Expression Cloning of a Human G\textsubscript{T3} Synthase

G\textsubscript{D3} AND G\textsubscript{T3} ARE SYNTHESIZED BY A SINGLE ENZYMEN*

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Gangliosides of the C series such as G\textsubscript{T3} are polysialylated glycosphingolipids whose synthesis is developmentally regulated. Here we report the expression cDNA cloning and characterization of G\textsubscript{T3} synthase that adds the second α-2,8-sialic acid to G\textsubscript{D3}. NeurNAc\textsubscript{2}→8NeurNAc\textsubscript{2}→3Gal\textsubscript{1}→4Glc→Cer, thus forming G\textsubscript{G3}→3NeurNAc\textsubscript{2}→8NeurNAc\textsubscript{2}→3Gal\textsubscript{1}→4Glc→Cer. Unexpectedly, the cloned cDNA was found to be identical to the cDNA that encodes G\textsubscript{D3} synthase. The newly identified enzyme was therefore named G\textsubscript{D3}/G\textsubscript{T3} synthase (G\textsubscript{D3}/G\textsubscript{T3}ST). G\textsubscript{D3}/G\textsubscript{T3}ST synthesized G\textsubscript{T3} most efficiently when G\textsubscript{M3} NeuNAc\textsubscript{2}→3Gal\textsubscript{1}→4Glc→Cer, was incubated as an acceptor, indicating that G\textsubscript{D3}/G\textsubscript{T3}ST is a polysialyltransferase that can transfer more than one sialic acid residue via α-2,8 linkage to gangliosides. Moreover, a longer period of incubation of G\textsubscript{D3} with G\textsubscript{D3}/G\textsubscript{T3}ST produced a significant amount of G\textsubscript{T3} and higher polysialogangliosides. Among various cell lines expressing G\textsubscript{D3}/G\textsubscript{T3}ST, higher polysialogangliosides including G\textsubscript{T3} were detected only in cell lines where the amount of G\textsubscript{D3}/G\textsubscript{T3} mRNA is sufficiently high. The expression of G\textsubscript{D3}/G\textsubscript{T3}ST mRNA among human tissues is highly restricted to fetal and adult brains. The G\textsubscript{D3}/G\textsubscript{T3}ST gene was found to be located at chromosome 12, region p12. Taken together, these results indicate that C series polysialogangliosides are synthesized by a ganglioside-specific polysialyltransferase, G\textsubscript{D3}/G\textsubscript{T3}ST, that is specifically expressed in neural tissues.

Glycoconjugates are major components of the plasma membrane of mammalian cells, and their carbohydrate structures change dramatically during development. Specific sets of carbohydrates are expressed in different stages of differentiation, and many of these carbohydrates are recognized by specific antibodies, thus providing differentiation antigens (Feizi, 1985; Fukuda, 1985). During the course of development, expression of distinct carbohydrates is eventually restricted to specific cell types, and aberrations in these cell surface carbohydrates are frequently observed in malignant cells (Hakomori, 1984). The functional significance of these cell type-specific carbohydrates and their alterations in malignancy is not well understood, although various reports suggest that some of these carbohydrates are involved in cell adhesion processes (Fukuda, 1992; Lowe, 1994).

Among gangliosides, gangliosides comprise a structurally diverse set of sialylated species and are enriched in nervous tissues. Gangliosides have been found to act as receptors for growth factors, toxins, and viruses and are apparently involved in cell adhesion. For example, cholera toxin binds to GM\textsubscript{3}, Gal\textsubscript{1}→3GalNAc\textsubscript{1}→4(NeuNAc\textsubscript{2}→3)Gal\textsubscript{1}→4Glcl\textsubscript{1}→Cer, before its entry into cells (Spiegel and Fishman, 1987). Influenza A virus binds to sialylparagloboside, NeuNAc\textsubscript{2}→3Gal\textsubscript{1}→4Glcl\textsubscript{1}→3Gal\textsubscript{1}→4Glcl\textsubscript{1}→Cer (Higa et al., 1985; Suzuki et al., 1986). In addition, there have been reports suggesting that gangliosides, G\textsubscript{D3}, in particular (see Fig. 1 for its structure) may play roles in cell-cell interaction. Cheshire et al. (1986) found that G\textsubscript{D3} and G\textsubscript{T3} facilitate the attachment of human melanoma and neuroblastoma cells to extracellular matrix proteins. Epithelial-mesenchymal interactions in embryonic kidney formation were perturbed by anti-G\textsubscript{D3} antibody, which reacted with G\textsubscript{D3} on the mesenchymal cells (Sariola et al., 1988). Gangliosides also modulate enzymatic activities. For example, G\textsubscript{M3} was found to inhibit epidermal growth factor receptor-mediated phosphorylation (Bremer et al., 1986), and G\textsubscript{O2a}b was shown to inhibit ADP-ribosyltransferases (Hara-Yokoyama et al., 1995).

Among gangliosides, increasing attention has been directed to the so-called C series polysialogangliosides, which have unique trisialyl residues, NeuNAc\textsubscript{2}→8NeurNAc\textsubscript{2}→8NeurNAc\textsubscript{2}→3Gal→R (Fig. 1). C series polysialogangliosides were found to be major constituents in adult fish brain. In higher vertebrates the C series polysialogangliosides comprise a minor proportion of total gangliosides present in the brain (Ando and Yu, 1979). However, a substantial amount of C series polysialogangliosides are present in fetal brain of higher vertebrates including human. They are also found in various neuroectodermal tumors, such as melanoma and glioma (Yates, 1988; Nakayama et al., 1993). In the early stages of neural development, G\textsubscript{D3} is predominantly expressed in the neural tube that consists of progenitor cells for neurons and macroglial cells. During the later stage of development, progenitor cells migrate and extend processes and finally differentiate to postmitotic neurons. In this developmental period, G\textsubscript{D3} decreases, and C series polysialogangliosides, such as G\textsubscript{T3}, increase (Rösner et al., 1985).

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBank®/EMBL Data Bank with accession number(s) L43494.

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1 The abbreviations for gangliosides are according to Svennerholm nomenclature (Svennerholm, 1964). The abbreviations used are: FITC, fluorescein isothiocyanate; FACS, fluorescence-activated cell sorting; PCR, polymerase chain reaction; HPTLC, high performance thin-layer chromatography; N-CAM, neural cell adhesion molecule; FISH, fluorescence in situ hybridization.
It has been generally accepted that each glycosyltransferase involved in the synthesis of gangliosides transfers only one sugar residue to form a specific linkage (Pohlentz et al., 1988) (Fig. 1). Until recently, the studies of C series polysialogangliosides have been limited to their structural analysis, since the enzyme responsible for GT3 synthesis (STII) was not purified or cloned. In order to understand the roles and synthesis of C series polysialogangliosides, it is critical to isolate a cDNA done of STII that forms GT3.

In this report, we describe the cloning of cDNA encoding GT3 synthase, STII, using a mammalian expression cloning with a newly devised modification. Surprisingly, the newly isolated cDNA was found to be identical to that of G0ST3 synthase, STII. By transfecting the newly isolated cDNA into HeLa and MeWo cells and assaying the activity of the soluble form of the enzyme, we demonstrated that a single enzyme encoded by the isolated cDNA forms both G0ST3 and GT3. We also found that GT3 synthase transcripts are expressed exclusively in neural tissues. The present study, STII and STIII were found to be the same enzyme in the present study, and STVI is probably the same enzyme as STII.

Two clones, named HeLa-62 and MeWo-62, were screened by immunofluorescent staining with M6704 antibody, and two clones, HeLa and MeWo, were established. Plasmid DNAs were rescued from the positive cells (Hirt, 1967) and transformed into the host E. coli JM107 (Ohta et al., 1993) and into COS-1 cells by electroporation (Cell-Porator, Life Technologies, Inc.). The transformed cells were placed into 20 plates, each containing about 500 colonies. Plasmid DNAs prepared from each plate were separately used for transfection by Lipofectamine into HeLa cells, and the transfected HeLa cells were examined by immunofluorescence staining using M6703 antibody. Sibling selection with sequentially smaller active pools identified a single plasmid, pcDNA-GT3ST, that determined the expression of G0ST3 at the cell surface.

Ganglioside-specific Polysialyltransferases

Antibodies—Monoclonal antibodies M6703 and M6704 were shown to react with G0ST3 and G0ST4, were established. Monoclonal antibodies R24 (anti-G0ST3), KM641 (anti-G0ST4), or M6703 antibody, followed by FITC-conjugated (Fab')2 fragment of goat anti-mouse IgG (Cappel) and thymus melanoma HeLa cells were examined by immunofluorescence staining using M6703 antibody. Sibling selection with sequentially smaller active pools identified a single plasmid, pcDNA-GT3ST, that determined the expression of G0ST3 at the cell surface.

Microfluorescence Microscopy—Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline and stained with mouse monoclonal antibodies R24 (anti-G0ST3), KM641 (anti-G0ST4), or M6703 (anti-G0ST3), followed by FITC-conjugated (Fab')2 fragment of goat anti-mouse IgG (Cappel). The cells were then examined under a Zeiss Axiophot microscope, as described previously (Williams and Fukuda, 1990).

Construction of a Truncated Form of G0ST/GT3ST—The cDNA encoding a truncated form of G0ST/GT3ST was prepared by polymerase chain reaction (PCR) using pcDNA1-GT3ST as a template. Upstream and downstream primers used were 5'-tagggggcGAGGGGCC-3' (HindIII site shown by underline) and 5'-taggggcGAGGGGCC-3' (NotI site shown by underline), respectively. The PCR product encompassed the sequence from nucleotide 38 to nucleotide 1,080. The nucleotide 38 is 8 nucleotides upstream from the second initiation methionine, and the nucleotide 1080 resides 9 nucleotides downstream from the stop codon. PCR was performed in a final volume of 100 μl using the primers (0.5 pmol each) for 30 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min. The amplified DNA fragment was digested with HindIII and NotI and cloned into the same sites of pcDNA1.

Establishment of Stable Transfectants Expressing G0ST/GT3ST—The two clones, named HeLa-G0ST3 and MeWo-G0ST4, were established. Thin-layer Chromatography of Glycospingolipids—Analytical thin-layer chromatography was carried out on precoated high performance thin-layer chromatography (HPTLC) plates (Si-HPF, J. T. Baker, Inc., Phillipsburg, N.J.). The solvent systems used were chloroform, methanol, and water, 1:1:2 by volume. The purified gangliosides were visualized by resorcinol/ HCl reagent.

A total of gangliosides from cells and TCL-immunostaining were performed as described previously (Hirabayashi et al., 1988). The purified gangliosides were applied onto a plastic plate (Poligram Sil G, Nagel, Doren, Germany) and developed under the same conditions as described above. The plate was subjected to immunostaining with R24 or M6703 antibody, followed by peroxidase-conjugated goat anti-mouse IgG antibody (Cappel). The peroxidase activity was visualized with 4-chloro-1-naphthol/H2O2.

In Vivo Sialyltransferase Assays and Product Characterization—The expression vector for protein A-G0ST/GT3ST fusion protein was constructed using pMA0 vector as described (Sasaki et al., 1994b). The cDNA in this pMA0-G0ST3 was excised by Sall and Asp718, filled in by the Klenow fragment, and subcloned into the EcoRI site of pcDNA1, resulting in pcDNA-proA-G0ST3. After confirming the correct orientation by sequencing, pcDNA-proA-G0ST3, or pPROTA (Kukawsk-Latallo et al., 1999) as a control was transfected into COS-1 cells. The protein A-G0ST3 cell dissociation solution (Specialty Media, Lavalliette, NJ). The detached cells were pooled and resuspended in cold phosphate-buffered saline (pH 7.4) containing 1% bovine serum albumin and were reacted with mouse monoclonal antibody M6703 at 1:200 dilution. After a 30-min incubation on ice, the cells were washed, and then fluorescein isothiocyanate (FITC)-conjugated (Fab')2 fragment of goat anti-mouse IgG (Cappel, Durham, NC) was added. After a 30-min incubation, the cells were washed and subjected to fluorescence-activated cell sorting (FACS) using FACStar (Becton-Dickinson, San Jose, CA). The sorting region was set where only strongly positive COS-1 cells were recovered. Plasmid DNAs were rescued from the positive cells (Hirt, 1967) and transformed into the host Esherichia coli MC1061/p3 cells by electroporation using Cell-Porator (Life Technologies, Inc.). The transformed cells were plated onto 20 plates, each containing about 500 colonies. Plasmid DNAs prepared from each plate were separately used for transfection by lipofectamine into HeLa cells, and the transfected HeLa cells were examined by immunofluorescent staining using M6703 antibody. Sibling selection with sequentially smaller active pools identified a single plasmid, pcDNA-GT3ST, that determined the expression of G0ST3 at the cell surface.
G\textsubscript{T2}ST fusion protein secreted into the culture medium was adsorbed to equilibrated IgG-Sepharose 6FF (Pharmacia Biotech Inc.) containing 0.05% Tween 20, washed nine times with 50 mM Tris-HCl buffer, pH 7.5, containing 1% bovine serum albumin and then two times with a 50 mM Tris-HCl buffer, pH 7.5, containing 5 mM CaCl\textsubscript{2} and 0.05% Tween 20, and finally suspended in Dulbecco's modified Eagle medium containing 10% fetal calf serum, as detailed previously (Kukowska-Latallo et al., 1990; Bierhuizen and Fukuda, 1992).

Sialyltransferase activity was measured as described previously (Sasaki et al., 1994a), after a 3-min sonication of 25 \mu l of 0.1 M sodium cacodylate buffer (pH 6.0) containing 20 \mu M MgCl\textsubscript{2}, 1% Triton CF-54, 2.4 mmoI of CMP-[\textsuperscript{3}H]NeuNAc, and 10 \mu g of a substrate with or without a competing substrate, 25 \mu l of the enzyme solution was added and incubated for 4, 12, or 24 h at 37 °C. At the end of the incubation period, 200 \mu l of phosphate-buffered saline was added to the incubation mixture, and the contents were applied to an Aspec Pak C18 cartridge (M & S, Tokyo, Japan), according to the procedure described (Williams and McEl broaden, 1980). After washing the column with water, glycosphin-golipids were eluted with 3 ml of chloroform-methanol (2:1 by volume). The sample was dried under nitrogen stream and then subjected to chromatography using an HPTLC plate under the same conditions as described above. Radioactive materials were visualized by fluorography after spraying an autoradiography enhancer (DuPont NEN). Standard and acceptor gangliosides were visualized by the resorcinol/HCl method.

Quantitation of G\textsubscript{D3}/G\textsubscript{T2}ST Transcripts Using Competitive PCR—The level of G\textsubscript{D3}/G\textsubscript{T2}ST transcript was measured by the competitive PCR using the CDNs, which were prepared by reverse transcription of total RNA, as detailed in the previous report (Sasaki et al., 1994a). For distinction of a target cDNA from its competitor DNA, G\textsubscript{D3}/G\textsubscript{T2}ST cDNA was truncated by deleting a 125-base pair EcoRI-2-Pull fragment of the cDNA from pUC-GD3SR (Sasaki et al., 1994b). The S' and 3' primers were S'-ACAGTATTACATCTACGCTGCT-3' and 5'-CATGAAAACACCTTTGACCATTCCCCT-3', respectively. The amount of amplified CDNs was calculated from the respective standard curves, converted into the values of molar numbers. As a control, the \beta-actin transcript was measured in the same CDN samples.

Northern Blot Analysis of Various Human Tissues—Poly(A)\textsuperscript{+} RNA from human fetal (19–23 gestational weeks) and adult brains purchased from Clontech (Palo Alto, CA) were electrophoresed in a 1.2% agarose gel containing 2.2 M formaldehyde and transferred to a nylon membrane filter (Micron Separation, Westboro, MA). Human multiple-tissue Northern blots of poly(A)\textsuperscript{+} RNA were purchased from Clontech, and these blots were hybridized with a gel-purified cDNA insert of pcDNAI-G\textsubscript{T2}ST after labeling with [\textsuperscript{32}P]dCTP by random oligonucleotide priming (Feinberg and Vogelstein, 1983) (Prime-It II labeling kit, Stratagene, San Diego, CA).

Fluorescence in Situ Hybridization Analysis of G\textsubscript{T2}ST Gene—Human genomic P1 plasmid library was screened by CRC as described (Onda and Fukuda, 1995). The S' and 3' primers for PCR correspond to the sequence of the nucleotides 1184–1203 and that of nucleotides 1424–1443 of the G\textsubscript{T2}ST sequence (see Sasaki et al., 1994b).

Purified DNA from one of the isolated P1 clones, clone 5459, was labeled with digoxigenin-DUTP by nick translation. Labeled probe was combined with sheared human DNA and hybridized to normal metaphase chromosomes derived from phytheramaglutinin-stimulated peripheral blood lymphocytes in a solution containing 50% formamide, 10% dextran sulfate and 2 × SSC. Specific hybridization signals were detected by incubating the hybridized slides in FITC-labeled anti-digoxigenin antibody followed by counterstaining with propidium iodide for one color experiment. Probe detection for two-color experiments was accomplished by cohybridizing the slides with a biotin-labeled probe, D1221-specific for centromere of chromosome 12 and the digoxigenin-labeled clone 5459. After incubating these slides with Texas Red-labeled avidin and FITC-labeled anti-digoxigenin antibody, they were counterstained with 4',6-diamidino-2-phenylindole (Rouquier et al., 1995).

RESULTS

Isolation of a cDNA Clone Encoding G\textsubscript{T3} Synthase—In order to encode G\textsubscript{T3} synthase, it was necessary to employ cells expressing a precursor ganglioside G\textsubscript{D3} that lacked G\textsubscript{T3} itself as recipients for transfection. The parent COS-1 cells did not react with M6703 (anti-G\textsubscript{T3}) or R24 (anti-G\textsubscript{D3}) monoclonal antibodies, indicating that G\textsubscript{D3} and G\textsubscript{T3} are not synthesized by COS-1 cells. Therefore, we transfected COS-1 cells with pHMa-\textsubscript{G\textsubscript{T3}} that harbors CDNAs encoding the G\textsubscript{D3} synthase and G418 resistance gene (Sasaki et al., 1994b), and isolated COS-1-G\textsubscript{D3} cells that were strongly stained by R24.

When the COS-1-G\textsubscript{D3} cells were tested for the presence of G\textsubscript{T3} by M6703 antibody, however, 3.5% of COS-1-G\textsubscript{D3} cells showed a strong positive signal for G\textsubscript{T3} judged in FACS analysis. We thus isolated COS-1-G\textsubscript{T3} cells, which barely reacted with M6703 antibody by FACS. The freshly sorted COS-1-G\textsubscript{T3} cells expressed only G\textsubscript{D3} and were expanded once up to 1.2 × 10\textsuperscript{6} cells in culture. Although a few of them still expressed G\textsubscript{T3} (Fig. 2C), they were used as recipient cells for expression cloning of G\textsubscript{T3} synthase.

COS-1-G\textsubscript{T3} cells were transfected with the SK-MEL-28 CDN library in pcDNAI. Sixty-two h after transfection, COS-1-G\textsubscript{T3} cells expressing G\textsubscript{T3} were enriched by FACS using a 1 × 10\textsuperscript{6} COS-1-G\textsubscript{T3} cells applied, 404 cells were sorted. Plasmid CDNs were rescued from these M6703-positive cells.

When COS-1-G\textsubscript{D3} cells were transiently transfected with a mixture of the above isolated plasmids, it was not possible to distinguish the cells that newly became G\textsubscript{T3}-positive from the cells that were endogenously G\textsubscript{T3}-positive by immunofluorescent staining with M6703 antibody. We reasoned that this failure was due to the high background expression of G\textsubscript{T3} in COS-1-G\textsubscript{D3} cells (see Fig. 2C). In order to overcome this problem, the plasmid CDNs were transfected into HeLa cells. The wild-type HeLa cells expressed detectable amounts of G\textsubscript{D3} as judged by immunofluorescent staining using another anti-G\textsubscript{D3} antibody, KM641 (Ohta et al., 1993) but were completely neg-
The transformed bacteria obtained after the Hirt procedure were thus divided into 20 pools, and the plasmid DNA from each pool was transfected separately into HeLa cells. The transfecants were screened by immunofluorescent staining using antibody M6703. Because of no background staining for M6703 in HeLa cells, we could identify two out of 20 plasmid pools that directed the expression of GT3 in HeLa cells. One of the plasmid pools, which produced strongly positive cell staining by M6703, was clearly seen on both COS-1 and HeLa cells (Fig. 2, A and J). Such a strong cell staining by M6703 was not seen on COS-1 cells transfected with pcDNAI-GD3/GT3ST, which produced a truncated GT3 (Fig. 2, B). These results indicate that GD3 synthase (STII) and GT3 synthase (STIII) are the same enzyme and suggest a possibility that a single enzyme catalyzes the reactions for the formation of disialosyl and trisialosyl groups. The newly identified enzyme is thus called GD3/GT3 synthase or GD3/GT3ST hereafter.

The above cDNA sequence shows a second initiation methionine, which resides 16 codons from the first initiation methionine. We synthesized the shorter cDNA encoding nucleotides 37–1,080 of the cDNA sequence by PCR, allowing the translation initiation only from the second initiation methionine (Fig. 3A). This truncated cDNA encoding GD3/GT3ST-S (Fig. 3B) was cloned into pcDNAI and expressed in both COS-1 and HeLa cells. The results obtained by immunofluorescent staining of the transfected cells clearly indicate that this truncated cDNA is also capable of GD3 and GT3 expression (data not shown). In fact, the nucleotide sequence surrounding the second initiation methionine, GCCATGG, is consistent with the consensus sequence, (A/G)CCATG, for optimal translation initiation (Kozak, 1991). In contrast, the nucleotide sequence surrounding the first methionine, GCCATGA, does not conform with the Kozak sequence. Moreover, the size of the cytoplasmic sequence in this shorter translated product is reasonably short (12 residues), which is characteristic for all glycosyltransferases cloned into pcDNAI promoter. The open reading frame in the sense orientation with respect to the pcDNAI promoter is consistent with the translated amino acid sequence shown below the nucleotide sequence. The translation initiation methionines are at residues 1 and 16, and they are shown in boldface type. The putative transmembrane/anchor domain is underlined by a solid line. The translation product starting from the second methionine is underlined by a dotted line. The rest of the sequence is shown by Sasaki et al. (1994b). The schematic representation of two GD3/GT3ST translation products and pro-GD3/GT3ST dimeric protein are shown. GD3/GT3ST and GD3/GT3ST-S start the translation in residues 1 and 16 shown in A and J, respectively. The cytoplasmic (Cyt), transmembrane/anchor (TM), tentative stem (Stem), and catalytic (Cata) domains are shown. Sialyloligosaccharide I (residues 137–182) and S (residues 273–295) are shown by cross-hatched boxes. The signal peptide sequence (Sig) of the human granulocyte colony-stimulating factor and IgG-binding domain of S. aureus protein A (ProA) was fused with a catalytic domain of GD3/GT3ST. The catalytic domain encompasses residue 57 (shown by the arrow in A) to the COOH terminus of the GD3/GT3ST.

The translated amino acid sequence is shown below the nucleotide sequence. The translation initiation methionines are at residues 1 and 16, and they are shown in boldface type. The putative transmembrane/anchor domain is underlined by a solid line. The translation product starting from the second methionine is underlined by a dotted line. The rest of the sequence is shown by Sasaki et al. (1994b). The schematic representation of two GD3/GT3ST translation products and pro-GD3/GT3ST dimeric protein are shown. GD3/GT3ST and GD3/GT3ST-S start the translation in residues 1 and 16 shown in A and J, respectively. The cytoplasmic (Cyt), transmembrane/anchor (TM), tentative stem (Stem), and catalytic (Cata) domains are shown. Sialyloligosaccharide I (residues 137–182) and S (residues 273–295) are shown by cross-hatched boxes. The signal peptide sequence (Sig) of the human granulocyte colony-stimulating factor and IgG-binding domain of S. aureus protein A (ProA) was fused with a catalytic domain of GD3/GT3ST. The catalytic domain encompasses residue 57 (shown by the arrow in A) to the COOH terminus of the GD3/GT3ST.
size $G_T$ even though a substantial amount of $G_D$ is synthesized (Fig. 4, E and F), the expression of $G_T$ in the MeWo cells should be solely due to the newly introduced $G_D/G_T$ ST cDNA, but not the accumulation of newly synthesized $G_D$. On the other hand, the enhanced expression of $G_D$ and the new synthesis of $G_T$ in the HeLa-$G_T$ cells were due to the newly introduced $G_D/G_T$ ST cDNA.

In order to confirm that the transfected cells synthesize both $G_D$ and $G_T$ gangliosides were isolated from the parent HeLa and MeWo cells and their stable transfectants. The thin-layer chromatogram of the gangliosides, detected by resorcinol reaction which reacts with sialic acid, showed that the HeLa-$G_T$ cells contained both $G_D$ and $G_T$, whereas the parent HeLa cells contained no $G_T$ (Fig. 4, lanes 1 and 2). Similarly, the MeWo-$G_T$ cells contained $G_T$ whereas the parent MeWo cells did not contain $G_T$ (see lanes 3 and 4 in Fig. 4I). These results were confirmed by immunostaining of newly synthesized gangliosides after separation by thin-layer chromatography. The parent HeLa cells express a very small amount of $G_T$, but HeLa-$G_T$ cells express a substantially increased amount of $G_D$ detected by R24 antibody (Fig. 4J, lanes 1 and 2). The parent MeWo cells, on the other hand, express a significant amount of $G_T$ (Fig. 4J, lane 3), but no $G_T$ was detected by M6703 antibody (Fig. 4K, lane 4). In contrast, both HeLa-$G_T$ and MeWo-$G_T$ cells express a large amount of $G_T$ (Fig. 4L, lanes 2 and 4). These results, taken together, clearly indicate that $G_D/G_T$ ST transfers an $\alpha$-2,8-linked sialic acid to $G_M$ and $G_D$, forming $G_D$ and $G_T$, respectively. The immunofluorescent stainings of HeLa-$G_T$ and MeWo-$G_T$ cells by M6703 were completely abolished by pretreatment of chloroform-methanol (2:1) extraction (data not shown), indicating that all of the newly formed trisialosyl groups are attached to glycosphingolipids. If some of them were attached to glycoproteins, some staining should remain because $G_D$ and $G_T$ cells express a variety of cells labeled on the top or standard $G_D/G_T$ ST cDNAs (50, 500, 2,500, and 5,000 fg) were mixed with 500 fg of the competitor cDNA (truncated $G_D/G_T$ ST cDNA) and subjected to 23 cycles of PCR as described under “Experimental Procedures.” The amplified products were separated by electrophoresis in 1.8% agarose gel and visualized by staining with ethidium bromide. Size markers, from the top were 4.3, 1.8, 1.1, 0.68, 0.38, 0.25, and 0.12 kb.

In order to formally prove if $G_D$ synthase also has $G_T$ synthase activity, a putative catalytic domain of this protein was expressed as a protein fused with the IgG-binding domain of Staphylococcus aureus protein A preceded by a signal peptide sequence (Sasaki et al., 1993) (see ProA-$G_D/G_T$ ST in Fig. 3B). The cDNA encoding this chimeric protein was cloned into pcDNA1 and expressed in COS-1 cells. The fusion protein secreted into the culture medium was absorbed to IgG-Sepharose and then incubated with $G_M$ or $G_D$ and the donor substrate CMP-[3H]NeuNAc. As shown in Fig. 5, lane 3, the soluble form of $G_D/G_T$ ST synthesized both $G_D$ and $G_T$ when incubated with $G_M$. These results establish that the newly identified enzyme, $G_D/G_T$ ST, is a polysialyltransferase that adds more than one sialic acid residue in $\alpha$-2,8-linkage.

The above experiments also suggested that $G_D/G_T$ ST added sialic acids much less efficiently when $G_M$ was used as an acceptor (Fig. 5, lane 5). However, the enzyme added sialic acid residues to $G_D$ (136 $\mu$M final concentration) after a longer period of incubation (Fig. 5, lanes 7 and 8). Under these conditions, $G_D/G_T$ ST also synthesized higher polysialogangliosides, which presumably have more than three sialic acid res-
We also tested if the product or an intermediate inhibits the enzymatic reaction as competing substrates. The results shown in Fig. 5 indicate that G2 (680 μM final concentration) inhibits the formation of G3 and G7 from G5 (Fig. 5, lane 10), while G2 (567 μM final concentration) inhibits the formation of G7 and higher polysialylgangliosides from G5 (Fig. 5, lane 11). These results taken together support the above conclusion that G2/G7 ST synthesizes G2 from G5 and then G2/G7 ST utilizes G2 as an acceptor to form G7.

The above results also suggested that the amount of G2/G7 ST mRNA transcript may be proportional to the amount of polysialylated gangliosides synthesized. In order to test this hypothesis, G2/G7 ST transcript was quantitated in the parent HeLa, HeLaG2, parent MeWo, and MeWoG2 cells. Fig. 4L shows that the HeLaG2 cells express a significant amount of the G2/G7 ST transcript (330 fg), while the parent HeLa cells scarcely express it. The MeWoG2 cells express approximately the same amount (370 fg) of the transcript as the HeLaG2 cells and about 15 times more than that in the parent MeWo cells (23 fg). As shown in Fig. 4, I, J, and K, the parent MeWo cells express G2 but barely express G7, while the MeWoG2 and HeLaG2 cells express both G2 and G7. These results clearly indicate that G7 is synthesized only when G2/G7 ST is abundantly present.

G2/G7 ST is expressed in both fetal and adult brains. To determine the tissue distribution of G2/G7 ST mRNA, Northern blots of poly(A) + RNA derived from various human tissues were examined. As shown in Fig. 6, a band of 2.3 kb was detected in the poly(A) + RNA isolated from the fetal and adult brains. The transcript was also detected in fetal lung. In adult tissues, the G2/G7 ST transcripts were detected in brain and very weakly in lung. Among different parts of the adult brain, a substantial amount of G2/G7 ST mRNA was detected inversely in different parts of brain (Fig. 6, right side). In some regions, a band of 9.5 kb was also detected. These two different sizes of the transcript might be produced due to the alternate usage of polyadenylation sites. The expression pattern of G2/G7 ST is different from that of the neural cell adhesion molecule (N-CAM)-specific polysialyltransferase (Nakayama et al., 1995), and G2/G7 ST expression is more restricted to brain.

G2/G7 ST Gene Is Mapped to Chromosome 12p12—The previous studies showed that the G2/G7 ST gene is present in chromosome 12, but no precise chromosomal location of this gene was reported (Sasaki et al., 1994b).

In order to localize precisely the G2/G7 ST gene, we utilized fluorescence in situ hybridization (FISH) procedures. First, P1 plasmid harboring G2/G7 ST gene, named clone 5459, was isolated, and genomic DNA was prepared from this P1 clone. Using this genomic DNA as a probe, the initial experiment resulted in specific labeling of the short arm of a group C chromosome. A second experiment was conducted in which a biotin-labeled probe (D1Z1) specific for the centromere of chromosome 12 was cohybridized with the digoxigenin-labeled clone 5459. This experiment resulted in the specific labeling of the centromere of chromosome 12 in red and the short arm of the same chromosome in green (Fig. 7A). Measurements of 10 specifically hybridized chromosome 12 demonstrated that the clone 5459 is located at a position that is 43% of the distance.

![Fig. 5. In vitro sialyltransferase assay using the protein A-G2/G7 ST chimeric protein.](image)

![Fig. 6. Northern blot analysis of G2/G7 ST in various human tissues.](image)
amino acid on the polymerized sialic acid residues such as colomic acid would be an acceptor for another reaction. Similarly, N-CAM-glycoprotein (polysialyltransferase) and glycosphingolipids linked sialic acid polymer in N-CAM (Eckhardt et al., 1996). These studies, taken together, strongly suggest that the slow migrating band may represent G\textsubscript{D3} shown in Fig. 1. It is possible that G\textsubscript{D3} is present as a very minor component so that it has escaped attention. Further studies are necessary to confirm the presence of this glycosphingolipid.

The present study indicates that the same enzyme is apparently capable of adding all of the $\alpha$-2,8-linked sialic acid, forming disialosyl, trisialosyl, and possibly tetrasialosyl residues in gangliosides. This finding is very similar to those reported for polysialylation of N-CAM. We and others have recently cloned a polysialyltransferase, which is responsible for polysialylation of N-CAM (Eckhardt et al., 1995; Nakayama et al., 1995). Although it has been suggested that the first disialosyl linkage is separated by an initiation enzyme (see Kitazume et al., 1994), the results obtained on mutant Chinese hamster ovary cells lacking polysialylation strongly suggest that polysialylation is catalyzed by single enzymes in both N-CAM glycoprotein (polysialyltransferase) and glycosphingolipids (G\textsubscript{D3}/GT\textsubscript{3}ST).

The present study demonstrated that G\textsubscript{D3}/GT\textsubscript{3}ST synthesizes G\textsubscript{D3} more efficiently from G\textsubscript{M3} than from G\textsubscript{D3}. It is tempting to speculate that G\textsubscript{D3}/GT\textsubscript{3}ST first binds to G\textsubscript{M3} and then continuously adds sialic acid residues until the binding of the enzyme to the product is weakened. In fact, the excess amount of G\textsubscript{D3} inhibited the formation of G\textsubscript{D3} from G\textsubscript{M3}, confirming that G\textsubscript{D3} is an intermediate in the polysialylation. Once the enzyme is released from the enzyme-acceptor complex, it is likely that the enzyme has much less affinity with the product, which would be an acceptor for another reaction. Similarly, N-CAM-specific polysialyltransferase was shown to scarcely add a sialic acid on the polymerized sialic acid residues such as colomic acid (McCoy et al., 1985). These results suggest that termination of polysialylation takes place when the enzyme no longer binds to G\textsubscript{M3}.

\[ 2 \text{Y. Hirabayashi, unpublished results.} \]
the polysialylated acceptor. It is apparent that G_{2y}/G_{T3}ST terminates its reaction early, most likely due to its inefficiency in binding to triosialyl or tetrasialyl residues.

It was shown recently that Neuro2a cells exhibited better neurite extension after the cells were stably transfected to express G_{2y}/G_{T3}ST (Kojima et al., 1994). Although these authors thought that this effect was due to the synthesis of G_{2y} and B series gangliosides, the effect may be due to the synthesis of G_{2y} and C series polysialogangliosides in the transfected Neuro2a cells. We have shown recently that neurite outgrowth in substratum cells is enhanced by the presence of polysialic acid in N-CAM (Nakayama et al., 1995). Previous studies showed that the presence of polysialic acid not only exhibits adhesive properties on homophilic N-CAM interaction but also influences cell-cell interactions carried by other cell surface receptors (Edelman, 1985; Rutishauser et al., 1988; Jessell et al., 1990). Further studies are thus needed to determine if the presence of polysialylated gangliosides influence the cell-cell interaction carried by other adhesive molecules.

Together with previously cloned polysialyltransferase cDNA that forms polysialic acid in glycoproteins, the cDNA doned in the present study will be a powerful tool to dissect the intricate and complex roles of polysialic acids attached to glycoproteins and glycoposphingolipids in cell-cell interactions during development.

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