In contrast, cells expressing the 14-kD MBP had a well-defined nuclear shadow (compare Fig. 3, *C* and *D*). Confocal microscopy was used to precisely map the MBP distributions within cell bodies.

Fig. 4 shows a confocal micrograph of a 21.5-kD MBP expressor. An optical section taken close to the coverslip reveals immunofluorescence throughout the cell with the greatest intensity at the ends of the processes (Fig. 4 *A*, *arrowheads*). Higher magnification demonstrated a finely granular, intracytoplasmic pattern in successive optical sections through the soma (Fig. 4, *B–D*). In 90% of the cells expressing this isoform, there was at least an equivalent level of fluorescence in the nucleus as in the cytoplasm, and in many cells, label was more intense in the nucleus. In all cells, a nuclear outline was discernible but difficult to identify. The presence of unlabeled nucleoli in sequential optical sections definitively revealed the position of the nucleus (Fig. 4, *C–D, arrowheads*).

All of the oligodendrocytes expressing the 17-kD MBP isoform displayed immunofluorescence in the cytoplasm as well as at the plasma membrane (Fig. 5). Plasma membrane fluorescence was usually more pronounced (Fig. 5 *C*, *arrow*), and the cytoplasmic label, more clumped than was the case with the 21.5-kD MBP (Fig. 4). 50% of the 17-kD expressors revealed MBP within the nucleus (Fig. 5 *C*). At all times, the nuclear fluorescence was less intense than the cytoplasmic fluorescence and therefore a nuclear outline was evident.

The 18.5- (Fig. 6) and the 14-kD (Fig. 7) MBP isoforms expressed in oligodendrocytes exhibited very strong fluorescence at the plasma membrane. Generally, cytoplasmic

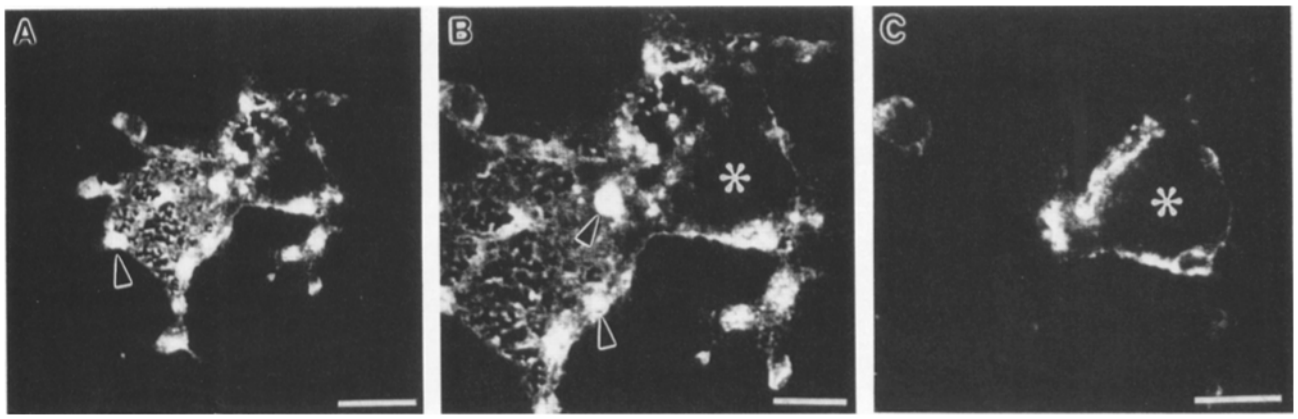

Figure 6. Optical sections of a transfected *shiverer* oligodendrocyte expressing the 18.5-kD MBP. Image recording was started at the level of the coverslip (*A* and *B*); (*A*) MBP distributes at the plasma membrane of the cell body and expanding membrane sheets. *B* is a magnification of the region of the soma containing the nucleus, and *C* is an image taken 1 μm above *B*. The protein clearly is distributed intensely in some regions of the plasma membrane (*A* and *B*, *arrowheads*). The nucleus and cytoplasm are devoid of MBP (B, C asterisks). Pixel spacing: (*A*) 2; (*B* and *C*) 1. Bars: (*A*) 20 μm; (*B* and *C*) 10 μm.

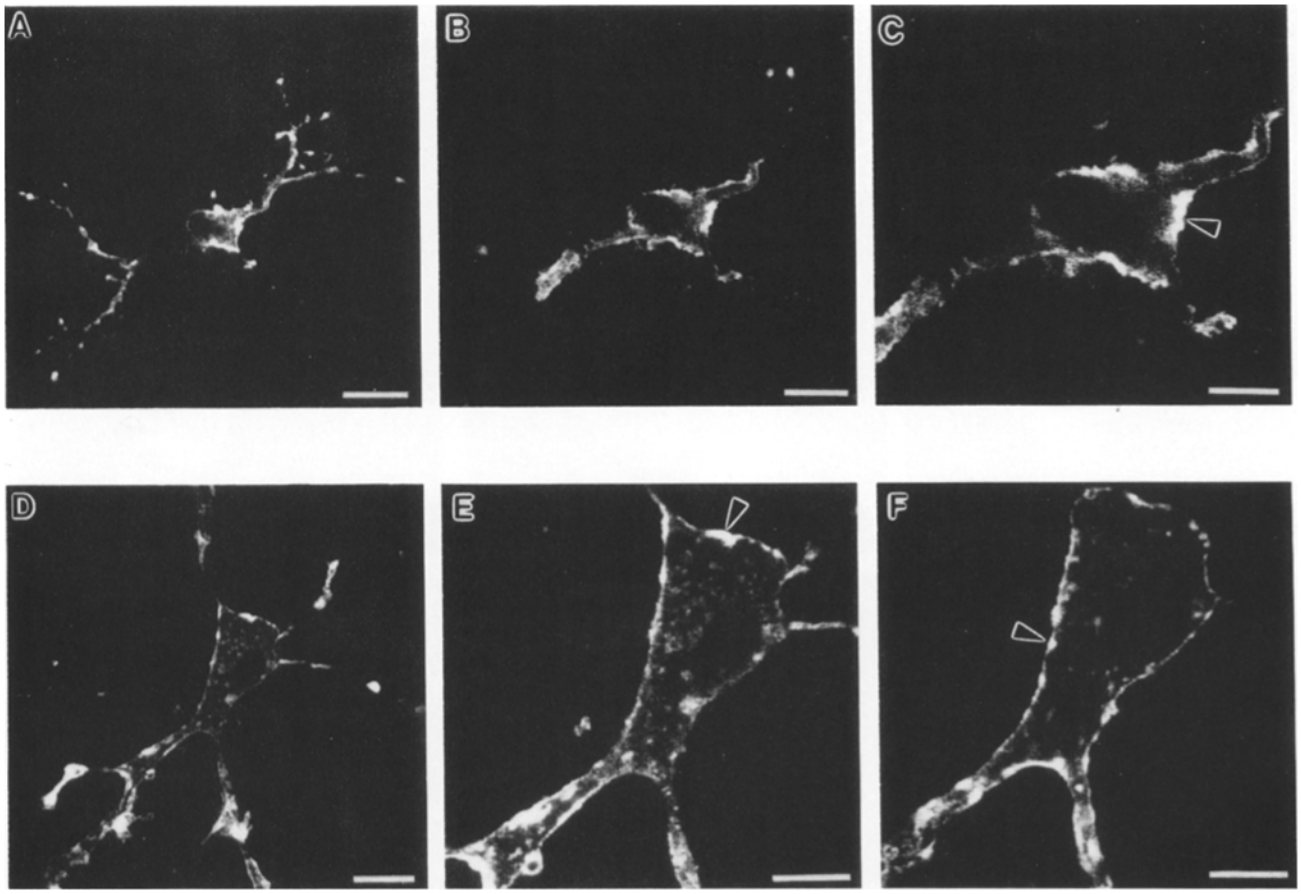

Figure 7. Optical sections of *shiverer* oligodendrocytes expressing the 14-kD MBP. (*A*, *B*, and *C*) The cDNA encoding the mouse full-length 14-kD MBP was used for transfection. This construct contained the complete 3′ noncoding region. (*D*, *E*, and *F*) The transfected cDNA encoded the rat 14-kD MBP and 3′ noncoding region was truncated. The MBPs encoded by these different constructs distribute identically at very high levels at the plasma membrane. "Patching" of label is seen at the cell membrane (*C*, *E*, and *F*, *arrowheads*). Pixel spacing: (*A*, *B*, and *D*) 2; (*C*, *E*, and *F*) 1. Bars: (*A*, *B*, and *D*) 20 μm; (*C*, *E*, and *F*) 10 μm.

fluorescence was absent (Fig. 6, *B* and *C*, *asterisks*) or extremely low in density, and labeling within the nucleus was never seen for these isoforms. The distribution of the 14-kD MBP was the same whether we used the full-length mouse 14-kD cDNA (Fig. 7, *A–C*) or a rat 14-kD cDNA that had been truncated at the 3′ end of the noncoding region (Fig. 7, *D–F*). Often MBP labeling was not uniformly distributed along the plasma membrane but instead was concentrated in highly fluorescent patches (Figs. 6, *A* and *B*, *arrowheads*; 7, *C*, *E*, and *F*, *arrowheads*), suggesting that the polypeptide is first inserted into restricted regions of the plasma membrane before diffusion over the entire surface.

Confocal Microscopy of Normal Mouse Oligodendrocytes

Other investigators have shown that MBP first appears in cell bodies of developing oligodendrocytes in normal brain (Hartman et al., 1979; Sternberger et al., 1978; Roussel and Nussbaum, 1981). In light of the patterns we observed in transfected *shiverer* oligodendrocytes, it was of interest to reexamine the distribution of MBP within normal oligodendrocytes by high-resolution confocal microscopy.

In tissue sections from 6- and 9-d mouse brains, numerous MBP-positive oligodendrocyte cell bodies (Fig. 8) with slender processes (Fig. 8, *D* and *E*, *arrowheads*) were commonly observed in the midbrain and thalamus. Sometimes, processes could be traced to thin, faintly fluorescent myelin sheaths. In these cells, MBP was consistently distributed in the cytoplasm in a finely granular pattern. Discrete plasma membrane fluorescence was not obvious in any cell body. Label was always detected in the nuclei of the most highly fluorescent cells (Fig. 8), but at levels somewhat less than in the surrounding cytoplasm. MBP distribution in the cell bodies of these maturing oligodendrocytes therefore closely resembled patterns obtained in the 21.5- and 17-kD MBP *shiverer* transfectants (c.f. Figs. 4, 5, and 8). In regions where intensely fluorescent, myelinated bundles were prominent virtually no cell bodies containing MBP were detected (data not shown).

The shift in MBP distribution from the cell body to the myelin sheath as the oligodendrocyte matures has been well documented, and in adult brain MBP is exclusively present in the sheath (Sternberger et al., 1978; Hartman et al., 1979; Roussel and Nussbaum, 1981). However, in cultured oligodendrocytes derived from adult brain, MBP becomes detectable throughout the cell (Barbarese and Pfeiffer, 1981; Szuchet et al., 1986; Dubois-Dalcq et al., 1986; Knapp et al., 1987, 1988; Dyer and Benjamins, 1989). Fig. 9 shows

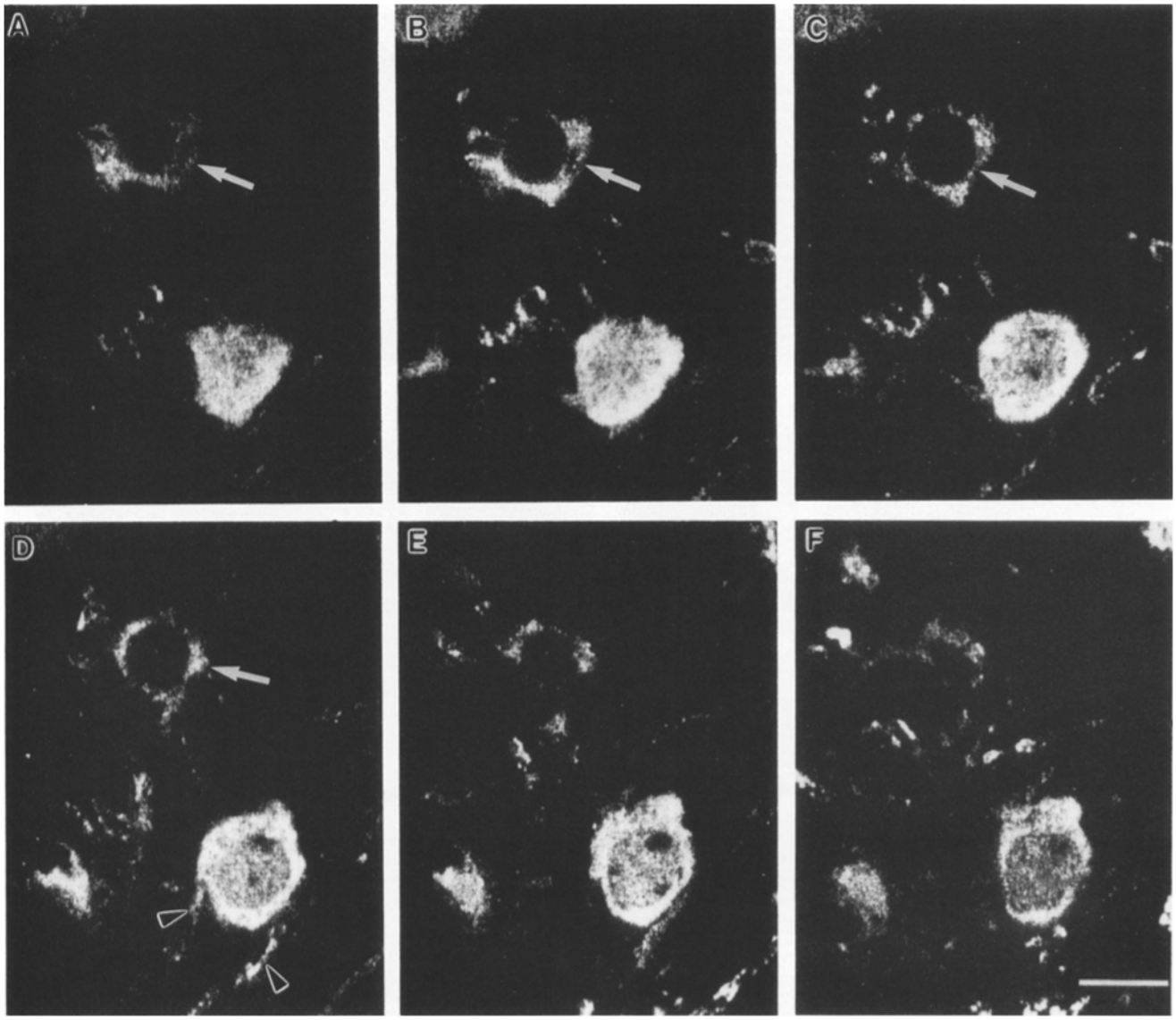

Figure 8. Confocal microscopy of MBP distribution in oligodendrocytes in situ in a 9-d-old normal mouse brain. Image recording was repeated at consecutive 1-μm increments (*A–F*). A diffuse localization of MBP throughout the cytoplasm is present in two cell bodies. In the upper cell (*arrow*), MBP fluorescence is weak and restricted to the cytoplasm. In the lower, clearly brighter cell, MBP was also present inside the nucleus. Small processes labeled with MBP (*arrowheads*) are visible (*D and E*). Pixel spacing, 1. Bar, 10 μm.

a normal adult oligodendrocyte cultured for 5 d. The soma of this cell is highly fluorescent, and in fact appears filled with MBP (Fig. 9 *A*, *arrow*). It is surprising therefore that confocal optical sections taken through the soma clearly show that MBP is confined to the plasma membrane (Fig. 9 *B*, *arrow*), and in many cells patches of intense fluorescence at the membrane were evident. This pattern was most frequently observed in cells with well-developed membrane expansions and was indistinguishable from that obtained on transfection of adult *shiverer* oligodendrocytes with either the 18.5- or the 14-kD MBPs (c. f. Figs. 6, 7, and 9). These data are consistent with the fact that these polypeptides form the overwhelming proportion of the MBPs synthesized by mature oligodendrocytes in culture (Barbarese and Pfeiffer, 1981).

Discussion

A complete understanding of the intracellular events that lead to the formation of the major dense line requires an analysis of the behavior of the MBPs under as close to physiological conditions as possible, and it was for this reason that the *shiverer* oligodendrocyte was chosen as a host cell for these transfection studies. Primary cultures of the MBP-deficient *shiverer* oligodendrocytes proved to be physiologically meaningful host cells, since the growth, morphology, and expression of other oligodendrocyte markers (Fig. 2) seem essentially unaffected by the absence of the MBPs.

MBP Distribution Patterns in Transfectants Correlate with MBP Expression in Myelinating Oligodendrocytes

By high-resolution confocal microscopy, two distinct patterns of intracellular polypeptide distribution were observed in transfected oligodendrocytes. The 14- and 18.5-kD isoforms were virtually absent from the cytoplasm and appeared to be sharply confined to the plasma membrane of transfected cells (c. f., Figs. 6 and 7). The predominant feature

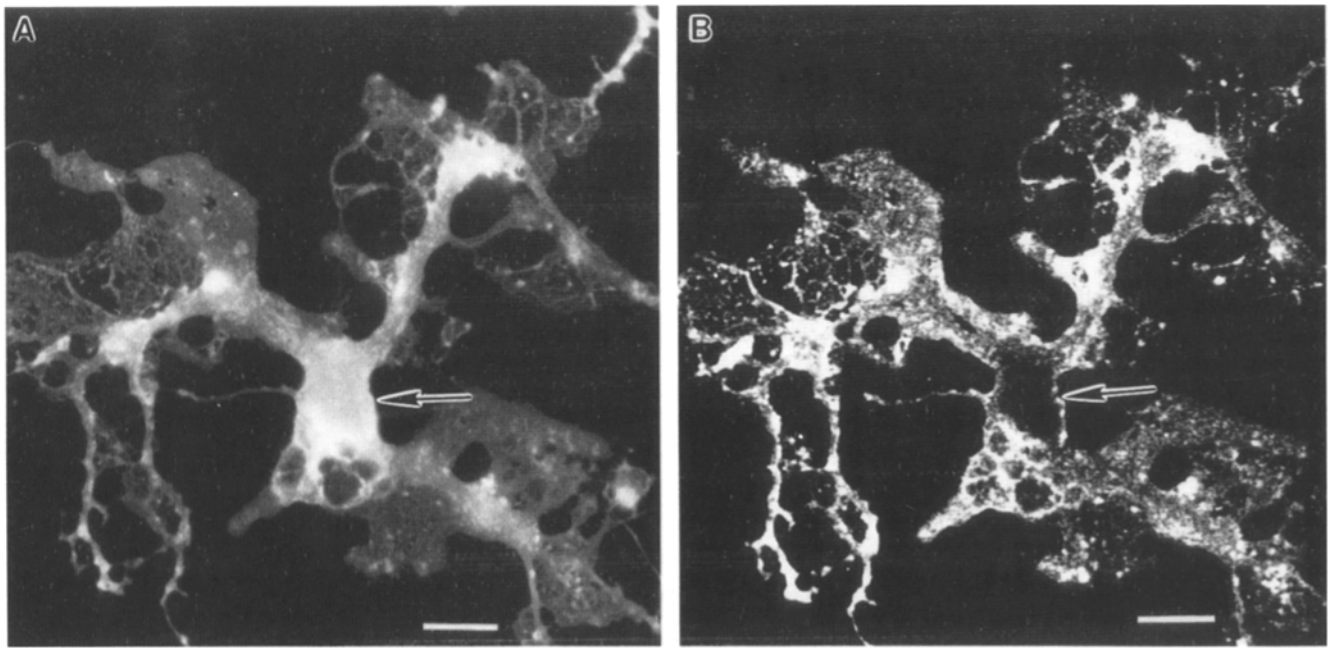

Figure 9. Comparison of MBP in a normal adult oligodendrocyte in culture viewed by conventional and confocal fluorescence microscopy. The same oligodendrocyte was observed by conventional (*A*) and by confocal (*B*) microscopy. An optical section (*B*) shows clearly the distribution of MBP at the plasma membrane and demonstrates that the intense fluorescence throughout the cell body (*arrows*) observed by conventional microscopy (*A*) results from the contribution of light from all parts of the plasma membrane in this region. Pixel spacing: (*B*) 2. Bar, 20 μm.

of the 17- and 21.5-kD isoforms, on the other hand, was extensive cytoplasmic and intranuclear distributions (Figs. 3 *A*, 4, and 5). These distributions observed in transfected oligodendrocytes may be correlated with patterns of MBP expression observed in normal, developing oligodendrocytes in brain.

It has been shown that MBP may be readily detected in oligodendrocyte cell bodies and processes, before the appearance of compact myelin in a given brain region (Sternberger et al., 1978; Hartman et al., 1979; Roussel and Nussbaum, 1981). Once the rapid myelination phase begins, however, MBP is lost from the cell bodies and processes, and the myelin sheaths are the only heavily labeled structures. Through the use of high-resolution confocal microscopy, we have been able to extend these earlier observations by demonstrating that in highly fluorescent young oligodendrocytes in 6- and 9-d-old mouse brain, MBP was not only abundant in the cytoplasm of the soma but was even detected in the nucleus (Fig. 8). Remarkably, this pattern is uniquely characteristic of the 17- or 21.5-kD isoforms in the transfectants (Figs. 3 *A*, 4, and 5). In fact, the absence of MBP at the cell body plasma membrane of normal young oligodendrocytes in situ (Fig. 8) makes it highly unlikely that the 14- or 18.5-kD MBPs are being synthesized in quantity in these cells at this time. Our data therefore suggest that the 21.5- and/or 17-kD MBPs may be expressed exclusively within oligodendrocytes at an early developmental stage.

It would appear at first that this conclusion cannot be easily reconciled with the observation that these polypeptides do not exceed >25% of the total MBP complement in young animals (Barbarese et al., 1978; Staugaitis et al., 1990). However, the absolute amount of each MBP isoform detected in any brain sample naturally reflects the sum of the contributions made by every oligodendrocyte, regardless of degree of maturity. At present, there is no evidence that all four major MBPs are simultaneously expressed in a single cell, and as we have noted above, the distinctive MBP distributions we have observed in tissue sections suggest that this may not occur. In individual cells, therefore, the developmental profile of MBP expression may undergo a much more dramatic shift than has been previously appreciated. The 17- and 21.5-kD isoforms that are detectable in the composite protein pattern at any developmental stage in brain may be uniquely contributed by those oligodendrocytes that are just entering the myelination program. The presence of low levels (~5%) of exon II–containing MBP mRNAs in normal adult CNS may therefore reflect the existence of a subpopulation of oligodendrocytes that are perhaps important in myelin remodeling or remyelination (Jordan et al., 1990).

The precise roles that the exon II–containing isoforms play in oligodendrocyte maturation are unknown. These polypeptides have only been detected in mammals (see Campagnoni, 1988) and appear to be absent in shark (Saavedra et al., 1989) and chicken (Zopf et al., 1989). Their early expression and limited capacity to form membrane associations make it unlikely that they function as major inducers of myelin compaction. Possibly, their presence in the cytoplasm might permit a protracted period of expansion of the oligodendrocyte membrane surface, and so engender greater process outgrowth. The presence of MBP in oligodendrocyte nuclei, in transfectants (Fig. 3 *A*, 4, and 5), and in situ (Fig. 8) raises the possibility that these isoforms may have regulatory roles in the implementation of the myelination program (Staugaitis et al., 1990). Alternatively, this observation may

merely reflect the fact that proteins of small molecular mass may enter into the nucleus passively and even be retained within this structure (Dingwall and Laskey, 1986).

Targeting of the MBPs to the Oligodendrocyte Plasma Membrane

It is now generally accepted that the MBPs are products of free polysomes that are selectively segregated in vivo to regions of the oligodendrocyte cytoplasm where myelin compaction is occurring (Colman et al., 1982; Trapp et al., 1987; Verity and Campagnoni, 1988, Jordan et al., 1989). We have suggested that by actively placing MBP-synthesizing polysomes at these loci, nascent MBPs can be directly incorporated into the forming major dense-line, and the nonspecific association of the most highly membrane-associative forms (14- and 18.5-kD MBPs) with nonmyelin intracellular membranes is prevented (Colman et al., 1982). When these polypeptides are expressed in HeLa cells, which presumably have not developed a mechanism to sequester MBP-synthesizing polysomes to the cell periphery, the 14- and 18.5-kD MBPs distribute in close association with perinuclear membranes (Staugaitis et al., 1990). Presumably, on exiting the HeLa cell nucleus, MBP mRNAs become incorporated into perinuclear polysomes and are translated in this region, where the newly synthesized proteins bind immediately to nearby membranes.

Given the virtually exclusive plasma membrane distribution of MBP in normal adult oligodendrocytes in culture (Fig. 9) and of the 14- and 18.5-kD MBP isoforms in the transfected oligodendrocytes (Figs. 6 and 7), it is therefore likely that MBP-synthesizing polysomes are actively placed at the plasma membrane in these cells. Translation at this locus would facilitate the association of the nascent polypeptides with the cell membrane, and the high affinity for membranes that these isoforms are known to possess would ensure that they remain there. In vivo, in the advanced stages of myelination, normal oligodendrocytes can apparently exclude MBP synthesis from the soma and confine it to the cell processes (Colman et al., 1982), since MBP mRNAs are not detectable by in situ hybridization within oligodendrocyte cell bodies (Trapp et al., 1987; Verity and Campagnoni, 1988; Jordan et al., 1989), nor are the MBP polypeptides detected by immunocytochemistry in the soma of mature oligodendrocytes in situ (Sternberger et al., 1978; Hartman et. al., 1979; Roussel and Nussbaum, 1981). It may be envisioned that the mRNAs encoding the 17- and 21.5-kD MBPs may be similarly segregated at the plasma membrane, but the inability of the expressed polypeptides to associate tightly with the membrane bilayer allows these isoforms to diffuse and distribute within the cytoplasm. This would explain why we observed strong fluorescence in the 17- and 21.5-kD transfectants throughout the cytoplasmic extensions as well as in the cytoplasm of the soma. The mechanism by which the eccentric distribution of MBP mRNAs is achieved is not known but could involve "signals" residing in the mRNA itself, or in the nascent polypeptide chains (Colman et al., 1982). Significantly, truncation of the 14-kD MBP mRNA at the 3′ end so that only ~200 nucleotides of noncoding sequence remained did not alter the distribution of the encoded polypeptide (Fig. 7). The 3′ nontranslated region therefore may not be involved in the movement of MBP mRNAs within the oligodendrocyte cytoplasm.

We have not directly examined the distribution of MBP mRNAs within the transfected oligodendrocytes. In situ hybridizations with cloned MBP DNA probes reveal silver grains overlying cell bodies and processes in cultured normal oligodendrocytes (Zeller et al., 1985; Shiota et al., 1989; Holmes et al., 1988). In these studies, precise intracellular distributions of MBP mRNAs could not be ascertained because of technical limitations in methodology. Therefore, the possibility that MBP mRNAs are distributed randomly throughout the cytoplasm must be considered. If this were so, it might be expected that the polypeptides, after release from polysomes, would use an intracellular (presumably vesicular) pathway to reach the plasma membrane, as seems to be the case in HeLa cells that are transfected with the 14- or 18.5-kD MBPs (Staugaitis et al., 1990). However, an association of any MBP with intracytoplasmic membranes was not observed in the present study. The fact that within the soma and processes of nontransfected *shiverer* oligodendrocytes (Fig. 2 *D*) as well as in normal oligodendrocytes (data not shown), intracellular membranes and vesicles containing the myelin proteolipid protein were readily detected (Fig. 2 *D*), strongly reinforces the idea (Benjamins et al., 1975; Hartman et al., 1979) that the biosynthetic pathways that ultimately deliver PLP and MBP to the myelin membrane are completely segregated up until the point of myelin sheath assembly at the compaction zones.

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References

Allinquant, B., D. D'Urso, S. M. Staugaitis, P. R. Smith, and D. R. Colman. 1989. Primary cultures of *shiverer* and normal mouse oligodendrocytes: immunocytochemical detection of myelin markers. *Trans. Am. Soc. Neurochem.* 20:122.

Barbarese, E., and S. E. Pfeiffer. 1981. Developmental regulation of myelin basic protein in dispersed cultures. *Proc. Natl. Acad. Sci. USA.* 78:1953–1957.

Barbarese, E., P. E. Braun, and J. H. Carson. 1977. Identification of prelarge and presmall basic proteins in mouse myelin and their structural relationship to large and small basic proteins. *Proc. Natl. Acad. Sci. USA.* 74:3360–3364.

Barbarese, E., J. H. Carson, and P. E. Braun. 1978. Accumulation of the four myelin basic proteins in mouse brain during development. *J. Neurochem.* 31:779–782.

Benjamins, J. A., M. Jones, and P. Morrell. 1975. Appearance of newly synthesized protein in myelin of young rats. *J. Neurosci.* 24:1117–1122.

Bernier, L., F. Alvarez, E. M. Norgard, D. W. Raible, A. Mentaberry, J. G. Schembri, D. D. Sabatini, and D. R. Colman. 1987. Molecular cloning of a 2′, 3′-cyclic nucleotide 3′-phosphodiesterase: mRNAs with different 5′ ends encode the same set of proteins in nervous and lymphoid tissues. *J. Neurosci.* 7:2703–2710.

Boggs, J. M., M. A. Moscarello, and D. Papahadjopoulos. 1982. Structural organization of myelin: role of lipid-protein interactions determined in model systems. *In* Lipid-Protein Interactions. Jost, P. C., and O. H. Griffiths, editors. John Wiley & Sons, Inc., New York. 1–51.

Campagnoni, A. T. 1988. Molecular biology of myelin proteins from the central nervous system. *J. Neurochem.* 51:1–14.

Chen, C., and H. Okayama. 1987. High-efficiency transformation of mammalian cells by plasmid DNA. *Mol. Cell. Biol.* 7:2745–2752.

Colman, D. R., G. Kreibich, A. B. Frey, and D. D. Sabatini. 1982. Synthesis and incorporation of myelin polypeptides into CNS myelin. *J. Cell Biol.* 95:598–608.

de Ferra, F., H. Engh, L. Hudson, J. Kamholz, C. Puckett, S. Molineaux, and R. A. Lazzarini. 1985. Alternative splicing accounts for the four forms of myelin basic protein. *Cell.* 43:721–727.

Dingwall, C., and Laskey, R. A. 1986. Protein import into the cell nucleus. *Annu. Rev. Cell Biol.* 2:367–390.

Dubois-Dalcq, M., T. Behar, L. Hudson, and R. A. Lazzarini. 1986. Emergence of three myelin proteins in oligodendrocytes cultured without neurons. *J. Cell Biol.* 102:384–392.

Dupouey, P., C. Jacque, J. M. Bourre, F. Cesselin, A. Privat, and N. Baumann. 1979. Immunochemical studies of myelin basic protein in *shiverer* mouse devoid of major dense line of myelin. *Neurosci. Lett.* 12:113–118.

Dyer, C. A., and J. A. Benjamins. 1989. Organization of oligodendroglial membrane sheets. I: Association of myelin basic protein and 2',3'-cyclic nucleotide 3'-phosphohydrolase with cytoskeleton. *J. Neurosci. Res.* 24:201–211.

Forman, B. M., C. Yang, F. Stanley, J. Casanova, and H. H. Samuels. 1988. c-erbA protooncogenes mediate thyroid hormone-dependent and independent regulation of the rat growth hormone and prolactin genes. *Mol. Endocrinol.* 2:902–911.

Gorman, C. M., B. H. Howard, and R. Reeves. 1983. Expression of recombinant plasmids in mammalian cells is enhanced by sodium butyrate. *Nucleic Acids Res.* 11:7631–7648.

Hartman, B. K., H. C. Agrawal, S. Kalmbach, and W. T. Shearer. 1979. A comparative study of the immunohistochemical localization of basic protein to myelin and oligodendrocytes in rat and chicken brain. *J. Comp. Neurol.* 188:273–290.

Hirayama, M., D. H. Silberberg, R. P. Lisak, and D. Pleasure. 1983. Long-term culture of oligodendrocytes isolated from rat corpus callosum by Percoll density gradient: lysis by polyclonal antigalactocerebroside serum. *J. Neuropath. Exp. Neurol.* 42:16–28.

Holmes, E., G. Hermanson, R. Cole, and J. de Vellis. 1988. Development expression of glial-specific mRNAs in primary cultures of rat brain visualized by in situ hybridization. *J. Neurosci. Res.* 19:389–396.

Jordan, C., V. Friedrich, and M. Dubois-Dalcq. 1989. *In situ* hybridization analysis of myelin gene transcripts in developing mouse spinal cord. *J. Neurosci.* 9:248–257.

Jordan, C. A., V. L. Friedrich Jr., F. de Ferra, D. G. Weismiller, K. V. Holmes, and M. Dubois-Dalcq. 1990. Development exon expression in myelin basic protein transcripts during central nervous system (CNS) remyelination. *Cell. Mol. Neurobiol.* 10:3–18.

Katsuki, M., M. Sato, M. Kimura, M. Yokoyama, K. Kobayashi, and T. Nomura. 1988. Conversion of normal behavior to shiverer by myelin basic protein antisense cDNA in transgenic mice. *Science (Wash. DC).* 241:593–595.

Kimura, M., M. Sato, A. Akatsuka, S. Nozawa-Kimura, R. Takahashi, M. Yokoyama, T. Nomura, and M. Katsuki. 1989. Restoration of myelin formation by a single type of myelin basic protein in transgenic shiverer mice. *Proc. Natl. Acad. Sci. USA.* 86:5661–5665.

Knapp, P. E., W. P. Bartlett, and R. P. Skoff. 1987. Cultured oligodendrocytes mimic in vivo phenotypic characteristics: cell shape, expression of myelin-specific antigens, and membrane production. *Dev. Biol.* 120:356–365.

Knapp, P. E., R. P. Skoff, and T. J. Sprinkle. 1988. Differential expression of galactocerebroside, myelin basic protein, and 2',3'-cyclic nucleotide 3'-phosphohydrolase during development of oligodendrocytes in vitro. *J. Neurosci. Res.* 21:249–259.

Lubetzki, C., P. Lombrail, J. J. Hauw, and B. Zalc. 1986. Multiple sclerosis: rat and human oligodendrocytes are not the target for CSF immunoglobulins. *Neurology.* 36:524–528.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. 545 pp.

Monge, M., D. Kadiiski, C. M. Jacque, and B. Zalc. 1986. Oligodendroglial expression and deposition of four major myelin constituents in the myelin sheath during development. An in vivo study. *Dev. Neurosci.* 8:222–235.

Privat, A., C. Jacque, J. M. Bourre, P. Dupouey, and N. Baumann. 1979. Absence of the major dense line in myelin of the mutant mouse *shiverer*. *Neurosci. Lett.* 12:107–112.

Raine, C. S. 1984. Morphology of myelin and myelination. *In* Myelin. Morell, P., editor. Plenum Publishing Corp., New York. 1–50.

Ranscht, B., P. A. Clapshaw, J. Price, M. Noble, and W. Seifert. 1982. Development of oligodendrocytes and Schwann cells studied with a monoclonal antibody against galactocerebroside. *Proc. Natl. Acad. Sci. USA.* 79:2709–2713.

Readhead, C., B. Popko, N. Takahashi, H. D. Shine, R. A. Saavedra, R. L. Sidman, and L. Hood. 1987. Expression of a myelin basic protein gene in transgenic *shiverer* mice: correlation of the dysmyelinating phenotype. *Cell.* 48: 703–712.

Roach, A., K. Boylan, S. Horvath, S. B. Prusiner, and L. E. Hood. 1983. Characterization of cloned cDNA representing rat myelin basic protein: absence of expression in brain of *shiverer* mutant mice. *Cell.* 34:799–806.

Roussel, G., and J. L. Nussbaum. 1981. Comparative localization of Wolfgram W1 and myelin basic proteins in rat brain during ontogenesis. *Histochem. J.* 13:1029–1047.

Saavedra, R. A., L. Fors, R. H. Aebersold, B. Arden, S. Horvath, J. Sanders, and L. Hood. 1989. The myelin proteins of the shark brain are similar to the myelin proteins of the mammalian peripheral nervous system. *J. Mol. Evol.* 29:149–156.

Shiota, C., M. Miura, and K. Mikoshiba. 1989. Developmental profile and differential localization of mRNAs of myelin proteins (MBP and PLP) in oligodendrocytes in the brain and in culture. *Dev. Brain Res.* 45:83–94.

Smith, R. 1977. Non-covalent cross-linking of lipid bilayers by myelin basic protein: a possible role in myelin formation. *Biochim. Biophys. Acta.* 470: 170–184.

Staugaitis, S. M., P. R. Smith, and D. R. Colman. 1990. Expression of myelin basic protein isoforms in nonglial cells. *J. Cell Biol.* 110:1719–1727.

Sternberger, N. H., Y. Itoyama, M. W. Kies, and H. deF. Webster. 1978. Immunocytochemical method to identify basic protein in myelin-forming oligodendrocytes of newborn rat C. N. S. *J. Neurocytol.* 7:251–263.

Szuchet, S., P. E. Polak, and S. H. Yim. 1986. Mature oligodendrocytes cultured in the absence of neurons recapitulate the ontogenic development of myelin membranes. *Dev. Neurosci.* 8:208–221.

Trapp, B. D., T. Moench, M. Pulley, E. Barbosa, G. Tennekoon, and J. Griffin. 1987. Spatial segregation of mRNA encoding myelin-specific proteins. *Proc. Natl. Acad. Sci. USA.* 84:7773–7777.

Verity, A. N., and A. T. Campagnoni. 1988. Regional expression of myelin protein genes in the developing mouse brain: in situ hybridization studies. *J. Neurosci. Res.* 21:238–248.

Zeller, N. K., T. N. Behar, M. E. Dubois-Dalcq, and R. A. Lazzarini. 1985. The timely expression of myelin basic protein gene in cultured rat brain oligodendrocytes is independent of continuous neuronal influences. *J. Neurosci.* 5:2955–2962.

Zopf, D., V. Sonntag, H. Betz, and E. D. Gundelfinger. 1989. Developmental accumulation and heterogeneity of myelin basic protein transcripts in the chick visual system. *Glia.* 2:241–249.