Genomic Structure and Promoter Activity of the Mouse Polysialic Acid Synthase Gene (mST8Sia II)

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The mouse ST8Sia II (mST8Sia II/STX) gene encodes a neural cell adhesion molecule-specific polysialic acid synthase whose expression is regulated during the developmental stages of mouse brain. To elucidate the molecular mechanism by which the expression is tissue-specifically and developmentally regulated, we isolated the complete genomic DNA and characterized the promoter of the gene for mST8Sia II. The gene encoding mST8Sia II was found to span about 80 kilobases and to be composed of six exons. Primer extension and S1 nuclelease protection analyses revealed that the transcription started from 167 nucleotides upstream of the translational initiation site. Promoter analyses of the 5′-flanking region of the mST8Sia II gene using a luciferase gene reporter system revealed strong promoter activity in retinoic acid-induced differentiated P19 cells, which highly express the mST8Sia II gene. Deletion analyses demonstrated that the minimal promoter activity detected for the proximal region 325 base pairs upstream from the translational initiation codon (−158 to +167) could be modulated by various sequences within the 9.5-kilobase 5′-flanking region. The minimal promoter was embedded in a GC-rich domain (74%, GC content), in which two Sp1 binding motifs as well as a long purine-rich region were found, but it lacked TATA and CAAT boxes. The positive regulatory region located between −159 and −659 contained two additional Sp1 binding motifs and a long pyrimidine-rich region. We also found that the minimal promoter region of the mST8Sia II gene was sufficient for expression of a reporter gene in mST8Sia II gene-expressing neural differentiated P19 cells but not in nonexpressing ones. Thus the TATA-less GC-rich minimal promoter region of mST8Sia II probably controls the cell type-specific expression of the mST8Sia II gene.

Polysialic acid (PSA) 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). The expression of PSA on N-CAM is developmentally regulated in the central and peripheral nervous systems (2–5), i.e. PSA on N-CAM is more abundant in the fetal than in the adult stage. Recent data imply important functions of PSA in the pathfinding and targeting on innervation of axons, migration of neuronal cells and tumor cells, and spatial learning and memory (6–8). PSA on N-CAM was shown to be synthesized through the action of specific sialyltransferase(s) (9, 10), but the mechanisms underlying the regulation of PSA synthesis and PSA expression remain poorly understood.

Recently, two cDNAs encoding α2,8-sialyltransferases named ST8Sia II/STX (11–13) and ST8Sia IV/PST-1 (14–16) were cloned. Mouse ST8Sia II (mST8Sia II) exhibits overall amino acid sequence identity of 56% with mouse ST8Sia IV (mST8Sia IV). Both sialyltransferases can synthesize PSA on α2,3-linked sialic acids on N-CAM without any initiators (16–20). Northern blot analysis indicated that expression of the mST8Sia II gene was restricted to the brain and testis, whereas the mST8Sia IV gene was expressed strongly in lung and heart rather than brain (13, 16). The expression of the mST8Sia II gene in the brain was strictly regulated during developmental stages (13), i.e. an mST8Sia II transcript could be detected in brain on embryonic day 14, which peaked on embryonic day 20 and then decreased progressively to an almost undetectable level by 2 weeks after birth. The expression of the mST8Sia IV gene was also higher in fetal than adult brain but was less regulated during brain development compared with that of the mST8Sia II gene (16). To elucidate the mechanisms underlying of their tissue- and development-specific expression, it is important to know the structures and activities of their promoters.

We showed recently that expression of the mST8Sia II gene increased in parallel with the increased activity of PSA synthase and the quantity of PSA N-CAM during neuronal differentiation of mouse teratocarcinoma P19 cells, which provides us with an in vitro model system for studying its promoter activity in the neural differentiation processes (21). In this paper, we describe the genomic structure and promoter sequence of the mST8Sia II gene and determination of the 5′-flanking region responsible for the cell type-specific promoter activity by means of transfection experiments using differentiated P19 cells.

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EXPERIMENTAL PROCEDURES

Isolation of Genomic and cDNA Clones Encoding mST8SiaII—A mouse genomic cosmid library was constructed and screened as described previously (22). The location of the exons of the mST8SiaII gene was determined by PCR (GeneAmp XL PCR kit; Perkin-Elmer) with specific oligonucleotide primers or by hybridizing radiolabeled tialase/laser cDNA to the same blots.

The 3’-untranslated region of the mST8SiaII II cDNA was isolated from a 3-day-old mouse brain cDNA library (23) by PCR using primers O1-3A (5’-GOTATACCCCTTGAAACTATAGCTCTG-3’; nucleotides +290 to +321) and O1-3C (5’-CTCAAGGTCTACCTGCCAC-3’; complementary to nucleotides +3420 to +3396).

PCR Amplification of the 5’ cDNA End (RACE)—mRNAs from newborn mouse brain were extracted by the guanidinium isothiocyanate method and purified with Oligotex-dT30 (Takara-Shuzo, Japan). Amplification of the 5’ end of mST8SiaII II cDNA was performed essentially according to the procedure of Frothman et al. (24). cDNA was synthesized by reverse transcription (Superscript II; Life Technologies, Inc.) of 5 μg of mouse brain poly(A) RNA using a primer O1-N6, 5’-TTAGATT-TGCTATGTAAGCTGTT-3’ (complementary to the mST8SiaII coding strand, nucleotides +1 to +8 and including an NcoI linker) and a 1 footprinting unit of recombinant Sp1. Five μl of freshly diluted Dnasafe (50 μg/ml) was added to the mixture for 60 s at 25°C, and then the reaction was stopped by the addition of 100 μl of 1% sodium dodecyl sulfate containing 20 mM EDTA and 200 mM NaCl. After phenol-chloroform extraction and ethanol precipitation, the reacted products were separated on a 6% sequencing gel along with a deoxyxynucleic acid chain-terminating reaction of pFT-O1, using O1-FT as the primer.

The amplified fragment was digested with KpnI within the pUC119 vector polylinker, blunt ended, and then digested with NcoI with within the AgI-ATGNeo primer. For construction of pBO1-EN1.8, the resultant fragment DNA was subcloned into Smal-NcoI-digested pIC-NeoI-2 (pIC-Neobasic II (pICPG1I, Toyo-ink, Japan); pBO1-NcoI was generated by subcloning the pBo1-EN1-1 and Smal-NcoI-digested PCR products into pICPG1I respectively.

Series of deletion plasmids were constructed by subcloning the restriction enzyme-digested PCR products. The primers and template plasmids used were pBO1-XN10.15, carrying a 0.15-kb XhoI-NcoI fragment amplified by using the primer set of O1-350X/O1-ATGNco; the sequences of the primers were 5’, including an NcoI linker) and 5’, including an XhoI linker) and O1-350X, 5’-TTGGCCGCTC- GAGTTAGTGGGAGGA-3’ (nucleotides 165 to 142, including an XhoI linker). Plasmid pO1-22E4.8 was used as the template. Plasmids pBO1-NH3.5, pBO1-RN5.5, and pBO1-EN9.6 were constructed as follows. An 8-kb EcoRI fragment from COS O1-22 (from 9600 to 16450 of 5’-ATGAGAACATCCATGCGCAGACGCGCCT-3’; complementary to the mST8SiaII coding strand, nucleotides +177 to +149 and including an NcoI linker) and a 1 footprinting unit of recombinant Sp1. Five μl of freshly diluted Dnasafe (50 μg/ml) was added to the mixture for 60 s at 25°C, and then the reaction was stopped by the addition of 100 μl of 1% sodium dodecyl sulfate containing 20 mM EDTA and 200 mM NaCl. After phenol-chloroform extraction and ethanol precipitation, the reacted products were separated on a 6% sequencing gel along with a deoxyxynucleic acid chain-terminating reaction of pFT-O1, using O1-FT as the primer.

Analysis of Promoter Activity—NIH 3T3 and undifferentiated P19 cells were seeded at 5 × 10⁵ cells/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 differentiation of P19 cells into neuronal cells, the cells were seeded into and aggregated in bacte-

rological grade dishes in the presence of 1 μM retinoic acid at the cell density of 1 × 10⁶/ml. After 3 days, the aggregates were trypsinized, and approximately 1 × 10⁵ cells/80-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 pSR2-Gal plasmid (0.5 μg) used as a co-transfection 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 (PGL-50; Toyo-ink). Luciferase activity was measured using a PicaGene Luciferase assay system (Toyo-ink) and a Luminescent Reader AB-2000 (ATTO, Japan). Light activity measurements were performed in quadruplicate, averaged, and then normalized as to β-galactosidase activity to correct for the transfection efficiency. β-Galactosidase activity was measured using a Luminescence β-Galactosidase Detection Kit (Clontech).

RESULTS

Isolation of mST8SiaII Genomic Clones—Screening of an NIH 3T3 cell cosmid library for mST8SiaII II cDNA resulted in the isolation of four independent clones. A restriction map of the approximately 100-kb region containing the mST8SiaII II gene is shown in Fig. 1.

We sequenced the exons, to determine their exact sizes, as...
well as the sequences of the intron/exon junctions (Table I). The sequences of all the intron-exon splice junctions conformed to the GT-AG rule (25). Introns were present only in the protein coding region of mST8SiaII. All of the splicing junctions of the mST8SiaII gene occurred after the second nucleotide of the amino acid codon. The mST8SiaII cDNA was divided into six exons, ranging from 63 to 4,340 bp, with intron sizes of about 4–39 kb and spanning approximately 80 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 32. This exon contained a cytoplasmic domain, a hydrophobic signal anchor sequence, and part of a stem domain. Exons 4–6 encoded the putative active domain of the enzyme, and exon 6 contained a large 3’–untranslated region. We reported previously mST8SiaII cDNA sequences lacking the whole 3’–untranslated region. Therefore, to determine the 3’ end of the 5-kb mST8SiaII 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 5,350 bp, thus it included a large 3’–untranslated region of 4055 bp. Poly(A) addition had occurred 18 nucleotides downstream of the sequence at the T residue of the polyadenylation signal (AATAAA).

Mapping of the Transcription Start Site—The transcription start site was determined by S1 nuclease mapping and primer extension with RNA recovered from 1-day mouse brain, in which the mST8SiaII gene was highly expressed (Fig. 2). A single stranded 32P-labeled S1 probe, corresponding to nucleotides −393 to +205 of the mST8SiaII gene, was hybridized with poly(A) RNA, followed by S1 nuclease digestion. The protected fragments were analyzed on a 6% sequencing gel. Yeast tRNA was used as a control to protect specificity. The end points of the protected fragments were determined by comparison with a sequence ladder derived from the same genomic DNA template (pO1-22E4.8) and the original primer, O1-EX3 (nucleotides +255 to +224, which is complementary to the mST8SiaII mRNA), used for synthesizing the S1 probe. The end point was determined to be at cytosine (+1), which corresponded to a position 167 nucleotides upstream from the initiation codon, ATG (Fig. 2A). Control experiments involving tRNA showed no such band. Primer extension with the same primer (O1-EX3) as used for S1 analysis resulted in extension products corresponding to the same cytosine (+1) (Fig. 2B). Consistent with the results of primer extension and S1 nuclease experiments, a predominant initiation site was found 167 nucleotides upstream of ATG, which gave the only comigrating product in both S1 nuclease protection and primer extension experiments. Moreover, we performed 5’ RACE-PCR on newborn mouse brain poly(A) RNA to identify the 5’ end of the mST8SiaII gene, and the longest RACE-PCR products corresponded to the transcription initiation site determined in the S1 nuclease protection and primer extension experiments.

Analysis of the 5’-flanking Region—Analysis of the sequence 2.5-kb upstream of the transcription initiation site revealed that the mST8SiaII gene promoter has no typical TATA or CAAT boxes (Fig. 3). As shown in Fig. 3, the sequence of the putative promoter region of the mST8SiaII gene was embedded in a GC-rich domain. The GC content of the sequence between immediately upstream of the transcription initiation site and the translational initiation site (nucleotides −175 to −168) was 74%. A pyrimidine-rich region (nucleotides −500 to −453) and a purine-rich region (nucleotides −83 to −11) were found in the 5’-flanking region. The TATA- and CAAT-less mST8SiaII gene promoter contains three Sp1 binding sequences, (G/T)GGGCGG(G/A)(G/A)(C/A), at positions −34 (matching 9–10), −80 (matching 9–10), and −170 (matching 6–10), and an inverted Sp1 binding site at position −573 (matching 8–10). To determine whether or not the proximal Sp1 binding sites upstream of the transcriptional initiation site are functional, we performed DNase I footprinting analysis with Sp1. As shown in Fig. 4, the Sp1 binding sites at positions −34 and −80 were both protected.

Demonstration of Promoter Activity—To determine mST8SiaII gene promoter activities, we used mouse embryonal car-

FIG. 1. Structure of the mST8SiaII gene. Panel A, schematic representation of the mST8SiaII gene locus with a restriction map. a, restriction map with EcoRI depicted. The shaded bars denote the four genomic clones (COS O1-22, COS O1-2, COS O1-1, and COS O1-14) isolated from a cosmid library. b, the six exons of the mST8SiaII gene are shown as filled rectangles and the 5’- and 3’-untranslated regions as open rectangles. The intronic sequences are indicated by the solid lines between the exons. Panel B, domain structure of mouse brain ST8SiaII. The boxes indicate translated sequences, and horizontal bars indicate untranslated sequences. A hydrophobic signal anchor sequence (transmembrane domain) and sialyl motifs L and S are denoted as TM, SM-L, and SM-S, respectively. The splicing sites are indicated by vertical arrows.
oligonucleotide (O1-EX3) complementary to mST8SiaII mRNA (nucleotides 1-224) was \( \text{[\gamma-32P]ATP end labeled, hybridized with 5' of poly(A) RNA isolated from 1-day mouse brain or 5' control plasmid, pSR poly(A) RNA from 5' treatment (21).}

The transcription start site has been mapped by means of both S1 nuclease protection (panel A) and primer extension (panel B) analysis. For S1 mapping, a 648-nucleotide single-strand DNA complementary to nucleotides 255 to 893 was generated as described under "Experimental Procedures" and hybridized with 5' of poly(A) RNA from 1-day mouse brain or 5' of yeast tRNA before the S1 nuclease reaction. For the primer extension reaction, a 32-mer oligonucleotide (O1-EX3) complementary to mST8Sia II mRNA (nucleotides 255 to 224) was \( \text{[\gamma-32P]ATP end labeled, hybridized with 5' of poly(A) RNA from 1-day mouse brain or 5' of yeast tRNA, and then reverse transcribed. The S1 nuclease-protected fragment as well as the primer-extended product were run on a sequencing gel along with a sequencing reaction of pO1-22E4.8, using O1-EX3 as the primer. In panel A, lane 1 is S1 nuclease reaction with 1-day mouse brain mRNA; lane 2, with yeast tRNA. In panel B, lane 1 is primer extension with 1-day mouse brain mRNA; lane 2, with yeast tRNA. The arrow indicates the position of the transcription start site.}

Additional text...
cells, indicating that the sequence between −158 and −9 is necessary for the minimal promoter activity. Two Sp1 binding sites, which were revealed to be functional on footprinting analysis (Fig. 4), and a purine-rich region were found in the region between −9 and −158. Interestingly, the promoter activities due to the pBO1-XN0.35 and pBO1-SN0.45 constructs were approximately 10-fold higher in differentiated P19 cells than undifferentiated P19 cells. On the other hand, all constructs gave hardly detectable activity in NIH3T3 cells, which do not express the mST8SiaII gene. These results suggest that the 325-bp sequence upstream from the translational initiation codon (nucleotides −158 to +167) is necessary for the minimum and cell type-specific promoter activity.

**DISCUSSION**

In this study, we determined the entire genomic organization of the mST8SiaII gene, characterized the functional promoter activity for the 5′-flanking region in transient transfection assays, and investigated the expression of the gene in mouse teratocarcinoma P19 cells. We demonstrated that the minimal promoter region of the mST8SiaII gene is sufficient to express cell type-specific promoter activity, which is correlated with mST8SiaII gene expression in the cells.

Previously, the genomic structures of five other sialyltransferase genes have been reported, i.e. rat galactoside α2,6-sialyltransferase (rST6GalI) (26, 27), human galactoside α2,3-sialyltransferase (hST3GalI) (28), human Galβ1,3GalNAc/β1,4GlcNAc α2,3-sialyltransferase (hST3Gal IV) (29), mouse N-acetylgalactosamidase α2,6-sialyltransferase (mST6GalNAc II) (30), and mouse sialoside α2,8-sialyltransferase (mST8SiaIII) (22). The genomic structure of the mST8SiaII gene was most similar to that of the mST8SiaIII gene, as follows (Fig. 6). The putative active domains of mST8SiaII and mST8SiaIII were encoded by only three and two exons, respectively, whereas those of the other genes consisted of at least five exons. In particular, the positions of the two exon-intron boundaries of the mST8SiaII gene (introns 3 and 5) and the mST8SiaIII gene (introns 2 and 3) were identical, and all of their splice junctions occurred after the second nucleotide of the amino acid codon. Although in mST8SiaIII sialyl motif L, a highly conserved putative nucleotide sugar binding domain (31) is encoded by one exon, the corresponding motif in mST8SiaII is encoded by discrete exons, like those observed in other genes. The similarity of the genomic organization and amino acid sequences between mouse ST8SiaII and ST8SiaIII suggests that these genes constitute a subgroup distinct from other sialyltransferase genes.

The results of primer extension and S1 protection analyses showed that mST8SiaII gene has a single transcription initiation site. In addition, we demonstrated that the 5′-flanking region of the mST8SiaII gene contained a functional promoter that was highly active in neural differentiated P19 cells, which strongly express the mST8SiaII gene. Deletion analysis clearly indicated that the minimal promoter, the region contained within the 158 bp upstream of the transcription start...
site (pBO1-XN0.35), exhibited substantial tissue specificity (Fig. 5). In differentiated P19 cells, an approximately 10-fold increase in promoter activity was observed with the pBO1-XN0.35 construct compared with that in undifferentiated P19 cells. We showed recently that the expression of the mST8SiaII gene in neural differentiated P19 cells increased.

**Fig. 5.** mST8SiaII gene promoter activity and identification of the regulatory regions. Shown is a schematic representation of DNA constructions containing various lengths of the mST8SiaII promoter linked to the luciferase gene (pPGBII). Each DNA fragment subcloned into the luciferase reporter plasmid is defined as to its position in the mST8SiaII gene promoter relative to the transcription start (+1). Five μg of each construct was transfected into NIH 3T3 (3T3), undifferentiated P19 (P19), or neural-differentiated P19 (P19(Dif)) cells. Luciferase activity was normalized as to β-galactosidase activity of a cotransfected internal control plasmid, pSRβ-Gal. The data are expressed as the fold increase in enzyme activity compared with transfections using a promoterless luciferase plasmid, pPGBII. Results are the average of five experiments.

| 3T3 | P19 | P19 (Dif) |
|-----|-----|----------|
| 1.2 | 3.2 | 13.1     |
| 2.1 | 5.1 | 17.4     |
| 2.0 | 5.1 | 30.1     |
| 2.4 | 5.3 | 30.3     |
| 2.9 | 8.4 | 32.4     |
| 2.2 | 5.2 | 55.2     |
| 1.1 | 3.1 | 28.3     |
| 0.9 | 0.8 | 1.0      |

**Fig. 6.** Comparison of the exon structure of the mST8SiaII gene with those of other sialyltransferase genes. The intron-exon structures of five sialyltransferase genes are presented (22, 26, 28–30). 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 identical positions of the two introns of the mST8SiaII and mST8SiaIII genes are indicated by dotted lines.
dramatically to a level approximately 10–20 times higher than that in undifferentiated cells (21). The increase in minimal promoter activity on neural differentiation is correlated with the gene expression of mST8SiaII in P19 cells. Moreover, the promoter activity was negligible in NIH 3T3 cells, in which the mST8SiaII gene was not expressed. Therefore, the minimal promoter region may possess the ability to initiate transcription in a cell-specific fashion.

The proximal region 158 bp upstream from the transcription initiation site was characterized as a minimal promoter that contained two functional Sp1 binding sites but lacked consensus TATA and CAAT boxes. In addition, the 5′-untranslated region of the mST8SiaII gene contained a large GC-rich domain with the characteristics of a CpG island. These structural features are usually associated with housekeeping gene promoters. However, recent studies have shown that many tissue-specific gene promoters, including neural cell-specific promoters, include the structural features of housekeeping gene-like promoters. Neuron-specific TATA-less promoters have been shown to have either a single transcription initiation site, as observed in the synapsin I gene (32), synapsin II gene (33), nerve growth factor receptor gene (34), and several olfactory neuron-specific genes (35), or to have multiple initiation sites, such as those found in the genes encoding synaptophysin, the type II sodium channel (36), the D1A dopamine receptor (37), and brain-specific aldolase C (38). Therefore, the mST8SiaII promoter may belong to the former group, i.e. GC-rich and TATA-less promoters with a single transcription initiation site.

Two Sp1 binding sites were found in the minimal promoter region, and DNase I protection assays with the purified Sp1 protein revealed that these two sites were functional (Fig. 4). Moreover, two additional Sp1 binding sites were present in the regulatory regions (−158 to −293 and −293 to −659). It was reported previously that the distantly located site functions synergistically with the promoter-proximal site to activate transcription strongly in vivo, and that this synergism is a direct consequence of interactions between remote and local Sp1s (39). Deletion of the sequence from positions −158 to −293 of mST8SiaII reduced the promoter activity, suggesting that Sp1 bound to the site at −170 probably interacts with another Sp1 in the minimal promoter region. The expression of Sp1 is ubiquitous, but it was reported previously that Sp1 levels appeared to be highest in developing hematopoietic cells, fetal cells, and spermatides in the mouse (40). The regulated expression of the mST8SiaII gene in fetal and newborn brain and testis seemed to correspond to that of Sp1. Although Sp1 was found to be expressed in NIH 3T3 and undifferentiated and differentiated P19 cells (data not shown), the expression level of the reporter gene fused to the minimal promoter sequence of ST8SiaII (−158/+168) was very low in undifferentiated P19 cells and hardly detectable in 3T3 cells. These results suggest that some cell-specific transcription factor is involved in differentiated P19 cells and that it might be required for interaction with Sp1 in the minimal promoter elements, which allows activation of the mST8SiaII gene promoter. Further studies involving identification of the regulatory elements and their binding factors will facilitate elucidation of the tissue-specific and developmental regulation of ST8SiaII gene expression.

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