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Expression cloning of a CMP-NeuAc:NeuAcα2-3Galβ1-4Glcβ1-1'Cer α2,8-sialyltransferase (GD3 synthase) from human melanoma cells

(glycosyltransferase/GD3 ganglioside/tumor antigen/melanoma)

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ABSTRACT **Using an expression cloning approach, we have isolated a cDNA encoding GD3 synthase (CMP-NeuAc:NeuAcα2-3Galβ1-4Glcβ1-1'Cer α2,8-sialyltransferase, EC 2.4.99.8), which is a key regulatory enzyme determining the prominence of the ganglioside biosynthesis pathway. The cloned cDNA encodes a 341-amino acid protein containing a single transmembrane domain at its N-terminal region, suggesting that the protein has a type II transmembrane topology. The sequence of α2,8-sialyltransferase showed a high level of similarity with other sialyltransferases at two conserved regions typical in the sialyltransferase family. Transfected cells containing the cloned cDNA expressed GD3 ganglioside on the cell surface, which was detectable with specific anti-GD3 antibody by immunofluorescence and immunostaining after separation of isolated glycolipids on thin-layer chromatography. The cDNA hybridized to a single mRNA species of 2.4 kb in melanoma cells. This sialyltransferase is distinctive in catalyzing the formation of the α2-8 linkage of sialic acids.**

Gangliosides are membrane-bound glycosphingolipids containing sialic acids that are found in high concentrations on the central nervous system. The carbohydrate moieties of gangliosides undergo profound changes during mammalian development, differentiation, and malignant transformation, suggesting that they may play fundamental roles in these processes (for reviews see refs. 2 and 3 and references therein). In particular, the GD3 ganglioside has been shown to be important for cell adhesion and growth of cultured malignant cells (4, 5) and the inductive epithelial–mesenchymal interaction in the kidney development (6). Although these ganglioside biological phenomena have been known, the mechanism regulating ganglioside expression remains unclear. To address this question, cloned genes that determine ganglioside expression are essential tools.

Ganglioside biosynthesis takes place in the Golgi apparatus, where glucosylceramide is glycosylated by sequential addition of galactose, *N*-acetylgalactosamine, and *N*-acetylneuraminic acid (7). These reactions are catalyzed by specific glycosyltransferases. Recently, several glycosyltransferase cDNAs responsible for glycolipids and glycoproteins have been cloned (for reviews, see refs. 8 and 9), and the regulation of cell-type-specific expression of glycosyltransferases is being intensively investigated (9). With regard to sialyltransferases, there is a family of more than 12 different enzymes showing different substrate specificities (9). To date, cDNAs of 5 different sialyltransferases have been cloned (10–16), but a sialyltransferase responsible for the sialyl-α2,8-sialyl linkage, which appears in poly(sialic acid) in glycoconjugates, has not yet been cloned from mammalian species. It is thus important to isolate the gene coding for α2,8-sialyltransferase for understanding of the biological roles of sialic acid in glycoconjugates.

To clone the cDNA of α2,8-sialyltransferase responsible for synthesis of GD3 ganglioside, which is the first ganglioside in the synthetic pathway having sialyl-α2,8-sialyl linkage, we utilized a transient expression cloning system (17–19) in polyoma large tumor antigen-expressing Chinese hamster ovary (CHO) cells (20). This method and adaptations of it have been successful in cloning other glycosyltransferases (21–23). By means of this system, cDNA coding the gene which determines the expression of GD3 was isolated from a cDNA library prepared from mRNA of the SK-Mel-28 human melanoma cell line (24, 25). Analyses of the newly expressed gangliosides and the sialyltransferase activity in transfected cells indicate that this cDNA encodes the CMP-NeuAc: NeuAcα2-3Galβ1-4Glcβ1-1'Cer α2,8-sialyltransferase (EC 2.4.99.8).§

EXPERIMENTAL PROCEDURES

Cells and Monoclonal Antibodies. Hamster CHOP cells were generously provided by J. Dennis (Samuel Lunenfeld Research Institute, Ontario, Canada) (20). Human SK-Mel-28 melanoma cells were a kind gift from R. Ueda (Aichi Cancer Center, Nagoya, Japan). The mouse IgG3 anti-GD3 monoclonal antibody R24 (26, 27) was used as the supernatant of hybridoma R_{24}, obtained from the American Type Culture Collection (ATCC no. HB8445). The mouse IgM anti-GM3 monoclonal antibody GMR6 was provided by T. Tai (Tokyo Metropolitan Institute of Medical Science) (28).

Construction of an Expression Library. Oligo(dT)-primed cDNA was synthesized from poly(A)$^+$ RNA isolated from human SK-Mel-28 cells. After addition of regenerative *Bst*XI adapters (Invitrogen), the cDNA was size-fractionated (>1 kb) and ligated into expression vector pCEV18, which is a derivative of expression vector pCEV4 (29), yielding a library of $\approx 3 \times 10^6$ primary recombinants.

Isolation of a Human α2,8-Sialyltransferase (h2,8ST) cDNA Clone. Twelve samples of 3×10^6 CHOP cells were transfected with 30 μg of plasmid DNA each in 0.8 ml of Hepes-buffered saline (20 mM Hepes, pH 7.05/137 mM NaCl/5 mM KCl/0.7 mM Na_2HPO_4/6 mM glucose) by electroporation using a Gene Pulser apparatus (Bio-Rad) at 400 V, 980 μF (30, 31). Three days after transfection, the exponentially growing cells were incubated with anti-GD3 monoclonal antibody R24

Abbreviations: h2,8ST, human CMP-NeuAc:NeuAcα2-3Galβ1-4Glcβ1-1'Cer α2,8-sialyltransferase; FITC, fluorescein isothiocyanate; the nomenclature used for gangliosides is based on the system of Svennerholm (1).

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§The sequence reported in this paper has been deposited in the GenBank data base (accession no. D26360).

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followed by fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG/IgA/IgM (Zymed). Positive cells were collected by sorting in a FACStar Plus cell sorter (Becton Dickinson). Plasmid DNA was extracted from the positive cells (32) and used to transform *Escherichia coli* DH10B (GIBCO) by electroporation. After two rounds of transfection and sorting, a pool of positive colonies was divided into subpools (four colonies per well in eight 96-well plates) and screened by sib selection until a single clone, pCEVh2,8ST, was isolated.

DNA Sequence and Northern Blot Analysis. Double-stranded sequencing was performed by the dideoxynucleotide chain-termination method with an Autocycle Sequencing Kit (Pharmacia) and a Pharmacia A.L.F. DNA sequencer. RNA blots were hybridized with a ^{32}P-labeled 1.7-kb *Bst*XI fragment of pCEVh2,8ST at 42°C in 50% (vol/vol) formamide/5× Denhardt's solution/2% SDS/5× SSPE (1× SSPE is 10 mM NaH_2PO_4, pH 7.4/0.15 M NaCl/1 mM EDTA). The hybridized blots were washed at 68°C in 1× SSC/0.1% SDS (1× SSC is 150 mM NaCl/15 mM sodium citrate, pH 7.0).

GD3 Synthase Assay. GD3 synthase activity was measured as described (33).

Isolation and Analysis of Gangliosides from Cells. Purification of gangliosides and TLC-immunostaining were performed by the procedure of Sanai *et al.* (34) and Hidari *et al.* (35), respectively.

Construction of the Staphylococcal Protein A-h2,8ST Fusion. The cDNA fragment containing nucleotide residues 102–1300 (see Fig. 2) of h2,8ST was prepared by digestion with restriction enzymes *Bsr*FI and *Xba* I and was blunted with T4 DNA polymerase for addition of *Eco*RI adapter. The design of *Eco*RI adapter (Pharmacia) was

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5'-AATTCGCGGCCGCT
      GCGCCGGCGA-5'.
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After addition of *Eco*RI adapter, the resultant fragment was inserted into the *Eco*RI site of pPROTA (36), to yield the expression plasmid pPROTAh2,8ST. pPROTA vector was provided by M. Fukuda (La Jolla Cancer Research Foundation, La Jolla, CA). The expression plasmid (50 μg) was transfected into 5 × 10^6 COS-7 cells, and after a 64-h expression period the cell supernatant was collected and processed exactly as described before (37).

RESULTS

Expression Cloning Strategy. The CHOP cell line established by Heffernan and Dennis (20) is a derivative of CHO cells that stably expresses polyoma large tumor antigen to amplify plasmids carrying the polyomavirus origin of replication. In our preliminary analysis using a cell sorter and anti-ganglioside monoclonal antibodies, CHOP cells were stained with monoclonal antibody GMR6 (anti-GM3) (28) but not with monoclonal antibody R24 (anti-GD3) (26, 27), suggesting that CHOP cells express GM3 but not GD3 on their cell surface. Moreover, the lysate of the CHOP cells contains no detectable GD3 synthase activity (data not shown). We therefore chose CHOP cells as a suitable host for the expression cloning of GD3 synthase.

Isolation of a Cloned cDNA That Determines Expression of the R24 Antigen in CHOP Cells. To clone the cDNA encoding a GD3 synthase, an expression library was prepared from human SK-Mel-28 melanoma cells, which contain a large amount of GD3 and a high level of GD3 synthase activity (24, 25). We first tried to isolate the clone by the panning method (17, 18), but it failed. Then we employed a fluorescence-activated cell sorting method in our expression cloning strategy. From a screen of ≈3 × 10^6 clones of the library as described in *Experimental Procedures*, a single clone (termed pCEVh2,8ST) was obtained. As shown in Fig. 1, flow cytometric analysis revealed that pCEVh2,8ST determined expression of the R24 antigen on the cell surface.

Primary Structure of h2,8ST cDNA. pCEVh2,8ST has a 1.7-kbp cDNA insert (Fig. 2). Two potential initiation codons are found within its first 151 nucleotides. Only the second methionine codon (ATG) at nucleotide position 149 is in a favorable context for translation initiation (38). Translation from this methionine predicts a protein 341 amino acids long with a molecular mass of 38,858 Da. Hydropathy analysis indicates the presence of a putative transmembrane domain at the N-terminal portion (residues 13–34) (39), suggesting that this protein has features of a type II transmembrane molecule, as has been shown for other glycosyltransferases cloned to date. There are two consensus sites for N-glycosylation as shown in Fig. 2. No extensive evidence of homology was observed when this sequence was compared to the GenBank sequences.

Comparison of the predicted amino acid sequence with other sialyltransferases cloned to date (10–16) reveals that the protein sequence exhibits 17–30% identities with the seven cloned sialyltransferases. The highest level of identity (30%) was found between the sequence and the rat brain sialyltransferase gene, *STX* (10); the level is indeed not so significant. However, the protein sequence showed marked homology among all cloned sialyltransferases at the domain, the so-called sialyl motif (10, 11, 40), in the center and the C-terminal portions of the protein (Fig. 3). These results suggest that this protein belongs to the sialyltransferase family.

Northern Blot Analysis of SK-Mel-28 mRNA. Fig. 4*A* shows blot hybridization analysis of poly(A)$^+$ RNA obtained from SK-Mel-28 cells. A single mRNA species corresponding to 2.4 kb was detected in SK-Mel-28 cells, as seen in lane 2. No signal was obtained from the CHOP cells used as host cell for the expression cloning system (lane 1).

pCEVh2,8ST Can Transfer CMP-NeuAc onto GM3 Ganglioside. To confirm the possibility that the cells transfected

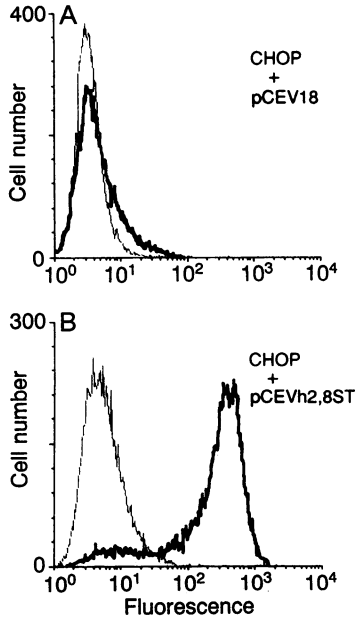

FIG. 1. Flow cytometric analysis of GD3 expression in transfected CHOP cells. Cells were stained with monoclonal antibody R24 followed by FITC-conjugated rabbit anti-mouse immunoglobulin (thick lines). The profile of cells stained with FITC-conjugated rabbit anti-mouse immunoglobulin alone are indicated as control (thin lines). (*A*) Sorting profiles of the cells transfected with pCEV18 vector. (*B*) Profiles of the cells transfected with pCEVh2,8ST.

FIG. 2. Nucleotide and predicted amino acid sequences of h2,8ST. The putative transmembrane domain is underlined. Potential N-glycosylation sites are indicated with asterisks. The domains showing high similarity with the sialyltransferases cloned to date are indicated by dashed lines.

with pCEVh2,8ST express GD3 synthase, sialyltransferase activity was assayed with ^{14}C-labeled CMP-NeuAc and GM3 as donor and acceptor substrates. As shown in Fig. 4*B*, a significant amount of sialyltransferase activity was detected in the cell extract of the transfectant. Specific activity is 3037 pmol of NeuAc incorporation per mg of protein per hour, which is comparable to the specific activities of the sialyltransferases reported previously (25). No GD3 was produced with extracts prepared from the CHOP cells transfected with pCEV18 vector alone. Due to transfer of labeled sialic acid to lactosylceramide by endogenous GM3 synthase, generation of small amounts of GM3 was also observed in both of the transfectants.

GD3 Expression on CHOP Cells Transfected with pCEVh2, 8ST. To gain further proof that the cloned cDNA encodes GD3 synthase, total ganglioside was extracted from CHOP cells transfected with pCEVh2,8ST and analyzed by TLC immunostaining using anti-GD3 monoclonal antibody R24. Fig. 5 demonstrates that GD3 was highly expressed on the cells transfected with pCEVh2,8ST (Fig. 5*A*, lane 4, and Fig. 5*B*, lane 2), whereas no detectable GD3 was expressed on the cells transfected with the vector alone (Fig. 5*A*, lane 5, and Fig. 5*B*, lane 1). Since the monoclonal antibody R24 recognizes the structure NeuAcα2-8Siaα2-3Galβ1-4Glc (or GlcNAc) (27, 41), pCEVh2,8ST encodes the α2,8-sialyltransferase involved in the biosynthesis of GD3 ganglioside.

Expression of a Soluble Form of the h2,8ST Protein. To eliminate the possibility that the isolated clone regulates the expression of the sialyltransferase in a trans-acting manner, we fused the putative stem and catalytic domain of the predicted protein to a secreted form of the IgG-binding domain of staphylococcal protein A. COS-7 cells were trans-

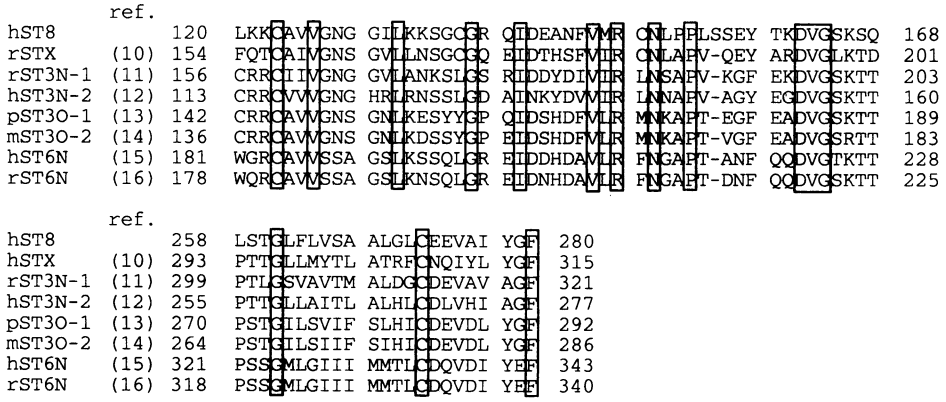

FIG. 3. Comparison in two conserved regions of the sialyltransferase family. hST8, h2,8ST; rSTX, rat *STX* gene (10); rST3N-1, rat Galβ1-3(4)GlcNAc α2,3-ST (11); hST3N-2, human Galβ(1-3/1-4)GlcNAc α2,3-ST (12); pST3O-1, porcine Galβ1-3GalNAc α2,3-ST (13); mST3O-2, mouse Galβ1-3GalNAc α2,3-ST (14); hST6N, human Galβ1-4GlcNAc α2,6-ST (15); and rST6N, rat Galβ1-4GlcNAc α2,6-ST (16). Identical amino acids in all sequences are boxed. Hyphens in the sequences indicate gaps introduced for alignment.

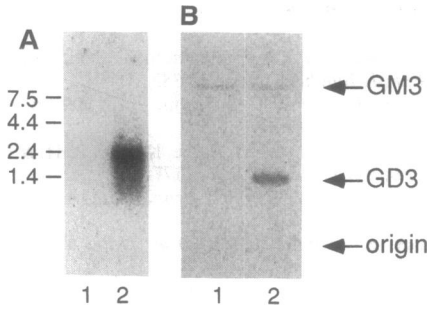

FIG. 4. (*A*) Northern blot analysis of h2,8ST. Poly(A)$^+$ RNA (2 μg per lane) prepared from CHOP cells (lane 1) and SK-Mel-28 cells (lane 2) was fractionated by formaldehyde/agarose gel electrophoresis and hybridized with the ^{32}P-labeled h2,8ST cDNA. The positions of RNA markers are indicated in the left margin (in kb). (*B*) GD3 synthase activity in CHOP cells transfected with pCEVh2,8ST. GD3 synthase activity transiently expressed in CHOP cells was measured by using CMP-[^{14}C]NeuAc and GM3 as donor and acceptor substrates. The reaction product was visualized by using a Fujix BAS 2000 Bio-Imaging Analyzer. Lane 1, CHOP cells transfected with pCEV18 vector alone. Lane 2, CHOP cells transfected with the sialyltransferase clone pCEVh2,8ST.

fected with pCEVh2,8ST or with pPROTAh2,8ST by electroporation. Culture media prepared from the cells transfected with pCEVh2,8ST or pPROTAh2,8ST contained significant GD3 synthase activity (Table 1). But the secreted activity generated by pPROTAh2,8ST could be bound to IgG-Sepharose, whereas the activity generated by pCEVh2, 8ST was not bound. These data show that the protein encoded by the cloned cDNA directly catalyzes GD3 formation.

To further characterize the product by the cDNA, the product of soluble h2,8ST-protein A fusion was treated with sialidases from *Arthrobacter ureafaciens* (42) and *Salmonella typhimurium* LT2 (43). Both GM3 and the [^{14}C]NeuAc-labeled product from GM3 ganglioside were sensitive to *A. ureafaciens* sialidase (35). However, the product was insensitive to *S. typhimurium* LT2 sialidase, whereas substrate GM3 was degraded by *S. typhimurium* LT2 sialidase (data not shown). *S. typhimurium* LT2 sialidase shows a high kinetic preference for sialyl α2-3 linkage compared with α2-6 and α2-8 linkage and does not cleave inner α2-3-sialyl linkages (35, 43). Therefore, these data showed the [^{14}C]NeuAc-labeled product from GM3 was protected from digestion with *S. typhimurium* LT2 sialidase by additional sialyl linkage,

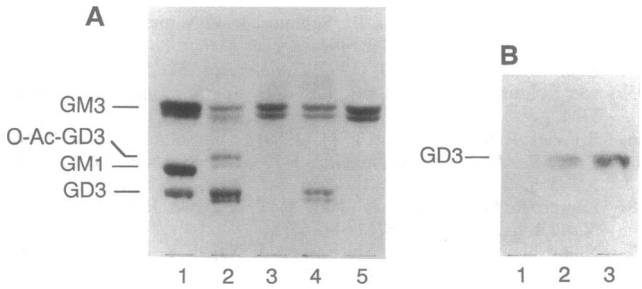

FIG. 5. Identification of the h2,8ST product in CHOP cells by orcinol staining (*A*) and TLC immunostaining analysis (*B*). (*A*) Gangliosides were prepared from SK-Mel-28 (lane 2), control CHOP cells (lane 3), CHOP cells transfected with the clone pCEVh2,8ST (lane 4), and CHOP cells transfected with pCEV18 vector alone (lane 5) and developed on a TLC plate. The samples were visualized with orcinol reagent. Authentic gangliosides migrated in lane 1 as standards. (*B*) Immunostaining analysis of the same samples in *A* with anti-GD3 monoclonal antibody R24. Lane 1, CHOP cells transfected with pCEV18 vector; lane 2, CHOP cells transfected with pCEVh2,8ST; and lane 3, standard gangliosides.

Table 1. Affinity chromatography of soluble GD3 synthase activity released from transfected COS-7 cells

	GD3 synthase activity, pmol/h					
	IgG-Sepharose			Sepharose		
Vector	Applied	Sup	Bound	Applied	Sup	Bound
pCEVh2,8ST	148	135	ND	148	148	ND
pPROTAh2,8ST	860	ND	457	860	860	ND

COS-7 cells were transfected with pCEVh2,8ST or pPROTAh2, 8ST. Culture media were subjected to chromatography on IgG-Sepharose or Sepharose. GD3 synthase activity was then determined on the matrix supernatants (Sup) and the washed matrices (Bound). Assay conditions were the same as for Fig. 4*B* except 4.15 μM CMP-[^{14}C]NeuAc (25×10^4 cpm) was used. Activity in 10 ml of culture medium is expressed as pmol of NeuAc incorporated in 1 h. ND, no detectable activity.

suggesting that the sialyltransferase transferred sialic acid to the terminal sialic acid residue of GM3. Monoclonal antibody R24 recognizes the structure NeuAcα2-8Siaα2-3Galβ1-4Glc (or GlcNAc) of GD3 (27, 41). These results indicate that the molecule encoded by this cloned cDNA is a sialyltransferase that transfers CMP-NeuAc to the terminal NeuAc residue of GM3 via an α2-8 linkage and generates GD3 ganglioside.

DISCUSSION

In this paper, we have isolated a cDNA encoding a CMP-NeuAc:NeuAcα2-3Galβ1-4Glcβ1-1'Cer α2,8-sialyltransferase (GD3 synthase) from human melanoma cells by using an expression cloning approach. Flow cytometric analysis, enzyme assay, and product characterization showed that the enzyme catalyzes the formation of GD3 by the transfer of a sialic acid molecule from CMP-NeuAc to the terminal sialic acid of GM3 via an α2-8 linkage. A GD3 synthase is a key enzyme in the "b" metabolic pathway of gangliosides (GD3, GD1b, GT1b, and GQ1b) which are associated with neural cell differentiation (44–47).

The deduced amino acid sequence indicates that the α2,8-sialyltransferase has two potential N-glycosylation sites. Hydropathy analysis suggests that the enzyme has a hydrophobic domain at the N terminus that may encode a transmembrane domain. Table 1 shows that the soluble form of h2,8ST protein that contains amino acids 35–341 is also catalytically active, as previously demonstrated for other glycosyltransferases cloned by the expression cloning strategy (19, 21, 37, 48). Therefore, we propose that the protein consists of a membrane-spanning domain, a stem domain, and a catalytic domain. This protein is relatively short in primary length compared with other sialyltransferases cloned to date. Apparently, the stem region is truncated because fewer amino acids (85 residues) exist between the membrane-spanning domain and a conserved region at the center of the protein (Fig. 2).

As shown in Fig. 3, a computer-assisted comparison of the sequence with the other sialyltransferases cloned to date revealed that the α2,8-sialyltransferase also contains the two conserved region, which is called the sialyl motif (11, 40). Although there are two possibilities as described by Wen *et al.* (11) that the sialyl motif bears a CMP-sialic acid binding site or a galactose acceptor binding site, its precise function remains unclear. The α2,3- and α2,6-sialyltransferases catalyze the transfer of sialic acid from CMP-sialic acid to terminal galactose of an acceptor, whereas the α2,8-sialyltransferase transfers sialic acid to the terminal sialic acid of an acceptor. It is therefore likely that the conserved regions are involved not so much in terminal galactoside acceptor binding as in CMP-sialic acid binding. Site-directed mutagenesis of the regions and cloning of the other type of

sialyltransferases exhibiting the different acceptor specificity are necessary to gain knowledge about the structure/function relationship of the sialyl motif.

GD3 is known as tumor-associated antigen of rat 3Y1 cells transformed with adenovirus *E1A* gene and *myc* (49–51). A high level of GD3 is expressed in human melanoma (24, 52). In addition, increase in the activity of sialyltransferase in melanoma is accompanied by GD3 expression (27). It is also reported that tumor necrosis factor-α-induced morphological changes of melanocytes occurs in parallel to increased expression of GD3 (53).

Multiple lines of investigation suggest that GD3 ganglioside plays an important role in growth, adhesion, and differentiation of not only melanoma cells but also normal cells, especially neuronal cells. Cell proliferation of melanoma cells is suppressed by treatment of anti-GD3 antibody (4). The carbohydrate moieties of GD3 and GD2 were shown to be involved in cell attachment of melanoma and neuroblastoma cells (5, 54). Furthermore, Sariola *et al.* (6) reported that anti-GD3 antibodies drastically inhibit the morphogenesis of epithelia and perturb the inductive epithelial–mesenchymal interactions during embryogenesis. Whether GD3 is directly involved in these cellular events is presently unknown. The cDNA reported here will enable us to determine expression patterns of GD3 synthase and the effects of the abolishment of this gene, and it will give clues to the biological functions of GD3 in mammalian cells.

In conclusion, we have isolated from human melanoma cells a cDNA encoding a 341-amino acid protein that directly causes transformed cells to express GD3 ganglioside. The sequence indicates that the enzyme belongs to a sialyltransferase family characterized by having the sialyl motif. This enzyme generates an α2-8 linkage of sialic acid. We expect that the cDNA cloned in this report will become an invaluable tool to elucidate the ganglioside functions.

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