Biosynthesis of Sialyl-Oligomeric-Lewis^X and VIM-2 Epitopes: Site Specificity of Human Milk Fucosyltransferase†

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ABSTRACT: In a previous study we have established the order of fucosylation of a trimer of Galβ1→4GlcNAc (LacNAc) linked to a synthetic hydrophobic aglycon, $(LacNAc)_3$-[(trifluoroacetamido)phenyl]ethyl, by a partially purified α3-fucosyltransferase preparation from normal human milk [De Vries, Th., Norberg, T., Lönn, H., & van den Eijnden, D. H. (1993) *Eur. J. Biochem. 216,* 769–777]. Using the same fucosyltransferase preparation, we have now studied the fucosylation of the oligosaccharide NeuAcα2→3$(LacNAc)_3$-Me. This compound was generated from the asialo analogue by use of an α3-sialyltransferase preparation from human placenta. The location of the fucose residues in the monofucosylated and difucosylated intermediate products was determined by analyzing digests obtained after endo-β-galactosidase treatment using HPLC on amino-bonded silica. In addition, the fucosylated NeuAcα2→3$(LacNAc)_3$-Me structures were characterized by high-pH anion-exchange chromatography with pulsed amperometric detection and were identified by 400-MHz ^{1}H-NMR spectroscopy. Intermediate products included oligosaccharides that contained the VIM-2, sialyl-Lewis^X, and sialyl-dimeric-Lewis^X epitopes. The final product was identified as the sialyl-trimeric-Lewis^X oligosaccharide. Kinetic analysis of the fucosylation reaction indicated that there is a significant difference in the rate of transfer of the first, second, and third fucose residues onto the acceptor molecule. Transfer of the first fucose occurred to either of the three GlcNAc residues in NeuAcα2→3$(LacNAc)_3$-Me with only a modest preference for the proximal and medial residues. A similar slight preference for these GlcNAc residues was found for the attachment of the second fucose residue. However, a single preferred pathway for fucosylation of the sialylated substrate was not found. These results are of relevance in understanding the role of α3-fucosyltransferases in the biosynthesis of sialyl-Lewis^X-related cell-surface glycoconjugates, which function as ligands for selectins and may play a role in the invasion and metastasis of certain carcinomas.

The sialyl-Lewis^X (sialyl-Le^X) epitope [NeuAcα2→3Galβ1→4(Fucα1→3)GlcNAc-R] is a carbohydrate ligand which is recognized by a family of adhesion molecules, the selectins (Phillips et al., 1990; Walz et al., 1990; Lowe et al., 1990; Tiemeyer et al., 1991; Polley et al., 1991; Tyrrel et al., 1991; Foxall et al., 1992; Berg et al., 1992). E-selectin functions in a key step of leukocyte extravasation (Stoolman, 1989; Brandley et al., 1990). Human neutrophils and monocytes constitutively express sialyl-Lewis^X epitopes on their cell surface (Macher & Beckstead, 1990). Upon activation of endothelial cells due to an inflammatory response, E-selectin becomes expressed on their cell surface, and subsequently binding of myeloid cells occurs (Bevilacqua et al., 1989). P-selectin is an integral membrane protein found in the granules of platelets and the Weibel–Palade bodies of endothelial cells. Within minutes after activation of these cells P-selectin is translocated and expressed on their surfaces to play a role in blood clotting and recruitment of leucocytes to sites of tissue injury (McEver et al., 1989; Furie et al., 1991). L-selectin is involved in the homing of lymphocytes to peripheral lymph nodes (Foxall et al., 1992; Berg et al., 1992).

Sialyl-Le^X and sialyl-dimeric-Le^X structures are abundantly expressed on many human tumors (Hakomori, 1981; Fukushi et al., 1984; Holmes & Levery, 1989; Holmes et al., 1986). Furthermore, the sialyl-dimeric-Lewis^X structure is expressed on CML cells but not on normal granulocytes (Fukuda et al., 1985). It has been suggested that human cancer cells utilize the interaction between selectins and sialyl-Le^X to metastasize. Indeed, a correlation has been found between invasive capacity and metastatic potential of colon and urinary bladder carcinoma cells and expression of the sialyl-dimeric-Lewis^X determinant (Matsushita et al., 1991; Matsusako et al., 1991). The lung colonization potential of human adenocarcinoma cell lines could also be associated with expression of this antigen (Inufusa et al., 1991).

Fucosylated carbohydrate antigens are all synthesized by the sequential action of a number of glycosyltransferases, of which a GDP-L-Fuc:Galβ1→4GlcNAc→R α(1→3)fucosyltransferase (α3-fucosyltransferase)[1] catalyzes the final and critical step. In the synthesis of sialylated structures, sialylation has to precede fucosylation (Holmes et al., 1986; Hanisch et al., 1988). The fine specificity of the fucosyltransferase involved determines if a Lewis^X, sialyl-Lewis^X, VIM-2, or sialyl-dimeric-Lewis^X epitope is formed. Humans possess at least four different types of α3-fucosyltransferase activities that can be discriminated by K_m values, cation requirement, sensitivity to inhibitors and detergents, tissue distribution, and in particular by their acceptor specificity (Mollicone et al., 1990; Macher et al., 1991; De Vries & van den Eijnden, 1992). Interestingly, α3-fucosyltransferases are capable of catalyzing fucose attachment as a branch to polylactosaminoglycan chains. These enzymes also appear

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[1] Abbreviations: α3-fucosyltransferase, GDP-Fuc:Galβ1→4GlcNAc→R α(1→3)fucosyltransferase; HPAEC, high-pH anion-exchange chromatography; PAD, pulsed amperometric detection.

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to have a distinct order (i.e., site specificity) in which they catalyze the attachment of fucosyl residues to those polylactosaminoglycans (Holmes & Levery, 1989; Holmes et al., 1985, 1986; De Vries et al., 1993a; Howard et al., 1987).

In an earlier report (De Vries et al., 1993a), we described the distinct order in which the human milk fucosyltransferase catalyzes the addition of fucosyl residues in α3 linkage to a timer of LacNAc to yield the trimeric-Le^X oligosaccharide. The aim of this work was to investigate whether the same enzyme (obtained from a nonmalignant source) can be used in the synthesis of the sialyl-dimeric- and sialyl-trimeric-Lewis^X determinants, which are often tumor-associated. To further characterize their biosynthesis, the order in which the fucosyl residues are attached to the different GlcNAc residues in the NeuAcα2→3(LacNAc)$_3$-Me molecule was established. The sialyl-oligomeric-Lewis^X oligosaccharides thus synthesized may be used as standards in future studies to characterize enzymes from tumor cells. In addition, these compounds may be of use for the further characterization of selectin-binding epitopes and for the development of antibodies, which can be useful in the early diagnosis of certain tumors.

EXPERIMENTAL PROCEDURES

Materials. Human milk was obtained from healthy lactating mothers and stored frozen until use. A partially purified preparation of human milk α3-fucosyltransferase was obtained by chromatography of delipidated milk on SP-Sephadex (Pharmacia) as described (De Vries et al., 1993a). Human placenta was obtained from the Department of Obstetrics and Gynecology, Academic Hospital of the Vrije Universiteit, Amsterdam, and stored frozen until use. Unlabeled GDP-fucose was kindly donated by Drs. H. Lönn and T. Norberg (BioCarb AB, Lund, Sweden). CMP-NeuAc was synthesized using a calf brain CMP-sialic acid synthetase preparation as described previously (van den Eijnden & van Dijk, 1972). GDP-[^{14}C]Fuc (250 Ci/mol) and CMP-[^{3}H]-NeuAc (18 900 Ci/mol) were purchased fron New England Nuclear Corp. (Boston, MA) and diluted with the unlabeled nucleotide sugar to obtain the desired specific radioactivity. (Galβ1→4GlcNAcβ1→3)$_3$-Me was a kind gift of Dr. A. Veyrières (Université de Paris-Sud, Orsay, France). Endo-β-galactosidase from *Bacteriodes fragilis* (EC 3.2.1.103) was purchased from Boehringer Mannheim. α_1-Acid glycoprotein was prepared from human plasma Cohn fraction V supernatant as described (Hao & Wickerhauser, 1973) and desialylated by mild acid hydrolysis (0.1 N trifluoroacetic acid, 1 h at 80 °C). All other chemicals were obtained from commercial sources and were of the highest purity available.

Preparation of NeuAcα2→3(Galβ1→4GlcNAcβ1→3)$_3$-Me. Human placenta was used as a source for α3-sialyltransferase (van den Eijnden & Schiphorst, 1981). Placenta membranes were prepared and the sialyltransferase activity was determined using asialo-α_1-AGP as an acceptor as described previously (Nemansky & van den Eijnden, 1993). Preparation of NeuAcα2→3(Galβ1→4GlcNAcβ1→3)$_3$-Me was essentially carried out as described for the sialylation of *N*-acetyllactosamine (De Vries et al., 1993b). (Galβ1→4GlcNAcβ1→3)$_3$-Me (2.47 mg, 2.2 μmol) and CMP-[^{3}H]-NeuAc (3.7 mg, 6 μmol, 0.1 Ci/mol) were lyophilized from small volumes together with 0.26 mmol of sodium cacodylate (pH 7.0), 10.6 μmol of ATP, 26 μmol of EDTA, and 5.3 mg of Triton X-100. α3-sialyltransferase (19 milliunits) in the form of placenta membranes was added and mixed with the other ingredients and 670 μL of water to yield a total volume of 2.6 mL. The mixture was then incubated at 37 °C for 3 h. After incubation, the reaction mixture was diluted with water to 12 mL and centrifuged for 15 min at 30000*g*. The supernatant was collected and the pellet was washed with another 10 mL of water and centrifuged again. The second supernatant was combined with the first, and the mixture was then lyophilized to dryness, taken up in a volume of 1 mL in H_2O and applied to a column (1.6 × 200 cm) of Bio-Gel P-6 (200–400 mesh, Bio-Rad) equilibrated and eluted at a flow of 8 mL/h with 50 mM ammonium acetate, pH 5.2, at 45 °C. Fractions (4 mL) were collected and counted for radioactivity. Fractions containing the product were pooled (nos 50–59), lyophilized, and desalted on a column (1 × 40 cm) of Bio-Gel P-2 (200–400 mesh) run in water. The fractions containing the sialyloligosaccharide were pooled and lyophilized. This resulted in a yield of 1.2 mg [1.1 μmol; 50% calculated from (Galβ1→4GlcNAcβ1→3)$_3$-Me].

Time Course of Fucosylation. The time course of incorporation of fucose into NeuAcα2→3(LacNAc)$_3$-Me was established as follows: A reaction mixture containing 55 nmol of NeuAcα2→3(LacNAc)$_3$-Me, 275 nmol of GDP-[^{14}C]Fuc (1 Ci/mol), 27.5 μmol of Mops/NaOH (pH 7.5), 2.75 μmol of $MnCl_2$, 55 μmol of NaCl, 2.0 μmol of ATP, and 0.8 milliunits of human milk fucosyltransferase in 550 μL was incubated at 37 °C. At intervals, aliquots (50 μL) were withdrawn from the reaction mixture and immediately frozen. Subsequently, the fucosylated products were separated from unreacted GDP-fucose on a MonoQ HR 5/5 anion-exchange column using a Pharmacia FPLC system. Elutions were carried out using a linear gradient running from 0 to 500 mM NaCl in water (5%/min, flow rate 1 mL/min). Eluates were counted for radioactivity, and appropriate fractions were pooled and desalted on a Bio-Gel P-2 column run in water. Fractions containing the fucosylated products were pooled and the materials were analyzed by HPLC.

Separation of Fucosylated NeuAcα2→3(LacNAc)$_3$-Me Products by HPLC. Unreacted NeuAcα2→3(LacNAc)$_3$-Me and the mono-, di-, and trifucosylated products formed from it were separated on a Spherisorb-NH_2 (PhaseSep, Clwyd, U.K.) column by elution with an 80:20 (v/v) mixture of acetonitrile and 15 mM potassium phosphate, pH 5.2, with a linear gradient of increasing buffer content at a rate of 0.3%/min. The flow rate was 2 mL/min. Eluates were monitored for absorbance at 195 nm. Radioactive compounds were quantified by liquid scintillation counting of the fractionated eluate.

Large-Scale Incubations. Two incubation mixtures were prepared. The first (A) contained in 1 mL 400 nmol of NeuAcα2→3(LacNAc)$_3$-Me, 800 nmol of GDP-[^{14}C]Fuc (0.1 Ci/mol), 1.4 milliunits of human milk fucosyltransferase, 5 μmol of $MnCl_2$, 4 μmol of ATP, 50 μmol of sodium cacodylate (pH 7.2), 0.1 mmol of NaCl, 5 μmol of $MgCl_2$, 50% glycerol, and 0.05% sodium azide. The second mixture (B) contained in 2 mL 500 nmol of NeuAcα2→3(LacNAc)$_3$-Me, 4.5 μmol of GDP-[^{14}C]Fuc (0.1 Ci/mol), 2.8 milliunits of human milk fucosyltransferase, 10 μmol of $MnCl_2$, 8 μmol of ATP, 0.1 mmol of sodium cacodylate (pH 7.2), 0.2 mmol of NaCl, 10 μmol of $MgCl_2$, 50% glycerol, and 0.05% sodium azide. Both mixtures were incubated at 37 °C for 48 h. Subsequently, each of the reaction mixtures was applied to a column (1.6 × 200 cm) of Bio-Gel P-6 (200–400 mesh) equilibrated and eluted at a flow of 8 mL/h with 50 mM ammonium acetate, pH 5.2, at 45 °C. Fractions (4 mL) were collected and monitored for radioactivity. Fractions containing the [^{14}C]-fucosylated products were pooled, lyophilized, and further desalted on a column (1 × 40 cm) of Bio-Gel P-2 (200–400

mesh) run in water. Fractions containing the fucosylated sialyloligosaccharides were pooled and lyophilized. The fucosylated oligosaccharides were separated into mono-, di-, and trifucosylated NeuAcα2→3(LacNAc)$_3$-Me products by high-pH anion exchange chromatography (HPAEC) with pulsed amperometric detection (PAD).

HPAEC-PAD of Sialylated, Fucosylated Oligosaccharide Products. The system used for HPAEC-PAD consisted of a Dionex Bio-LC gradient pump, CarboPac PA-1 column (9.0 × 250 mm), and a Model PAD 2 detector. The following pulse potentials and durations were used for detection: E_1 = 0.05 V (t_1 = 480 ms); E_2 = 0.60 V (t_2 = 120 ms); E_3 = –0.60 V (t_3 = 60 ms). The response time of the detector was set to 1 s. Samples were dissolved in water and injected via a Dionex microinjection valve equipped with a 25-μL sample loop. The chromatographic data were integrated and plotted using a Shimadzu C-R5A integrator. A Dionex eluant degas module was employed to saturate the eluants with helium in order to degas and to minimize absorption of CO_2. For the separation of the products, isocratic elution was conducted with 0.1 mol/L NaOH for 10 min, whereafter a gradient of sodium acetate (0–0.1 mol/L in 40 min) in 0.1 mol/L NaOH was applied. The flow rate was 5 mL/min and fractions of 2.5 mL were collected in tubes containing 2.5 mL of 200 mM ammonium acetate, pH 5.2, and counted for radioactivity.

Endo-β-galactosidase Digestion. Endo-β-galactosidase digestion of mono-, di-, and trifucosylated NeuAcα2→3(LacNAc)$_3$-Me (5 nmol) was carried out in 20 μL containing 2 μmol of sodium acetate, pH 5.7, 4 μg of bovine serum albumin, and 5 milliunits of endo-β-galactosidase. After a 24-h incubation at 37 °C, digests were concentrated to dryness and analyzed by HPLC on a Spherisorb column.

400-MHz ^{1}H-NMR Spectroscopy. Prior to ^{1}H-NMR spectroscopic analysis, the substrate and the fucosylated products (300–500 nmol) were desalted on a Bio-Gel P-2 column and exchanged in D_2O three times at room temperature with intermediate lyophilization. Finally, each sample was redissolved in 400 μL of D_2O (99.96 atom % D; Aldrich, Milwaukee, WI). ^{1}H-NMR spectroscopy was performed on a Bruker MSL 400 spectrometer, operating at 400 MHz at a probe temperature of 300 K. Resolution enhancement was achieved by Lorentzian to Gaussian transformation. Chemical shifts are expressed in parts per million downfield from internal sodium 4,4-dimethyl-4-silapentane-1-sulfonate but were actually measured by reference to internal acetone (δ = 2.225 ppm in D_2O).

RESULTS

Synthesis of NeuAcα2→3(LacNAc)$_3$-Me. For the synthesis of NeuAcα2→3(LacNAc)$_3$-Me, CMP-NeuAc was used in excess (2.7-fold) over the trimeric *N*-acetyllactosamine oligosaccharide. After incubation the product was isolated by gel filtration. The yield of α3-sialylated product was 50%, based on the oligosaccharide as the limiting substrate. The ^{1}H-NMR spectrum of the sialylated compound obtained by this procedure indicated a high degree of purity (>95%, Figure 4A).

Time Course of Fucosylation of NeuAcα2→3(LacNAc)$_3$-Me. Fucosylated products formed after incubation for different times were quantified after separation by HPLC using Spherisorb-NH_2. The different peaks were identified as mono-, di-, and trifucosyl-NeuAcα2→3(LacNAc)$_3$-Me on the bases of their ratio of absorbance at 195 nm and ^{14}C radioactivity. Addition of each fucose residue resulted in an increase of 7–9 min in retention time. Progress curves were

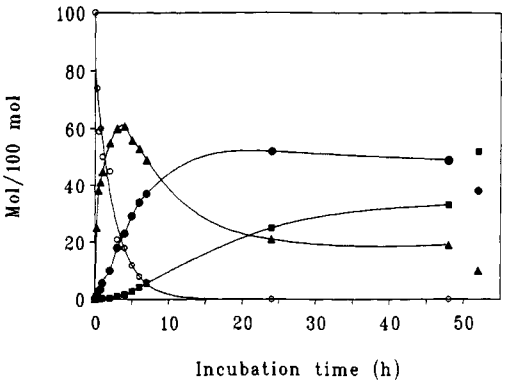

FIGURE 1: Time course of incorporation of ^{14}C-labeled fucose into NeuAcα2→3(LacNAc)$_3$-Me. NeuAcα2→3(LacNAc)$_3$-Me (5 nmol) was incubated with 25 nmol of GDP-fucose (1 Ci/mol) and 0.07 milliunits of human milk fucosyltransferase for the times indicated. Fucosylated products were isolated by anion-exchange chromatography and were separated by HPLC as described under Experimental Procedures. NeuAcα2→3(LacNAc)$_3$-Me (○); monofucosylated NeuAcα2→3(LacNAc)$_3$-Me (▲); difucosylated NeuAcα2→3(LacNAc)$_3$-Me (●); trifucosylated NeuAcα2→3(LacNAc)$_3$-Me (■).

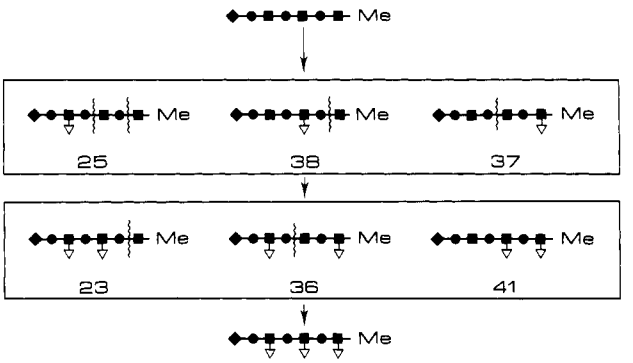

FIGURE 2: Fucosylation pathway of NeuAcα2→3(LacNAc)$_3$-Me. The location of fucose was established by digestion with endo-β-galactosidase as described under Experimental Procedures. The relative yield of each fragment obtained by digestion allowed calculation of the ratio of isomers in each fucosylated NeuAcα2→3(LacNAc)$_3$-Me intermediate. The wavy lines indicate the cleavage sites in the various fucosylated products. ♦, NeuAc; ●, Gal; ■, GlcNAc; ▽, Fuc; numbers indicate the percentage of isomers.

obtained for mono-, di-, and trifucosyl-NeuAcα2→3(LacNAc)$_3$-Me (Figure 1). From these curves a significant difference in the rate of transfer of the first, second, and third fucose residues to the sialylated (LacNAc)$_3$-Me acceptor (7:4:1, respectively) could be calculated. Transfer of the third fucose was found to proceed at a low rate. Even with a large excess of GDP-fucose (9 mol/mol of acceptor substrate) and prolonged reaction time (52 h) it was not possible to obtain more than a 57% overall conversion into trifucosylated product.

Identification of Fucosylated Products by Digestion with Endo-β-galactosidase. To determine the location of the fucose residues in the monofucosylated and difucosylated intermediate fractions, the products were digested with endo-β-galactosidase. This enzyme cleaves Galβ1→4 linkages in ...GlcNAcβ1→3Galβ1→4GlcNAc... but not in ...GlcNAcβ1→3Galβ1→4[Fucα1→3]GlcNAc... sequences (Kannagi et al., 1982) nor in a NeuAcα2→3Galβ1→4GlcNAc... sequence (Fukuda, 1981). The sites of cleavage in each fucosylated product are indicated in Figure 2. Table 1 shows the retention times on Spherisorb-NH_2 of the [^{3}H]sialylated and/or [^{14}C]-fucosylated fragments obtained after digestion with endo-β-galactosidase, along with their relative yields and their tentative structures. Identification was based on the ratio of ^{3}H/^{14}C radioactivity and the elution position of the fragments. For

Table 1: Characteristics of Diagnostic Fragments Obtained by Endo-β-galactosidase Digestion of Fucosylated Products[a]

[^{14}C]fucosylated, [^{3}H]NeuAcα2→3-(LacNAc)$_3$-Me product	characteristics of ^{3}H- and/or ^{14}C-labeled fragments		
	HPLC retention time (min)	tentative structure	relative yield of [^{14}C]Fuc after digestion (%)
monofucosylated-	29	GN-G-[F]GN-Me	37
	45	SA-G-[F]GN-G	25
	62	SA-G-GN-G-[F]GN-G	38
difucosylated-	29	GN-G-[F]GN-Me	18
	45	SA-G-[F]GN-G	18
	66	SA-G-GN-G-[F]GN-G-[F]GN-Me	41
	71	SA-G-[F]GN-G-[F]GN-G	23
trifucosylated-	75	SA-(G-[F]GN-)$_3$-Me	100

[a] The mono-, di-, and trifucosylated NeuAcα2→3(LacNAc)$_3$-Me intermediate fractions were subjected to endo-β-galactosidase digestion as described in Experimental Procedures. Fragments were analyzed by HPLC on a Spherisorb-NH_2 column. SA-, [^{3}H]NeuAcα2→3; G-, Galβ1→4; F, [^{14}C]Fucα1→3; GN-, GlcNAcβ1→3. The fragments are characterized on the basis of their ratio of ^{3}H/^{14}C radioactivity and their retention times. The yield is expressed relative to the total of each (monofucosylated and difucosylated) intermediate.

monofucosylated NeuAcα2→3(LacNAc)$_3$-Me the relative yields of fragments appeared to be slightly higher for those formed from the isomers with a fucose attached to the proximal (GN1) and medial (GN3) (cf. Table 2) GlcNAc residues. For the difucosylated products the relative yield of the endo-β-galactosidase-resistant isomer SA-G-GN-G-[F]GN-G-[F]-GN-Me, among the fragments originating from the nonresistant difucosylated products, was slightly higher (41% over 23%) than the fragment that was obtained from the product fucosylated at GN5 and GN3 (SA-G-[F]GN-G-[F]GN-G) (Table 1). The relative yields of the fragments allowed the calculation of the ratio of isomers in each fucosylated NeuAcα2→3(LacNAc)$_3$-Me intermediate mixture and the identification of the major steps in the fucosylation pathway (Figure 2). These results indicate that in the intermediate products the fucose residues have been attached to the different acceptor sites in the molecule with only a modest preference for the proximal (GN1) and medial (GN3) GlcNAc residues. It thus appears that there is not a single preferred pathway for fucosylation of the NeuAcα2→3(LacNAc)$_3$-Me structure, although there is a slight preference for a route leading to the trifucosylated structure through prior fucosylation of GlcNAc residues GN1 and GN3.

Large-Scale Synthesis of Fucosylated Products. The different products obtained in the two incubations performed on a large scale were isolated by HPAEC-PAD. The elution profiles are shown in Figure 3 for the incubations with a 2-fold (A) and 9-fold (B) molar ratio of GDP-fucose to acceptor substrate, respectively. The retention time of the acceptor substrate NeuAcα2→3(LacNAc)$_3$-Me was 24.2 min. On the basis of the ratio of ^{3}H/^{14}C radioactivity, peak 1 can be identified as the trifucosylated product, peaks 2 and 3 as difucosylated products, and peak 4 as a mixture of monofucosylated products. Addition of one fucose residue to the acceptor molecule causes no significant difference in retention time, while addition of two and three fucose residues resulted in a decrease of 14.0 and 20.5 min in retention time, respectively. Yields were, for mixture A, 67% (268 nmol) monofucosylated products, 32% (128 nmol) difucosylated products, and 1% (4 nmol) trifucosylated NeuAcα2→3(LacNAc)$_3$-Me; and for mixture B, 3% (15 nmol) monofucosylated products, 38% (190 nmol) difucosylated products, and 59% (295 nmol) trifucosylated NeuAcα2→3(LacNAc)$_3$-Me.

400-MHz ^{1}H-NMR Spectroscopy. The ^{1}H-NMR spectra of the acceptor substrate NeuAcα2→3(LacNAc)$_3$-Me and the enzymically fucosylated products are shown in Figure 4. The relevant ^{1}H chemical shifts are given in Table 2, along with the numbering system of the monosaccharide residues. In the spectrum of NeuAcα2→3(LacNAc)$_3$-Me (Figure 4A), three different H-1 signals of the three galactose residues are observed, along with three different H-1 signals of the three GlcNAc residues. The H-1 signal of the galactose G6 residue is found at a relatively downfield position (δ = 4.555 ppm) due to the presence of a NeuAc in α2→3 linkage (Vliegenthart et al., 1983). The H-1 signal of the GlcNAc GN1 residue is found upfield (δ = 4.459 ppm) due to its attachment to the Me group. The assignment of resonance signals of the other galactose and GlcNAc residues is based on the assignment of the signals of a neutral trimer of LacNAc (De Vries et al., 1993a). The H-1 signal of galactose G4 is located downfield of the H-1 signal of galactose G2 (De Vries et al., 1993a; Hokke et al., 1993).

Addition of a fucose residue in α3 linkage to a GlcNAc residue results in an upfield shift of the H-1 signal of the adjacent galactose of ~0.02 ppm and of the GlcNAc *N*-acetyl signal of ~–0.01 ppm (Vliegenthart et al., 1983). The spectrum of the monofucosylated products of peak 4 (Figure 3) is shown in Figure 4B. In the α-anomeric region three different H-1 doublets can be observed (δ = 5.119, 5.114, and 5.086 ppm). These can be assigned as H-1 signals of fucose residue F5, F3, and F1, respectively, by analogy with the neutral trimeric-Lewis^{X} oligosaccharide (De Vries et al., 1993a). Two doublets of the H-1 of galactose G6 can be observed at δ = 4.526 and 4.557 ppm. The signal at δ = 4.526 ppm has the same intensity as the fucose H-1 signal at δ = 5.119 ppm, which hence is concluded to belong to galactose G6 of an isomer fucosylated at GlcNAc GN5. Also, for each of the galactose residues G4 and G2 two sets of doublets can be recognized, for a galactose in a -Galβ1→4[Fucα1→3]-GlcNAc-element (δ 4.450 and 4.431 ppm for G4 and G2, respectively) and for a galactose in a -Galβ1→4GlcNAc-element (δ 4.466 and 4.447 ppm for G4 and G2, respectively). A small doublet appears at δ = 4.471 ppm, which can be assigned to the H-1 signal of fucosylated GN1 and is consistent with a downfield shift caused by an attached fucose (Vliegenthart et al., 1983). However, such a downfield shift is not observed for the H-1 resonances of GN3 and GN5. Two fucose H-6 doublets can be observed; the smaller one at δ = 1.167 ppm can be assigned to F5 and the other one at δ = 1.151 ppm to F3 and F1. The signal at δ = 3.505 ppm, belonging to the Me group, is shifted by the presence of a fucose residue on GlcNAc GN1 (to δ 3.498 ppm). In conclusion, this spectrum appears to consist of a mixture of all three possible isomeric structures with fucose residues on GlcNAc GN5, GN3, and GN1 in a ratio of 26:42:32, which is consistent with the results obtained by endo-β-galactosidase digestion.

Table 2: Relevant ^1H Chemical Shifts of Structural Reporter Groups of NeuAcα2→3triLacNAc-Me (I) and Its Fucosylated Derivatives Obtained by Incubation with Human Milk Fucosyltransferase (II-VIII)[d]

Reporter group	Residue	Chemical shift for structural reporter groups of I	II	III	IV	V	VI	VII	VIII
H-1	GN1	4.459[a]	4.459	4.459	4.471	4.459	4.471	4.472	4.471
	G2	4.457[a]	4.447	4.447	4.431	4.444	4.433	4.431	4.430
	GN3	4.699	4.698	4.698	4.698	4.700	4.700	4.707[b]	4.701[c]
	G4	4.466	4.466	4.450	4.466	4.453	4.465	4.450	4.446
	GN5	4.699	4.698	4.698	4.698	4.700	4.700	4.698[b]	4.706[c]
	G6	4.555	4.526	4.557	4.557	4.527	4.527	4.558	4.530
	F1	-	-	-	5.086	-	5.086	5.086	5.086
	F3	-	-	5.114	-	5.114	-	5.122	5.120
	F5	-	5.119	-	-	5.125	5.119	-	5.126
$H\text{-}3_{eq}$	SA	2.758	2.764	2.758	2.758	2.764	2.764	2.759	2.765
$H\text{-}3_{ax}$	SA	1.796	1.792	1.796	1.796	1.792	1.792	1.797	1.792
CH_3	F1	-	-	-	1.151	-	1.147	1.145	1.144
	F3	-	-	1.151	-	1.149	-	1.152	1.147
	F5	-	1.167	-	-	1.167	1.167	-	1.167
	Me	3.505	3.505	3.505	3.498	3.505	3.498	3.499	3.498
NAc	GN1	2.030	2.030	2.030	2.021	2.030	2.020	2.020	2.020
	GN3	2.030	2.030	2.021	2.025	2.020	2.026	2.016	2.015
	GN5	2.030	2.021	2.025	2.030	2.014	2.020	2.025	2.015
	SA	2.032	2.030	2.030	2.030	2.031	2.031	2.031	2.031

[a-c] Assignments may have to be interchanged. [d] Chemical shifts in parts per million are given downfield from sodium 4,4-dimethyl-4-silapentane-1-sulfonate in D_2O at 300 K, acquired at 400 MHz (but were actually measured relative to internal acetone, δ = 2.225 ppm). In the table heading, the structures are represented by symbolic notation: ◆, NeuAc; ●, Gal; ■, GlcNAc; ▽, Fuc; –, not applicable. The monosaccharides are numbered as follows:

SA G6 GN5 G4 GN3 G2 GN1

NeuAcα2→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAcβ1→3Galβ1→4GlcNAc-Me

3 3 3

↑ ↑ ↑

Fucα1 Fucα1 Fucα1

F5 F3 F1

The spectrum of the difucosylated product of peak 2 (Figure 3) is shown in Figure 4C. Four different H-1 doublets of fucose can be observed (δ = 5.125, 5.119, 5.114, and 5.086 ppm), three of which are located at the same position as in Figure 4B. The H-1 signal of galactose G6 can be observed at δ = 4.527 ppm, indicating that this spectrum results from compounds of which all GlcNAc GN5 residues are fucosylated. Furthermore, a mixture of four H-1 doublets of galactose residues can be observed at δ = 4.465, 4.453, 4.444, and 4.433 ppm; these signals can be assigned to residues G4 with (4.453 ppm) and without (4.465 ppm) a fucose at GlcNAc GN3 and residues G2 with (4.433 ppm) and without (4.444 ppm) fucose at GlcNAc GN1. Three fucose H-6 doublets can be

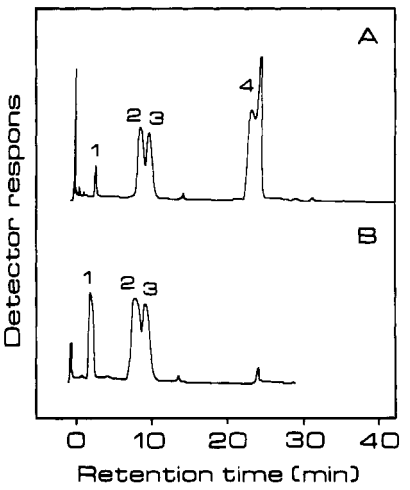

FIGURE 3: HPAEC-PAD profiles of large-scale incubation mixtures. (A) Elution profile of incubation mixture A. NeuAcα2→3(LacNAc)$_3$-Me (400 nmol) was incubated with 800 nmol of GDP-[^{14}C]fucose (0.1 Ci/mol) and 1.4 milliunits of human milk fucosyltransferase as described under Experimental Procedures. (B) Elution profile of incubation mixture B. NeuAcα2→3(LacNAc)$_3$-Me (500 nmol) was incubated with 4.5 μmol of GDP-[^{14}C]fucose (0.1 Ci/mol) and 2.8 milliunits of human milk fucosyltransferase as described under Experimental Procedures. Peak 1 is characterized as trifucosylated NeuAcα2→3(LacNAc)$_3$-Me, peaks 2 and 3 as difucosylated products, and peak 4 as monofucosylated products.

observed: two small signals at δ = 1.147 ppm (F1) and at δ = 1.149 ppm (F3) and a larger one at δ = 1.167 ppm (F5). On the basis of the position of the fucose H-1 signals, the position of the galactose G6 H-1 signal, the ratio of galactose G4 and G2 H-1 doublets, and the fucose H-6 doublets, the spectrum appears to result from a mixture of two difucosylated isomers, fucosylated at GN5 and GN3 and at GN5 and GN1, respectively, in a ratio of ~1:1. It should be noted that the fucose residues influence each other's H-1 signals. When F3 is present the signal belonging to F5 is shifted downfield (from δ = 5.119 to 5.125 ppm).

Figure 4D shows the spectrum of the material of peak 3 (Figure 3). Two doublets can be observed at δ = 5.122 and 5.086 ppm, which can be assigned as the H-1 resonances belonging to fucose residues F3 and F1, respectively. Furthermore, the galactose G6 H-1 signal is found at δ = 4.558 ppm, indicating that GlcNAc GN5 is not fucosylated. The H-1 resonances from galactose residues G4 and G2 are found at δ = 4.450 and 4.431 ppm, respectively, indicating that both GlcNAc residues GN3 and GN1 are fucosylated. Moreover, the position of the H-1 doublet of GlcNAc GN1 (δ = 4.472 ppm) is typical for the presence of a fucose residue at GlcNAc GN1. Two fucose H-6 doublets can be recognized at δ = 1.152 and 1.145 ppm, resulting from F3 and F1, respectively. In conclusion, this spectrum appears to be of a single isomer, fucosylated at GN1 and GN3.

Figure 4E shows the spectrum of the trifucosylated compound (peak 1, Figure 3). All three different fucose H-1 and H-6 resonances can be observed, in addition to an upfield shift of each galactose H-1 signal and a downfield shift of the GlcNAc GN1 H-1 signal.

The assignment of resonance signals to the CH_3 protons of the *N*-acetyl groups for the GlcNAc residues is complicated by the fact that attachment of a fucose residue not only affects the *N*-acetyl signal of the GlcNAc to which it is introduced ($\Delta\delta \approx -0.01$ ppm) but also has an effect on the signal of the more distally located neighbor GlcNAc ($\Delta\delta$ = –0.005 ppm). For instance, in the spectrum of the starting material, NeuAcα2→3(LacNAc)$_3$-Me (Figure 4A), the chemical shifts

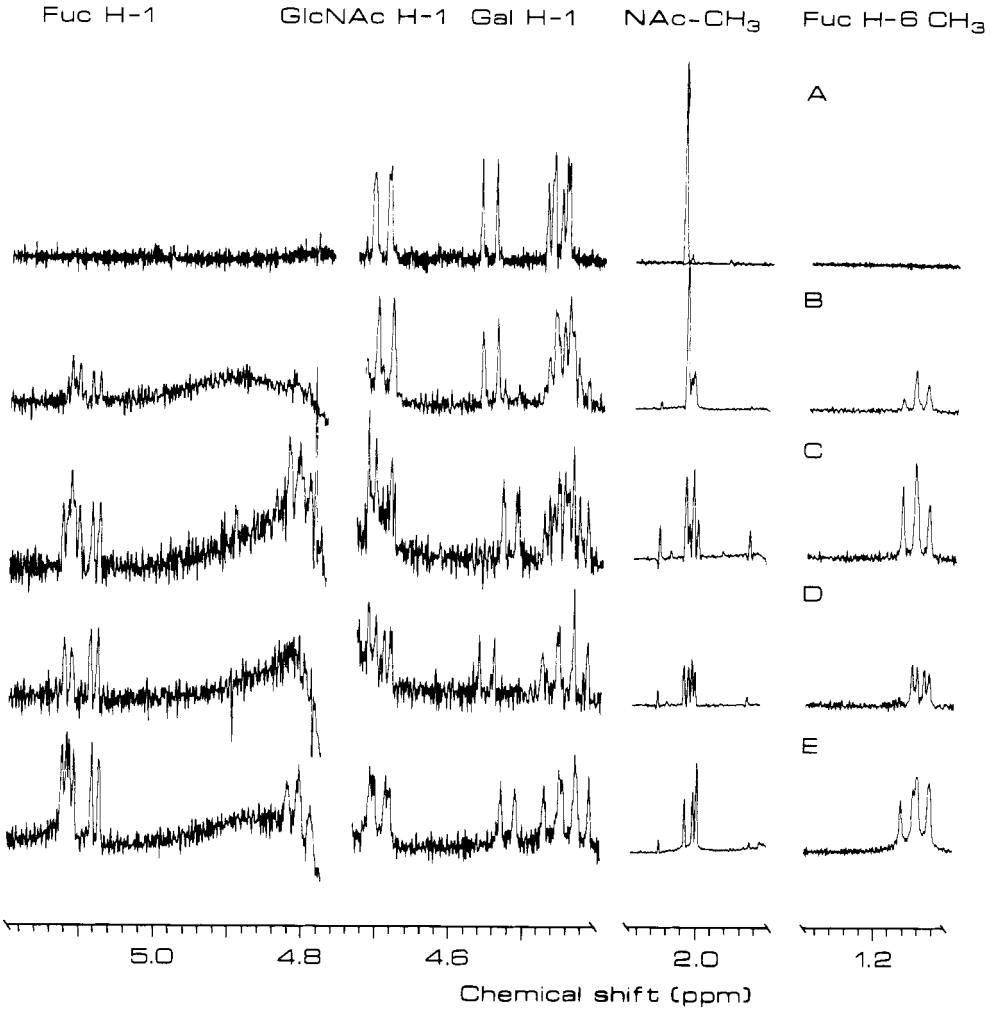

FIGURE 4: Comparison of the structural reporter group regions of the 400-MHz ^{1}H-NMR spectra of NeuAcα2→3(LacNAc)$_3$-Me and fucosylated products thereof. (A) Substrate; (B) monofucosylated fraction (peak 4 in Figure 3); (C, D) difucosylated products (peaks 2 and 3, respectively, in Figure 3); (E) trifucosylated NeuAcα2→3(LacNAc)$_3$-Me (peak 1 in Figure 3). The relative intensity of the NAc region is ×0.25, and of the fucose H-6 CH_3 region, ×0.33.

of all three GlcNAc CH_3 protons can be found at δ = 2.030 ppm. In the spectrum of the monofucosylated compounds, Figure 4B, the *N*-acetyl signal of a GlcNAc residue in a -Galβ1→4[Fucα1→3]GlcNAc- element is found at δ = 2.021 ppm, whereas the signal of a distally located nonfucosylated neighbor GlcNAc residue is found at δ = 2.025 ppm.

DISCUSSION

In an earlier report we demonstrated that human milk fucosyltransferase catalyzes the attachment of fucose residues to a trimer of LacNAc in a distinct order (De Vries et al., 1993a). The first fucose residues appeared to be transferred with equal preference to the proximal (GN1) and medial GlcNAc (GN3) residues and with far less efficiency to the distal GlcNAc (GN5). The fucosyltransferase catalyzing this reaction was concluded to be a plasma-type enzyme and not the Lewis α3/1,4-fucosyltransferase, also present in normal human milk (De Vries et al., 1993a). In the present study we used the same fucosyltransferase preparation to establish the order of fucose attachment to a sialylated trimer of LacNAc, NeuAcα2→3(LacNAc)$_3$-Me. It appears that for this substrate a preferred biosynthetic pathway to the trifucosylated structure is less apparent. The first fucose is attached with only a slight preference to the proximal and medial GlcNAc residues. Thus, there is not a specific site of initial fucosylation. Probably, the presence of the sialic acid residue, or just the fact that galactose residue G6 is substituted at carbon 3 (as is the case for an internal -Galβ1→4GlcNAc- unit), is sufficient to render the distal GlcNAc residue a relatively better substrate site than in the asialo compound. The second step in the biosynthetic pathway also shows only a slight preference for fucosylation of the medial and proximal GlcNAc residues. Since no more than 59% of conversion into the trifucosylated product could be obtained, the possibility was considered that one of the three difucosylated products was a poor substrate for further fucosylation. However, this was concluded not to be the case because no accumulation of a single isomer in the pools of mono- or difucosylated products was found.

Several other studies on site specificity of α3-fucosyltransferase have been carried out, which are summarized in Table 3. It should be noted, however, that direct comparisons between different studies are difficult to make. Nonidentical methods and acceptors have been used. For instance, in studies performed with glycosphingolipids as acceptor substrates, incubations were conducted in the presence of detergents (Holmes & Levery, 1989; Holmes et al., 1985, 1986; Holmes & Macher, 1993; Basu et al., 1991; Howard et al., 1987), which have been shown to influence the site of initial fucosylation (Holmes & Macher, 1993). Partially purified enzymes (De Vries et al., 1993a; Basu et al., 1991) as well as cell homogenates and extracts (Holmes & Levery, 1989; Holmes et al., 1985, 1985; Holmes & Macher, 1993; Howard et al., 1987) have been used. The latter may have contained

Table 3: Site Specificity of α3-Fucosyltransferase[a]

enzyme source	preferred GlcNAc residue in acceptor substrate		reference
	nLc_6Cer	$VI^3NeuAc/GcnLc_6Cer$	
FucTI (LEC11 cells)	proximal GlcNAc	proximal GlcNAc	Howard et al., 1987
FucTII (LEC12 cells)	distal GlcNAc	no substrate	Howard et al., 1987
HL-60 cells	distal GlcNAc	proximal GlcNAc	Holmes & Macher, 1993
PC9 cells	distal GlcNAc	distal GlcNAc	Holmes et al., 1986
NCI-H69 cells	distal GlcNAc	nd	Holmes et al., 1985
Colo 205 cells	proximal GlcNAc	distal GlcNAc	Holmes & Levery, 1989; Basu et al., 1991

[a] The preferred GlcNAc residues for initial fucosylation. All studies were performed on glycolipids. The proximal GlcNAc residue is the III-GlcNAc and the distal GlcNA residue is the V-GlcNAc. nd, not determined.

factors that influenced the site specificity. Some enzymes used were membrane forms (Holmes & Levery, 1989; Holmes et al., 1985, 1985; Holmes & Macher, 1993; Basu et al., 1991; Howard et al., 1987), whereas other preparations contained soluble enzyme forms (De Vries et al., 1993a). It is not known whether the presence of a transmembrane domain in the polypeptide chain of an α3-fucosyltransferase is of influence on the site specificity. Furthermore, acceptors with different aglycons were used: different ceramide moieties (Basu et al., 1991), a synthetic [(trifluoroacetamido)phenyl]ethyl group (De Vries et al., 1993a), or a single methyl group as aglycon (this study). No data have been presented on site specificity with respect to polylactosaminoglycan chains on glycoproteins. It is conceivable, though, that the presence of a bulky mannose-containing core structure is yet another factor influencing the site of preference.

Nonetheless, it is of interest to compare the site specificities of different α3-fucosyltransferases with nLc_6Cer and its sialylated derivative. Several patterns for the preferred sites of fucose addition to these substrates can be distinguished. The myeloid-type fucosyltransferases, as found in HL-60 (Holmes & Macher, 1993) and LEC12 cells (Howard et al., 1987), preferentially catalyze the attachment of a fucosyl residue to the distal V-GlcNAc of neutral substrates, but initial fucosylation is shifted to the proximal III-GlcNAc when the substrate is sialylated (if transfer can occur at all; Howard et al., 1987). The fucosyltransferase found in LEC11 cells, FucTI, which shows acceptor specificity characteristics very similar to the plasma-type enzyme, prefers proximal III-GlcNAc residues with both types of substrates (Howard et al., 1987). However, other fucosyltransferases resembling the plasma-type enzyme, as found in PC9 (Holmes et al., 1986) and NCI-H69 cells (Holmes et al., 1985), show a preference for the distal V-GlcNAc, whether the substrate is sialylated or not. The enzyme found in human colonic adenocarcinoma Colo 205 cells (Holmes & Levery, 1989; Basu et al., 1991) shows a behavior opposite to that of the first group. In case of a neutral substrate the preferred GlcNAc is the proximal III-GlcNAc. No data have been presented on the site of fucosylation of $VI^3NeuAcnLc_6$-Cer by this fucosyltransferase. However, Basu et al. (1991) reported that 90% of the product of fucose transfer to $VI^3NeuGcnLc_6$-Cer by the Colo 205 cell enzyme stained with the anti-sialyl-Lewis^x antibody, CSLEX1, suggesting that with a sialylated substrate initial transfer takes place at the V-GlcNAc. Our results obtained with the human milk fucosyltransferase preparation demonstrate that the site specificity of human milk fucosyltransferase resembles that of the Colo 205 cell enzyme. The preferred sites of initial fucosylation were in the case of the neutral trimeric LacNAc substrate the medial (GN3) and proximal (GN1) GlcNAc residues (De Vries et al., 1993a), whereas with the sialylated trimeric LacNAc acceptor substrate also the distal (GN5) GlcNAc becomes a site of initial fucosylation.

Molecular cloning of α3-fucosyltransferase genes has suggested the existence of two closely related α3-fucosyltransferases (FucTV and FucTVI), the latter of which is the plasma-type enzyme (Weston et al., 1992b). Recombinant forms of these two enzymes can be distinguished by their capability to act on 2′-fucosyllactose (Weston et al., 1992a,b; Koszdin & Bowen, 1992). FucTV is capable of transferring fucose to 2′-fucosyllactose with good efficiency (Weston et al., 1992a). The properties of FucTVI are more similar to those described for the purified plasma enzyme from serum (Sarnesto et al., 1992) and hepatocytes (Jezequel-Cuer et al., 1993). LacNAc and sialyl-LacNAc are used by this enzyme with high efficiency, but 2′-fucosyllactose is a relatively poor acceptor (Weston et al., 1992b). It is interesting to note that FucTVI, when expressed in transfected cells, does not support the synthesis of the VIM-2 epitope, while precursors for this epitope are present (Weston et al., 1992b). However, high amounts of surface-localized sialyl-Lewis^x and sialyl-dimeric-Lewis^x determinants occur on these cells. This suggests that initial fucosylation by FucTVI takes place at distal GlcNAcs exclusively or that VIM-2 epitopes are converted into sialyl-dimeric-Lewis^x determinants with high efficiency. Immunostaining of the products formed by incubation of $VI^3NeuAcnLc_6Cer$ with recombinant FucTV using the antibodies CSLEX1 and VIM-2 revealed that initial fucosylation occurred both at the distal V-GlcNAc and at the proximal III-GlcNAc, with a slight preference for the latter residue (De Vries and Macher, unpublished observation). On the basis of these data, it cannot be assessed whether FucTV, FucTVI, or both occur in human milk. However, if one compares the relative activities of the human milk fucosyltransferase toward lacto-*N*-biose I, 2′-fucosyllactose, and LacNAc (De Vries et al., 1993a; Johnson et al., 1992) with those of recombinant FucTIII, FucTV, and FucTVI (Weston et al., 1992b), it can be estimated that the fucosyltransferase responsible for the α3-fucosylation reactions we studied is most probably FucTVI. If FucTV were present in human milk, a much higher relative activity with 2′-fucosyllactose should have been found. Alternatively, it also remains possible that the human milk α3-fucosyltransferase is encoded by another, yet uncharacterized, fucosyltransferase gene. Furthermore, it is not impossible that the human milk enzyme, being a soluble form of a fucosyltransferase without transmembrane region, has a slightly altered (acceptor) site specificity, compared to the full-length form of the enzyme. It cannot be excluded that factors outside the catalytic domain play a role in the recognition of specific GlcNAc residues and thus help to determine site specificity.

The products obtained in this study will be of value in future studies on the site specificity of recombinant α3-fucosyltransferases. Such studies, in combination with a molecular genetic approach, will allow identification of the genes coding for the α3-fucosyltransferase activities in human milk.

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