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Gene Expression During Peripheral Nerve Remyelination

by

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To Mom and Dad

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ABSTRACT

The expression of myelin-specific genes was investigated in two models of peripheral neuropathy. The distal segment of the crush-injured rat sciatic nerve presents an opportunity to study molecular events during transient degeneration and subsequent remyelination. The distal segment of the permanently transected nerve is however incapable of remyelination in the absence of Schwann cell-axonal contact. The temporal course of expression of the genes encoding the major peripheral nervous system (PNS) myelin-specific proteins PO (the major PNS myelin glycoprotein), the myelin basic proteins (MBP) and the myelin-associated glycoprotein (MAG) suggested that PO and MBP genes are co-regulated in the peripheral nerve, and their expression is induced 10 to 14 days post crush-injury. The MAG gene is regulated in a different manner, and may be induced prior to the major myelin genes during remyelination. In contrast, the gene encoding the myelin enzyme 2',3'-cyclic nucleotide 3'-phosphodiesterase (CNPase) was induced over adult levels in the period following crush-injury as well as after permanent transection. The expression of the CNS myelin proteolipid protein (PLP) gene in the developing, degenerating and regenerating PNS was also examined and compared to the that of other peripheral nerve myelin-specific or myelin-associated genes. PLP gene expression in the peripheral nerve remains relatively dissociated from axonal influences. The effect of a permanent transection on a crushed, regenerating sciatic nerve was also evaluated to establish the degree of axonal control exerted upon Schwann cells that were previously induced to remyelinate. The induction of myelin gene expression observed during peripheral nerve regeneration is dependent on continuous signals from the ingrowing axons. The crush-transected model permits the classification of myelin genes, according to their response to Schwann cell-axonal contact. PO and MBP genes appear to be co-regulated and are expressed at basal levels in the absence of axonal contact. Levels of MAG encoding transcripts were reduced to undetectable levels after transection and after crush-transection, suggesting that the MAG gene is stringently controlled by signals emanating from the axon. CNPase coding transcripts, in contrast, were induced to higher than normal levels after all forms of nerve injury, although CNPase enzyme activities followed the pattern of demyelination and remyelination in these experimental models. The PLP gene was also atypical, in that its expression appeared to be relatively independent of axonal control. These results demonstrate the presence of diverse modes of regulation of myelin genes at the levels of transcription, post-transcription, translation, and post-translation. They are consistent with the concept that both remyelination and myelination during neural development are cellular processes under hierarchical control.

List of Abbreviations:

AMP	adenosine monophosphate
ANSA	1-amino-2-napthol-4-sulfonic acid
AP	alkaline phosphatase
apoE	apolipoprotein E
ATP	adenosine 5' triphosphate
BCIP	5-bromo-4-chloro-3-indolyl phosphate
bp	base pairs
cAMP	cyclic 3'-5' adenosine monophosphate
cDNA	complementary DNA
CNPase	2',3'-cyclic nucleotide phosphodiesterase
CNS	central nervous system
CRE	cAMP regulatory element
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EDTA	ethylene diamine tetraacetic acid
EGTA	ethylene glycol bis(β-aminoethyl ether)N,N,N',N'-tetraacetic acid
EtOH	ethanol
GAP	growth associated protein
GC	galactocerebroside
HDL	high density lipoprotein
HrP	horseradish peroxidase
IgG	immunoglobulin G
KGB	potassium glutamate buffer
LMP	low melting point
MAG	myelin-associated glycoprotein
MBP	myelin basic protein
mRNA	messenger RNA
MS	multiple sclerosis
NBT	nitroblue tetrazolium
NCAM	neural cell adhesion molecule
NGF	nerve growth factor
PBS	phosphate buffered saline
PLP	proteolipid protein
PNS	peripheral nervous system
RNA	ribonucleic acid
rRNA	ribosomal RNA
S.D.	standard deviation
SC	Schwann cell
SDS	sodium dodecyl sulfate
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SN	sciatic nerve
SSC	saline sodium citrate
TBS	tris-buffered saline
TBST	tris-buffered saline with tween-20
TCA	trichloroacetic acid
TE	10mM tris-Cl pH7.4, 1mM EDTA
TFMS	trifluoromethanesulfonic acid
Tris	tris (hydroxymethyl) aminomethane
UV	ultraviolet light
VLDL	very low density lipoprotein
YT	yeast extract-tryptone broth

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1. INTRODUCTION

1.1 The structure, function and formation of the myelin sheath.

The myelin sheath of the central nervous system (CNS) and the peripheral nervous system (PNS) is a modified plasma membrane that serves to increase the velocity of nerve impulses through axons. The events which lead to the formation of the compact myelin sheath have been the source of inspiration for investigators for over a century. Despite great advances in our understanding of the process of myelination, little is known about the molecular mechanisms by which the glial cells of the CNS and PNS extend their plasma membranes and physically ensheathe axons.

Although the myelin sheaths of the CNS and PNS resemble one another in many ways, there are basic differences in the process of myelination in the CNS and PNS as well as in the molecular structure of CNS and PNS myelin sheath. This section of the introduction is devoted to a brief description of the structure of the myelin sheath, its molecular organization and the events which lead to the formation of compact myelin in the peripheral nerve.

i. The structure of the myelin sheath.

The myelin sheath is formed by two distinct cell types in the CNS and PNS [for review see Raine, 1984]. Oligodendrocytes in the CNS extend their plasma membrane over several axons in a spiral wrapping, to form the compact myelin sheath. In the peripheral nerve,Schwann cells form the myelin sheath, although in contrast to oligodendrocytes, one Schwann cell is capable of ensheathing only a single axon in the

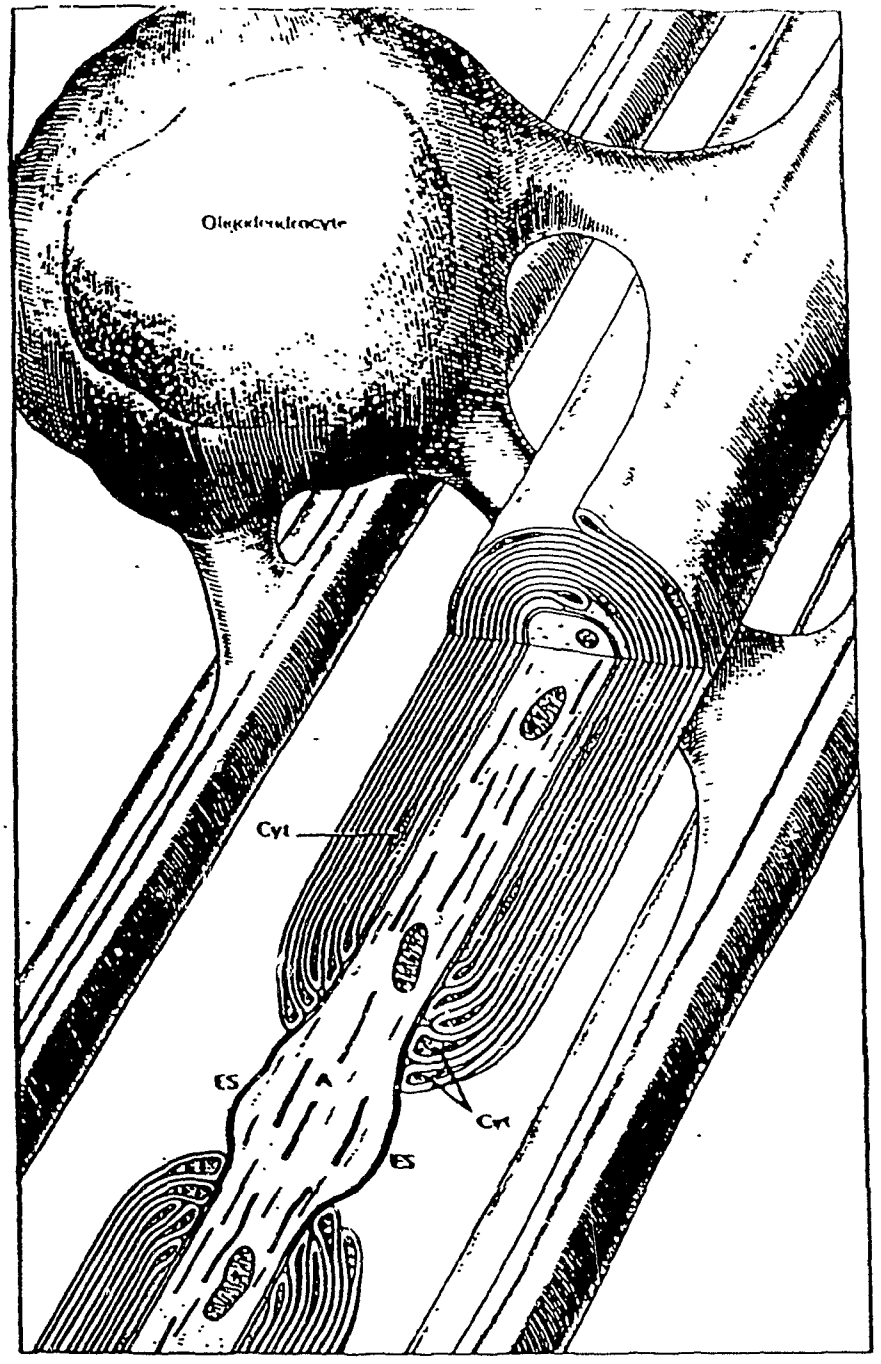


Figure 1.1 The myelin sheath of the CNS.

The same oligodendrocyte forms myelin sheaths for several nerve fibres as shown. The nodes f Ranvier in the CNS are exposed to the extracellular space (ES). The myelin sheath also contains channels of oligodendroglial cytoplasm (Cyt). Adapted from Bunge et al., [1961].

peripheral nerve. As can be seen in Figure 1.1, several axons in the CNS may be ensheathed by distinct myelin sheaths produced a single oligodendrocyte. The myelin sheaths from adjacent Schwann cells or oligodendrocytes are in close proximity with one another in a distinct structure known as the node of Ranvier.

Figure 1.2 shows in a schematic fashion how the myelin sheath is formed around the axon by the glial cell Axons are invested by the Schwann cell, and a loose wrap of the glial cell plasma membrane and cytoplasm takes place to completely engulf the axon. Subsequently, multiple wrappings of the axon occur, and extrusion of the cytoplasm leads to the compaction of several lipid bilayers to form the compact myelin sheath. Under the electron microscope, two distinct features of compact myelin may be observed in cross-sectional views. The apposition of the cytoplasmic surfaces forms the major period line, while the apposition of extracellular surfaces results in the formation of the intraperiod line. As may be seen in Figure 1.2, compacted myelin contains cytoplasmic regions. For example the inner tongue of the sheath known as the Schwann cell cytoplasmic collar contains cytoplasm and apposes the axolemma. Another schematic depiction of the glial cell and the axons of the neuronal cell body is shown in Figure 1.3. In this diagram the myelin sheath has been unwrapped to demonstrate the presence of other cytoplasmic channels within compact myelin, known as the Schmidt-Lanterman incisures, as well as the outlying cytoplasmic channel known as the lateral loop. As can be seen in Figure 1.3, the nucleus of the glial cell is segregated from the axon that is ensheathed by the compact myelin sheath. Thus communication between the axon and the nucleus of the glial cell may take place through the cytoplasmic channels, the Schmidt-Lanterman incisures and the lateral loops.

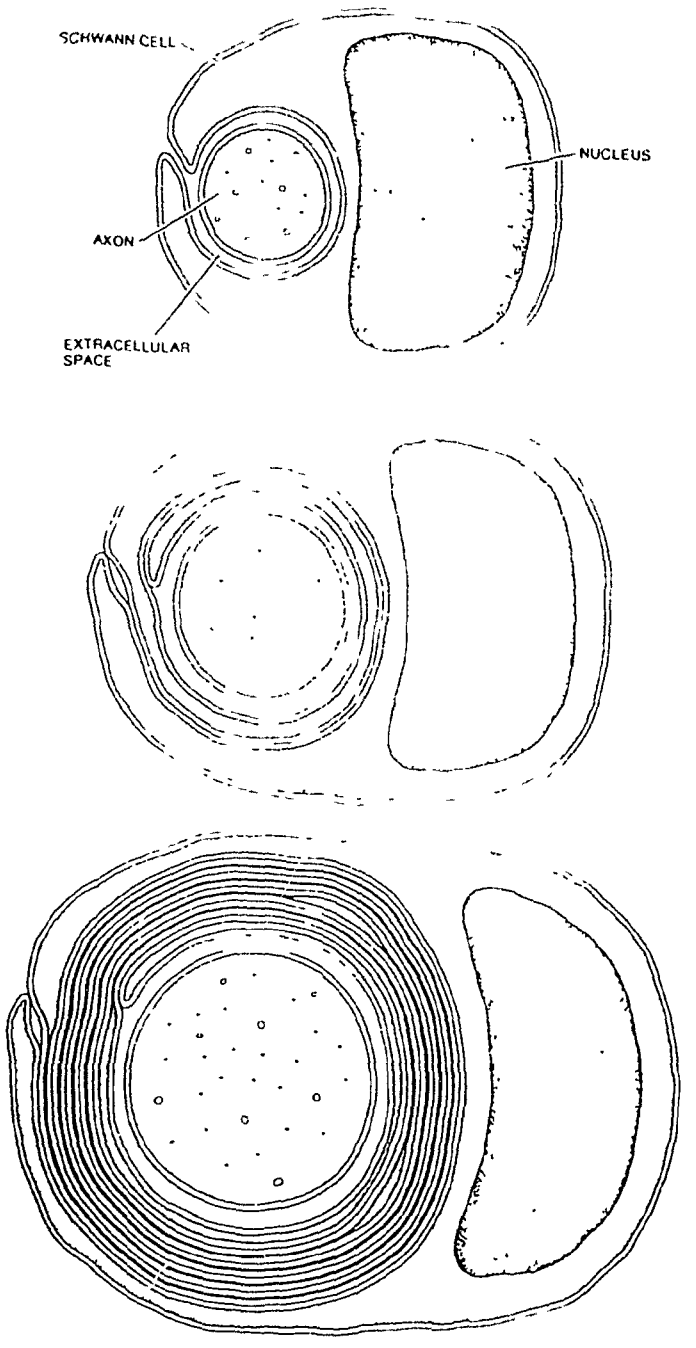


Figure 1.2 A schematic representation of the formation of the myelin sheath. The glial cell invest the axon in the initial phase of myelin formation. Myelinogenesis commences with the first spiral wrap around the axn. The spiralling of the cytoplasm and membrane, and cytoplasmic extrusion from the inner layers eventually results in the formation of compact myelin. (adapted from Raine [1984])

ii. Myelinogenesis in the PNS.

Myelin formation in the peripheral nerve occurs postnatally in rodents [for reviews see Raine, 1984 and Webster and Favilla, 1984]. Schwann cells are of neuroectodermal origin and migrate from the neural crest to the peripheral nerve during development. Axons leave the CNS and enter the PNS and it is still a matter of debate whether Schwann cells lead axons into the peripheral nerve or follow axons during neural development [for review see Keynes, 1987]. A large bundle or fascicle of unsheathed axons is subsequently invested by a single layer of Schwann cells. As the Schwann cells proliferate, they each send processes into the fascicle, slowly separating one axon from another. This process of Schwann cell proliferation and segregation of axons continues until each axon is encompassed by a single Schwann cell plasma membrane. The entire surface of the Schwann cell at this point is covered by a basement membrane that is synthesized by the Schwann cell and is continuous from one Schwann cell to the adjacent Schwann cell. When the diameter of the axon is 1 -2 μm, and each axon is enveloped by a single Schwann cell, myelinogenesis commences.

The primary event in PNS myelination is the formation of the first spiral wrap around the axon. One cytoplasmic ridge is tucked underneath the process on the other side of the axon to form the first complete wrapping of the axon. The spiralling of the cytoplasm and plasma membrane around the axon continues and the cytoplasm is slowly extruded until membrane compaction takes place. Bunge and co-workers [1989] have recently demonstrated that the movement of the Schwann cell nucleus does not contribute significantly to ensheathment of the axon. The myelin sheath is thus formed by the advance of the inner tongue in a spiral fashion, drawing with it the extended plasma membrane. Thus, compacted myelin so formed around the axon, the

Figure 1.3 The presence of cytoplasmic channels in myelin.

In this schematic representation, the myelin sheath has been unwrapped to demonstrate the presence of cytoplasmic channels in compact myelin, namely the Schmit-Lanterman incisures, as well as the outlying lateral loop.

Schwann cell cytoplasm and the basement membrane surrounding the Schwann cell-axon unit form the basic unit of the complex peripheral nerve. A more detailed description of the organization of multiple units of Schwann cells and axons is presented in Section 1.3.

iii. The molecular organization of myelin.

The molecular composition of the myelin sheath is quite unique as compared to other cell membranes [for review see Braun, 1984]. The myelin sheath is composed of about 70% lipid and 30% protein, and the molar proportion of phospholipids, glycolipids and cholesterol in the lipid fraction of the myelin sheath is approximately 4:2:4. One of the glycolipids that is typical of the myelin sheath is galactosylceramide (or cerebroside). This lipid is also present in the sulphated form known as sulphatide. Other lipids in myelin include plasmalogens, phosphatidylcholine, phosphatidylserine, phosphatidyl-ethanolamine and sphingomyelin. Both phosphoinositol and polyphosphoinositides are present in myelin, and remain associated with myelin proteins. There are also complex sialic acid-containing glycosphingolipids, or gangliosides, present in myelin. The monosialoganglioside GM_1 is the major ganglioside in myelin .

The protein components of CNS and PNS myelin are different. As shown in Figure 1.4, the major proteins in CNS myelin are the proteolipid protein (PLP), the family of myelin basic proteins (MBPs), the minor but functionally important myelin associated glycoprotein (MAG) and the myelin enzyme 2'3'-cyclic nucleotide phosphodiesterase (CNPase) (EC 3.1.4.37). The PLP, a hydrophobic integral membrane protein of about 26 kDa, forms about 55% of the total myelin proteins. The family of myelin basic proteins (MBP) forms about 30% of the total proteins in CNS myelin and about 15 - 20% of the total proteins of PNS myelin. PO is the major myelin protein of PNS myelin and is thought to perform an analogous role to PLP, which includes compaction of the multiple

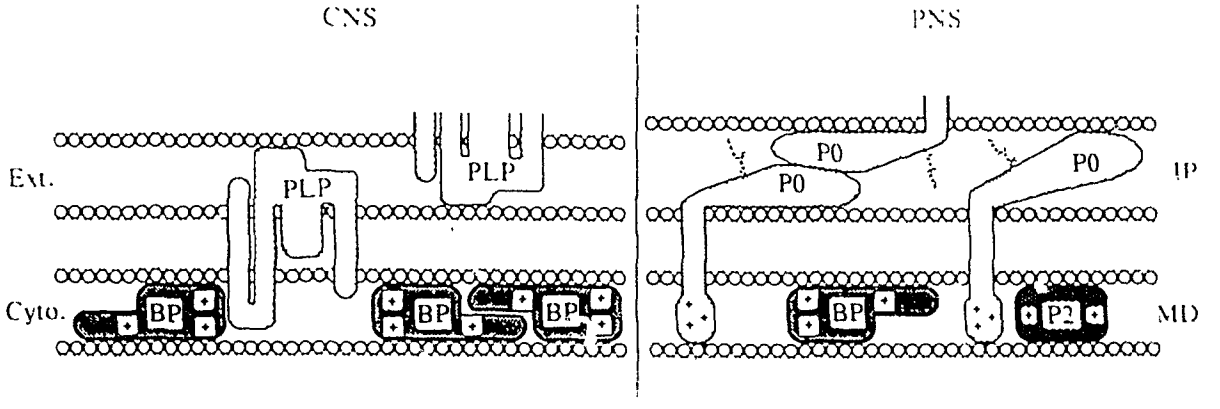


Figure 1.4 The molecular organization of compact CNS and PNS myelin

Apposition of the extracellular surfaces (Ext) of the glial cell membranes from the intraperiod line (IP). Apposition of the cytoplasmic surfaces (Cyt) of the membranes of the myelin-forming cells forms the major dense line (MD). The PLP and PO protein are thought to occupy analogous positions in CNS and PNS myelin respectively. BP: basic proteins P2: PNS specific basic protein [Morell et al, 1989]

membrane bilayers through hydrophobic interactions. Figure 1.4 shows a contemporary view of the organization of these proteins in the myelin sheath. Interactions between PLP molecules on adjacent membrane bilayers may lead to compaction of the myelin sheath. It is possible that interactions between the MBP located at the major dense line and PLP also serve to enhance compaction of the myelin sheath. In an analogous fashion, the PO molecule in PNS myelin may mediate membrane compaction through homophilic or heterophilic interactions. It is likely that the glycosidic moieties on PO may be involved in intermolecular interactions. MAG has been localized to the Schwann cell cytoplasmic collar and is thought to interact with an as yet uncharacterized receptor on the axolemmal surface. It is possible that MAG plays a structural role in maintaining the 12 - 14 nm space between the Schwann cell cytoplasmic collar and the axonal surface.

1.2 The organization of the genes encoding CNS and PNS myelin proteins.

The formation of the myelin sheath during neural development and regeneration involves a complex and coordinated synthesis of myelin proteins and lipids. In the past several years, the study of myelin proteins has been facilitated by the use of modern molecular biological approaches. Human and rodent genes encoding myelin proteins (PLP, MBP, MAG, PO, and CNPase) have been isolated, sequenced and characterized.

The expression of the genes encoding myelin proteins has in general been found to be more complex that was expected [reviewed by Lemke, 1989]. Myelin basic protein, for example, was found to exist in six different isoforms in the mouse through isolation of encoding cDNAs, although only four isoforms are observed by electrophoretic separation of the basic proteins [deFerra et al., 1985, Newman et al., 1987, for review see Lees and Brostoff, 1984]. These variant MBP cDNAs are formed from a primary transcript by

alternative splicing, a process which in itself is developmentally regulated [Takahashi et al., 1985]. Modern molecular genetic techniques have allowed chromosomal mapping of these genes, identification of the mutations in several dysmyelinating mutants on a molecular level, and even the curing of a dysmyelinating phenotype (the shiverer) through physical insertion of a normal allele in a transgenic animal.

A summary of the organization of the genes encoding myelin proteins follows, with a brief description of their expression in normal and mutant animals. Myelin gene organization has been reviewed recently by Campagnoni [1987], Lemke [1989], and Sutcliffe [1989].

i. The proteolipid protein (PLP) gene.

The PLP gene is quite unique, in that it encodes a protein which is highly conserved over evolution [reviewed in Nave and Milner, 1989]. The PLP and its smaller isoform, DM-20 are encoded by a single gene which encompasses 17 kb and is comprised of seven exons [(Figure 1.5) Diehl et al., 1986, Macklin et al., 1987, and Ikenaka et al., 1988]. There are multiple transcriptional start sites, but the major variability of transcript size arises from the use of three different polyadenylation signals 3' of the coding region [Ikenaka et al., 1988, Milner et al., 1985, Macklin et al., 1987, Puckett et al. ,1987]. Several laboratories have isolated and sequenced cDNA clones encoding PLP [Milner et al., 1985, Branks and Wilson, 1986, Dautigny et al., 1985, Naismith et al., 1985, Gardinier et al., 1986, Schaich et al., 1986, and Hudson et al., 1986]. The most abundant PLP transcript in the rat is 3.2 kb, and another 1.6 kb and a minor transcript of 2.4 kb are observed on Northern analysis. The open reading frame encoding PLP is 831 nucleotides in length, followed by approximately a 2.2 kb 3' non-coding region. Long 3' untranslated regions

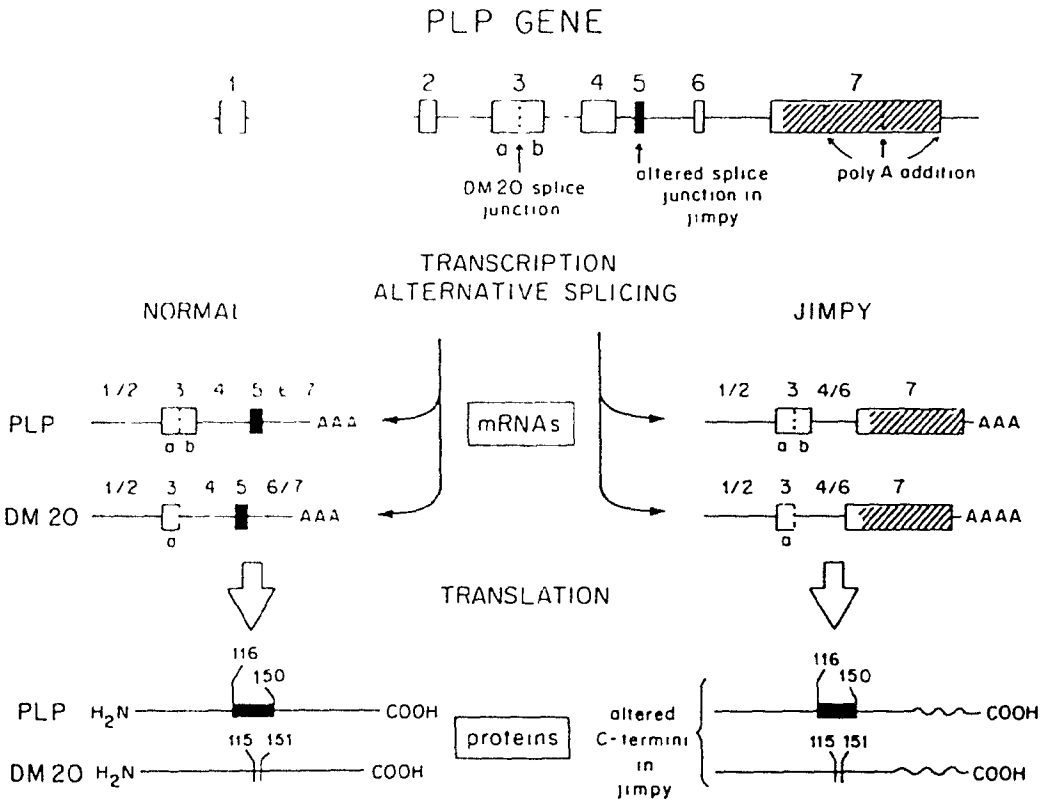


Figure 1.5 The PLP gene and its products in normal and jimpy mice.

The dotted line in exon 3 refers to the splice site used in the production of the DM-20 transcript. The sites of polyadenylation are indicated in exon 7 to produce PLP mRNAs of different sizers. The wavy line in the jimpy PLP and DM-20 protein refers to the altered C-terminal region produced when exon 5 is spliced to exon 7 in the jimpy mutant [reviewed by Campagnoni, 1988].

appear to be characteristic of messages derived from neural tissue [Milner and Sutcliffe, 1983].

The DM-20 protein is encoded by a transcript which is colinear with PLP mRNA [Nave et al., 1986, Simons et al., 1987]. Alternative splicing produces a PLP transcript [with exons 3A and 3B] and a DM-20 transcript [with exon 3A only]. The DM-20 transcript is 105 nucleotides shorter and encodes a corresponding protein shorter than PLP by 35 amino acid residues. The presence or absence of a 35 amino acid stretch which comprises a major hydrophilic domain may dramatically affect the function of the resultant protein. It remains to be ascertained, however, that PLP and DM-20 serve the same function in the CNS.

Other alternative splicing events of primary PLP transcripts cannot be ruled out. In this regard, smaller proteolipid proteins of 16 and 14 kDa have been isolated with sequences common to the C-terminus and N-terminus of PLP respectively [for review see Campagnoni, 1987]. The putative encoding transcripts are probably too low in abundance to be detected by Northern blot analysis. Developmental expression of the PLP gene has been studied in detail in the CNS. PLP may be detected in newborn rats and mice. The expression of PLP and DM-20 increases over neural development, with a peak of expression at about 21-22 days. The relative expression of PLP and DM-20 has been investigated in the developing spinal cord [Kronquist et al., 1987] and in the developing mouse brain [Gardinier and Macklin, 1988], and DM-20 expression appears to precede that of PLP.

Another unique feature of the PLP gene is its physical localization to the middle of the long arm of the X-chromosome (between bands Xq13 and Xqter) [Willard and Riordan, 1985]. This finding allowed the subsequent characterization of the aberrant phenotype in several X-linked myelin disorders. The jimpy (*jp*) mouse [Phillips, 1954] and its allelic form, the myelin synthesis-deficient (*msd*) [Meier and MacPike, 1970,

Eicher and Hoppe, 1973] are models for a similar human condition known as Pelizaeus-Merzbacher (PM) disease, first described a century ago [Pelizaeus, 1885, Merzbacher, 1910]. The dysmyelinating phenotype in *jp* is complex, but primarily arises from a point mutation. Such point mutations have recently been described in the PLP locus of PM patients [Hudson et al., 1989]. The linkage of PM disease to the PLP gene has thus provided a starting point for the understanding of inherited neurological diseases.

ii. The myelin basic protein (MBP) gene.

The family of basic proteins forms a major protein, comprising 30-40% of CNS and 5-15% of PNS myelin proteins [reviewed by Lees and Brostoff, 1984, Campagnoni, 1988]. Historically, the 18.5 kDa MBP was the first myelin protein whose sequence was completely deduced by peptide sequencing [Eylar et al., 1971, Carnegie, 1971] .

The MBP gene has been characterized and studied by several laboratories. Both rodent and human copies of the MBP gene have been isolated [(Figure 1.6) deFerra et al., 1985, Takahashi et al., 1985, Kamholz et al., 1988, Streicher and Stoffel, 1989]. The single copy gene has been mapped to the distal end of chromosome 18 [Roach et al., 1985, Saxe et al., 1985, Sparkes et al., 1987]. The MBP gene contains seven exons which span 30 kb in the mouse [deFerra et al., 1985, Takahashi et al., 1985] and 32 to 34 kb in the human in one study [Streicher and Stoffel, 1989] and 45 kb in another study [Kamholz et al., 1988]. The organization of the MBP gene is rather remarkable, as the exons are as small as 33 bp and the intervening introns span relatively large distances. Exon 7 is the largest exon, spanning 1600 bp, and contains a very long 3' untranslated region [deFerra et al., 1985].

Complementary DNAs encoding the MBPs have been isolated by several groups [Roach et al., 1983, Zeller et al., 1984, Mentaberry et al., 1986, Roth et al., 1986,

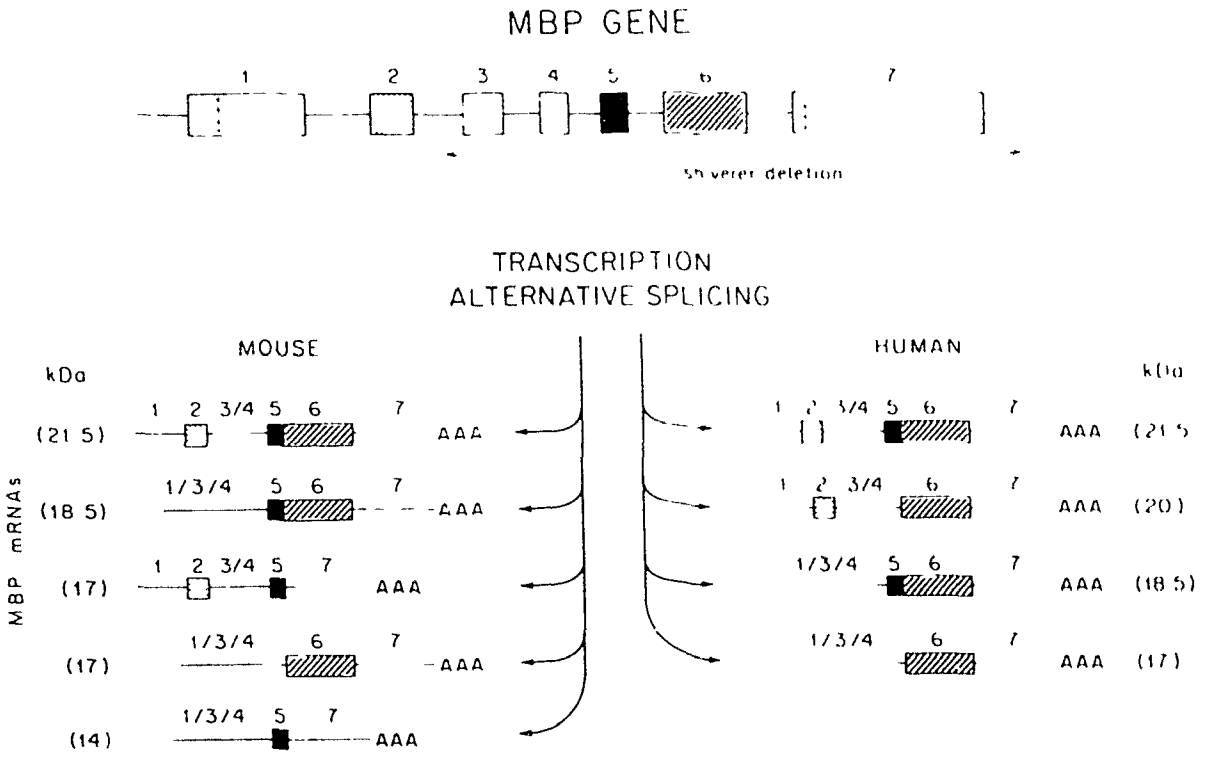


Figure 1.6 Structure and expression of the MBP gene

Alternative splicing of the MBP primary transcript gives rise several different transcripts in rodents and in the human. The numbers above the transcripts indicate the presence of those exons in the mRNA. The size of the MBPs encoded by each mRNA species is indicated in brakets [adapted from Campagnoni, 1988].

Kamholz et al., 1986, Newman et al., 1987] and the sequences shown to be identical in the CNS and PNS [Mentaberry et al., 1986]. Two polyadenylation signals have been described and both appear to be utilized [Roth et al., 1987]. There are, to date, reports of at least six isoforms of MBP in the mouse, and four in the human, formed by alternative splicing of the seven exons. The splicing event(s) that results in such diverse isoforms, is developmentally regulated [Barbarese et al., 1978]. Exon 2-containing transcripts [which encode the 17 kDa and 21.5 kDa MBPs] are found to be more abundant during myelinogenesis, and the relative levels of these isoforms drop in the adult mouse. Kamholz et al. [1986] proposed that the amino acid sequence motif encoded by exon 2 forms an extra loop that can interact with PLP and mediate compaction of newly formed myelin. A more detailed study of the abundance and distribution of the various MBP mRNAs and protein during myelination needs to be carried out to validate this hypothesis. Developmental expression of the MBPs has been investigated in detail in the CNS and PNS [reviewed by Campagnoni, 1988]. MBPs like PLP may be detected in newborn rats and mice. MBP expression peaks prior to that of the PLP and DM-20. The expression of the various isoforms of MBPs is developmentally and spatially regulated [Kronquist et al., 1987, Roth et al., 1987, Kamholz et al., 1986, Jordan et al., 1987].

Two lethal recessive murine mutations, the shiverer mouse (*shi*) and its allelic form, the myelin-deficient mouse (*mld*), have been shown to involve the MBP locus [Doolittle and Schweikart, 1977, Doolittle et al., 1981]. In *shi* mice the molecular defect was found to be the deletion of exons 3 to 7 [Kimura et al., 1985, Roach et al., 1985, Molineaux et al., 1986]. No MBP is detected in the mutant mouse, and this is further manifested by the absence of a major dense line in the myelin sheath. The MBP gene in the *mld* mouse, on the other hand, is partially duplicated with an inverted gene segment of exons 3 to 7 appearing upstream of the normal gene [Okano et al., 1988, Popko et al., 1988]. No human diseases have yet been localized to chromosome 18. Trisomy 18,

which might very well involve the MBP locus in many patients, causes many phenotypic defects including mental deficiency. It is possible that overproduction of MBP may interfere with proper neural development.

A landmark event in the field of molecular genetics has been the cure of the shiverer deletion by a molecular approach. In this endeavour, Readhead and co-workers [1987] introduced a wild-type MBP copy into the genome of transgenic shiverer mice. Clinical symptoms of the shiverer phenotype were largely eliminated in the transgenic mice, although levels of MBP mRNA were only 25% that of normal. A second transgenic approach using an MBP promoter upstream of an MBP cDNA in a target 'minigene' has also led to a successful cure of the shiverer phenotype . These approaches have thus marked the introduction of gene therapy to neurological disorders.

iii. The myelin-associated glycoprotein (MAG) gene.

The myelin-associated glycoprotein (MAG) is a large glycoprotein of approximately 100 kDa, that is found in both the PNS and CNS. This protein has recently become the subject of much interest, as recent investigations have suggested MAG is a cell-adhesion molecule that may play an important role in initiation of myelination [Quarles, 1989].

Complementary DNAs encoding MAG were isolated from rat and mouse cDNA libraries using nucleic acid and immunological screening strategies [Lai et al., 1987, Arquint et al., 1987, Salzer et al., 1987]. Very recently, human cDNAs for MAG have been isolated and characterized [Spagnol et al., 1989, Sato et al., 1990]. From the deduced amino acid sequence, it was apparent that two MAG isoforms of molecular weight 67 and 72 kDa [named p67MAG and p72MAG, respectively] are encoded by mRNAs that are formed by alternative splicing at the 3' end of a primary transcript (Figure 1.7). Thus p67MAG is encoded by an mRNA containing exon 12, while p72MAG arises from an

mRNA that lacks exon 12. Differential splicing is developmentally regulated, as p72MAG-encoding mRNAs are predominant early in neural development in the CNS, while the major MAG transcripts in the adult CNS encode p67MAG [Lai et al., 1987, Tropak et al., 1988, Fujita et al., 1998]. Tropak and co-workers [1988] have examined the expression of MAG mRNAs during PNS and CNS development. They have identified another 5' splicing event that does not affect the coding region of the transcript, and appears to be independent of 3' splicing events. This has been corroborated in a recent abstract from another laboratory [Fujita et al., 1990]. The expression of the MAG gene is also regulated in a tissue-specific fashion at the level of transcription. Transcripts encoding p67MAG are the major MAG mRNAs found in the PNS at all stages of neural development [Frail and Braun, 1985, Tropak et al., 1988, Salzer et al., 1987].

It has been suggested recently that the two isoforms of MAG play distinct roles, and that MAG may mediate the initiation of myelination by modulation of adhesion between the axon and the ensheathing Schwann cell or oligodendrocyte [Attia et al., 1989]. In support of this hypothesis, Edwards and co-workers [1989] have reported that p72MAG is selectively phosphorylated in the CNS. There are several lines of evidence that suggest that the isoforms of MAG may not be functionally distinct. The variation in splicing events observed in rodent MAG gene expression does not appear to take place in humans. Sato et al. [1990] have reported the isolation of several human cDNAs encoding MAG from an adult brain cDNA library, that all encode p72MAG. This preliminary report suggests that p72MAG may be the only isoform present throughout human CNS development. Noronha and co-workers [1990] have reported that MAG in the rat PNS (which is predominantly p67MAG) is phosphorylated, suggesting that selective phosphorylation observed in the rat CNS may not be essential for modulated cell adhesion as previously proposed.

The MAG gene is by far the most fragmented myelin gene to be characterized. Of the 13 exons spanning 16 kb, the first three are not involved in the coding region of the

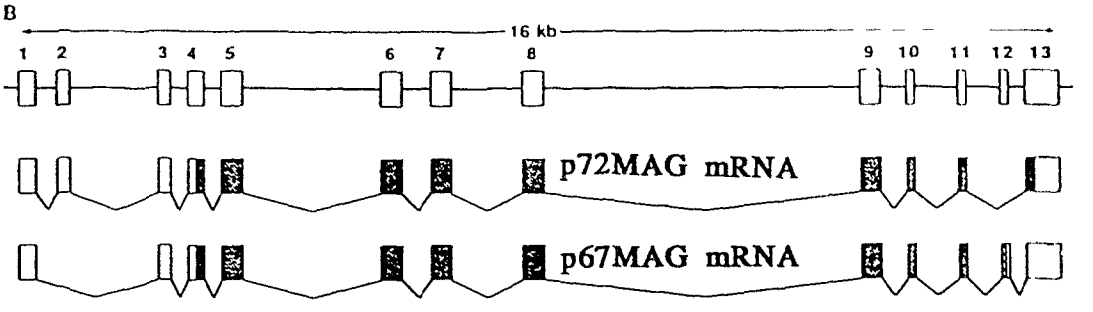


Figure 1.7 Structure and expression of the MAG gene

The structure of the MAG gene is presented in this diagram. Two alternatively spliced mRNAs are formed by the alternative splicing of exons 2 and 12. Of these two exons, only exon 12 affects the coding region (shown by the shaded region) of the MAG mRNAs. Thus p72MAG is encoded by a transcript lacking exon 12. The MAG isoform, p67MAG is encoded by a transcript with exon 12. See text for details [adapted from Lai, et al, 1987].

transcript [Lai et al., 1987]. Abnormal expression of the MAG gene has been reported in the CNS of quaking mice. The predominant MAG mRNA at 15 and 25 days of age in the quaking CNS encodes p67MAG [Frail and Braun, 1984, Fujita et al., 1988]. Although the genetic defect underlying the quaking phenotype is as yet unknown, several post-transcriptional abnormalities in MAG expression have been reported. The molecular weight of MAG in quaking mice appears to be altered, while decreased fucosylation of the polypeptide also appears to take place [Matthieu et al., 1974, Inuzuki et al., 1987].

iv. The 2'3' cyclic nucleotide 3'-phosphodiesterase (CNPase) gene.

CNPase (EC 3.1.4.37) is one of the most active enzymes in myelin. It catalyses the conversion of 2'3' cyclic AMP to 2'-AMP, although it is quite likely that cyclic AMP is not the physiological substrate for the enzyme [see recent reviews by Vogel and Thompson, 1989, Sprinkle, 1989].

Complementary DNAs encoding the two isoforms of CNPase (of 44 and 48 kDa) in the rat, bovine and human CNS were recently isolated by several laboratories [Kurihara et al., 1987, Bernier et al., 1987, Vogel and Thompson, 1987, Kurihara et al., 1988]. Bernier and co-workers [1987] also reported the expression of an mRNA encoding CNPase in the thymus. The mRNA expressed in the thymus is of an intermediate size (2.6 kb) compared to neural CNPase mRNAs (2.4 and 2.8 kb), and is probably formed by alternative splicing at the 5' end of the primary transcript. Another splicing event results in the formation of the two mRNAs that encode the CNS isoforms of CNPase [Kurihara et al., 1987, Bernier et al., 1987, Kurihara et al., 1990].

Kurihara and co-workers [1990] have recently reported the organization of the mouse CNPase gene. The gene has a relatively simple structure, in that three exons are separated by two introns, and the CNPase gene spans 6 kb. All three exons comprise the

coding region of the gene. These results suggest that the CNPase gene may fall in the class of myelin genes, where alternative splicing of the primary transcript occurs in a tissue-dependent manner. In contrast to the expression of the MBP, PLP and MAG genes, splicing events in the expression of the CNPase gene do not appear to be developmentally regulated [Bernier et al., 1987].

v. The PO gene.

PO is one of the few characterized PNS myelin proteins that is not expressed in the CNS. There is reason to believe that of the genes encoding the major myelin proteins, PLP, MBP and PO, the last may be the most ancient. This has been suggested through phylogenetic examination of the myelin proteins in the nervous system of invertebrates and lower vertebrates [reviewed by Waehneldt et al., 1986]. For example the shark CNS contains PO-like proteins while the CNS of more evolved fish, e.g. lungfish, show the presence of both PO-like and PLP-like proteins. Waehneldt and co-workers[1986] have proposed that PO in the CNS of lower vertebrates was replaced by PLP during evolution. Examination of the structure of PO protein as deduced from the sequence of the polypeptide [Uyemura et al., 1987] and from encoding cDNAs [Lemke and Axel, 1985], suggest that PO is a member of the immunoglobulin gene superfamily [Uyemura, 1988]. This role of PO as a putative cell-adhesion molecule has been further supported by recent studies from this laboratory [Földvári et al., 1990] and from other groups [D'Urso et al., 1990, Filbin et al., 1989].

A full length cDNA encoding PO was isolated by Lemke and Axel [1985] using a differential screening approach. The 1.85 kb mRNA encodes a 28 kDa polypeptide, which is proposed to have a hydrophobic and glycosylated extracellular domain, a single membrane spanning domain and a basic intracellular domain. The extracellular domain is

thought to contain one disulphide bridge and thus resembles a variable chain of an immunoglobulin.

The PO gene is approximately 7 kb in length and contains six exons [(Figure 1.8) Lamar et al., 1988]. A unique feature of the PO gene is the scission of the immunoglobulin domain over two exons (exons 2 and 3). This is consistent with previous concepts that the progenitor gene of the immunoglobulin gene superfamily was assembled by duplication of a half-domain [Bourgois, 1975]. Such half-domains have also been identified within the genes of other members of the immunoglobulin gene superfamily, including NCAM [Owens et al., 1987] and CD4 [Littman and Gettner, 1987].

1.3 Regeneration in the peripheral nervous system.

A singular feature of the PNS is its capacity to regenerate after considerable nerve damage has occurred. This remarkable phenomenon was investigated as early as the turn of this century, with the pioneering studies by Ramon y Cajal in the formation of axonal sprouts on proximal segments of injured nerves and their growth towards a peripheral target. The process of regeneration has just begun to be understood at the level of the cell and efforts are underway to unravel the molecular basis of regeneration and remyelination of the peripheral nerve.

Molecular biological approaches to the characterization of a biological system may often lead to a clearer understanding of events at the level of the individual molecule. However, experiments with a molecular biological approach are often quite detached from the events that actually take place at the cellular level. An understanding of the morphological changes that take place is critical to the appreciation of the results from such investigations. It is with this view in mind that the present section of the introduction is devoted to cellular aspects of peripheral nerve regeneration, and a description of changes that take place in various components of the peripheral nerve in response to nerve injury.

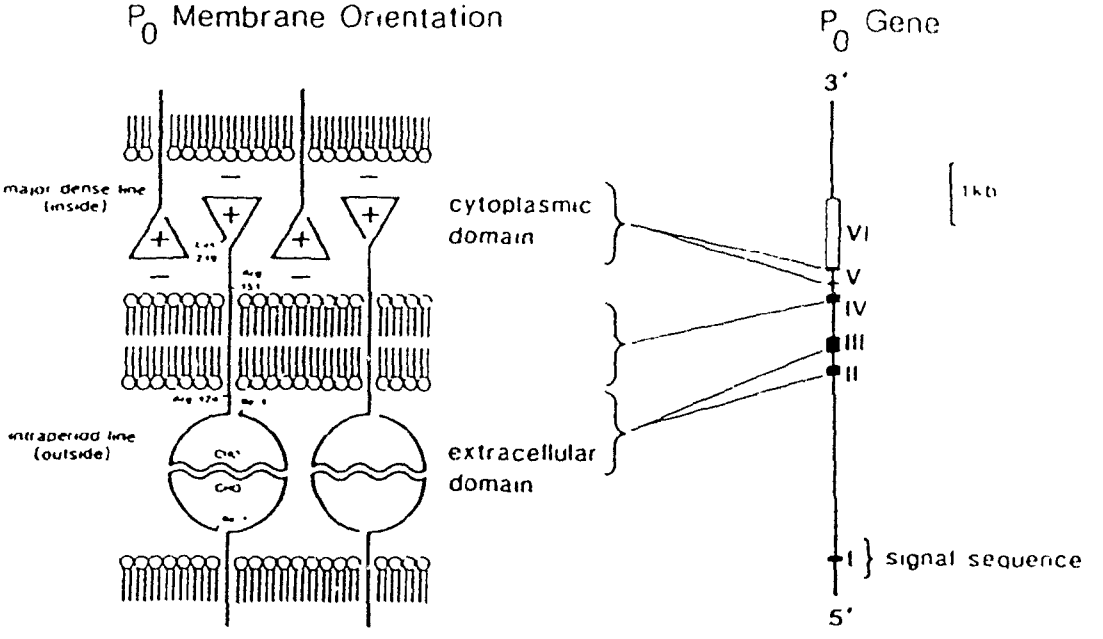


Figure 1.8 Structure of the PO gene.

In this diagram, the rat PO gene has been aligned with a representation of the putative orientation of PO protein in the PNS myelin.The PO protein is depicted as spanning the membrane once, with the amino terminus positioned extracellularly. The proposed extracellular domain of PO protein is encoded.by two exons (II and III) as depicted. [Lemke et al, 1988]

i. Microscopic anatomy of the peripheral nerve trunk.

The microscopic and ultrastructure of nerve fibres has been reviewed by Thomas and Ochoa [1984] and by Lundborg [1988]. Ensheathment of the axon by the multilamellar myelin sheath allows fast conduction of saltatory impulses from the neuronal cell body to peripheral target cells. Although conduction velocities in myelinated nerve fibres are very much higher than in unmyelinated fibres, both forms of nerve fibres are found in the PNS. If fact, it is possible to find certain regions of an axon that are myelinated and others that are unmyelinated, suggesting that axonal signals are critical for their ensheathment by Schwann cells and the consequent formation of the myelin sheath.

Schwann cells are arranged in a longitudinal fashion along the axon, and meet each other at the nodes of Ranvier, where the processes from adjacent Schwann cells interdigitate. Ions are transported in and out of the axon at the nodes of Ranvier along the nerve fibre, allowing saltatory propagation of an impulse from one node to the next along the nerve fibre. The axon and the surrounding concentric layers of the Schwann cell and the myelin sheath are further ensconced by a continuous Schwann cell-plasma membrane and its basal lamina. The reticular network and collagenous matrix surrounding fascicles of nerve fibres form the endoneurial sheath. The endoneurial sheath is thus an anatomical component of the nerve trunk that consists mainly (80-90%) of Schwann cells, as well as endothelial cells, mast cells, fibroblasts and a capillary network. The endoneurial sheath represents an easily dissectable component of the nerve that may be used to study the metabolism of Schwann cells in vivo [discussed in Yao, 1984].

Nerve fibres are organized in each endoneurial compartment in bundles, and each fascicle is further surrounded by a dense sheath of considerable tensile strength called the perineurium. The perineurium consists of a number of layers of flattened cells which possess a basement membrane on both the inner and outer aspects. The number of layers

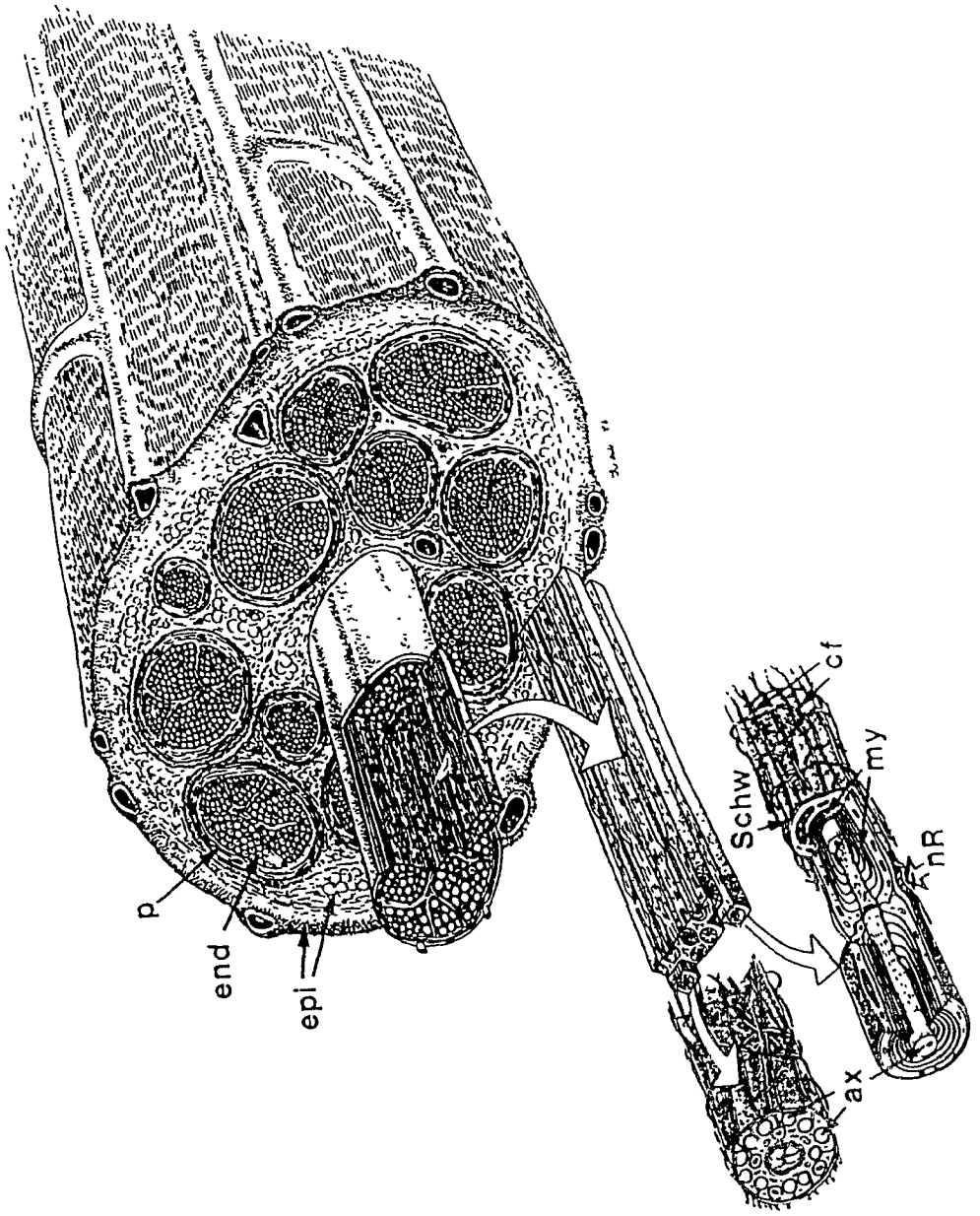


Figure 1.9 Microscopic anatomy of the peripheral nerve trunk.

In an individual nerve fibre, the axon (ax) is ensheathed by the myelin sheath (my) synthesised by the Schwann cell (Schw). Connective fibres (cf) are run in a network around the nerve fibre to form part of the endoneurial sheath (end). The fascicles of nerve fibres are further ensconced in the perineurium (p) and epineurium (epi). [Lundborg, 1988].

forming the perineurium may be as many as 15 [Thomas and Olsson, 1984], as seen in mammalian nerve trunks. These flattened cells form a tight barrier isolating the endoneurial space from the external milieu. The perineurium therefore appears to serve a dual function of creating a diffusion barrier to the endoneurial compartment and protecting the endoneurial sheath from mechanical stress.

Several bundles of nerve fibres are loosely placed in a connective sheath called the epineurium. The epineurium serves as yet another protective barrier for the nerve fibres. The epineurium contains a well-developed network of blood vessels which feed the intricate network of capillaries within the endoneurial compartments.

The conduction of nerve impulses through peripheral nerves and transport of macromolecules through the axon both demand a continuous supply of energy. This is supplied through the complex microvasculature in the endoneurium, the perineurium and the epineurium, first described in detail by Adams [1942]. Longitudinal channels of blood vessels are located in the epineurium and are connected to parallel channels in the perineurium and in the endoneurial compartments. The microvasculature within the peripheral nerve is highly complex and differentiated in function in each compartment. For example, the blood-nerve barrier is maintained in the endoneurial compartment, inasmuch as certain macromolecules are not capable of diffusion from the capillaries situated within. It is thought that tight junctions in the plasma membranes of endothelial cells prevent the diffusion of substances into the endoneurial space and into the vicinity of nerve fibres. On the other hand, the vasculature in the epineurium is relatively leaky, allowing passive diffusion of macromolecules.

The peripheral nerve is thus a complex tissue comprised of several cell types and compartments. Although the major glial cell in the peripheral nerve is the Schwann cell, it is important to keep in mind that all Schwann cells are not involved in myelination. Almost two-thirds of the Schwann cells in the sciatic nerve are non-myelin forming Schwann cells

[Jessen et al., 1985]. In addition, the relative numbers of cell types may change with development and during injury. For example, the invasion of macrophages into the peripheral nerve after nerve injury is well-documented and it is likely that these cells play an important role in the regenerative process [Liu, 1974, Boyles et al., 1989, Clemence et al., 1989].

ii. The temporal sequence of events following crush-injury and permanent transection.

Peripheral nerve injuries have been classified by the degree of damage caused to the nerve fibres [Sunderland 1978]. Nerve injuries vary in degree of severity from a simple compression of the nerve trunk, classified as a Sunderland type 1 lesion, to a permanent transection classified as Sunderland type 3 - 5 lesions. In a Sunderland type 1 lesion, the integrity of the tissue is preserved but a block of axonal transport takes place. In cases where there is no disruption of axonal continuity, removal of the compression leads to complete recovery of the tissue within weeks or months. Crush-injuries may be largely classified as type 2 lesions, where the axonal continuity is lost, demyelination of the segment distal to the site of injury takes place, and axonal regrowth into the distal segment is required for complete regeneration. It is possible to further classify such injuries on the severity of the damage to the endoneurial tubes. If the endoneurial sheaths remain intact after crush-injury, the regrowing axon is capable to re-innervating the proper targets due to the correct guidance offered by the endoneurial sheaths. However, more severe damage to the inner compartments of the peripheral nerve trunk lead to misguided axonal outgrowth and incomplete regeneration of the distal segments. A permanent transection of the peripheral nerve may be classified as Sunderland type 3-5 lesions, depending on the severity of the damage. In cases where the proximal and distal segments of the nerve are

physically separated, the proximally located axonal sprouts cannot obtain proper guidance for regrowth, and regeneration and remyelination are prevented.

Disruption of axonal continuity in a peripheral nerve fibre, as caused by a crush-injury or permanent transection, has a profound effect on the neuronal cell body. The neuronal cell body,or soma, is the major site of biosynthesis of macromolecules. Loss of transport of macromolecules to the periphery through axons results in profound metabolic changes in the neuron. It may be stressed at this juncture, that one of the unique features of PNS regeneration is that it involves cellular repair, wherein the neuronal cell body is suddenly depleted of axoplasmic volume and subsequently compensates for this loss through axonal regrowth and re-establishment of proper channels for impulse conduction. This is in contrast to tissue damage, where a large number of cells are destroyed and have to be replaced during repair.

Figure 1.10 depicts the sequence of events that follow crush-injury of the peripheral nerve or after a transection that is not permanent. Loss of axonal contact leads to demyelination and degeneration in the distal segment of the nerve shortly after crush. Myelin debris is phagocytosed by Schwann cells and macrophages in the distal segment. Other changes also occur at this stage as depicted in Figure 1.10c, including demyelination of the proximal segment to the nearest node of Ranvier, increases in the cell volume of the neuronal cell body, displacement of the nucleus to the periphery of the cell, and loss of basophilic material from the cytoplasm of the neuron. Transient demyelination after crush is followed by Schwann cell proliferation in the distal segment [Figure 1.10d] and the formation of discrete bands of Bungner. These channels of Schwann cells serve to guide the regrowing axonal sprouts from the proximal segment of the nerve. The re-

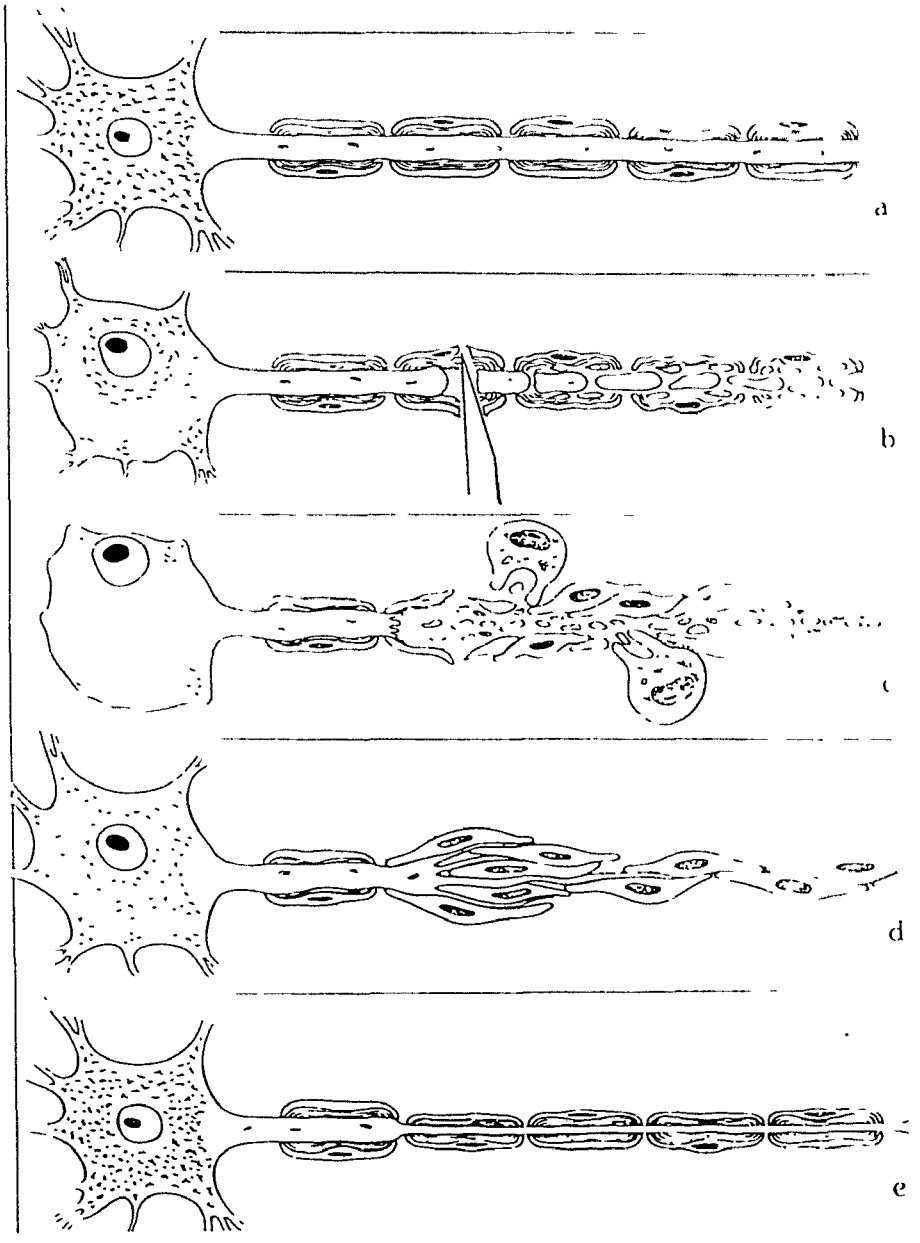


Figure 1.10 Degeneration and regeneration of myelinated nerve fibres.

a. Normal appearance of a myelinated fibre. b. Transection of the fibre results in distal fragmentation of axon and myelin. In the proximal segment , some degeneration occus at least to the nearest node of Ranvier. c. In the distal segment Schwann cells proliferate. Macrophages and Schwann cells phagocytose debris material. d. The Schwann cells in the distal segment line up in bands of Büngner. e. Axonal connection with the distal fragment leads to regeneration and remyelination [Lundborg, 1988].

instated Schwann cell-axonal contacts lead to remyelination of the nerve fibres progressing to complete regeneration within 3 to 4 months [Figure 1.10d].

Gershenbaum and Roisen [1978] were able to depict such cellular events using scanning electron microscopy to observe initial degeneration followed by regeneration of nerve fibres in the distal segment of the crush-injured nerve. As shown in Figure 1.11, obvious morphological changes occurred by 3 days post-crush in the distal segment of the sciatic nerve. The myelinated axons dissociated into elliptical segments of varying size, and several constrictions were observed along the nerve fibre. Demyelination and phagocytosis were quite extensive by 9 days post-crush. Large spherical globules of myelin debris were observed at this stage. There was also an increase in the connective tissue content of the distal segment. By fifteen days post -crush, there were indications of regeneration, although myelin globules were still apparent. At twenty days post-crush, the debris from the demyelinating phase was decreased, and regeneration was apparent. By thirty days post-crush, Gershenbaum and Roisen [1978] were able to demonstrate the presence of several regenerated nerve fibres, indicating that the distal segment was well on the way of regeneration.

iii. The response of the neuron to nerve injury.

The loss in axoplasmic volume following peripheral nerve injury results in dramatic changes in the metabolism of the neuronal cell body. In order to recover, the neuronal cell extends axonal sprouts in the direction of the target tissue. The changes that occur at the tip of each individual neurite are just beginning to be understood with recent advances in the elucidation of the growth cone, a structure thought to be largely responsible for the motility and growth of extending neurites.

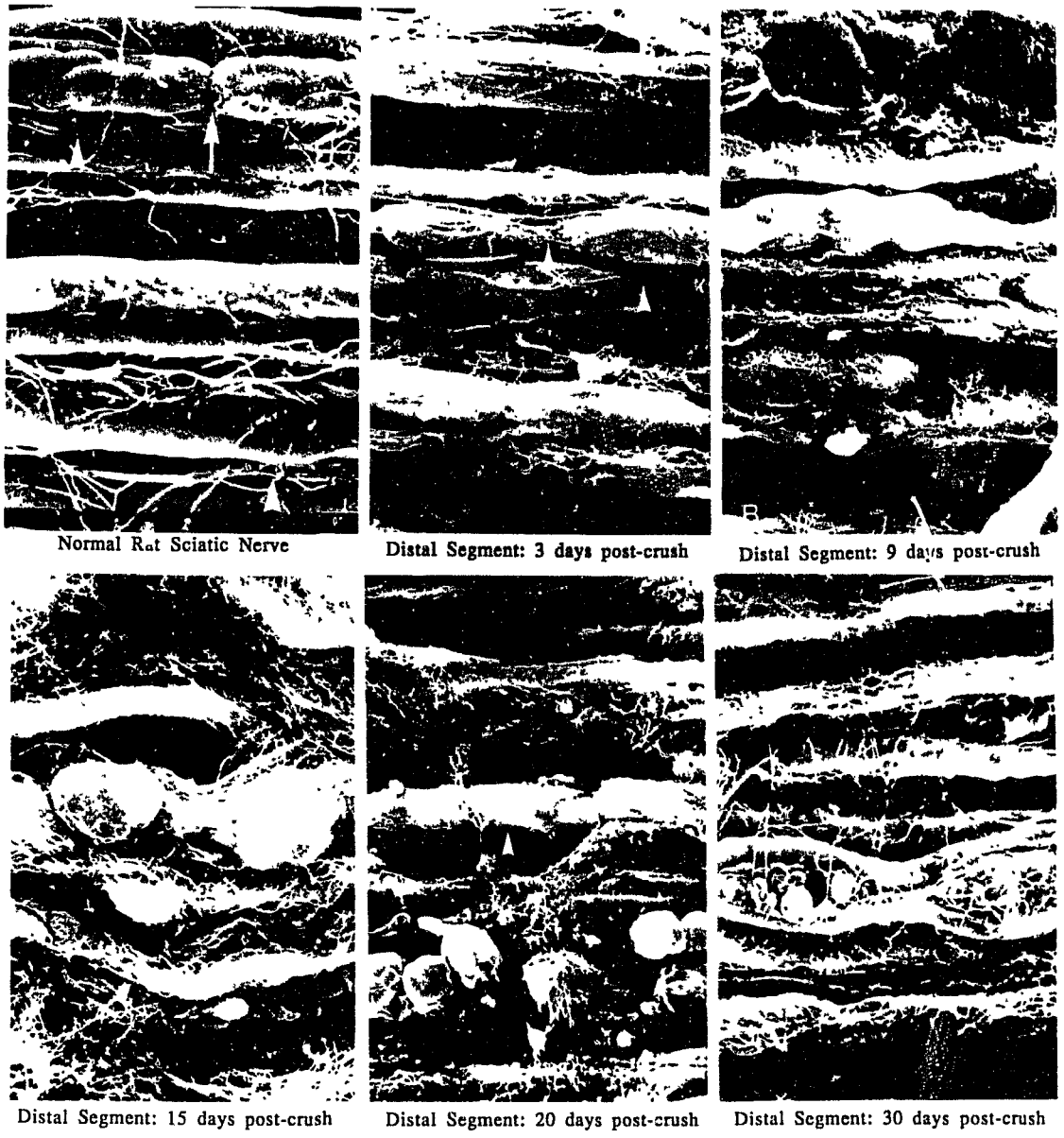

Normal Rat Sciatic Nerve
Distal Segment: 3 days post-crush
Distal Segment: 9 days post-crush
Distal Segment: 15 days post-crush
Distal Segment: 20 days post-crush
Distal Segment: 30 days post-crush

Figure 1.11

The structure and possible modes of function of the growth cone have been recently reviewed by Gordon-Weeks [1989]. The growth cone has been proposed to have two major functions. First, the growth cone converts external guidance cues that may be proferred by cell surfaces or by neurotrophic factors into changes in motile behaviour. Second, it has been implicated in determining the direction of neurite outgrowth. Studies are in progress to examine the molecular connection between these two functions of the growth cone.

At the periphery of the growth cone are finger-like extensions called filopodia, and broad, sheet-like structures called lamellopodia. As the growth cone advances, the filopodia encounter new territory and subsequently change the direction of growth towards a preferred target. The neurite itself provides material for new growth at the tip of the cell soma via axoplasmic transport of membrane components through bundles of microtubules that form the axoplasmic transport system. Thus growth occurs at the growth cone through the insertion of transported membrane components.

Cheng and Reese [1988] recently observed through electron microscopic examination of growth cones, that the bundles or fascicles of microfilaments end at the periphery of the growth cone and fan out as single microtubules into each filopodium. It is possible that the actin cytoskeleton in each filopodium presents a physical barrier for the microtubules. The actin cytoskeleton in itself is important in growth cone motility, as disruption of the actin network has been shown to result in undirected growth at the tip of each neurite. At the molecular level, large pools of unpolymerized tubulin have been detected in each filopodia. This form of tubulin is tyrosinated at the C terminus and is unstable in polymerized microtubular structures. It was suggested that exchange of tubulins at the filopodia undermines microtubule stability at this location while allowing microtubules to remain stable and active in the neurite.

The current view, therefore, of the tip of the growth cone is that it consists of highly motile filopodia that determine the direction of growth and express motility through the presence of an actin-based cytoskeleton. The change in direction of growth as well as cues from the external environment result in incorporation of membrane components delivered to the site by a transport system consisting of microtubules, and subsequent growth at the tip of the extending neurite towards a preferred target tissue.

In addition to changes at the tip of extending neurites, rapid alterations take place in the cell soma, which is the major site of biosynthesis in neurons. Perhaps one of the best demonstrations of significant changes in neuronal phenotype through nerve injury is in investigations involving conditioning lesions [reviewed by Lundborg, 1988]. Several investigators have reported that a conditioning lesion modifies the response of the nerve fibre to subsequent test lesions [reviewed by Lundborg, 1988]. Although the relative positions of the conditioning and test lesions do not appear to affect the regenerative process, Jenq and co-workers [1988] were able to demonstrate that a minimum of 2 days between lesions was required to observe accelerations in regeneration. They also reported that the number of regenerating axons was increased by conditioning lesions. It is thought that accelerated regeneration by conditioning lesion may either be due to changes in the local environment of regrowing axons, or due to primary changes in the metabolism of the neuronal cell body [Brown and Hopkins, 1981, McQuarrie, 1985]. In support of the possible priming of the neuron to subsequent injuries, Jenq and co-workers [1988] have suggested that a retrograde transport signal from the site of the conditioning lesion leads to alterations in the phenotype of the neuronal cell in a time frame of 2 days. This change may involve increases in synthesis of cytoskeletal proteins [McQuarrie and Grafstein, 1982], or in specific proteins that may be associated with neurite outgrowth.

Recent searches for neuronal proteins that may be involved in neurite outgrowth both in the CNS and PNS have resulted in the characterization of a group of growth-

associated proteins (or GAPs). These proteins are developmentally regulated, and hypothesized to be essential for the development of neural circuitry. GAP-43 has recently come into prominence, as a 43-48 kDa protein that was found to be associated with axonal outgrowth during development in lower and higher vertebrates [reviewed by Snipes et al., 1987, Benowitz and Routtenberg, 1989, Skene, 1989]. Skene and Willard [1981a] initially reported greater than 15 to 20 fold enhancement of synthesis of this protein during regeneration of the optic nerve of the toad. GAP-43 was subsequently found to be transported down the axon at rates up to 100 fold greater during axonal outgrowth, suggesting that this protein was required in some way during neuronal outgrowth [Skene and Willard, 1981b, Skene and Kalil, 1984]. GAP-43 has subsequently been shown to be identical to previously identified proteins described in growth cone membranes during embryogenesis, [Meiri et al., 1986, Skene et al., 1986], in presynaptic membranes in the regulation of phosphoinositide turnover [Jacobson et al., 1986, Perrone-Bizzozero, et al., 1986], and in hippocampal tissue during long-term potentiation [Nelson and Routtenberg, 1985]. Accumulation of GAP-43 has also been observed in growth cones by immunofluorescence microscopy [Meiri et al., 1986], an observation that has been confirmed by biochemical studies [Meiri et al., 1986, Skene et al., 1986].

GAP 43, a highly acidic protein that is phosphorylated, was originally thought to be an integral membrane protein, as it could not be extracted from membranes by high salt washes. However, analysis of GAP43 encoding cDNAs from the rat [Karns et al., 1987, Basi, et al., 1987], mouse [Cimler et al., 1987] and human [Kosik et al., 1988, Ng et al., 1988] have indicated that there appears to be no obvious membrane-spanning domain. Recently, Skene and Virag [1989] have shown that GAP43 is covalently linked to two palmitate moieties via thioester linkages on adjacent cysteine residues at the N-terminus of the protein. This may provide the mechanism for incorporation of GAP43 into the membrane. This finding is significant as they were also able to demonstrate turnover of

fatty acid moieties on the protein in the growth cone. In contrast, Zuber and co-workers [1989] reported that the amino terminus of GAP-43 contains a membrane targeting signal. Using laser-scanning confocal microscopy, they examined the localization of fusion proteins containing N-terminal sequences of GAP-43, and concluded that a short stretch was sufficient to direct accumulation of these proteins in growth-cone membranes, especially in filopodia.

These reports support the current theory that GAP-43 is involved in the response of the growth cone to external stimuli . It has been proposed that GAP-43 may alter the rate of addition of membrane components at the growth cone, thereby playing an important role in neurite outgrowth [Gordon-Weeks, 1989]. Moreover, its function in the growth cone may be modulated post-translationally through changes in degree of phosphorylation, and in alterations in fatty-acylation of the protein [Skene and Virag, 1989].

In summary, GAP-43 exemplifies a set of neuronal proteins that may be part of the neuronal response to guidance cues from the external environment. Although the functions of such growth-associated proteins have yet to be clarified, it is possible that their expression mediates neurite outgrowth during development, regeneration, and possibly during synaptic remodelling [Skene et al., 1989]. A second question that remains unanswered is the mechanism by which the expression of these proteins is repressed in mature neurons, and the signals which trigger their re-expression during synaptic remodelling and nerve regeneration.

iv. The influence of the extracellular matrix on PNS regeneration.

Individual axons in the peripheral nerve are ensheathed by Schwann cells, and Schwann cell-neurite units are further enveloped by a covering of basal lamina. This unit of the peripheral nerve fibre is further suspended in a matrix consisting mainly of collagen

fibrils, known as the extracellular matrix (or ECM). Investigations of the linkage between the production of the ECM and the myelination of axons have been carried out extensively in tissue culture paradigms [reviewed by Bunge et al., 1986]. These experiments have allowed the in vitro manipulation of Schwann cell-axonal contact and the production of the the ECM, and have conclusively demonstrated that both processes are required for myelination. The role of the ECM in peripheral nerve regeneration is also under intense scrutiny by several laboratories, as it appears that the presence of a favourable substratum is critical for neurite outgrowth after nerve damage. Thus, the ECM in the peripheral nerve may be a potential determinant of regenerative capacity [reviewed by Bunge, 1989].

The ECM is a complex matrix, which may consist of as many as 25 polypeptides, as observed by the measurement of the release of these molecules into the medium by Schwann cell-neuronal co-cultures [Carey and Bunge, 1981]. The major components of the ECM are collagens type I, III, IV and V, laminin, entactin, heparan sulfate proteoglycan and fibronectin. A variety of biochemical studies have shown that collagen fibrils are formed from collagen secreted by Schwann cells in the presence of neurons [Carey et al., 1983, Bunge et al.,1986]. Bunge and co-workers [1986] reported that substituting dialyzed serum compromised the formation and assembly of basal lamina by Schwann cells. The ability of the Schwann cells to form the basal lamina could be restored by the addition of 50 μg/ml ascorbate to the dialyzed serum. This may be explained by the established role of ascorbate in collagen synthesis. The proline residues in procollagen are converted to hydroxyproline by the action of hydroxylases, which require ascorbate as a reducing agent in the enzymatic process. Normal triple helical organization of the procollagen molecule is not possible without the formation of hydroxyproline [Prockop et al., 1976]. When the secretion of collagen is perturbed in co-cultures of Schwann cells and neurons using dialyzed serum, or by the addition of the proline analogue cis-

hydroxyproline, the ensuing ensheathment of axons is abnormal and ensheathment and myelination of axons are deficient [Copio and Bunge, 1980, Bunge et al., 1986].

The linkage between the production of the ECM and the ensheathment of the axon by Schwann cells was further indicated by the changes in Schwann cell function when deprived of ECM. Bunge and Bunge observed at an early stage of their investigations [1978] that in Schwann cell-neuronal co-cultures during neurite outgrowth, the neurites were lifted off the culture dish and became suspended in the culture medium. Under such conditions, the Schwann cells attached to neurites aggregated into grape-like clusters and failed to ensheathe the axons. This was easily corrected by forcing the neurites onto the provided substratum, and myelination then proceeded normally. Bunge and co-workers [1986] interpret these observations as a demonstration of the requirement of the Schwann cell to be in contact with two dissimilar surfaces, one provided by the axon to be ensheathed and the other provided by the ECM. It is likely that the Schwann cell, like many epithelial cells, needs such a disparity in contact surfaces to develop sidedness and polarization. The work of Bunge and co-workers strongly supports the linkage between the production of ECM, the ensuing ensheathment of the axon and subsequent myelination.

Laminin is a major component of the basal lamina, and has been characterized as a cruciform-shaped 900 kDa glycoprotein that mediates cell-cell and cell-substratum interactions [Timpl et al., 1979]. The functions of several domains of laminin have been elucidated [for review see von der Mark and Kuhl, 1985]. Several in vitro studies demonstrated that laminin is responsible for neurite outgrowth-promoting activity in a variety of conditioned media [Lander et al., 1985, for review see Lander, 1987].

Another ECM component that may play an important role in neurite outgrowth during peripheral nerve regeneration is fibronectin. Fibronectin consists of two disulphide-linked polypeptide chains of approximately 220 kDa each in molecular weight. Multiple isoforms of fibronectin have been identified and are formed by alternatively spliced

fibronectin mRNAs and through post-translational modification of the encoded peptides. The significance of the different isoforms of fibronectin is just beginning to be examined [Humphries et al., 1986, Jones et al., 1986, Schwarzbauer et al., 1987, Humphries et al., 1988].

Although several reports in recent literature note the increase in neurite outgrowth in the presence of fibronectin [Akers et al., 1981, Gundersen, 1987, among others], there are two major differences between neurite outgrowth promoted by laminin and by fibronectin. The response to fibronectin is weaker than the response to laminin in tissue culture paradigms [Baron-van Evercooren, 1982, Gundersen, 1987] and the proportion of neurons that respond to fibronectin is fewer than those that respond to treatment with laminin [reviewed in Lander, 1987].

One of the most striking advances in understanding the role of the ECM in neurite outgrowth was the discovery that a family of cell-surface receptors known as integrins [reviewed by Hynes, 1987]. Members of this family mediate cell-adhesion to ECM components such as fibronectin and the collagens through recognition of the specific three amino acid sequence Arg-Gly-Asp or (the RGD sequence). The integrin molecules are non-covalently linked heterodimers with α and β subunits. At present three distinct but homologous integrin families with differing α and β subunits have been identified [reviewed by Reichardt et al., 1989]. Integrins have been found to mediate the effects of laminin and fibronectin on neuronal attachment [Bozyczko and Horwitz, 1986], although there is sufficient evidence regarding the advancement of the growth cone across ECM substrata to suggest that binding of integrins to laminin may occur through some site other than an RGD sequence recognition [Letourneau et al., 1988]. A number of laboratories are actively pursuing the isolation of the laminin receptor on neurons [reviewed by Edgar, 1989].

The isolation and characterization of ligands in the ECM and their neuronal receptors has led to elegant hypotheses of the molecular mechanisms underlying the growth-promoting activities of the ECM components. However the concept that such cell-substratum adhesion mediates neurite outgrowth is falling out of favour. It is becoming evident that increased adhesiveness to the substratum is not the cause of growth cone advancement. In this regard, Gundersen [1987] measured growth cone substratum adhesion by sensory neurites to substrata coated with ECM components and found no increase in adhesion of the growth cones. Current thinking favours the hypothesis that the possible stabilization of filopodia and lamellopodia of the growth cones occurs by some mechanism other than increased adhesion [Lander, 1987]. Clearly, the molecular picture on the nature of neurite outgrowth promoted by the ECM is incomplete.

In summary, the ECM appears to have two distinct roles in the peripheral nerve both during development and during regeneration. Components of the ECM mediate neurite outgrowth and provide favourable substrata for the migration of the growth cone towards the peripheral target. The physical basis of this remains nebulous, although there have been proposals that the ECM components may change growth cone adherance through modifications in the dynamics of extension and retraction of filopodia and lamellopodia in the advancing growth cone. The integrin family of receptors and other uncharacterized receptors may also participate in migration of the growth cone by sending favourable signals to the neuronal cell soma through retrograde axonal transport. Secondly, ECM components may play an important role in Schwann cell-axonal interactions, through modulations in the polarization of the Schwann cell. There is a strong case, thus, for the ECM as a key player in the regenerative processes after peripheral nerve injury.

v. The role of neurotrophic factors in PNS regeneration.

Nerve Growth Factor (NGF) is by far the best characterized neurotrophic factor. NGF was first identified as a substance released from mouse sarcomas, that caused sensory and sympathetic neurite outgrowth towards the tumours [Levi-Montalcini, 1952]. The development of an in vitro assay enabled the purification of NGF [Levi-Montalcini, 1954]. β-NGF is a dimer of a 13 kDa polypeptide that is the active component of the NGF complex. Although NGF has an intrinsic neurite outgrowth promoter function, it also increases the survival of NGF-responsive neurons [reviewed by Yankner and Shooter, 1982]. This observation forms the basis of the neurotrophic hypothesis, which states that neuronal survival is dependent on and regulated by factors secreted by peripheral targets [reviewed by Davies, 1988]. The molecular mechanisms by which NGF acts on responsive neurons have just begun to be characterized with the isolation and cloning of an NGF receptor [Johnson et al., 1986, Radeke et al., 1987]. NGF bound to its receptor is internalized by receptor-mediated endocytosis and is transported in a retrograde fashion to the neuronal cell body [Hendry et al., 1974, Stöckel et al., 1974, 1976, Schwab et al., 1979, Johnson et al., 1987]. There are two forms of the NGF receptor with high and low affinity binding for NGF. It appears that the high affinity NGF receptors are largely responsible for the responsiveness of neurons to NGF [Green et al., 1986].

NGF receptors are also expressed by Schwann cells although their function on this cell type is as yet unclear. Schwann cell growth and survival are not dependent on NGF, nor are Schwann cells affected in morphology when treated with NGF in culture. However, loss of Schwann cell-axonal contact through transection of the peripheral nerve results in an induction of NGF receptor gene expression in Schwann cells [Taniuchi et al., 1986, Heumann et al., 1987].

It is possible that the receptors for NGF on the Schwann cell perform a physiological function during regeneration [Taniuchi et al., 1986]. Schwann cells may increase the number of low affinity cell surface receptors for NGF as well as NGF

production after transection. The interaction of the neurotrophic factor with the autocrine receptor would result in accumulation of NGF in the vicinity of regenerative processes. The NGF molecules may then be transferred to high affinity receptors on ingrowing neurites and result in enhancement of neurite outgrowth. In this light, Schwann cells may participate in a trophic guidance mechanism during peripheral nerve regeneration.

The list of identified neurotrophic factors continues to grow. Although several trophic factors are novel proteins (eg. brain-derived neurotrophic factor, purpurin), several known proteins with postulated functions have been shown to possess neurotrophic activity (eg. neuroleukin, apolipoprotein E). Several growth factors may be potential neurotrophic factors (eg. epidermal growth factor, insulin-like growth factor, insulin and the fibroblast growth factors). The neurotrophic activities of these proteins have been reviewed [Walicke, 1989].

vi. Identification of inhibitors of neurite outgrowth in the CNS.

A central issue in the field of regeneration has been the significant lack of neurite outgrowth in the CNS of higher vertebrates. CNS axons readily regenerate into peripheral nervous tissue [Benfey and Aguayo, 1982, Richardson et al., 1984, So and Aguayo, 1985]. PNS neurons, in a converse fashion, fail to extend processes into the CNS, outlining the basic difference in CNS and PNS tissue with respect to regeneration [Aguayo et al., 1978, Weinberg and Spencer, 1979].

The non-permissiveness of the CNS to neurite outgrowth may be passive but it is also likely that the CNS contains active inhibitors of neurite outgrowth [reviewed by Chiquet , 1989]. To examine the latter possibility, Caroni and Schwab [1988] assayed the influence of membrane fractions derived from the CNS on neurite outgrowth using in vitro assays. With this experimental paradigm, they identified two minor membrane-bound

proteins of 35 and 250 kDa in CNS myelin that inhibit neurite outgrowth. They showed that CNS myelin and oligodendrocytes are non-permissive substrates for neurite outgrowth in vitro [Schwab and Caroni, 1998], and that the identified inhibitors of neurite outgrowth were absent in the CNS myelin in fish and in frogs, two species in which CNS regeneration occurs [Caroni and Schwab, 1988]. In recent studies Schnell and Schwab [1990] employed a hybridoma producing monoclonal antibodies against CNS inhibitors of neurite outgrowth to neutralise these antigens in vivo. When such hybridomas were transplanted at the site of a CNS lesion, regeneration and sprouting of neurites up to 7 to 11 mm was observed, as opposed to 1 mm in control lesions. These data highlight the physiological significance of negative regulators of neurite outgrowth and strongly suggest that these as yet uncharacterized proteins may be important in the inhibition of regeneration in the CNS.

vii. The molecular response of glial cells to peripheral nerve injury.

One of the most striking primary responses to nerve injury is the rapid proliferation of Schwann cells [Abercrombie and Johnson, 1946]. The source of the mitogenic signal for Schwann cells is, however, unclear. It is evident from the investigations from several laboratories that this proliferative event is critical for ensuing regeneration, especially with the identification of possible trophic guidance mechanisms proferred by the Schwann cells to the ingrowing axon through modulated release of NGF [Taniuchi et al., 1987, Heumann et al., 1987].

During the degenerative phase of crush-injury or after permanent transection of the peripheral nerve, myelin debris is removed both by Schwann cells and by macrophages . Electron microscopic studies have shown myelin fragments in both of these cell types [Ohmi, 1961, Nathaniel and Pease, 1963, O'Daly and Imaeda, 1967, Williams and Hall,

1971]. A rapid proliferation of Schwann cells also follows the phagocytosis of myelin [Fisher and Turano, 1963, Bradley and Ashbury, 1970, Thomas, 1970, Romine et al., 1976]. The mitogenic response of Schwann cells in vitro after myelin phagocytosis has been well-documented [Meador-Woodruff et al., 1985, Bigbee et al., 1987]. Macrophages may also mediate Schwann cell mitogenicity by presenting the Schwann cell with a mitogenic factor produced from myelin [Baichwal et al., 1988]. A mitogen from myelin that may be responsible for macrophage-mediated Schwann cell proliferation has been shown to be derived from myelin basic protein [Baichwal and DeVries, 1989].

In vivo models of PNS regeneration have also been crucial in characterizing Schwann cell proliferation after nerve injury. LeBeau and co-workers [1988] employed an experimental model in which the sciatic nerve was allowed to regenerate across a silicone tube spanning 10 mm. Soluble factors that promoted Schwann cell adhesion, migration and proliferation in in vitro assays were discovered in the conditioned fluid obtained from the silicone tube. The nature of the active components of the conditioned fluid remains uncharacterized. The results of these experiments from an in vivo situation underline the importance of secreted factors on Schwann cell function during PNS regeneration.

Other non-glial cells also undergo proliferation as a result of nerve injury. The proliferation of macrophages in the distal segment of the degenerating peripheral nerve has been found to occur as early as 2 days post-injury [Liu 1974, Boyles et al., 1989, Clemence et al., 1989]. An increase in macrophages may reflect the need of the tissue to remove myelin debris, and subsequently cause proliferation of Schwann cells through mitogenic factors released by macrophages as noted above.

A secondary response to peripheral nerve injury by Schwann cells is an alteration in phenotype. Galactocerebroside (GC) has been considered a marker for myelin, but was shown also to be a component of the plasma membrane of non-myelin forming Schwann cells [Jessen et al., 1985]. In recent studies by Jessen and co-workers [1987], the

dependence of GC expression by Schwann cells on axonal signals was examined. The sciatic nerve, which comprises both myelin-forming and non-myelin forming Schwann cells, was crush-injured or permanently transected to examine the effects of loss of Schwann cell-axonal contact. In an analogous fashion, the cervical sympathetic trunk wherein 99% of axons are unmyelinated [Aguayo et al., 1972],was crushed or permanently transected. The transient disappearance and subsequent reappearance of galactocerebroside after crush-injury was observed in both nerves. This suggests that axonal signals direct the differentiation of both myelin-forming and non-myelin forming Schwann cells during regeneration.

viii. Alterations in lipid metabolism during peripheral nerve regeneration.

Mechanical injury to the peripheral nerve results in the release of large amounts of lipid from the degenerating axonal membrane and from myelin. The changes in lipid metabolism that ensue after crush-injury have been extensively reviewed by Yao [1984].

During the degenerative phase after crush-injury, the content of phospholipid, cerebrosides, sphingomyelin, sulfatides and mono-unsaturated fatty acids decreases by 6 days post-crush, reaching a minimum by 12 days post-crush. The levels of lipid begin to increase by a month after crush-injury, marking the regeneration of the peripheral nerve. Cholesterol released by the degradation of myelin and axonal membranes, on the other hand, appears to be stored in the resident cell population of the nerve and is re-utilized during remyelination [Simon, 1966, Rawlins et al., 1970, Rawlins et al., 1972]. There is good evidence that de novo biosynthesis of cholesterol also takes place in the crush-injured sciatic nerve, increasing between 7 and 16 days post-crush. There is, however, a decrease in the free cholesterol content of the injured nerve, with a concomitant increase in content of cholesteryl esters. The level of cholesteryl esters in the nerve only decreases to normal

levels after 3 months, when remyelination is complete [Yao, 1980]. It is likely that cholesterol is stored in the form of esters [Mezei, 1970] and is re-utilized in the remyelination process after hydrolysis to free cholesterol. It is also possible that formation of cholesteryl esters serves to decrease free fatty acid content in the degenerating peripheral nerve [Yao, 1988].

Axonal degeneration and degradation of myelin in the crush-injured peripheral nerve are also accompanied by dramatic changes in the metabolism of the resident cell population. At the molecular level, one of the most striking alterations observed in the distal segment of the crush-injured nerve was the increased synthesis of a 37 kDa protein by over two orders of magnitude [Skene and Shooter, 1983]. This 37 kDa protein was later identified to be apolipoprotein E [Ignatius et al., 1986], a lipoprotein associated with very low density lipoprotein particles (VLDL) or a subclass of high density lipoprotein (HDL_2) [for review see Dolphin, 1985]. ApoE in mammalian species is primarily synthesized by the liver and in some peripheral tissues [Williams et al., 1985, Elshourbagy et al., 1985, Newman et al., 1985, Lenich et al., 1988]. In the injured sciatic nerve, invading and/or resident macrophages synthesize apoE [Snipes et al., 1986, Stoll and Müller, 1986, Müller et al., 1986, Boyles et al., 1989], which is secreted in lipoprotein-like particles [Basu et al., 1982]. The apoE enhances the uptake of cholesterol esters by HDL particles [Gordon et al., 1983, Koo et al., 1985] and it is thought that these cholesterol-loaded particles in the crush-injured sciatic nerve are the receptacles for lipid storage during Wallerian degeneration. Subsequently, the cholesterol is transferred to the growth cones of ingrowing axonal tips by receptor-mediated endocytosis [Ignatius et al., 1987a] and also to the Schwann cell [Mueller and Rothe, 1988]. Such internalized lipids may then be re-utilized in membrane biogenesis [Ignatius et al., 1987b].

Müller and co-workers [1985] noted that after permanent transection of the peripheral nerve, levels of apoE were still higher in this tissue than in the distal segment of

the crush-injured sciatic nerve when compared two months post-operation. These results suggested that axonal signals might down-regulate the expression of apoE by resident macrophages. LeBlanc and Poduslo [1990] have examined the regulation of apoE gene expression in the permanently transected and crush-injured sciatic nerve. Their results suggest that the increase in steady-state levels of apoE mRNAs in the two experimental models largely parallel the temporal pattern of macrophage infiltration. Thus transcriptional regulation of apoE gene expression does not appear to be mediated by the presence or absence of Schwann cell-axonal contact. Since apoE is synthesised and secreted in uninjured peripheral nerves [LeBlanc and Poduslo, 1990], it is also possible that apoE may play a role in lipid homeostasis in the adult peripheral nerve.

ix. Cascades of cell adhesion molecules during peripheral nerve regeneration.

Previous sections of this introduction have described components of the ECM and neurotrophic factors that are essential during nerve development and during nerve regeneration. Another class of molecules that have been recognized as important molecular determinants of nerve development and repair, is the cell adhesion molecules.

The cell adhesion molecules, NCAM, L1, MAG, and the calcium-dependent cell adhesion molecule, N-cadherin, are important for PNS regeneration. These proteins are large integral membrane proteins that span the membrane once, or as in the case of one isoform of the neural cell adhesion molecules (or NCAM), are covalently attached to a glycolipid moiety in the membrane. NCAM is a polypeptide that is part of the immunoglobulin gene superfamily, as its extracellular domain contains five domains similar to the immunoglobulin constant region domain. The NCAM gene expresses three alternatively spliced transcripts that give rise to three variants of NCAM of molecular wt. 120, 92, and 80 kDa respectively. Polysialic acid residues are also incorporated into

NCAM in a developmentally regulated fashion, giving rise to variant forms of this cell adhesion molecule that may differ in function [reviewed by Cunningham et al., 1987]. The L1 molecule, like NCAM, is a member of the immunoglobulin gene superfamily, and has six immunoglobulin-like domains. This 200 kDa protein is thought to be involved in homophilic interactions both between neurons, and between neurons and glial cells. L1 and NCAM are spatially and temporally restricted in their expression, suggesting that their functions may be complementary in both the PNS and CNS [reviewed recently by Salzer and Colman, 1989].

MAG is expressed on glial cells and has recently been included in the immunoglobulin gene superfamily [see Section 1.2]. During development, MAG is expressed after NCAM and L1, suggesting that it functions as a distinct cell adhesion molecule [Martini and Schachner, 1986]. A cell adhesion function of MAG has been observed in in vitro assays with proteoliposomes containing recombinant MAG [Johnson et al., 1989]. The neuronal receptor for MAG, if one exists, still remains to be identified.

Reports from several laboratories have suggested changes in the level of expression of these cell adhesion molecules during peripheral nerve regeneration. The localization of NCAM, L1 and MAG in the distal segment of the regenerating sciatic nerve was determined by immunoelectron microscopy [Martini and Schachner, 1988]. The conclusions from these investigations were that in general, the temporal and spatial expression of these cell adhesion molecules during regeneration is similar to that observed during development of the sciatic nerve. The Schwann cell population of the distal segment of the injured nerve expressed both L1 and NCAM in the absence of axonal contact. With the ingrowth of the regenerating axons, fasciculation of the axons took place, followed by myelination. At the onset of remyelination L1 expression on the Schwann cell and on neurons diminished to undetectable levels. The resumption of Schwann cell-axonal contact down-regulated the expression of NCAM by Schwann cells.

In this regard, Jessen and co-workers [1987] have recently qualified the degree of control exerted by axons on myelin-forming and non-myelin forming Schwann cells. NCAM expression in non-myelin forming Schwann cells was found to be unaffected by the presence or absence of contact with the axon. The cell adhesion molecule, MAG, was expressed when the Schwann cell completed 1.5 loops around the axon to be ensheathed [Martini and Schachner, 1988]. These observations are consistent with the concept that MAG is a distinct cell adhesion molecule that may be important in initiating myelination through modulated adhesion [Attia et al., 1989].

The list of cell adhesion molecules that may be important in PNS development and regeneration is growing. Some of these and their putative function have been described in recent reviews [Lander, 1987, Schachner, 1989, Salzer and Colman, 1989].

x. Biochemical aspects of peripheral nerve regeneration.

The crush-injured sciatic nerve and the permanently transected sciatic nerve provide excellent model systems to study the consequences of the interaction of two distinct cell types, the Schwann cell and the axon connected to the neuronal cell body. These in vivo experimental paradigms offer contrasting situations, the crush-injured nerve in which remyelination takes place, and the transected nerve in which no myelin assembly takes place.

In the past decade, advances in biochemical and molecular biological techniques have allowed the careful examination of events at various levels of expression of cellular genes. The elucidation of biochemical changes in the transected and crush-injured nerve was first initiated at the level of post-translational modifications and has now progressed to a stage at which regulation at the more complex levels (at the level of transcription, during post-transcriptional events and at the level of translation) may be addressed. A review of

the regulation of Schwann cell expression of myelin-specific glycoproteins and glycolipids has appeared in recent literature [Poduslo, 1986].

The major PNS myelin protein PO is highly modified at the post-translational level. It is glycosylated [Everly et al., 1973, Wood and Dawson, 1973], phosphorylated [Wiggins and Morell, 1980, Gilbert et al., 1982], sulfated [Matthieu et al., 1975] and linked to fatty acyl moieties in mammalian species [Agrawal et al., 1983, Sakamoto et al., 1986]. Changes in the glycosylation pattern of PO were found to occur in Schwann cells in the distal segment of the sciatic nerve after transection [Poduslo, 1984]. The oligosaccharide moiety was converted from a complex oligosaccharide in the normal nerve to a mannose-rich oligosaccharide in the distal segment of the transected sciatic nerve [Poduslo, 1985]. This led to targeting of the PO protein to the lysosome by a pathway that is distinct from the mannose-6-phosphate receptor-mediated degradative pathway. Thus, permanent transection of the sciatic nerve led to an alteration in the glycosylation of PO and a subsequent degradation of de novo synthesised PO protein in the distal segment of the injured nerve. A similar situation was found in cultures of primary Schwann cells in the absence of neuronal influences [Poduslo et al., 1985]. These results suggest that axonal signals in some way affect the post-translation modification mechanisms in Schwann cells.

The PO protein is also phosphorylated [Singh and Spritz, 1976, Wiggins and Morell, 1980]. The intracellular site of phosphorylation of PO was determined by using the crush-injured and transected models of peripheral nerve regeneration. Using pulse-chase labelling techniques, Brunden and Poduslo [1987] ascertained that phosphorylation of PO occurs only in the crush-injured but not in transected sciatic nerve. Thus, the phosphorylation of PO was dependent on myelin assembly. These results suggest that PO phosphorylation occurs after the biosynthetic and maturation events are complete, possibly in intact myelin. A phorbol ester-sensitive kinase was found to be involved in the phosphorylation of PO in myelin [Brunden and Poduslo, 1987]. More recent experiments

from this laboratory have focussed on the sulphation of PO in the crush-injured and permanently transected sciatic nerve [Poduslo and Berg, 1990]. Sulphation of PO was found to be absent in the transected nerve, in the absence of myelin assembly. It was postulated that lack of sulphation may result in a default targeting of PO to the lysosome for degradation. However, when sulphation in the crush-injured sciatic nerve was inhibited with a specific inhibitor of ATP-sulphurylase, no degradation of the PO could be detected after long periods of chase. These results suggest that though there is a lack of sulphation of PO in the transected nerve, it does not result in the targeting of the major PNS myelin glycoprotein to the lysosome.

In summary, the crush-injured and transected sciatic nerves provided excellent model systems to study the effect of axonal signals on the metabolism of the Schwann cell. The alterations in post-translation events in the metabolism of PO as described above led to further examination of possible regulation at other levels of myelin gene expression in these experimental model systems.

1.4 Objectives of the current investigations.

The crush-injured and permanently transected sciatic nerve provide excellent models to study the effect of axonal signals on the metabolism of Schwann cells The presence of post-translational mechanisms to regulate the expression of the major PNS myelin protein PO, led to the supposition that regulation may occur at other levels, namely at the level of transcription, during post-transcriptional processing of PO-encoding transcripts, as well as during translation of the PO mRNAs. Such investigations of regulation of myelin gene expression would allow further comprehension of the nature of the interactions between two distinct cell types, the Schwann cell and the neuronal cell body, and the consequent formation of a modified plasma membrane.

An examination of the molecular events surrounding the process of peripheral nerve remyelination would also form a basis for future clinical studies in the field of regeneration. Multiple sclerosis (MS) is the most common type of demyelinating disease which mainly affects the CNS [Morell, 1984]. Its etiology remains unknown, but genetic disposition, viral infection and possibly environmental factors, followed by a poorly regulated immune response occurring in the CNS have been proposed as the most important theories. Contrary to the previous concept that remyelination is not possible in the CNS, it has been shown that under certain conditions CNS remyelination may occur [Benfey and Aguayo, 1982]. It has been demonstrated, that spinal cord MS lesions often contain regenerating myelin sheaths formed by Schwann cells [Itoyama et al., 1983], indicating that Schwann cells can myelinate CNS axons [Blakemore, 1975]. In order to effectively assess the mechanisms of remyel ation and implement treatment regimes, it is important to understand these processes in molecular terms. In this regard, the crush-injured and permanently transected sciatic nerves are excellent models for such purposes.

Preliminary studies on the regulation of PO and MBP gene expression in the crush-injured and permanently transected sciatic nerve were initiated in this laboratory in collaboration with Dr. Joseph Poduslo at the Mayo Clinic. Steady-state levels of PO and MBP mRNAs were determined in the distal segments of the crush-injured and permanently transected sciatic nerve, 35 days post-operation [LeBlanc et al., 1987]. The levels of PO and MBP mRNAs in the distal segment of the crush-injured sciatic nerve were not found to be significantly different from that found in the normal adult sciatic nerve. This observation suggested that any alterations that may have occurred in levels of PO and MBP gene expression as a result of crush-injury were no longer evident after 35 days. On the other hand, the levels in the distal segment of the permanently transected nerve were reduced to basal levels compared to that of the control adult sciatic nerve. These results suggested that transcriptional regulation of the PO and MBP genes by the axon was likely

in the peripheral nerve. In vitro translation of poly(A) + RNA from the distal segments of the crush-injured and permanently transected nerves were carried out, and the PO product immunoprecipitated. This technique permitted evaluation of the translational efficiency of the PO mRNAs present in the regenerating and degenerating sciatic nerve. It was found that levels of PO product obtained after in vitro translation were similar to those obtained by dot blot analysis of steady state levels of PO mRNAs. These results implied that major regulation of gene expression did not occur through modulation of rates of translation. In summary, these preliminary studies strongly pointed towards transcriptional regulation of expression of myelin-specific genes in the presence and absence of myelin assembly.

In light of these data, the initial goal of the present investigations was to delineate the expression of PO and MBP genes in the distal segment of the sciatic nerve over the first three weeks after crush-injury. Such an examination of the temporal course of gene expression of the major PNS myelin genes would allow comparison of changes in encoding mRNA levels with the well-characterized morphological sequence of events following crush-injury. A similar experimental protocol was applied to examine the steady-state levels of PO- and MBP-coding transcripts using dot-blot analysis, as well as determination of translational efficiency of PO mRNAs in the distal segment of the crush-injured nerve. The steady-state levels of PO protein would also be determined by Western blot analysis to observe whether regulation occurs at the level of translation or post-translation in vivo.

While these studies were in progress, the sequence of cDNAs encoding the myelin-associated glycoprotein (MAG) were published [Salzer et al., 1987, Arquint et al., 1987, Lai et al., 1987]. MAG was thought to be a cell-adhesion molecule from the amino acid sequence elucidated from the cDNA sequence, and it was postulated that MAG may play a role in the initiation of myelination. In light of these data, and the results that were obtained from the delineation of PO and MBP gene expression in the sciatic nerve, following crush-

injury, the examination of levels of MAG mRNAs and MAG protein in the crush-injured model as well as in the transected sciatic nerve were carried out. These experiments would shed light on possible differential regulation of genes that encode proteins thought to play a functional role in remyelination as compared to those encoding structural proteins. In this context, when the cDNAs encoding the myelin-specific enzyme 2'3'cyclic nucleotide phosphodiesterase (CNPase) became available, the levels of expression of CNPase mRNAs were also determined in the sciatic nerve following crush-injury.

In 1987, Puckett and co-workers published an exciting report of the presence of mRNAs encoding the CNS myelin protein, proteolipid protein, in the peripheral nerves of the rat and the human. Although the PLP gene was expressed in the PNS, the encoded protein was not incorporated into PNS myelin. An examination of the expression of PLP mRNAs in the developing, degenerating and regenerating peripheral nerve was initiated to compare the atypical expression of a gene encoding a CNS myelin protein, with that of PNS myelin-specific genes.

The final study of the present investigations arose as a direct consequence of results from the delineation of expression of the PO, MBP, MAG, and CNPase genes in the distal segment of the crush-injured sciatic nerve as carried out in the preliminary investigations. It was apparent from these studies that there was a tight regulation of myelin gene expression by the presence or absence of Schwann cell-axonal contact. The question was addressed whether this regulation of myelin gene expression during remyelination, depended on a continuous inductive signal from the ingrowing axon. To investigate the characteristics of axonal modulation of myelin genes, the sciatic nerve was first crushed and allowed to regenerate for 12 days. In this period, axonal degeneration and demyelination is followed by a period of rapid remyelination. At this stage of crush-injury, the sciatic nerve was permanently transected, to remove Schwann cell-axonal contact. After 9 days, the distal and proximal segments of the doubly operated nerve were dissected

and levels of mRNAs encoding various myelin genes were determined and compared to levels of coding transcripts in the distal segment of the sciatic nerve, at 12 and 21 days post-crush, and at 21 days post-transection. Thus, the effect of a permanent transection on the regenerating sciatic nerve, with a concomitant loss of Schwann cell-axonal contact was determined at the molecular level. This novel experimental model allowed an assessment of the effect of Schwann cell-axonal contact on myelin gene expression in vivo.

In summary, the primary aim of these investigations was to delineate the expression of two PNS myelin genes (PO and MBP) in the distal segment of the sciatic nerve following crush-injury. However, the availability of cDNAs for other myelin genes, allowed the direct comparison of expression of genes encoding proteins which might be functionally important in the process of myelination and remyelination (MAG), as well as proteins whose function in the PNS remain to be determined (CNPase and PLP). The development of another experimental model of peripheral nerve regeneration permitted the evaluation of axonal modulation of gene expression in the regenerating peripheral nerve.

Some of these results have appeared in publications in peer-reviewed journals. The studies on PO and MBP gene expression in the distal segment of the sciatic nerve following crush-injury were reported [Gupta, S.K., Poduslo, J.F., and Mezei, C.(1988) Mol. Brain Res. 4, 133 - 141.]. The expression of the MAG gene in the presence and absence of Schwann cell-axonal contact were reported in a recent publication [Gupta, S.K., Poduslo, J.F., Dunn, R., Roder, J., and Mezei, C. (1990) Dev. Neurosci 12, 22 - 33]. At the time of submission of this thesis, the studies on PLP gene expression in the developing, degenerating and regenerating peripheral nerve have appeared in an abstract [Gupta, S.K., Pringle, J., Poduslo, J.F., and Mezei, C. (1990) Trans. Amer. Soc. Neurochem. 21, 101] and were submitted for review in the Journal of Neurochemistry [Gupta, S.K., Pringle, J., Poduslo, J.F., and Mezei, C. (1990) J. Neurochem. (submitted)] while the studies on the novel crush-transected model and axonal modulation of myelin gene expression are to be

submitted to Glia [Gupta, S.K., Pringle, J., Poduslo, J.F., and Mezei, C. (1990) Glia (manuscript in preparation)].

2. MATERIALS AND METHODS

2.1 Surgical procedures.

Sciatic nerves of adult Sprague-Dawley rats were crushed as described by Poduslo [1984] and these operations were carried out in his laboratory. Sciatic nerve tissue was pooled from 15 - 20 animals from each stage following crush-injury. The tissue was rapidly frozen in liquid N_2 and not allowed to thaw prior to RNA preparation. Sciatic nerves and brain tissue from adult and 21-23 day old rats were collected in this laboratory.

i. Crush-injury of the adult rat sciatic nerve.

The sciatic nerves were crushed below the sciatic notch with a pair of fine forceps for 5 sec followed by closure of the wound. At each time interval under investigation , the animals were anesthetized with sodium pentobarbital, and the entire length of the distal segment of the sciatic nerve including the tibial, peroneal and sural branches was removed and processed. The proximal segments of crush-injured sciatic nerves were also collected for analysis.

ii. Permanent transection of the adult sciatic nerve.

Rats were anesthetized with intraperitoneal sodium pentobarbital. The sciatic nerve was exposed below the sciatic notch and ligated by tying two pairs of 4/0 sterile sutures around the nerve trunk. The nerve was then cut between each pair of sutures. Each end was then undercut for a distance of 10 mm, repositioned by 180 degrees, and tied to

adjacent muscle. This permanent transection of the sciatic nerve is designed to prevent the escape of regenerating axons from the proximal segment and their subsequent entry into the distal segment.

iii. Crush-transection of the adult sciatic nerve.

The crush-transection procedure was carried out as follows: the sciatic nerve was crushed below the sciatic notch with a fine pair of forceps for 5 seconds followed by closure of the wound. The nerve was subsequently transected distal to the site of crush, 12 days later (as described in the previous section). Proximal and distal segments were dissected 9 days post-transection.

2.2 Preparation of RNA.

i. Preparation of RNA by the guanidinium thiocyanate-hot phenol method.

Summary: Pools of rapidly frozen tissue were ground in a mortar with a pestle under liquid N_2. The powder was further homogenized in a solution containing the strong denaturant, guanidinium thiocyanate. DNA was sheared by passing the homogenate repeatedly through a syringe fitted with an 18 gauge needle. Extractions with hot phenol, phenol: $CHCl_3$, and $CHCl_3$ were followed by EtOH precipitation of RNA. The EtOH precipitate was subsequently washed with 3M CH_3COONa, pH 5.0 to solubilize remaining traces of DNA. After two washes with 70% EtOH, the RNA preparation was suitable for preparation of poly(A)$^+$ RNA or for use in Northern and dot blot analysis.

Total cellular RNA was extracted from pooled samples of rapidly frozen sciatic nerves and other tissue as described in Maniatis et al. [1982], with modifications. The tissue was ground to a free-flowing powder, in a pre-cooled mortar (-20°C) with a pestle, under liquid N_2. The powder was quickly transferred to a 15 ml glass homogenizer (Kontes Glass Co, Vineland, NJ, U.S.A.) containing 5 ml/g wet weight tissue of 5M guanidinium thiocyanate, 50 mM Tris-HCl pH 7.6, 2% (w/v) sodium N-lauryl sarkosinate and 1% (v/v) β-mercaptoethanol. The tissue was completely dispersed and then homogenized using a mechanical homogenizer (Fisher Dyna-Mix, Fisher-Scientific, U.S.A.). The homogenate was heated to 60°C in a water bath, and sheared by drawing 25-30 times through an 18 gauge needle into a glass syringe. An equal volume of hot (60°C) TE-saturated phenol was added and the emulsion drawn into the syringe 5 more times. Two and a half ml/g tissue of 100 mM CH_3COONa, 10 mM Tris-HCl, 1mM EDTA pH 7.4 was added to the homogenate. The homogenate was extracted in a 50 ml glass-stoppered centrifuge tube (Kontes Glass Co, Vineland, NJ, U.S.A) with an equal volume of $CHCl_3$: isoamyl alcohol (24:1) at 60°C, while the pressure generated during extraction was frequently released. The solution was cooled on ice, and centrifuged at low speed (2000 rpm, IEC) for 10 min at 4°C. The aqueous phase was transferred to a new centrifuge tube, while the organic phase was re-extracted with 1 ml/g tissue of 100 mM Tris-HCl pH 9.0, and an equal volume of $CHCl_3$: isoamyl alcohol. The aqueous phases were pooled and extracted twice with $CHCl_3$: isoamyl alcohol . The aqueous phase after the final extraction was precipitated with two volumes of 95% EtOH, at -20°C. The pellet was washed with 2 ml of 3M CH_3COONa, pH 5.0, and re-centrifuged at 7000 g (7,700 rpm, JA-20 rotor, J2-21 centrifuge, Beckman, Fullerton, CA, U.S.A.) for 5 min. The pellet was then washed twice with 70% EtOH, and stored under 95% EtOH at -70°C.

ii. Preparation of poly(A)$^+$ RNA by oligo(dT) cellulose chromatography.

Summary: Poly(A)$^+$ RNA was prepared from total RNA by two successive cycles of oligo(dT) cellulose chromatography. The RNA was dissolved in a salt-containing buffer, denatured, and loaded onto a 1 ml column of oligo(dT) cellulose. After several column volumes of washes, the poly(A)$^+$ RNA was specifically eluted at 37°C using a salt-free elution buffer. The purification was enhanced by a second cycle of chromatography. From 1000 µg of total RNA, the poly(A)$^+$ RNA yield from the first cycle of chromatography was approx 100 µg, and between 15-30 µg from the second cycle. The poly(A)$^+$ RNA thus prepared was suitable for in vitro translation, Northern blot and dot blot analyses.

Poly(A)$^+$ RNA was prepared from total RNA by two successive cycles of oligo(dT) cellulose chromatography as described in Maniatis [1982]. Chromatography was carried out in 1 ml plastic syringes, using 70 mg oligo(dT) [Pharmacia, Canada] in the first cycle, and 40 mg oligo(dT) in the second cycle of purification. Prior to loading, the oligo(dT)cellulose was suspended in 5 ml of binding buffer (0.5 M NaCl, 10 mM Tris-HCl pH 7.4, 1 mM EDTA, 0.5% SDS), applied to the column, washed with 3 ml sterile dH_2O, 1 ml of 0.1M NaOH-5 mM EDTA, and washed with sterile dH_2O until the eluate was neutral. The columns were then washed with 10 ml of binding buffer. Prior to loading, the RNA pellets (containing up to 1000 µg) were dissolved in 500 µl of sterile dH_2O, and 500 µl of 2X binding buffer (1M NaCl , 20 mM Tris-HCl pH 7.4, 2 mM EDTA, 1% SDS) was added. RNA was denatured at 60°C for 4 min, and quickly cooled on ice. The RNA solution was then loaded onto the column and the eluate recycled three times through the column to ensure complete absorption. The column was then washed with 8 ml of binding buffer, and 4 ml of washing buffer (100 mM NaCl, 10 mM Tris-HCl, pH 7.4, 1 mM

EDTA, 0.5% SDS). The column was equilibrated at 37 °C for 10 min and the poly(A)$^+$ RNA eluted with 1 ml of elution buffer (10 mM Tris-HCl, pH 7.4, 1 mM EDTA, 0.05% SDS) also at 37°C. For the second cycle of chromatography, the poly(A)$^+$ RNA-enriched fraction was made up to appropriate salt concentrations by adding an equal volume (1 ml) of 2X binding buffer. The RNA was denatured at 60°C for 4 min and quickly cooled on ice. The RNA was loaded onto the second column and the eluate recycled three times through the column. The column was washed with 7 ml of binding buffer, and 4 ml of washing buffer. The column was then allowed to equilibrate at 37°C for 10 min, and the poly(A)$^+$ RNA eluted with 1 ml of elution buffer also at 37°C. The eluate was centrifuged for 10 min at 4°C at 16,000*g* (Eppendorf 5415 microcentrifuge) to remove any oligo(dT) cellulose, and the supernatant made up to 0.3 M CH_3COONa, pH 5.0 and precipitated with 2 volumes of 95% EtOH at -20°C. At each step of the chromatography, aliquots were taken to determine yields by spectrophotometry at 260 and 280 nm [1 A unit at 260 nm = 40 µg/ml RNA].

iii. Preparation of total RNA by the guanidinium thiocyanate-CsCl method.

Summary: This method of RNA preparation was initially developed for tissues which are rich in ribonuclease. The tissue was ground in a mortar with a pestle, under liquid N_2, and further homogenized in a strong denaturing solution of guanidinium thiocyanate using the Kinematica polytron mechanical disruptor. The homogenate was centrifuged at low speed to remove nuclear membranes, and loaded onto a cushion of CsCl. Under the conditions of centrifugation, RNA is precipitated and pelleted through the CsCl cushion while cellular DNA remains above the CsCl cushion. The pellet was recovered, dissolved, and extracted once with phenol:$CHCl_3$, made up to 0.3M CH_3COONa, pH 5.0 and precipitated with 2

volumes of 95% EtOH. This procedure was found to be particularly advantageous when dealing with larger numbers of samples, and was not as labour intensive as the guanidinium thiocyanate-hot phenol method described above. The number of preparations that were carried out at a time was limited to eight by the space in the fixed angle 80Ti ultracentrifuge rotor.

RNA was prepared by the guanidinium thiocyanate-CsCl method [Chirgwin et al, 1979] with the following modifications. Tissue (400 - 700 mg) was ground to a powder in a mortar with a pestle under liquid N_2. The powder was transferred to 7 ml of guanidinium thiocyanate solution (4M guanidinium thiocyanate, 25 mM sodium citrate, 0.1M β-mercaptoethanol, and 0.5% N-laurylsarkosine, pH 7.0) and 200 µl Antifoam A (Sigma, U.S.A.). The suspension was homogenized at full speed for 2 min in a Kinematica GMBH polytron homogenizer (Brinkman Instruments, Rexdale, Ont. Canada). The homogenate was centrifuged at 3000*g* (2500 rpm, IEC centrifuge) for 10 min (4°C), and the supernatant was layered on a 5 ml cushion of CsCl (5.7M CsCl, 1mM EDTA). The samples were centrifuged at 28,000*g* (20,000 rpm) for 20 h at 22°C in the 80Ti fixed angle rotor. The following day, the pellets were recovered from the bottom of each tube and resuspended in 500 µl TE, pH 7.4 (10 mM Tris-HCl, 1 mM EDTA, pH 7.4) at 65°C. The pellets were allowed to dissolve at 65°C for 15 min, and incubated with phenol:chloroform: isoamyl alcohol (25:24:1 by volume) at 65°C for 15 min. The phases were separated by centrifugation at 3000*g* (2500 rpm, IEC centrifuge) and the aqueous phase was precipitated in 0.3M CH_3COONa, pH 5.0 with 2 volumes of 95% EtOH. After overnight precipitation at -20°C, the RNA pellets were recovered by centrifugation at 16,000*g* (15,000 rpm, Eppendorf 5415 microcentrifuge) for 30 min at 4°C. The pellets were dissolved in TE pH 7.4 and then re-precipitated under the same conditions. Total

RNA thus prepared (30 to 45 μg) was suitable for Northern analysis using formaldehyde gels.

iv. Estimation of total cellular DNA and extraction of total RNA.

Summary: This procedure allowed for the determination of DNA content in a tissue of interest, prior to extraction of total cellular RNA. No EtOH precipitation steps were required, resulting in no losses of DNA or RNA. The tissue was homogenized in a lysing solution containing heparin, and an aliquot of the homogenate was used to estimate DNA in a fluorimetric assay. The homogenate was subsequently treated with a commercially available RNAse-free preparation of DNAse I. The RNA remaining was solubilized and denatured in the presence of formaldehyde, quantitated by spectrophotometry and subsequently used for dot blot analysis.

Total cellular RNA was extracted without EtOH precipitation steps, by the method described by Krawczyk and Wu [1987]. The pooled samples of sciatic nerves (60 -100 mg) were ground in a mortar with a pestle under liquid N_2. The powder was transferred to a 3 ml ground glass homogenizer (Kontes) and thoroughly homogenized in 200 - 400 μl of lysing solution (10 mM Tris-HCl pH 7.4, 2 mM $MgCl_2$, 1 mM $CaCl_2$, 1.5 mg/ml heparin (Sigma Chemical Co, St. Louis, MO, U.S.A.), 1u/ml RNAsin (Promega, Madison, WI, U.S.A.). The homogenate was vortexed vigorously for 2 min, and frozen in liquid N_2. The homogenate was allowed to thaw and the exact volume of the homogenate was measured. An aliquot (10 μl) of homogenate was kept aside for DNA measurement. One hundred units of RQ1 DNAse (Promega, Madison, WI, U.S.A.) was added to the homogenate and allowed to incubate at room temperature for 60 min. Four volumes of

denaturing buffer (1.3M NaCl, 65 mM sodium phosphate, pH 7.0, 7.8% (v/v) formaldehyde) were added and a precipitate allowed to form on ice for 10 min. The precipitate was pelleted by centrifugation at 16,000*g* at 4°C for 30 min (Eppendorf 5415 microcentrifuge). The solution was denatured at 65°C for 5 min, incubated on ice for 10 min and re-centrifuged as mentioned above. The supernatant was filtered through glass wool and transferred into a new tube. Aliquots were taken for RNA estimation, and the supernatant was to be used for dot blot analysis. However, the RNA preparation as described above was found to be degraded on Northern blot analysis. This procedure was therefore used only for the determination of DNA and RNA content in sciatic nerve tissue (see Section 3.1 of Results).

DNA estimation was carried out using a fluorimetric assay based on the enhancement of fluorescence of bis-benzimidazole on binding to DNA. This assay was found to be applicable to the quantitative estimation of DNA content of crude cellular extracts, from which RNA has not been removed [Labarca and Paigen, 1980]. A standard curve was prepared using calf thymus DNA (Sigma Chemical Co, St Louis, MO, U.S.A.), from 150 ng to 2500 ng in a total volume of 2 ml of 1X TNE (10 mM Tris, 1 mM EDTA, 1 M NaCl, PH 7.4) containing 0.1 μg/ml of bis-benzimidazole dye (also known as Hoechst 33258, Sigma Chemical Co, St Louis, MO, U.S.A.). The SLM/AMINCO fluorimeter was used so that the excitation spectrum was in the 365 nm range and the emission spectrum peaked in the 460 nm range. For determination of the DNA content of crude homogenates, duplicate samples containing 4 μl of the homogenate were assayed in 2 ml. The number of cells in the tissue sample was determined by estimating the DNA content of rat cells to be approximately 6.6 pg [Shapiro, 1975].

2.3 Preparation of protein extracts.

i. Preparation of protein extracts from sciatic nerve and brain tissue.

Summary: The pooled samples of sciatic nerve or brain tissue were ground in a mortar with a pestle under liquid N_2 and the resulting powder was homogenized in Tris-HCl, pH 7.4. The membranous material was solubilized by the addition of sodium deoxycholate. To prepare samples for SDS-PAGE under reducing conditions, β-mercaptoethanol and SDS were added to the extract, and the samples were boiled for 2 min. Prior to loading on polyacrylamide gels, glycerol and dye were added to the sample.

Pools of sciatic nerves or brain tissue were ground in a mortar with a pestle under liquid N_2, and the resulting powder was thoroughly homogenized in 10 volumes of 100 mM Tris-HCl, pH 7.4 in a ground-glass hand homogenizer of the appropriate size (Kontes Glass Co, Vineland, NJ, U.S.A) on ice (10 mg tissue was thus homogenized in 100 µl of 100 mM Tris-HCl, pH 7.4) as described by Nunn and Mezei [1984]. Fifteen volumes of 1% sodium deoxycholate were added to solubilize membranous material, and the suspension was re-homogenized on ice. Aliquots of these extracts (5 and 10 µl) were digested in 50 µl of 0.1M NaOH, and subsequently assayed for protein by the method of Lowry [1951].

To prepare samples for Western blotting, protein solubilizer (containing 4% (v/v) β-mercaptoethanol, 0.25 M Tris pH 8.6, 1.92M glycine, 1% SDS) was added to make the samples contain 90% protein solubilizer. The samples were boiled for 2 min. Prior to SDS-PAGE, a loading buffer (75% glycerol (v/v) and 0.125%(w/v) bromophenol blue)

was added to allow the sample to contain 9.4% glycerol (v/v) and 0.16% (w/v) bromophenol blue and 0.2 to 1 μg/μl protein.

In experiments involving chemical deglycosylation, extracts of protein in Tris-deoxycholate were freeze-dried prior to the deglycosylation procedure. The deglycosylated samples were dried and dissolved in a composite sample buffer (0. 1M Tris-HCl, pH 6.8, 10% SDS, 20% (v/v) glycerol, 4% (v/v) β-mercaptoethanol, 0.003% (w/v) bromophenol blue) to contain 2.5 to 5.0 μg/μl total protein. Samples were boiled for 2 min before loading on the SDS-polyacrylamide gel.

2.4 Specific methods.

i. Northern blot analysis of poly(A)$^+$ RNA and total RNA using formaldehyde gels.

Summary: Poly(A)$^+$ RNA and total RNA were denatured in deionized formamide and formaldehyde, and fractionated on a denaturing agarose gel containing formaldehyde. This procedure allows the efficient separation of RNA based on molecular weight and is largely independent of anomalies in electrophoretic mobility of RNA due to secondary structure. After electrophoresis, the agarose gel was treated with dilute NaOH to partially hydrolyze RNA into smaller fragments to improve transfer. The gel was subsequently neutralized and the RNA allowed to migrate to a superposed nitrocellulose membrane through capillary transfer using a high salt buffer. The nitrocellulose blots thus prepared were baked in a vacuum oven, to fix the transferred RNA, and subsequently used for filter hybridizations. The formaldehyde gel electrophoresis of RNA is one of the simplest electrophoretic procedures presently available and resolution of RNAs differing by 100 bases is possible.

Northern analysis of poly(A)$^+$ RNA was carried out on formaldehyde gels as described by Maniatis et al [1982], with the following modifications. EtOH-precipitated RNA was pelleted by centrifugation for 30 min at 16,000*g* at 4°C (Eppendorf 5415 microcentrifuge). The pellets were washed in 500 µl of 70% EtOH, and re-pelleted for 5 min at 16,000*g* at 4°C. The pellets were dried on the speed-vac concentrator (Savant Instruments, U.S.A.). The RNA pellets were dissolved in sterile dH_2O on ice for at least 30 min. Total RNA (30 to 45 µg) or 2 µg of poly(A)$^+$ RNA were denatured at 65°C for 12 min in 50% formamide, 2.2 M formaldehyde, 0.5X running buffer (1X running buffer is 20 mM 3-[N-morpholino]-propanesulphonic acid, 5 mM sodium acetate, 1 mM Na_2EDTA, pH 7.0), and quickly cooled on ice for 2 min. A 1.3% agarose gel containing 2.2 M formaldehyde, 1X running buffer and 0.5 µg/ml ethidium bromide was prepared and samples were electrophoresed in 1X running buffer at 40 v for 18 h. After electrophoresis, the agarose gel was photographed through a UV transilluminator to visualize and mark the positions of the 28S and 18S rRNA markers. The gel was treated with 50 mM NaOH, 10 mM NaCl for 45 min, to partially hydrolyze the RNA. The gel was then neutralized with 0.1M Tris-HCl, pH 7.4 for 45 min. The gel was soaked for 2 h in 20XSSC (1X SSC is 150 mM NaCl, 15 mM Na citrate, pH 7.0). The RNA was allowed to transfer to nitrocellulose (0.45 µm, BioRad Laboratories, Richmond CA, U.S.A.) overnight using 10X SSC as the capillary transfer buffer. The blot was rinsed the next day with 3X SSC, allowed to air-dry, and baked for 2 h at 80°C in a vacuum oven The blot was ready at this stage for filter hybridization.

ii. Northern blot analysis of total RNA using vertical formaldehyde gels.

Summary: Vertical formaldehyde gels have been found to be technically advantageous for Northern blot analysis [Dycaico, 1989]. Although the vertical

format agarose gels are harder to pour than the horizontal counterparts and are relatively more difficult to handle, the gels are thinner (1.5 mm as opposed to 4-5 mm for horizontal gels), leading to more efficient transfer of RNA. Electrophoretic times are reduced to 3 h for vertical gels compared to overnight electrophoresis for horizontal gels. Capillary transfers may take only 6 h in the case of vertical gels, and abundant messages may be detected in as little as 0.5 µg of electrophoretically fractionated total RNA using vertical gels. Vertical gels were found to be more practical in these studies, considering the limited amount of RNA available from sciatic nerve tissue.

A 1% agarose gel containing 2.2M formaldehyde, 1X running buffer (20 mM 3-[N-morpholino]-propanesulphonic acid, 5 mM sodium acetate, 1 mM Na_2EDTA, pH 7.0) and 0.5 µg/ml ethidium bromide was poured in a vertical gel apparatus (V-16, Bethesda Research Laboratories, Gaithersburg, MD) using 1.5 mm spacers and a 10-well comb. Once the gel was set, the plates were set flat and separated carefully with a spatula. This allowed easy removal of the comb by lifting it up vertically. Residual agarose in the wells was removed with a spatula. The plates were reset, ensuring that the spacers were once again flush against the gel. The gel and plates were immobilized in the apparatus in the vertical position with an agarose dam in the bottom chamber, as suggested by the manufacturer. RNA samples were prepared as described above for horizontal gels, except that 5 µg of total RNA was used per sample.The formaldehyde gel was electrophoresed in 1 X running buffer at 100v for 3 h, and destained in dH_2O, for 30 min to visualise the position of the rRNA bands. The gels were then denatured in 50 mM NaOH, 150 mM NaCl for 15 min and neutralized in 100 mM Tris-HCl, 150 mM NaCl, pH 7.5 for 15 min. Capillary transfers to nitrocellulose (0.45 µm, Stratagene Cloning Systems, La Jolla, CA) were carried out overnight in 10 X SSC (1 X SSC is 150 mM NaCl, 15 mM sodium

citrate, pH 7.0). The blots were rinsed in 3 X SSC for 5 min, allowed to air-dry, and UV cross-linked at 254 nm with 0.12J/cm^2 irradiant energy employing a commercial UV cross-linker (Stratalinker ^{TM}, Stratagene Cloning Systems, La Jolla, CA).

iii. Dot and slot blot analysis of poly(A)$^+$ RNA and total RNA.

Summary: Dot and slot blot analyses of RNA allow facile estimation of relative steady-state levels of mRNAs under investigation. RNA was denatured in a salt-containing buffer with formaldehyde and quickly cooled on ice. The RNA samples were then serially diluted and applied to nitrocellulose sheets. The dots or slots were then used for filter hybridization. Spectrophotometric determinations of the RNA concentration prior to denaturation were used for quantitation. The slot blot format allowed easier densitometric scanning of the autoradiograms for subsequent quantitation.

Dot and slot blots were prepared by the method described by Cheley and Anderson [1984]. Poly(A)$^+$RNA or total RNA was pelleted from EtOH precipitates, washed in 70% EtOH, and dried on a speed-vac concentrator as described above. RNA was dissolved in sterile dH_2O and the concentration was determined by spectrophotometry (1 A unit at 260 nm = 40 µg/ml). Poly(A)$^+$ RNA (1 to 3 µg) or 15 -30 µg of total RNA was denatured for 15 min at 50°C in 10X SSC, 15% (v/v) formaldehyde and then quickly cooled on ice. Serial dilutions (one in two) of the RNA were then carried out in 10X SSC. Each dilution was filtered through a nitrocellulose membrane (0.45 µm, BioRad Laboratories, Richmond, CA or Stratagene Cloning Systems, La Jolla, CA) fitted in a dot-blot or slot-blot apparatus (BioRad Laboratories, Richmond, CA). The wells were washed twice with 250 µl of 10X SSC. The nitrocellulose membrane was removed and allowed to air-dry.

The membrane was baked for 2 h at 80°C under vacuum. Alternatively, the RNA on the membrane was UV- cross-linked at 254 nm, with 0.12J/cm^2 irradiant energy employing a commercial UV cross-linker (Stratalinker ^{TM}, Stratagene Cloning Systems, La Jolla, CA). The dot or slot blots thus prepared were ready for filter hybridization.

iv. In vitro translation of poly(A)$^+$ RNA.

Summary: In vitro translation of poly(A)$^+$ RNA was carried out in a cell-free rabbit reticulocyte lysate system from a commercial source. The kit contained aliquots of rabbit reticulocyte lysate, as well as a translation cocktail containing creatine phosphate, spermidine, guanosine triphosphate and dithiothreitol to provide optimized in vitro translation. The translation of EGTA-washed poly(A)$^+$ RNA was carried out in the presence of 50 µCi of ^{35}S-methionine for 60 min at 37°C and the products were analysed by SDS-PAGE and fluorography.

Translation of poly(A)$^+$ RNA and analysis of the products of translation were carried out in a rabbit reticulocyte lysate system as described by Pelham and Jackson [1976] and modified in this laboratory [LeBlanc and Mezei, 1985]. Poly(A)$^+$ RNA was pelleted from EtOH precipitates by centrifugation for 30 min at 4°C at 16,000*g* (Eppendorf 5415 microcentrifuge). The pellets were washed twice with 66% EtOH, 5mM EGTA, and re-pelleted by centrifugation at 16,000*g* for 5 min after each wash. The pellets were lyophilized or dried in the speed-vac concentrator (Savant Instruments, U.S.A.) and dissolved in sterile dH_2O at 2 µg/µl. RNAsin inhibitor (Promega Madison, WI, U.S.A.) and EGTA were added to obtain final concentrations of 1.5 µg/µl RNAsin and 5 mM EGTA, respectively. The in vitro translation was carried out using a commercial kit (NEN, Boston, MA, U.S.A.), which provides reticulocyte lysate and a translation mix to form a

complete cell-free translation system, relatively free of endogenous message. Poly(A)$^+$ RNA (1 to 2 µg) were translated in reaction volume of 25 µl containing 10 µl of the reticulocyte lysate and 13 µl of translation cocktail (88 mM CH_3COOK, 0.7 mM $Mg(CH_3COO)_2$, 50µCi ^{35}S-Methionine (1000Ci/mmol) and 5.5 µl of a solution containing creatine phosphate, spermidine, guanosine triphosphate and dithiothreitol, supplied by the manufacturer, NEN, Boston, MA, U.S.A.). The translation was allowed to proceed at 37°C for 1 h, at which point a 2 µl aliquot was taken for electrophoresis of the total translation products. This aliquot was diluted with 18 µl of protein solubilizer (4% (v/v) β-mercaptoethanol, 0.25 M Tris pH 8.6, 1.92M glycine, 1% SDS). The samples were boiled for 2 min. Prior to SDS-PAGE, a loading buffer (75% glycerol (v/v) and 0.125%(w/v) bromophenol blue) was added to allow the sample to contain 9.4% glycerol (v/v) and 0.16% (w/v) bromophenol blue. The remainder of the translation reaction mixture was centrifuged at 16,000*g* at 4°C for 10 min and the supernatants were transferred to new tubes. The samples were then processed for immunoprecipitation as described below.

To determine the efficiency of translation, 1 µl aliquots of the translation reaction mixture were taken at the start of the assay and at 60 min. These aliquots were spotted on Whatman filter paper discs (Grade 3, 2.3 cm diameter). The discs were boiled for 10 min in 10% trichloroacetic acid, quickly cooled with the addition of ice, and washed successively, twice with dH_2O, twice with EtOH, and twice with acetone. The filters were allowed to dry and counted in 300 µl of protein solubilizer (Protosol, NEN, Boston, MA, U.S.A.), 5 ml of scintillation fluid (Econofluor, NEN, Boston, MA, U.S.A.) and 4 drops of glacial acetic acid (to reduce quenching).

v. Immunoprecipitation of in vitro translated PO protein.

Summary: The products from in vitro translation using the cell-free rabbit reticulocyte lysate system were immunoprecipitated with an anti-chick PO IgG, and the immune complexes were recovered by adsorption to Protein A-Sepharose. The immunoprecipitates were washed extensively and then dissociated from Protein A-Sepharose by boiling in a reducing sample buffer. The immunoprecipitates were analyzed by SDS-PAGE and fluorography.

To the supernatants of the centrifuged translation reaction mixture, SDS was added to obtain a final concentration of 5% (w/v), and the samples were boiled for 2 min and quickly cooled on ice. To a sample volume of 30 µl, were added 180 µl of incubation buffer containing 1% Triton X-100, 10 mM EDTA pH 8.0, 0.2% (w/v) NaN_3 and 200 µg/ml phenylmethylsulfonyl flouride (Sigma Chemical Co., St. Louis, MO, U.S.A.) in phosphate-buffered saline (PBS, Dulbecco and Voght, 1954). To eliminate non-specific binding to Protein A-Sepharose, 50 µl of a 10% suspension of Protein A-Sepharose (Pharmacia, Canada) were added to the sample, and the tube rotated at room temperature for 30 min. The protein A-Sepharose was subsequently removed by centrifugation at 4°C for 5 min (16,000*g*, Eppendorf 5415 centrifuge). Anti-chick-PO IgG [Nunn and Mezei, 1984] (5 µl containing 16 µg IgG/µl) were added to the supernatants, and the samples were rotated at 37°C for 1 h and overnight at 4°C. The following day, 100 µl protein A-Sepharose suspension were added and the samples rotated for 1 h at room temperature. The immunoprecipitates were collected by centrifugation (16,000*g*, Eppendorf 5415 microcentrifuge) for 2 min at 4°C. The immunoprecipitates were washed three times with 300 µl of incubation buffer (1% Triton X-100, 10 mM EDTA pH 8.0, 0.2% (w/v) NaN_3), three times with 300 µl of PBS, and once with dH_2O, with recovery of pellets at each step

by microcentrifugation. The immunoprecipitate was recovered by boiling for 5 min in 35 µl sample buffer (125 mM Tris-HCl pH 6.8, 4% β-mercaptoethanol, 2% SDS, and 50% (v/v) glycerol). Samples were then centrifuged for 10 min at 4°C (16,000*g*, Eppendorf 5415 microcentrifuge) and to the supernatants were added 5 µl of dye-glycerol (75% glycerol (v/v) and 0.125%(w/v) bromophenol blue). The samples were processed by SDS-PAGE and fluorography.

vi. Chemical deglycosylation of protein extracts from sciatic nerve and brain tissue.

Summary: Chemical deglycosylation of protein extracts from sciatic nerve and brain tissue was carried out using trifluoromethanesulphonic acid (TFMS). Freeze-dried extracts were dissolved in anisole and TFMS treatment was carried out in an inert (N_2 gas) atmosphere, on ice for 2 h. The unreacted TFMS was neutralized with pyridine on dry ice. The white precipitate of the pyridinium salt of TFMS and the biological material were collected by centrifugation, dried and the proteins precipitated with trichloroacetic acid. The pellets were recovered by centrifugation, washed with acetone, and dried. Samples were then prepared for SDS-PAGE and Western blotting.

Protein extracts in 40 mM Tris-HCl, pH 7.5, 0.6% (w/v) sodium deoxycholate (25 - 50 µg) were lyophilized in screw-cap 1.5 ml Eppendorf microcentrifuge tubes. The dried samples were dissolved in 5 µl anisole (BDH Chemicals, Dartmouth, N.S., Canada) and 45 µl of freshly opened TFMS (Aldrich Chemicals, Milwaukee, WI, U.S.A.) were added to each sample. The tubes were flushed with nitrogen before being tightly capped. The tubes were vortexed to dissolve the samples and kept on ice for 2 h. Ice-cold Et_2O (800 µl) was added and the samples were kept on dry ice for 2 min, followed by the dropwise

addition of 100 µl of pyridine. Samples were left on dry ice for another 60 min and the resultant precipitates were collected by centrifugation at 4°C for 10 min at 16,000*g* (Eppendorf 5415 microcentrifuge). Pressure in the tubes was released and the supernatant pipetted off. Precipitates were dried in a speed-vac concentrator (Savant Instruments, U.S.A.) for 20 min to remove excess pyridine. The pellets were dissolved in 800 µl of 1% (v/v) Triton X-100 and TCA was added to 13% (w/v). (A 100 % (w/v) TCA stock may be prepared by dissolving 500 g of TCA in 227 ml dH_2O , Maniatis et al,1982). The precipitation was allowed to proceed on ice for 20 min, and the precipitates collected by centrifugation at 4°C for 15 min at 16,000*g* (Eppendorf 5415 microcentrifuge). The pellet was washed twice with 1 ml in acetone (kept at -20°C) with centrifugation. The pellet was dried in the speed-vac concentrator and dissolved in a composite sample buffer (0. 1M Tris-HCl, pH 6.8, 10% SDS, 20% (v/v) glycerol, 4% (v/v) β-mercaptoethanol, 0.003% (w/v) bromophenol blue). Samples were boiled for 2 min before loading on the SDS-polyacrylamide gel.

vii. SDS-polyacrylamide gel electrophoresis and fluorography.

SDS-polyacrylamide gel electrophoresis was carried out essentially according to Laemmli [1970] using a vertical slab gel apparatus with a cooling chamber (Protean II, BioRad Laboratories, Richmond, CA). The slab gels were 1.5 mm in thickness. The stacking gel was composed of 3% (w/v) acrylamide and was 3 cm high, while the running gel was composed of 10% (w/v) acrylamide and was 10 cm high. The electrophoresis buffer was 25 mM Tris, pH 8.6, 192 mM glycine, 0.1%(w/v) SDS and was chilled prior to use. Electrophoresis was carried out with ice-cold water circulated through the inner chamber as a coolant. A constant current of 40 mA was applied per two slab gels until the samples containing the bromophenol blue tracking dye reached the running gel. The

current was then increased to 80 mA and electrophoresis continued for about 2 h. Gels that were to be stained were fixed and stained with Coomassie blue as described previously [Fairbanks, 1981].

For analysis of fractionated labelled proteins as obtained on SDS-PAGE of in vitro translation products and immunoprecipitates, gels were fixed, stained with Coomassie blue [Fairbanks, 1981] and fluorographed in EN^{3}HANCE autoradiography enhancer (Dupont Canada, Quebec) according to the manufacturer's instructions.

For chemical deglycosylation experiments, SDS-PAGE was carried out on a mini-vertical slab gel apparatus (Mini-Protean II, BioRad Laboratories, Richmond, CA). The slab gels were 0.75 mm in thickness. The stacking gel was composed of 4% (w/v) acrylamide and was 1.5 cm in height. The running gels were composed of 10% (w/v) or 12%(w/v) acrylamide and were 5 cm in height. The electrophoresis buffer was 25 mM Tris, pH 8.3, 192 mM glycine, 0.1% SDS and was chilled prior to use. All solutions were made up with Milli-Q water (Millipore Corporation, Bedford, MA, U.S.A.) Electrophoresis was carried out with tap water running through the inner chamber as coolant. A constant current of 100 mA per two slab gels was applied until the samples containing bromophenol blue tracking dye reached the bottom of the gel. Alternatively, electrophoresis was monitored by the progress of prestained molecular weight markers (Rainbow Markers, Amersham, Ont., Canada) wherein each standard is covalently linked to a differently coloured dye. The Rainbow markers were also useful in determining efficiency of electrophoretic transfer onto nitrocellulose in subsequent Western blot analysis.

viii. Western blot analysis of proteins.

Summary: Protein extracts from nerve homogenates or brain tissue, or chemically deglycosylated preparations were fractionated by SDS-PAGE. The gels were immediately transferred to nitrocellulose using electrophoretic transfer in a methanol-containing buffer. The nitrocellulose sheets were then blocked from non-specific binding to antibodies by treatment with gelatin or bovine serum albumin. The nitrocellulose blot was immunostained with anti-chick PO or anti-rat MAG antibodies, and developed using an enzyme-coupled assay and employing second indicator antibodies linked either to horseradish peroxidase (HrP) or to alkaline-phosphatase (AP). An enzyme-catalysed reaction involving the production of an insoluble, coloured product was used to develop the Western blot.

Immediately after SDS-PAGE the proteins in the gel were transferred onto nitrocellose sheets according to a slightly modified method of Towbin et al.[1979]. The stacking gel was removed and discarded. The running gel was placed on a Scotch Brite pad lined with soaked 3M Whatman chromatography paper, supported by a stiff plastic grid. The pad was soaked in blotting buffer containing 25 mM Tris, pH 8.3, 192 mM glycine, and 20% (v/v) redistilled methanol. A sheet of nitrocellulose was soaked in blotting buffer and overlaid on the gel, carefully avoiding air bubbles between the nitrocellulose and the slab gel. A second piece of 3M paper soaked in blotting buffer, the second Scotch Brite pad, also soaked in blotting buffer and a covering plastic grid were added in sequence and the composite 'sandwich' placed into an electrophoretic transfer chamber (TransBlot 1200, BioRad Laboratories, Richmond, CA) with the nitrocellulose facing the anode. The chamber was completely filled with blotting buffer and the transfer

carried out for 60 min at 60 v at 4°C. Electrophoretic transfer of proteins for Western blot analysis of MAG was carried out for 90 min.

HrP-coupled goat anti-rabbit IgG staining: This procedure was carried out for Western blot analysis of PO protein. The procedure is outlined by Hawkes et al. [1982] as well as in the Immunoblot Assay Kit (BioRad Laboratories, Richmond, CA, U.S.A.). The nitrocellulose sheet containing the transferred proteins was placed in 100 ml of TBS (20 mM Tris, 0.5M NaCl, pH 7.5) containing 3%(w/v) gelatin (BioRad Laboratories, Richmond, CA, U.S.A.) for 1 h. This prevents non-specific binding of antibodies to the Western blot. The blots were then incubated overnight at room temperature in TBS buffer (100 µl TBS /cm^2 of nitrocellulose) containing 1% gelatin and rabbit anti-chick PO IgG (containing 16 µg IgG/µl) at a dilution of 1:10,000. After at least 12 h of incubation, the blots were washed six times in 20 ml of TBS buffer, with each wash lasting 5 min. The blot was then incubated with the second antibody, goat anti-rabbit IgG linked to HrP (BioRad Laboratories, Richmond, CA, U.S.A.). This was carried out in TBS buffer (100 µl TBS /cm^2 of nitrocellulose) containing 1% (w/v) gelatin at a 1:2000 dilution of the antibody for at least 4 h. The blots were then washed similarly six times with TBS. The PO band coupled to HrP was visualized by staining with a solution of 0.05% (w/v) 4-chloronaphthol, 0.015%(v/v) hydrogen peroxide and 20%(v/v) methanol in TBS buffer. When the colour reaction was sufficiently complete to detect the bands, the blots were rinsed several times in dH_2O and dried on filter paper.

Immunostaining with AP-linked indicator antibodies: This procedure is modified from protocols for immunoscreening λgt11 cDNA libraries (Promega Madison, WI, U.S.A.) After electrophoretic transfer of the proteins to nitrocellulose, the blots were incubated for 1 h in 100 ml of TBST (10 mM Tris, pH 8.0, 150 mM NaCl, 0.05% Tween 20) containing 1% (w/v) bovine serum albumin. The blots were then transferred to TBST buffer containing 1:10,000 dilution of rabbit anti-chick PO (16 µg/µl) or 1:7500 dilution of

the GenS3 monoclonal mouse anti-rat MAG antibody overnight at room temperature. The following day the blots were washed six times in TBST buffer, each wash lasting 5 min. The blots were incubated with AP-linked indicator antibody either directed against the rabbit or mouse IgG (1: 10,000 dilution, Promega Madison, WI, U.S.A.), for at least 4 h at room temperature. The blots were then washed six times as before with TBST buffer. The color development was carried out using 20 ml alkaline phosphatase buffer (100 mM Tris, pH 9.5, 100 mM NaCl, 5 mM $MgCl_2$) containing 6.6 mg 5-bromo-4-chloro-3-indolyl phosphate (BCIP) and 3.3 mg nitroblue tetrazolium (NBT). After sufficient coloration had been achieved, the blots were rapidly rinsed in dH_2O and permitted to dry on filter paper.

ix. 2',3'-cyclic nucleotide 3'-phosphodiesterase activity (EC 3.1.4.37).

CNPase activity was assayed according to the procedure of Drummond and Dean [1971]. The enzyme activity was determined using a coupled system involving the hydrolysis by the endogenous enzyme of the 3'-phosphate bond of 2',3'-cAMP to form the corresponding 2'-ester followed by hydrolysis of the 2'-phosphate ester by an excess of added alkaline phosphatase. The release of inorganic phosphate was then determined. An aliquot of protein extract in 40 mM Tris-HCl, pH 7.5, 0.6% sodium deoxycholate corresponding to 10 µg to 50 µg total protein was taken. Each assay contained 0.1 ml of 50 mM Tris-HCl, pH 7.5, containing 6.25 mg/ml egg albumin, 30 µl of 120 µg/ml alkaline phosphatase and 20 µl of 4mM 2',3'-cAMP. The final volume was obtained by adding dH_2O to 250 µl. The tubes were incubated for exactly 20 min at 37°C. The reaction was terminated by adding 1 ml of ice-cold 3% (w/v) trichloroacetic acid. Tubes were centrifuged at low speed (2500 rpm, IEC) for 5 min at 4°C and 500 µl aliquots of the supernatant were used for inorganic phosphate determination. To each aliquot were added

50 µl of 2.5% ammonium molybdate in 5N H_2SO_4. and 430 µl dH_2O. Color was developed by addition of 20 µl 1-amino-2-naphthol-4-sulfonic acid (ANSA) (154 mg/ml). The absorbance at 720 nm was recorded after 10 min. A sample containing substrate and no tissue homogenate served as a control for 2'-AMP contamination of the substrate. A sample containing the homogenate but no substrate served as a control for phosphate present in the tissue fractions. Activity was expressed as µmol of 2',3'-cAMP hydrolysed/h/ mg protein and normalized to values obtained for the adult sciatic nerve. the enzymatic determinations were found to be linear with time and concentration of tissue homogenate.

x. Analysis of data.

Results were expressed as a range for 2 separate experiments or mean ± S.D. for 3 or more determinations. In cases where data were compared to the normal adult sciatic nerve (as in dot blot analysis of steady-state level of encoding mRNAs, or levels of protein as determined by Western blot analysis), the value obtained for the adult sciatic nerve was taken arbitrarily to be one and the values from other tissue compared to this value. The statistical significance of difference was determined by the Student's t-test and represented as p values.

Estimation of steady-state levels of mRNAs were carried out by dot blot and slot blot analysis. In order to demonstrate the treatment of raw data obtained from dot blots, a sample calculation of steady-state levels of MBP mRNAs in distal segments of the sciatic nerve 21 days post-crush and in the adult sciatic nerve is presented below.

Dot blots were prepared with serial dilutions of 2 µg poly(A)$^+$ RNA from the distal segment of the sciatic nerve 21 days post-crush. The dot blots were hybridized to ^{32}P-labelled MBP cDNA probe, washed, and autoradiographed. The autoradiogram from

which these data are derived is shown in Figure 3.4 in the Results section. Densitometric tracings of the blots provided the following intensities (peak areas) for each dilution of RNA:

	1.0 µg	0.5 µg	0.25 µg	0.125 µg
Distal segment				
21 days post-crush	0.645	0.314	0.104	0.054

Regression analyses of the peak areas and the amount of RNA in each dot was carried out . A line c best fit was selected. In most analyses, a suitable line of best fit with a coefficient of regression ($r^2 \geq 0.99$) was obtained, demonstrating the linearity of response. The peak area per 1 µg poly(A)$^+$ RNA was determined in each case using the line of best fit. Thus, in the present analysis:

	peak area /µg poly(A)$^+$ RNA	Level of MBP mRNA/µg relative to adult SN
Distal segment		
21 days post-crush	0.64	1.52
Adult SN	0.42	1.00

The peak area/µg for each sample was thus normalized to values obtained for the adult sciatic nerve in this experiment. In order to carry out statistical analysis, several dot blot analyses were carried out using biologically independent samples and the statistical significance of difference determined using the Student's t-test. A discussion on the statistical treatment of data from slot-blot analyses is presented in Appendix II.

2.5 Preparation of probes for hybridization.

i. Plasmid preparation by alkaline lysis.

Summary: Cells containing extrachromosomal plasmid were lysed in alkaline SDS, and the chromosomal DNA selectively precipitated using CH_3COOK. The plasmid in the supernatant after centrifugation was precipitated with EtOH. RNA was subsequently salted out from the preparation by treatment with 2.5M CH_3COONH_4. This crude preparation of plasmid DNA was further purified by banding on CsCl gradient by equilibrium centrifugation.

Plasmid DNA was prepared by the alkaline lysis method described by Birnboim and Doly [1979]. Host cells (JM101) were transformed with the plasmid containing the cDNA clone by the method described by Dagert and Erhlich [1974]. Positive transformants were selected for ampicillin resistance by plating overnight at 37°C on YTA plates (0.8%(w/v) bactotryptone, 0.5% (w/v) yeast extract, 0.5% (w/v) NaCl, 1.5% (w/v) bacto-agar, and 75 μg/ml ampicillin). A single colony was streaked out on another YTA plate and incubated at 37°C overnight. An individual colony from this plate was used to inoculate 2 ml of 2X YT (1.6% (w/v) bactotryptone, 1% (w/v) yeast extract, 0.5% (w/v) NaCl, 50 mM Tris-HCl, pH 7.5) containing 100 μg/ml ampicillin to prepare an overnight culture. One litre of 2X YT was inoculated with a 1/1000 inoculum of an overnight culture of cells. The culture was shaken at 200 rpm for 24 h, at 37°C in the presence of 100 μg/ml ampicillin. The cells were centrifuged for 5 min at 4°C at 4400*g* (5,000 rpm, JA-10 rotor, Beckman, Fullerton, CA, U.S.A.) and the supernatant discarded. The cells were resuspended in 20 ml of solution I (50 mM glucose, 25 mM Tris pH 8.0, 10 mM EDTA, 4 mg/ml lysozyme) by trituration. The cell suspension was allowed to stand on ice for 10

min. Solution II (0.2 N NaOH, 1% SDS) (40 ml) was added and the cells were allowed to lyse by gentle inversion of the centrifuge tube. Lysis was allowed to take place for 10 min on ice. Solution III (3M CH_3COOK, 5 M CH_3COOH) (15 ml) was added, the solution was vigorously shaken and placed on ice for 10 min to allow the precipitate to form. The solution was centrifuged at 14,000*g* (9,000 rpm, JA-10 rotor, Beckman, Fullerton, CA, U.S.A.) and the supernatant filtered through cheese cloth to remove any remaining particulate matter. Two volumes of 95% EtOH were added, and the plasmid DNA allowed to precipitate at -20°C for 1 h. The DNA was pelleted by centrifugation at 9800g for 10 min at 4°C. The pellet was resuspended in 5 ml of TE pH 8.0 (10mM Tris, 1 mM EDTA, pH 8.0) and 2.5 ml of 7.5 M CH_3COONH_4 were added. The residual RNA formed a precipitate on ice in 30 min, and was removed by centrifugation at 27,000*g* (15,000 rpm, JA-20 rotor, Beckman, Fullerton, CA, U.S.A.) for 5 min at 4°C. The supernatant containing the plasmid DNA was precipitated with 2 volumes of 95% EtOH at -20°C for at least 1 h. The DNA was pelleted at 27,000*g* (15,000 rpm, JA-20 rotor, Beckman, Fullerton, CA, U.S.A.) for 30 min at 4°C and resuspended in TE pH8.0. This plasmid DNA preparation was suitable for further purification by CsCl equilibrium centrifugation. Typical yields of 1 - 10 mg DNA per litre culture were obtained by this procedure.

ii. CsCl equilibrium gradient centrifugation of plasmid DNA.

Summary: Plasmid DNA prepared by alkaline lysis was further purified by equilibrium centrifugation in CsCl. After centrifugation, the plasmid DNA was visualised by UV light in the presence of ethidium bromide, and the band corresponding to supercoiled plasmid was extricated from the ultracentrifuge tube. Ethidium bromide was removed from the preparation by repeated extraction with isopropanol. The DNA was further purified by ethanol precipitation, and phenol

and phenol:$CHCl_3$ extractions, followed by another EtOH precipitation Plasmid DNA was quanititated by spectrophotometry and found to be suitable for use in restriction analysis and preparation of probes.

Plasmid DNA prepared by alkaline lysis was further purified by banding on a CsCl gradient as described by Maniatis [1982]. Plasmid DNA and 10 g of CsCl were dissolved in 11 ml TE pH 8.0 (10 mM Tris, 1 mM EDTA, pH 8.0). Ethidium bromide, 250 µl (10 mg/ml), was added and the plasmid solution was subsequently kept in the dark. The solution was centrifuged at 17,400*g* (12,000 rpm, JA-20 rotor, Beckman, Fullerton, CA, U.S.A.) for 5 min at room temperature, and the supernatant loaded in ultracentrifuge tubes (Quik-Seal polyallomer tubes, Beckman, Fullerton, CA, U.S.A.), balanced and sealed using a Quik-Seal Tube Sealer (Beckman, Fullerton, CA, U.S.A.). The tubes were centrifuged at 150,000*g* (45,000 rpm, 80Ti rotor, Beckman, Fullerton, CA, U.S.A.) for 40 h at 15°C, allowing the CsCl gradient to form. The ultracentrifuge was stopped without a brake to prevent any disruption of the gradient. The bands in the tube were observed either in visible light or visualised by brief exposures to UV. The lower fluorescent band corresponding to supercoiled plasmid DNA was collected carefully with a 5 ml syringe and 18 gauge needle, taking care not to take more than 1 ml of the CsCl solution. The purified plasmid was extracted 4 to 5 times in the dark, with isopropanol saturated with salt-saturated TE pH8.0 to remove ethidium bromide. The DNA solution (1 volume) was diluted with 5 volumes of dH_2O, and precipitated with 12 volumes of 95% EtOH, at -20°C, overnight. The DNA precipitate was collected by centrifugation at 27,000*g* (15,000 rpm, JA-20 rotor, Beckman, Fullerton, CA, U.S.A.) for 30 min at 4°C, resuspended in 0.5 ml of TE pH 8.0 and subjected to extraction with equal volumes of TE-saturated phenol:$CHCl_3$:isoamyl alcohol (25:24:1 v/v), and $CHCl_3$:isoamyl alcohol (24:1). The DNA was made up to 2.5 M CH_3COONH_4 and precipitated with 2 volumes of 95% EtOH

at -20°C overnight. The DNA was centrifuged at 16,000*g* (Eppendorf 5415 microcentrifuge) at 4°C for 30 min; the following day, it was resuspended in 500 µl of TE pH 8.0 and quantitated by spectrophotometry. One A unit at 260 nm was taken to be equivalent to a concentration of 50 µg/ml DNA.

iii. Restriction analysis of plasmids.

Restriction of plasmid DNA for analysis or for excision and purification of a particular fragment was performed essentially as described by Davis and co-workers [1986]. In a typical restriction digest, 1 - 2 µg of purified DNA were incubated with 3 -10 units of the desired restriction enzyme in a suitable buffer, in a total volume of 40 µl. For most routine restriction digests, the potassium glutamate buffer (KGB) was found to be convenient, as a large number of common restriction enzymes are active in this buffer system [McClelland, 1988]. A 20X KGB stock (2M potassium glutamate, 0.5 M Tris-acetate, 0.2 M $Mg(CH_3COO)_2$) was prepared and used at 1X or 0.5X concentrations in the final volume of the restriction digest. The digests were routinely incubated at 37°C for 1.5 h, and heat-inactivated at 65°C for 5 min. Loading dye (8 µl) (containing 15% (w/v) Ficoll (MW 400,000), 30 mM EDTA and 0.1 % (w/v) of bromophenol blue and xylene cyanole) was added prior to agarose gel electrophoresis.

iv. Agarose gel electrophoresis.

Agarose gel electrophoresis was carried out essentially as described in Maniatis et al. [1982] or in a more recent handbook [Ogden and Adams, 1987]. To separate restriction fragments in the range of 0.8 kb - 10 kb, 0.7% agarose gels were poured in a horizontal format, containing 0.5 µg/ ml ethidium bromide and electrophoresis buffer (89mM Tris-

borate, pH 8.3, 25 mM EDTA). Short gels with a length of 10 cm were conveniently run in a commercially available submarine gel apparatus (Mini-sub cell, BioRad Laboratories, Richmond, CA, U.S.A.) or for increased separation 20 cm long agarose gels were used in the larger submarine gel apparatus (Sub-cell electrophoresis cell, BioRad Laboratories, Richmond, CA, U.S.A.). The gels were allowed to set for 30 min, after pouring, and electrophoresis was normally carried out at low voltage gradients (2-5 v/cm). For low-melting point agarose gels, lower voltage gradients (1-2 v/cm) were employed and the electrophoresis was carried out at 4°C. After electrophoresis, photographs of the gels were taken using a UV transilluminator.

v. Purification of inserts by low melting point agarose gel electrophoresis and phenol extraction.

This method of purification is described in Maniatis et al. [1982]. Plasmids containing the insert cDNA to be used as a probe were digested with a suitable restriction enzyme as described above. The restriction digest was normally fractionated on 0.7% or 1.0% low-melting point agarose gels (Ultrapure LMP agarose, Bethesda Research Laboratories, Bethesda, MD, U.S.A.) at low voltage gradients (1-2 v/cm) and at 4°C. Separation of the fragment to be isolated from the plasmid was achieved in 2 - 3 h of electrophoresis. The bands were visualised on the UV transilluminator, and the fragment to be purified was excised with a clean scalpel. In an Eppendorf microcentrifuge tube, the agarose containing the DNA was melted at 65°C for 10 min with 200 µl TE pH 8.0 (10 mM Tris, 1 mM EDTA, pH 8.0). TE-saturated phenol (500 µl) was added and the tube vortexed. The phases were separated by centrifugation at 16,000*g* for 5 min at room temperature (Eppendorf 5415 microcentrifuge). The aqueous phase was transferred to a new tube, while the phenolic phase was re-extracted with 100 µl TE pH 8.0. The aqueous

phases from these extractions were pooled and further extracted once with an equal volume of phenol:$CHCl_3$: isoamyl alcohol (25:24:1 v/v), and once with $CHCl_3$: isoamyl alcohol (24:1 v/v). The aqueous phase was made up to 2.5M CH_3COONH_4, and precipitated at -20°C with 2 volumes of 95% EtOH, for at least 1 h. The DNA was recovered by centrifugation at 16,000g (Eppendorf 5415 microcentrifuge) at 4°C for 30 min, washed twice with 70% EtOH, and dried on the Speed-vac concentrator (Savant Instruments) for 20 min. The DNA was re-dissolved in 20 - 30 µl and its concentration determined by spectrophotometry.

vi. Nick translation of insert DNA.

Summary: Nick translation is a specific method to incorporate radioactive nucleotides into double-stranded DNA. DNA polymerase I which has 3'-polymerase activity, also possesses 5'-exonuclease activity. Thus in combination with specific amounts of DNAse I, which serves to form random nicks in the double-stranded DNA, DNA polymerase I may be used to introduce radioactive nucleotides at the 3' ends of nicked strands while eliminating 5'-end nucleotides by the inherent exonuclease activity.

Nick translation of cDNA probes was carried out by the method of Kelly et al. [1970] as modified by Fuscoe et al. [1983]. The reaction was carried out in nick translation buffer (10mM Tris-HCl pH 7.5, 5 mM $MgCl_2$, 1 mg/ml BSA) with 30 µM each of dATP, dGTP, dTTP (Pharmacia, Quebec, Canada) and 100 µCi of [α-32P]-dCTP (3000 Ci/ mmol, DuPont Canada Inc., Quebec), 0.1 ng DNAse I (Sigma, U.S.A.) and 20 units of DNA polymerase I (*E. coli* minimal nuclease, PL Biochemicals, Milwaukee, WI, U.S.A.) for 1 µg of purified insert in a volume of 50 µl. Total incoporation of the labelled

nucleotide was measured by acid precipitation as described by Maniatis et al. [1982]. An aliquot (1 µl) of the reaction mixture was precipitated in the presence of 40 ng of carrier sheared salmon sperm DNA (Sigma Chemical Co., St. Louis, MO, U.S.A.) with 3 ml of 1N HCl, 1% (w/v) NaH_2PO_4 and 1% (w/v) $Na_2H_2P_2O_7$. The precipitate was collected on Whatman GF/C filter disks and the incorporation determined by liquid scintillation. For each preparation of a purified DNA fragment, a time course of incorporation was determined to obtain a peak specific activity of the probe. This pre-determined time corresponding to peak incorporation of radiolabel was used in further preparations of probe from a particular preparation.

vii. Random primer labelling of DNA fragments.

Summary: Random primer labelling of DNA was used to prepare probes of high specific activity (10^8 -10^9 cpm/ µg DNA). The DNA to be labelled was first denatured and then mixed with hexadeoxyribonucleotides of random sequence. The random hexamers anneal to random sites on the DNA template and serve as primers of DNA-directed DNA synthesis by the Klenow fragment of *E.coli* DNA polymerase I. With very small quantities of template (30 - 50 ng), probes of high specific activity were routinely obtained using this procedure.

Gel-purified DNA fragments were labelled by random primer labelling [Feinberg and Vogelstein, 1984] using a commercial kit (Oligolabelling ^{TM} kit, Pharmacia Inc, Quebec). DNA (50 ng) was denatured in dH_2O at 90°C for 15 min and transferred to ice for 5 min. A reagent mix containing dATP, dGTP, dTTP and random hexamers in an appropriate buffer was added. Labelling was carried out in the presence of 50 µCi [α-^{32}P]-

dCTP (3000 Ci/mmol, Amersham Canada) and 5 units of Klenow fragment of DNA polymerase I. Incorporation was determined as described above for nick-translated probes.

viii. Purification of probes using Sephadex G-50 spin columns and push columns.

Probes were purified by exclusion chromatography on Sephadex G-50 as described by Maniatis et al. [1982]. A spin column was prepared by loading a 1 ml syringe plugged with glass wool with a suspension of Sephadex G-50 in TE pH 7.5 (10 mM Tris-HCl, 1 mM EDTA, pH 7.5) and centrifuging the column for 5 min at low speed (2500 rpm, IEC). The column was washed twice with 200 µl of TE pH 7.5, 0.1% SDS by centrifugation. The crude reaction mix from nick translation or random primer labelling was loaded in a total volume of 200 µl and spun for 10 min at low speed (2500 rpm, IEC). The eluate was collected in a 1.5 ml Eppendorf microcentrifuge tube and recovery of radiolabelled probe was determined by acid precipitation as described above. In more recent experiments, a modification of this procedure using positive pressure displacement instead of centrifugation was employed. Commerically available columns (Push-Columns, Stratagene Cloning Systems, La Jolla, CA, U.S.A.) were used according to the manufacturer's instructions.

2.6 Filter hybridization.

i. Hybridization of blots using formamide.

Northern blots and dot or slot blots were prehybridized and hybridized according to the method described by Thomas [1983]. Prehybridization of blots was carried out for 18 - 24 h at 42°C in 50% deionized formamide, 5 X SSC, 1 X Denhardt's (0.02% BSA,

0.02% polyvinyl pyrrolidone, 0.02% Ficoll), and 250 µg/ml salmon sperm DNA. Hybridization was carried out for 24 h at 42 °C in fresh prehybridization buffer containing 1 X 10^6 acid precipitable cpm /ml prehybridization buffer. For each cm^2 of nitrocellulose membrane to be probed, 100 µl of hybidization buffer were used. Care was taken to remove air bubbles from plastic bags containing the blot in prehybridization and hybridization buffers.

ii. Washing of blots.

Northern, dot and slot blots were washed with agitation, four times in 200 ml of 2 X SSC, 0.1% (w/v) SDS at room temperature, with each wash lasting 5 min. Two washes with 200 ml of 0.1 X SSC, 0.1% (w/v) SDS were carried out at 50°C, each wash lasting 30 min. Blots were allowed to air-dry for at least 1 h, and autoradiographed using Kodak XAR-5 or XK-1 film with intensifying screens (DuPont, Canada) at -70°C for appropriate lengths of time (ranging from 1 hr to 2 weeks for estimation of levels of high and low abundance mRNAs respectively).

iii. Rehybridization of blots after removal of probe.

Probes were removed from blots by repeated washes with 0.1 X SSC, 0.1% SDS at near boiling temperatures. This was done by boiling 0.1 X SSC, 0.1% SDS, and pouring the solution over the blot, and washing with agitation at room temperature for 15 min. Most probes were completely removed after three washes. The blots were dried, checked for residual probe with a Geiger counter, and then prehybridized and hybridized as described above. Nitrocellulose blots that had been UV cross linked as described in the preparation of Northern blots and dot blots were found to retain the immobilized RNA even after such harsh stripping procedures.

3. RESULTS

3.1 The temporal course of myelin gene expression in the crush-injured sciatic nerve.

i. The pattern of PO and MBP gene expression follows demyelination and remyelination in the crush-injured sciatic nerve.

The molecular response of the sciatic nerve to injury has been reported [Oderfeld-Nowak and Niemeierko, 1969]. In order to assess changes in cellular metabolism in the injured nerve, RNA, poly(A)$^+$ RNA and DNA content were measured in the distal segments of the nerve at various times following crush-injury. In this series of experiments, RNA was isolated by the guanidinium thiocyanate-hot phenol method from pooled samples of distal segments of sciatic nerve at various times after crush-injury. The yield of total cellular RNA from sciatic nerve was significantly higher at all times following crush injury compared to normal adult nerve (Table 3.1). Furthermore, poly(A)$^+$ RNA-enriched fractions were isolated from the total RNA extracts of tissues obtained at each time-point under investigation after two cycles of oligo(dT) cellulose chromatography. The amount of poly(A)$^+$ RNA at each stage was variable, but generally higher than that in the adult control nerve. The total amounts of RNA and poly(A)$^+$ RNA from the young, rapidly myelinating sciatic nerve were 2 and 2.5 fold higher, respectively, than those obtained from the adult.

In a subsequent set of experiments, total cellular DNA was measured prior to RNA extraction using the Heparin-DNAse method described in Materials and Methods. To account for heterogeneity in cell populations between the proximal and distal segments of the sciatic nerve, total cellular DNA content was determined in pools of proximal and distal segments of crush-injured sciatic nerves. Total cellular DNA content was also estimated for control sciatic nerves similarly dissected into proximal and distal segments. The RNA

Table 3.1 Content of total RNA and poly(A)$^+$ RNA in the sciatic nerve following crush-injury.

Total cellular RNA was extracted from normal adult, 21-23 day old, and distal segments of sciatic nerves of adult rats at 1 to 21 days following crush-injury. The poly(A)$^+$ RNA was purified by two cycles of oligo(dT) cellulose chromatography. The amount of RNA was determined by spectrophotometry at 260 nm [1 A unit = 40 μg RNA/ml]. The ratio of absorbance at 260/280 nm was $\geq$ 1.8 for all preparations. Results are expressed as a range for 2 separate experiments or mean ± S.D. of RNA preparations from 3-6 separate experiments. The numbers in parentheses indicate the number of separate experiments for each time following crush injury or control preparations. Sciatic nerves of 15-20 animals were pooled for each experiment. The effect of crush injury at various time intervals was compared to values obtained for normal adult nerves and statistical significance (p values) was calculated by the Student's t-test.

Table 3.1

Content of total RNA and poly(A)$^+$ RNA in the sciatic nerve following crush-injury

Time after crush-injury (days)	µg RNA/ g wet weight tissue		µg poly(A)$^+$ RNA/ g wet weight tissue	
1	803-1026	(2)	29-71	(2)
2	1309-1362	(2)	17-121	(2)
4	1501±488^b	(3)	25±6^c	(3)
7	1410±310^d	(3)	55±58^a	(3)
10	1838-2033	(2)	27-42	(2)
14	1668±255^c	(3)	33±5^d	(3)
21	1413±289^d	(3)	35±10^b	(3)
Adult	700±171	(6)	9.1±3.0	(6)
Young	1581±706^c	(6)	24.9±3.7^e	(6)

a: $P<0.25$ b: $P<0.05$ c: $P<0.025$ d: $P<0.01$ e: $P<0.005$

content of each tissue was subsequently determined. These results are presented in Table 3.2 and represent, in most cases, data from two biologically independent pools. Increased DNA content or cell number, reflecting an increase in mitotic activity, was observed in the distal segment of the crush-injured nerve by 10 days post-operation, but not in the corresponding distal control. The number of cells remained relatively unchanged in both the proximal segment of the crush-injured sciatic nerve and in the corresponding proximal control over the period of investigation. Levels of RNA were comparable or higher in proximal segments at all stages under investigation, compared to the corresponding distal tissue. This may reflect disparities in the metabolism of cells in proximal and distal segments of the sciatic nerve. In summary, the results of these experiments demonstrate alterations in the mitotic activity and possibly the metabolism of resident cell populations after damage to the peripheral nerve.

Northern blot analysis was carried out to assess the quality of messages encoding myelin-specific proteins in the preparations of poly(A)$^+$ RNA from the crush-injured sciatic nerve. Figure 3.1 shows the size of the single PO transcript is approximately 1.9 kb which is consistent with previous observations [Lemke and Axel, 1985]. Under the conditions employed for Northern blot analysis, only differences greater than 100 bases in molecular weight may be resolved. At this level of resolution, the size of the PO message did not change over the time periods following nerve injury. The trends in steady-state levels of PO-coding transcripts were apparent from Northern blot analysis. As seen in Figure 3.1, the level of PO message decreased sharply after crush-injury reaching a minimum at 7 days. This was followed by an increase which was the greatest between 10 and 14-days (compare lanes E and F, Figure 3.1).

To determine whether the expression of another myelin-specific gene changed in a similar manner during the time course of crush-injury, the quality of the transcripts and the steady-state levels of MBP were also estimated in the above tissues (Figure 3.2). Northern analysis indicated that the MBP-cDNA probe hybridized to poly(A)$^+$ RNA species with an

Table 3.2 Content of DNA and RNA/DNA ratios in proximal and distal segments of crush-injured and control sciatic nerves.

DNA and RNA content of proximal and distal segments of crush-injured and control sciatic nerves was determined by the Heparin-DNAse method. DNA content was estimated by comparison with known concentrations of calf thymus DNA, using a fluorimetric assay. Cell number was determined by estimating DNA content of rat cells to be 6.6 pg (see Materials and Methods). RNA content was estimated by spectrophotometry at 260 nm [1 A unit = 40μg RNA/ml] after treatment of the tissue homogenates with RQ1-DNAse. Values for cell number and RNA/DNA ratios are expressed as ranges for 2 experiments. Numbers in parentheses indicate the numbers of biologically independent pooled samples assayed.

Table 3.2

Content of DNA and RNA/DNA ratios in proximal and distal segments of crush-injured and control sciatic nerves

	Distal Tissue		Proximal Tissue	
Days post crush-injury	cells/ mg tissue (x 10^{-4})	RNA/ DNA	cells/ mg tissue (x 10^{-4})	RNA/ DNA
1	2.0 - 4.0(2)	11.0 - 11.4(2)	0.8 - 3.8(2)	10.5 (1)
2	2.9 - 5.3(2)	4.9 - 9.9(2)	1.4 - 2.3(2)	27.5 - 30.5(2)
4	3.4 - 4.9(2)	6.1 - 15.6(2)	2.5 - 3.5(2)	14.8 - 17.8(2)
7	4.9(1)	9.9(1)	3.3 - 3.9(2)	14.1 - 18.3(2)
10	5.2 - 9.0(2)	5.6 - 13.6(2)	4.1 - 4.4(2)	12.8 - 14.3(2)
12	5.9 - 6.7(2)	7.3 - 13.6(2)	5.4 - 7.0(2)	9.3 - 13.5(2)
14	4.4 - 8.8(2)	12.4 - 15.6(2)	4(1)	6.2 - 12.0(2)
21	4.1(1)	11.8(1)	2.6 - 2.8(2)	22.5 - 25.5(2)
Control Tissue				
1	1.1 - 2.0(2)	14.7 - 22.9(2)	1.0 - 4.5(2)	17.5 - 38.4(2)
2	2.5 - 2.6(2)	12.3 - 16.8(2)	1.0 - 1.4(2)	26.1-42.9(2)
4	1.3 - 2.1(2)	18.3 - 35.3(2)	1.6 - 3.4(2)	12.3 - 35.4(2)
7	2.4 - 2.7(2)	9.0 - 15.5(2)	2.3(1)	2.7(1)
10	2.4 - 3.3(2)	10.2 - 13.4(2)	2.0 - 4.2(2)	11 - 32.6(2)
12	1.6 - 3.7(2)	10.7 - 15.9(2)	3.4 - 4.3(2)	12.8 - 24.6(2)
14	2.0 - 3.3(2)	12.4 - 15(2)	3.9 - 6.4(2)	10.1 - 19.2(2)
21	1.7 - 2.0(2)	18.0 - 20.9(2)	2.7(1)	23.4(1)

Figure 3.1. Northern blot analysis of PO coding transcripts in the distal segments of the sciatic nerve following crush-injury.

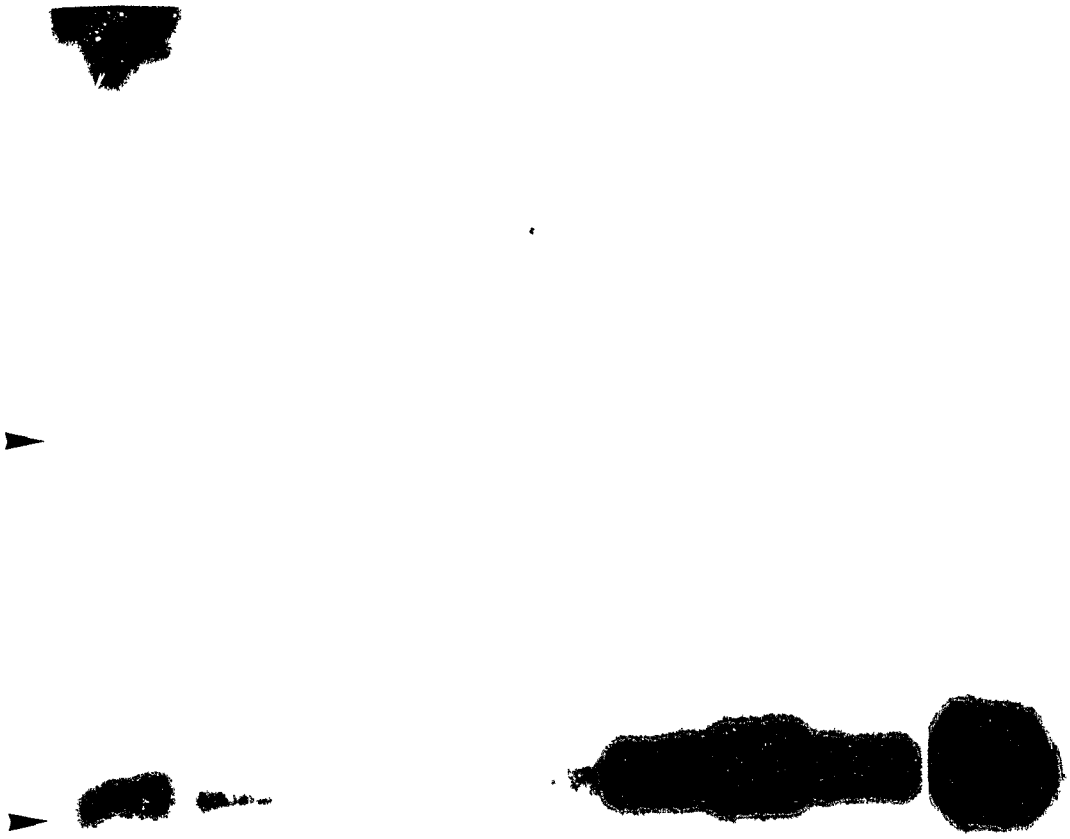
Hybridization was carried out with ^{32}P-labelled rat PO-cDNA probe as described in Materials and Methods. The amount of fractionated poly(A)$^+$ RNA in each lane was 2 µg. Lanes A-G are poly (A)$^+$ RNA from the distal segment of crushed nerves, 1,2,4,7,10,14 and 21 days post crush-injury respectively. Lane H is poly(A)$^+$ RNA from adult sciatic nerve.

A B C D E F G H

Figure 3.1

Figure 3.2 Northern blot analysis of MBP coding transcripts in the distal segment of the sciatic nerve following crush-injury

Hybridization was carried out with ^{32}P-labelled MBP-cDNA probe as described in Materials and Methods. The amount of fractionated poly(A)$^+$ RNA in each lane was 2 μg. Lanes A-G are poly(A)$^+$ RNA from the distal segments of crushed nerves 1,2,4,7,10,14 and 21 days post-injury respectively. Lanes H and I are poly(A)$^+$ RNA from adult sciatic nerve and 21-23 day old rat brain respectively.



A B C D E F G H I

Figure 3.2

approximate size of 2.1 kb under high stringency conditions. The size of the MBP message appeared to be the same in each tissue. Although the MBP message is known to be differentially spliced to yield at least six alternative transcripts, Northern analysis under these conditions does not permit resolution of these messages into distinct bands. The steady-state levels of MBP transcripts during the course of the crush-injury were found to be similar to those obtained for the PO message (Figure 3.2). The amount of MBP mRNA(s) decreased until 4-7 days post-crush, followed by an increase, with steady-state levels approaching or sometimes surpassing that of the normal adult by 21-days post-injury.

To quantitate the relative levels of PO message during the time course following crush-injury, dot-blot analysis was carried out. Patterns of gene expression inferred from Northern analysis were confirmed, as shown in a typical experiment in Figure 3.3. Crushed nerves after 4-7 days of injury contained only 8% of the PO message present in normal adult nerves. The level of PO mRNA in this tissue is significantly different from the normal adult ($P < 0.005$) (Table 3.3). The crushed nerves at 21 days have levels of PO mRNA which approached that of the normal adult, while young sciatic nerves showed the highest amount ($P < 0.05$). The steady-state levels of MBP coding transcripts followed the trends observed for PO transcripts (see Figure 3.4), although relatively higher levels were obtained in the distal segments at 4 to 7 days post-operation (16 to 23 % for MBP as opposed to 8 % for PO mRNAs). Similar trends were obtained when the PO and MBP steady state levels were expressed per gram of wet tissue weight (Table 3.4).

The variability in determining levels of PO and MBP mRNAs per gram of wet tissue weight was probably due to the difficulty in rapidly determining the weight of tissue that was frozen in liquid N_2 and could not be allowed to thaw prior to preparation of RNA. Variability in yield of RNA and poly(A)$^+$ RNA, as reflected in Table 3.1, may also have contributed to error in the normalization of the data presented in Table 3.4. These results

Figure 3.3 Dot blot analysis of PO mRNAs following crush-injury

Serial dilutions (one in two) of approximately 1 μg poly(A)$^+$ RNA were immobilized on nitrocellulose membrane as described in Materials and Methods. The dots on the nitro-cellulose sheet were hybridized with ^{32}P-labelled rat PO-cDNA probe, washed and subjected to autoradiography. Spectrophotometric readings of poly(A)$^+$ RNA before spotting were used for quantitation. Lanes A-G are serial dilutions of poly(A)$^+$ RNA from the distal segment of crushed nerves, 1, 2, 4, 7, 10, 14 and 21 days post crush-injury respectively. Lane H contains serial dilutions of poly(A)$^+$ RNA from the proximal segment of the sciatic nerve 7 days post-crush. Lanes I, J are serial dilutions of poly(A)$^+$ RNA from adult sciatic nerve and 21-23 day old sciatic nerve respectively.

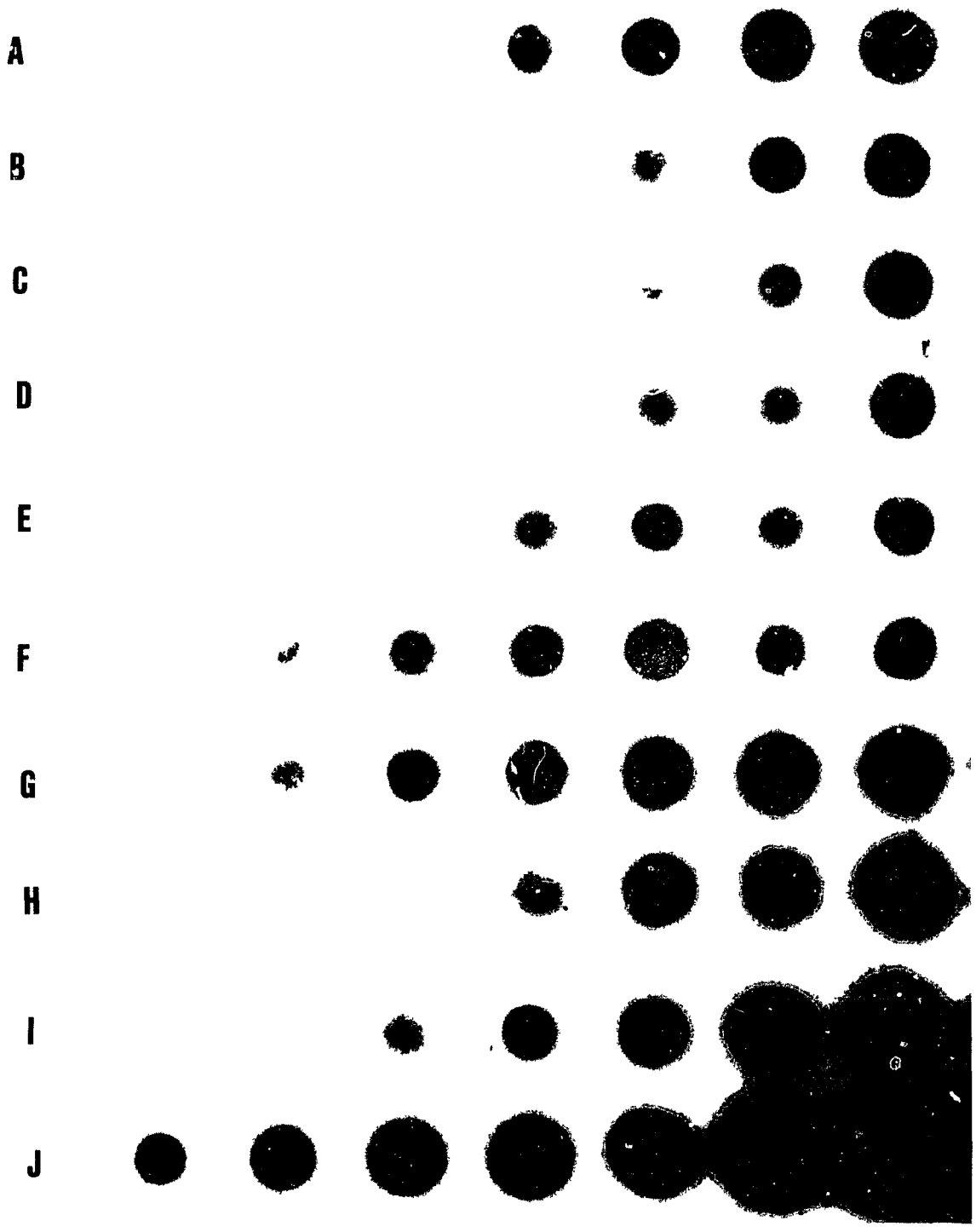


Figure 3.3

Table 3.3 Estimation of the steady-state level of PO and MBP mRNA by dot blot assay.

Poly(A)+ RNA was extracted and purified from the distal segments of crushed sciatic nerves at various times following injury, from normal adult, and from 21-23 day old rat sciatic nerves. Serial dilutions (one in two) of the RNA were filtered through nitrocellulose sheets, and the dots on the nitrocellulose sheet were hybridized with either the ^{32}P-labelled rat PO-cDNA or with the MBP-cDNA probe as described in Materials and Methods. The washed and dried nitrocellulose blots were autoradiographed, and the intensity of each dot was measured by densitometry. Peak area were determined by planimetry. The units of peak area were divided by the amounts of poly(A)+ RNA in the dot to give units per μg of poly(A)+ RNA. The amount obtained for the normal adult sciatic nerve was designated to be one and results from all other tissues were compared to that value in each experiment. Results are expressed and analyzed as described in the legend of Table 3.1.

Table 3.3

Estimation of the steady-state level of PO and MBP mRNA by dot blot assay

Time after crush-injury (days)	Units PO mRNA/ μg poly(A)$^+$ RNA	Units MBP mRNA/ μg poly(A)$^+$ RNA
1	0.61 - 0.66 (2)	0.80 - 0.95 (2)
2	0.23 - 0.37 (2)	0.73 - 0.90 (2)
4	0.08 ± 0.05^d (3)	0.16 - 0.21 (2)
7	0.08 ± 0.07^d (3)	0.18 - 0.23 (2)
10	0.11 - 0.21 (2)	0.23 - 0.39 (2)
14	0.70 ± 0.22^c (3)	0.68 - 1.07 (2)
21	0.91 ± 0.17^a (3)	0.95 - 1.54 (2)
Adult	1.00 (3)	1.00 (2)
Young	2.23 ± 0.18^b (3)	2.80 - 4.21 (2)

a: $p<0.25$ b: $p<0.05$ c: $p<0.025$ d: $p<0.005$

Figure 3.4 Dot blot analysis of MBP mRNAs following crush-injury

Serial dilutions (one in two) of approximately 1 μg poly(A)$^+$ RNA were immobilized on nitrocellulose membrane as described in Materials and Methods. The dots on the nitro-cellulose sheet were hybridized with ^{32}P-labelled MBP-cDNA probe, washed and subjected to autoradiography Spectrophotometric readings of poly(A)$^+$ RNA before spotting were used for quantitation. Lanes A-G are serial dilutions of poly (A)$^+$ RNA from the distal segment of crushed nerves, 1, 2, 4, 7, 10, 14 and 21 days post crush-injury respectively.Lane H contains serial dilutions of poly(A)$^+$ RNA from the proximal segment of the sciatic nerve 7 days post-crush. Lanes I, J are serial dilutions of poly(A)$^+$ RNA from adult sciatic nerve and 21-23 day old sciatic nerve respectively.

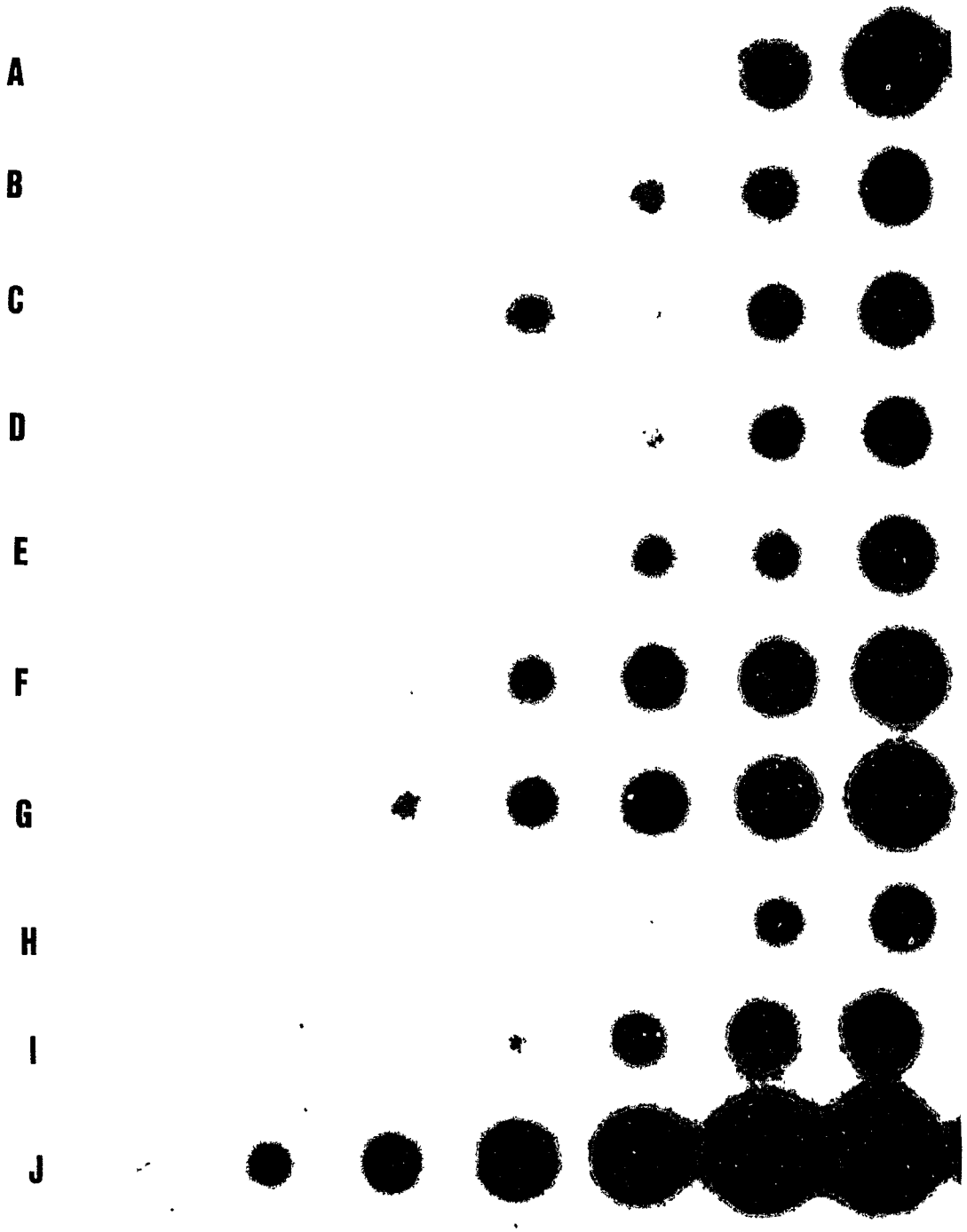


Figure 3.4

Table 3.4 Estimation of the steady-state level of PO and MBP mRNA by dot blot assay, normalized to wet weight.

Steady-state levels of PO and MBP mRNAs were estimated by dot blot analysis as described in the legend of Table 3.3. For each determination, the units of peak area corresponding to PO or MBP mRNA levels were divided by the amounts of poly(A)+ RNA in the dot to give units per μg of poly(A)+ RNA. This value was then multiplied by the yield of poly(A)+ RNA in each preparation per g tissue, to obtain units of peak area per g tissue. The amount obtained for the normal adult sciatic nerve was arbitrarily designated to be one and results from all other tissues were compared to that value in each experiment. Statistical analysis was carried out as described in the legend of Table 3.1

Table 3.4

Estimation of the steady-state level of PO and MBP mRNA
by dot blot assay normalized to wet weight

Time after crush-injury (days)	Units PO mRNA/ g tissue		Units MBP mRNA/ g tissue	
1	1.17 - 4.68	(2)	1.85 - 6.04	(2)
2	2.96 - 4.72	(2)	1.06 - 9.44	(2)
4	0.19 ± 0.13[a]	(3)	0.22 - 0.74	(2)
7	0.04 - 0.54	(2)	0.25 - 2.85	(2)
10	0.39 - 0.54	(2)	0.70 - 1.03	(2)
14	1.71 ± 0.39[b]	(3)	2.20 - 2.87	(2)
21	2.65 ± 1.19[c]	(3)	2.76 - 4.52	(2)
Adult	1.00	(3)	1.00	(2)

a: $0.0005 < p \leq 0.005$, b: $0.025 < p \leq 0.05$, c: $0.05 < p \leq 0.1$

reflected the need for an internal molecular standard that would remain largely unperturbed by nerve damage.

The replication-independent histone variant, H3.3 cDNA [Engel et al., 1982], was tested as a 'constitutive' control in these experiments. The expression of the histone H3.3 variant gene was previously proposed to be independent of cell cycle [Brush et al., 1985] and differentiation [Brown et al., 1985]. This gene has been used as a control in other investigations [Brown et al., 1985; Vardimon et al., 1986]. Levels of H3.3 coding transcripts were lower than adult levels 1 day after crush-injury, and increased slowly over the period under investigation as depicted in Figure 3.5. This contrasted with the patterns observed for the PO and MBP coding transcripts. These results showed that alterations in levels of PO and MBP mRNAs were a selective phenomenon, and not applicable to levels of mRNAs encoding a histone variant.

ii. The expression of the PO gene in the crush-injured sciatic nerve is mainly under transcriptional control.

To confirm that the RNA transcripts encoding the PO gene were translatable, poly(A)$^+$ RNA preparations from the distal segments of crush-injured sciatic nerves were translated in vitro using a heterologous translation system. Aliquots of the in vitro translated product were separated by SDS-PAGE, as shown in the Figure 3.6 and subsequently autoradiographed. A variety of high and low molecular weight species were observed in each lane, indicating the intactness of the poly(A)$^+$ RNA preparations. The in vitro translated products were immunoprecipitated with a rabbit anti-chick PO IgG fraction and the immunoprecipitated products were resolved by SDS-PAGE. The immunoprecipitated products in these experiments showed several bands, as demonstrated in the representative autoradiograph in Figure 3.7A. The PO protein was identified as the major band migrating at approximately 30 kDa, and in this experiment had a mobility of

Figure 3.5 Dot blot analysis of H3.3 mRNAs in the distal segment of sciatic nerves following crush-injury

Serial dilutions (one in two) of approximately 1 μg poly(A)$^+$ RNA were immobilized on nitrocellulose membrane and hybridized to ^{32}P labelled H3.3 cDNA probe as described in Materials and Methods. Spectrophotometric readings of poly(A)$^+$ RNA before spotting were used for quantitation. Lanes A-G are poly(A)$^+$ RNA from distal segments of the sciatic nerve 1, 2, 4, 7, 10, 14, and 21 days post crush-injury. Lane H contains poly(A)$^+$ RNA from the proximal segment of the sciatic nerve 7 days post-crush. Lanes I and J are poly(A)$^+$ RNA from normal adult and 21-23 day old sciatic nerve respectively.

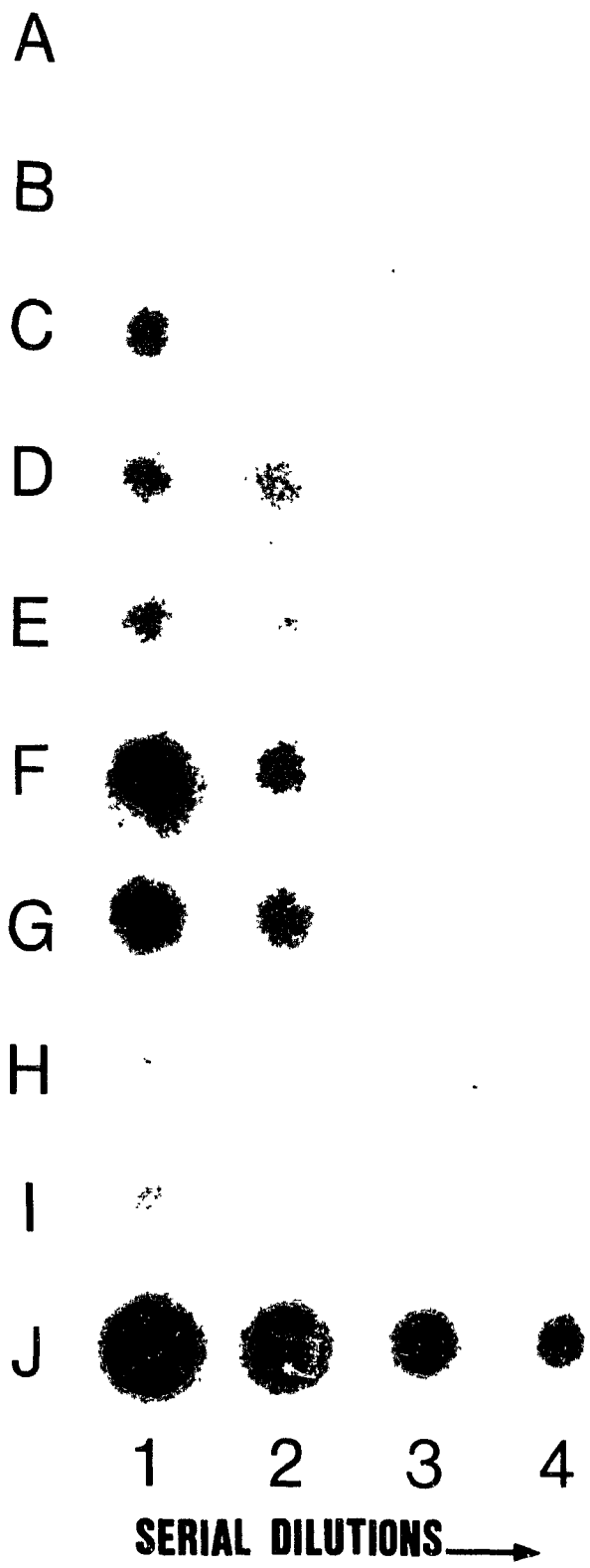


Figure 3.5

Figure 3.6 SDS-polyacrylamide gel electrophoresis of in vitro translation products of poly (A)$^+$ RNA from distal segment of the crush-injured sciatic nerve.

Poly(A)$^+$ RNA was extracted and translated in a reticulocyte lysate cell-free system. Translation products were fractionated on 10% SDS-polyacrylamide gels and subsequently processed for fluorography. Lanes 1-7 are total translation products of poly(A)$^+$ RNA from distal segments of crush-injured sciatic nerves 1, 2, 4, 7, 10, 14, and 21 days post-injury, respectively. Lanes 8, 9, and 10 are total translation products of poly(A)$^+$ RNA from the proximal segment of the sciatic nerve 7 days post-injury, from adult sciatic nerve, and from 21-23 day old sciatic nerve respectively. Arrowheads indicate the positions of molecular weight standards.

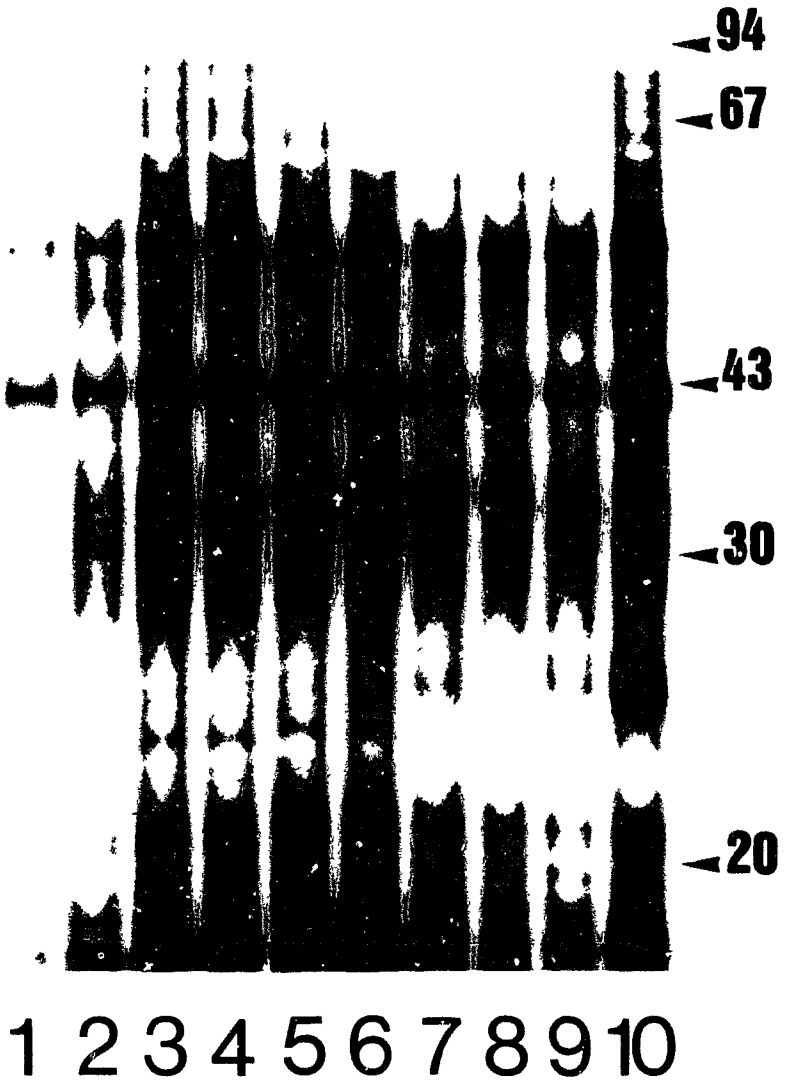


Figure 3.6

Figure 3.7. SDS-polyacrylamide gel electrophoretic pattern of immuno-precipitates from in vitro translation of poly(A)$^+$ RNA.

A. In vitro translation products of poly(A)$^+$ RNA from distal segments of crush-injured sciatic nerves were precipitated with anti-chick PO antibody, and the resultant immunoprecipitates were fractionated on 10% SDS polyacrylamide gels and processed for fluorography. Lanes 1-7 are immunoprecipitated translation products of poly(A)+ RNA from distal segments of sciatic nerves 1, 2, 4, 7, 10, 14, 21 days post crush-injury, respectively. Lanes 8, 9, 10 are immunoprecipitates from translation of poly(A)+ RNA from proximal segments 7 days post-crush, adult sciatic nerve, and 21-23 day old sciatic nerve. Arrowheads indicate the positions of molecular weight standards as shown previously in Figure 3.6. The small arrow indicates the position of the in vitro translated PO immunoprecipitate. **B.** Mobilities of the molecular weight standards on the fluorograph in Figure 3.7A were subjected to regression analysis. The open squares represent the mobilities of the molecular weight standards through which a line of best fit ($r^2 = 0.98$) was drawn. The mobility of the immunoprecipitate, shown by the arrow, was used to calculate the relative molecular weight of the in vitro translated PO product.

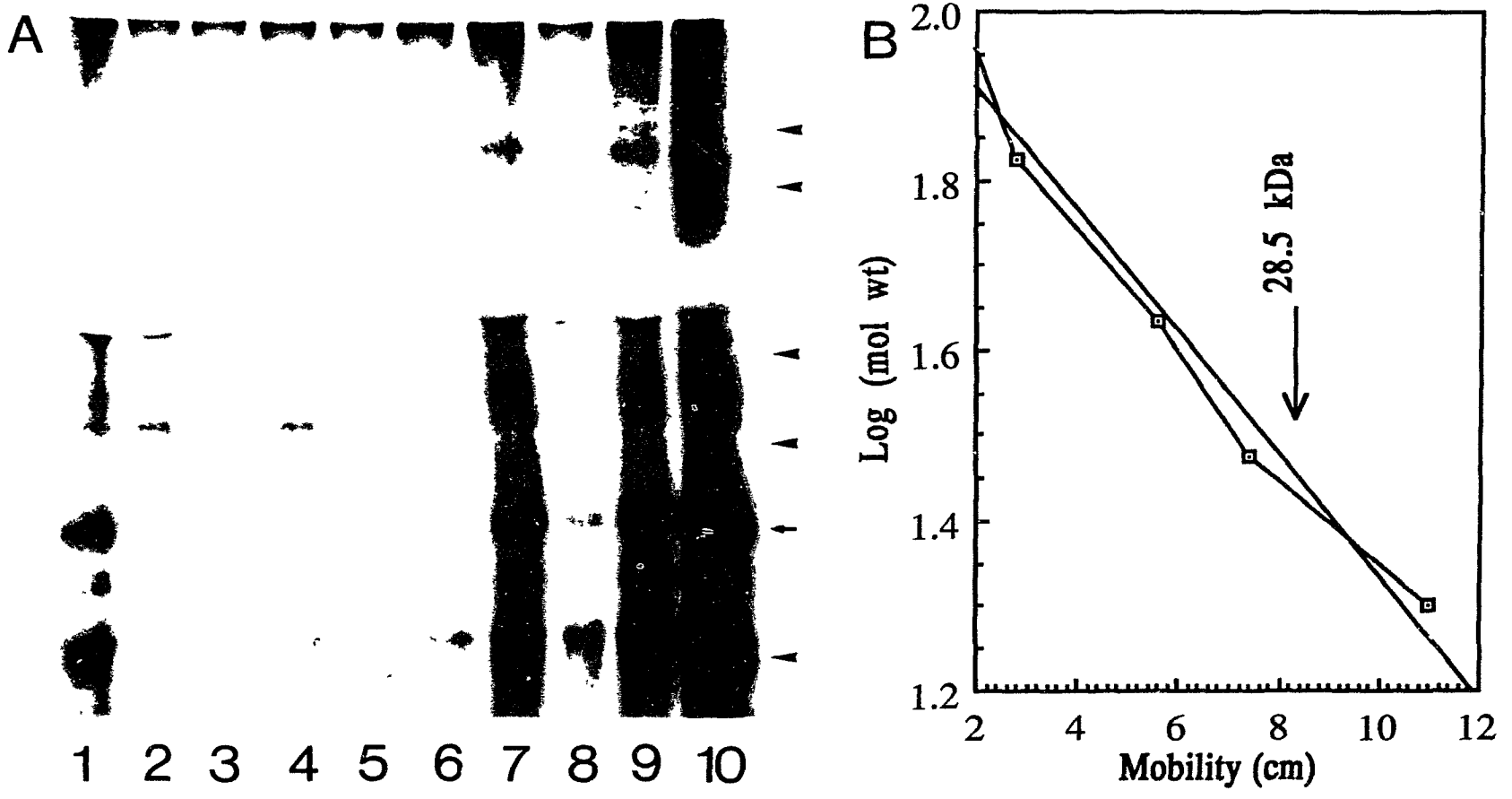

A
1 2 3 4 5 6 7 8 9 10
B
2.0
1.8
1.6
1.4
1.2
Log (mol wt)
28.5 kDa
2 4 6 8 10 12
Mobility (cm)

Figure 3.7

28.5 kDa, when compared with mobilites of applied standards (Figure 3.7B). The PO protein shows different mobilities in its oxidized and reduced form [Cammer, 1980; Brunden et al., 1990], and this may explain the presence of the lower molecular weight (~25 kDa) band in certain lanes. Bands migrating lower than 28.5 kDa may also represent degradation products of the in vitro translated product. The intensity of the 28.5 kDa band was quantitated by densitometric analysis. Although only one representative set of experiments is shown in Table 3.5, immunoprecipitation of in vitro translated PO product with anti-chick PO antibody showed a similar pattern of PO levels in several experiments using biologically independent pools of sciatic nerves. However, the translatable PO message appears to drop more sharply at 1 and 2 days post-injury than the steady-state levels of PO transcripts as measured by dot-blot analysis. The in vitro translation experiments also indicated that the translation efficiency of poly(A)$^+$ RNA preparations, expressed as incorporation of precursor ^{35}S-methionine into acid-insoluble protein and normalized to units of exogenous template poly(A)$^+$ RNA, was higher than that of adult for all stages of the crushed model (Table 3.6). The translation efficiency of the preparations showed greater variability, and it is possible that this reflects the degree of contamination of the poly(A+) RNA preparations with ribosomal RNA.

In summary, in vitro translation and immunoprecipitation of PO from poly(A)$^+$ RNA preparations confirmed in a qualitative manner the trends observed from Northern and semi-quantitative dot-blot analysis. The results from these experiments suggested that levels of PO product translated in vitro were determined by the steady-state levels of PO coding mRNAs and therefore, control of PO gene expression was primarily exerted at the level of transcription.

Table 3.5 Estimation of the steady-state levels of PO mRNA in a heterologous in vitro translation system following crush-injury

Levels of anti-chick PO immmunoprecipitated translation product were estimated as described in Materials and Methods The amount obtained for the normal adult sciatic nerve was designated to be one and all other tissues were compared relative to that value in each experiment. These results are from one representative experiment. Sciatic nerves of 15 - 20 animals were pooled for each separate experimental stage.

Table 3.5

Estimation of the steady-state level of PO mRNA in a heterologous in vitro translation system following crush-injury

Time after crush-injury (days)	Relative level of Immunoprecipitated in vitro product of PO mRNA
1	0.07
2	0.02
4	0.03
7	Not detected
10	0.15
14	0.18
21	0.46
Adult	1.00
Young	6.63

Table 3.6 Estimation of the relative translation efficiency of poly(A)$^+$ RNA from the distal segment of the sciatic nerve after crush-injury.

In vitro translations were carried out in a cell-free rabbit reticulocyte lysate system, using 1-3 μg poly(A)$^+$ RNA prepared from the distal segment of the sciatic nerve at various stages after crush-injury. The translation efficiency was determined as the TCA precipitable cpm per μg poly(A)$^+$ RNA. The value obtained for the normal adult sciatic nerve was arbitrarily designated to be one, and results from all other tissues were compared relative to that value in each experiment. Statistical significance (p values) was calculated by the Student's t-test. The number in parenthesis indicates the number of separate experiments for each time following crush-injury or control preparations.

Table 3.6

Estimation of the relative translation efficiency of poly(A)$^+$ RNA from the distal segment of the sciatic nerve after crush-injury

Days post crush-injury	Relative translational efficiency
1	1.63 ± 1.31^a (3)
2	2.37 ± 1.82^a (3)
4	2.58 ± 1.95^a (4)
7	1.32 ± 0.65^a (4)
10	2.56 ± 2.45^a (3)
12	7.24 (1)
14	2.73 ± 1.66^b (4)
21	1.90 ± 0.95^b (4)
Adult	1.00 (4)
Young	2.61 ± 1.79^b (4)

a: $0.1 < p < 0.375$ b: $0.05 < p < 0.1$

iii. The reappearance of PO protein in the distal segment after crush-injury lags behind the recovery in levels of the PO transcript.

To estimate the in vivo levels of PO protein following crush-injury, a solid phase immunoassay was carried out on total protein extracts from distal and proximal segments of the crushed nerves. The levels of in vivo PO protein in the distal segments followed the same trend as that obtained for the PO mRNA, although a delay was apparent (Figure 3.8). Levels of PO decreased until 14 days, and began to increase by 21 days after nerve-crush. Decreases in the level of PO protein were also apparent in the proximal segments, but were generally not as severe as in the distal segments. These general trends were the same whether the results were expressed on the basis of total cellular protein or unit wet weight of tissue (Table 3.7). The PO protein shows different mobilities in its oxidized and reduced form, and this may explain the presence of a closely migrating doublet in certain lanes [Cammer, 1980; Brunden et al., 1990]. Figure 3.8 also revealed high-molecular weight immuno-crossreactive proteins. In this regard, the anti-chick PO polyclonal antibody used for Western blot analysis has been shown to cross-react with other glycoconjugates in nerve tissue [Nunn et al., 1987].

In summary, Western blot analysis suggested a delay in the degradation and biosynthesis of PO protein during the demyelinating and remyelinating phases of crush-injury, with respect to the levels of PO transcripts (see Figure 3.9). It is likely that additional translational effects or post-translational effects take place in the regulation of PO gene expression in vivo.

Figure 3.8 Western blot analysis of PO protein in the distal and proximal segments of the sciatic nerve following crush-injury

Proteins were extracted, fractionated by 10% SDS-PAGE and electroblotted as described. The nitrocellulose sheets were immunostained with anti-chick PO γ-globulins. Approximately 5 μg of total nerve protein was applied on each lane. Lane A and S contain 50 ng and 100 ng purified chick PO protein respectively; lanes B-I are the distal segment of crushed nerve 1,2,4,7,10,12,14,21 days post-injury respectively; J, the normal control adult nerve; and lanes K-R are the corresponding proximal segments of crushed nerve 1-21 days post-injury.

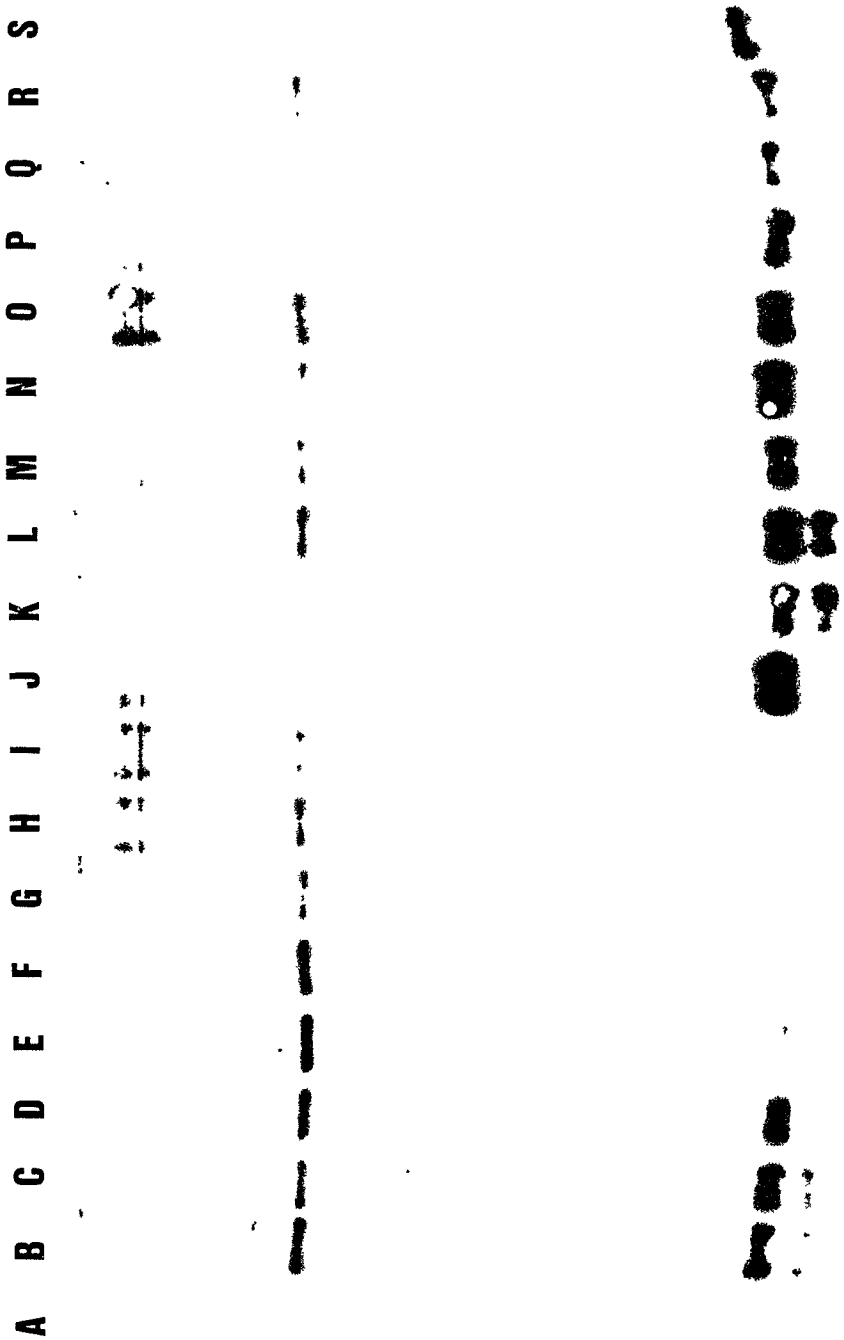


Figure 3.8

Table 3.7 Levels of PO protein in the proximal and distal segments of the sciatic nerve following crush-injury

The amount of PO protein was determined from immunoblots probed with anti-chick PO γ-globulins as described in the legend for Figure 3.8. Total protein (5 μg)was applied on each lane from tissue extracts of distal and proximal segments of crush-injured sciatic nerves obtained at various times post-injury. The intensity of each immunostained protein band was measured by densitometry and quantitated by planimetry. To estimate absolute levels of PO protein, intensities were compared to that obtained for purified chick PO protein, and then expressed as a percentage of the total protein applied. Levels of PO were also compared to that obtained from the normal adult sciatic nerve, per μg protein, and per unit wet weight of tissue used to prepare the extracts.

Table 3.7

Levels of PO protein in the proximal and distal segments of the sciatic nerve following crush-injury

Time after crush-injury	(days)	Levels of PO protein (% of total protein)	Levels relative to adult SN Levels/μg protein	Levels/mg tissue
Distal	1	3.3	0.68	0.69
	2	2.7	0.55	0.45
	4	2.8	0.59	0.16
	7	1.3	0.26	0.29
	10	0.8	0.17	0.16
	12	0.5	0.11	0.13
	14	0.5	0.11	0.11
	21	1.1	0.23	0.21
Proximal	1	4.4	0.90	0.88
	2	6.4	1.30	1.34
	4	4.7	0.95	0.83
	7	5.4	1.09	0.45
	10	4.4	0.90	0.55
	12	3.7	0.75	0.72
	14	1.5	0.30	0.28
	21	1.6	0.34	0.11
	Adult	4.9	1.00	1.00

Figure 3.9 Levels of PO protein and mRNA at various times following crush-injury.

The amount of PO protein was determined from immunoblots immunostained with anti-chick PO γ-globulins as described in the legend of Table 3.7. Estimation of the steady-state level of PO mRNA is described in the legend of Table 3.3. Steady-state levels of H3.3 mRNA were estimated by quantitation of dot blots as exemplified in Figure 3.5 and as described in Materials and Methods. The levels of PO protein and mRNA and H3.3 mRNA obtained for the normal adult sciatic nerve were designated to be one and results from all other tissues were compared to that value in each experiment.

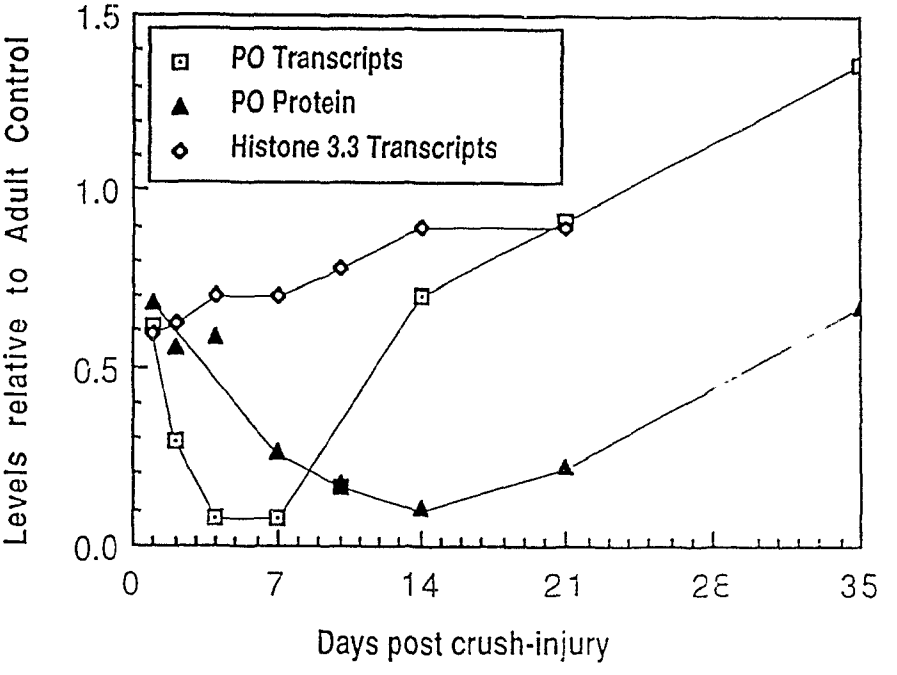

PO Transcripts
PO Protein
Histone 3.3 Transcripts
Levels relative to Adult Control
1.5
1.0
0.5
0.0
0
7
14
21
28
35
Days post crush-injury

FIGURE 3.9

iv. The MAG gene is expressed prior to PO and MBP genes during PNS remyelination.

The following experiments were performed as a corollary to the investigations with PO and MBP gene expression after crush-injury. The time course of gene expression of the putative cell adhesion molecule MAG, in the presence and absence of Schwann cell-axonal contact, was determined using experimental techniques similar to those described in preceding sections and was compared to the expression of the major PNS myelin genes.

To assess the size and quality of MAG transcripts, total RNA preparations from the distal segments of rat sciatic nerves at various stages of crush-injury and at 35 days post-transection were analyzed (Figures 3.10 A, B and C). The size of the MAG transcript was 2.5 kb as previously reported [Lenoir et al., 1986; Lai et al., 1987; Salzer et al., 1987]. The alternately spliced mRNAs coding for different MAG isoforms (p67MAG and P72MAG) differ by 45 bases and therefore could not be resolved under these experimental conditions. A second, larger MAG coding transcript of 3.0 kb was observed in the brain of 21-23 day old rats (Figure 3.10B, lane K) and has been reported previously by other investigators [Lenoir et al., 1986; Arquint et al., 1987; Salzer et al., 1987] in the developing rat brain. In the crush-injured sciatic nerve, the levels of MAG message decreased sharply 2 days after the operation. Although Figure 3.10A, lane C indicates a slight increase in message levels 4 days post-crush, statistical analysis of 4 biologically independent samples indicated that the elevation of message levels at 4 days was not significant (see Table 3.8). Levels at 21 days post crush-injury were not significantly higher than adult ($0.1 < p \leq 0.375$). The level of MAG mRNA in the distal segment of the transected nerve remained undetectable on Northern analysis when compared to that of normal adult (Figure 3.10C, lane N). This infers distinct transcriptional control of MAG gene expression. Reprobing this blot with ^{32}P-labelled PO cDNA indicated the presence

Figure 3.10. Northern blot and slot blot analyses of MAG-coding transcripts in the distal segments of the crush-injured and permanently transected sciatic nerve

Hybridization was carried out with ^{32}P-labelled rat MAG-cDNA as described in Materials and Methods. Arrowheads mark the position of 18S and 28S rRNA on Northern blots on panels A,B, and C. **A.** The amount of fractionated total RNA in each lane was 45 μg. Lanes A-G contain RNA from distal segments of nerves, 1,2,4,7, 10, 14 and 21 days post crush-injury, respectively. **B.** The amount of fractionated total RNA in each lane was 30 μg. Lane H contains RNA from 21-23 day old rat sciatic nerve, Lane I from adult sciatic nerve, Lane J from adult brain, Lane K from 21-23 day old rat brain and Lane L contains wheat rRNA. **C.** Lane M contains 30 μg total RNA from 21-23 day old rat sciatic nerve, and Lane N contains 6 μg poly(A)$^+$ RNA from the distal segment of sciatic nerve 35 days post-transection. **D.** Slot blot analysis of total RNA . Serial dilutions (one in two) of 15 μg of total RNA were immobilized on nitrocellulose membrane and analyzed as described in Materials and Methods. Spectrophotometric readings of total RNA before spotting were used for quantitation. The slots on the nitrocellulose were hybridized with ^{32}P labelled rat MAG-cDNA probe. Lanes A-E and F-L constitute separate experiments probed at different times. Lanes A and J contain RNA from adult sciatic nerve, Lanes B-H contain RNA from distal segments of nerves 2, 4, 7, 10, 12, 14 and 21 days post crush-injury respectively. Lane I contains RNA from 21-23 day old rat sciatic nerve, Lane K contains RNA from adult brain, and Lane L from 21-23 day old rat brain.

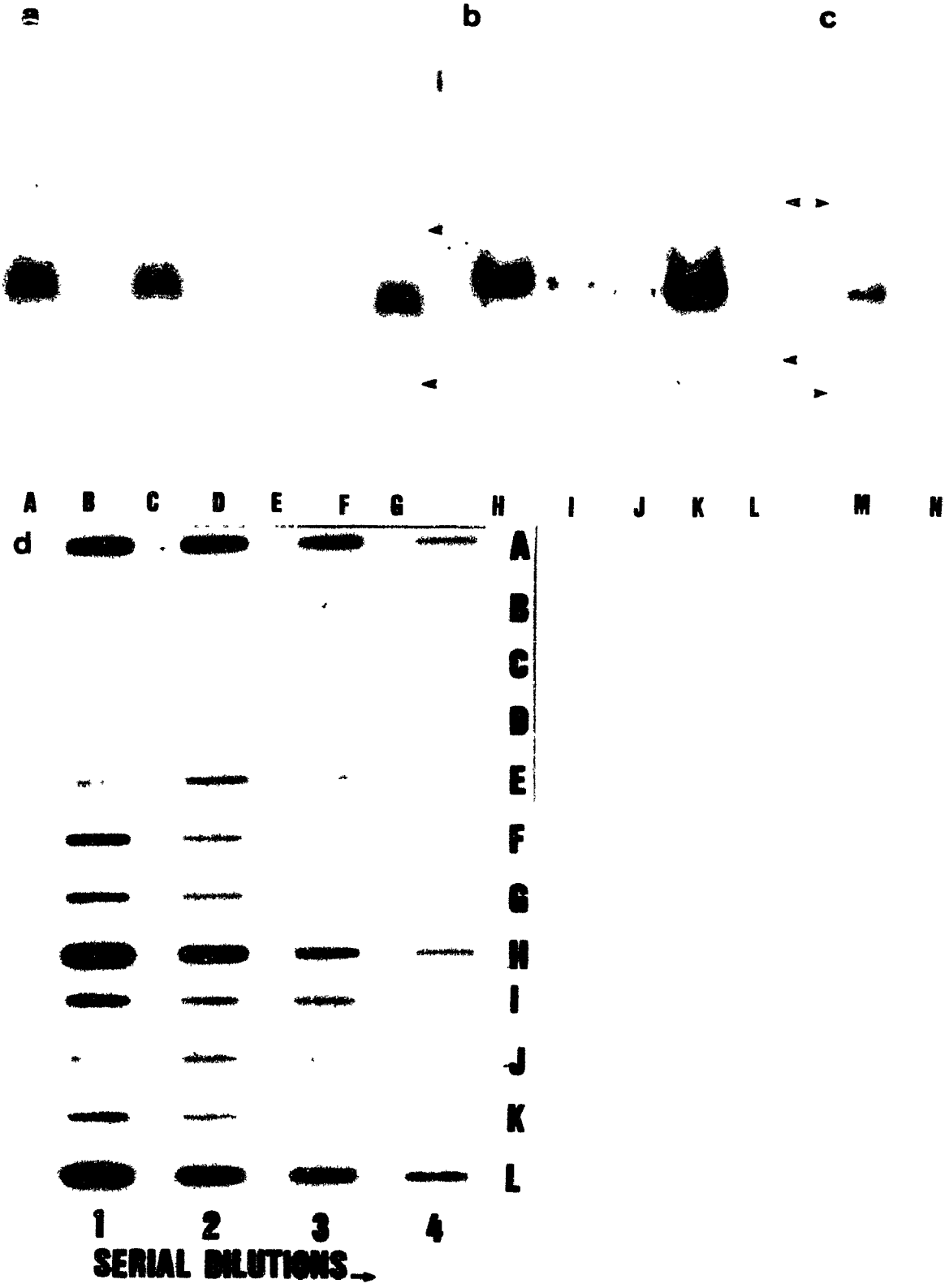


Figure 3.10

Table 3.8 Estimation of the steady-state level of MAG mRNAs by slot blot assay

Total RNA was extracted from the distal segments of crushed sciatic nerves at various times following crush injury, from normal adult nerves and from 21-23 day old rat brain. Serial dilutions (one in two) of the RNA were filtered through nitrocellulose sheets, and the slots on the nitrocellulose sheet were hybridized with ^{32}P MAG cDNA probe as described in Materials and Methods. The washed and dried nitrocellulose blots were autoradiographed and the intensity of each slot was measured by densitometry. The units of peak area were divided by the amount of total RNA in the slot to give units per μg total RNA. The amount obtained for the normal adult sciatic nerve was arbitrarily designated to be one. Results are expressed as mean ± S.D. of RNA preparations for 3 to 5 experiments. The numbers in parentheses indicate the number of separate experiments using sciatic nerves pooled from 6-8 animals for each time point following crush-injury or control preparations. One 21-23 day old rat brain was used for preparation of total RNA in each experiment. Results from slot blot analysis of RNA from crush-injured nerves, and from 21-23 day old rat brain were compared to values obtained for normal adult nerves and statistical significance (p values) was calculated by the Student's t-test.

Table 3.8.

Estimation of the steady state level of MAG mRNA by slot blot assay

Time after crush-injury (days)	Relative units MAG mRNA/μg total RNA
1	0.88 ± 0.33^{a} (3)
2	0.22 ± 0.12^{c} (5)
4	0.26 ± 0.23^{c} (4)
7	0.27 ± 0.10^{c} (5)
10	0.34 ± 0.04^{c} (5)
12	0.37 (1)
14	0.41 ± 0.05^{c}(3)
21	1.13 ± 0.47^{a}(3)
Adult	1.00 (5)
21-23 day rat brain	3.05 ± 1.45^{b}(4)

a: $0.1 < p \leq 0.375$ b: $0.005 < p \leq 0.01$ c: $p \leq 0.0005$

of low amounts of undegraded PO mRNA indicating the intactness of the poly(A)$^+$ RNA preparation (results not shown, but see Section 3.2, Figure 3.15).

Since Northern blots are not easily quantitated, slot blot analysis was carried out on at least three biologically independent samples. A typical representation is shown in Figure 3.10D. At 2 days, distal segments of crush-injured nerves contained only 22% of the MAG message present in normal adult nerves (Table 3.8). This value was different from that of the 1-day post-crush tissue and of the adult control ($p \leq 0.005$ and $p \leq 0.0005$ respectively). Levels of message in the distal segment rose steadily after 2 days post-crush until 14 days post-injury (22-41%) while the largest increase occurred between 14 and 21 days post-operation. After 21 days of crush the distal segments showed normal or higher than normal levels. MAG message levels in the brains of young rats were found to be 2-3 fold higher and different from normal adult sciatic nerve ($0.005 \leq p < 0.01$).

These results suggest that the MAG gene is expressed prior to PO and MBP genes in the remyelinating phase of crush-injury. Moreover, the complete down-regulation of MAG gene expression to undetectable levels after permanent transection of the sciatic nerve contrasts with basal levels of expression of the major myelin genes in the absence of Schwann cell-axonal contact [LeBlanc et al., 1987; see Section 3.3].

To examine the in vivo levels of translation products of the MAG gene in the crush-injured and transected sciatic nerve, Western blot analysis of MAG was carried out. Total protein extracts from the crushed and permanently transected nerves at various stages of injury were used. The levels of anti-MAG immunoreactive protein followed the same pattern as the corresponding MAG mRNA, with an approximate delay of 7 days (Figure 3.11). Levels of the immunoreactive antigen were decreased until 12 days post-injury (Figure 3.11, lanes A-F), and began to increase only 14 days after nerve crush (Figure 3.11, lanes G, H). No MAG could be detected by the Western blotting technique in the distal segment of permanently transected nerve 35 days post-operation (Figure 3.11, lane L), while near normal levels of MAG were visible in extracts from the corresponding

Figure 3.11 Western blot analysis of MAG in the distal segment of the crush-injured and permanently transected sciatic nerve.

Proteins were extracted, fractionated by 10% SDS polyacrylamide gel electrophoresis and electroblotted as described. The nitrocellulose sheets were immunostained with mouse anti-rat MAG monoclonal antibody GenS3. Approximately 75 μg of total nerve protein was applied on each lane. The arrowhead marks the immunostained 100kDa MAG protein. The arrows mark an immunocrossreactive doublet discussed in the Results section. Lanes A-H are the distal segments of nerves, 1,2,4,7, 10, 12, 14 and 21 days post crush-injury respectively. Lane I contains the adult sciatic nerve, Lane J is from 21-23 day old rat sciatic nerve and Lane K from 21-23 day old rat brain. Lanes L and M are the distal and proximal segments of adult sciatic nerve, 35-days post-transection respectively.

Figure 3.11

proximal segment (Figure 3.11, lane M). A lower molecular weight immuno-crossreactive doublet was observed on the Western blots from all operated nerves, probably corresponding to the deglycosylated forms of MAG [Frail and Braun, 1984]. A similar pattern was obtained when total proteins from endoneurial extracts were subjected to Western blot analysis (results not shown).

Glycosylated MAG isoforms cannot be resolved by SDS-PAGE and run as a single broad band at 100 kDa. To examine the differential expression of MAG polypeptides after crush-injury, nerve extracts were chemically deglycosylated using trifluoromethane sulphonic acid [Horvath et al., 1989]. The samples were analyzed by Western blot analysis and immunostained for MAG polypeptides. The deglycosylation procedure was found to be reproducibly complete, as exemplified by the conversion of glycosylated MAG in the 21-23 day rat brain (Figure 3.12A, lane 1) to the deglycosylated form (Figure 3.12A, lane 2). In a subsequent experiment, the relative mobilities of MAG polypeptides in the developing CNS, adult PNS and in the regenerating PNS were determined. A sharper resolution of the MAG isoforms was not obtained in this series of experiments, although a variety of polyacrylamide gel concentrations were tested. The MAG isoform expressed in the adult sciatic nerve (Figure 3.12B, lane 6) was smaller than that found in the 21-23 day rat brain (Figure 3.12B, lane 7). In contrast, the MAG polypeptides expressed over crush-injury (Figure 3.12B, lanes 1-5) were found to be of an intermediate molecular weight. The MAG isoform in the distal segment of the sciatic nerve 21 days after crush-injury (Figure 3.12B, lane 5) was at least 2 kDa larger than that found in the normal adult sciatic nerve (Figure 3.12B, lane 6). Tropak and co-workers [1988] have suggested through characterization of MAG mRNAs, that p67MAG is the major isoform expressed in both the developing and adult PNS. The present investigations suggest that MAG expression is altered during PNS remyelination, and that the MAG polypeptide in the remyelinating PNS may be larger than that in the adult peripheral nerve. It is not possible to rule out other alternative splicing events that may lead to changes in length of MAG polypeptides in the

Figure 3.12 Western blot analysis of chemically deglycosylated MAG in the crush-injured and adult sciatic nerve

Extracts from the sciatic nerve or brain were prepared and chemically deglycosylated as described in Materials and Methods. The treated samples were fractionated by SDS -PAGE and electroblotted onto nitrocellulose sheets. The nitrocellulose membranes were immunostained with mouse anti-rat MAG monoclonal antibody GenS3. Arrows indicate the leading edges of the molecular weight standards. **A.** The samples were fractionated by 10% SDS-PAGE. Lanes 1 and 2 are untreated and deglycosylated extracts from the 21-23 day rat brain, while Lane 3 depicts the molecular weight standards. **B.** The samples were fractionated by 12 % SDS-PAGE. Lanes 1-5 contain chemically deglycosylated extracts from the distal segment of the sciatic nerve, 1, 2, 12, 14, and 21 days post crush-injury, respectively. Lanes 6 and 7 contain the deglycosylated extracts from the adult sciatic nerve and the 21-23 day old rat brain, respectively. Lane 8 shows the molecular weight standards.

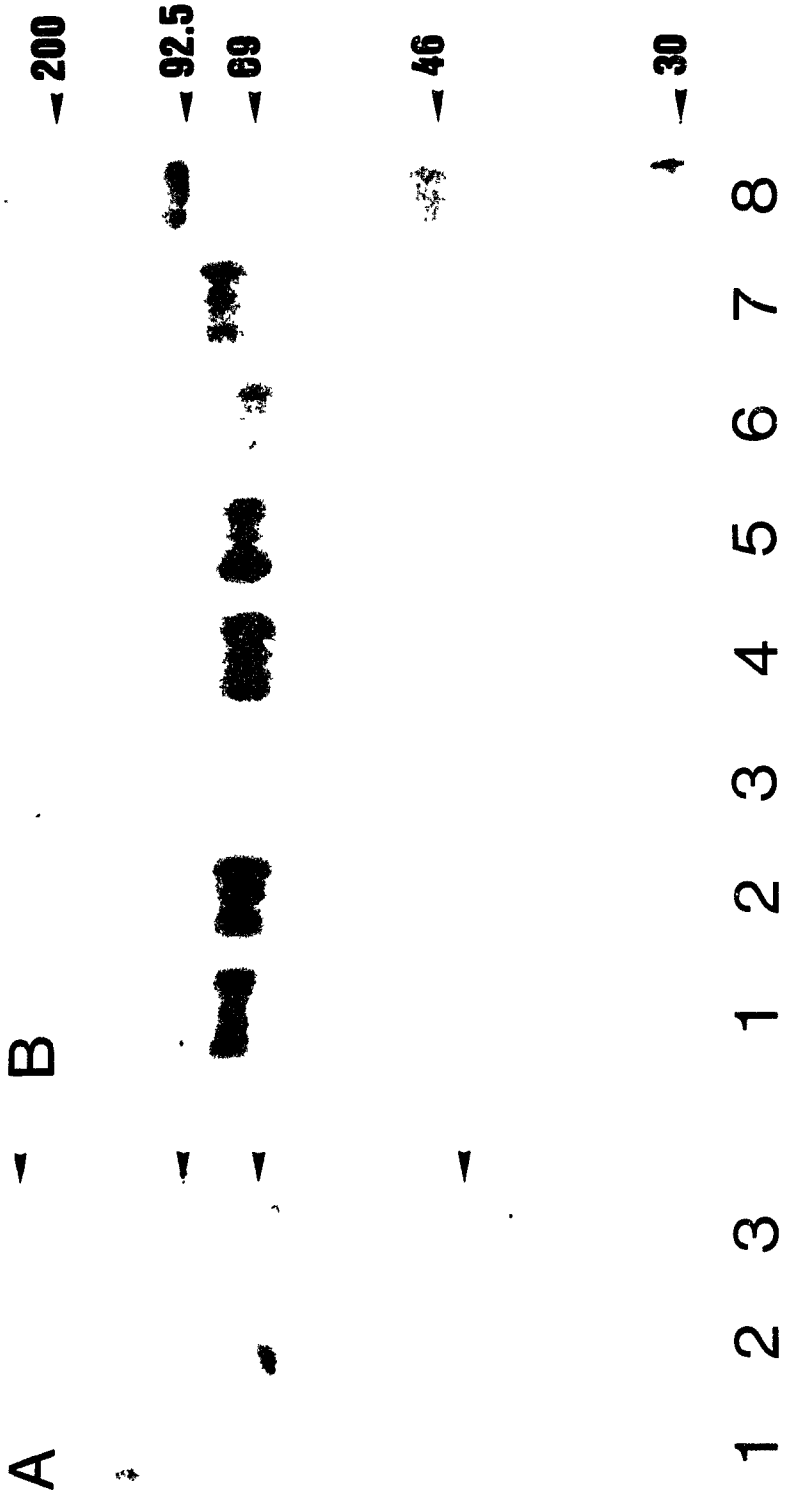


Figure 3.12

PNS. The present Western blotting results point out that MAG expression in the PNS may differ quite significantly from that in the CNS. A closer examination of MAG mRNAs in the PNS, or alternatively, isolation, purification, and characterization of the glycoprotein from the PNS will be necessary to clarify the results of the present studies.

v. The CNPase gene is induced after mechanical injury of the peripheral nerve, but is also subject to post-transcriptional regulation.

The results obtained so far indicated that genes encoding myelin-specific proteins were dramatically down-regulated as a result of loss of axonal contact during the transient phase of demyelination after crush-injury. The growth of the axon into the distal stump resulted in rapid increases in expression of the MAG gene followed by the PO and MBP genes. LeBlanc and Poduslo [1989] recently reported that the CNPase gene was quite atypical in its response to mechanical injury. Levels of CNPase mRNAs were increased in the distal segment as a consequence of both crush and permanent transection of the sciatic nerve. These results were confirmed through slot-blot analysis of CNPase gene expression over the first three weeks after crush-injury. As shown in Figure 3.13 and quantitated in Table 3.9, levels of CNPase mRNAs were greater than those found in the normal adult nerve, by 2 days after crush-injury. The trends were reproducible in two independent determinations with pooled biologically independent samples (Table 3.9).

Mezei and co-workers had previously reported that CNPase activities were reduced after permanent transection of the rabbit and chick sciatic nerve [Mezei et al., 1974]. In light of these data, the results from slot-blot analysis of CNPase mRNA levels were intriguing. CNPase activities were determined essentially as described by Mezei and co-workers. The CNPase activity in the distal segment of crush-injured sciatic nerve followed the pattern of demyelination and remyelination, as described in Table 3.9. Thus, CNPase

Figure 3.13 Slot blot analysis of CNPase coding transcripts in the distal segment of the sciatic nerve following crush-injury.

Serial dilutions (one in two) of 36μg of total RNA were immobilized on nitrocellulose filters and hybridized with a ^{32}P-labelled CNPase cDNA. Spectrophotometric readings of total RNA before spotting were used for quantitation. Lanes A-D contain RNA from distal segments of sciatic nerves 2, 4, 7, and 10 days post crush-injury respectively. Lanes E and F contain RNA from the adult sciatic nerve and from 21-23 day old rat brain, respectively. Lane G contains wheat rRNA.

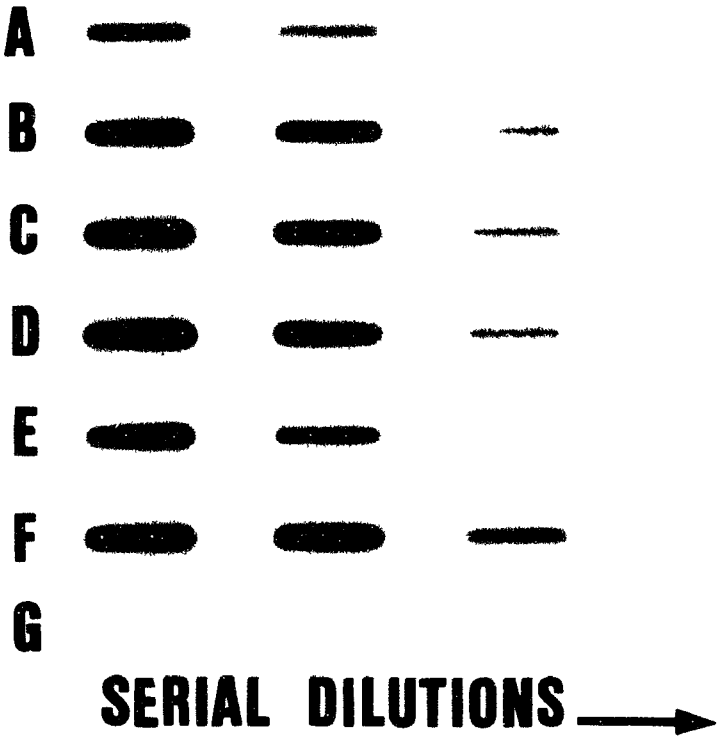


Figure 3.13

Table 3.9 Estimation of the steady-state levels of CNPase mRNAs by slot blot assay and levels of CNPase activity at various stages after crush-injury

Total RNA was extracted from the distal segments of crushed sciatic nerves, at various times following crush injury, from normal adult nerves and from 21-23 day old rat brain. Serial dilutions (one in two) of the RNA were filtered through nitrocellulose sheets, and the 'slots' on the nitrocellulose sheet were hybridized with the ^{32}P CNP cDNA probe as described in Materials and Methods. The washed and dried nitrocellulose blots were auto-radiographed and the intensity of each slot was measured by densitometry. The units of peak area were divided by the amount of total RNA in the slot to give units per μg total RNA. The amount obtained for the normal adult sciatic nerve was arbitrarily designated to be one. Results are expressed as a range. The numbers in parentheses indicate the number of separate experiments using sciatic nerves pooled from 6-8 animals for each time point following crush-injury or in control preparations. One 21-23 day old rat brain was used for preparation of total RNA in each experiment. CNPase activity in tissue homogenates was expressed as μmol product formed/hour/mg protein. The value in adult sciatic nerve was designated to be one, and the results from other tissues were expressed relative to this value.

Table 3.9.

Estimation of the steady state level of CNPase mRNA by slot-blot assay and levels of CNPase activity at various stages after crush injury.

Time after crush-injury (days)	Relative units CNPase mRNA /μg total RNA	CNPase activity/mg protein Distal	Proximal
1	nd	0.47 - 0.81 (2)	0.71 (1)
2	0.58 - 0.67(2)	0.31 - 0.49 (2)	0.79 (1)
4	1.35 - 1.91 (2)	0.19 - 0.37 (2)	0.82 (1)
7	2.12 - 2.28 (2)	0.17 - 0.17 (2)	0.68 (1)
10	2.64 - 2.86 (2)	n.d	nd
12	1.22 - 1.32 (2)	0.55 (1)	nd
21	1.45 -1.61 (2)	0.80 (1)	nd
Adult SN	1.00 (2)	1.00 (1)	nd
21 - 23 day rat brain	1.76 - 2.70 (2)	nd	nd

nd: not determined

gene expression after mechanical injury was found to be quite atypical of the myelin genes, and may also be subject to modulation at post-transcriptional levels.

3.2 The expression of the PLP gene in the developing, demyelinating and remyelinating peripheral nerve.

Studies described in Section 3.1 have examined the regulation of genes encoding myelin-specific and myelin-associated proteins in two contrasting experimental paradigms. In the peripheral nerve, it appears that a 'continuous' exchange of information between the Schwann cell and the axon is necessary, and a disruption leads to a rapid down-regulation of transcripts encoding myelin-specific proteins. In this study, the possibility that PLP expression in the PNS was also under axonal control was investigated, by using previously characterized models of peripheral nerve development, degeneration and regeneration.

i. PLP transcripts are expressed in the PNS and CNS during neural development.

Myelination during neural development is marked by an increased biosynthesis of specific proteins and lipids in Schwann cells and oligodendrocytes. This is also reflected in increases in steady-state levels of transcripts encoding myelin-specific proteins. We first compared the levels of PO, MAG and PLP mRNAs during PNS and CNS development.

In these experiments, RNA was isolated by the guanidinium thiocyanate-CsCl method as described in Materials and Methods. Figure 3.14A represents a Northern blot analysis of PO mRNA in the 21-23 day old rat sciatic nerve (Figure 3.14A, lane 1) and in the adult sciatic nerve (Figure 3.14A, lane 2). Levels of the 1.9 kb PO transcripts in the rapidly myelinating nerve were estimated to be 2.2 times those in the adult nerve (see Section 3.1, Table 3.3). No hybridization to rat brain mRNA was observed either in the adult (Figure 3.14A, lane 3) or in the 21-23 day old rat (Figure 3.14A, lane 4), indicating

that PO gene expression is indeed specific to the peripheral nerve . Wheat rRNA (30μg) was used in this Northern blot as a negative control (Figure 3.14A, lane 5).

The MAG gene is expressed both in the PNS and in the CNS [Lai et al., 1987; Salzer et al., 1987; Tropak et al., 1988; Section 3.1]. The levels of the 2.5 kb MAG transcript were much higher in developing sciatic nerve (Figure 3.14B, lane 1) and brain (Figure 3.14B, lane 4) than in the respective adult tissues (Figure 3.14B, lanes 2 and 3).

The major PLP transcript in the developing rat brain was 3.2 kb, as previously reported, while other minor forms were also detected of 1.6 kb and 2.4 kb (Figure 3.14C, lane 4) [Milner et al., 1985; Gardinier et al., 1986]. These are thought to be formed through the use of the three polyadenylation signals found in the 3' non-coding region of the PLP transcript [Puckett et al., 1987]. PLP mRNA levels were lower in the adult brain (Figure 3.14C, lane 3) than in the 21-23 day old rat brain (Figure 3.14C, lane 4) and were even lower in the sciatic nerve of the 21-23 day old rat (Figure 3.14C, lane 1) and in the adult (Figure 3.14C, lane 2). Moreover, in sharp contrast to PO and MAG gene expression as represented in Figures 3.14A and Figure 3.14B , PLP transcripts remained unchanged during PNS development. This was subsequently confirmed by semi-quantitative slot-blot analysis of levels of PLP mRNA (Table 3.10).

ii. PLP transcripts are observed in the distal segment of the permanently transected sciatic nerve, in the absence of Schwann cell-axonal contact.

The expression of myelin genes in the distal segment of the transected sciatic nerve was examined, wherein the resident cell population comprises about 90% Schwann cells. The Schwann cell population in the distal segment of the transected sciatic nerve is, however, incapable of myelin assembly. PO transcript levels in the transected nerve are decreased to basal levels, 35 days post-transection [LeBlanc et al., 1987]. This is shown in Figure 3.15A. PO transcripts, which were abundant in 21-23 day old rat sciatic nerve

Figure 3.14 Northern blot analysis of PO, MAG, and PLP transcripts during neural development

The amount of RNA in each lane was 30 μg. Lane 1 contains RNA from 21-23 day old rat sciatic nerve, Lane 2 from adult rat sciatic nerve, Lane 3 from adult rat brain, Lane 4, 21-23 day old rat brain and Lane 5 contains wheat rRNA. The arrowheads show the positions of the 28S and 18S rRNA in each panel. **Panel A** was hybridized to a rat PO-specific cDNA probe (pSN63c). **Panel B** was hybridized to a rat MAG-specific cDNA probe (pMAG 1.2). **Panel C** was hybridized to a rat PLP-specific probe (pLP 1.5). The nitrocellulose blot was allowed to decay between hybridizations.

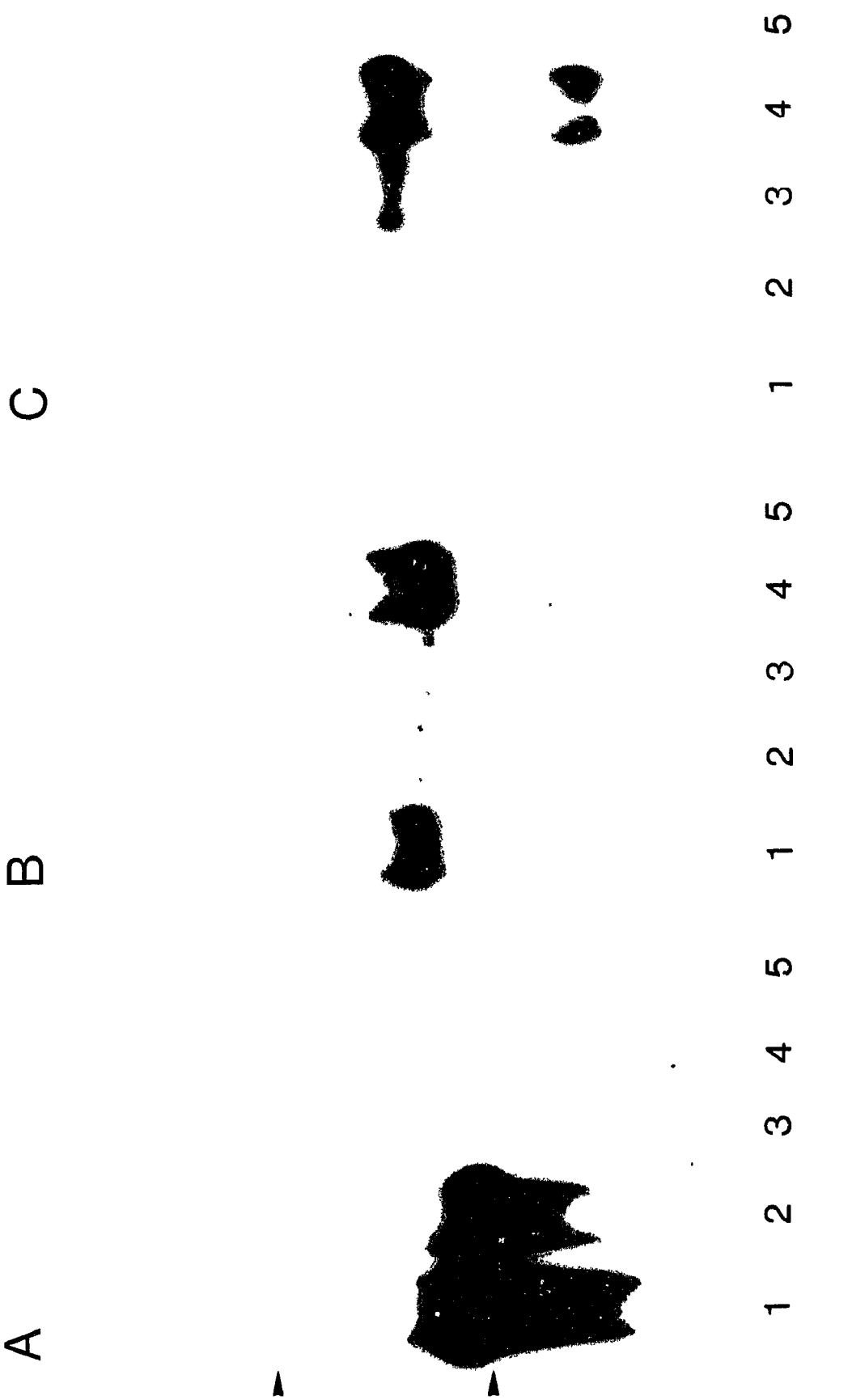


Figure 3.14

Table 3.10. Estimation of the steady state level of PLP mRNAs by slot-blot assay.

Total RNA was extracted from the distal segments of crushed sciatic nerves at various times following crush injury, from normal adult nerves and from 21-23 day old rat brain. Serial dilutions (one in two) of the RNA were filtered through nitrocellulose sheets, and the 'slots' on the nitrocellulose sheet were hybridized with ^{32}P labelled PLP cDNA probe as described in Materials and Methods. The washed and dried nitrocellulose blots were autoradiographed and the intensity of each slot was measured by densitometry. The units of peak area were divided by the amount of total RNA in the slot to give units per μg total RNA. The amount obtained for the normal adult sciatic nerve was designated to be one. Results are expressed as a range for 2 separate experiments or as mean ± S.D. of RNA preparations for 3 experiments. The numbers in parentheses indicate the number of separate experiments using sciatic nerves pooled from 6-8 animals for each time point following crush-injury or control preparations. One 21-23 day old rat brain was used for preparation of total RNA in each experiment. Results from slot blot analysis of RNA from crush-injured nerves, and from 21-23 day old rat brain were compared to values obtained for normal adult nerves and statistical significance (p values) was calculated by the Student's t-test.

Table 3.10.

Estimation of the steady state level of PLP mRNAs by slot-blot assay

Time after crush-injury (days)	Relative units PLP mRNA/μg total RNA
1	0.973 (1)
2	0.40 - 0.46 (2)
4	0.58 - 0.82 (2)
7	0.82 ± 0.42^a (3)
10	1.00 - 1.02(2)
12	0.82 ± 0.32^a (3)
14	1.26 - 1.32 (2)
21	1.69 ± 0.42^b (3)
Developmental profile:	
21 - 23 day rat SN	0.94 - 1.15 (2)
Adult SN	1.00 (3)
Adult rat brain	2.83 (1)
21 - 23 day rat brain	5.70 ± 1.74^c (3)
Levels after permanent transection:	
distal segment, 21 days post-transection	0.55 - 0.68 (2)
proximal segment, 21 days post-transection	0.95 (1)

a: $0.1 < p \leq 0.375$ b: $0.05 < p \leq 0.1$ c: $0.01 < p \leq 0.025$

Figure 3.15 Northern blot analysis of PO, MAG, and PLP transcripts after permanent nerve transection

The amount of total RNA in each lane was 5 μg. Lane 1 contains RNA from 21-23 day old rat sciatic nerve, Lane 2 from adult rat sciatic nerve, Lane 3 from the distal segment of sciatic nerve, 21 days after transection, and Lane 4 contains RNA from adult rat liver. **Panel A** was probed for PO mRNA, **Panel B** was probed for MAG mRNA, and **Panel C** was probed for PLP mRNA. The blots were prepared in triplicate and each was probed separately.

Figure 3.15

Figure 3.16 Northern blot analysis of PO and PLP transcripts after crush-injury.

Panel A. Lanes 1-7 contain 4 μg of poly(A)$^+$ RNA from distal segments of sciatic nerves 1, 2, 4, 7, 10, 14 and 21 days after crush-injury, respectively. Lane 8 contains 4 μg of poly(A)$^+$ RNA from adult sciatic nerves.The Northern blot was probed for PO mRNA. **Panel B.** Lanes 1-7 contain 30 μg of total RNA from distal segments of sciatic nerves 1, 4, 7, 10, 12, 14 and 21 days after crush-injury, respectively. The Northern blot was probed for PLP mRNA.

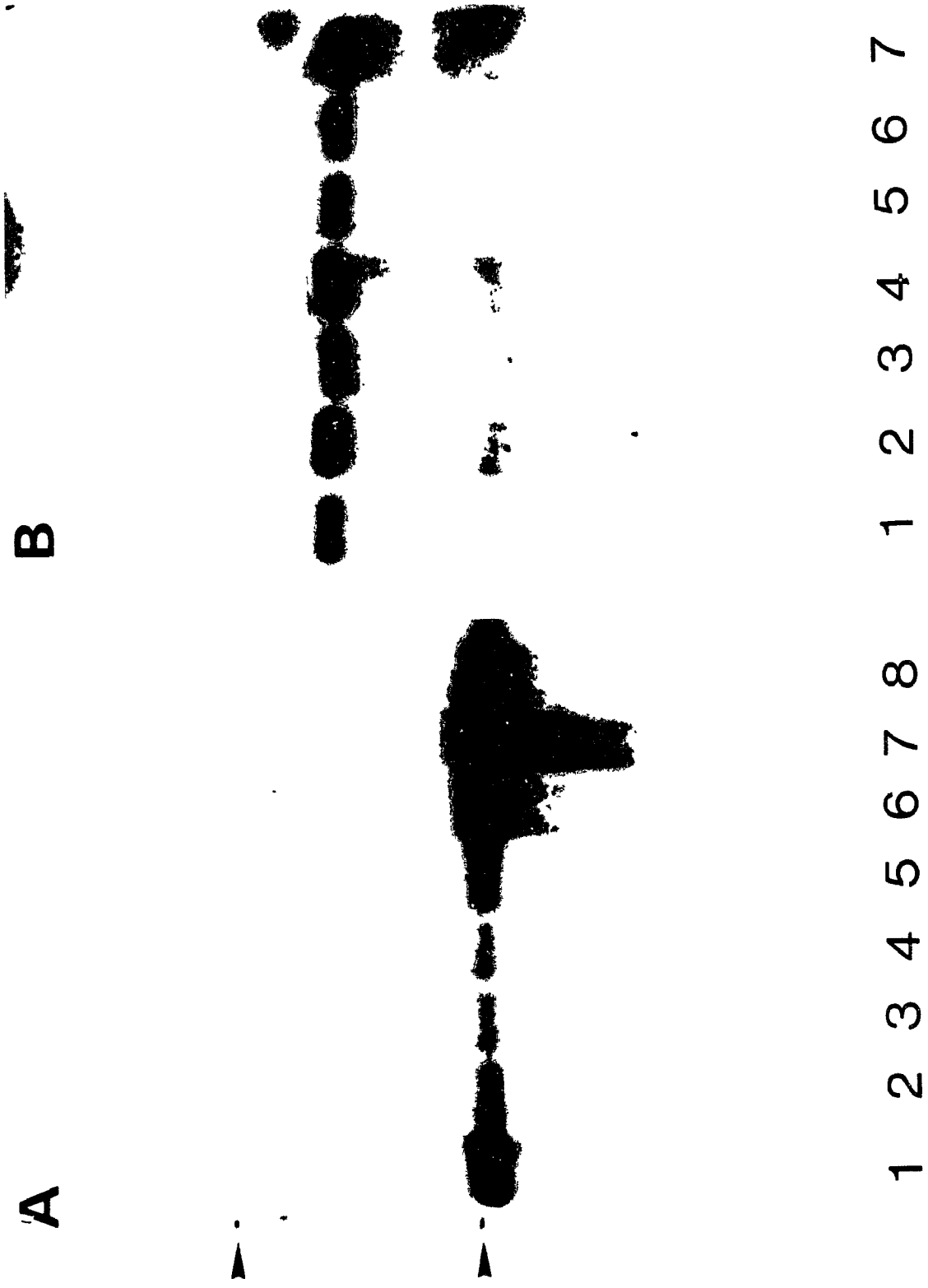


Figure 3.16

These observations suggest a relative dissociation of the PLP gene expression in the sciatic nerve from axonal control.

3.3 Axonal modulation of myelin gene expression in the crush-transected model.

i. Induction of myelin genes in the remyelinating nerve requires a continuous signal from the ingrowing axon.

Mechanical and neurotoxic damage to the sciatic nerve are both marked by rapid and significant changes in the steady-state levels of mRNAs encoding myelin-specific genes [LeBlanc et al., 1987; Veronesi et al., 1989; Section 3.1]. In this series of experiments, the effect of a permanent transection on a remyelinating nerve was examined to obtain further information about axonal modulation of myelin gene expression using an in vivo model. The adult sciatic nerve was crushed, and the distal segment was allowed to regenerate for 12 days. The sciatic nerve was then transected distal to the site of crush to disrupt the Schwann cell-axonal contacts that had reformed. Levels of transcripts coding for five myelin proteins were assayed in the distal segment of the crush-transected nerve after 9 days and were compared with corresponding levels in the distal segments of sciatic nerves at 12 and 21 days post-crush, and at 21 days post-transection using Northern blot and slot-blot analysis.

Figure 3.17 represents Northern blot analysis of RNA prepared from the sciatic nerve, after crush, transection and crush-transection. Rapid remyelination in the distal segment of the crush-injured sciatic nerve occurs 10 to 14 days after crush-injury with the re-introduction of contact of the Schwann cell population by the ingrowing axons (as described in Section 3.1). This is depicted in Figure 3.17A, showing dramatic increases in levels of the 1.9 kb PO transcripts in distal segments 12 days (lane 1), 21 days (lane 2)

Figure 3.17 Northern blot analyses of PO, MAG, CNPase and PLP transcripts in sciatic nerves after crush-injury, after permanent transection, after crush-transection

Total RNA (5 μg) was fractionated in each lane. The arrowheads mark the positions of the 18S and 28S rRNAs. Lanes 1, 2, 3 are from distal segment of the sciatic nerve, 12, 21, and 28 days post crush-injury respectively. Lane 4 is from the distal segment of the sciatic nerve, 21 days post-transection. Lane 5 contains RNA from the distal segment of the sciatic nerve, 21 days post crush-transection (the sciatic nerve was crushed; a permanent transection was carried out distal to the site of crush after 12 days; the segment was dissected 9 days later, distal to the site of transection). Lanes 6 and 7 contain RNA from the adult sciatic nerve and 21-23 day old rat sciatic nerve, respectively. Lane 8 is from the proximal segment of the sciatic nerve, 21 days post crush-transection. Lane 9 is from the proximal segment of the sciatic nerve, 21 days post-transection; Lane 10 contains RNA from the adult rat liver. **Panel A** was probed for PO mRNAs, **Panel B** for MAG mRNAs, and **Panel C** for CNPase mRNAs. **Panel D**: Total RNA (5 μg) was fractionated in each lane. Lanes 1 and 2 contain RNA from the distal segment of the sciatic nerve, 12, and 21 days post crush-injury. Lane 3 is from the distal segment of the sciatic nerve, 21 days post-transection. Lane 4 contains RNA from the distal segment of the sciatic nerve, 21 days post crush-transection. Lanes 5 and 6 contain RNA from the adult sciatic nerve and 21-23 day old rat sciatic nerve, respectively. Lane 7 contains RNA from the proximal segment of the sciatic nerve, 21 days post crush-transection. Lane 8 is from the proximal segment of the sciatic nerve, 21 days post-transection; Lane 9 contains RNA from the adult rat liver. **Panel D** was probed for PLP mRNAs.

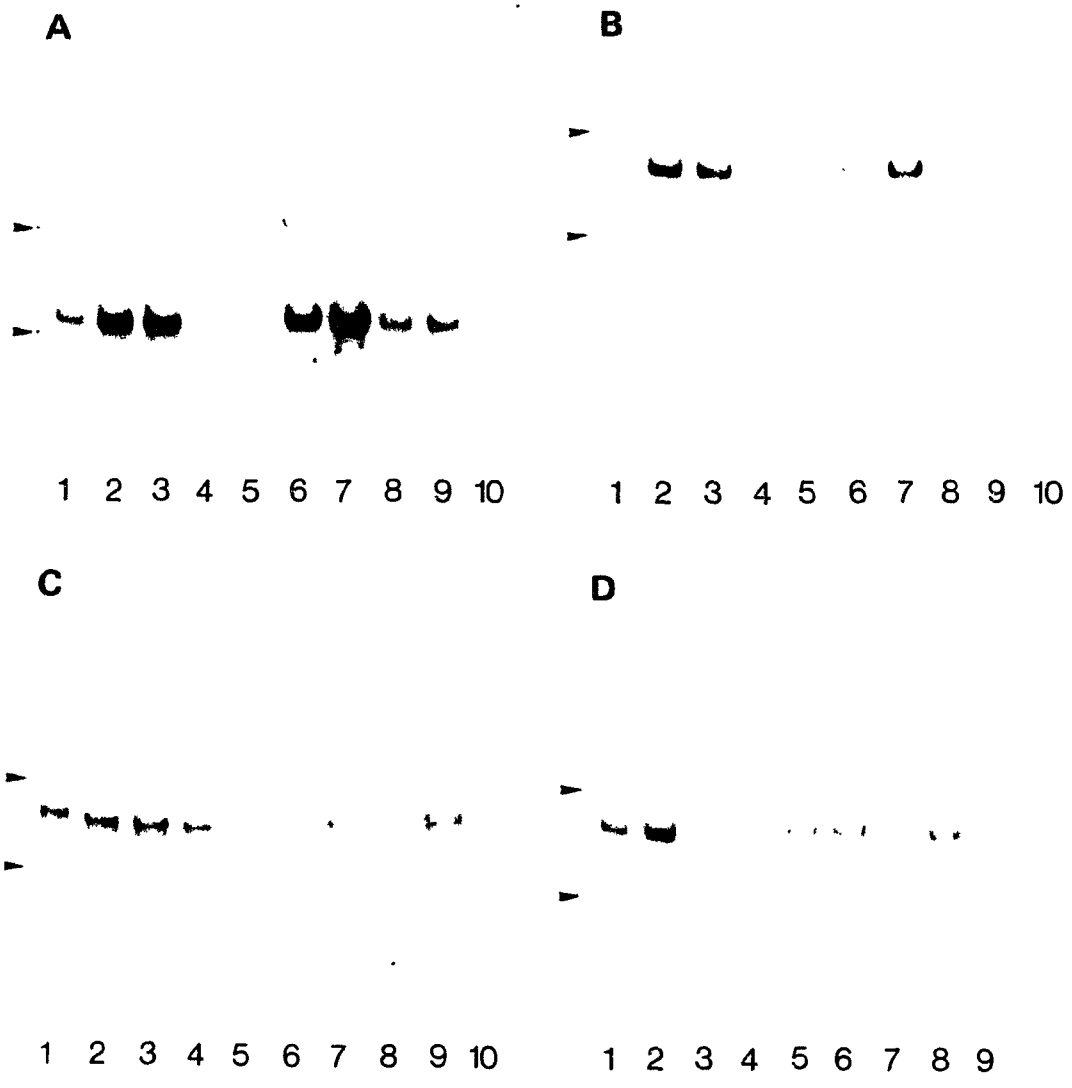


Figure 3.17

and 28 days (lane 3) post crush-injury. Levels of PO transcripts in the distal segment were comparable to that in the adult nerve (lane 6), within three weeks of peripheral regeneration. High levels of PO transcripts were also observed in the rapidly myelinating sciatic nerve at 21-23 days post-natal (lane 7). In contrast, levels of PO transcripts decrease in the distal segment of the transected sciatic nerve 21 days after injury (lane 4). The 12 day crush-injured sciatic nerve was transected distal to the site of crush, and proximal and distal segments were sampled 9 days post-transection. The loss of axonal contact to the remyelinating peripheral nerve resulted in rapid down-regulation of the expression of the PO gene. Thus, levels of PO mRNA in the distal segment of crush-transected nerve 9 days post-transection (lane 5) were low and not significantly different from the transected nerve 21 days after transection (lane 4). Bands representing PO mRNA were detectable on over-exposure of this blot. Levels of PO mRNAs were quantitated subsequently by slot-blot analysis (see Figure 3.18). The proximal segments of both the crush-transected nerve and the transected nerve contain PO mRNA levels comparable to the adult control (Figure 3.17A, lanes 8 and 9, respectively). No detectable hybridization was observed to RNA prepared from the adult liver (lane 10). Northern analysis of PO transcripts thus provided general trends in myelin-specific messages in this experimental paradigm, and indicated the RNA preparations were not degraded.

A similar trend was observed on probing a duplicate Northern blot with the MAG cDNA (Figure 3.17B). Levels of the 2.5 kb MAG message were increased in the remyelinating phase of crush-injury (lanes 1-3). MAG transcripts were about twice as abundant in the developing nerve when compared to the adult sciatic nerve (lanes 7 and 6 respectively, and as described in Section 3.1). MAG transcripts, in contrast to the trends observed for PO mRNAs, were undetectable in distal segments of the transected and crush-transected nerves (lanes 4 and 5, respectively), even after over-exposure of the blot (10 days). Levels of MAG mRNA in the proximal segments of the crush-transected and

Figure 3.18 Slot blot analysis of PO, MBP, MAG, CNPase, PLP and p1B15 transcripts in sciatic nerves after crush-injury, after permanent transection and after crush-transection.

Serial dilutions (one in two) of 10 μg of total RNA were immobilized on nitrocellulose membranes and hybridized to different probes as described in Materials and Methods. Spectrophotometric readings of total RNA before spotting were used for quantitation. This is a composite representation of several slot-blot analyses with different cDNA probes. Thus, all slots in one column represent equal quantities of immobilized RNA, and pictorially depict the relative steady-state levels of a specific message in sciatic nerves after crush-injury, after permanent transection, after crush-transection and in adult and young sciatic nerves. Lanes 1 and 2 are from the distal segment of the sciatic nerve, 12, and 21 days post crush-injury. Lane 3 contains RNA from the distal segment of the sciatic nerve 21 days post-transection. Lane 4 contains RNA from the distal segment of the sciatic nerve 21 days post crush-transection (the sciatic nerve was crushed; 12 days after, a permanent transection was carried out distal to the site of crush; the segment was dissected 9 days later, distal to the site of transection). Lanes 5 and 6 contain RNA from the adult sciatic nerve and 21-23 day old rat sciatic nerve, respectively. Lane 7 contains RNA from the proximal segment of the sciatic nerve 21 days post crush-transection. Lane 8 is from the proximal segment of the sciatic nerve 21 days post-transection. Lane 9 contains adult rat liver RNA.

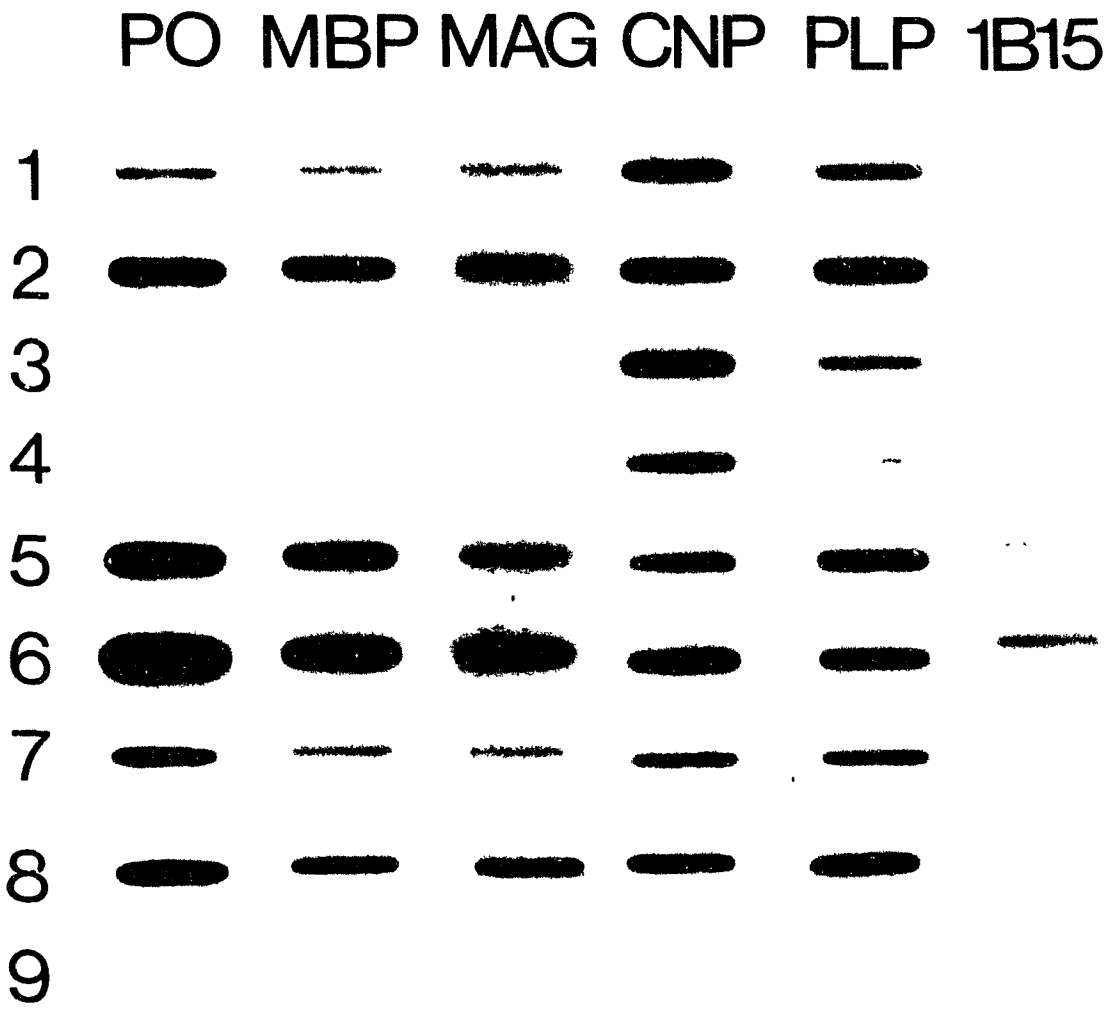


Figure 3.18

transected nerves were comparable to levels found in the adult (compare lanes 8 and 9 with lane 6, respectively).

The CNPase gene is expressed in both the CNS and PNS. Two transcripts of 2.4 and 2.8 kb are formed by alternative splicing at the 5' end of the primary transcript and are thought to occur in the 5' non-coding regions of the CNPase mRNAs [Bernier et al., 1987]. The sizes of the CNPase messages in the rat sciatic nerve were confirmed through Northern blot analysis (Figure 3.17C). The steady-state levels of CNPase mRNAs have also been shown to be induced during Wallerian degeneration [LeBlanc and Poduslo, 1989]. This was confirmed in these experiments with levels of CNPase mRNAs induced over adult levels in the distal segments of crush-injured (lanes 1-3), transected (lane 4) and to a smaller extent in the distal segments of the crush-transected (lane 5) sciatic nerve. In contrast, levels of CNPase mRNAs in the proximal segments of the crush-transected and transected nerve were comparable to those found in the normal adult control (lanes 8 and 9, respectively) . These observations suggest that disruption of Schwann cell-axonal contact affects the expression of the CNPase gene quite unlike other myelin genes.

The PLP gene was recently found to be expressed in the rat and human PNS, although the resultant gene product is not incorporated into peripheral nerve myelin [Puckett et al., 1987]. As was shown in Section 3.2, the expression of PLP in the PNS is relatively dissociated from Schwann cell-axonal contact . The Northern blot depicted in Figure 3.17D confirmed these observations. The level of PLP mRNAs remained the same in the developing sciatic nerve as in the adult sciatic nerve (lanes 6 and 5, respectively). The level of PLP-coding transcripts in the distal segment of the sciatic nerve increased slightly over crush-injury (lanes 1 and 2), and decreased slightly after transection (lane 3), but in no case was the alteration in levels as striking as that found for PO and MAG. The distal segment of the crush-transected nerve also contained levels of PLP transcripts comparable to those in the distal segment of the transected nerve (lanes 4 and 3,

respectively). These result suggest that PLP gene expression is relatively unaffected by manipulations of Schwann cell-axonal contact.

ii. Loss of Schwann cell-axonal contact affects the expression of myelin genes in at least four different ways.

The trends observed in Northern blots were further substantiated by semi-quantitative analysis using slot-blots. In addition to the probes mentioned above, the slot-blots were also probed with MBP and pIBI5, a cDNA originally isolated from a rat brain cDNA library [Milner and Sutcliffe, 1985; Danielson et al., 1988]. The p1B15 cDNA clone encodes cyclophilin, a protein that is ubiquitous in tissue. The mRNA encoding cyclophilin has previously been detected in nearly all tissues in the rat and the monkey [Danielson et al., 1988], and was used in the present investigation as a constitutive control. A composite representation of several slot-blot analyses with different cDNA probes is shown in Figure 3.18. Thus, all slots in one column represent equal quantities of immobilized RNA and pictorially depict the relative steady-state levels of a specific message in sciatic nerves at 12 and 21 days after crush-injury (lanes 1 and 2), after permanent transection (lane 3), after crush-transection (lane 4) and in adult and young sciatic nerves (lane 5 and 6, respectively). The levels of mRNAs encoding different myelin proteins were also determined in the proximal segments of the crush-transected (lane 8) and transected nerves (lane 9) and compared to levels found in the adult sciatic nerve (lane 5). The patterns of hybridization of various probes to RNA from proximal segments of crush-transected and transected nerves demonstrate that alterations in gene expression are distinct from those observed in RNA prepared from the distal counterparts. Levels of mRNAs in different tissues remained relatively constant for the constitutive control p1B15, although levels in the 21-23 day old sciatic nerve were higher than those found in the adult control (compare lanes 6 and 5, respectively). No detectable hybridization to RNA prepared from

the adult liver (lane 9) was observed for any cDNA probe, though low levels were observed in the case of p1B15 on over-exposure of the blot. Levels of mRNAs encoding the various myelin proteins were determined relative to those found in the adult sciatic nerve for biologically independent samples, and the results are summarized in Table 3.11. Relative levels of PO- and MBP-coding transcripts were very similar after crush, after transection, and after crush-transection, suggesting that these genes may be co-regulated in the peripheral nerve [LeBlanc et al., 1987; Sutcliffe et al., 1989; Section 3.1].

Slot blot analysis of MAG gene expression in this experimental paradigm also demonstrated distinct levels of MAG mRNAs after crush, after transection and after crush-transection. While MAG mRNA levels increased during the regenerative phase of crush injury (44% at 12 days post-crush, to 113% at 21 days post-crush), MAG mRNAs remained undetectable in the distal segment of the transected and crush-transected sciatic nerve (Figure 3.18, and Table 3.11). This cannot be attributed to lack of sensitivity of the technique, as p1B15 mRNAs were detected in these tissues and levels of MAG and p1B15 mRNAs in the adult peripheral nerve appear to be comparable. In relative terms, MAG gene expression was more stringently controlled by Schwann cell-axonal contact than the expression of PO and MBP genes.

Slot blot analysis also confirmed previous observations (Section 3.2) that PLP gene expression remains relatively unaffected by loss of Schwann cell-axonal contact. As may be seen in Table 3.11, changes in PLP mRNA levels in the distal segment of the sciatic nerve after crush, after transection and after crush-transection were not as striking as those observed for steady-state levels of PO, MBP and MAG mRNAs.

The effect of mechanical injury on the expression of the CNPase gene was quite atypical of the myelin genes under investigation. The results of the present investigation, which showed an induction of message levels after crush, after transection and after crush-transection, were particularly intriguing, as levels of CNPase activity actually decrease in the rabbit and chicken sciatic nerve after permanent transection [Mezei et al., 1974].

Table 3.11 Estimation of levels of PO, MBP, MAG, CNPase, PLP, and 1B15 mRNAs and CNPase activity in the sciatic nerve after crush, after permanent transection, and after crush-transection

Total RNA was extracted from sciatic nerve tissue pooled from 6 - 8 animals 12 and 21 days after crush-injury, 21 days after permanent transection, and 21 days after crush-transection (the sciatic nerve was crushed; 12 days after, a permanent transection was carried out distal to the site of crush; the segment was dissected 9 days later, distal to the site of transection). Serial dilutions (one in two) of the RNA were filtered through a slot-blot manifold onto nitrocellulose sheets. The blots were hybridized to a variety of ^{32}P-labelled cDNA probes, washed and autoradiographed as described in Materials and Methods. The nitrocellulose blots were prepared in triplicate and stripped between successive hybridizations. The intensity of each slot after autoradiography was measured by densitrometry. The units of peak area were divided by the amount of total RNA in the slot to give units per microgram total RNA. The amount obtained for the normal adult sciatic nerve was arbitrarily designated to be one for each message, and the results were calculated relative to the value obtained for the adult sciatic nerve. CNPase activity in tissue homogenates was expressed as μmol product formed/hour/mg protein. The value in adult sciatic nerve was designated to be one, and the results from other tissues were expressed relative to this value.

Table 3.11

Levels of PO, MBP, MAG, CNP, PLP and 1B15 mRNAs and CNPase activity in the sciatic nerve after crush, after transection, and after crush-transection

Steady-state levels of encoding mRNAs relative to adult control

	PO	MBP	MAG	CNP	PLP	1B15	CNPase activity
The distal segment of the sciatic nerve							
12 days post-crush	0.15 - 0.23(2)	0.12 - 0.17(2)	0.44 ± 0.06^{a}(3)	1.23 - 1.32(2)	0.823 ± 0.315^{c} (3)	0.59(1)	0.54(1)
21 days post-crush	0.83 ± 0.14^{b}(4)	1.05 ± 0.34^{d}(4)	1.13 ± 0.35^{c}(5)	1.45 - 1.61(2)	1.688 ± 0.421^{b} (3)	0.78(1)	0.80(1)
21 days post-transection	0.13(1)	0.03 -0.06(2)	n.d.*(2)	1.49 - 1.74(2)	0.55 - 0.68(2)	0.76(1)	0.33(1)
21 days post crush-transection	0.04(1)	0.03(2)	n.d.(2)	1.12 - 1.24(2)	0.34 - 1.40(2)	0.41(1)	0.28(1)
The proximal segment of the sciatic nerve							
21 days post-transection	0.36(1)	0.33(1)	0.89(1)	1.08(1)	0.95(1)	0.73(1)	-
21 days post crush-transection	0.33(1)	0.24(1)	0.26(1)	0.83(1)	0.57(1)	0.85(1)	-
Controls							
Adult sciatic nerve	1.00(4)	1.00(4)	1.00(5)	1.00(2)	1.00(2)	1.00(1)	1.00(1)
21-23 day old rat sciatic nerve	2.32 ± 0.14^{a} (3)	1.56 - 1.76(1)	1.18 - 1.66(2)	1.80 - 1.94(2)	0.94 - 1.14(2)	1.50(1)	1.15(1)

* n.d.: not detected, a: $0.0005<p\leq0.005$, b: $0.05<p\leq0.1$, c: $0.1<p\leq0.375$, d: $p>0.4$

Levels of CNPase activity were assayed in the distal segments of the crush-injured, the transected and the crush-transected sciatic nerve, and were found to be lower in each case than the adult control (Table 3.11). These results further substantiate differential regulation of CNPase gene expression in the presence and absence of Schwann cell-axonal contact.

In summary, the results of these investigations strongly point towards four distinct modes of regulation of expression of myelin genes by the axon. PO and MBP gene expression is up-regulated in the regenerating sciatic nerve only in the continuing presence of axon in the peripheral nerve. MAG mRNA levels in the peripheral nerve appear to be more stringently controlled by Schwann cell-axonal contact, while PLP mRNA levels remain relatively detached from axonal influences. The CNPase gene represents a fourth distinct class of myelin genes that are repressed in their expression by the axon. The novel crush-transected model has permitted the characterization and classification of axonal influences on myelin gene expression in the regenerating peripheral nerve.

4. DISCUSSION

4.1 Myelin gene expression in the crush-injured peripheral nerve is controlled by a hierarchy of regulatory mechanisms.

The crush-injured sciatic nerve provides an excellent model to study Schwann cell regulation of myelin gene expression during the process of demyelination and remyelination. As a preliminary step towards characterizing the crush model, the temporal course of expression of the major PNS myelin-specific genes was investigated, as described in Section 3.1. In order to investigate the possible transcriptional regulation of myelin gene expression, the quantity, quality and translational efficiency of PO- and MBP-coding messages were investigated as a function of time following crush-injury. In subsequent investigations, the expression of the gene encoding the intriguing cell adhesion molecule MAG was delineated in the distal segment of the crush-injured nerve over the first three week post-operation, and compared to the patterns previously obtained for the expression of the major PNS myelin genes. The results of these studies suggested that PO and MBP genes are co-regulated in the peripheral nerve, while the MAG gene is regulated in a different manner and may be induced prior to the major myelin genes during remyelination. A completely different pattern of regulation in the PNS was exemplified by the temporal course of CNPase gene expression over crush-injury. PO, MBP and MAG genes were down-regulated during demyelination and subsequently increased during remyelination, while CNPase mRNA levels were induced over levels found in the adult sciatic nerve by 4 days post-crush. CNPase mRNA levels were also increased above normal adult levels, in the distal segment of the sciatic nerve, 21 days post-transection, wherein the resident Schwann cell population is quiescent and incapable of myelin assembly. These observations strongly suggested that the CNPase gene is atypical and that its expression is induced as a result of mechanical injury to the peripheral nerve.

The molecular response of the sciatic nerve to injury is reflected in the rapid changes of both DNA and RNA content as soon as two hours after nerve injury [Oderfeld-Nowak and Niemeierko, 1969]. Alberghina and co-workers [1983] reported that the RNA content increased 400% at 14 days post-injury, but then decreased rapidly by 30 days and gradually over 60-120 days. Overall increases in total cellular RNA content and DNA content per wet weight tissue were also observed in the present studies over the three week time period under investigation (Tables 3.1 and 3.2). The yield of poly(A+) RNA was also increased at all stages, indicating increased metabolic activity of the cell populations in the distal segment in response to the injury. In addition to Schwann cell proliferation, proliferation of endothelial cells, pericytes, and fibroblasts also takes place, as well as an influx of macrophages [Smith and Benjamins, 1984]. The peak in macrophage proliferation during Wallerian degeneration has been reported to be as early as two days post-injury [Liu, 1974; Boyles et al., 1989; Clemence et al., 1989].

Regulation of myelin gene expression may occur at four main levels: at the level of transcription, post-transcriptional processing of the message, translation and post-translational modifications of the encoded polypeptide. Transcriptional control may be exerted through a variety of mechanisms affecting rates of transcription, transcript stability and processing of transcripts prior to translation. In the context of the complexity and dynamic nature of the crush-injured sciatic nerve, it was necessary to clearly distinguish between changes in steady-state levels of mRNAs that were directly involved with myelination and those which reflected a general increase in cell number in the tissue under investigation. With the proliferation of different cell types, gene products not directly involved with myelination or the maintenance of the myelin sheath were expected to increase and then reach a steady state level

The expression of the histone H3.3 variant gene was previously proposed to be independent of cell cycle [Brush et al., 1985] and differentiation [Brown et al., 1985]. This gene has been used as a control in other investigations [Brown et al., 1985; Vardimon

et al., 1986]. Using the chick histone H3.3 cDNA as a probe, levels of histone H3.3 mRNAs transcripts were found to be lower than that of the adult control nerve 1 day after crush injury (Figure 3.5). The invasion of non-glial cells into the distal segment of the crush-injured sciatic nerve might explain the drop in H3.3 mRNA levels, if the level of expression is lower in these cell types than in Schwann cells. Although H3.3 mRNA levels did display a pattern of expression quite distinct from that observed for levels of PO- and MBP-coding transcripts, an alteration in mRNA levels did not favour the use of H3.3 in subsequent investigations as a constitutive control. The cyclophilin cDNA, p1B15, was previously used as a control in experiments involving CNS gene expression in cell culture paradigms [Saneto et al., 1988]. This cDNA was used in the subsequent investigation of myelin gene expression in the crush-transected model. The p1B15 cDNA was more acceptable as a constitutive control as levels of p1B15 mRNAs did not change over PNS development to a great extent, or after mechanical injury. Lower levels of p1B15 mRNAs were, however, found in the RNA preparation from adult rat liver. This indicates that the degeneracy in levels of p1B15 mRNAs in all tissues may not be correct. Though slow increase to normal levels of H3.3 transcripts was observed over the period of investigation, myelin-specific transcripts were expected to reflect the demyelinating and remyelinating phases observed after crush-injury. The present investigation confirmed this trend for PO and MBP mRNAs. Northern and dot blot analysis of PO- and MBP-coding transcripts indicated that these messages dropped rapidly within 4-7 days of crush-injury (Figures 3.1-3.4, Table 3.3). The demyelinating phase was thus reflected in the decrease in levels of transcripts coding for the major PNS myelin proteins. A subsequent, rapid recovery in levels of these messages was observed between 10 and 14 days after crush-injury. The steady-state levels of PO and MBP message may thus be used as a measure of the functional activity of the Schwann cell population in this experimental model. It is generally assumed that the specific interaction of the distal segment of the crushed nerve with the regenerating axon from the proximal site results in enhanced synthesis of myelin

components [Edmonds-Smith et al., 1983; Politis et al., 1982]. Therefore, the period between 10 and 14 days after crush injury can be designated as the most rapid stage of regeneration in the injured peripheral nerve. Message levels approached those of the control nerve after 21 days. These results, in conjunction with previous findings from this laboratory [LeBlanc et al., 1987], indicate that the injured nerve appears to be well on the way to regeneration in the period 3 to 5 weeks after injury.

Northern and dot blot analyses also indicated that the expression of PO- and MBP-coding transcripts follows similar trends during the time course of crush-injury (Figures 3.1- 3.4, Table 3.3). It is not unreasonable to assume that the amounts of two myelin-specific proteins which are required for the formation and maintenance of compact PNS myelin would be controlled at some higher level than pool size within the cell. On the basis of these results and previous studies on the crushed and transected model [LeBlanc et al., 1987], it may be postulated that these genes are co-regulated.

To assess whether such regulation at the level of transcription applied to a minor but functionally important protein, the temporal course of expression of the MAG gene was followed in the crush-injured and transected model. The HNK-1 carbohydrate epitope present on MAG [McGarry et al., 1983] was demonstrated to possess adhesion properties [Riopelle et al., 1986] and to be involved in cell interactions [Kunemund et al., 1988]. Since MAG has been implicated in mediating cell-cell interactions in the CNS [Poltorak et al., 1987; Johnson et al., 1989], its role in the process of PNS remyelination may be quite significant.

Northern and slot blot analysis of MAG mRNAs in the distal segment of the sciatic nerve, showed that a sharp decrease in levels of MAG-coding message took place shortly after disruption of Schwann cell-axonal contact (Figure 3.10, Table 3.8). This was followed by a slow rise in the levels of MAG message within a week after crush injury indicating the start of the axonal sprouting into the distal segment and initiation of remyelination. MAG mRNAs were undetectable in the distal segments 35 days post-

transection. This finding is consistent with previous results (Figure 3.10) and indicates the MAG gene, like most myelin-specific genes, was down-regulated under experimental conditions where Schwann cell-axonal contact as well as subsequent myelin assembly is prevented [LeBlanc et al., 1987]. In quantitative terms, though, the MAG gene appeared to be more stringently down-regulated than the major PNS myelin genes. Where levels of PO and MBP mRNAs were low but detectable in the transected sciatic nerve, MAG mRNAs were undetectable. This observation is consistent with current thoughts on the role of MAG in cell-cell interactions and as a mediator of initiation of myelination in the CNS and PNS [Poltorak et al., 1987; Lai et al., 1987a,b; Attia et al., 1989]. Primary control during PNS regeneration appears to be effected through the steady-state levels of mRNAs encoding diverse myelin-specific and myelin-associated genes. Thus, transcripts encoding proteins which may be important for the process of initiation of remyelination are induced prior to those encoding structural proteins. In this context, the results of Dubois-Dalcq et al. [1986] also indicate that MAG is the first myelin protein detected in the processes of cultured oligodendrocytes, prior to the appearance of MBP and proteolipid protein. In a related model of peripheral nerve regeneration, Willison et al. [1988] have demonstrated that when freshly severed proximal axons penetrate a permanently transected distal stump, a spatial separation of the myelination process may be observed as a gradient down the distal segment. In this model, Willison and co-workers [1988] find higher relative levels of MAG than PO in the most proximal regenerating zone, while elevated levels of MAG are sustained further into the distal stump than PO. These observations are consistent with the results from the present investigation inasmuch as MAG mRNA is expressed earlier than PO mRNA during remyelination and that higher relative levels of MAG mRNA than PO mRNA are maintained between 4 and 10 days after crush-injury as seen in the schematic diagram in Figure 4.1.

The results obtained from Northern analysis do not exclude the possibility that regulation can also occur at the level of post-transcriptional processing. The experimental

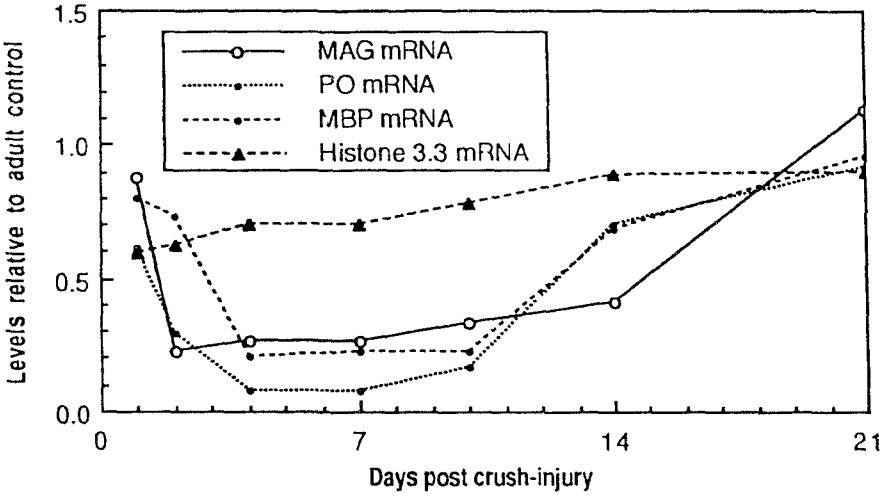


Figure 4.1 The temporal course of expression of PO, MBP, MAG and Histone H3.3 genes after crush-injury. Levels of PO, MBP, and MAG mRNA are taken from Table 3.2 and Table 3.4 in the Results section. In order to simplify this schematic representation of the temporal course of gene expression, error bars where applicable, were not shown.

conditions under which Northern blot analysis was performed do not permit the resolution of mRNAs which differ by less than 100 bases. For example, although the MBP primary transcript is known to be differentially spliced to yield at least six isoforms [deFerra et al., 1985; Newman et al., 1987a; Newman et al., 1987b], the transcripts migrate as a single broad band on Northern blots (Figure 3.2). It is quite likely that the relative proportion of the individual forms of MBP mRNAs changes during PNS remyelination, as has been shown to occur in the CNS during development [Carson et al., 1978; Barbarese et al., 1983]. It has also been shown recently that MAG transcripts coding for the two isoforms are formed by alternative splicing, resulting in a divergence in the amino acid sequence at the carboxyl-terminus of the two peptides [Lai et al., 1987a]. The two messages are developmentally regulated [Frail and Braun, 1985; Matthieu et al., 1986]. The MAG mRNA encoding the 72 kDa protein appears to be more abundant at earlier ages in the rat hindbrain, peaking at 17-19 days post-natal, while the MAG mRNA encoding the 67 kDa protein is at low levels at younger ages and steadily increases in level to day 50 [Lai et al., 1987a]. Although previous investigations [Frail et al., 1985] and the recent experiments of Tropak et al. [1988] indicate that only the smaller form of MAG (with a relative molecular weight of 67 kDa) is predominant in the PNS at all ages, it is possible that there is altered expression of MAG isoforms in the crushed or permanently transected nerve. The size of the transcripts detected in the present investigation is approximately 2.5 kb (Figures 3.10a, b, c). This size remains relatively unchanged from that of the normal control and was observed at several stages after crush-injury. The extra (3 kb) MAG transcript in the actively myelinating 21-23 day old rat brain is however, resolved under these conditions (Figure 3.10a, lane K) and has been observed previously by other investigators [Lenoir et al., 1986; Arquint et al., 1987; Salzer et al., 1987]. The origin and function of this mRNA species is unclear at present [Lai et al., 1987b]. The 3.0 kb transcript was not observed in the developing PNS in these studies or by other investigators, and also was not detected in the regenerating PNS in the present investigation.

It was also not possible to rule out the possibility of post-transciptional regulation of the myelin genes. Regulation of MBP mRNAs in the CNS at the level of translation by brain-specific factors has been demonstrated by Campagnoni and co-workers [1987]. It was quite possible that the steady-state levels of PO and MBP mRNAs observed at any particular stage of crush-injury might not directly reflect the actual amount of translated product. In vitro translation of poly(A)+ RNA obtained from distal segments of the sciatic nerve at various time points after crush-injury and immunoprecipitation of the translated PO product, were carried out to determine possible regulation of the PO mRNAs at the level of translation. The in vitro translation assay showed reasonable agreement with the results obtained by dot-blot analysis, indicating that increments in message levels were concomitant with increases in levels of in vitro translated PO polypeptides (Table 3.5). The efficiency of translation of poly(A)$^+$ RNA derived from the distal segments of the crush-injured nerve were not significatly different from the adult (Table 3.6). However, the in vitro translation assay indicated that the distal portion of crushed nerves contain much lower amounts of functional PO mRNA than those obtained from dot blot analysis. The reason for this discrepancy might be the greater susceptibility of the in vitro translation system to experimental manipulations which might damage mRNA structure and function. The lack of endogenous, sciatic nerve-specific translation factors might also result in poor translation of PO mRNA in the heterologous in vitro translation system. Recently, Verdi and co-workers [1989] have reported corticosteroid-mediated regulation of MBP and PLP synthesis in the CNS, at the level of initiation of translation. The results from the in vitro translation assay nevertheless demonstrated that the RNA preparations from the distal segment of the sciatic nerve were biologically active in a heterologous in vitro translation system.

To investigate possible regulation of myelin genes at the level of translation, solid-phase immunoassays of PO and MAG proteins were carried out over the period of crush-injury under investigation. The results from these assays indicated that PO protein levels

sharply decrease in the distal segment between 7 and 14 days after crush-injury, followed by an increase at 21 days (Figure 3.8). During the course of demyelination and remyelination in the crushed sciatic nerve, the degradation and biosynthesis of PO protein appears to be delayed (Figure 3.9). This contrasts with the trends observed for PO mRNA levels where the recovery is maximal between 10 and 14 days. These results might indicate regulation of PO gene expression at the translational or post-translational level. In this respect, Poduslo [1984] has shown that the lower level of newly synthesized PO protein in the permanently transected model is regulated post-translationally. Schwann cells in this neuropathy synthesize an immature type of PO protein with an incomplete oligosaccharide chain, which is routed to lysosomes shortly after biosynthesis [Brunden and Poduslo, 1987a]. The absence of appreciable phosphorylation of PO in this model as opposed to the phosphorylation of PO in the sciatic nerve 35 days post crush provides further evidence for regulation at the level of post-translational modification [Brunden and Poduslo, 1987a,b].

Solid-phase immunoassay of total protein extracts from distal segments of crushed nerves showed that the level of the MAG gene translation products follows the trends of MAG messages, although a delay of 7 days is once again apparent (Figure 3.11). Figlewicz and co-workers [1983] followed the incorporation of radioactive fucose into MAG as a function of time after crush-injury and reported a decrease in incorporation by 3 days, with a recovery in levels by 30 days post-crush. The discrepancy between the present results and those of Figlewicz and co-workers [1983] may be explained by the fact that solid-phase immunoassay detects the proteinaceous debris from the disrupted myelin sheath, whereas the precursor incorporation experiments are not affected by the remaining, undegraded MAG. However, both approaches point towards an apparent delay in recovery of steady-state levels of protein with respect to recovery of levels of transcripts.

In summary, delays in the reappearance of PO and MAG protein are observed in the distal segment of the crush-injured sciatic nerve during PNS remyelination, when

compared to the re-expression of the encoding mRNAs. These observations are presented in a schematic form in Figure 4.2. A similar temporal lag between levels of MBP mRNAs and protein was found to occur in this experimental model [LeBlanc and Poduslo, 1989]. These findings corroborate the results of Mitchell and co-workers [1990] who examined spatial relationships between reappearance of transcripts and axonal regrowth into the distal segment of the sciatic nerve, using in situ hybridization of teased nerve fibres. These investigators have reported that up-regulation of PO mRNA in fixed sections lags behind the leading axonal edge, while compact myelin is found still further from the axonal tip. Northern blot, slot blot analysis, and solid-phase immunoassay as performed in the present investigation provide a global but much more quantitative aspect to expression of transcripts and encoded protein, while in situ hybridization and immunohistochemistry describe spatial and morphological relationships. The results from both approaches point towards a lag between re-appearance of myelin transcripts and the detection of encoded proteins. Such a delay in translation of myelin transcripts reflects the temporal dissociation of induction of myelin genes through Schwann cell-axonal contact, and the formation of remyelinated fibres in the distal segment of the crush-injured nerve.

A more detailed examination of the expression of the MAG gene at the level of the translated product was carried out by chemical deglycosylation of homogenates of distal segments of the crush-injured sciatic nerve, fractionation through SDS-PAGE, and probing for MAG peptides using solid-phase immunoassay techniques. The facile and complete deglycosylation of glycoproteins with trifluoromethane sulfonic acid [Horvath et al., 1989], allowed the subsequent determination of the mobilities of the MAG peptides in the crush-injured nerve and comparison to MAG found in the adult sciatic nerve and in the developing sciatic nerve. Figure 3.12 shows that the the isoform of MAG expressed during PNS development and over crush-injury is of a higher relative molecular weight than that found in the adult sciatic nerve, by approximately 2 kDa. Although splicing of exon 12, leading to the two major isoforms of MAG in the CNS (p72MAG and p67MAG)

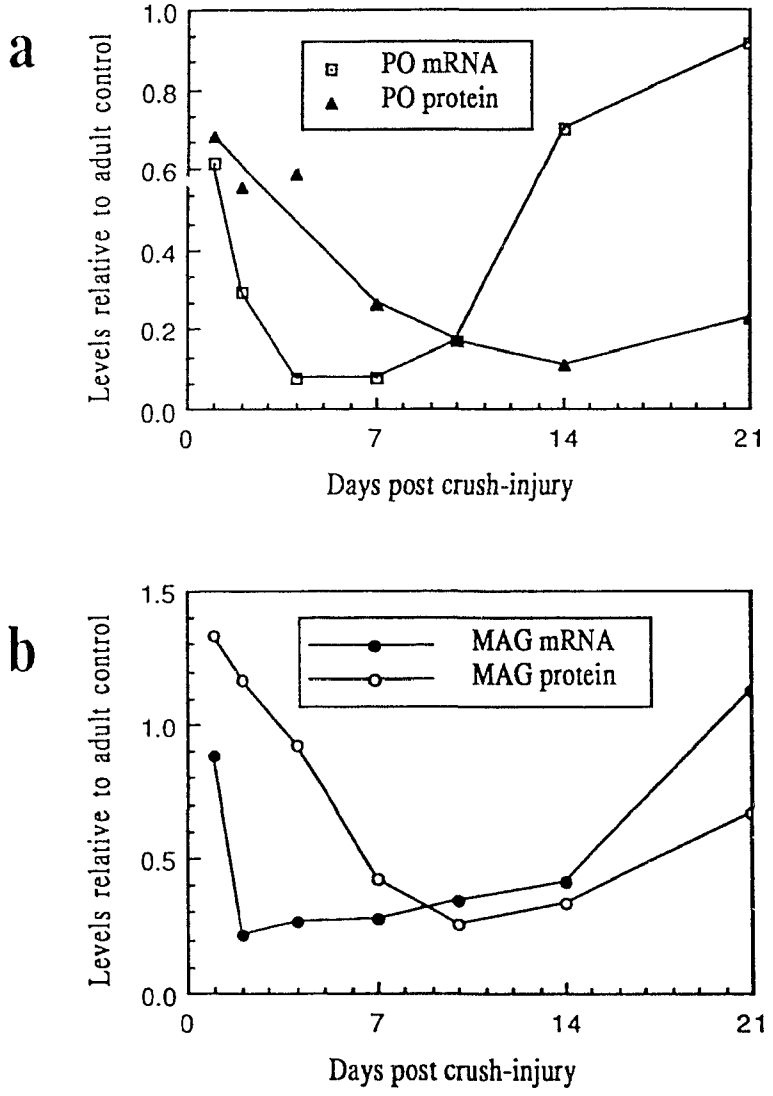


FIGURE 4.2 a. Levels of PO protein and mRNA in the distal segment of the sciatic nerve following crush-injury. The data were taken from Tables 3.3 and 3.7. Error bars where applicable, are omitted to simplify the representation. **b.** Levels of MAG protein and mRNA in the distal segment of the sciatic nerve following crush-injury. The data were taken from Tables 3.8 and densitometric quantitation of the Western blot shown in Figure 3.11. Error bars where applicable, are omitted to simplify the representation.h

may not be in effect in the PNS [Frail et al., 1985; Tropak et al., 1988] it is not possible to rule out alternative splicing events that may lead to changes in the length of MAG polypeptides. Sequences of human cDNAs encoding MAG suggest that p72MAG may be the predominant isoform in the human CNS at all stages of development and in the adult human. It is likely that splicing patterns differ between the human and the rat with respect to MAG gene expression [Sato et al., 1989]. Post-translational proteolytic cleavage of MAG may also lead to such differences in molecular weight. A closer examination of MAG mRNAs in the PNS or alternatively, isolation, purification and characterization of PNS MAG will be necessary to substantiate these preliminary results.

The pattern of expression of PO, MBP and MAG genes indicated that genes encoding myelin-specific proteins are dramatically down-regulated as a result of loss of axonal cc tact during the transient phase of demyelination after crush-injury. The ensuing growth of the axon from the proximal segment into the distal stump leads to rapid increases in steady-state levels of MAG mRNAs followed by increases in PO and MBP gene expression. In sharp contrast, the levels of CNPase mRNAs are increased over adult levels in the distal segment as a consequence of crush-injury [LeBlanc and Poduslo, 1989; Figure 3.13 and Table 3.9]. The physiological function of this myelin-specific enzyme in the PNS and CNS is as yet unknown [Sprinkle, 1989]. It is apparent however, from these results and from those reported by LeBlanc and Poduslo [1989] that the expression of this gene is induced as a result of mechanical injury. It is also intriguing in this context, that the effect of crush-injury on the activity of the enzyme in the rat sciatic nerve follows a pattern quite opposite to that described by steady-state levels of CNPase mRNAs. Levels of the CNPase activity dropped in the distal segment after crush-injury during transient demyelination, and returned to normal levels with the remyelination of the sciatic nerve. These data are presented in Table 3.4 and the patterns of CNPase expression after crush are depicted in Figure 4.3. Post-translational modifications of CNPase, such as phosphorylation [Sprinkle et al., 1990] and acylation [Agarwal et al., 1990],may be likely

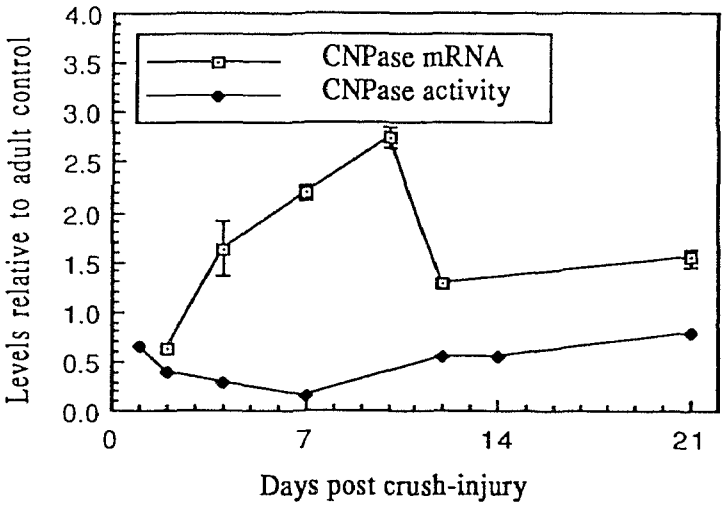


Figure 4.3 Levels of CNPase mRNA and activity at varous times post crush-injury. See Table 3.5 for numerical values of CNPase mRNAlevels and CNPase activity.

modes of modulation of CNPase activity in vivo. It may be noted that incongruous expression of the CNPase gene has been previously reported in the CNS. While PLP and MBP mRNAs are positively regulated by corticosteroids at the level of translation, translation of CNPase mRNAs is inhibited under similar conditions [Verdi et al., 1989]. The CNPase gene is also distinct from other myelin genes in that it is expressed in non-neural tissue [reviewed by Sprinkle, 1989]. A CNPase transcript alternatively spliced at the 5' region has been detected in lymphoid tissue, suggesting tissue-selective post-transcriptional regulation of this gene takes place in the rodent. These results taken in their entirety strongly implicate the atypical regulation of the CNPase gene when compared to other PNS and CNS myelin genes.

In conclusion, these investigations have established that a major point in the hierarchy of control of myelin gene expression exists at the level of transcription. It remains to be determined whether this control is effected through alterations in the rate of transcription, as a consequence of a specific axonal signal, or through changes in the rate of degradation of myelin transcripts. In addition, these studies have shown that transcripts encoding a cell adhesion molecule MAG are induced prior to those encoding structural proteins such as PO and MBP. A second point of control of myelin gene expression during remyelination has been identified at the level of translation, with delays in the reappearance of two myelin proteins during regeneration when compared to the re-expression of the encoding transcripts. CNPase gene expression in the crush-injured sciatic nerve has also been studied in some detail, and the present studies suggest that the expression of this myelin gene is atypical. Complex forms of regulation of this gene at the level of transcription and at a post-transcriptional level are inferred. The crush-injured sciatic nerve has thus provided an excellent model system to compare and contrast the behaviour of several myelin genes following disruption of Schwann cell-axonal contact and has permitted the identification of possible points of regulation in their expression.

4.2 The expression of a CNS myelin gene in the peripheral nerve is detached from axonal control.

It is quite remarkable that the myelin sheaths of the mammalian CNS and PNS, though functionally identical, differ in so many respects. Not only are CNS and PNS myelin derived from different glial cells, but they also differ in the protein composition. It is interesting but not unexpected, in this context, that a myelin gene may be differently regulated in the CNS and PNS.

Puckett and co-workers [1987] recently reported that PLP transcripts are expressed by Schwann cells. These investigators showed by Northern blot analysis that PLP transcripts were present in rat and rabbit sciatic nerve, as well as in human acoustic neuromas derived from Schwann cells. PLP protein was detectable on Western blot analysis in sciatic nerve homogenates, but not in purified PNS myelin. In support of these findings, Griffiths and co-workers [1989] have pinpointed a perinuclear cytoplasmic location of PLP transcripts in the sciatic nerve using in situ hybridization techniques, while Baron and co-workers [1989] have isolated and characterized PLP cDNAs derived from the peripheral nerve. These observations raise the possibility that PLP may also have a role distinct from maintaining the integrity of the myelin sheath.

A curious observation by Puckett and co-workers [1987] was the expression of PLP transcripts in acoustic neuromas, with the concomitant down-regulation of PO mRNA expression. The experiments described in Section 3.1 have demonstrated the tight regulation of PO, MBP, and MAG gene expression in the PNS by Schwann cell-axonal contact. In contrast, when PLP gene expression was investigated in the previously characterized models of peripheral nerve degeneration and regeneration, it was found that PLP transcription in the peripheral nerve remains relatively dissociated from axonal influences (see Section 3.2).

The elucidation of the genomic sequences of the PLP, PO and MBP loci may provide an insight into how these genes are regulated at the molecular level [Sutcliffe, 1988]. There are several upstream shared sequences between PO and MBP genes and PLP and MBP genes, 5' to the coding regions. These sequences may constitute response or enhancer elements to which putative signal transducers may bind. There are preliminary reports of the induction of the PO gene through treatment with forskolin, which increases intracellular cAMP via a direct action on adenyl cyclase. These observations suggest the existence of a signal transduction pathway between the axon and the Schwann cell involving adenyl cyclase and interacting G-proteins [Lemke, et al.,1988; LeBlanc and Poduslo, 1990].

In light of these data it is intriguing that while PO and MAG genes are regulated during neural development in the PNS, levels of PLP mRNA remain unchanged between the rapidly myelinating and adult sciatic nerve [Section 3.2]. Moreover, the expression of the PLP gene is relatively impervious to the presence or absence of Schwann cell-axonal contact, while PO, MBP and MAG genes remain under tight axonal control. It is likely that axonal control takes place at the level of transcription of these myelin genes. The response elements that mediate axonal influences on PO and MBP transcription in the Schwann cell are probably absent in the regulatory region of the PLP gene. PO and MBP genes show sequence similarity in 19 out of 25 nucleotides downstream of the transcription initiation sites, between nucleotides +16 and +40 [Lemke, et al., 1988]. Conversely, sequences which are important for the 'programmed' expression of PLP and MBP in oligodendrocytes must necessarily be absent in the regulatory region of the PO gene. In this regard PLP and MBP loci share a nine nucleotide purine nucleotide sequence in the region between -70 and -40, upstream of the transcription initiation sites [Ikenaka et al., 1988]. At this point, it is difficult to predict whether such putative elements are positive or negative regulators of transcription. An analysis of PLP, PO and MBP gene expression

in oligodendrocyte chimeras may help to pinpoint such putative inducer or suppressor response elements in these myelin-specific genes.

The actual function of PLP in the peripheral nervous system remains a mystery. Although PLP in the CNS is thought to be primarily a structural component of myelin, there have been reports suggesting alternative functions of the proteolipid protein. Ting-Beall and co-workers [1979] reported formation of voltage-dependent channels upon incorportaion of the myelin proteolipid apoprotein into planar lipid bilayers. In subsequent investigations, Lin and Lees [1982] demonstrated the occurrence of proton transport in reconstituted vesicles containing the apoprotein, and the inhibition of such ionophoric activity with a specific inhibitor of proton translocation, dicyclohexylcarbodiimide. They identified specific binding sites for the inhibitor on the proteolipid apoprotein [Lin and Lees, 1984]. These results strongly suggest that PLP may serve an additional role in ion transport in the nervous system. In this context, ion channels with diverse characteristics have been detected in glial cells of the CNS and PNS. It is possible that these channels play an important physiological role in the proper functioning of ensheathed axons [Gray and Ritchie, 1985, Barres et al., 1990].

The study of myelination in certain dysmyelinating mutants has provided ample evidence to suggest that PLP is essential for the maintenance of normal CNS myelin [reviewed by Nave and Milner, 1989]. Its absence in the *jimpy* mutant, for example, severely compromises the structure and function of the myelin sheath [Duncan et al., 1989]. On the other hand, primary defects in the PLP locus lead to multiple phenotypic effects, not all of which may be explained by the accepted structural role of PLP in CNS myelin [reviewed by Nave and Milner, 1989]. For instance, the oligodendrocytes in the *jimpy* mutant die prematurely, prior to the death of the animal. There are two possible explanations for this. Cell death may be a direct result of the abnormal PLP in *jimpy* or it may be an indirect result of the lack of PLP. These observations in the *jimpy* mutant thus suggest an additional role for PLP in glial cells.

It is also unclear why PLP is incorporated into CNS but not into PNS myelin. Perhaps the autoacylation of PLP observed in the CNS, and thought to be important in the proper incorporation of PLP into the myelin sheath, is somehow inhibited in the peripheral nerve [Bizzozero et al., 1987]. In this regard, Bizzozero and co-workers [1987] have suggested that acyl groups may participate in myelin compaction by changing the conformation of PLP or altering the interaction of PLP with other membrane components. It is likely that an altered acylation of PLP in the peripheral nerve would affect the assembly and incorporation of this lipophilic protein into compact myelin.

These observations suggest PLP may be a component more essential to the nervous system than was previously thought. Perhaps careful localization of PLP transcripts and protein in Schwann cells will provide a better understanding of the function of PLP in the central and peripheral nervous system.

4.3 Induction of myelin genes during peripheral nerve remyelination requires a continuous signal from the ingrowing axon.

The present investigations strongly suggest that up-regulation of myelin gene expression during peripheral nerve regeneration is dependent on continual Schwann cell-axonal contact. This was demonstrated in a crush-transected experimental model of peripheral nerve regeneration. The adult sciatic nerve was first crushed, and the distal segment allowed to regenerate for 12 days. The sciatic nerve was then transected distal to the site of crush to disrupt the Schwann cell-axonal contacts that had reformed. In the distal segment of the crush-transected nerve after 9 days post-transection, levels of transcripts coding for five myelin proteins were determined and were compared with corresponding levels in the distal segments of sciatic nerves at 21 days post-crush and 21 days post-transection using Northern blot and slot-blot analysis. If myelin gene expression during the rapid phase of remyelination was up-regulated by a single inductive signal from

the ingrowing axon, the effect of a subsequent permanent transection on the steady-state levels of myelin transcripts would not be drastic. In such a circumstance, the levels of message for the major myelin proteins in the distal segment of the doubly operated nerve would resemble those in the distal segment of the sciatic nerve, 21 days post-crush. If, on the other hand, a continuous signal were required from the axon to up-regulate myelin gene expression, the crush-transected nerve would resemble a transected nerve with respect to the steady-state levels of myelin coding mRNAs.

PO and MBP mRNAs in the distal segment of the crush-transected nerve decreased to basal levels found in the distal stump of the transected nerve, indicating a rapid loss in the ability of the Schwann cell population to express transcripts encoding myelin components at substantial levels. The molecular basis of these effects are as yet unknown, although there are indications that axonal induction of myelin genes is mediated through increases in levels of intracellular cAMP [Bansal and Pfeiffer, 1987; Lemke and Chao, 1988; Kreider et al., 1988; Behrman et al., 1989; LeBlanc et al., 1990].

Several lines of evidence indicate that Schwann cells in the developing peripheral nerve are quite distinct from those found in the adult sciatic nerve. Schwann cells in the developing sciatic nerve are relatively immature, express high levels of mRNAs encoding myelin components, and assemble compact myelin [reviewed by Benjamins and Smith, 1984; Mezei et al., 1987] . On the other hand, the Schwann cells in the adult peripheral nerve that maintain the myelin sheath appear to be more differentiated, and metabolically less active. Myelin gene expression in either Schwann cell population is nevertheless dependent on contact with the axon [LeBlanc et al., 1987; Trapp et al., 1988; Section 3.1].

The results in Section 3.3 show that the effect of transection on a regenerating nerve is similar to that on an adult peripheral nerve. Distal segments of crush-transected nerves displayed steady-state levels of mRNAs encoding the major myelin genes, similar to those of the distal segments of the transected nerve (see Table 3.11). The values obtained for PO and MBP mRNA levels after transection are comparable to those obtained by Trapp et al.

[1988] in transection studies of the neonatal rat sciatic nerve. It is therefore plausible that the Schwann cells in the rapid phase of remyelination are committed to respond to axonal contact (or the loss of axonal contact) in the same way as the Schwann cells of the neonatal nerve. Permanent transection therefore has a similar effect on the adult, the developing and the regenerating nerve. However major differences in the response of these Schwann cell populations may be manifested not at the level of gene expression, but through various post-transcriptional, translational and post-translational mechanisms [Poduslo and Windebank, 1985].

The results of these investigations also suggest that the major myelin genes may be classified in four groups according to their response to loss of Schwann cell-axonal contact. PO and MBP gene expression is down-regulated to basal levels after transection of the adult or the regenerating peripheral nerve. These findings substantiate previous proposals that these genes encoding structural proteins of peripheral myelin may be co-regulated [LeBlanc et al., 1987; Section 3.1]. The MAG gene appears to fall in a separate class, as MAG mRNA levels decrease to undetectable levels after axonal disruption [LeBlanc et al., 1989]. Substantial evidence suggests that the myelin-associated glycoprotein is important in the initial phase of myelination [Owens and Bunge, 1989] and that it mediates cell-cell interactions [Johnson et al., 1989; Attia et al., 1989]. The present studies imply that axonal control of MAG gene expression is much more stringent than axonal control of PO and MBP gene expression. Studies of the 5' promoter region of the MAG gene may identify critical sequence motifs that are involved in such rigorous control by the axon.

The incongruous behaviour of two other myelin genes has also been examined in this novel experimental paradigm. CNPase mRNAs appear to be up-regulated by nerve injury, although the levels of CNPase activity actually decreased in the distal segment of the sciatic nerve after crush, after transection, and after crush-transection. Post-transcriptional regulation of the CNPase gene may be postulated to cause such diverse

patterns of encoding mRNA levels and the activity of the ultimate gene product. As discussed previously [Section 4.1], anomalous expression of the CNPase gene has been previously reported [Verdi et al., 1989; Bernier et al., 1987; reviewed by Sprinkle, 1989].

The PLP gene falls in a class of its own, inasmuch as it is relatively dissociated from axonal control (see Section 4.2). The levels of PLP mRNAs decrease slightly in the distal segment of the sciatic nerve following transection and crush-transection, but these effects are not as drastic as those observed for PO and MBP mRNAs. The function of this important CNS myelin protein in the peripheral nerve remains to be determined [Nave et al., 1989].

In conclusion, these studies have shown that myelin genes are up-regulated in the regenerating sciatic nerve only under the continuing presence of Schwann cell axonal contact. It was possible, using this novel experimental model, to further characterize and classify the influence of the axon on myelin gene expression in the regenerating peripheral nerve. An investigation of the molecular basis of these observations will undoubtedly enhance current ideas about the process of nerve regeneration, and the consequences of cell-cell interactions in the peripheral nervous system.

4.4 Axonal modulation of Schwann cell gene expression.

Schwann cells possess a remarkable capacity to alter their metabolism and cellular function as a result of their interactions with the extracellular matrix and the neuronal cell body [Aguayo et al., 1976; Weinberg and Spencer, 1976; Mirsky et al., 1980; Politis et al., 1982; Bunge et al., 1989a,b]. Axonal contact can lead to diverse changes in Schwann cell phenotype, including proliferation, differentiation and ultimately, elaboration and modification of the Schwann cell plasma membrane to form compact myelin.

Several experimental paradigms have been employed recently to investigate the pleiotropic effects of Schwann cell-axonal contact, and these effects are just beginning to be understood at a molecular level. Recent observations from several laboratories have

implicated increases in intracellular cAMP levels in the induction of myelin-specific genes [Bansal and Pfeiffer, 1987; Lemke and Chao, 1988; Kreider et al., 1988; Behrman et al., 1988; LeBlanc et al., 1990]. It is difficult, however, to propose a general model of axonal modulation of Schwann cell expression mediated through increases in levels of intracellular cAMP and the consequent change in gene expression. One is confronted with the fact that cAMP is an ubiquitous regulatory molecule, that is capable of exerting diverse effects on eukaryotic metabolism and that there are multiple and diverse effects of axonal contact on Schwann cell expression. It is essential, in a careful examination of axonal control of the Schwann cell phenotype, to discriminate those effects of Schwann cell-axonal contact that occur through elevations in intracellular cAMP levels, and those effects that might be mediated through other mechanisms.

Axonal contact triggers a marked proliferation of Schwann cells [DeVries et al., 1982; Cassel et al., 1982; Sobue et al., 1983; Yoshino et al., 1984; Sobue and Pleasure, 1985, DeCoster and DeVries, 1989], and there are indications that this occurs without elevations in levels of cAMP within the Schwann cell [Meador-Woodruff, 1984]. Schwann cell proliferation also occurs after nerve transection and after crush-injury [Oderfeld-Nowak and Niemierko, 1969]. Macrophages are also involved in this proliferative process, as myelin debris has been found to be processed by macrophages and presented as a mitogen to Schwann cells after peripheral nerve injury [Baichwal et al., 1988]. However, both myelin-forming as well as non-myelin forming Schwann cells proliferate with the resumption of Schwann cell-axonal contact [Jessen et al., 1985]. These observations suggest that axon-mediated Schwann cell proliferation is a general effect that is quite distinct from Schwann cell differentiation and expression of the myelin-forming phenotype.

Schwann cell-axonal contact causes differentiation of the glial cell and induces expression of galactocerebroside (GC) [Ranscht et al., 1982, Jessen et al., 1987]. Studies of the metabolism of GC indicate that these effects might be mediated through activation of

Schwann cell adenyl cyclase and elevation in cAMP levels. Treatment with agents that elevate intracellular cAMP stimulated GC biosynthesis and its expression on the cell surface [Sobue and Pleasure, 1984; Sobue et al., 1986]. In vivo, both myelin-forming Schwann cells in the sciatic nerve and non-myelinating Schwann cells in the cervical sympathetic trunk, respond to the ingrowing axon during development and after crush-injury by expressing GC, thus indicating that GC expression is also independent of the ability of the Schwann cell to form the myelin sheath [Jessen et al., 1985; Jessen et al., 1987].

Neurotrophic factors such as NGF play a critical role in axonal regeneration and in the reestablishment of Schwann cell-axonal contacts. NGF has been shown to be synthesised by Schwann cells and levels of NGF mRNA and protein are increased during development [Buck et al., 1987, Ernfors et al., 1988], on nerve transection or after crush-injury [Heumann et al., 1987] . In an analogous fashion, NGF receptor expression in Schwann cells is induced after transection or nerve crush [Heumann et al., 1987; Taniuchi et al., 1986, 1988]. Although the NGF receptor expressed on Schwann cells is of the low affinity form [Yasuda et al., 1987; DiStefano and Johnson, 1988; Green and Greene, 1986], it has been suggested that these receptors serve to concentrate NGF on the SC surface after nerve injury, prior to transfer to high affinity NGF receptors on regenerating axons [Taniuchi et al., 1986; DiStefano and Johnson, 1988]. Levels of NGF and NGF receptor mRNAs in Schwann cells decrease with axonal ingrowth into the distal segment of the crush-injured peripheral nerve [Heumann et al., 1987; Taniuchi et al., 1986, 1988]. Mokuno and co-workers [1989] demonstrated that treatment of cultured Schwann cells with diffusible analogues of cAMP or with forskolin, a potent mobilizer of intracellular cAMP, results in diminution of NGF receptor mRNA levels. These observations conflict with recent findings of Lemke and Chao [1988] that NGF receptor mRNA levels were not down-regulated by forskolin treatment. It is possible that these conflicting results arise

from differing culture conditions and/or concentrations of forskolin used for induction of gene expression.

There are indications that axonal induction of myelin genes occurs through increases in levels of intracellular cAMP [Bansal and Pfeiffer, 1987; Lemke and Chao, 1988; Kreider et al., 1988; Behrman et al., 1989; LeBlanc et al., 1990]. Treatment with forskolin increased levels of PO and MBP mRNAs in primary Schwann cell cultures [Lemke and Chao, 1988; Behrman et al., 1989]. These studies suggest that PNS myelin gene expression is primarily controlled at the level of transcription through a signal transduction pathway involving cAMP. LeBlanc and co-workers [1990] have also reported that increases in levels of PO and MBP gene expression occurred after treatment of short-term nerve explant cultures with forskolin. This increase was observed only in the distal segment of the crush-injured sciatic nerve and not in the quiescent Schwann cell population of the distal segment of the transected nerve [LeBlanc et al., 1990]. Their results reflect the requirement of the axon (or axolemma) in the induction of myelin genes in an experimental paradigm which may be more representative of the physiological situtation than dissociated Schwann cell cultures.

The present investigations with the crush-transected model of peripheral nerve injury have highlighted two aspects of axonal modulation of Schwann cell gene expression. It was demonstrated that, in vivo, induction of myelin genes during peripheral nerve remyelination requires a continual signal from the ingrowing axon. Myelin genes were also classified by their response to axonal contact. Within the class of genes down-regulated to basal levels by loss of axonal contact are PO and MBP genes [present studies, LeBlanc et al., 1987; Trapp et al., 1988] and the genes encoding S-100 [Freeman et al., 1989] and GFAP [Mokuno et al., 1989; Freeman et al., 1989]. The MAG gene appears to form a class of its own in that it is completely down-regulated by loss of Schwann cell-axonal contact. PLP mRNA levels are relatively unaffected by alterations in neuronal-glial cell interactions and thus the PLP gene typifies a distinct class of unresponsive genes.

Expression of the CNPase gene [present studies, LeBlanc and Poduslo, 1989] and the NGF and NGF receptor genes [Heumann et al., 1987, Mokuno et al., 1989] is induced by nerve injury and these genes represent a separate class of genes responsive to Schwann cell-axonal contact.

Mechanisms of cAMP-mediated alterations in eukaryotic gene transcription are just beginning to be elucidated [for review see Roesler et al., 1988]. Two cAMP regulatory sequences in promoters of responsive genes and the corresponding DNA binding proteins have been identified. Although cAMP-responsive genes may be classified as rapidly responding (where increases in mRNA levels take place within minutes) or slowly responding (increases in mRNA levels occur over several hours), much of the work to date has involved characterization of rapidly responding genes. cAMP regulatory elements (CREs) have the properties of enhancers in that they regulate transcription from promoters in a manner independent of position or orientation. One of the two identified CREs, AP-2, is also a basal transcription enhancer and is inducible by phorbol esters and cAMP. Current models of cAMP mediated induction of eukaryotic genes thus involve ligand-(or hormone-) mediated activation of a plasma membrane-bound adenyl cyclase, with increases in concentrations of intracellular cAMP over several orders of magnitude. This in turn activates cAMP-dependent protein kinase within the cell, and results in phophorylation of regulatory proteins. Such regulatory proteins may affect gene transcription through increases in binding affinity to CREs located near responsive genes. Alternatively, Roesler and co-workers [1988] have postulated that phosphorylation may result in increased affinity of a cAMP-DNA binding protein for the transcription initiation complex and a consequent increase in rate of transcription.

Although model building with very limited and, at times, conflicting information may be compared to building a castle of cards, such an exercise often serves to highlight the present state of knowledge and channel thoughts to future lines of investigation. In light of the current models of cAMP-mediated induction of eukaryotic genes, it is quite

likely that one of the ways in which the axon mediates up- or down-regulation of myelin gene expression may involve similar mechanisms of induction at the level of transcription. Such a model is presented schematically in Figure 4.4 An unidentified signal from the axon, whose action can be mimicked by forskolin, might be proposed to interact with a receptor on the surface of the Schwann cell and result in activation of adenyl cyclase. In light of the observations of LeBlanc et al. [1990] regarding the requirement of the axon in forskolin-mediated induction, it is possible that the axolemma is somehow involved in presentation of the hormone or ligand to the Schwann cell, and that physical contact between the axon and the Schwann cell is necessary for the primary signalling event .

The axonal diameter plays a critical role in the commitment by the Schwann cell to myelinate. Schwann cells will thus myelinate axons with diameters greater than 0.7 μm [Duncan, 1934; Matthews, 1968;Windebank et al., 1985], though the molecular basis of this criterion remains unknown. It is interesting to note in this regard, that axonal diameter is also affected by the size of the innervated peripheral target [Voyvodic,1989]. This implies that myelination may ultimately be controlled through retrograde signals from the peripheral target, and that the structure of the PNS is intimately modulated by growth of target tissues. In peripheral nerve regeneration, the segment distal to the site of crush may be considered by the axon as a 'substitute' target, and may thus provide signals to the ingrowing axon that render it of the 'proper' calibre to allow remyelination to occur.

The activation of adenyl cyclase in the Schwann cell in this model, thus results in large and rapid increases in intracellular cAMP, the activation of cAMP-dependent kinase, and the subsequent phosphorylation of regulatory proteins. The simplest case of control of multiple myelin genes may involve a common regulatory protein for the induction of transcription. If the transcriptional activity is largely determined by the affinity of the activated (phosphorylated) form of regulatory protein for the CRE of a particular gene, a lower Kd for the PO and MBP CREs than for the MAG CRE might explain the greater stringency exerted by Schwann cell axonal contact on MAG gene expression. Thus, at

Figure 4.4 A model of cAMP mediated induction of myelin gene expression.

A model for the induction of myelin gene expression as modulated by Schwann cell-axonal contact is presented. An unidentified signal from the axon, whose action can be mimicked by forskolin, might be proposed to interact with a receptor on the surface of the Schwann cell and result in activation of adenyl cyclase. It is possible that the axolemma is involved in the presentation of the hormone or ligand to the Schwann cell, and that physical contact between the axon and the Schwann cell is necessary for the primary signalling event. The increase in intracellular cAMP results in a cascade of phosphorylation and may eventually result in the activation of a regulatory protein. The transcriptional activity of the various myelin genes may be up- or down-regulated in the presence of the activated regulatory protein, and a putative activated transcription initiation complex.

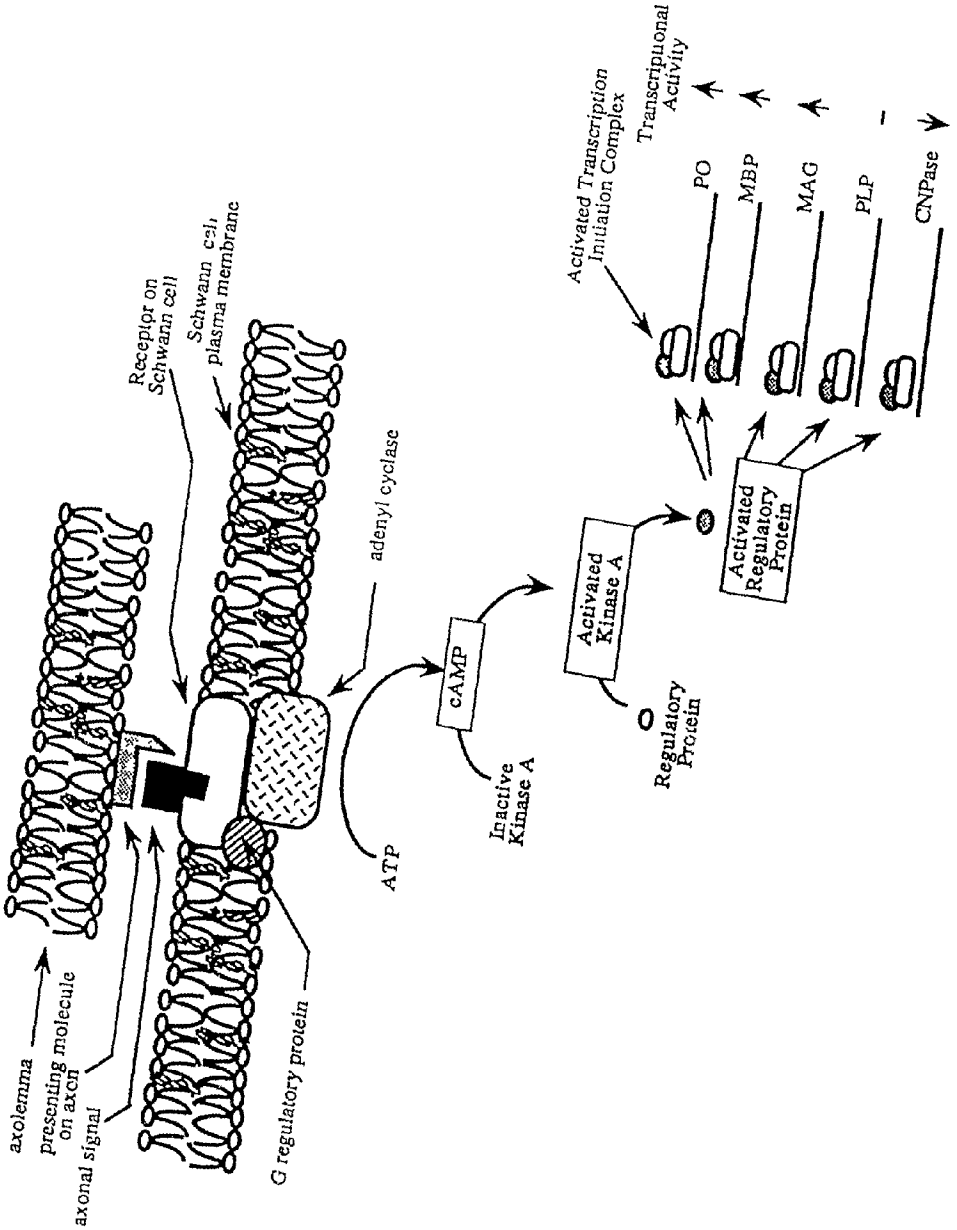

axolemma
presenting molecule on axon
axonal signal
G regulatory protein
ATP
adenyl cyclase
Receptor on Schwann cell
Schwann cell plasma membrane
cAMP
Inactive Kinase A
Activated Kinase A
Regulatory Protein
Activated Regulatory Protein
Activated Transcription Initiation Complex
Transcriptional Activity
PO
MBP
MAG
PLP
CNPase

Figure 4.4

lower concentrations of the activated (phosphorylated) form of the regulatory protein, as might be found in the absence of axonal contact, the activated regulatory protein would preferentially bind to PO and MBP CREs and induce PO and MBP gene transcription over the MAG gene. In an analogous fashion, the activated (phosphorylated) form of the regulatory protein might inhibit CNPase gene expression (or conversely, the unphosphorylated form of the protein might induce CNPase gene transcription), and this might explain the atypical behaviour of this class of myelin genes. In the PNS, the PLP gene may be unresponsive to regulatory effects of this putative protein, and alternate CNS-specific mechanisms of induction of PLP gene expression may be expected to take place. This model is simplistic in its outlook, but does focus on one of the major aspects of the present investigations, namely that a continuous and possibly common signal from the axon is necessary to induce expression of several myelin genes.

A model of cAMP-mediated regulation of Schwann cell gene expression might be further incorporated into a broader summary of the hierarchy of control mechanisms that have been demonstrated in the peripheral nerve as a result of the present studies and from the work of other investigators [LeBlanc et al., 1987; Trapp et al., 1988; LeBlanc and Poduslo, 1989]. As shown schematically in Figure 4.5, the growth of the axon from the proximal segment of the crush-injured sciatic nerve into the distal segment, largely affects the expression of myelin genes at the level of transcription. The primary transcripts may further be modified post-transcriptionally through mechanisms of alternative splicing, ultimately yielding gene products that may differ at the level of the peptide sequence. Translational control has been shown in the crush-injured sciatic nerve, possibly through cell-specific control of initiation of translation of the available pools of mRNAs. Post-translational processing of translated peptides and underlying axonal control of this process has been demonstrated in detail in the transected model, both through altered glycosylation of PO and through decreases in phosphorylation and sulphation of PO.

Figure 4.5 Axonal modulation of Schwann cell gene expression.

This schematic figure represents the current information available on the modulation of Schwann cell gene expression by contact with the axon. Regulation may take place at the level of transcription as described in detail in figure 4.4, at the level of post-transcription, at the level of translation and at post-translational levels. Please see Section 4.4 for detailed description of the regulatory phenomena and appropriate references.

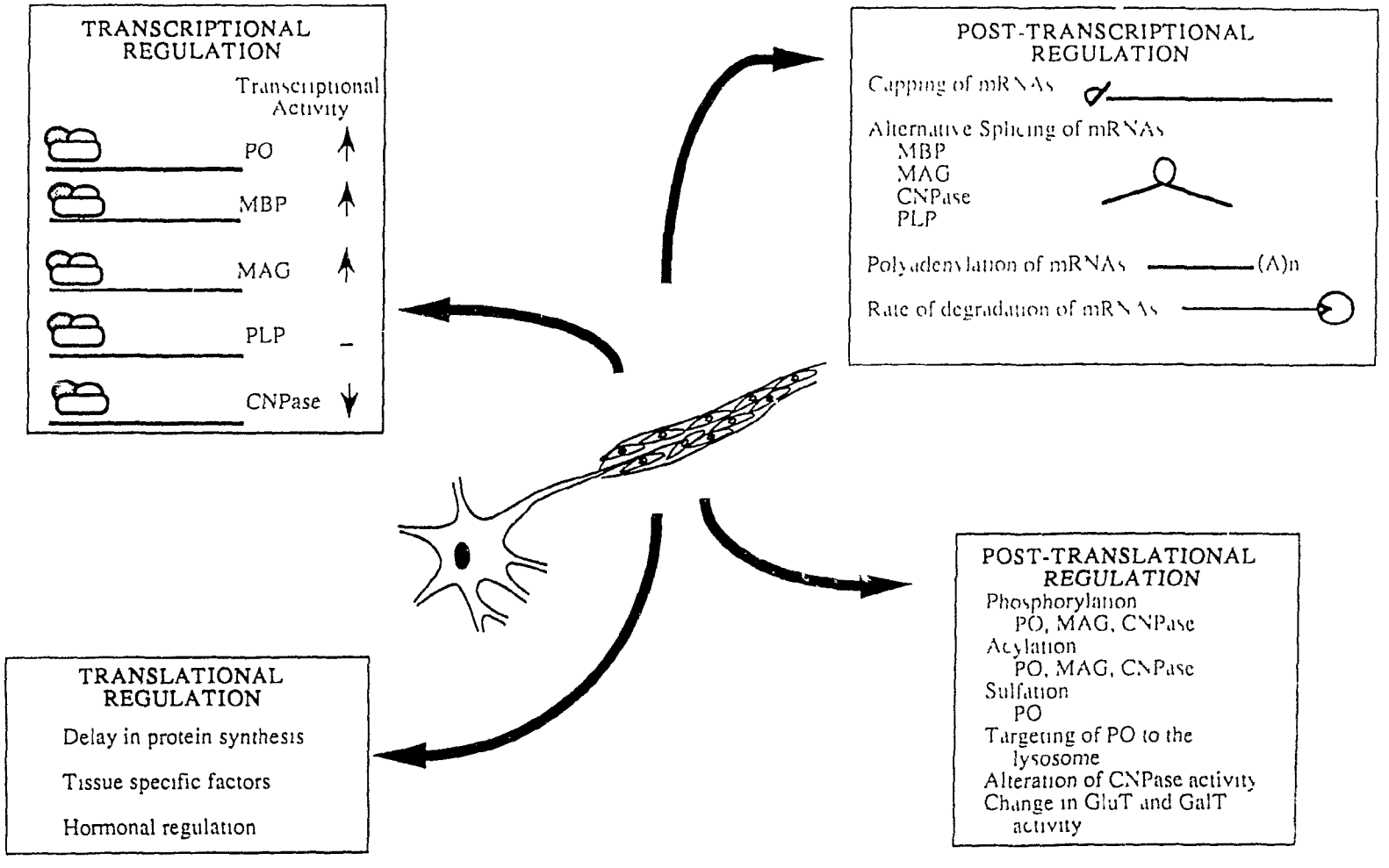

TRANSCRIPTIONAL REGULATION
Transcriptional Activity
PO
MBP
MAG
PLP
CNPase
POST-TRANSCRIPTIONAL REGULATION
Capping of mRNAs
Alternative Splicing of mRNAs
MBP
MAG
CNPase
PLP
Polyadenylation of mRNAs
(A)n
Rate of degradation of mRNAs
POST-TRANSLATIONAL REGULATION
Phosphorylation
PO, MAG, CNPase
Acylation
PO, MAG, CNPase
Sulfation
PO
Targeting of PO to the lysosome
Alteration of CNPase activity
Change in GluT and GalT activity
TRANSLATIONAL REGULATION
Delay in protein synthesis
Tissue specific factors
Hormonal regulation

FIGURE 4.5

In conclusion, the present studies have centered on the critical interaction of the Schwann cell and the neuronal cell body in forming and maintaining the myelin sheath. Future studies will undoubtedly clarify the molecular basis of this phenomenon, and may provide sufficient information to formulate hypotheses of the molecular basis of cellular interactions, and the evolution of hierarchical control of gene expression in the peripheral nervous system.

4.5 Concluding remarks.

The studies presented in this thesis exemplify the complex nature of the cellular interactions in the PNS. The delineation of PO and MBP gene expression during regeneration of the crush-injured sciatic nerve laid the foundation for further investigations of the expression of other myelin genes. The presence of regulation at the levels of transcription, post-transcription, translation, and post-translation, is consistent with the concept that myelination during neural development and remyelination are cellular processes under hierarchical control.

The molecular mechanism(s) of axonal modulation of Schwann cell gene expression demonstrated in the present investigations on the crush-transected model should be elucidated in the near future. These mechanisms will shed new light on the fundamental nature of the problem - the interaction of two distinct cell types and the resultant synthesis of a biological membrane. Such studies will also have practical implications, as factors that induce remyelination may eventually be characterized, isolated and employed to reverse the effects of demyelinating diseases.

There are a number of avenues that may be explored to further characterize regulation of myelin gene expression. Steady-state levels of mRNAs as employed in the present studies do not provide complete evidence to validate the hypothesis that primary regulation of gene expression occurs at the level of transcription. Alterations in rates of

transcription, mRNA stability, and rates of degradation of specific mRNAs are embodied in steady-state analyses. The development of a reliable in vitro transcription assay using the sciatic nerve would allow one to make more definite conclusions about precise regulatory mechanisms. A nuclear run-off transcription assay, wherein nuclei containing incomplete mRNAs are pulse labelled, and the resulting radioactive RNAs analysed for rate of transcription of specific message by filter hybridization has been described in liver [Spindler et al., 1982] and used in cell culture models [Obara et al., 1988]. Development of this assay in the sciatic nerve was one of the primary objectives of this thesis. However, the preparation of viable nuclei from the sciatic nerve proved to be difficult. The sciatic nerve is rich in connective tissue and preparations of nuclear extracts from the tissue were not transcriptionally active, possibly due to the damage caused to nuclei during tissue homogenization. Desheathing the endoneurium from the sciatic nerves was also attempted as a means to reduce connective tissue content, but was too time-consuming a procedure to obtain active nuclei. Such technical difficulties necessitated that this project be abandoned and more productive avenues of research be sought. It is possible that a similar run-off assay may be carried out in short-term organ cultures by pulse-labelling the dissected sciatic nerve with radioactive RNA precursors. However, incorporation of radiolabel in a short incubation period, may be too low to detect transcription of specific messages. It is possible that this technique would prove to be an alternate way to address the question of alterations in rates of transcription of myelin genes.

Another avenue that may provide more information on events taking place at the molecular level of remyelination is the establishment of cDNA libraries from the rapidly regenerating sciatic nerve. The distal segment of the sciatic nerve undergoes a marked up-regulation in the expression of PO and MBP mRNAs in the period of 10 to 14 days post-injury. In contrast, the levels of mRNAs for PO and MBP are basal in the distal segment of the permanently transected sciatic nerve. Since both cell populations are involved in growth and repair, factors involved in such cellular processes would be present in both

RNA populations. The difference between these two RNA populations would therefore be a set of mRNAs that are induced during remyelination and regeneration. This may form the basis of a subtractive differential screening approach to isolate, identify and characterize transiently expressed mRNAs in the regenerating sciatic nerve.

The discovery that the CNS myelin protein, PLP, is expressed in the peripheral nerve and that its expression is dissociated from axonal influences, has been the most intriguing finding of these studies. It is necessary to determine the subcellular localization of PLP in the Schwann cell in an effort to understand its mysterious presence and propose roles for PLP in the PNS. It is also important to elucidate whether the isoform of PLP, the DM-20, is also expressed in the PNS. Efforts are presently underway in this laboratory to resolve these issues.

One may also address the presence of the PLP in the PNS in an evolutionary context. It is possible that PLP arose in the PNS during evolution, subsequently adopted a structural role in the CNS myelin, and remained in the mammalian peripheral nerve, not as a myelin protein but as an evolutionary artifact. It is equally likely that PLP arose in the CNS, and was introduced in the PNS relatively recently. PLP may thus take over the role of PO in the PNS as a recapitulation of events in the CNS early in evolution. Perhaps these questions may be addressed by determining whether PLP mRNAs and protein are found in the PNS of less evolved vertebrates.

The experimental models developed and employed in the course of these studies have shed some light on the possible modes of regulation of myelin gene expression during regeneration. Future studies using these models will undoubted provide exciting information regarding the molecular mechanism(s) underlying regeneration in the peripheral nerve.

5. APPENDIX I: The use of a heterologous cDNA probe in the isolation of the chick PO cDNA

5.1 Introduction:

PO is a highly hydrophobic, integral membrane protein which is specifically expressed in PNS myelin [for review see Lees and Brostoff, 1984]. The hydrophobic nature of PO, and its tendency to form aggregates in solution, have been major stumbling blocks in the characterization of this intriguing protein. The protein has been purified from the mammalian PNS [Kitamura et al., 1976; Eylar et al., 1979] and partially purified from avian PNS myelin in this laboratory [Mezei and Verpoorte, 1981]. The first insight into the molecular nature of this protein was, however, obtained by the isolation and characterization of rat PO-encoding cDNAs by Lemke and Axel [1985]. The deduced amino acid sequence from the rat PO cDNA [Lemke and Axel, 1985], as well as from the sequence subsequently derived from the purified bovine PO protein [Uyemura et al., 1987], suggested that PO spans the membrane bilayer once. The proposed extracellular domain of this protein has been predicted to contain eight β-pleated sheets [Lemke and Axel, 1985]. The extracellular domain of PO also contains potential sites for glycosylation, and this has been recently corroborated with the isolation and characterization of the glycosidic moieties from human PO [Uyemura et al., 1990]. The intracellular domain of PO was found to contain several charged residues, suggesting that electrostatic interactions may play a role in PO-mediated compaction of the myelin sheath [Lemke and Axel, 1985].

From an evolutionary standpoint, it is possible that PO is more closely related to the proposed ancestral progenitor than other members of the Ig gene superfamily. NCAM, L1 and MAG possess multiple Ig-domains, that suggest duplication events in the evolution of these genes from the ancestral progenitor gene. PO on the other hand, has only a single Ig

domain that has been proposed to arise from a duplication of a half-domain. PO may therefore represent a more primitive member of the Ig gene superfamily.

It has been known for some time that PO is more archaic than its CNS counterpart, the proteolipid protein, PLP. CNS myelin in lower vertebrates has been found to contain proteins that are immuno-crossreactive with mammalian PO [Franz et al., 1981; Tai and Smith, 1983; Waehneldt et al., 1985; Jeserich and Waehneldt , 1986; Matthieu et al., 1986]. Waehneldt [1986] suggested that PO was gradually lost in the CNS during evolution and may have been replaced in function by the proteolipid protein. It is interesting to note in this regard, that the PLP is highly conserved in evolution [for review see Nave and Milner, 1990]. This sequence conservation may be critical to PLP function in the CNS, while the more plastic nature of PO gene over evolution suggests that stringent sequence conservation may not be required for the role that PO plays in the PNS.

This laboratory has been involved in a long range investigation into the molecular basis of myelination of the peripheral nerve [reviewed in Mezei, 1987]. In an effort to characterize the PO protein in the avian peripheral nerve, and exploit its full potential as a biochemical marker of PNS myelination, the PO protein was isolated, and partially purified from the chick sciatic nerve [Mezei and Verpoorte, 1981]. A solid-phase immunoassay of PO was developed with the production of high-titre polyclonal antisera to PO [Nunn and Mezei, 1984]. This technique provided a means to evaluate the degree of myelination in several experimental paradigms involving neural development, and in dystrophic mutants [Nunn and Mezei, 1984; Lewis and Mezei, 1985].

The PO protein was isolated and partially purified from the chick sciatic nerve myelin a number of years ago [Mezei and Verpoorte, 1981]. One of the major obstacles in its characterization was its propensity to aggregate into oligomeric forms in solution. The PO protein from highly purified myelin was purified by gel chromatography and a band corresponding to 28 kDa was gel purified and subsequently used to raise high titre polyclonal antisera in rabbits [Nunn and Mezei, 1984].

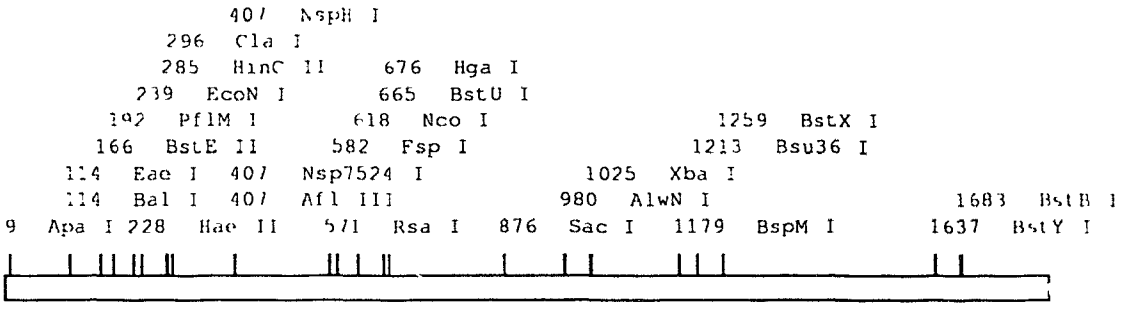
In the meantime, more information was obtained in this laboratory about the nature of the chick PO gene and the encoded protein [LeBlanc and Mezei, 1987]. The rat PO cDNA and fragments derived from this cDNA were used as probes to specifically detect chick PO mRNAs on Northern blots. Figure 5.1 shows the structure of the rat PO cDNA, and also marks the restriction sites. The 604 bp 5'-coding region probe (named PSN604) was obtained by digesting the rat PO cDNA with PstI . Another potential probe (called PSN 296) was the 296 bp 3'-coding region probe also obtained by digestion with PstI. The rat PO cDNA probe which was full-length detected a mRNA species of 1.85 kb in rat sciatic nerve and did not detect any mRNA species in RNA from the chick sciatic nerve under conditions of medium stringency. However, a smaller band of 1.6 kb was detected on hybridization under conditions of low stringency of a Northern blot containing RNA from chick sciatic nerve with a probe encompassing the 5'-coding region of the rat cDNA (PSN 604)[LeBlanc and Mezei, 1987]. These results suggested that some sequence similarity existed between the coding region of the rat and the chick PO cDNAs. Based on the size of the mRNA detected (1.6 kb) and the known size of the chick PO protein (28 kDa), the coding region of the chick PO was expected to be between 800 and 900 bases, followed by a non-coding tail of at least 600 - 700 bases. Such long 3' non-coding regions are a characteristic of mRNAs in the nervous system.

At the level of the genome, neither the rat PO cDNA probe, nor component probes could detect bands on a Southern blot containing chick genomic DNA, while bands were readily detected in other mammalian genomic DNAs [LeBlanc and Mezei, 1987]. This suggested that at the level of the genome, sequence differences precluded detection of bands by hybridization at medium stringency. It is possible that the intron-exon arrangements differ in the chick PO gene to an extent that obviated the detection of bands with a heterologous cDNA clone in Southern blot analysis.

In vitro translation of poly(A)$^+$RNA prepared from both the chick and rat sciatic nerves was carried out and the translation products were specifically immunoprecipitated

Figure 5.1 Restriction Map of the Rat PO cDNA

The rat PO cDNA is presented with the position of the unique restriction sites. The Pst 1 fragment from 1 to 604 bp forms the 5' coding region probe (PSN604) while the Pst 1 fragment from 605 to 900 bp forms the 3' coding region probe (PSN 296).



Rat PO cDNA complete 1834 base pairs Unique Sites

FIGURE 5.1

with polyclonal anti-chick PO antisera. Both chick PO and rat PO were immunoprecipitable with this antisera, implying that common epitopes exist between the two polypeptides [LeBlanc and Mezei, 1987]. Isoelectric focussing of the peptides showed that the chick PO polypeptide was relatively more acidic than the mammalian counterpart.

In summary, these studies demonstrated a divergence at the level of the PO gene between the rat and the chicken. In addition, similarities were manifested in the encoded PO polypeptides in the two species. Therefore, the polyclonal antibody against the chick PO protein [Nunn and Mezei, 1984] was used as an immunological screen in the primary attempt to isolate cDNAs encoding the chick PO [LeBlanc, 1987, Ph.D. Thesis]. A λgt11 library representing the poly(A)$^+$ RNA population from the rapidly myelinating chick sciatic nerve was immunoscreened with the anti-chick PO antibody. Several putative positives were obtained using this screening approach. However, sequencing of these clones and larger cross-hybridizing clones obtained from a pUC8-based cDNA library from chick sciatic nerve, showed that a majority of these cDNAs encoded the apolipoprotein A-I (apo A-I), a serum lipoprotein thought to be important in lipid transport and cholesterol homeostasis [LeBlanc et al., 1989]. This unexpected finding was resolved in subsequent experiments from this laboratory which demonstrated that apoA-I is expressed in the rapidly myelinating avian peripheral nerve, and that this 28 kDa protein was a highly antigenic contaminant in the partially purified PO protein. The polyclonal antibodies raised against the gel-purified PO also cross-reacted against apoA-I and this eventually resulted in the isolation of apoA-I cDNAs using the immunoscreening approach. In this regard, it is also likely that λgt11 recombinants expressing chick PO were more toxic to the bacterial host system than other recombinants and this made the isolation of chick PO cDNA clones in λgt11 more difficult. Since it was demonstrated that the rat PO cDNA probe could cross-hybridize with the 1.6 kb PO mRNA derived from the chick sciatic nerve, the rat PO cDNA or parts of the cDNA were thought to be potential heterologous probes in another attempt to isolate of cDNA.

5.2 Results:

i. The 3'-coding region probe from the rat PO cDNA (PSN 296) detects chick PO mRNA.

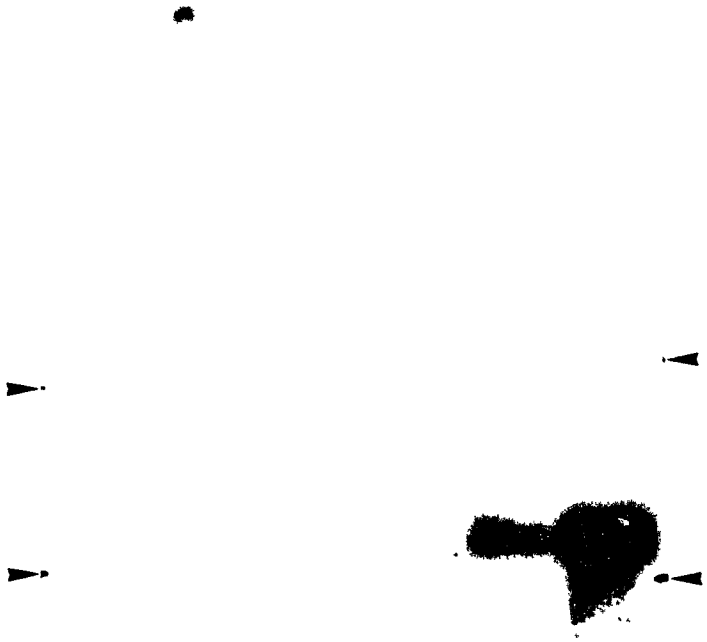
Heterologous probes may be employed to detect sequences that are similar by carrying out hybridizations and washes of blots under reduced stringency [Wahl and Berger, 1987]. There are a number of equations that relate the melting temperature of DNA-DNA and DNA-RNA hybrids to the salt concentration, the length of the DNA strands and the percentage of formamide in the hybridization buffer. However, empirical determination of hybridization conditions is critical to the success of such experiments.

Northern blot analysis was carried out on total RNA prepared from the brain and sciatic nerve of the chicken and the rat. The probe used was the 3'-coding region probe PSN296 and the blot was hybridized in an aqueous hybridization buffer at 37°C as described in Materials and Methods. Washes were carried out at successively increasing temperatures, in a low salt buffer, until a specific band was detected in the RNA from the chick sciatic nerve.

Figure 5.2 shows the final results of such a Northern blot analysis. A specific band at 1.9 kb corresponding to PO mRNA was detected in the adult rat sciatic nerve (lane 5) and in the 21 - 23 day old rat sciatic nerve (Lane 6). A small amount of cross-hybridization was observed to the 28S rRNA band in both these lanes. The 1.9 kb band was not detected in RNA prepared from the 21 - 23 day old rat brain (lane 3) or from the adult rat brain (lane 4). This demonstrated the specificity of hybridization of the PSN296 probe to RNA from rat sciatic nerve and brain. The PSN296 probe also detected two bands in the 19 day embryonic chick sciatic nerve RNA (lane 1) which represents an RNA population in the rapidly myelinating peripheral nerve. One band co-migrated with the 28S rRNA while

Figure 5.2 Northern blot analysis of chick and rat sciatic nerve RNA.

The Northern blot was probed with the PSN296 probe as described in the Results section. Lane 1. 19 day embryonic chick sciatic nerve. Lane 2. 19 day embryonic chick brain. Lane 3. 21-23 day old rat brain. Lane 4. Adult rat brain. Lane 5. Adult rat sciatic nerve. Lane 6. 21-23 day old rat sciatic nerve. Each lane contains 30 μg of fractionated total RNA. Arrowheads on the left and right hand sides represent the rRNA markers from chick and rat respectively.



1 2 3 4 5 6

Figure 5.2

the other was below the 18S rRNA and was sized as approximately 1.6 kb relative to the rRNA markers. The single band detected in chick brain RNA from the same developmental stage was of the same molecular weight as the 28S rRNA (lane 2). In this regard, the chick 28S rRNA was also found to be slightly smaller than that from rat. These results showed that although chick PO mRNA was detected with the PSN296 probe under these conditions, there was significant cross-hybridization to 28S rRNA. However, it was also apparent that the 3' coding region probe could detect a specific band in electrophoretically fractionated chick sciatic nerve RNA, that was of the same molecular weight as previously detected with the 5'-coding region probe, PSN 604 [LeBlanc and Mezei, 1987]. These results suggested that both the PSN296 and PSN604 were potential probes for the isolation of the chick PO cDNA.

ii. Isolation of putative positive cDNAs from a 1 day chick sciatic nerve plasmid cDNA library.

In order to isolate the chick PO cDNA using heterologous probes, the pUC8-based cDNA library prepared from 1 day chick sciatic nerve poly(A)$^+$ RNA was used [LeBlanc, 1987, Ph.D.Thesis]. Initially, control experiments were performed using colony blot hybridization techniques to show that under the conditions established by Northern blot analysis, no hybridization to the pUC vector sequences would take place.

PSN296 was gel-purified twice to remove any contaminating vector sequences. This step has been noted to be critical in reducing background in colony blot hybridizations [Wahl and Berger, 1987]. Colonies containing the rat PO cDNA clone in pUC8 (named pSN63c), the mouse MBP cDNA in pUC8 (pHF43) and colonies containing pUC8 were transferred onto membranes, denatured and the immobilized plasmid DNA was hybridized to the PSN296 probe. Only hybridization to pSN63c-containing colonies was observed

under these conditions. The MBP plasmid-containing colony served as an additional negative control in these experiments.

Colony blots containing 3000 independent recombinants from the pUC8-based chick sciatic nerve cDNA library were picked and gridded onto nylon membrane sheets and subsequently hybridized to both PSN296 and PSN604. The positive and negative controls in each case were pSN63c-, pHF43- and PUC19 vector-containing colonies. Each colony was blotted in duplicate on the nylon membranes.

Positive recombinants were chosen from these hybridization experiments, under the following three criteria:

1. The signal derived from the colonies was significantly over that observed for the negative controls.
2. The signal was positive in both colonies representing the same recombinant.
3. Hybridization was over background to at least one of the two rat PO cDNA probes.

Although the level of the signal was determined qualitatively, a rough approximation of the level of the signals is presented in Figure 5.4. Thus, 30 colonies were selected over two experiments, representing 1% of the population gridded out in colony blots. Based on the total number of recombinants initially produced in this library ($3\text{-}4 \times 10^4$), the number of selected cDNAs was approximately 0.075% of the total mRNA population represented in the library.

iii. Analysis of putative positive cDNAs by Northern blot.

Several approaches were available for the rapid analysis of the putative positive cDNAs isolated. The sizes of the inserts were from 0.1 kb to 1.3 kb. This implied that none of the clones corresponded to the full-length of the chick PO mRNA. Since the

library was prepared using oligo(dT) as a primer, the recombinants were biased towards the 3' ends of mRNAs. One approach to analysis of these clones was by direct sequencing and would involve comparison of the sequences of the isolated positive cDNAs with the 3' region of the rat PO cDNA. In a second approach, Northern blot analysis of chick sciatic nerve RNA with probes from the isolated positives would allow identification of clones which recognized an mRNA species of approximately 1.6 kb.

Northern analysis of RNA from 19 day embryonic chick sciatic nerve was carried out with inserts from some of the putative positive cDNA clones. These Northern blots are depicted in Figure 5.3. The mRNAs detected by these cDNAs varied in size. The isolated cDNAs detected mRNAs of 0.8 to 1.0 kb in length. Isolate 2/13 detected a 0.8 kb band as well as two other transcripts of 2.5 and 3.0 kb in length. 0.8 kb mRNAs were also detected by 2/14 and 2/15 although these cDNAs were subsequently shown to be distinct from 2/13 by direct sequencing. Some cDNAs did not hybridize to any mRNAs under high stringency (see Northern blots probed with inserts of 2/4, 2/10, 3/4, and 3/13). The probes prepared were of high specific activity (10^7 cpm/μg), suggesting that these cDNAs correspond to mRNAs of low abundance. The cDNAs that did hybridize to mRNAs near 1.6 kb were 3/10 (a faint signal) and 3/14 (the band was slightly larger than the 18S rRNA).

iv. Analysis of putative positive cDNAs by direct sequencing.

The second approach to identification of the putative positives was initiated by rapid sequencing of the plasmids containing insert cDNAs. A number of cDNA clones were sequenced using the Sanger dideoxy method on alkali-denatured plasmids [Sanger et al., 1977, Chen and Seeburg, 1985]. In cases where the insert was too large to sequence from each end, a combination of double-stranded sequencing methods as well as subcloning restriction fragments in M13, followed by single-stranded sequencing of the subclones was

carried out. A summary of the stretches of cDNAs sequenced in isolated cDNAs is presented in Figures 5.4 and 5.5. Certain isolates did not hybridize to any band on Northern blots and were eliminated from subsequent screening procedures as false positives. Sequencing of these cDNAs was not carried out.

v. Analysis of sequence data.

The sequence data obtained from the bank of isolated positive cDNAs were analysed on the University of Wisconsin analysis package using FASTA software [Pearson and Lipman, 1988]. In the first instance, sequence similarity at the level of the peptide is often significantly higher than that found at the level of the encoding DNA sequences. Thus, the rat PO cDNA including the non-coding region was transformed into an open reading frame by ignoring the stop codons. This peptide sequence was then compared to all six open reading frames obtained from the sequencing of a particular isolate, as a means of identification of sequence similarity.

Figure 5.6 shows the stretch of translated sequence obtained from isolate 3/1 (a 500 bp insert cDNA) from the second ORF that showed some sequence similarity in amino acid residues 228 to 279 of the rat PO. This level of sequence similarity is considered to be low as the length of sequence involved is only 53 amino acid residues. Figure 5.7 shows the restriction map of the sequence of 3/1 while Figure 5.8 shows the sequence of 300 bp from isolate 3/1. In subsequent experiments isolate 2/6 was found to be identical to 3/1. However, when the sequence of 3/1 was compared to sequences in the GenBank, it was found to be 97.4 % identical to the residues 3908 to 4718 of the rat 28S rRNA (Figure 5.9). In retrospect, this finding is not so unexpected, as preliminary experiments had established cross-hybridization of the PSN296 probe with 28S rRNA.

Other sequences from isolated positive cDNAs were subsequently compared to the sequence data bank GenBank in an effort to identify the unknowns. Another isolate that

Figure 5.3 Northern blot analysis of chick sciatic nerve RNA with putative positive cDNAs

RNA from 19 day embryonic chick sciatic nerve (10 µg) was fractionated on formaldehyde gels and transferred to nitrocellulose as described in Materials and Methods. Individual lanes were probed with the inserts from putative postive cDNAs. In this composite figure, the arrowheads mark the positions of the chick rRNA markers.

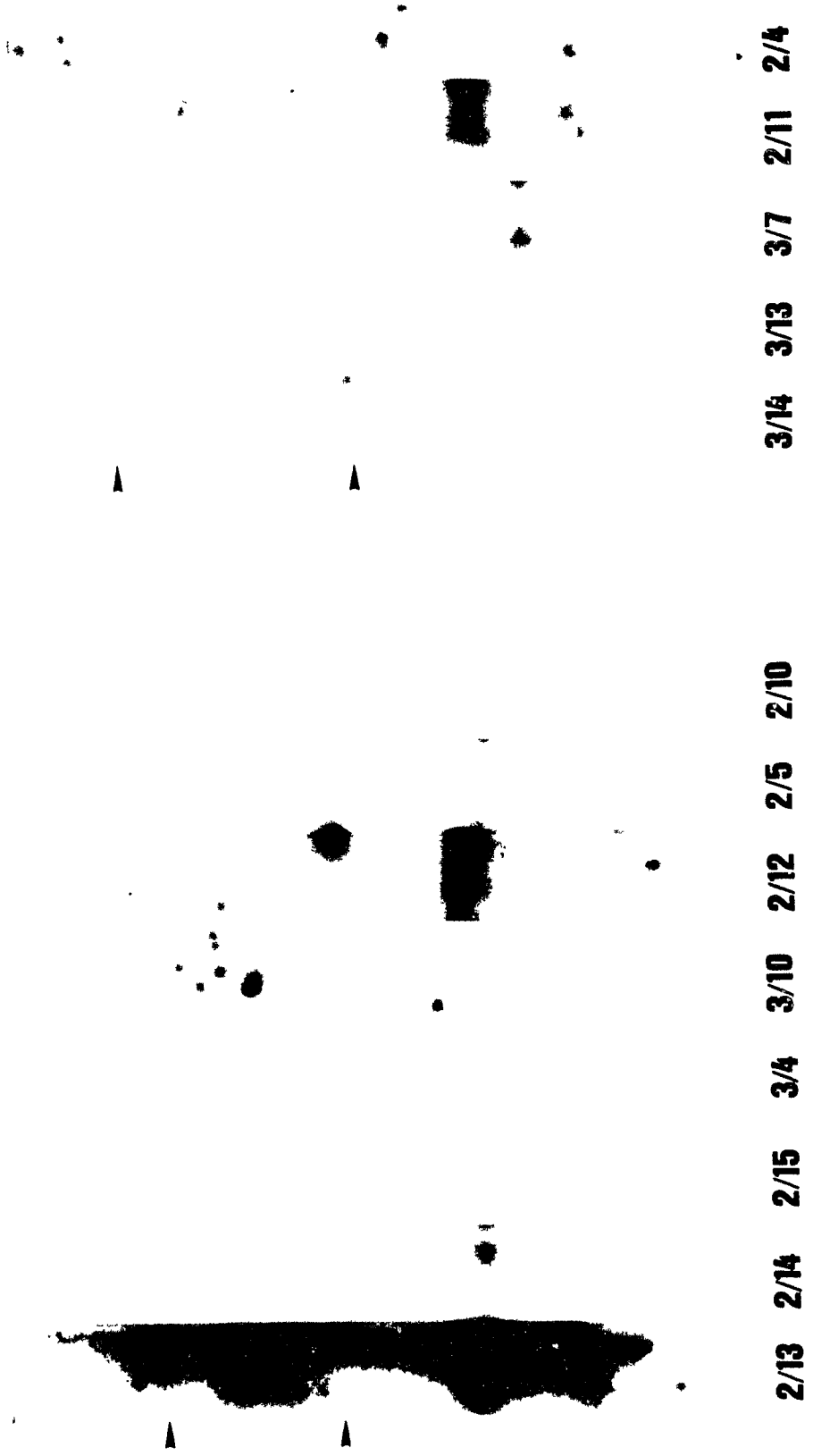


Figure 5.3

Figure 5.4 Analysis of putative positive cDNAs by sequencing.

This schematic diagram represents the summary of sequencing of several putative cDNAs. The shaded boxes represent the intensity of the signal obtained from colony hybridization experiments. The arrows show the direction of sequence generated and the numbers above the arrows indicate the bases read. In certain cases, sequencing of the inserts was not carried out, as explained in the Section 5.2.

Levels of hybridization of plasmids to ratPO cDNA probes

Name	Size	PSN 296	PSN604	Sequencing done
3/6	0.8			321 → ← 168
3/9	0.5			264 →
3/10	0.8			177 → H ← 341 ← 152
2/12	0.22			← 171
3/5	0.27			136 → ← 211
2/13	0.17			← 151+p(A)
2/9	0.9			
2/7	0.26			240 +p(A) →
2/15	0.9			218 → (2/15S)
3/1	0.5			← 300
3/2	0.5			200 →
3/4	0.5			223 → ← 138
3/7	0.3			
3/8	0.3			

FIGURE 5.4

Figure 5.5 Analysis of putative positive cDNAs by sequencing.

This figure is a continuation of data presented in Figure 5.4. The shaded boxes represent the intensity of the signal obtained from colony hybridization experiments. The arrows show the direction of sequence generated and the numbers above the arrows indicate the bases read. In certain cases, sequencing of the inserts was not carried out, as explained in the Section 5.2.

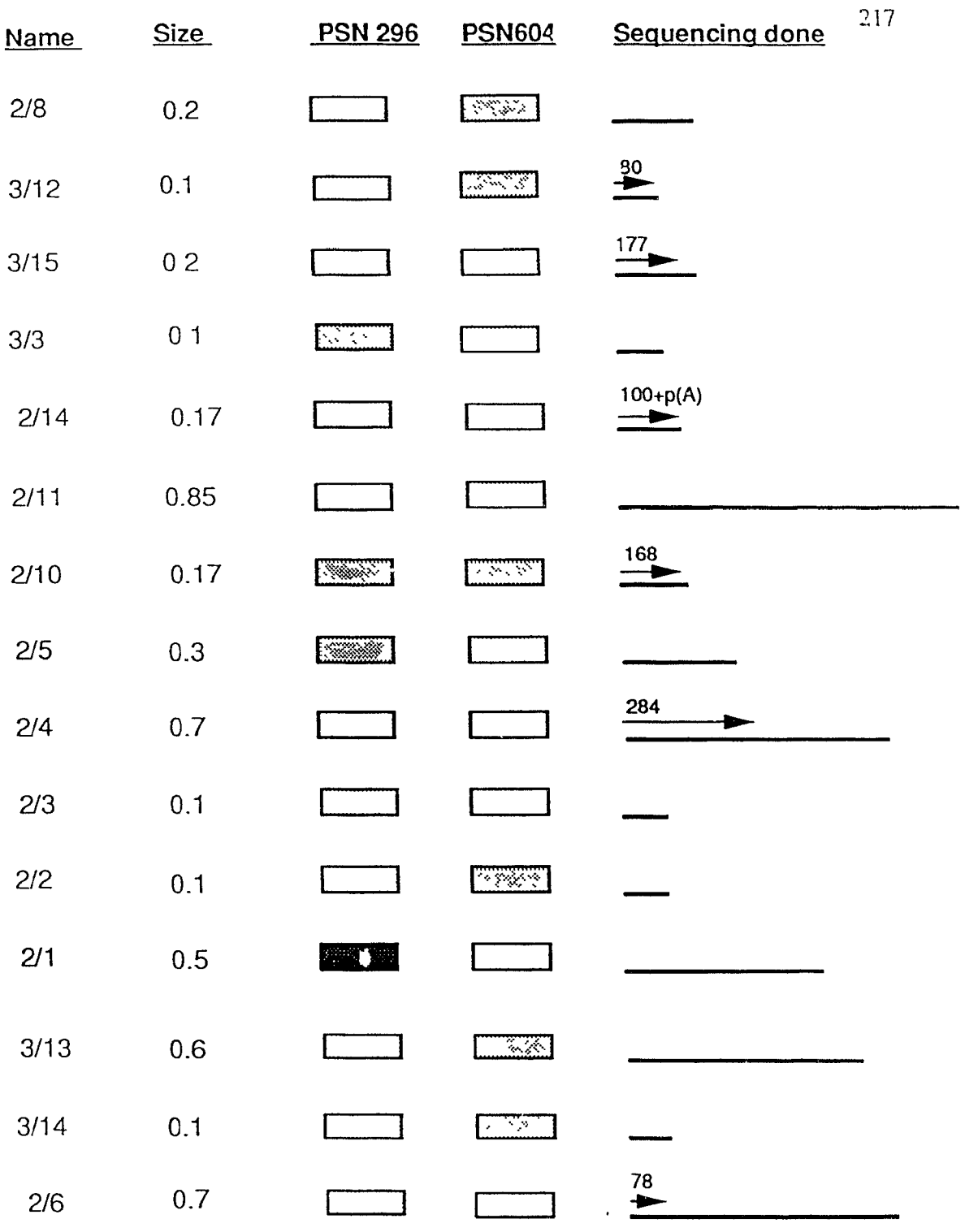

Name
Size
PSN 296
PSN604
Sequencing done
2/8
0.2
3/12
0.1
80
3/15
0 2
177
3/3
0 1
2/14
0.17
100+p(A)
2/11
0.85
2/10
0.17
168
2/5
0.3
2/4
0.7
284
2/3
0.1
2/2
0.1
2/1
0.5
3/13
0.6
3/14
0.1
2/6
0.7
78

FIGURE 5.5

Figure 5.6 Sequence comparison of isolate 3/1 and ratPO cDNA

The open reading frame obtained from direct sequencing of isolate 3/1 was compared with that from the ratPO cDNA, encoding the rat PO protein. The top line of the comparison depicts the one-letter amino acid code for rat PO, while the bottom line represents that from one of the open reading frames of isolate 3/1. As shown, there were 18 matches over 53 amino acids with the introduction of 2 gaps.

Sequence comparison of isolate 3/1 and rat PO cDNA

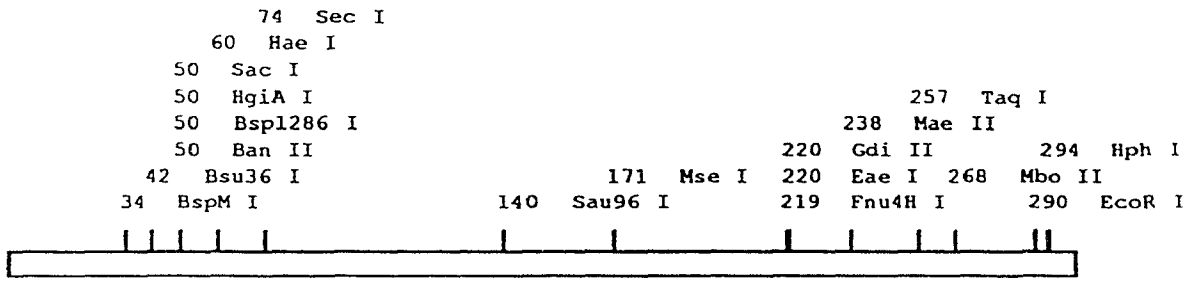
rat PO	228	V	L	Y	A	M	L	D	H	S	R	S	T	K	A	A		S	E	K	K
3/1	53	V	S	U	L	T	L	I	A	L	R	S	L	P	A	F	Q	S	G	N	L
			*	*	*	*		*	*	*			*	*		*	*		*	*	*
	247	S	K	G	L	G	E	S	R	K	D	K	K	U	R	L	A	G	R	A	G
	73	S	C	Q	L	H	U	U	I	G	Q	R	A	G	R	G	G	L	R	I	G
			*	*		*	*	*	*	*	*	*	*	*		*	*	*		*	
	267	G	R	G	S	A	W	S	L	P	K	A	L	R		279					
	93		R	S	S	A	S	S	L	T	D	S	L	R		104					
		*		*			*			*	*	*									

Matches = 18 Length = 53 Matches/length = 34.0 percent

FIGURE 5.6

Figure 5.7 Restriction map of isolate 3/1.

A restriction map of isolate 3/1 is presented with the postions of the unique restriction sites. This cDNA was eventually identified to encode chick rRNA as described in Section 5.2.



CSCN 3.1 REV SEQ19 300 base pairs Unique Sites

FIGURE 5.7

Figure 5.8 Sequence of the isolate 3/1.

The isolate 3/1 was sequenced by double-stranded sequencing as described in Section 5.2. The sequence read (300 bases) is presented with the position of restriction sites. Unique restriction sites are underlined.

CSCN 3.1 REV SEQ19 -> Full Restriction Map

DNA sequence 300 b.p. TTGACTGGGGCG ... AGAATTCACCAA linear

Positions of Restriction Endonucleases sites (unique sites underlined)

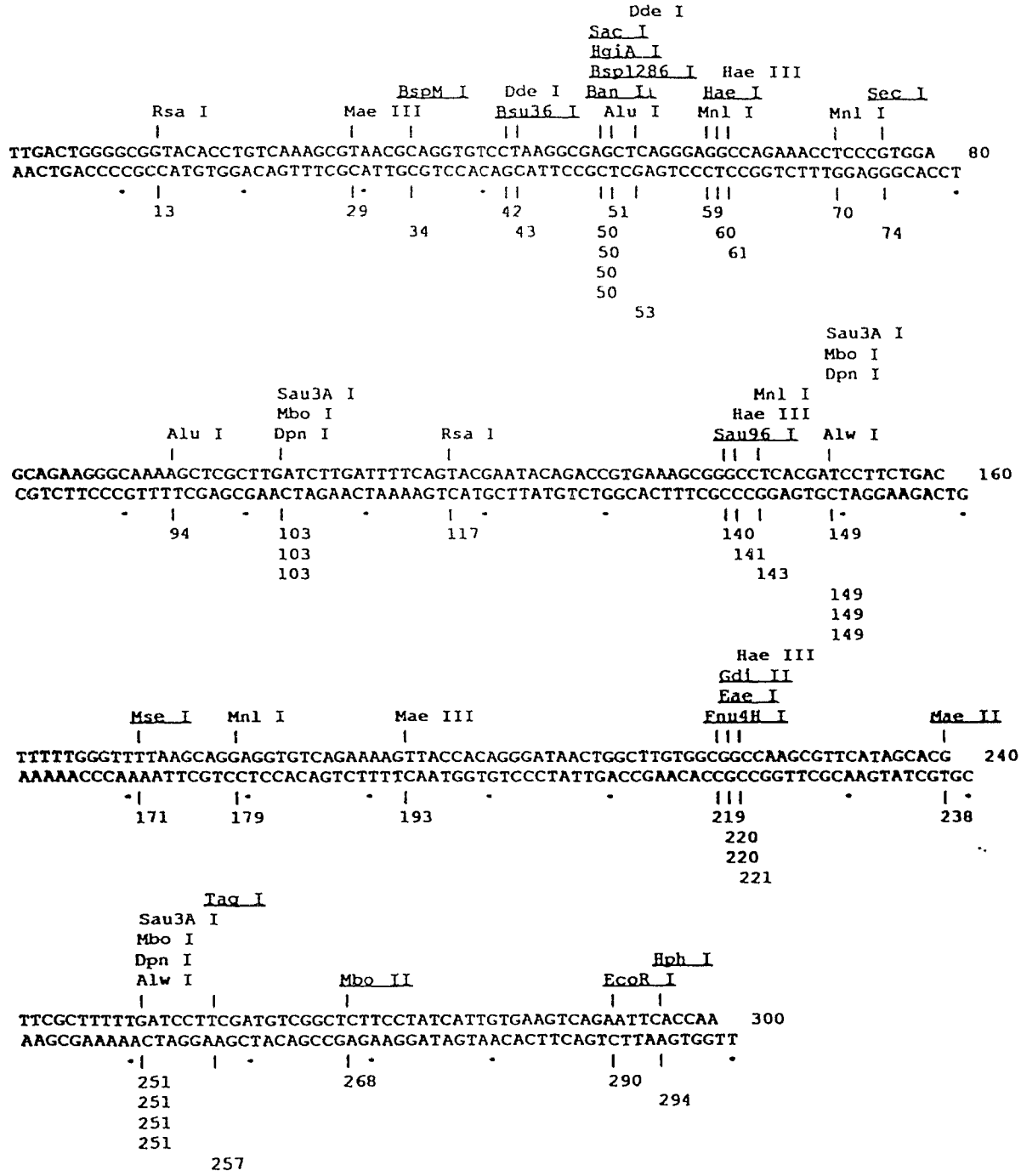


FIGURE 5.8

Figure 5.9 Comparison of the sequence from isolate 3/1 and rat rRNA.

An alignment of the derived sequence from isolate 3/1 and rat ribosomal RNA is presented. Asterisks mark the positions of mismatches between the two sequences.

Isolate 3.1
28S rRNA

```
TTGACTGGGGCGGTACACCTGTCAAACGGTAACGCAGGTGTCCTAAGGCGAGCTCAGGGAGGCCAGAAACCTCCCGTGGA
TTGACTGGGGCGGTACACCTGTCAAAGCGTAACGCAGGTGTCCTAAGGCGAGCTCAGGGAGGACAGAAACCTCCCGTGGA
                              **                                    *


GCCGAAGGGCAAAAGCTCGCTTGATCTTGATTTTCAGTACGAATACAGACCGTGAAAGCGGGGCCTCACGATCCTTCTGAC
GCCGAAGGGCAAAAGCTCGCTTGATCTTGATTTTCAGTACGAATACAGACCGTGAAAGCGGG CCTCACGATCCTTCTGAC
                                                                  *


TTTTTGGGTTTTAAGCAGGAGGTGTCAGAAAAGTTACCACAGGGATAACTGGCTTGTGGCGGCCAAGCGTTCATAGCACG
CTTTTGGGTTTTAAGCAGGAGGTGTCAGAAAAGTTACCACAGGGATAACTGGCTTGTGGCGGCCAAGCGTTCATAGCACG
*

TTCATAGC ACGTTCGCTTTTTGATCCTTCGATGTCGGCTCTTCCTATCATTGTGAAGTCAGAATTCACCAA
TTCATAGCGACGT CGCTTTTTGATCCTTCGATGTCGGCTCTTCCTATCATTGTGAAG CAGAATTCACCAA
        *    *                                                 *
```

FIGURE 5.9

was identified by this means was 2/13 which was found to encode the mitochondrial cytochrome c oxidase subunit II. As shown in Figure 5.10, short stretches of sequence similarity between the known sequence from *Xenopus laevis* and *Drosophila melanogaster* and the isolated cDNA from the chick were apparent.

In summary, a heterologous cDNA probe was identified for the isolation of the chick PO cDNA. A plasmid cDNA library prepared from the poly(A)$^+$ RNA fraction of the 1 day old chick sciatic nerve [LeBlanc , 1987, Ph.D. Thesis] was screened by colony blot hybridization for putative positives, using low stringency hybridizations and washes. Two approaches to analysis of the isolated putative positives for the chick PO cDNA were adopted. Northern blot analysis was carried out to identify any clones that hybridized to a 1.6 kb band. In addition a number of the cDNAs were sequenced and compared at the level of ORFs to the known ORF of rat PO cDNA. No positive cDNA for chick PO could be isolated by these experiments. Two clones that may be useful in normalization of steady-state level of mRNAs in the chick were identified: one encoding the partial sequence for chick 28S rRNA and one encoding partial sequence of the cytochrome c oxidase subunit II mRNA.

5.3 Discussion:

In January 1990, the cDNA sequence for the chick PO was published by Barbu [1990]. At this time, the isolation and characterization of putative positives for the chick PO had been set aside to explore more fruitful avenues in peripheral nerve regeneration, as discussed in the main chapter of the thesis. However, the sequence data published by Barbu did provide some possible explanations for the failure in the present attempt to isolate the PO cDNA from the chick sciatic nerve.

Barbu [1990] isolated and characterized the chick PO protein by an immunoscreening approach. A λgt11 cDNA library prepared from the poly(A)$^+$ RNA

```
               10        20        30        40
orf2.s NQTSFITTRPGVFYGQCSEICGRNHTYIPIVVESTPZNTLKPDPHYCHL
       X:.:...:::.:::::::::: ::...:::.:X.: :..
C25797 NQTNFFINRPGLFYGQCSEICGANHSFMPIVIESVPVNNFIKWISSNNS
     180       190       200       210       220

;OBHU2 Cytochrome c oxidase (EC 1.9.3.1), polypeptide I 141  141  160
  56.5% identity in 46 aa overlap

               10        20        30        40
orf2.s NQTSFITTRPGVFYGQCSEICGRNHTYIPIVVESTPZNTLKPDPHYCH
       X:.:..:::::.:::::::: ::...::X.: .: .... .: .
OBHU2  NQTTFTATRPGVYYGQCSEICGANHSFMPIVLELIPLKIFEMGPVFTL
     180       190       200       210       220
```

Figure 5.10 Comparison of the sequence from isolate 2/13 and cytochrome oxidase subunit II.

An alignment of the open reading frame derived from the sequence of isolate 2/13 and that of the cytochrome oxidase subunit II is presented. C25797 represents the sequence from *X. laevii*, while OBHU2 represents the human sequence of cytochrome oxidase subunit II.

from the 1-month old chick sciatic nerve was immunoscreened with a polyclonal antibody raised against rabbit PO. A single 210 bp cDNA was isolated on primary screening and two larger clones were subsequently obtained from the same library on secondary screening with the primary cDNA positive. The mature protein was found to be 220 amino acids long with an N-terminal signal sequence of 29 amino acids. The overall sequence similarity at the protein level was found to be 78% to the rat PO and 77% to the bovine PO. While regions thought to form the extracellular and cytoplasmic domains of PO were similar to 80%, the transmembrane region was only 58% similar. In this regard, PO cDNA from the shark CNS was recently cloned [Saavedra et al., 1989]. The shark diverged from other vertebrate classes over 400 million years ago; however they are the oldest known living vertebrates that have multilamellar organization of myelin around nerve fibres. In this respect, the sequence similarity between the shark PO protein and the rat PO protein was found to be 46%. The sequences representing the extracellular and cytoplasmic domains were 58% and 55% similar while the transmembrane domains were 22% similar. These results represent the degree of divergence in the sequence of the PO protein over vertebrate evolution.

Sequence similarities between the rat PO and the chick PO cDNAs were determined and are displayed in Figure 5.11. The overall sequence similarity in the 840 bases of the chick PO cDNA reported by Barbu [1990] and that reported for the rat PO cDNA [Lemke and Axel, 1985], was found to be 70% at the level of encoding mRNAs. The sequence similarity in the region corresponding to PSN296 was also analysed and found to be 70%.

These results point towards a higher degree of sequence similarity between chick PO and rat PO cDNAs than was expected from empirical observations from this laboratory [LeBlanc and Mezei, 1987] and in the present studies. This may provide an explanation for the failure to isolate the chick PO cDNA in this set of experiments.

The degree of sequence similarity that was expected between the chick PO cDNA and the rat PO cDNA probe PSN296 was approximately 40%. Although Northern analysis

```
      Gap Weight:  5.000      Average Match:  1.000
   Length Weight:  0.300   Average Mismatch:  0.000

         Quality:  155.1             Length:    251
           Ratio:  0.660               Gaps:      1
Percent Similarity: 70.306   Percent Identity: 70.306

Sgchkpo x Sgratpo         March 15, 1990  21:16  ..

                .         .         .         .         .
596 AGGCGGTGCTGCAGAGGCGGCTGAGCGCCATGGAGAAGGGGAAGCTGCAG 645
                 |||| .|||| || ||||||||||||||||  | ||
604 ............GAGGAGGCTCAGTGCCATGGAGAAGGGGAAATTTCAC 640
               .         .         .         .         .
646 AGGTCGGCCAAGGACGCGTCCAAGCGCAGCCGGCAGCCTCCCGTGCTTTA 695
    | |||  | |||||| | || |||||| | |||||| | || ||||| ||
641 AAGTCTTCTAAGGACTCCTCGAAGCGCGGGCGGCAGACGCCAGTGCTGTA 690
              .         .         .         .         .
696 CGCCATGCTGGATCACAGCCGCAGCGCCAAAGCGGCCGCCGAGAAGAAAA 745
     |||||||||||| |||||||| ||| ||||||| |||   |||||||||
691 TGCCATGCTGGACCACAGCCGAAGCACCAAAGCTGCCAGTGAGAAGAAAT 740
              .         .         .         .         .
746 GCAAAGGAGCTCCGGGTGAAGCCCGCAAGGACAAGAAATAGCGGTTAGAA 795
      |||||    | ||| ||  | ||||||||| ||||||||||||||||
741 CTAAAGG...GCTGGGGGAGTCTCGCAAGGATAAGAAATAGCGGTTAGCG 787
              .         .         .         .         .
796 GGCAGGGAAGGTGCTCTCCTCGTCCTCGGGGGGGGTCGGGGCACC..... 840
    ||| |||  || | ||            || |   ||      |
788 GGCCGGGCGGGGGGTCGGGGGTCTGCATGGAGTCTTCCAAAGGCTCTCAG 837
```

Figure 5.11 Comparison of the sequences of chick PO and rat PO cDNAs over the 3' coding region.

The sequences reported by Lemke and Axel [1985] for the rat PO cDNA and by Barbu [1990] for the chick PO cDNA were aligned for the region corresponding the the PSN296 probe obtained from the rat PO cDNA. As indicated, the sequence similarity between the two sequences over 251 bases was 70.3%, with the introduction of a single gap.

was carefully carried out with washes at increasing temperatures to establish proper conditions of hybridization to specifically detect the PO mRNA in the chick sciatic nerve, it was never carried out to a stringency level that corresponds to 70% sequence similarity. As a result, a large number of false positives which were similar in sequence to PSN296 to the extent of 40% were isolated. As can be seen in Figure 5.12, the sequences derived from a number of isolated positives showed sequence similarity to the PSN296 probe to the extent of 40%. The error was further compounded by the number of positive recombinants selected for characterization. Although the number of isolates corresponded to 1% of the total cDNA population plated out for colony blots, the false positives from low stringency hybridization eliminated by sheer numbers the chance of obtaining a true positive cDNA encoding the chick PO.

Thus in retrospect, the principles behind colony blot hybridization and the use of the heterologous probe must be qualified by a strict examination of the degree of stringency required to obtain a specific signal. In practice, this may be determined by carrying out Northern blot analysis on multiple blots hybridized at the same temperature, but each separately washed at increasing temperatures until the signal is not detected at a particular level of stringency. Under such stringent conditions large numbers of false positive would have been eliminated. This elimination process at the very outset of the experiment is undoubtedly the key to future success in isolation of the cDNA of interest.

The publication of the chick PO cDNA clone by Barbu [1990] brought about an end to the attempts to clone the elusive chick PO cDNA in this laboratory. The degree of sequence similiarity of the mature PO protein in the chicken and in the rat contrasts with the previous indirect observations in this laboratory of sequence divergence between the PO genes of the two species [LeBlanc and Mezei, 1987]. It is not possible to rule out changes in the intron-exon structure of the two genes, which was previously observed [LeBlanc and Mezei, 1987]. This aspect of organization of the PO gene over evolution may be addressed in future studies.

Figure 5.12 Comparison of the complete sequences for rat and chick PO cDNA.

The sequences for the rat PO cDNA [Lemke and Axel, 1985] and chick PO cDNA [Barbu, 1990] were aligned and are presented. A single gap at the 3' end of the cDNAs was introduced in the alignment. The sequences were approximately 70% similar.

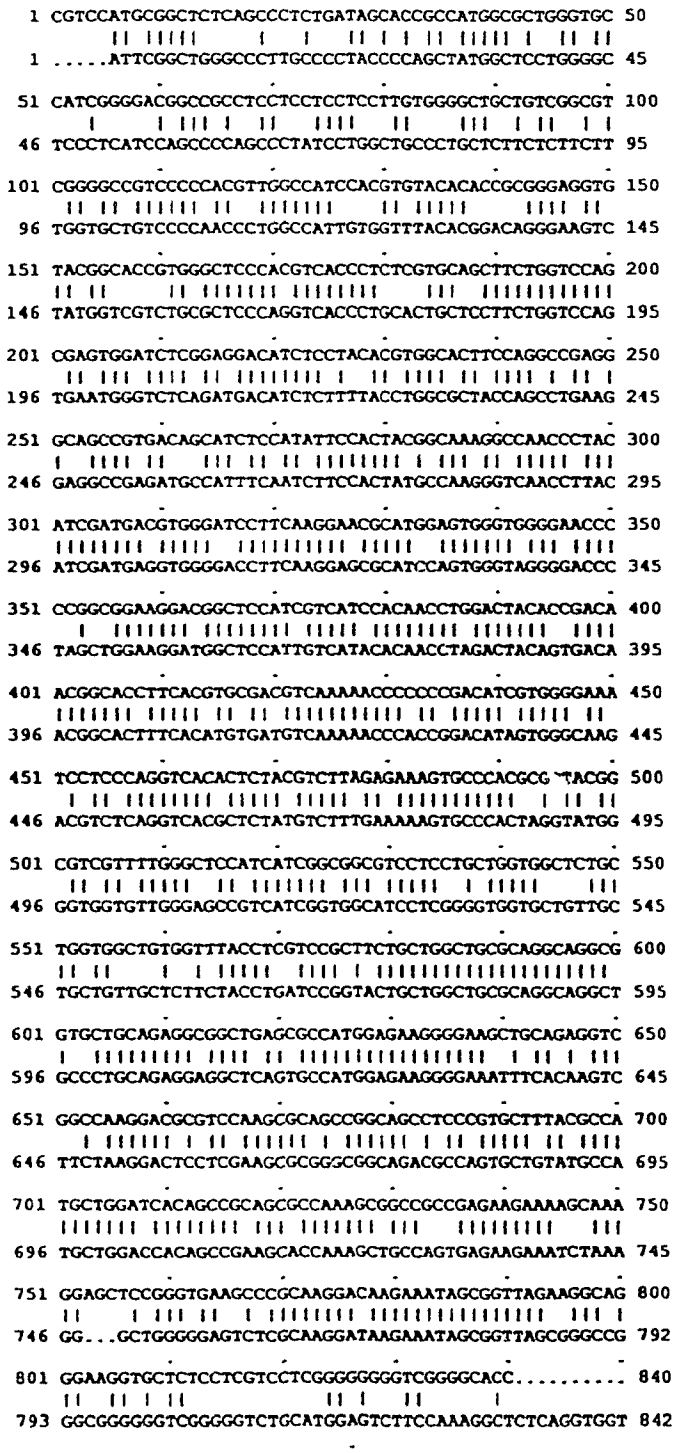


FIGURE 5.12

The establishment of organ-culture systems in the chicken for the study of myelinogenesis is presently one of the primary aims of this laboratory. In practical terms, the degree of sequence similarity between the chick PO cDNA and the rat PO cDNA may also allow the facile use of the rat PO cDNA as a specific probe in avian experimental models once proper conditions are established. The use of the cloned chick PO cDNA, or the rat PO cDNA under proper stringency, or synthetic oligonucleotide probes to the chick PO cDNA will certainly play an important role in future studies on the regulation of gene expression during myelination of the avian peripheral nerve.

6. APPENDIX II: Statistical treatment of data from slot blots

Dot blot and slot blots were used in the estimation of levels of mRNAs encoding numerous PNS myelin proteins in the present studies. In each experiment, biologically independent samples were obtained by pooling the distal segments of sciatic nerves at various stages following crush-injury and after permanent nerve transection. The RNA preparations from these pools were then immobilized on nitrocellulose sheets, probed with radiolabelled cDNA encoding the protein of interest, and autoradiographed to obtain an estimate of the levels of mRNAs present in the distal segment of the nerve after nerve injury. An RNA preparation from a pool of normal adult rat sciatic nerves was used in each case as a control. For semi-quantitative estimation, as described in Materials and Methods, the levels of mRNAs in the adult sciatic nerve RNA preparation were taken arbitrarily to be one.

A question was raised at the oral defense of this thesis as to the suitability of subjecting such an estimation to statistical analysis. As all values in each experiment were normalized to the value obtained in the adult sciatic nerve preparation, the deviation in the adult sciatic nerve population was not taken into account. The two-sample t test, however, assumes that both samples considered come at random from normal populations with equal variances [Zar, 1984]. Only when such criteria are fulfilled may the t-test provide valid infomation about the statistical significance of difference between two populations.

In the light of these questions, it was necessary to address the validity of the statistical treatment of slot blots presented in this thesis. A constitutive mRNA was chosen to serve as a internal standard to measure relative changes in levels of other myelin specific

Table 6.1 Ratio of levels of MAG mRNAs to PLP mRNAs in the distal segment of the sciatic nerve following crush-injury

Total RNA was extracted from the distal segments of crushed sciatic nerves at various times following crush-injury, and from adult sciatic nerves. Serial dilutions (one in two) of the RNA were filtered through nitrocellulose sheets, and slots were hybridized with ^{32}P MAG cDNA and subsequently with ^{32}P PLP cDNA as described in Materials and Methods. The washed and dried nitrocellulose blots were autoradiographed and the intensity of each slot was measured by densitometry. The units of peak area were divided by the amount of total RNA in the slot to give units per μg total RNA. Results are expressed as a ratio of levels of MAG mRNAs to levels of PLP mRNAs. Results are expressed as mean ± S.D. of RNA preparations for 3 to 5 experiments. The numbers in parenthesis indicate the number of separate experiments using sciatic nerves pooled from 6-8 animals for each time point following crush-injury or control preparations. Statistical significance (p values) were calculated by comparison to the adult control using a two-sample paired Student's t test on raw values of ratios of levels of MAG mRNAs to PLP mRNAs. The data was subsequently normalized with the vaule for the adult sciatic nerve taken to be one.

Ratio of levels of MAG mRNAs to PLP mRNAs in the distal segment of sciatic nerves following crush-injury

Table 6.1

Days following crush-injury	Levels of MAG mRNAs/levels of PLP mRNAs						Levels relative to adult control
	Expt 1	Expt 2	Expt 3	Expt 4	Expt 5	Mean ratio	
1	0.63	-	-	-	-	0.63 (1)	0.74
2	-	0.22	0.68	-	-	0.22 - 0.68 (2)	0.18 - 0.59
4	-	0.07	0.12	-	-	0.07 - 0.12 (2)	0.06 - 0.10
7	0.23	0.36	0.13	-	-	0.24 ± 0.12^{a}(3)	0.28 ± 0.16
10	0.20	0.40	0.17	-	-	0.26 ± 0.12^{a}(3)	0.30 ± 0.16
12	-	-	-	0.62	0.29	0.29 - 0.62 (2)	0.43 - 0.88
21	0.47	-	-	0.74	0.16	0.46 ± 0.29^{b}(3)	0.71 ± 0.43
Adult SN	0.55	1.18	1.15	0.70	0.67	0.85 ± 0.29 (5)	1.00 ± 0.34

a: $0.025 < p \leq 0.05$, b: $0.1 < p \leq 0.375$

mRNAs following crush-injury. The studies presented in this thesis have demonstrated that the levels of PLP mRNAs remain relatively unchanged following changes in Schwann cell-axonal contact as exemplified by crush-injury and permanent transection [Sections 3.2 and 4.2] Thus, statistical analyses were repeated using levels of PLP mRNAs as internal standards to assay alterations in levels of MAG mRNAs in the distal segment of the sciatic nerve at various times following crush-injury.

In each experiment , levels of MAG mRNAs/μg total RNA and levels of PLP mRNAs/μg total RNA were determined by slot blot analysis. As shown in Table 6.1, the ratio of these parameters demonstrated an alteration in the expression of the MAG gene relative to the expression of the PLP gene following crush-injury of the sciatic nerve. The ratio of levels of MAG mRNAs/levels of PLP mRNAs in the distal segment of the sciatic nerve following crush-injury were then compared in a two sample t test to the ratio found in the adult sciatic nerve as shown in Table 6.1. The data was subsequently normalized with the mean value found in the adult sciatic nerve taken to be one. The results of such an analysis were comparable to those presented in Section 3.1. MAG mRNAs levels dropped sharply within 2 days following crush-injury. Levels of MAG mRNAs were increased by 7 days post crush-injury, prior to observed increases in PO- and MBP- coding transcripts in the distal segment of the crush-injured sciatic nerve.

The present statistical treatment however, points out the need for an internal control in slot blot analyses, if the data is to be used subsequently for statistical analysis. Without such an internal (presumed invariant) standard, such a semi-quantitative estimation does not allow an accurate assessment of the variation in the normal population. As described in Section 4.1, certain constitutive controls such as cDNAs encoding the histone 3.3 or p1B15 were used in the present studies. The analysis presented in this section suggests that in the context of axonal modulation of Schwann cell gene expression, the levels of PLP mRNAs may also be used as a relatively invariant molecular marker.

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