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Linkage-specific Action of Endogenous Sialic Acid *O*-Acetyltransferase in Chinese Hamster Ovary Cells*

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Wei-Xing Shi, Roger Chammas‡, and Ajit Varki§

From the Glycobiology Program, University of California at San Diego Cancer Center, Division of Cellular and Molecular Medicine, University of California at San Diego, La Jolla, California 92093

9-*O*-Acetylation of sialic acids shows cell type-specific and developmentally regulated expression in various systems. In a given cell type, *O*-acetylation can also be specific to a particular type of glycoconjugate. It is assumed that this regulation is achieved by control of expression of specific 9-*O*-acetyltransferases. However, it has been difficult to test this hypothesis, as these enzymes have so far proven intractable to purification or molecular cloning. During a cloning attempt, we discovered that while polyoma T antigen-positive Chinese hamster ovary cells (CHO-Tag cells) do not normally express cell-surface 9-*O*-acetylation, they do so when transiently transfected with a cDNA encoding the lactosamine-specific α2–6-sialyltransferase (Galβ1–4GlcNAc:α2–6-sialyltransferase (ST6Gal I); formerly ST6N). This phenomenon is reproducible by stable expression of ST6Gal I in parental CHO cells, but not upon transfection of the competing lactosamine-specific α2–3-sialyltransferase (Galβ1–(3)4GlcNAc:α2–3-sialyltransferase; (ST6Gal III) formerly ST3N) into either cell type. Further analyses of stably transfected parental CHO-K1 cells indicated that expression of the ST6Gal I gene causes selective 9-*O*-acetylation of α2–6-linked sialic acid residues on *N*-linked oligosaccharides. In a similar manner, while the α2–3-linked sialic acid residue of the endogenous G_{M3} ganglioside of CHO cells is not *O*-acetylated, transfection of an α2–8-sialyltransferase (G_{M3}:α2–8-sialyltransferase (ST8Sia I); formerly G_{D3} synthase) caused expression of 9-*O*-acetylation of the α2–8-linked sialic acid residues of newly synthesized G_{D3}. These data indicate either that linkage-specific sialic acid *O*-acetyltransferase(s) are constitutively expressed in CHO cells or that expression of these enzymes is secondarily induced upon expression of certain sialyltransferases. The former explanation is supported by a low level of background 9-*O*-acetylation found in parental CHO-K1 cells and by the finding that *O*-acetylation is not induced when the ST6Gal I or ST8Sia I cDNAs are overexpressed in SV40 T antigen-expressing primate (COS) cells. Taken together, these results indicate that expression of sialic acid 9-*O*-acetylation can be regulated by the action of specific sialyltransferases that alter the predominant linkage of the terminal sialic acids found on specific classes of glycoconjugates.

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§ To whom correspondence should be addressed: Cancer Center, 0687, UCSD School of Medicine, La Jolla, CA 92093-0687. Tel.: 619-534-3296; Fax: 619-534-5611; E-mail: avarki@ucsd.edu.

Sialic acids are a family of 9-carbon carboxylated sugars usually found at terminal positions of mammalian cell-surface sugar chains. Sia residues can be attached to underlying oligosaccharides in different linkages and can also be modified in a variety of ways (1, 2). It is now clear that the different types of Sia residues are more widely distributed than previously thought (2, 3). These modifications tend to be tissue-specific and developmentally regulated. Different linkages of the Sia residues to the underlying sugar chain further increase the diversity of Sia presentation. In mammalian systems, these α-linkages are usually to Gal or GalNAc and, less commonly, to GlcNAc or to another Sia residue (1, 4–6). A further structural subdivision can be made according to the type of underlying oligosaccharide chain involved. Thus, Sia residues may be found at the termini of the *N*-glycosidically linked oligosaccharides of many glycoproteins of the "complex" type. This group includes Sia residues α2–3-linked to Galβ1–(3)4GlcNAcβ1- or α2–6-linked to Galβ1–4GlcNAcβ1-. Common Sia linkages on *O*-glycosidically linked chains include α2–3-Sia residues capping Galβ1–3GalNAc-*O*-Ser/Thr units and/or α2–6-Sia linkages to the GalNAc-*O*-Ser/Thr residue. In the majority of mammalian gangliosides (sialylated ceramide-linked glycosphingolipids) so far discovered, the Sia residues are α2–3-linked to Gal or α2–8-linked to other Sia residues. However, many of these "oligosaccharide class-specific" linkages can be found on more extended forms of the other classes, *e.g.* the α2–3- and α2–6-sialylation of Galβ1–4GlcNAc (type 2 lactosamine) units common on *N*-linked chains can also occur at the termini of extended *O*-glycosidically linked chains or neolacto series glycosphingolipids (1, 2, 5–7).

In general, each Sia linkage type is generated by one or more unique sialyltransferases, several of which have been recently cloned (4–6, 8–18). Thus, a family of distinct sialyltransferase gene products has been identified that can transfer Sia residues onto glycoprotein or ganglioside oligosaccharides, each showing specificity not only for the linkage formed, but also for the acceptor structure (4–6, 8–18). In some cases, multiple gene products with overlapping specificities can catalyze synthesis of the same gene products, *e.g.* there are so far three reported sialyltransferases that can transfer Sia in α2–3-linkage to lactosamine units (6).

The most common modifications of Sia residues are *O*-acetyl substitutions at C-7 or C-9 (1–3, 19, 20). Since *O*-acetyl esters at C-7 spontaneously migrate to C-9 at physiologic extracellular pH, 9-*O*-acetylated Sia residues predominate on cell-surface glycoconjugates (21–23). Current data indicate that most, if not all, Sia *O*-acetylation takes place within the lumen of the Golgi pathway after the transfer of Sia residues to glycoconjugates by *O*-acetyltransferases by a novel mechanism, probably involving a transmembrane transfer of acetate groups to Sia residues (23–26). In some cell types, *O*-acetylation may be restricted to certain Sia residues on certain glycoconjugates. For example,

in human melanoma cells (27), 9(7)-*O*-acetyltransferases seem to act primarily on Siaα2–8Siaα2–3Gal sequence in gangliosides, while in rat liver (23), 9-*O*-acetylation is primarily found on Siaα2–6Galβ1–4GlcNAcβ1- sequences of *N*-linked oligosaccharides. The latter structure (which can be a ligand for the I-type lectin CD22 of B cells) can be masked by 9-*O*-acetylation in various lymphoid cells (28). Such findings predict highly specific roles for these modifications in tissue development and/or organization and the occurrence of specific enzymatic mechanisms for their generation and regulation.

The *O*-acetyltransferase(s) responsible for 9(7)-*O*-acetylation of Sia residues have so far proven intractable to purification or cloning. Since the Sia residues on *N*-linked oligosaccharides of rat liver have high levels of *O*-acetylation (23), we attempted expression cloning of the relevant *O*-acetyltransferase by transfection of a rat liver cDNA library into CHO[1] cells carrying the polyoma T antigen (CHO-Tag cells). CHO cells are known to have Sia residues on *N*-linked oligosaccharides that are almost exclusively in the Siaα2–3Galβ1–4GlcNAcβ1- sequence (29). In contrast, rat hepatocytes carry 9(7)-*O*-acetylation predominantly on Siaα2–6Galβ1–4GlcNAcβ1- sequences (23, 30, 31). To maximize the probability of success in expression cloning, we decided to first stably transfect the ST6Gal I cDNA into CHO-Tag cells. To our surprise, we found that while these cells were now appropriately expressing the Siaα2–6Galβ1–4GlcNAcβ1- sequence, they were also expressing 9-*O*-acetylation. To pursue this observation, we studied the effects of overexpression of several different sialyltransferases in CHO-Tag cells, parental CHO cells, and COS cells. We show here that endogenous *O*-acetyltransferase(s) present in CHO cells can act specifically on Sia residues in certain linkages.

EXPERIMENTAL PROCEDURES

Materials—Most of the chemicals and some of the reagents used were from the Sigma. The following materials were obtained from the commercial sources indicated: diisopropyl fluorophosphate, Aldrich; HPLC solvents, Fisher; and FITC-conjugated goat anti-human IgG (H + L) and FITC-conjugated goat anti-mouse IgG, Pharmingen. Full-length cDNAs encoding ST6Gal I (formerly ST6N) and ST3Gal III (formerly ST3N) in the pcDM8 or pBluescript vector, respectively, were graciously provided by J. Paulson and E. Sjoberg (Cytel, Inc., San Diego, CA). All other chemicals were of reagent grade or better and were from commercial sources.

Cell Lines—Parental CHO-K1 cells were cultured in α-minimum Eagle's medium with 10% heat-inactivated fetal calf serum. CHO-Tag cells (CHO cells permanently transfected with the polyoma T antigen; kindly provided by Dr. J. Lowe, University of Michigan, Ann Arbor, MI) were grown in α-minimum Eagle's medium containing ribonucleosides and deoxyribonucleosides (Life Technologies, Inc.) with 10% heat-inactivated fetal calf serum, 0.4 mg/ml Geneticin (G418, Life Technologies, Inc.). COS cells were propagated in α-minimum Eagle's medium supplemented with 10% heat-inactivated fetal calf serum.

Antibodies and Probes—The mouse anti-G_{D3} monoclonal IgG_3 antibody R24 (American Type Culture Collection) and the anti-9-*O*-acetyl-G_{D3} monoclonal IgG_3 antibody 27A (kindly provided by M. Farquhar, University of California at San Diego, La Jolla, CA) were used in the form of hybridoma supernatants. Recombinant human CD22βRg (a fusion protein containing Ig domains 1–3 of human CD22β fused to the Fc portion of mouse IgG_1) (32) was kindly provided by I. Stamenkovic (Massachusetts General Hospital, Boston) and was produced in CHO cells as described (33). The soluble chimeric protein CHE-Fc, consisting of the extracellular domain of influenza C hemagglutinin esterase fused to the Fc portion of human IgG_1, was generated and characterized as described (3). The modified form CHE-FcD was generated by treating CHE-Fc with 1 mM diisopropyl fluorophosphate to inactivate the esterase activity as described (3). CHE-Fc specifically releases 9-*O*-acetyl esters from Sia residues (*i.e.* it functions as an esterase), whereas CHE-FcD specifically recognizes and binds to 9-*O*-acetylated Sia residues (for more details, see Ref. 3). Binding of CD22βRg or CHE-FcD to cells can be detected with appropriate secondary reagents directed toward the IgG Fc tails. Biotinylated *Maackia amurensis* lectin (34, 35) and *Sambucus nigra* lectin (36) were obtained from EY Laboratories, Inc. Table I summarizes all the probes used, the methods of detection, and the effects of base (de-*O*-acetylation) on binding.

Plasmid Construction—An *Eco*RI fragment containing the entire coding sequence of ST3Gal III was ligated into the *Eco*RI site of the pcDNA3 plasmid (Invitrogen). An *Xba*I fragment containing the entire coding sequence of ST6Gal I was excised from a pcDM8-based construct and ligated into the *Xba*I site of the pcDNA3 plasmid. Restriction analysis was used to determine correct orientation of both cDNAs relative to the cytomegalovirus promoter. A cDNA encoding the ST8Sia I (formerly G_{D3} synthase) sequence was amplified by polymerase chain reaction using oligonucleotide primers based on the previously reported sequence of human ST8Sia I (13, 16). Total RNA was derived from human melanoma cells using TRIzol reagent (Life Technologies, Inc.). Following reverse transcription-polymerase chain reaction, the reaction mixture was analyzed on a 1% agarose gel, and the expected 1.1-kilobase fragment was gel-purified and ligated into the TA cloning vector pCR-II (Invitrogen). The identity of the clone was confirmed by restriction analysis and sequencing. A *Hin*dIII/*Xho*I fragment containing the entire coding sequence of ST8Sia I was excised from pCR-II-ST8Sia I and ligated into the *Hin*dIII/*Xho*I site of the pcDNA3 or pcDM8 plasmid. The subclones gave the expected patterns on restriction analysis.

Transient Expression of Sialyltransferases—Plasmids carrying the ST6Gal I, ST8Sia I, or ST3Gal III cDNA under control of a cytomegalovirus promoter were transiently overexpressed in CHO-Tag or COS cells (polyoma *ori*- or SV40 *ori*-based episomal replication, respectively) using Lipofectin reagent (Life Technologies, Inc.). Transient expression of gene activity was tested for 72 h after the start of transfection.

Isolation of CHO-K1 Clones Stably Expressing Sialyltransferases—Stable expression of the same cDNAs was obtained by transfecting pcDNA3-based constructs (containing the *neo* gene) into parental CHO-K1 cells using the same protocol. In this case, 72 h after transfection, cells were passaged 1:10 into selective medium containing 1 mg/ml G418. Cells resistant to G418 were subcloned by limiting dilution, and levels of sialylation expressed by individual clones of cells were analyzed.

α2–3-Sialyltransferase Assay—CHO-K1 clones stably transfected with full-length ST3Gal III cDNA were grown to confluence and harvested by scraping into PBS. Pelleted cells were washed with PBS and resuspended in 1 ml of 1% Triton X-100, 50 mM NaCl, 5 mM $MnCl_2$, 25 mM MES, pH 6.0. This crude homogenate was centrifuged for 10 min at 10,000 × *g*, and the supernatant was removed and used directly as the enzyme source. The assay mixture consisted of 50 μM CMP-Neu5Ac, 0.1% Triton CF-54, 20 mM cacodylate buffer, pH 6.0, and 10 μl of enzyme extract in a 30-μl reaction mixture. Reactions were performed in a shaking incubator at 37 °C for 20 min. A sensitive ELISA-based assay (37, 38) was used to detect ST3Gal III activity. The substrate (asialo-α_1-acid glycoprotein, 20 μg/ml) was coated to ELISA plate wells at 4 °C overnight. The reaction mixture was added to the plate and incubated at 37 °C for 20 min. At the end of the incubation period, the reaction mixture was removed, and the wells were washed several times with PBS and treated with 5% BSA in PBS at room temperature for 2 h to block nonspecific binding. Biotinylated *M. amurensis* lectin, which recognizes α2–3-linked Sia, was added to each well and incubated at room temperature for 1 h. After washing in PBS, horseradish peroxidase-conjugated streptavidin was added to the wells and incubated for 30 min at room temperature, followed by a PBS wash. The amount of biotinylated enzyme product was measured by adding OPD substrate and monitoring product formation at 490 nm using an ELISA plate reader. Under the conditions used, product formation was linear with time and amount of cell lysate added.

Flow Cytometric Analysis of Sialylation or O-Acetylation in Trans-

[1] The abbreviations used are: CHO, Chinese hamster ovary; HPLC, high performance liquid chromatography; HPTLC, high performance thin-layer chromatography; FITC, fluorescein isothiocyanate; G_{D3}, Siaα2–8Siaα2–3Galβ1–4Glcβ1–1′-ceramide; G_{M3}, Siaα2–3Galβ1–4Glcβ1–1′-ceramide; PBS, phosphate-buffered saline; MES, 4-morpholineethanesulfonic acid; Neu5Ac, 5-*N*-acetylneuraminic acid; Neu5Gc, 5-*N*-glycolylneuraminic acid; ELISA, enzyme-linked immunosorbent assay; BSA, bovine serum albumin; mAb, monoclonal antibody; DMB, 1,2-diamino-4,5-methylene-dioxybenzene dihydrochloride; Cer, ceramide. Specific Sia residues are designated by combinations of Neu (neuraminic) or Gc (glycolyl) and Ac (acetyl), *e.g.* Neu5,9Ac_2 is 9-*O*-acetyl-5-*N*-acetylneuraminic acid. The sialyltransferases are denoted by the recently developed nomenclature (Ref. 6; J. C. Paulson, personal communication) as follows: ST6Gal I, Galβ1–4GlcNAc:α2–6-sialyltransferase (formerly ST6N); ST3Gal III, Galβ1–(3)4GlcNAc:α2–3-sialyltransferase (formerly ST3N); ST8Sia I, G_{M3}:α2–8-sialyltransferase (formerly G_{D3} synthase).

fected Cells—Expression of transfected ST6Gal I or ST8Sia I was examined by flow cytometric analysis on a Becton Dickinson FACscan machine. Transfected cells (10^6) were released from the culture plate with 0.5 mM EDTA in PBS and incubated with mAb R24 (recognizes G_{D3}) or CD22βRg (recognizes Siaα2–6Gal), followed by goat anti-mouse IgG secondary antibody. After final washing, the cells were analyzed by flow cytometry. For analysis of cell-surface *O*-acetylation, 1×10^6 cells were washed in ice-cold PBS, 0.02% sodium azide and incubated on ice in 100 μl of PBS, 0.02% sodium azide, 1% BSA containing 30 μg/ml CHE-FcD for 120 min. Cells were then washed with ice-cold PBS, 0.02% sodium azide and resuspended in 100 μl of PBS, 0.02% sodium azide, 1% BSA containing FITC-conjugated goat anti-human IgG (1:50 dilution of 1 mg/ml). After incubation for 30 min, cells were washed once in PBS, 0.02% sodium azide and fixed in PBS, 0.02% sodium azide containing 2% formaldehyde. Stained fixed cells were analyzed by flow cytometry.

Extraction of Glycoproteins and Glycolipids—Washed cell pellets were sequentially extracted by 10–20 volumes of $CHCl_3$/MeOH (2:1, 1:1, and 1:2, v/v) using brief homogenization. The final pellet was extracted with 95% ethanol, and the combined extracts containing lipids were dried down. After removal of the 95% ethanol, the residue containing proteins was immediately homogenized into 100 mM Tris-HCl, pH 7.5.

ELISA Plate Assay for 9-O-Acetylation in Gangliosides—Total ganglioside extracts prepared as described above were studied by lipid ELISA as detailed previously (39). Briefly, lipids were applied to the plates in 45% methanol, allowed to dry, and then blocked with 5% BSA in PBS overnight. The effects of base (de-*O*-acetylation) on reactivity were assessed by treatment with 0.1 M NaOH at 4 °C for 30 min. After treatment, the plates were extensively washed with PBS, blocked with 2% BSA in PBS for 1 h, and then incubated with the mAb 27A hybridoma supernatant or CHE-FcD (20 μg/ml) for 2 h. After washing three times with 1% BSA, horseradish peroxidase-conjugated goat anti-mouse IgG (1:1000 dilutions) was added for 1 h. After washing, the reaction was developed as described above. Background levels determined with the secondary antibody alone were subtracted.

HPTLC Immuno-overlay of Gangliosides—Gangliosides were separated on Silica LHP-KF HPTLC plates (Whatman) using chloroform, methanol, 0.02% $CaCl_2$ in water (60:40:9), and the plates were plasticized and overlaid with antibodies overnight at 4 °C. Horseradish peroxidase-conjugated goat anti-mouse IgG recognizing mAb 27A was reacted with the plate for 1 h at 4 °C. After washing, the reaction was developed with diaminobenzidine. For de-*O*-acetylation, gangliosides were spotted on HPTLC plates and exposed to ammonia vapors overnight prior to separation.

Peptide N-Glycosidase F Treatment—Protein samples were denatured in 0.5 M Tris-HCl, pH 8.0, containing 0.1 mM 2-mercaptoethanol, 0.5% SDS at 100 °C for 3 min. The supernatant was collected and incubated overnight at 37 °C with 1 milliunits of peptide *N*-glycosidase F. The enzyme was heat-inactivated for 3 min at 100 °C in SDS-polyacrylamide gel electrophoresis sample buffer.

SDS-Polyacrylamide Gel Electrophoresis and Western Blot Analysis—Proteins were separated by SDS-polyacrylamide gel electrophoresis on 7.5% polyacrylamide gels under reducing conditions and transferred to nitrocellulose membranes (Bio-Rad), and the blots were blocked with 5% BSA in PBS and incubated overnight at 4 °C with CHE-FcD (10 μg/ml) in PBS, 1% BSA. Glycoproteins reacting with the chimera were colorimetrically detected with a goat anti-human IgG antibody conjugated with alkaline phosphatase.

Enzymatic and Acidic Release of Sialic Acids—Sia residues were released from lipid extracts or glycoproteins by incubation with 5–10 milliunits of *Arthrobacter ureafaciens* sialidase in 100 μl of 100 mM sodium acetate, pH 5.5, for 14–16 h or with Newcastle disease virus sialidase under the same conditions for 2 h at 37 °C under a toluene atmosphere. Alternatively, Sia release was achieved by heating in 2 M acetic acid at 80 °C for 3 h. These conditions for release have been previously shown to avoid destruction of *O*-acetyl esters and to limit migration of 7-*O*-acetyl esters to the 9-position (30).

Metabolic Labeling—Parental or stably transfected CHO-K1 cells (1×10^6) were labeled by incubation with 10 ml of α-minimum Eagle's medium containing 100 μCi of [6-^{3}H]$GlcNH_2$ for 3 days, chased in fresh medium overnight, and washed three times with ice-cold PBS. The labeled cell pellet was used for glycolipid extraction and glycoprotein production as described above.

Separation of O-Acetylated Sialic Acids by Paper Chromatography—Released Sia residues were dried, resuspended in 20 μl of distilled water, and separated by descending paper chromatography on Whatman No. 3MM paper eluted with pyridine/ethyl acetate/acetic acid/water (5:5:1:3) for 16 h. In this system, Neu5Ac and Neu5Gc comigrate, but they separate from Neu5,9Ac_2 and other *O*-acetylated Sia residues. In some cases, an aliquot of the Sia residues was pretreated with 0.1 M NaOH at 37 °C for 15 min to attain complete de-*O*-acetylation. After the run, strips of 1 cm were cut and soaked in 500 μl of water, and radioactivity was monitored by liquid scintillation counting.

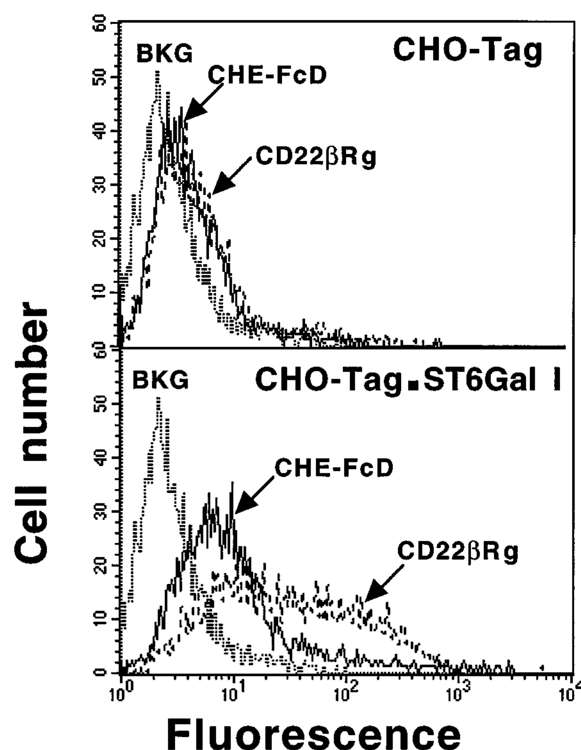


FIG. 1. **Flow cytometric study of CHO-Tag cells transiently transfected with α2–6-sialyltransferase.** CHO-Tag cells were transiently transfected with ST6Gal I and stained with CD22βRg or CHE-FcD using fluorescently tagged secondary antibodies as described under "Experimental Procedures." *BKG*, background staining with secondary antibody alone. The *upper panel* shows CHO-Tag cells undergoing transfection with the vector alone, and the *lower panel* shows cells transfected with vector containing the ST6Gal I cDNA. See Table I for the binding specificity of all probes used.

HPLC Analysis of Released Sialic Acids—Acid-released Sia residues were derivatized with DMB and analyzed by reverse-phase HPLC on a C_{18} column as described previously (40). DMB derivatives were eluted from the C_{18} column using acetonitrile/methanol/water (9:7:84, v/v/v) at room temperature. Derivatization with DMB was done before and after de-*O*-acetylation (30), and the changes in peak area were monitored to confirm the identification of the different Sia residues. If reagent peaks partly overlapped with *O*-acetylated Sia peaks, the change in the combined peak area after de-*O*-acetylation was used to calculate the amount of Sia.

RESULTS AND DISCUSSION

CHO-Tag Cells Transiently Transfected with Lactosamine-specific α2–6-Sialyltransferase Also Newly Express Cell-surface 9-O-Acetylation—CHO cells express primarily α2–3-linked Sia residues on their *N*-linked oligosaccharides (29). In keeping with this (Fig. 1, *upper panel*), CHO cells expressing the polyoma large T antigen (CHO-Tag cells) did not show high level binding of CD22βRg, which is known to require Siaα2–6Galβ1–4GlcNAc sequences for recognition (33) (see Table I for a listing of all the probes used in this study). When these cells were transfected with a plasmid carrying the polyoma origin of replication and a cDNA encoding full-length ST6Gal I, overexpression of the enzyme caused an increase of cell-surface ligands for CD22βRg (Fig. 1, *lower panel*). Increases in staining with *S. nigra* lectin were also seen (data not shown). The influenza C hemagglutinin-derived probe CHE-FcD can be used to detect 9-*O*-acetylation of cell-surface Sia residues regardless of their linkage (3). While this probe does not show

TABLE I
Probes used and structures detected

Probe	Structure(s) detected	Secondary detection method	Effect of de-*O*-acetylation (base) on binding
CHE-FcD	9-*O*-Ac-Siaα2-*X*	Anti-human IgG	Abolished
CD22βRg	Siaα2–6Galβ1–4GlcNAcβ1- and Siaα2–6GalNAc (?)	Anti-mouse IgG	Increase if Sia was 9-*O*-acetylated
mAb R24 (anti-G_{D3})	Siaα2–8Siaα2–3Galβ1–4Glcβ1–1′-Cer	Anti-mouse IgG	Increase if Sia was 9-*O*-acetylated
mAb 27A (anti-*O*-Ac-G_{D3}	9-*O*-AcSiaα2–8Siaα2–3Galβ1–4Glcβ1–1′-Cer	Anti-mouse IgG	Abolished
Biotinylated MAA[a]	Siaα2–3Galβ1–4GlcNAc	Streptavidin or secondary FITC tag	Unknown
FITC-SNA	Siaα2–6Galβ1–4GlcNAcβ1- and Siaα2–6GalNAc	Primary FITC tag	Unknown

[a] MAA, *M. amurensis* lectin; SNA, *S. nigra* lectin.

binding to the untransfected cells, it detects 9-*O*-acetyl groups on the transfected cells (Fig. 1, compare *upper* and *lower panels*). Such an induction of cell-surface 9-*O*-acetylation upon expression of a sialyltransferase is not expected based on prior literature.

Transient Transfection of the Lactosamine-specific α2–3-Sialyltransferase Does Not Cause Expression of Cell-surface 9-O-Acetylation in CHO-Tag Cells—The appearance of 9-*O*-acetylation noted above seems to be related to expression of ST6Gal I since sham-transfected cells also do not show CHE-FcD binding (data not shown). To see if this is a general result of overexpressing a Golgi sialyltransferase, we carried out the same experiment with the cDNA encoding the lactosamine-specific α2–3-sialyltransferase ST3Gal III. As shown in Fig. 2 (*upper panel*), this overexpression did not result in detectable CHE-FcD binding (the low level of CD22βRg binding shown in this panel is sometimes seen even in untransfected CHO cells and may represent a low level of endogenous α2–6-sialylation of Gal or GalNAc).

CHO-Tag Cells Transiently Transfected with α2–8-Sialyltransferase (G_{D3} Synthase) Express Cell-surface 9-O-Acetyl-G_{D3}—The major endogenous ganglioside of CHO cells is known to be G_{M3} (41–43). This common ganglioside is not usually 9-*O*-acetylated. In some cell types (*e.g.* human melanoma), the addition of another Sia residue in α2–8-linkage gives G_{D3}, and the outer Sia residue of this structure is frequently 9-*O*-acetylated (27, 44–47). We therefore studied the effects of overexpressing ST8Sia I (G_{D3} synthase) in the CHO-Tag cells. As shown in Fig. 2 (*lower panel*), expression of this enzyme resulted in expression of G_{D3} (detected by mAb R24) as well as expression of 9-*O*-acetyl-G_{D3} (detected by mAb 27A). In keeping with this, CHE-FcD staining was also noted (data not shown), although not to the same level as seen with ST6Gal I expression. The staining of the mock transfectants is the same as the background (data not shown).

Stable Transfection of Parental CHO Cells with Sialyltransferases Reproduces the Effects Seen with Transient Expression in CHO-Tag Cells—Transient overexpression of plasmids in CHO-Tag cells involves plasmid hyper-replication and eventual cell death. Also, these cells were grown in a modified medium with G418. To rule out the possibility that expression of 9-*O*-acetylation is related to these issues, we prepared stably transfected cloned cell lines derived from parental CHO-K1 cells. Clones stably expressing ST6Gal I or ST8Sia I could be easily selected by the appearance of cell-surface staining with CD22βRg or mAb R24, respectively (Fig. 3). In contrast, parental CHO-K1 cells are already known to express endogenous ST3Gal III (29). Thus, there is already strong staining of wild-type cells with *M. amurensis* lectin (data not shown), and overexpression could not be easily detected with this probe. We therefore studied individual isolated clones for expression of ST3Gal III enzyme activity. Several clones were detected that overexpressed ST3Gal III in comparison with the parental cells. One clone that expressed up to 4-fold increased transfer-

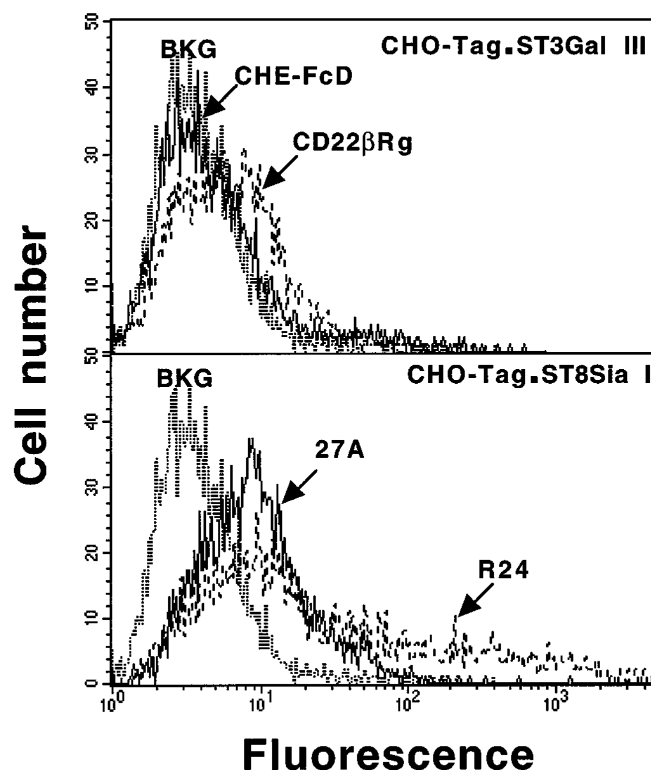


FIG. 2. **Flow cytometric study of CHO-Tag cells transiently transfected with α2–3- or α2–8-sialyltransferase.** CHO-Tag cells were transiently transfected with ST3Gal III or ST8Sia I; stained with CHE-FcD, CD22βRg, mAb R24, or mAb 27A; and studied by flow cytometry as described for Fig. 1. See Table I for the binding specificity of all probes used. Mock-transfected cells stained with mAb R24 or mAb 27A gave staining very similar to the background (data not shown). *BKG*, background staining with secondary antibody alone.

ase activity (data not shown) was picked for further study. As shown in Fig. 3, the stably transfected CHO-K1 cell lines reproduced the same expression patterns seen with the transient transfection, *i.e.* CHE-FcD staining with ST6Gal I expression and mAb 27A staining with ST8Sia I expression. Again, ST3Gal III expression did not cause the appearance of 9-*O*-acetylation.

Sialyltransferase-specific Expression of 9-O-Acetylation Occurs on N-Linked Oligosaccharides or on Gangliosides—Proteinase K treatment of ST6Gal I-transfected CHO cells resulted in a loss of CHE-FcD staining, indicating that the 9-*O*-acetylation was carried on glycoproteins (data not shown). In contrast, similar protease treatments of ST8Sia I-expressing cells caused an increase in staining with either CHE-FcD or mAb 27A (data not shown). This is consistent with the fact that gangliosides would be resistant to proteolysis and, indeed, should become more exposed to the probes by proteases. To confirm these findings, we carried out a Western blot analysis

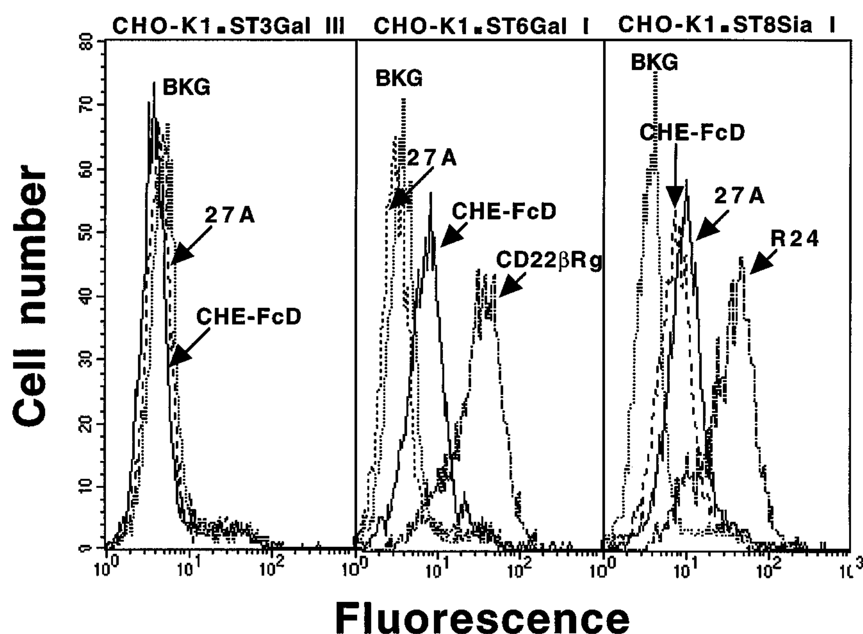


FIG. 3. **Flow cytometric study of CHO-K1 cells stably transfected with sialyltransferases.** CHO-K1 clones stably transfected with each of the three sialyltransferases indicated were stained with CHE-FcD, CD22βRg, mAb R24, or mAb 27A and studied by flow cytometry as described for Fig. 1. See Table I for the binding specificity of all probes used. *BKG*, background staining with secondary antibody alone.

of CHO cell glycoproteins probed with CHE-FcD. As shown in Fig. 4, only ST6Gal I-transfected cells showed easily detectable glycoproteins that carried 9-*O*-acetylation (prolonged development of the blot picked up low levels of staining in the other cells; data not shown). As shown in Fig. 4, treatment of the glycoproteins with peptide *N*-glycosidase F eliminated the CHE-FcD staining of the glycoproteins from ST6Gal I-transfected cells. This indicates that the 9-*O*-acetylated Sia residues are carried primarily on *N*-linked oligosaccharides.

To confirm the presence of 9-*O*-acetyl-G_{D3} in the ST8Sia I-transfected cells, we extracted total gangliosides and studied them by ELISA or by thin-layer chromatography with detection using mAb 27A. As shown in Fig. 5, both assays showed evidence for mAb 27A binding that was labile to base treatment, which eliminates *O*-acetyl groups. In the latter case, the overlay showed a base-labile band migrating in the position expected for authentic 9-*O*-acetyl-G_{D3}.

Evidence for Selective O-Acetylation of α2–6-Linked Sialic Acids in ST6Gal I-transfected CHO Cells—Up to this point, all demonstrations of the presence of 9-*O*-acetyl groups relied mainly upon the CHE-FcD and mAb 27A probes. While these probes are highly specific, it is necessary to confirm the presence of 9-*O*-acetylated Sia residues by direct analysis. Furthermore, it is of interest to determine the nature of the linkages of the *O*-acetylated Sia residues on the *N*-linked chains. Finally, it is necessary to see if other related *O*-acetylation reactions (7-*O*-acetylation) are taking place, and if this is selectively occurring on Neu5Ac or Neu5Gc. Stably transfected CHO cells were therefore metabolically labeled with $[6\text{-}^3H]GlcNH_2$, which selectively labels hexosamines and Sia residues (48). After a 3-day labeling and overnight chase, the glycolipids were extracted, and glycoproteins were prepared from the resulting residue. The latter should contain all of the *N*-linked oligosaccharides that were noted to be 9-*O*-acetylated in Fig. 4. Aliquots of these labeled glycoproteins were treated with specific sialidases, and the released Sia residues were detected by descending paper chromatography. Some examples of the results are shown in Fig. 6. It can be seen that only the *A. ureafaciens* sialidases (which cleave α2–6-, α2–3-, and α2–8-linked Sia residues) released radioactivity that ran in the positions expected for *O*-acetylated species (as expected, base treatment eliminated these peaks). In contrast, the Newcastle disease virus sialidase released only non-*O*-acetylated Sia residues. The latter enzyme can only release α2–3- and α2–8-linked Sia residues (*O*-acetylated or non-*O*-acetylated) under the conditions used. Thus, we can conclude that the *O*-acetyl groups are primarily carried on α2–6-linked Sia residues that were synthesized by the transfected ST6Gal I enzyme. In contrast, glycoproteins from CHO-K1 cells stably transfected with ST3Gal III or ST8Sia I did not carry *O*-acetylated Sia residues (data not shown). However, in the case of ST8Sia I, labeled *O*-acetylated Sia residues were found in the glycolipid fraction (data not shown). These data are completely consistent with the earlier results using the CHE-FcD and mAb 27A probes (Figs. 1–5).

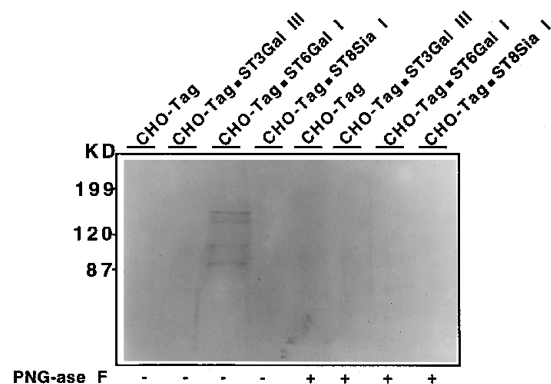


FIG. 4. **Study of glycoprotein 9-*O*-acetylation by Western blot analysis with CHE-FcD.** Proteins from cell lysates were separated by SDS-polyacrylamide gel electrophoresis under reducing conditions and transferred to nitrocellulose membranes, and the blot was probed with CHE-FcD as described under "Experimental Procedures." As indicated (+ or −), some samples were predigested with peptide *N*-glycosidase F (*PNG-ase F*) to release *N*-linked oligosaccharides.

Quantitation and Characterization of O-Acetylation of Sialic Acids in Transfected CHO Cells—The paper chromatography system used above does not completely resolve the different species of modified Sia residues. Also, the metabolic labeling study presented above might not be completely quantitative if labeling had not reached equilibrium in 3 days. Direct chemical quantitation of total Sia residues from the different transfected cells indicated no major changes in Sia content (data not shown). This is not surprising for ST6Gal I and ST3Gal III since the overexpressed enzymes would simply be competing

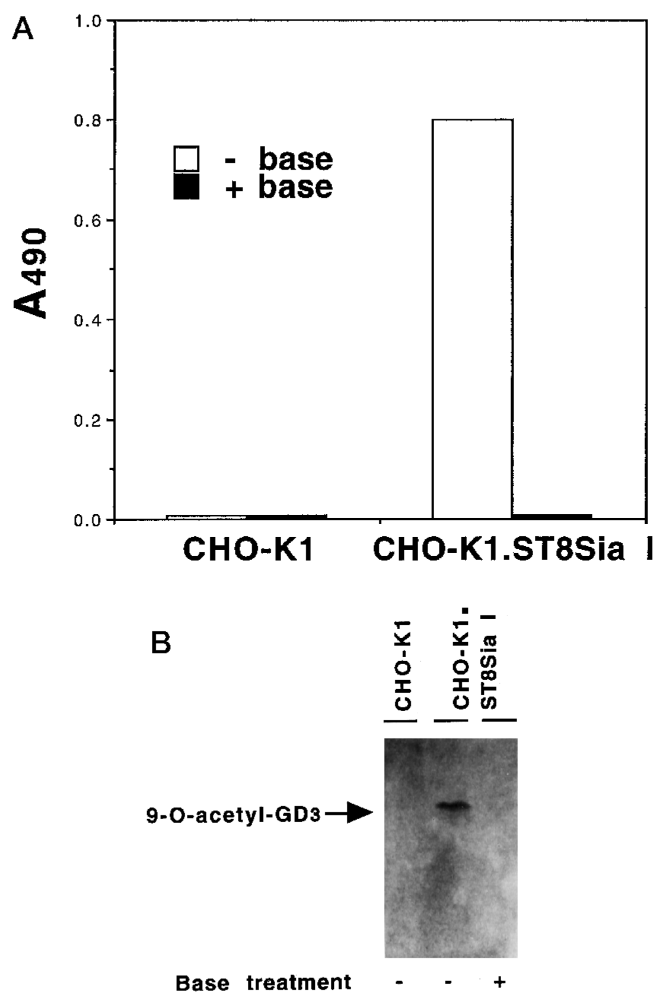


FIG. 5. **Study of ganglioside 9-*O*-acetylation in CHO-K1 clones stably transfected with ST8Sia I.** Total glycolipids were extracted from the cells and studied by ELISA or HPTLC immuno-overlay as described under "Experimental Procedures." *A*, glycolipids dried on 96-well plates were probed with mAb 27A, with or without prior base treatment for de-*O*-acetylation. *B*, glycolipids were separated by HPTLC, and the plates were probed with mAb 27A, with or without prior base treatment for de-*O*-acetylation.

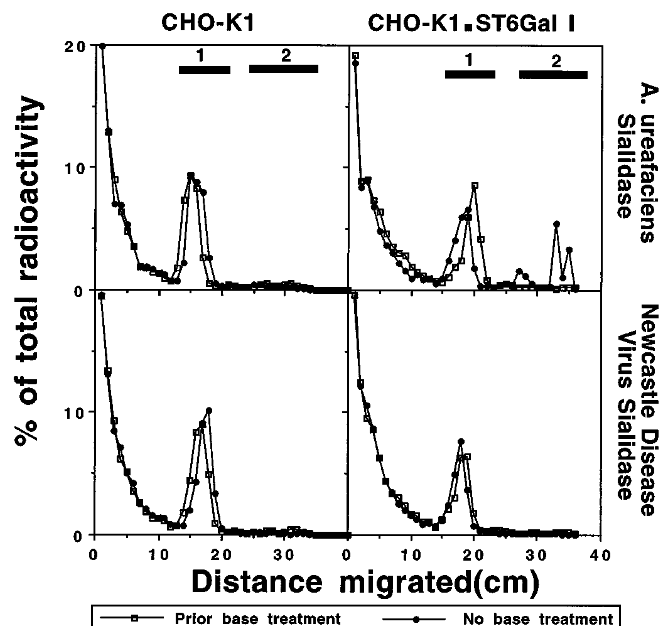


FIG. 6. **Paper chromatography of labeled sialic acids released from glycoproteins and gangliosides by sialidases.** CHO cells stably transfected with various sialyltransferases were metabolically labeled with [6-^{3}H]GlcNH$_2$, and glycoproteins were prepared and treated with sialidase as described under "Experimental Procedures." The reaction mixtures were spotted onto Whatman No. 3MM paper and studied by descending paper chromatography as described under "Experimental Procedures." Aliquots of each sample were subjected to mild base treatment for de-*O*-acetylation prior to separation. Control incubations minus sialidase gave radioactivity that either remained at the origin or migrated up to 10 cm (data not shown). The marked areas indicate the position of migration of non-*O*-acetylated Sia residues (*bar 1*) and *O*-acetylated Sia residues (*bar 2*). Examples are shown from the cells transfected with ST6Gal I. All the other transfected CHO cells gave profiles similar to those obtained with the parental line (data not shown). For convenient comparison, the data are shown as percentage of total radioactivity recovered in each lane. Note that the extent of sialidase release of the metabolically incorporated label ranged from 30–35% (*A. ureafaciens* sialidase) to 25–30% (Newcastle disease virus sialidase). This is expected since only a portion of the [6-^{3}H]GlcNH$_2$ would be expected to enter into sialic acids.

for Galβ1–4GlcNAc sites, which were already mostly sialylated in the wild-type cells. In the case of ST8Sia I, this enzyme could not more than double the quantity of glycolipid-bound Sia since the endogenous substrate is monosialylated G_{M3}. To more precisely characterize the type of *O*-acetylated Sia residues in the transfected CHO cells, we prepared lipid and protein fractions from unlabeled cells, released the total Sia residues by mild acid hydrolysis, and studied them with a sensitive method that separates fluorescent derivatives of each of the different types of Sia residues. Examples of the HPLC profiles are shown in Fig. 7, and complete quantitative data are presented in Table II. It can be seen that the wild-type parental cells express predominantly Neu5Ac and a small fraction of Neu5Gc. Interestingly, this analysis also shows a trace amount of 9-*O*-acetylated Sia (Neu5,9Ac$_2$) that was not picked up by the other methods (other than Western blotting). This also fits some recent reports of low levels of 9-*O*-acetylation found on recombinant erythropoietin synthesized by CHO cells (49). As predicted from the earlier studies, substantial increases in this peak are seen only upon transfection of ST6Gal I (glycoprotein fraction only) or ST8Sia I (glycolipid fraction only). Small amounts of other related *O*-acetylated molecules (Neu5Gc9Ac, Neu5,7Ac$_2$, and Neu5,7(8)9Ac$_3$) are also seen. These results fit prior data predicting that 9-*O*-acetyltransferases can work on both Neu5Ac and Neu5Gc and can also transfer to the 7-position (23–26). With the latter reaction, the spontaneous migration of *O*-acetyl esters from the 7- to the 9-position makes it difficult to accurately quantitate the relative ratios in the two positions. The presence of small amounts of the di-*O*-acetylated species is also not surprising, if the enzyme works on both the 7- and 9- positions. Interestingly, the data also indicate a strong preference for 9-*O*-acetylation of Neu5Gc over Neu5Ac. Also, as mentioned in Table II, the ST6Gal I-transfected cells contained another unknown alkali-labile peak that accounted for ~11.5% of the total area. Since this peak did not migrate in the position of any known *O*-acetylated Sia residues, we did not include it in our calculations. Regardless, the overall data are completely consistent with the conclusion that ST6Gal I transfection induces 9(7)-*O*-acetylation in glycoproteins and that ST8Sia I does the same in glycolipids, while ST3Gal III does not do either.

Transient Overexpression of ST6Gal I or ST8Sia I cDNA in COS Cells Does Not Induce Expression of 9-O-Acetylation—The data presented above indicate either that linkage-specific sialic acid *O*-acetyltransferase(s) are constitutively expressed in CHO cells or that expression of these enzymes is secondarily induced upon expression of certain sialyltransferases. The former explanation is favored by the low level of background 9-*O*-acetylation noted in parental cells. To explore the latter possibility further, we transiently overexpressed ST6Gal I or

ST8Sia I cDNA in COS cells, which can support episomal replication of plasmids with SV40 *ori*. These cells are also similar to CHO-Tag cells in that they do not normally express ST6Gal I or ST8Sia I. As shown in Fig. 8, expression of these cDNAs resulted, as expected, in the appearance of CD22βRg or mAb R24 binding. However, in neither case was there any evidence for expression of 9-*O*-acetylation (Fig. 8). Thus, the endogenous *O*-acetyltransferase(s) found in CHO cells are not present in COS cells.

Conclusions and Perspectives—It was previously assumed that the tissue-specific and developmentally regulated expression of Sia *O*-acetylation was regulated by the presence or absence of specific *O*-acetyltransferases (1, 2). However, since these enzymes have so far proven intractable to purification or molecular cloning, it has not been possible to test this assumption. Taken together, the results presented here indicate that expression of Sia 9(7)-*O*-acetylation can be regulated by the action of specific sialyltransferases that alter the predominant linkage of the terminal Sia residues found on specific classes of glycoconjugates. Thus, the pathway shown below on the left (Reaction 1) can be functionally activated by the expression of the α2–6-specific sialyltransferase, ST6Gal I:

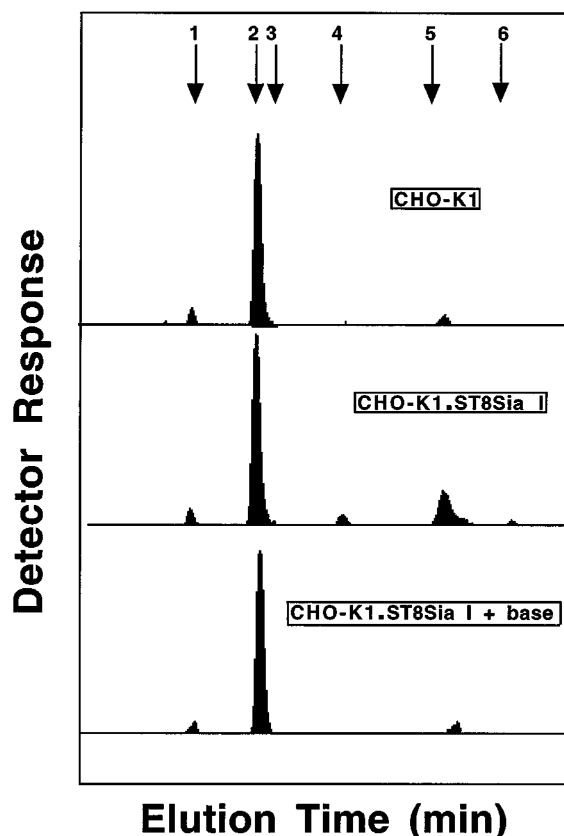


FIG. 7. **Examples of HPLC analysis of sialic acids from CHO cells after DMB derivatization.** The lipid and protein fractions from unlabeled CHO cells were isolated, and total Sia residues were released by mild acid hydrolysis, derivatized with DMB before or after de-*O*-acetylation, and studied by reverse-phase HPLC as described under "Experimental Procedures." The arrows mark the positions of elution of Neu5Gc (*arrow 1*), Neu5Ac (*arrow 2*), Neu5,7Ac$_2$ (*arrow 3*), Neu5Gc9Ac (*arrow 4*), Neu5,9Ac$_2$ plus reagent peak (*arrow 5*), and Neu5,7(8)9Ac$_3$ (*arrow 6*). Some examples of the data are shown here, and the overall results are presented in Table II. *Upper trace*, Sia residues from the lipid fraction of parental CHO-K1 cells; *middle trace*, Sia residues from the lipid fraction of ST8Sia I-transfected CHO-K1 cells; *lower trace*, Sia residues from the lipid fraction of ST8Sia I-transfected CHO-K1 cells subjected to base treatment (de-*O*-acetylation) before DMB derivatization.

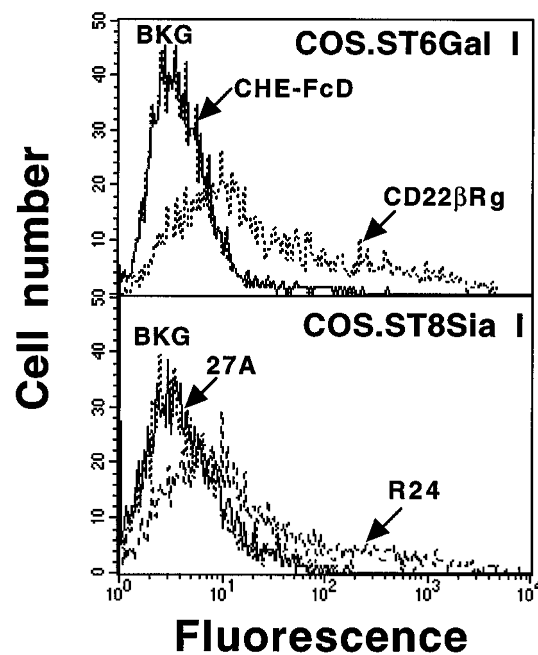


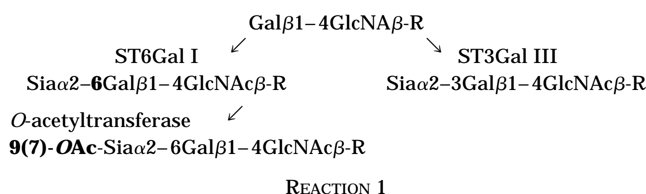
FIG. 8. **Flow cytometric study of COS cells transiently transfected with sialyltransferases.** COS cells were transiently transfected with each of the two sialyltransferases indicated; stained with CHE-FcD, CD22βRg, mAb R24, or mAb 27A; and studied by flow cytometry as described for Fig. 1. See Table I for the binding specificity of all probes used. *BKG*, background staining with secondary antibody alone.

TABLE II
Quantitation of sialic acids in stably transfected CHO cells

Total glycolipids and glycoproteins were prepared from stably transfected CHO-K1 cells; sialic acids were released by acid hydrolysis; and aliquots of each hydrolysate were derivatized with DMB and analyzed by HPLC as described under "Experimental Procedures." The individual sialic acids were identified based upon their elution position relative to known standards and the effect of prior base treatment (de-*O*-acetylation) (see Fig. 7 for examples of the profiles obtained). For purposes of relative comparison, it is assumed that each sialic acid derivative gives the same detector response.

Transferase transfected	Fraction	Percentage of total sialic acids					
		Neu5Ac	Neu5,9Ac$_2$	Neu5,7Ac$_2$	Neu5,7(8)9Ac$_3$	Neu5Gc	Neu5Gc9Ac
None	Lipids	93.0	3.0	<0.1	<0.1	4.0	<0.1
	Proteins	92.6	2.4	<0.1	<0.1	5.0	<0.1
ST6Gal I	Lipids	93.7	2.2	<0.1	<0.1	4.3	<0.1
	Proteins[a]	71.6	10.7	2.5	2.6	6.2	6.0
ST3Gal III	Lipids	92.0	3.5	<0.1	<0.1	4.5	<0.1
	Proteins	92.2	2.7	<0.1	<0.1	3.8	1.3
ST8Sia I	Lipids	73.0	18.8	<0.1	0.8	4.4	3.4
	Proteins	91.3	2.4	<0.1	<0.1	6.3	<0.1

[a] Proteins from the cells transfected with ST6Gal I gave an additional alkali-labile fluorescent peak that accounted for 11.5% of the total peak areas. Since this peak did not coincide with any known *O*-acetylated sialic acid, it was not included in the calculation.

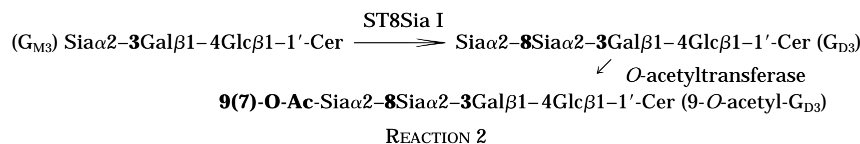


REACTION 1

where R is usually an *N*-linked chain, but can be an extended *O*-linked chain or a neolactoganglioside.

It is of interest to note that tissue-specific expression of ST6Gal I under control of different promoters has been identified (14, 50–53), and activation and/or cell-cycle dependent expression in various cell types has been demonstrated (54–60). Under these circumstances, the changes in sialyltransferase could in turn affect the level of 9(7)-*O*-acetylation. Of course, this does not rule out the possibility that α2–3-linked Sia residues can be *O*-acetylated at a much less efficient rate. Indeed, small quantities of such sequences were reported recently in recombinant erythropoietin synthesized by CHO cells (49).

Regarding glycosphingolipids, the present data indicate that the following pathway (Reaction 2) can be functionally activated by expression of ST8Sia I (G_{D3} synthase).



REACTION 2

As with ST6Gal I, ST8Sia I expression is also known to be inducible under various circumstances (61–64). Our data thus fit the correlation reported in many studies between the expression of G_{D3} and its 9(7)-*O*-acetylated counterpart (27, 46, 47). On the other hand, there are also clear examples in which these two structures are differentially regulated in expression (44, 45). Thus, in at least some instances, the presence of 9-*O*-acetylation might be controlled primarily by expression of the *O*-acetyltransferase(s).

In this study, we have primarily focused upon *N*-linked oligosaccharides and gangliosides. *O*-Acetylation has also been reported on *O*-linked oligosaccharides, *e.g.* on bovine submaxillary mucin (65) and on murine glycophorins (66–68), which carry large numbers of clustered *O*-linked chains. In contrast, parental CHO cells are known to have short sialylated *O*-linked oligosaccharides (49, 69, 70), which evidently do not carry 9-*O*-acetyl groups (since they were not detected in this study). In keeping with this, we have recently noted that overexpression of an α2–3-sialyltransferase directed against Galβ1–3GalNAc units of *O*-linked chains in CHO cells did not induce *O*-acetylation.[2] Studies of the other sialyltransferases capable of selectively acting upon *O*-linked chains are currently underway. It is also necessary to study the other rarer Sia linkages found on *N*-linked chains, *e.g.* Sia α2–6-linked to GlcNAc (71).

These findings could also be of practical relevance regarding the specificity of sialic acid-binding lectins. Some endogenous mammalian lectins (*e.g.* the I-type lectins CD22 and sialoadhesin), viral hemagglutinins (*e.g.* influenza), and other lectins used for analytical purposes (*e.g.* plant and crab lectins) are known to recognize the side chain of Sia, in addition to the underlying linkage. Thus, the addition of a bulky *O*-acetyl group to this side chain can be expected to alter binding. This has indeed been documented in some cases (28, 72). It is also worth pointing out that these are not the first studies to overexpress these particular sialyltransferases in CHO cells. Indeed, prior studies (including our own) carried out similar transfections for unrelated reasons, but missed the appearance of *O*-acetylation (73, 74). This exemplifies the fact that *O*-acetylation is easily missed when using conventional methods to study Sia residues. Indeed, it is the availability of the CHE-FcD probe that has allowed us to detect 9-*O*-acetylation here and to show that this modification may be much more common than previously realized. Thus, development of specific probes for many other known forms of modified sialic acids (*e.g.* 4-*O*-acetylation) would be quite valuable.

Finally, these studies still leave open the question of how many *O*-acetyltransferases are involved in these reactions. Are there separate enzymes specific for glycoproteins and gangliosides? Are there separate *O*-acetyltransferases for 7- and 9-*O*-acetylation? Molecular cloning of the cDNAs encoding these enzymes will be needed to conclusively address these issues.

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[2] W. Shi and A. Varki, unpublished data.

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