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Isolation of G_{D3} synthase gene by expression cloning of G_{M3} α-2,8-sialyltransferase cDNA using anti-G_{D2} monoclonal antibody

(sialyl motif/ganglioside)

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ABSTRACT **For the isolation of ganglioside G_{D3} synthase (EC 2.4.99.8) cDNA, we developed an expression cloning approach that used an anti-G_{D2} monoclonal antibody for selection. A host recipient cell line that we have named KF3027-Hyg5 was also utilized. This cell line expresses high levels of G_{M2} as well as G_{M3} but no G_{D3} or G_{D2} and was constructed from mouse B16 melanoma cells transfected with the polyoma large tumor antigen gene (KF3027) and the previously cloned β-1,4-*N*-acetylgalactosaminyltransferase (EC 2.4.1.92) cDNA. Four rounds of transfection, monoclonal antibody 3F8 panning, and Hirt extraction resulted in the isolation of two cDNA clones, transfection of which directed the expression of G_{D3} in KF3027 and B16 melanoma cells and G_{D3} and G_{D2} in KF3027-Hyg5 cells. The cDNA contained a 1650-bp insert and a single open reading frame. The deduced amino acid predicted a type II membrane topology consisting of cytoplasmic (14 aa), transmembrane (18 aa), and catalytic (309 aa) domains. The sequence also predicted the presence of a sialyl motif similar to that found in the other sialyltransferases cloned so far. As expected, mRNA of this gene (2.6 kb) was strongly expressed in human melanoma lines.**

Gangliosides are glycosphingolipids containing sialic acids. They have been studied as molecules characteristically expressed in brain tissues of various mammals (1) and also as tumor markers of neuroectoderm-derived malignant cells (2) such as melanomas (3, 4) and neuroblastomas (5). Gangliosides,[§] especially G_{D3}, are highly expressed in human melanoma tissues and melanoma cell lines (7–9). Although G_{D3} is a relatively minor species among gangliosides present in the adult brain (10, 11), it is a major ganglioside in early stages of the development of fetal rat brain (12). Furthermore, G_{D3} appears in activated human T lymphocytes (13, 14) as well as in T-cell acute lymphoblastic leukemia cells (15, 16). To analyze mechanisms for the expression of G_{D3} in these biologically important systems, it would be helpful to isolate the G_{D3} synthase gene.

Although genes of a number of glycosyltransferases have recently been cloned (17, 18), glycosyltransferase genes responsible for the synthesis of gangliosides have not yet been isolated except for the β-1,4-*N*-acetylgalactosaminyltransferase (GalNAc-T; EC 2.4.1.92) gene (19). Since the utilization of the sialyl motif was introduced by Paulson's group as an efficient method to clone sialyltransferase genes using PCR (20), several sialyltransferase genes have been isolated by his group (20) and others (21).

In this study, we have developed a strategy to isolate the cDNA clones of G_{D3} synthase gene[¶] by using a modification of the eukaryotic expression cloning system originally established by Seed and Aruffo (22, 23). Using a similar approach, we previously cloned GalNAc-T cDNAs (19). To clone G_{D3} synthase cDNA, we used the mouse melanoma line B16 transfected with both the polyoma large tumor antigen and GalNAc-T cDNA as a recipient cell. For panning, we used the anti-G_{D2} monoclonal antibody (mAb), rather than anti-G_{D3}. This mAb reacts with G_{D2} converted from the direct product of the transfected G_{D3} synthase using preexisting GalNAc-T. The isolated G_{M3} α-2,8-sialyltransferase (Sia-T; EC 2.4.99.8) gene codes for a protein that contains a partial sialyl motif and is predicted to have a type II membrane topology characteristically found in glycosyltransferases.

MATERIALS AND METHODS

Preparation of a Recipient Cell Line for the Transfection. The KF3027 cell line, which had been used for the expression cloning of GalNAc-T (19), was used after transfection with pM2T1-1/MIK Hyg (G_{M2}/G_{D2} synthase cDNA clone inserted in pMIK Hyg). The newly established line (named KF3027-Hyg5) expressed G_{M2}, as well as G_{M3}, but not G_{D3} or G_{D2}. The cell line synthesized polyoma large tumor antigen.

Cloning of Sia-T cDNA. A cDNA library prepared from mRNA of YTN17 was kindly provided by Hatakeyama (Osaka University; ref. 19). YTN17 extracts showed moderate levels of Sia-T. Plasmid DNA was prepared from the library and was transfected into KF3027-Hyg5 using DEAE-dextran as described (19). The transfected cells were treated with mAb 3F8 at 5 μg/ml and panned on dishes coated with goat anti-mouse IgG as described (19). Transformation of *Escherichia coli* MC1061/P3 by Hirt extracts, transfection of the expanded plasmids into KF3027-Hyg5, and panning were repeated four times. Then, after screening 500 colonies divided into 20 groups, several clones that directed the expression of G_{D2} on KF3027-Hyg5 were isolated using microscale transfection and immunofluorescence assay.

DNA Sequencing. The cDNA insert was isolated by *Hin*dIII and *Not* I digestion and then transferred into phagemid BlueScript SK−. Deletion mutants of this BlueScript SK− clone were prepared with a deletion kit (Takara Shuzo, Kyoto). Dideoxynucleotide termination sequencing was per-

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Abbreviations: GalNAc-T, β-1,4-*N*-acetylgalactosaminyltransferase; Sia-T, G_{M3} α-2,8-sialyltransferase; mAb, monoclonal antibody; FITC, fluorescein isothiocyanate.

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§Ganglioside nomenclature is based on that of Svennerholm (6): G_{M3}, NeuAcα2-3Galβ1-4GlcCer; G_{M2}, GalNAcβ1-4(NeuAcα2-3)Galβ1-4GlcCer; G_{D3}, NeuAcα2-8NeuAcα2-3Galβ1-4GlcCer; G_{D2}, GalNAcβ1-4(NeuAcα2-8NeuAcα2-3)Galβ1-4GlcCer.

¶The nucleotide sequence reported in this paper has been deposited in the GenBank data base (accession numbers L32867).

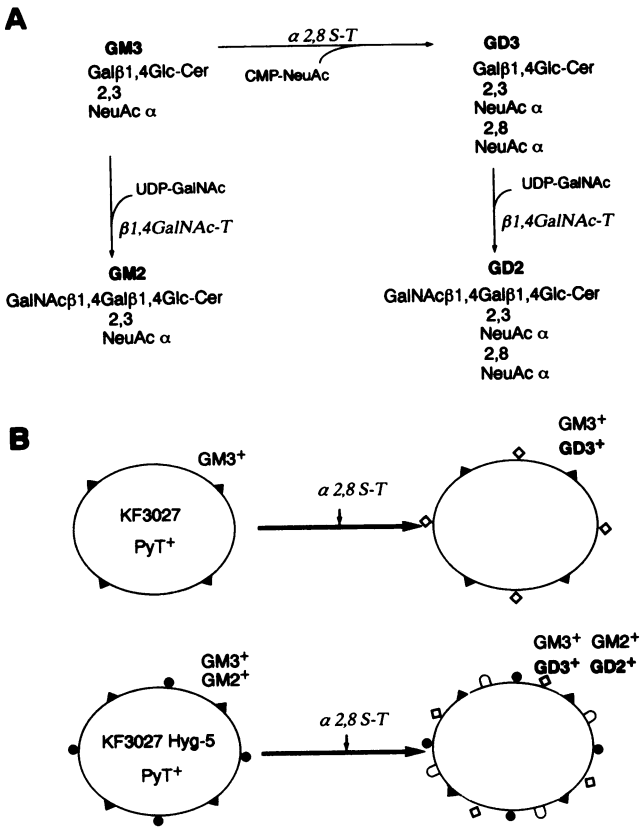

FIG. 1. Strategies for expression cloning of G_{D3} synthase cDNA. (*A*) Synthetic pathway of gangliosides G_{M2}, G_{D3}, and G_{D2}. (*B*) Strategies for the isolation of G_{D3} synthase cDNA by using anti-G_{D3} mAb (*Upper*) or anti-G_{D2} mAb (*Lower*). KF3027 is B16 melanoma transfected with the polyoma large tumor antigen gene (19), and KF3027-Hyg5 is a transfectant of KF3027 with pM2T1-1 (G_{M2}/G_{D2} synthase cDNA).

formed by using dye terminators with the Applied Biosystems model 373A DNA sequencing system.

Cell Lines. Human cell lines (obtained from L. J. Old at the Memorial Sloan–Kettering Cancer Center) were cultured as described (19).

Stable Transfectant Cell Lines. The cDNA clone pD3T-31 was cotransfected with pSV2Neo into B16 melanoma cells (B78) by calcium phosphate precipitation as described (19), and stable transfectant cell lines were obtained by selection with G418.

Flow Cytometry. Ganglioside expression was analyzed using mouse mAb 3F8 (anti-G_{D2}; ref. 5), mAb R24 (anti-G_{D3}; ref. 8), and mAb 10-11 (anti-G_{M2}; ref. 24) and fluorescein isothiocyanate (FITC)-conjugated second antibodies on a FACScan (Becton Dickinson) as described (19).

Sia-T Assay. cDNA clone pD3T-31 or pCDM8 was transfected into KF3027 or KF3027-Hyg5 cells in 10-cm dishes transiently by the DEAE-dextran method. After culture for 3 days, cells were harvested and used for the enzyme assay. The enzyme activity of Sia-T was determined according to the method described previously (25, 26). Separated products were analyzed by TLC using chloroform/methanol/2.5 M NH_4OH (60:35:8, vol/vol), and autoradiography.

Northern Blotting. The poly(A)$^+$ RNA fraction was isolated by an mRNA isolation kit (Pharmacia) from frozen cell pellets. Northern blotting was carried out as described (19).

Extraction of Gangliosides and TLC Immunostaining. Gangliosides were extracted from B78 cells stably transfected with pD3T-31 and pSV2Neo or pSV2Neo alone and then analyzed by TLC using a resorcinol spray or immunostaining as described (27).

RESULTS

Cloning Strategy for Isolation of cDNAs That Determine the Expression of G_{D3}. In the expression cloning of glycosyltransferases, a recipient host cell and a specific ligand for detection of the product are required. As we demonstrated in the cloning of GalNAc-T cDNA (19), B16 melanoma cells almost exclusively expressed G_{M3}. This profile of ganglioside expression seemed suitable for the expression cloning of Sia-T as well as GalNAc-T cDNA. However, trials using anti-G_{D3}

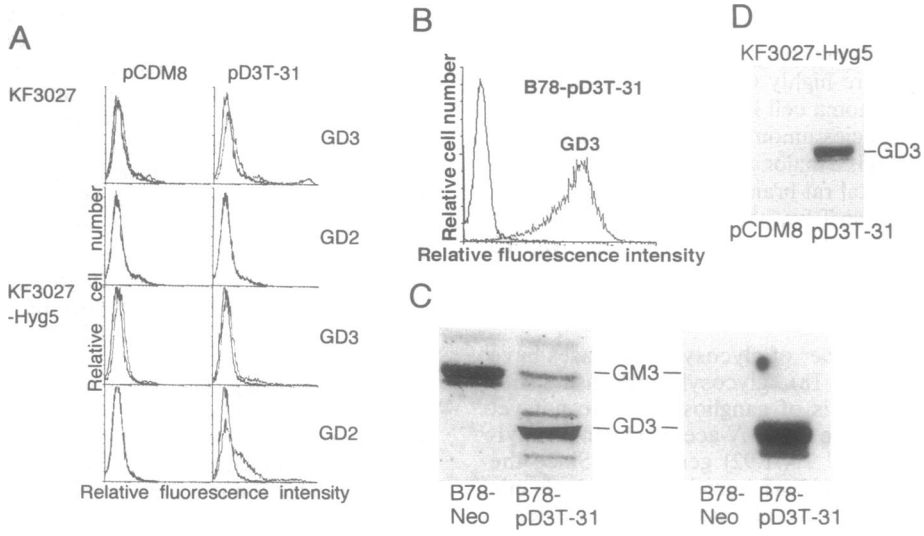

FIG. 2. Cloned pD3T-31 determines the expression of G_{D3} or G_{D3}/G_{D2} when introduced into KF3027 or KF3027-Hyg5, respectively. (*A*) Flow cytometry of transiently transfected KF3027 and KF3027-Hyg5 with pD3T-31 or pCDM8. Anti-G_{D3} (mAb R24) or anti-G_{D2} (mAb 3F8) was incubated with the transfectants, and antibody binding was detected by fluorescein isothiocyanate-conjugated anti-mouse IgG (thick lines). Thin lines represent controls performed with the second antibody alone. (*B*) G_{D3} expression on a stable transformant line of B16 (B78) with pD3T-31. G_{D3} was detected as in *A*. (*C*) Gangliosides extracted from stable transfectants. (*Left*) Resorcinol spray. (*Right*) TLC immunostaining with mAb R24. Establishment and ganglioside analysis of stable transfectants were done as described in *Materials and Methods*. (*D*) TLC pattern of enzyme assay products using extracts from the transient transfectants. KF3027 and KF3027-Hyg5 were transfected with pCDM8 or pD3T-31; then membrane fractions were prepared and analyzed for Sia-T activity in the extracts as described in *Materials and Methods*, and the products were analyzed by TLC. KF3027 transfectants showed identical results.

mAb R24 did not yield positive transfectants. We then changed the cloning strategy to isolate transfectants expressing an indirect product—i.e., G_{D2}—as shown in Fig. 1*B* (*Lower*).

Isolation of cDNA That Determines the Expression of G_{D2} in KF3027-Hyg5. After repeating the steps of transfection, panning, and Hirt extraction four times, two cDNA clones directing the expression of G_{D2} in KF3027-Hyg5 were obtained. As shown in Fig. 2*A*, KF3027 transiently transfected with pD3T-31 expressed G_{D3} but not G_{D2}, whereas KF3027-Hyg5 expressed both G_{D3} and G_{D2}. Although the positive staining for G_{D3} and/or G_{D2} was not striking, 5–10% positive cells was consistently detected. KF3027 and KF3027-Hyg5 transfected with pCDM8 alone did not show any G_{D3} or G_{D2} positivity. Mouse B16 melanoma cells stably transfected with the pD3T-31, on the other hand, stained uniformly and positively for G_{D3} (Fig. 2*B*). Gangliosides extracted from these stably transfected cells showed a strong G_{D3} resorcinol-positive band in TLC (Fig. 2*C Left*), and specifically stained with mAb R24 by TLC immunostaining (Fig. 2*C Right*). These results strongly suggest that this clone derives from the G_{D3} synthase gene. Analysis of the extracts from transiently transfected cells for Sia-T activity showed very high activity of G_{D3} synthase in both KF3027 and KF3027-Hyg5 (Fig. 2*D*). The enzyme activity in the KF3027-Hyg5 transfected with pD3T-31 shown in Fig. 2*D* was 29,833 units (pmol per mg per h), whereas that with pCDM8 was not detectable. These results support the expected identity of the clone.

The deduced protein sequence of the cloned cDNA predicts a type II membrane topology and the presence of a partial sialyl motif (Fig. 3). The cDNA in pD3T-31 is 1650 nucleotides long and consists of a 112-bp 5′-untranslated region and a continuous open reading frame of 1023 bp. The

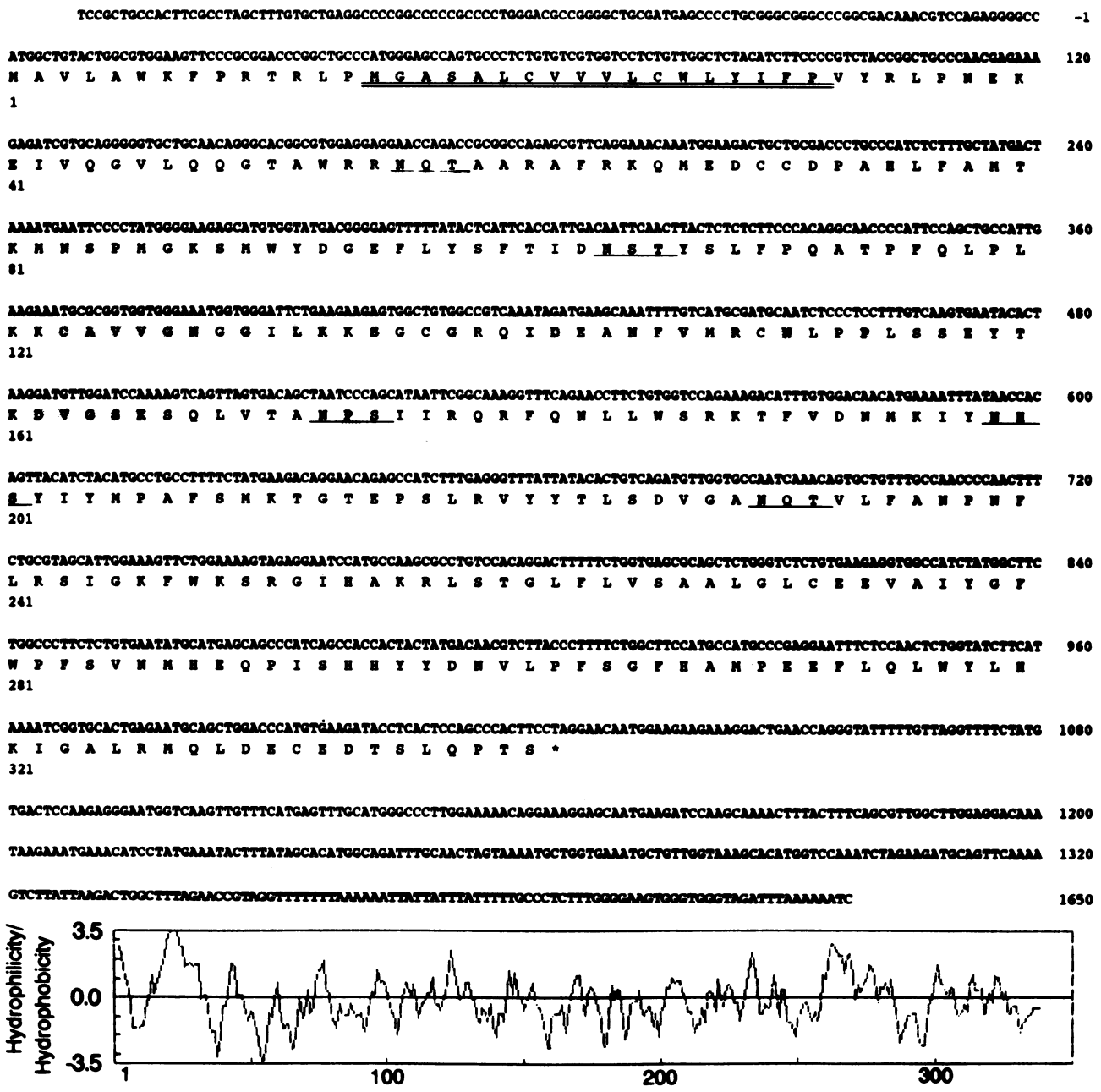

FIG. 3. (*Upper*) Nucleotide and deduced amino acid sequence of the cloned pD3T-31. The transmembrane region is underlined with double lines. Five potential N-glycosylation sites are underlined. Regions containing homology with the reported sialyl motif (20) are indicated by shaded squares. (*Lower*) Hydropathy of the predicted amino acid sequence, analyzed by the method of Kyte and Doolittle (28).

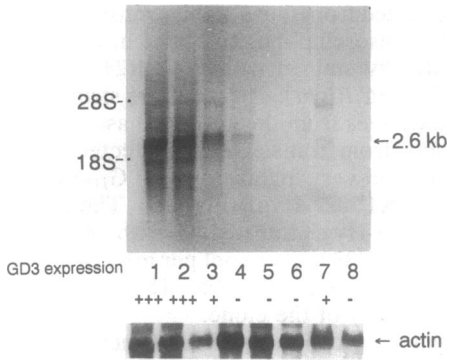

FIG. 4. mRNA expression of G_{D3} synthase gene in cancer cell lines. Poly(A)$^+$ RNA (10 μg) was separated in a formamide/agarose gel and blotted onto a nylon membrane. Then the membrane was hybridized with the ^{32}P-labeled cDNA insert of pD3T-31 as described in *Materials and Methods*. The sources of poly(A)$^+$ RNA are as follows: lane 1, SK-MEL-23; lane 2, SK-MEL-28; lane 3, SK-MEL-31 (these three lines are human melanomas); lane 4, ATN-1 (adult T-cell leukemia); lane 5, CCRF/CEM (T-cell acute lymphoblastic leukemia); lane 6, K562 (erythroleukemia); lane 7, YTN-17 (natural killer-like line); lane 8, B78 (B16 melanoma). G_{D3} expression of each cell line was graded as +++ (70–100%), ++ (40–70%), + (10–40%), and − (<10%) based on the results of flow cytometry.

initiation codon at the beginning of the open reading frame is embedded within a sequence similar to the Kozak consensus initiation sequence (29, 30). This open reading frame predicts a 341-aa protein with a molecular mass of 38,903 Da.

Searches of currently available nucleic acid and protein data bases (GenBank Release 80; Swiss-Prot Release 27) identified no genes with high nucleotide sequence homology (>50%) to this cDNA. However, a rat cDNA of Sia-T family (31) and a human Sia-T cDNA from submaxillary gland (M.-L. Chang and J. T. Lau, personal communication) showed significant homology in the sialyl motif region (20) of 48.7% and 45.4%, respectively. In this region, from amino acids 123 to 166, there are sequences relatively homologous with all the known sialyltransferases. Sequences from 123 to 128 and from 162 to 166 were very similar to the ends of the sialyl motif.

Inspection and hydropathy analysis of the predicted protein sequence suggested that this protein maintains a structural organization similar to those of known glycosyltransferases. There is a single hydrophobic segment near the amino terminus, which is made up of 18 aa and is flanked by basic residues. This putative signal-anchor sequence would place 14 aa within the cytosolic compartment and 309 residues within the Golgi lumen.

Northern Blots Revealed Strong mRNA Expression in Human Melanoma Cell Lines. As shown in Fig. 4, cell lines expressing G_{D3}, particularly melanoma lines, showed strong mRNA bands at 2.6 kb. In ATN-1, a faint band was detected, although G_{D3} could not be detected in this cell line. However, the expression of G_{D2} in ATN-1 suggests that this cell line does contain G_{D3} synthase.

DISCUSSION

The expression cloning of glycosyltransferase genes is an indirect approach in which cells expressing carbohydrate antigens generated by direct products of transfected genes are selected. Host recipient cells are, therefore, required to contain sufficient amounts of the precursor structure and no or low level of the target structure. Since the approach of expression cloning to isolate Sia-T cDNA by using anti-G_{D3} mAb R24 was not successful, we changed the strategy to select the more complex ganglioside G_{D2} instead of G_{D3}. We took advantage of dual function of GalNAc-T, cDNA of which can determine the expression of both G_{M2} and G_{D2} when introduced in the cells with appropriate precursors (18). As shown in Fig. 1, B16 transfected by pM2T1-1 (G_{M2}/G_{D2} synthase cDNA) expresses G_{M2}, but not G_{D2}, in the absence of G_{D3} synthase. However, when G_{D3} synthase cDNA plasmids are introduced, the cells should synthesize both G_{D3} and G_{D2}. Apparently because of the superiority of anti-G_{D2} mAb over the anti-G_{D3} for panning, we were able to successfully clone an Sia-T cDNA.

Using the sialyl motif reported by Paulson and coworkers (20), several sialyltransferase cDNAs have been cloned by PCR (20, 21). In the predicted amino acid sequence of the cloned Sia-T, there are also sequences fitting the sialyl motif between amino acids 123 and 166. As shown in Fig. 5, the homology between the cloned Sia-T (ST8G) and other sialyltransferases is slightly lower than the homology among the other three sialyltransferases. In addition, Sia-T has one more amino acid in the sequence of the sialyl motif in comparison to the other sialyltransferases. This may be a specific characteristic of sialyltransferases that catalyze the synthesis of gangliosides. Molecular cloning of other sialyltransferases involved in ganglioside synthesis will clarify this point.

There have been many studies on the characteristic expression of ganglioside G_{D3} in development (12, 32) and malignant transformation (3, 4, 7–9). In addition to human melanoma, acute T-cell leukemia and adult T-cell leukemia cells also express G_{D3} (15, 16, 33). Moreover, normal T lymphocytes can be activated by anti-G_{D3} mAb, resulting in the increase of G_{D3} expression (13, 14, 34). Expression of G_{D3} in a fetal rat cell line transfected with an adenovirus gene coding E1A protein has also been reported (35, 36). These findings suggest that G_{D3} expression is strongly associated with cell activation or transformation. The regulatory mechanisms for the expression of G_{D3} and the biological roles of

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ST3N156  CRRCIIVGNGGVLANKSLGSRIDDYDIVIRLNSAP
ST3O142  CRRCAVVGNSGNLKESYYGPQIDSHDFVLRMNKAP
ST6N178  YQRCAVVSSAGSLKNSQLGREIDNHDAVLRFNGAP
ST8G120  LKKCAVVGNGGILKKSGCGRQIDEANFVMRCNLPP

ST3N191  VKGFE   KDVGSKTTLRITYPE
ST3O177  TEGFE   ADVGSKTTHHFVYPE
ST6N213  TDNFQ   QDVGSKTTIRLMNSQ
ST8G155  LSSEYT  KDVGSKSQLVTANPS
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FIG. 5. Partial amino acid sequence of the G_{D3} synthase deduced from the pD3T-31 insert showing the homology with other sialyltransferases. Amino acids from 120 to 175 were compared with corresponding portions of other sialyltransferases. Shaded letters represent highly homologous positions (sialyl motif) (20). ST3N represents rat Gal(β1-3/1-4)GlcNAc(α2-3) sialyltransferase. ST30 indicates porcine Gal(β1-3)GalNAc(α2-3) sialyltransferase, and ST6N is rat Gal(β1-4)GlcNAc(α2-6) sialyltransferase. ST8G is human G_{D3} synthase.

G_{D3} can now be investigated more precisely by using the Sia-T cDNA clones.

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