Role of Sp Proteins and RORα in Transcription Regulation of Murine Prosaposin*

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Prosaposin is the precursor of four low molecular weight sphingolipid-activating proteins (SAPs) or saposins. These four proteins function as intracellular activators of several lysosomal enzymes involved in the degradation of glycosphingolipids, and prosaposin itself has neurite outgrowth effects. Expression of prosaposin is regulated in a temporal and spatial manner with expression in specific brain neurons and visceral cell types. Here a major regulatory fragment was characterized within 310 bp 5′ to the transcription start site. Using electrophoretic mobility shift assay (EMSA) and DNA footprinting, members of the Sp family (Sp1, Sp3, and Sp4), the orphan nuclear receptor (RORα), and an unknown transcription factor (U; TGGGGGAG) were shown to bind to this region. To evaluate the role of such transcription factor binding sites for this locus, a series of mutant constructs was generated within this region, and their function was evaluated in cultured NS20Y neuroblastoma cells. A 3′ Sp1 site, a 5′ Sp1/U cluster and the RORα binding sites were functional. The data are consistent with a model in which the factors that bind to the Sp1/U cluster and RORE site interact negatively to diminish promoter activity to a background level that is determined primarily by the 3′ Sp1 site. These interactions depend on the tissue-specific repertoire of transcription factors leading to differential expression of this locus.

Prosaposin is a multifunctional protein with specific intracellular and extracellular functions. It is the precursor of four 80-amino acid glycoprotein activators (1–4). In humans, rats, and mice the prosaposin mRNA encodes in tandem the highly similar saposins A, B, C, and D that participate in the sequential degradation of glycosphingolipids to sphingosine and fatty acids (2–5). In the rat, prosaposin is designated SGP-1, sulfated glycoprotein 1, since it is a major sulfated glycoprotein in Sertoli cells (5). In addition to prosaposin’s essential role in glycosphingolipid degradation, it has in vivo neurite outgrowth effects. When placed in the media surrounding neuroblastoma cells, prosaposin facilitates neurite outgrowth (6–8), and prosaposin facilitates in vivo regeneration of the sciatic nerve following injury (9). Prosaposin also functions in glycosphingolipid transfer between artificial membranes (10). Targeted disruption of the murine prosaposin gene resulted in a complex phenotype including severe central nervous system disease and widespread storage of multiple sphingolipids (11).

The human gene for prosaposin has been partially characterized to contain 13 (or 14) exons and 12 (or 13) introns (12, 13). An alternatively spliced 9-bp1 “exon” is present in the saposin B region of the prosaposin gene (12). The 13 (or 14) exons are spread over ~30 kb of human chromosome 10. Interestingly, the prosaposin gene, a presumed “housekeeping” gene, displays temporally and spatially regulated expression (14). Despite the ubiquitous role of lysosomal hydrolases in all tissue types, the level of prosaposin expression is highly dependent on the cell type and maturation. The highest levels of expression are in specific neurons of the adult cerebrum, the Purkinje cell layer of the cerebellum, and neurons of the lateral regions of the spinal cord (14). Components of the hind brain also show higher levels of mRNA expression early in embryogenesis (15).

Previously, we characterized the 5′ region of the murine prosaposin gene including the first exon that contains the translation initiation site (16). The first intron of the mouse prosaposin locus is unusually large, almost 15 kb, and constitutes nearly 60% of the gene. Like many other housekeeping genes, the promoter region of the murine prosaposin gene is “TATA-less” and GC rich. The prosaposin gene has a major and a minor transcription start site. Transfection of deletion constructs containing reporter genes into NS20Y, NIH-3T3, or SF-7 (Sertoli) cells showed positive and negative regulatory elements within 2,400 bp 5′ to the transcription start sites (16).

In this paper, we report the characterization of a major regulatory fragment located within 310 bp 5′ to the murine prosaposin gene transcription start site. DNA footprinting, electrophoretic mobility shift assays (EMSA), and site-directed mutagenesis were used to analyze in vitro the interaction of transcription factors that bind to this fragment. Sp members 1, 3, and 4; RORα (the orphan nuclear receptor); and an unknown transcription factor (U) were identified to be involved in the regulation of the murine prosaposin gene. A complex interaction of multiple transcription factors is proposed to modulate gene expression from one essential Sp1 binding site within 16 bp 5′ to the transcription start site.

EXPERIMENTAL PROCEDURES

Materials—The following materials were from commercial sources: Wizard™ PCR Prep DNA kit, pGL2B Luciferase Reporter Vectors, the luciferase assay system, and the β-galactosidase enzyme assay system (Promega, Madison, WI); restriction enzymes and Taq DNA polymerase (New England Biolabs, Beverly, MA); Sequenase™ version 2.0 DNA sequencing kit (U.S. Biochemical Corp.); oligonucleotide synthesis, poly(dI-dC), poly(dA-dT), NAP-10, and NAP-5 columns (Amersham

1 The abbreviations used are: bp, base pair(s); kb, kilobase pair(s); EMSA, electrophoretic mobility shift assay; DMEM, Dulbecco’s modified Eagle’s medium; RORE, ROR response element; MZF, myeloid zinc finger; O1–O4, oligonucleotides 1–4, respectively; MO1 and MO4, mutant oligonucleotides 1 and 4, respectively.
Prosaposin Promoter: Role of Sp1 and RORa

Pharmacia Biotech; anti-Sp1, -Sp2, -Sp3, and -Sp4 antibodies (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA); GeneClean Kit (BIO 101, Inc., Vista, CA); QIAGEN Plasmid Midi Kit (QIAGEN, Chatsworth, CA); Microcin microcentrator 3 (Amicon, Inc., Beverly, MA); Lipo-fectamine Plus-Opti-MEM, DEMEM-free medium, Life Technologies Inc.; Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA); isotope, [\( ^{32}P \)]ATP (NEN Life Science Products). Cell line NS20Y was from Dr. Marshall Nirenberg (National Institutes of Health).

Double-stranded Oligonucleotides—Single-stranded oligonucleotides were synthesized on an Amersham Pharmacia Biotech DNA synthesizer. After purification with NAP-10 columns, the complementary oligonucleotides were heated at 95 °C for 5 min in the annealing buffer (20 mM Tris, pH 7.4, 2 mM MgCl\(_2\), and 50 mM NaCl) and then cooled to room temperature. The annealed oligonucleotides were purified by electrophoresis in polyacrylamide gels.

Nuclear Extract Preparation—Nuclear extracts were prepared using a minipreparextract procedure (17) with minor modifications. Nuclear extracts were desalted and concentrated using Microcon microcentrator 3 (Amicon, Inc.). Protein concentrations were estimated by the Bradford method (18).

EMSA—The double-stranded oligonucleotides were labeled with [\( ^{32}P \)]ATP and T4 polynucleotide kinase. The probes were purified on NAffino columns. The labeled double-stranded oligonucleotides were incubated with 3–6 μg of nuclear extract at room temperature for 20 min in 20 μl containing 5% glycerol, 1 mM MgCl\(_2\), 0.5 mM dithiothreitol, 0.5 mM EDTA, 50 mM NaCl, 10 mM Tris-HCl (pH 7.5), and 0.5 mM fresh phenylmethylsulfonyl fluoride. Poly(dI-dC) or poly(dA-dT) was used as heterologous competitor in the reaction (800 ng/reaction). For antibody supershift assays, the extract was incubated overnight at 4 °C with the specific anti-Sp antibodies. Extract-antibody mixtures were then incubated with the probe. Bound and free probes were resolved by nondeaturing electrophoresis in 6% polyacrylamide gels.

DNase I Footprinting—Polymerase chain reaction products were generated, and wild-type parental DNA template was digested, and the circular, nicked circular strand that was then digested with DpnI. The methylated, wild-type parental DNA template was digested, and the circular, nicked double-stranded DNA was transformed into XL2-Blue ultracompetent cells. Plasmid DNA from positive clones was digested with DpnI and HindIII and then recloned into the pGL2-basic vector. Each mutant was normalized to the individual transfection experiments. The results represent the means of three independent experiments conducted in triplicate.

RESULTS

DNA Footprint Analysis—A 408-bp segment (−310 to +98) from the 5’ end of the murine prosaposin gene contained promoter activity in a number of different cell types (16). This was added to the cells. At 65 h after transfection, the cells were washed twice with phosphate-buffered saline by centrifugation (1000 × g, 5 min) and then incubated in the 200 μl of reporter lysis buffer (25 mM Tris-phosphate, pH 7.5, 2 mM EDTA, 2 mM dithiothreitol, 10% glycerol, 1% Triton X-100) at room temperature for 15 min with shaking. The cell lysate was collected, transferred into microcentrifuge tubes, vigorously agitated (15 s), and centrifuged (12,000 × g, 2 min). The supernatants were transferred to fresh tubes for luciferase and β-galactosidase activity assays. For luciferase assays, lysate (20 μl) was mixed with luciferase assay reagent (100 μl). Light emission was quantified at room temperature. For the β-galactosidase assay, lysate (10 μl) was diluted into lysis buffer (150 μl) and mixed with 150 μl of 2 × assay solution (120 mM Na\(_2\)HPO\(_4\), 80 mM NaH\(_2\)PO\(_4\), 2 mM MgCl\(_2\), 100 mM β-mercaptoethanol, and 1.33 mg/ml O-nitrophenyl-β-D-galactopyranoside). The mixture was incubated (37 °C, 20 min) and stopped with 1 mM Na\(_2\)CO\(_3\) (0.5 ml). The absorbance was determined at 420 nm. Luciferase activity was normalized to β-galactosidase activity for each lysate collected from the individual transfection experiments. The results represent the means of three independent experiments conducted in triplicate.

| Mutant construct | Template | Designation | Sequence (5’ → 3’) |
|------------------|----------|-------------|-------------------|
| m43m             | m43      | M1S         | CGCCGACGGTTCCTGG  |
| m114m            | m114     | M1R         | CGCAAGCGCGACGCT   |
| m234m            | m234     | M2S         | GTGGTGGGTGGTCCTGG |
| m234m3           | m234     | M2R         | GTCACTGGGGGACGCC  |
| m310m            | m310     | M3S         | CGGGGCGGGCGCGCT   |
| m310mB           | m310     | M3R         | CGGGGCGGGCGCGCT   |
| m310c            | m310     | M4S         | CGGGGCGGGCGCGCT   |
| m310mD           | m310     | M4R         | CGGGGCGGGCGCGCT   |
| m310mE           | m310     | M5S         | CGGGGCGGGCGCGCT   |
| m310mF           | m310     | M5R         | CGGGGCGGGCGCGCT   |
| m310mG           | m310     | M6S         | CGGGGCGGGCGCGCT   |
| m310mH           | m310     | M6R         | CGGGGCGGGCGCGCT   |
| m310mI           | m310     | M7S         | CGGGGCGGGCGCGCT   |
| m310mJ           | m310     | M7R         | CGGGGCGGGCGCGCT   |
| m310mK           | m310     | M8S         | CGGGGCGGGCGCGCT   |
| m310mL           | m310     | M8R         | CGGGGCGGGCGCGCT   |
| m310mM           | m310     | M9S         | CGGGGCGGGCGCGCT   |
| m310mN           | m310     | M9R         | CGGGGCGGGCGCGCT   |
| m310mO           | m310     | M10S        | CGGGGCGGGCGCGCT   |
| m310mP           | m310     | M10R        | CGGGGCGGGCGCGCT   |
| m310mQ           | m310     | M11S        | CGGGGCGGGCGCGCT   |
| m310mR           | m310     | M11R        | CGGGGCGGGCGCGCT   |
| m310mS           | m310     | M12S        | CGGGGCGGGCGCGCT   |

Primers are numbered from Table I.

Mutations sites are underlined.

| Primer Primersa | Designation | Sequence (5’ → 3’) |
|-----------------|-------------|-------------------|
| m43m            | M1S         | CGCCGACGGTTCCTGG  |
| m114m           | M1R         | CGCAAGCGCGACGCT   |
| m234m           | M2S         | GTGGTGGGTGGTCCTGG |
| m234m3          | M2R         | GTCACTGGGGGACGCC  |
| m310m            | M3S         | CGGGGCGGGCGCGCT   |
| m310mB           | M3R         | CGGGGCGGGCGCGCT   |
| m310c            | M4S         | CGGGGCGGGCGCGCT   |
| m310mD           | M4R         | CGGGGCGGGCGCGCT   |
| m310mE           | M5S         | CGGGGCGGGCGCGCT   |
| m310mF           | M5R         | CGGGGCGGGCGCGCT   |
| m310mG           | M6S         | CGGGGCGGGCGCGCT   |
| m310mH           | M6R         | CGGGGCGGGCGCGCT   |
| m310mI           | M7S         | CGGGGCGGGCGCGCT   |
| m310mJ           | M7R         | CGGGGCGGGCGCGCT   |
| m310mK           | M8S         | CGGGGCGGGCGCGCT   |
| m310mL           | M8R         | CGGGGCGGGCGCGCT   |
| m310mM           | M9S         | CGGGGCGGGCGCGCT   |
| m310mN           | M9R         | CGGGGCGGGCGCGCT   |
| m310mO           | M10S        | CGGGGCGGGCGCGCT   |
| m310mP           | M10R        | CGGGGCGGGCGCGCT   |
| m310mQ           | M11S        | CGGGGCGGGCGCGCT   |
| m310mR           | M11R        | CGGGGCGGGCGCGCT   |
| m310mS           | M12S        | CGGGGCGGGCGCGCT   |

a Primers are numbered from Table I.
promoter region is shown in Fig. 1 (GenBank™ accession number AF037437). Sequence analysis of the 310 bp 5' to the transcription start site revealed four potential Sp1 binding sites, three of which are overlapping in a 5' cluster, and GATA, AP-1, and RORα binding sites.

DNA footprint analysis of this region (Fig. 2) showed three DNase I protected regions when using nuclear extracts from NS20Y cells and the sense probe. The control reactions contained bovine serum albumin. The most 3'-protected region (~32 to ~12) is GC-rich and contains a Sp1 binding site. In particular, the AP-1 site did not footprint. A strongly protected region was at ~184 to ~164. This region has high homology to the RORα binding site (also termed ROR response element (RORE)) that is composed of a half-site PuGGTCA preceded by a 6-bp AT-rich region (21, 22). A weakly protected region was at ~267 to ~294 and contained three overlapping Sp1 binding sites. Using the antisense probe, similar results were obtained, but weaker protection was observed at the RORE and the 3' Sp1 binding sites (data not shown).

**EMSA**—To confirm the DNA footprinting results, EMSA was performed with several double-stranded oligonucleotides (Table III) that covered the protected regions. When EMSA was conducted with a probe (oligonucleotide 1, O1) covering bases ~35 to ~3, two major DNA-protein complexes were observed with NS20Y cell nuclear extracts (Fig. 3, A and B (lanes 1 and 2)). Formation of DNA-protein complexes was competed by an excess of unlabeled O1, but not by MO1, an oligonucleotide containing a mutated Sp1 binding site (Table III; Fig. 3B, lanes 3 and 4). The addition of excess cold Sp1 consensus oligonucleotide competed with labeled O1 for binding to the protein(s) (Fig. 3B, lane 5). When purified Sp1 protein was incubated with the same probe, a single DNA-protein complex was detected (Fig. 3B, lane 6). O3 was synthesized to cover three overlapping Sp1 binding sites from ~298 to ~263. Three DNA-protein complexes were resolved when this probe was incubated with NS20Y cell nuclear extracts (Fig. 3C, lanes 1 and 2). The formation of DNA-protein complexes was competed off by the addition of cold O3, but not by MO3 (Fig. 3C, lanes 3 and 4). MO4 contains a mutated RORα binding site (Table III). These results indicate that RORE is functional but that the GATA binding site is not.

The Sp1 multigene family includes Sp1 and three other genes encoding the Sp2, Sp3, and Sp4 proteins. The consensus binding sequences of these proteins are very similar (23, 24). No cross-reaction was detected among the antibodies for the individual Sp proteins, and none of them bound to O1 or O3 to form DNA-protein complexes (data not shown). Polyclonal antibodies against Sp1, Sp2, Sp3, or Sp4 were used for EMSA. Preincubation of anti-Sp1 or anti-Sp3 antibodies with NS20Y cell nuclear extracts produced a DNA-protein supershift using O1 and O3 (Fig. 5). A similar shift was obtained with Sp4 antibody using O1, but not with O3 (Fig. 5A, lane 6). For O1, the Sp1 and Sp3 antibodies appeared to supershift different bands and, by inference, interacted with different proteins or complexes. The Sp1 antibody supershifted a slower migrating band (lane 3), whereas the Sp3 antibody supershifted a faster migrating band (lane 5).

**Definition of an Unknown Transcription Factor Binding Site**—In the EMSA experiment using O3 as probe (Fig. 3C, lane 4), the addition of cold Sp1 consensus oligonucleotides eliminated the top band, but not the middle and bottom bands. This result shows that a transcription factor(s), in addition to Sp1, binds to this region and that this binding is independent of that for Sp1. In an attempt to define this transcription factor binding site, a series of mutant O3s were made to densely cover this region (Fig. 6A) and were used as probes during EMSA. NS20Y nuclear extracts were used. Three similar bands were obtained with the oligonucleotides 3, 3A–3D, and 3F. Oligonucleotide 3E produced only two supershifted bands, and one major band was missing, i.e. a transcription factor binds to the mutated region that was not covered in the oligonucleotide 3E region (Fig. 6B, lane 7). Competition assays using cold oligonucleotides 3A–3F against their radioactive derivatives showed that the bindings of each of the mutant O3s were specific (data not shown). A competition of the transcription factor binding site data bases for the 3E region (TGGGGGAG) showed high homology to the myeloid zinc finger (MZF) protein consensus binding sequences (25). Cold MZF consensus oligonucleotides were synthesized and did not compete with labeled O3 for the formation DNA-protein complexes (data not shown). Thus, MZF is unlikely to bind to the region covered by O3. We defined this region as an un-
Fig. 2. DNase I footprint analysis of the prosaposin promoter region shown in Fig. 1. The in vitro DNase I digestion pattern of the sense strand of the promoter region was in the presence of NS20Y nuclear extract or of bovine serum albumin (BSA). The triangle indicates an increasing amount of DNase I in the digestion. The G-A lane is the specific Gilbert sequencing reaction products for G and A. The boxes on the right indicate the regions protected from DNase I digestion by NS20Y cell nuclear extract. The filled rectangles are strongly protected regions. The hatched box was a region that was less strongly protected. Analyses were in −238 to +1 (A) and −213 to −300 (B).

Table III
Sequences of double-stranded oligonucleotides

| Name               | Sequence                                      |
|--------------------|-----------------------------------------------|
| Oligonucleotide 1  | 5′-'GGCCCCAGTTTGGGGGGGGGGCCCTGCGCA-3′          |
| -35/-3             | 3′-'GGGGGGGAAGATGTTCTGATATTAGGTTGGTGG-5′      |
| MO1*               | 5′-'GGCCCCAGTTTTTTTTTGGGGGGGGCCCTGCGCA-3′    |
| Oligonucleotide 2  | 3′-'GGGCTCAGGGTGGGTGGGTTGGGGAGCACAAGA-3′     |
| −168/−133          | 5′-'GGGCCCTAGCTTGATATTAGTCCACGA-3′           |
| Oligonucleotide 3  | 3′-'GGGCCCTAGCTTGATATTAGTCCACGA-3′           |
| −298/−263          | 5′-'GGGCCCTAGCTTGATATTAGTCCACGA-3′           |
| Oligonucleotide 4  | 3′-'GGGCCCTAGCTTGATATTAGTCCACGA-3′           |
| −186/−159          | 5′-'GGGCCCTAGCTTGATATTAGTCCACGA-3′           |
| MO4               | 5′-'GGGCCCTAGCTTGATATTAGTCCACGA-3′           |
| Sp1                | 3′-'GGGCCCTAGCTTGATATTAGTCCACGA-3′           |
| Consensus oligonucleotide | 3′-'GGGCCCTAGCTTGATATTAGTCCACGA-3′ |

* MO1, mutant oligonucleotide 1 as underlined (mutated Sp1 binding site).

** MO4, mutant oligonucleotide 4 as underlined (mutated RORα binding site).

known, or U, region (−270 to −277).

Mutational Analysis—The function and potential interaction of the transcription factors that bind to the identified sites across this region were evaluated by mutagenesis and transfection/expression analyses (Fig. 7). For these studies, luciferase was used as the reporter gene, and NS20Y cells were transfected. Deletion constructs were made to contain −43, −114, −234, and −310 bp 5′ and +98 bp 3′ to the major transcription start site (+1). The constructs are arranged in groups to display the effects of the identified transcription factor binding sites up to the 5′ Sp1/U cluster. The results with the first eight constructs show that destruction of the most 3′ Sp1 site caused a ≥80% reduction in activity. Lengthening of the construct from −43 to −234 bp resulted in small (~15%) increases in absolute promoter activity until the RORE site was included (~45% increase). Destruction of the RORE site reduced activity to nearly those observed with m114, whereas loss of the Sp1 site alone or together with RORE mutagenesis reduced activity to that observed with the m43m. These findings indicate that within the context of the m234 construct the 3′ Sp1 is nearly essential for activity, and RORE adds an additional ~30% of activity. The sequences between the 5′ end of the Sp1 site and −114 have minor, but consistent, positive effects, although this region did not footprint; nor are these known transcription factor binding sites in this region. Also, construct m310mC, which contains an additional 5′ 76 bp and a destroyed 3′ Sp1 site, gave promoter activity that was ~80% reduced relative to the wild-type sequence (m310) and about 45% of that for the shortest construct, m43. These results indicate the major importance of the most 3′ Sp1 (designated D) site to the promoter of prosaposin.

The next series of constructs evaluated the effects of the 5′ Sp1/U cluster in the context of intact 3′ Sp1 and RORE. For clarity, the overlapping 5′ Sp1 sites have been designed from 5′ to 3′ A, B, and C. Destruction of the Sp1 and U sites individually or in several combinations led to increased activity over the wild-type sequence (m310). The largest increases were found when site Sp1B and -C were destroyed alone (m310mA) or together with U (m310mD). Obliteration of Sp1A produced a small increase (m310mI), as did combinations of the Sp1A, -B, and -C mutagenesis with (m310mG) or without (m310mI) mutagenesis of U.

Similar analyses were conducted when the RORE site was mutated (m310mK backbone). Compared with wild-type sequence, the mutation of RORE enhanced activity, but the presence of additional mutations in the 5′ Sp1/U cluster modulated the observed level and pattern of increased activity. Within the context of a mutant RORE, individual mutation of Sp1C with or without Sp1B or U mutagenesis (m310mC or P) in the cluster produced small decreases (20–50%) in activity. This
contrasts with 90–160% increases in the context of a wild-type RORE. Destruction of the Sp1A, -B, and -C sites (m310mQ) produced a 90% increase in activity that was similar to that observed in the presence of a wild-type RORE site. The addition of a mutated U site (m310mR) produced the same level of activity as in a construct with an intact RORE (m310mI).

The final series of mutant constructs were made on the m310 backbone Sp1D destroyed. Compared with m310, this led to a substantial decrease in overall promoter activity (20–94%). Mutagenesis of all Sp1 sites and of the U and RORE sites (m310mM) gave absolute basal promoter activities that were very similar to those in m43m, m114m, and m234m3. Compar-
ison of m310mC and m310mN shows a 3-fold increase in activity of m310mN that has a mutagenized RORE. Thus, the m310mN reflects the basal activity of the 5′ Sp1/U cluster and that an intact RORE has a negative influence. The U region (m310mL) supports promoter activity at a level that is nearly equivalent to that observed with m114, i.e. with only Sp1D present. Taken in totality, these results support a promoter region within the first 310 bp 5′ to the transcription start site that has complex, and mostly negative, interactions between elements. Each of these promoter elements alone has substantial ability to promote prosaposin expression and modulates basal promoter activity on the Sp1D background.

**DISCUSSION**

The multifunctional properties of prosaposin, its intracellular and extracellular functions, and the temporal and spatial regulation of its locus provided the rationale for the current studies to define the elements that facilitate expression of the prosaposin gene. Of lysosomal loci, prosaposin (14), lysosomal acid lipase (26), and acid α-glucosidase (27) have shown sufficient temporal and spatial variation in transcript levels to permit differential detection by in situ hybridization. Thus, elucidation of the control elements for transcription of the prosaposin locus is essential to understanding the relationship of this regulation, biological function, and the modulation of its activities in various tissues. For the current studies, we have focused on the 310-bp 5′ to the transcriptional start site, since initial analysis in transgenic mice indicates that this is an important region for promoter activity in the central nervous system. The present results indicate a potential for complex regulation based upon the milieu of transcription factors present in various tissues. The current studies define an essential Sp1 binding site (Sp1D) just 5′ to the transcription start site a n d a 5′ functional Sp1/U cluster (Sp1A, -B, and -C, and an unknown factor (U)), and RORE. All of these participate in the modulation of prosaposin transcriptional activity. Furthermore, Sp1 and Sp3 interact at sites in the Sp1/U cluster to modulate prosaposin transcriptional activity. Sp1, Sp3, and Sp4 bind to the Sp1D site. Other potential binding sites in this region, i.e. AP-1 and GATA, were nonfunctional and apparently play little role in the modulation of transcriptional activity of this locus.

The most 3′ Sp1 site, just 16 bp upstream from the major transcriptional start site, is essential to promoter activity. From EMSA, the Sp1, Sp3, and Sp4 members of the Sp family of transcription factors bind to this region. Although Sp1 and Sp3 are ubiquitously expressed with some temporal and spatial
FIG. 7. Mutagenesis of major transcriptionally active binding sites of the prosaposin promoter. Constructs refer to Table II where the designations and exact mutagenesis sequences are indicated and refer to the primers in Table I. The symbols refer to the designated transcription factor binding sites identified as participating in transcriptional modulation by DNA footprinting and/or EMSA (Figs. 2–6). The most 5′ region represents three overlapping Sp1 consensus sequences, the most 3′ of which overlaps the U (unknown) region. Together they constitute the Sp1/U cluster (Fig. 1). The Sp1 sites within m310 from 5′ to 3′ are labeled as A, B, C, and D. The open and closed symbols designate the wild-type and mutagenized sequences. The mutations obliterate the indicated binding sites. The horizontal lines represent sequences with identified (i.e. GATA or AP-1) or no identifiable transcription factor binding sites that are minor or nonfunctional by DNA footprinting, EMSA, or expression analysis. The mutagenized constructs are arranged in groups to highlight the effects of obliteration of the Sp1D site alone or in the presence or absence of wild-type or mutant RORE (first eight constructs) or the effects of systematic destruction of one or more of the 5′ Sp1/cluster sites. This was with the RORE and Sp1D intact (seven constructs). The next five constructs underwent similar analyses as the previous seven but with the RORE site destroyed. The last eight constructs underwent similar analyses with Sp1D destroyed but with the RORE site intact (5 constructs) or destroyed (3 constructs). For each group, a promoter activity value of 100% was assigned for the wild-type sequence, and the absolute value is provided for the wild-type constructs for reference. All absolute values of luciferase activity were normalized to β-galactosidase activity in lysates from individual co-transfection experiments. GL-2B and GL-2C are promoterless and SV40 promoter/enhancer-driven plasmid constructs, respectively, as negative and positive controls for the experiments.
regulation of expression (28), Sp4 expression appears to be limited to brain and the reproductive system (29). Thus, the binding of Sp1, Sp3, and Sp4 at the Sp1D site depends on the transcription factor milieu that may determine the tissue specific expression of prosaposin. The NS20Y line used here has cholinergic properties and prosaposin exerts neurite outgrowth effects in this particular NS20Y subculture (8). Also, a high level of expression of the prosaposin gene was observed in NS20Y cells by immunofluorescence analyses.2 Thus, we selected this cell type to assess the specific effects of our constructs in nervous system tissues, since primary cultures of various neurons are not clearly adaptable to transfection assays. It appears that the NS20Y cells contain at least some complement of the endogenous transcription factors present in specific neurons in the brain and probably are not representative of all specific neuronal types throughout the brain substance. Ongoing transgenic analyses in these laboratories show that the Sp1D site is also essential to prosaposin expression in transgenic animals (30). Based upon the ubiquitous expression of Sp1 and Sp3, we would propose that the Sp1D site is responsible for basal expression of prosaposin in a variety of tissues. Thus, the basal expression provided by this Sp1 site could be modulated by varying and competitive Sp3 or Sp4 levels. The Sp1D site would provide for necessary lysosomal functions of the derived saposins on glycosphingolipid hydrolases throughout the body. This Sp1 site would require interaction with other promoter components for tissue-specific modulation of transcription and activity observed throughout development and in a variety of cellular types, particularly in the brain and reproductive system.

The Sp1A, -B, and -C; U; and RORE sites appear to have a primarily negative regulatory effects on the basal activity of Sp1D in NS20Y cells. This conclusion is based upon the findings that individual mutations of these binding sites lead to an enhanced activity of the reporter gene when the Sp1D site is functional. However, when the Sp1D site is mutated, these upstream sites have some positive effects on the reporter gene transcription and can provide a substantial level expression. Mutation of all of the sites, the 5’ Sp1/U cluster, RORE, and the Sp1D, nearly obliterates activity. The negative modulatory effects on the Sp1D site of these upstream sites are supported by the relatively weak protection in DNA footprinting of the most Sp1A, -B, and -C sites and the U site. Indeed, an explanation for this relatively weak footprint is the presence of competing transcription factors Sp1 and Sp3, and potentially U, for this region leading to a less clear footprint than might be anticipated. Strong and very distinct footprints are obtained in the 5’ Sp binding regions with purified Sp1 protein. The EMSA assays clearly show specific binding to sites in this Sp1/U cluster region and competition between Sp1 and Sp3. Although the Sp1C and U sites overlap, EMSA analysis indicated they have separable activities. The U site is not an MZF binding site as is evident from competition assays with cold oligonucleotides. Thus, the transcription factor binding to the U site remains unidentified. However, the mutagenesis, supershift, and competition assays support interaction of Sp1, Sp3, and U in a complex manner for the binding sites at this upstream region. The mutagenesis of individual Sp1 and the U sites within this cluster leads to increased promoter activity over the wild-type sequence. The largest increases were observed with mutagenesis of the Sp1B and -C sites in the cluster with or without U mutations. Lower promoter activity was found with two of these cluster Sp1 sites, with or without U being obliterated. These results indicate a negative interaction between the factors binding to the Sp1B or -C sites and the U site in the cluster. The increase in absolute activity between m234 and m310 shows a positive overall effect of the presence of this 5’ cluster. The RORE has additive effects (23%) on promoter activity in the context of the Sp1D site. In constructs containing the 5’ Sp1/U cluster, the effects are less than expected. For example, m310mK increased promoter activity relative to wild type when RORE was mutated; i.e. this indicates a negative interaction between factors binding within the RORE and the 5’ Sp1/U cluster. The greater activity with m310mQ than m310mG suggests that negative interaction is primarily via the Sp1A and -B sites in the cluster, but U is not involved. Thus, it is likely that the tissue milieu of transcription factors that compete for these upstream sites could provide a variety of suppressive interactions for the Sp1D site and modulate the background activity provided by an unmodulated Sp1D site.

The RORα nuclear receptor is expressed primarily in neurons of the brain and provides a tempting target for the neuronal specific expression modulation of prosaposin. In particular, the recent identification of the RORα gene deletion in the staggerer mouse indicates the profound effects of the absence of this receptor (31). In the staggerer mouse, Purkinje cell development and migration as well as olivo-pontine neurons degenerate progressively through adulthood (32). Thus, some target

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2 P. Jin, Y. Sun, and G. A. Grabowski, unpublished observation.
sequences for RORα have a maintenance and, potentially, trophic effect upon these specific types of neurons during brain development. The massive destruction of neurons in the brains of the prosaposin knockout make it difficult to assess trophic effects of prosaposin deficiency in that animal model, but the staggerer mouse may provide an interesting model system for the evaluation of prosaposin trophic effects on cerebellar development and maintenance.

The mutagenesis and expression studies support a complex model of prosaposin promoter function within the first 310 bp 5′ to the transcription start site. A model is proposed in Fig. 8 that indicates a basal functional activity of the 3′ Sp1 site and a combination of transcription factor interactions at the more upstream sites that are primarily negative in their modulatory functions. The negative effects are primarily mediated by a negative (competitive) interaction of factors binding to the Sp1/U cluster, since enhanced promoter activity is observed when any of the sites in this cluster are obliterated. RORE factors also negatively interact with this cluster, since enhanced activity of m310 is apparent when RORE is destroyed. For the Sp1/U cluster, we showed competition of Sp1 and Sp3. We would propose that such competition could be extended to Sp4 at the Sp1D site and that the occupancy of various transcription sites, depending upon the chromatin and DNA structure in the region, could interfere or interact with each other to down- or up-regulate the basal Sp1D site function. These studies have not defined factors that are clearly tissue-specific for the modulation of prosaposin function in neurons, the reproductuctive tract, or specific epithelial cells throughout the body. Our previous in vitro studies indicate negative regulatory elements within the first 741 bp 5′ to the transcription start site and the large first intron (~15 kb) provide additional targets for tissue specificity and/or facilitator functions of prosaposin expression in more physiologic systems. However, our preliminary transgenic data (30) show that the first 310 bp 5′ to the start site contains elements that promote prosaposin expression in neurons of the central nervous system and that the interaction of the factors described here must have a significant effect on the modulation of regional prosaposin expression in that organ system.

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