Permanent exposure of mucin-secreting HT-29 cells to benzyl-*N*-acetyl-α-D-galactosaminide induces abnormal O-glycosylation of mucins and inhibits constitutive and stimulated MUC5AC secretion

Sylviane HENNEBICQ-REIG*, Thécla LESUFFLEUR†, Calliope CAPON‡, Carmen DE BOLOS§, Isabelle KIM*, Odile MOREAU*, Colette RICHET*, Brigitte HÉMON*, Marie-Ange RECCHI‡, Emmanuel MAËS‡, Jean-Pierre AUBERT*, Francisco X. REAL§, Alain ZWEIBAUM†, Philippe DELANNOY‡, Pierre DEGAND* and Guillemette HUET*[1]

*Unité de Recherches sur la Biologie et la Physiopathologie des Cellules Mucipares, INSERM U377, Place de Verdun, 59045 Lille cedex, France, †Unité de Recherches sur la Différenciation Cellulaire Intestinale, INSERM U178, 16 Avenue Paul Vaillant Couturier, 94807 Villejuif cedex, France, ‡Laboratoire de Chimie Biologique, CNRS UMR 111, Université des Sciences et Technologies de Lille, 59655 Villeneuve d'Ascq cedex, France, and §Unitat de Biologia Cellular i Molecular, Institut Municipal d'Investigacio Medica, Universitat Autonoma de Barcelona, Spain

Previous work has shown that treatment of HT-29 methotrexate (MTX) cells with benzyl-*N*-acetyl-α-D-galactosaminide results in profound changes in mucin oligosaccharide chains. To analyse in depth the effect of this drug, we first determined the structure of mucin oligosaccharide chains synthesized by HT-29 MTX cells and the changes induced by permanent drug exposure. Mucins from untreated cells contained nine monosialylated structures (core types 1, 2, 3 and 4) and four disialylated structures (types 1, 2 and 4). Core 1 structures predominated, in particular NeuAcα2–3Galβ1–3GalNAc-ol. Exposure of HT-29 MTX cells to benzyl-*N*-acetyl-α-D-galactosaminide from days 2–21 resulted in a decrease in intracellular mucins and both their sialic acid and galactose content, and an increased T (Galβ1–3GalNAcα-*O*-Ser/Thr) and Tn (GalNAcα-*O*-Ser/Thr) antigenicity. A 3-fold increase in both Galβ1–3GalNAc α2,3-sialyltransferase activity and mRNA expression was detected. At the ultrastructural level, T-antigen was not detectable in mucin droplets in control cells, but was strongly expressed in intracytoplasmic vesicles in treated cells. In these cells, *MUC1* and *MUC3* transcripts were up-regulated, whereas *MUC2*, *MUC5B* and *MUC5AC* were down-regulated. Furthermore, constitutive and secretagogue-induced MUC5AC secretion was reduced and no mucus layer was detected. In conclusion, benzyl-*N*-acetyl-α-D-galactosaminide induces abnormal O-glycosylation and altered regulation of MUC5AC secretion.

INTRODUCTION

Mucins are secreted or membrane-bound glycoproteins produced by epithelial cells of normal and malignant tissues. The mucins constitute a family of heavily glycosylated high-molecular-mass glycoproteins [1].

To date, nine human mucin genes (*MUC1* to *MUC8*) have been identified. Although more than one mucin gene can be expressed in a given tissue, the *MUC* genes display different expression patterns in normal epithelia: *MUC1* in the breast and gastrointestinal tract [2], *MUC2* and *MUC3* in the small and large intestines [3,4], *MUC4* in the tracheobronchus and colon [5], *MUC5AC* in the stomach and tracheobronchus [6,7], *MUC5B* in the submaxillary glands, tracheobronchus and cervix [6], *MUC6* in the stomach and gallbladder [8,9], *MUC7* in salivary glands [10] and *MUC8* in the tracheobronchus [11]. In addition, mucins also show a large heterogeneity, as found for the O-glycosylation pattern of the mucin core peptides [12]. The oligosaccharides have been classified in eight types of core structure [13]. In colon, the oligosaccharide chains are mainly based on the core 3 structure [14,15].

Malignant transformation of epithelial cells is accompanied by changes in the biochemical characteristics of mucins. The changes include both an altered expression of mucin genes and an aberrant glycosylation of mucin core peptides [13,16]. Both abnormal mucin gene expression and O-glycosylation have also been found in mucins synthesized by cultured colon carcinoma cells of mucin-secreting phenotypes: HT-29 methotrexate (MTX) cells, selected from the HT-29 cell line by culture in the presence of MTX [17], express low levels of *MUC3*, a gene that is normally expressed in the colon, and high levels of *MUC5AC*, a gene that is normally expressed in the stomach [18,19]. The HT-29 MTX subpopulation and a mucin-secreting clonal derivative of the HT-29 cell line, Cl.16E, produce acidic sialylated mucins sharing epitopes with normal gastric mucins [20,21]. Structural investigations carried out on mucins secreted by Cl.16E cells have shown that the oligosaccharide chains are based on core types 1, 2 and 4 and that the elongation of

Abbreviations used: MTX, methotrexate; Gal, D-galactose; GalNAc, *N*-acetyl-D-galactosamine; core 1 β3-Gal-T, UDP-Gal:glycoprotein-GalNAc-R β1,3-galactosyltransferase;GalNAcα-*O*-benzyl, 2-acetamido-α-2-deoxy-D-galactopyranoside; HPAEC–PAD, high-pH anion-exchange chromatography–pulsed amperometric detection; MALDI–TOF, matrix-assisted laser-desorption ionization–time-of-flight; AE–HPLC, anion-exchange–HPLC; MAA, *Maackia amurensis* agglutinin; ST3 Gal I, CMP-NeuAc:Galβ1–3GalNAc α2,3-sialyltransferase; GlcNAc, *N*-acetyl-D-glucosamine; ST3 Gal IV, CMP-NeuAc:Galβ1–4GlcNAc α2,3-sialyltransferase; polypeptide α-GalNAc-T, UDP-GalNAc:polypeptide α-*N*-acetylgalactosaminyltransferase; Neu5Ac, *N*-acetylneuraminic acid; PNA, *Peanut* agglutinin; RT, reverse transcriptase; FITC, fluorescein isothiocyanate. The abbreviated nomenclature for cloned sialyltransferases follows the system of Tsuji et al. [S. Tsuji, A. K. Datta and J. C. Paulson (1996) Glycobiology **6**, 7: v–vii].

[1] To whom correspondence should be addressed (e-mail huet@lille.inserm.fr).

oligosaccharide chains is truncated by addition of sialic acid in α2–3 linkage to galactose (Gal)β1–3R and Galβ1–4R residues [22], where R indicates that Gal is linked to an oligosaccharide.

Inhibitors of O-glycosylation, such as aryl-*N*-acetyl-α-galactosaminides, have been recently described. These compounds were used initially as potential competitors of the glycosylation of *N*-acetyl-D-galactosamine (GalNAc) residues linked to the core protein and have been shown to inhibit UDP-Gal:GalNAc-R β1–3-galactosyltransferase (core 1 β3-Gal-T; EC 2.4.1.122) *in vitro* [23]. However, it was observed that a short-term treatment (24 h) with 2-acetamido-α-2-deoxy-D-galacto-pyranoside (GalNAcα-*O*-benzyl) of mucin-secreting colon cancer cells in culture led to the increased expression not only of Tn antigen, but also of T antigen [24,25]. Subsequent analyses revealed that GalNAcα-*O*-benzyl is metabolized in the cells to Galβ1–3GalNAcα-*O*-benzyl, which behaves as a competitor of further elongation of T antigen [24,26]. In HT-29 MTX cells, which synthesize highly sialylated mucins, GalNAcα-*O*-benzyl mainly acts as a competitive substrate of Galβ1–3GalNAc α2,3-sialyltransferase activity, leading to a dramatic decrease in the sialic acid content of mucins [25,26].

The purpose of the present work was to analyse in depth the effect of GalNAcα-*O*-benzyl on mucin synthesis and secretion, both constitutive and secretagogue-induced, in HT-29 MTX cells when present permanently in the culture medium, i.e. from the second day after seeding, when the cells do not yet synthesize mucins, up to day 21, when they are fully differentiated. As a prerequisite for this study, and because sialylation of mucins from HT-29 MTX cells has been only indirectly appreciated from their sialic acid content [25] and lectin reactivity [18], the sialylated oligosaccharide structures of HT-29 MTX mucins were fully characterized.

MATERIALS AND METHODS

Materials

Monoclonal antibodies anti-Tn (Tn-5; [27]) and anti-Thomsen-Friedenreich (BM 22.19; [28]) were a gift from Dr. Danuta Dus (Ludwik Hirszfeld Institute of Immunology and Experimental Therapy, Wroclaw, Poland). Rabbit polyclonal anti-(apomucin MUC5AC) antibody (LUM5–1), raised against a synthetic peptide with the sequence RNQDQQGPFKMC corresponding to a non-glycosylated part of MUC5AC apomucin, was a gift from Dr. Ingemar Carlstedt (Department of Cell and Molecular Biology, Lund University, Sweden) [29].

Cell culture

HT-29 MTX cells, derived from parental HT-29 cells by selection with the anti-cancer drug MTX (10^{-6} and 10^{-5} M) [17], were grown in drug-free Dulbecco's modified Eagle's minimal essential medium (Eurobio, Paris, France), supplemented with 10 % (v/v) heat-inactivated (30 min, 56 °C) fetal-calf serum. Cells were seeded at 2×10^4 cells/cm^2 and cultured at 37 °C in a 10 % CO_2/90 % air atmosphere. The medium was changed daily. Control cells were cultured up to late post-confluent period, when all cells display a mucin-secreting phenotype [17]. Chronic GalNAcα-*O*-benzyl treatment was carried out by the addition of 2 mM GalNAcα-*O*-benzyl to the culture medium from day 2 after seeding up to the late post-confluent period.

Different investigations were performed in order to affirm the viability and health of the cells that had been treated for such a long period of time (21 days) with GalNAcα-*O*-benzyl. The cell viability was evaluated by Trypan Blue exclusion and found to be 94.7 % (versus 93.2 % for control cells). The viability and health of the treated cells were also evaluated through the capacity of the treated cells to restart growth in standard medium after trypsin treatment. The cell growth was similar to control cells and confluence was reached 7 days after seeding.

Structural oligosaccharide analysis

Release of oligosaccharide chains from mucins with alkaline borohydride treatment

Mucins were purified from post-confluent (day 21) HT-29 MTX cell lysates by ultracentrifugation through a caesium bromide gradient. The purified mucins were submitted to β-elimination under reductive conditions (0.1 M KOH containing 1 M KBH_4) for 24 h at 45 °C [30].

Chromatography of oligosaccharides

The purified oligosaccharide alditols obtained after β-elimination were fractionated by anion-exchange chromatography according to Baenziger et al. [31] on a 10 μm Micro-Pak AX-10 column (50 cm × 0.8 cm I.D., Varian, CA, U.S.A.).

The monoacidic fraction, FI, was fractionated further on primary amino-bonded silica (5 μm Spheri-5 column 250 mm × 4.6 mm I.D., Brownlee Labs, CA, U.S.A.). The column was equilibrated with the initial solvent [acetonitrile/15 mM KH_2PO_4 adjusted to pH 5.2 (75:25, v/v)]. After the injection, isocratic conditions were applied with the initial solvent for 25 min followed by a linear gradient to acetonitrile/15 mM KH_2PO_4 (35:65, v/v) for 60 min, and then isocratic conditions for 30 min. The flow rate was 1 ml/min. The collected fractions were purified on a Bio-Gel P2 column.

The diacidic fraction, FII, was further fractionated by high-pH anion-exchange chromatography–pulsed amperometric detection (HPAEC–PAD). Elution of oligosaccharide alditols contained in fraction FII was performed at a flow rate of 1 ml/min in 100 mM NaOH for 5 min, followed by a linear gradient of sodium acetate (to 100 mM NaOH/50 mM sodium acetate at 16 min, to 100 mM NaOH/70 mM sodium acetate at 35 min and to 100 mM NaOH/400 mM sodium acetate at 80 min), by the method of Lo-Guidice et al. [32]. Each fraction collected after HPAEC–PAD was neutralized immediately with 30 % (v/v) acetic acid before purification on a Bio-Gel P2 column.

Carbohydrate analysis

The molar carbohydrate composition of oligosaccharide alditols was determined using gas chromatography by the method of Montreuil et al. [33].

Matrix-assisted laser desorption ionization–time-of-flight (MALDI-TOF) MS analysis

Molecular masses of the oligosaccharides were measured by MALDI–TOF MS analysis on a Vision 2000 TOF instrument (Finnigan Mat, Bremen, Germany) equipped with a 337 nm UV laser. The mass spectra were measured in reflectron mode under 6 kV accelerating voltage and either positive or negative detection. The sample was dissolved in water at a concentration of 100 pmol · μl^{-1}. Aliquots (2 μl) of the analyte solution were mixed with an equal vol. of the matrix solution. The sample/matrix crystallization was performed using the method of Papac et al. [34]. External calibration was performed using angiotensin I standard (M_r 1296.7), purchased from Sigma (St. Louis, MO, U.S.A.).

400 MHz ^{1}H-NMR spectroscopy

Before ^{1}H-NMR spectroscopic analysis, the fractionated oligosaccharide alditols were exchanged twice with ^2H_2O at room temperature and pD 6.5. After each exchange treatment (1 h) the materials were freeze-dried. Finally, each sample was redissolved in 0.5 ml of ^2H_2O (99.96 atom % ^{2}H, Centre d'Energie Atomique, Saclay, France). NMR experiments were performed on a BRUKER AM-WB spectrometer operating in the Fourier transform mode at a probe temperature of 27 °C.

Characterization of mucins

Isolation of mucins

Mucins from both control cells and GalNAcα-*O*-benzyl-treated cells were isolated from 10 75-cm^2 flasks and purified by ultracentrifugation and anion-exchange HPLC (AE-HPLC) by the method of Huet et al. [25].

Biochemical analysis

For amino acid analyses, samples were hydrolysed in 5.6 M HCl for 24 h under a vacuum and processed using a 7300 Beckman amino acid analyser (Palo Alto, CA, U.S.A.), equipped with a high-performance sodium column (4 mm × 120 mm) (Beckman, Fullerton, CA, U.S.A). Carbohydrate analysis was carried out by GLC of trimethylsilyl derivatives of methylglycosides formed by methanolysis in 0.5 M HCl in methanol at 80 °C for 24 h [35]. All compositional analyses were performed at least twice to confirm the reproducibility of the results.

ELISAs

Mucins (isolated as described above), in amounts from 0–100 ng, or culture media (collected as described below) normalized for cellular protein content were plated overnight at 4 °C on 96-well Maxisorp™ immunoplates. Unbound sites were blocked with 4 % (w/v) BSA in PBS. After washing with PBS/0.05 % (v/v) Tween 20 (six times), antibodies or lectin [biotinylated *Maackia amurensis* agglutinin (MAA), Vector Laboratories, Burlingame, CA, U.S.A.] were added and left for 2 h at 37 °C. After washing (six times), peroxidase-conjugated anti-mouse antibody or peroxidase-conjugated streptavidin was applied for 1 h at 37 °C. Plates were washed and developed using *o*-phenylenediamine (1 mg·ml^{-1}) in 0.1 M phosphate/citrate buffer, pH 5.5, with 0.03 % (v/v) hydrogen peroxide. The reaction was terminated with 1 M HCl and the A_{492} was measured.

Western blot analysis

For the characterization of baseline or secretagogue-induced secreted mucins, culture media (collected as described below) normalized to cellular proteins were analysed using SDS/PAGE (2–10 % gradient gels) under reducing conditions [25]. After transfer to a Hybond C extra membrane (Amersham, Aylesbury, Bucks., U.K.), the membrane was treated with the rabbit anti-MUC5AC antibody LUM5-1 diluted 1000-fold and then with the peroxidase-conjugated anti-rabbit antibody (Sigma, St. Louis, MO, U.S.A.) diluted 4000-fold. Detection was carried out by luminescence using the ECL Western blotting system (Amersham).

RNA extraction and analysis

Samples of total RNA, isolated after cell lysis in guanidinium isothiocyanate and centrifugation through a CsCl gradient, were fractionated by electrophoresis through 1 % agarose gels and transferred to nylon (Hybond N, Amersham).

Filters were prehybridized and hybridized with the ^{32}P-labelled probes for 20 h at 42 °C using a classical procedure. Blots were washed in standard conditions followed by a last wash with 0.1 × SSC (where 1 × SSC is 0.15 M NaCl/0.015 M sodium citrate)/0.1 % (w/v) SDS at 65 °C for 15 min. To normalize for RNA, filters were dehybridized and hybridized with an antisense 22-mer oligonucleotide (5′-ACGGTATCTGATCGTCTTCGA-A-3′) specific for 18 S rRNA sequence. CMP-NeuAc:Galβ1–3GalNAc α2,3-sialyltransferase (ST3 Gal I) was detected with a 537-bp PCR-amplified cDNA (from +361 to +898 in the open reading frame) prepared using HepG2 cell RNA as the template [36]. *MUC1* was detected using cDNA PUM24P [37] obtained from Dr. D. Swallow (MRC, London, U.K.), *MUC2* with cDNA SMUC41 [3] and *MUC3* with cDNA SIB 124 respectively [4], obtained from Dr. Y. Kim (VA Hospital, San Francisco, CA, U.S.A.), *MUC5AC* with cDNA JER58 [38] or cDNA L31 [21], *MUC5B* with cDNA JER57 [39] and villin with cDNA V19 [40] obtained from Dr. D. Louvard (Institut Curie, Paris, France).

The PCR-based detection of ST3 Gal I and CMP-NeuAc:Galβ1–4 *N*-acetyl-D-glucosamine (GlcNAc) α2,3-sialyltransferase (ST3 Gal IV) transcripts was performed by 25 cycles of annealing/extension using 0.6 μM specific 22-mer oligonucleotide primers (5′-$_{361}$TCAGAGTGGTGCCTGGGAATGT-3′ as sense and 3′-$_{877}$CGTTTCCCTTGACCGTGGTGAT-5′ as anti-sense for ST3 Gal I; 5′-$_{322}$CCCAAGAACATCCAGAGCCTCA-3′ as sense and 3′-$_{759}$CTAATTCGTCTTCGGGTGGTGC-5′ as anti-sense for ST3 Gal IV), as described previously by Recchi et al. [36]. Amplified products (537 bp and 458 bp for ST3 Gal I and ST3 Gal IV respectively) were separated on an 8 % polyacrylamide gel and stained with ethidium bromide. Quantification was performed by computerized densitometric scanning of the gel under UV light. The intensity of the signal for ST3 Gal I was expressed as the relative intensity of the signal using ST3 Gal IV as an internal standard in these experiments.

Measurement of glycosyltransferase activities

Glycosyltransferase activities were assayed using microsomal suspensions as enzyme sources. Microsomal suspensions were prepared as reported previously [41] and the protein concentration was determined by the method of Peterson [42]. All glycosyltransferase assays were performed at least in duplicate. To achieve the appropriate concentration of each donor substrate, the different radioactive nucleotide sugars were used after isotopic dilution with the corresponding non-radiolabelled compound.

UDP-GalNAc:polypeptide α-*N*-acetylgalactosaminyltransferase (polypeptide α-GalNAc-T; EC 2.4.1.41) assay

Polypeptide α-GalNAc-T activity was assayed on the synthetic peptide substrate TTSAPTTS (Neosystem, Strasbourg, France), which corresponds to the *MUC5AC* tandem repeat [41]. The purity of the peptide was confirmed by amino acid sequence determination, HPLC analysis and electrospray MS. Microsomal suspensions were incubated with 3 mM peptide as exogenous substrate, and UDP-[^{3}H]GalNAc (1 mM, 662 MBq·mmol^{-1}; 27.8 kBq/42 μl) for 2 h at 37 °C.

Core 1 β3-Gal-T assay

The assays were performed using GalNAcα-*O*-benzyl (2 mM) as

acceptor and UDP-[^{14}C]Gal (1 mM, 9.34 MBq·mmol^{-1}, 392 Bq/42 μl) as substrate for 2 h at 37 °C [41].

ST3 Gal I assay

Microsomal fractions were incubated for 0.5 to 1 h at 37 °C using Galβ1–3GalNAcα-*O*-benzyl (1 mM) as acceptor substrate and CMP-[^{14}C]*N*-acetylneuraminic acid (Neu5Ac) (52.9 μM, 0.58 GBq·mmol^{-1}; 1.83 kBq/60 μl) as described previously [26].

Electron microscopy

For morphological analysis, control or treated cells grown in 25-cm² flasks were fixed in 2 % (v/v) glutaraldehyde, post-fixed in 1 % (w/v) osmium, embedded in Epon and re-embedded in order to make sections perpendicular to the bottom of the flask as reported previously [17].

For ultrastructural lectin labelling, cells grown in 25-cm² flasks were rinsed three times in PBS, fixed in 0.1 M phosphate buffer containing 0.05 % (v/v) glutaraldehyde and 4 % (v/v) *p*-formaldehyde. The cell layer was scraped with a rubber policeman and the cell pellet was dehydrated and embedded in LR White. Ultrathin sections were successively incubated with biotinylated lectin [MAA or *Peanut* agglutinin (PNA), Vector Laboratories), streptavidin, rabbit anti-streptavidin antibody and 15-nm gold-conjugated goat anti-rabbit antibody (Amersham). The lectins, streptavidin and antibodies were diluted in 0.1 M Tris buffer, pH 7.4, containing 1 % (w/v) BSA, 1 % (v/v) normal goat serum and 0.09 % (w/v) NaCl. The grids were finally counterstained with uranyl acetate and lead citrate and observed using a Zeiss 902 electron microscope.

Metabolic labelling

The baseline secretion of mucins was evaluated throughout cell culture comparatively in control cells until day 21, in treated cells until day 21 and in cells treated for 21 days and then reverted to standard medium until day 25. Continuous metabolic labelling with [^{3}H]threonine (1.7 GBq·mmol^{-1}; 462.5 kBq/500 μl) in threonine-free medium was carried out in triplicate for 24 h every 2 days. The medium was then collected and cells were lysed in Ripa buffer [0.01 M Tris/HCl (pH 8.0)/0.01 M NaCl/0.1 % (w/v) SDS/1 % (v/v) Triton X-100/0.5 % (w/v) sodium deoxycholate/1 % (w/v) PMSF/0.001 M Na-EDTA]. Protein concentration in the cell lysates was determined by the method of Peterson [42]. Secreted mucins were analysed by SDS/PAGE with 2–10 % gradient polyacrylamide gels, and by autoradiography [25]. The amounts of medium used were normalized with reference to the same amount of cellular proteins.

To analyse mucin biosynthesis, cells were cultured on 6-well plates either in standard medium or in medium containing 2 mM GalNAcα-*O*-benzyl until day 15. Subsequently, untreated cells were pulse-labelled for 15 min with [^{3}H]threonine (1.7 GBq·mmol^{-1}, 7.4 MBq/1 ml) in threonine-free medium and then chased for various times up to 48 h (1 h, 2 h, 6 h, 24 h, 48 h) with 1 ml of threonine in regular medium. The same protocol was applied to treated cells, except for the presence of 2 mM GalNAcα-*O*-benzyl throughout the experiment. Cell-culture media were collected and cells were rinsed in PBS and lysed in 1 ml of Ripa buffer. Cell-culture media and cell lysates were analysed by SDS/PAGE (2–10 % gels) and autoradiography. The samples were normalized with reference to the same amount of cellular proteins.

To analyse stimulated mucin secretion, cells were cultured on 6-well plate filters (Transwell, Corning Costar Acton, MA, U.S.A.) either in standard medium or in medium containing 2 mM GalNAcα-*O*-benzyl until day 21. Subsequently, untreated and treated cells were cultured for 24 h with [^{3}H]threonine in threonine-free medium to label stored mucins. The labelling medium was removed and the cells were washed in standard medium to eliminate constitutive secretion of mucins. Mucin secretion was then induced for 45 min at 37 °C with either the Ca^{2+} ionophore A23187 (25 μg·ml^{-1}) + PMA (25 μM) [43] or the Ca^{2+} ionophore A23187 (4×10^{-5} M) + forskolin (10^{-4} M) [22]. Media were then harvested and the cells were carefully washed further with medium to collect all secreted mucus. The medium used for rinsing was pooled with the first harvested medium. Aliquots of pooled media were analysed by SDS/PAGE (2–10 % gels) and autoradiography. The amounts of medium used were normalized with reference to the same amount of cellular proteins. To analyse the mucin secretion by Western blotting and ELISA, the same experiment was carried out without labelling.

RESULTS

Structural characterization of carbohydrate chains of HT-29 MTX mucins

Oligosaccharide alditols, released by base-borohydride treatment and desalting, were obtained in a yield of 27.7 %. The molar carbohydrate composition of released oligosaccharides was in agreement with that of native mucins reported previously [25] (Table 1).

The oligosaccharide alditols were first fractionated by quaternary amine anion-exchange chromatography (Figure 1A) into one neutral (FN) and three acidic fractions (FI–FIII). The fractions were collected and analysed for their carbohydrate composition (Table 1). The structures present in the two major fractions FI and FII representing 74 % of total oligosaccharides were studied further after fractionation of FI by HPLC on amine-bounded silica (Figure 1B) and FII by high-pH anion-exchange chromatography (Figure 1C). Fractions were subjected to MALDI–TOF MS (Table 2). The carbohydrate composition of each oligosaccharide was checked by GLC analysis (results not shown). The oligosaccharide alditols were subjected to ^{1}H-NMR spectroscopy and the structural analysis is given for the major oligosaccharide alditols in Table 3.

The oligosaccharides ranged in size from two to seven monosaccharides. The elongation of chains was performed by addition of lactosamine units Galβ1–3GlcNAc of type 1 or Galβ1–4GlcNAc of type 2. Substitution by sialic acid was mainly in α2–3 linkage on a terminal Gal residue and also in α2–6 linkage on the GalNAc.

In total, 13 oligosaccharide alditol structures were identified;

Table 1 Carbohydrate composition of native MTX mucins, β-eliminated mucins and fractions obtained after anion-exchange chromatography

FN, neutral fraction; FI, monoacidic fraction; FII, diacidic fraction; FIII, triacidic fraction.

	Man	Gal	GalNAc	GlcNAc	NeuAc	GalNAc-ol	Mass (mg)
Native mucins*	0.3	1.3	1.0	0.8	1.7	–	100
β-Eliminated mucins†	0.4	2.1	0.2	0.9	1.8	1.0	27.7
FN	0.2	0.4	0.2	0.2	–	1.0	2.2
FI	–	0.9	–	0.3	1.0	1.0	5.0
FII	–	1.1	0.1	0.8	2.2	1.0	6.6
FIII	0.4	3.1	0.4	2.0	2.8	1.0	1.9

* GalNAc was taken as 1; † GalNAc-ol was taken as 1.

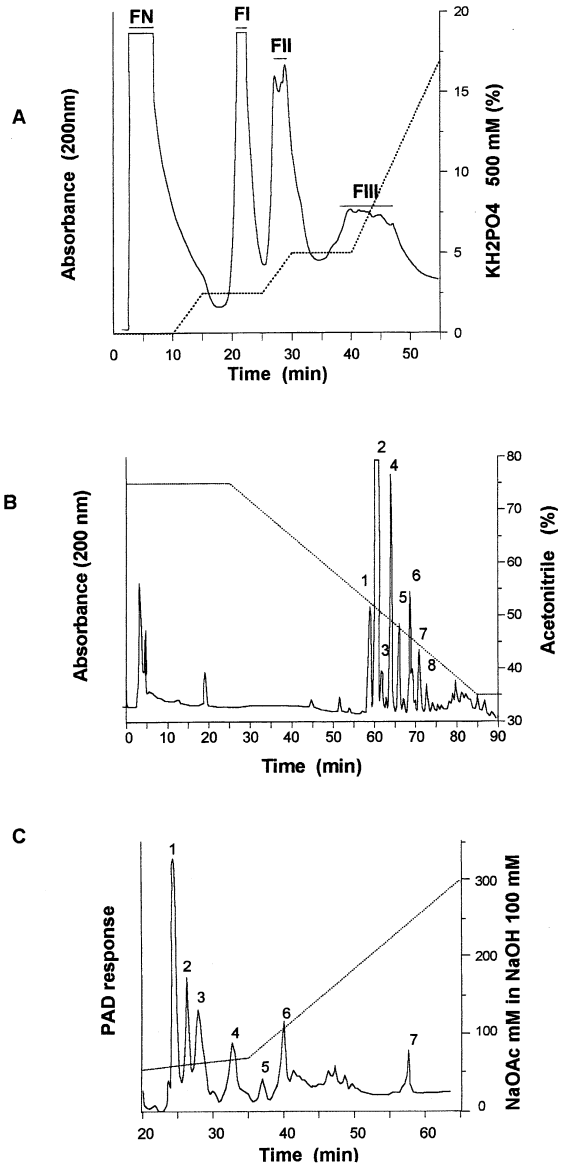


Figure 1 Fractionation of oligosaccharide alditols obtained after β-elimination of HT-29 MTX mucins

(**A**) The oligosaccharides were loaded on to a 10-μm AX-10 column equilibrated in water and eluted with solvent containing an increasing percentage of 500 mM KH_2PO_4 (dotted line). The collected fractions are indicated by the horizontal bars. (**B**) Fractionation of the monoacidic fraction FI obtained after anion-exchange chromatography. The oligosaccharide alditols were chromatographed on an amino-bonded silica column eluted with decreasing concentrations of acetonitrile in 15 mM KH_2PO_4 (dotted line). (**C**) Fractionation of the diacidic fraction FII by HPAE-PAD chromatography. Increasing concentrations of NaOAc were applied for elution (dotted line).

nine monosialylated structures and four disialylated structures (Table 2). Core structures of types 1, 2, 3 and 4 were found. However, the core types 1 and 2 were predominant and represented 57.5% and 20.5% of FI/FII oligosaccharides respectively. The core type 1 was mainly represented by the linear trisaccharide NeuAcα2–3Galβ1–3GalNAc-ol (41% of FI/FII oligosaccharides). The core type 2 mainly corresponded to a branched hexasaccharide alditol: NeuAcα2–3Galβ1–4GlcNAcβ1–6 [NeuAcα2–3Galβ1–3] GalNAc-ol (17% of FI/FII).

Table 2 MALDI–TOF analysis of acidic oligosaccharide alditols from fractions FI and FII

			Molar ratio				
Compound	*m/z**	H/Na†	Gal	GlcNAc	NeuAc	GalNAc-ol	Mass (μg)
FI-1	513.1	−H	–	–	1	1	20
FI-2	674.6	−H	1	–	1	1	500
FI-4	877.9	−H	1	1	1	1	100
FI-5	675.0	−H	1	–	1	1	50
FI-6	1039.9	−H	2	1	1	1	135
FI-7	1040.0	−H	2	1	1	1	25
FI-8	1243.1	−H	2	2	1	1	100
FII-1	1332.0	−H	2	1	2	1	100
FII-2	966.7	−H	1	–	2	1	30
FII-3	1354.1	−H + Na	2	1	2	1	50
	1719.7	−H + Na	3	2	2	1	50
FII-4	1556.8	−H + Na	2	2	2	1	60

* *m/z* values refer to chemical masses; † fractions were analysed in negative mode and the number of H or Na atoms subtracted and/or added in the pseudomolecular ions are indicated.

Carbohydrate changes of HT-29 MTX mucins after GalNAcα-*O*-benzyl treatment

The intracellular mucins of control and GalNAcα-*O*-benzyl-treated cells were analysed after isolation by ultracentrifugation through a CsBr gradient. Mucins were obtained from 10 75-cm^2 flasks of control and GalNAcα-*O*-benzyl-treated cells (75 mg and 19 mg respectively). Mucins were separated further from proteoglycans by AE-HPLC chromatography, as described previously [25]. The chemical composition of the mucin fraction is given in comparison with the control HT-29 MTX mucin fraction (Table 4).

Only slight changes in amino acid composition were found in mucins of treated cells. In contrast, the relative amounts of both GalNAc and GlcNAc, expressed as the molar ratio to hydroxy amino acids, were increased by approx. 2-fold. Carbohydrate compositions, given as the molar ratio of monosaccharides to GalNAc, showed that GalNAcα-*O*-benzyl treatment led to a 2.6-fold decrease in the relative content of sialic acid and a 1.5-fold decrease in the relative content of Gal.

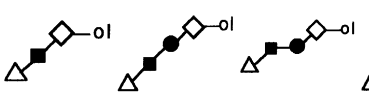
Mucin carbohydrate changes induced by GalNAcα-*O*-benzyl were analysed using lectins and antibodies. Carbohydrate analysis data led us to assay MAA, which reacts with NeuAcα2–3Gal-R, anti-T antibody, which reacts with Galβ1–3GalNAc-R, and anti-Tn antibody, which reacts with GalNAc-R. Assays were performed on the purified intracellular mucins by ELISA (Figure 2). In agreement with the carbohydrate data showing a high proportion of oligosaccharide chains terminated by α2–3 sialic acid and, in particular, the trisaccharide NeuAcα2–3Galβ1–3GalNAc, mucins of control HT-29 MTX cells reacted strongly with MAA. On GalNAcα-*O*-benzyl treatment, this MAA reactivity was greatly decreased, whereas the mucins greatly expressed the T-antigen. Control mucins displayed a slight Tn antigenicity, which became markedly increased by GalNAcα-*O*-benzyl treatment of the cells.

GalNAcα-*O*-benzyl influences the activity of glycosyltransferases

The changes in mucin glycosylation on GalNAcα-*O*-benzyl treatment led us to investigate the levels of glycosyltransferase activities involved primarily in the glycosylation of HT-29 MTX mucins, i.e. polypeptide α-GalNAc-T (evaluated on a MUC5AC

Table 3 ^{1}H-NMR chemical shifts of structural reporter-group protons of constituent monosaccharides for major oligosaccharide alditols of HT-29 MTX mucins

The monosaccharides are represented by the following symbolic notation: ◇–ol = GalNAc–ol; △ = αNeuAc; ■ = βGal; and ● = GlcNAc. The linkage position is specified by the direction of the connecting bars as follows: 6 ↘, 4 →, 3 ↗ and n.d. = not determined.

		Chemical shift in compound						
		FI-2	FI-4-A	FI-4-B	FI-6-A	FI-6-B	FI-8	FII-1
								
Residue	Reporter group							
GalNAc–ol	H-2	4.386	4.291	4.291	4.394	4.394	4.281	4.388
	H-3	4.072	4.013	4.013	4.050	4.070	n.d.	4.068
	H-4	3.498	3.554	3.554	3.499	3.441	n.d.	3.443
	H-5	4.186	4.141	4.141	4.184	4.267	4.224	4.264
	NAc	2.045	2.033	2.038	2.050	2.064	2.043	2.066
Gal^3	H-1	4.545	–	–	4.467	4.532	–	4.533
	H-3	4.120	–	–	n.d.	4.123	–	4.114
	H-4	3.931	–	–	4.123	3.932	–	3.928
Gal^{3,3}	H-1	–	4.530	–	4.519	–	4.452	–
	H-3	–	4.096	–	4.087	–	n.d.	–
	H-4	–	3.941	–	3.941	–	3.919	–
Gal^{4,3}	H-1	–	–	4.530	–	–	–	–
	H-4	–	–	3.954	–	–	–	–
Gal^{4,6}	H-1	–	–	–	–	4.470	4.550	4.546
	H-3	–	–	–	–	n.d.	4.112	4.114
	H-4	–	–	–	–	3.922	3.959	3.959
GlcNAc^3	H-1	–	4.653	4.624	–	–	4.650	–
	NAc	–	2.074	2.079	–	–	2.068	–
GlcNAc^{3,3}	H-1	–	–	–	4.721	–	–	–
	NAc	–	–	–	2.036	–	–	–
GlcNAc^6	H-1	–	–	–	–	4.560	4.556	4.555
	H-6	–	–	–	–	n.d.	n.d.	4.006
	NAc	–	–	–	–	2.064	2.058	2.062
NeuAc^3	H-3a	1.798	1.784	1.798	1.784	1.798	1.798	1.800
	H-3e	2.773	2.766	2.758	2.761	2.771	2.759	2.777
	NAc	2.034	2.031	2.031	2.030	2.030	2.032	2.777
NeuAc^{3,4}	H-3a	–	–	–	–	–	–	1.800
	H-3e	–	–	–	–	–	–	2.757
	NAc	–	–	–	–	–	–	2.032

peptide sequence, because *MUC5AC* is a gene primarily expressed in HT-29 MTX cells [18,19]), core 1 β3-Gal-T and ST3 Gal I (Table 5).

The activities of both polypeptide α-GalNAc-T and ST3 Gal I were significantly increased (by 1.9-fold and 2.8-fold respectively). The core 1 β3-Gal-T activity was also increased, but to a lesser extent (by 1.5-fold).

ST3 Gal I was further studied at the mRNA level. Northern blots (Figure 3) were carried out from control HT-29 MTX cells, GalNAcα-*O*-benzyl-treated cells and reverted cells. The results obtained showed an increased expression of ST3 Gal I transcripts in treated cells.

In parallel, the relative expression of ST3 Gal I mRNA was analysed after reverse transcriptase (RT)-PCR amplification, with reference to that of ST3 Gal IV mRNA, which is not affected by GalNAcα-*O*-benzyl treatment. Results showed that GalNAcα-*O*-benzyl led to a relatively enhanced expression of ST3 Gal I comparatively with ST3 Gal IV (2.7–3.2 fold), which fitted well with the increased enzymic activity of transfer of Neu5Ac on to Galβ1–3GalNAcα-*O*-benzyl. After reversion, the level of ST3 Gal I mRNA decreased to that of the control cells.

Inhibition of both constitutive and secretagogue-induced MUC5AC secretion upon GalNAcα-*O*-benzyl treatment

Constitutive mucus secretion was evaluated by 24-h continuous metabolic labelling with [^{3}H]threonine in GalNAcα-*O*-benzyl-treated cells from day 3 to day 21. From day 7 onwards, control cells began to secrete high amounts of mucins appearing as a diffuse high-molecular-mass band of much larger size than the 200 kDa molecular-mass marker (Figure 4A). Maximal secretion was observed at days 9–11. The level of secreted mucins decreased progressively from day 13 to day 21. In contrast, the mucin secretion of treated cells remained at a very low level at day 7 and

Table 4 Amino acid and osamine composition of mucins from control HT-29 MTX cells and GalNAcα-*O*-benzyl-treated HT-29 MTX cells

The amino acids of importance, serine and threonine, are underlined.

	Control HT-29 MTX mucins	GalNAcα-*O*-benzyl-treated HT-29 MTX mucins
Cys	0.73	0.48
Asp	4.80	3.71
Thr	24.18	25.66
Ser	15.87	19.08
Glu	7.17	6.60
Pro	11.83	10.35
Gly	6.62	8.40
Ala	7.12	7.86
Val	5.18	3.61
Ile	2.40	2.16
Leu	3.11	2.31
Tyr	0.08	1.00
Phe	2.20	1.34
His	1.55	1.40
Lys	2.59	3.50
Arg	4.57	2.53
Osamines (in molar ratio to hydroxy amino acids)		
GalNAc	0.45	0.84
GlcNAc	0.20	0.47
Carbohydrates (in molar ratio to GalNAc)		
Galactose	1.4	0.9
GalNAc	1	1
GlcNAc	0.6	0.4
Sialic acid	1.3	0.5

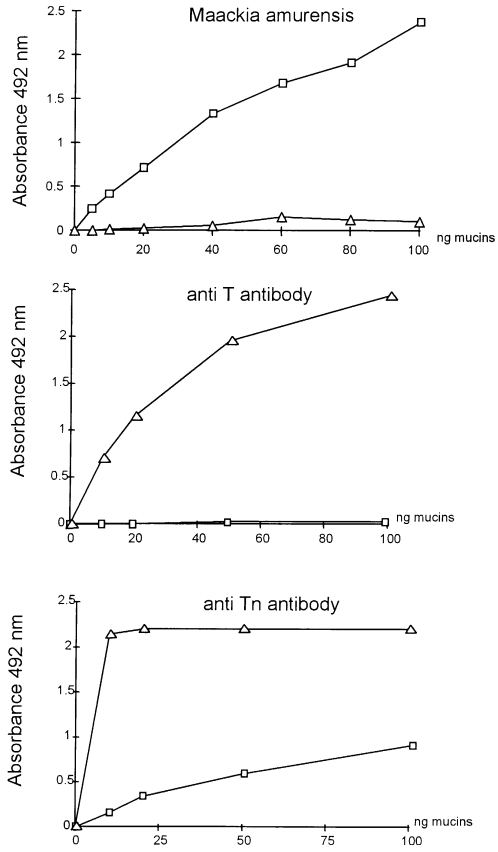


Figure 2 ELISA of mucins purified from post-confluent (day 21) control and GalNAcα-*O*-benzyl-treated HT-29 MTX cells

Increasing amounts of purified mucins (0–100 ng) from control HT-29 MTX cells (□) or from GalNAcα-*O*-benzyl-treated cells (△) were coated on to wells of the immunoplate and assayed with lectins or glycan-epitope-specific antibodies: MAA lectin (NeuAcα2–3Gal-R; upper panel), anti-T antibody (Galβ1–3GalNAc-R; middle panel) and anti-Tn antibody (GalNAc-R; lower panel).

Table 5 *In vitro O*-glycosylation assays using HT-29 MTX cells at day 21, cultured with (treated cells) or without benzyl GalNAc (control cells)

	Activity (nmol/h · mg^{-1} protein)	
	Control cells	Treated cells
Polypeptide α-GalNAc-T (substrate TTSAPTTS)	62.5	121.1
β1–3 galactosyltransferase (substrate benzyl α-GalNAc)	29.3	43.1
α2-3ST(O) (substrate Galβ1–3GalNAc-α-*p*-nitrophenol)	46.7	130.0

even gradually decreased up to day 21 (Figure 4B). The reversion of treated cells in standard medium without GalNAcα-*O*-benzyl resulted in the reappearance of larger amounts of secreted mucins. When samples of culture media from control, treated and reverted cells were run on the same gel, the apparent M_r of mucins from treated cells was higher in comparison with control cells. After reversion of treated cells to a standard medium, the mucin bands reverted to the apparent molecular mass of control HT-29 MTX mucin.

Since the constitutive secretion of mucins from treated cells was found to be strongly inhibited, we examined the potential secretory response elicited by the combined action of Ca^{2+} ionophore and PMA, or Ca^{2+} ionophore and forskolin, after metabolic labelling with [^{3}H]threonine. Results (Figure 4C) showed that the secretory response of treated cells was much lower than that of control cells, whatever the combination of secretagogues used. Secreted mucins from treated cells also displayed a lower mobility in SDS/PAGE than secreted mucins from control cells.

Secreted mucins were then assayed by Western blot analysis with an antiserum detecting an epitope in the non-glycosylated C-terminus of MUC5AC apomucin, because MUC5AC is the major mucin expressed by HT-29 MTX cells (Figure 4D). Results showed that mucin bands reacted with the anti-MUC5AC antibody and that both constitutive and stimulated secretion of MUC5AC was much lower in treated cells than in control cells. The MUC5AC bands and the radiolabelled bands displayed the same electrophoretic mobility.

Culture media normalized to cellular protein level were further evaluated by ELISA with lectins or glycan-epitope-specific antibodies and with the anti-MUC5AC antibody (Figure 5). A striking difference was observed with MAA lectin. For control cells, a high spontaneous secretion was obtained which was, on average, enhanced 4.1-fold after stimulation. For treated cells, neither spontaneous nor stimulated secretion of MAA-reactive material was detected. For the anti-T antibody and the anti-Tn antibody, control cells displayed a low basal secretion, which was increased after stimulation on average by 1.6-fold and 3-fold respectively, and treated cells displayed a higher basal secretion that was increased after stimulation, on average, by 2.1-fold and 2.6-fold respectively. For the anti-MUC5AC antibody, we observed a striking difference between untreated and treated cells. For control cells, a high spontaneous secretion was

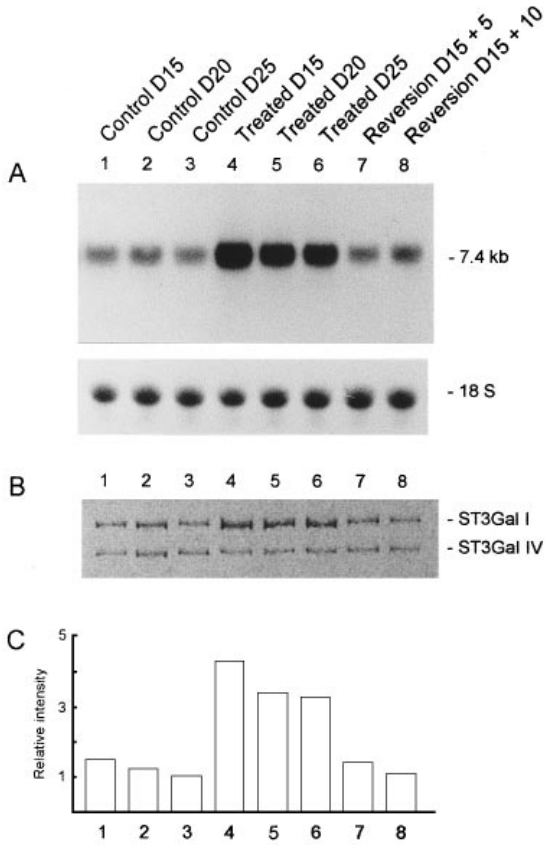


Figure 3 Analysis of ST3 Gal I mRNA expression in control, GalNAcα-*O*-benzyl-treated and reverted HT-29 MTX cells

Total RNA was isolated from control HT-29 MTX cells at days (D) 15 (lane 1), 20 (lane 2) and 25 (lane 3), from 2 mM GalNAcα-*O*-benzyl-treated cells at days 15 (lane 4), 20 (lane 5) and 25 (lane 6) and from reverted cells in standard medium after a further 5 days (day 15 + 5, lane 7) and 10 days (day 15 + 10, lane 8) after 15 days of GalNAcα-*O*-benzyl treatment. (**A**) Northern blot analysis of ST3 Gal I mRNA was performed using 40 μg of total RNA and a ^{32}P-labelled ST3 Gal I cDNA probe. The amount of RNA in each lane was normalized with reference to 18 S RNA. (**B**) RT-PCR co-amplification of ST3 Gal I and ST3 Gal IV. After separation on an 8% (v/v) polyacrylamide gel, amplified fragments were stained by ethidium bromide [the position of ST3 Gal I and ST3 Gal IV fragments (537 bp and 458 bp respectively) is indicated at the right side of the Figure]. (**C**) Relative intensity of ST3-Gal-I-amplified fragment normalized with reference to ST3 Gal IV.

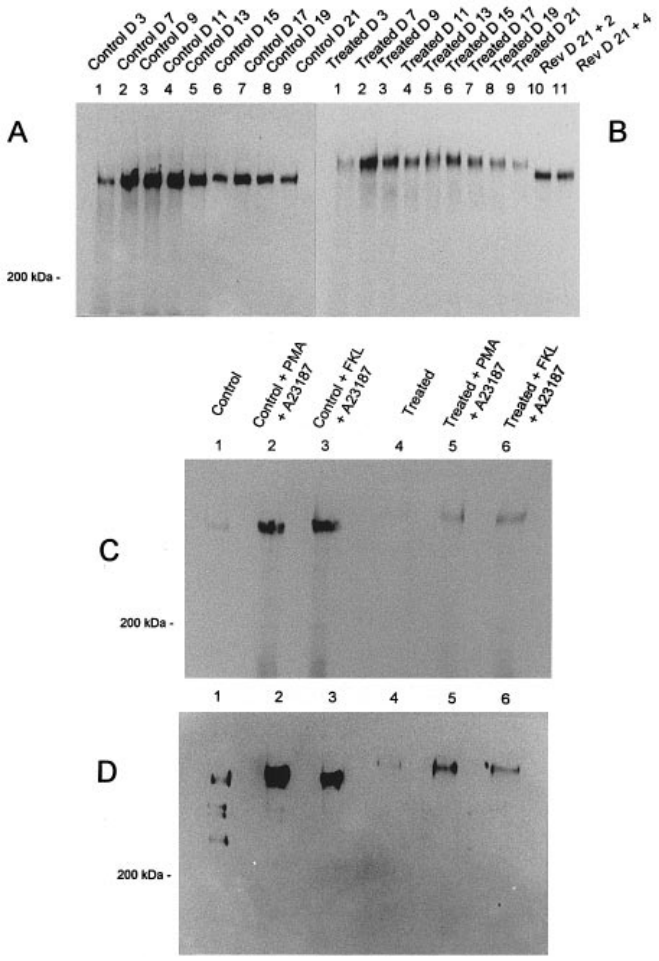


Figure 4 Analysis of constitutive and secretagogue-induced mucin secretion

For constitutive secretion, HT-29 MTX cells were continuously labelled for 24 h and the medium was then collected and analysed by SDS/PAGE (2–10% gradient gels). The labelling was carried out on control cells (**A**) and GalNAcα-*O*-benzyl-treated cells (**B**) at days 3, 7, 9, 11, 13, 15, 17, 19 and 21 (lanes 1–9), and also on reverted HT-29 MTX cells in standard medium after 21 days of GalNAcα-*O*-benzyl treatment at days (D) 23 (lane 10) and 25 (lane 11). For secretagogue-induced secretion (**C** and **D**), control and GalNAcα-*O*-benzyl-treated HT-29 MTX cells at day 21 with (**C**) or without (**D**) a previous 24-h incubation with [^{3}H]threonine to label stored mucins were subjected to secretagogue stimulation for 45 min. Media of control cells (lanes 1–3) and GalNAcα-*O*-benzyl-treated cells (lanes 4–6) were then collected without (lanes 1 and 4) or with secretagogue stimulation by either PMA + Ca^{2+} ionophore (lanes 2 and 5) or forskolin + Ca^{2+} ionophore (lanes 3 and 6), and these were analysed by SDS/PAGE (2–10% gradient gels) followed by autoradiography (**C**) or immunoblotting with the anti-(apomucin MUC5AC) antibody LUM5–1 (**D**).

obtained, which was enhanced by 2.9-fold after stimulation. For treated cells, both spontaneous and stimulated secretions remained at a very low level, despite a slight increase under secretagogue treatments. Considering this latter result, we investigated the presence of detectable MUC5AC apomucin within the GalNAcα-*O*-benzyl-treated cells by ELISA with the anti-MUC5AC antibody. MUC5AC apomucin was detected in an amount 5-fold lower than in the control cells (results not shown), a result in agreement with the lower (3.8-fold) amount of mucins collected in the treated cells.

Inhibition of the spontaneous or secretagogue-induced formation of a viscous mucus layer

No mucus layer was detectable on GalNAcα-*O*-benzyl-treated cells at day 21. The treated cells were also analysed by histochemical staining of sections of cell layers (results not shown). The mucus layer, revealed by Alcian Blue staining in control cells, was not detectable on monolayers of treated cells. Reversion of treated cells in standard medium without GalNAcα-*O*-benzyl resulted in the reappearance of the mucus layer.

The secretagogue-induced effects were studied on thin sections of the cell layer by immunofluorescence with MAA-Texas Red and PNA-fluorescein isothiocyanate (FITC) (Figure 6).The response of untreated cells to the secretagogues was clearly revealed by the appearance of a thick mucus layer covering the apical side of the cells; this was labelled with MAA (Figures 6b and 6c). In contrast, for secretagogue-stimulated GalNAcα-*O*-benzyl-treated cells, no thick layer was detected over the cells and no labelling was observed with either MAA-Texas Red (results not shown) or PNA-FITC (Figures 6e and 6f).

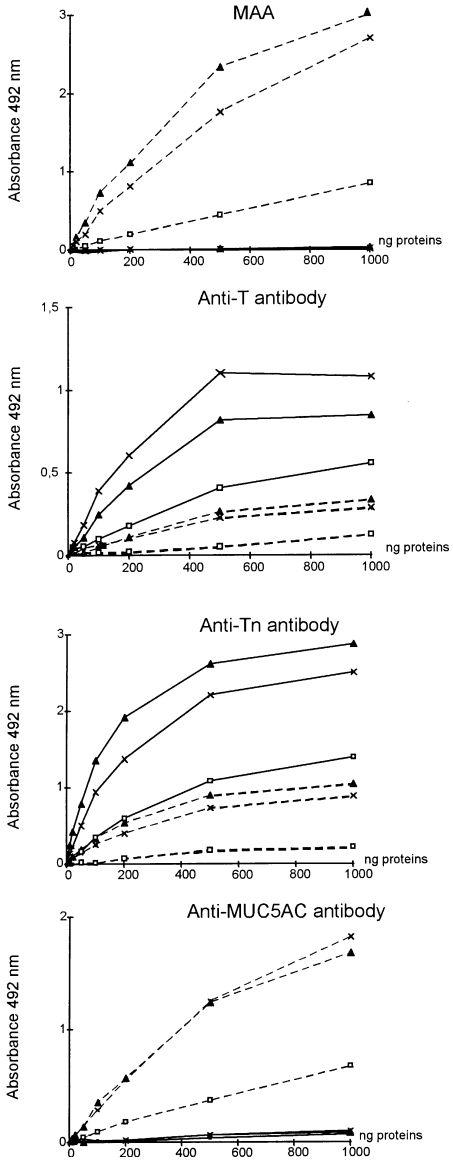


Figure 5 Analysis by ELISA of secreted material with or without secretagogue stimulation

The spontaneous or stimulated secretion by control (dashed line) and GalNAcα-*O*-benzyl-treated (continuous line) HT-29 MTX cells at day 21 was studied in collected media after incubation for 45 min. Non-stimulated control cells (□), control cells stimulated by PMA + Ca^{2+} ionophore (▲) or forskolin + Ca^{2+} ionophore (×), non-stimulated GalNAcα-*O*-benzyl-treated cells (□) and GalNAcα-*O*-benzyl-treated cells stimulated by PMA + Ca^{2+} ionophore (▲) or forskolin + Ca^{2+} ionophore (×) were studied comparatively for glycan epitopes with MAA lectin, anti-T and anti-Tn antibodies and for the MUC5AC mucin with the anti-(apomucin MUC5AC) antibody.

Morphological changes after GalNAcα-*O*-benzyl treatment

In the control HT-29 MTX cells, the mucins are stored within apical vacuoles of about 1 μm size, as shown in Figure 7(a). The MAA lectin intensely labelled the inside of the vacuoles (Figure 7c). No labelling was observed with PNA (results not shown).

GalNAcα-*O*-benzyl induced morphological changes on HT-29 MTX cells, as revealed by transmission electron microscopy of treated cells (Figure 7b), which showed the appearance of numerous vesicles (sizes ranging from 0.1 to 0.4 μm) in the cytoplasm. Ultrastructural immunolabelling was performed in order to localize the immature mucins expressing the T-antigen inside the treated cells. The PNA lectin (which reacts with Galβ1–3 GalNAc-R) was used because the mucins of treated cells were not more reactive towards the MAA lectin, but strongly expressed T-antigen (Figure 2). Results showed a strong labelling in the small vesicles (Figure 7d), but only weak labelling at the periphery of the vacuoles (Figure 7d). No labelling with MAA was observed (results not shown).

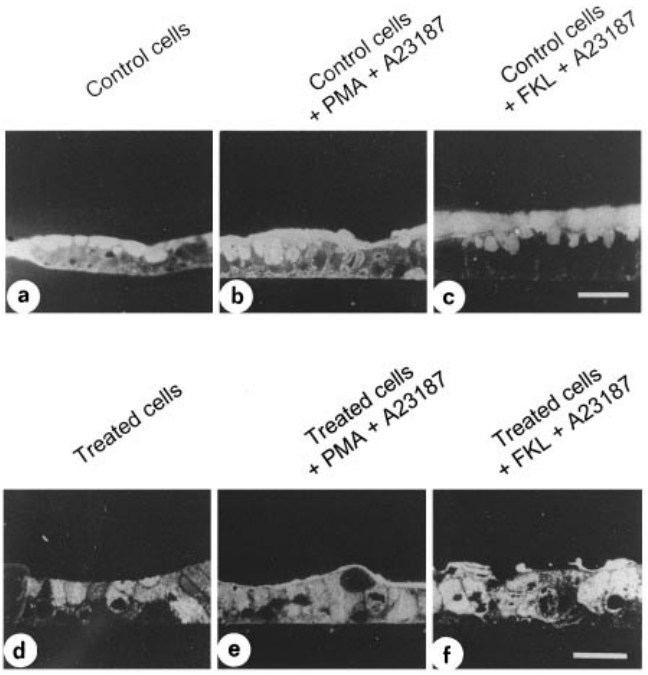


Figure 6 Immunofluorescence analysis of stimulated secretion from post-confluent (day 21) control and GalNAcα-*O*-benzyl-treated HT-29 MTX cells on semi-thin sections

(**a**–**c**) MAA-Texas Red on control cells and (**d**–**f**) PNA-FITC on treated cells. The stimulated secretion is clearly visible on control cells as a viscous gel labelled by the MAA lectin appearing on the apical side of the cells. In contrast, no secretion can be detected on top of the stimulated GalNAcα-*O*-benzyl-treated cells with the PNA lectin (bar = 20 μm).

Effect of GalNAcα-*O*-benzyl treatment upon the expression of *MUC* genes

The steady-state levels of mucin gene transcripts present in HT-29 MTX cells were analysed (i.e. *MUC1*, *MUC2*, *MUC3*, *MUC5AC* and *MUC5B*) (Figure 8). The expression of *MUC* genes was compared with the expression of the non-glycosylated protein villin, which is revealed to be two mRNA bands of 4 and 3.2 kb respectively [40]. In GalNAcα-*O*-benzyl-treated cells, reproducible changes in *MUC* gene expression were detected, i.e. an increase in *MUC1* and *MUC3* transcripts, and a decrease in the expression of *MUC5AC* and, to a lesser extent, of *MUC2* and *MUC5B*,. The expression of *MUC1* was revealed by two transcripts of 4 and 6.5 kb related to the allelic variation by the variable number of tandem repeats [44] and the expression of the other *MUC* genes was revealed by larger-sized mRNA transcripts, estimated to be between 14 and 22 kb [45]. Both types of changes were reversible after removing the GalNAcα-*O*-benzyl, except for *MUC2*.

In order to consider the possibility that the observed decreased secretion might result from decreased synthesis, the biosynthesis

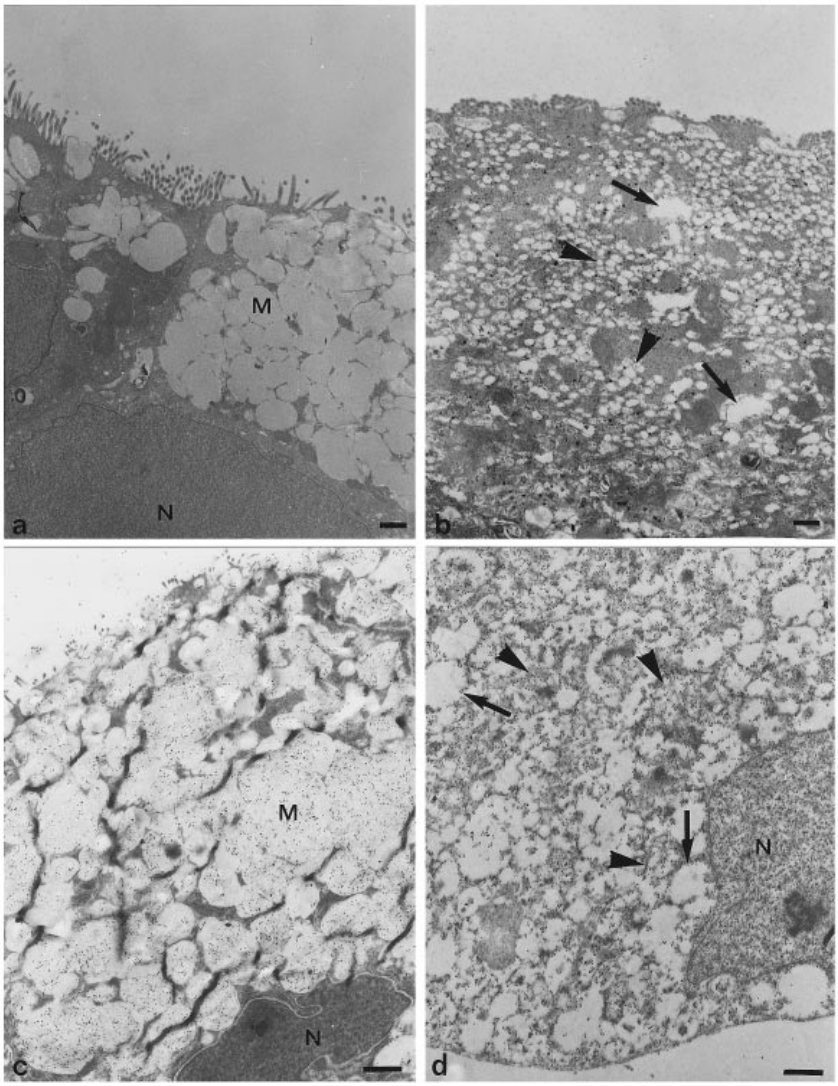


Figure 7 Transmission electron microscopy of post-confluent (day 21) control and GalNAcα-*O*-benzyl-treated HT-29 MTX cells

(**a**) and (**b**) Ultrathin sections of HT-29 MTX cells perpendicular to the bottom of the flask: (**a**) control cells showing a goblet-cell organization with mucus vacuoles (M) of size about 1 μm in the supranuclear compartment of the cells (where N denotes the nucleus) and (**b**) GalNAcα-*O*-benzyl-treated cells filled with vesicles (shown by arrowheads) between 0.1 and 0.4 μm diameter. Some vacuoles are also detected (shown by arrows). The bar shown represents 1 μm. (**c**) and (**d**) LR-White-embedded HT-29 MTX cells with ultrastructural post-embedding-immunogold-lectin labelling [MAA in (**c**) and PNA in (**d**)]: (**c**) the lectin MAA densely labels the mucus vacuoles of control cells, and (**d**) the lectin PNA labels the vesicles (shown by arrowheads) and the periphery of the vacuoles (shown by arrows). The bar shown represents 1 μm.

and secretion of mucins were studied in cells permanently exposed to GalNAcα-*O*-benzyl, in comparison with control cells, using pulse–chase experiments with [^{3}H]threonine (Figure 9). In control cells, the mucins were secreted in a large amount from 6 h onwards and remained in the culture medium up to 48 h, whereas in treated cells, the mucins were secreted at a much lower level up to 48 h. However, the biosynthesis of mucins did not appear to be markedly decreased in the treated cells, showing that the main effect of GalNAcα-*O*-benzyl was on mucin secretion. Moreover, we found that the intracellular mucins were retained by treated cells longer than control cells.

DISCUSSION

Our results show that culture of HT-29 MTX cells in the permanent presence of the sugar analogue GalNAcα-*O*-benzyl resulted: (i) in an alteration of the glycosylation of synthesized mucins, and (ii) in a marked inhibition of both the constitutive and stimulated secretion of MUC5AC mucin, which is the major mucin expressed in these cells [18,19]. Two important points to consider first are (i) that this long-time exposure to GalNAcα-*O*-benzyl did not affect the cell viability, and (ii) that all the major observed processes were completely reversible after reversion of the cells to a medium devoid of GalNAcα-*O*-benzyl.

We have found previously [25] that a subpopulation of HT-29 MTX cells synthesizes mucins containing a high content of sialic acid, which are devoid of fucose. Structural investigations reported here have determined that oligosaccharides of HT-29 MTX mucins consist of short sialylated structures (two to seven residues), mainly of core types 1 (57.5%) and 2 (20.5%). Incorporation of sialic acid occurs primarily via an α2–3 linkage to a terminal Gal residue. More precisely, the major structure

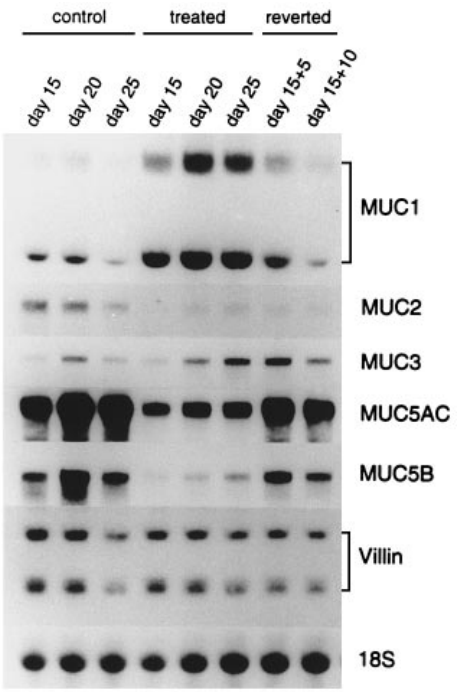


Figure 8 Expression of *MUC* genes in control, GalNAcα-*O*-benzyl-treated and reverted HT-29 MTX cells

Northern blots were carried out in control HT-29 MTX cells and GalNAcα-*O*-benzyl-treated HT-29 MTX cells at days 15, 20 and 25 and in HT-29 MTX cells reverted to standard medium for 5 and 10 days after 15 days of GalNAcα-*O*-benzyl treatment. RNA (20 μg) was hybridized with the indicated probes (see the Materials and methods section).

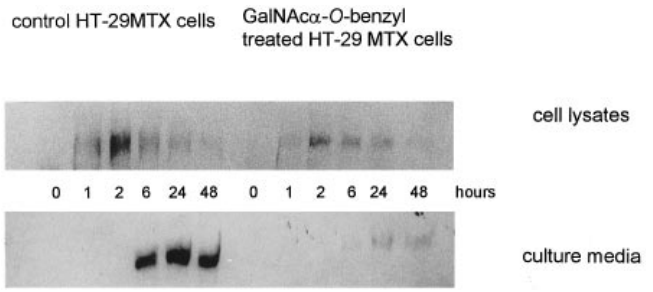


Figure 9 Biosynthesis and secretion of mucins in control and GalNAcα-*O*-benzyl-treated HT-29 MTX cells

Cells were pulse-labelled with [^{3}H]threonine for 15 min, subjected to various chase periods up to 48 h (1 h, 2 h, 6 h, 24 h and 48 h) and both cell-culture media and cell lysates were analysed by SDS/PAGE (2–10% gradient gels) and autoradiography.

found in HT-29 MTX cells was the monosialylated trisaccharide of core type 1, i.e. NeuAcα2–3Galβ1–3GalNAc. These core types 1 and 2 are not found in normal human colonic mucins, which contain core type 3 oligosaccharides. Moreover, sialylation of normal colonic mucins occurs solely via an α2–6 linkage to a terminal Gal residue or to GalNAc [14,15]. Short oligosaccharide stuctures truncated by addition of sialic acid in an α2–3 linkage have been also described in the clonal HT-29-derived cell line, Cl.16E [22]. However, while core type 1 structures are predominant in HT-29 MTX cells, core type 2 structures predominate in Cl.16E cells.

The high proportion of core 1 structures and, in particular, of the trisaccharide NeuAcα2–3Galβ1–3GalNAc in HT-29 MTX cells can be related to the high expression level of ST3 Gal I activity [26,46]. Accordingly, ST3 Gal I is the major sialyltransferase expressed in HT-29 MTX cells and its expression increases during the course of the differentiation process [47]. Our investigations have shown previously [26] that GalNAcα-*O*-benzyl treatment of post-confluent HT-29 MTX cells for 24 h resulted in a marked inhibition of ST3 Gal I activity via the competitive effect of Galβ1–3GalNAcα-*O*-benzyl synthesized *in situ*. Consequently, this inhibitor of glycosylation in HT-29 MTX cells provides a means for significantly inhibiting the terminal elongation of major oligosaccharide chains of mucins in order to study the biological effects of such impairment of glycosylation.

To determine whether chronic exposure to GalNAcα-*O*-benzyl leads to modifications in the glycosylation of mucins, as we have shown previously for short-term exposure, we have isolated intracellular mucins and investigated the changes in mucin glycosylation induced by GalNAcα-*O*-benzyl treatment from day 2 up to day 21. A significant amount of intracellular mucins was collected. The sialic acid content in mucins of GalNAcα-*O*-benzyl-treated cells was actually decreased (by 2.6-fold). As a consequence, mucins expressed T antigen. This decrease in the sialic acid content of mucins was paradoxically accompanied by an increased expression of ST3 Gal I, suggesting a regulation of this sialyltransferase by its substrates.

Another glycosylation change affects GalNAc residues. The marked increase in the expression of Tn antigenicity could result from the competition between GalNAcα-*O*-benzyl and the O-linked GalNAc of mucins during the process of elongation carried out by glycosyltransferases synthesizing core structures (galactosyltransferases or *N*-acetylglucosaminyltransferases). This hypothesis is strengthened by the fact that the Gal content is decreased in mucins of treated cells. Another explanation could be that an increased transfer of GalNAc occurs on to the mucin core peptides, in agreement with our O-glycosylation experiments *in vitro*, which show an increased transfer of GalNAc on to a MUC5AC peptide sequence for treated cells. The modification in the glycosylation of mucins in treated cells has been also detected by the increased ratio of both GalNAc and GlcNAc to Ser/Thr. The latter change might indicate that different types of oligosaccharides are being made. However, although the *MUC5AC* gene remained the most highly expressed *MUC* gene in treated cells, we cannot exclude the possibility that some modification in glycosylation could also be related to changes in the synthesis of different mucins.

The major biological effect observed after chronic administration of GalNAcα-*O*-benzyl to HT-29 MTX cells was the strong inhibition of both the constitutive and secretagogue-induced secretion of mucins. The viscous mucus layer was no longer apparent on treated cells either spontaneously or after stimulation. On SDS/PAGE, the HT-29 MTX mucins of either the baseline or the secretagogue-induced secretion appear as a similar diffuse high-molecular-mass band. On GalNAcα-*O*-benzyl treatment, this band was replaced by a much weaker band of lower electrophoretic mobility. This mobility difference probably results from the decreased sialic acid content in the secreted mucins from treated cells (as shown by ELISA with MAA lectin), because the intrinsic negative charge of mucins might influence their migration on SDS/polyacrylamide gels. The extensively sialylated form of MUC1 has a higher mobility on SDS/PAGE than the incompletely sialylated premature form [48]. By Western blot analysis using the anti-(apomucin MUC5AC) antibody, the secreted mucin bands have been

identified as MUC5AC, a result in agreement with the fact that *MUC5AC* is the major mucin gene expressed in HT-29 MTX cells [18,19]. However, until now we could not exclude the putative presence of other mucin(s) with a similar electrophoretic mobility. The inhibition of both constitutive and secretagogue-induced MUC5AC secretion on GalNAcα-*O*-benzyl treatment has been further confirmed by ELISA. Therefore GalNAcα-*O*-benzyl-treated cells secrete a very small amount of abnormally glycosylated MUC5AC mucin, which fails to constitute the viscous mucus layer on the cells.

Such a decrease in both constitutive and secretagogue-induced mucin secretion led us to investigate the *MUC* gene expression and the synthesis of the apomucins. We observed a down-regulation of the *MUC* genes corresponding to secreted mucins (*MUC5AC* particularly, but also *MUC5B* and *MUC2*), whereas the transmembrane mucin gene *MUC1* was highly up-regulated. This paradoxical effect of GalNAcα-*O*-benzyl on the regulation of *MUC* genes at the mRNA level could be relevant to their different localization/functions. The *MUC5AC* gene is still transcribed on GalNAcα-*O*-benzyl treatment, but at a lower level. The reduced mRNA level probably accounts for the reduction in apomucin synthesis in the treated cells, as detected by pulse–chase experiments with [^{3}H]threonine. The latter experiment also showed that the main effect of GalNAcα-*O*-benzyl treatment was a dramatic inhibition of the process of mucin secretion. Consequently, it was important to know if the effect of GalNAcα-*O*-benzyl was specific for mucins or whether the secretion process of other glycoproteins was also affected. We have shown that the secretion of two N-linked glycoproteins (procathepsin B and procathepsin L) was not inhibited in GalNAcα-*O*-benzyl-treated cells (results not shown).

Consequently, our results strongly suggest that the modification of mucin O-glycosylation is responsible for the inhibition of constitutive and stimulated MUC5AC mucin secretion. In intestinal goblet cells, mucins intended for constitutive or stimulated secretion are condensed into storage granules [49,50]. It may be inferred from the ultrastructural data for GalNAcα-*O*-benzyl-treated cells that abnormally glycosylated secretory mucins could be diverted to the detected intracytoplasmic vesicles and therefore not reach their storage granules.

Our results show that glycosylation of mucins plays a key role in the regulation of their secretion process. Even under high stimulation, abnormally glycosylated HT-29 MTX mucins are not secreted from HT-29 MTX cells and are unable to form a viscous mucus layer. Consequently, the secretory potential of mucins seems to be highly related to their glycosylation state.

We are grateful to Dr. Ingemar Carlstedt for his gift of the anti-MUC5AC antibody, LUM5–1. We thank Marie-José Dejonghe and Dominique Demeyer for excellent technical assistance, Françoise Roussez and Marie-Jeanne Freymont for typing the manuscript. This work was supported in part by grant #2209 from the Association pour la Recherche sur le Cancer. Work at the Institut Municipal d'Investigacio Medica was supported in part by grants 94/1128 from Fondo de Investigacion Sanitaria and SAF97–0085 from CICYT.

REFERENCES

1 Roussel, P., Lamblin, G., Lhermitte, M., Houdret, N., Lafitte, J. J., Perini, J. M., Klein, A. and Scharfman, A. (1988) Biochimie **70**, 1471–1482
2 Siddiqui, J., Abe, M., Hayes, D., Shani, E., Yunis, E. and Kufe, D. (1988) Proc. Natl. Acad. Sci. U.S.A. **85**, 2320–2323
3 Gum, J. R., Byrd, J. C., Hicks, J. W., Toribara, N. W., Lamport, D. T. A. and Kim, Y. S. (1989) J. Biol. Chem. **264**, 6480–6487
4 Gum, J. R., Byrd, J. C., Hicks, J. W., Swallow, D. M., Lagace, R. L., Byrd, J. C., Lamport, D. T. A., Siddiki, B. and Kim, Y. S. (1990) Biochem. Biophys. Res. Commun. **171**, 407–415
5 Porchet, N., Nguyen, V. C., Dufossé, J., Audié, J. P., Guyonnet-Dupérat, V., Gross, M. C., Denis, C., Degand, P., Bernheim, A. and Aubert, J. P. (1991) Biochem. Biophys. Res. Commun. **175**, 414–422
6 Audié, J. P., Janin, A., Porchet, N., Copin, M. C., Gosselin, B. and Aubert, J. P. (1993) J. Histochem. Cytochem. **41**, 1479–1485
7 Guyonnet-Dupérat, V., Audié, J. P., Debailleul, V., Laine, A., Buisine, M. P., Galiegue-Zouitina, S., Pigny, P., Degand, P., Aubert, J. P. and Porchet, N. (1995) Biochem. J. **305**, 211–219
8 Toribara, N. W., Roberton, A. M., Ho, S. B., Kuo, W. L., Gum, E., Hicks, J. W., Gum, J. R., Byrd, J. C., Siddiki, B. and Kim, Y. S. (1993) J. Biol. Chem. **268**, 5879–5885
9 De Bolos, C., Garrido, M. and Real, F. X. (1995) Gastroenterology **109**, 723–734
10 Bobeck, L. A., Tsai, H., Biesbrock, A. R. and Levine, M. J. (1993) J. Biol. Chem. **268**, 20563–20569
11 Shankar, V., Gilmore, M. S., Elkins, R. C. and Sachdev, G. P. (1994) Biochem. J. **300**, 295–298
12 Lamblin, G., Lhermitte, M., Klein, A., Houdret, N., Scharfman, A., Reuben, R. and Roussel, P. (1991) Am. Rev. Respir. Dis. **144**, S19–S24
13 Kim, Y. S., Gum, J. R. and Brockhausen, I. (1996) Glycoconjugate J. **13**, 693–707
14 Podolsky, D. K. (1985) J. Biol. Chem. **260**, 8262–8271
15 Podolsky, D. K. (1985) J. Biol. Chem. **260**, 15510–15515
16 Lesuffleur, T., Zweibaum, A. and Real, F. X. (1994) Crit. Rev. Oncol. Hematol. **17**, 153–180
17 Lesuffleur, T., Barbat, A., Dussaulx, E. and Zweibaum, A. (1990) Cancer Res. **50**, 6334–6343
18 Lesuffleur, T., Porchet, N., Aubert, J. P., Swallow, D., Gum, J. R., Kim, Y. S., Real, F. X. and Zweibaum, A. (1993) J. Cell Sci. **106**, 771–783
19 Lesuffleur, T., Roche, F., Hill, A. S., Lacasa, M., Fox, M., Swallow, D. M., Zweibaum, A. and Real, F. X. (1995) J. Biol. Chem. **270**, 13665–13673
20 Lesuffleur, T., Barbat, A., Luccioni, C., Beaumatin, J., Claire, M., Kornowski, A., Dussaulx, E., Dutrillaux, B. and Zweibaum, A. (1991) J. Cell Biol. **115**, 1409–1418
21 Maoret, J. J., Augeron, C., Codogno, P., Bauvy, C., Aubery, M. and Laboisse, C. (1989) Biochem. J. **258**, 793–799
22 Capon, C., Laboisse, C. L., Wieruszeski, J. M., Maoret, J. J., Augeron, C. and Fournet, B. (1992) J. Biol. Chem. **267**, 19248–19257
23 Kuan, S. F., Byrd, J. C., Basbaum, C. and Kim, Y. S. (1989) J. Biol. Chem. **264**, 19271–19777
24 Huang, S., Byrd, J. C., Yoor, W. H. and Kim, Y. S. (1992) Oncol. Res. **4**, 507–515
25 Huet, G., Kim, I., de Bolos, C., Lo-Guidice, J. M., Moreau, O., Hemon, B., Richet, C., Delannoy, P., Real, F. X. and Degand, P. (1995) J. Cell Sci. **108**, 1275–1285
26 Delannoy, P., Kim, I., Emery, N., de Bolos, C., Verbert, A., Degand, P. and Huet, G. (1996) Glycoconjugate J. **13**, 717–726
27 Duk, M., Steuden, F., Dus, D., Radzikowski, C. and Lisowska, E. (1995) Glycoconjugate J. **9**, 148–153
28 Steuden, I., Duk, M., Czerwinski, M., Radzikocoski, C. and Lisowska, E. (1985) Glycoconjugate J. **2**, 303–314
29 Hovenberg, H. W., Davies, J. R. and Carlstedt, I. (1996) Biochem. J. **318**, 319–324
30 Aminoff, D., Gathmann, W. D., McLean, C. M. and Yadoma, T. (1980) Anal. Biochem. **101**, 44–53
31 Baenziger, J. U. and Natowicz, M. (1981) Anal. Biochem. **110**, 357–361
32 Lo-Guidice, J. M., Wieruszeski, J. M., Lemoine, J., Verbert, A., Roussel, P. and Lamblin, G. (1994) J. Biol. Chem. **269**, 18794–18813
33 Montreuil, J., Bouquelet, S., Debray, H., Fournet, B., Spik, G. and Strecker, G. (1986) in Carbohydrate Analysis: a Practical Approach (Chaplin, M. F. and Kennedy, J. F., eds.), pp. 43–204, IRL Press, Oxford
34 Papac, D. I., Wong, A. and Jones, J. S. (1996) Anal. Chem. **68**, 3215–3223
35 Lamblin, G., Boersma, A., Klein, A., Roussel, P., Van Halbeek, H. and Vliegenthart, J. F. G. (1984) J. Biol. Chem. **259**, 9051–9058
36 Recchi, M. A., Harduin-Lepers, A., Boilly-Morer, Y., Verbert, A. and Delannoy, P. (1998) Glycoconjugate J. **15**, 19–27
37 Yonezawa, S., Byrd, J. C., Dahiya, R., Ho, J. J. L., Gum, J. J., Griffiths, B., Swallow, D. M. and Kim, Y. S. (1991) Biochem. J. **276**, 599–605
38 Crépin, M., Porchet, N., Aubert, J. P. and Degand, P. (1990) Biorheology **27**, 471–484
39 Dufossé, J., Porchet, N., Audié, J. P., Guyonnet-Dupérat, V., Laine, A., Van-Seuningen, I., Marrakchi, S., Degand, P. and Aubert, J. P. (1993) Biochem. J. **293**, 329–337
40 Pringault, E., Arpin, M., Garcia, A., Finidori, J. and Louvard, D. (1986) EMBO J. **5**, 3119–3124
41 Hennebicq-Reig, S., Tetaert, D., Soudan, B., Kim, I., Huet, G., Briand, G., Richet, C., Demeyer, D. and Degand, P. (1997) Exp. Cell Res. **235**, 100–107
42 Peterson, G. L. (1977) Anal. Biochem. **83**, 346–356
43 Lencer, W. I., Reinhart, F. D. and Neutra, N. R. (1990) Am. J. Physiol. **258**, G96–G102

44 Gendler, S. J., Lancaster, C. A., Taylor-Papadimitriou, J., Duhig, T., Peat, N., Burchell, J., Pemberton, L., Lalani, E. and Wilson, D. (1990) J. Biol. Chem. **265**, 15286–15293
45 Debailleul, V., Laine, A., Huet, G., Mathon, P., Collyn d'Hooghe, M., Aubert, J. P. and Porchet, N. (1998) J. Biol. Chem. **273**, 881–890
46 Dall'Olio, F., Malagolini, N., Guerrini, S. and Serafini-Cessi, F. (1993) Biochem. Biophys. Res. Commun. **196**, 714–720
47 Huet, G., Hennebicq-Reig, S., De Bolos, C., Ulloa, F., Lesuffleur, T., Barbat, A., Carrière, V., Kim, I., Real, F. X., Delannoy, P. and Zweibaum, A. (1998) J. Cell Biol., in the press
48 Hilkens, J. and Buijs, F. (1988) J. Biol. Chem. **263**, 4215–4222
49 Specian, R. D. and Oliver, M. G. (1991) Am. J. Physiol. **260**, C183–C193
50 Forstner, J. F. and Forstner, G. (1994) in Physiology of the Gastrointestinal Tract, 3rd edn. (Johnson, L. R., ed.), pp. 1255–1283, Raven Press, New York

Received 25 February 1998/19 May 1998; accepted 10 June 1998