Genomic Structure and Promoter Activity of the Mouse Polysialic Acid Synthase (mST8Sia IV/PST) Gene*

(Received for publication, November 10, 1997, and in revised form, January 12, 1998)

Shou Takashima‡, Yukiko Yoshida‡§, Tae Kanematsu, Naoya Kojima, and Shuichi Tsuji¶

From the Department of Molecular Glycobiology, Frontier Research Program, The Institute of Physical and Chemical Research (RIKEN), Wako, Saitama 351-01, Japan

The mouse gene encoding ST8Sia IV/PST, one of two polysialic acid synthases, was isolated and characterized. The mST8Sia IV/PST gene was found to comprise over 60 kilobases and to be composed of five exons. Primer extension analysis revealed that transcription started from 333 nucleotides upstream of the translational initiation site. Transfection with nested deletion mutants of the 5′-flanking region fused to the luciferase reporter gene revealed that the promoter activity of the −107/+145 region was correlated with the gene expression of mST8Sia IV/PST in embryonal carcinoma P19 and neuroblastoma F11 cells. This proximal promoter region lacks an apparent TATA box but has putative binding sites for transcription factors Sp1 and NF-Y (CCAAT binding protein) at nucleotide positions −66/−57 and −47/−37, respectively. Individual deletions and mutations of the inverted Sp1 binding site or inverted NF-Y binding site caused significant reduction of the promoter activity, indicating that each binding site was involved in essential transcription control. Mobility shift assaying also revealed that Sp1 and NF-Y in a nuclear extract of P19 cells bind to the promoter region of the mST8Sia IV/PST gene. Deletion of the region from −60 to −40, which contains parts of both the Sp1 and NF-Y binding sites, completely abolished the promoter activity, suggesting that both Sp1 and NF-Y are synergistically involved in transcription regulation of the mST8Sia IV/PST gene in P19 and F11 cells. Although the overall structures of the two polysialic acid synthase genes (ST8Sia II/STX and IV/PST) are very similar, there is no extensive sequence homology between the 5′-flanking regions of the ST8Sia II/STX and IV/PST genes, suggesting that these two genes are expressed under different regulatory systems.

Polysialic acid (PSA)3 is a linear homopolymer of α2,8-sialic acid residues mainly associated with the neural cell adhesion molecule (N-CAM) in mammalian cells and is implicated in the reduction of N-CAM adhesion through its large negative charge (1). In the late embryonic and early postnatal stages, neurons mainly express the highly polysialylated form of N-CAM (2, 3). However, in the course of neural development, the content of PSA associated with N-CAM decreases, resulting in an increase in the adhesive ability of the N-CAM itself (3–5). Recent data imply important functions of PSA in the pathfinding and targeting in the innervation of axons, migration of neuronal cells and tumor cells, and spatial learning and memory (6–8).

In 1995, Eckhardt et al. (9) cloned the cDNA of a sialyltransferase, which is the key enzyme for PSA expression in Chinese hamster ovary cells, and named the enzyme polysialyltransferase-1 (PST-1). We independently cloned a mouse cDNA encoding an α2,8-sialyltransferase, ST8Sia IV, whose amino acid sequence exhibits 99.8% identity to that of hamster PST-1, and showed that ST8Sia IV is a PSA synthase (10). On the other hand, we demonstrated that ST8Sia II/STX is another PSA synthase (12). In the mouse, the amino acid sequences of the two types of PSA synthases, ST8Sia II/STX and IV/PST, exhibit 56% identity, which is the highest score among the sialyltransferases cloned so far. Northern blot analysis indicated that expression of the ST8Sia II/STX gene was restricted to the brain and testis, whereas the ST8Sia IV/PST gene was expressed strongly in the lung, spleen, and heart, rather than the brain (10, 12). Expression of the ST8Sia II/STX gene in the brain was strictly regulated during development (12). Expression of the ST8Sia IV/PST gene was also higher in fetal than adult brain but was less regulated during brain development as compared with that of the ST8Sia II/STX gene (10). Our recent results indicated that ST8Sia II/STX and IV/PST synthesize PSA of different sizes in vitro and in vivo (17, 18). However, it is not clear why two types of PSA synthases exist and how they are differently expressed.

To elucidate the mechanisms underlying the differential expression of the mST8Sia II/STX and IV/PST genes, it is important to know the structures and activities of their promoters. We recently reported the entire genomic organization and the promoter structure of the mST8Sia II/STX gene (19). We demonstrated that the minimal promoter region of the mST8Sia II/STX gene conferred the cell type-specific expression in the reporter gene. The minimal promoter was embedded in a GC-rich domain (GC content, 74%), in which two Sp1 binding motifs as well as a long purine-rich region were found, but it lacked TATA and CAAT boxes. In the present study, we describe the genomic structure of the mST8Sia IV/PST gene and cell adhesion molecule; kb, kilobase(s); bp, base pair(s); PCR, polymerase chain reaction; RACE, rapid amplification of cDNA ends; nt, nucleotide(s); the abbreviated nomenclature for cloned sialyltransferases follows the system of Tsuji et al. (31).
its promoter sequence involved in the regulation of transcription activity.

**EXPERIMENTAL PROCEDURES**

Isolation of Genomic and cDNA Clones Encoding mST8Sia IV/PST—A mouse genomic cosmid library was constructed and screened as described previously (20). The locations of the exons of the mST8Sia IV/PST gene were determined by PCR (GeneAmp XL PCR Kit, Perkin-Elmer) with specific oligonucleotide primers or by hybridizing radiolabeled cDNA to the same blots.

Amplification of the 5′-cDNA End (RACE)—A mouse genomic cosmid library was constructed and screened as described previously (20). The locations of the exons of the mST8Sia IV/PST gene were determined by PCR (GeneAmp XL PCR Kit, Perkin-Elmer) with specific oligonucleotide primers or by hybridizing radiolabeled cDNA to the same blots.

**TABLE I**

| Primer | Sequence | Strand | Position |
|--------|----------|--------|----------|
| O5-3A  | 5′-CTGAGCTAATGTGATCGTCTGAT-3′ | Sense   | +5447 to +5740 |
| O5-3B  | 5′-ACCGAGAAGACCTTGCTGACAC-3′ | Antisense | +5922 to +5899 |
| O5-3C  | 5′-CGAGCAGCTAATGTGATCGTCTGAT-3′ | Sense   | +5603 to +5626 |
| O5-EX2 | 5′-CCGGGGAATTCTTGCTGACAC-3′ | Antisense | +325 to +293 |
| O5-N5  | 5′-GGGAGCTGATCCTGCTGACAC-3′ | Antisense | +194 to +152 |
| O5-530X| 5′-GGGGCTGATCCTGCTGACAC-3′ | Antisense | +298 to +179 |
| O5-440X| 5′-GCTACCTGAGTCCAGACGAGCAGA-3′ | Sense | -165 to -84 |
| O5-350N| 5′-TTCTCCCTGCTAGGGCAGAAGAGGGCGAGTGA-3′ | Sense | -22 to +9 |
| O5-310X| 5′-GCCGGCACTTCTGAGAAGAGGGCGAGTGA-3′ | Sense | +18 to +49 |
| O5-150H| 5′-GGGCCGAGCTTCTGAGAAGAGGGCGAGTGA-3′ | Sense | +151 to +122 |
| Sp^+~N | 5′-TGCTGGAATGAGGGCGAGCAGGAG-3′ | Sense | +65 to -37 |
| Sp^+~C | 5′-GCCCTGGAAATGAGGGCGAGCAGGAG-3′ | Sense | +64 to -95 |
| BSp^+~N | 5′-AAGGCGGAATGAGGGCGAGCAGGAG-3′ | Sense | -49 to -21 |
| BSp^+~C | 5′-CAGGCGGCAGGAGCAGGAG-3′ | Sense | -70 to -43 |
| C-N   | 5′-TACACCTGAGGCCCCGAGCAGGAG-3′ | Sense | +61 to +39 |
| C-N   | 5′-CGACCTGAGGCCCCGAGCAGGAG-3′ | Sense | +68 to -40 |
| O-N   | 5′-TACACCTGAGGCCCCGAGCAGGAG-3′ | Sense | -68 to -33 |
| D-N   | 5′-GACCGGCACTTCTGAGAAGAGGGCGAGTGA-3′ | Sense | -64 to -37 |
| D-N   | 5′-GCCGGCACTTCTGAGAAGAGGGCGAGTGA-3′ | Sense | -29 to -29 |
| D-N   | 5′-GCCGGCACTTCTGAGAAGAGGGCGAGTGA-3′ | Sense | -59 to -34 |
| D-N   | 5′-GCCGGCACTTCTGAGAAGAGGGCGAGTGA-3′ | Sense | -52 to -27 |
| E-N   | 5′-CAAGGGCAAACTGCGCTGAGGA-3′ | Sense | -56 to -31 |
| E-N   | 5′-CGGTTAATGTTTGGCTGCTTAT-3′ | Sense | -49 to -25 |
| F-N   | 5′-AGAGGCCAACTGCGCTGAGGA-3′ | Sense | -55 to -29 |
| F-N   | 5′-GGTTAATGTTTGGCTGCTTAT-3′ | Sense | -47 to -22 |
| Ap^+~N| 5′-CTCTCCCTAGATGCCCCAGGCTTCTGAGAAGAGGGCGAGTGA-3′ | Antisense | +93 to +62 |
| Ap^+~C| 5′-AACCTGGATGCCCCAGGCTTCTGAGAAGAGGGCGAGTGA-3′ | Sense | +76 to +107 |
| FAp^+~N| 5′-TGCAATGAGGATGCTGCTGAGAAGAGGGCGAGTGA-3′ | Sense | +53 to +22 |

Analysis of Promoter Activity—To obtain various lengths of the 5′-flanking region of the mST8Sia IV gene, PCR with Tth DNA polymerase (GeneAmp XL PCR Kit, Perkin-Elmer) was performed using two primers, O5–ATGNg0 (Table I) and a reverse sequencing primer, with pO5–22E1.5 as the template. The mST8Sia IV/PST-luciferase fusion gene expression plasmids were constructed by subcloning the following restriction fragments from the PCR products into pBluescript (pBluescript II, Toyko, Japan): pBo5–BN0.87 carries a 0.87-kb BamHI-Nco fragment and pBo5–SaN0.16 carries a 0.16-kb SacI-Nco fragment, respectively. Other series of deletion plasmids were constructed by subcloning the restriction enzyme-digested PCR products amplified using the primer set of O5–ATGNg0 and the restriction enzyme site introducing mutagenic primers into pBluescript. The primers and template plasmids used as follows were: pBo5–XN0.53 carries a 0.53-kb XhoI-Nco fragment amplified by using the primer set of pO5–530X/O5–ATGNg0; pBo5–XN0.44 carries a 0.44-kb XhoI-Nco fragment amplified by using the primer set of pO5–440X/O5–ATGNg0; pBo5–XN0.35 carries a 0.35-kb XhoI-Nco fragment amplified by using the primer set of pO5–350X/O5–ATGNg0; pBo5–XN0.31 carries a 0.31-kb XhoI-Nco fragment amplified by using the primer set of pO5–310X/O5–ATGNg0; and pBo5–XH0.25 carries a 0.25-kb XhoI-HindIII fragment amplified by using the primer set of pO5–250X/O5–ATGNg0. Each clone was subcloned into pBo5–XN0.25 digested with the same restriction enzyme. The pBo5–XH0.25 fragment was amplified by using the primer set of pO5–250X/O5–ATGNg0.

In all the cell lines tested, pBluescript II was inactive to the expression of luciferase activity, whereas pBluescript caused a high level of expression.

NIH3T3, F11, undifferentiated-P19, Neuro2a, F9, B16, LL2, C2C12, and NMuMG cells were seeded at 5 × 10⁵ cells per 60-mm diameter dish in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum 24 h prior to transfection, respectively. For the neural
diffuion of P19 cells into neuronal cells, the cells were seeded into and aggregated in bacteriological grade dishes in the presence of 1 mm retinoic acid at the cell density of 1 x 10^6/ml. After 3 days, the aggregates were trypsinized, and then approximately 1 x 10^6 cells per 60-mm diameter dish (tissue culture grade dishes) were plated in Dulbecco's modified Eagle's medium, 10% fetal calf serum 24 h prior to transfection.

The luciferase plasmid (5 μg) used as the reporter and the pBNG-gal plasmid (0.5 μg) used as an internal control for transfection efficiency were transfected into the cells by means of LipofectAMINE (Life Technologies, Inc.). After 48 h transfection, the cells were washed three times with phosphate-buffered saline and then lysed with cell lysis buffer (PB-50, Toyo-Ink, Japan). Luciferase activity was measured using a PicoGene Luciferase Assay System (Toyo-ink) and a Luminescence AB-2000 (ATTO, Japan). Light activity measurements were performed in quadruplicate, averaged, and then normalized to the β-galactosidase activity to correct for the transfection efficiency. β-Galactosidase activity was measured using a Luminescent β-Galactosidase Detection Kit II (CLONTECH).

The primers used for the construction of the Sp1 site-replaced mutants and site-directed deletion mutants of the Sp1, NF-Y, and AP-2 binding sites—Binding Sites—The primers used for the construction of site-replaced mutants and site-directed deletion mutants of the Sp1, NF-Y, and AP-2 binding sites are listed in Table I. The AP-2 binding site-replaced mutants, pBO5-XN0.63 (Sp1*, NF-YB*, and AP2*) and pBO5-XN0.53 (Sp1*, NF-YB*, and AP2*), were constructed by subcloning into pGEMII an XhoI-EcoRI fragment amplified with the primer set of Ap*-C/O5-ATGNco. All fragments were verified by restriction mapping and sequencing.

Cloning of the NF-Y Gene—The NF-YA gene was cloned from P19 cells by reverse transcriptase-PCR using primers 5'-GAAGCTTCAG-GACTCTTA1A3' and 5'-TGACTGACGTCTCTGGCCAGC3' (22). PCR products were cloned into pBluescript II SK+ and then sequenced. The NF-YB gene (22) was cloned from an adult mouse brain cDNA library by plaque hybridization and then sequencing.

In vitro Transcription and Translation—In vitro transcription of the NF-YA and NF-YB genes was performed using an mCAP mRNA capping kit (Strategene) according to the manufacturer's instructions. The resulting mRNA samples (2 μg) were applied to a rabbit reticuloocyte lysate system (Amersham Corp.) for in vitro translation.

Gel Shift Assay—The DNA fragment from -107 to -16 was prepared from pBO5-XN0.44 by digestion with XhoI and PstI and then end-labeled with [32P]dCTP using Klenow polymerase. Binding assays were performed with a labeled probe (10–20 k rpm) in the presence of 2 μg of poly(dI-dC)poly(dI-dC) (Pharmacia) and 2 μg of a nuclear protein extract on an appropriate volume of the products translated in vitro. Binding reactions were carried out for 30 min at 0°C in 25 mM HEPES-KOH (pH 7.9), 0.5 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 10% glycerol. Competitor fragments or anti-Sp1 antibodies (Santa Cruz Biotechnology) were included where indicated. The DNA fragment from -339 to -221, which was prepared from pBO5-XN0.63 by digestion with XhoI and BglII, and the DNA fragment from -107 to -16 were used as nonspecific and specific competitors, respectively. The synthetic DNA fragments, 5'-GGCG-CCCTTCAGACGCGGTATGGCGTG-3' (nucleotide positions from -62 to -36) and 5'-AGGCCGACTACCTAGTGGCAAGGGG-3' (complementary to nucleotide positions from -60 to -33), were used as competitors after the two synthetic DNAs had been annealed. After incubation, the samples were loaded onto a 4% polyacrylamide gel (acrylamide:bisacrylamide, 19:1) in 0.5X TBE. The gel was run in the cold at 200 V and dried, and then the radioactivity was detected with a BAS 2000 image analyzer (Fuji Film, Ltd.).

RESULTS

Isolation of mST8Sia IV/PST Genomic Clones—The screening of an NIH3T3 cell cosmid library with mST8Sia IV/PST cDNA resulted in the isolation of three independent genomic clones. A restriction map of the approximately 100-kb region containing the mST8Sia IV/PST gene is shown in Fig. 1. The locations of the mST8Sia IV/PST exons were determined by PCR and Southern blot hybridization using a variety of oligonucleotides designed according to the known mST8Sia IV/PST DNA sequence. Since cross-hybridization experiments indicated that CosO5-12 and CosO5-19 did not overlap, further screening of 5 x 10^6 genomic clones (inserts ranging from 30 to 40 kb in size) was performed with a 2-kb fragment of CosO5-12 including exon 4 and a 1.3-kb fragment of the T3 primer end of the mST8Sia IV/PST gene.
CosO5-19 as probes. However, no overlapping clones were obtained. Southern blot analyses of the NIH3T3 genomic DNA using the above two probes showed that the distance between the termini of CosO5-12 and -19 was over 12 kb (data not shown).

We sequenced the exons to determine their exact sizes and the intron/exon junctions (Table II). The sequences of all the intron/exon splice junctions conformed to the GT-AG rule (23). The mST8Sia IV/PST cDNA was divided into 5 exons, ranging from 132 to 6566 bp, with intron sizes of 8–24 kb, and spanning more than 60 kb of genomic DNA (Fig. 1). Exon 1 contained the entire 5′-untranslated region and the beginning of the coding region to amino acid residue 37, containing a cytoplasmic domain of the enzyme, and exon 5 contained a large 3′-untranslated region. Exons 3–5 encoded the putative active main, a short hydrophobic signal anchor sequence, and a part of the stem domain. Exons 3–5 encoded the putative active domain of the enzyme, and exon 5 contained a large 3′-untranslated region. We previously reported mST8Sia IV/PST cDNA sequences lacking the whole 3′-untranslated region. Therefore, to determine the 3′ end of the 5-kb mST8Sia IV/PST transcript, the mouse brain cDNA library was screened by PCR using primers distributed along the 3′ part of the gene. Sequence analyses of the PCR products revealed that the size of the transcribed RNA was 6786 bp, thus it included a large 3′- untranslated region of 5372 bp. Poly(A) addition occurred 23 nucleotides downstream of the transcription initiation site (T residue) of the polyadenylation signal (AATAAA).

**Mapping of the Transcription Initiation Site**—The transcription initiation site was determined by primer extension with RNA recovered from 1-day-old mouse brain, in which the mST8Sia IV/PST gene was expressed (Fig. 2). Northern blot analysis indicated that the mST8Sia IV/PST gene gave a single transcript, whose size was about 5 kb. The primer extension products obtained with primer O5-EX2 were analyzed on a 6% sequencing gel. The end points of the extension were determined by comparison with a sequencing ladder derived from the same genomic DNA template and the original primer, O5-EX2. The end point was determined to be a guanine (+1), which corresponded to a position 333 nucleotides upstream from the initiation codon, ATG. Moreover, we performed 5′-RACE-PCR on newborn mouse brain poly(A) RNA to identify the 5′ end of the mST8Sia IV/PST gene, and the longest RACE-PCR product corresponded to the transcription initiation site determined in the primer extension experiments. Therefore, mST8Sia IV/PST mRNA was transcribed at a single position 333 nucleotides upstream from the initiation codon at least in mouse brain and gave a single transcript.

**Analysis of the 5′-Flanking Region**—Analysis of the sequence immediately upstream of the transcription initiation site revealed that the mST8Sia IV/PST gene promoter consists of a G+C-rich sequence lacking a canonical TATA box (Fig. 3). In this promoter, an apparent G+C-rich region extends from −100 to +150 (GC content, 64%). The G+C-rich region of the mST8Sia IV/PST gene promoter is shorter and its GC content is lower than those of the mST8Sia II/STX gene promoter (nt −175 to +168, 74%) (18). The TATA-less mST8Sia IV/PST gene promoter contains an inverted Sp1 binding site at positions −66 to −57 (8 of 10 matching), an inverted NF-Y (CCAAT binding protein) binding site at positions −47 to −37 (9 of 11 matching), and an AP-2 binding site, CC/G/C/CA/G/C/GC, at positions +84 to +91 (7 of 5 matching). The 1-kb 5′-flanking sequence of the mST8Sia IV/PST gene does not exhibit extensive homology with the upstream region of the mST8Sia II/STX gene.

**Demonstration of Promoter Activity**—To characterize the regions regulating the transcription activity of the gene, we constructed a series of chimeric plasmids containing different lengths of the 5′-flanking region of the mST8Sia IV/PST gene fused to the promoterless luciferase gene in pPGBII (Fig. 4). One of the constructs, pBO5-NKN3.5, was assayed for promoter activity by transient transfection into several cell lines at first
Fig. 3. Nucleotide sequence of the 5′-flanking region of the mST8Sia IV/PST gene. The transcription initiation site (+1) is indicated by a vertical arrow. The sequence of the first exon is shown in capital letters and those of the untranscribed regions in lowercase letters. The coding sequence of the first exon is shown as codon triplets. The putative binding sites for several transcription factors are indicated. The O5-EX2 primer is indicated by arrows. For the detection of promoter activity, the start point of each construction is indicated by an arrowhead.

(Table III). Of these cell lines, embryonal carcinoma P19 cells showed the highest promoter activity, neuroblastoma F11 cells showed a moderate level of promoter activity, and NIH3T3 fibroblast cells showed a very low level of promoter activity. The level of endogenous mST8Sia IV/PST gene expression in P19 cells was similar to that in F11 cells, but NIH3T3 cells did not express the mST8Sia IV/PST gene at all (data not shown). Thus, we decided to use P19, F11, and NIH3T3 cells for the experiment. For the detection of promoter activity, the start point of each construction is indicated by an arrowhead.

To clarify the involvement of the Sp1 binding site, we constructed a series of internal deletion mutants of pBO5-XN0.53, one of which lacked the inverted Sp1 binding site and its upstream sequence (nt 47–53). A mobility shift experiment involving a synthetic DNA fragment corresponding to nucleotide positions 53–60, which failed to bind to recombinant Sp1 (data not shown). The deletion downstream of the inverted Sp1 binding site and its downstream sequence (nt 60–75) decreased the promoter activity from one-fourth to one-fifth of the wild-type construct. The promoter activity of the Sp1-mutated construct (pBO5-XN0.53 (Sp1*)) was also reduced to 40–50% (Fig. 5). In contrast, the deletion downstream of the inverted Sp1 binding site (construct pBO5-XN0.53 (Δ−60/−40)) caused a drastic decrease in the promoter activity to the basal level in all cells examined (Fig. 5). A mobility shift experiment involving a nuclear protein extract of P19 cells revealed that only one shifted band disappeared in the presence of the synthetic DNA fragment corresponding to nucleotide positions 62 to 33, suggesting the nuclear protein of P19 cells bound in this region (Fig. 6, lane 5).

To clarify the involvement of the downstream of the Sp1 binding site, we constructed a series of internal deletion mutants of pBO5-XN0.53 and analyzed their promoter activities. Each 3-base deletion from nucleotide positions –53 to −45 of pBO5-XN0.53 (constructs pBO5-XN0.53 (Δ−53/−51), pBO5-XN0.53 (Δ−50/−48), and pBO5-XN0.53 (Δ−47/−45)) had little effect on the promoter activity (Fig. 5). In contrast, the deletion of the sites from −41 to −39 and from −39 to −37 (constructs pBO5-XN0.53 (Δ−41/−39) and pBO5-XN0.53 (Δ−39/−37)) led to a reduction of the promoter activity to 42% (in the case of F11 cells), 15% (in the case of undifferentiated P19 cells), and 20% (in the case of differentiated P19 cells) as compared with the wild-type construct. The promoter activity of the Sp1-mutated construct (pBO5-XN0.53 (Sp1*)) was also reduced to 40–50% (Fig. 5). In contrast, the deletion downstream of the inverted Sp1 binding site (construct pBO5-XN0.53 (Δ−60/−40)) caused a drastic decrease in the promoter activity to the basal level in all cells examined (Fig. 5). A mobility shift experiment involving a nuclear protein extract of P19 cells revealed that only one shifted band disappeared in the presence of the synthetic DNA fragment corresponding to nucleotide positions 62 to 33, suggesting the nuclear protein of P19 cells bound in this region (Fig. 6, lane 5).
The mutation of the AP-2 binding site (pBO5-XN0.53 (Ap2*)) had little effect on the promoter activity. On the other hand, the deletion of the region between nucleotide positions −65 to −57 did not result in a consistent decrease in promoter activity, indicating that these regions are not critical for the function of the promoter in these cells.

The mutation of the AP-2 binding site (pBO5-XN0.53 (Ap2*)) had little effect on the promoter activity. On the other hand, the deletion of the region between nucleotide positions +42 and +87 increased the promoter activity (Fig. 5).

**Involvement of Sp1 and NF-Y in Transcription of the mST8Sia IV/PST Gene—**To determine whether or not the inverted CCAAT motif, which corresponds to the NF-Y binding site, was included in the site from −44 to −37, we analyzed whether or not this motif was recognized by NF-Y. In the mobility shift experiment involving the DNA fragment from −107 to −16, NF-Y translated in vitro bound to the DNA fragment (Fig. 7, lane 3), whose mobility corresponded to that of band C observed when a nuclear extract of P19 cells was used (Fig. 6, lane 2, and Fig. 7, lane 2). The shifted band was not abolished by the nonspecific competitor (DNA fragment from −339 to −221) but completely disappeared in the presence of the non-labeled specific competitor (synthetic DNA fragment from −62 to −33; Fig. 6, lane 5, and Fig. 7, lane 5). Thus, the inverted CCAAT motif at −44 to −37 was recognized by NF-Y. These results suggested that Sp1 and NF-Y are involved in the transcription of mST8Sia IV/PST mRNA.

**DISCUSSION**

We recently reported the genomic organization and promoter activity of the mST8Sia II/STX gene, a PSA synthase gene, whose expression is highly regulated during brain development (19). In the present study, we showed that the genomic organization of the mST8Sia IV/PST gene, another PSA synthase gene, is highly similar to that of the mST8Sia II/STX gene, whereas the sequence of the 5′-flanking region of the mST8Sia IV/PST gene does not exhibit extensive homology with the upstream region of the mST8Sia II/STX gene. We showed that...
the proximal promoter region of the mST8Sia IV/PST gene has the ability to express the transcriptional activity, which correlated with the endogenous mST8Sia IV/PST gene expression in several cell lines.

So far, the genomic organizations of six other sialyltransferase genes have been reported (19, 20, 25–29). Among them, the genomic structures of the mST8Sia II/STX gene is fairly similar to that of the mST8Sia IV/PST gene. In particular, three introns are inserted into the regions coding for the putative active domains of the enzymes, mST8Sia II/STX and IV/PST (Fig. 8A). The entire amino acid sequence of mST8Sia IV/PST shows 56% identity with that of mST8Sia II/STX, and its putative active domain exhibits higher similarity to that of mST8Sia II/STX (Fig. 8B). However, the amino acid sequences of exons 1 and 2 in mST8Sia IV/PST are not conserved in the corresponding exons of mST8Sia II/STX. On the other hand, both the genomic organization, and the amino acid sequence of the mST8Sia IV/PST gene showed no similarity, except in the sialyl motifs, to other known α2,3- and α2,6-sialyltransferase genes. These observations suggest that the mST8Sia II/STX and IV/PST genes are evolutionarily related and distant from other sialyltransferase genes.

In this study, we mapped a highly active promoter region of the mST8Sia IV/PST gene by transient transfection of a series of deleted promoter sequences in P19 and F11 cells (Fig. 4). Deletion analyses demonstrated that the promoter sequence from −2107 to −115 is critical for the function of the promoter, because its removal effectively reduced the reporter gene expression. The promoter activity was measured as luciferase activity, which was normalized to the β-galactosidase activity of a cotransfected internal control plasmid, pSRβ-Gal. The values are presented as percentages of the promoter activity due to pO5-XN0.53, from which was subtracted the basal activity due to pO5-XN0.31. The activities of the promoter of pO5-XN0.53 relative to those of pBSV in F11, undifferentiated P19, and differentiated P19 cells were 93.6 ± 13.6, 109.1 ± 13.4, and 126.3 ± 10.9%, respectively. The basal activities due to pO5-XN0.31 relative to those of pBSV in F11, undifferentiated P19, and differentiated P19 cells were 15.0 ± 3.7, 21.4 ± 1.4, and 19.5 ± 5.2%, respectively.

![Diagram](image-url)
inverted CCAAT motif (NF-Y binding site, nt 247 to 237). A mobility shift assay showed that the inverted Sp1 binding site was functional in the examined cells (Fig. 6). The results of deletion and mutation of the Sp1 binding site suggested that the Sp1 binding site is partly involved in transcription regulation in P19 and F11 cells (Fig. 5). We also showed the involvement of NF-Y in the transcriptional regulation, NF-Y binding to the inverted CCAAT motif located in the region from 244 to 237 in mobility shift assays (Fig. 7). It should be noted that deletion of the 260 to 240 region (pBO5-XN0.53(D260/240)) abolished the promoter activity almost completely, whereas deletion of either the inverted Sp1 site (264/257) or the inverted NF-Y site (244/237) reduced the promoter activity only partly (about 40% as compared with the wild type), and deletion of the site from 253 to 245 had little effect on the promoter activity. Thus, the two different sites, the inverted Sp1 site and the inverted NF-Y binding site, are required for the promoter activity in P19 and F11 cells. Probably, the synergetic effect of Sp1 and NF-Y is essential for the transcription of mST8Sia IV/PST mRNA. Sp1 and NF-Y are thought to be ubiquitous transcription factors. In fact, NIH3T3 cells express Sp1 and NF-Y at almost the same levels to P19 and F11 cells (data not shown), although the promoter activity in NIH3T3 cells is very low. Therefore, the minimal promoter region identified in this study seems to be an essential transcription unit, and some other transcription factors may be involved in the specific promoter activity in P19 and F11 cells.

FIG. 6. Gel shift assays of the mST8Sia IV/PST proximal promoter region with a nuclear extract of P19 cells. The 5′-end-labeled DNA fragment from −107 to −16 (lane 1) was incubated with the nuclear extract of P19 cells either alone (lane 2) or with 25 times the amount of the non-labeled specific competitor (DNA fragment from −107 to −16, lane 3), 25 times the amount of the non-labeled nonspecific competitor (DNA fragment from −339 to −221, lane 4), and 16 pmol of the non-labeled specific competitor (synthetic DNA fragment from −62 to −33, lane 5), the anti-Sp1 antibodies (lane 6), and then subjected to the gel shift assay. Lane 7 shows the results of a gel shift assay involving 0.4 footprinting units of recombinant Sp1 instead of the nuclear extract of P19 cells.

FIG. 7. Gel shift assays of the mST8Sia IV/PST proximal promoter region with NF-Y translated in vitro. The 5′-end-labeled DNA fragment from −107 to −16 (lane 1) was incubated with the nuclear extract of P19 cells (lane 2). The labeled DNA fragment was also incubated with NF-Y translated in vitro either alone (lane 3), with 25 times the amount of the non-labeled nonspecific competitor (DNA fragment from −339 to −221, lane 4), or with 20 pmol of the non-labeled specific competitor (synthetic DNA fragment from −62 to −33, lane 5) and then subjected to the gel shift assay.

inverted CCAAT motif (NF-Y binding site, nt −47 to −37). A mobility shift assay showed that the inverted Sp1 binding site was functional in the examined cells (Fig. 6). The results of deletion and mutation of the Sp1 binding site suggested that the Sp1 binding site is partly involved in transcription regulation in P19 and F11 cells (Fig. 5). We also showed the involvement of NF-Y in the transcriptional regulation, NF-Y binding to the inverted CCAAT motif located in the region from −44 to −37 in mobility shift assays (Fig. 7). It should be noted that deletion of the −60 to −40 region (pBO5-XN0.53(D−60/−40)) abolished the promoter activity almost completely, whereas deletion of either the inverted Sp1 site (−64/−57) or the inverted NF-Y site (−44/−37) reduced the promoter activity only partly (about 40% as compared with the wild type), and deletion of the site from −53 to −45 had little effect on the promoter activity. Thus, the two different sites, the inverted Sp1 site and the inverted NF-Y binding site, are required for the promoter activity in P19 and F11 cells. Probably, the synergetic effect of Sp1 and NF-Y is essential for the transcription of mST8Sia IV/PST mRNA. Sp1 and NF-Y are thought to be ubiquitous transcription factors. In fact, NIH3T3 cells express Sp1 and NF-Y at almost the same levels to P19 and F11 cells (data not shown), although the promoter activity in NIH3T3 cells is very low. Therefore, the minimal promoter region identified in this study seems to be an essential transcription unit, and some other transcription factors may be involved in the specific promoter activity in P19 and F11 cells. The mobility shift experiment indicated the occurrence of other nuclear proteins that specifically bind to the proximal promoter region of mST8Sia IV/PST. This may suggest the existence of other sites that are required for the transcriptional regulation of the mST8Sia IV/PST gene in the proximal promoter region. However, we could not identify such additional sites at this stage. Identification of such sites is required.

FIG. 8. Comparison of the genomic structures of the mST8Sia IV/PST and II/STX genes. A, intron/exon structures of the mST8Sia IV/PST and II/STX genes. The protein domain structure is represented schematically by a rectangle, which is subdivided to show the major structural elements of the protein. Sialyl motifs L and S are underlined. The nucleotide sequences of the exons at identical positions in the exon/intron junctions of the mST8Sia IV/PST and II/STX genes are shown. The derived amino acids are indicated. B, comparison of the deduced amino acid sequences of the five exons of the mST8Sia IV/PST gene with those of the corresponding exons of the mST8Sia II/STX gene.
CREB, cAMP-responsive element-binding protein. Transcription factors are shown schematically. The minimal promoter regions of the mST8Sia IV/PST and II/STX genes are shown by bold lines.

We recently demonstrated that the minimal promoter region of the mST8Sia II/PST gene conferred cell type-specific expression in the reporter gene. The minimal promoter was embedded in a GC-rich region (GC content, 74%), in which two Sp1 binding motifs as well as a long purine-rich region were found, but it lacked TATA and CAAT boxes. Comparison of the promoter regions of the mST8Sia II/STX and IV/PST genes revealed no extensive sequence homology (Fig. 9). However, both the promoters of these two genes have functional Sp1 binding site(s) but lack canonical TATA boxes. This type of promoter is usually associated with housekeeping genes but has also been found in a number of tissue-specific genes, including the neuronal cell-specific promoters of the neuron-specific enolase, type II somatostatin, synapsins I and II, and D1A dopamine receptor genes (30). Although Sp1 binding sites are found in the proximal promoter regions of the mST8Sia II/STX and IV/PST genes, NF-Y binding sites are not found in the proximal promoter region of the mST8Sia II/STX gene (−158/+167). In addition to the difference that NF-Y is involved in the regulation of the expression of the mST8Sia IV/PST gene, but not in that of the mST8Sia II/STX gene, identification of other factors that interact with the proximal promoter regions of the mST8Sia II/STX and IV/PST genes may facilitate understanding of the differential regulation of the two genes. For example, there is a putative cAMP-responsive element-binding protein binding site in the proximal promoter regions of both genes. Now, we are trying to identify regulatory factors, including cAMP-responsive element-binding protein, that interact with the proximal promoter regions of the mST8Sia II/STX and IV/PST genes.

Acknowledgments—We are grateful to Dr. Yoshitaka Nagai, Director of the Glycoscience Research Group, and Dr. Tomoya Ogawa, Coordinator of the Group, Frontier Research Program of the Institute of Physical and Chemical Research (RIKEN), for their continued support and encouragement regarding our research.

REFERENCES
1. Troy, F. A. (1992) Glycobiology 2, 5–23
2. Finne, J., Finne, U., Deagostomo-Bazin, H., and Goridis, C. (1983) Biochem.
3. Hoffman, S., Sorkin, B. C., White, P. C., Brackenbury, R., Mailhammer, R., Rutishauser, U., Cunningham, B. A., and Edelman, G. M. (1982) J. Biol. Chem. 257, 7720–7729
4. Hoffman, S., and Edelman, G. M. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 5762–5766
5. Sadoul, R., Hirn, M., Deagostomo-Bazin, H., Rougon, G., and Goridis, C. (1983) Nature 304, 474–479
6. Cremer, H., Lange, R., Christoph, A., Plomann, M., Vopper, G., Roes, J., Brown, R., Baldwin, S., Krammer, P., Scheef, S., Barths, D., Rajewsky, K., and Wille, W. (1994) Nature 367, 455–459
7. Tomasiwierz, H., Ono, K., Yee, D., Thompson, C., Goridis, C., Rutishauser, U., and Magnuson, T. (1993) Neuron 11, 1163–1174
8. Takamatsu, K., Auerbach, B., Gerard, S. R., Eckhardt, M., Jaques, G., and Madry, N. (1994) Cancer Res. 54, 2509–2513
9. Eckhardt, M., Muhlenhoff, M., Bethke, A., Koopma, J., and Gerard, S. and Schachn, M. F. R. (1995) Nature 373, 715–718
10. Yoshida, Y., Kojima, N., and Tsuji, S. (1995) J. Biochem. (Tokyo) 118, 655–664
11. Livingston, B. D., and Paulson, J. C. (1993) J. Biol. Chem. 268, 11564–11569
12. Kojima, N., Yoshida, Y., Kurosawa, N., Lee, Y.-C., and Tsuji, S. (1995) FEBS Lett. 360, 1–4
13. Kojima, N., Yoshida, Y., and Tsuji, S. (1995) FEBS Lett. 373, 119–122
14. Scheidegger, E. P., Sternberg, L. R., Roth, J., and Lowe, J. B. (1995) J. Biol. Chem. 270, 22685–22688
15. Nakayama, J., Fukuda, M. N., Fretette, B., Ranscht, B., and Fukuda, M. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 7031–7035
16. Nakayama, J., and Fukuoka, M. (1996) J. Biol. Chem. 271, 1829–1832
17. Kojima, N., Tachida, Y., Yoshida, Y., and Tsuji, S. (1996) J. Biol. Chem. 271, 19457–19463
18. Kojima, N., Tachida, Y., and Tsuji, S. (1997) J. Biochem. (Tokyo) 122, 1265–1273
19. Yoshida, Y., Kurosawa, N., Kanasue, T., Kojima, N., and Tsuji, S. (1996) Glycobiology 6, 773–780
20. Deleted in proof
21. Deziel, J. F., and Senaphathy, S. (1997) Nucleic Acids Res. 15, 7155–7174
22. Kojima, N., Kono, M., Yoshida, Y., Nakafuku, M., and Tsuji, S. (1996) J. Biol. Chem. 271, 30167–30173
23. Yoshida, Y., Kurosawa, N., Kanasue, T., Arita, M., Kojima, N., and Tsuji, S. (1996) Glycobiology 6, 773–780
24. Kojima, N., and Tsuji, S. (1996) J. Biol. Chem. 271, 20258–20262
25. Svensson, E. C., Soreghan, B., and Paulson, J. C. (1990) J. Biol. Chem. 265, 20863–20869
26. Wang, X., O'Hanlon, T. P., Young, R. F., and Lau, J. T. (1990) Glycobiology 1, 5–23
27. Chang, M. L., Eddy, R. L., Shows, T. B., and Lau, J. T. (1995) Glycobiology 5, 319–325
28. Kitagawa, H., Mattei, M.-G., and Paulson, J. C. (1996) J. Biol. Chem. 271, 3851–3858
29. Kurosawa, N., Inoue, M., Yoshida, Y., and Tsuji, S. (1996) J. Biol. Chem. 271, 15109–15116
30. Twyman, R. M., and Jones, E. A. (1995) J. Neurogenet. 10, 67–101
31. Tsuji, S., Datta, A. K., and Paulson, J. C. (1996) Glycobiology 6, v–vii
Genomic Structure and Promoter Activity of the Mouse Polysialic Acid Synthase (mST8Sia IV/PST) Gene
Shou Takashima, Yukiko Yoshida, Tae Kanematsu, Naoya Kojima and Shuichi Tsuji

J. Biol. Chem. 1998, 273:7675-7683.
doi: 10.1074/jbc.273.13.7675

Access the most updated version of this article at http://www.jbc.org/content/273/13/7675

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 30 references, 13 of which can be accessed free at http://www.jbc.org/content/273/13/7675.full.html#ref-list-1