Multiple Regulatory Elements Control Transcription of the Peripheral Myelin Protein Zero Gene*

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The gene encoding protein zero (P0), the most abundant protein of peripheral nervous system myelin, is expressed uniquely in Schwann cells. Previous studies have demonstrated that much of the cell type specificity of this expression is due to transcriptional control elements in the 1.1-kilobase pair 5′-regulatory region of the gene. We have now analyzed this region and have identified a set of functional elements in the 500 base pairs proximal to the transcription start site. DNA sequence conservation within the 5′ regions of the human, mouse, and rat P0 genes correlates closely with the results of promoter deletion analysis of the 1.1-kilobase pair region assayed in Schwann cell cultures and reveals a potent proximal region from position −350 to +45. Sites of protein/DNA interaction within the proximal 500 base pairs of the promoter were identified by footprinting assays. Functional transcriptional elements were identified within the protected regions in the proximal promoter by mutation and transient transfection analysis in P0-expressing cell lines. The core (or basal) P0 promoter is identified as two regulatory elements, a GC-rich element that binds nuclear factor Sp1 and a CAAT box that binds NF-Y. These core elements are essential for the transcription observed from the transfected promoter in cultured Schwann cells.

The myelin sheath is a specialized membranous organelle of the vertebrate nervous system. Elaborated by oligodendrocytes in the central nervous system and by Schwann cells in the peripheral nervous system (PNS), this organelle consists of a large sheet of plasma membrane that is repeatedly wrapped and very tightly compacted around axons (1). Myelin is essential for the rapid conduction of action potentials in vertebrates, and human diseases that compromise the integrity of the sheath (e.g. peripheral neuropathies in the PNS and multiple sclerosis in the central nervous system) are generally debilitating (2). The elaboration of myelin requires a substantial biosynthetic up-regulation of plasma membrane lipids and proteins, several of which are unique to the myelin sheath (3). In the PNS, the most abundant of these is protein zero (P0), a 31-kilodalton, immunoglobulin-related, transmembrane glycoprotein that accounts for greater than 50% of the protein in mammalian PNS myelin (4). The mRNA encoding this protein is estimated to account for nearly 8% of the poly(A) RNA in actively myelinating Schwann cells (5).

A variety of studies have demonstrated that P0 is essential for peripheral nerve development and function. Mice in which the P0 gene has been deleted exhibit a severe peripheral neuropathy that includes hypomyelination, loss of motor control, tremors, and grossly abnormal myelin sheaths (6, 7). Furthermore, mutations in the P0 gene account for a variety of inherited human peripheral neuropathies, including Charcot-Marie-Tooth disease type 1B, Dejerine-Sottas syndrome, and congenital hypomyelination (8–12).

High level P0 expression is a distinctive feature of terminal differentiation in myelinating Schwann cells. Expression of P0 mRNA peaks during the period of active peripheral myelination (the first 3 postnatal weeks in rodents) and is maintained at lower steady-state levels into adulthood (5, 13). During Schwann cell development, the P0 gene is coordinately induced together with genes encoding other myelin-specific proteins, such as myelin basic protein, PMP-22, and myelin-associated glycoprotein (14). In contrast to these proteins, however, P0 is not expressed by oligodendrocytes in the central nervous system. The pronounced up-regulation of P0 biosynthesis observed at the onset of overt myelination appears to be triggered by a signal associated with the surface of axons (15, 16). This signal may be transduced intracellularly through elevation of cyclic AMP: in cultured Schwann cells, cAMP-elevating agents such as forskolin potentiate P0 gene expression and strongly potentiate expression of less abundantly expressed myelin-specific genes (16, 17).

P0 regulation during Schwann cell development reflects a combination of transcriptional and translational controls, although the former appears to predominate. Cell culture and transgenic mouse studies have demonstrated that major features of this regulation are controlled by a 1.1-kilobase pair region flanking the transcription start site of the P0 gene. When linked to heterologous reporter genes, this 1.1-kilobase pair DNA fragment drives Schwann cell-specific, forskolin-inducible transcription upon transient transfection into cultured Schwann cells (18) and has been shown to target a number of transgenes exclusively to myelinating Schwann cells, on an appropriate developmental schedule, in transgenic mice (19, 20). The 5′-DNA region therefore contains many if not most of the elements required for tissue-specific and developmentally accurate P0 expression. To determine the cis-acting regulatory elements and transcription factors that control this expression, we examined protein/DNA interactions and promoter function of the P0 5′-regulatory region in rat Schwann cells. An array of protected regions was detected on the proximal promoter, and among these a region of cell type-specific differences between P0-expressing and nonexpressing cell lines was noted. Muta-

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† The abbreviations used are: PNS, peripheral nervous system; P0, protein zero; bp, base pair(s); CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus; DMS, dimethyl sulfate; C/EBP, CAAT enhancer-binding protein.
transcriptional analysis of sequences within DNase I-protected regions revealed multiple functional elements, each of which is responsible for a different level of transcription. The core promoter is defined as the two proximal elements located within the protections at positions −48 to −59 and −66 to −79, which bind nuclear factors Sp1 and NF-Y, respectively.

EXPERIMENTAL PROCEDURES

Plasmids—The P0 gene, 5′-regulatory sequences, and the P0 CAT plasmids pPCATX6a and pPCATHA16 have been previously described (18). The P0−2 promoter plasmids from −915 to +45 were generated from the pPCATX6 plasmid by BglII digestion, and the 5′ end was confirmed by sequencing. The P0−2 luciferase plasmid, −915P0LUC, was generated by cloning the −915 to +45 P0 promoter fragment (obtained by digesting pPCATHA16 (Ref. 18) with XbaI and HindIII) into the NheI and HindIII sites of the promoterless luciferase reporter vector, pGL2-B (Promega).

Cell Cultures—Rat Schwann cell cultures were prepared and maintained in media with forskolin and glial factor as described previously (21). Briefly, Schwann cells were prepared from 3–4-day-old Sprague-Dawley rat sciatic nerves. Cells were treated by anti-thy 1 and complement lysis to remove contaminating fibroblasts. Schwann cells were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum, penicillin, and streptomycin; 2 μM forskolin; and either 13 μM 2′-bromo-2′-deoxyuridine for 5 days or 10 ng/ml recombinant neuregulin (kind gift of Dr. Kuo-Fen Lee). Bovine pituitary extracts were prepared by methods previously described (22). The rat cell line B103 (23) is a P0-positive transformed rat Schwann cell line that expresses Schwann cell properties (24). B103, HeLa, and the fibroblast cell line Rat2 were maintained in Dulbecco’s modified Eagle’s medium with 10% fetal bovine serum and antibiotics.

Footprinting—DNase I footprinting assays were performed using −915P0LUC plasmid to generate radiolabeled probes of the proximal P0 promoter (−550 to +45) using published protocols (25). The P0 promoter was first digested with HindIII and XbaI and used to produce footprints most proximal to the transcription start site (proximal probes). The proximal, noncoding strand probe was made by end labeling with kinase and [γ-32P]ATP, and the proximal, coding strand probe was made by fill in, using Klenow and [α-32P]dATP. The DNA was then digested with BglII and the 600-bp fragment was purified by polyacrylamide gel electrophoresis. The distal P0 probes were generated by digestion with BglII to generate the 5′-end of the probe and radiolabeling as above and then digestion with HindIII and purification by gel electrophoresis. Nuclear extracts were prepared from subcultured cell lines as described methods (26). Rat Schwann cell cultures were induced to high levels of P0 expression with 20 μM forskolin for 2 days before extracts were made. The footprinting assay was performed by incubating radiolabeled probe with nuclear extracts for 10 min and digesting for 1 min on ice with DNase I. Appropriate dilutions of DNase I were predetermined for both probe alone and probe with various nuclear extracts. The reaction products were separated on 6% polyacrylamide sequencing gels using wedge spacers. The results were visualized by autoradiography.

In vivo DMS footprinting assays were performed in Schwann cell cultures treated with 2 days with 20 μM forskolin utilizing the ligation-mediated polymerase chain reaction method as described previously (27, 28). Briefly, the procedure was as follows. Schwann cell cultures were treated with dimethyl sulfoxide (DMS), in parallel with untreated cultures, and the methylated genomic DNA was isolated and purified of protein. The untreated DNA was DMS-treated in vitro, and both samples were piperidine-cleaved. These DNA fragments were amplified by ligation-mediated polymerase chain reaction and radiolabeled with [γ-32P]ATP using sets of three nested P0-specific primers. DMS-treated samples were electrophoresed on 6% sequencing gels. The primers for the coding strand were as follows: primer 1, 5′-CTGGGCTAAGGGGCAAGG; primer 2, 5′-TGGGAGAGGAGGGGGGACAGG; primer 3, 5′-GGGGTGGAGAGGAGGGGGGCAAGGAC. The primers for the noncoding strand were as follows: primer 1, 5′-ACAATGCCCCCTTCGTCT; primer 2, 5′-CTGGCCACCCCTCCCAAC; primer 3, 5′-CACCACCCGCCACCCAC.

SEQUENCES—Specific substitution and deletion mutations were generated in the −915P0LUC plasmid using two mutagenic oligonucleotides of the coding strand sequence, T4 DNA polymerase and the Transformamutagenesis kit (CLONTECH). The specific sequence changes are described in Fig. 4A. Although the mutations in these plasmids were generated by a single round of synthesis and not by polymerase chain reaction, we observed that in these two different mutantogenized colonies targeted by the same mutantogen oligo yielded significantly different luciferase activities. Consequently, 500-bp fragments containing only the desired mutation were excised from all of the mutated plasmids and cloned into the corresponding site in −915P0LUC to eliminate the possibility of secondary mutations.

Transient Transfection and Reporter Gene Assay—Transfection with CAT plasmids was performed using a standard calcium phosphate procedure (18, 29). Schwann cells were grown in medium with serum (2 μM forskolin and 13 μg/ml glial growth factor) and transfected in media alone, and after transfection, cells were incubated in medium with 2 μM forskolin and without glial growth factor. Ten-μg P0 CAT 5′ deletion plasmids were co-transfected with 2 μg of RSV-luciferase plasmid as a measure of transfection efficiency. Cell extracts were made, and luciferase activity was measured for CAT activity using [14C]chloramphenicol and thin layer chromatography as described previously (18, 30). Results are shown relative to the activity of the full-length plasmid, pPCATX6a.

Transfection of cell lines with luciferase reporter plasmids was performed by the polyethylenimine (Fluka) complex formation method as described previously (31). In brief, DNA and polyethyleneimine complexes were formed in 0.15 M NaCl for 30 min and applied to cell cultures grown to approximately 50% confluence. Complexes and cells were incubated for 3–4 h, the medium was removed, and fresh medium was applied. P0-luciferase plasmids were co-transfected with RSV/β-galactosidase plasmid used to normalize for transfection efficiency. Before transfection, Schwann cells were grown in medium containing 2 μM forskolin and 10 ng/ml recombinant neuregulin. After transfection, cells were incubated in medium with 10 μM forskolin and without recombinant neuregulin. Schwann cell lysates were made approximately 25 h later, and B103 cell lysates were prepared 42–44 h later. Luciferase assays and β-galactosidase assays were performed as described previously (18), or alternatively the Dual Light assay for both tests was used (Tropix, Inc.). Light units were normalized to β-galactosidase units, and the results with the mutant plasmids were expressed relative to −915P0LUC, which was set to 100. Shown are the results of five experiments, each performed in duplicate.

Electrophoretic Mobility Shift Assay (EMSA)—Nuclear extracts for band shift were prepared as for DNase I footprinting experiments or by the rapid method as described previously (32). EMSAs were performed by mixing 4 μg of nuclear extracts with 1 ng of radiolabeled oligonucleotide probe in the following buffer (2 mM Tris, pH 7.5, 50 μM KCl, 10 μM MgCl2, 10 μM ZnSO4, 1 mM EDTA, 1 mM dithiothreitol, 10% Ficoll, and 0.2 mM phenylmethylsulfonyl fluoride). Unlabeled oligonucleotide competitors were used at an 80-fold molar excess relative to the labeled probe. Antibody supershift reactions were performed by adding antibody and protein to interact before incubation with probe. Reactions were incubated on ice for 30 min and electrophoresed on 5% polyacrylamide gels in 0.5 × TBE buffer, and the gels were dried and exposed to autoradiographic film. The sequences of the P0 EMSA probes are as follows: probe 3, 5′-GTCACCTGGCTAGGGCAGCCTCCCCCTTCACTGCT; probe 4, 5′-CAGGCTGGAATTGCTGCTGTCAG; probe 5, 5′-CACCTCTGAAATTGCTGAGCTG; probe 6, 5′-GGCTCACTGGCCCTTGCAG; probe 7, 5′-GAGACTCGTTGCTGCTGCTG; probe 8, 5′-CCTGTGTAAGGGGTG; probe 9, 5′-TTGGGGAGTTGCTCTAGGAAG; probe 13, 5′-GCCAGGAAGGATGGAGAGAGTG; probe 4+B; 5′-CCCTCTCAAACTTCCAGCAGCTCAGAGTGGCTGCTG; probe 5′-AGACTGGACTGGACAACTTCACTT; probe 6′-GCCAGGACAGGGCCGGGACAGG; probe 7′-GGGGTGGAGAGGAGGGGGGACAGG; probe 8′-GGGGTGGAGAGGAGGGGGGCAAGGAC.

RESULTS

Sequence Conservation and Promoter Deletion Analyses Reveal a Potent Proximal Promoter—We first examined sequence conservation between the aligned 5′-flanking regions of the human, rat, and mouse P0 genes. These regions, including exon 1 and extending into intron 1, were pairwise aligned mouse to human and rat to human. Sequence homologies were determined by calculating the percentage of nucleotide identity within a 40-bp window whose 5′-end was located at the position relative to the transcription start site that is shown on the x
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FIG. 1. Sequence conservation correlates with transcriptional function. A, comparison of P<sub>0</sub> gene sequence between aligned human and rodent species. Percentages of nucleotide identity were determined within 40 nucleotide windows analyzed at 5-nucleotide intervals from -900 to +400. The dotted line is comparison between rat and human, and the solid line is the comparison between mouse and human. The y axis shows the percentage of nucleotide identity, and the x axis denotes the position of the 5' nucleotide of each 40-bp window analyzed, relative to the transcription start site. B, transient transfection analysis of 5' deletion mutants of the P<sub>0</sub> promoter. Ten μg of each P<sub>0</sub>CAT plasmid and 2 μg of RSV-luciferase plasmid were co-transfected into rat Schwann cell cultures. Promoter activity is expressed as a percentage relative to the full-length plasmid (−915/+45), which was set to 100%. Note that the x axis of Fig. 1B (transcriptional activity) is aligned with the x axis of Fig. 1A (sequence conservation). Sequential 5' deletions from −915 to −400 resulted in a progressive, although modest, decrease in promoter activity up to the −400 region. At position −400, about 60% of the promoter activity remained. Within the −400 to −200 region, however, each deletion resulted in a substantial loss of CAT activity, indicating that important regulatory elements were progressively deleted (Fig. 1B). Deletions from −200 to −100 reduced activity to near background levels. The presence of negative regulatory elements, as indicated by an increase in reporter activity upon deletion, was not observed. The relative promoter activities of the 5' deletion mutants correlate well with the amount of sequence identity within the P<sub>0</sub> promoter (Fig. 1, compare A and B), in that the region between −350 and −300 where sequence conservation rises to >80% is the same region in which 5' deletions begin to remove strong regulatory elements. A 3' deletion series starting at position +45, (whose 5'-end was fixed at +915) revealed a modest diminution of activity until the deletions extended through the putative TATAA element at −23 to −28, at which point all transcriptional activity was lost (data not shown).

DNase I Footprint Analysis Reveals Sites of Protein/DNA Interactions—Given the results of the deletion survey, we performed in vitro binding studies on the proximal P<sub>0</sub> promoter from −550 to +45 to delineate sites of protein/DNA interaction.

In these experiments, nuclear extracts were prepared from two P<sub>0</sub>-expressing cell lines: 1) primary cultures of rat Schwann cells grown in 20 μM forskolin and 2) B103 cells, a P<sub>0</sub>-positive transformed rat cell line. Extracts were also prepared from two nonexpressing cell lines, Rat-2 fibroblasts and HeLa cells. Extracts from B103 cells produced a number of distinct protections on the proximal 600-bp coding strand probe (Fig. 2A shows several of these). Protected regions labeled 7, 9, and 10 were consistently strong and distinct; however, some sites, such as sites 8 and 6, were weak in comparison. Similar contacts were observed in nuclear extracts from cultures of rat Schwann cells (Fig. 2B), and labeling the noncoding strand of the proximal probe revealed protected regions that confirmed the boundaries of the protections observed with the coding strand probe (Fig. 2B). Footprint analysis using the distal probes detected (in addition to protection labeled 10) three additional protections on the promoter from −393 to −493, which were designated 11, 12, and 13 (summarized in Fig. 3). Hypersensitive bands were noted flanking regions 5, 9, 10, and 11 (Fig. 2, arrows). Thus, 13 sites of protein/DNA contact on the proximal promoter from the start site to −500 were delineated. Surrounding protections, such as sites 7 and 8, and overlapping

vious work has suggested that this sequence does indeed function as a TATAA element (18). When the three CAAT boxes at bp −78, −98, and −145 are compared, the sequences at bp −78 and at bp −145 are entirely conserved. It should be noted that the high sequence conservation observed over the entire proximal P<sub>0</sub> promoter, a conservation as high as that for the coding sequences in exon 1, is unusual.

To correlate the regions of high sequence identity with transcriptional function, 5' truncation mutants were made of the P<sub>0</sub> promoter from position −915 to −133 and linked to the CAT reporter gene. The 3'-end of the series was fixed at position +45, within the P<sub>0</sub> 5'-untranslated region. The regulatory activity of the 5' deletion mutants was analyzed by transient transfection and CAT assay in primary cultures of rat Schwann cells. The results are shown in Fig. 1B. The x axis of Fig. 1 denotes the 5'-end of the P<sub>0</sub> promoter sequence in each deletion plasmid, and the y axis denotes percentage of CAT activity relative to the full-length plasmid (−915/+45), which was set to 100%. Note that the x axis of Fig. 1B (transcriptional activity) is aligned with the x axis of Fig. 1A (sequence conservation). Sequential 5' deletions from −915 to −400 resulted in a progressive, although modest, decrease in promoter activity up to the −400 region. At position −400, about 60% of the promoter activity remained. Within the −400 to −200 region, however, each deletion resulted in a substantial loss of CAT activity, indicating that important regulatory elements were progressively deleted (Fig. 1B). Deletions from −200 to −100 reduced activity to near background levels. The presence of negative regulatory elements, as indicated by an increase in reporter activity upon deletion, was not observed. The relative promoter activities of the 5' deletion mutants correlate well with the amount of sequence identity within the P<sub>0</sub> promoter (Fig. 1, compare A and B), in that the region between −350 and −300 where sequence conservation rises to >80% is the same region in which 5' deletions begin to remove strong regulatory elements. A 3' deletion series starting at position +45, (whose 5'-end was fixed at +915) revealed a modest diminution of activity until the deletions extended through the putative TATAA element at −23 to −28, at which point all transcriptional activity was lost (data not shown).

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sites, such as sites 3–5, may be the result of binding by one protein or by multiple proteins.

The $P_0$ promoter mediates strong cell type-specific expression. Protein/DNA contacts were compared using nuclear extracts from $P_0$-expressing and nonexpressing cell lines. Extracts from the fibroblast cell line Rat2 produced similar protections over several regions (Fig. 2B). However, the protections created by Rat2 extracts appeared to be considerably weaker than the Schwann cell and B103 protections on site 4 and, even more so, on site 5. HeLa cell extracts were used as an additional nonexpressing cell type in footprint experiments utilizing both the distal and proximal end probes. Distinct differences in the boundaries for protections 3–5 were again observed with these extracts (Fig. 2C). Site 3 in B103 extracts is only weakly protected, while in HeLa it is quite strong. Also, a boundary is not evident between sites 3 and 4 with HeLa extracts, but it is present with B103 cell extracts. Similarly, a boundary of unprotected sequence appears between sites 4 and 5 with HeLa extracts but not with B103 extracts, and the protection of site 5 itself is again much weaker with HeLa extracts. These results show that while several sites of protein/DNA contact observed on the $P_0$ proximal promoter are the same for extracts prepared from expressing and nonexpressing cells, cell-specific differences in binding intensity and boundaries are detected, particularly at sites 3–5. A summary of protected sites created by expressing and nonexpressing cell types is included in graphic form in Fig. 3.

The importance of the DNase I-protected sequences was confirmed in a series of in vivo footprinting experiments using primary cultures of rat Schwann cells. The in vivo assay has the advantage of detecting protein/DNA contacts on the promoter in the nucleus of living cells that are actively expressing the gene. The DMS treatment allows subsequent cleavage by piperidine at guanine, and sometimes adenine, residues. The region from $-250$ to $+45$ was analyzed, and the protections observed in vitro within this region overlapped with the protected sequence noted by genomic footprinting. A selected ex-
ample of the protections observed in vivo on sites 3–5 is shown (Fig. 2). The two G residues in 5'CAATTGG (site 4) are strongly reduced, and two G residues in the 3'end of site 5 are also protected on the coding strand (Fig. 2D). In addition, the run of five G residues of site 3 is strongly protected on the noncoding strand (Fig. 2E). The protections observed in vivo in Schwann cells suggest that interactions detected in vitro are biologically relevant in the context of living cells.

Since the protected regions revealed by DNase I and genomic footprinting are probably binding sites for transcription factors, the 600-bp proximal promoter was scanned for known transcription factor binding sites within the protected sequences. This search again identified potential CAAT box binding sites located in protected sites 4 and 5 for known CAAT-binding proteins including NF-Y (see Fig. 3). In addition, immediately downstream of the CAAT sequence in site 5 is a CACATG motif, a match for the E box consensus sequence, CANNTG. Protected sites 3, 6, 7, and 9 are G/C-rich and were identified as potential AP-2, T-antigen, or PER2 consensus binding sites. In addition, a sequence homology within site 7 was also identified as a possible binding site for sterol response element-binding protein. Footprint region 10 contains a 34-bp palindromic sequence; however, potential DNA-binding factors for this site were not identified. This site and other P0 promoter binding sites may therefore correspond to recognition sequences for novel transcription factors. It is interesting to note that site 10 displays significant sequence similarity to a footprint in another myelin gene promoter, the proteolipid protein gene (26).

**Transcriptional Activities of Protein Binding Sites**—We measured the contribution of each of the 13 protected regions to transcriptional activity of the promoter by performing a mutational analysis of individual sites in the context of the full-length P0 promoter linked to a luciferase reporter gene (Fig. 4). Most sites were altered by substitution mutagenesis, and four large protections were completely deleted (sites 7, 9, 10, and 13). Site 5 was divided into two separate mutants corresponding to sequence homologies in the 3' (CANNTG) and 5' (CAAT) regions of the protection and are referred to as 5A and 5B, respectively (see Fig. 4A). Site 1 overlaps the putative cap site, and site 2 overlaps the putative TATAA element; these sites are not shown in this study. Each construct was introduced by transient transfection into rat Schwann cells, and cell extracts were subsequently assayed for luciferase activity and normalized for transfection efficiency using β-galactosidase activity and reported as percentages of the wild type activity obtained with the parental plasmid, -915P0LUC.
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sites, site 3 and site 4, had the strongest effects on promoter activity. Mutation of site 3, a potential G/C box, and of site 4, a potential CAAT box, reduced activity to 21 and 6% of the wild type levels, respectively. Mutation of elements 7, 8, and 9 also had a substantial effect on luciferase expression and reduced activity to 52, 55, and 67% of the wild type activity, respectively. Mutation of sites 5A, 5B, 6, 11, and 13 individually reduced activity minimally, resulting in activities between 70 and 80% of the wild type. Mutation of sites 10 and 12 had little or no effect, reducing activity to 88 and 100% of wild type, respectively. In summary, five individual mutations resulted in a significant loss in promoter activity (mutations 3, 4, 7, 8, and 9), while six of the other mutations had a more modest effect on transcription (mutations 5A, 5B, 6, 10, 11, and 13).

To confirm and extend these results, the mutant promoter constructs were also analyzed by transient transfection in B103 cells (Fig. 4, gray bars). The effects of mutations in sites 3, 4, 5A, 6, 7, 8, 10, 11, and 12 were similar (±20%) to the results in rat Schwann cells, and mutations in sites 3 and 4 again had the greatest effect on luciferase activity. Significant differences in promoter activity were observed with sites 5B, 9, and 13. These differences may reflect biological differences between the B103 tumor cell line and primary Schwann cells. In this regard, it should be noted that 100% wild type activity of -915P₀LUC in Schwann cells corresponds to ~60,000 light units/20-μl sample, while B103 cells yield ~4,000 light units/20-μl sample for the same construct. Maximal promoter usage reflected by absolute light units measured could be dependent on the activity of elements 5B, 9, and 13, whose functions appear to be altered in B103 cells.

Differences in the boundaries of binding sites 3–5 were observed when comparing nuclear extracts from P₀-expressing cells and nonexpressing cells in DNase I footprinting experiments. We further examined the cell type specificity of these promoter elements by comparing their activity in transient transfections in nonexpressing Rat2 cells and in Schwann cells. The region from -113 to +45 (including sites 3–5) was cloned upstream of the luciferase reporter gene and tested as described in Fig. 4. When luciferase activities in both cell types were normalized to the background vector alone (pGL2-B), transcriptional activity from this minimal promoter construct was 5.0 ± 1.0 relative light units in Schwann cells compared with 4.5 ± 0.5 units in Rat2 cells. In contrast to the full-length 1.1-kilobase pair P₀ regulatory region (18), the P₀ promoter from -113 to +45 appeared to have very similar activities in expressing and nonexpressing cell lines, indicating that strong, independent Schwann cell-specific elements were not included in this construct. In addition, when plasmids Mut 3 and Mut 4 were assayed in Rat2 cells luciferase activity dropped to 34 and 29% of wild type promoter activity, respectively, indicating that these elements were necessary for the low levels of transcription detected in nonexpressing cells. This confirms that most of the exuberant cell type specificity of the 5’ P₀ regulatory region lies upstream of the core promoter.

Electrophoretic Mobility Shift Assays—Binding activities of the functional P₀ promoter elements were further examined by EMSAs, in which radiolabeled oligonucleotide probes of the protected sites were used to identify protein complexes in Schwann cell nuclear extracts. P₀ probes detected strong DNA binding activity in Schwann cell extracts (Fig. 5), with the exception of probes for sites 5 and 8; weak complexes were detected with site 5 only upon 10 times longer exposure of the gel. Since cAMP regulators, such as forskolin, modestly up-regulate P₀ expression in cultured Schwann cells, we used EMSA to test whether forskolin-inducible complexes could be detected on the P₀ promoter elements exhibiting in vivo function.
protection 4, referred to as probe 4 + 5. In addition to the C1 doublet, the 4 + 5 probe bound an additional complex with a slower mobility in the gel, designated C2. Complex C2 was eliminated by cold 4 + 5 competitor and was weakly competed by cold site 4 alone but not by an irrelevant oligo competitor, Sp1 (Fig. 7B, lanes 5–7 and 9). Cold competitor composed of site 5 alone did not effect C2 formation, indicating that the complex does not bind to the upstream CAAT sequence in site 5 (lane 8). When competition assays were performed with the 4 + 5 probe using known CAAT box binding sites as competitors, C2 was competed by the NF-Y binding site (lanes 10 and 11), but competitors of NF-1 and C/EBP binding sites did not compete (data not shown). NF-Y is a widely expressed transcription factor composed of three subunits, NF-YA, NF-YB, and NF-YC, and was detected in cultured Schwann cell nuclei by immunofluorescent staining (data not shown). This prompted us to probe the C1 and C2 complexes in supershift experiments with a well characterized antibody specific for the NF-YA subunit (35). While normal rabbit serum and AP-2-specific antibody did not react with either C1 or C2, NF-YA antibody supershifted the C2 complex, indicating that this complex in Schwann cell extracts contains NF-Y (lanes 12–16). The NF-Y control probe (major histocompatibility complex class II Y box sequence) bound a complex of the same mobility in Schwann cell extracts and was also supershifted by the NF-Y antibody (Fig. 7C, lanes 17–20). Notably, this antibody did not alter the probe 4-specific doublet, C1 (lane 14) and did not supershift C1 on the probe containing site 4 alone (data not shown). Whereas C1 reflects the binding of an as yet unidentified protein, these results suggest that C2 contains transcription factor NF-Y in Schwann cell nuclear extracts.

DISCUSSION

A variety of previous studies have demonstrated that most of the specificity of Po gene expression is due to transcriptional control and that the kilobase of DNA flanking the 5′-end of Po carries the requisite elements for Schwann cell-specific expression and appropriate developmental regulation of this gene. In particular, mouse transgenes that are placed under the control of this regulatory region are expressed exclusively in Schwann cell nuclear extracts by EMSA. Probe 3 (GTCACGGCAGCAGCCCCTCATGCT) was incubated with rat Schwann cell nuclear extracts (lanes 1–9). Competitions were performed using cold oligonucleotides as indicated (lanes 2–4). Antibody supershift experiments were performed with normal rabbit serum control (NRS, lane 5), α-Krox-20 (lane 6), and α-Sp1 (lane 7). The Krox-20 polyclonal rabbit serum does not create a supershifted band; however, it does block complex formation on Krox-20 consensus probes using Schwann cell extract and using recombinant Krox-20 (not shown). The control probe, Sp1, was incubated with Schwann cell extracts and normal rat serum (lane 8) or α-Sp1 (lane 9). The arrow indicates the location of the Sp1-supershifted band in the gel.

Protein complexes on Po site 4 and site 4 + 5. A, complexes bind probe site 4 (CAGGCTGCAATTGGTCACTGGCTCAG). Probe 4 was incubated with Schwann cell nuclear extract, and competitions were performed using oligonucleotides as indicated (lanes 1–4). The doublet with sequence specificity is indicated by the nomenclature C1. B, Po site 4 + 5 (CTCTTCATGGCTCAGCCCCTCATGCT) binds NF-Y. Competition experiments were performed by incubating probe 4 + 5 with Schwann cell extracts in the presence of the unlabeled competitor as indicated (lanes 5–11). Antibody supershift experiments were performed by incubating probe 4 + 5, Schwann cell nuclear extracts, and antibody with normal rabbit serum (NRS, lane 11), α-NF-Y (lane 12), and α-AP-2 (lane 13). Lane 14 is probe and α-NF-Y without nuclear extract. C, NF-Y probe binds NF-Y in Schwann cell nuclear extracts. NF-Y control probe was incubated with cold competitor (lane 18) or antibody as indicated. The arrow indicates the position of the NF-Y-supershifted band.
Regulate P0 mRNA and protein in cultured Schwann cells with limited concerning a powerful and compact regulatory region when used in a several of the coding regions of the P0 gene. The 5' conservation observed between rat (or mouse) and human for 90% for a 40-bp window, slightly higher than the sequence species. This nucleotide conservation is typically in the range of the transcription start site is highly conserved between three mammalian isotypes or in the functional assays of identified footprint sites. Although a number of neuronal genes appear to be regulated in a cell type-specific manner by silencer elements (36), such elements were not detected in the promoter truncation experiments or in the functional assays of identified footprint sites.

Substantial transcriptional activity was localized to the region from -500 to +45. Both this region and regions upstream were analyzed by in vitro DNase I footprinting, and 13 protected sites were detected using nuclear extracts from cultured Schwann cells and B103 cells. The protected regions may contain individual transcription factor binding sites or may be composite sites containing recognition sequences for more than one factor. In addition, neighboring sites such as 7 and 8 may be the result of the binding of one protein. The region most proximal to the transcription start, sites 3–5, contains a G/C-rich sequence, two CAAT boxes, and an E box homologue. Mutations in site 3, the most proximal G/C box, and site 4, the most proximal CAAT box, had the most significant effects on promoter activity, each reducing activity to less than 20%, and were analyzed further by in vitro binding assays. Based on their position relative to the transcription start site, their essential functional activity, and their sequence, we have assigned sites 3 and 4 as the core or basal promoter of the P0 gene.

While the 1.1-kilobase pair P0 promoter has been shown to be a powerful and compact regulatory region when used in a number of transgenic mouse experiments, our knowledge is limited concerning in vivo regulation of P0 transcription. Recent studies suggest that the steroid hormone progesterone may play a role in myelination (37). It will also be of interest to determine if the sequence at site 7 with similarity to the sterol response element on the coding strand (9/11) is the other 48 to 59) is the other 130, as is the GCAAT element on the coding strand (9/11 versus 6/11 nucleotide similarity). A number of CAAT box binding factors and their isoforms have been identified and cloned, among them C/EBP, NF-Y, and NF-I (40–43). Our results demonstrate that the site 4 + 5 probe binds NF-YA or a closely related protein complex in Schwann cell extracts. NF-Y is a ubiquitously expressed heteromeric complex of three subunits, all of which are required for binding to DNA (44). NF-Y binding sites are found in a number of promoters, including those of tissue-specific genes, and are frequently positioned between -60 and -130, as is the case for P0 site 4 (45). NF-Y functions in several promoters through protein/protein interactions with transcription factors positioned on neighboring sites and has been shown to interact with factors in a cooperative manner (35, 39, 46, 47).

The G/C-rich protection at site 3 (-48 to -59) is the other important element identified in the core promoter. Mutation of four of the cytosines reduced promoter activity to one-fifth of the wild type promoter activity. While Sp1 is the most common factor binding the G/C box in proximal promoters of genes analyzed, Krox-20 recognizes G/C-rich sequences quite similar to the Sp1 site (48). Krox-20 has previously been shown to have an important role in Schwann cell development demonstrated by the Krox-20 knockout mice, which have a severe defect in PNS myelination and barely detectable P0 expression (49). However, competition experiments with consensus binding sites as well as antibody supershift experiments with specific antisera demonstrate that Sp1 or an Sp1-related protein binds site 3. As is the case for similarly positioned Sp1 sites in other genes, Sp1 may play a role in regulating basal transcription of the P0 gene. In addition, Sp1 controls basal transcription via a delayed time course (33, 38), we examined changes in protein binding on the P0 promoter elements upon forskolin treatment and saw modest changes with two P0 probes. An induced DNA-binding activity was formed on site 7, and an increase in band intensity was observed on site 11 that parallels the amount of P0 up-regulation observed at the mRNA level. The proteins that recognize these sites may therefore be subject to indirect cAMP regulation. Other changes in promoter activity may not be detected at the level of protein/DNA interaction because they may involve either the modification of existing factors or the interaction of proteins off the DNA.

We propose that as with other tissue-specific promoter regions, specificity is influenced by the binding of a particular set of factors and the precise orientation of these factors on the DNA. In this respect, the footprinting results suggested sites 3–5 as a region of cell type-specific differences within the P0 promoter. However, further transfection analysis reveals that the core elements, sites 3–5, are functional elements in a non-expressing cell line. The differences in binding properties observed may reflect the binding of related but distinct factors in different cell types or interactions between factors on the core promoter and those on upstream elements. Alternatively, the footprint differences may reflect post-translational modifications of the same factors or the presence of tissue-specific protein partners. It is also possible that a Schwann cell-specific enhancer may lie in the upstream regulatory region from -500 to -1100.

The mutation with the most effect on promoter function is a 4-base pair change in the GCAATT sequence at protected site 4 (-66 to -79). This alteration also changes three nucleotides of the overlapping sequence CCAATT, on the opposite DNA strand and in the opposite orientation. Although according to our data either of the CAAT homologues may be the functional binding site, the CCAAT on the noncoding strand (-64 to -77) is more similar in sequence to the consensus NF-Y element, (A/G)×(G/A)×CCAAT(C/G)×(A/G)×G(C/A) (39), than is the GCAAT element on the coding strand (9/11 versus 6/11 nucleotide similarity). A number of CAAT box binding factors and their isoforms have been identified and cloned, among them C/EBP, NF-Y, and NF-I (40–43). Our results demonstrate that the site 4 + 5 probe binds NF-YA or a closely related protein complex in Schwann cell extracts. NF-Y is a ubiquitously expressed heteromeric complex of three subunits, all of which are required for binding to DNA (44). NF-Y binding sites are found in a number of promoters, including those of tissue-specific genes, and are frequently positioned between -60 and -130, as is the case for P0 site 4 (45). NF-Y functions in several promoters through protein/protein interactions with transcription factors positioned on neighboring sites and has been shown to interact with factors in a cooperative manner (35, 39, 46, 47).

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interaction of its glutamine-rich activation domain with the TATAA-binding protein-associated factors (50). In this regard, it would be interesting to determine if P0 transcription is mediated by an Sp1/NF-Y interaction or alternatively an Sp1/TAF interaction.

In summary, a composite of regulatory elements are found within the 500-bp proximal P0 promoter, with various elements exhibiting a range of transcriptional and protein binding activities. The P0 basal or core promoter is composed of a CAAT element that binds NF-Y and a G/C element that binds Sp1. We now need to identify the factors binding the P0 regulatory elements, to assess the extent to which functional interactions occur between these proteins, and to define their precise roles in regulating P0 transcription. Since P0 expression is increased substantially in myelinating peripheral nerves compared with Schwann cells maintained in culture, our future plans are to test the significance of these factors for P0 production during myelination. The identification of the DNA binding proteins and the factors that activate P0 will allow us to begin to understand the cell type specificity and developmentally regulated expression of this major myelin protein.

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