Bovine colostrum CMP-NeuAc:Gal β (1 $\rightarrow$ 4)GlcNAc-R α (2 $\rightarrow$ 6)-sialyltransferase is involved in the synthesis of the terminal NeuAc α (2 $\rightarrow$ 6)GalNAc β (1 $\rightarrow$ 4)GlcNAc sequence occurring on *N*-linked glycans of bovine milk glycoproteins

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Bovine colostrum CMP-NeuAc:Gal β (1 $\rightarrow$ 4)GlcNAc-R α (2 $\rightarrow$ 6)-sialyltransferase (α 6-sialyltransferase) appears to be capable of catalysing α 6-sialylation of the disaccharide GalNAc β (1 $\rightarrow$ 4)GlcNAc to yield the trisaccharide NeuAc α (2 $\rightarrow$ 6)GalNAc β (1 $\rightarrow$ 4)GlcNAc. This provides an enzymic basis for the occurrence of this sialylated structure on the *N*-linked glycans of a number of bovine milk glycoproteins. Competition experiments using Gal β (1 $\rightarrow$ 4)GlcNAc and GalNAc β (1 $\rightarrow$ 4)GlcNAc as acceptors indicate that both substrates are recognized by a single active site on the α 6-sialyltransferase. Extrapolation of these results suggests that the NeuAc α (2 $\rightarrow$ 6)GalNAc β (1 $\rightarrow$ 4)GlcNAc structural element occurring on the *N*-linked glycans of several human glycoproteins are similarly synthesized by the action of a Gal β (1 $\rightarrow$ 4)GlcNAc-R α (2 $\rightarrow$ 6)-sialyltransferase.

INTRODUCTION

In several studies, the GalNAc β (1 $\rightarrow$ 4)GlcNAc disaccharide unit has been detected as a novel structural element on *N*-linked glycans of several bovine milk glycoproteins [1–3]. The latter glycoproteins contain this unit at the termini of branched complex-type and hybrid-type glycans. This structural element has earlier been described to occur in a sulphated form on the *N*-linked glycans of pituitary hormones of several mammals [4]. In bovine milk glycoproteins, however, capping of this disaccharide instead occurs by an α 6-linked sialic acid residue [2,3]. Recently, the sequence NeuAc α (2 $\rightarrow$ 6)GalNAc β (1 $\rightarrow$ 4)GlcNAc-R has also been reported to occur on *N*-linked glycans of human lutropin [5] and Bowes melanoma tissue plasminogen activator (tPA) [6].

The occurrence of these structures raises the question of whether a novel GalNAc β (1 $\rightarrow$ 4)GlcNAc-R-specific α (2 $\rightarrow$ 6)-sialyltransferase is responsible for the sialylation of the GalNAc β (1 $\rightarrow$ 4)GlcNAc units [5,6]. Alternatively, it is possible that these structures are produced by the action of the Gal β (1 $\rightarrow$ 4)GlcNAc-R α (2 $\rightarrow$ 6)-sialyltransferase. This would imply that this transferase is capable of recognizing GalNAc β (1 $\rightarrow$ 4)GlcNAc in addition to Gal β (1 $\rightarrow$ 4)GlcNAc units.

In the present study we show that the Gal β (1 $\rightarrow$ 4)GlcNAc-R α (2 $\rightarrow$ 6)-sialyltransferase from bovine colostrum indeed is capable of catalysing the production of the sialylated trisaccharide NeuAc α (2 $\rightarrow$ 6)GalNAc β (1 $\rightarrow$ 4)GlcNAc with high efficiency. This result, together with data from competition experiments, suggests that the bovine milk enzyme is responsible for the α (2 $\rightarrow$ 6)-sialylation of the GalNAc β (1 $\rightarrow$ 4)GlcNAc units on the glycans of bovine milk glycoproteins.

EXPERIMENTAL

Materials

CMP-[14 C]NeuAc (specific radioactivity 4.1 Ci/mol) was obtained from New England Nuclear (Boston, MA, U.S.A.) and diluted with unlabelled CMP-NeuAc [7] to the desired specific

radioactivity. CMP-NeuAc:Gal β (1 $\rightarrow$ 4)GlcNAc-R α (2 $\rightarrow$ 6)-sialyltransferase (α 6-sialyltransferase) was partially purified from bovine colostrum, using a CDP-ethanolamine–Sephacryl column eluted with NaCl as the major purification step. This yielded a 2000–3000-fold purified enzyme preparation [8]. Enzyme activity was assayed with asialo- α ₁-acid glycoprotein as an acceptor [9]. The disaccharide [3 H]GalNAc β (1 $\rightarrow$ 4)GlcNAc (0.1 Ci/mol) was a gift from Dr. A. P. Neeleman, Department of Medical Microbiology and Parasitology, Vrije Universiteit, Amsterdam, The Netherlands.

Large-scale incubation with α 6-sialyltransferase

The incubation mixture contained in a volume of 1 ml: 1.35 μ mol of [3 H]GalNAc β (1 $\rightarrow$ 4)GlcNAc (0.1 Ci/mol), 1.65 μ mol of CMP-[14 C]NeuAc (0.22 Ci/mol), 0.1 mmol of sodium cacodylate, pH 6.7, and 35 munits of α 6-sialyltransferase. This mixture was incubated at 37 °C for 45 h. A drop of toluene was added in order to minimize bacterial growth. After incubation, the mixture was applied to a column (1.6 cm $\times$ 200 cm) of Bio-Gel P-6 (200–400 mesh) equilibrated and eluted with 50 mM-ammonium acetate at pH 5.2 and room temperature, at a flow rate of 8 ml/h. Fractions of 2 ml were collected and assayed for 14 C and 3 H radioactivity by liquid-scintillation counting. Fractions containing the sialylated product were pooled and freeze-dried.

1 H n.m.r. spectroscopy

Before 1 H n.m.r. spectroscopic analysis, the samples were desalted on a column (0.7 cm $\times$ 45 cm) of Bio-Gel P-4 (200–400 mesh) run in water. Samples were repeatedly treated with 2 H₂O (99.75 atom %; Merck, Darmstadt, Germany) at p²H7 and room temperature. After each exchange treatment, the samples were freeze-dried. Finally, each sample was redissolved in 360 μ l of 2 H₂O (99.95 atom %; Aldrich, Milwaukee, WI, U.S.A.). 1 H n.m.r. spectroscopy was performed at 400 MHz on a Bruker MSL-400 spectrometer (facility of the Department of Physics, Vrije Universiteit, Amsterdam, The Netherlands) operating in

Abbreviations used: α 6-sialyltransferase, CMP-NeuAc:Gal β (1 $\rightarrow$ 4)GlcNAc-R α (2 $\rightarrow$ 6)-sialyltransferase (EC 2.4.99.1); CMP-NeuAc, cytidine 5'-monophospho- β -N-acetylneuraminic acid; tPA, tissue plasminogen activator.

the Fourier-transform mode. The probe temperature was kept at 300 K. Resolution enhancement of the spectra was achieved by Lorentzian-to-Gaussian transformation. Chemical shifts are expressed downfield from internal 4,4-dimethyl-4-silapentane-1-sulphonate, but were actually measured by reference to internal acetone ($\delta = 2.225$ p.p.m.) with an accuracy of 0.002 p.p.m.

Kinetic parameters of $\alpha 6$ -sialyltransferase and substrate competition experiments

K_m and V_{max} values for Gal β (1 $\rightarrow$ 4)GlcNAc and GalNAc β (1 $\rightarrow$ 4)GlcNAc as acceptors were determined at a concentration of 1 mM-CMP-NeuAc. Reaction mixtures contained, in a total volume of 10 μ l, 10 nmol of CMP-[14 C]NeuAc (6.78 Ci/mol), 1 μ mol of sodium cacodylate, pH 6.7, 42.6 μ units of $\alpha 6$ -sialyltransferase (0.016 mg), 0.4 μ l of glycerol and Gal β (1 $\rightarrow$ 4)GlcNAc or GalNAc β (1 $\rightarrow$ 4)GlcNAc at concentrations varying from 0.80 to 16.0 mM. For the competition experiments both acceptor substrates were used in the sialyltransferase assays in the same incubation at various concentrations, keeping the total acceptor substrate concentration at 20 mM. The mixtures were incubated for 30 min at 37 $^{\circ}$ C, whereafter the reactions were stopped on ice. The mixtures were diluted with 500 μ l of 5 mM-sodium phosphate, pH 6.8, at 0 $^{\circ}$ C. Incorporation of [14 C]NeuAc into the acceptors was assayed by applying the mixtures to a column (1.6 cm $\times$ 200 cm) of Bio-Gel P-6 or P-4 (200–400 mesh) as described above, yielding the separation of the precursor label (CMP-[14 C]NeuAc), the acceptor substrates and the products. Radioactive counts in the fractions containing the products were used to calculate the incorporation of [14 C]NeuAc. The kinetic parameters K_m and V_{max} were estimated from Lineweaver–Burk plots of $1/v$ against $1/[S]$ in which each point represented the average of two independent assays.

RESULTS

The trisaccharide NeuAc α (2 $\rightarrow$ 6)GalNAc β (1 $\rightarrow$ 4)GlcNAc was obtained by sialylation of the disaccharide acceptor [3 H]GalNAc β (1 $\rightarrow$ 4)GlcNAc by using bovine colostrum $\alpha 6$ -sialyltransferase and CMP-[14 C]NeuAc. The trisaccharide was produced in a yield of 49 % with respect to the disaccharide acceptor. After incubation, the product was purified by gel filtration on Bio-Gel P-6 (Fig. 1). Fractions containing the sialylated products were identified by the presence of both 14 C and 3 H radioactivity (indicated in Fig. 1 by a bar). The peaks eluted in the fractions at 300 and 330 ml could be identified as

excess CMP-[14 C]NeuAc (300 ml fraction) and its degradation product [14 C]NeuAc (330 ml fraction) as well as remaining acceptor disaccharide [3 H]GalNAc β (1 $\rightarrow$ 4)GlcNAc (330 ml fraction). Fractions containing the trisaccharide product were pooled and analysed by 400 MHz 1 H n.m.r. spectroscopy to identify its structure.

The 1 H n.m.r. data of both the acceptor disaccharide and the sialylated product are summarized in Table 1, and in Fig. 2 the relevant structural reporter-group regions of the 1 H n.m.r. spectra of GalNAc β (1 $\rightarrow$ 4)GlcNAc and NeuAc α (2 $\rightarrow$ 6)GalNAc β (1 $\rightarrow$ 4)GlcNAc are compared with each other.

The signals in the 1 H n.m.r. spectrum of the disaccharide substrate and the trisaccharide product could be assigned by comparison with the chemical-shift data of Gal β (1 $\rightarrow$ 4)GlcNAc

Fig. 1. Purification of the product from the incubation of $\alpha 6$ -sialyltransferase, with [3 H]GalNAc β (1 $\rightarrow$ 4)GlcNAc and CMP-[14 C]NeuAc as substrates, on a column (1.6 cm $\times$ 200 cm) of Bio-Gel P-6 (200–400 mesh)

The column was run in 50 mM-ammonium acetate, pH 5.2, at a flow rate of 8 ml/h. Fractions of 2 ml were collected and were assayed for 14 C (●) and 3 H (○) radioactivity by liquid-scintillation counting. The bar indicates the fractions that were pooled for analysis by 1 H n.m.r. spectroscopy.

Table 1. 400 MHz 1 H n.m.r. chemical-shift values (p.p.m.) and $J_{1,2}$ constants (Hz) of structural reporter-group protons of the constituent monosaccharides of the disaccharide substrate and the $\alpha 6$ -sialylated trisaccharide product

The enzymic synthesis, the isolation of the product and the analysis by 1 H n.m.r. spectroscopy are described in the Experimental section. Chemical shifts are given at 300 K by reference to internal acetone ($\delta = 2.225$ p.p.m.).

Reporter group	Residue	GalNAc β (1 $\rightarrow$ 4)GlcNAc	NeuAc α (2 $\rightarrow$ 6)GalNAc β (1 $\rightarrow$ 4)GlcNAc
H-1	GlcNAc α	5.194 ($J_{1,2} = 2.8$)	5.181 ($J_{1,2} 3.6$)
	β	4.702 ($J_{1,2} = 8.3$)	4.725 ($J_{1,2} 7.9$)
	GalNAc α	4.532 ($J_{1,2} = 8.4$)	4.508 ($J_{1,2} 8.5$)
	β	4.522 ($J_{1,2} = 8.4$)	4.501 ($J_{1,2} 8.5$)
H-3e	NeuAc	—	2.664
H-3a	NeuAc	—	1.725
NAc	GlcNAc α	2.037	2.063*
	β	2.037	2.067†
	GalNAc α	2.067	2.070*
	β	2.066	2.070†
	NeuAc	—	2.030

* and †, Assignments may have to be interchanged.

Fig. 2. 400 MHz ^1H n.m.r. spectra comparing the structural reporter-group regions of the substrate GalNAc $\beta(1\rightarrow 4)$ GlcNAc (a) with those of the product of the incubation with bovine colostrum $\alpha 6$ -sialyltransferase, NeuAc $\alpha(2\rightarrow 6)$ GalNAc $\beta(1\rightarrow 4)$ GlcNAc (b)

The asterisk denotes an impurity. The numbers at the resonances refer to the following residues: 1, GlcNAc; 2, GalNAc.

[10] and the NeuAc $\alpha(2\rightarrow 6)$ GalNAc $\beta(1\rightarrow 4)$ GlcNAc-R unit [5]. The spectrum of the disaccharide shows signals typical of the protons of the reducing GlcNAc residue: H-1 GlcNAc- α , $\delta = 5.194$ p.p.m.; and H-1 GlcNAc- β , $\delta = 4.702$ p.p.m. Because of the differences in the amounts of the α - and β -anomers (approximately 2:1), the H-1 signals of GalNAc could be assigned: H-1 GalNAc- α , $\delta = 4.532$ p.p.m., H-1 GalNAc- β , $\delta = 4.522$ p.p.m. It should be noted that these signals are at lower field than those of Gal in Gal $\beta(1\rightarrow 4)$ GlcNAc ($\Delta\delta \approx 0.053$ p.p.m.), which is consistent with the difference in chemical-shift values of the anomeric protons of GalNAc and Gal in their $\alpha 6$ -sialylated forms [5]. By similar comparisons, the resonances of the CH_3 - protons of the NAc- groups of GalNAc and GlcNAc could be assigned as follows: NAc GlcNAc- α/β , $\delta = 2.037$, NAc GalNAc α,β , $\delta = 2.067$ and 2.066 p.p.m. respectively.

The spectrum of the trisaccharide product shows signals typical of an $\alpha 6$ -linked NeuAc (NAc NeuAc $\delta = 2.030$ p.p.m., H-3e $\delta = 2.664$ p.p.m. and H-3a $\delta = 1.725$ p.p.m. [11]). The intensity ratio of these signals to those of the other residues in the spectrum of this compound reveals that 1 mol of NeuAc is introduced per mol of GalNAc $\beta(1\rightarrow 4)$ GlcNAc. This was also supported by the molar ratio of [^{14}C]NeuAc and [^3H]GalNAc in the product [^{14}C]NeuAc $\alpha(2\rightarrow 6)$ [^3H]GalNAc $\beta(1\rightarrow 4)$ GlcNAc, which appeared to be 1.2:1.0. Together with the appearance of these signals, a change occurs in the chemical shifts of the H-1 and

NAc protons of the GalNAc and the GlcNAc residue. Both the H-1 α and the H-1 β signals of the GalNAc residue shift upfield ($\Delta\delta = -0.024$ p.p.m. and $\Delta\delta = -0.021$ p.p.m. respectively), quite analogous to the effect of addition of $\alpha 6$ -linked NeuAc to the Gal $\beta(1\rightarrow 4)$ GlcNAc termini in oligosaccharides or glycopeptides [12]. Also the H-1 signals of GlcNAc move to another position (H-1 α $\Delta\delta = -0.013$ p.p.m.; H-1 β $\Delta\delta = 0.023$ p.p.m.). In addition, the NAc signal of GlcNAc shows a significant downfield shift ($\Delta\delta \approx 0.030$ p.p.m.), consistent with the earlier described effect of $\alpha 6$ -sialylation on the corresponding chemical-shift value of GlcNAc in Gal $\beta(1\rightarrow 4)$ GlcNAc-R [11,12]. Because this NAc signal and that of GalNAc are found at almost the same position, it is hard to estimate from our n.m.r. data whether the exact positions of the NAc GlcNAc α,β signals are at $\delta = 2.063$ and 2.067 p.p.m., or at $\delta = 2.070$ p.p.m. Therefore the assignments of the NAc signals in the sialylated product to GalNAc and GlcNAc are tentative and might have to be interchanged.

Comparison of the ^1H n.m.r. data of the *N*-linked diantennary glycan of bovine α -lactalbumin, which carries two terminal GalNAc residues $\beta(1\rightarrow 4)$ -linked to GlcNAc [1], with those of mono- and di-antennary glycans of human lutropin, containing $\alpha(2\rightarrow 6)$ -sialylated GalNAc $\beta(1\rightarrow 4)$ GlcNAc-R structures [5], shows that differences in the chemical-shift values found are very similar to those occurring on $\alpha 6$ -sialylation of the GalNAc $\beta(1\rightarrow 4)$ GlcNAc disaccharide. It is therefore concluded

Table 2. K_m and V_{max} values of $\alpha 6$ -sialyltransferase for the two disaccharide acceptor substrates Gal β (1 $\rightarrow$ 4)GlcNAc and GalNAc β (1 $\rightarrow$ 4)GlcNAc

Incubation conditions and determination of incorporation of [14 C]NeuAc into the disaccharide acceptors are described in the Experimental section. The kinetic parameters K_m and V_{max} were calculated from Lineweaver–Burk plots of $1/v$ against $1/[S]$.

Acceptor substrate	K_m (mM)	V_{max} (nmol/min per mg)	Relative acceptor activity (V_{max}/K_m)
Gal β (1 $\rightarrow$ 4)GlcNAc	7.03	8.14	1.16
GalNAc β (1 $\rightarrow$ 4)GlcNAc	13.05	5.92	0.45

that the product formed in the present incubation has indeed the structure NeuAc α (2 $\rightarrow$ 6)GalNAc β (1 $\rightarrow$ 4)GlcNAc.

By analysing the rate of formation of sialylated products at various concentrations of the disaccharide acceptors Gal β (1 $\rightarrow$ 4)GlcNAc and GalNAc β (1 $\rightarrow$ 4)GlcNAc, the K_m and V_{max} values for the two substrates were determined (Table 2). The affinity of the sialyltransferase for Gal β (1 $\rightarrow$ 4)GlcNAc was about twice as high as that for GalNAc β (1 $\rightarrow$ 4)GlcNAc and the V_{max} with the former substrate was 1.4 times higher than with the latter. This resulted in a 2.6 times higher kinetic efficiency for the transfer of a sialic acid to Gal β (1 $\rightarrow$ 4)GlcNAc than the transfer to GalNAc β (1 $\rightarrow$ 4)GlcNAc.

In order to investigate whether GalNAc β (1 $\rightarrow$ 4)GlcNAc is sialylated by the same sialyltransferase that catalyses the transfer of sialic acid to Gal β (1 $\rightarrow$ 4)GlcNAc, a competition experiment was conducted. In this experiment both substrates, at various relative concentrations, were combined in a single sialyltransferase assay, while the total substrate concentration was kept at 20 mM. For two independent enzymes, each recognizing only one of the substrates A and B, the total velocity v_t can be calculated as the sum of two partial velocities v_A and v_B given by their respective Michaelis–Menten equations [eqn. (1)]:

$$v_t = v_A + v_B = \frac{V_{max,A}}{1 + \frac{K_{m,A}}{[A]}} + \frac{V_{max,B}}{1 + \frac{K_{m,B}}{[B]}} \quad (1)$$

When a single enzyme acts on two different substrates, which both are present in the incubation, each will act as a competitive inhibitor with respect to the other. The total reaction velocity is given by eqn. (2) [13]:

Fig. 3. Competition between the substrates Gal β (1 $\rightarrow$ 4)GlcNAc and GalNAc β (1 $\rightarrow$ 4)GlcNAc in the $\alpha 6$ -sialyltransferase assay

Both acceptors were present in the assay mixtures at various concentrations, the total substrate concentration being kept at 20 mM. Total reaction velocities determined experimentally (●) or calculated from eqn. (1) or (2), using the kinetic parameters shown in Table 2, are plotted against the substrate concentration. The upper curve represents v_t as calculated from eqn. (1) (two different enzymes), and the lower curve shows v_t as calculated from eqn. (2) (one enzyme).

$$v_t = \frac{V_{max,A} \frac{[A]}{K_{m,A}} + V_{max,B} \frac{[B]}{K_{m,B}}}{1 + \frac{[A]}{K_{m,A}} + \frac{[B]}{K_{m,B}}} \quad (2)$$

The results of the competition experiment are shown in Fig. 3. The experimental values are in agreement with those calculated using eqn. (2) but not with those using eqn. (1), indicating that Gal β (1 $\rightarrow$ 4)GlcNAc and GalNAc β (1 $\rightarrow$ 4)GlcNAc are recognized by a single active site on the $\alpha 6$ -sialyltransferase of bovine colostrum.

DISCUSSION

Studies on the *N*-linked glycans of different glycoproteins revealed four major forms of negatively charged, sialylated or

Table 3. Variant forms of sialylated or sulphated terminal disaccharide sequences occurring at the *N*-linked glycans of glycoproteins

Terminal sequence	Occurrence	Reference
NeuAc α (2 $\rightarrow$ 3/6)Gal β (1 $\rightarrow$ 4)GlcNAc-R	Ubiquitous	[27]
NeuAc α (2 $\rightarrow$ 6)GalNAc β (1 $\rightarrow$ 4)GlcNAc-R	Pituitary hormones	[5]
	Bowes melanoma tPA	[6]
	Bovine lactotransferrin	[2]
	Epithelial glycoprotein IV (CD36)	[3]
SO $_4^-$ $\rightarrow$ 3Gal β (1 $\rightarrow$ 4)GlcNAc-R	Pig thyroglobulin	[28]
	Human and calf thyroglobulin	[29]
	Human Tamm–Horsfall glycoprotein	[30]
SO $_4^-$ $\rightarrow$ 4GalNAc β (1 $\rightarrow$ 4)GlcNAc-R	Pituitary hormones	[4]
	Human Tamm–Horsfall glycoprotein	[30]

sulphated disaccharide sequences occurring at the non-reducing ends of their *N*-linked glycans (Table 3). Some of the enzymes responsible for sialylation [8,14] or sulphation [15,16] of these structures have been identified. With respect to the occurrence of one of these sequences, NeuAc α (2 $\rightarrow$ 6)GalNAc β (1 $\rightarrow$ 4)GlcNAc, on the *N*-linked glycans of several glycoproteins [2,3,5,6], the question was raised whether or not a hitherto unknown GalNAc β (1 $\rightarrow$ 4)GlcNAc-R α (2 $\rightarrow$ 6)-sialyltransferase would be involved in the synthesis of these structures [5,6]. In this paper we show that bovine colostrum CMP-NeuAc:Gal β (1 $\rightarrow$ 4)GlcNAc-R α (2 $\rightarrow$ 6)-sialyltransferase catalyses the *in vitro* synthesis of NeuAc α (2 $\rightarrow$ 6)GalNAc β (1 $\rightarrow$ 4)GlcNAc. This provides an enzymic basis for the occurrence of these structures on bovine milk glycoproteins. Although the $\alpha 6$ -sialyltransferase shows both a lower affinity and a lower $V_{\max}$ with GalNAc β (1 $\rightarrow$ 4)GlcNAc in comparison with Gal β (1 $\rightarrow$ 4)GlcNAc as acceptor, it was possible to synthesize the trisaccharide in a high yield. Thus the need to invoke a novel sialyltransferase is abolished.

Competition experiments using Gal β (1 $\rightarrow$ 4)GlcNAc and GalNAc β (1 $\rightarrow$ 4)GlcNAc as substrates strongly suggest that $\alpha 6$ -sialylation of both substrates is catalysed at the same active site of the $\alpha 6$ -sialyltransferase. Although it is likely that a single $\alpha 6$ -sialyltransferase is involved, such experiments, however, never completely rule out the possibility of a second enzyme, even when carried out with highly purified preparations. Similar kinetics could have been obtained with two closely related enzymes, each of which are specific for one substrate and are competitively inhibited by the other [13]. In this context it is interesting to note that the bovine colostrum enzyme has been obtained in two forms differing in molecular mass, but, as far as tested, not in their apparent kinetic properties [8,17]. Such isoforms of glycosyltransferases, which may be the result of alternative splicing [18,19] or alternative promoter usage [20], could, in principle, show subtle differences in their acceptor properties. On the other hand, it was ruled out that another $\alpha 6$ -sialyltransferase [CMP-NeuAc:GalNAc-Ser/Thr α (2 $\rightarrow$ 6)-sialyltransferase] which is involved in the synthesis of *O*-linked NeuAc α (2 $\rightarrow$ 6)GalNAc disaccharide units [21,22], was responsible for the sialylation of GalNAc β (1 $\rightarrow$ 4)GlcNAc. The latter sialyltransferase, isolated in a highly purified and active form from pig submaxillary glands [23], showed no reaction with the disaccharide acceptor (results not shown).

A loose specificity of the Gal β (1 $\rightarrow$ 4)GlcNAc-R $\alpha 6$ -sialyltransferase of rat liver has been suggested before, on the basis of its ability to catalyse the sialylation of the disaccharide Man β (1 $\rightarrow$ 4)GlcNAc [24]. Although the efficiency of this process appears very low, this result, together with our finding that bovine colostrum $\alpha 6$ -sialyltransferase is capable of sialylating GalNAc β (1 $\rightarrow$ 4)GlcNAc units, shows that neither the axial/equatorial position of the hydroxy groups at C-2 and C-4 of the terminal sugar, nor the presence of the NAc group at C-2 of this residue, seems to be of vital importance for substrate recognition by these enzymes. It should be noted, however, that the specificity of the $\alpha 6$ -sialyltransferase is very strict toward the linkage by which the accepting sugar is attached to the penultimate sugar as well as toward the nature of the latter [17,25] (M. Nemansky, W. E. C. M. Schiphorst and D. H. Van den Eijnden, unpublished work). In addition, the enzyme from bovine colostrum seems to specifically recognize an extended portion of branched complex-type oligosaccharide substrates including at least one GlcNAc residue of the core, which leads to the highly ordered addition of NeuAc to the different branches [12,26].

Extrapolating our findings, we speculate that the NeuAc α (2 $\rightarrow$ 6)GalNAc β (1 $\rightarrow$ 4)GlcNAc termini occurring on human lutropin [5] and Bowes melanoma tPA [6] are similarly

synthesized by Gal β (1 $\rightarrow$ 4)GlcNAc-R α (2 $\rightarrow$ 6)-sialyltransferases in human pituitary glands and melanoma cells.

Note added in proof (received 14 July 1992)

Recently a NeuAc α 2 $\rightarrow$ 3GalNAc β 1 $\rightarrow$ 4GlcNAc terminus has been described on the *N*-linked glycans of a pit-viper serine proteinase [31]. It is unknown whether a CMP-NeuAc:Gal β 1 $\rightarrow$ 4GlcNAc-R α 2 $\rightarrow$ 3-sialyltransferase is involved in the synthesis of this structure.

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