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Isolation and Analysis of the Gene Encoding Peripheral Myelin Protein Zero

Greg Lemke, Elise Lamar, and John Patterson
Molecular Neurobiology Laboratory
The Salk Institute
San Diego, California 92138

Summary

We have isolated the gene encoding the Schwann cell glycoprotein P_0, the major structural protein of the peripheral myelin sheath. In rats and mice, this gene is split into six exons distributed over 7 kb of DNA. The segregation of these exons is consistent with the functional segregation of the P_0 protein into extracellular, membrane-spanning, and cytoplasmic domains. We find that the P_0 extracellular domain is similar in structure to a single immunoglobulin variable region domain. In contrast to prototypical immunoglobulin domains, however, this P_0 domain is encoded by two exons, the partitioning of which provides genetic evidence for the evolution of immunoglobulin-related domains from an ancestral half-domain. We also describe procedures for transfection of cultures of nontransformed rat Schwann cells and use these procedures to show that the Schwann cell–specific expression of the P_0 gene is controlled by *cis*-acting elements localized upstream of exon I.

Introduction

The rapidly conducting axons of vertebrates are surrounded by myelin, a multilayered, membranous sheath of insulation that serves to greatly increase the velocity of nerve impulse propagation (Ranvier, 1878; Ritchie, 1983). In the peripheral nervous system (PNS) this sheath is elaborated by Schwann cells as the result of a contact-dependent interaction with axons (Bray et al., 1981). This interaction has two notable metabolic consequences: an enormous increase in Schwann cell plasma membrane biosynthesis (Webster, 1971) and the induction and high level expression of a set of proteins unique to myelin-forming cells (Lemke, 1986).

The most abundant of these induced proteins is P_0, a small integral membrane glycoprotein that accounts for over 50% of the protein present in peripheral myelin (Ishaque et al., 1980). This protein has been the subject of considerable interest owing both to its hypothesized role as an organizer of the peripheral myelin sheath and to the marked cellular specificity and inducibility of its expression (Lemke, 1986). The primary structure of P_0 has been deduced from cloned cDNAs (Lemke and Axel, 1985) and directly determined by protein sequencing (Sakamoto et al., 1987). Analysis of this structure has suggested a model for P_0 transmembrane orientation and function in which distinct domains of the protein play essential roles in both the elaboration and the subsequent compaction of the myelin sheath. One of these P_0 domains is positioned extracellularly, at the intraperiod line of the myelin sheath, and has been hypothesized to bring about myelin compaction through homotypic interactions with like domains expressed on the surface of apposed myelin lamellae (Lemke and Axel, 1985). This domain has recently been found to exhibit significant similarity to members of the immunoglobulin superfamily (Lai et al., 1987) that are known to mediate a variety of homotypic and heterotypic interactions in the immune and nervous systems (Barclay et al., 1987).

P_0 expression is restricted to myelinating Schwann cells. The protein is not produced by oligodendrocytes, the myelinating glia of the central nervous system (CNS) (Greenfield et al., 1973), or by those Schwann cells that do not elaborate myelin (Brockes et al., 1980). Schwann cell expression of the P_0 gene is initiated as the result of, and remains dependent upon, contact with appropriate peripheral axons (Politis et al., 1982; Trapp et al., unpublished data). Experiments in vitro indicate that this induction and continued dependence may be mediated intracellularly by cAMP (Lemke and Chao, 1988). These data suggest that the *cis*-acting elements controlling transcription of the P_0 gene must be both cell-specific and inducible.

We have undertaken the isolation and analysis of the P_0 gene in order to address issues of structure, function, and regulation in detail. We find that the genomic organization of this gene supports our previously described model for the functional segregation and transmembrane orientation of P_0 protein domains. This organization also provides genetic evidence for the evolution of immunoglobulin domains from an ancestral half-domain and suggests that P_0 is among the most primitive of the immunoglobulin-related proteins. We describe methods for the identification of regulatory elements of myelin genes using nontransformed cultures of Schwann cells and demonstrate that for the P_0 gene these elements reside within a 1 kb region of DNA upstream of the coding sequences.

Results

Isolation and Structure of the Rat and Mouse P_0 Genes

We isolated clones encoding the rat and mouse P_0 genes by screening two genomic libraries with the full-length rat P_0 cDNA. These clones were then restriction mapped using synthetic RNA probes transcribed from selected regions of the rat cDNA (see Experimental Procedures). These mapping studies allowed us to determine the gross pattern of exon–intron segregation within both the rat and the mouse genes. We then used dideoxy sequencing methods to determine the precise structure of selected portions of the rat gene.

The results of these studies are presented in Figures 1 and 2. In contrast to the myelin basic protein gene (Takahashi et al., 1985), both the rat and the mouse P_0 genes

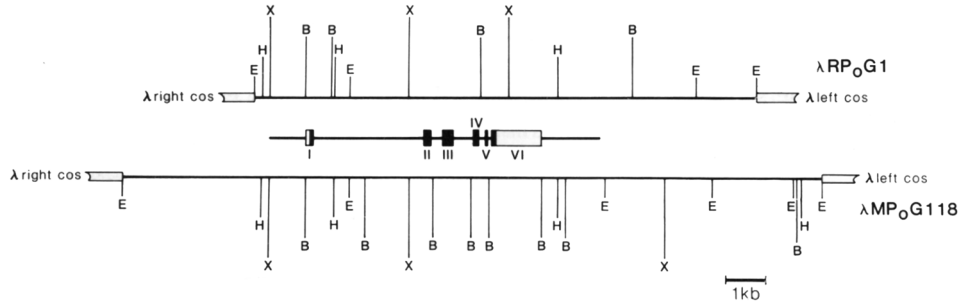

Figure 1. Restriction Map and Exon Segregation of the Rat and Mouse P_0 Genes

Charon 4A genomic clones containing the rat P_0 (λRP_0G1) and mouse P_0 (λMP_0G118) genes were restriction mapped with BamHI (B), EcoRI (E), HindIII (H), and XbaI (X) as described in Experimental Procedures. Roman numerals indicate exons I through VI, which are aligned to show correct spacing relative to the λRP_0G1 map. Approximately the same alignment holds for the λMP_0G118 map, although exon–intron boundaries for the mouse gene have not been sequenced. Solid black and solid white boxes indicate protein coding and untranslated sequences, respectively. For both clones, the 5′ end of the gene is ligated to the right phage arm.

are relatively small (<7 kb) and are carried on single λ phage clones. Each gene is interrupted by five introns, the first of which is much larger than the remaining four.

Comparison of the rat genomic and deduced protein sequences reveals that the segregation of exons within the P_0 gene is well correlated with the proposed segrega-

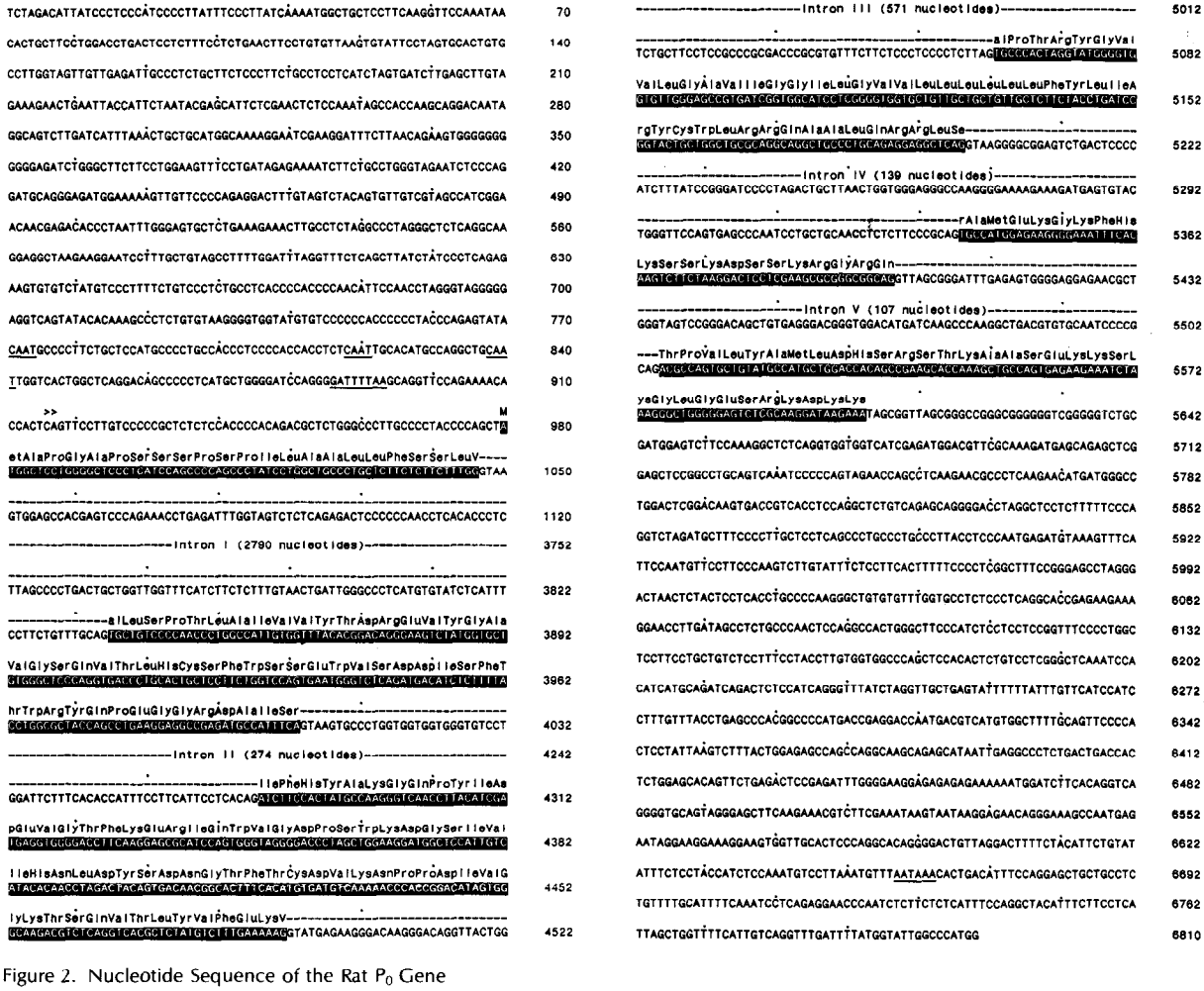

Figure 2. Nucleotide Sequence of the Rat P_0 Gene

The nucleotide sequence of indicated portions of λRP_0G1 was determined as described in Experimental Procedures. The sequence of exon-coding regions is displayed as white characters on black, with the deduced amino acid sequence of these regions printed above. Not displayed but determined are the complete sequences of introns II and III (available upon request). Underlined are three CAAT elements at nucleotides 771, 818, and 838, a presumptive TATA element (see text) at nucleotide 886, and a polyadenylation signal (AATAAA) at nucleotide 6659. The transcription start site of this cloned gene, as determined by primer extension (see text and Figure 4) is indicated (>>). Numbering begins with the 5′-most XbaI site indicated in Figure 1.

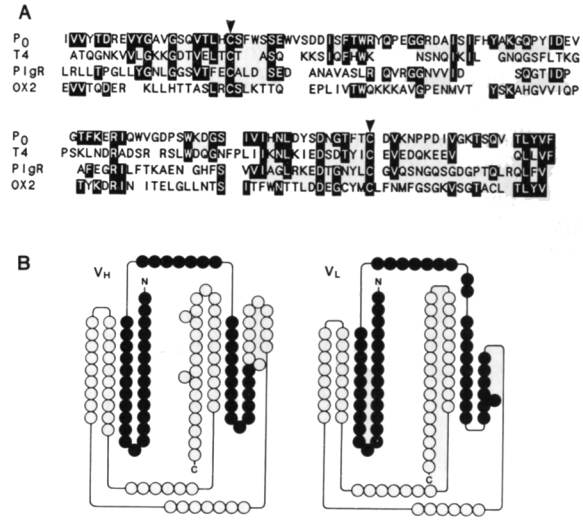

Figure 3. Immunoglobulin Relatedness of the P_0 Extracellular Domain

(A) Alignment of the amino acid sequence of the rat P_0 extracellular domain with the sequences of three related members of the immunoglobulin superfamily (see text). Alignments were performed and evaluated using the computer program ALIGN as described in Experimental Procedures. Matches between P_0 residues and those of aligned sequences are displayed as white characters on black. Gaps have been introduced in this figure to maintain the register between all four displayed sequences; individual pairwise alignments involve the introduction of fewer gaps. The paired cysteine residues characteristic of immunoglobulin domains are arrowed.

(B) Superposition of aligned amino acid residues encoded by exons II and III of the rat P_0 gene onto the secondary structure of the heavy (V_H) and light (V_L) chain variable region domains of human immunoglobulin Fab New (see text). This figure was generated by aligning the amino acid sequences of the Fab New V_H and V_L domains with the sequence of the P_0 extracellular domain. Shown are schematic diagrams of the V_H and V_L secondary structures in which filled and open circles represent immunoglobulin residues upstream and downstream, respectively, of the aligned position of the second P_0 intron. Antiparallel β-strands of the V_H and V_L domains are oriented vertically. N and C indicate the amino and carboxyl terminus of each domain. Adapted from Amzel and Poljak (1979).

tion of functional domains of the protein. Thus exon I encodes 5′-untranslated mRNA sequences together with most of the protein's amino-terminal signal sequence (residues −29 to −7), while exons II and III together encode the P_0 extracellular domain (residues −6 to 49 and 50 to 120). Similarly, exon IV encodes the transmembrane domain of the protein (residues 121 to 165), exon V a portion of the cytoplasmic domain (residues 166 to 186), and exon VI the remainder of this domain (residues 187 to 219) along with the complete 3′-untranslated region. Each of these exons is bounded by appropriate consensus splice sites (Mount, 1982), with the junctions between exons II and III and exons V and VI falling between codons. The boundary between the extracellular and membrane-spanning domains is delimited almost precisely by the intron separating exons III and IV. The boundary between the membrane-spanning and cytoplasmic domains is less clearly marked in the rat gene, in that 15 of the 69 amino acids of the latter domain are encoded in exon IV. The nucleotide sequence of the coding exons presented in Figure 2 is in exact agreement with our previously published sequence for the rat P_0 cDNA. The genomic organization of the six P_0 exons is consistent with the generally advanced hypothesis that the exons of eukaryotic genes often encode discrete functional units (Gilbert, 1978; Blake, 1978; Go, 1983).

The existence and location of the intron separating exons II and III are of particular interest with respect to the structure of the P_0 extracellular domain. In agreement with the observations of Lai and colleagues (1987), we find that this domain is significantly similar to certain members of the immunoglobulin gene superfamily (Williams, 1985; Hood et al., 1985) and consists of a single variable-like immunoglobulin domain. It carries nearly all of the essential skeletal features of prototypical immunoglobulin domains, including 2 cysteine residues, separated by 76 amino acids and presumed to be disulphide-bonded (Cammer et al., 1980), an intervening Arg-Asp salt bridge, and reiterated stretches of alternating hydrophobic residues. We have examined this similarity in detail by aligning the amino acid sequence of the P_0 extracellular domain with several immunoglobulin-related sequences. The three alignments that exhibited the greatest statistical similarity in a computer-assisted search (see Experimental Procedures) are illustrated in Figure 3A. These alignments indicate that the P_0 domain is most similar to the first variable region domain of the human T cell glycoprotein T4 (which serves as the receptor for the human immunodeficiency virus) (Maddon et al., 1986); domain 3 of the polyimmunoglobulin receptor (Mostov et al., 1984); and domain 1 of the brain–thymus antigen OX2 (Clark et al., 1985). Each of these comparisons yields alignment scores greater than 6.0 standard deviations above mean random scores, with amino acid identities in the range of 22%–29%. The values for the P_0–polyimmunoglobulin receptor alignment, for example, are 7.2 (alignment score) and 29% (residue matches after the introduction of seven gaps). These values are indicative of sequences that are distantly yet unambiguously related (see Experimental Procedures). Comparisons with several other members of the immnoglobulin superfamily, including Thy-1, the myelin-associated glycoprotein, and light and heavy chain immunoglobulin variable region domains, yield somewhat lower but nonetheless significant alignment scores. The degree of amino acid sequence identity and the spacing between disulphide-bonded cysteine residues (arrowed in Figure 3A) clearly place the P_0 extracellular domain among variable-like as opposed to constant-like immunoglobulin domains (Barclay et al., 1987).

In immunoglobulin and closely related genes, these domains are encoded by single exons (Evans, 1987). Both primary and secondary structure analyses, however, have suggested that immunoglobulin domains may have arisen from an ancestral half-domain that evolved through duplication and subsequent adjoining. One prediction of this scenario is that genes encoding more primitive immunoglobulin-related polypeptides might

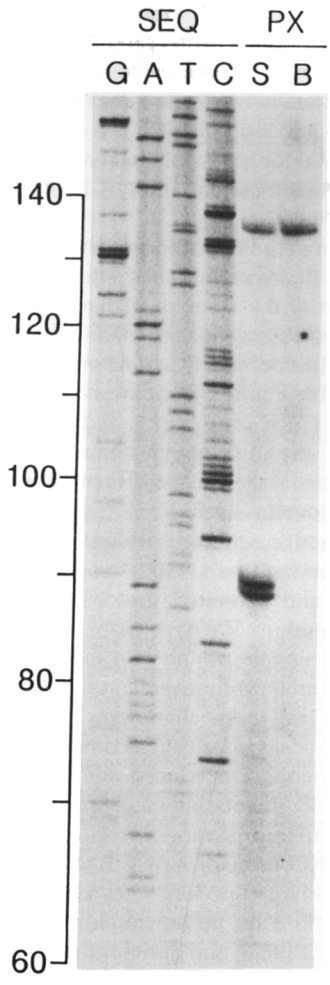

Figure 4. Primer Extension Mapping of the 5′ End of the Rat P_0 Gene

A radiolabeled oligonucleotide complementary to the 5′ end of the rat P_0 cDNA (Lemke and Axel, 1985) was hybridized to either sciatic nerve or BC_3H1 mRNA and then extended using reverse transcriptase as described in Experimental Procedures. Shown are the radiolabeled extension products as resolved on a denaturing polyacrylamide gel adjacent to the dideoxy sequencing ladder of a fragment whose sequence we have determined on both strands. SEQ, sequencing reactions; PX, primer extension products generated from sciatic nerve (S) or BC_3H1 (B) mRNAs. (BC_3H1 muscle cells do not express the P_0 message.) The scale at left indicates the length of resolved sequencing fragments in nucleotides.

retain remnants of these ancestral half-domains encoded in separate exons. As illustrated in Figure 2, the second intron of the rat and mouse P_0 genes splits sequences encoding the variable-like extracellular domain into two such exons. We have aligned the amino acid sequence of this domain with those of two human immunoglobulin variable region domains whose secondary structures have been solved (the heavy and light chain variable regions of Fab New) (Amzel and Poljak, 1979). The position at which the second P_0 intron would divide these aligned domains is illustrated schematically in Figure 3B. For both of these structures this intron divides the aligned variable region fold into two roughly equivalent subfolds (of four anti-parallel β-strands) joined by a connecting bridge. For the aligned light chain variable region this division is an exact one. Although the secondary structure of the P_0 extracellular domain has not been determined directly, we have observed that predictive algorithms (Chou and Fassman, 1978) yield remarkably comparable secondary structures when applied to the P_0 and human immunoglobulin Fab New amino acid sequences.

One additional, highly conserved feature of immunoglobulin-related genes is the pattern of RNA splicing between exons encoding individual immunoglobulin-related domains. This splicing occurs between the second and third nucleotide of the junctional codon for all known cases (Evans, 1987), a pattern that is broken in the P_0 gene. As illustrated in Figure 2, the splicing between P_0 exons III and IV occurs between the first and second nucleotides of the codon.

Boundaries of the P_0 Gene

In addition to addressing questions relating to protein structure, function, and evolution, isolation of the P_0 gene allows for analysis of the *cis*- and *trans*-acting elements that control its cell-specific expression. To pursue these studies, we first needed to determine the 5′ and 3′ limits of the gene. The latter can be inferred from the position of a consensus polyadenylation signal (AATAAA) at nucleotide 6659 (underlined in Figure 2). There are no other polyadenylation signals (Birnstiel et al., 1985) downstream of the P_0 stop codon, and poly(A) addition near nucleotide 6659 would generate an mRNA of approximately 2 kb, the size observed for the bona fide P_0 message (Lemke and Axel, 1985). We determined the 5′ end of the P_0 gene by primer extension, using reverse transcriptase to extend a radiolabeled synthetic oligonucleotide primer (complementary to nucleotides 976–1006 in Figure 2) following hybridization to poly(A)$^+$ RNA isolated from either rat Schwann cells or the BC_3H1 muscle cell line. The extension products were resolved on a denaturing polyacrylamide gel, and their lengths were measured relative to the migration of an adjacent dideoxy sequencing ladder. The results of this analysis are illustrated in Figure 4. Two major extension products of 89 and 90 bp are observed. Given the length and sequence position of the oligonucleotide primer, these products mark a stuttered 5′ cap site 63 and 64 nucleotides upstream of the initiator ATG. These positions correspond to the dinucleotide CA, a common site for the initiation of RNA polymerase II transcripts (Breathnach and Chambon, 1981). We have isolated three independent rat P_0 cDNAs that extend to within 5 nucleotides of these positions. The sequence at the 5′ ends of these cDNA clones is colinear with that of the cloned rat P_0 gene, thus excluding the possibility of an additional intron interrupting genomic sequences encoding 5′-untranslated RNA. In addition to the 89/90 bp primer extension product, a second, longer product is also present in the gel shown in Figure 4. This product is also observed with control mRNA isolated from BC_3H1 muscle cells, which do not express the P_0 message (Lamar and

Lemke, unpublished data), and must therefore result from hybridization of the oligonucleotide primer to a ubiquitous non-P_0 RNA.

Identification of the 5′ end of the rat P_0 gene has allowed us in turn to identify several intriguing sequence similarities between the upstream and 5′-untranslated regions of this gene and corresponding regions from the other major myelin genes, those encoding myelin basic protein (MBP) and the proteolipid protein (PLP). The P_0 and MBP genes are coordinately regulated in Schwann cells in the PNS, whereas the PLP and MBP genes are coordinately regulated in oligodendrocytes in the CNS (Lemke, 1986). The most extensive stretch of sequence similarity (19 identities out of 25 nucleotides) is found within the 5′-untranslated regions of both the P_0 and MBP genes (Figure 2, nucleotides 941–965):

P_0 **ACCCCACAG**ac**GCTC**t**GG**gcc**CTTG**
MBP **ACCCCACAG**ca**GCTC**c**GG**agg**CTTG**

In addition, we note that the sequence at and around the transcription start site of the rat P_0 gene (TCAG) is the same as that determined for the mouse MBP gene (Takahashi et al., 1985) and estimated for the rat PLP gene (Milner et al., 1985). The upstream regions of neither the P_0 gene nor the MBP gene exhibit a conventional TATA box (Corden et al., 1980). The TA-rich element that is likely to serve this function for the P_0 gene (GATTTTAA) is located at position −30 to −23 (nucleotides 886–893, underlined in Figure 2). The sequence around this region is also similar to what are thought to be the equivalent elements of the mouse MBP and human PLP genes and is identical to the TATA box known to regulate the transcription of several major histocompatibility genes, including the murine E_α gene (Dorn et al., 1987). The P_0 upstream region contains three CAAT sequences positioned 78, 98, and 145 nucleotides upstream of the transcription start site, none of which are canonical (Jones et al., 1987). An inverted canonical sequence is present on the noncoding strand 72 nucleotides upstream of the start site. The CAAT sequence at −98 is preceded by a 9 bp element (GCCACCCTC, nucleotides 797–805 in Figure 2), which is similar to a critical upstream promoter element similarly situated in the mouse β major globin gene (Myers et al., 1986). This element is in turn preceded by two nearly identical direct repeats of 10 bp each. Although we have found that Schwann cell expression of the P_0 gene is strongly potentiated by elevated levels of intracellular cAMP (Lemke and Chao, 1988), the cAMP response element identified by Montminy and colleagues (1986) is not present within the 1 kb of DNA preceding the P_0 transcription start site.

Schwann Cell Expression of the P_0 Gene

Ideally, one would like to be able to assess directly the functional relevance of each of the above sequences with respect to the cell specificity, inducibility, and coordinate regulation of the genes they may control. This goal has in general proven elusive for the large number of specialized genes expressed by eukaryotic cell types whose growth in vitro is limited by availability or culture conditions. For those genes expressed by Schwann cells, recent advances in cell biology have overcome these limitations. These cells can be prepared as an essentially pure population that, upon the addition of appropriate mitogens, can be expanded at will (Brockes et al., 1979; Lemke and Brockes, 1984; Porter et al., 1986). We have found that these purified cells can be maintained as a dividing population in a nontransformed state for many months in culture and can be frozen in liquid nitrogen and thawed with >90% viability.

In attempting to identify *cis*-acting elements that control the Schwann cell–specific expression of the P_0 gene, we first analyzed the activity of DNA segments located immediately upstream of the transcription start site. These segments were cloned into the plasmid pSVO-CAT (Gorman et al., 1982) adjacent to the bacterial chloramphenicol acetyltransferase (CAT) gene and transfected into Schwann cells (see Experimental Procedures). As a control for variations in transfection efficiency, a second plasmid containing the bacterial β-galactosidase (βgal) gene linked to the Rous sarcoma virus promoter (pRSV-βgal) (Edlund et al., 1985) was cotransfected into each experimental plate. Sixty hours following transfection, cells were harvested and assayed for βgal activity. βGal equivalents of each transfection extract were then assayed for CAT activity (see Experimental Procedures).

Figure 5 illustrates the result of introducing constructs in which three different pairs of upstream segments of the rat P_0 gene are linked to the CAT gene. Two of these construct pairs (HA5/16 and XA2/6) contain fragments whose 3′ ends are within sequences encoding 5′-untranslated RNA (Figure 2, the ApaI site at nucleotide 957). These fragments extend 5′ to either a HindIII site (HA5/16) or an XbaI site (XA2/6) 1092 and 915 bp upstream of the transcription start site, respectively. The third pair of CAT constructs (HB19/27) contains a fragment whose 3′ end is 39 bp upstream of the transcription start site (Figure 2, the BamHI site at nucleotide 877) and that extends 5′ to the upstream HindIII site. These paired constructs, which correspond to sense and antisense orientations relative to the CAT gene, are diagrammed in Figure 5A. Their activity profile upon transfection into Schwann cells is presented in Figure 5B.

This profile demonstrates that the HA16 and XA6 segments are potent stimulators of CAT transcription when introduced into Schwann cells. These constructs, which contain the identified CAAT and TATA elements along with approximately two-thirds of the MBP-homologous 5′-untranslated element, routinely generate levels of CAT activity 30- to 80-fold above those of the promoterless reference plasmid pSVO-CAT. This activity is comparable to that generated by the strong promoters of the SV40 and Rous sarcoma viruses (Gorman et al., 1982) (see Figure 5B), an effect consistent with the high level of endogenous P_0 expression in Schwann cells. While both the HA16 and the XA6 constructs are transcriptionally active, their inverted partners, HA5 and XA2, do not

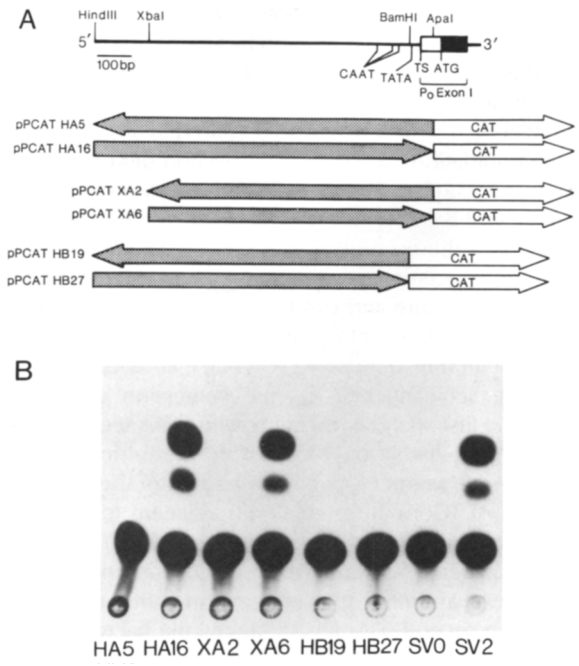

Figure 5. Structure and Transcriptional Activity of P_0 Promoter Constructs

(A) Indicated regions of the rat P_0 gene (λRP_0G1) were subcloned in both orientations upstream of the bacterial CAT gene at the HindIII cloning site of pSVO-CAT (Gorman et al., 1982). The upper P_0 gene map is in exact alignment with the lower construct diagrams. (B) Activity profile of the CAT fusion constructs diagrammed in (A). Constructs were transfected into cultured Schwann cells, and CAT activity was assayed as described in Experimental Procedures. HA5/16, XA2/6, and HB19/27 refer to the plasmids diagrammed in (A). SVO, promoterless pSVO-CAT reference plasmid; SV2, pSV2-CAT (Gorman et al., 1982), a plasmid in which the bacterial CAT gene is driven by the SV40 early promoter. All transfections were performed in medium containing 4 μM forskolin (see text).

exhibit any activity above that of the SVO reference plasmid. The third segment tested (HB19/27) has its 3′ terminus immediately upstream of the presumptive TATA element (thereby also excluding the MBP-related element). This segment is completely devoid of transcriptional activity in either orientation (Figure 5B).

The expression of these hybrid constructs is Schwann cell–specific to the extent that we have not observed any transcriptional activity when they are introduced into seven different glial and nonglial cell lines (Table 1). This specificity is particularly striking in the case of the Rat2 and NIH3T3 cell lines, in light of the fact that these fibroblast lines have been found to express a variety of transfected genes that are not expressed endogenously. Since Schwann cells develop from the neural crest, we also transfected cells lines derived from tumors of two other crest derivatives, melanocytes and chromaffin cells. Neither B78H1 melanoma cells (Graf et al., 1984) nor PC12 pheochromocytoma cells (Greene and Tischler, 1976) support transcription from the HA16 or XA6 promoter segments (Table 1).

As noted above, Schwann cell expression of the major myelin genes is dependent upon contact with neuronal axons. Experiments in vitro have suggested that this dependence may in part derive from the ability of axons to raise intracellular levels of cAMP: addition of a low concentration (1 μM) of forskolin, a specific and reversible activator of adenylate cyclase (Seamons et al., 1981), results in a 10-fold increase in steady-state P_0 mRNA levels in Schwann cells cultured in the absence of axons (Lemke and Chao, 1988). The HA16 and XA6 hybrid constructs show a very similar cAMP dependence. Schwann cells transfected with these constructs and cultured in the absence of forskolin do not express appreciable levels of CAT activity (Table 1). This effect is most clearly seen in the relative activities of the P_0 and SV40 promoters. As illustrated in Figure 5 and Table 1, these promoters show roughly comparable activities when introduced into Schwann cells cultured in the presence of forskolin. Although the activity of both promoters is reduced in cells withdrawn from forskolin, the magnitude of this reduction is 7- to 10-fold greater for the P_0 promoter than for its SV40 counterpart (Table 1). Taken together, these data indicate that we have delineated a set

Table 1. Cell Specificity and Inducibility of the P_0 Promoter

		HA16/SVO		SV2/SVO	
Cell Line	Cell Type	−F	+F	−F	+F
PC12[a]	Rat pheochromocytoma	0.7	0.4	14.9	28.2
B78H1[a]	Mouse melanoma	ND	1.4	ND	162.9
C6[a]	Rat CNS glioma	ND	2.0	ND	152.0
BC_3H1[b]	Mouse muscle	1.2	1.6	21.2	24.9
NIH 3T3[b]	Mouse fibroblast	0.8	0.7	44.1	50.0
Rat2[a]	Rat fibroblast	ND	1.1	ND	63.7
RSC[a]	Rat Schwann cells	2.2	71.8	20.3	86.6

The SV40 promoter is active in all cell lines transfected, but the P_0 promoter is active only in Schwann cells cultured in the presence of forskolin. CAT activity ratios were calculated from the combined radioactivity present in the 1-acetyl and 3-acetyl [^{3}H]chloramphenicol spots of thin layer chromatograms, as determined by liquid scintillation counting, or densitometric scans of autoradiograms. Each cell line was transfected with pPCATHA16 (P_0 promoter), pSV2-CAT (SV40 early promoter), or pSVO-CAT (no promoter) in the presence (+F) or absence (−F) of 4 μM forskolin. Variation in the SV2:SVO ratio between negative cell lines reflects cell-specific variation in the basal level of CAT expressed by pSVO-CAT transfectants, transfection efficiency, and expression level of the SV40 promoter.

ND, not determined.

[a] CAT activity determined by liquid scintillation counting.

[b] CAT activity determined by densitometric scans of autoradiograms.

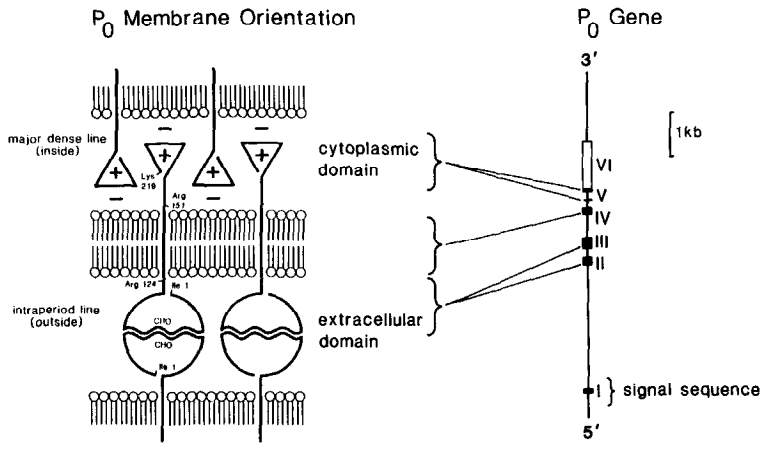

Figure 6. Schematic Representation of the Segregation of P_0 Protein Domains with Exons of the P_0 Gene

For this diagram, the rat P_0 gene map has been aligned with a representation of P_0 protein orientation in the peripheral myelin membrane. In agreement with biochemical and sequence data, the P_0 protein is depicted as spanning this membrane once, with the amino terminus positioned extracellularly. The extracellular domain is depicted as bringing about extracellular membrane adhesion through homotypic interactions, and the cytoplasmic domain is depicted as promoting intracellular membrane adhesion through electrostatic protein–lipid interactions. (Biochemical and genetic evidence supports the hypothesis that these domains function as adhesion molecules, but there is no direct evidence to support the mechanisms depicted.) The membrane stacking shown is repeated many times in the peripheral myelin membrane.

of *cis*-acting elements that regulate the cell-specific and inducible transcription of the rat P_0 gene.

Discussion

The Structure, Function, and Evolution of P_0

P_0 has been hypothesized to function as a cell adhesion molecule during the elaboration and compaction of the peripheral myelin sheath (Braun, 1984; Lemke and Axel, 1985). This compaction must be achieved at two distinct membrane appositions. The first of these corresponds to the joining of apposed cytoplasmic membrane faces and results in what structural biologists have termed the major dense line of myelin. Compaction at this apposition is probably achieved through the combined action of MBP and the cytoplasmic domain of P_0. The second region of myelin compaction corresponds to the joining of apposed extracellular membrane faces to form the myelin intraperiod line. In the PNS, this joining is hypothesized to result from hydrophobic, homotypic interactions between P_0 domains expressed at the extracellular surfaces of apposed myelin lamellae (Lemke and Axel, 1985). This mechanism is analogous to that utilized by the neural cell adhesion molecule (N-CAM) and other cell adhesion proteins (Edelman, 1983).

Our analysis of the structure of the P_0 gene supports and extends this hypothesis in several respects. The hypothesized configuration of the P_0 protein within the myelin membrane – a single extracellular domain of 124 amino acids, a single membrane-spanning domain of 26 amino acids, and a single cytoplasmic domain of 69 amino acids – is generally consistent with the segregation of exons within the rat and mouse P_0 genes (Figure 6). Most importantly, our results demonstrate that the P_0 extracellular domain is homologous to the variable region fold of immunoglobulins, a property shared by a growing number of neuronal and glial cell surface adhesion molecules (Lai et al., 1987). Of the three members of the immunoglobulin superfamily to which the P_0 extracellular domain is most similar, two – the polyimmunoglobulin receptor and the T4 protein – function as binding or adhesion molecules. The polyimmunoglobulin receptor is a protein that transports immunoglobulin molecules across epithelial cells and is thought to bind IgA and IgM via interactions between the immunoglobulin domains present on both the receptor and the antibody molecules it recognizes (Mostov et al., 1984). The glycoprotein T4 is a T cell surface antigen thought to function as an adhesion molecule by interacting with the immunoglobulin-related major histocompatibility class II antigens of macrophages (Gay et al., 1987). (The function of the OX2 antigen is not known.) The fact that these two proteins function as recognition molecules in which immunoglobulin-related domains interact with immunoglobulin-related domains provides support for the hypothesis that the extracellular domain of P_0 functions by recognizing itself rather than through direct interaction with lipids of the apposed myelin membrane.

The position of P_0 within the immunoglobulin superfamily is unique. It is the only member of this family that contains a single immunoglobulin domain and is hypothesized to function through self-recognition. These are the properties one would predict for a structure whose gross features are minimally diverged from those of the primordial recognition molecule from which both the immune and the nervous systems are thought to have evolved (Evans, 1987). This notion is supported by the structure of the P_0 gene, in which sequences encoding the variable-like extracellular domain are split between two exons. Until recently, all immunoglobulin-related domains have been found to be completely encoded within single exons. This has proven to be the case both for simple, single domain structures such as Thy-1 (Ingraham et al., 1986) and for complex, multi-

domain structures such as immunoglobulins (Honjo, 1983). However, the split exons of the P_0 gene suggest that these structures may not have evolved from a single ancestral domain, but rather from the duplication and joining of an ancestral half-domain. This hypothesis has been advanced previously on the basis of amino acid sequence similarity between the two halves of heavy chain variable region domains (Bourgois, 1975) and from consideration of the bilaterally symmetric secondary structures of the immunoglobulin domains of immunoglobulins and major histocompatibility complex antigens (Amzel and Poljak, 1979). We propose that the second intron of the P_0 gene would split these secondary structures almost precisely into two symmetric subfolds (Figure 3B).

A partitioning of the immunoglobulin fold has also been observed for exons encoding each of the immunoglobulin-related domains of N-CAM (Owens et al., 1987), a protein whose multi-domain structure appears to be intermediate between P_0 and the more complex immunoglobulins. Similarly, Littman and Gettner (1987) and Maddon and colleagues (1987) have observed an intron interrupting sequences encoding the first of the four variable-like domains of the T4 protein. The position of the T4 intron is similar to the position of the second P_0 intron: in the aligned sequences shown in Figure 3A, this T4 intron occurs 11 amino acid residues downstream of its P_0 counterpart. Two of the five introns interrupting N-CAM immunoglobulin domains are also positioned similarly to the P_0 intron, although the remaining introns (those interrupting domains 1, 3, and 5) exhibit wide positional variation. This variation could be accounted for by an intron sliding mechanism (Marchionni and Gilbert, 1986; McKnight et al., 1986; Craik et al., 1983), and does not in itself argue against the evolutionary scenario we propose. Littman and Gettner (1987) have advanced an alternative model for the evolution of immunoglobulin related proteins based on the suggestion that the first immunoglobulin-related domain of T4 may have evolved from the insertion of intron sequences into a single, preexisting variable-like exon. Our observation of a similarly positioned intron in the gene encoding a far simpler member of the immunoglobulin superfamily makes this possibility less likely.

The Regulation of P_0 Gene Expression

A second important feature of the evolution of the P_0 gene must have involved the restriction of its expression to myelin-forming cells and eventually to myelin-forming cells in the PNS (Tai and Smith, 1984). Although a priori one might imagine several mechanisms that could account for such a restriction, the transfection studies presented above demonstrate that *cis*-acting elements of this gene play a key role in controlling the specificity and inducibility of its expression. Our identification of the P_0 regulatory region and demonstration that nontransformed Schwann cells provide a workable transfection host will allow for a detailed analysis of these myelin regulatory elements.

The results of our first assays of the P_0 promoter region are generally consistent with the organization and endogenous expression of the P_0 gene. Our identification of the transcription start site of this gene and of an upstream TATA element, for example, are supported by the failure of the HB27 construct to drive Schwann cell–specific transcription. Similarly, the orientation dependence of the HA16 and XA6 constructs almost certainly reflects their inclusion of this TATA element. The robust transcriptional activity, Schwann cell specificity, and cAMP dependence of these constructs accurately mirror the regulatory behavior of the endogenous gene.

Inspection of the P_0 promoter sequence reveals a relative lack of generic transcription elements: complete copies of the AP1 (Lee et al., 1987), MTLF (Chodish et al., 1986), SP1 (Briggs et al., 1986), NFIII (Sen and Baltimore, 1986), AP2 (Mitchell et al., 1987), and AP3 (Johnson et al., 1987) elements are not present on either strand. An incomplete representation of the AP2 element (6 out of 7 nucleotides) is present on the noncoding strand at nucleotides 401–407. This sequence has recently been shown to mediate cAMP regulation of the human metallothionein II_A gene (Imagawa et al., 1987). A similarly incomplete version of the AP3 element is present at nucleotides 207–215 and 733–741. As noted above, the P_0 promoter lacks the core regulatory element (TGACGTCA) described for a variety of cAMP-responsive genes (Montminy et al., 1986). Although small fragments of this sequence or its opposite strand complement can be identified within the promoter, it seems likely that cAMP regulation of the P_0 gene involves the action of novel cAMP-responsive elements.

An important outstanding question concerns the extent to which these and other P_0 regulatory elements are utilized in the regulation of the other myelin-specific genes—those encoding MBP, PLP, the myelin-associated glycoprotein (Lai et al., 1987), etc.—and of those non-myelin-specific genes coordinately up-regulated during myelination (e.g., the HMG-CoA reductase gene) (Lemke and Axel, 1985). The transfection procedures we have developed should permit us to address this question directly and to determine why certain myelin genes are restricted to either central or peripheral glia while others are not. The 5′-untranslated element shared between the P_0 and MBP genes, for example, is a good candidate for an element conveying PNS specificity in that it is not present in the PLP gene. This element could conceivably play a role in either the induction or specificity of gene transcription, as is the case for similarly situated elements in Drosophila heat shock and viral genes, or in the regulation of translation.

Axons exert a dramatic and general effect on Schwann cell gene expression in addition to their effect on expression of the major myelin genes. Plastic expression of the NGF and NGF receptor genes, for example, has been found to be a function of axons, the withdrawal of which activates expression of these genes (Taniuchi et al., 1986; Lemke and Chao, 1988). The identification of myelin regulatory elements should therefore permit us to deter-

mine the extent to which these and related elements play a role in axonal regulation in general.

Experimental Procedures

RNA Isolation

RNA was isolated by LiCl precipitation of guanidinium thiocyanate homogenates as described by Cathala et al. (1983). Poly(A)$^+$ RNAs were enriched for by oligo(dT) cellulose chromatography (Aviv and Leder, 1972).

Genomic Library Screening

Two λ Charon 4A genomic libraries were screened for P_0 sequences: a partial EcoRI library prepared from rat liver DNA (Sargent et al., 1979) and a partial HaeIII/EcoRI-linkered library prepared from mouse myeloma DNA (Seidman and Leder, 1978). Both libraries were screened with ^{32}P-labeled synthetic SP6 RNA probes (Melton et al., 1984) of the complete SN63 P_0 cDNA (Lemke and Axel, 1985). Filter hybridizations were performed in 5× SSPE, 0.2% SDS, 10% dextran sulfate, 15% formamide, at 65°C. Screening of 6×10^5 clones from each library resulted in the isolation of 6 rat and 5 mouse P_0 clones. All rat and all mouse clones were identical as judged by EcoRI restriction maps, with the exception of 5′ or 3′ additions. Restriction digests of DNA from these clones were blotted and probed with synthetic RNA probes corresponding to a 5′-specific EcoRI–ClaI fragment of the rat P_0 cDNA, a 3′-specific NcoI–EcoRI fragment of the same cDNA, or the complete cDNA (see cDNA restriction map in Lemke and Axel, 1985). For mapping of the mouse gene, a BamHI fragment specific for exon I of the rat gene (Figure 2, nucleotides 876–1506) was also used.

DNA Sequence Analysis

Selected regions of rat P_0 genomic clone λRP$_0$G1 were subcloned into M13mp18/19 (Yanisch-Perron et al., 1985). DNA sequences were determined on both strands by the dideoxy chain termination method (Sanger et al., 1977).

Amino Acid Sequence Comparisons

The amino acid sequences used for similarity comparisons with the P_0 extracellular domain were obtained from the National Biomedical Research Foundation database or were entered directly from published data. Sequence alignments were generated using the computer programs ALIGN (Dayhoff et al., 1979) and IFIND/ALIGN (Intelligenetics). Both programs generated similar alignments. For comparisons employing the ALIGN program, similarities were scored using the mutation data matrix (MDM) established by Dayhoff and colleagues (1979), with a matrix bias parameter of 6 and a gap penalty of −6. An alignment score was obtained for each comparison performed. This score corresponds to the number of standard deviations that a given total score exceeds the mean total score generated by 100 repeated comparisons of randomly permuted versions of the aligned sequences. Using this procedure, alignment scores greater than 6 indicate that the probability of aligned sequences being related by chance is less than 1×10^{-9} (Dayhoff et al., 1983).

Primer Extension

A 30-mer oligonucleotide complementary to the 5′ end of the rat P_0 cDNA (Figure 2, nucleotides 976–1006) was synthesized on a Systec oligonucleotide synthesizer. This oligonucleotide was end-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, hybridized (at 65°C for 75 min, followed by slow cooling to room temperature over 3 hr) to 2 μg of poly(A)$^+$ RNA isolated from either 2 day rat sciatic nerve or BC_3H1 muscle cells, and then extended with unlabeled deoxynucleotides and AMV reverse transcriptase at 37°C for 1 hr according to standard procedures (Epstein et al., 1986). Extended products were resolved on an 8 M urea, 6% polyacrylamide gel adjacent to a dideoxy sequencing ladder read from a BamHI fragment of clone λRP$_0$G1 (Figure 2, nucleotides 876–1506) subcloned into M13mp18.

Schwann Cell Culture and Transfection

Schwann cells were dissociated from neonatal rat sciatic nerves and purified by immunoselection (Brockes et al., 1979). Purified cells were triggered to divide by the addition of glial growth factor (Lemke and Brockes, 1984) and 2 μM forskolin as described by Porter and colleagues (1986). Three weeks after the addition of mitogens, approximately 1.5×10^8 cells were frozen in liquid nitrogen (in aliquots of 3×10^6 cells) for subsequent use in transfection experiments. Transfections were performed using 1×10^6 cells per 5 cm dish. DNAs (5–10 μg) were precipitated according to standard calcium phosphate procedures (Graham and Van der Eb, 1973) and applied to cells in 4 ml of culture medium for 4 hr. (We have found that purified Schwann cells can also be readily transformed by DEAE-dextran procedures, although cell viability following transfection is substantially reduced.) These cells were washed once, the culture medium was replaced, and the cells then grown for an additional 60 hr. During these manipulations, we found that it was critical to maintain the pH of culture media below 8.0 to avoid Schwann cell death. Three micrograms of a plasmid composed of the Rous sarcoma virus promoter linked to the bacterial βgal gene (Edlund et al., 1985) was cotransfected into each experimental plate to normalize for variations in transfection efficiency. After 60 hr, cell extracts were prepared as described by Gorman and colleagues (1982). The protein concentration of each extract was estimated by the method of Lowry et al. (1951), and a constant amount of extracted protein (usually 20 μg) was then assayed for βgal activity by standard procedures (Norton and Coffin, 1985). βGal equivalents of each extract were subsequently assayed for CAT activity by the procedures of Gorman and colleagues (1982), except that the incubation time for the assay was extended to 2 hr. For transfections performed in the absence of forskolin, frozen cells were plated in medium lacking the drug and cultured for 2 days prior to use. (Forskolin-mediated increases in intracellular cAMP levels are reversed upon withdrawal of the drug [Seamons et al., 1981].) The CAT plasmids described in Figure 5 were prepared by excising the indicated regions from λRP$_0$G1, blunt-ending the excised fragments with T4 DNA polymerase, and then subcloning the blunt-ended fragments into the HindIII cloning site of pSVO-CAT using HindIII linkers.

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Note Added in Proof

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