J. Biochem. **134**, 287–296 (2003)
DOI: 10.1093/jb/mvg142

Comparison of the Enzymatic Properties of Mouse β-Galactoside α2,6-Sialyltransferases, ST6Gal I and II

Shou Takashima[*,1], Shuichi Tsuji[2] and Masafumi Tsujimoto[†,1]

[1]Laboratory of Cellular Biochemistry, RIKEN, 2-1 Hirosawa, Wako, Saitama 351-0198; and [2]Molecular Glycobiology, The Glycoscience Institute, Ochanomizu University, Otsuka, Bunkyo-ku, Tokyo 112-8610

Received May 7, 2003; accepted June 17, 2003

The cDNA encoding a second type of mouse β-galactoside α2,6-sialyltransferase (ST6Gal II) was cloned and characterized. The sequence of mouse ST6Gal II encoded a protein of 524 amino acids and showed 77.1% amino acid sequence identity with human ST6Gal II. Recombinant ST6Gal II exhibited α2,6-sialyltransferase activity toward oligosaccharides that have the Galβ1,4GlcNAc sequence at the nonreducing end of their carbohydrate groups, but it exhibited relatively low and no activity toward some glycoproteins and glycolipids, respectively. On the other hand, ST6Gal I, which has been known as the sole member of the ST6Gal-family for more than ten years, exhibited broad substrate specificity toward oligosaccharides, glycoproteins, and a glycolipid, paragloboside. The ST6Gal II gene was mainly expressed in brain and embryo, whereas the ST6Gal I gene was ubiquitously expressed, and its expression levels were higher than those of the ST6Gal II gene. The ST6Gal II gene is located on chromosome 17 and spans over 70 kb of mouse genomic DNA consisting of at least 6 exons. The ST6Gal II gene has a similar genomic structure to the ST6Gal I gene. In this paper, we have shown that ST6Gal II is a counterpart of ST6Gal I.

Key words: cDNA cloning, genomic organization, oligosaccharide, sialyltransferase, ST6Gal II.

Abbreviations: The ganglioside designations are according to the nomenclature of Svennerholm (*1*). The cloned sialyltransferase designations are according to the nomenclature of Tsuji *et al*. (*2*). The abbreviations used are: BSM, bovine submaxillary mucin; EST, expressed sequence tag; G3PDH, glyceraldehyde 3-phosphate dehydrogenase; HPTLC, high performance thin layer chromatography; MES, 4-morpholineethanesulfonic acid; NANase, *N*-acetylneuraminidase; RT, reverse transcription; Sia, sialic acids.

Sialic acids (Sia) are negatively charged acidic sugars, and usually occur at the terminal ends of the carbohydrate groups of glycoproteins and glycolipids. Because of their negative charge and their wide occurrence at exposed positions on cell-surface molecules, sialic acids function as key determinants of oligosaccharide structures that may mediate a variety of biological phenomena, such as cell–cell communication, cell–substrate interaction, adhesion, and protein targeting. For the synthesis of sialylglycoconjugates, a family of glycosyltransferases called sialyltransferases catalyzes the transfer of the sialic acid from CMP-Sia to an acceptor carbohydrate. All mammalian sialyltransferases characterized to date have a type II transmembrane topology, and contain highly conserved motifs called sialyl motifs L (Long), S (Short), and VS (Very Short) (*3–5*). Sialyl motif L is characterized by a 45–60 amino acid region in the center of the protein and has been shown to be involved in the binding of the donor substrate, CMP-Sia (*6*). Sialyl motif S is located in the COOH-terminal region and consists of a 20–30 amino acid stretch. It has been shown to be involved in the binding of both donor and acceptor substrates (*7*). Sialyl motif VS is also located in the COOH-terminal region, within which one glutamic acid residue is always found separated by four amino acid residues from a highly conserved histidine residue. This motif is thought to be involved in the catalytic process (*5*, *8*). Based on the high sequence conservation of sialyl motifs L and S, PCR-based cloning of sialyltransferase cDNAs has been extensively performed (reviewed in Refs. *9* and *10*). In addition, some sialyltransferase cDNAs have been cloned efficiently by using sequence information derived from the expressed sequence tag (EST) database (*11–15*). So far, the cDNA cloning of 20 members of the mammalian sialyltransferase family has been performed and they have been grouped into four subfamilies according to the carbohydrate linkages they synthesize: α2,3-sialyltransferases (ST3Gal I-VI), α2,6-sialyltransferases (ST6Gal I and II), GalNAc α2,6-sialyltransferases (ST6GalNAc I-VI), and α2,8-sialyltransferases (ST8Sia I-VI) (*14*).

Among them, ST6Gal I has been known as the sole member of the α2,6-sialyltransferase family for more than ten years. But recently, a novel member of the α2,6-sialyltransferase family, named ST6Gal II, was cloned from man (*16*, *17*). Human ST6Gal II exhibits activity toward oligosaccharides that have the Galβ1,4GlcNAc sequence at the nonreducing end of their carbohydrate groups, but it exhibits relatively low and no activity toward some glycoproteins and glycolipids, respectively. Therefore, it seems that ST6Gal II is an oligosaccharide-specific enzyme compared with ST6Gal I, which exhibits

[*]Special postdoctoral researcher from RIKEN.
[†]To whom correspondence should be addressed. Tel: +81-48-467-9370, Fax: +81-48-462-4670, E-mail: tsujimot@postman.riken.go.jp.

broad substrate specificity toward glycoproteins, glycolipids, and oligosaccharides. Although the enzymatic properties of human ST6Gal II have been analyzed, its physiological function has not been fully elucidated yet. To determine the physiological function of ST6Gal II, it may be advantageous to analyze the mouse ST6Gal II gene, as some experiments such as ones involving gene-targeting can not be easily performed with the human ST6Gal II gene. The existence of mouse ST6Gal II has been expected from EST clones and genomic sequences (*16*, *17*). However, the cDNA cloning of mouse ST6Gal II has not been performed yet. Thus, it is not clear whether or not the mouse ST6Gal II gene encodes a functional enzyme which has similar enzymatic properties to human ST6Gal II. We think it is necessary to clone mouse ST6Gal II and to characterize it as the first steps for elucidation of the physiological function of ST6Gal II. Here, we describe the cloning of mouse ST6Gal II, characterization of its enzymatic properties, and genomic organization of the gene.

MATERIALS AND METHODS

Materials—Fetuin, asialofetuin, bovine submaxillary mucin (BSM, type I-S), α1-acid glycoprotein, ovomucoid, lactosyl ceramide (LacCer), GA1, GM3, GM1a, Galβ1,3GalNAc, Galβ1,3GlcNAc, Galβ1,4GlcNAc, β-galactosidase (from bovine testes), and Triton CF-54 were purchased from Sigma (St. Louis, MO). Paragloboside and lactose were from Wako (Tokyo). CMP-[^{14}C]-NeuAc (12.0 GBq/mmol, 925 kBq/ml) was from Amersham Biosciences (Piscataway, NJ). Lacto-*N*-tetraose, lacto-*N*-neotetraose, and sialidases (NANases I and II) were from Glyko (Novato, CA). [α-^{32}P]dCTP was from PerkinElmer Life Sciences (Boston, MA). The mouse multiple tissue cDNA panel I and mouse multiple tissue Northern blot were from Clontech (Palo Alto, CA). Asialo-BSM, asialo-α1-acid glycoprotein, and asialo ovomucoid were prepared as described previously (*18*, *19*).

Isolation of ST6Gal II cDNA—Mouse EST sequences (GenBank™ accession Nos. BB552328, BB651169, and BU055532) and mouse genomic sequences (GenBank™ accession Nos. AC125224 and NW_000132) exhibiting similarity to human ST6Gal II were identified through BLAST searches against the dbEST and genomic sequence databases at the National Center for Biotechnology Information, respectively. To obtain the entire coding region, reverse transcription polymerase chain reaction (RT-PCR) was performed with primer sets, 5′-CAATGAAACCACACTTGAAGCAATGGCGAC-3′ (CB241, nucleotides 484–508 in Fig. 1A) and 5′-CGCAACAAAAAAATAGCTATCTTCCTCGGG-3′ (CB244, complementary to nucleotides 862–891), 5′-GACAATGGGGATGAGTTTTTTACATCCCAG-3′ (CB243, nucleotides 802–831) and 5′-CGATTTCCTCCCCCAAGGAGGAGTTCAGG-3′ (CB239, complementary to nucleotides 1383–1411), and 5′-ACGTTGGACGGCAGAGAGGCGCCCTTCTCG-3′ (CB238, nucleotides 1255–1284) and 5′-ACCTTATTGCACATCAGTTCCCAAGAGTTC-3′ (CB240, complementary to nucleotides 2063–2092), with the first strand cDNA of mouse brain as a template. The above PCRs were performed as follows: 94°C for 60 s; 45 cycles of 94°C for 60 s, 50°C for 60 s, and 72°C for 90 s; and 72°C for 10 min. The PCR products were cloned into the pBluescript II SK(+) vector, and then combined using the *Aor*51HI and *Kpn*I sites. The nucleotide sequence was confirmed by the dideoxy termination method using an ABI PRISM 377 DNA sequencer (Applied Biosystems, Foster City, CA).

Construction of Expression Vectors—We constructed an expression vector encoding the soluble ST6Gal II. A *Mun*I site was introduced at nucleotide position 568 by using a mutagenic primer, 5′-CATCCAATTGACCAACAGCAATCCTGCGGC-3 (CB245, corresponds to nucleotides 564–593; the synthetic *Mun*I site is underlined). Then the *Mun*I–*Xho*I fragment encoding a truncated form of ST6Gal II lacking the first 28 amino acids was prepared from the pBluescript II SK(+) vector harboring the *Mun*I site introduced-ST6Gal II cDNA and subcloned into the *Eco*RI–*Xho*I site of expression vector pcDSA. The resulting plasmid, designated as pcDSA-mST6Gal II, encodes a soluble fusion protein consisting of the IgM signal peptide, the *Staphylococcus aureus* protein A IgG-binding domain, and a truncated form of ST6Gal II.

An expression vector for soluble mouse ST6Gal I was constructed as follows. The DNA fragment encoding a truncated form of mouse ST6Gal I lacking the first 30 amino acids was amplified by PCR using primers 5′-AGCGAATTCGAGGCTCTTACATTGCAAGCC-3′ (CB246, nucleotides 391–420 of GenBank™ accession No. D16106; the synthetic *Eco*RI site is underlined) and 5′-GGATCTCGAGCTCAACAGCGATTGTTCCGG-3′ (CB247, complementary to nucleotides 1497–1526) with mouse liver cDNA as a template, and then cloned into the pBluescript II SK(+) vector. Then the 1.1 kb *Eco*RI–*Xho*I fragment encoding a truncated form of ST6Gal I was prepared and subcloned into the *Eco*RI–*Xho*I site of pcDSA, the resulting plasmid being designated as pcDSA-mST6Gal I.

Preparation of Soluble Sialyltransferases—For the production of soluble forms of sialyltransferases, COS-7 cells were transfected with the above pcDSA-vectors using LipofectAMINE™ Reagent (Invitrogen, Carlsbad, CA), and cultured as described previously (*18*). The protein A-fused sialyltransferases expressed in the medium were adsorbed to IgG-Sepharose gel (Amersham Biosciences) and then used as the enzyme sources.

Sialyltransferase Assays and Product Characterization—Sialyltransferase assays were performed as described previously (*20*, *21*). In brief, enzyme activity was measured in 50 mM MES buffer (pH 6.0), 1 mM $MgCl_2$, 1 mM $CaCl_2$, 0.5% Triton CF-54, 100 μM CMP-[^{14}C]NeuAc, acceptor substrate, and enzyme preparation, in a total volume of 10 μl. As acceptor substrates, 10 μg of glycoproteins, 5 μg of glycolipids, or 10 μg of oligosaccharides were used. The enzyme reaction was performed at 37°C for 3–20 h. For glycoproteins, the reaction was terminated by the addition of SDS-PAGE loading buffer, and the reaction mixtures were directly subjected to SDS-PAGE. For glycolipids, the reaction mixtures were applied to a Sep-Pak Vac C_{18} column (100 mg; Waters, Milford, MA), and the purified glycolipids were subjected to high-performance thin-layer chromatography (HPTLC, Silica-Gel 60; Merck, Darmstadt, Germany) with a solvent system of chloroform, methanol, and 0.02% $CaCl_2$ (55:45:10). For oligosaccharides, the reaction mixtures were directly subjected to HPTLC with a solvent system of 1-propanol, aqueous ammonia, and water (6:1:2.5). The radioactive

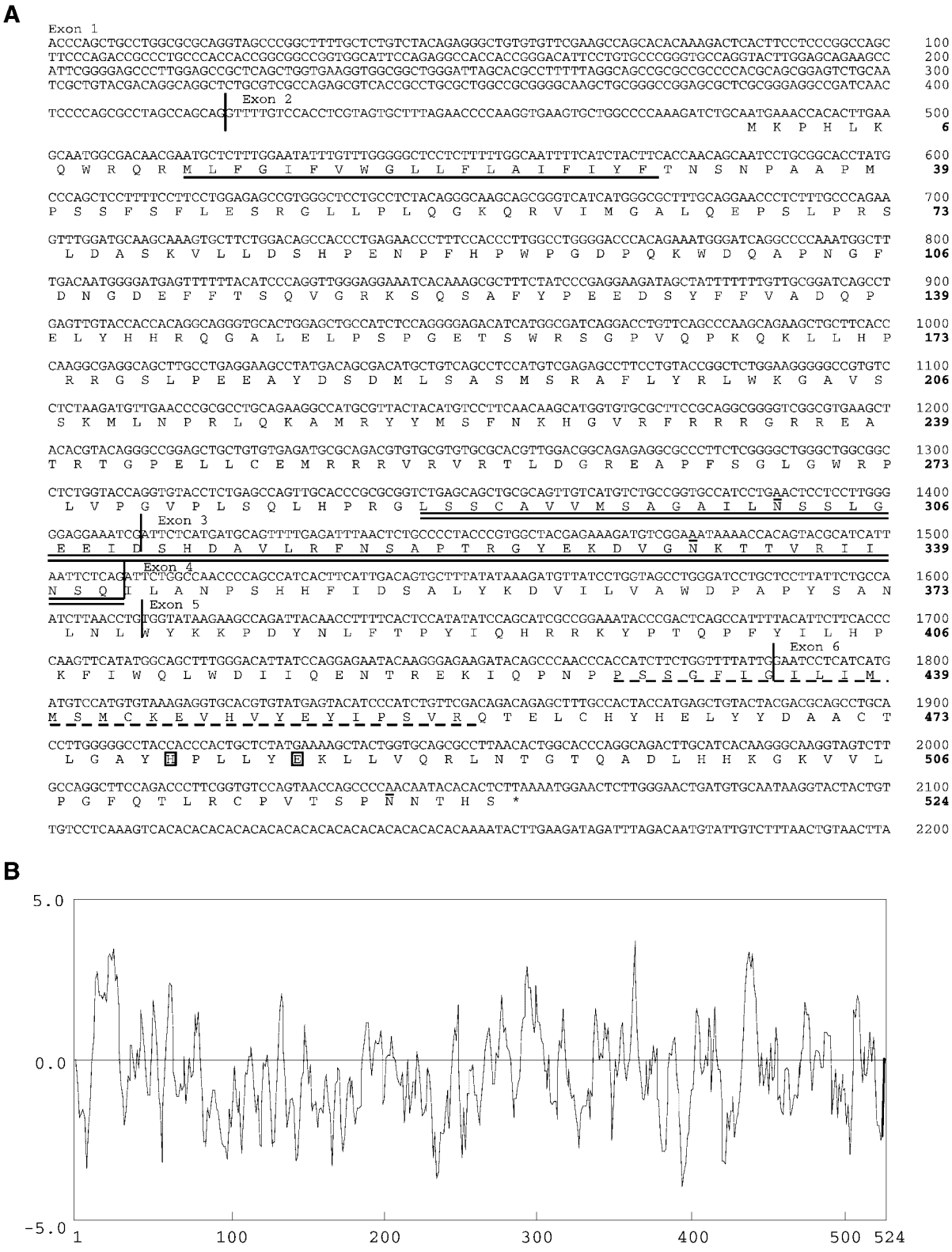

Fig. 1. **Nucleotide and deduced amino acid sequences of mouse ST6Gal II, and hydropathy plot of the protein.** A, the deduced amino acid sequence is shown below the nucleotide sequence. The putative transmembrane domain is underlined. Sialyl motifs L and S are double underlined and dashed underlined, respectively. The conserved His and Glu residues in sialyl motif VS are boxed. Three potential *N*-linked glycosylation sites are overlined. Junctions between exons are indicated by vertical lines. B, the hydropathy plot was calculated by the method of Kyte and Doolittle (*23*).

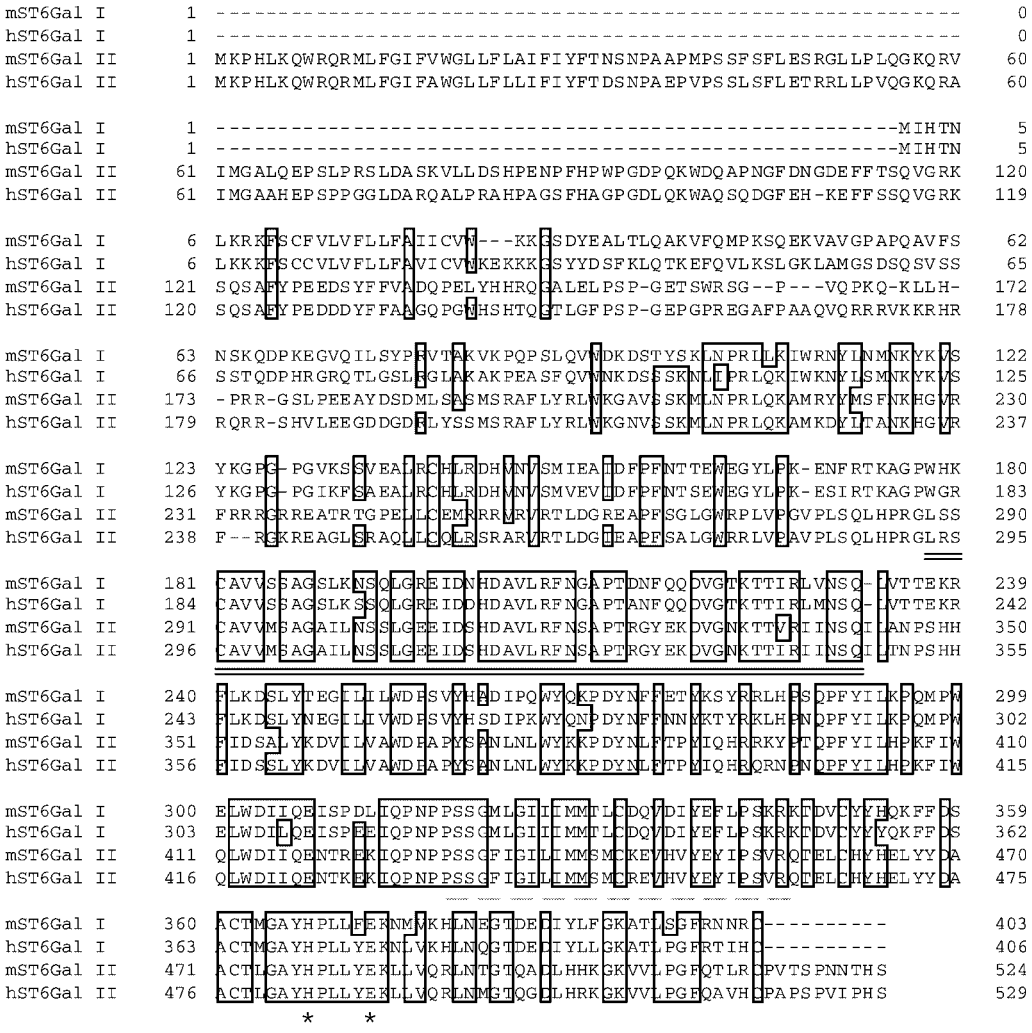

Fig. 2. **Sequence comparison of mouse and human ST6Gal I and II.** The conserved amino acid residues are boxed. Sialyl motifs L and S are double underlined and dashed underlined, respectively. The conserved His and Glu residues in sialyl motif VS are indicated by asterisks.

materials were visualized and quantified with a Fuji BAS2000 radioimage analyzer. The intensity of the radioactivity was converted into moles using the radioactivities of various amounts of CMP-[^{14}C]NeuAc (12.0 GBq/mmol, 925 kBq/ml) as standards. Quantification was performed within the linear range of the standard radioactivity.

For kinetic analysis, the reaction was performed as described above except for the use of various concentrations of acceptor substrates. Under these conditions, the product formation from the individual acceptor substrates was linear up to 4 h. Kinetic parameters were determined by means of Lineweaver-Burk plots.

For linkage analysis of sialic acids, [^{14}C]NeuAc-incorporated Galβ1,4GlcNAc with ST6Gal I or II was digested with β-galactosidase (from bovine testes; Sigma), or a linkage-specific exosialidase: NANase I (specific for α2,3-linked sialic acids; Glyko) or NANase II (specific for α2,3- and α2,6-linked sialic acids; Glyko). After the above treatment, the reaction mixtures were subjected to HPTLC with a solvent system of 1-propanol/aqueous ammonia/water (6:1:2.5). The radioactive materials were visualized with the BAS2000 radioimage analyzer.

Analysis of ST6Gal I and II Gene Expression in Various Mouse Tissues—For Northern blotting, a mouse Multiple Tissue Northern (MTN™) Blot was purchased from Clontech. Each lane contained approximately 2 μg of poly(A)+ RNA . The blot was probed with [α-^{32}P]dCTP-labeled ST6Gal II cDNA or β-actin cDNA according to the manufacturer's instructions. The relative expression levels of ST6Gal I and II mRNAs were estimated by RT-PCR using mouse multiple tissue cDNA panel I (Clontech) and the first strand cDNA of lactating mammary gland as templates. For the analysis of ST6Gal II gene expression, ST6Gal II-specific primers CB241 and CB244 were used. For the analysis of ST6Gal I gene expression, ST6Gal I-specific primers 5′-ATGATTCATACCAACTTGAAG-3′ (CB142, nucleotides 304–324 of GenBank™ accession No. D16106) and 5′-GGTGCCCCATTAAACCTCAG-3′ (CB143, complementary to nucleotides 919–938) were used. As a control, glyceraldehyde 3-phosphate dehydrogenase (G3PDH) gene expression was also measured using

G3PDH specific primers 5′-GGATCCACCACAGTCCATGCCATCAC-3′ and 5′-AAGCTTTCCACCACCCTGTTGCTGTA-3′ (*22*). PCRs were performed as follows: 94°C for 60 s; 35–40 cycles of 94°C for 60 s, 50°C for 60 s, and 72°C for 90 s for the ST6Gal I and II genes, and 25 cycles for the G3PDH gene; and 72°C for 10 min. The PCR products were electrophoresed on a 2% agarose gel, stained with ethidium bromide, and then visualized under UV light.

RESULTS

Cloning and Nucleotide Sequencing of the Mouse ST6Gal II cDNA—Using the mouse EST and genomic sequence databases, we found some sequences [GenBank™ accession Nos. BB552328, BB651169, and BU055532 (EST clones), and AC125224 and NW_000132 (genomic sequences)] exhibiting similarity to human ST6Gal II. Then we performed RT-PCR to obtain the entire coding region of this clone with the mouse brain first strand cDNA as a template. We amplified it as three DNA fragments, as described under "MATERIALS AND METHODS," and each fragment was cloned into the pBluescript II SK(+) vector. Then the fragments were combined and the DNA fragment encoding mouse ST6Gal II was obtained.

The nucleotide sequence of mouse ST6Gal II cDNA and its deduced amino acid sequence are shown in Fig. 1A. The predicted protein consists of 524 amino acids with a calculated molecular mass of 60,122 Da, with three potential *N*-linked glycosylation sites. Hydropathy analysis (*23*) indicated one prominent hydrophobic sequence of 19 amino acids in length in the NH_2-terminal region, predicting that the protein has the type II transmembrane topology characteristic of many other glycosyltransferases cloned to date (Fig. 1B). The overall amino acid sequence of mouse ST6Gal II showed 77.1%, 46.4%, and 46.2% sequence identity with human ST6Gal II, human ST6Gal I, and mouse ST6Gal I, respectively (Fig. 2). But it showed relatively low sequence identity (22.1–30.8%) with members of the mouse ST3Gal-, ST6GalNAc-, and ST8Sia-families.

Enzymatic Activity of Mouse ST6Gal II—To facilitate determination of the enzymatic activity of mouse ST6Gal II, we constructed expression plasmid pcDSA-mST6Gal II, which allows expression of ST6Gal II lacking the transmembrane domain as a secretable protein fused with the IgG-binding domain of *Staphylococcus aureus* protein A. The plasmid was then transfected into COS-7 cells, and the protein A-fused ST6Gal II expressed in the medium was adsorbed to IgG-Sepharose resin, which was used as the enzyme source. For comparative analysis, protein A-fused mouse ST6Gal I was also prepared. As shown in Table 1 and Fig. 3, ST6Gal II exhibited activity toward oligosaccharides Galβ1,4GlcNAc and lacto-*N*-neotetraose, both of which have the Galβ1,4GlcNAc structure at the nonreducing end of their carbohydrate groups. The apparent K_m value of mouse ST6Gal II for Galβ1,4GlcNAc was estimated to be 1.25 mM, which was significantly lower than that of ST6Gal I for Galβ1,4GlcNAc (2–10 mM) (*24*). However, ST6Gal II did not exhibit activity toward oligosaccharides such as Galβ1,3GalNAc, Galβ1,3GlcNAc, lactose, and lacto-*N*-tetraose, all of which do not have the Galβ1,4GlcNAc structure at the nonreducing end of their carbohydrate groups. ST6Gal II also exhibited relatively low activity toward some glycoproteins, which are considered to have the Galβ1,4GlcNAc structure at the nonreducing end of their carbohydrate groups. But ST6Gal II did not exhibit activity toward the glycolipids examined in this study, including paragloboside, which has the Galβ1,4GlcNAc structure at the nonreducing end of its carbohydrate group.

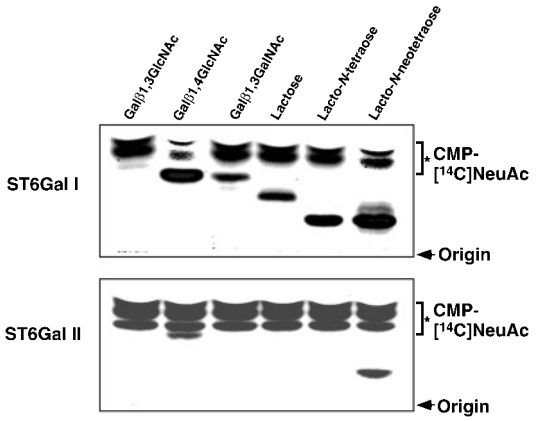

Fig. 3. **Incorporation of sialic acids into various oligosaccharides by mouse ST6Gal I and II.** The various oligosaccharides (10 μg/lane) indicated were incubated with ST6Gal I or II, and the resulting oligosaccharides were analyzed by HPTLC with a solvent system of 1-propanol, aqueous ammonia, and water (6:1:2.5).

On the other hand, ST6Gal I exhibited more or less activity toward all oligosaccharides examined in this study (Fig. 3 and Table 1). ST6Gal I also exhibited relatively high activity toward some asialoglycoproteins. In addition, although it was low, ST6Gal I exhibited activity toward a glycolipid, paragloboside. As the specific activity of ST6Gal I used in these assays was about 18.4 times higher than that of ST6Gal II (Table 1), we also prepared highly concentrated recombinant ST6Gal II and used it for assays. But we could not detect weak or residual activity of ST6Gal II, and the substrate specificity of ST6Gal II did not change (data not shown). These findings indicate that the substrate specificity of ST6Gal II is narrower than that of ST6Gal I.

Linkage Specificity of ST6Gal II—The linkages of the incorporated sialic acids were also examined. Galβ1,4GlcNAc was sialylated with ST6Gal II and then treated with linkage-specific exosialidases (Fig. 4A). The incorporated [^{14}C]NeuAc was resistant to treatment with α2,3-specific exosialidase (NANase I), but it was digested by α2,3- and α2,6-specific exosialidase (NANase II). The sialylated product generated in this experiment comigrated with 6′-sialyl-*N*-acetyllactosamine on TLC (data not shown), and was also resistant to treatment with β-galactosidase (Fig. 4B), suggesting that the incorporated [^{14}C]NeuAc binds to galactose, but not *N*-acetylglucosamine, through an α2,6-linkage. Moreover, these resulting patterns of ST6Gal II were virtually identical to those of ST6Gal I (Fig. 4, A and B). These results indicated that mouse ST6Gal II transfers sialic acid to galactose of the Galβ1,4GlcNAc structure through an α2,6-linkage, and this enzyme certainly belongs to the

Table 1. **Acceptor substrate specificities of mouse ST6Gal I and II.** Various acceptor substrates were incubated in the standard assay mixture using soluble sialyltransferase fused with protein A as an enzyme source. Each substrate was used at the concentration of 0.5 mg/ml for glycolipids, and 1 mg/ml for glycoproteins and oligosaccharides. Relative rates were calculated as a percentage of the incorporation obtained with Galβ1,4GlcNAc. R represents the remainder of the *N*-linked oligosaccharide chain.

Acceptors	Representative structures of carbohydrates	Relative rate (%)	
		ST6Gal I	ST6Gal II
Oligosaccharides			
Type II	Galβ1,4GlcNAc	100[a]	100[b]
Type I	Galβ1,3GlcNAc	1.8	0
Type III	Galβ1,3GalNAc	11.4	0
Lactose	Galβ1,4Glc	15.5	0
Lacto-*N*-tetraose	Galβ1,3GlcNAcβ1,3Galβ1,4Glc	36.8	0
Lacto-*N*-neotetraose	Galβ1,4GlcNAcβ1,3Galβ1,4Glc	106.2	128.8
Glycoproteins			
Fetuin	NeuAcα2,3Galβ1,3GalNAc-*O*-Ser/Thr NeuAcα2,3Galβ1,3(NeuAcα2,6)GalNAc-*O*-Ser/Thr NeuAcα2,6(3)Galβ1,4GlcNAc-R	5.9	0
Asialofetuin		105.6	21.0
BSM	NeuAcα2,6GalNAc-*O*-Ser/Thr GlcNAcβ1,3(NeuAcα2,6)GalNAc-*O*-Ser/Thr	0	0
Asialo-BSM		0	0
Ovomucoid	NeuAcα2,3Galβ1,4GlcNAc-R	8.4	0
Asialoovomucoid		9.1	0
α1-Acid glycoprotein	NeuAcα2,6(3)Galβ1,4GlcNAc-R	20.6	0.75
Asialo- α1-Acid glycoprotein		175.6	12.3
Glycolipids			
Lactosylceramide	Galβ1,4Glcβ1-Cer	0	0
GA1	Galβ1,3GalNAcβ1,4Galβ1,4Glcβ1-Cer	0	0
GM1a	Galβ1,3GalNAcβ1,4(NeuAcα2,3)Galβ1,4Glcβ1-Cer	0	0
GM3	NeuAcα2,3Galβ1,4Glcβ1-Cer	0	0
Paragloboside	Galβ1,4GlcNAcβ1,3Galβ1,4Glcβ1-Cer	2.0	0

[a]50.3 pmol/h/ml medium. [b]2.74 pmol/h/ml medium.

ST6Gal-family. It should also be noted that the above enzymatic properties of mouse ST6Gal II are virtually identical to those of human ST6Gal II (*16*, *17*).

Expression of the ST6Gal II Gene in Mouse Tissues—To examine the expression of the ST6Gal II gene in various adult mouse tissues, Northern blot analysis was performed (Fig. 5A). A faint band corresponding to about 7.5 kb was detected for brain, but the expression levels of the ST6Gal II gene in other tissues were too low to be detected on Northern blotting. Thus we also performed semi-quantitative RT-PCR to examine the expression of the ST6Gal II gene in various tissues (Fig. 5B). The expression of the ST6Gal II gene was detected on RT-PCR in brain, and 11-, 15-, and 17-day embryos, and very low expression was also detected in spleen, lung, and skeletal muscle. It should also be noted that the ST6Gal II gene is expressed in the pregnant mouse oviduct, judging from some ST6Gal II-related EST clones. On the other hand, the expression of the ST6Gal I gene was detected ubiquitously. Although the expression levels of the ST6Gal I gene varied among tissues, they were higher than those of the ST6Gal II gene.

As shown in this and previous studies (*16*), both mouse and human ST6Gal I and II can synthesize some kinds of 6′-sialyloligosaccharides that are contained in milk. But in our previous study on human ST6Gal I and II (*16*), we could not determine which enzyme is responsible for the synthesis of 6′-sialyloligosaccharides in milk because human lactating mammary gland tissue was unavailable for us. Alternatively, we analyzed it using mouse lactating mammary gland tissue in this study. In this case, the expression of the mouse ST6Gal I gene in lactating mammary gland was detected, whereas no expression of the ST6Gal II gene was observed (Fig. 5B), suggesting that 6′-sialyloligosaccharides in mouse milk are mainly synthesized by ST6Gal I.

Genomic Organization of the ST6Gal II Gene—To determine the genetic and evolutional relations of the ST6Gal II gene with other sialyltransferase genes, we analyzed the genomic organization of the ST6Gal II gene by means of a data base search. The genomic sequence containing the ST6Gal II gene (GenBank™ accession No. AC125224) was obtained and analyzed by means of a BLAST search of the mouse genome data base using ST6Gal II-related cDNA sequences (GenBank™ accession Nos. AB095093, BB666153, and BU055532) as queries. A schematic representation of the most probable genomic structure of the ST6Gal II gene is shown in Fig. 6. We found that the ST6Gal II gene is located on chromosome 17 (17C) and spans over 70 kb of mouse genomic DNA consisting of at least 6 exons (Fig. 6A). The sequences of the splice junctions of the ST6Gal II gene obey the GT-AG rule (25, Fig. 6B). Some amino acid residues at the exon/intron boundaries of the ST6Gal I and II genes are highly conserved (Fig. 6B). In our previous study, we found that codons for Arg in sialyl motif L are

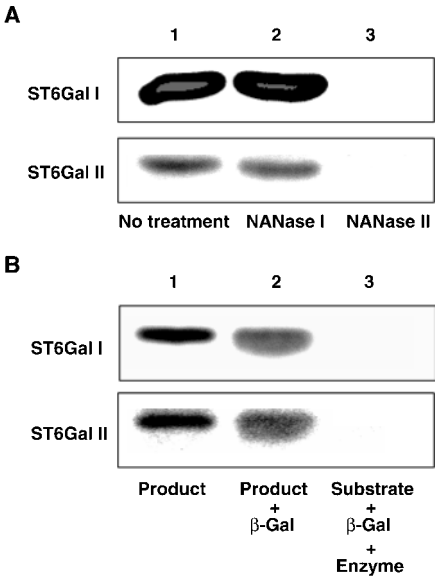

Fig. 4. **Linkage analysis of incorporated sialic acids with mouse ST6Gal I and II.** A, [^{14}C]NeuAc-incorporated Galβ1,4GlcNAc (lane 1) sialylated with ST6Gal I (upper panel) or ST6Gal II (lower panel) was treated with α2,3-specific exosialidase (NANase I, lane 2) or α2,3- and α2,6-specific exosialidase (NANase II, lane 3), and then analyzed by HPTLC. B, [^{14}C]NeuAc-incorporated Galβ1,4GlcNAc (product, lane 1) sialylated with ST6Gal I (upper panel) or ST6Gal II (lower panel) was treated with β-galactosidase (β-Gal, lane 2), and then analyzed by HPTLC. As a control, Galβ1,4GlcNAc (Substrate) was treated with β-galactosidase, and then incubated with ST6Gal I or ST6Gal II (Enzyme) and [^{14}C]NeuAc (lane 3).

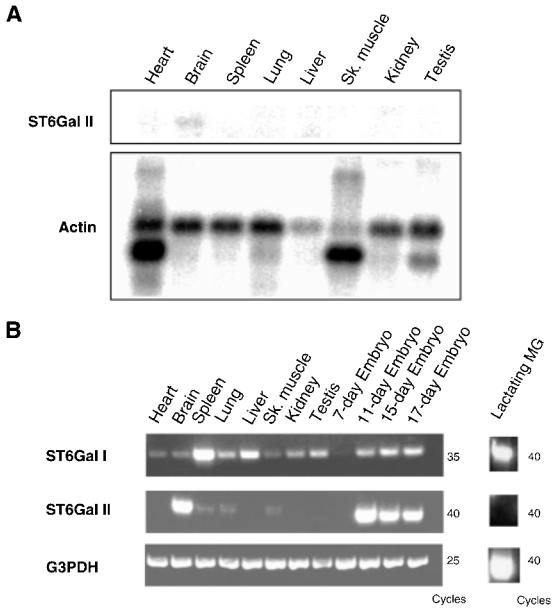

Fig. 5. **Expression analysis of the mouse ST6Gal II gene.** A, Northern blotting with approximately 2 μg of poly(A)+ RNA from various adult mouse tissues was performed as described under "MATERIALS AND METHODS." B, the relative expression levels of the ST6Gal I and II genes in various mouse tissues were measured by semi-quantitative RT-PCR as described under "MATERIALS AND METHODS." Sk. muscle, skeletal muscle; MG, mammary gland.

highly conserved as splice junctions in many mouse and human sialyltransferase genes. But exceptionally, the codons for Asp in sialyl motif L are conserved as splice junctions in members of the mouse and human ST6Gal-families (*16*, *26*). We also found that the codon for Asp in sialyl motif L is a splice junction in the mouse ST6Gal II gene (Figs. 6B and 7). In addition, the split patterns of the coding sequences for sialyl motif S of the ST6Gal I and II genes are different from those of the ST3Gal I and II genes, and the ST6GalNAc I and II genes (Fig. 7). Com-

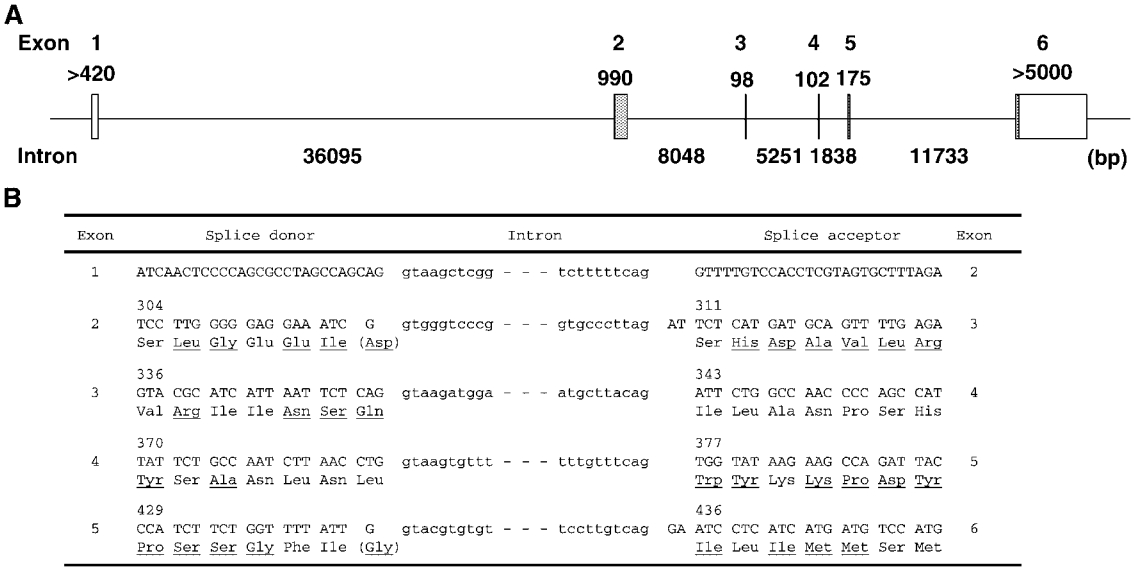

Fig. 6. **Genomic organization of the mouse ST6Gal II gene.** A, the genomic organization of the ST6Gal II gene predicted by the BLAST search is shown. The protein coding region and untranslated region are shown by filled rectangles and open rectangles, respectively. B, the nucleotide sequences comprising the splice sites are shown. The derived amino acid sequence is shown below the nucleotide sequence. The conserved amino acid residues in the mouse ST6Gal I and II genes are underlined. The numbering of amino acid residues starts at the initiator methionine, as +1.

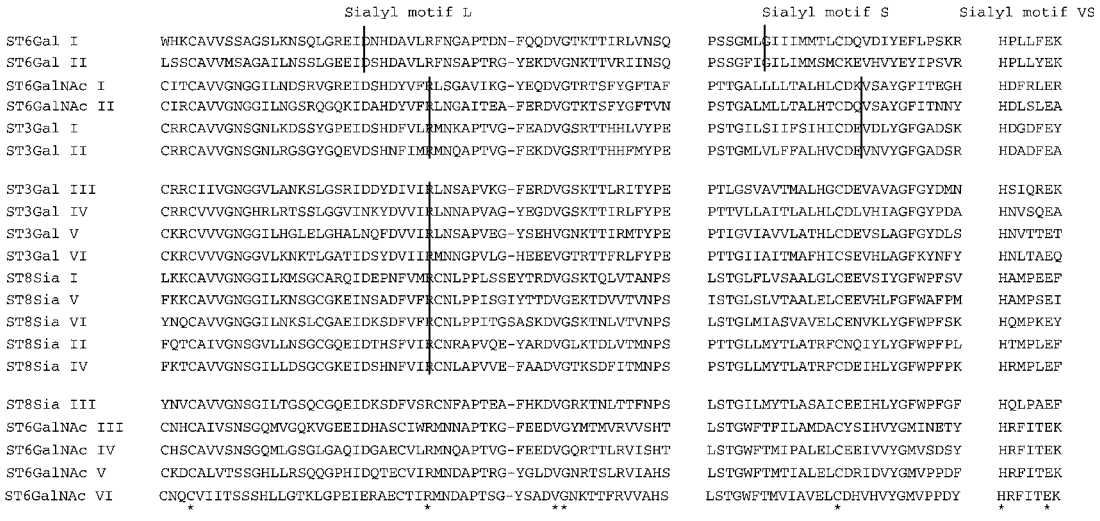

Fig. 7. **Split patterns of sialyl motifs.** The split positions of exons encoding sialyl motifs are indicated by vertical lines. The highly conserved amino acid residues are indicated by asterisks.

parison of the exon/intron boundaries and exon sizes indicated that the ST6Gal I and II genes have similar genomic structures (Figs. 6 and 8).

DISCUSSION

In this study, we have cloned a second type of mouse β-galactoside α2,6-sialyltransferase, ST6Gal II, and characterized it. As shown in this study, mouse ST6Gal II exhibits activity toward oligosaccharides containing the Galβ1,4GlcNAc structure at the nonreducing end of their carbohydrate groups, but exhibits weak or no activity toward glycoproteins and glycolipids, respectively. This substrate specificity is similar to that of human ST6Gal II (*16*, *17*). On the other hand, ST6Gal I has been shown to exhibit broad substrate specificity toward glycoproteins, glycolipids, and oligosaccharides. In addition, the expression patterns of the ST6Gal I and II genes are different. These findings suggest that physiological functions of ST6Gal I and II may be different from each other. For example, ST6Gal I knockout mice exhibited great loss of cell surface Siaα2,6Galβ1,4GlcNAc structures and hallmarks of severe immunosuppression (*27*). These findings indicate that ST6Gal II is hardly involved in the production of cell surface Siaα2,6Galβ1,4GlcNAc structures and can not compensate for the missing ST6Gal I activity in the immune system. Judging from the *in vitro* substrate preference of ST6Gal II, it is possible that its main physiological substrates are oligosaccharides containing the Galβ1,4GlcNAc structure at the nonreducing end. However, although it was relatively weak, ST6Gal II exhibited activity toward some glycoproteins *in vitro* (Table 1). So there remains the possibility that some glycoproteins and/or glycolipids, which were not examined in this study, are specifically sialylated by ST6Gal II *in vivo*. Since the expression of the ST6Gal II gene is very restricted, the expression of such substrate molecules may also be very restricted. Therefore, sialylglycoconjugates produced by ST6Gal II may not have been detected on analyses of ST6Gal I knockout mice so far. The generation of knockout mice and identification of *in vivo* substrates of ST6Gal II are needed for elucidation of the physiological function of ST6Gal II.

Although the main substrates of ST6Gal I *in vivo* have been considered to be glycoproteins, it is also likely that ST6Gal I is significantly involved in the synthesis of sialyloligosaccharides in some tissues. Because our *in vitro* analysis showed that ST6Gal I can sialylate not only Galβ1,4GlcNAc and lacto-*N*-neotetraose but also Galβ1,3GlcNAc, Galβ1,3GalNAc, lactose and lacto-*N*-tetraose, whereas ST6Gal II cannot sialylate Galβ1,3GlcNAc, Galβ1,3GalNAc, lactose or lacto-*N*-tetraose (Fig. 3 and Table 1). These findings suggest that some kinds of sialyloligosaccharides are produced by ST6Gal I only. For example, 6′-sialyllactose in mouse milk should be synthesized by ST6Gal I judging from the substrate specificity of this enzyme and the expression of this gene in the lactating mammary gland (Figs. 3 and 5B, and Table 1).

Sialyloligosaccharides are considered to play important roles in physiological functions in infancy, such as in growth and development (*28*). Moreover, it has been reported that some sialyloligosaccharides in human milk have growth-promoting effects on bifidobacteria and lactobacilli present in the intestinal flora and inhibitory activity against the binding of cholera toxin B subunit to its receptor, GM1 (*28*). The predominant bifidobacteria flora in the intestinal tract is considered to inhibit the growth of harmful bacteria, such as pathogenic strains of *Escherichia coli*, and to protect infants against gastrointestinal diseases. Many Sia-binding pathogens exhibit a preference for the α2,3-sialyl linkage (*29*), but it is considered that compounds containing an α2,6-sialyl linkage may act as decoys or smoke screens to foil potential pathogens (*30*). Therefore, it is possible that sialyloligosaccharides produced by ST6Gal I in mouse milk contribute to the growth, development and maintenance of the intestinal flora, and protection against enteric infections.

On the other hand, the biological importance of sialyloligosaccharides produced by ST6Gal II is unclear at present. But expression of the ST6Gal II gene seems to be developmentally or tissue-specifically regulated (Fig. 5), suggesting that sialyloligosaccharides produced by

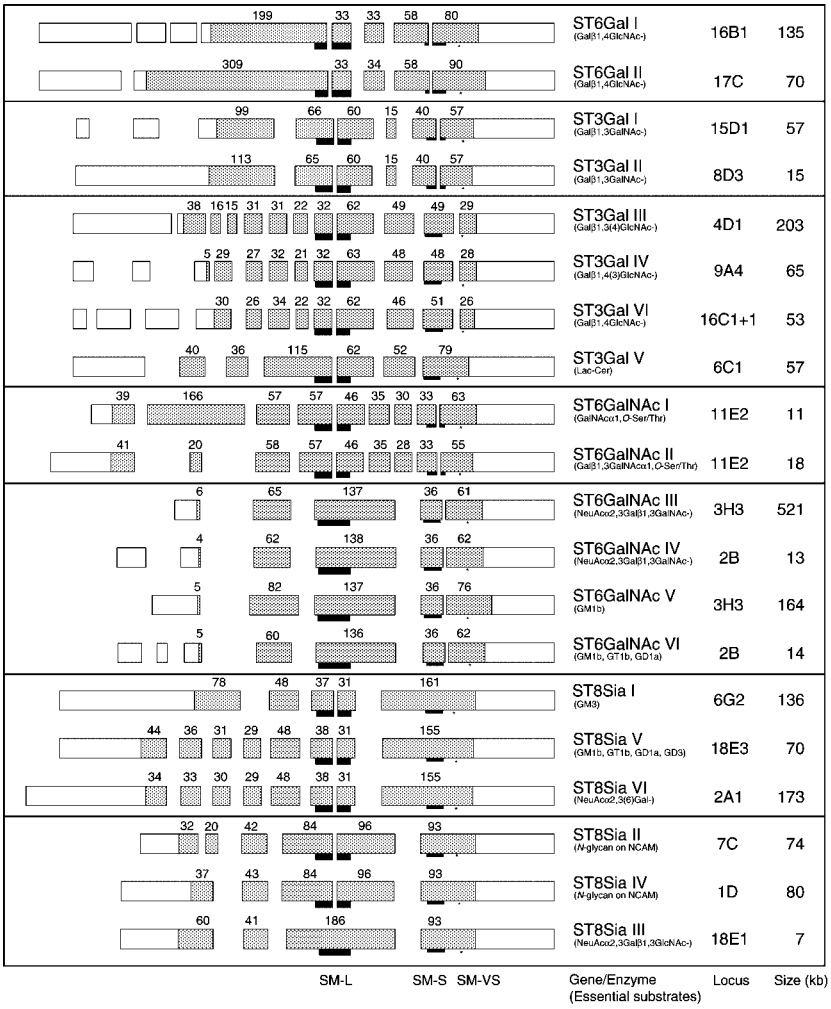

Fig. 8. **Comparison of the genomic structures of the mouse sialyltransferase genes.** The genomic structures of 20 mouse sialyltransferase genes are presented. The protein coding region and untranslated region are shown by filled rectangles and open rectangles, respectively. Untranslated regions are not necessarily shown to scale. It should be noted that the genomic structure of the ST3Gal V gene would show more similarity to those of the ST3Gal III, IV, and VI genes if exons 4 and 7 of the ST3Gal V gene were split at appropriate positions. It should also be noted that the genomic structure of the ST8Sia III gene would show more similarity to those of the ST8Sia II and IV genes if exon 3 of the ST8Sia III gene was split at an appropriate position. Sialyl motifs L (SM-L) are underlined in bold. Sialyl motifs S (SM-S) are underlined. Sialyl motifs (VS) are indicated by asterisks. The sialyl motifs L and S of some genes are split by introns.

ST6Gal II may also play important roles in various biological phenomena, such as in growth and development. We are trying to analyze the physiological function of ST6Gal II in detail now.

At present, 20 mouse sialyltransferase genes have been identified. To clarify the genetic and evolutional relations of these genes, we have performed an extensive data base search, and obtained information on the chromosomal localization and genomic organization of these genes. This is the first report on the chromosomal localization and genomic organization of the 20 mouse sialyltransferase genes. The results are summarized in Fig. 8. Genomic structural analysis of the ST6Gal II gene revealed that it has a similar genomic structure to the ST6Gal I gene, suggesting that these genes have a common ancestral gene. The split patterns of the coding sequences for sialyl motifs L and S of these genes are different from those of other sialyltransferase genes (Fig. 7), also suggesting that the ST6Gal I and II genes may have evolved independently or differently from the most ancestral sialyltransferase gene. Besides the ST6Gal I and II genes, there are several sets of sialyltransferase genes that encode similar enzymes and have similar genomic structures. Among them, the ST6GalNAc I and II genes, ST6GalNAc III and V genes, and ST6GalNAc IV and VI genes are located close to each other on chromosomes 11, 3, and 2, respectively (Fig. 8), suggesting that each gene pair is closely related from an evolutional standpoint. Probably each gene pair arose from a common ancestral gene through tandem duplication. It should be noted that the genome sizes of each gene pair are also relatively similar to each other (Fig. 8). This situation as to the ST6GalNAc-family has also been observed for the human genome (*16*). On the other hand, other pairs of similar sialyltransferase genes, such as the ST6Gal I and II genes, are not located on the same chromosome. This suggests that these genes arose from a common ancestral gene through gene duplication and were subsequently dispersed in the mouse genome through translocation. From a genomic structural point of view, the four sialyltransferase families (ST3Gal-, ST6Gal-, ST6GalNAc-, and ST8Sia-families) can be further subdivided into seven groups (Fig. 8). The members of each group show similar genomic structures and substrate specificities. This also supports the idea that the members of each group arose from a common ancestral gene. It is interesting that all the so far cloned sialyltransferases each have a counterpart with similar enzymatic properties and genomic structure. The biological significance of these multiple genes is unclear at present. One interpretation is that they may be important for fine control of the expression of sialylglycoconjugates, result-

ing in a variety of developmental stage-specific and tissue-specific glycosylation patterns. Characterization of each sialyltransferase and analysis of the transcriptional regulation of each gene will help elucidate the biological significance of each sialyltransferase and the sialylglycoconjugates produced by them.

This work was supported in part by a grant for the "Chemical Biology Research Program" from RIKEN. The nucleotide sequence reported in this paper has been submitted to the GenBank™/EBI/DDBJ Data Bank under accession number AB095093.

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