Polysialic acid (PSA) is an important regulator of cellular interactions. Two enzymes (ST8SiaII and ST8SiaIV) are capable of synthesizing PSA. In the present study, the gene encoding the murine ST8SiaIV (PST-1) has been isolated and characterized. In contrast to the ST8SiaII (STX) gene which contains six exons and spans about 50 kb, the ST8SiaIV gene comprises only five exons spanning over at least 55 kb. However, alignment of the two genes revealed that exon–intron boundaries of exons 2–5 of ST8SiaIV and exons 3–6 of ST8SiaII are located at identical sites. Differences are restricted to the 5′-region encoded by one exon in the case of ST8SiaIV, whereas the corresponding region of ST8SiaII is interrupted by a very long intron. 5′-RACE analysis of the ST8SiaIV transcript using mRNA from AtT20 cells identified two transcription start sites at positions -324 and -204 relative to the translation start codon. The promoter region of ST8SiaIV lacks TATA- and CAAT-like sequences and is enriched in G+C (60%). The promoter contains putative Sp1, AP-1, AP-2, and PEA3 binding sites, as well as a purine- and a pyrimidine-rich region. Luciferase reporter gene assays demonstrated that the region between nucleotides -443 and -162 is sufficient to direct gene expression. The induction of luciferase activity was 30- and 10-fold in the PSA-positive AtT20 and CHO cells, but only 5- and 7-fold in the PSA-negative NIH-3T3 cells and in a PSA-negative subline of AtT20. Thus, although decreased in activity in PSA-negative cell lines, the basal promoter is not sufficient for the strong cell-type and tissue specific regulation of the ST8SiaIV gene, suggesting regulatory elements in the more upstream 5′-region.

Key words: polysialic acid/polysialyltransferase/genomic structure

Introduction

Polysialic acid (PSA) is a differentially expressed posttranslational modification of the neural cell adhesion molecule (NCAM). PSA-expression is highly regulated during development (Seki and Arai, 1993; Rutishauser, 1996) and changes in the amount of PSA seem to be important for plastic processes in the central nervous system (Kiss and Rougon, 1997). Recently, it has been demonstrated that PSA is necessary for the two paradigms used to determine activity-dependent synaptic plasticity, i.e., long term potentiation and long-term depression (Becker et al., 1996; Muller et al., 1996). PSA modulates the neurite outgrowth-promoting effect of NCAM (Doherty et al., 1990) and is believed to be essentially involved in axon fasciculation (Cremer et al., 1997).

Two enzymes, ST8SiaIV (PST-1) (Eckhardt et al., 1995; Nakayama et al., 1995; Yoshida et al., 1995) and ST8SiaII (STX) (Livingston and Paulson, 1993; Kojima et al., 1995a; Scheidegger et al., 1995), are able to synthesize PSA (for a recent review, see Kiss and Rougon, 1997). The acceptor structure recognized by both enzymes is NCAM that carries terminal sialic acid linked α2,3 or α2,6 to galactose (Kojima et al., 1995b; Mühlenhoff et al., 1996a,b). Additional enzymatic activities are not required to polysialylate NCAM under in vitro conditions (Kojima et al., 1995b; Mühlenhoff et al., 1996b; Nakayama and Fukuda, 1996).

The expression patterns of the polysialyltransferases closely parallel that of polysialylated NCAM (Kuroswa et al., 1997; Phillips et al., 1997). However, in the developing rat brain some neuroepithelial areas were found to be PSA-negative although mRNAs for both enzymes and NCAM were present (Phillips et al., 1997; Wood et al., 1997). In early developmental phases the two enzymes are expressed in the brain and in many non-neural tissues (Angata et al., 1997; Phillips et al., 1997). The level of ST8SiaII mRNA is thereby multiple times higher than the level of ST8SiaIV mRNA (Angata et al., 1997). In the adult, polysialyltransferases become restricted to discrete areas in the brain (Phillips et al., 1997). Northern blot and in situ hybridization studies were carried out to analyze the tissue-specific and developmental expression of these genes (Angata et al., 1997; Kuroswa et al., 1997; Phillips et al., 1997). Results arising from these studies are in part contradicting, especially with respect to the relative expression levels of the two genes. However, a direct comparison of the results is difficult, since different species were analyzed (human, mouse, and rat, respectively) and differences might be due to species specific expression patterns. Nevertheless, all studies clearly show that in PSA-positive tissues the amount of PSA correlates with the level of polysialyltransferase mRNAs expressed. Furthermore, the expression levels correlate with polysialyltransferase activities determined in different cell lines and during differentiation of P19 cells (Mühlenhoff, 1996; Kojima et al., 1996).

In contrast, data concerning the expression level of the proteins are not available. This fact together with the observation that the polysialyltransferases exhibit strong differences in their enzymatic activity in vitro (Kojima et al., 1997; Mühlenhoff et al., 1997), does not allow an evaluation to which extent an individual polysialyltransferase contributes to the polysialylation of NCAM. The situation is further complicated by the fact that very little information exists on the signal transduction pathways involved in the regulation and activity of polysialyltransferases. A recent study suggests regulation via an increase in the intracellular calcium concentration and activation of protein kinase C (Rafuse and Landmesser, 1996). To get further insight into the developmental and tissue-specific regulation of polysialyltransferases, the structural organization of the genes and the identification of
regulatory elements is required. Yoshida et al. (1996b) reported the structure of the murine ST8SiaII gene and demonstrated that the basal promoter of 158 bp upstream from the transcription start site is sufficient to promote cell-type specific expression of ST8SiaII. In the present report, we describe the genomic structure of the murine ST8SiaIV gene and demonstrate considerable similarity to the organization of the ST8SiaII gene.

Results

Isolation and characterization of the ST8SiaIV gene

A genomic DNA bacteriophage λFIXII library of mouse strain 129/Sv was screened by hybridization with a digoxigenin labeled RNA probe complementary to the 2 kb hamster ST8SiaIV cDNA described previously (Eckhardt et al., 1995). Approximately 10^6 plaques were screened and five different lambda phages containing the 5′-region, including the first three exons, and the 3′-end of the gene were obtained. In order to identify the missing part of the gene, a digoxigenin labeled DNA probe of exon 4 was generated by PCR, and the phage library was rescreened. One positive clone was identified which was found to contain exon 4 and adjacent intronic sequences.

Phage clones were subjected to restriction mapping and Southern blot analysis. Fragments encoding exon sequences were subcloned into the vector pBluescript and the exact size of exons and the sequences of exon–intron boundaries were determined by sequencing. From these data the organization of the ST8SiaIV gene could be deduced as shown in Figure 1. Southern blot analysis of mouse genomic DNA digested with different restriction endonucleases confirmed this genomic map (data not shown). The length of introns 1 and 2 was deduced from the restriction map and verified by PCR. Since the phage harboring exon 4 does not overlap with the other phage clones long range PCR was applied to determine the length of intron 1 and 2. A specific product of approximately 23 kb was obtained for intron 3 but no product corresponding to intron 4 was found. From Southern blot analyses with different restriction enzymes, we estimated the size of the latter to be at least 10 kb.

The ST8SiaIV gene is divided into five exons, spanning over more than 55 kb. The exon-intron boundaries (Table II) were found to be consistent with the splice acceptor and donor consensus sequences (Breathnach and Chambon, 1981) and correspond to the GT-AG rule (Mount, 1982). All introns interrupt exons after the second nucleotide of a codon. Exon 1 and exon 2 encode the cytosolic amino-terminus and the transmembrane domain, respectively. The catalytic domain of ST8SiaIV is encoded by exons 3 to 5 (Table I). Exons 3 and 4 harbor the sialyl motif L, which is involved in CMP-sialic acid binding (Datta and Paulson, 1995). Exon 5 contains the sialyl motif S and the recently identified sialyl motif VS (Geremia et al., 1997).

Northern blot analysis of murine tissues gives an hybridization signal of approximately 5.5 kb (Eckhardt et al., 1995; Yoshida et al., 1995), suggesting a long 3′-untranslated region, which is not contained in the published mouse ST8SiaIV cDNA sequence (Yoshida et al., 1995). To identify the polyadenylation site of the gene, 3′-RACE was carried out using a primer deduced from the known sequence of exon 5 (corresponds to nucleotides +3467 to +3489). A single 1.6 kb PCR product was obtained and characterized by sequencing. The polyadenylation site was found to be located 3995 bp downstream of the stop codon. Polyadenylation occurs 18 nucleotides downstream of an AAUAAA sequence (data not shown).

<table>
<thead>
<tr>
<th>Exon</th>
<th>Size (bp)</th>
<th>Amino acids</th>
<th>Features</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>437</td>
<td>1–38</td>
<td>5′-UTR, transmembrane domain</td>
</tr>
<tr>
<td>2</td>
<td>132</td>
<td>39–82</td>
<td>Stem region</td>
</tr>
<tr>
<td>3</td>
<td>258</td>
<td>83–168</td>
<td>Catalytic domain/L-motif</td>
</tr>
<tr>
<td>4</td>
<td>294</td>
<td>169–266</td>
<td>Catalytic domain/L-motif</td>
</tr>
<tr>
<td>5</td>
<td>4278</td>
<td>267–359</td>
<td>Catalytic domain/S-motif, 3′-UTR</td>
</tr>
</tbody>
</table>

Identification of an alternative polyadenylation signal

Northern blot analysis of the murine cell line AtT20 using an RNA probe transcribed from the complete cDNA of hamster ST8SiaIV revealed not only the 5.5 kb mRNA normally found in murine tissues, but an additional 1.4 kb band (Figure 2A). The latter signal is absent from Northern blots hybridized with a mouse exon 5 specific probe (data not shown). In order to further characterize this mRNA a cDNA phage library created from AtT20 cells was screened and a cDNA that lacks the sequence encoded by exon 5 could be isolated. Comparison of the cDNA with the genomic ST8SiaIV sequence revealed that the 1.4 kb mRNA is generated by using an alternative polyadenylation signal that is present in intron 4, 95 nucleotides downstream of exon 4 (Figure 2B). Since this variant lacks a large part of the sequence encoding the catalytic domain, including the conserved short and very short sialyl motifs, this mRNA should not give rise to an active enzyme. To confirm this hypothesis, the cDNA was subcloned into the eukaryotic expression vector pCDM8 and transiently transfected into the ST8SiaV-negative mutant 2A10 (Eckhardt et al., 1995). As expected, the cDNA was unable to correct the PSA-negative phenotype of 2A10 cells (data not shown). Until now the short mRNA variant has not been found.

Fig. 1. Genomic structure of the mouse ST8SiaIV gene. A schematic representation of the exon/intron organization is shown at the top. Boxes represent exons; solid boxes correspond to sequences encoding amino acids of ST8SiaIV, open boxes indicate the noncoding regions at the 5′- and 3′-end. Restriction sites recognized by XhoI (X) and SacI (S) are shown. The size of intron 3 could be estimated by PCR to be approximately 23 kb. Based on Southern blot analysis of genomic DNA, the size of intron 4 is beyond 10 kb. The positions of genomic phage clones are indicated below.
in murine tissues, but mRNAs of similar size have been detected in human cell lines (Eckhardt, 1995; Mühlhoff, 1996) and in peripheral blood leukocytes (Angata et al., 1997), demonstrating that differential polyadenylation occurs also under physiological conditions in normal tissues. Usage of different polyadenylation sites could be involved in regulating the expression level of functional active ST8SiaIV.

Identification of the transcription initiation site and sequence analysis of the promoter region

To identify the transcription start site of the gene, we used the 5'-RACE technique. mRNA from AtT20 cells was reverse transcribed and subjected to two consecutive PCRs as described in Materials and methods (Figure 3A). Sequence analysis of PCR products revealed the presence of two different transcription initiation sites, 324 and 204 nucleotides upstream of the translation start codon (Figure 3A). 5'-RACE carried out with a primer (P3; Figure 3B), which anneals four nucleotides upstream of the first identified transcription start site gave no product. This result strongly suggests the absence of further upstream located transcription start sites in AtT20 cells.

The promoter region (Figure 4) lacks a TATA box and does not contain a CCAAT motif. The sequence around the transcription initiation site has a high G+C content (60% between nucleotides -660 and -180). The promoter region is also enriched in CpG dinucleotides, and 58 CpG dinucleotides are present between position -1230 and -1 (CpG to GpC ratio = 0.73). A purine-rich region (93% purines in nucleotides -1212 to -1157) is followed by a pyrimidine-rich region (93% pyrimidines in nucleotides -1117 to -1078). Partial sequencing of the more upstream 5'-region revealed the presence of a [TG]20 microsatellite app. 2.4 kb upstream of the translation start codon. Potential transcription factor binding sites in the promoter sequence include an Sp1 binding motif (Faisst and Meyer, 1992) “CCTCGGC” at position -395/-386, and an AP-2 site (Williams and Tjian, 1991) “GGCTGCC” at position -370/-363. Furthermore, AP-1 (Wasylyk et al., 1989) “TGAGTCAC” and PEA3 (Faisst and Meyer, 1992) “CAGGAAGT” binding motifs are present in nucleotides -1554 to -1547 and -1423 to -1416, respectively.
Fig. 4. Nucleotide sequence of the promoter region and exon 1 of mouse ST8SiaIV. The sequence was numbered relative to the translation start site. The two transcription start sites are indicated by arrows. Restriction sites and the position of primer ME119 used for the construction of the reporter gene plasmids are shown. The purine- and pyrimidine-rich regions are marked by dotted lines. Putative binding sites for Sp1, AP1, AP2, and PEA3 are underlined.

Table II. Exon-intron borders in the mouse ST8SiaIV gene

<table>
<thead>
<tr>
<th>5′-Exon/splice donor</th>
<th>Size of intron</th>
<th>Splice acceptor / 3′-exon</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTC ATC GG gtaatgcat</td>
<td>Intron 1: 6 kb</td>
<td>tctttcag A GAT GGT</td>
</tr>
<tr>
<td>Leu Ile Gly(38)</td>
<td></td>
<td>Asp Gly</td>
</tr>
<tr>
<td>GAG ATA AG gigagttct</td>
<td>Intron 2: 7.5 kb</td>
<td>ccaatacag G AAG AAC</td>
</tr>
<tr>
<td>Glu Ile Arg(82)</td>
<td></td>
<td>Lys Asn</td>
</tr>
<tr>
<td>GTA ATA AG gigagcatc</td>
<td>Intron 3: 23 kb</td>
<td>ttcttcag G TGC AAT</td>
</tr>
<tr>
<td>Val Ile Arg(168)</td>
<td></td>
<td>Cys Asn</td>
</tr>
<tr>
<td>GTC AGA GG gtaatgctt</td>
<td>Intron 4: &gt;10 kb</td>
<td>ttcttcag A TAC TGG</td>
</tr>
<tr>
<td>Val Arg Gly(266)</td>
<td></td>
<td>Tyr Trp</td>
</tr>
</tbody>
</table>

Intron sequences are represented by small and exon sequences by capital letters. The position of amino acids encoded by the last two nucleotides of each exon and the first nucleotide of the following exon are indicated in parentheses.
et al. (Eckhardt et al., 1995) Murine and hamster cell lines with the phenotype NCAM+/PSA+. Demonstration of promoter activity ST8SiaIV (Eckhardt et al., 1995, and data not shown). For the promoter studies a 1.4 kb sequence covering nucleotides -1609 to -162 of the ST8SiaIV gene was cloned into plasmid pGL2Basic in front of the luciferase gene. The resulting construct pGL2PST2 together with the β-galactosidase expressing vector pCMVlacZ was transiently transfected into the cell lines mentioned above. Luciferase activity was determined 48 h later and normalized to β-galactosidase activity. All cell lines tested showed significant promoter activity irrespective of whether they express PSA or not (Figure 5). However, the promoter was more active in PSA-positive cell lines. Compared to the promoterless plasmid pGL2Basic a 10-fold induction was obtained in PSA-positive CHO-cells and a 30-fold induction in PSA-positive AtT20 cells. In the PSA-negative subclone of AtT20 a 7-fold induction and NIH-3T3 cells a 5-fold induction was observed. Truncations up to nucleotide -668 (pGL2PST3) and -443 (pGL2PST4) had only minor effects on the promoter activity. In contrast, deletion of the nucleotide fragment -443 to -162 containing the transcription start site reduced the activity in CHO and AtT20 cells by 75% and 90%, respectively. The above results demonstrate that the promoter region extending from nucleotide -443 to -162 is sufficient to promote gene expression. Putative transcription factor binding sites that could be involved in activation of the minimal promoter include an Sp1 motif at position -395/-386, and an AP2 motif at position -370/-363 (see Figure 4). The activity of the promoter is higher in cell lines expressing ST8SiaIV, but is significant also in PSA- and ST8SiaIV-negative cell lines. This result suggests that additional upstream elements exist that control the cell-type-specific expression of ST8SiaIV.

**Discussion**

Sialyltransferases form a family of at least 15 different members that transfer sialic acid in α2,3-, α2,6-, or α2,8-linkage to galactose or sialic acid (for review, see Tsuji, 1996). The genomic structure has been elucidated for six sialyltransferases (Svensson et al., 1990; Wang et al., 1990; Chang et al., 1995; Kitagawa et al., 1996; Kuroswa et al., 1996; Yoshida et al., 1996a, b). All are divided into several (4–10) exons, spanning over up to 100 kb. A related structural composition has been found for the polysialyltransferase genes ST8SiaII (Yoshida et al., 1996b) and ST8SiaIV (this study). ST8SiaIV and ST8SiaII are unique in comparison with other sialyltransferases because of their ability to polymerize sialic acid and because of their restricted substrate specificity. The only identified cellular substrate recognized by both polysialyltransferases is the neural cell adhesion molecule NCAM. Furthermore, in contrast to other sialyltransferases, where primary sequence similarity is restricted to the sialyl motifs L, S (Drickamer, 1993), and VS (Geremia, 1997), ST8SiaIV and ST8SiaII exhibit more similarity is restricted to the sialyl motifs L, S (Drickamer, 1993), and VS (Geremia, 1997). Despite these similarities in structure and substrate specificity, the polysialyltransferases are differentially expressed with respect to cell types and developmental stages (Angata et al., 1997; Kuroswa et al., 1997; Phillips et al., 1997), suggesting separate signal transduction pathways and transcription factors to be involved in the regulation of these genes.

In addition to their high amino acid sequence homology, which probably reflects a close evolutionary relationship, the genomic structures of ST8SiaIV and ST8SiaII are very similar. Exon-intron boundaries between exons 2–5 of ST8SiaIV and exons 3–6 of ST8SiaII are located at identical sites and the sequences around the splice donor and acceptor sites show a high degree of similarity. Differences are found in the 5′-region of the genes. While transmembrane and stem region of ST8SiaIV are encoded by a single exon, the corresponding part of ST8SiaII is split by a very long intron resulting in 6 exons for ST8SiaII.

**Demonstration of promoter activity**

Murine and hamster cell lines with the phenotype NCAM+/PSA+ (CHO (Eckhardt et al., 1995) and AtT20 (Alcaraz and Goridis, 1991)) and NCAM+/PSA- (NIH-3T3 (Eckhardt et al., 1995) and a PSA-negative subline of AtT20 (unpublished observations)) were used to determine activity of the ST8SiaIV promoter. In Northern blot analysis the PSA-negative cell lines were shown not to express ST8SiaIV, but is significant also in PSA- and ST8SiaIV-negative cell lines. This result suggests that additional upstream elements exist that control the cell-type-specific expression of ST8SiaIV.

**Fig. 5.** Activity of the mouse ST8SiaIV gene promoter in different cell lines. Promoter/luciferase gene constructs are shown on the left. At the top, restriction sites and promoter regions used to generate the 5′- and 3′-truncated promoter fragments (see Materials and methods) and the transcription start site at -324 are indicated. CHO (solid bars), NIH-3T3 (open bars), PSA-positive AtT20 (striped bars), and PSA-negative AtT20 cells (gray bars) were transiently cotransfected with the plasmids indicated and pCMVlacZ. Luciferase activity was determined 48 h later and normalized to the β-galactosidase activity. Results are expressed as the fold induction in luciferase activity compared to the activity obtained by transfecting the promoterless plasmid pGL2Basic. The data are representative of three independent experiments, each performed in duplicate.
(Yoshida et al., 1996b). As is the case for ST8SiaII, no TATA or CAAT boxes were found in the promoter region of the ST8SiaIV gene. The region around the transcription initiation sites has a high GC content (60%) and is enriched in CpG dinucleotides, i.e., shows the characteristics of CpG islands. Taken together, the promoter of ST8SiaIV exhibits the structural characteristics of other sialyltransferase genes including ST8SiaII (Yoshida et al., 1996b, and references therein).

Analysis of AtT20 cells using the 5′-RACE technique revealed the presence of two transcriptional start sites at positions -324 and -204 relative to the translation start. This result is consistent with the observation that many promoters lacking a TATA box have multiple initiation sites (Geng and Johnson, 1993; Haun et al., 1993). Luciferase gene assays clearly demonstrated that the region from nucleotide -443 to -162 is sufficient to initiate transcription in PSA-positive CHO and AtT20 cells. However, the minimal promoter is also able to promote gene expression in the PSA-negative AtT20 subclone and in NIH-3T3 cells, albeit at a lower level. These results strongly suggest the existence of regulatory elements outside the 1.4 kb fragment tested. However, it seems worthwhile to mention that the artificial systems used to determine promoter activities do not respect the role of the chromatin structure (Smith and Hager, 1997). Promoter activity observed in ST8SiaIV negative cells may therefore result form the artificial assay system. In contrast, minimal promoter was undetectable in NIH-3T3 cells, which are negative for ST8SiaII and ST8SiaIV (Yoshida et al., 1996b).

Two functional Sp1 sites have been identified in the ST8SiaII minimal promoter (Yoshida et al., 1996b). Putative binding sites in the basal promoter of ST8SiaIV that could be involved in the expression of the gene also include one Sp1 site. Further support for the involvement of Sp1 sites in the regulation of polysialyltransferases comes from the observation that Sp1 expression in many tissues parallels the expression of the enzymes. Sp1 expression is high in lung, thymus, and fetal cells, but low in several adult organs, e.g., liver and kidney (Saffer et al., 1991). ST8SiaII and ST8SiaIV are highly expressed in different fetal tissues (Angata et al., 1997; Phillips et al., 1997) as well as in adult lung and thymus, but are low or undetectable in adult liver and kidney (Yoshida et al., 1995; Angata et al., 1997). Thus, the different expression levels of ST8SiaIV in these organs may in part be due to changes in the Sp1 level. However, the identification of the factors involved in the highly restricted cell-type-specific and developmental regulation of ST8SiaIV requires the analysis of further upstream elements and the application of analytical methods which allow to determine the promoter activity in its "in vivo environment."

The sequence data reported in this paper have been submitted to the EMBL/GenBank data bank under accession numbers AJ223955 and AJ223956.

### Materials and methods

#### Isolation and characterization of genomic and cDNA clones of ST8SiaIV

A λFix II phage genomic library of mouse strain 129/Sv (Stratagene) was screened by hybridization with a digoxigenin-labeled RNA probe transcribed from the cloned hamster ST8SiaIV cDNA (Eckhardt et al., 1995). Hybridizations were performed overnight at 60°C in 5x SSC, 50% formamide, 7% SDS, 50 mM sodium phosphate, 1% blocking reagent (Boehringer Mannheim). Filters were then washed twice in 2x SSC, 0.1% SDS at room temperature and twice in 0.5x SSC, 0.1% SDS for 20 min at 65°C. Bound probes were detected by incubation with anti-digoxigenin Ig-alkaline phosphate conjugate (Boehringer Mannheim) and chemiluminescence detection using di-sodium-3-(4-methoxyisopro-1,2-dioxetane-3,2′-dioxo)tricyclo[3.3.1.13,7]decane]-4-ylphenylphosphate (CSPD; Boehringer Mannheim) as a substrate. The phage library was rescreened with an exon 4 specific DNA probe, which was generated by PCR using the primers ME87 (5′-CTCTCTGTGAGGATTCGCTGATGTG-3′) and ME88 (5′-TCTGACTGCGATATGAAGTGGATGGA TAG-3′) and a PCR DIG probe synthesis kit (Boehringer). A βZAP cDNA library of the murine cell line AtT20 was screened essentially as described for the genomic DNA library using a digoxigenin labeled ST8SiaIV hamster antisense RNA probe. To determine the length of intron 3 and 4 long range PCR was performed using the long template PCR system of Boehringer Mannheim. Primers used were: ME83 (5′-GATTGCACACCAACCACTTCTTATAGTGAGC-3′) and ME84 (5′-ACATCGACCCGAACTCCACAGAG- GC-3′) to amplify intron 3, and ME85 (5′-TCTTCACTTTCGACTTGCCACAGG-3′) and ME86 (5′-GTGAA TTTATCAGGAGATACTGGTGCCAAATG-3′) to amplify intron 4.

#### Southern blot analysis

Genomic DNA was isolated from AtT20 cells by proteinase K digestion and phenol/chloroform extraction (Sambrook et al., 1989). DNA was digested with restriction endonucleases BamHI, EcoRI, SacI, and XhoI. Samples (10 µg per lane) were electrophoresed in a 0.8% agarose gel and transferred to a nylon membrane using standard procedures (Sambrook et al., 1989). The membrane was hybridized with digoxigenin labeled DNA probes, prepared by PCR using exon specific primers. Hybridization and probe detection procedure was the same as described above for the phage library screening.

#### Northern blot analysis

Total RNA was isolated from CHO cells by CsCl gradient centrifugation of guanidinium isothiocyanate lysates (Sambrook et al., 1989). RNA (5 µg) was electrophoresed in a 1% agarose/1 M formaldehyde gel in 20 mM MOPS (pH 7.0), 10 mM sodium acetate, 1 mM EDTA and transferred to a nylon membrane (Qiagen). Nylon filters were hybridized overnight at 65°C in 5x SSC, 50% formamide, 50 mM sodium phosphate, 7% SDS, 1% blocking reagent (Boehringer Mannheim) to a digoxigenin-labeled antisense RNA probe of the hamster ST8SiaIV cDNA.
(Eckhardt et al., 1995). After hybridization the filters were washed twice in 2x SSC, 0.1% SDS at room temperature for 5 min and twice in 0.1x SSC, 0.1% SDS at 65°C for 20 min. Bound probes were detected by chemiluminescence detection as described above.

Rapid amplification of cDNA ends (RACE)

RNA was isolated from AtT20 cells and enriched for poly(A)+ RNA as described (Eckhardt et al., 1996). The RACE protocol follows the procedure of Frohman (1993). To amplify the 5'-end of ST8SiaIV 1 µg mRNA was reverse transcript for 1 h at 42°C using the antisense primer RT (5'-CCCTATGCGAGATGGTCC-3': corresponds to nucleotides +41 to +23; +1 = A of the start codon) and Superscript II RNase H' reverse transcriptase (Gibco). Thereafter, primers were removed passing the sample through a Glassmax spin column (Gibco). The cDNA was tailored using dATP and terminal transferase (Boehringer Mannheim). Second strand synthesis was done using the primer ME44 (5'-GGCGATCTCGAGTCGAC-3': and Superscript II RNase H' reverse transcriptase (Gibco) for 30 min at 42°C. Again, excess primers were removed by using Glassmax spin column. The cDNA was subjected to PCR (Saiki et al., 1988) using Taq DNA polymerase, the forward primer ME45 (5'-CGGTTTCTAATGGAGGCTAC-3'): complementary to nucleotides +20 to +1), and the gene specific primers P1 (5'-TGGCAGAGGAGCCGTGACGAC-3'; corresponds to nucleotides -102 to -122), P2 (5'-GGCGTTCTCTGACGCTG-3'; corresponds to nucleotides -289 to -308), and P3 (5'-CCTCTTCTGCGATCAG-3'; corresponds to nucleotides -328 to -347), respectively. PCR products were gel purified using Qiaquick gel purification kit (Qiagen) and sequenced.

3'-RACE was performed similar to the 5'-RACE protocol. mRNA (1 µg) was reverse transcripted with the primer ME44 and primers were removed by centrifugation through a Glassmax spin column (Gibco). The cDNA was subjected to PCR using primer ME45 and the gene specific primers P1 (5'-TGCGAGGAGGAGCCGTGACGAC-3': corresponds to nucleotides +3467 to +3489) for 40 cycles of 30 s at 68°C, 1 min at 72°C, 30 s at 94°C. One microliter of the PCR product was further amplified using the same forward primer (ME45) and the gene specific primers P1 (5'-TGCGAGGAGGAGCCGTGACGAC-3'; corresponds to nucleotides +102 to +122), P2 (5'-GGCGTTCTCTGACGCTG-3'; corresponds to nucleotides -289 to -308), and P3 (5'-CCTCTTCTGCGATCAG-3'; corresponds to nucleotides -328 to -347), respectively. PCR products were gel purified using Qiaquick gel purification kit (Qiagen) and sequenced.

Construction of luciferase gene plasmids

To generate the luciferase reporter gene construct pGL2PST1, the 1.16 kb XhoI–SacI fragment of phase A14.1.1, which is located 120 bp upstream of the transcription start site was subcloned into the SmaI and SacI sites of pGL2Basic (Promega). A fragment containing sequence between nucleotide -848 and -161 was amplified by PCR using the primers ME75 (5'-GGCGAGATCAGCTCATACG-3') and ME1I (5'-CGAAGCTTTGCTAGCTCTCCGATGTTCTCCGAC-3'), introducing NheI and HindIII restriction sites (underlined) to facilitate subcloning. The 280 bp SacI–NheI fragment of the PCR product was subcloned into the SacI and NheI sites of pGL2Basic to generate construct pGL2PST4, and into the same sites of pGL2PST1 to create plasmid pGL2PST2. Furthermore, plasmid pGL2PST2 was prepared by subcloning the 500 bp XhoI–HindIII fragment of the PCR product into the XhoI and HindIII sites of pGL2Basic. All constructs were confirmed by sequencing.

Transfection and luciferase assay

Chinese hamster ovary (CHO) cells were maintained in Dulbeccco’s modified Eagle’s medium (DMEM) Nut Mix F12 (Gibco) supplemented with 5% fetal calf serum (FCS), 2 mM glutamine, 1 mM sodium pyruvate, 100 µM penicillin, and 100 µg/ml streptomycin. AtT20(+) and AtT20(-), showing the phenotype NCAM+PSA+ and NCAM+PSA-, respectively, and NIH-3T3 cells were grown in DMEM with Glutamax (Gibco) supplemented with 5% FCS, 100 µM penicillin, and 100 µg/ml streptomycin. All cells were maintained at 37°C in a 5% CO2 incubator. Approximately 2 x 105 cells were seeded on 35 mm cell culture dishes and transfected 24 h later using Lipofectamine (Gibco) following the instructions of the manufacturer. Cells were transfected with equimolar amounts of the luciferase gene plasmids constructs (∼0.5 µg DNA) and 0.5 µg pCMVlucZ. After 2 days cells were lysed in 25 mM Tris-H3PO4 (pH 7.8), 2 mM EDTA, 2 mM DTT, 10% glycerol, and 1% NP-40. Aliquots of the lysate were analyzed for luciferase activity in 25 mM glycelyglycine (pH 7.8), 15 mM MgSO4, 2 mM ATP, 250 µM coenzyme A, and 45 µM luciferase (Sigma) using a Berthold Multi-biolumat LB 9505C. To measure β-galactosidase activity, aliquots of the lysates were incubated in 100 mM sodium phosphate (pH 7.0), 10 mM KCl, 1 mM MgSO4, 2 mM DTT, and 1 mg/ml o-nitrophenyl-β-galactopyranoside. The reaction was stopped with Na2CO3 and absorbence was measured at 405 nm.

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Abbreviations

NCAM, neural cell adhesion molecule; PCR, polymerase chain reaction; PSA, polysialic acid; RACE, rapid amplification of cDNA ends; ST8Sial II, CMP-Neu5Ac:(Neu5Ac)n Neu5Acβ2,3sialyltransferase II; ST8Sial IV, CMP-Neu5Ac:(Neu5Ac)α Neu5Acα2,3Gal α2,8 sialyltransferase IV.

References


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References


1171
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