In situ Hybridization Analysis of Myelin Gene Transcripts in Developing Mouse Spinal Cord

Craig Jordan, Victor Friedrich, Jr., and Monique Dubois-Dalcq

Laboratory of Molecular Genetics, National Institute of Neurological and Communicative Disorders and Stroke, National Institutes of Health, Bethesda, Maryland 20892

We analyzed the location and abundance of transcripts for the 4 CNS myelin protein genes, myelin basic protein (MBP), proteolipid protein (PLP), myelin-associated glycoprotein (MAG), and 2',3'-cyclic nucleotide phosphohydrolase (CNP), in the mouse cervical spinal cord from the time of rapid myelination until adulthood (8–45 d). In the white matter, maximal levels of transcripts were found for each of the myelin genes at the peak of myelination (8 d). Total MBP and PLP mRNAs stayed high until 20 d and showed a minor decrease thereafter. In contrast, MAG and the MBP exon 2 containing transcripts (coding for the 21.5 and 17 kDa MBP isoforms) decreased sharply between 8 and 20 d, suggesting that high levels of these transcripts are needed primarily during the initiation of myelination. CNP transcripts were less abundant, maintained high expression until 20 d, and then decreased sharply. PLP, MAG, and CNP transcripts were clustered in the oligodendrocyte cell body, while MBP mRNAs were scattered throughout the cell body and processes. In contrast to the white matter, all these myelin specific transcripts in the gray matter showed a marked increase from 8 to 20 d, as did the number of oligodendrocytes identified by CNP immunostaining. MAG transcripts were found in white matter and in satellite and other oligodendrocytes of the gray matter but not in neurons identified by their expression of neurofilament transcripts. The results of our quantitative *in situ* hybridization study are in good agreement with those of previous molecular studies and provide new information on the cellular and topographic distribution of myelin-specific mRNAs during myelination.

Oligodendrocytes are multipolar glial cells of the CNS that synthesize, transport, and integrate myelin components in their processes before wrapping them around axons to form multilamellar myelin internodes (reviewed in Benjamins and Smith, 1984; Raine, 1984). The genetic program controlling the expression of myelin proteins in oligodendrocytes appears tightly coordinated and regulated (reviewed by Lemke, 1986; Sutcliffe, 1987). With the recent cloning and sequencing of myelin protein genes, it became possible to identify the various mRNA species

encoding one or several isoforms of 4 myelin proteins: proteolipid protein (PLP, 2 isoforms), myelin basic protein (MBP, at least 4 isoforms), 2',3'-cyclic nucleotide phosphohydrolase (CNP, 2 isoforms), and myelin-associated glycoprotein (MAG, 2 isoforms) (Zeller et al., 1984; de Ferra et al., 1985; Naismith et al., 1985; Takahashi et al., 1985; Diehl et al., 1986; Arquint et al., 1987; Bernier et al., 1987; Lai et al., 1987). PLP is synthesized on rough endoplasmic reticulum and is the major CNS myelin protein (50% of total myelin protein) (reviewed by Lees and Brostoff, 1984; Norton and Cammer, 1984). It is an acylated, very hydrophobic protein that spans the myelin membrane and probably plays an essential structural role in myelin compaction (Duncan et al., 1987). MBP (30% of total myelin protein) is synthesized on free ribosomes, probably close to the cytoplasmic face of the oligodendrocyte membrane, and it is involved in compaction of the major dense line of myelin (Privat et al., 1979; Colman et al., 1982; Omlin et al., 1982). Although CNP (5% of total myelin protein) is found in both CNS and PNS myelin, its function is unknown (reviewed by Norton and Cammer, 1984). Finally, MAG (1% of total myelin protein) is structurally similar to several known cell adhesion molecules (reviewed by Williams, 1987) and appears to mediate contacts between oligodendrocyte processes and axons before being excluded from compact myelin (reviewed by Trapp and Quarles, 1984; Quarles, 1985). These 4 myelin proteins emerge almost synchronously in oligodendrocytes within each CNS tract shortly before the start of myelination (Sternberger et al., 1979; Rousset and Nussbaum, 1981; Hartman et al., 1982; Monge et al., 1986). The timely expression of myelin protein genes appears to be independent of continuous neuronal influences since it also occurs *in vitro* when oligodendrocytes are cultured without neurons (Zeller et al., 1985; Dubois-Dalcq et al., 1986).

Expression of the major myelin protein genes appears to be regulated at the level of transcription (Zeller et al., 1984, 1985; Hudson et al., 1987; Trapp et al., 1987). MBP and PLP transcripts are differentially distributed within the oligodendrocyte cytoplasm as observed by *in situ* hybridization. Using double-stranded cDNA probes, MBP mRNAs are first detected in the cell body of oligodendrocytes and later in the processes (Zeller et al., 1985; Kristensson et al., 1986; Trapp et al., 1987), while PLP transcripts stay confined to the cell body (Branks and Wilson, 1986; Trapp et al., 1987). We have now performed a comparative analysis of transcript location and abundance of 4 myelin genes—MBP, PLP, CNP, and MAG—in the developing CNS utilizing single-stranded cRNA and oligonucleotide probes labeled with ³⁵S to equal specific activity. Single-stranded probes offer several advantages over cDNA double-stranded probes: They have a higher hybridization efficiency, are not susceptible

Received Feb. 19, 1988; revised May 31, 1988; accepted June 1, 1988.

We would like to thank Drs. L. D. Hudson, R. A. Lazzarini, and K. Koller for the gift of probes; Dr. W. S. Young III for advice and use of the Loats image-analysis system; Drs. F. de Ferra, N. K. Zeller, and L. D. Hudson for helpful discussions; Mr. R. Rusten for photographic printing; and Mrs. J. Carrillo for painstaking manuscript preparation.

Correspondence should be addressed to Dr. Craig Jordan, NIH/NINCDS, Building 36, Room 4A01; Bethesda, MD 20892.

0270-6474/89/010248-10\$02.00/0

Figure 1. Oligonucleotide probe specificities and Northern analysis of both DNA and RNA probes. **A.** The myelin genes for MBP, PLP, and MAG are schematically represented (genes and exons are not to scale) in order to show the position of the oligonucleotide probes along the genome (shaded boxes). Oligonucleotide probes for CNP 3' and 5' regions (shaded boxes) are positioned along a cDNA clone (see details in Materials and Methods) (Bernier et al., 1987). **B.** Northern blot demonstrating specific transcripts detected by the following probes: MAG exon 7–8 (lanes a, b), PLP exon 2 (lanes c, d), PLP exon 3 (lanes e, f), MBP exon 2 (lanes g, h), and MBP exon 1 (lanes i, j) were hybridized to 5 µg/lane of total brain RNA (lanes a, c, e, g, i) or total liver RNA (lanes b, d, f, h, j) from 20-d-old mice. Note that MBP exon 1 probe (lane i) detected mRNA trapped in ribosomal RNA markers, and this accounts for the additional bands (which were not seen when poly A-selected RNA was used). **C.** Northern blot of poly(A)⁺ RNA from 20-d-old mouse brain (lane a, 10 µg; lane b, 5 µg) hybridized with a mixture of CNP 3' and 5' probes (lane a) and with cRNA PLP probe (lane b).

to hyperpolymer formation, which may influence quantitation, and anti-sense probes can be used for control of specificity (Haase et al., 1981; Cox et al., 1984). In addition, oligonucleotide probes can be designed to differentiate between different types of transcripts. In mouse, alternative splicing generates 4 MBP transcripts, each of which codes for 1 of the 4 MBP isoforms (Zeller et al., 1984; de Ferra et al., 1985). Exon 2-containing transcripts code for 2 of these MBP isoforms (21.5 and 17 kDa) whose expression appears developmentally regulated (Barbarese et al., 1978; Carson et al., 1983); in this study we have utilized exon 2-specific probes to study the expression of early developmental forms of MBP mRNA directly *in situ*.

Here we describe the distribution and relative abundance of transcripts for 4 myelin protein genes in the mouse spinal cord from 8 to 45 d after birth. In the white matter, the peak expression of each type of transcript coincides with the peak of myelination. However, subsequent expression of the different transcripts varies: high levels of MAG and MBP exon 2-containing transcripts are observed only during the most active myelination period while mature MBP and PLP transcripts are still steadily expressed in the adult white matter. CNP expression shows a more complex pattern. The expression of all 4 myelin specific genes is delayed in gray matter as compared to white matter. Our results indicate that *in situ* hybridization is a most sensitive and precise method to study regional distribution and relative abundance of myelin gene transcripts during development.

Materials and Methods

Tissue preparation. C57B1/6N mice 8, 20, and 45 d old were anesthetized with methoxyflurane followed by chloral hydrate administered intraperitoneally (0.35–0.5 mg/gm body weight). Mice were perfused with 4% formaldehyde freshly prepared from paraformaldehyde in 0.1 M phosphate buffer (pH 7.2), and spinal cords were dissected out, cut into transverse pieces, cryoprotected overnight in 15% sucrose (RNase free in the same buffer, frozen in liquid nitrogen, and stored at –70°C until sectioning. Eight to ten micron sections were cut on a cryostat and thaw-mounted on gelatin-coated slides that had been heated 2 hr at 170°C to inactivate possible traces of ribonuclease (RNase). Sections were stored up to 3 weeks at –20°C before use.

Probes. Single-stranded RNA probes were prepared in the following way. A 930 base pair fragment corresponding to the mouse PLP-coding region and some 5'-untranslated sequence was subcloned into pGEM 3 (kindly supplied by L. D. Hudson; Hudson et al., 1987), which contains SP6 and T7 promoters. The PLP plasmid was linearized with Eco-R1 and transcribed with SP-6 polymerase, following the conditions recommended by Promega Biotec (Madison, WI) in the presence of 30 µCi of (α-³²S) UTP (1250 Ci/mmol, Amersham Corp., Arlington Heights, IL). The RNA probes were purified on G-50 Sephadex columns, alkaline-hydrolyzed to 200–400 base fragments (Cox et al., 1984), and stored with 100 mM dithiothreitol (DTT) and 1200 U/ml RNase-inhibitor (Boehringer Mannheim Biochemicals, Indianapolis, IN).

Oligonucleotide probes (48–54 nucleotides) were synthesized on an Applied Biosystems DNA synthesizer following the manufacturer's recommendations and published sequences for PLP (exon 2, nucleotides no. 220–267; exon 3, nucleotides no. 514–561, following the numbering of Hudson et al., 1987), MBP (exon 1, nucleotides no. 38–85; exon 2, nucleotides no. 238–285, following the numbering of de Ferra et al.,

Figure 2. Comparison of cRNA and oligonucleotide DNA probes by *in situ* hybridization. Twenty-day-old mouse spinal cord cross sections were hybridized with the cRNA PLP probe (*a*), oligonucleotide probes for PLP exon 2 and exon 3 (*b*), and an oligonucleotide complementary to the PLP exon 3 probe as a control (*c*). Note the typical clustering of grains in both white and gray matter (see also Fig. 4). The cRNA probes showed some background binding over the gray matter between the clusters of grains. All sections were treated identically, exposed for 14 d and photographed in dark-field illumination as are Figures 3–5. Scale bar, 500 μ m.

1985), MAG (exon 7–8 junction, nucleotides no. 958–1007, following the numbering of Arqint et al., 1987), and CNP (5'-end probe, nucleotides no. 46–93; 3'-end probe, nucleotides no. 1087–1134, following the numbering of Bernier et al., 1987). A synthetic oligonucleotide corresponding to the mouse small neurofilament (NF) gene (the kind gift of K. Koller) was made following the published sequence (nucleotides no. 117–170, from the cDNA clone of Lewis and Cowan, 1985). The oligonucleotides were usually purified on 8 M urea/10% acrylamide gels. Labeling was accomplished with terminal deoxynucleotidyl-transferase and (35 S) dATP (1200 Ci/mmol, Amersham Corp.) (Zeller et al., 1985). Probes had tail lengths in the range of 15–25 residues and were stored at 4°C with 100 mM DTT for up to 6 weeks.

Northern analysis. RNA was extracted from brains and livers of 20-d-old C57Bl/6N mice with guanidinium thiocyanate (Maniatis et al., 1982). Poly A⁺ mRNA was selected with oligo (dt)-cellulose, electrophoresed on formaldehyde/agarose gels, and transferred to nitrocellulose

membranes. Filters were hybridized overnight at 40°C with the RNA or DNA probes, washed, and visualized by autoradiography.

In situ hybridization. The technique is basically that of Young et al. (1986). The cryostat sections were removed from -20°C storage and placed at room temperature for 10 min. Sections were then fixed again in 4% formaldehyde in PBS, rinsed, acetylated, dehydrated, delipidated, and air-dried. Sections were then hybridized with a mixture containing 50% deionized formamide (Fluka Chemical Corp., Ronkonkoma, NY), 4 $\times$ saline sodium citrate or SSC (1 $\times$ SSC consists of 150 mM NaCl, and 15 mM sodium citrate), 500 $\mu\text{g}/\text{ml}$ sheared single-stranded salmon sperm DNA, 250 $\mu\text{g}/\text{ml}$ yeast t-RNA (both from Sigma Chemical Company), 1 $\times$ Denhardt's solution (0.02% ficoll, 0.02% polyvinyl pyrrolidone, 0.02% BSA fraction V), 10% dextran sulfate (MW = 500,000), 100 mM DTT, and $1\text{--}2 \times 10^6$ cpm of probe per 50 μl . Usually, groups of 2–4 spinal cord sections were hybridized with 20 μl of the mixture, covered with a small piece of parafilm, and incubated overnight in a humid chamber at 37°C.

After hybridization the parafilm coverslips were removed in 1 $\times$ SSC followed by 3 quick rinses in 1 $\times$ SSC. At this point, specimens hybridized with cRNA probes were treated differently than those with DNA oligonucleotide probes. Sections hybridized with cRNA probes were treated with (1) RNase A (100 $\mu\text{g}/\text{ml}$) and RNase T₁ (10 U/ml) (both from Boehringer Mannheim, Indianapolis, IN) in 1 mM EDTA, 0.5 M NaCl, 10 mM Tris, pH 8.0, for 30 min at 37°C; (2) 1 $\times$ SSC; (3) 2 $\times$ SSC with 50% formamide at 40°C for two 15 min changes; and (4) 0.1 $\times$ SSC at 50°C for two 15 min changes. Sections hybridized with DNA oligonucleotide probes were washed in (1) 2 $\times$ SSC with 50% formamide at 40°C for four 15 min changes; (2) 1 $\times$ SSC at room temperature for two 30 min changes. All slides were rinsed in H₂O followed by ethanol and air-dried. Dried slides were dipped into Kodak NTB2 emulsion diluted 1:1 with H₂O at 45°C and allowed to expose at 4°C for 14–40 d. The emulsion was developed with D-19 (Kodak, 1/2 strength) and Kodak fixer before being washed and counterstained with cresyl violet (0.5% in 0.1 M sodium acetate, pH 4). Sections were partially destained with 5 mM of the sodium acetate buffer in 95% ethanol.

Densitometric analysis. Autoradiograms were analyzed with a Loats image-analysis system (Loats, Westminster, MD) using dark-field illumination and measurement windows from $2\text{--}40 \times 10^3 \mu\text{m}^2$. Two to four optical density determinations were made from each of 6 standard locations in 2 sections for each age/probe combination. For comparison of developmental patterns among the different probes (Fig. 8), values were corrected at each age for background (binding to substantia gelatinosa and central canal) and data for each probe scaled to give a value of 100% for the most intense binding.

Immunocytochemistry. Sections were immunolabeled for CNP by the immunoperoxidase method (Sternberger et al., 1970). Sections were immersed in acetone at -20°C for 10 min, rinsed with 0.5 M Tris buffer, pH 7.6 (TB), treated for 15 min at room temperature with TB containing 0.1% gelatin and 0.1% sodium azide (TBG) with 3% normal goat serum, and left overnight at 4°C in rabbit anti-CNP serum (kindly provided by F. A. McMorris; McMorris et al., 1984) diluted 1:300 in TBG. The following day, sections were treated at 20°C with the following: TB, 15 min; goat anti-rabbit IgG, 150 $\mu\text{g}/\text{ml}$ in TBG, 30 min; TB, 15 min; rabbit horseradish peroxidase-antiperoxidase complex, 35 $\mu\text{g}/\text{ml}$ in TBG without azide, 30 min; TB, 15 min; 0.01% hydrogen peroxide and 0.05% diaminobenzidine in TB, 8 min; PBS, 15 min; 0.02% osmium tetroxide in PBS, 1 min; PBS, 5 min. Sections were then dehydrated, and coverslips were mounted with Permount. For counting, only profiles with a clearly visible nucleus and immunostained cytoplasm were counted. This criterion was necessary because of the concurrent myelin membrane staining and resulted in conservative estimates of cell numbers.

Results

Characterization of the probes

Figure 1*A* represents the position of the MBP exon 1- and exon 2-specific oligonucleotide probes along the MBP genome. Both oligonucleotide MBP probes hybridized on Northern blots of mouse total brain RNA to a wide band of approximately 2.2 kilobase (kb), as expected for rodent MBP (Fig. 1*B*, *g* and *i*) (Zeller et al., 1984; Mentaberry et al., 1986; Popko et al., 1987). Signal from the exon 2 probe (Fig. 1*B*, *g*) is less intense than that from the exon 1 probe (Fig. 1*B*, *i*) since the exon 2 probe binds only to the 2 early forms of MBP mRNAs (coding for the

Figure 3. Differential expression of MBP exon 1- and exon 2-containing transcripts. Cervical spinal cord sections from 8-, 20-, and 45-d-old mice were hybridized with either a MBP exon 1-specific (*left side*) or a MBP exon 2-specific probe (*right side*). While binding sites in the white matter are very high with exon 1 probe at 8 and 20 d, exon 2 binding sites dramatically decrease from 8 to 20 d. In contrast, gray matter signal increased substantially with both probes from 8 to 20 d (see also Figs. 7 and 8 for quantitation data). With exon 1 probe at day 20, grains are seen aligned along myelinating fibers crossing from the dorsal funiculi to the dorsal horns (*arrows*). Although MBP signal is predominantly detected as a diffuse grain pattern in the white matter, clusters of grains are seen at day 8 in the gray matter. For all procedures, sections were treated identically and exposed for 14 d. Different age cross sections are scaled to equivalent sizes. Scale bar, 1 mm.

21.5 and 17 kDa proteins), while the exon 1 probe binds to all 4 major MBP mRNA forms.

We used 3 PLP probes (1 cRNA, 2 exon-specific oligonucleotide probes) (see Materials and Methods). The diagram in Figure 1*A* shows the position of the oligonucleotide probes for exon 2 and exon 3 along the PLP genome. The exon 3 probe corresponds to a region deleted in one type of PLP transcript (referred to as DM20). The 2 oligonucleotide probes were mixed together for *in situ* hybridization to increase the hybridization signal. When the cRNA probe or the oligonucleotide probes were hybridized to mouse brain mRNA on Northern blots (Fig. 1*B*, *c* and *e*; and 1*C*, *b*), they revealed 2 strong bands and 1 weak band corresponding to the expected sizes (4.8, 3.2, and 2.4 kb) for mouse PLP mRNAs (Hudson et al., 1987). These transcripts result from the use of alternative polyadenylation

sites but cover the same coding region (Milner et al., 1985; Hudson et al., 1987).

The single probe used for MAG transcript detection is an oligonucleotide that spans the junctions of exons 7 and 8 (Fig. 1*A*). This region is common to both the early and late forms of MAG (Lai et al., 1987), corresponds to an extracellular portion of the MAG molecule located between 2 immunoglobulin-like domains, and has less than 35% nucleotide homology to N-CAM, another member of the Ig superfamily expressed in the CNS (reviewed by Williams, 1987). When hybridized on Northern of mouse brain RNA, a single species of approximately 3.0 kb was recognized (Fig. 1*B*, *a*). This message is of the appropriate size, although we were not able to resolve the 2 MAG messages described as a doublet in the 2.5–3.5 kb range (Lenoir et al., 1986; Arquint et al., 1987; Salzer et al., 1987).

Figure 4. Expression of PLP and MAG transcripts. As in Figure 3, cervical spinal cord sections from 8-, 20-, and 45-d-old mice were hybridized with either a combination of PLP exon 2 and exon 3 probes (*left side*) or the MAG exon 7–8 probe (*right side*) in the same conditions used for MBP probes. Transcripts are organized in discrete clusters both in gray and white matter at all ages. White matter signal decreased earlier with MAG (8–20 d) than with PLP (20–45 d). Gray matter signal increased dramatically from 8 to 20 d for both transcripts with a peak at 20 d. Scale bar, 1 mm.

The 2 oligonucleotide probes for CNP correspond to 3' and 5' regions of a known cDNA clone (Fig. 1*A*). For hybridization, the 2 probes were mixed to maximize signal and increase the amount of genetic information detected since splicing is likely to occur with CNP (Bernier et al., 1987). As can be seen from the Northern blot in Figure 1*C, a*, these oligonucleotide probes detected a broad band probably corresponding to the 2.4 and 2.8 kb transcripts described by Bernier et al. (1987). [The bands are not distinct because we used more poly A selected RNA (10 μ g) in this lane to enhance its detection.]

Comparison between RNA and oligonucleotide probes

cRNA probes corresponding to full-length coding sequences for PLP and MBP were compared with 48 nucleotide DNA oligonucleotide probes in order to evaluate relative levels of background and sensitivity during *in situ* hybridization. Results obtained with both single-stranded RNA and DNA probes were consistent and reproducible (Fig. 2).

With the same short autoradiographic exposure time, cRNA probes displayed higher sensitivity than the oligonucleotide probes. This is most likely due to the much shorter sequence represented in the oligonucleotide probes. After longer exposure times (Fig. 2), the difference in sensitivity was no longer striking, but cRNA probes produced a higher background, especially noticeable in the gray matter between oligodendrocytes. This increased background was also seen with the MBP cRNA probe (data not shown) and is probably due to the non-specific binding of very small partial transcripts resulting from the alkaline hydrolysis performed after cRNA labeling (see Materials and Methods). Since cRNA probes cause increased background, require a cDNA clone as a template, and are susceptible to endogenous RNase digestion, we have conducted our developmental experiments with oligonucleotide cDNA probes. In some cases, we mixed 2 of these probes to increase the number of binding sites on the transcripts to increase the hybridization signal.

Figure 5. Expression of CNP transcripts. Cervical spinal cord sections from 8-, 20-, and 45-d-old mice were hybridized with a combination of CNP 3' and 5' probes. As with PLP and MAG transcripts (Fig. 4), the hybridization signal is mostly arranged in clusters, although not as tightly organized (see also Fig. 6). White matter signal decreases with age in general, with the exception of the dorsal funiculus signal, which is still high at 20 d. As with the other genes, gray matter signal increases from day 8 to day 20 with a peak at day 20. All procedures were the same as for Figures 3 and 4 except for the exposure time, which was increased to 40 d. Scale bar, 1 mm.

In order to minimize variability and allow comparative analysis of data, all *in situ* hybridizations depicted in Figures 2–5 were performed simultaneously on proximate sections from one block of tissue for each age; these sections were treated with the same reagents, exposed for the same time (except where noted), and developed with the same chemicals.

MBP transcripts

As mentioned above, exon 2 is present only in the 2 mRNA forms coding for the 21.5 and 17 kDa MBPs while exon 1 is present in all 4 forms of MBP transcripts. Cryostat sections from 8-, 20-, and 45-d-old mouse cervical spinal cords were hybridized separately with MBP probes specific for exon 1 and exon 2 (Fig. 3). Both probes bound to similar regions of the spinal cord during development and were more abundant in white matter than in gray matter. In white matter, the amount of probe binding decreased with age with both exon 1 and exon 2 oli-

Figure 6. Differential localization of myelin gene transcripts as seen in bright-field microscopy. Hybridization patterns for the different probes in spinal cord ventral horn which contains large neuron cell bodies stained by cresyl violet (arrowheads). In addition, a neurofilament probe detected a large number of transcripts in these neurons (e). In a, MBP exon 2 probe, 20 d tissue. The diffuse silver grains are seen around the neurons and in higher number in the lower-left portion of the picture, which shows part of the white matter. In b, CNP 3' and 5' probes, 20 d tissue. Grain clusters are associated with the stained nuclei of putative oligodendrocytes, some of them closely associated with neurons. These grains are not as densely packed as those seen with PLP or MAG (compare with c and d). In c, PLP exon 2 and 3 probes, 45 d tissue. Grains are clustered around nuclei sometimes closely associated with neurons and are tightly organized. In d, MAG exon 7–8 probe (45 d tissue) also shows transcripts in tight clusters around nuclei (arrows), although the signal is less intense than with PLP. In e, NF probe, 8 d tissue. Only the neurons are labeled, a pattern never seen with any of the myelin-specific probes tested here. Scale bars, 20 μ m.

gonucleotides; however, the exon 2-containing transcripts decreased earlier, more rapidly, and to a significantly lower level than exon 1-containing MBP transcripts. In contrast, MBP transcripts increased in the gray matter, peaking at 20 d. This increase was especially noticeable in areas where myelinated fibers pass from the dorsal columns to the dorsal horn.

Figure 7. Densitometric measurement of MBP exon 1 probe binding. Images of MBP exon 1 probe binding to 8-, 20-, and 45-d-old spinal cord tissue were digitized and the optical density was measured. Three white matter areas (dorsal, ventral, and lateral funiculi) and three gray matter regions (ventral horn: *ven. horn*; central canal: *cent. can.*; and substantia gelatinosa: *subst. gel.*) were measured. Points represent average optical densities from 2–4 measurements for each of 2 sections. The vertical axis is oriented so that maximum probe binding is at the top.

PLP and MAG transcripts

When PLP and MAG probes were hybridized to sections of 8-d-old mouse spinal cord, very intense binding was seen throughout the white matter, with grains clustered around and above oligodendrocyte cell bodies (Figs. 4 and 6). This localization in the perikaryon was strikingly different from the diffuse binding seen with MBP specific probes (Fig. 3). For both PLP and MAG, grain clusters in white matter were close to each other at 8 d and became more widely spaced at 20 d (Fig. 4). However, the intensity of individual clusters and of overall white matter binding remained high for PLP at 20 d, while decreasing markedly for MAG (Fig. 4). PLP binding decreased later, between 20 and 45 d. Thus, in their white matter abundance, MAG transcripts resemble MBP exon 2 transcripts in showing a striking decline between 8 and 20 d, while PLP transcripts, like total MBP transcripts, decrease later.

Grain clusters were also seen in the gray matter of PLP- and MAG-labeled sections; the clusters increased in number between days 8 and 20 (Fig. 4). A neuron-specific probe (NF) was hybridized to nearby sections in order to compare the pattern of labeling of neurons and oligodendrocytes in the gray matter. As expected, intense binding of the NF probe to neurons was seen, especially in ventral horn cells (Fig. 6e). This pattern of labeling did not overlap with that of MAG or PLP mRNA, which were only detected in satellite and other oligodendrocytes (Fig. 6, c, d).

CNP transcripts

In initial experiments, the level of CNP probe binding was low after the standard 14 d exposure. This suggests that the level of

CNP transcripts is lower than that of the other myelin genes. Therefore, the emulsion exposure was prolonged to 40 d and gave the results shown in Figure 5. The general pattern of CNP transcript distribution was similar to that of the other myelin gene transcripts. In white matter the transcripts varied in abundance from region to region but as a whole were maximally expressed at 8 and 20 d and then decreased. As with the other myelin gene transcripts, CNP transcripts in the gray matter region increased from 8 to 20 d and then decreased. CNP transcripts were detected as perinuclear clusters, which were more loosely organized than the clusters seen with MAG and PLP (Fig. 6b). The low abundance of CNP transcripts as compared with PLP and MBP transcripts might be expected since CNP constitutes only 5% of myelin protein. A discrepancy between levels of message and protein could exist due to differences in turnover rates.

Quantitative and comparative analysis of in situ hybridization data

To confirm and extend the qualitative observations presented above, we measured grain densities in several regions of spinal cord using an image analyzer (as described in the Materials and Methods). The results for MBP exon 1 are presented in detail in Figure 7, which shows the highest binding in the 3 white matter areas measured (dorsal, lateral, and ventral funiculi) and the lowest binding over the substantia gelatinosa and central gray matter. Myelin and oligodendrocytes are almost completely absent from the latter 2 regions, and grains over those regions represent background; consequently, optical densities over other areas were corrected for this background. The 3 white matter regions showed similar grain densities (Fig. 7); in order to simplify comparison between different probes, we combined data from the 3 white matter regions.

Analyzed in this way, the densitometric data revealed different developmental patterns of transcript expression (Fig. 8). As expected, white matter always showed greater binding than gray matter. In white matter, MBP exon 1, PLP and CNP probe binding showed relatively little change from 8 to 20 d; by contrast, MBP exon 2 and MAG probe decreased dramatically during this period. Binding of all probes decreased in white matter between 20 and 45 d; this decrease was most pronounced for MBP exon 2 and CNP transcripts. The pattern exhibited in gray matter was substantially different. Binding increased dramatically between 8 and 20 d for all probes, including those whose binding to white matter decreased during the same interval (MBP exon 2 and MAG).

Densitometric analysis determines overall binding intensity but gives no information about the clustering of transcripts observed with certain genes. These clusters, which are found around the nuclei of small cells, most likely localized in the perikaryon of individual oligodendrocytes. Grain clusters were most clearly seen in the gray matter, where oligodendrocytes are scattered and can thus be counted easily. We counted oligodendrocytes in the gray matter after immunostaining for CNP and observed an increase of about 2.5-fold in number per spinal cord cross section between days 8 and 20 (data not shown). We then counted the number of grain clusters in gray matter after *in situ* hybridization with the CNP probe and observed a similar increase. Finally, we compared the number of grain clusters in spinal cord cross sections seen with CNP, PLP, and MAG (Fig. 9). All 3 probes yielded similar developmental curves: the number of clusters visualized was lowest at 8 d, increased several

Figure 8. Densitometric measurement of probe binding for the different myelin gene transcripts. Grain density was measured for 5 different probes as shown for MBP exon 1 probe in Figure 7. Data for each probe was corrected and normalized independently. The central canal and substantia gelatinosa values were averaged and subtracted from all other values as a correction for background binding (see Fig. 7). All 3 white matter regions were averaged to yield the plotted white matter values $\pm$ SE (vertical bars). Ventral horn data is the average of multiple measurements $\pm$ SE. All values for a particular probe were normalized as a percentage of the maximal binding (100%). A, MBP exon 1 probe. B, MBP exon 2 probe. C, PLP exon 2 and exon 3 probes. D, MAG exon 7-8 probe. E, CNP 3' and 5' probes.

fold at 20 d, and remained unchanged thereafter (Fig. 9). However, the absolute number of grain clusters detected at each age differed markedly among the 3 probes. This variation may reflect differences between the myelin genes in absolute number of transcript copies per cell and in the degree to which transcripts are confined to the cell body and excluded from processes. As shown in Figure 6, grain clusters in the PLP preparations were intense and more defined than those in the CNP sections, and many clusters in the CNP sections may not have been detected.

Discussion

In this *in situ* hybridization study of mouse spinal cord, we compared the location and abundance of transcripts coding for 4 CNS myelin protein genes from the myelination period to adulthood. Similar conditions of fixation, cryocutting, and hybridization with single-stranded probes of matched specific activity were rigorously applied to CNS tissues taken at 3 different time points. The quantitative observations made here by *in situ* hybridization parallel changes in transcript abundance observed by others using Northern blot analysis. In such a molecular

analysis, PLP transcripts in adult were approximately 50% less than in 18-d-old mouse brain (Hudson et al., 1987; L. Hudson, personal communication); we found the same decrease in spinal cord with *in situ* hybridization. Total MBP mRNAs also remain high in adult CNS (Zeller et al., 1984), a pattern consistent with the substantial binding of exon 1 probe to adult spinal cord (Fig. 8). The sharp decrease of exon 2-containing MBP transcripts in the white matter observed here by *in situ* hybridization was also demonstrated by RNase protection experiments (de Ferra, in preparation).

In situ hybridization revealed a progressive decrease of MAG transcripts in spinal cord white matter with age, although less dramatic than that of MBP exon 2. According to Lai and co-workers (1987), MAG transcripts lacking exon 12 information predominate in the postnatal period while transcripts containing exon 12 predominate in adults. Our probes did not distinguish between these 2 transcripts; it seems likely that the substantial levels observed at 45 d correspond to the "late" MAG mRNAs.

The 35 S-labeled probes used in our experiments yielded sufficient resolution to reveal information about the cellular lo-

Figure 9. Number of grain clusters (representing putative oligodendrocyte perikaryons) as detected by *in situ* hybridization. Grain clusters over gray matter regions were counted in mouse spinal cord sections (8, 20, and 45 d of age) that had been hybridized with probes for PLP, MAG, and CNP. Grain clusters from the CNP probe were less tightly organized, and this may have resulted in an underestimate of their numbers. The plotted points represent the mean $\pm$ SE for counts from 2–4 sections each.

calization of the myelin transcripts. We found PLP and MAG transcripts concentrated in cell bodies, as would be expected for mRNAs associated with rough endoplasmic reticulum and as described previously for PLP in the CNS and P_0 in the PNS (Trapp et al., 1987). MBP *in situ* hybridization revealed that both exon 1 and exon 2 transcripts exhibit a diffuse pattern consistent with the presence of these mRNAs in oligodendrocyte processes (Colman et al., 1982; Zeller et al., 1985; Kristensson et al., 1986; Trapp et al., 1987). CNP, like MBP, is synthesized on free ribosomes (Karin and Waehneldt, 1985; Bernier et al., 1987); surprisingly, CNP showed some clustering of its transcripts within the oligodendrocyte perikaryon. The distribution of probe binding for the 4 myelin gene transcripts was in all cases comparable to that of oligodendrocytes visualized in proximate sections of the spinal cord with CNP immunostaining and different from neurons revealed by NF probe binding. In comparing these preparations, we found no evidence for expression of MBP, PLP, MAG, or CNP in cells other than oligodendrocytes.

MAG was detected only in oligodendrocytes when antibodies to the purified protein were used (Sternberger et al., 1979). Our localization of MAG transcripts (in the spinal cord) to oligodendrocytes is in good agreement with the immunolabeling data. Previous immunolabeling and *in situ* hybridization studies with a brain-specific cDNA clone (1B236), now known to correspond to MAG (Lai et al., 1987), have demonstrated expression first in myelinating regions and later in neuron-rich regions of the rat brain (Sutcliffe et al., 1983; Lenoir et al., 1986; Milner et al., 1987; Wilson et al., 1987). Although we cannot exclude the possibility that some neurons produce low levels of mRNAs for MAG or some closely related molecule, or that neurons differ from spinal cord and brain, it appears likely that the signal in cortical regions described in these previous studies could correspond to late-developing satellite oligodendrocytes.

The evidence that oligodendrocytes in gray matter regions may indeed differentiate later than those in the white matter is compelling, at least for mouse spinal cord. We found an increase in myelin-specific transcripts in the gray matter between 8 and 20 d. At the same time, the same transcripts remain constant or decrease in white matter, indicating that myelination of spinal

cord gray matter is delayed relative to white matter. Since the increase in gray matter myelin-specific mRNAs parallels an increase in the number of oligodendrocytes (revealed by CNP immunostaining or by *in situ* grain clusters), it seems likely that oligodendrocytes are generated or mature later in gray than in white matter. There is mounting evidence that the different myelination schedules of each tract of the CNS depend on differences in the timing of migration and/or mitosis of the progenitor cell of oligodendrocytes in these specific regions (Small et al., 1987).

In comparing transcript levels in white matter, one can distinguish 2 types of transcripts. MAG and MBP exon 2-containing transcripts are maximal at 8 d, a time when most tracts are starting to myelinate in the spinal cord, and are decreased substantially at 20 d. In contrast, PLP and MBP transcripts devoid of exon 2 information remain at a high level or increase between 8 and 20 d, and decrease moderately thereafter. CNP transcripts exhibit an intermediate pattern; at 20 d the average white matter binding is equal to that at 8 d (Fig. 8), but individual regions show substantial variation (Fig. 5). This difference in developmental pattern suggests that the level of expression of different transcripts is regulated independently. MAG has been implicated in initial recognition and adhesion between axons and oligodendrocyte processes (reviewed by Trapp and Quarles, 1984; Quarles, 1985). Exon 2 of MBP codes for a sequence of amino acids whose folding might be critical for interaction with myelin lipids and/or PLP in the early events of myelination (discussed in Kamholz et al., 1986). Thus, both MAG and MBP exon 2 transcripts might be critically required in the early events of myelination. On the other hand, PLP and mature forms of MBP make up the bulk of proteins within the myelin sheath (reviewed by Lees and Brostoff, 1984), they undergo some turnover in the adult and may therefore require sustained synthesis of their transcripts for both myelin compaction and maintenance.

Thus, *in situ* hybridization for myelin genes can be used as a reliable and convenient way to analyze gene expression at the level of single cells and fiber tracts *in vivo*. It provides information on *in vivo* events of differentiation and cellular and topographic organization not available from Northern blot analysis. Our approach also appears well suited to the analysis of myelin gene transcripts in focal lesions during the course of a demyelinating disease (Jordan et al., unpublished observations).

References

- Arquint, M., J. Roder, L.-S. Chia, J. Down, D. Wilkinson, H. Bayley, P. Braun, and R. Dunn (1987) Molecular cloning and primary structure of myelin-associated glycoprotein. *Proc. Natl. Acad. Sci. USA* 84: 600–604.
- 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., and M. E. Smith (1984) Metabolism of myelin. In *Myelin*, P. Morell, ed., pp. 225–258, Plenum, New York.
- 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.
- Branks, P. O., and M. C. Wilson (1986) Patterns of gene expression in the murine brain revealed by *in situ* hybridization of brain-specific mRNAs. *Mol. Brain. Res.* 1: 1–16.
- Carson, J. H., M. L. Nielson, and E. Barbarese (1983) Developmental regulation of myelin basic protein expression in mouse brain. *Dev. Biol.* 96: 485–492.
- 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.
- Cox, K. H., D. V. DeLeon, L. M. Angerer, and R. C. Angerer (1984) Detection of mRNAs in Sea Urchin embryos by *in situ* hybridization using asymmetric RNA probes. *Exp. Biol.* 101: 485-502.
- 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.
- Diehl, H.-J., M. Schaich, R.-M. Budzinski, and W. Stoffel (1986) Individual exons encode the integral membrane domains of human myelin proteolipid protein. *Proc. Natl. Acad. Sci. USA* 83: 9807-9811.
- 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.
- Duncan, I. D., J. P. Hammang, and K. F. Jackson (1987) Jimpy myelin lacks PLP and has a defect in the intraperiod line. *Soc. Neurosci. Abstr.* 13: 118.
- Haase, A. T., P. Ventura, C. J. Gibbs, Jr., and W. W. Tourtellotte (1981) Measles virus nucleotide sequences. Detection by hybridization *in situ*. *Science* 212: 672-675.
- Hartman, B. K., H. C. Agrawal, D. Agrawal, and S. Kalmbach (1982) Development and maturation of central nervous system myelin: Comparison of immunohistochemical localization of proteolipid protein and basic protein in myelin in oligodendrocytes. *Proc. Natl. Acad. Sci. USA* 79: 4217-4220.
- Hudson, L. D., J. Berndt, C. Puckett, C. A. Kozak, and R. A. Lazzarini (1987) Aberrant splicing of proteolipid protein mRNA in the dysmyelinating jimpy mutant mouse. *Proc. Natl. Acad. Sci. USA* 84: 1454-1458.
- Kamholz, J., F. de Ferra, C. Puckett, and R. Lazzarini (1986) Identification of three forms of human myelin basic protein by cDNA cloning. *Proc. Natl. Acad. Sci. USA* 83: 4962-4966.
- Karin, N. J., and T. V. Waehneltdt (1985) Biosynthesis and insertion of Wolfram protein into optic nerve membranes. *Neurochem. Res.* 10: 897-907.
- Kristensson, K., N. K. Zeller, M. E. Dubois-Dalcq, and R. A. Lazzarini (1986) Expression of myelin basic protein gene in the developing rat brain as revealed by *in situ* hybridization. *J. Histochem. Cytochem.* 34: 467-473.
- Lai, C., M. A. Brow, K.-A. Naxe, A. B. Noronha, R. H. Quarles, F. E. Bloom, R. J. Milner, and J. G. Sutcliffe (1987) Two forms of IB236/myelin-associated glycoprotein, a cell adhesion molecule for postnatal neural development, are produced by alternative splicing. *Proc. Natl. Acad. Sci. USA* 84: 4337-4341.
- Lees, M. B., and S. W. Brostoff (1984) Proteins of myelin. In *Myelin*, P. Morell, ed., pp. 197-224, Plenum, New York.
- Lemke, G. (1986) Molecular biology of the major myelin genes. *TINS* 9: 266-270.
- Lenoir, D., E. Battenberg, M. Kiel, F. E. Bloom, and A. J. Milner (1986) The brain specific gene IB236 is expressed postnatally in the developing rat brain. *J. Neurosci.* 6: 522-530.
- Lewis, S. A., and N. J. Cowan (1985) Genetics, evolution and expression of the 68,000-mol-wt neurofilament protein: Isolation of a cloned cDNA probe. *J. Cell Biol.* 100: 843-850.
- Maniatis, T., E. F. Fritsch, and J. Sambrook (1982) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory, NY.
- McMorris, F. A., S. V. Kim, and T. J. Sprinkle (1984) Intracellular localization of 2',3'-cyclic nucleotide 3'-phosphohydrolase in rat oligodendrocytes and C₆ glioma cells and effect of cell maturation and enzyme induction on location. *Brain Res.* 292: 123-131.
- Mentaberry, A., M. Adesnik, M. Atchison, E. M. Norgard, F. Alvarez, D. D. Sabatini, and D. R. Colman (1986) Small basic proteins of myelin from central and peripheral nervous systems are encoded by the same gene. *Proc. Natl. Acad. Sci. USA* 83: 1111-1114.
- Milner, R. J., G. A. Higgins, H. Schmale, and F. E. Bloom (1987) Localization of cell-type-specific mRNAs by *in situ* hybridization. In *In Situ Hybridization Applications to Neurobiology*, K. L. Valentine, J. H. Eberwine, and J. D. Barchas, eds., pp. 163-178, Oxford U. P., New York.
- 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.
- Naismith, A. L., E. Hoffman-Chudzik, L.-C. Tsui, and J. R. Riordan (1985) Study of the expression of myelin proteolipid protein (lipophilin) using a cloned complementary DNA. *Nucleic Acids Res.* 13: 7413-7425.
- Norton, W. T., and W. Cammer (1984) Isolation and characterization of myelin. In *Myelin*, P. Morell, ed., pp. 147-195, Plenum, New York.
- Omlin, F. X., H. deF. Webster, C. G. Paklovitz, and S. R. Cohen (1982) Immunocytochemical localization of basic protein in major dense line regions of central and peripheral myelin. *J. Cell Biol.* 95: 242-248.
- Popko, B., C. Puckett, E. Lai, H. D. Shine, C. Readhead, N. Takahashi, S. W. Hunt, III, R. L. Sidman, and L. Hood (1987) Myelin deficient mice: Expression of myelin basic protein and generation of mice with varying levels of myelin. *Cell* 48: 713-721.
- Privat, A. C., J. M. Jacque, P. 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.
- Quarles, R. H. (1985) Myelin-associated glycoprotein in development and disease. *Dev. Neurosci.* 6: 285-303.
- Raine, C. S. (1984) Morphology of myelin and myelination. In *Myelin*, P. Morell, ed., pp. 1-50, Plenum, New York.
- Roussel, G., and J. L. Nussbaum (1981) Comparative localization of Wolfram W1 and myelin basic protein in the rat brain during ontogenesis. *Histochem. J.* 13: 1029-1047.
- Salzer, J. L., W. P. Holmes, and D. R. Colman (1987) The amino acid sequences of the myelin-associated glycoprotein: Homology to the immunoglobulin gene super family. *J. Cell Biol.* 104: 957-965.
- Small, R. K., P. Riddle, and M. Noble (1987) Evidence for migration of oligodendrocyte Type 2 astrocyte progenitor cells into the developing rat optic nerve. *Nature* 328: 155-157.
- Sternberger, L. A., P. H. Hardy, Jr., J. J. Cuculis, and H. G. Meyer (1970) The unlabeled antibody enzyme method of immunohistochemistry—Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish) and its use in identification of spirochetes. *J. Histochem. Cytochem.* 18: 315-333.
- Sternberger, N. H., R. H. Quarles, Y. Itoyama, and H. deF. Webster (1979) Myelin-associated glycoprotein demonstrated immunocytochemically in myelin and myelin-forming cells of developing rat. *Proc. Natl. Acad. Sci. USA* 76: 1510-1514.
- Sutcliffe, J. G. (1987) The genes for myelin. *TIG* 3: 73-76.
- Sutcliffe, J. G., R. J. Milner, T. M. Shinnick, and F. E. Bloom (1983) Identifying the protein products of brain-specific genes with antibodies to chemically synthesized peptides. *Cell* 33: 671-682.
- Takahashi, N., A. Roach, D. B. Teplow, S. B. Prusiner, and L. Hood (1985) Cloning and characterization of the myelin basic protein gene from mouse: One gene can encode both 14 kd and 18.5 kd MBPs by alternate use of exons. *Cell* 42: 139-148.
- Trapp, B. D., and R. H. Quarles (1984) Immunocytochemical localization of the myelin-associated glycoprotein. Fact or artifact? *J. Neuroimmunol.* 6: 231-249.
- Trapp, B. D., T. Moeuch, M. Pulley, E. Barbosa, G. Tennekoon, and J. Griffith (1987) Spatial segregation of mRNA encoding myelin-specific proteins. *Proc. Natl. Acad. Sci. USA* 84: 7773-7777.
- Williams, A. F. (1987) A year in the life of the immunoglobulin superfamily. *Immunol. Today* 8: 298-303.
- Wilson, M. C., G. A. Higgins, H. Schmale, F. E. Bloom, and R. J. Milner (1987) Neuroanatomical and developmental expression of novel brain mRNAs determined by *in situ* hybridization. In *In Situ Hybridization in Brain*, G. R. Uhl, ed., pp. 117-133, Plenum, New York.
- Young, W. S., III, T. I. Bonner, and M. R. Brann (1986) Mesencephalic dopamine neurons regulate the expression of neuropeptide mRNAs in the rat forebrain. *Proc. Natl. Acad. Sci. USA* 83: 9827-9831.
- Zeller, N. K., M. J. Hunkeler, A. T. Campagnoni, J. Sprague, and R. A. Lazzarini (1984) Characterization of mouse myelin basic protein messenger RNAs with a myelin basic protein cDNA clone. *Proc. Natl. Acad. Sci. USA* 81: 18-22.
- 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.