

Expression Cloning of a Human G_{T3} Synthase

G_{D3} AND G_{T3} ARE SYNTHESIZED BY A SINGLE ENZYME*

(Received for publication, August 16, 1995, and in revised form, November 6, 1995)

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Gangliosides of the C series such as G_{T3} are polysialylated glycosphingolipids whose synthesis is developmentally regulated. Here we report the expression cDNA cloning and characterization of G_{T3} synthase that adds the second α -2,8-sialic acid to G_{D3}, NeuNAc α 2 \rightarrow 8NeuNAc α 2 \rightarrow 3Gal β 1 \rightarrow 4Glc \rightarrow Cer, thus forming G_{T3}, NeuNAc α 2 \rightarrow 8NeuNAc α 2 \rightarrow 8NeuNAc α 2 \rightarrow 3Gal β 1 \rightarrow 4Glc \rightarrow Cer. Unexpectedly, the cloned cDNA was found to be identical to the cDNA that encodes G_{D3} synthase. The newly identified enzyme was therefore named G_{D3}/G_{T3} synthase (G_{D3}/G_{T3}ST). G_{D3}/G_{T3}ST synthesized G_{T3} most efficiently when G_{M3}, NeuNAc α 2 \rightarrow 3Gal β 1 \rightarrow 4Glc \rightarrow Cer, was incubated as an acceptor, indicating that G_{D3}/G_{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_{D3} with G_{D3}/G_{T3}ST produced a significant amount of G_{T3} and higher polysialogangliosides. Among various cell lines expressing G_{D3}/G_{T3}ST, higher polysialogangliosides including G_{T3} were detected only in cell lines where the amount of G_{D3}/G_{T3} mRNA is sufficiently high. The expression of G_{D3}/G_{T3}ST mRNA among human tissues is highly restricted to fetal and adult brains. The G_{D3}/G_{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_{D3}/G_{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 those 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 glycosphingolipids, 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 G_{M1},¹ Gal β 1 \rightarrow 3GalNAc β 1 \rightarrow 4(NeuNAc α 2 \rightarrow 3)Gal β 1 \rightarrow 4Glc β 1 \rightarrow Cer, before its entry into cells (Spiegel and Fishman, 1987). Influenza A virus binds to sialylparagloboside, NeuNAc α 2 \rightarrow 3Gal β 1 \rightarrow 4GlcNAc β 1 \rightarrow 3Gal β 1 \rightarrow 4Glc β 1 \rightarrow Cer (Higa *et al.*, 1985; Suzuki *et al.*, 1986). In addition, there have been reports suggesting that gangliosides, G_{D3} in particular (see Fig. 1 for its structure) may play roles in cell-cell interaction. Cheresch *et al.* (1986) found that G_{D3} and G_{D2} 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_{D3} antibody, which reacted with G_{D3} on the mesenchymal cells (Sariola *et al.*, 1988). Gangliosides also modulate enzymatic activities. For example, G_{M3} was found to inhibit epidermal growth factor receptor-mediated phosphorylation (Bremer *et al.*, 1986), and G_{Q1b} 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 trisialosyl residues, NeuNAc α 2 \rightarrow 8NeuNAc α 2 \rightarrow 8NeuNAc α 2 \rightarrow 3Gal \rightarrow 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_{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_{D3} decreases, and C series polysialogangliosides, such as G_{T3}, increase (Rösner *et al.*, 1985).

* This work was supported by Grants RO1 CA33895 (to M. F.) and DK 37016 (to M. N. F.) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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.

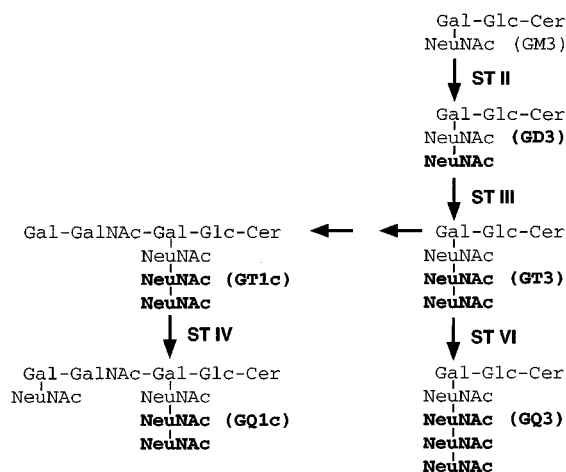


FIG. 1. **Synthetic pathways of C series polysialogangliosides.** α -2,8-sialic acid residues are shown in **boldface type**. The rest of the sialic acid residues are α -2,3-linked. In MeWo cells, the synthesis of G_{T1c} and G_{Q1c} does not take place, since β -1,4-*N*-acetylgalactosaminyltransferase is absent. G_{Q3} and STVI are newly proposed in 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.

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 G_{T3} synthesis (STIII) was not purified or cloned. In order to understand the roles and synthesis of C series polysialogangliosides, it is critical to isolate a cDNA clone of STIII that forms G_{T3} .

In this report, we describe the cloning of cDNA encoding G_{T3} synthase, STIII, using a mammalian expression cloning with a newly devised modification. Surprisingly, the newly isolated cDNA was found to be identical to that of G_{D3} 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 G_{D3} and G_{T3} . We also found that G_{T3} synthase transcripts are expressed exclusively in neural tissues and that its gene is located at chromosome 12, region p12. These results, taken together, indicate that polysialogangliosides are synthesized by a single enzyme, G_{D3}/G_{T3} synthase, which is specifically expressed in neural tissues.

EXPERIMENTAL PROCEDURES

Antibodies—Monoclonal antibodies M6703 and M6704 were shown to react with G_{T3} having NeuNAc α 2 \rightarrow 8NeuNAc α 2 \rightarrow 8 NeuNAc α 2 \rightarrow 3Gal β 1 \rightarrow R structure of C series polysialogangliosides (Hirabayashi *et al.*, 1988). Monoclonal antibodies R24 (Pukel *et al.*, 1982) and KM641 (Ohta *et al.*, 1993) were shown to react with G_{D3} . NeuNAc α 2 \rightarrow 8NeuNAc α 2 \rightarrow 3Gal β 1 \rightarrow 4Glc \rightarrow Cer. R24 antibody was obtained from American Type Culture Collection, and KM641 antibody was kindly provided by Drs. Nobuo Hanai and Kenya Shitara at the Kyowa Hakko Kogyo, Japan.

Construction of Stably Transfected COS-1 Cells Expressing G_{D3} —COS-1 cells were transfected with pAMo-GD3 (Sasaki *et al.*, 1994b) and selected for G418 resistance. The transfected COS-1 cells expressing G_{D3} were selected by immunofluorescent staining with anti- G_{D3} antibody, R24, and one clone named COS-1- G_{D3} cells was isolated.

Expression Cloning of a Human G_{T3} ST cDNA—A mammalian expression vector, pcDNAI-based cDNA library, pcDNAI-SK-MEL-28 constructed from poly(A)⁺ RNA isolated from human melanoma SK-MEL-28 cells, was purchased from Invitrogen (San Diego, CA). SK-MEL-28 cells express a significant amount of G_{T3} (Dubois *et al.*, 1986). COS-1- G_{D3} cells (1.2×10^7) were transfected with 20 μ g of pcDNAI-SK-MEL-28 using lipofectamine (Life Technologies, Inc.). After 62 h, the transfected cells were detached at 37 °C in Hanks'-based

cell dissociation solution (Specialty Media, Lavallete, 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 on ice, 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 *Escherichia coli* MC1061/p3 cells by electroporation using 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 immunofluorescent staining using M6703 antibody. Sibling selection with sequentially smaller active pools identified a single plasmid, pcDNAI- G_{T3} ST, that determined the expression of G_{T3} at the cell surface.

Immunofluorescence Microscopy—Cells were fixed with 4% paraformaldehyde in phosphate-buffered saline and stained with mouse monoclonal antibodies R24 (anti- G_{D3}), KM641 (anti- G_{D3}), or M6703 (anti- G_{T3}), followed by FITC-conjugated (Fab')₂ fragment of goat anti-mouse IgG (Cappel). The cells were then examined under a Zeiss Axioplan microscope, as described previously (Williams and Fukuda, 1990).

Nucleotide Sequence Analysis—The cDNA insert of pcDNAI- G_{T3} ST was sequenced by the dideoxy nucleotide chain termination method (Sanger *et al.*, 1977) using oligonucleotide primers, as described (Bierhuizen *et al.*, 1993). The sequencing was initially carried out by using DyeDeoxy terminator cycle sequencing kit and DNA autosequencer (Applied Biosystems, Foster City, CA). The sequence was confirmed by using [³⁵S]dATP and a Sequenase sequencing kit (Amersham Corp.).

Construction of a Truncated Form of G_{D3}/G_{T3} ST, G_{D3}/G_{T3} ST-S—The cDNA encoding a truncated form of G_{D3}/G_{T3} ST was prepared by polymerase chain reaction (PCR) using pcDNAI- G_{T3} ST as a template. Upstream and downstream primers used were 5'-cccaagctt-GAGGGGCC-3' (*Hind*III site shown by underline) and 5'-atagtt-tagcgggcgcCCATTGTTCC-3' (*Not*I site shown by underline), respectively. The PCR product encompasses 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 μ l 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 *Hind*III and *Not*I and cloned into the same sites of pcDNAI.

Establishment of Stable Transfectants Expressing G_{T3} —Human cervical epitheloid carcinoma HeLa cells and human melanoma MeWo cells were cotransfected with pcDNAI- G_{D3}/G_{T3} ST and pSV2neo (10:1) and selected by G418 resistance. The transfected cells expressing G_{T3} were screened by immunofluorescent staining with M6704 antibody, and two clones, named HeLa- G_{T3} and MeWo- G_{T3} , were established.

Thin-layer Chromatography of Glycosphingolipids—Analytical thin-layer chromatography was carried out on precoated high performance thin-layer chromatography (HPTLC) plates (Si-HPF, J.T. Baker, Inc., Phillipsburg, NJ). The solvent systems used were chloroform, methanol, 14 mM MgCl₂ in water (5:4:1 by volume) for the first development and chloroform, methanol, water, 15 M NH₄OH (50:40:8:2 by volume) for the second development. Gangliosides were visualized by resorcinol/HCl reagent.

Isolation of gangliosides from cells and TLC-immunostaining were performed as reported 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 Vitro Sialyltransferase Assays and Product Characterization—The expression vector for protein A- G_{D3}/G_{T3} ST fusion protein was constructed using pAMoA vector as described (Sasaki *et al.*, 1994b). The cDNA in this pAMoA-GD3 was excised by *Sal*I and *Asp*718, filled in by the Klenow fragment, and subcloned into the *Eco*RV site of pcDNAI, resulting in pcDNA-proA- G_{D3} . After confirming the correct orientation by sequencing, pcDNAI-proA- G_{D3} or pPROTA (Kukowska-Latallo *et al.*, 1990) as a control was transfected into COS-1 cells. The protein A- $G_{D3}/$

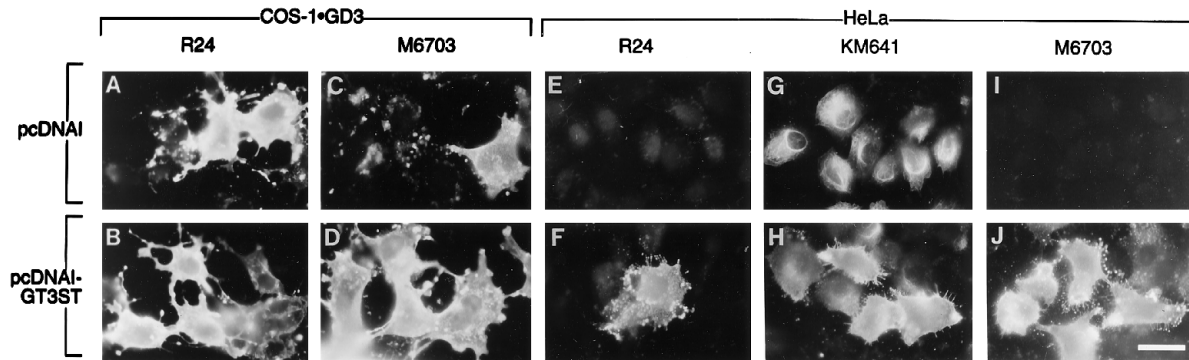


FIG. 2. **Expression of polysialylated gangliosides by pcDNAI- $G_{T3}ST$.** COS-1- G_{D3} cells (A–D) and HeLa cells (E–J) were transfected with pcDNAI- $G_{T3}ST$ (B, D, F, H, J) or with control pcDNAI vector (A, C, E, G, I). Sixty-two h after transfection, the cells were fixed and stained with monoclonal antibodies R24 (A, B, E, F), M6703 (C, D, I, J), and KM641 (G, H), followed by FITC-conjugated (Fab')₂ fragment of goat anti-mouse IgG antibody. COS-1- G_{D3} cells are positive for the M6703 antibody before transfection with pcDNAI- $G_{T3}ST$ (C), while HeLa cells were negative for the M6703 antibody before transfection with the same vector (I). Bar, 50 μ m.

$G_{T3}ST$ 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 20 mM Tris-HCl buffer, pH 7.5, containing 5 mM CaCl₂ 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.*, 1994b). Briefly, after a 1-min sonication of 25 μ l of 0.1 M sodium cacodylate buffer (pH 6.0) containing 20 mM MgCl₂, 1% Triton CF-54, 2.4 nmol of CMP-[¹⁴C]NeuNAc, and 10 μ g of a substrate with or without a competing substrate, 25 μ 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 μ l of phosphate-buffered saline was added to the incubation mixture, and the contents were applied to an Aspec Pak tC18 cartridge (M & S, Tokyo, Japan), according to the procedure described (Williams and McCluer, 1980). After washing the column with water, glycosphingolipids 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 $G_{D3}/G_{T3}ST$ Transcripts Using Competitive PCR. The level of $G_{D3}/G_{T3}ST$ transcript was measured by the competitive PCR using the cDNAs, 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, $G_{D3}/G_{T3}ST$ cDNA was truncated by deleting a 125-base pair *EcoT221-PvuII* fragment of the cDNA from pUC-GD3R (Sasaki *et al.*, 1994b). The 5' and 3' primers were 5'-ACAGTTACATCTACATGCCTGCCTT-3' and 5'-CATGAAACAACCTGACCATTCCCTC-3', respectively. The amount of amplified cDNAs was calculated from the respective standard curves, converted into the values of molar numbers. As a control, the β -actin transcript was measured in the same cDNA samples.

Northern Blot Analysis of Various Human Tissues. Poly(A)⁺ 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 filter (Micron Separation, Westboro, MA). Human multiple-tissue Northern blots of poly(A)⁺ RNA were purchased from Clontech, and these blots were hybridized with a gel-purified cDNA insert of pcDNAI- $G_{T3}ST$ after labeling with [α -³²P]dCTP by random oligonucleotide priming (Feinberg and Vogelstein, 1983) (Prime-It II labeling kit, Stratagene, San Diego, CA).

Fluorescence in Situ Hybridization Analysis of $G_{T3}ST$ Gene. Human genomic P1 plasmid library was screened by PCR as described (Onda and Fukuda, 1995). The 5' and 3' primers for PCR correspond to the sequence of the nucleotides 1184–1203 and that of nucleotides 1424–1443 of the $G_{T3}ST$ 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 phytohemagglutinin-stimulated pe-

ripheral blood lymphocytes in a solution containing 50% formamide, 10% dextran sulfate and 2 \times 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, D12Z1-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_{T3} Synthase.—In order to clone G_{T3} synthase, it was necessary to employ cells expressing a precursor ganglioside G_{D3} but lacked G_{T3} itself as recipients for transfection. The parent COS-1 cells did not react with M6703 (anti- G_{T3}) or R24 (anti- G_{D3}) monoclonal antibodies, indicating that G_{D3} and G_{T3} are not synthesized by COS-1 cells. Therefore, we transfected COS-1 cells with pAmo- G_{D3} , which harbors cDNAs encoding the G_{D3} synthase and G418 resistance gene (Sasaki *et al.*, 1994b), and isolated COS-1- G_{D3} cells that were strongly stained by R24.

When the COS-1- G_{D3} cells were tested for the presence of G_{T3} by M6703 antibody, however, 3.5% of COS-1- G_{D3} cells showed a strong positive signal for G_{T3} judged in FACS analysis. We thus isolated COS-1- G_{D3} cells, which barely reacted with M6703 antibody by FACS. The freshly sorted COS-1- G_{D3} cells expressed only G_{D3} and were expanded once up to 1.2×10^7 cells in culture. Although a few of them still expressed G_{T3} (Fig. 2C), they were used as recipient cells for expression cloning of G_{T3} synthase.

COS-1- G_{D3} cells were transfected with the SK-MEL-28 cDNA library in pcDNAI. Sixty-two h after transfection, COS-1- G_{D3} cells expressing G_{T3} were enriched by FACS using M6703 antibody under the conditions where only highly positive cells were selected. From 2.6×10^6 COS-1- G_{D3} cells applied, 4,044 cells were sorted. Plasmid DNAs were rescued from these M6703-positive cells.

When COS-1- G_{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_{T3} -positive from the cells that were endogenously G_{T3} -positive by immunofluorescent staining with M6703 antibody. We reasoned that this failure was due to the high background expression of G_{T3} in COS-1- G_{D3} cells (see Fig. 2C). In order to overcome this problem, the plasmid DNAs were transfected into HeLa cells. The wild-type HeLa cells expressed detectable amounts of G_{D3} as judged by immunofluorescent staining using another anti- G_{D3} antibody, KM641 (Ohta *et al.*, 1993) but were completely neg-

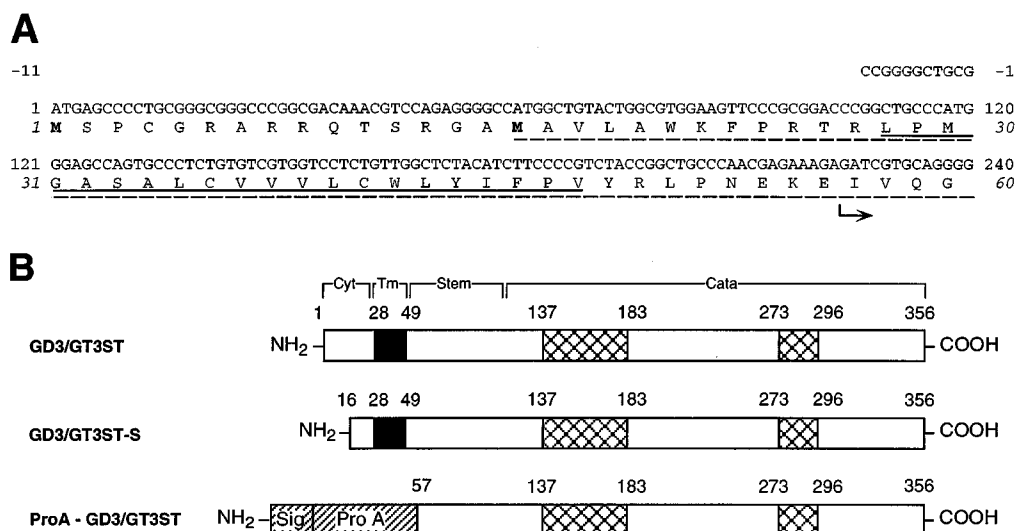


FIG. 3. The nucleotide sequence of $G_{T3}ST$ and schematic representation of $G_{D3}/G_{T3}ST$ protein and its derivatives. A, the nucleotide sequence of the 5'-terminal region of the cDNA insert of pcDNAI- $G_{T3}ST$ is shown. The translated amino acid sequence is shown below the nucleotide sequence. The possible 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). B, the schematic representation of two $G_{D3}/G_{T3}ST$ translation products and proA- $G_{D3}/G_{T3}ST$ chimeric protein are shown. $G_{D3}/G_{T3}ST$ and $G_{D3}/G_{T3}-S$ start the translation in residues 1 and 16 shown in A, respectively. The cytoplasmic (Cyt), transmembrane/anchor (Tm), tentative stem (Stem), and catalytic (Cata) domains are shown. Sialylmotif L (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 $G_{D3}/G_{T3}ST$. The catalytic domain encompasses residue 57 (shown by the arrow in A) to the COOH terminus of the $G_{D3}/G_{T3}ST$.

ative for G_{T3} (Fig. 2, G and I).

The transformed bacteria obtained after the Hirt procedure were thus divided into 20 pools, and the plasmid DNA from each plate was transfected separately into HeLa cells. The transfectants 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 G_{T3} in HeLa cells. One of the plasmid pools, which produced strongly positive cell staining by M6703, was selected, and subsequent rounds of sibling selection with sequentially smaller, active pools identified a single plasmid, pcDNAI- $G_{T3}ST$, that directed the expression of G_{T3} at the cell surface of HeLa cells.

COS-1- G_{D3} and HeLa cells were transiently transfected with pcDNAI- $G_{T3}ST$, and the transfected cells were examined for the expression of G_{D3} and G_{T3} by immunofluorescent staining. Fig. 2, D and J, show that the expression of G_{T3} , detected by M6703, was clearly seen on both COS-1- G_{D3} and HeLa cells after the transfection. The expression of G_{D3} was also notably increased in some of the transfected HeLa cells (Fig. 2, F and H).

Sequencing of the isolated cDNA in pcDNAI- $G_{T3}ST$ revealed an insert of 1622 base pairs in size encoding a single open reading frame in the sense orientation with respect to the pcDNAI promoter. The open reading frame predicts a protein of 356 amino acids in length with a calculated molecular mass of 40,517. When this cDNA sequence was compared with other cDNAs in the data base, it was found to be identical to that encoding another α -2,8-sialyltransferase, G_{D3} synthase (Harauchi *et al.*, 1994; Nara *et al.*, 1994; Sasaki *et al.*, 1994b) (Fig. 3A). The newly isolated sequence is, however, shorter in the 5'-flanking sequence and starts with 11 base pairs upstream from the initiation methionine (Fig. 3A). The 3'-flanking sequence just before poly(A) was shorter by 3 base pairs compared with the reported G_{D3} synthase (Sasaki *et al.*, 1994b). The deduced amino acid sequence predicts that this protein has a type II membrane topology, which has been found in all mammalian glycosyltransferases cloned (Schachter, 1994).

These results indicate that G_{D3} synthase (STII) and G_{T3} synthase (STIII) are the same enzyme and suggests a possibility that a single enzyme catalyzes the reactions for the formation of disialosyl and trisialosyl groups. The newly identified enzyme is thus called G_{D3}/G_{T3} synthase or $G_{D3}/G_{T3}ST$ 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 $G_{D3}/G_{T3}ST-S$ (Fig. 3B) was cloned into pcDNAI and expressed in both COS-1- G_{D3} and HeLa cells. The results obtained by immunofluorescent staining of the transfected cells clearly indicate that this truncated cDNA is also capable of G_{D3} and G_{T3} expression (data not shown). In fact, the nucleotide sequence surrounding the second initiation methionine, GCCATGG is consistent with the consensus sequence, (A/G)CCATGG for optimal translation initiation (Kozak, 1991). In contrast, the nucleotide sequence surrounding the first methionine GCGATGA 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 to date (Schachter, 1994). These results suggest that actual initiation of translation starts probably from this downstream methionine coding for a protein of 341 amino acids with a molecular mass of 38,901.

Synthesis of G_{D3} , G_{T3} , and Possibly Higher Polysialogangliosides by G_{D3}/G_{T3} Synthase—In order to determine whether or not $G_{D3}/G_{T3}ST$ catalyzes the formation of G_{D3} and G_{T3} , HeLa and MeWo cells were transfected with pcDNAI- $G_{D3}/G_{T3}ST$, and the resultant transfected cells, termed HeLa- G_{T3} and MeWo- G_{T3} , were analyzed for the synthesis of gangliosides. The results shown in Fig. 4, C and D, indicate that the HeLa- G_{T3} cells synthesized not only G_{T3} but also G_{D3} , although the expression of G_{T3} was stronger. Similarly, the MeWo- G_{T3} cells synthesized a significant amount of G_{T3} together with G_{D3} (Fig. 4, G and H). Since the parent MeWo cells barely synthe-

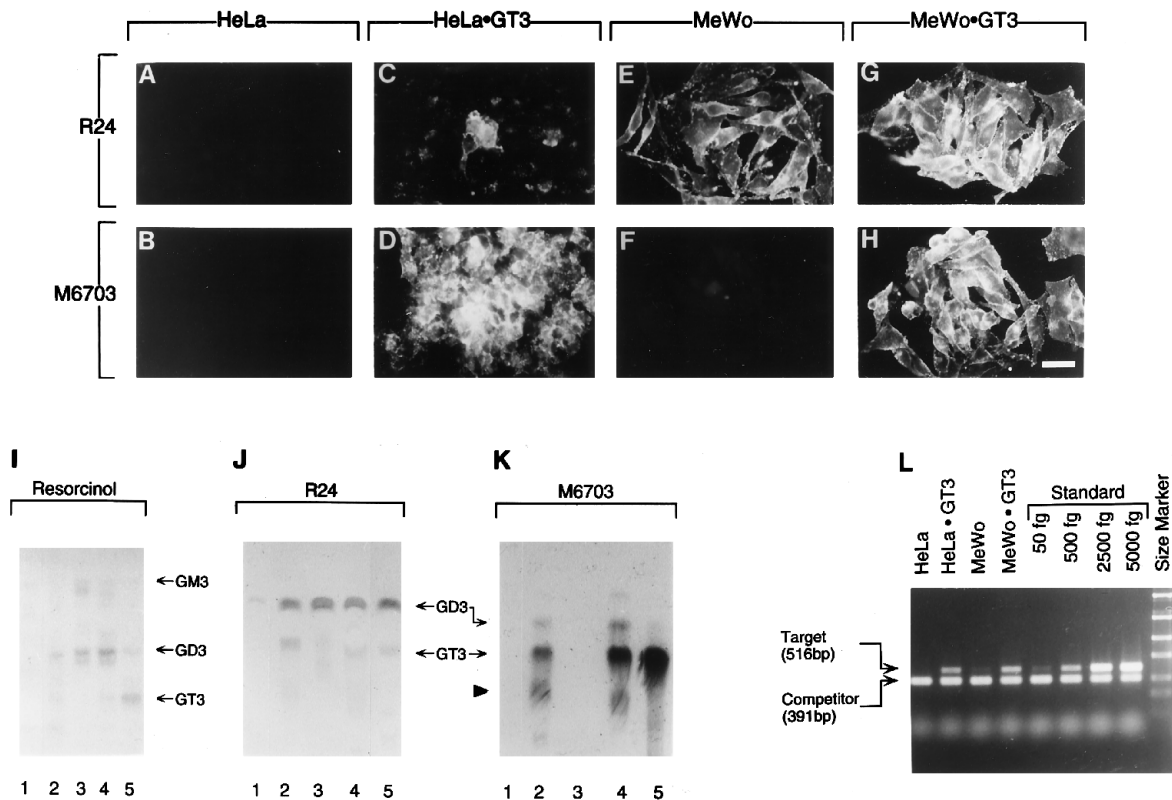


FIG. 4. **Expression of polysialylated gangliosides in stably transfected HeLa and MeWo cells.** A–H, the parent HeLa (A, B) and MeWo (E, F) cells and those stably transfected HeLa-GT₃ (C, D) and MeWo-GT₃ (G, H) cells were examined by immunofluorescent staining using R24 (anti-G_{D3}) antibody (A, C, E, G) or M6703 (anti-G_{T3}) antibody (B, D, F, H). Bar, 50 μ m. I–K, gangliosides expressed in HeLa-GT₃ and MeWo-GT₃ cells were analyzed by TLC. Gangliosides were extracted from HeLa (lane 1), HeLa-GT₃ (lane 2), MeWo (lane 3), and MeWo-GT₃ (lane 4) cells and subjected to HPTLC as described under “Experimental Procedures.” Lane 5 represents ganglioside standards. The plate was then visualized by resorcinol/HCl (I), by R24 (anti-G_{D3}) antibody (J), or by M6703 (anti-G_{T3}) antibody (K) followed by a peroxidase-conjugated goat anti-mouse IgG. L, G_{D3}/G_{T3}ST transcripts in various cells were analyzed by competitive PCR analysis. Single-stranded cDNAs reverse-transcribed from total RNA of a variety of cells labeled on the top or standard G_{D3}/G_{T3}ST cDNAs (50, 500, 2,500, and 5,000 fg) were mixed with 500 fg of the competitor cDNA (truncated G_{D3}/G_{T3}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.

size G_{T3} even though a substantial amount of G_{D3} is synthesized (Fig. 4, E and F), the expression of G_{T3} in the MeWo-GT₃ cells should be solely due to the newly introduced G_{D3}/G_{T3}ST cDNA, but not the accumulation of newly synthesized G_{D3}. On the other hand, the enhanced expression of G_{D3} and the new synthesis of G_{T3} in the HeLa-GT₃ cells were due to the newly introduced G_{D3}/G_{T3}ST cDNA.

In order to confirm that the transfected cells synthesize both G_{D3} and G_{T3}, 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-GT₃ cells contained both G_{D3} and G_{T3}, while the parent HeLa cells contained no G_{T3} (Fig. 4I, lanes 1 and 2). Similarly, the MeWo-GT₃ cells contained G_{T3}, whereas the parent MeWo cells did not contain G_{T3} (see lanes 3 and 4 in Fig. 4I). These results were confirmed by immunostaining of gangliosides after separation by thin-layer chromatography. The parent HeLa cells express a very small amount of G_{D3}, but HeLa-GT₃ cells express a substantially increased amount of G_{D3}, detected by R24 antibody (Fig. 4J, lanes 1 and 2). The parent MeWo cells, on the other hand, express a significant amount of G_{D3} (Fig. 4J, lane 3), but no G_{T3} was detected by M6703 antibody (Fig. 4K, lane 3). In contrast, both HeLa-GT₃ and MeWo-GT₃ cells express a large amount of G_{T3} (Fig. 4K, lanes 2 and 4). These results, taken together, clearly indicate that G_{D3}/G_{T3}ST transfers an α -2,8-linked sialic acid to G_{M3} and G_{D3}, forming G_{D3} and G_{T3}, respectively. The immunofluorescent stainings of HeLa-GT₃

and MeWo-GT₃ 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 M6703 also reacts with trisialosyl residues in glycoproteins (Nakayama *et al.*, 1993).

In order to formally prove if G_{D3} synthase also has G_{T3} 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_{D3}/G_{T3}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_{M3} or G_{D3} and the donor substrate CMP-[¹⁴C]NeuNAc. As shown in Fig. 5, lane 3, the soluble form of G_{D3}/G_{T3}ST synthesized both G_{D3} and G_{T3} when incubated with G_{M3}. These results establish that the newly identified enzyme, G_{D3}/G_{T3}ST, is a polysialyltransferase that adds more than one sialic acid residue in α -2,8-linkage.

The above experiments also suggested that G_{D3}/G_{T3}ST added sialic acids much less efficiently when G_{D3} was used as an acceptor (Fig. 5, lane 5). However, the enzyme added sialic acid residues to G_{D3} (136 μ M final concentration) after a longer period of incubation (Fig. 5, lanes 7 and 8). Under these conditions, G_{D3}/G_{T3}ST also synthesized higher polysialogangliosides, which presumably have more than three sialic acid res-

idues, as shown in lanes 7 and 8 of Fig. 5.

We also tested if the product or an intermediate inhibits the enzymatic reaction as competing substrates. The results shown in Fig. 5 indicate that G_{D3} (680 μ M final concentration) inhibits the formation of G_{D3} and G_{T3} from G_{M3} (Fig. 5, lane 10), while G_{T3} (567 μ M final concentration) inhibits the formation of G_{T3} and higher polysialogangliosides from G_{D3} (Fig. 5, lane 11). These results taken together support the above conclusion that $G_{D3}/G_{T3}ST$ synthesizes G_{D3} from G_{M3} and then $G_{D3}/G_{T3}ST$ utilizes G_{D3} as an acceptor to form G_{T3} .

The above results also suggested that the amount of $G_{D3}/G_{T3}ST$ mRNA transcript may be proportional to the amount of polysialylated gangliosides synthesized. In order to test this hypothesis, $G_{D3}/G_{T3}ST$ transcript was quantitated in the parent HeLa, HeLa- G_{T3} , parent MeWo, and MeWo- G_{T3} cells. Fig. 4L shows that the HeLa- G_{T3} cells express a significant amount of the $G_{D3}/G_{T3}ST$ transcript (330 fg), while the parent HeLa

cells scarcely express it. The MeWo- G_{T3} cells express approximately the same amount (370 fg) of the transcript as the HeLa- G_{T3} 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 G_{D3} but barely express G_{T3} , while the MeWo- G_{T3} and HeLa- G_{T3} cells express both G_{D3} and G_{T3} . These results clearly indicate that G_{T3} is synthesized only when $G_{D3}/G_{T3}ST$ is abundantly present.

$G_{D3}/G_{T3}ST$ Is Expressed in both Fetal and Adult Brains—To determine the tissue distribution of $G_{D3}/G_{T3}ST$ 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 $G_{D3}/G_{T3}ST$ transcripts were detected in brain and very weakly in lung. Among different parts of the adult brain, a substantial amount of $G_{D3}/G_{T3}ST$ mRNA was detected invariably 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 $G_{D3}/G_{T3}ST$ is different from that of the neural cell adhesion molecule (N-CAM)-specific polysialyltransferase (Nakayama *et al.*, 1995), and $G_{D3}/G_{T3}ST$ expression is more restricted to brain.

$G_{D3}/G_{T3}ST$ Gene Is Mapped to Chromosome 12p12—The previous studies showed that the $G_{D3}ST$ 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 $G_{D3}/G_{T3}ST$ gene, we utilized fluorescence *in situ* hybridization (FISH) procedures. First, P1 plasmid harboring $G_{D3}/G_{T3}ST$ 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 (D12Z1) 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

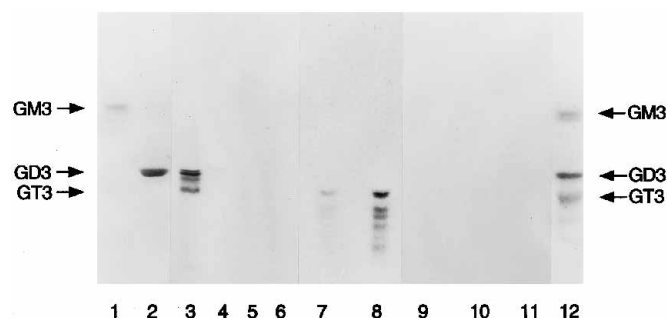


FIG. 5. *In vitro* sialyltransferase assay using the protein A- $G_{D3}/G_{T3}ST$ chimeric protein. The chimeric soluble $G_{D3}/G_{T3}ST$ was incubated for 4 h with CMP-[¹⁴C]NeuNAc and 10 μ g of G_{M3} (lane 3) or 10 μ g of G_{D3} (lane 5), and the reaction products were subjected to HPTLC followed by fluorography. Lanes 4 and 6 represent the experiments that used a control vector, pPROTA, that lacks $G_{D3}/G_{T3}ST$ cDNA, and G_{M3} (lane 4) or G_{D3} (lane 6). The products are shown for those experiments after 12 h (lane 7) and 24 h (lane 8) incubation using G_{D3} as the acceptor. Lane 9 represents the experiment under the same conditions as lane 8 except that pPROTA was used. Lane 10 shows the products when 10 μ g of G_{M3} was incubated for 4 h with CMP-[¹⁴C]NeuNAc together with 50 μ g of G_{D3} , and lane 11 shows the products when 10 μ g of G_{D3} was incubated for 24 h with CMP-[¹⁴C]NeuNAc together with 50 μ g of G_{T3} . Lanes 1 and 2 represent the G_{M3} and G_{D3} used as acceptors, and lane 12 is a mixture of standard gangliosides, detected by resorcinol/HCl.

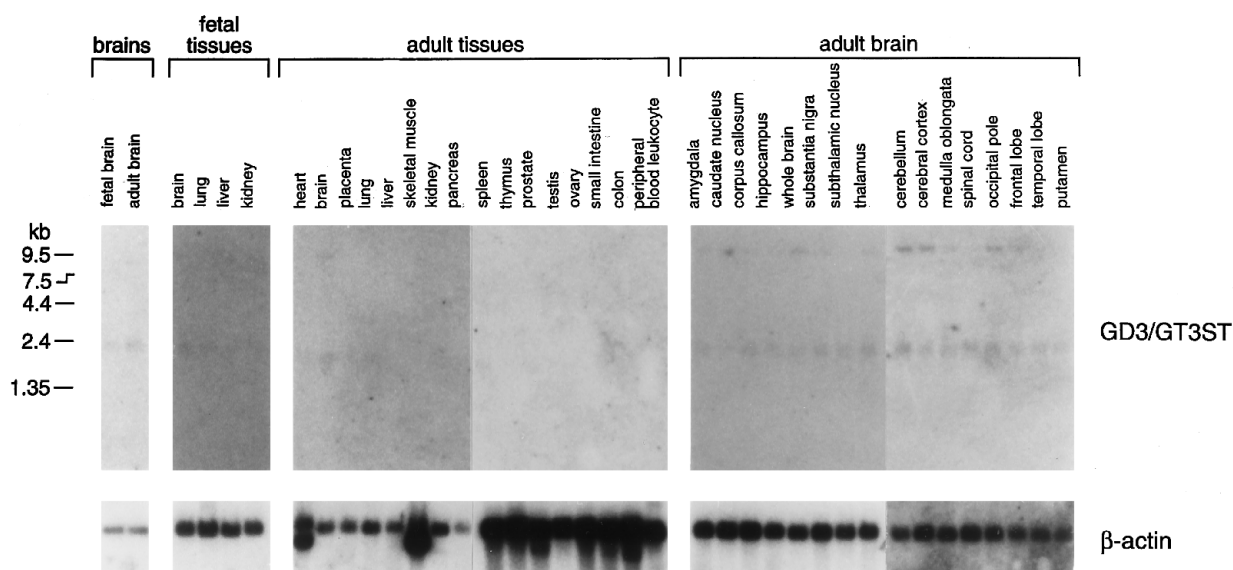


FIG. 6. Northern blot analysis of $G_{D3}/G_{T3}ST$ in various human tissues. Each lane contained 2 μ g of poly(A)⁺ RNA. The blot for the first two lanes at the far left was made separately and apparently contained less poly(A)⁺ RNA than the other Northern blots. The same blots were probed by ³²P-labeled $G_{D3}/G_{T3}ST$ cDNA ($G_{D3}/G_{T3}ST$) or β -actin cDNA (β -actin).

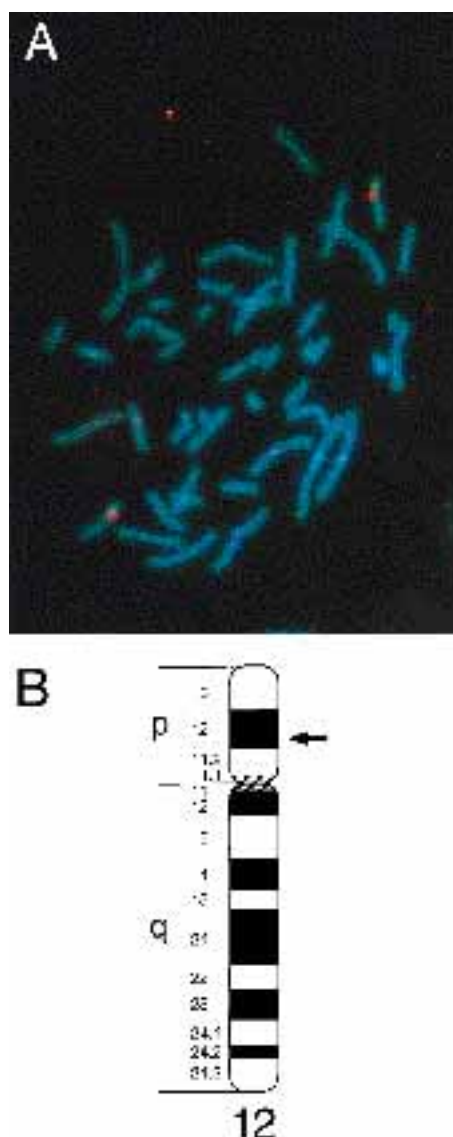


FIG. 7. Chromosomal localization of $G_{D3}/G_{T3}ST$ gene as revealed by FISH. A, two-color FISH analysis on metaphase chromosomes using a digoxigenin-labeled P1 clone 5439 encoding $G_{T3}ST$ (stained as green signal) and biotin-labeled D12Z1, a specific probe for the centromere of chromosome 12 (stained as red signal). A discrete signal is discernible on all chromosome 12 chromatids for each probe. DNA was counterstained with 4',6-diamidino-2-phenylindole. B, the specific hybridization occurs on chromosome 12, region p12.

from the centromere to the telomere of short arm, an area that corresponds to 12p12 (Fig. 7B). This location is close to that of the *c-Ki-ras* protooncogene (Muleris *et al.*, 1993) and human islet amyloid polypeptide gene (Christmanson *et al.*, 1990).

DISCUSSION

The present study describes the isolation of a cDNA clone encoding G_{T3} synthase, the key enzyme responsible for C series polysialogangliosides using expression cloning with a newly devised modification. We utilized COS-1 cells as recipient cells for the enrichment of plasmids that directed the expression of G_{T3} . Since the large T antigen is synthesized in COS-1 cells, plasmids such as pcDNA1 that contain the replication origin of SV40 are amplified in the cells. In order to test whether or not plasmids isolated from sorted COS-1- G_{D3} cells could convert G_{D3} to G_{T3} , COS-1- G_{D3} cells were not suited as recipient cells because of a substantial background of G_{T3} expression. In contrast, HeLa cells do not express G_{T3} but synthesize a small

amount of G_{D3} . Therefore HeLa cells should be able to synthesize G_{T3} once a cDNA encoding G_{T3} synthase is introduced. By using HeLa cells, we thus could identify a pool of plasmids that directed G_{T3} expression. This is the first report where recipient cells for enriching plasmids differ from cells used for testing the enriched plasmids that direct the expression of a desired gene.

It was generally accepted that G_{D3} synthase (STII) produced the first disialosyl linkage, forming NeuNAc α 2 \rightarrow 8NeuNAc α 2 \rightarrow 3Gal β 1 \rightarrow 4Glc \rightarrow Cer, and then another enzyme, G_{T3} synthase (STIII), added one more sialic acid, forming NeuNAc α 2 \rightarrow 8NeuNAc α 2 \rightarrow 3Gal β 1 \rightarrow 4Glc \rightarrow Cer (Fig. 1). The present study, however, demonstrates that the same enzyme can form disialosyl and trisialosyl residues.

It was also noted that the stable transfectants of both HeLa and MeWo cells acquired gangliosides larger than G_{T3} (see the arrowhead in Fig. 4K). Since MeWo cells lack β -1,4-*N*-acetylgalactosaminyltransferase (Yamashiro *et al.*, 1995), G_{T1c} , or G_{Q1c} can not be formed in MeWo- G_{T3} cells (see Fig. 1 for the structure of G_{T1c} and G_{Q1c}). In addition, M6703 recognizes not only trisialosyl residues but also tetrasialosyl residues.² When G_{D3} was incubated with the soluble $G_{D3}/G_{T3}ST$, slow migrating gangliosides were also produced in addition to G_{T3} (Fig. 5, lanes 7 and 8). Since this assay was performed in the absence of other enzymes, these higher gangliosides are most likely polysialogangliosides having more than three sialic acid residues. The results, taken together, strongly suggest that the slow migrating band may represent G_{Q3} shown in Fig. 1. It is possible that G_{Q3} 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 α -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 separately formed by an initiation enzyme (see Kitazume *et al.*, 1994), the results obtained on mutant Chinese hamster ovary cells lacking polysialylation strongly suggest that polysialyltransferase carries out all reactions that form α -2,8-linked sialic acid polymer in N-CAM (Eckhardt *et al.*, 1995). Moreover, it has been demonstrated recently that polysialyltransferase can add all α -2,8-linked sialic acid residues necessary for polysialic acid formation (Nakayama and Fukuda, 1996). These studies, taken together, strongly suggest that the polysialylation is catalyzed by single enzymes in both N-CAM glycoprotein (polysialyltransferase) and glycosphingolipids ($G_{D3}/G_{T3}ST$).

The present study demonstrated that $G_{D3}/G_{T3}ST$ synthesizes G_{T3} more efficiently from G_{M3} than from G_{D3} . It is tempting to speculate that $G_{D3}/G_{T3}ST$ first binds to G_{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_{D3} inhibited the formation of G_{T3} from G_{M3} , confirming that G_{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

² Y. Hirabayashi, unpublished results.

the polysialylated acceptor. It is apparent that G_{D3}/G_{T3}ST terminates its reaction early, most likely due to its inefficiency in binding to trisialosyl or tetrasialosyl residues.

It was shown recently that Neuro2a cells exhibited better neurite extension after the cells were stably transfected to express G_{D3}/G_{T3}ST (Kojima *et al.*, 1994). Although these authors thought that this effect was due to the synthesis of G_{D3} and B series gangliosides, the effect may be due to the synthesis of G_{T3} 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 antiadhesive 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 cloned in the present study will be a powerful tool to dissect the intricate and complex roles of polysialic acids attached to glycoproteins and glycosphingolipids in cell-cell interactions during development.

Acknowledgments—We thank Drs. Kenneth Lloyd for the kind gift of MeWo cells, Nobuo Hanai and Kenya Shitara for KM641 antibody, Joseph Trotter for carrying out the flow cytometry analysis, David Small for carrying out FISH analysis, and Yu Yamaguchi for critical reading of the manuscript. We also thank Susan Greaney for secretarial assistance and the members of the laboratories for useful discussion.

REFERENCES

- Ando, S., and Yu, R. K. (1979) *J. Biol. Chem.* **254**, 12224–12229
- Bierhuizen, M. F. A., and Fukuda, M. (1992) *Proc. Natl. Acad. Sci. U. S. A.* **89**, 9326–9330
- Bierhuizen, M. F. A., Mattei, M.-G., and Fukuda, M. (1993) *Genes & Dev.* **7**, 468–478
- Bremer, E. G., Schlessinger, J., and Hakomori, S. (1986) *J. Biol. Chem.* **261**, 2434–2440
- Cheresh, D. A., Pierschbacher, M. D., Herzig, M. A., and Mujoo, K. (1986) *J. Cell Biol.* **102**, 688–696
- Christmanson, L., Rorsman, F., Stenman, G., Westermark, P., and Betsholtz, C. (1990) *FEBS Lett.* **267**, 160–166
- Dubois, C., Magnani, J. L., Grunwald, G. B., Spitalnik, S. L., Trisler, G. D., Nirenberg, M., and Ginsburg, V. (1986) *J. Biol. Chem.* **261**, 3826–3830
- Eckhardt, M., Mühlenhoff, M., Bethe, A., Koopman, J., Frosch, M., and Gerardy-Schahn, R. (1995) *Nature* **373**, 715–718
- Edelman, G. M. (1985) *Annu. Rev. Biochem.* **54**, 135–169
- Feinberg, A. P., and Vogelstein, B. (1983) *Anal. Biochem.* **132**, 6–13
- Feizi, T. (1985) *Nature* **314**, 53–57
- Fukuda, M. (1985) *Biochim. Biophys. Acta* **780**, 119–150
- Fukuda, M. (ed) (1992) *Cell Surface Carbohydrates and Cell Development*, CRC Press, Boca Raton, FL
- Hakomori, S. (1984) *Annu. Rev. Immunol.* **2**, 103–126
- Haraguchi, M., Yamashiro, S., Yamamoto, A., Furukawa, K., Takamiya, K., Lloyd, K. O., Shiku, H., and Furukawa, K. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 10455–10459
- Hara-Yokoyama, M., Hirabayashi, Y., Irie, F., Syuto, B., Moriishi, K., Sugiyama, H., and Furuyama, S. (1995) *J. Biol. Chem.* **270**, 8115–8121
- Higa, H. H., Rogers, G. N., and Paulson, J. C. (1985) *Virology* **144**, 279–282
- Hirabayashi, Y., Hirota, M., Matsumoto, M., Tanaka, N., Obata, K., and Ando, S. (1988) *J. Biochem. (Tokyo)* **104**, 973–979
- Hirt, B. (1967) *J. Mol. Biol.* **26**, 365–369
- Jessell, T. M., Hynes, M. A., and Dodd, J. (1990) *Annu. Rev. Neurosci.* **13**, 227–255
- Kitazume, S., Kitajima, K., Inoue, S., Inoue, Y., and Troy, F. A., II (1994) *J. Biol. Chem.* **269**, 10330–10340
- Kojima, N., Kurosawa, N., Nishi, T., Hanai, N., and Tsuji, S. (1994) *J. Biol. Chem.* **269**, 30451–30456
- Kozak, M. (1991) *J. Biol. Chem.* **266**, 19867–19870
- Kukowska-Latallo, J. F., Larsen, R. D., Nair, R. P., and Lowe, J. B. (1990) *Genes & Dev.* **4**, 1288–1303
- Lowe, J. B. (1994) in *Molecular Glycobiology* (Fukuda, M., and Hindsgaul, O., eds) pp. 163–205, Oxford University Press, Oxford
- McCoy, R. D., Vimr, E. R., and Troy, F. A. (1985) *J. Biol. Chem.* **260**, 12695–12699
- Muleris, M., Laurent-Puig, P., Salmon, R.-J., Thomas, G., and Dutrillaux, B. (1993) *Cancer Genet. Cytogenet.* **69**, 161–162
- Nakayama, J., and Fukuda, M. (1996) *J. Biol. Chem.* **271**, 1829–1832
- Nakayama, J., Katsuyama, T., Sugiyama, E., and Hirabayashi, Y. (1993) *J. Histochem. Cytochem.* **41**, 1563–1572
- Nakayama, J., Fukuda, M. N., Fredette, B., Ranscht, B., and Fukuda, M. (1995) *Proc. Natl. Acad. Sci. U. S. A.* **92**, 7031–7035
- Nara, K., Watanabe, Y., Maruyama, K., Kasahara, K., Nagai, Y., and Sanai, Y. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91**, 7952–7956
- Ohta, S., Honda, A., Tokutake, Y., Yoshida, H., and Hanai, N. (1993) *Cancer Immunol. Immunother.* **36**, 260–266
- Onda, M., and Fukuda, M. (1995) *Gene (Amst.)* **159**, 225–230
- Pohlentz, G., Klein, D., Schwarzmann, G., Schmitz, D., and Sandhoff, K. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 7044–7048
- Pukel, C. S., Lloyd, K. O., Travassos, L. R., Dippold, W. G., Oettgen, H. F., and Old, L. J. (1982) *J. Exp. Med.* **155**, 1133–1147
- Rösner, H., Al-Aqtum, M., and Henke-Fahle, S. (1985) *Brain Res.* **350**, 85–95
- Rouquier, S., Lowe, J. B., Kelly, R. J., Fertitta, A. L., Lennon, G. G., and Giorgi, D. (1995) *J. Biol. Chem.* **270**, 4632–4639
- Rutishauser, U., Acheson, A., Hall, A. K., Mann, D. M., and Sunshine, J. (1988) *Science* **240**, 53–57
- Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74**, 5463–5467
- Sariola, H., Aufderheide, E., Bernhard, H., Henke-Fahle, S., Dippold, W., and Ekblom, P. (1988) *Cell* **54**, 235–245
- Sasaki, K., Kurata, K., Funayama, K., Nagata, M., Watanabe, E., Ohta, S., Hanai, N., and Nishi, T. (1994a) *J. Biol. Chem.* **269**, 14730–14737
- Sasaki, K., Kurata, K., Kojima, N., Kurosawa, N., Ohta, S., Hanai, N., Tsuji, S., and Nishi, T. (1994b) *J. Biol. Chem.* **269**, 15950–15956
- Schachter, H. (1994) in *Molecular Glycobiology* (Fukuda, M., and Hindsgaul, O., eds) pp. 88–162, Oxford University Press, Oxford
- Spiegel, S., and Fishman, P. H. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 141–145
- Suzuki, Y., Nagao, Y., Kato, H., Matsumoto, M., Nerome, K., Nakajima, K., and Nobusawa, E. (1986) *J. Biol. Chem.* **261**, 17057–17061
- Svennerholm, L. (1964) *Lipids* **5**, 145–162
- Williams, M. A., and McCluer, R. H. (1980) *J. Neurochem.* **35**, 266–269
- Williams, M. A., and Fukuda, M. (1990) *J. Cell Biol.* **111**, 955–966
- Yamashiro, S., Haraguchi, M., Furukawa, K., Takamiya, K., Yamamoto, A., Nagata, Y., Lloyd, K. O., Shiku, H., and Furukawa, K. (1995) *J. Biol. Chem.* **270**, 6149–6155
- Yates, A. J. (1988) *Neurochem. Pathol.* **8**, 157–180