

Human α 2,3-Sialyltransferase (ST3Gal II) Is a Stage-specific Embryonic Antigen-4 Synthase*

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Monosialosyl globopentaosylceramide (MSGb5), originally described as stage-specific embryonic antigen-4, is expressed in testicular germ cell tumors and in aggressive cases of human renal cell carcinoma (RCC). Clarification of the molecular mechanisms regulating synthesis of MSGb5 is very important to understand testicular carcinogenesis and the malignant progression of human RCC. For this purpose, we have investigated α 2,3-sialyltransferase involved in the synthesis of MSGb5. We used the method of expression cloning combined with polymerase chain reaction targeted to sialylmotif to isolate a cDNA clone from RCC cell line ACHN library. The cloned cDNA was found to be identical to the previously cloned ST3Gal II in sequence. A soluble recombinant form of the protein in COS-1 cells showed an enzyme activity of α 2,3-sialyltransferase toward globopentaosylceramide (Gb5) in addition to asialo-GM1 and GM1a. Transient transfection of COS-7 and ACHN cells with this cDNA induced an increase of MSGb5, whereas stable transfection of antisense ST3Gal II cDNA suppressed expression of MSGb5 in ACHN cells. The ST3Gal II mRNA level was increased in 7 of 8 RCC cell lines and in all six RCC tissues surgically obtained, although it was not necessarily consistent with the MSGb5 level in RCC cell lines. This study indicates that ST3Gal II is a MSGb5 (stage-specific embryonic antigen-4) synthase and that its increased expression level is closely related to renal carcinogenesis.

Gangliosides are glycosphingolipids containing sialic acids, which are classified as ganglio-, lacto-, and globo-series, depending on the core structure. Gangliosides undergo qualitative and quantitative changes during development, differentiation, and carcinogenesis (1). In relation to malignant progression, they play roles in tumorigenicity (2), in the inhibition of T cell function (3, 4), and as adhesion receptors followed by activation of signal transducers leading to enhanced motility and invasiveness (5).

Monosialosyl globopentaosylceramide (MSGb5)¹ also known

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¹ The abbreviations used are: MSGb5, monosialosyl globopentaosylceramide; SSEA-4, stage-specific embryonic antigen-4; Gb5, globopen-

as stage-specific embryonic antigen-4 (SSEA-4) (6, 7), is expressed maximally in murine four-cell stage embryo (8), in testicular germ cell tumors (9, 10) and in aggressive cases of human renal cell carcinomas (RCC) (11, 12). Recent work on MSGb5 showed that monoclonal antibody (mAb) RM1 specific to MSGb5 stimulated invasive properties of human breast carcinoma cell line MCF-7 (13). On the other hand MSGb5 (SSEA-4) is also important as a cell surface marker for embryonic stem cells (14, 15). Therefore, cloning of α 2,3-sialyltransferase determining synthesis of MSGb5 may contribute to understanding not only of testicular carcinogenesis and malignant progression of RCC and breast carcinoma but also of mouse embryogenesis and stem cell research.

To clone the cDNA of α 2,3-sialyltransferase responsible for synthesis of MSGb5, which is the first ganglioside in the globoseries ganglioside pathway (Fig. 1), we utilized a transient expression cloning system in COS-7 cells according to the procedure by Seed and Aruffo (16). By means of this system combined with polymerase chain reaction targeted to sialylmotif, cDNA coding the gene that determines the expression of MSGb5 was isolated with mAb RM1, from a cDNA library of human RCC cell line ACHN. The cloned cDNA was found to be identical to ST3Gal II (17) in sequence. Analyses of the newly expressed gangliosides and the sialyltransferase activity in transfected cells indicate that ST3Gal II is a SSEA-4 (MSGb5) synthase. We also found that the ST3Gal II mRNA level was indeed increased in most RCC cell lines and renal tumor tissues examined.

EXPERIMENTAL PROCEDURES

Materials—Asialo-GM1, GM1, GM2, and GD1a were purchased from Sigma. Globopentaosylceramide (Gb5), monosialosyl globopentaosylceramide (MSGb5), and GM3 were prepared from pooled renal tumor tissues as described previously (18).

Cells and Monoclonal Antibodies—The human RCC cell line ACHN was purchased from Dainihonsei-yaku Co. (Osaka, Japan). Human RCC cell lines SMKT-R2, -R3, and -R4 were kindly provided by Dr. N. Miyao (Sapporo Medical College) (19, 20), and TOS-1, -2, and -3 and 3LN were established in our laboratory (21). COS-7 and COS-1 cells were obtained from Human Science Research Resources Bank (Osaka, Japan), and CV-1 cells were from Riken Cell Bank (Wako, Japan). Mouse IgM was purchased from Sigma. The mouse IgM anti-SSEA3 mAb (MC631) was purchased from the Developmental Studies Hybridoma Bank at the University of Iowa. The mouse IgM anti-MSGb5 mAb RM1 (18) was purified by ionic exchange chromatography (Takara Shuzo, Kyoto, Japan). Goat anti-mouse IgM was purchased from Zymed Laboratories, Inc. (San Francisco, CA).

Construction of cDNA Library—Double-stranded cDNAs were synthesized with oligo(dT) primers using poly(A⁺) RNA prepared from human ACHN cells. After addition of *Bst*XI adapters (Invitrogen), the cDNA was size-fractionated (>0.4 kb) and ligated into expression vector

taosylceramide; RCC, renal cell carcinoma; mAb, monoclonal antibody; FACS, Fluorescence-activated cell sorter analysis; PBS, phosphate-buffered saline; ST, sialyltransferase; TLC, thin layer chromatography.

pME18S, which was kindly provided by Dr. Maruyama (Tokyo Medical and Dental College). The library contained 2×10^6 independent clones in *Escherichia coli* strain DH10B (Invitrogen).

Isolation of a Human $\alpha 2,3$ -Sialyltransferase cDNA Clone—TLC immunostaining and fluorescence-activated cell sorter (FACS) analysis

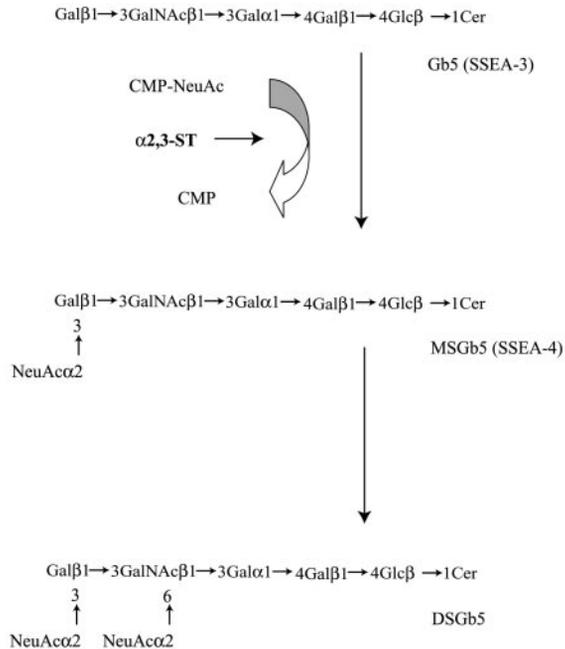
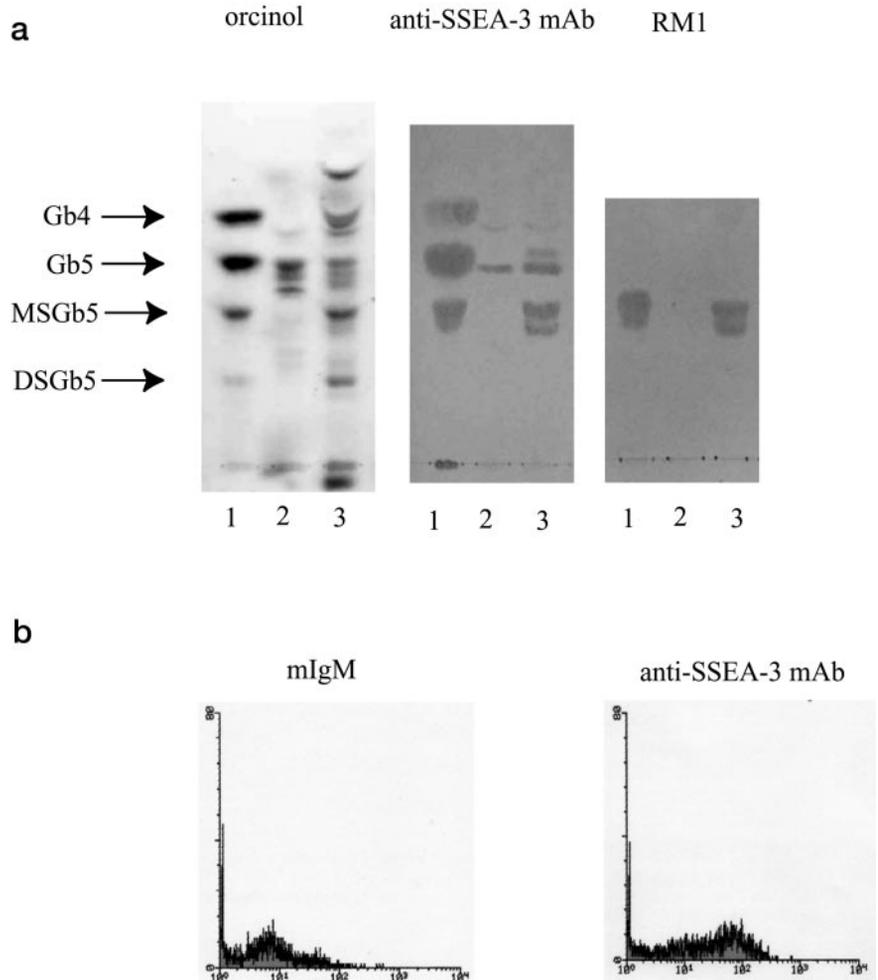


FIG. 1. Synthetic pathway of globo-series gangliosides.

showed that COS-7 cells express Gb5 and the precursor of MSGb5 but not MSGb5 (Fig. 2). Eight samples of 7×10^6 COS-7 cells were transfected with 30 μ g of plasmid DNA each in 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 II (Bio-Rad) at 220 V and 975 microfarad. Three days after transfection, the exponentially growing cells were harvested, blocked with 5% fetal calf serum in PBS for 1 h, and incubated with anti-MSGb5 mAb RM1 (10 μ g/ml) for 1 h. The cells were washed twice with PBS and incubated in a Petri dish (Falcon 1007) coated with anti-mouse IgM goat IgG (Zymed Laboratories Inc.). Plasmid DNA was rescued from adherent cells by Hirt extraction (22) and used to transform *E. coli* DH10B (Invitrogen) by electroporation. Adherent cells were observed under the inverted microscope to increase from 100–200/dish after the first round of transfection and panning and about 500–1000 after the second round to $\sim 1 \times 10^4$ after the fifth round. After seven rounds of transfection and panning, we tried to isolate a cDNA clone that directs synthesis of MSGb5 by sibling selection but failed to identify positive transfectants. We then employed PCR with degenerate primers to detect a sialylmotif (23) that might be concentrated as the round of transfection and panning advanced. The primers were sense 5'-TGCCGSCGCTGYGTGGTGTGGGG and the antisense 5'-GGTGGTYYKGSWSCCAACATC (S = C, G; Y = C, T; B = C, G, T; K = G, T; W = A, T). PCR was carried out with Ampli Taq Gold (Perkin-Elmer) by 40 cycles of 94 $^{\circ}\text{C}$ for 30 s, 50 $^{\circ}\text{C}$ for 1 min, and 72 $^{\circ}\text{C}$ for 1 min. The PCR product was gradually concentrated as the cycles of transfection and panning advanced. The concentrated PCR product was isolated and cloned to pBluescript, and the 14 samples were sequenced. All of the samples of PCR product were identical to the sialylmotif of ST3Gal II in sequence. Then we isolated a cDNA of 2.3 kilobase pairs (named pME-Gb5ST) identical to ST3Gal II in sequence using the amplified PCR product as a probe.

PCR of the β -Galactoside $\alpha 2,3$ -Sialyltransferase cDNAs—So far the three β -galactoside $\alpha 2,3$ -sialyltransferases including ST3Gal II have been reported (17, 24, 25). To determine which sialyltransferase was most enriched during the cycles of transfection and panning, we examined the

FIG. 2. TLC immunostaining of upper phase glycolipids extracted from COS-7 cells and ACHN cells (a) and FACS analysis of Gb5 expression in COS-7 cells by anti-SSEA-3 mAb (b). a, left panel, orcinol staining. Middle panel, immunostaining by anti-SSEA-3 (Gb5) mAb. Right panel, immunostaining by anti-SSEA-4 (MSGb5) mAb RM1. Lanes 1, reference glycolipids; lanes 2, glycolipids extracted from COS-7 cells; lanes 3, glycolipids extracted from ACHN cells. COS-7 cells express Gb5, but not MSGb5. b, expression of Gb5 was observed in COS-7 cells by FACS analysis. DSGb5, disialosyl globopentaosylceramide.



level of the three sialyltransferase cDNA (ST3Gal I, ST3Gal II, and ST3Gal IV) by PCR in the plasmids extracted from the adherent cells every cycle of transfection and panning. The primers were sense 5'-CCC-GAATTCAAGATGGTGACCCTGCGGAAG and antisense 5'-CCCTCAGATCATCTCCCCTTGAAGATAAG for ST3Gal I, sense 5'-CCCGGATCCGGCCTGCAGCGCCTCAGCAAG and antisense 5'-CCCGGATCCGCCCCGGTGCATAGATGG for ST3Gal II, and sense 5'-ATGTGTCCTGCAGGCTGGAAGCTC and antisense 5'-TCAGAAGGACGTGAGGTCTTTGAT for ST3Gal IV. PCR was carried as described above for ST3Gal I and ST3Gal IV and by 30 cycles for ST3Gal II.

DNA Sequence—The cDNA was sequenced with a model 4200L-2 DNA autosequencer (LI-COR) using a Thermo Sequenase cycle sequencing kit (Amersham Biosciences) with fluorescence-labeled M13 forward and reverse primers.

Construction of the Sialyltransferase Ligated into pCEP4 Vector—To examine whether the Gb5ST cDNA encodes the enzyme in RCC cell lines, the cDNA was excised from pME-Gb5ST with *Xho*I and subcloned into pCEP4 vector (Invitrogen), and the sense and the antisense Gb5ST cDNA ligated into pCEP4 were respectively obtained.

Construction of a Soluble Form of the Sialyltransferase—A truncated form of Gb5ST, which lacks 55 amino acids from the NH₂ terminus of the open reading frame, was prepared by PCR as described previously using a 5' primer containing an in-frame *Bam*HI site and a 3' primer located 50 bp downstream of the stop codon with *Bam*HI site (17). The PCR fragment was subcloned into *Bam*HI site of the pSVL vector containing the protein A (pSVL-protA) kindly provided by Dr. J. C. Paulson (Scripps Research Institute) to yield expression plasmid pSVL-protA-Gb5ST.

Expression of a Soluble Form of the Sialyltransferase and the Enzyme Activity Assay—The expression plasmid (1 μ g) was transfected into COS-1 cells on a 60-mm culture dish using Effectene transfection reagent (Qiagen). After 48 h, the cell culture medium collected from three dishes was combined (15 ml), and 5 ml of the culture medium was concentrated by ultrafiltration using a Centricon 10 (Amicon). The remaining 10 ml of the culture medium was mixed with 100 μ l of equilibrated IgG-Sepharose beads (Amersham Biosciences), followed by gentle shaking for 12 h at 4 °C. The Sepharose beads were collected by centrifugation and washed three times with serum-free Dulbecco's modified Eagle's medium. The beads were then resuspended in 200 μ l of serum-free medium and used for sialyltransferase assay. A part (5 ml) of the medium after incubation with the beads was also collected and concentrated as described above. The original culture medium concentrated, the culture medium concentrated after incubation with the beads, and the beads after incubation with the original culture medium were assayed for sialyltransferase activity using glycolipids as acceptor substrate. The reaction mixture contained 10 mM MgCl₂, 0.2% Triton X-100, 50 mM sodium cacodylate buffer, pH 6.5, 2 mM CaCl₂, 0.2 mM 2,3-dehydro-2-deoxy-*N*-acetylneuraminic acid (Sigma), 10 μ l of 1 mM CMP-[¹⁴C]NeuAc (Amersham Biosciences), 23.5 μ l of the concentrated culture medium or 10 μ l of the beads plus 13.5 μ l of the medium, and 0.1 mM acceptor in total volume of 50 μ l. After incubation at 37 °C for 2 h, the reaction was terminated by adding 200 μ l of PBS. The product was isolated by C18 Sep-Pak cartridge (Waters, Milford, MA) and subjected to chromatography on a high performance thin layer chromatography plate (Baker) with a solvent system of chloroform, methanol, and water (50:40:10) containing 0.05% CaCl₂. The radioactivity on a plate was visualized with a BAS 2000 image analyzer (Fuji Film, Tokyo, Japan).

Stable Transfection with the Sense or Antisense Gb5ST cDNA Ligated into pCEP4—The sense or antisense Gb5ST cDNA was stably transfected into ACHN cells to examine whether the sense cDNA further increases MSGb5 level and whether the antisense cDNA down-regulates MSGb5 level. For transfection, Effectene transfection reagent was used as recommended by the supplier. Stable transfectants were selected under hygromycin (300 μ g/ml).

RNA Preparation and Northern Blot Analysis—Total RNA was prepared respectively from RCC cell lines, nontumor kidney tissues, and renal tumor tissues by the acid guanidium-phenol-chloroform method (26). 5 μ g of total RNA from cell line or 15 μ g of that from surgically obtained tissue was separated on 1.0% agarose-formaldehyde gel and then transferred to Hybond-N+ membrane (Amersham Biosciences). The truncated form of ST3Gal II was used as a probe. The membrane was hybridized with labeled cDNA probe at 65 °C in a solution containing 136 mM Na₂HPO₄, 158 mM NaH₂PO₄, 7% SDS, 1 mM EDTA, and 10 μ g/ml salmon sperm DNA and then washed in 2 \times SSC solution containing 0.1% SDS at 65 °C. The radioactivity was visualized with a BAS 2000 image analyzer (Fuji Film). We obtained the informed consent from the patients prior to the experiments.

FACS Analysis—Cells untreated or transfected with the plasmids were harvested by 0.02% EDTA in PBS, incubated with primary anti-

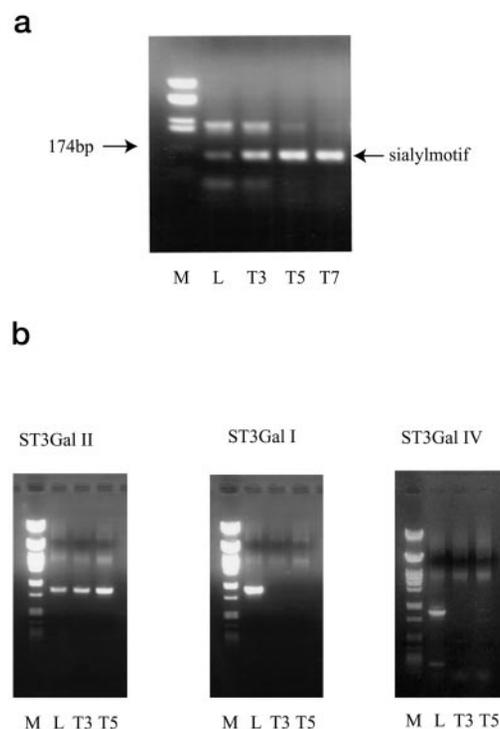


FIG. 3. Changes of PCR products targeted to sialylmotif (a) and to the β -galactoside α 2,3-sialyltransferase cDNAs (b) during the expression cloning procedure. M, marker (pUC/*Hae*III for figure 3a and *λ* PstI for figure 3b); L, T3, T5, and T7, template plasmids; L, ACHN cDNA library plasmids; T3, T5, and T7, plasmids rescued from COS-7 cells after three, five, and seven rounds of transfection and panning, respectively. PCR amplification was 40 cycles for ST3Gal I and ST3Gal IV and 30 cycles for ST3Gal II.

body for 90 min on ice, washed twice with 1% bovine serum albumin in PBS, and then incubated with fluorescein isothiocyanate-conjugated goat F(ab')₂ directed to mouse IgG and IgM (Tago, Burlingame, CA) for 45 min on ice. After washing twice, the cells resuspended in PBS were subjected to FACS analysis.

Isolation and Analysis of Gangliosides from Cells—Purification of gangliosides and TLC immunostaining were performed as described before (18).

RESULTS

Molecular Cloning of a MSGb5 (SSEA-4) Synthase—In the expression cloning of sialyltransferase, the host cells that express acceptor, but no or little if any sialyl-acceptor (product) and a ligand highly specific for the product are required. COS-7 cells were positively stained by anti-SSEA-3 mAb but not by RM1 (anti-MSGb5 mAb) as demonstrated in Fig. 2. Therefore, we used COS-7 cells as a recipient host for the expression cloning of MSGb5 synthase and mAb RM1 as a specific ligand for the product (MSGb5). We identified a cDNA directing MSGb5 synthesis with combination of PCR using degenerate primers targeted to sialylmotif. The PCR product was gradually concentrated as the cycles of transfection and panning advanced (Fig. 3a). Using the amplified PCR product as a probe, a cDNA of 2.3 kilobase pairs (named pME-Gb5ST) identical to ST3Gal II in nucleotide sequence was obtained. Because so far the three β -galactoside α 2,3-sialyltransferases (ST3Gal I, ST3Gal II, and ST3Gal IV) have been cloned, we examined which sialyltransferase cDNA was concentrated after each round of transfection and panning. Although the PCR product of ST3Gal II was concentrated, those of ST3Gal I and ST3Gal IV were rather lost after repeating the procedures of transfection and panning (Fig. 3b).

Expression of MSGb5 Synthase cDNA—To investigate whether the cloned cDNA directs MSGb5 expression, the ex-

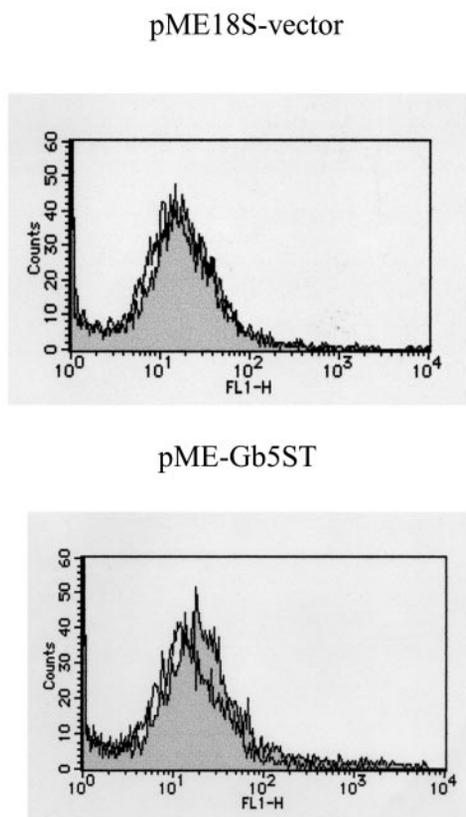


FIG. 4. **MSGb5 expression in COS-7 cells transiently transfected with Gb5ST cDNA.** COS-7 cells transiently transfected by pME-18S vector (*upper panel*) or pME-Gb5ST (*lower panel*) with sialidase inhibitor. *Shaded area*, RM1 as a primary antibody; *solid line*, mouse IgM as a negative control. With the inhibitor, a 10–14% increase of MSGb5 by transfection of pME-Gb5ST was observed reproducibly in four experiments, whereas without the inhibitor the increase of MSGb5 was only 2–5% (data not shown).

pression plasmids pME-Gb5ST and pCEP4-Gb5ST were transiently transfected into COS-7 and ACHN cells, respectively. Because COS-7 cells were found to have high sialidase activity, COS-7 cells transiently transfected by pME-Gb5ST were cultured with sialidase inhibitor, 2,3-dehydro,2-deoxy-*N*-acetylneuraminic acid (Sigma) (200 μ M). The culture medium with the inhibitor was changed every 24 h before FACS analysis. After 48 h the cells were harvested, incubated with RM1, and subjected to FACS analysis. With sialidase inhibitor, COS-7 cells transfected with Gb5ST cDNA showed a significant increase of MSGb5 compared with those transfected with vector (Fig. 4), which was reproducible in four experiments even though the difference was not remarkable (10–14%). On the other hand, the increase of MSGb5 was only 2–5% for COS-7 cells without sialidase inhibitor and ~7% for ACHN cells compared with those transfected with vector (data not shown).

To confirm that the cDNA clone encodes MSGb5 synthase, we constructed a soluble recombinant form of the enzyme. As shown in Fig. 5 (*a* and *b*), the concentrated cell culture medium from COS-1 cells transiently transfected with the plasmid (pSVL-protA-Gb5ST) showed the transfer activity of [14 C]NeuAc to Gb5, asialo-GM1, and GM1a, but no radioactivity was detected toward the other glycolipids. To further verify that the sialyltransferase activity toward Gb5 was derived from pSVL-protA-Gb5ST, the culture medium after incubation with IgG-Sepharose beads and the beads after incubation with the original culture medium were used for sialyltransferase assay. Sialyltransferase activity was only detected for the fractions adsorbed to the beads but not in the nonadsorbed fractions (the

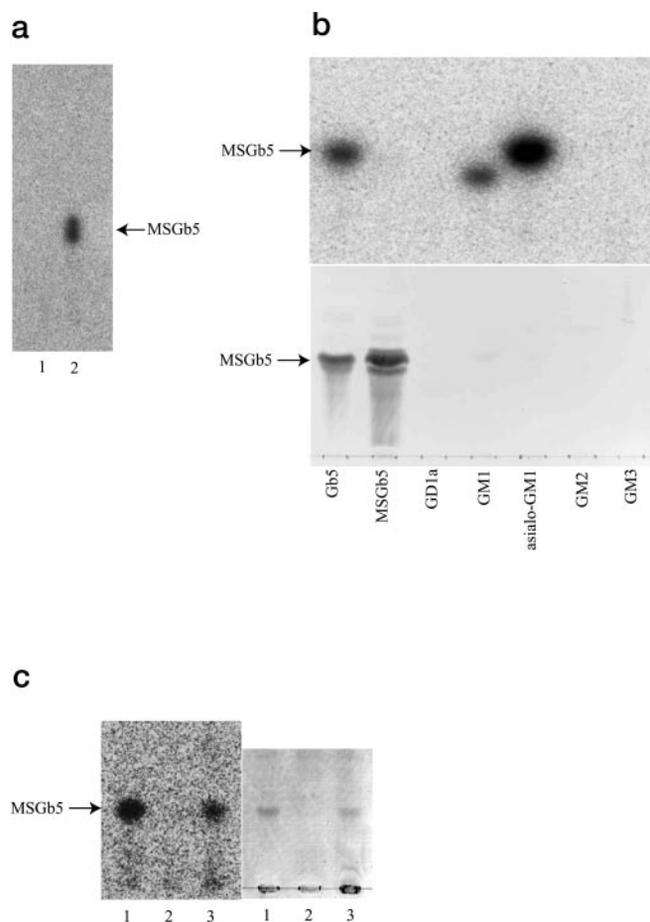


FIG. 5. **Sialyltransferase activity of the soluble recombinant form of ST3Gal II (pSVL-protA-Gb5ST).** *a*, ST activity toward Gb5 using the concentrated culture medium from COS-1 cells transfected with vector (pSVL-protA) (*lane 1*) or with pSVL-protA-Gb5ST (*lane 2*). Transfer of [14 C]NeuAc to Gb5 was observed with a BAS 2000 image analyzer. *b*, ST activity toward various glycolipids using concentrated culture medium. *Upper panel*, radioactivity of [14 C]NeuAc transferred to various glycolipids. *Lower panel*, TLC immunostaining by RM1. The same plate was used for detection of radioactivity and immunostaining. *c*, ST activity toward Gb5 using IgG-Sepharose beads. *Left panel*, radioactivity of [14 C]NeuAc transferred to Gb5. *Right panel*, TLC immunostaining by RM1. The same plate was used for the detection of radioactivity and immunostaining. *Lanes 1*, the original culture medium concentrated. *Lanes 2*, the culture medium concentrated after incubation with IgG-Sepharose beads. *Lanes 3*, IgG-Sepharose beads after incubation with the original culture medium.

culture medium after incubation with the beads) (Fig. 5c). In this experiment, less than 40% of the total activity in the culture medium was recovered in the adsorbed fractions, the low recovery being probably due to partial inhibition of the reactions by the beads themselves. These data indicate that the secreted proteins encoded by the cDNA directly catalyze MSGb5 formation.

The antisense Gb5ST cDNA ligated into pCEP4 was then stably transfected into ACHN cells to confirm whether the antisense cDNA down-regulates the MSGb5 level. FACS analysis showed that expression of MSGb5 was suppressed in the stable transfectants with the antisense cDNA (Fig. 6a). To further confirm that the level of MSGb5 was decreased, TLC analysis was carried out. Total glycolipids corresponding to the same wet weight (10 mg) from the stable transfectants were subjected to chromatography on the TLC plate and stained by orcinol sulfuric acid. MSGb5 level from each transfectant was determined by comparing with standard MSGb5, the concentration of which was calculated in advance. The densitometric

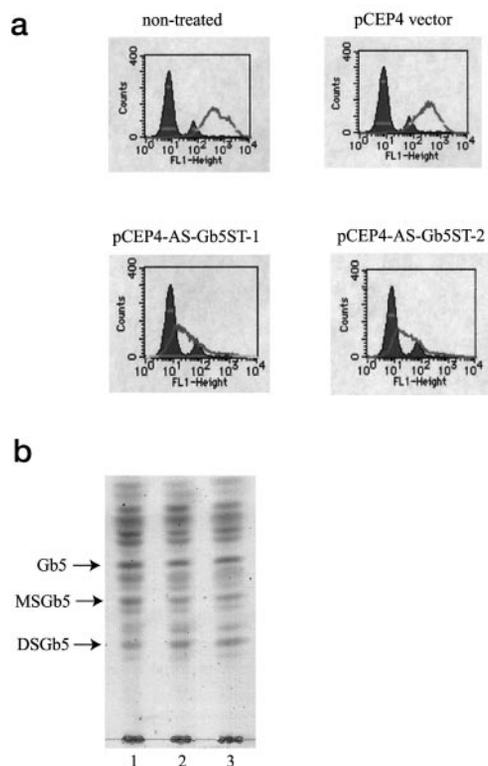


FIG. 6. Suppression of MSGb5 expression in ACHN cells stably transfected with the antisense Gb5ST cDNA. *a*, FACS analysis; *b*, TLC pattern. *a*, RM1 (solid line) was used as primary antibody, and mouse IgM (shaded area) as a negative control. Cell surface MSGb5 expression was suppressed in the stable transfectants with the antisense Gb5ST cDNA (pCEP4-AS-Gb5ST-1 and -2). *b*, TLC pattern of total glycolipids extracted from cell pellets. Lane 1, ACHN cells stably transfected with pCEP4 vector; lanes 2 and 3, stable transfectants with pCEP4-antisense Gb5ST (pCEP4-AS-Gb5ST-1 and -2 as shown in *a*). TLC confirmed the significant decrease in MSGb5 synthesis by the antisense cDNA.

analysis were performed using Scion image (Scion Co.), and the values were normalized to Gb5 showing almost the same level in all the transfectants. The assessed amounts of MSGb5 from the stable transfectants were 71 (vector), 48 (AS-Gb5ST-1), and 52 (AS-Gb5ST-2) pmol/total glycolipid from 10 mg of cell pellets, respectively. (Fig. 6*b*). Thus, TLC confirmed the significant decrease in MSGb5 synthesis by the antisense cDNA. Unexpectedly, precursor (Gb5) accumulation was not observed with the antisense cDNA compared with vector (Fig. 6*b*). In an attempt to further increase MSGb5 level in ACHN cells, stable transfection with the sense cDNA was performed. However, isolation of stable transfectants with the sense cDNA has not been successful because of reduced viabilities.

ST3Gal II Level in RCC Cell Lines and Renal Tumor Tissues—To examine whether the sialyltransferase is involved in renal carcinogenesis, we analyzed the levels of ST3Gal II mRNA along with those of MSGb5 in human RCC cell lines. A high level of ST3Gal II mRNA was detected in all human RCC cell lines except SMKT-R2, although levels of ST3Gal II mRNA were not necessarily consistent with those of MSGb5 (Fig. 7). When the ST3Gal II mRNA level was examined in nontumor kidney and renal tumor tissues, the level was higher in all six cases of RCC than nontumor kidney tissues (Fig. 8).

DISCUSSION

The present study described the isolation of a cDNA clone directing synthesis of MSGb5 (SSEA-4), which was found to be identical to ST3Gal II. Although COS-7 cells which express Gb5 but not MSGb5 seemed to be an appropriate recipient

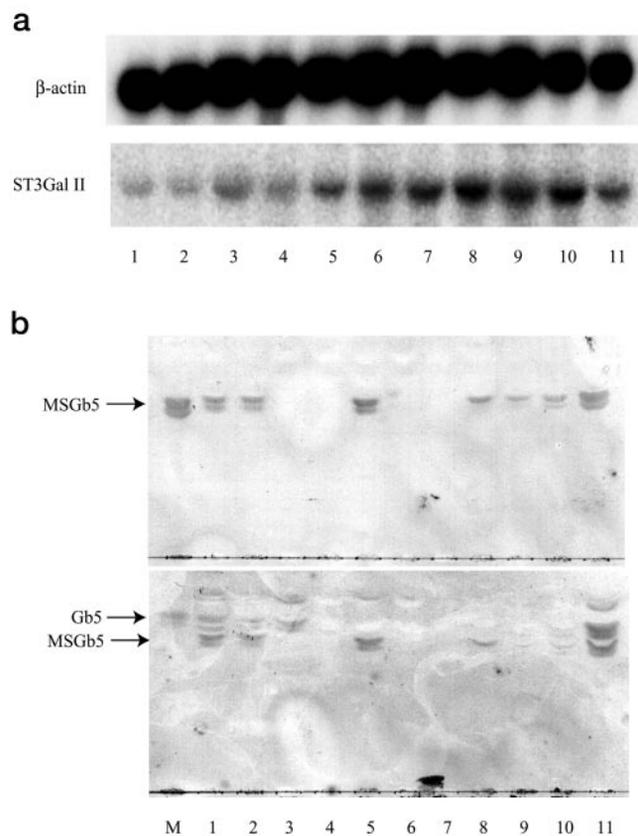


FIG. 7. Relationship between levels of ST3Gal II mRNA and of MSGb5 in monkey kidney cell lines and human RCC cell lines. *a*, Northern blotting of ST3Gal II mRNA. *b*, upper panel, TLC immunostaining of MSGb5 by RM1. Lower panel, TLC immunostaining of Gb5 by anti-SSEA-3 mAb. Lanes 1, CV-1; lanes 2, COS-1; lanes 3, COS-7; lanes 4, SMKT-R2; lanes 5, SMKT-R3; lanes 6, SMKT-R4; lanes 7, TOS-1; lanes 8, TOS-2; lanes 9, TOS-3; lanes 10, TOS-3LN; lanes 11, ACHN. Lane M, marker glycolipids (MSGb5 in upper panel and Gb5 in lower panel, respectively). The levels of ST3Gal II mRNA are not necessarily consistent with those of MSGb5.

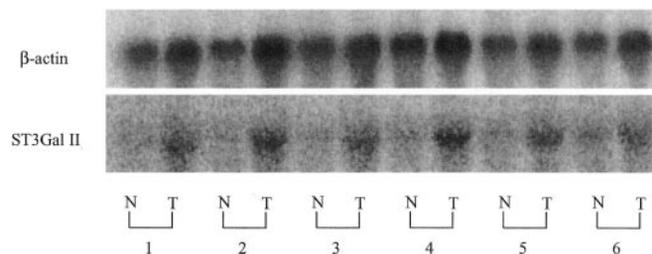


FIG. 8. Increased level of ST3Gal II mRNA in human RCC cases. Northern blotting of ST3Gal II mRNA was conducted in human RCC tissues. N, nontumor kidney; T, primary renal tumor. Each pair of N and T is from the same case.

cells, we failed to isolate a MSGb5 synthase gene by the conventional expression cloning and sibling selection method. The major problem may be due to high sialidase activity in COS-7 cells that may reduce the amount of the product (MSGb5).

Nevertheless, the gradual increase of cells (COS-7 cells expressing SSEA-4) adherent to the Petri dish was observed under the microscope during the expression cloning procedure, and therefore we attempted to isolate a positive clone by utilizing the adherent cells. For this purpose we used the information of the sialylmotif (23) with the expectation that if a sialyltransferase cDNA directs synthesis of MSGb5, enrichment of this cDNA should be observed as the round advances. With degenerate primers, we found that only a sialylmotif

identical to that of ST3Gal II was concentrated but not the other types of β -galactoside α 2,3-sialyltransferase.

In confirmation of the clone directing MSGb5 synthesis, we transfected the cDNA into COS-7 cells and ACHN cells. Only a slight increase of MSGb5 was observed in COS-7 cells transiently transfected with the ST3Gal II cDNA, but the addition of a sialidase inhibitor resulted in a significant increase (10–14%) of MSGb5. Detection of a higher amount of the product with the inhibitor is probably due to protection from degradation. We do not know at present why transfection of the sense cDNA in COS-7 cells led to a smaller amount of MSGb5 synthesis than expected even with a sialidase inhibitor. However, these results indicate that in addition to the specific antibody RM1, the sialylmotif was a powerful tool to identify the clone with a slight increase of MSGb5 expression.

On the other hand, the MSGb5 level was suppressed in the stable transfectants with the antisense Gb5ST cDNA in RCC cell line ACHN. Unexpectedly, anticipated accumulation of Gb5 was not observed in these stable transfectants compared with that with vector. A possible mechanism for this result may be due to physiological importance to maintain Gb5 under a certain level in ACHN cells.

Transfection of the soluble recombinant form of ST3Gal II demonstrated the sialyltransferase activity toward Gb5, asialo-GM1, and GM1a in the culture medium. Concentration of the activity with IgG-Sepharose beads indicates that it is derived from soluble recombinant form of ST3Gal II (pSVL-protA-Gb5ST). Thus, the results of our study shows that ST3Gal II has a role of catalyzing transfer of sialic acid to Gal β 1,3GalNAc epitope of Gb5 in addition to that of asialo-GM1 and GM1a (17), which suggests that the different glycolipid series, *i.e.* globo- and ganglio-series, could be sialylated by the same enzyme. Recently α 2,3-sialylation of GalNAc β 1,3Gal determinant by ST3Gal II was demonstrated (28), indicating that ST3Gal II is a multifunctional enzyme. The fact that levels of ST3Gal II mRNA are not necessarily consistent with those of MSGb5 in RCC cell lines may also suggest that the enzyme plays the other roles.

The level of ST3Gal II mRNA was not necessarily consistent with that of MSGb5 in each RCC cell line. This may also mean that not only sialyltransferase but also the other glycosyltransferases involved in synthesis of globo-series glycolipid (29, 30) or sialidases determine the level of MSGb5. Interestingly all but one of the RCC cell lines examined possessed a high level of ST3Gal II mRNA. Moreover, the levels of ST3Gal II mRNA were higher in all six cases of human RCC tissues than nontumor kidney tissues, which possessed little if any ST3Gal II mRNA. Along with the fact that ST3Gal II mRNA level was very low in normal kidney (17), an increased level of this sialyltransferase mRNA may be related to carcinogenesis of the kidney, although further studies on many cases are necessary. However, surgically obtained renal tissues could not exhibit the exact levels of mRNA, because the time from ligation of renal artery to extirpation usually requires 1 to 2 h or more in radical nephrectomy, and thus mRNAs in renal tissues surgically obtained might be degraded. We have to take this possibility into consideration in analyzing the mRNA level in human materials.

We previously demonstrated that the higher gangliosides are related to the metastatic potential of human RCC (11, 12, 18, 27, 31). Globo-series gangliosides including MSGb5 are the major components of these gangliosides. In this regard decreased motility of ACHN cells stably transfected with pCEP4 antisense Gb5ST was observed compared with those stably transfected with pCEP4 only,² consistent with the findings by

Steelant *et al.* (13). Thus, increased levels of ST3Gal II mRNA may be involved not only in carcinogenesis of kidney but also in the malignant progression of human RCC. This may indicate that at the time of carcinogenesis, as suggested by increased level of ST3Gal II mRNA, renal carcinoma cells may be ready for the next malignant progression. Studies on the levels of ST3Gal II mRNA in many cases of RCC and on the roles of the enzyme will further shed light on the nature of carcinogenesis as well as the malignant potential of RCC. So far there has been only a study on sialyltransferase in human RCC (32). Down-regulation of ST3Gal IV mRNA was associated with the malignant progression of RCC, and the cases with high level of ST3Gal IV mRNA showed a favorable prognosis (32). In this context, the expression level of ST3Gal II mRNA is likely in inverse relation with that of ST3Gal IV mRNA in human RCC. Because regulated expression of sialyltransferases was observed in human normal tissues (24), regulation mechanisms of sialyltransferase expression were assumed to be involved in carcinogenesis. Elucidation of the mechanisms may be essential to understand the nature of the malignant progression.

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