Molecular Cloning of a Developmentally Regulated N-Acetylgalactosamine α2,6-Sialyltransferase Specific for Sialylated Glycoconjugates*

Eric R. Sjoberg¶§, Hiroshi Kitagawa¶§, John Glushka, Herman van Halbeek‡, and James C. Paulson**

From the Cyte Corporation and the Departments of Chemistry and Molecular Biology, Scripps Research Institute, San Diego, California 92121 and the Complex Carbohydrate Research Center and Department of Biochemistry, The University of Georgia, Athens, Georgia 30602

A cDNA encoding a novel sialyltransferase has been isolated employing the polymerase chain reaction using degenerate primers to conserved regions of the sialylmotif that is present in all eukaryotic members of the sialyltransferase gene family examined to date. The cDNA sequence revealed an open reading frame for 305 amino acids, making it the shortest sialyltransferase cloned to date. This open reading frame predicts the second in the carboxyl-terminal portion of the cDNA. When compared with all other sialyltransferase cDNAs, the predicted amino acid sequence displays the lowest homology in the sialyltransferase gene family. Northern analysis shows this sialyltransferase to be developmentally regulated in brain with expression persisting through adulthood in spleen, kidney, and lung. Stable transfection of the full-length cDNA in the human kidney carcinoma cell line 293 produced an active sialyltransferase with marked specificity for the sialoside, Neu5Acα2,3Galβ1,3GalNAc and glycoconjugates carrying the same sequence such as GM3b and fetuin. The disialylated tetrasaccharide formed by reacting the sialyltransferase with the aforementioned sialoside was analyzed by one- and two-dimensional 1H and 13C NMR spectroscopy and was shown to be the Neu5Acα2,3-Galβ1,3(Neu5Acα2,6)GalNAc sialoside. This indicates that the enzyme is a GalNAcα2,6-sialyltransferase. Since two other ST6GalNAc sialyltransferase cDNAs have been isolated, this sialyltransferase has been designated ST6GalNAc III. Of these three, ST6GalNAc III displays the most restricted acceptor specificity and is the only sialyltransferase cloned to date capable of forming the developmentally regulated ganglioside GD1a from GM3b.

positioned in the protective and interactive glyocalyx of the cell surface, sialylated glycoconjugates are optimally situated to mediate initial communication events between two cells. Examples demonstrating the significance of biological events mediated by these interactions range from neurodevelopment where sialosides confer migratory properties to cells to trafficking of leukocytes from blood to sites of inflammation and lymphoid organs (1–4). These interactions are controlled by the structural diversity of sialosides that undergo dramatic alterations throughout ontogeny and also by the site-specific expression of sialic acid recognition molecules such as the selectins and t-type lectins (1, 5–10, 55, 60).

Sialosides are generated by a family of glycosyltransferases termed the sialyltransferases by the transfer of sialic acid from its high energy donor CMP-sialic acid, to the nonreducing terminus of oligosaccharides (33, 34). Sialyltransferases generate considerable structural diversity by transferring sialic acid with remarkable specificity for the underlying oligosaccharide substrate (33, 34). The regulated expression of sialosides is dependent on many factors including the availability of sugar nucleotide transporters within a particular Golgi cisternae, and transit time of acceptors through the Golgi apparatus (56–59). However, the greatest determinant of sialoside expression is probably the site-specific expression observed for each member of the sialyltransferase gene family (30). Based on known structures for sialylated glycoconjugates, the sialyltransferase gene family has been estimated to consist of 10–12 independent gene products, although it is becoming evident that the final number of sialyltransferases may ultimately prove to be larger. To date, 11 enzymatically distinct mammalian sialyltransferases have been cloned by direct and indirect methods (10–24, 27–29, 42). Analysis of their amino acid sequences has revealed two conserved motifs. The longest is characterized by a 48–50-amino acid region centrally located, and the shorter motif consists of a 20–24-amino acid stretch and is found at opposing ends of L-sialylmotif enabling the use of sugar nucleotide transporters within a particular Golgi cisternae, and transit time of acceptors through the Golgi apparatus (33, 34). The regulated expression of sialosides is dependant on many factors including the availability of sugar nucleotide to the Golgi lumen; competing glycosyltransferases; co-localization of appropriate acceptors, transferases, and sugar nucleotide transporters within a particular Golgi cisternae, and transit time of acceptors through the Golgi apparatus (56–59). However, the greatest determinant of sialoside expression is probably the site-specific expression observed for each member of the sialyltransferase gene family (30). Based on known structures for sialylated glycoconjugates, the sialyltransferase gene family has been estimated to consist of 10–12 independent gene products, although it is becoming evident that the final number of sialyltransferases may ultimately prove to be larger. To date, 11 enzymatically distinct mammalian sialyltransferases have been cloned by direct and indirect methods (10–24, 27–29, 42). Analysis of their amino acid sequences has revealed two conserved motifs. The longest is characterized by a 48–50-amino acid region centrally located, and the shorter motif consists of a 20–24-amino acid stretch (14, 16, 26). These have been designated L-sialylmotif and S-sialylmotif respectively (13, 20). Site-directed mutagenesis of L-sialylmotif indicates that it plays a role in the recognition of the sugar nucleotide donor common to all sialyltransferases, CMP-Neu5Ac (61). When the eukaryotic sialyltransferase cDNAs are compared, the greatest conservation of amino acids is found at opposing ends of L-sialylmotif enabling the use of PCR1 to clone additional members of this gene family.

* The abbreviations used are: PCR, polymerase chain reaction; kb, kilobase(s); MES, 4-morpholineethanesulfonic acid; HPLC, high-performance liquid chromatography; NDV, Newcastle disease virus; TLC,
Using degenerate oligonucleotide primers derived from the most conserved regions of L-sialylomof, we have cloned a novel developmentally regulated sialyltransferase that forms the Neu5Acα2,6GalNAc linkage and utilizes sialylated glycoproteins as well as the ganglioside G4,1ed as acceptors generating the sequence Neu5Acα2,3Galβ1,3(Neu5Acα2,6)GalNAc.

**EXPERIMENTAL PROCEDURES**

Materials—αZAPII vector, Escherichia coli strain XL-1 Blue, Giga- pack II packaging extracts, and Bluescript plasmid vector were purchased from Stratagene (Stratagene). The resultant library was packaged using a Stratagene E. coli strain XL-1 Blue, Giga-pack II packaging extracts, and Bluescript plasmid vector were purchased from Stratagene. [55x348]

Isolation of RNA—Total RNA from rat tissues was prepared as described previously (25). Poly(A)+ mRNA was selected by two cycles of binding to oligo(dT)-cellulose type 2 (Collaborative Research) as described previously (11).

PCR Cloning with Degenerate Oligonucleotides—Based on the sequence information of the sialylomof (14), two degenerate oligonucleotides (27, 28) (Table 1) were designed on the basis of the 5′ and 3′ primer and can be used to amplify fragments in a manner similar to the high-throughput gene cloning procedures. The primers were then ligated into plasmid constructs and used to amplify fragments of the putative sialylomof gene. Restricted enzyme digestion was performed with various restriction endonucleases as well as the ganglioside G4,1ed as acceptors generating the sequence Neu5Acα2,3Galβ1,3(Neu5Acα2,6)GalNAc.

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3GalNAc1-benzyl—20 mg of Galβ1,3GalNAc1-benzyl was sialylated to near completion using 70 ml units of recombinant ST3Gal I purified from baculovirus supernatants by CDP-hexanolamine chromatography as described previously (65). Briefly, 49 mg of CMP-Neu5Ac (20 mM final), 25 mM sodium cacodylate, pH 6.0, 0.5% Triton CF-54, and 70 ml units of ST3Gal I were mixed in a total reaction volume of 4 ml. The progress of the reaction was monitored by TLC analysis, and the mixture was sialylated overnight at 37°C. The reaction was lyophilized, resuspended in 20 ml of ethanol, and finally eluted with 20% methanol. The reverse phase purification step was passed over a 5-ml C-18 column, washed with 20 ml of water, and approximately 95% complete as judged by TLC analysis. This material was then recovered in the run through/wash fractions and could be desalted and purified by anion-exchange HPLC as described below.

Synthesis and Purification of the Disialylated Tetrasaccharide Formed by STY—293 cells stably transfected with full-length STY cDNA were grown to confluence in three 225-cm2 tissue culture plates. Cells were harvested and solubilized as described above prior to cDNA preparation. The STY cDNA was cloned into the expression plasmid pCDM6 (29) and the recombinant protein was expressed in insect cells transformed with baculovirus using the baculovirus expression vector system (17). The expression level of STY was monitored by following absorbance at 214 nm. To avoid overloading the column, 4–5 separate runs were performed. Elution buffers were 1 mM Tris, pH 8.0 (Buffer A) and 1 mM Tris, pH 8.0, 0.2M NaCl (Buffer B), utilizing a gradient of 0%–100% Buffer B over 20 cm. The run through and wash fractions were lyophilized, resuspended in 600 μl of 20% ethanol, and desalted over a Bio-Gel P-2 column equilibrated in 20% ethanol. 0.5-mL fractions were collected, and elution of the P-2 column (1.8 x 20 cm) was monitored by spotting every other fraction on a TLC plate and subsequent detection by resorcinol spray. Fractions containing the STY sialoside were dried for further purification. This column provided substantial enrichment of the sialylated tetrasaccharide over its monosialylated trisaccharide precursor. The desalted fractions containing the STY sialoside were resuspended in 2 ml of Milli Q water and fractionated over a strong anion HPLC column (HEMA-IC, Alltech) utilizing a 500-μl injection loop. Elution was monitored by following the absorbance at 214 nm. To avoid overloading the column, 4–5 separate runs were performed. Elution buffers were 20 mM Tris, pH 8.0, 2 mM butyrate, 1 mM Tris, pH 8.0, 0.2 mM NaCl (Buffer B), utilizing a gradient of 0%–22% over 12 ml, and then to 90% over 17 min, at 1.2 ml/min and collecting 0.6-mL fractions. Fractions containing STY sialoside were dried, resuspended in 20% ethanol, and desalted over a Bio-Gel P-2 column. Those fractions containing STY sialoside were pooled and dried for analysis by NMR. This system easily resolved the disialylated STY sialoside from its monosialylated predecessor, CMP-Neu5Ac, and any free sialic acid.

Nuclear Magnetic Resonance Spectroscopy—Samples (~2 mmol) of the trisaccharide precursor and the STY-produced tetrasaccharide product were first dissolved in D2O (99.6% d) at pH 7.0, lyophilized, and then resuspended in D2O. NMR spectra were recorded at 23 °C on Bruker AM-500 and AMX-600 spectrometers, operating at frequencies of 500 and 600 MHz, respectively, for 1H NMR. In all experiments, low power presaturation was employed to suppress residual HDO signals. 1H chemical shifts are expressed in ppm downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate, with an accuracy of 0.002 ppm, and were measured relative to internal acetonitrile at 2.225 ppm. 13C chemical shifts are expressed in ppm downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate, with an accuracy of 0.02 ppm, and were measured from indirect 1H detection experiments with the RF carrier at a position calibrated to be 80.0 ppm (relative to the methyl signal of internal acetonitrile at 3.290 ppm).

One-dimensional TOCSY (43, 46), and ROESY (45) experiments were performed with selective excitation of accessible structural-reporter group signals by DANCE pulse trains (50). The one-dimensional TOCSY pulse program contained a 100-ms DIPSI-2 mixing sequence (52). The one-dimensional ROESY experiments used a 240 ms, 2.2 kHz, CW spin-lock pulse train flanked by two 90° pulses for offset compensation (47). Two-dimensional double quantum filtered COSY (51), HSQC (44), and HMOC-TOCSY (48) datasets were processed typically with a Lorentzian-to-Gaussian weighting function applied in the t1 dimension, and a shifted squared sine bell function and zero-filling was applied in the t2 dimension. Processing was performed with the Felix software package, version 2.3 (BioSym Technologies, Inc.) on a Sun Sparc workstation.

RESULTS

PCR Amplification of a Sialylmotif Fragment—To extend the PCR homology approach for cloning additional members of the family gene, rat brain cDNA was used as a template for PCR reactions, since it is an abundant source of sialyloligosaccharides that are synthesized by sialyltransferases that have not yet been cloned. The PCR experiments resulted in the amplification of a 150-base pair band equal to the size of the highly conserved region of the sialylmotif. Subcloning and sequencing of the amplified PCR products revealed that the band was a mixture of three DNA fragments. Of 50 clones characterized, 26 corresponded to the sialylmotif sequences of the previously cloned sialyltransferases, ST6Gal II, and ST3Gal III. The remaining 24 clones encoded a new sialylmotif fragment, designated SMY, which contained 7 of 8 amino acids that were found to be invariant in the 12 previously cloned sialyltransferases (Fig. 1). The greatest homology is observed between SMY and the sialylmotif of ST3Gal III (54% identity), while the lowest homology is observed between SMY and the sialylmotif of ST8Sia I (33% identity).

Primary Structure of the STY cDNA—In order to clone the complete coding sequence of the gene containing the new sialylmotif, the SMY 150-base pair fragment was used to screen a rat brain cDNA library. Five positive clones, STY-1–STY-5, were isolated. Characterization of the positive clones revealed that clone STY-1 contained a 2.5-kb insert, clones STY-2 and -3 were 2.4 kb, clone STY-4 was 1.8 kb, and clone STY-5 was 1.3 kb in length. Northern analysis indicated that the STY mRNA was 3.0 kb (see below), suggesting that clone STY-1 was near full-length. Sequence analysis revealed that clones STY-1, -2, and -5 contained the complete open reading frame. STY-5 had the longest 5'-untranslated region, which contained one in-frame ATG codon and three additional upstream ATG codons that were followed by a short open reading frame ("mini-cistrons") ranging from 10 amino acids to 37 amino acids up to the stop codons, while the 5' ends of STY-1 and -2 were ~51 and ~47, respectively (Fig. 2). We assigned the second in-frame ATG codon of STY-5 as the initiator codon, which was embedded within a better sequence for translation initiation than the upstream ATG, based on Kozak's rules (31). Additionally, STY-1, lacking the first initiation site and STY-5, containing...
The entire 5' end, including the first potential initiation site, were stably transfected into 293 cells. The result showed that STY-1 cell extracts had considerably more activity than STY-5 extracts did when identical amounts of protein were assayed, providing preliminary evidence that the appropriate start site is most likely the second in-frame ATG site (data not shown).

The open reading frame encodes a protein of 305 amino acids and two N-linked glycosylation sites (Fig. 2). Hydropathy analysis (32) revealed one potential membrane-spanning region consisting of 17 hydrophobic residues, located 8 residues from the amino terminus (Fig. 2). This structural feature suggests that the STY protein has a type II membrane topology characteristic of all other glycosyltransferases cloned to date (33).

Comparison of the primary structure of STY protein and the 11 other cloned sialyltransferases is shown in Table I.

Table I: Comparison of the primary structure of STY protein and the 11 other cloned sialyltransferases

| Clones     | Sequences | Residues | Ref. |
|------------|-----------|----------|------|
| STYGal     | PSSGMGI... | 338-340 | 11   |
| STYGalII   | PTTGILS... | 270-292 | 12   |
| II         | PTGLS...   | 277-299 | 13   |
| III        | PTLGS...   | 299-321 | 14   |
| IV         | PTGLLLA... | 258-280 | 15,23|
| STYGalNAC1 | PSTGALM... | 333-355 | 17   |
| STYGalNAC2 | LSTGWF...  | 253-275 | 20   |
| STYGalNAC3 | LSTGLMF... | 293-315 | 19   |
| STYGalNAC4 | LSTGMLF... | 309-329 | 20   |
| STYGalNAC5 | LSTGLMF... | 276-300 | 21   |

The 11 previously cloned sialyltransferases are the rat ST6Gal I (11), the rat ST3Gal I (12), the mouse ST3Gal II (13), the rat ST3Gal III (14), the human ST3 Gal IV (15, 23), the chick ST6GalNAc I (16), the chick ST6GalNAc II (17), the rat ST6GalNAc III (this publication), the mouse ST8Sia I (18, 22, 24), the rat ST8Sia II (19), the mouse ST8Sia III (20), and the hamster ST8Sia IV (21). The sialyltransferase motifs are grouped by the linkage that they form. Consensus sequences were designated using the following rules: 1) If 12 sialylmotifs contain the indicated amino acid at a particular aligned site, 2) if only two amino acids are found at a particular aligned site, the designated amino acid(s) must be present in at least three sialylmotifs.

Expression of Full-length STY Yields Sialyltransferase Activity—Although insertion of an epitope-tagged soluble form of STY into an expression vector resulted in the translation of secreted STY when transfected into COS cells, no sialyltransferase activity was detected. Since the length of the transmembrane domain is only predicted and the stem region of STY is predicted to be relatively short, it is possible that the DNA was truncated within the catalytic domain of STY precluding the detection of activity. Therefore the entire open reading frame of STY was inserted into the mammalian expression vector, pcDNA3 (Invitrogen) and stably transfected into the kidney carcinoma cell line 293. Triton extracts from these cells displayed strikingly elevated levels of a unique sialyltransferase activity relative to 293 cells stably transfected with the vector alone. As displayed in Table I, STY transferred sialic acid only to sialylated glycoconjugates displaying the Neu5Acα2,3Galβ1,3GalNAc sequence including α2,3-sialylated anti-freeze glycoprotein, the ganglioside GM1b, and fetuin. In contrast, the asialo derivatives of these substrates were not acceptors for STY. While the ganglioside GM1b was a good acceptor for STY, other gangliosides containing the Neu5Acα2,3Galβ1,3GalNAc sequence such as GD1a and GT1b, were not acceptors (Table I). Since GD1a and GT1b both contain a terminal Neu5Acα2,3Galβ1,3GalNAc sequence, it is clear that the α2,3-linked sialic acid attached to the internal galactose of GD1a and GT1b (see Table I for structures) abolishes the ability of the sialyltransferase to transfer sialic acid to this sequence.

To confirm that Neu5Acα2,3Galβ1,3GalNAc is required for activity with STY, we analyzed several sialylated oligosaccharides at a concentration of 0.1 mM to test for their ability to act as STY acceptors. As displayed in Table I, the best acceptor for STY is Neu5Acα2,3Galβ1,3GalNAc sialoside acceptor for STY. While the ganglioside GM1b was a good acceptor for STY, other gangliosides containing the Neu5Acα2,3Galβ1,3GalNAc sequence such as GD1a and G1b, both contain a terminal Neu5Acα2,3Galβ1,3GalNAc sequence, it is clear that the α2,3-linked sialic acid attached to the internal galactose of GD1a and G1b (see Table I for structures) abolishes the ability of the sialyltransferase to transfer sialic acid to this sequence.

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above data, the acceptor specificity of STY is restricted to sialylated glycoconjugates carrying the Neu5Ac\(^{a2,3}\)Gal\(^{b1,3}\)GalNAc sialoside in which the GalNAc is either \(\alpha\)-linked to serine or threonine or \(\beta\)-linked to galactose (GM1\(b\)). Based on the acceptor specificity of STY and previously described sialyltransferase activities, we reasoned that STY formed either Neu5Ac\(^{a2,3}\)Gal\(^{b1,3}(\text{Neu5Ac}\(^{a2,6}\))GalNAc common found \(\text{O}\)-linked to glycoproteins as well as on the ganglioside GD1\(a\) or possibly the sialoside Neu5Ac\(^{a2,8}\)Neu5Ac\(^{a2,3}\)Gal\(^{b1,3}\)GalNAc found on the ganglioside GD1\(c\) (39). Differential sialidase and mild periodate experiments failed to elucidate the linkage formed by STY. To resolve this issue, the STY sialosidewas extensively characterized by one- and two-dimensional \(^1\)H and \(^13\)C NMR experiments as described below.

**One- and Two-Dimensional \(^1\)H and \(^13\)C NMR Analysis Establishes That STY Forms an \(\alpha\)\(^{2,6}\)-GalNAc Linkage—**

To unambiguously determine the structure of sialyloligosaccharide generated by STY, we produced approximately 3 mg of purified disialylated STY tetrasaccharide utilizing Neu5Ac\(^{a2,3}\)Gal\(^{b1,3}\)GalNAc\(^b\) as an acceptor and detergent extracts from 293 cells transfected with STY as an enzyme source. After purification, the STY disialylated tetrasaccharide was analyzed by a variety of NMR experiments as described below. The one-dimensional \(^1\)H NMR spectra of the acceptor monosialoside Neu5Ac\(^{a2,3}\)Gal\(^{b1,3}\)GalNAc\(^b\) and its disialyl tetrasaccharide product, recorded at 600 MHz in D\(_2\)O at pH 6.7, are shown in Fig. 3 (\(\text{a}\) and \(\text{b}\)). The signals on the tetrasaccharide trace that are indicative of the presence of a second sialic acid residue not present in the acceptor trisaccharide include the H-3ax (\(\text{d}\); 1.72), H-3eq (\(\text{d}\); 5.53), and H-5 (\(\text{d}\); 4.98). The discrete pattern of the other peaks on the tetrasaccharide spectrum in comparison to the monosialoside spectrum indicates the presence of another Neu5Ac residue linked to the GalNAc residue.

**Fig. 2. Nucleotide and deduced amino acid sequences of the ST6GalNAc III protein.** The putative signal-anchor domain of the ST6GalNAc III sialyltransferase is boxed, the potential N-glycosylation sites are marked with asterisks, and the ATG codons upstream from the sialyltransferase translation initiation are underlined. The sequences are numbered relative to the translation initiation site, which begins at the second in-frame ATG codon. 

*Downloaded from http://www.jbc.org*
Various glycoconjugate acceptors at 0.5 mM concentrations were incubated in the standard assay mixture with cellular extracts from 293 cells stably transfected with STY or the expression vector itself. Incorporation of label using extracts from 293 cells expressing vector alone (ranged from 0 to 250 cpm depending on the acceptor. * activity is most likely due to endogenous ST3GalI activity present in 293 cells expressing ST6GalNAcIII. Reaction mixtures were separated by anion exchange HPLC and CPM eluting in the disialylated region was used to determine the relative activities. Assays performed with extracts from cells transfected with vector alone displayed incorporation rates ranging from 0 to 250 cpm depending on the acceptor. * activity is most likely due to endogenous ST3GalI activity present in 293 cells forming the acceptor sialoside for STY.

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**Table I**

Glycoconjugate acceptors

| Acceptor | Structure(s) | Relative Rate |
|----------|--------------|---------------|
| Asialo fetuin | Galβ1,3GalNAca1, O-Thr/Ser | 14 |
| Asialo a1-Acid glycoprotein | Galβ1, 4GlcNAcβ1-R | 1 |
| Human transferrin | Galβ1, 4GlcNAcβ1-R | 5 |
| Ovine submaxillary mucin | Galβ1, 4GlcNAcβ1-R | 1 |
| Tamm Horsfall protein | Galβ1, 4GlcNAcβ1-R | 35 |
| Asialo fetuin | Galβ1,3GalNAca1, O-Thr/Ser | 2 |
| Asialo a1-Acid glycoprotein | Galβ1, 4GlcNAcβ1-R | 0 |
| Human transferrin | Galβ1, 4GlcNAcβ1-R | 0 |
| Ovine submaxillary mucin | Galβ1, 4GlcNAcβ1-R | 0 |
| Tamm Horsfall protein | Galβ1, 4GlcNAcβ1-R | 0 |

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**Table II**

Activity of STY with various free oligosaccharides

| Acceptor | CPM | Relative rate % |
|----------|-----|-----------------|
| Neu5AcO2,3Galβ1,3GalNAc | 46766 | 100 |
| Neu5AcO2,3Galβ1,3GalNAc | 280 | 0 |
| Neu5AcO2,3Galβ1,3GalNAc1,4-Lactose | 1290 | 3 |
| Neu5AcO2,3Galβ1,3GalNAc1,4-Lactose | 0 | 0 |
| Neu5AcO2,3Galβ1,3GalNAc1,4-Lactose | 0 | 0 |
| Neu5AcO2,3Galβ1,3GalNAc1,4-Lactose | 0 | 0 |
| Neu5AcO2,3Galβ1,3GalNAc1,4-Lactose | 218 | 0 |

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Fig. 3. 600-MHz one-dimensional 1H NMR of sialosides. One-dimensional 1H-NMR spectra of the acceptor trisaccharide (a) and the product tetrasaccharide (b) in D2O at pH 6.7, recorded at 600 MHz. The structural reporter group signals are marked.
charide) (see Table III). Having obtained the partial assignment of the 1H NMR spectra of the two saccharides, we then attempted to assign their 13C NMR spectra. Fig. 4 shows the pertinent portion of the two-dimensional 1H,13C HSQC spectrum of the tetrasaccharide. An HSQC experiment as conducted here is a two-dimensional 1H,13C correlation experiment that connects signals belonging to 1H and 13C nuclei that are directly (through one bond) attached to each other in the chemical structure. First of all, such an HSQC spectrum provides the assignments of the 13C signals from already assigned 1H signals through one-on-one connectivities. Furthermore, HSQC spectra can provide the missing entries to complete the assignment of the 1H spectra, by revealing the C-6 (or C-9, in case of Neu5Ac) methylene protons. Not only are methylene carbons coupled to two protons each, it also happens that the carbon signals of carbohydrate CH2 groups in 13C NMR spectra (55–70 ppm) are well separated from the CH signals (70–110 ppm). Thus, the HSQC experiment provides a relatively convenient way of assigning the CH2 protons in the 1H spectra. The HSQC CH2 correlations are marked in Fig. 4. The spectrum of the acceptor trisaccharide shows three CH2 signals (at δ 63.40, 63.41, and 64.94), while that of the product tetrasaccharide shows four of them (δ 63.56, 65.08, 65.27, and 66.11). Their 1JCH coupled protons in the 1H spectra were found at the positions listed in Table III.

To assign the CH2 signals to specific glycosyl residues, the 13C signals need to be “linked” to 1H signals already assigned by COSY, TOCSY, and/or ROESY experiments. Two-dimensional HMOC-TOCSY experiments were conducted to provide these links for two of the observed 13C CH2 signals, namely to the Gal and GalNAc H-5 signals (results not shown). The HMOC-TOCSY spectra show partial TOCSY spectra in 1H rows superimposed on 1H,13C correlations. For example, the tetrasaccharide CH2 signal at δ 66.11 ppm showed a HMOC connectivity to two C-6 protons (at δ 3.66 and 3.98 ppm) that in turn are TOCSY-correlated to a proton signal at 3.76 ppm; the latter had been assigned to GalNAc H-5 by a one-dimensional ROESY experiment (see Table III). Analogously, the C-6 signals of Gal in the tri- and tetrasaccharide and C-6 of GalNAc in

| Residue/Site | 1H Chemical shift (ppm) | 13C Chemical Shift (ppm) | Δ δ Tetra vs. Tri |
|-------------|--------------------------|--------------------------|-------------------|
| GalNAc      | 4.531                    | 4.521                    | 0.11              |
| GalNAc      | 4.003                    | 3.989                    | 0.13              |
| GalNAc      | 3.803                    | 3.780                    | 0.11              |
| GalNAc      | 4.165                    | 4.183                    | 0.02              |
| GalNAc      | 3.682                    | 3.763                    | 0.20              |
| GalNAc      | 3.775, 3.824              | 3.655, 3.977              | 2.70              |
| GalNAc      | 1.906                    | 1.879                    | -0.95             |
| Gal         | 4.643                    | 4.449                    | -0.10             |
| Gal         | 3.516                    | 3.516                    | 0.10              |
| Gal         | 4.043                    | 4.038                    | 0.06              |
| Gal         | 3.920                    | 3.914                    | 0.13              |
| Gal         | 3.608                    | 3.603                    | 0.04              |
| Gal         | 3.70, 3.70                | 3.691, 3.724              | 0.16              |
| Neu5Ac      | 1.762, 2.740              | 1.777, 2.742              | -0.08             |
| Neu5Ac      | 3.666                    | 3.663                    | 0.10              |
| Neu5Ac      | 3.833                    | 3.834                    | 0.29              |
| Neu5Ac      | 3.604                    | 3.605                    | 0.06              |
| Neu5Ac      | 3.584                    | 3.586                    | 0.22              |
| Neu5Ac      | 3.850                    | 3.851                    | 0.06              |
| Neu5Ac      | 3.628, 3.834              | 3.631, 3.836              | 0.14              |
| Neu5Ac      | 2.024                    | 2.024                    | 0.20              |
| Neu5Ac      | 3.70, 3.70                | 3.691, 3.724              | 0.16              |
| Neu5Ac      | 1.718, 2.728              | 42.91                    |
| Neu5Ac      | 3.692                    | 70.87                    |
| Neu5Ac      | 3.845                    | 54.41                    |
| Neu5Ac      | 3.733                    | 75.24                    |
| Neu5Ac      | 3.586                    | 70.78                    |
| Neu5Ac      | 3.913                    | 74.33                    |
| Neu5Ac      | 3.652, 3.899              | 65.27                    |
| Neu5Ac      | 2.042                    | 24.69                    |
| Bn          | 4.691, 4.899              | 4.688, 4.894              | 73.86              |

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**Fig. 4.** Two-dimensional HSQC spectra of the disialyl tetrasaccharide. Portions of the two-dimensional 1H,13C HSQC spectrum of the STY disialyl tetrasaccharide, recorded at 600 MHz in D2O and pH 6.7. The CH2 connectivities are marked.
the trisaccharide were assigned. By default, the Neu5Ac C-9 signals were identified as the remaining CH₂ signals in the 13C spectra. These CH₂ signals are displayed in Table IV.

Thus, it became obvious that the crucial difference between the 13C NMR spectra of the tri- and tetrasaccharide was the chemical shift increment of ΔC₂ = 2.7 ppm shown by the GalNAc C-6 signal, which is typical for glycosylation at that position. Additionally, the C-5 of the GalNAc unit is shifted 1.5 ppm, which is characteristic of glycosylation at the C-6 position of GalNAc(69). No other 13C signals in the spectrum of the acceptor trisaccharide underwent a similar chemical shift increment (see Table I). It was deduced that the newly introduced sialic acid residue in the tetrasaccharide is attached to the C-6 position of GalNAc. Therefore, the product disialoside formed by the acceptor trisaccharide underwent a similar chemical shift increment (see Table III). The signals were identified as the remaining CH₂ signals in the 13C spectra. These CH₂ signals are displayed in Table IV.

Expression of ST6GalNAc III in Adult and Newborn Tissues—In order to determine the pattern of expression and message size of the ST6GalNAc III gene, Northern blots with mRNA from various adult and newborn tissues were probed with a probe for ST6GalNAc III, as described under “Experimental Procedure.” The acceptor specificity of ST6GalNAc III most closely resembles sialytransferase activity described in fetal liver by Bergh et al. (66) and in adult rat brain by Baubichon-Cortay et al. (67). Like ST6GalNAc III, both of these tissues express a 2,6-GalNAc sialyltransferase activity that utilizes the Neu5Ac₂2,3Galβ1,3GalNAc₁,0-Thr/Ser glycoconjugate as an acceptor but not its asialo derivative.

The most striking enzymatic difference between ST6GalNAc III and other members of the N-acetylgalactosaminide 2,6-sialyltransferase family is that ST6GalNAc III is the only sialyltransferase doted to date capable of forming the developmentally regulated ganglioside G_{D₁} from G_{M₁} and G₁₁ do not utilize G_{M₁₂} as an acceptor (17). Thus ST6GalNAc I and II transfer sialic acid to α-linked GalNAc (GalNAc₁,0-Thr/Ser) but not β-linked GalNAc such as is found in G₁₁. Unlike G₁₁, they are not acceptors for ST6GalNAc III. The only difference between G₁₁ and G₁₂ is a sialic acid residue linked to the internal galactose of G₁₂. Thus this sialic acid residue abolishes the catalytic activity of ST6GalNAc III perhaps by sterically hindering the access of the acceptor to the C-6 position of GalNAc. It has recently been shown that an α,2,6-GalNAc sialyltransferase activity exists in rat liver that utilizes G₁₁ and G₁₂ as acceptors forming respectively G₁₁ and G₁₂. Since ST6-

### TABLE IV

| Pertinent C¹³ NMR CH₂ chemical shifts |
|--------------------------------------|
| CH₂ group | Chemical shift in |
| --- | --- |
| Trisaccharide | Tetrasaccharide |
| GalNAc C-6 | 63.41 | 66.11 |
| Gal C-6 | 63.40 | 63.56 |
| Neu5Ac₂(2,3) C-9 | 64.94 | 65.08 |
| Second Neu5Ac | 65.27 |

**Fig. 5.** Differential expression of ST6GalNAc III in various rat tissues. Northern blots with mRNA from various adult and newborn rat tissues were hybridized with a probe for ST6GalNAc III, as described under “Experimental Procedures.”

### TABLE V

| Glycoconjugate acceptor specificities of the three cloned ST6GalNAc sialyltransferases |
|-----------------------------------------|
| Acceptor | ST6GalNAc I | ST6GalNAc II | ST6GalNAc III |
| --- | --- | --- | --- |
| GalNAc₁,0-Thr/Ser | + | – | – |
| Galβ₁,3GalNAc₁,0-Thr/Ser | + | + | – |
| Neu5Ac₂,3Galβ₁,3GalNAc₁,0-Thr/Ser | + | + | + |
| G₁₁ | – | – | – |

### DISCUSSION

With the isolation of ST6GalNAc III cDNA, 12 enzymatically distinct sialyltransferases have been isolated. Three of these, including ST6GalNAc III, encode N-acetylgalactosaminide 2,6-sialyltransferases (16, 17). As summarized in Table V, each ST6GalNAc sialyltransferase utilizes the Neu5Ac₂2,3Galβ₁,3GalNAc₁,0-Thr/Ser glycoconjugate as an acceptor. However the acceptor specificity of ST6GalNAc III is considerably more restricted than that of ST6GalNAc I and II. While ST6GalNAc I and II utilize various asialo O-linked structures as acceptors, ST6GalNAc III displays an absolute requirement for the sialylated structure (16, 17). Indeed, the only acceptor for ST6GalNAc III to be identified in the current study is Neu5Ac₂2,3Galβ₁,3GalNAc both in its free oligosaccharide form and as attached to glycoconjugates. The next best oligosaccharide acceptor, LSTa (see Table II for structure) has an incorporation rate of only 3% of that of Neu5Ac₂2,3Galβ₁,3GalNAc, suggesting that transfer to glycoconjugates carrying the LSTa sequence is unlikely to be of physiological relevance. The acceptor specificity of ST6GalNAc III most closely resembles sialytransferase activity described in fetal liver by Bergh et al. (66) and in adult rat brain by Baubichon-Cortay et al. (67). Like ST6GalNAc III, both of these tissues express a α,2,6-GalNAc sialytransferase activity that utilizes the Neu5Ac₂2,3Galβ₁,3GalNAc₁,0-Thr/Ser sialoside of fetuin as an acceptor but not its asialo derivative.

3. S. Tsuji, personal communication.
GalNAc III does not utilize Galβ1,4Glc as acceptors, it is clear that another ST6GalNAc sialyltransferase exists in addition to the three cDNAs already isolated. At this point, it is unclear if gangliosides such as G3H, are acceptors for ST6GalNAc III (39).

Previously, sialosides generated by novel cDNAs relied on indirect methods such as sialidase treatments for linkage analysis. In the current studies, we were unable to confidently characterize the sialoside generated by ST6GalNAc III with strictly sialidase treatments, forcing us to synthesize and purify quantities of sialoside sufficient for NMR analysis. NMR analysis confirmed that the acceptor monosialoside utilized in these studies was Neu5Acα2,3Galβ1,3GalNAcβ1,0-benzyl. Furthermore, the product tetrasaccharide was identified by a combination of one-dimensional and two-dimensional 1H and 13C NMR experiments as Neu5Acα2,3Galβ1,3(3 Neu5Acα2,6)GalNAcβ1,0-benzyl.

Careful comparison of the HSQC spectra of the tri- and tetrasaccharide revealed that one of the CH2 signals in the 13C spectrum of the trisaccharide had undergone a chemical shift increment typical for glycosylation at that site. That CH2 group was attributed to GalNAc based on an HMBC-TOCSY experiment. Thus, the linkage position of the second Neu5Ac residue was identified, not as would usually be the case) by an HMBC experiment (as Neu5Ac does not have an anomeric proton), but by a 13C-edited TOCSY experiment. Synthesis of quantities of ST6GalNAc III sialoside for these experiments was made possible by employing an expression vector in which the entire open reading frame of ST6GalNAc III was placed under control of the cytomegalovirus promoter. Stable transfection of this construct into 293 cells yielded remarkably high levels of ST6GalNAc III sialyltransferase activity that allowed for the enzymatic synthesis of milligram quantities of the ST6GalNAc III sialoside using only detergent lysates as an enzyme source. Indeed, levels of ST6GalNAc III were high enough to render endogenous sialidase and sialyltransferase activities insignificant relative to the recombinant sialyltransferase activity. This expression system may be of future utility for expression of sialyltransferases particularly when relatively high levels of enzyme activity are required.

In certain instances, sialyltransferases share sequence identity outside the sialylmotifs, greatly enhancing the probability that they form identical linkages. This is apparent when the sequences of four α2,8-sialyltransferase cDNAs are compared with one another. Throughout their open reading frames, they share a 60–28% identity, with the closest identity occurring between ST6Sia IV and ST6Sia II (19, 21, 42). Two distinct ST6GalNAc transfers (ST6GalNAc I and II) differing slightly in their substrate specificity share 32% sequence identity throughout their coding region. The identity increases to 48% when the sequences are compared from the beginning to the end of their coding region. The identity increases to 48% when the sequences are compared from their respective open reading frames, they
development of milligram quantities of the ST6GalNAc III sialoside using only detergent lysates as an enzyme source. Indeed, levels of ST6GalNAc III were high enough to render endogenous sialidase and sialyltransferase activities insignificant relative to the recombinant sialyltransferase activity. This expression system may be of future utility for expression of sialyltransferases particularly when relatively high levels of enzyme activity are required.

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Molecular Cloning of a Developmentally Regulated N-Acetylgalactosamine 2,6-Sialyltransferase Specific for Sialylated Glycoconjugates
Eric R. Sjoberg, Hiroshi Kitagawa, John Glushka, Herman van Halbeek and James C. Paulson

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