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Spatial segregation of mRNA encoding myelin-specific proteins

(oligodendrocytes/Schwann cells/*in situ* hybridization/immunocytochemistry)

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ABSTRACT **The cellular and subcellular distributions of mRNAs encoding three myelin-specific proteins—myelin basic protein (MBP), proteolipid protein (PLP), and Po protein—were studied in tissue sections of developing rat nervous systems by *in situ* hybridization. The developmental appearance of these mRNAs closely paralleled the appearance of the proteins they encode as determined by immunocytochemistry. mRNA encoding the extrinsic membrane protein, MBP, was concentrated around oligodendrocyte and Schwann cell nuclei during initial stages of myelination; as myelination proceeded, MBP mRNA became distributed diffusely over myelinated fibers. In contrast, mRNAs encoding the intrinsic membrane proteins, PLP and Po, remained concentrated around oligodendrocyte (PLP) and Schwann cell (Po) nuclei at all stages of myelination. These results establish that myelinating cells spatially segregate certain myelin-specific mRNAs. The presence of MBP mRNA within the cytoplasmic domains of myelin internodes indicates that protein sorting during myelination involves transportation of mRNA to specific subcellular sites.**

Myelin, a multilamellar compact membrane, surrounds many axons in the central and peripheral nervous systems (CNS and PNS). CNS myelin is formed by oligodendrocytes, which individually have the potential to form 30–40 different myelin internodes by extending long slender cytoplasmic processes that ensheath and spirally wrap around axons to form compact myelin. In the PNS, Schwann cells form single myelin internodes. The protein composition of CNS and PNS myelin is well characterized (1). Ultrastructural studies have shown that CNS and PNS myelination occurs in a systematic and predictable manner (2, 3), suggesting that myelin-forming cells utilize very efficient mechanisms for synthesis, transport, and integration of myelin components to accomplish the massive expansion of membrane.

Formation of myelin represents a terminal phenotypic expression of oligodendrocytes and Schwann cells that must involve the expression of a myelin-specific genetic program. DNAs complementary (cDNA) to the mRNAs that encode several myelin proteins have been cloned recently (4–10). Using these clones, the time courses for expression of myelin-specific mRNAs have been analyzed during development (4–10). The present study focuses on the cellular distribution of three myelin-specific mRNAs. Using *in situ* hybridization, we determined when these mRNAs could first be detected during early stages of myelination and asked whether spatial segregation of certain mRNAs might occur. Our results indicate a close relationship between initial transcription and translation of myelin gene products. Some myelin proteins are synthesized in the perikaryon and are subsequently transported to myelin membranes for insertion. mRNA for one protein (myelin basic protein; MBP) was diffusely distributed throughout myelin-forming cells, suggesting that MBP is synthesized near the site of its insertion into myelin. Results of this study have been presented in abstract form (11, †).

MATERIALS AND METHODS

Tissues. Sprague–Dawley rats (10- and 35-day-old and adults) were perfused with 4% paraformaldehyde in 0.08 M phosphate buffer. Lumbar and cervical spinal cord, medulla, pons, and cerebral hemispheres including diencephalon, trigeminal nerves, and liver were removed, placed in fixative overnight, and processed to paraffin by standard procedures. All tissues from individual animals were embedded in a single block. Serial 6-μm-thick sections were cut, placed on glass slides, deparaffinized, and rehydrated before use. *In situ* hybridization and immunocytochemistry were performed separately on adjacently cut sections.

***In Situ* Hybridization.** Sections on slides were hybridized according to the method of Moench *et al.* (12) with minor modifications. Prehybridization treatment consisted of 0.2 M HCl for 20 min at room temperature followed by protease K (25 μg/ml) for 15 min at 37°C. Hybridization consisted of incubating the sections for 16 hr at room temperature with recombinant DNA (0.2 μg)labeled with ^{35}S by nick-translation in a standard hybridization buffer (13). Posthybridization washes included incubating the sections in 2× SSC (1× SSC = 0.3 M NaCl/0.03 M sodium citrate, pH 7.4) in 1 mM EDTA for 1 hr at 55°C and in 50% formamide containing 0.3 M NaCl, 1 mM EDTA, and 5 mM Tris (pH 8) for 30 min at room temperature. Autoradiography was performed by standard procedures with use of NTB-3 nuclear track emulsion (Kodak) melted at 43°C and diluted 1:1 with 0.6 M ammonium acetate. After development of the emulsion, the sections were stained with hematoxylin, dehydrated, and overlaid with coverslips. Sections were photographed with a Zeiss photomicroscope using bright-field and dark-field optics.

The cDNA clones used have been well characterized and described elsewhere: proteolipid protein (PLP) (6), MBP (4), and Po (8). The specificity of cDNA hybridization was demonstrated by pretreating control tissue sections with RNase.

Immunocytochemistry. Sections were stained by the peroxidase-antiperoxidase procedure as described (14). The antisera directed against MBP (15) and Po protein (16) have been well characterized. PLP antiserum was kindly provided by Jean Marie Matthieu (University of Lausanne, Lausanne, Switzerland). Immunostained sections were photographed under bright-field optics.

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Abbreviations: MBP, myelin basic protein; PLP, proteolipid protein; CNS, central nervous system; PNS, peripheral nervous sytem.

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RESULTS

Specificity of mRNA Hybridization. To determine the specificity of our *in situ* hybridization procedure, the distributions of MBP, PLP, and Po mRNAs were compared to the tissue distributions of the proteins they encode in sections of trigeminal nerve from 35-day-old rats (Fig. 1). The trigeminal nerve was well-suited for this study because it contains both CNS and PNS myelin. As expected, MBP antiserum stained both CNS and PNS myelin (Fig. 1*A*), whereas PLP and Po antisera stained CNS and PNS myelin, respectively (data not shown). Silver grains produced by MBP cDNA were diffusely distributed over both CNS and PNS myelin (Fig. 1*B*). Similar to MBP immunoreaction product, the concentration of MBP mRNA was greater in the CNS than in the PNS. Silver grains produced by PLP cDNA were restricted to the CNS and concentrated around oligodendrocyte nuclei (Fig. 1*C*). Silver grains produced by Po cDNA were restricted to the PNS and concentrated around Schwann cell nuclei (Fig. 1*D*).

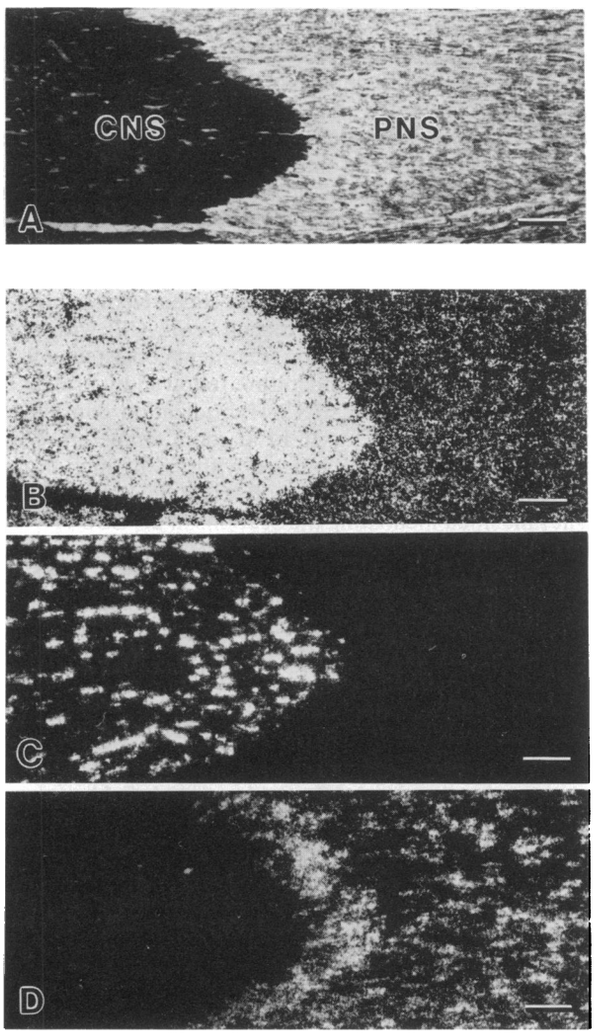

FIG. 1. Comparison of the tissue distribution of MBP (*A*), MBP mRNA (*B*), PLP mRNA (*C*), and Po mRNA (*D*) at the CNS–PNS junction of 35-day-old rat trigeminal nerve. The intensity of dark immunoreaction product (*A*) reflects the higher concentration of MBP in CNS myelin when compared to PNS myelin. MBP mRNA visualized as bright spots of silver grains photographed in dark field (*B*) is diffusely distributed over myelinated fibers in both CNS and PNS and is detected at a higher concentration in the CNS. PLP mRNA is restricted to the CNS, where it is concentrated around oligodendrocyte nuclei (*C*). Po mRNA is restricted to the PNS, where it is concentrated around Schwann cell nuclei (*D*). (*A*) Bright-field image. (*B–D*) Dark-field images. (Bar = 100 μm.)

Sections of liver were included on all sides hybridized and never contained grain counts above background. Pretreatment of slides with RNase abolished specific binding of all cDNA probes.

Developmental Appearance of MBP and PLP mRNAs. Because rostral regions of the rat CNS begin to myelinate during the first two postnatal weeks (15, 17, 18), we began our developmental study by testing for the initial appearance of MBP and PLP mRNAs in sections from the telencephalon of 10-day-old animals (Fig. 2 *A* and *B*). Regions of the telencephalon just beginning to myelinate were identified by faint immunoreaction product produced by PLP and MBP antisera. When sections cut adjacent to those immunostained were hybridized with PLP and MBP cDNA, silver grains were readily detectable around oligodendrocyte nuclei (Fig. 2*C*) that were located in the same areas as immunoreaction product (Fig. 2*D*). Cells in the periventricular zone, the presumed origin of oligodendrocyte precursors, were not labeled by either PLP or MBP cDNAs. These results established that PLP and MBP mRNAs could be detected during initial stages of myelination.

In addition to the general caudal-to-rostral gradient in the initiation of CNS myelination, different fiber tracts at any one level of the rat CNS can begin to myelinate at different times. For example, MBP immunoreaction product indicated that myelination was well-advanced in the upper portion of the dorsal column from 10-day-old rat spinal cord (Fig. 3*A*). In contrast, myelination was just beginning in the lower half. Sections cut serial to Fig. 3*A* were hybridized with MBP and PLP cDNAs. Both MBP (Fig. 3*B*) and PLP (Fig. 3*C*) mRNAs were found at higher concentrations over the actively myelinating region. MBP mRNA was distributed diffusely in a pattern similar to MBP immunoreaction product. In contrast, PLP mRNA was found in clusters around oligodendrocyte nuclei. Fewer silver grains were found over the region undergoing initial stages of myelination. PLP mRNA and the majority of MBP mRNA were concentrated around oligodendrocyte nuclei.

The above results established that the developmental appearance of MBP and PLP mRNAs closely paralleled the appearance of the proteins they encode and that PLP mRNA was always concentrated around oligodendrocyte nuclei. MBP mRNA was concentrated around cell nuclei during early stages of myelination, but during more active stages it was diffusely distributed over myelinated fibers. This is clearly shown when Fig. 3*B* is viewed at higher magnification with bright-field optics (Fig. 3*D*). Intermediate stages in the transition of MBP mRNA distribution were found. For example, in a region of pons from a 10-day-old rat MBP mRNA was concentrated around oligodendrocyte nuclei and distributed diffusely along longitudinally oriented fiber tracts (Fig. 3*E*).

Since the majority of CNS myelination occurs in the rat during the first postnatal month, we wanted to determine whether MBP and PLP mRNAs were present at detectable levels in tissue sections from adult animals. Both MBP (Fig. 4*A*) and PLP (Fig. 4*B*) mRNAs were readily detectable in sections from adult brains. MBP mRNA was distributed over myelinated fibers, while PLP mRNA was concentrated around oligodendrocyte nuclei.

Satellite Oligodendrocytes and Schwann Cells. Oligodendrocytes and Schwann cells can be found in close apposition to neuronal perikarya. Since the myelinating capabilities of these satellite cells have been questioned, we investigated whether they expressed myelin-specific mRNAs. Satellite oligodendrocytes were labeled intensely by PLP cDNA (Fig. 4*C*). These cells also were labeled by MBP cDNA during

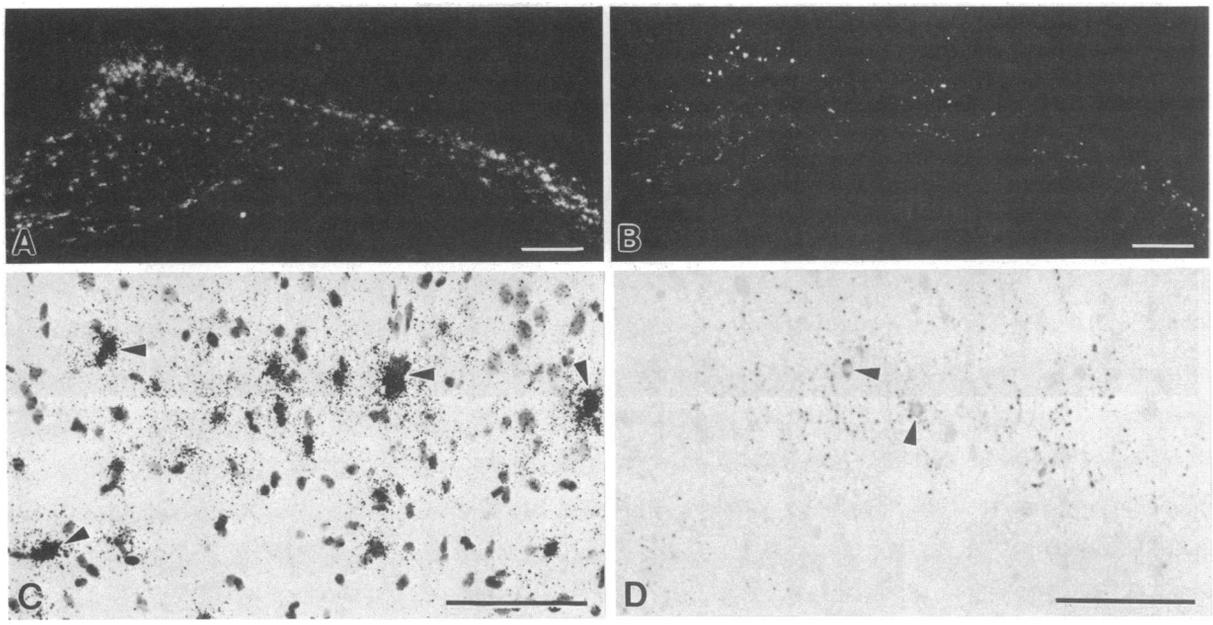

FIG. 2. Sections of 10-day-old rat brain hybridized with MBP cDNA (*A* and *C*), PLP cDNA (*B*), and immunostained with MBP antiserum (*D*). MBP (*A*) and PLP (*B*) mRNAs are present within the corpus callosum. At higher magnification, MBP mRNA (*C*) is shown to be concentrated around oligodendrocyte nuclei (arrowheads). Adjacent sections of regions containing MBP mRNA are stained by MBP antisera (*D*). Particulate staining represents thin myelin sheaths; oligodendrocyte cytoplasm is faintly stained (arrowheads). (*A* and *B*) Dark-field images. (*C* and *D*) Bright-field images of adjacently cut sections. (*A* and *B*, bars = 500 μm; *C* and *D*, bars = 75 μm.)

early stages of myelination (data not shown). In contrast, Po mRNAs were detected in satellite Schwann cells (Fig. 4*D*).

DISCUSSION

mRNAs encoding MBP, PLP, and Po proteins were localized in paraffin sections of developing rat nervous systems by *in situ* hybridization. The developmental appearance of PLP and MBP mRNAs in different brain regions reflected the known caudal-to-rostral progression as well as local asynchrony in the process of CNS myelination. The initial appearance of these mRNAs closely paralleled the initial appearance of the proteins they encode. Although silver

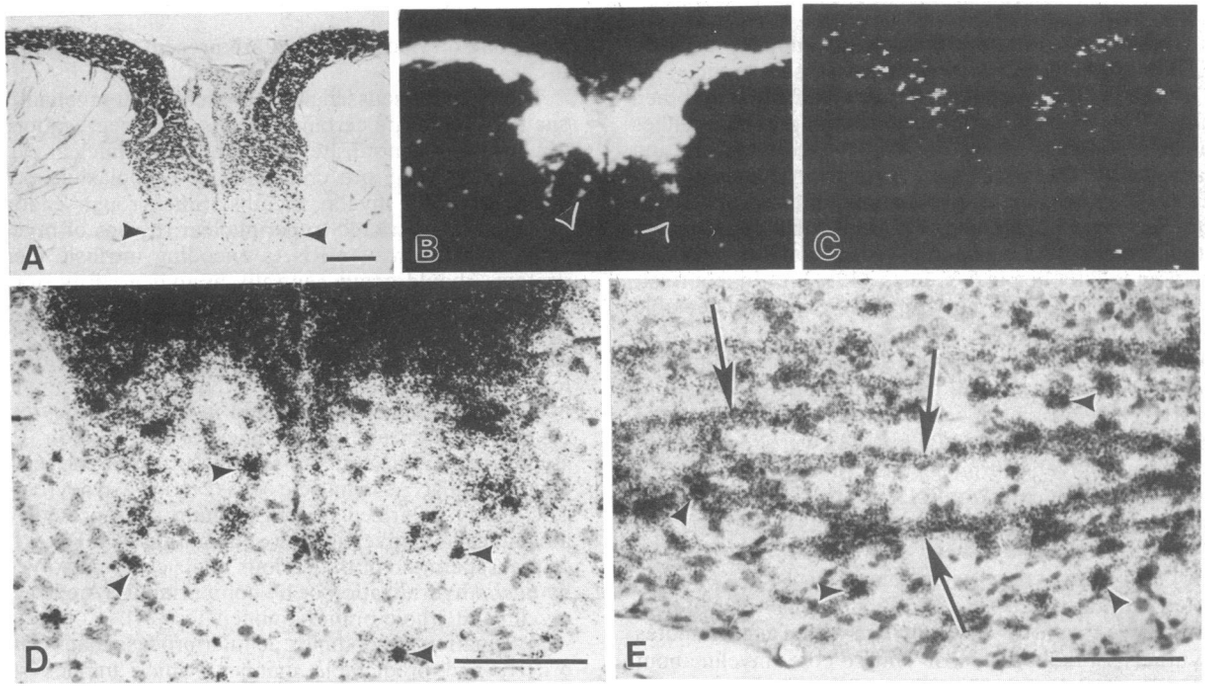

FIG. 3. Paraffin sections of 10-day-old rat brain. The distributions of MBP (*A*), MBP mRNA (*B*), and PLP mRNA (*C*) are compared in serial sections of the dorsal spinal cord. The intensity of MBP immunoreaction product (*A*) indicates variations in the extent of myelination. MBP (*B*) and PLP (*C*) mRNAs are enriched in the actively myelinating regions; MBP mRNA is distributed diffusely, whereas PLP mRNA is concentrated around oligodendrocyte nuclei. Regions just beginning to myelinate (*A*, arrowheads) contain less MBP and PLP mRNAs; both are concentrated around oligodendrocyte nuclei. (*D*) Bright-field image of a portion of *B*. In a section of pons (*E*), MBP mRNA is enriched around oligodendrocyte nuclei and distributed diffusely along longitudinally oriented myelinated tracts. (*A*, *D*, and *E*) Bright-field images; (*B* and *C*) dark-field images. (Bars = 100 μm.)

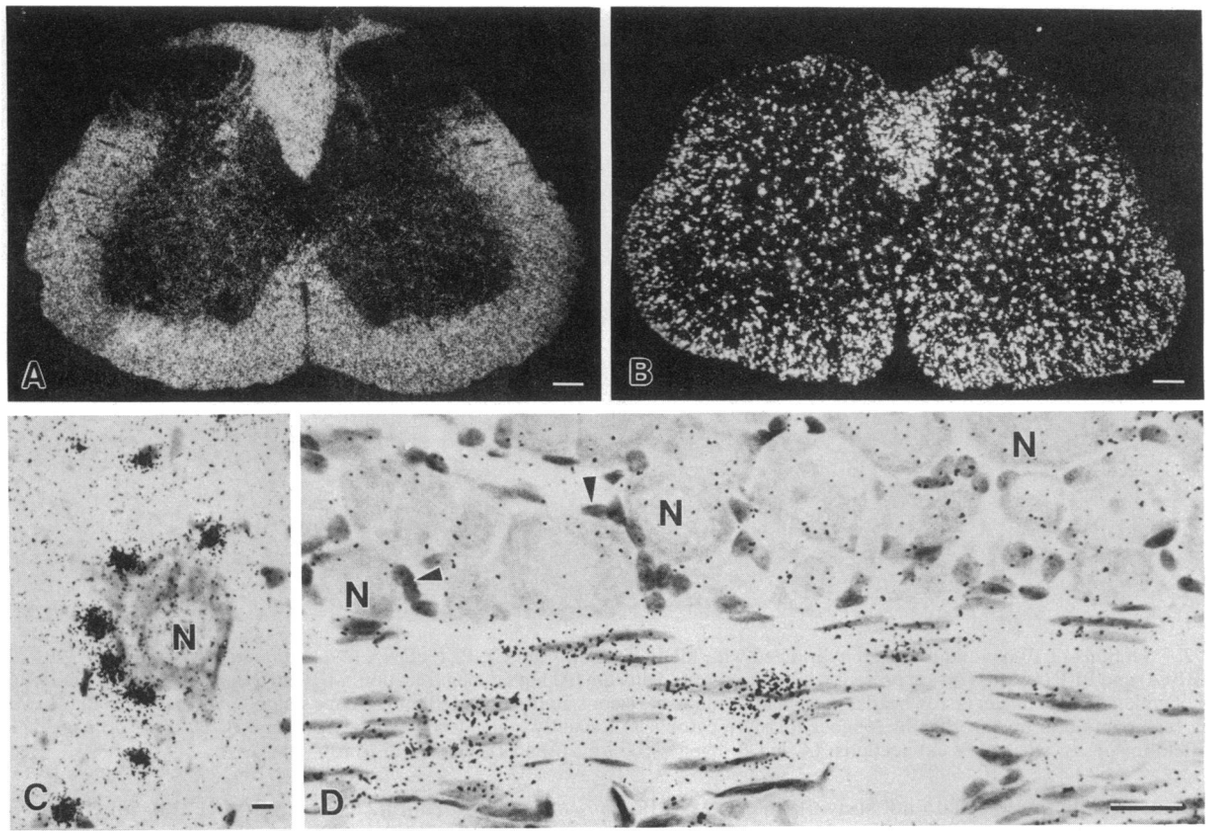

FIG. 4. In transverse sections of adult rat spinal cord, MBP mRNA is distributed over myelinated fibers in white and grey matter (*A*), whereas PLP mRNA is concentrated around oligodendrocyte nuclei (*B*). Oligodendrocytes closely apposing neurons in the adult spinal cord are labeled intensely by PLP cDNA (*C*). In longitudinal sections of 35-day-old trigeminal nerve (*D*), Po mRNA is concentrated around Schwann cell nuclei in myelinated tracts but is not detected in Schwann cells that closely appose neurons (arrowheads). N, neuronal perikarya. (*A* and *B*) Dark-field images, (*C* and *D*) bright-field images. (*A* and *B*, bars = 200 μm; *C* and *D*, bars = μm.)

grains were not quantitated, the general intensity of PLP, MBP, and Po hybridization signal increased to maximal levels during the most active stages of myelination and then decreased. These results are consistent with previous studies (4–10), suggesting a close relationship between transcription and translation of PLP, MBP, and Po gene products throughout the process of normal myelination. Intense labeling of satellite oligodendrocytes with PLP and MBP cDNAs supports their myelinating capabilities as demonstrated by previous immunocytochemical studies (19, 21). In contrast, satellite Schwann cells do not appear to express detectable amounts of Po or MBP mRNAs.

A major finding of this study was the diffuse distribution of MBP mRNA over myelinated fibers. Diffuse distribution of MBP mRNA in white matter was apparent in previous studies (22, 23). In contrast, PLP and Po mRNAs were restricted to the perikarya of myelin-forming cells. These results support the hypothesis that MBP mRNA is transported to and translated at sites near compact myelin. This hypothesis was based on a 20-fold enrichment of MBP mRNA in RNA extracts of myelin fractions when compared to RNA extracts of whole brain homogenates (24). Biochemical studies (24–26) have shown that MBP enters myelin within a few minutes after synthesis. In contrast, PLP and Po enter myelin about 30 min after synthesis. The sites of synthesis of these molecules (MPB close to myelin, PLP and Po far from myelin) are probably responsible for their kinetics of entry into myelin. MBP immunoreaction product cannot be detected in perinuclear regions of oligodendrocytes during active stages of myelination (17, 18). Therefore, the majority of MBP synthesis is most likely to occur along the myelin internode on ribosomes that are present in the outer tongue processes (2).

The results discussed above indicate that myelinating cells spatially segregate certain mRNAs. This segregation can be divided into a central site consisting of perinuclear cytoplasm and a peripheral site consisting of cytoplasmic processes associated with myelin sheaths. Since rough endoplasmic reticulum is restricted to perinuclear regions of myelinating cells, translation of mRNAs encoding intrinsic membrane proteins should occur centrally as indicated by our results with PLP and Po. In contrast, mRNAs encoding extrinsic membrane proteins could be located centrally and peripherally (free ribosomes are present at both locations). These locations need not be mutually exclusive. For example, some MBP mRNA may be translated in oligodendrocyte perinuclear regions during active stages of myelination. Previous studies have indicated site-specific transport or segregation of certain mRNAs in muscle cells (27) and astrocytes (28, 29). The highly polarized myelin-forming cells provide several advantages for investigating segregation of mRNA because spatial separation of the various pools can be resolved easily by *in situ* hybridization or by comparing RNA extracts from myelin and whole brain homogenates (24).

Myelination is a complex cellular process that occurs in an orderly and predictable manner, which implies stringent regulation, probably involving many intricately related control mechanisms. The apparent close parallel between the expression of the major structural myelin proteins and the expression of their mRNAs during normal myelination suggests that transcriptional control mechanisms are a major regulator. Since oligodendrocytes can form 40 different myelin internodes and myelin internodes in the PNS can be

2 mm long, additional control mechanisms operating close to myelin sheaths may exist. Translational regulation of mRNA within the cytoplasmic domains of myelin internodes could potentially play a role in such regulation.

Among the higher vertebrates, intracellular transport within myelin-forming cells is probably only exceeded by that within neurons. However, compared to the neuron very little is known about how myelin components are transported. Three major categories of transport should be considered: (*i*) vesicular transport of integral membrane proteins; (*ii*) transport of peripheral membrane proteins (as soluble molecules or in association with other structures); and (*iii*) transport of mRNA encoding peripheral membrane proteins. Why and how myelin-forming cells preferentially transport mRNA rather than the translational product remains to be determined. Identification and characterization of mRNAs, other than MBP, that are preferentially located centrally or peripherally within myelin-forming cells will help address these questions.

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