

# Characterization of a Novel Peripheral Nervous System Myelin Protein (PMP-22/SR13)

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**Abstract.** We have recently described a novel cDNA, SR13 (Welcher, A. A., U. Suter, M. De Leon, G. J. Snipes, and E. M. Shooter. 1991. *Proc. Natl. Acad. Sci. USA.* 88:7195–7199), that is repressed after sciatic nerve crush injury and shows homology to both the growth arrest-specific mRNA, *gas3* (Manfioletti, G., M. E. Ruaro, G. Del Sal, L. Philipson, and C. Schneider. 1990. *Mol. Cell Biol.* 10:2924–2930), and to the myelin protein, PASII (Kitamura, K., M. Suzuki, and K. Uyemura. 1976. *Biochim. Biophys. Acta.* 455:806–816). In this report, we show that the 22-kD SR13 protein is expressed in the compact portion of essentially all myelinated fibers in the peripheral nervous system. Although SR13 mRNA was found in the central nervous system, no corresponding SR13

protein could be detected by either immunoblot analysis or by immunohistochemistry. Northern and immunoblot analysis of SR13 mRNA and protein expression during development of the peripheral nervous system reveal a pattern similar to other myelin proteins. Furthermore, we demonstrate by in situ mRNA hybridization on tissue sections and on individual nerve fibers that SR13 mRNA is produced predominantly by Schwann cells. We conclude that the SR13 protein is apparently exclusively expressed in the peripheral nervous system where it is a major component of myelin. Thus, we propose the name Peripheral Myelin Protein-22 (PMP-22) for the proteins and cDNAs previously designated PASII, SR13, and *gas3*.

**M**YELIN is a highly specialized extension of the plasma membrane of Schwann cells in the peripheral nervous system (PNS)<sup>1</sup> and of oligodendrocytes in the central nervous system (CNS). Its characteristic multilaminated structure is produced by the wrapping of the plasma membrane of myelin-forming cells around axons, forming a cylindrical sheath which is divided longitudinally into discontinuous segments, interrupted by the nodes of Ranvier (for a detailed description see Peters et al., 1976). When viewed in cross section, myelin is composed of alternating apposing cytoplasmic and extracellular surfaces of the plasma membrane which give rise to the major dense line and the intraperiod line, respectively (Napolitano and Scallen, 1969). This highly ordered membranous sheath facilitates the electrical conduction velocity of myelinated axons (Ritchie, 1984).

Peripheral and central nervous system myelin have been extensively studied and, although their general organization is quite similar, they differ with regards to morphological ap-

pearance and protein composition (Morell et al., 1989). In the CNS, each oligodendrocyte produces up to 30 internodal myelin segments which tend to have fewer lamellae than PNS myelin. As opposed to the oligodendrocyte, a single Schwann cell can produce only one internodal myelin segment around a single axon in the PNS. Additional morphological differences between central and peripheral myelin have been described (Peters et al., 1976).

The major structural myelin proteins in the CNS include proteolipid protein (PLP), the myelin basic proteins (MBP), and myelin-associated glycoprotein (MAG) (for review see Campagnoni, 1988). PLP is a highly hydrophobic transmembrane protein which projects into both the major dense line, and the intraperiod line, and has been implicated in maintaining the apposition between the extracellular faces (the intraperiod line) of myelin (Hudson et al., 1987). In contrast, MBP is a highly charged soluble intracellular protein whose expression is limited to the major dense line. MAG is a glycoprotein that is structurally related to the immunoglobulin gene superfamily (Salzer et al., 1987). This protein has been hypothesized to play a role in myelin-axon interactions because of its homology to molecules involved in cellular recognition and adhesion and its immunolocalization to the axoplasmic surface of myelin, although this localization is controversial (Trapp and Quarles, 1984). Recent experiments examining recombinant retrovirus-mediated

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1. *Abbreviations used in this paper:* CNS, central nervous system; MAG, myelin-associated glycoprotein; MBP, myelin basic proteins; PLP, proteolipid protein; PMP-22, peripheral myelin protein-22 kilodaltons; PNS, peripheral nervous system; Po, Protein zero; SCP, spinal cord preparation.

MAG overexpression in mixed Schwann cell neuron cultures also provide support for the hypothesis that MAG is involved in axon-Schwann cell interactions (Owens et al., 1990).

Protein zero (P0), MBP, and MAG are the major protein components of PNS myelin (reviewed in Morell et al., 1989; Lemke, 1988). P0, a transmembrane glycoprotein which, like MAG, belongs to the immunoglobulin gene superfamily (Lai et al., 1987; Lemke et al., 1988) is the most abundant protein in PNS myelin. The immunoglobulin-like extracellular domain of P0 is located in the intraperiod line and has led to the hypothesis that P0 may be responsible for the adhesion between the extracellular surfaces of the myelin plasma membrane. Recent studies on cultured cells clearly demonstrate the capacity of P0 to undergo homophilic interactions (Filbin et al., 1990; Schneider-Schaulies et al., 1990; D'Urso et al., 1990).

The regulation of myelin protein expression is under exquisite control because of the highly specialized function of myelin in the nervous system. In development and after injury to the PNS, cessation of Schwann cell proliferation is followed by myelin formation (Asbury, 1967). The synthesis of the major myelin proteins correlates closely with the formation of myelin during the development of both the CNS and PNS (Uyemura et al., 1979; Lampert et al., 1990; Kronquist et al., 1987; Stahl et al., 1990). After peripheral nerve injury, myelin protein expression is quickly diminished, presumably because of transcriptional regulation initiated by loss of axonal contact. Myelin protein synthesis resumes in crush-lesioned peripheral nerves with a time course comparable to the remyelination of regenerating axons (Trapp et al., 1988; LeBlanc and Poduslo, 1990; Mitchell et al., 1990). Thus, myelin protein expression shows a similar pattern of regulation both during development and during nerve regeneration.

Recently, we described the cloning and initial characterization of a putative myelin protein, designated SR13, which was isolated by differential screening of cDNA libraries from injured versus uninjured rat sciatic nerves (Welcher et al., 1991b). The SR13 cDNA sequence predicts a 160-amino acid protein of 18 kD. Nucleotide sequence comparisons revealed an extensive homology of SR13 with the growth-arrest specific gene *gas3* (Manfioletti et al., 1990) and considerable amino acid identity with the partial amino acid sequence of PAS-II, a protein previously isolated from bovine peripheral myelin (Kitamura et al., 1976). Based on these findings and preliminary immunohistochemical studies, we suggested that SR13 is a myelin protein (Welcher et al., 1991b). Furthermore, since *gas3* has been proposed as a regulator of cell growth in tissue culture fibroblasts, we have been interested in examining the possibility of a similar regulatory function for SR13 in vivo. Because of the association of SR13/*gas3* with growth arrest, it was also of interest to examine the expression of SR13 during periods of cell division in development and after nerve injury.

We have characterized the time course of SR13 expression and its anatomical localization during myelination as well as after nerve injury. In these studies, we have demonstrated that SR13 is a 22-kD myelin protein which is expressed exclusively in the PNS. Thus, we propose the name Peripheral Myelin Protein 22 (PMP-22) for this protein that was previously designated SR13, *gas3*, and PAS-II.

## Materials and Methods

### Animal Care and Surgery

All surgical procedures followed the National Institutes of Health (NIH) guidelines for the care and use of laboratory animals at Stanford University. Male Sprague-Dawley rats (6-wk old; Bantin and Kingman, Inc., Fremont, CA) were anesthetized by intraperitoneal injection of a mixture of ketamine and chloral hydrate. The right sciatic nerves were exposed and crushed for 30 s with No. 5 jeweler's forceps ~2-mm distal to the hip joint. In a similar fashion, the contralateral nerve was exposed but not crushed. At timed intervals, the crush-lesioned animals and developing rat pups were euthanized in a CO<sub>2</sub> atmosphere. Sciatic nerves, brains, and spinal cords were quickly removed and snap frozen on dry ice or placed immediately into 4% paraformaldehyde in 0.1 M sodium phosphate, pH 7.4, for paraffin embedding or in isotonic glutaraldehyde buffer (0.33 M sodium cacodylate, 2.7% glutaraldehyde, pH 7.4) followed by postfixation in aqueous 2% osmium tetroxide before embedding in LX112 resin. Selected tissue blocks were processed for EM.

### Preparation of Anti-PMP 22 Antibodies

Two peptides were selected using hydrophilicity and surface probability predictions based on the primary amino acid sequence of PMP-22/SR13.

Peptide 1 <sup>27</sup>Gln-Trp-Leu-Val-Gly-Asn-Gly-His-Arg-Thr-Asp-Leu-Trp-Gln-Asn-Cys<sup>42</sup>-COOH

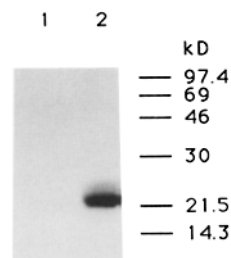
Peptide 2 <sup>117</sup>Tyr-Thr-Val-Arg-His-Ser-Glu-Trp-His-Val-Asn-Asp-Tyr-Ser-Tyr<sup>133</sup>-Cys-COOH

(A carboxy-terminal cysteine was added for cross-linking purposes). Amino acid numbering refers to the primary amino acid sequence of PMP-22/SR13 (Welcher et al., 1991b). Both peptides were synthesized on an automated peptide synthesizer (Milligen/Bioscience, Burlington, MA) and cross-linked to keyhole limpet hemocyanin (Calbiochem-Behring Corp., San Diego, CA) as follows: 250  $\mu$ l keyhole limpet hemocyanin (20 mg/ml in 50 mM sodium phosphate, pH 6) was mixed with 25  $\mu$ l m-maleimidobenzoyl-N-hydroxysuccinimide ester (Calbiochem-Behring Corp.; 100 mg/ml in tetrahydrofuran). 250  $\mu$ l 50 mM sodium phosphate, pH 6, was added and the mixture was incubated for 30 min at room temperature with gentle agitation. 1-ml peptide solution (5 mg/ml in 50 mM sodium phosphate, pH 6) was added and the mixture was incubated with rocking for another 3 h at room temperature. The cross-linked proteins were then dialyzed against PBS for 48 h at 4°C with several buffer changes. Insoluble material was removed by centrifugation and the volume adjusted to 2 ml with PBS. 250  $\mu$ l of the conjugate was combined with 350  $\mu$ l free peptide solution (3 mg/ml in PBS) and 600  $\mu$ l Freund's complete adjuvants (Sigma Chemical Co., St. Louis, MO) was added. This cocktail was used for primary immunization of female New Zealand rabbits. The rabbits were boosted 14, 38, and 58 d after the initial immunization with the same solution except that Freund's incomplete adjuvant was used. Both peptides gave rise to comparable antisera in two different rabbits as judged from solid-phase ELISA.

### Isolation of Proteins and Immunoblot Analysis

20 to 150 mg of frozen tissue was added to 1 ml of a PBS-1% SDS solution. The tissue was disrupted using a polytron (Brinkmann Instruments, Inc., Westbury, NY) at the highest setting for 10 s, after which the sample was placed in a 100°C water bath, and boiled for 3 min. Insoluble material was pelleted by centrifugation at 5,000 rpm for 5 min at room temperature in a low speed centrifuge (Beckman Instruments, Inc., Fullerton, CA). The supernatant was removed to a microfuge tube and centrifuged at 10,000 rpm for 10 min at room temperature. Dilutions of the supernatants were used to determine the protein concentration by the BCA protein assay system (Pierce Chemical Co., Rockford, IL) using the manufacturer's reagents and instructions.

Equivalent amounts of the protein samples were added to sample buffer containing 0.5% 2-mercaptoethanol, electrophoresed through 12.5% SDS-polyacrylamide gels, and transferred to nitrocellulose as described previously (Welcher et al., 1991a). The filters were blocked with a solution of PBS-0.05% Tween-5% nonfat milk (Blotto), incubated with antiserum to peptide 1 diluted 1:1,000 in Blotto, followed by incubation with affinity purified <sup>125</sup>I-protein A (Amersham Corp., Arlington Heights, IL; 45



**Figure 1.** Anti-PMP-22 peptide antisera recognize a 22-kD protein from rat sciatic nerve. Proteins (50  $\mu$ g per lane) from adult rat sciatic nerves were electrophoresed through a SDS-polyacrylamide gel, transferred to nitrocellulose, and the membrane was cut in half. One half (lane 1) was incubated with pre-blocked (30 min, 1 mg/ml peptide 2) anti-peptide 2 antisera (final concentration 1:1,000). The other half (lane 2) was

incubated with anti-peptide 2 antisera only (1:1,000). Bound antibody was detected with  $^{125}$ I-Protein A followed by autoradiography. Position of the molecular weight standards is indicated on the right.

mCi/mg) at a concentration of 2  $\mu$ Ci/10 ml of Blotto solution. All incubations were at room temperature for 1 h each. The filters were autoradiographed, and films scanned as described for the Northern blots.

### Immunohistochemistry

Immunohistochemistry for the PMP-22 protein on 4% paraformaldehyde-fixed paraffin-embedded tissue was performed as previously described (Welcher et al., 1991b) except that both primary antipeptide antibodies were used simultaneously at a dilution of 1:100 and the blocking and primary antiserum solutions contained 0.1% Triton X-100 (Sigma Chemical Co.). Rabbit anti-human MBP serum (DAKOPATTS, Copenhagen) was used at a dilution of 1:60. For high resolution light microscopic and immunoperoxidase studies, nerves were embedded in LX 112 (Ladd Research Industries, Inc., Burlington, VT) and 0.5- $\mu$ m-thick sections were cut on an ultramicrotome (Reichert Jung, Vienna) and either stained with 1% toluidine blue or etched with sodium hydroxide-saturated ethanol for 20 s as described by Baskin et al. (1979) and processed for immunohistochemistry as described above.

### RNA Isolation and Northern Blots

Total RNA was extracted from at least six pooled sciatic nerves according to the method of Chomczynski and Sacchi (1987) and analyzed by Northern blotting using Hybond-N membranes (Amersham Corp.). Total RNA was quantitated initially by optical density measurements at 260 nm and verified by ethidium bromide staining. Blots were probed with a  $^{32}$ P-labeled PMP-22/SRI3 cDNA probe (hexanucleotide labeling kit; Boehringer Mannheim Corp., Indianapolis, IN), washed under high stringency conditions, and exposed to XAR5 films (Eastern Kodak Co., Rochester, NY). Quantitative analysis was performed by densitometric scanning of appropriate (linear

range exposed) autoradiograms using a laser densitometer (LKB Instruments, Gaithersburg, MD).

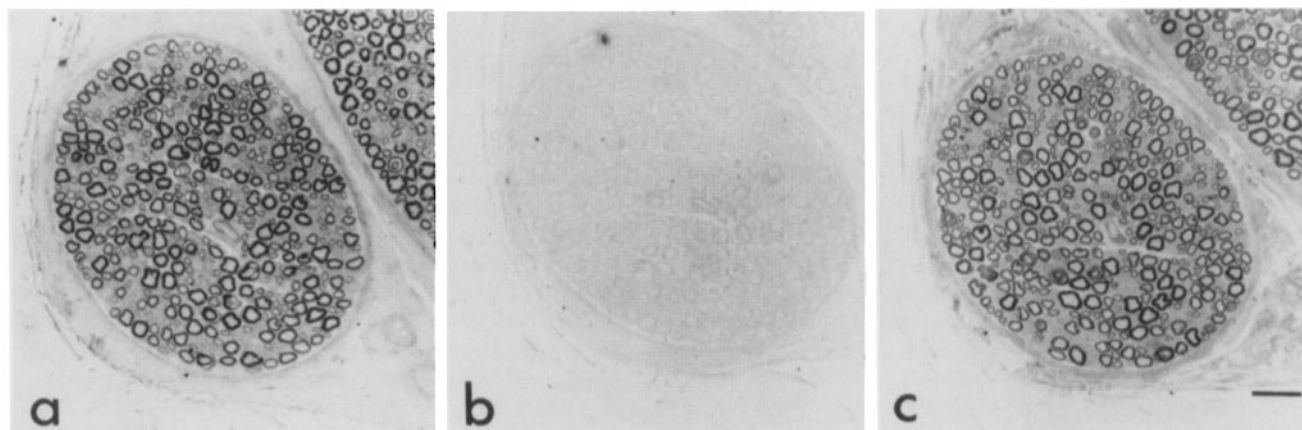
### In Situ mRNA Hybridization

The 1.6-kb XbaI fragment of the PMP-22/SRI3 cDNA was subcloned in both orientations into the XbaI site of the pSP72 (Promega Biotec, Madison, WI) plasmid. Sense and antisense riboprobes, containing  $^{35}$ S CTP (Amersham Corp.), were synthesized according to the manufacturer's instructions. Paraffin sections cut onto 3-aminopropyltriethoxysilane-treated glass slides (Rentrop et al., 1986) were deparaffinized in xylenes, rehydrated through graded ethanol solutions, and treated sequentially with 4% paraformaldehyde (5 min), 20 mM HCl (3 min), 0.01% Triton X-100 (3 min), 1  $\mu$ g/ml proteinase K (10 min; 50 mM Tris, 3 mM EDTA, pH 8.0), and 4% paraformaldehyde (5 min) each separated by two washes in PBS (3 min each; supplemented with 0.2% glycine after the proteinase K incubation). The sections were then dehydrated through graded ethanol solutions and air dried. For nerve tease experiments, normal sciatic nerves were fixed in buffered formalin, teased onto silane-treated slides, and dried in a 37°C oven for 3 h. The teased nerves were treated as described above, starting with the 4% paraformaldehyde solution. Sections were prehybridized with 25 mM Tris, 0.75 M NaCl, 25 mM EDTA, 1 $\times$  Denhardt's solution, 50% formamide, 0.2% SDS, 225  $\mu$ g/ml sheared salmon sperm DNA, 225  $\mu$ g/ml poly A, 15 mM DTT at 42°C for 3 h and hybridized for 16 h at 53°C in prehybridization solution containing 10<sup>6</sup> cpm sense or antisense probes and 5% dextran sulfate. Hybridization signals were detected with  $\beta$ -max high resolution film (Amersham Corp.) or with photographic emulsion. For double label experiments, the sections were first hybridized with the PMP-22 sense and antisense probes then immunolabeled with rabbit anti-bovine S-100 (1:100, Dako Corp., Santa Barbara, CA) using the hybridization and peroxidase antiperoxidase techniques described above.

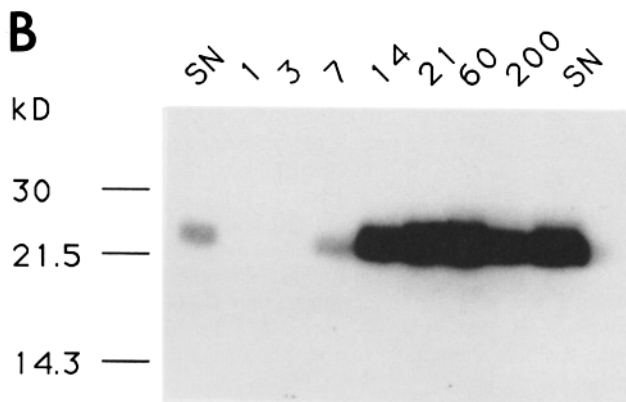
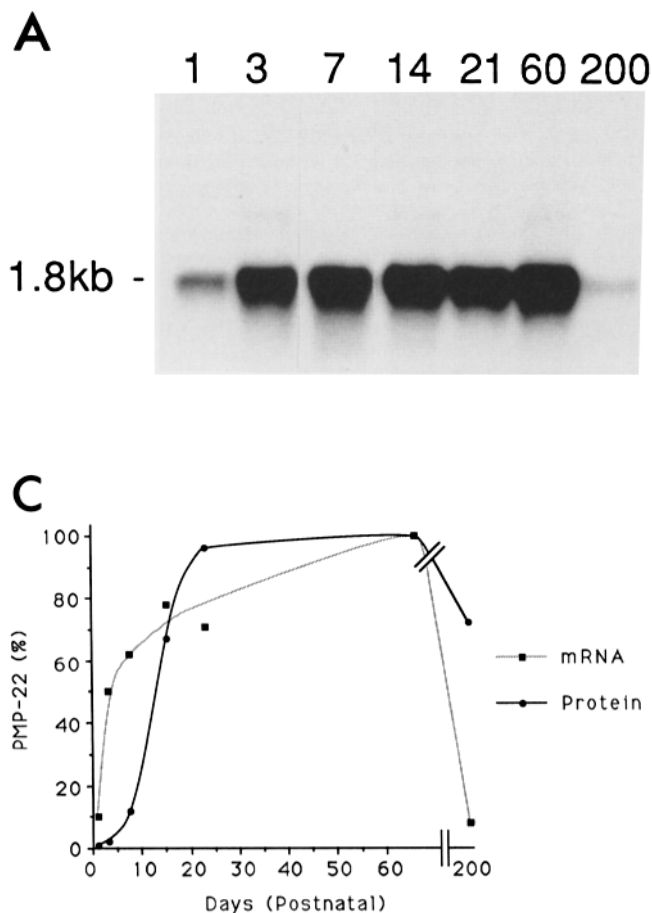
### Results

#### *PMP-22, a 22-kD Protein, Is a Component of all Myelin Sheaths in the Peripheral Nervous System*

Synthetic peptides corresponding to the two predicted major hydrophilic regions of the PMP-22 molecule were conjugated to keyhole limpet hemocyanin and used to immunize rabbits. The antisera to both peptides were found to be specific for a 22-kD protein on electrophoretic transfers (immunoblots) of total protein isolated from rat sciatic nerves. Fig. 1 shows that the anti-PMP-22 peptide 1 antiserum specifically recognizes a 22-kD protein in sciatic nerves



**Figure 2.** Immunoperoxidase detection of the PMP-22 protein in 0.5- $\mu$ m cross sections of normal rat sciatic nerve using pooled antipeptide antibodies (a) or preimmune sera (b), both diluted 1:50. Sciatic nerve from 30-d-old rats was fixed with isotonic glutaraldehyde and embedded in LX 112. For immunohistochemistry, 0.5  $\mu$ m sections were etched with ethanolic sodium hydroxide for 20 s before immunoperoxidase staining by the peroxidase-antiperoxidase method. Toluidine blue stain highlights myelin in adjacent plastic sections (c). Bar, 20  $\mu$ m.



**Figure 3.** Developmental expression of PMP-22 mRNA and protein in the sciatic nerve. (A) RNA was isolated from rat sciatic nerves, fractionated by formaldehyde-agarose gel electrophoresis (5  $\mu$ g total RNA per lane), transferred to a nylon membrane, hybridized with a  $^{32}$ P-labeled PMP-22 probe, and autoradiographed. The ages of the rats (in days) are indicated above the lanes. mRNA size was determined using RNA molecular weight standards. (B) Proteins were isolated from rat sciatic nerves, fractionated by SDS-polyacrylamide electrophoresis (50  $\mu$ g of protein per lane), and transferred to nitrocellulose. The membrane was incubated consecutively with antisera 2 (1:1,000),  $^{125}$ I-Protein A, and visualized by autoradiography. The ages of the rats (in days) are indicated above the lanes. SN refers to proteins (1  $\mu$ g, left side; 50  $\mu$ g, right side) isolated from 60-d-old rats. Position of the protein molecular weight standards is indicated on the left. (C) Quantitation of PMP-22 mRNA and protein expression. Autoradiographs were scanned and the expression plotted relative to the expression at 60 days (100%). (■) RNA expression; (●) protein expression.

(Fig. 1, lane 2) which is abolished by preincubating the antiserum with PMP-22 peptide 1 (Fig. 1, lane 1). The PMP-22 protein is predicted to have a molecular mass of 18,000 based on the peptide sequence and contains a consensus sequence for N-linked glycosylation (Welcher et al., 1991b) which predicts a total molecular mass consistent with a 21–22-kD protein. Both antipeptide antisera had identical specificities on immunoblots and in immunohistochemical studies. Control blots using preimmune serum were consistently negative (data not shown).

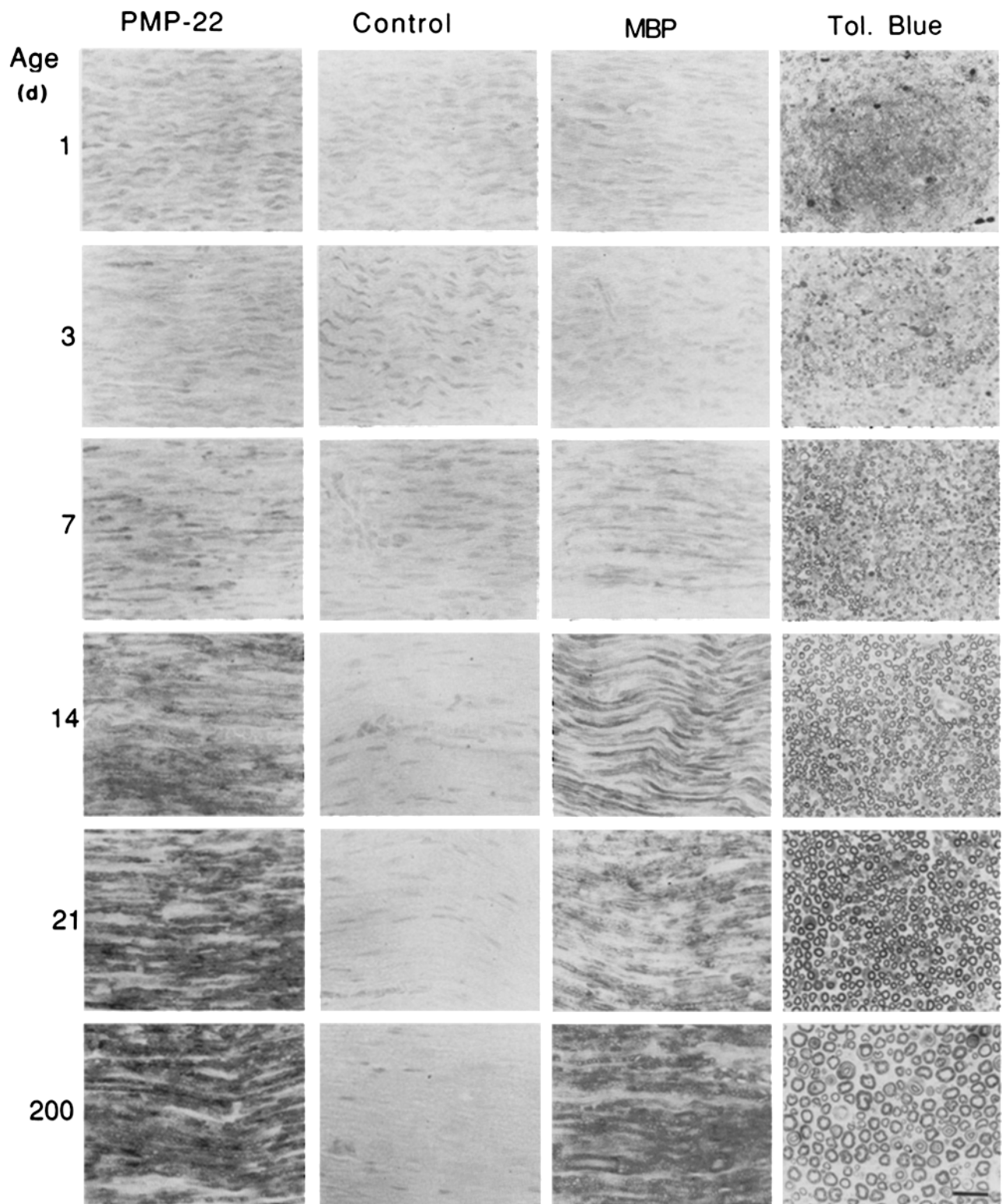
While PMP-22 is expressed at high levels in normal adult rat sciatic nerves, Northern blot analysis showed that PMP-22 mRNA expression was undetectable in liver and kidney but was present in trace levels in heart and skeletal muscle (Welcher et al., 1991b). Immunoblot and immunohistochemical analysis of the PMP-22 protein agreed with the previous results and identified no detectable PMP-22 protein expression in a variety of tissues including heart, gut, lung, adrenal gland, kidneys, skeletal muscle, thymus, and spleen except in the myelin of the innervating nerves (data not shown). Initial immunohistochemical results had demonstrated that PMP-22 was associated with the myelin sheaths of axons in the sciatic nerve (Welcher et al., 1991b). This finding was confirmed by immunoperoxidase studies on 0.5- $\mu$ m plastic sections which localized the PMP-22 protein to the compact portion of the myelin sheaths of essentially all myelinated axons in the sciatic nerve (Fig. 2 a) when compared to toluidine blue-stained adjacent plastic sections (Fig. 2 c). It was concluded that PMP-22 protein expression is ap-

parently restricted to the nervous system where it is associated with myelin sheaths.

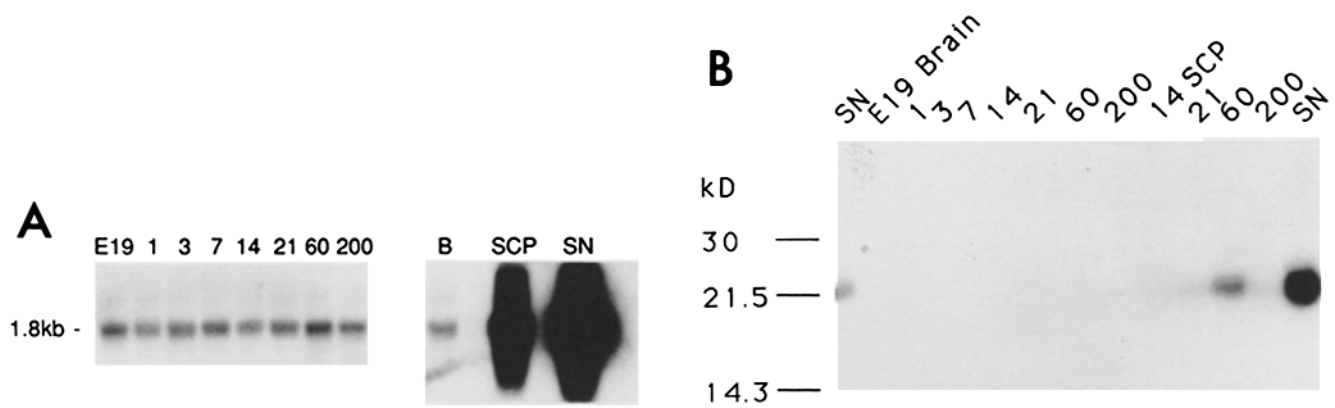
### **PMP-22 Expression Correlates with Myelin Formation during Sciatic Nerve Development**

After having identified PMP-22 as a putative myelin protein, it was important to compare its expression with other myelin proteins to establish the role of PMP-22 in myelin formation and to investigate its role in cellular growth arrest. Thus, a time course study of PMP-22 expression was undertaken during the immediate postnatal period to adulthood, the time interval in which Schwann cell proliferation ceases and myelination ensues in the rat sciatic nerve (Friede and Samorajski, 1968). Northern blot analysis of total RNA isolated from sciatic nerves at different time points in development showed that a single 1.8-kb PMP-22 mRNA species is initially expressed at low levels in the immediate postnatal period (10% of maximal) but is rapidly induced to adult levels over the first three postnatal weeks (Fig. 3 A). Densitometric analysis of the RNA blots demonstrated that PMP-22 mRNA expression reached half-maximal adult levels between postnatal days two to seven and increased to near maximal levels by postnatal day 21 (Fig. 3 C).

Parallel immunoblot analysis of PMP-22 protein expression in the developing rat sciatic nerve revealed that, as expected, the production of PMP-22 protein lags temporally behind PMP-22 mRNA expression (Fig. 3 B). Overall, however, protein and mRNA display parallel expression pat-



**Figure 4.** Developmental expression of PMP-22 in the rat sciatic nerve. Sciatic nerves were collected from 0, 3, 7, 14, 21, and 200-d-old rats and fixed by immersion in 4% paraformaldehyde in PBS and processed for paraffin embedding. 5- $\mu\text{m}$  paraffin sections were cut and processed for PMP-22 (1:100 each) and MBP (1:60) immunohistochemistry as described in Materials and Methods. Control sections were reacted with the corresponding preimmune sera (1:100 each). Toluidine blue-stained 0.5- $\mu\text{m}$  plastic sections from each nerve sample are shown at the right for comparison. Bar, 25  $\mu\text{m}$ .



**Figure 5.** Developmental expression of PMP-22 mRNA and protein in brain and spinal cord preparations. (A) RNA was isolated from the brain or spinal cord, fractionated by formaldehyde-agarose gel electrophoresis (5  $\mu$ g total RNA per lane), and analyzed as described in Fig 3. The left panel shows mRNA from the brain; the ages of the rats (in days) are indicated above the lanes. mRNA size was determined using RNA molecular weight standards. (Right) Relative expression of PMP-22 mRNA in brain (B), spinal cord preparation (SCP), and sciatic nerve (SN) in 5  $\mu$ g of total RNA per lane. (B) Proteins were isolated from the brain or spinal cord, and analyzed as described in Fig 3. The ages of the rats (in days) are indicated above the lanes. SN refers to proteins (1  $\mu$ g, left side; 50  $\mu$ g, right side) isolated from 60-d-old rats. Brain samples (E19–200) contained 100  $\mu$ g of protein. Spinal cord preparation samples (SCP, 14–200) contained 50  $\mu$ g of protein. Positions of the protein molecular weight standards are indicated on the left.

terns. PMP-22 protein levels are below detectable limits in the immediate postnatal period but reach half-maximal values between postnatal days 10 and 15 and maximal levels by postnatal day 21 (Fig. 3 C). Immunohistochemical analysis showed that PMP-22 protein expression is restricted to myelin and correlates temporally with the formation of myelin when compared to the expression of MBP, another component of compact myelin, and to myelin formation as monitored using toluidine blue-stained plastic sections of developing rat sciatic nerves (Fig. 4).

#### **Expression of PMP-22 Is Dramatically Lower in the Central Nervous System than in the Peripheral Nervous System**

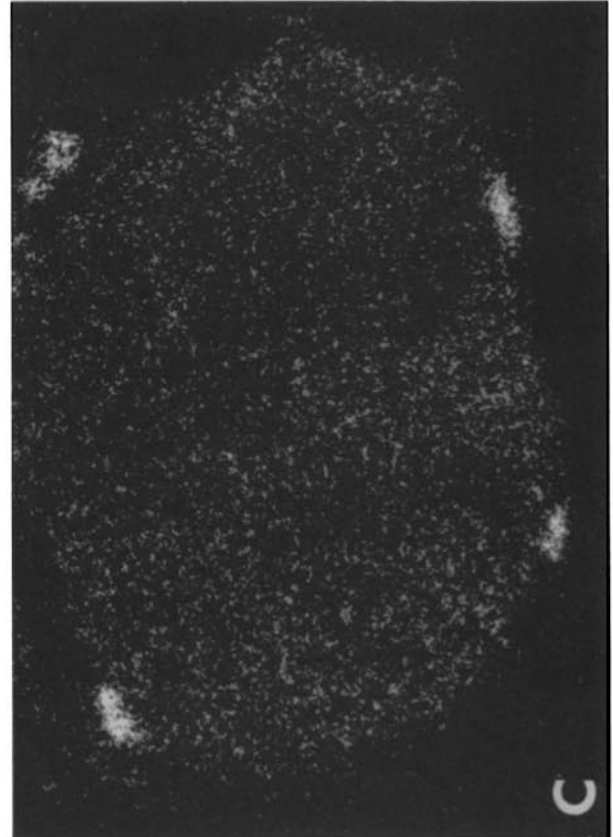
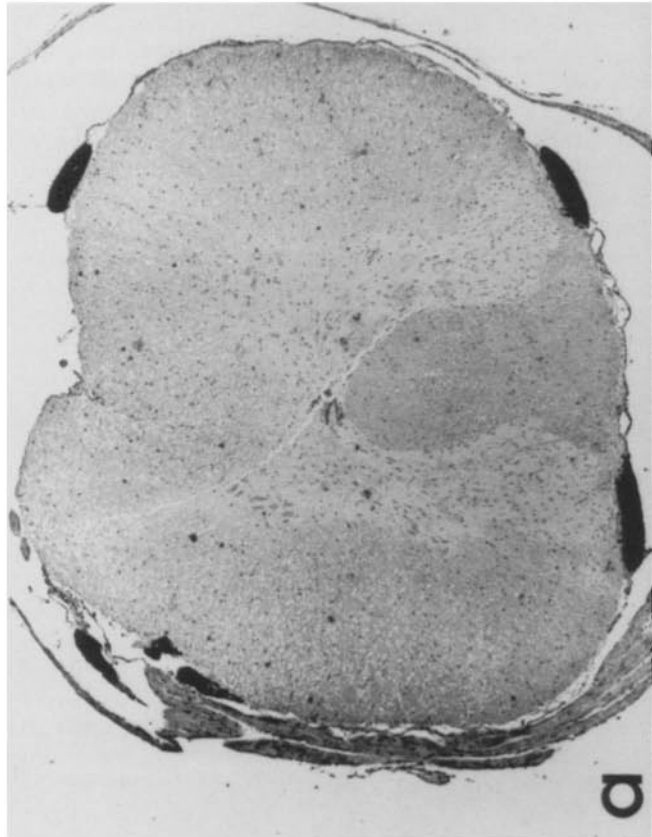
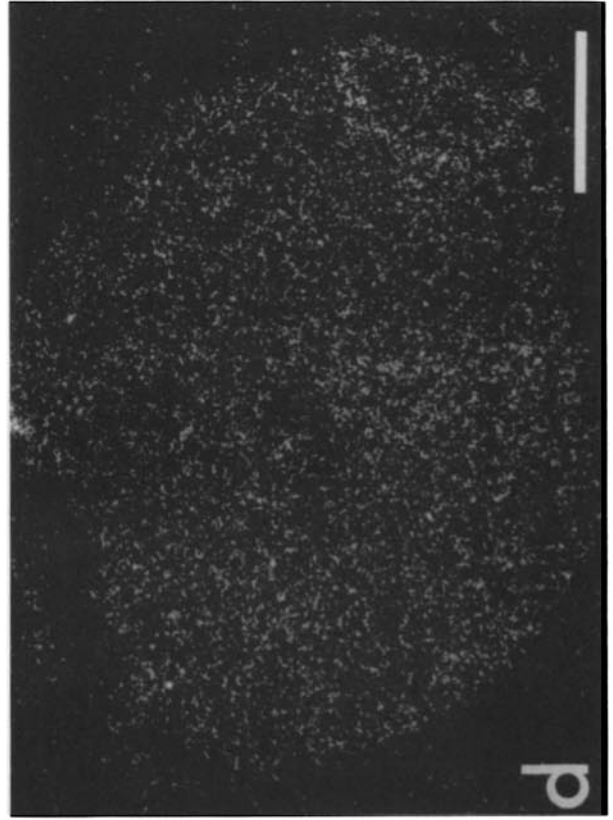
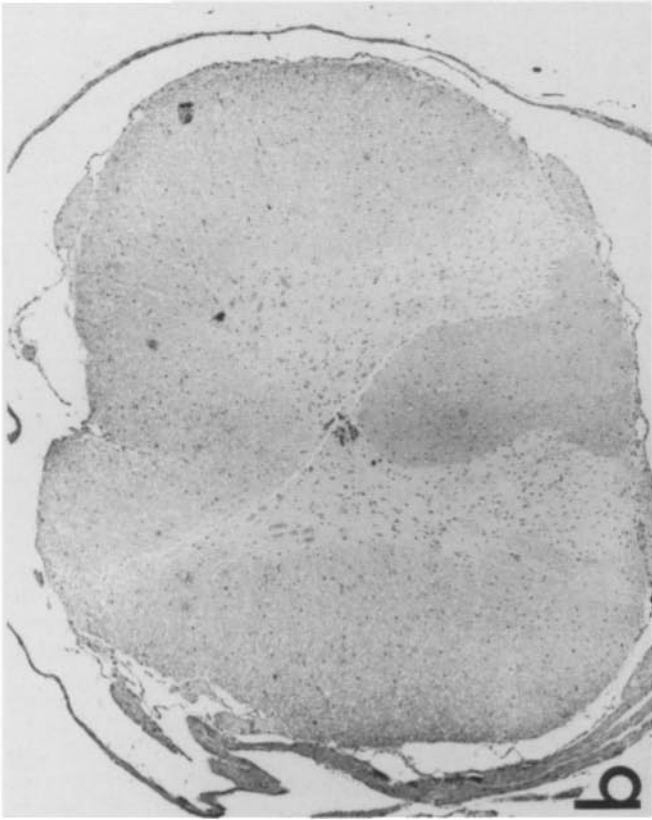
Previous studies indicated that PMP-22 mRNA is present in low levels in the brain (Welcher et al., 1991b). Thus, it was of interest to determine if PMP-22 was also a component of myelin in the CNS. Initial attempts to visualize PMP-22 protein in the brain using immunohistochemical methods were unsuccessful, a finding which was subsequently explained by Northern and immunoblot analysis. Quantitation of Northern blots of total RNA isolated from the brain revealed that the mRNA for PMP-22 is  $\sim$ 300-fold less abundant in brain than in sciatic nerve (Fig. 5 A, lanes B versus SN). Furthermore, in contrast to PMP-22 mRNA expression in peripheral nerve, the brain PMP-22 mRNA levels are not developmentally regulated (Fig. 5 A, lanes E19–200). No PMP-22 protein was detectable in 100  $\mu$ g of brain tissue at any time point under experimental conditions that were able to detect PMP-22 protein from 1  $\mu$ g of nerve tissue (Fig. 5 B). It is concluded that PMP-22 protein levels in the brain are

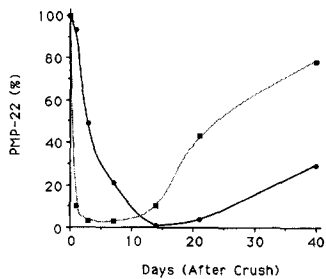
at least 100-fold lower than in peripheral nerve. As a second CNS tissue, spinal cord preparations (SCP) were examined for PMP-22 expression. Initial quantitative Northern blot analysis of SCP-derived RNA revealed appreciable levels of PMP-22 mRNA expression (Fig. 5 A, lane SCP). Similarly, relatively high levels of PMP-22 protein were detected in SCP by immunoblot analysis (Fig. 5 B). In situ mRNA hybridization (Fig. 6 a) and immunohistochemistry (Fig. 6 c), however, clearly demonstrated the expression of PMP-22 protein and mRNA in the PNS-derived dorsal and ventral spinal roots but failed to provide evidence of significant PMP-22 expression in the spinal cord. These results suggest that some, if not most, of PMP-22 mRNA and protein detected in the SCP is because of PMP-22 expression in the spinal roots of peripheral nerves. It remains possible that the absolute levels of PMP-22 mRNA and protein in the spinal cord differs from the low levels observed in the brain since a direct comparison between these structures cannot be made from these results. We conclude that, while PMP-22 mRNA may be expressed in the CNS at very low levels in a nondevelopmentally regulated manner, PMP-22 protein and mRNA levels are expressed at much higher levels in the PNS. Although we cannot exclude regional expression of PMP-22 in the brain, the protein is not a major component of CNS myelin.

#### **PMP-22 Expression Correlates with Myelin Degradation and Remyelination during Sciatic Nerve Regeneration**

PMP-22 was originally identified based on its precipitous down regulation after sciatic nerve crush injury. We have

**Figure 6.** PMP 22 immunohistochemistry and in situ mRNA hybridization on cross sections of the spinal cord. (a) PMP-22 immunoperoxidase studies performed on cross sections of spinal cord demonstrate dark staining for PMP-22 protein in the dorsal and ventral nerve roots, but no immunoreactivity is present within the spinal cord. (b) Control slides reacted with preimmune sera show no significant immunoreactivity. These sections are lightly counterstained with hematoxylin. The pattern of mRNA expression parallels the protein expression as demonstrated by in situ mRNA hybridization on adjacent sections of spinal cord probed with  $^{35}$ S-labeled PMP-22 antisense (c) and sense (d) RNA. c and d are negative images of the resulting autoradiogram (i.e., the portion of the autoradiography film exposed by the radioactive probe is white). Bar, 500  $\mu$ m.





**Figure 7.** Expression of PMP-22 mRNA and protein during sciatic nerve regeneration. Protein and mRNA were isolated from rat sciatic nerves at various days after a crush injury. Samples were analyzed by blotting as described in Fig. 3. Autoradiographs were scanned, and the expression plotted relative to the expression in the contralateral, uninjured sciatic nerves (100%). (■) mRNA expression; (●) protein expression.

now characterized in more detail the expression of PMP-22 after sciatic nerve crush to compare it to similar studies which have been performed using other known myelin proteins. Sciatic nerves were crushed several millimeters distal to the hip joint and marked with a loosely tied silk suture. At predetermined times after crush injury, 1–2-cm segments of sciatic nerve distal to the site of crush injury along with sham-operated contralateral nerves were harvested. Special care was taken to avoid the immediate area around the site of injury to eliminate the effects of local inflammation. Northern blot analysis confirmed the previously described rapid decline of PMP-22 mRNA to <10% of normal adult levels by 3–7 d after crush injury. This decline in the levels of PMP-22 mRNA is paralleled by a slower decline of the 22-kD PMP-22 protein expression which reaches similar low levels during days 10 to 20 after crush injury. Although PMP-22 mRNA and protein have started to approach normal levels by 40 d after crush (Fig. 7), the expression of protein lagged behind.

### Morphological Studies on PMP-22 after Sciatic Nerve Injury

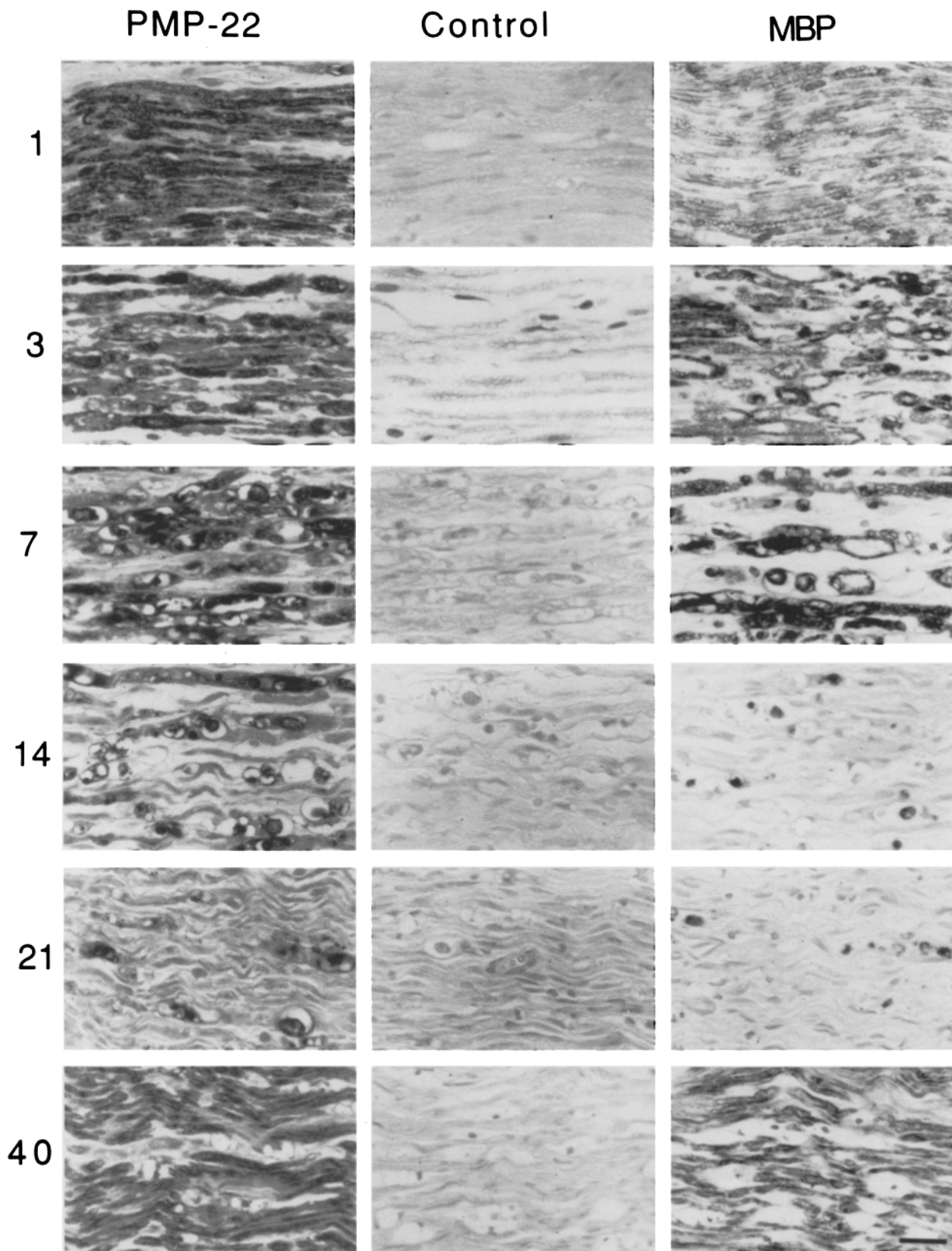
Although the morphologic events after peripheral nerve injury have been well described (for a historical perspective see Weller and Cervos-Navarro, 1977), we have performed anatomic studies in parallel with the protein and mRNA detection experiments. This strategy allowed us to more precisely correlate the morphological changes with the pattern of expression of the PMP-22 protein in the particular nerve segments used for biochemical and molecular analysis. Fig. 8 shows the results of the immunohistochemical study of PMP-22 and MBP expression after nerve injury. On the first day after crush injury, the only pathological alteration in the nerve segment distal to the site of crush injury is a slight loosening of the myelin sheath. At this time, PMP-22 immunoreactivity is more evident in the myelin sheaths distal to the site of crush injury than in the proximal nerve segment. This is probably because of loss of myelin integrity and consequent increased antibody penetration since a similar increase in immunoreactivity was noted in nerve fibers undergoing spontaneous Wallerian degeneration in aged rats. Three days after crush injury, neurofilament immunoreactivity is greatly diminished (data not shown) as the axons degenerate. At this time point, the PMP-22 mRNA level has fallen to 10% of precrush levels as the myelin sheaths are disrupted and are being digested by the Schwann cells as re-

flected in the persistently elevated levels of immunostaining for PMP-22 and MBP. By 7 d after crush injury, PMP-22 and MBP are found increasingly in “digestion chambers of Cajal,” a structure composed of Schwann cell cytoplasm surrounding degenerating myelin ovoids. In situ mRNA hybridization for PMP-22 mRNA at 14 d after crush injury illustrates the downregulation of PMP-22 mRNA in the portion of the sciatic nerve distal to the injury site when compared to the proximal portion (Fig. 9). Wallerian degeneration continues through the first 2 wk after injury as the absolute PMP-22 protein levels and PMP-22 immunoreactivity decline. In our system, axonal regeneration is evident 21 d after crush injury as shown by the presence of thinly myelinated axons in toluidine blue-stained plastic sections and electron micrographs (not shown). As demonstrated on immunoblots, PMP-22 protein levels start to increase by 21 d after crush injury. Axon regeneration continues through at least 40 d after crush injury as demonstrated by the Bielschowski silver stain for axons (data not shown). By 40 d after crush, the PMP-22 immunoreactivity is present predominantly in the many newly formed myelin sheaths and occasionally in residual “digestion chambers of Cajal.” This finding correlates well with the Northern and immunoblot results which demonstrate increased levels of PMP-22 protein and mRNA 40 d after crush injury.

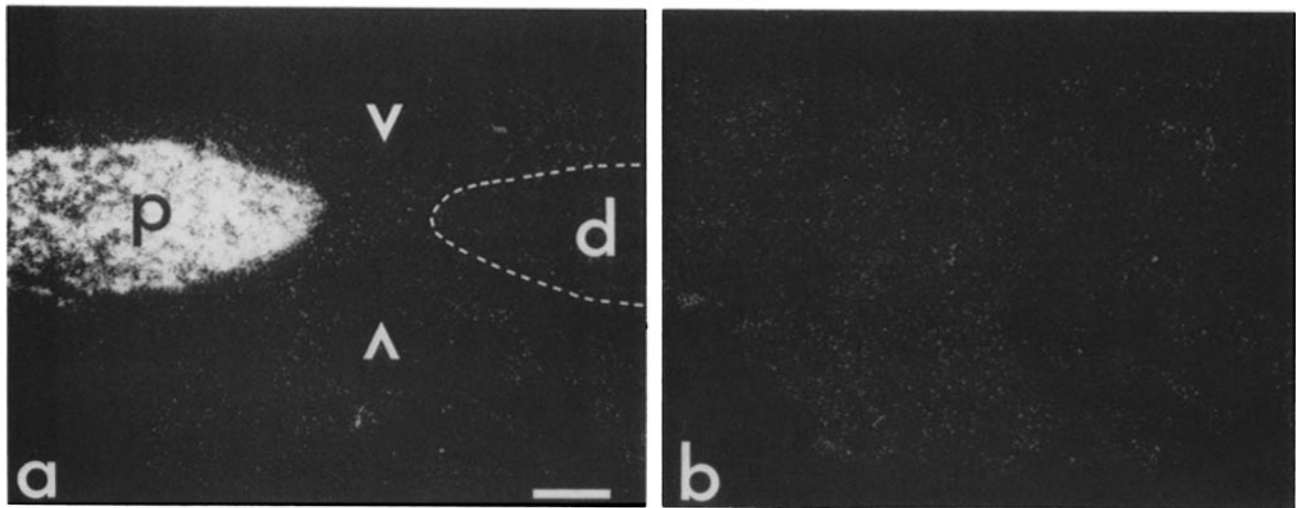
### PMP-22 Is Produced by Schwann Cells

Our initial characterization of PMP-22 provided no evidence for significant expression of the PMP-22 protein outside of the PNS. In addition, there is compelling evidence that PMP-22 is a myelin protein and, therefore, must be synthesized by Schwann cells. Yet, after clarification of a sequencing error in the mouse *gas3* cDNA, it is clear that PMP-22 and *gas3* share 98% amino acid identity over the complete protein sequence (data not shown). Thus, PMP-22 is the rat homologue to the mouse *gas3* cDNA which was isolated from growth-arrested fibroblasts (Schneider et al., 1988; Manfioletti et al., 1990). Since fibroblasts are also a component of the nerve sheath, it seemed possible that endoneurial fibroblasts could be the major source of PMP-22 expression. Two experiments were carried out to identify the cells that are mainly responsible for PMP-22 expression. First, we performed in situ mRNA hybridization for PMP-22 on teased nerve preparations, reasoning that if Schwann cells are synthesizing PMP-22, a signal should be observed with a longitudinal periodicity corresponding to the length of the internodal segment. Such an anticipated periodicity is evident in Fig. 10 c which shows PMP-22 mRNA localized to the perinuclear cytoplasm of a Schwann cell from a single myelin internode. This result was confirmed using double labeling combining in situ hybridization for PMP-22 mRNA and immunoperoxidase staining for S-100 protein, a specific marker for Schwann cells in peripheral nerves (Stefansson et al., 1982). In control experiments (not shown), the in situ hybridization procedure selectively diminished the subsequent immunostaining for the S-100 protein in the cytoplasm more than the nucleus, although specific staining of the nuclei could clearly be seen. The double-labeling studies demonstrated that S-100 positive Schwann cells also produce PMP-22 mRNA (Fig. 10 a). While all of the S-100 positive cells appear to express PMP-22 mRNA, there is a small





**Figure 8.** Expression of PMP-22 protein and MBP detected by immunohistochemistry in the distal portion of the rat sciatic nerve at various stages of Wallerian degeneration. Both PMP-22 and MBP can be detected in degenerating myelin sheaths and in myelin ovoids within “digestion chambers of Cajal” in these longitudinal sections of the rat sciatic nerves taken at 1, 3, 7, 14, 21, and 40 d after sciatic nerve crush. Control sections are reacted with PMP-22 preimmune (MBP nonimmune) rabbit sera as the primary antisera. All sections are lightly counterstained with hematoxylin. Bar, 25  $\mu$ m.



**Figure 9.** In situ mRNA hybridization performed 14 d after crush injury demonstrates the marked downregulation of PMP-22 mRNA in a longitudinal section of the sciatic nerve distal (*d*), but not proximal (*p*), to the site of crush injury (between arrowheads). Antisense (*a*) and sense (*b*)  $^{35}\text{S}$  RNA probes were hybridized to crush-injured rat sciatic nerves and detected by autoradiography. As in Fig. 6, the negative image of the autoradiogram is shown. Bar, 0.5 mm.

population of cells that are producing PMP-22 but do not stain for S-100. These PMP-22<sup>+</sup>/S-100<sup>-</sup> cells may represent rare fibroblasts or Schwann cells whose nuclei are out of the plane of section. In teased nerve preparations, occasionally more than one nucleus was observed associated with a single internodal myelin segment, but there was never more than one cell per internode that hybridized with PMP-22 antisense mRNA. Thus, we conclude that Schwann cells are responsible for synthesizing most, if not all of PMP-22. We cannot exclude, however, the possibility that there is a small population of other cell types expressing PMP-22.

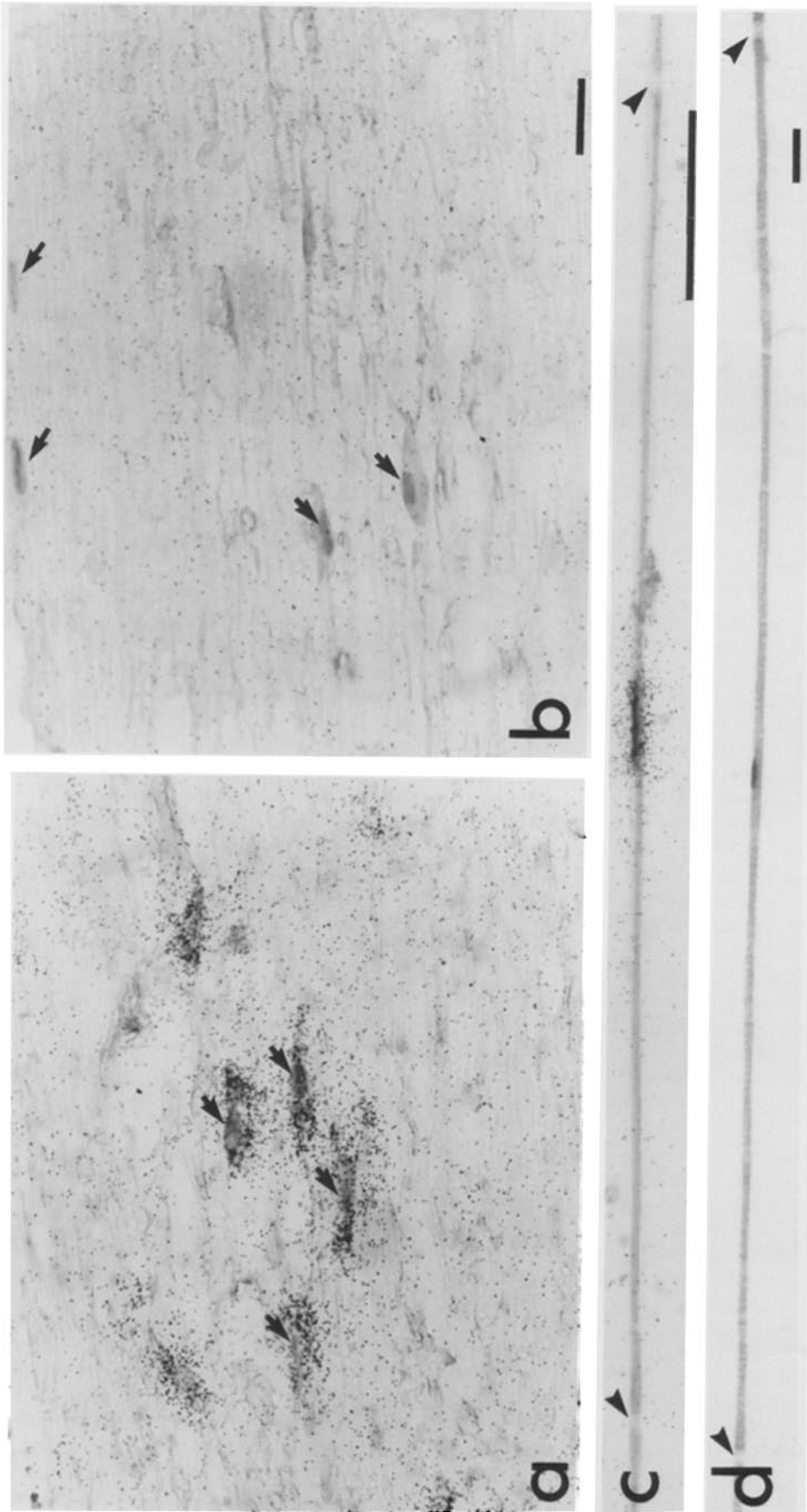
## Discussion

In this report, we have established that the recently described PMP-22 gene product is a component of peripheral myelin. This conclusion is based, in part, on immunohistochemistry and in situ mRNA hybridization studies which indicate that PMP-22 mRNA is produced predominantly by Schwann cells and that the PMP-22 protein is localized to the compact portion of the myelin sheath. The assignment of PMP-22 as a peripheral myelin protein is further supported by the finding that the regulation of PMP-22 expression during development and after nerve injury is similar to that of other myelin proteins.

The pattern of PMP-22 expression in the PNS during development is essentially identical to other proteins of PNS myelin, such as P0 and MBP (Stahl et al., 1990; Wiggins et al., 1975; Lees and Brostoff, 1984). Comparative side by side immunohistochemical localizations of PMP-22 and MBP during development reveal identical patterns of expression and correlate well with the formation of peripheral myelin as demonstrated using toluidine blue-stained plastic sections. Previous studies have also shown that P0 has a similar pattern of expression as MBP (and therefore PMP-22) during the development of the PNS (Garbay et al., 1989; Stahl et al., 1990). In the CNS, however, PMP-22 expression does not correlate with development and the formation of CNS myelin. The lack of regulation of PMP-22 expression

in the CNS is in marked contrast to the upregulation of the major CNS myelin protein, PLP, during CNS development. Gardinier et al. (1986) have shown that PLP mRNA is present in the CNS at 3 d after birth and reaches a broad peak at 2 to 4 wk of age. This expression pattern correlates well with the progression of rat CNS myelin formation. Thus, during the myelination period in the rat CNS, PLP mRNA increases from undetectable at birth to adult levels by 28-d postpartum (Naismith et al., 1985), while PMP-22 mRNA levels are unchanged and PMP-22 protein is undetectable. Interestingly, PLP mRNA and protein have been detected in the peripheral nervous system although the PLP protein apparently is not incorporated into PNS myelin (Puckett et al., 1987). In the PNS, PLP mRNA levels are not regulated during development and after nerve injury (Gupta et al., 1991), analogous to that reported here for PMP-22 mRNA in the CNS.

The pattern of expression of PMP-22 in the distal nerve stump after unilateral sciatic nerve crush was also comparable to the expression of other PNS myelin proteins. Trapp et al. (1988) have shown that, like PMP-22 mRNA, the mRNA encoding MBP and P0 decreased 40-fold by 5 d after crush injury. By 21 d after nerve injury, the MBP and P0 mRNA levels began to increase again although no morphologically detectable regeneration was observed (Trapp et al., 1988). Similarly, we show in this study that PMP-22 mRNA and protein expression are rising at 21 d after crush, a time at which we and others (Nichols et al., 1968) were able to observe axon sprouting. Trapp et al. (1988) also demonstrated that P0 immunoreactivity, like PMP-22 and MBP shown here, was localized to degenerating myelin ovoids at 21 d after crush injury. Interestingly, P0 expression is not completely repressed by loss of axonal contact as demonstrated by long-term tissue culture studies which indicated that small amounts of P0 are expressed constitutively by mature Schwann cells (Poduslo and Windebank, 1985). Given its possible role in cellular growth arrest, it would be of considerable interest to determine if PMP-22 was constitutively expressed in mature nonmyelinating Schwann cells.



**Figure 10.** Schwann cells synthesize PMP-22 mRNA. Longitudinal sections of normal rat sciatic nerves show that Schwann cells, identified by their S-100 immunoreactivity (arrows), also label for PMP-22 anti-sense (a), but not sense (b), mRNA as detected by in situ mRNA hybridization and visualized by emulsion autoradiography. In situ mRNA hybridization demonstrates a perinuclear localization of PMP-22 mRNA at the center of the myelin segment (the internode) between two nodes of Ranvier (arrowheads) from an individual myelinated nerve fiber (c). Nerve fibers reacted with the labeled sense probe showed no hybridization signal (d). Teased nerves are counterstained with hematoxylin and eosin. Bar, 25  $\mu$ m.

Overall, our results suggest that the regulation of PMP-22 after nerve injury is similar to other peripheral myelin proteins.

We have previously shown that PMP-22 (SR13) has sequence homology with two partial peptide sequences of PASII, a glycoprotein isolated from PNS myelin preparations. Although the reported molecular weight of PASII (13,000, more recently 19,000; Uyemura and Kitamura, 1991) is considerably different from PMP-22, the sequence identity, data indicating that PASII and PMP-22 are glycoproteins (see below), and the fact that PASII and, now, PMP-22 have been shown to be PNS specific, indicate that PASII and PMP-22 are very likely to be identical proteins. It is a plausible explanation that the smaller observed molecular weight of PASII may be due to proteolytic degradation. If PASII and PMP-22 are indeed the same protein, it is of interest that PASII (PMP-22) has been isolated from purified myelin preparations and identified as one of four major PNS myelin proteins (Uyemura et al., 1978). Densitometric scanning of Coomassie-stained SDS-polyacrylamide gels of human peripheral myelin proteins suggest that the levels of the comigrating PMP-22 and P2 proteins may be up to 10–20% that of P0 (Uyemura and Kitamura, 1991). This notion is further supported by the high abundance of PMP-22 cDNA clones in a sciatic nerve library (De Leon et al., 1991) and the relative ease of PMP-22 detection in immunoblots using low quantities of sciatic nerve homogenates. We conclude that PMP-22 is a major component of PNS myelin.

The assertion that PMP-22 is a major component of the PNS myelin sheath raises the question why this protein has not been widely recognized earlier. Although we cannot offer a definitive answer, there are several features of PMP-22 that may have obscured its detection. First, PMP-22 has an apparent molecular weight of 22,000 and thus would be expected to comigrate with the high molecular weight MBP (21.5 kD) on SDS-polyacrylamide gels as suggested by Uyemura et al. (1979) and does comigrate with another peripheral myelin protein, P<sub>2</sub> (Uyemura and Kitamura, 1991). Secondly, during two dimensional-PAGE analysis of PMP-22, we noticed a tendency of PMP-22 to form aggregates in the absence of SDS which could possibly be attributed to the highly hydrophobic nature of the PMP-22 protein (data not shown). Similar unusual biochemical characteristics are described for other myelin proteins (e.g., PLP; see Agrawal and Hartman, 1980).

Comparison of the details of PMP-22 mRNA and protein localization with other proteins expressed by Schwann cells provides additional insight into the probable localization of PMP-22 within the myelin sheath. First, the perinuclear *in situ* mRNA localization of PMP-22 in the nerve teased preparation and on tissue sections is consistent with the hypothesis that PMP-22 is an integral membrane protein. Two patterns of mRNA localization for other myelin proteins have been described; namely, a perinuclear localization exemplified by P0 and PLP mRNAs and a diffuse cytoplasmic localization, often prominent in the paranodal regions of the Schwann cell, as demonstrated for MBP mRNA (Trapp et al., 1987; Griffiths et al., 1989). These patterns of mRNA expression are thought to reflect the fact that P0 and PLP are integral membrane proteins and must undergo intracellular processing through the RER and Golgi apparatus as opposed to the soluble MBPs which are presumably synthesized on free

ribosomes (Trapp et al., 1987). In support of this hypothesis, Lamperth et al. (1990) have provided a direct demonstration of P0 mRNA processing using mRNA hybridization at the ultrastructural level to show that P0 mRNA is localized to the RER. Secondly, we noticed by immunohistochemistry that PMP-22 was not highly expressed in the Schwann cell cytoplasm and was excluded from the cytoplasmic invaginations into the myelin sheath known as Schmidt-Lanterman incisures. Such a pattern of expression is reciprocal to that of proteins like S-100, which is found in the Schwann cell nucleus, cytoplasm (Stefansson et al., 1982), and Schmidt-Lanterman incisures (unpublished observations). Additional evidence that PMP-22 is an integral membrane protein is provided by analysis of the PMP-22 amino acid sequence which reveals four hydrophobic stretches that may function as transmembrane spanning regions as well as a consensus site for N-linked glycosylation. *gas3* mRNA (PMP-22) translated *in vitro* in the presence of microsomes has been shown to produce a endoglycosidase H-sensitive, proteinase K-insensitive protein (Manfioletti et al., 1990). Taken together, these studies suggest that PMP-22 is a transmembrane glycoprotein expressed in PNS myelin and, as such, probably has domains which project into both the major dense line and the intraperiod line of myelin.

The protein composition of CNS and PNS myelin are significantly different. There is, however, a pervasive notion in the literature that both myelin structures are sufficiently similar that two sets of analogous proteins serving similar functions must exist in both systems. According to this paradigm, P0 has been proposed to be the PNS homologue of PLP. This hypothesis was based on the fact that both proteins are relatively abundant, have similar molecular masses (PLP = 30,000, Lemke, 1988; P0 = 28,000, Sakamoto et al., 1987) and display reciprocal patterns of expression in PNS and CNS. In addition, the glycoprotein P0 is a member of the immunoglobulin gene superfamily of cell surface receptors (Lemke and Axel, 1985) and has been proposed to bind apposing membranes of the myelin sheath intraperiod line (contiguous with the extracellular space) through homophilic mechanisms (Lemke, 1988). PLP may have a similar function in the CNS as suggested by studies of the *jimpy* mutation, a point mutation of a splice acceptor site in the PLP gene (Hudson et al., 1987) which causes specific intraperiod line abnormalities in affected mice. Structurally, however, PLP and P0 are quite dissimilar. PLP contains four highly hydrophobic regions and has been proposed to have up to four membrane-spanning regions (Popot et al., 1991). P0, on the other hand, has a relatively hydrophobic, but glycosylated, extracellular domain, a single transmembrane domain, and a very basic cytoplasmic domain (Sakamoto et al., 1987; Lemke and Axel, 1985). As shown here, PMP-22 also displays a reciprocal pattern of expression compared to PLP and, like PLP, has four hydrophobic domains which may serve as membrane-spanning regions. Thus, we propose that PMP-22 may serve, at least in part, as the PNS analogue of PLP.

It was one of the goals of this study to examine the correlation of PMP-22 expression with Schwann cell proliferation because of the hypothesized role of *gas3* (PMP-22) in cellular growth arrest. *gas3* mRNA is induced in quiescent (growth-arrested) fibroblasts and is repressed in proliferating cells (Schneider et al., 1988; Ciccarelli et al., 1990). Our

studies reveal no relationship between PMP-22 expression in the CNS and the oligodendrocyte proliferation which occurs postnatally in the rat. In the PNS, however, PMP-22 expression correlates inversely with Schwann cell division during development. The situation after nerve injury is less clear. Pelligrino and Spencer (1985) examined Schwann cell proliferation after nerve injury using [<sup>3</sup>H]thymidine uptake visualized by autoradiography. Two peaks of Schwann cell proliferation were identified. The first peak appeared at ~5 d after injury and the second coincided with axon-glia contact (Pellegrino et al., 1982). Trapp et al. (1988) also noted that the density of Schwann cells in the distal nerve stump after nerve injury increased between 5–20 d. Our results demonstrate that PMP-22 mRNA expression is minimal 5 d after nerve injury and starts to increase by ~20 d after crush injury. The decrease in PMP-22 protein level lags significantly behind the mRNA such that there is still a significant amount of PMP-22 protein detectable by immunoblot during the first 10 d after nerve injury. Our immunohistochemistry results indicate that most of the PMP-22 protein observed during the second week after nerve crush injury is localized to degenerating myelin ovoids where it may be unavailable to serve a regulatory function. These results can be interpreted in several ways: First, PMP-22 (*gas3*) expression may not be directly related to in vivo growth regulation and may reflect irrelevant gene transcription in tissue culture. Indeed, there is no direct evidence that *gas3* is involved in regulating growth arrest. Alternatively, however, since PMP-22 mRNA (and to a lesser extent, protein) expression is correlated with the differentiation of Schwann cells, it remains possible that PMP-22 RNA negatively regulates cell division in Schwann cells as well as in other cell types. To accommodate the latter hypothesis, we would speculate that the lack of correlation of PMP-22 mRNA expression with the second wave of Schwann cell division during nerve regeneration is because of dilution of the relatively small number of Schwann cells in contact with the advancing tips of regenerating axons as compared with the whole nerve sample. Although we do not have direct evidence to support the idea that PMP-22 serves as both a myelin protein and as a regulator of cell growth, it should be pointed out that oligodendrocytes from mutant mice deficient in PLP (*jimpy*) proliferate more rapidly than normal (Skoff, 1982), but most of these cells ultimately die (Knapp et al., 1986). The existence of these mutations suggests that PLP may play a more fundamental role in oligodendrocyte biology than functioning as a myelin structural protein. Likewise, the possibility remains that PMP-22 may also subserve a similar dual function.

In conclusion, these studies indicate that the PMP-22 protein is synthesized by Schwann cells and is a major component of PNS, but not CNS myelin. Like other myelin proteins, PMP-22 mRNA and protein expression is actively regulated and correlates with myelin production during periods of myelination and Wallerian degeneration. Examination of the predicted structure of the PMP-22 protein reveals similarities to the predicted structure of PLP. The fact that both proteins have multiple membrane-spanning domains suggests that these proteins project into both the major dense line and the intraperiod line of myelin where they may serve similar functions. Whether PMP-22 or PLP are involved in the regulation of the cell cycle awaits further investigations.

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