Isolation and characterization of the human prosaposin promoter

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Abstract

Prosaposin is a multifunctional protein that encodes four glycoproteins, named saposins A, B, C and D. They participate in the catabolism of glycosphingolipids in lysosomes. When secreted, intact prosaposin may function as a neuritogenic factor. Human and mouse prosaposin displayed similar temporal and spatial regulation of expression. To gain insight into the transcriptional regulation of this locus, the 5′∞ region was characterized from the human prosaposin gene. The putative human promoter was shown to be TATA-less, i.e. it belonged to the TATA-less housekeeping gene family. The transcription initiation sites were localized to −23, −27, −31 and −83 bp 5′∞ to ATG, compared to −87 and −94 bp in the mouse. In SK-N-SH neuroblastoma cells, positive regulatory elements were detected −343 to −813 bp upstream of ATG. A negative regulatory region existed between −813 and −2170 bp using SK-N-SH, H441 and NS20Y cells. EMSA and DNA-footprint analysis showed that Sp1 and Sp3 are involved in human prosaposin gene regulation. Compared to the mouse promoter, the human promoter is missing a Sp1 cluster within a 310-bp upstream segment, and has AP-1, Oct-1 and two RORα sites that are protected from DNaseI by selected nuclear extracts. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Gene expression; Promoter analysis; Regulation of prosaposin gene; RORα; Sp1

1. Introduction

In humans, rats and mice, a precursor protein, prosaposin, encodes in tandem the highly homologous saposins A, B, C and D, that participate in sequential degradation of glycosphingolipids to sphingosine and fatty acids. These four saposins are proteolytically cleaved from the 508–511-amino-acid precursor (Gavrieli-Rorman and Grabowski, 1989; Nakano et al., 1989) to their mature ~80-amino-acid length through several pathways in many mammalian cells (Leonova et al., 1996). However, several tissues also secrete intact prosaposin (Collard et al., 1988; Kondoh et al., 1991), indicating the cellular control of proteolytic processing.

A specific deficiency of saposin B leads to a clinical syndrome that is similar to metachromatic leukodystrophy resulting from the deficiency of the cognate lysosomal hydrolase, arylsulphatase A. By comparison, saposin C deficiency leads to a Gaucher-like disease, i.e. acid β-glucosidase deficiency, with accumulation of glucosylceramide (Zhang et al., 1990; Holtschmidt et al., 1991b; Schnabel et al., 1991; Rafi et al., 1993). A complete deficiency of prosaposin, due to an initiation codon mutation, results in the storage of multiple glycosphingolipid substrates resembling a multilysosomal hydrolase deficiency (Harzer et al., 1989; Paton et al., 1992; Schnabel et al., 1992). The severe accumulation of these glycosphingolipids in the brain and consequent architectural distortion resulted in an early demise. This phenotype is mimicked by that of the prosaposin knockout mouse (Fujita et al., 1996).

Prosaposin is also a secretory protein present in several body fluids showed neuritogenic activity both in vivo and ex vivo. When placed in media of neuroblastoma cells, prosaposin facilitates neurite outgrowth in selected cholinergic subtypes of neurons (O’Brien et al., 1994, 1995; Qi et al., 1996). This neurite outgrowth effect is mediated via sequences in the NH2-terminal 50% of saposin C (O’Brien et al., 1995; Qi et al., 1996). Prosaposin also facilitates glycosphingolipid transfer in vitro (Hiraiwa et al., 1992), but the functional link between this property and the neuritogenic effect is unknown.

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The human gene for prosaposin has been partially characterized and contains 13 (or 14) exons and 12 (or 13) introns over about 20–40 kb on chromosome 10 (Holschmidt et al., 1991a; Gavrieli-Rorman et al., 1992). An alternatively spliced 9 bp ‘exon’ is present in the saposin B region of the prosaposin gene (Holschmidt et al., 1991b). Several large introns are located toward the more 5′ regions that encompass the exons coding for saposin A and B (Holschmidt et al., 1991a; Gavrieli-Rorman et al., 1992), but the 5′ end and promoter region have not been characterized.

The mouse locus displays highly regulated temporal and spatial expression (Sun et al., 1994). The highest levels of expression were observed in specific neurons of the adult cerebrum, the Purkinje cell layer of the cerebellum, and neurons of the lateral regions of the spinal cord (Sun et al., 1994). Components of the hindbrain also show higher levels of mRNA expression early in embryogenesis (Sprecher-Levy et al., 1993). To gain an insight into this control, we characterized the murine prosaposin promoter structure (Sun et al., 1997). It is a TATA-less promoter and contains GC box. The 5′ flanking region was shown to contain positive and negative transcription regulatory elements within 2.5 kb 5′ of the transcription start site. A major regulatory fragment was located within 310 bp 5′ to the murine prosaposin transcription start site (Jin et al., 1998), and the Sp members 1, 3 and 4, RORα (the orphan nuclear receptor) and an unidentified transcription factor (U) were involved in the regulation of this locus.

In this paper, we present the organization of human prosaposin promoter and identify that Sp1, Sp3, RORα, AP1 and Oct-1 may participate in the regulation of the human prosaposin gene. Knowledge of the entire genomic organization should facilitate the understanding of the biology of this locus.

2. Materials and methods

2.1. Materials

The following were from commercial sources: pGL2B Luciferase Reporter Vectors, Luciferase assay system, β-Galactosidase enzyme assay system, Primer extension kit and αX174 HindIII DNA markers (Promega, Madison, WI), Restriction enzyme and Taq polymerase (New England Biolabs, Beverly, MA), PromoterFinder DNA Walking Kits (Clontech, Palo Alto, CA), Sequenase® and Sequenase kit (USB, Cleveland, OH), pCRII® vector and Fast Track 2.0 mRNA isolation kit (Invitrogen, San Diego, CA), Oligonucleotide Synthesis reagents, poly(dI–dC), NAP-5 and NAP-10 columns (Pharmacia, Piscataway, NJ), Sp1, Sp2, Sp3 and Sp4 antibodies (Santa Cruz Biotechnology, Santa Cruz, CA), pNoTA/T7 vector (5 Prime–3 Prime, Boulder, CO), SI nuclease, lipofectamine, OPTI-MEM serum-free medium, DMEM and DH5α competent cells (Gibco, Grand Island, NY), Isotopes, ATP (γ32P) and dATP-dCTP (α32P) (DuPont, NEN Research Products, Boston, MA). The cell line NS20Y was from Dr Marshall Nirenberg, National Institutes of Health.

2.2. PCR cloning of the promoter fragments of human prosaposin

Using PromoterFinder DNA Walking Kits (Clontech), the human prosaposin promoter fragments were amplified from five libraries of uncloned, adapter-ligated genomic DNA fragments. The nested gene specific primers HE1 and HE2 correspond to −1 to +25 nt and −28 to −7 nt in exon 1, respectively as follows: HE1 (5′ to 3′) TGGCCAGGGAGAAG-GGGCGTACATA and HE2 (5′ to 3′) GTCT-GACTCCGCAGCTGCAAT. The anchor primers, AP1 and AP2, are supplied by the Kit. The protocol consisted of two PCR reactions with 35 or 24 cycles each. The primary PCR reaction uses the outer primers AP1 and HE1. Aliquots of primary reaction (1 μl) were used as templates in the secondary PCR reaction with nested primers AP2 and HE2. The PCR reactions were run at 95 °C for 1 min, 95 °C for 30 s, 68 °C for 5 min, and extended for 8 min at 68 °C. The solutions contain 1.1 mM Mg(OAc)2, 0.2 mM dATP, 1 × Tth PCR reaction buffer, 10 pmol of each primer, 32 units/mL Tth DNA polymerase and 8.8 μg/ml Tth Start antibody. The final products were then cloned into pCRII® vector for characterization.

2.3. Screening of PAC genomic libraries

Two probes, H9-4 and exon 2, were used to screen a human P1 plasmid library and PAC library (Genomic Systems, St. Louis, MO). H9-4 is a PCR clone of the prosaposin promoter fragment containing 360 bp upstream from exon 1. The primers made to cover exon 2 of the human prosaposin gene were used to generate exon 2 PCR product. The primers were HE2F (5′ to 3′) TGAGGACTGCGCTGACGTA and HE2R (5′ to 3′) CACTGTGGCGTTGTTCCAA. Only one clone from human PAC library hybridized to both H9-4 and the exon 2 probes. This clone was further characterized.

Bacteria carrying the positive PAC plasmids were seeded in 20 ml of LB media containing 25 μg/ml of kanamycin overnight at 37 °C. Following centrifugation, the bacterial pellets were re-suspended in 1 ml GTE (1% sucrose, 50 mM Tris–Cl, pH 8.0 and 0.01 M EDTA) with lysozyme and then incubated at room temperature (5 min). After adding 0.2 M NaOH containing 1% SDS (2 ml), the suspensions were placed on ice (5 min), and 1.5 ml of 5 M potassium acetate was added. The suspension was centrifuged (10 000 × g; 10 min), the supernatants transferred to new tubes, and incubated at 37 °C.
(30 min) with 50 μg/ml of RNase A. The DNA was extracted once with phenol/chloroform (1:1), and pre-cipitated in isopropl alcohol. The purified PAC plasmid DNA was used for restriction digestes. The DNA fragments that hybridize to the H9-4 or exon 2 probes were subcloned into Bluescript (±) vector for analysis.

2.4. Transient transfection

Based on the human prosaposin gene 5’ sequences from the PAC clones, a series of PCR primers containing required 5’ restriction enzyme sites were designed to make deletion constructs (Table 1). PCR products were directionally cloned into pNOsTA/T7 vector using Primer PCR Cloner Cloning System (5Prime→3Prime). Following restriction digestion, the PCR products were released from the vector and then cloned into the KpnI and HindIII sites of the pGL2-basic vector. These were upstream of the luciferase cDNA. HEK293 competent cells were used for transformation during cloning. The sequences of each construct were determined using the Sequenase® kit.

Confluent SK-N-SH, H441 or NS20Y cells were seeded at a density of 1 × 10⁶ cells per well into 12-well plates and incubated for 18 h at 37°C. For each deletion construct, 0.75 μg of test DNA and 0.25 μg of β-galactosidase DNA were diluted into 50 μl of OPTI-MEM serum-free medium. This solution then was mixed with lipofectamine (4 μl of lipofectamine in 50 μl DMEM). The mixture was incubated at room temperature for 30 min to allow DNA–liposome complexes to form. The complexes then were mixed with 0.4 ml of DMEM (Dulbecco’s modified Eagle’s media) and overlaid on to the DMEM rinsed cells. After incubation for 5 h at 37°C, the transfection mixtures were removed, and 1 ml of complete DMEM (with 10% fetal serum and 1% penicillin/streptomycin) was added to the cells. The cell extracts were assayed 65 h after transfection for luciferase and β-galactosidase activity.

2.5. Reporter assays

Luciferase and β-galactosidase activities were determined using the luciferase assay and β-galactosidase enzyme assay systems as described by Sun et al. (1997).

2.6. Primer extension

A primer extension analysis was carried out using the AMV Reverse Transcriptase Primer Extension System based on the human prosaposin gene 5∞ sequences from the PAC clones, a series of PCR primers containing (Promega). Primer HE1R (5’ to 3’, AGGAGGCTG-GCCAGGAGGAA) was end-labeled with (c-32P)ATP required 5∞ restriction enzyme sites were designed to make deletion constructs (Table 1). PCR products were directionally cloned into pNOsTA/T7 vector using Primer PCR Cloner Cloning System (5Prime→3Prime). Primer extension reaction was carried out at 42°C for 1 h with 1 unit of AMV reverse transcriptase in the solution containing 50 mM Tris–Cl, pH 8.3, 50 mM KCl, 10 mM MgCl₂, 10 mM DTT, 1 mM dATP, 0.5 mM spermidine and 2.8 mM sodium pyrophosphate. The products were resolved in 8% denaturing polyacrylamide gels.

2.7. S1 nuclease digestion

S1 nuclease digestion was conducted as described by Sun et al. (1997). A 61-bp-long oligonucleotide starting −57 to +4 nt of the human DNA sequence was labeled and hybridized to the mRNA purified from H441 cells. The primer extension reaction was carried out at 45°C for 1 h with 1 unit of AMV reverse transcriptase in the solution containing 50 mM Tris–Cl, pH 8.3, 50 mM KCl, 10 mM MgCl₂, 10 mM DTT, 1 mM dATP, 0.5 mM spermidine and 2.8 mM sodium pyrophosphate. The products were resolved in 8% denaturing polyacrylamide gels.

2.8. Double-strand oligonucleotides

Single-strand oligonucleotides were synthesized on a Pharmacia DNA synthesizer. After purification using the NAP-10 column, the complementary oligonucleotides were heated at 95°C for 5 min in the annealing buffer (20 mM Tris, pH 7.4, 2 mM MgCl₂, and 50 mM NaCl), then cooled to room temperature. The annealed

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Table 1

<table>
<thead>
<tr>
<th>Primers for generating deletion constructs</th>
<th>Nucleotide number* (5’ to 3’)</th>
<th>Sequence</th>
<th>Construct name</th>
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<tr>
<td>−2500 to −2477</td>
<td>CAGAGGTACCGTCGATTCTTCTTCTTCTCACATCT</td>
<td>H2.5K</td>
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</tr>
<tr>
<td>−818 to −790</td>
<td>CAGAGGTACCGGTTTAAGCTTTCTGGCCCTCT</td>
<td>H113</td>
<td></td>
</tr>
<tr>
<td>−499 to −476</td>
<td>CAGAGGTACCGGTTTAAGCTTTCTGGCCCTCT</td>
<td>H493</td>
<td></td>
</tr>
<tr>
<td>−348 to −326</td>
<td>CAGAGGTACCGGTTTAAGCTTTCTGGCCCTCT</td>
<td>H243</td>
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<td>−101 to −79</td>
<td>CAGAGGTACCGGTTTAAGCTTTCTGGCCCTCT</td>
<td>H95</td>
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</tr>
</tbody>
</table>

* Nucleotide number is derived from Fig. 4.
oligonucleotides were purified by electrophoresis in polyacrylamide gels.

2.9. DNase I footprinting and electrophoretic mobility shift assays (EMSA)

Nuclear extracts from H441 and NS20Y cell lines were used in these assays. Nuclear extract preparation, DNA-footprinting and EMSA were performed as described by Jin et al. (1998).

3. Results

3.1. Structure of the 5' region of the human prosaposin gene

PCR amplification and direct cloning from plasmid libraries were used to obtain the 5' region of the prosaposin gene. PCR reactions were accomplished with uncloned, adapter-ligated genomic DNA templates. Nested primers, corresponding to −1 to +25 and −28

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Fig. 1. PCR products of 5' flanking sequence of human prosaposin (A). PCR products were generated using nested primers from exon 1 and adapter primers from PromoterFinder DNA Walking kit. The sizes of the PCR products are indicated on the right. (B) Southern blot analysis of a PAC clone. Following digestion with the indicated restriction enzymes, the PAC clone was subjected to Southern blot analysis using probes H9-4, exon 2 PCR product or 3 kb fragment of 3' end of clone H9. Three fragments were cloned into pBluescript vector. H9 was released by HindIII and hybridized to H9-4; E5 was released by EcoRI and hybridized to exon 2. B4 was released by BglII and hybridized to a 3 kb fragment. The sizes of the DNA markers are indicated on the right.
to −7 (ATG = +1) in exon 1, were used to obtain two PCR products, termed H9-4 (369 bp) and H11-9 (~2.5 kb) (Fig. 1A). From the sequence analysis, these fragments were found to contain 5′ flanking regions to exon 1.

To obtain the entire prosaposin gene and extended 5′ flanking sequence, the human P1 and PAC plasmid libraries were screened with H9-4 and a PCR product of exon 2. From a PAC clone that hybridized to both probes, HindIII, (H9), and EcoRI, (E5) fragments were cloned into pBluescript (Fig. 1B). H9 contained 3.5 kb of flanking sequence 5′ to exon 1, and 6 kb of intron 1. E5 included exons 2 and 3, and 2.8 kb of intron 1. To define the length of intron 1, a BglII fragment (Bgl4), that hydridizes to a 3-kb fragment from the 3′ end of clone H9, was shown to contain ~18 kb of intron 1. A map of the ~25 kb of intron 1 is shown in Fig. 2. About 2 kb of this intron could not be cloned.

### 3.2. Transcription initiation site and analyses of the 5′ sequence

Primer extension and S1 nuclease mapping were used to identify the transcription initiation site of human prosaposin mRNA. Primer extension assays used labeled 20-nt primer HE1R (+13 to +32) for annealing to mRNA purified from H441 cells. A 115-bp fragment and several shorter products were suggested from primer extension (Fig. 3A). The 115-bp extension product located the transcription initiation site at 83 nt upstream from the translation start codon, ATG. From S1 nuclease studies using a 61-nt oligonucleotide, the shorter products were resolved and mapped to minor transcription initiation sites about 30 nt 5′ to the ATG (Fig. 3B). Three S1 digestion products were obtained and located the minor sites to −23, −27 and −31 nt 5′ to the ATG (Fig. 4).

### 3.3. Functional analysis of the prosaposin promoter region

A set of 5′ deletion fragments (95 bp to 2.5 kb) were cloned individually into the GL2B vector. Promoter activity was tested in SK-N-SH (human neuroblastoma), H441 (human lung papillary adenocarcinoma) and NS20Y (mouse neuroblastoma) cells. The promoterless GL2B was used as a negative control.

The trends of luciferase expression were different in two human cell lines (Fig. 5). In SK-N-SH cells, the constructs H213 and H343 showed an approximately 50-fold increase in promoter activity compared to H9. The constructs H493 and H813 produced the greatest activities (~150-fold). These results show that a potential positive regulatory elements (possibly Sp1) is located between −343 and −493 nt. The construct H2.5K showed approximately 60% diminished activity relative to H813 or H493, indicating that the sequence from −813 to −2500 may contain the negative regulatory elements. A similar pattern of expression was observed in mouse NS20Y cells. In H441 cells, the pattern of promoter activity was different. With constructs H213, H343, H493 and H813, approximately 170- to 220-fold increases of luciferase activity were observed. The positive effect of the 343–493 sequence observed in SK-N-SH cells was not present in H441. Also, H2.5K did not show a suppression effect in H441. All con-

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**Fig. 2.** Restriction map of the 5′ region of human prosaposin gene. Human prosaposin clones contain 3.5 kb of the 5′ flanking sequence from ATG and 25 kb of the first intron. The subclones H9, E5 and Bgl4 were derived from PAC clones and subcloned individually into Bluescript vector. PCR fragments, H9-4 and H11-9, were generated using PromoterFinder DNA Walking kits with nested primers from exon 1. The size of each clones is indicated on the right. The exons are represented by black rectangles and numbered above.
Fig. 4. Nucleotide sequence of human prosaposin promoter region. Bold letters indicate the coding sequence. Nucleotides upstream of ATG have negative numbers. The computer-identified transcription factor binding sites are underlined. The potentially functional transcription factor binding elements are boxed. The dotted line below the sequence indicates the primers used for primer extension analysis. The double-dotted line shows the 61 oligonucleotide sequence used in S1 nuclease mapping. The arrows represent the transcription start sites identified by primer extension and/or S1 mapping. The sequence has been deposited in GenBank under Accession No. AF057307.

Fig. 3. Localization of the transcription initiation sites of human prosaposin gene. Primer extension and S1 nuclease mapping analyses were used to identify the start site(s). The mRNA from H441 was used in both reactions (A). Primer extension. The extension reaction stopping at G is labeled with **. The nucleotide sequence is on the left. X174 locations of the Sp-1, RORα, and SRY in the human Hinf I size markers are on the right. (B) S1 protection mapping produced partial digestion of a 61 nucleotide annealed fragment resulting in three products (arrows).

structs, except H95, gave approximately 200- to 300-fold increases in luciferase activities. Background activity was obtained from construct H95, which has 18 nt upstream from the Cap site, in all three cell lines.

3.4. Identification of transcription factors involved in the regulation of human prosaposin gene

From a computer analysis, the nucleotide sequence upstream to the transcription initiation site was found to contain a GC box, Sp1 site at position −20 nt 5’ from the transcription initiation site. TATA and CAAT boxes were not identified (Fig. 4). The human prosaposin promoter region up to −818 bp contains sites for RORα, GATA, AP-1, Lyf-1, CdxA and SRY. The locations of the Sp-1, RORα and SRY in the human and mouse gene are in the same relative positions (Fig. 6). The mouse gene has a Sp1 cluster of three Sp1 half sites in the 5’ region. This is not present in the human sequence. Two RORα sites were predicted by sequence analysis in the human sequence, whereas only one is present in the mouse. The AP-1 site in the mouse is not functional. The overall nucleotide identity of these regions of human and mouse gene is 45%.

Transient transfection results show that there exists positive regulatory element(s) within 343 bp to the translation start site. In order to determine the factor(s) that may bind to this region, DNA-footprinting analysis was performed (Fig. 7). The analysis showed several DNase I protected regions within 343 bp fragment when
Fig. 5. Deletion analysis of human prosaposin promoter activity. The deletion constructs were cloned just 5' to the luciferase cDNA, and the length of the prosaposin 5' sequence inserted is shown in (A). The fold increases of luciferase activity in (B) mouse NS20Y cells, (C) H441 cells, and (D) human SK-N-SH are shown in the bar graph together with the standard errors. The average fold increase for each construct is indicated to the right of each bar. Experiments were performed in triplicates of three independent experiments. Luciferase activities of the test samples were referenced to those from promoterless GL2B. The transfection efficiency was normalized to β-galactosidase activity from co-transfected constructs containing the β-galactosidase cDNA driven off the CMV promoter.

Fig. 6. Comparison of 5' regions of human and mouse prosaposin gene. The exons are represented by a black rectangle and numbered above. The transcription factor binding elements are labeled above. Their relative nucleotide positions to ATG are indicated below. A of ATG is numbered as +1. Only the major transcription initiation site is indicated by the arrow. The Ub and Um refer to unknown transcription factor binding sites.
Fig. 7. DNase I footprinting analysis of human prosaposin promoter region. In-vitro DNase I digestion of the sense strand of the human promoter region was done in the presence of H441 or NS20Y cell nuclear extracts. Bovine serum albumin (BSA) was used as the control. The triangle indicates increasing amounts of different nuclear extracts. The G+A lane represents the Gilbert sequencing reaction products for G and A. The boxes on the right indicate the regions protected from DNase I digestion by different nuclear extracts. The filled rectangles are strongly protected regions. The hatched boxes were regions that were less strongly protected. The dotted boxes were regions that were only protected with H441 cell nuclear extract. The blank box was a region that was protected only with NS20Y cell nuclear extracts. The transcription factor binding sites are labeled on the right.

using nuclear extract from H441 and NS20Y cells and sense probe. The control reactions contain BSA. Two strongly protected regions (−321 to −309 and −114 to −97) were observed in both cell nuclear extracts. These contain sequences similar to those for Oct-1 and Sp1 binding sites, respectively (Fig. 7). Several weakly protected regions were detected in both cell lines, including −304 to −252 (RORα binding sites), −151 to −136 (AP-1 binding site) and −203 to −190 (an unknown transcription factor binding site).
Interestingly, the protection at the second ROR\(\alpha\) site (−274 to −252) was only observed with HH441 nuclear extract. At the most 3′ region, two protected regions were observed in HH441 and NS20Y cell nuclear extract. By using the antisense probe, these two protected regions were close to transcription start site (data not shown). It is possible that this protection resulted from RNA polymerase II binding to the template.

In order to define which transcription factor(s) binds to the 3 Sp1 binding site, electrophoretic mobility shift assays (EMSA) were performed with the double-strand oligonucleotide, HSP (Fig. 8A), that covers this site, and different nuclear extracts. When EMSA was conducted with probe HSP and HH441 nuclear extract, several major DNA-protein complexes were observed (Fig. 8B, lanes 1 and 2). The formation of a DNA-protein complex was competed by an excess of unlabeled HSP, but not by MHSP, which is a HSP oligonucleotide containing a mutated Sp1 binding site (Fig. 8B, lane 3 and lane 4). The addition of excess cold Sp1 consensus oligonucleotide competed with labeled HSP for binding to the protein(s) (Fig. 8B, lane 5). 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 (Hagen et al., 1992; Kinsley and Winoto, 1992). Polyclonal antibodies against Sp1, Sp2, Sp3 and Sp4 were used for EMSA. Preincubation of anti-Sp1 or anti-Sp3 antibodies with HH441 nuclear extract produced a supershift (Fig. 8B, lanes 6 and 8). Similar results were observed with NS20Y cell nuclear extract (Fig. 8C).

4. Discussion

Human prosaposin, like its mouse homologue (Sun et al., 1997), belongs to the TATA-less house-keeping gene group. It contains a GC box as the proximal promoter. The human gene also has a larger first intron (~25 kb) than that in the mouse (~15 kb). The pres-

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Fig. 8. Electrophoretic mobility shift assay (EMSA) with HSP (HSP oligonucleotide, −118 to −86 bp) and different cell nuclear extracts. Labeled oligonucleotide was incubated with HH441 or NS20Y cell nuclear extracts in the presence or absence of 100-fold excess of unlabeled oligonucleotides or anti-Sp antibodies. Poly (dI–dC) (800 ng) was added to each reaction. (A) DNA sequences of double-stranded oligonucleotides used in the reactions. (B) EMSA with HSP and HH441 cell nuclear extract. The legend above each lane indicates the contents of each reaction in addition to radiolabeled HSP. 100 x cold HSP, MHSP and Sp1 refer to the molar excess over the amount of radiolabeled HSP, a mutated HSP with destroyed Sp1 binding site and consensus Sp1, respectively. The amount of antibody in each reaction from lane 6 to lane 9 is 0.5 μg. Lane 5 contains only radiolabeled HSP. (C) EMSA with HSP and NS20Y cell nuclear extracts. The legend above indicates the contents of the reactions in each lane in addition to radiolabeled HSP. Lane 1 contains the HSP probe only. F is the unbound probe.
ence of this large intron 1 contributed to the difficulty in cloning of the promoter and 5' flanking sequence of prosaposin. Multiple transcriptional initiation sites were defined upstream in the human gene at −23, −27, −31 and −83 bp of 5' to translation start ATG, whereas only two were present in the mouse. Like the mouse counterpart, positive and negative regulatory elements were found in the human prosaposin using SK-N-SH cells. The region of 493 to 813 nt increased promoter activity by 70% compared to the construct H213 that contains only Sp1 and Ap1 binding sites. However, the facilitator activity within 493 to 813 nt was not observed in H441 and NS20Y cells. Either the compositions of the transcriptional factors in these two cell lines are different from those in SK-N-SH, or the weak positive effect was masked by potent proximal promoter activity. A repressor element within 2.5 kb decreased the activity by 60% in all three cell lines. Curiously, an approximately 20-fold greater promoter activity was obtained with the human deletion constructs in NS20Y cells when compared to similar mouse constructs (Sun et al., 1997).

These results indicate that the major control elements in humans and mice may be similar. Searches showed that the 5' flanking regions of human and mouse prosaposin gene shared a relatively low (~45%) homology. However, the human and mouse genes have some transcription factor binding sites in common, including Sp1, \( \text{ROR} \alpha \) and SRY. In the mouse gene, the Sp1 and \( \text{ROR} \alpha \) are protected by nuclear extracts from NS20Y cells. Mutation of these elements significantly diminishes promoter activity (Jin et al., 1998). DNaSE I footprinting showed that both \( \text{ROR} \alpha \) binding sites in the human gene were protected by nuclear extracts from H441, but only one was protected by NS20Y extracts. In contrast to the mouse promoter region, the human gene has DNaSE protected AP-1 and Oct-1 sites. The major difference between the promoters of these two species is the lack of a three-Sp1 half-site cluster within 400 bp of the ATG in mice. This difference and the presence of an additional \( \text{ROR} \alpha \) and functional AP-1 and Oct-1 sites in the human promoter indicates a potential significant difference in the regulation of this locus in these two species.

Current evidence suggests that prosaposin is essential for the degradation of glycosphingolipids by lysosomal enzymes, and may function as a neurotrophic factor (O'Brien et al., 1994; Qi et al., 1996) and in nerve regeneration (Kotani et al., 1996). In addition, prosaposin binds to gangliosides and functions in vitro as a transport protein for such lipids (Hiraiwa et al., 1992). These functions relate to glycosphingolipid metabolism. Interestingly, the developmental expression of prosaposin correlates with that of brain gangliosides. The expression of these lipids is tightly controlled, temporally and spatially, in the developing brain (Vanier et al., 1971). In humans, total gangliosides remain low during early gestation, increase approximately threefold from the 10th gestational week to 5 years of postnatal life (Svennerholm et al., 1989), and then remain constant throughout life. The developmental profiles in rat brain are similar (Vanier et al., 1971). The brain prosaposin expression has a similar pattern. Low levels of prosaposin mRNA expression were present in mice during gestation. After birth, the mRNA levels increased by at least two- to threefold (Sun et al., 1994).

Increased gangliosides are associated with human primary brain tumors (Fredman et al., 1988). Indeed, Fredman (1994) proposed that the uncontrolled growth and metastatic properties of such tumor cells may be a consequence of an aberrant overabundance of gangliosides. Interestingly, a high level of expression of prosaposin was detected in some human tumors, including neuroblastoma, ganglioneuroblastoma and adrenal corticocarcinomas. Low levels were found in poorly differentiated Wilm's tumors and megakaryocytic leukemias (data not shown). However, the causal link between these phenomena and prosaposin remain undefined.

Recently, prosaposin was suggested to be a myelotrophic factor (Hiraiwa et al., 1997; Campana et al., 1998). Prosaposin stimulated sulfatide synthesis, a major component of myelin, in Schwann cells and oligodendrocytes through a G-protein MAPK pathway (Campana et al., 1998). In contrast, hypomyelination was observed in prosaposin deficient humans (Harzer et al., 1989) and mice (Fujita et al., 1996). Prosaposin is also regulated during recovery from nerve injury. Rat prosaposin mRNA was decreased for the first 2 days following crush injury to the sciatic nerve. This was followed by a sixfold increase in prosaposin mRNA after 7 days, and then by a decline to control levels by 4 weeks (Gillen et al., 1995). Together these as-yet mechanistically non-unified observations suggest the intriguing possibility of a feedback regulation by sphingolipids to alter the expression of prosaposin.

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References


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