THE JOURNAL OF BIOLOGICAL CHEMISTRY
Vol. 276, No. 5, Issue of February 2, pp. 3564–3573, 2001

Printed in U.S.A.

β1,3-Galactosyltransferase β3Gal-T5 Acts on the GlcNAcβ1→3Galβ1→4GlcNAcβ1→R Sugar Chains of Carcinoembryonic Antigen and Other *N*-Linked Glycoproteins and Is Down-regulated in Colon Adenocarcinomas*

Received for publication, July 26, 2000, and in revised form, October 6, 2000
Published, JBC Papers in Press, October 31, 2000, DOI 10.1074/jbc.M006662200

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We attempted to determine whether β1,3-galactosyltransferase β3Gal-T5 is involved in the biosynthesis of a specific subset of type 1 chain carbohydrates and expressed in a cancer-associated manner. We transfected Chinese hamster ovary (CHO) cells expressing Fuc-TIII with β3Gal-T cDNAs and studied the relevant glycoconjugates formed. β3Gal-T5 directs synthesis of Lewis type 1 antigens in CHO cells more efficiently than β3Gal-T1, whereas β3Gal-T2, -T3, and -T4 are almost unable to direct synthesis. In the clone expressing Fuc-TIII and β3Gal-T5 (CHO-FT-T5), sialyl-Lewis a synthesis is strongly inhibited by swainsonine but not by benzyl-α-GalNAc, and sialyl-Lewis x is absent, although it is detected in the clones expressing Fuc-TIII and β3Gal-T1 (CHO-FT-T1) or Fuc-TIII and β3Gal-T2 (CHO-FT-T2). Endo-β-galactosidase treatment of *N*-glycans prepared from clone CHO-FT-T5 releases (±NeuAcα2→3)Galβ1→3[Fucα1→4]GlcNAcβ1→3Gal but not GlcNAcβ1→3Gal or type 2 chain oligosaccharides, which are found in CHO-FT-T1 cells. This result indicates that β3Gal-T5 expression prevents poly-*N*-acetyllactosamine and sialyl-Lewis x synthesis on *N*-glycans. Kinetic studies confirm that β3Gal-T5 prefers acceptors having the GlcNAcβ1→3Gal end, including lactotriosylceramide. Competitive reverse transcriptase mediated-polymerase chain reaction shows that the β3Gal-T5 transcript is expressed in normal colon mucosa but not or poorly in adenocarcinomas. Moreover, recombinant carcinoembryonic antigen purified from a CHO clone expressing Fuc-TIII and β3Gal-T5 reacts with anti-sialyl-Lewis a and carries type 1 chains on oligosaccharides released by endo-β-galactosidase. We conclude that β3Gal-T5 down-regulation plays a relevant role in determining the cancer-associated glycosylation pattern of *N*-glycans.

Type 1 chain oligosaccharides found in *N*- and *O*-glycans, as well as in glycolipids, contain the distinctive Galβ1→3GlcNAc disaccharide as their core structure. It is synthesized by β1,3-galactosyltransferases (β1,3Gal-Ts),[1] a family of enzymes whose genes have been cloned very recently (1). The functional role of type 1 chains is not known, but several studies have indicated that some of them are differentially expressed in cancer. In particular, CEA expressed in colon cancer is characterized by the absence of such chains (2), which are abundantly present on the *N*-glycans of its normal counterparts synthesized by healthy colon mucosa, also referred to as the nonspecific cross-reacting antigen-2 (3) and the normal fecal antigen-2 (4). At this regard, a β1,3Gal-T activity measured using GlcNAcβ1→3Galβ1→4Glc as acceptor was found lower in adenocarcinomas than in normal mucosa (5). However, type 1 chain Lewis antigens sialyl-Lewis a (sLe^a) and Le^b are considered tumor markers (6), and serum levels of sLe^a (CA19-9 antigen) are used for clinical diagnosis and follow-up of epithelial cancers of the gastrointestinal tract (7). sLe^a from cancer patient serum was found on mucins (8), and it is reported on both mucins (9) and glycolipids (10) in adenocarcinoma cell lines. Five β3Gal-T cDNAs are presently available. β3Gal-T1, cloned first from melanoma cells (11) and then from colon carcinoma cells (12), as well as β3Gal-T2 (13), were found to synthesize sLe^a in CHO cells but to be very poorly expressed in cancer cell lines expressing sLe^a. β3Gal-T3 and -T4 (14) were found expressed in various tissues, including colon and pancreas and in some cancer cell lines, but the expression levels do not correlate with those of type 1 chain Lewis antigens. Moreover, there is no evidence yet for their ability to synthesize the type 1 chain. In particular, β3Gal-T3 was very recently reported as a GalNAc transferase involved in globoside biosynthesis and renamed β3GalNAc-T1 (15). β3Gal-T5, cloned from colon carcinoma cells, was found to direct synthesis of sLe^a in different cell lines, and its expression levels correlate with those of Lewis type 1 antigens in cancer cell lines from colon and pancreas (16). This enzyme was suggested to be the one previously characterized as the β1,3Gal-T active on glycolipids (17) and GlcNAc (18, 19), and involved in the synthesis of core

* This work was supported in part by a grant from the Associazione Italiana Ricerca sul Cancro (to M. T.) and by a grant from the University of Insubria (FAR 1999). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: Gal-T, galactosyltransferase; GnT, *N*-acetylglucosaminyltransferase; FUT2, secretor type α1,2-fucosyltransferase; Fuc-TIII, α1,(3/4)-fucosyltransferase; sLe^a, sialyl-Lewis a (NeuAcα2→3Galβ1→3[Fucα1→4]GlcNAc); Le^a; Lewis a (Galβ1→3[Fucα1→4]GlcNAc); Le^b, Lewis b (Fucα1→2Galβ1→3[Fucα1→4] GlcNAc); sLe^x, sialyl-Lewis x (NeuAcα2→3Galβ1→4[Fucα1→3]GlcNAc); CEA, carcinoembryonic antigen; RT-PCR, reverse transcriptase mediated-polymerase chain reaction; lactotriosylceramide, GlcNAcβ1→3Galβ1→4Glcβ1→1′Cer; lacto-*N*-tetraosylceramide, Galβ1→4GlcNAcβ1→3Galβ1→4Glcβ1→1′Cer; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; HPTLC, high performance thin layer chromatography; PAGE, polyacrylamide gel electrophoresis; FITC, fluorescein isothiocyanate; CHO-T-FT, CHO cells expressing Polyoma virus T antigen and Fuc-TIII; CHO-FT-T1, CHO clone permanently expressing Fuc-TIII and β3Gal-T1; CHO-FT-T2, CHO clone permanently expressing Fuc-TIII and β3Gal-T2; CHO-FT-T5, CHO clone permanently expressing human Fuc-TIII and β3Gal-T5.

TABLE I
Oligonucleotide primers used for competitive RT-PCR analysis

Target gene	Primer pairs[a]	PCR products		Restriction sites for competitor
		Target	Competitor	
		bp		
β3Gal-T2	F: 5′-GCCAAAAGGTCTCTGTTCCGCACTC-3′ R: 5′-GATTGGGTGCATATCCTCGCATTAGG-3′	817	556	*Nde*I/*Pst*I
β3Gal-T3	F: 5′-CTGGTGACCTCCCACCCTTCAGATG-3′ R: 5′-GCATGTGGTGTTCCTTAGCATGACCTGC-3′	740	632	*Xmn*I/*Pst*I
β3Gal-T4	F: 5′-CCAGAGAAACGCCATTCGGGCTTCGTG-3′ R: 5′-AGGGTGGAAAGGGGCCCCAGGTG-3′	526	416	*Sma*I/*Eco*RV
β3Gal-T5	F: 5′-CAGATACAGACTGCAGGCAGACACCTCC-3′ R: 5′-GCAGGCCACGATCCTCCTGAAGAGGC-3′	709	480	*Xmn*I
Mouse β-actin	F: 5′-GCACCCTGTGCTGCTCACCGAGGC-3′ R: 5′-GTGCACGATGGAGGGGCCGGACTC-3′	813	534	*Rca*I

[a] F, forward primer; R, reverse primer.

3 *O*-glycans (20). The above data led to the working hypothesis that the biosynthesis of different type 1 chain oligosaccharides may depend on the expression of different and differentially regulated β3Gal-Ts. In particular, an enzyme able to act on *N*-glycans would be expected to be less active in cancer than in normal mucosa, whereas another active on *O*-glycans should be more expressed in cancer.

To evaluate whether β3Gal-T5 is actually involved in the biosynthesis of a specific subset of type 1 chain oligosaccharides and expressed in a cancer-associated manner, we have transiently transfected CHO cells expressing Fuc-TIII with β3Gal-T cDNAs and compared the relative ability of enzymes to direct synthesis of Lewis type 1 antigens. We then constructed CHO clones permanently expressing Fuc-TIII and β3Gal-T5, Fuc-TIII and β3Gal-T2, or Fuc-TIII and β3Gal-T1, determined the effect of drugs affecting glycosylation on antigen expression, and characterized the oligosaccharides that became radioactive in some clones upon metabolic labeling with tritiated Gal. Moreover, we studied the substrate specificity of β3Gal-T5 by calculating the kinetic constants toward different acceptors, and measured the expression levels of the transcript in colon adenocarcinomas and surrounding normal mucosa by competitive RT-PCR. We further modified the CHO clone expressing β3Gal-T5 to make it able to stably express human CEA and investigated the presence of type 1 chains in CEA purified from such clone.

EXPERIMENTAL PROCEDURES

Materials—GlcNAcβ1→3Galβ1→4Glc was prepared by digesting lacto-*N*-tetraose (IsoSep, Lund, Sweden), 5 mg/ml, in 0.1 M citrate phosphate buffer, pH 4.5, with 40 milliunits/ml bovine testis β-galactosidase (Sigma), for 20 h at 37 °C. The obtained trisaccharide was purified from Gal and unreacted lacto-*N*-tetraose by repeated Bio-Gel P-2 columns monitored by HPTLC, as reported (21). Lactotriosylceramide was prepared by digesting lacto-*N*-neotetraosylceramide, 4 mg/ml, in 50 mM cacodylate/HCl buffer, pH 6.5, with *Diplococcus pneumoniae* β-galactosidase (Sigma), 0.2 units/ml for 20 h at 37 °C. The obtained compound was purified by a Silica-Gel column (0.7 × 50 cm) using chloroform/methanol/water, 55:20:3 (v/v), as the eluting solvent system. Lacto-*N*-neotetraosylceramide was prepared by digesting sialyl-lacto-*N*-neotetraosylceramide, 5 mg/ml, in 0.1 M sodium cacodylate buffer, pH 6.0, with *Clostridium perfringens* sialidase, 1 unit/ml, for 20 h at 37 °C. Ganglioside sialyl-lacto-*N*-neotetraosylceramide was purified from bovine erythrocytes using the procedure reported by Chien *et al*. (22). GlcNAcβ1→2Man was from Dextra Laboratories (Reading, UK), GlcNAcβ1→3Galβ1→*O*-methyl and GlcNAcβ1→6Manα→*O*-methyl were from Sigma. Anti-sLe^x monoclonal antibody was precipitated from the culture media of hybridoma CSLEX1 (ATCC HB-8580) by ammonium sulfate, resuspended, dialyzed, and kept at a concentration of 2 mg/ml. Anti-Le^a and anti-sLe^a monoclonal antibodies were as reported (12, 19). Mouse monoclonal anti-CEA (clone COL-1) was from NeoMarkers (Lab Vision, Italy).

Cell Cultures, Transfections, and Treatments—CHO cells expressing Polyoma virus T antigen and Fuc-TIII (CHO-T-FT), COLO-205, and WM266-4 cells were cultured as described previously (12, 19). Human gastric adenocarcinoma MKN-45 cells (a gift of C. Ponzetto, University of Turin), were cultured in RPMI 1640 medium containing 10% fetal bovine serum, 100 units/ml penicillin, 1.0 mg/ml streptomycin, and 2 mM L-Glu. For transient transfection, 2.0×10^5 CHO-T-FT cells were plated in 12-well plates 20 h before transfection, washed with serum free α-minimal essential medium, and incubated with 0.5 ml of transfection solution for 3 h under usual growing conditions. Transfection solutions, containing 1 μg/ml β3Gal-T cDNA in pcDNAI or pCDM8 vectors plus 0.065 μg/ml pcDNAI-Luc and 18 μl/ml DOTAP (Roche Molecular Biochemicals) were prepared and used as reported previously (12). Seventy-two hours after transfection, cells were harvested, washed, and resuspended with PBS. One-tenth of resuspended cells was processed for luciferase activity determination, using a commercial kit (Luciferase assay system, Promega) according to the manufacturer's recommendations. The remaining material was stained and analyzed by flow cytometry as previously reported (12, 19). For treating cell lines and clones with drugs affecting glycosylation, 1×10^5 cells were plated in 12-well plates and incubated overnight, and the medium was replaced with medium containing 0.1 μg/ml swainsonine (Sigma), 2 mM benzyl-α-GalNAc (Sigma), or 2 mM sodium butyrate. After 48 h, medium was replaced with fresh medium containing drugs. After additional 48 h, cells were collected, stained, and analyzed by flow cytometry (19).

Construction of CHO Clones—CHO clones expressing either Fuc-TIII and β3Gal-T5 (CHO-FT-T5), Fuc-TIII and β3Gal-T2 (CHO-FT-T2), or Fuc-TIII and β3Gal-T1 (CHO-FT-T1) were obtained by the calcium phosphate transfection method (23), using a modification of the procedure reported (24). Briefly, the DNA mixture (20 μg) contained 1 μg of *Eco*RI-linearized pSV2Neo, 10 μg of *Sca*I-linearized pcDNAI/Fuc-TIII, and 10 μg of *Sca*I-linearized pCDM8/β3Gal-T5, or 10 μg of *Kpn*I-linearized pCDM8/β3Gal-T2, or 10 μg of *Sca*I-linearized pcDNAI/β3Gal-T1, respectively. Upon selection with 0.4 mg/ml active G418, 30 (CHO-FT-T5 and CHO-FT-T1) or 60 colonies (CHO-FT-T2) were collected using cloning cylinders, grown in tissue culture slides, stained with anti-sLe^a or anti-Le^a antibody followed by secondary FITC-conjugated anti-mouse IgG, and analyzed by fluorescence microscopy. Two or three positive colonies were subcloned by limiting dilution in 96-well plates, and several subclones were analyzed as above. sLe^a and Le^a expression on positive colonies was quantitated by flow cytometry. Single colonies expressing a constant level of sLe^a and Le^a, named CHO-FT-T5, CHO-FT-T2, and CHO-FT-T1, respectively, were selected and used for further characterization and experiments. CHO cells expressing CEA (CHO-FT-T5-CEA) were obtained from clone CHO-FT-T5 by the transfection procedure described above but using 20 μg of *Kpn*I-linearized pCDM8-CEA and 1 μg of pHA58 plasmid in the DNA mixture, and 0.5 mg/ml hygromycin B (Roche Molecular Biochemicals) for selection. pHA58 plasmid, a generous gift of P. Morandini (University of Milan), was derived from pSV72 vector and had the hygromycin B gene under the control of mouse phosphoglycerate kinase-1 promoter. Monoclonal anti-CEA antibody was used, at 1:100 dilution, for cell staining.

DNA Preparation—β3Gal-T1 cloning in pcDNAI vector has been previously reported (12). For β3Gal-T2, -T3, -T4, -T5, and CEA cloning, the corresponding coding sequences were amplified using single-stranded cDNA as template, obtained by reverse transcription of total RNA extracted (12) from WM266-4 (β3Gal-T2), MKN-45 (β3Gal-T3 and CEA), or COLO-205 cells (β3Gal-T4 and -T5), in a reaction mixture containing, in 40-μl volume, 50 mM Tris-HCl, pH 8.3, 50 mM KCl, 5 mM $MgCl_2$, 1000 units/ml human placental ribonuclease inhibitor, 1 mM of each deoxynucleotide triphosphate, 0.4 μM oligo$(dT)_{16}$ primer, 1000

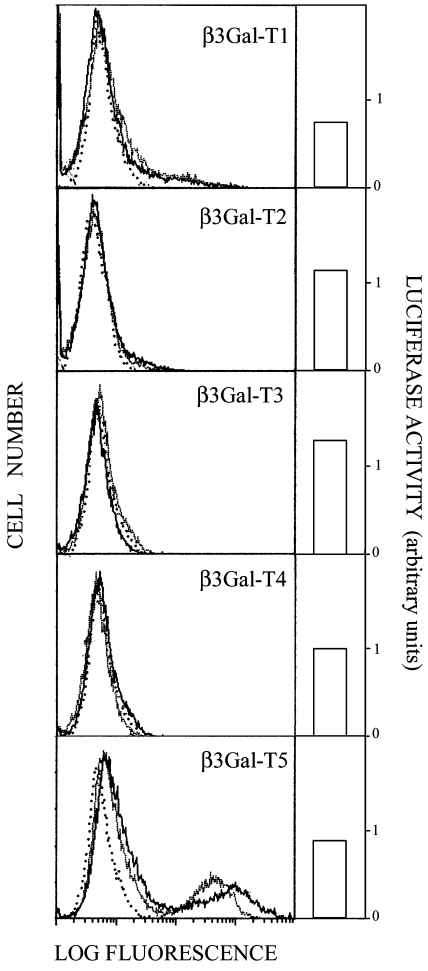

FIG. 1. **Flow cytometry analysis of CHO-T-FT cells transiently cotransfected with different β3Gal-T and luciferase cDNAs.** CHO-T-FT cells, expressing Polyoma virus T antigen and human Fuc-TIII, were transfected using liposomes containing a 15:1 mixture of individual β3Gal-T and luciferase cDNAs, each cloned in pCDM8 or pcDNAI expression vectors. Seventy-two hours later, cells were harvested and stained with anti-sLe^a (*solid line*) or anti-Le^a (*stippled line*) monoclonal antibodies followed by FITC-conjugate anti-mouse IgG secondary antibody, or with secondary antibody alone (*dotted line*), and analyzed by flow cytometry. An aliquot of the same cells was homogenized and used for luciferase activity assay.

units/ml avian myeloblastosis virus reverse transcriptase (Amersham Pharmacia Biotech), and freshly denatured total RNA (250 μg/ml) as template. Reactions were kept for 90 min at 42 °C. Amplifications were performed in the presence of a commercially available "high fidelity" *Taq* polymerase (LA *Taq*, Takara), according to the manufacturer's protocol, using 2.5 μg/ml of each oligonucleotide primer, deduced from published sequences, and 4 μl of reverse transcription reaction for each 100 μl of amplification mixture. PCR reactions were incubated as follows: a single treatment at 94 °C for 3.5 min followed by a cycle consisting of 1.5 min at 94 °C (melting), 2.0 min at 62 °C (annealing), and 2.5 min at 72 °C (extension), repeated 30 (β3Gal-Ts) or 15 times (CEA); a final extension step was performed at 72 °C for 8 min. The amplified DNA was digested with *Hin*dIII and *Xba*I (β3Gal-T3) or ligated to *Bst*XI adaptors (all others), and cloned in pcDNAI (β3Gal-T3) or pCDM8 vectors (all others) using a procedure reported previously (12). Direct DNA sequencing of the obtained constructs, performed by the dideoxynucleotide chain-termination method using an automated procedure, indicated that the coding sequences are identical to those published. pcDNAI-Luc, expressing the luciferase gene, was constructed by removing the luciferase coding sequence from plasmid pGL3 (Promega) using *Hin*dIII and *Xba*I, and subcloning into the corresponding sites of pcDNAI vector.

Enzyme Assays and Reaction Product Characterization—β3Gal-T5 and Fuc-TIII were assayed in cell clones as previously reported (19). For kinetic analysis, β3Gal-T5 was assayed upon transfection of pCDM8-β3Gal-T5 in COS-7 cells. Cells were transfected, harvested, washed, resuspended, and used as the enzyme source, as described previously (21). Enzyme activity was determined in a reaction mixture containing, in a final volume of 10 μl, 0.1 M Tris/HCl buffer, pH 7.0, 10 mM $MnCl_2$, 0.5 mg/ml Triton X-100, 1 mg/ml α-lactalbumin, 5 mM CDP-choline, 0.8 mM donor UDP-[^{3}H]Gal, specific radioactivity (10 mCi/mmol), and 0.2–1.0 mg/ml cell protein, in the presence of different acceptors at various concentrations. In the case of glycolipid acceptor, it was dissolved in chloroform/methanol, 4:1 (v/v), mixed in the reaction tube with 15 μg of Triton CF-54 dissolved in the same solvent, and dried before adding the reaction mixture. Incubations were done at 37 °C for 60 min. At the end of incubation, reaction products were assayed by Dowex chromatography (oligosaccharide acceptors) or descending paper chromatography (glycolipid acceptor) according to previously reported protocols (19, 25). The oligosaccharide reaction products were identified by pooling several Dowex eluates, which were lyophilized and purified by Bio-Gel P-2 chromatography (21). The obtained saccharides were treated with *D. pneumoniae* or *Xanthomonas manihotis* β-galactosidases, specific for β1,4- and β1,3-linkages, respectively, and analyzed using a Bio-Gel P-2 column (19). Reaction product with glycolipid acceptor, pooled from several reactions, was purified by Sep-Pack C-18 cartridge, desalted by partitioning in chloroform/methanol/water, 2:1:1 (v/v), and analyzed by HPTLC, using chloroform/methanol/water, 60:35:8 (v/v), as eluting solvent system, and visualized by fluorography (25).

Metabolic Labeling and Carbohydrate Analysis—CHO clones (2.0×10^6 cells) were plated in 60-mm dishes containing 0.1 mCi of [^{3}H]Gal (Amersham Pharmacia Biotech) in 2.5 ml of culture medium and incubated 40 h under regular conditions. Labeled cells were harvested as for immunostaining, resuspended in PBS at a density of 4×10^6 cells/0.1 ml, and kept frozen until used. *N*-Glycans were released from cell suspension by *N*-glycanase (Glyko) digestion of the material denatured by heating in the presence of SDS, under the conditions recommended by the manufacturer. Released *N*-glycans were isolated by a Sep-Pak C-18 cartridge and further purified by Sephadex G-50 chromatography, as reported (24). Oligosaccharides released by endo-β-galactosidase were characterized following the procedure reported by Seuyoshi *et al*. (26) with some modifications. Purified *N*-glycans were passed through a Bio-Gel P-4 column (0.7×50 cm), and only the radioactivity eluted with water as a peak close to the exclusion volume was collected, lyophilized, and used for characterization. Endo-β-galactosidase digestion was performed on radioactive *N*-glycans, 10,000–20,000 cpm/μl, using the enzyme from *Bacteriodes fragilis* (Glyko), 0.5 milliunit/μl, in the buffer supplied by the manufacturer, for 20 h at 37 °C. The reaction mixture was then applied to the same Bio-Gel P-4 column as above, and the radioactivity was eluted with the exclusion volume collected and referred to as the endo-β-galactosidase resistant *N*-glycans, whereas the radioactivity eluted as oligosaccharides was applied to a QAE-Sephadex column to separate neutral and charged sugars, according to a previous procedure (26). Material collected in the flow-through was referred to as the neutral oligosaccharides, whereas that eluted with NaCl was collected, desalted on a Bio-Gel P-2 column, and treated with Newcastle disease virus sialidase. Neutral and de-sialylated oligosaccharides were analyzed by a Bio-Gel P-4 column (0.7×100 cm), eluted with water at a flow rate of 0.1 ml/min, 4.5 min/fraction. The obtained peaks were collected, lyophilized, and treated with glycohydrolases for characterization. *X. manihotis* β-galactosidase (Glyko), almond meal α-fucosidase (Oxford Glycosystem), *D. pneumoniae* β-*N*-acetylglucosaminidase (Glyko), *D. pneumoniae* β-galactosidase (Sigma), and Newcastle disease virus sialidase (Glyko) digestions were performed on radioactive oligosaccharides, 1000–2000 cpm/μl, according to the procedures reported (19, 26). The amount of Gal released by glycohydrolase treatment of endo-β-galactosidase-resistant *N*-glycans was determined as reported (19). Glycohydrolase digestions of the tetrasaccharide obtained from clone CHO-FT-T5 were analyzed using a Bio-Gel P-2 column (0.7×100 cm) eluted with water at a flow rate of 0.1 ml/min, 4.5 min/fraction, whereas those of the peaks were collected from CHO-FT-T1 using a Bio-Gel P-4 column (0.7×100 cm) under the same conditions.

CEA Purification and Characterization—CEA was extracted and purified following a published procedure (27). Briefly, about 2.5×10^7 metabolically labeled cells, prepared as above described, were diluted to 1.5×10^7 cells/ml in PBS, homogenized by vortexing 1 min, and treated with 0.1 unit/ml phosphatidylinositol phospholipase C (Glyko) at 37 °C for 2 h. Total reaction was spun at 14,000 rpm for 10 min, and the supernatant was collected, made 0.6 M with perchloric acid, and centrifuged as above. The clear supernatant was neutralized with NaOH, freeze-dried, resuspended, and loaded onto a Sepharose CL6B column equilibrated and eluted with 50 mM PBS, pH 5.2. Elution was monitored by liquid scintillation counting, and relevant fractions were analyzed by SDS-PAGE and visualized by fluorography. Western blotting

was performed as previously reported (24) using monoclonal anti-CEA (1:200), anti-sLe^a (1:500), and anti-sLe^x (1:100) antibodies, followed by peroxidase-conjugated goat anti-mouse immunoglobulin (Amersham Pharmacia Biotech) (1:2000). ECL was developed with Renaissance chemiluminescence reagent (PerkinElmer Life Sciences). Homogeneous fractions were collected, lyophilized, and desalted on a Bio-Gel P-2 column. Purified [^{3}H]CEA was treated with *C. perfringens* sialidase and almond meal α-fucosidase, and then with endo-β-galactosidase, under the conditions reported for *N*-glycan digestion. The reaction mixture was then applied to a Bio-Gel P-2 column (0.7 × 50 cm), eluted with water at a flow rate of 0.13 ml/min, 2.5 min/fraction. The obtained peaks, as well as the total purified CEA, were treated with β-galactosidases and analyzed by Bio-Gel P-2 chromatography as above described.

Competitive RT-PCR Analysis—For the analysis of human samples, bioptic specimens were collected at surgery, immediately frozen in dry ice, and placed in liquid nitrogen until used. For RNA extraction, 1–2 mm^3 of material was homogenized with a rotary homogenizer in 0.5 ml of the lysis buffer from a Qiagen RNeasy minikit and processed in the presence of DNase according to the manufacturer's recommendations. First strand cDNA was synthesized by Moloney murine leukemia virus reverse transcriptase (Amersham Pharmacia Biotech) as previously reported (12). Control reactions were prepared by omitting the reverse transcriptase in the mixture. First strand cDNA was amplified in a 50-μl reaction and in the presence of 20 fg of competitor for 35 cycles (glycosyltransferases) or 5 pg of competitor for 25 cycles (β-actin), under the conditions reported (12). No amplification was detected when the control reactions were used as template. Human β-actin, Fuc-TIII, FUT2, and β3Gal-T1 competitors and oligonucleotide primers used were the same as those described in a previous study (12). β3Gal-T2, -T3, -T4, -T5, and mouse β-actin oligonucleotide primers, as well as the restriction enzymes used for competitor preparation, are listed in Table I. Quantification of human β3Gal-T5, β3Gal-T2, β3Gal-T1, and Fuc-TIII transcripts in CHO clones was performed by the same procedure but using 60 fg of each glycosyltransferase competitor, or 20 pg of mouse β-actin competitor, in 50-μl reactions that were amplified for 30 (glycosyltransferases) or 25 cycles (β-actin).

RESULTS

Biosynthesis of sLe^a and Le^a in CHO Cells Transiently Transfected with β3Gal-T cDNAs—The ability of different cloned β3Gal-Ts to synthesize the type 1 chain was assessed to determine the amount of sLe^a and Le^a antigens expressed upon cDNA transfection on the surface of CHO-T-FT cells, a clone permanently expressing Fuc-TIII and Polyoma virus T antigen. For this purpose, cells were transiently cotransfected with β3Gal-T and reporter luciferase cDNAs both placed in expression vectors having the Polyoma virus origin of replication. Luciferase activity from different transfections ranged between 0.6 and 1.4 units. Despite such differences in transfection efficiency, both sLe^a and Le^a antigens were easily detected in cells transfected with β3Gal-T5 (very bright) and β3Gal-T1, poorly detected with β3Gal-T2, and not at all detected with β3Gal-T3 and β3Gal-T4. In a typical experiment (Fig. 1), a peak representing up to 35% of cells transfected with β3Gal-T5 appears intensely bright mostly after anti-sLe^a antibody staining. Fluorescence intensity on positive cells was much lower upon β3Gal-T1 transfection, and faintly or not detectable in the other cases, although transfection efficiency was similar or sometimes higher, as assessed by luciferase activity assay. These results suggest that different substrate specificity exists among the different β3Gal-Ts, and only β3Gal-T5 finds large amount of suitable acceptors in CHO cells.

Construction and Characterization of CHO Clones Expressing β3Gal-T5 and Fuc-TIII, β3Gal-T2 and Fuc-TIII, or β3Gal-T1 and Fuc-TIII—To evaluate the hypothesis that the amount of sLe^a and Le^a antigens expressed in CHO cells transfected with β3Gal-Ts reflects the availability of the proper precursors, we constructed three CHO clones permanently expressing the antigens upon stable transfection with either β3Gal-T5 and Fuc-TIII, β3Gal-T2 and Fuc-TIII, or β3Gal-T1 and Fuc-TIII. All clones, named CHO-FT-T5, CHO-FT-T2, and CHO-FT-T1, respectively, express human Fuc-TIII transcript in a similar amount, as determined by competitive RT-PCR, whereas each clone expresses only its corresponding human β3Gal-T transcript (Fig. 2*A*). Flow cytometry analysis (Fig. 2*B*) of the obtained clones shows intense and homogeneous staining with anti-sLe^a and anti-Le^a antibodies in CHO-FT-T5 cells, weak and heterogeneous staining in CHO-FT-T1, and very faint staining in CHO-FT-T2 despite the high expression level of the transcript, thus providing fluorescence patterns very similar to

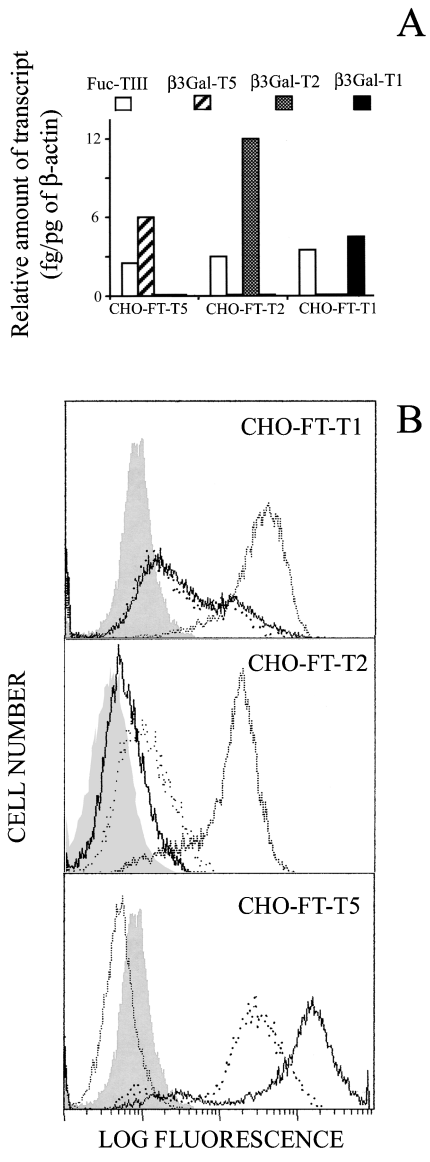

FIG. 2. **Characterization of CHO-FT-T5, CHO-FT-T2, and CHO-FT-T1 clones.** CHO clones permanently expressing human Fuc-TIII and β3Gal-T5 (*CHO-FT-T5*), Fuc-TIII and β3Gal-T2 (*CHO-FT-T2*), or Fuc-TIII and β3Gal-T1 (*CHO-FT-T1*) were constructed as described under "Experimental Procedures." *A*, total RNA was extracted from the clones and reverse-transcribed, and the obtained first strand cDNA was amplified by PCR using primers specific for human glycosyltransferases or mouse β-actin, in the presence of the proper competitor DNA. One-fifth aliquot of each PCR reaction was analyzed by 1% agarose gel electrophoresis, visualized by staining with ethidium bromide, and photographed, and the spots were quantitated by densitometric scanning of the negative film. The amounts of amplified target cDNAs were calculated from their respective standard curves and normalized by those for β-actin. *B*, cell clones were stained with monoclonal anti-sLe^a (*solid line*), anti-Le^a (*dotted line*) (both IgG), or anti-sLe^x (*stippled line*) (IgM) monoclonal antibody, followed by FITC-conjugate anti-mouse IgG or IgM, respectively, and analyzed by flow cytometry. Negative controls (*shaded areas*) represent the cell clones stained with FITC-conjugate anti-mouse IgG alone.

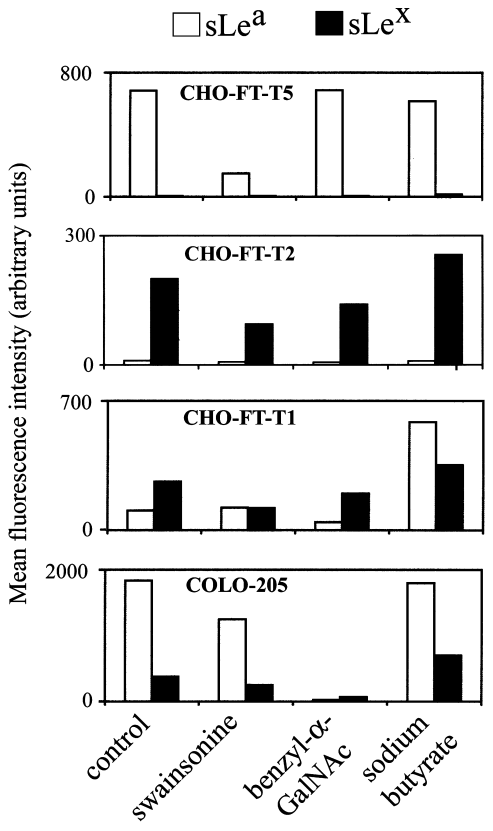

FIG. 3. **Effect of drugs affecting glycosylation on the expression of sLe^a and sLe^x antigens in different cells.** Cells were grown under regular conditions (controls), in the presence of 0.1 μg/ml swainsonine, 2 mM benzyl-α-GalNAc, or 2 mM sodium butyrate. At the end of incubation, cells were harvested, stained, and analyzed by flow cytometry as in Fig. 2.

TABLE II
Radioactivity distribution in CHO clones metabolically radiolabeled with [^{3}H]Gal

Values are expressed as cpm × 10^6/mg cell protein.

	CHO-FT-T5 (%)	CHO-FT-T1 (%)	CHO-T-FT (%)
Total cell incorporation	6.2 (100)	8.4 (100)	7.9 (100)
N-Glycans	5.2 (83.8)	6.4 (76.2)	6.2 (78.4)
Oligosaccharides			
Endo-β-galactosidase-sensitive	1.2 (19.3)	1.6 (19.0)	1.5 (18.9)
Neutral[a]	0.84 (13.5)	1.0 (11.9)	0.92 (11.6)
Charged[b]	0.39 (6.3)	0.52 (6.2)	0.55 (6.9)

[a] Unbound to QAE-Sephadex.
[b] Bound to QAE-Sephadex.

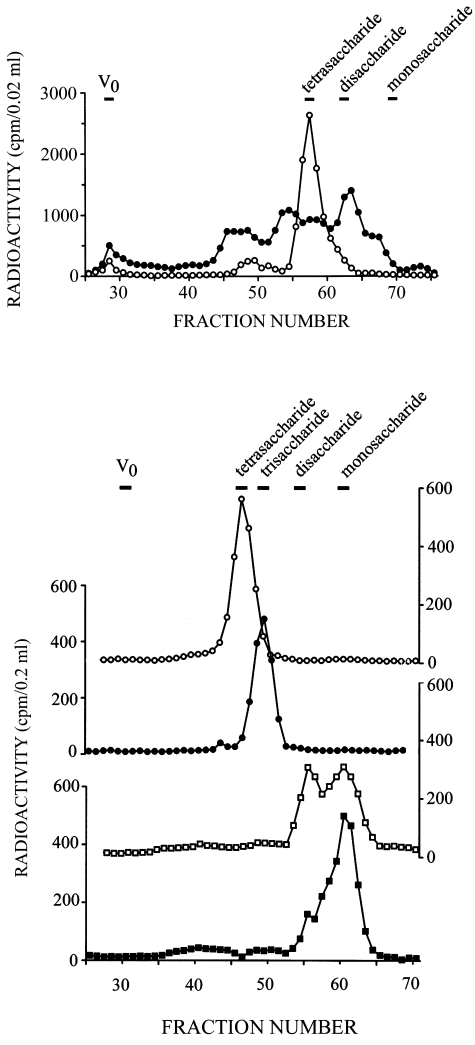

FIG. 4. **Characterization of radioactive oligosaccharides released by endo-β-galactosidase.** *Upper panel,* the neutral radioactive oligosaccharides released by endo-β-galactosidase from the *N*-glycans isolated from CHO-FT-T5 (*empty circles*) or CHO-FT-T1 (*solid circles*), were submitted to Bio-Gel P-4 filtration. *Lower panel*, the peak from CHO-FT-T5 cells eluted at the tetrasaccharide size (*fractions 55–59* in *upper panel*) was submitted to Bio-Gel P-2 chromatography upon no treatment (*empty circles*), almond meal α-fucosidase digestion (*full circles*), almond meal α-fucosidase plus *X. manihotis* β-galactosidase digestion (*empty squares*), or to almond meal α-fucosidase plus *X. manihotis* β-galactosidase and *D. pneumoniae* β-*N*-acetylglucosaminidase digestion (*full squares*). Calibration of each column is indicated at the *top* of each *panel*. *Tetrasaccharide*, Galβ1→4GlcNAcβ1→3Galβ1→4Glc; *trisaccharide*, Fucα1→2Galβ1→3GlcNAc; *disaccharide*, Galβ1→3GlcNAc; *monosaccharide*, Gal.

those obtained by the transient transfection experiments (Fig. 1). Moreover, CHO-FT-T2 and CHO-FT-T1 cells are homogeneously bright with anti-sLe^x staining, as expected in CHO cells expressing Fuc-TIII, whereas CHO-FT-T5 cells are totally negative (Fig. 2*B*). This last result is surprising and suggests a competition between Gal-Ts in the synthesis of type 1 *versus* type 2 chain Lewis antigens.

Effect of Drugs Affecting Glycosylation on the Expression of sLe^a and sLe^a in CHO Clones—CHO cells are known to express mostly complex type *N*-glycans and simple core 1 type *O*-glycans (28). Because the latter are not precursors of type 1 chains, our findings suggest that β3Gal-T5 has a distinctive ability to act on complex type *N*-glycans. To address this issue and to study the contribute of *N*- and *O*-linked glycosylation on the expression of sLe^a and sLe^x, cells were treated with swainsonine and benzyl-α-GalNAc, selectively affecting complex type *N*-glycan and *O*-glycan biosynthesis, respectively, before staining with anti-sLe^a or anti-sLe^x antibodies. sLe^a staining is 80% inhibited by swainsonine treatment in CHO-FT-T5, whereas benzyl-α-GalNAc treatment is ineffective and sLe^x staining is absent (Fig. 3). Conversely, in clone CHO-FT-T2 sLe^a staining is almost absent, whereas sLe^x staining is reduced by swainsonine treatment (about 65%) and slightly stimulated by sodium butyrate. In clone CHO-FT-T1 sLe^a staining is almost 70% inhibited by benzyl-α-GalNAc, whereas swainsonine treatment is ineffective; moreover, sodium butyrate treatment stimulates sLe^a synthesis 5-fold. sLe^x staining in this clone is reduced by swainsonine treatment and slightly stimulated by sodium butyrate, as in CHO-FT-T2. In control COLO-205 cells, sLe^a and sLe^x reactivity is totally abolished by benzyl-α-GalNAc, and only about 30% inhibited by swainsonine treatment (Fig. 3). These results indicate that sLe^a expressed in CHO-FT-T5 cells is carried by complex type *N*-glycans, presumably

TABLE III
[^{3}H]Gal released by D. pneumoniae or X. manihotis β-galactosidase treatment of endo-β-galactosidase resistant N-glycans prepared from CHO clones

The whole reaction mixtures (about 5000 cpm) were applied to Bio-Gel P-2 column, and radioactivity in the eluted fractions was determined by liquid scintillation counting. Results are the means for two independent experiments and are presented as percentage of recovered radioactivity in the galactose peak.

Condition	CHO-FT-T5		CHO-FT-T1	
	D. pneumoniae	*X. manihotis*	*D. pneumoniae*	*X. manihotis*
	%			
Alone	<5	<5	7.4	<5
Plus sialidase	50.5	17.4	45.3	<5
Plus fucosidase	<5	<5	6.8	<5
Plus sialidase and fucosidase	48.2	14.3	47.2	<5

the same carrying the sLe^x epitope in CHO-FT-T1 and CHO-FT-T2 cells.

Characterization of Complex N-Glycans Formed in Clone CHO-FT-T5—To investigate the actual nature of the saccharides used as substrates by β3Gal-T5 in CHO cells, we studied the radioactive structures formed upon metabolic labeling with [^{3}H]Gal in CHO-FT-T5 as well as in CHO-FT-T1 and CHO-T-FT cells, a clone not expressing β1,3Gal-T but Fuc-TIII only. The *N*-glycans were prepared from the total incorporated radioactivity and treated with endo-β-galactosidase, and the released oligosaccharides were separated in the neutral and charged fractions. As shown in Table II, the radioactivity recovered after each step is rather similar in the three clones. On the other hand, the composition of the neutral saccharides released by endo-β-galactosidase treatment of CHO-FT-T5 *N*-glycans is dramatically different from that of CHO-FT-T1 *N*-glycans. In fact, almost all radioactivity derived from clone CHO-FT-T5 is eluted as a single peak in the area of tetrasaccharides by Bio-Gel P-4 fractionation, whereas that from clone CHO-FT-T1 is distributed in several peaks, including some of higher molecular weight as well as one in the disaccharide area (Fig. 4, *upper panel*). The radioactive peak obtained from CHO-FT-T5 *N*-glycans is converted to a trisaccharide by almond meal α-fucosidase, to an equal mixture of disaccharide and monosaccharide by the action of both almond meal α-fucosidase and *X. manihotis* β-galactosidase, and to a monosaccharide by the combination of the above enzymes and *D. pneumoniae* β-*N*-acetylglucosaminidase (Fig. 4, *lower panel*). Digestion of the tetrasaccharide peak with almond meal α-fucosidase and *D. pneumoniae* β-galactosidase determines the formation of a single peak in the area of trisaccharides but not detectable monosaccharides (not shown). The elution profile obtained after de-sialylation of the charged oligosaccharides is identical, indicating that Galβ1→3[Fucα1→4]GlcNAcβ1→3Gal and NeuAcα2→3Galβ1→3[Fucα1→4]GlcNAcβ1→3Gal account for the vast majority of oligosaccharides released by endo-β-galactosidase digestion of the *N*-glycans formed in clone CHO-FT-T5. However, the elution profile of the oligosaccharides released by endo-β-galactosidase treatment of CHO-FT-T1 and CHO-T-FT cells are very similar. Among such peaks, the one eluted as a disaccharide was identified as GlcNAcβ1→3Gal, because it is converted to a monosaccharide by *D. pneumoniae* β-*N*-acetylglucosaminidase. Such a disaccharide is formed by the action of endo-β-galactosidase on multiple lactosamine repeats (29). The peak eluted at the size of tetrasaccharides was identified as Galβ1→4[Fucα1→3]GlcNAcβ1→3Gal, because it is converted to a monosaccharide only by the sequential action of almond meal α-fucosidase, *D. pneumoniae* β-galactosidase, and *D. pneumoniae* β-*N*-acetylglucosaminidase. The larger peaks (fractions 51–55 and 46–50 in Fig. 4, *upper panel*) contain one and two other major oligosaccharides, respectively. They were identified by sequential exoglycosidase digestions and Bio-Gel P-4 filtration as the pentasaccharide, hexasaccharide, and heptasaccharide already reported (26). Such oligosaccharides contain double lactosamine repeats fucosylated on the inner GlcNAc, a substitution that makes the Galβ1→4GlcNAc linkage resistant to endo-β-galactosidase under usual reaction conditions (30). These results strongly suggest that the (±NeuAcα2→3)Galβ1→3[Fucα1→4]GlcNAcβ1→3Gal oligosaccharide synthesized in CHO-FT-T5 replaces the poly-*N*-acetyllactosamine chains and sLe^x structure present in CHO-FT-T1 as well as in CHO cells expressing Fuc-TIII only. To assess the presence of type 1 chain carbohydrates in endo-β-galactosidase-resistant *N*-glycans, we have determined the amount of radioactive Gal released by *X. manihotis* β-galactosidase. As shown in Table III, *X. manihotis* β-galactosidase is active only on the glycans derived from CHO-FT-T5, but not on those from CHO-FT-T1. The amount of Gal released by the enzyme is strongly dependent on the concurrent action of sialidase, but not on that of α-fucosidase, and is much lower than that released by *D. pneumoniae* β-galactosidase under the same reaction conditions. These results indicate that type 1 chains synthesized on endo-β-galactosidase-resistant *N*-glycans by β3Gal-T5 are present in limited amount and are not significantly substituted by fucose residues.

TABLE IV
Kinetic properties of β3Gal-T5

Substrate	K_m	V_{max}	V_{max}/K_m
	mM	*nmol/mg protein/h transferred Gal*	
UDP-Gal	0.29		
GlcNAc	161	853	5.3
GlcNAcβ1→3Galβ1→*O*-methyl	0.45	788	1751
GlcNAcβ1→3Galβ1→4Glc	1.91	755	395
GlcNAcβ1→2Man	20.9	825	39.5
GlcNAcβ1→6Manα1→*O*-methyl	34.7	1034	29.8
Lactotriosylceramide	0.34	1347	3961

In Vitro Properties of β3Gal-T5—Using a cell homogenate prepared from COS cells transiently transfected with β3Gal-T5 cDNA as the enzyme source, we determined the optimal reaction conditions using GlcNAc as acceptor and found that the activity requires Mn^{2+}, is maximal at neutral pH, is saturated by donor UDP-Gal concentrations above 0.5 mM, and is not affected by α-lactalbumin. Under the same reaction conditions, various oligosaccharides are also used. The obtained reaction products are over 95% affected by *X. manihotis* β-galactosidase and almost unaffected by *D. pneumoniae* β-galactosidase. In the presence of the proper detergent concentration, the same homogenate very efficiently transfers Gal to the glycolipid lactotriosylceramide, and the reaction product was identified as lactotetraosylceramide (Galβ1→3GlcNAcβ1→3Galβ1→4Glcβ1→1′Cer) by HPTLC mobility and differential sensitivity to β-galactosidases. The calculated K_m and V_{max} values (Table IV) indicate that the enzyme prefers the GlcNAcβ1→3Gal end but also distinguish among the different carrier molecules,

TABLE V
Major clinical features of the patients with colon adenocarcinoma whose biopsy were analyzed by competitive RT-PCR

Case	Age	Sex[a]	Localization	Grade[b]	Stage (Dukes')	CEA[c]	CA19.9[d]
						ng/ml	*units/ml*
1	61	F	Splenic flexure	G2	C2	5.3	72.9
2	75	M	Hepatic flexure	G2	C2	53.2	84.6
3	69	M	Rectum	G2	B2	NA[e]	NA
4	76	F	Cecum	G3	C2	60.5	19.2
5	64	F	Ascending colon	G2	B2	4.7	115
6	78	M	Ampulla recti	G2	B2	NA	NA
7	77	M	Cecum	G2	D	NA	NA
8	77	F	Ascending colon	G2	B2	NA	NA
9	66	F	Sigmoid colon	G2	B2	NA	NA
10	65	F	Rectum	G3	C1	NA	NA

[a] M, male; F, female.
[b] G2, moderately differentiated; G3, poorly differentiated.
[c] Normal range <5.0 ng/ml.
[d] Normal range <37 units/ml.
[e] NA, not available.

with a preference for the glycolipid acceptor. GlcNAc linked to α-Man through β1,2- or β1,6-linkages is also a suitable acceptor, but with much lower affinity. These *in vitro* results are in good agreement with those obtained by the structural analysis of CHO-FT-T5 clone, where we found that endo-β-galactosidase-sensitive saccharides, having the GlcNAcβ1→3Gal acceptor sequence, are preferentially operated upon by β3Gal-T5, whereas endo-β-galactosidase-resistant *N*-glycans, mostly having GlcNAcβ1→2/6Man as acceptor sequences, are poorly utilized.

Expression of β3Gal-T5 Transcript in Normal Colon Mucosa and in Adenocarcinomas—To evaluate whether the β3Gal-T5 transcript is differentially expressed during carcinogenesis, we analyzed its amount by competitive RT-PCR performed on total RNA extracted from human colon specimens collected at surgery, representing both adenocarcinomas and surrounding normal mucosa. Clinical features and tumor staging are outlined in Table V. The β3Gal-T5 transcript is detected in all normal mucosa samples, although it is faintly detectable or undetectable in adenocarcinomas whose cDNA is well amplified using control primers such as those for β-actin or other glycosyltransferases (Fig. 5). In quantitative terms (Fig. 6), the β3Gal-T5 transcript is on the average 30-fold less expressed in adenocarcinomas than in normal mucosa. In individual cases, the levels in adenocarcinomas range from 4-fold to over 100-fold less than in normal mucosa. For comparison we looked at the expression levels of the other β3Gal-T transcripts, as well as of Fuc-TIII and FUT2, two fucosyltransferases involved in type 1 chain fucosylation. β3Gal-T4 transcript is expressed at high and heterogeneous levels in normal mucosa and remains detectable in all adenocarcinomas. β3Gal-T1 transcript is detectable in eight normal mucosa and four adenocarcinoma cases, whereas β3Gal-T2 transcript is detectable in four normal mucosa and seven adenocarcinoma cases. β3Gal-T3, Fuc-TIII, and FUT2 transcripts are heterogeneously expressed in both normal mucosa and adenocarcinomas. Altogether, these data suggest that the β3Gal-T5 transcript, almost undetectable in colon adenocarcinomas, is strongly down-regulated during carcinogenesis.

Characterization of CEA Purified from a CHO Clone Expressing β3Gal-T5—To study the ability of β3Gal-T5 to act on CEA, a CHO clone permanently expressing this antigen, as well as β3Gal-T5 and Fuc-TIII, was obtained by transfecting CHO-FT-T5 cells with CEA (CHO-FT-T5-CEA). As assessed by flow cytometry, the expression levels of sLe^a and Le^a antigens in this clone are identical to those of the starting one, whereas those of CEA were comparable to that of human cancer cell lines. In particular, we measured a mean fluorescence intensity

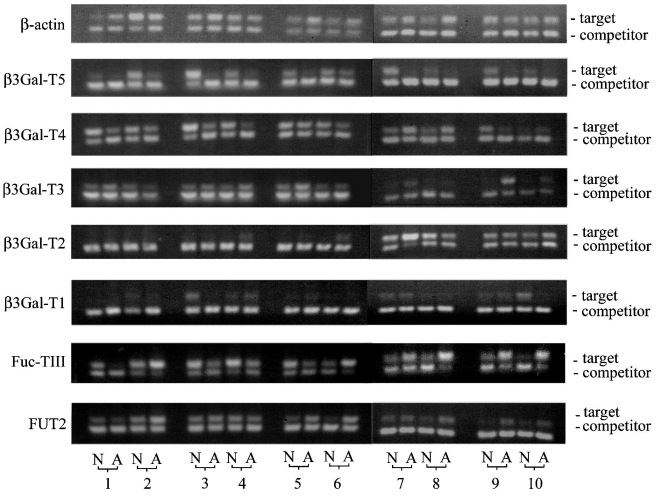

FIG. 5. **Competitive RT-PCR analysis of β3Gal-T5 transcript in normal mucosa and in adenocarcinomas.** RNA extracted from adenocarcinomas and surrounding normal mucosa, collected at surgery, was reverse-transcribed, and the obtained first strand cDNA was mixed with competitor (truncated) cDNAs (5 pg and 20 fg for β-actin and glycosyltransferases, respectively), and subjected to PCR (25 and 35 cycles for β-actin and glycosyltransferases, respectively). Primers and PCR product length are indicated in Table I. One-fifth aliquot of each amplification reaction was electrophoresed in 1% agarose gel and visualized by staining with ethidium bromide. *N*, normal mucosa; *A*, adenocarcinoma. *Numbers* denote patients according to Table V. *Samples 1–6* and *7–10* were run on different gels.

lower than in MKN-45 cells, but higher than in COLO-205 cells (not shown). The tritiated material purified from clone CHO-FT-T5-CEA metabolically radiolabeled with [^{3}H]Gal runs on SDS-PAGE as a single radioactive band of an apparent molecular mass of 200 kDa (Fig. 7). Immunoblot analysis shows that this band strongly reacts with anti-CEA antibody. Sequential stripping and reprobing of the filter with anti-Lewis antigen antibodies reveals that the purified CEA reacts with anti-sLe^a but not with anti-sLe^x antibodies, indicating that type 1 chains are specifically present on CEA expressed in the clone. To directly assess the presence of type 1 chain carbohydrates on CEA and to determine whether they are preferentially bound to GlcNAcβ1→3Galβ1→4GlcNAcβ1→R outer sequences, the purified [^{3}H]CEA, once de-fucosylated and de-sialylated, was treated with different β-galactosidases before and after endo-β-galactosidase treatment. To this end, the endo-β-galactosidase reaction mixture was submitted to Bio-Gel P-2 filtration and the endo-β-galactosidase-resistant and -sensitive radioactivity was recovered with the exclusion volume and at the size

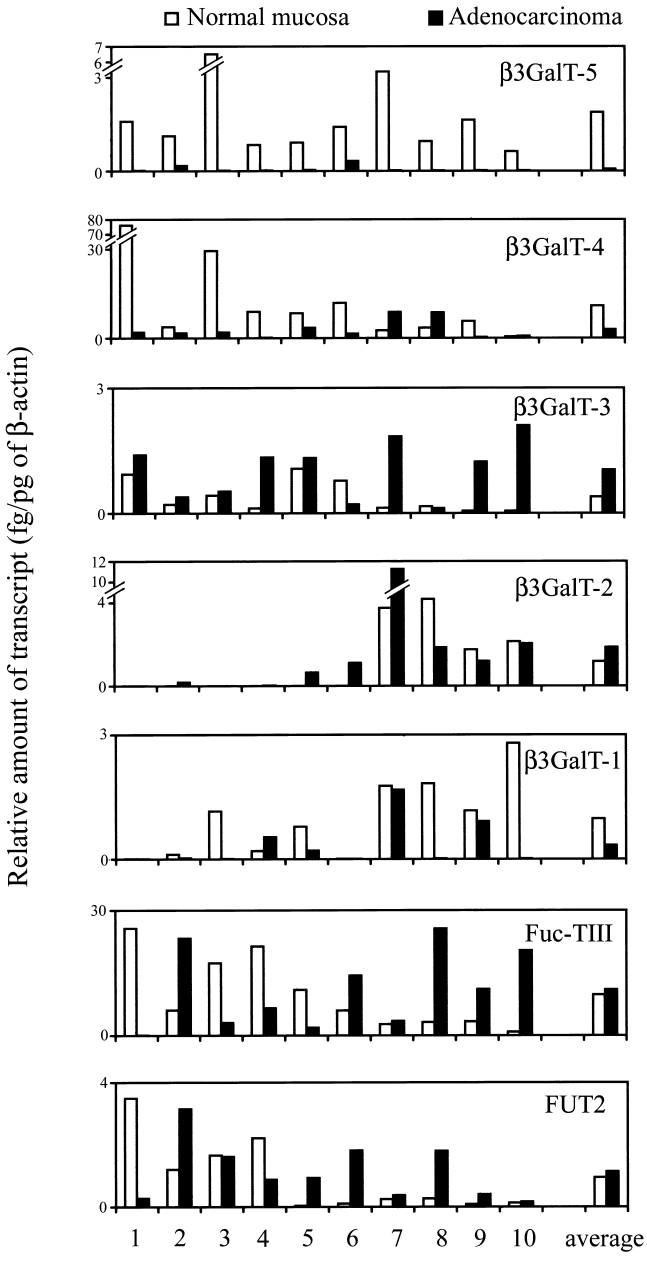

FIG. 6. **Competitive RT-PCR quantification of glycosyltransferase transcripts expressed in normal colon mucosa and in adenocarcinomas.** Quantification was performed by densitometric scanning of the negative films of gels in Fig. 5. The amounts of amplified target cDNAs were calculated from their respective standard curves and normalized by those for β-actin. *Numbers* denote patients according to Table V. Note the different scales used.

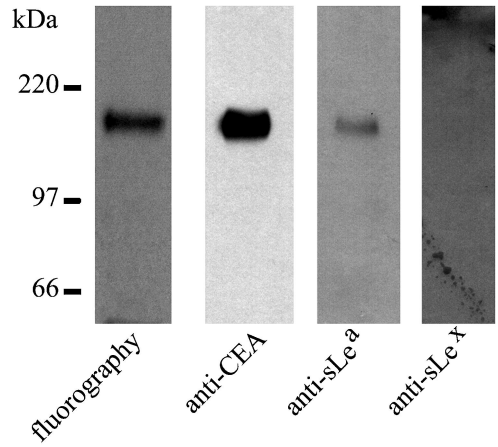

FIG. 7. **SDS-PAGE of CEA purified from clone CHO-FT-T5-CEA.** CHO-FT-T5 cells were transfected with human CEA cDNA and a clone expressing CEA, named CHO-FT-T5-CEA, was isolated and metabolically radiolabeled with [^{3}H]Gal. Radioactive material recovered upon phosphatidylinositol-specific phospholipase C extraction, perchloric acid precipitation, and Sepharose CL6B chromatography was desalted on a Bio-Gel P-2 column, and 10,000-cpm aliquots were loaded on two lanes of a 6% SDS-PAGE. After the run, one lane was processed for fluorography using liquid En^{3}Hance (PerkinElmer Life Sciences) (10-day exposure). The other one was blotted onto a nitrocellulose filter that was probed with monoclonal anti-CEA antibody using peroxidase-conjugated goat anti-mouse Ig as secondary antibody, and visualized by ECL. The same filter was stripped and reprobed sequentially with monoclonal anti-sLe^a and anti-sLe^x antibodies.

of trisaccharides, respectively (Fig. 8, *upper panel*). A small but detectable amount of Gal was removed by *X. manihotis* β-galactosidase from total de-sialylated and de-fucosylated CEA, and the amount released from the endo-β-galactosidase-resistant material (*fractions 18–22* in Fig. 8) was minimal, whereas that released by *D. pneumoniae* β-galactosidase was more consistent in both cases. Conversely, almost 40% of the radioactivity from the endo-β-galactosidase-sensitive trisaccharide (*fractions 32–39* in Fig. 8) was released by *X. manihotis* β-galactosidase, but poorly affected by the diplococcal enzyme (Fig. 8, *lower panel*). These data indicate that β3Gal-T5 preferentially acts on the GlcNAcβ1→3Galβ1→4GlcNAcβ1→R side chains present in CEA.

DISCUSSION

This report shows that β3Gal-T5, in transfected CHO cells, directs synthesis of type 1 chain oligosaccharides on CEA and other *N*-glycans having the GlcNAcβ1→3Galβ1→4GlcNAcβ1→R outer sequence, prevents poly-*N*-acetyllactosamine and sLe^x synthesis on *N*-glycans, and is down-regulated in colon adenocarcinomas.

We have found that β3Gal-T5 synthesizes sLe^a and Le^a antigens very efficiently in CHO cells, much more than β3Gal-T1, which is still more efficient than β3Gal-T2, whereas β3Gal-T3 and -T4 are unable to synthesize these antigens at all. In the stable clone CHO-FT-T5, sLe^a expression is selectively affected by swainsonine, an inhibitor of complex type *N*-glycan processing (31), but not by benzyl-α-GalNAc, an inhibitor of *O*-glycan biosynthesis (32), although the opposite occurs in clone CHO-FT-T1. These data support the hypothesis that β3Gal-T5 acts on complex type *N*-glycans in CHO cells and suggest that β3Gal-T1 presumably affects unknown *O*-glycans available in these cells in a low amount. In this regard, it is interesting that sLe^a expression in CHO-FT-T1 is strongly stimulated by sodium butyrate, a commonly used activator reported to enhance core 2 GnT activity in CHO cells (33). However, results obtained with CHO-FT-T2 suggest that β3Gal-T2 requires dedicated substrates not available in these cells. Moreover, CHO-FT-T1 cells, as well as CHO-FT-T2, were found to express a large amount of sLe^x that is undetectable in CHO-FT-T5. We found that the oligosaccharides released from *N*-glycans by endo-β-galactosidase are completely different in the clones. In fact, (±NeuAcα2→3)Galβ1→3[Fucα1→4]GlcNacβ1→3Gal is the only oligosaccharide identified in CHO-FT-T5, whereas in clone CHO-FT-T1 several oligosaccharides are released, which correspond to those reported in CHO expressing Fuc-TIII only (26). They include the sLe^x tetrasaccharide as well as poly-*N*-acetyllactosamine side chains, but not type 1 chain oligosaccharides. Among endo-β-galactosidase-resistant *N*-glycans, type 1 chain oligosaccharides are present in small amounts and not fucosylated in clone CHO-FT-T5 but not detectable in CHO-FT-T1 cells. On this basis we conclude that the expression of

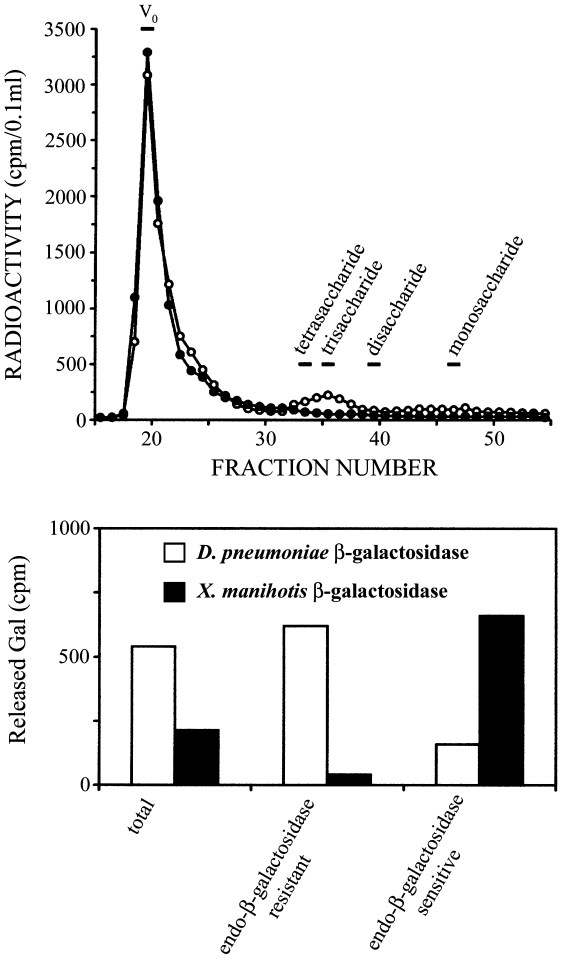

FIG. 8. **Sensitivity to endo-β-galactosidase and different β-galactosidases of tritiated CEA purified from clone CHO-FT-T5-CEA labeled with [^{3}H]Gal.** *Upper panel,* radioactive CEA prepared as described in Fig. 7 was de-sialylated, de-fucosylated, and then subjected to Bio-Gel P-2 chromatography before (*full circles*) and after (*empty circles*) endo-β-galactosidase treatment. Column calibration is indicated at the *top* of the *panel*, standards are as in Fig. 4. *Lower panel*, the total de-fucosylated and de-sialylated material, as well as endo-β-galactosidase-resistant (*fractions 19–22* in *upper panel*) and -sensitive (*fractions 32–38* in *upper panel*) radioactivity were treated with *D. pneumoniae* or *X. manihotis* β-galactosidases, and the reaction mixtures were analyzed by Bio-Gel P-2 chromatography. Released Gal was quantitated by liquid scintillation counting.

β3Gal-T5 in CHO cells inhibits the synthesis of poly-*N*-acetyllactosamines and sLe^x in *N*-glycans, replacing them with a short type 1 chain, whereas that of β3Gal-T1 does not affect *N*-glycans. Expression of *N*-linked poly-*N*-acetyllactosamines, which are often modified to express functional oligosaccharides such as sLe^x, is considered to be associated with tumor progression and malignancy (34). Recent work on poly-*N*-acetyllactosamine extension in *N*-glycans (35) demonstrated that it is achieved mainly by β4Gal-T1 and β1,3-*N*-acetylglucosaminyltransferase iGnT (36) and is favored by β1,6GlcNAc branching, because the branched structure serves as a much better acceptor for such enzymes (37). Expression of GlcNAc-TV, responsible for branching, also correlates with metastatic potential of cell lines (38) as well as with metastasis and poor prognosis in colon cancer (39). Because β3Gal-T5 has a very high affinity for acceptors having the GlcNAcβ1→3Gal outer sequence, but much lower for those with the GlcNAcβ1→2Man or GlcNAcβ1→6Man sequence, the following scenario can be envisaged in CHO cells. In the presence of β3Gal-T5, β4Gal-T1 still acts on GlcNAc linked to Man, and complex type *N*-glycans are elongated by a single lactosamine unit. This is acted upon by iGnT, presumably on a β1,6-branched chain, which forms the GlcNAcβ1→3Galβ1→4GlcNAcβ1→R outer group. At this stage, β3Gal-T5 competes with β4Gal-T1 adding a β1,3-galactosyl residue that prevents further chain elongation by iGnT. Consequently, poly-*N*-acetyllactosamine chains could not be efficiently extended nor sLe^x synthesized.

It is worth noting that the K_m values of β3Gal-T5 for GlcNAcβ1→3Galβ1→4Glc and GlcNAcβ1→2Man are virtually identical to those reported for a β1,3Gal-T expressed in normal colon mucosa whose activity was found low in adenocarcinomas (5). These data prompted us to evaluate the expression level of β3Gal-T5 transcript in normal mucosa *versus* colon adenocarcinomas. Competitive RT-PCR analysis performed on 10 colon adenocarcinoma cases indicate that β3Gal-T5 transcript is constantly down-regulated in cancer, confirming that it is the enzyme previously detected by Seko *et al*. (5) and corroborating the hypothesis that *in vivo* it would prevent the expression of poly-*N*-acetyllactosamines and sLe^x on *N*-glycans. This behavior is unique among β3Gal-T and other glycosyltransferase transcripts studied to date (40–43).

Because CEA synthesized in normal mucosa is reported to express *N*-glycans having type 1 chains as Galβ1→3GlcNAcβ1→3Galβ1→4GlcNAcβ1→R outer sequences (3, 4), whereas CEA produced by colon adenocarcinomas lacks type 1 chains but have poly-*N*-acetyllactosamine and β1,6 branched type 2 chains on *N*-glycans (2, 44), our results suggest that β3Gal-T5 may act on CEA *in vivo*. To assess this hypothesis, we permanently expressed CEA in clone CHO-FT-T5 (CHO-FT-T5-CEA). We found that CEA purified from the clone reacts with anti-sLe^a antibody by Western blot. Moreover, the type 1 chain carbohydrates present in the molecule are more abundant in endo-β-galactosidase-sensitive oligosaccharides than in those resistant. These results confirm that β3Gal-T5 acts on CEA and suggest that it is responsible for the differential glycosylation pattern of this protein in colon cancer.

The kinetic data calculated in this paper for lactotriosylceramide, as well as those reported (20) for GlcNAcβ1→3GalNAc as acceptors, suggest that β3Gal-T5 is able to act on glycolipids as well as on core 3 *O*-linked glycoproteins. The possibility of a broad range of specificity of the enzyme is confirmed by the recent report that it also acts on the GalNAc end of globotetraosylceramide (45). On this basis, the down-regulation of β3Gal-T5 in cancer is expected to affect other glycoconjugates synthesized by the enzyme in normal mucosa. However, β3Gal-T5 expression is maintained in some colon adenocarcinoma cell lines (16), and we suggest that the results obtained with these cells must be cautiously interpreted, or reinterpreted (12, 19). In particular, further experimental data are needed to establish whether or not the CA19.9 antigen present in patient serum is synthesized by β3Gal-T5 and secreted from the tumor mass.

Acknowledgments—We thank Marco Bellaviti for computer artwork, Dr. Nozomu Hiraiwa (Aichi Cancer Center, Nagoya, Japan) for helpful suggestions, and Dr. Fabio Dall'Olio (Department of Experimental Pathology, University of Bologna, Italy) for critical reading of the manuscript.

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