The β1,6-GlcNAc transferase activity present in hog gastric mucosal microsomes catalyses site-specific branch formation on a long polylactosamine backbone

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Abstract We find that the β1,6-GlcNAc transferase activity present in hog gastric mucosal microsomes converts the linear pentasaccharide GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc (1) in a site-specific way to the branch-bearing hexasaccharide GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc (2). The product is a positional isomer of GlcNAcβ1-3Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc (3), reportedly formed from 1 by another polylactosamine β1,6-GlcNAc transferase activity present in human serum (Leppänen et al., *Biochemistry*, 30 (1991) 9287). Combined use of the two kinds of activities gave in the present experiments the heptasaccharide GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc (4), in which one of the branches occupies the position of the branch in 2 and the other the position of the branch in 3.

Key words: Polylactosamine; Backbone branching; β1,6-GlcNAc transferase; Site-specificity

1. Introduction

Multiply branched polylactosaminoglycan backbones are expressed on proteins of murine and human embryonal carcinoma cells [1–3], human erythrocytes [4] and the protozoan *Trypanosoma brucei* [5]. They are present also in glycolipids of human and rabbit erythrocytes [6,7], human placenta [8,9] and pancreatic carcinoma cells [10]. Most of them represent long polylactosamine chains, bearing *short* branches.

The backbone branching is developmentally regulated in the course of murine embryonic development [11], in embryonal carcinoma cells [1,11] and in human erythrocytes [4,12]. A polylactosamine, carrying several short branches and decorated by multiple sialyl Lewis x groups, was recently shown to be a *nanomolar* inhibitor of L-selectin-mediated adhesion of lymphocytes to the activated endothelium of blood vessel walls in vitro [13,14]. These observations prompt interest in synthesis of multiply branched polylactosamines.

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Abbreviations: G and Gal, D-galactose; GlcNAc and GN, *N*-acetyl-D-glucosamine; Lac, lactose; LacNAc, Galβ1-4GlcNAc; NMR, nuclear magnetic resonance, MALDI, matrix-assisted laser desorption/ionization; ManNAc, *N*-acetyl-D-mannosamine; MH, maltoheptaose; MP, maltopentaose; MT, maltotriose; MTet, maltotetraose; MS, mass spectrometry; TOF, time-of-flight

Their biosynthesis involves main chain assembly by alternating β1,3-GlcNAc- and β1,4-Gal-transfers, and ensuing branch generation by β1,6-GlcNAc transferases. One type of the branch-forming enzymes transfers characteristically to the inner Gal residue of Galβ1-4GlcNAcβ1-3**Gal**β1-4GlcNAc [15–19], while the other type transfers to the Gal-residue of the trisaccharide GlcNAcβ1-3**Gal**β1-4GlcNAc [20–23]. Here, we show that the latter type of activity, present in hog gastric mucosal microsomes [20], converts the linear pentasaccharide GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc (**1**) in a site-specific way to the hexasaccharide GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc (**2**). Glycan **2** is an isomer of GlcNAcβ1-3Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc (**3**), which is formed from **1** by the mid-chain β1,6-GlcNAc transferase activity of human serum [15]. By combining the use of the two activities, we converted in the present experiments glycan **1** to the doubly branched heptasaccharide GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc (**4**), that bears both branches found in **2** and **3**. A preliminary report of some of the data has appeared [24].

2. Materials and methods

2.1. Oligosaccharides

GlcNAcβ1-3[^{3}H]Galβ1-4GlcNAcβ1-3[^{14}C]Galβ1-4GlcNAc (**1**) was synthesized as in [15] by using UDP-[^{14}C]Gal in the first, and UDP-[^{3}H]Gal in the second galactosylation step; for the synthesis of the isotopomer GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4[^{14}C]GlcNAc, [^{14}C]GlcNAc was used as acceptor in the first galactosylation step. UDP-Gal, UDP-GlcNAc, D-galactose, lactose and malto-oligosaccharides were purchased from Sigma. UDP-[^{14}C]Gal [^{14}C]GlcNAc and UDP-[^{3}H]Gal were from Amersham.

2.2. Enzymic methods

β1,6-GlcNAc transferase (EC 2..4.1.148) reactions were performed by incubating the saccharides with UDP-GlcNAc and hog gastric mucosal microsomes as in [25]. Treatment with endo-β-galactosidase (EC 3.2.1.103) from *Bacteroides fragilis* (Boehringer, Mannheim, Germany) was performed as in [15]. Jack bean β-*N*-acetylhexosaminidase (EC 3.2.1.30) digestions were performed as in [26].

2.3. Partial acid hydrolysis

Partial acid hydrolysis was performed as in [25].

2.4. Chromatographic methods

Paper chromatography was performed as in [15] by using the upper phase of *n*-butanol/acetic acid/water 4:1:5 by vol. (solvent A) or *n*-butanol/ethanol/water 10:1:2 by vol. (solvent E). Biogel P-4 chromatography was as in [26].

2.5. ^{1}H-NMR experiments

The ^{1}H-NMR experiments were carried out as in [17].

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2.6. MALDI-TOF mass spectra

The MALDI-TOF mass spectra were recorded as in [13].

3. Results

3.1. Enzyme-assisted synthesis of hexasaccharide **2**

Incubation of the doubly labeled pentasaccharide GlcNAcβ1-3[^{3}H]Galβ1-4GlcNAcβ1-3[^{14}C]Galβ1-4GlcNAc (**1**) with UDP-GlcNAc and hog gastric mucosal microsomes, which contain β1,6-GlcNAc transferase activity [20], gave a product chromatographing like a hexasaccharide (Fig. 1A). Its partial acid hydrolysis gave ^{3}H-labelled, but not ^{14}C-labelled glycans that contained the newly formed GlcNAcβ1-6Gal bond. The cleavage products included the disaccharide GlcNAcβ1-6[^{3}H]Gal, the trisaccharides GlcNAcβ1-6[^{3}H]Galβ1-4GlcNAc and GlcNAcβ1-3(GlcNAcβ1-6)[^{3}H]Gal as well as the tetrasaccharide GlcNAcβ1-3(GlcNAcβ1-6)[^{3}H]Galβ1-4GlcNAc (Fig. 2A). This established that the new branch had been formed solely at the ^{3}H-labelled galactose-4 of the acceptor (for numbering of the individual monosaccharide residues, see Table 1), generating GlcNAcβ1-3(GlcNAcβ1-6)[^{3}H]Galβ1-4GlcNAcβ1-3[^{14}C]Galβ1-4GlcNAc (**2**).

Preparative scale incubation of pentasaccharide **1** with UDP-GlcNAc and the gastric mucosal microsomes gave 130 nmol of hexasaccharide **2** that was isolated by Bio-Gel P-4 chromatography. The ^{1}H-NMR spectrum of **2** (Fig. 3A and Table 2) confirmed its structure. The spectrum revealed reporter group signals similar to those of glycan **1**, but contained also an additional one-proton doublet at 4.592 ppm, assigned to H-1 of the β1,6-bonded GlcNAc-7. Interestingly, this signal is distinct from the H-1 resonance of the β1,6-linked GlcNAc in the tetrasaccharide GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc (δ = 4.585 ppm, [21]); it differs also from the H-1 signal of the β1,6-bonded GlcNAc-6 in the isomeric hexasaccharide GlcNAcβ1-3Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc (**3**) (δ = 4.584

Table 1
Structures of the key saccharides and denotation of their monosaccharide residues

No. and saccharide	
1	GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc (residues: 5 GlcNAcβ1, 4 Gal, 3 GlcNAc, 2 Gal, 1 GlcNAc)
2	GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc (residues: 7 GlcNAcβ1 (→6), 5 GlcNAcβ1 (→3), 4 Gal, 3 GlcNAc, 2 Gal, 1 GlcNAc)
3	GlcNAcβ1-3Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc (residues: 6 GlcNAcβ1 (→6 of Gal 2), 5 GlcNAcβ1, 4 Gal, 3 GlcNAc, 2 Gal, 1 GlcNAc)
4	GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc (residues: 7 GlcNAcβ1 (→6 of Gal 4), 6 GlcNAcβ1 (→6 of Gal 2), 5 GlcNAcβ1, 4 Gal, 3 GlcNAc, 2 Gal, 1 GlcNAc)

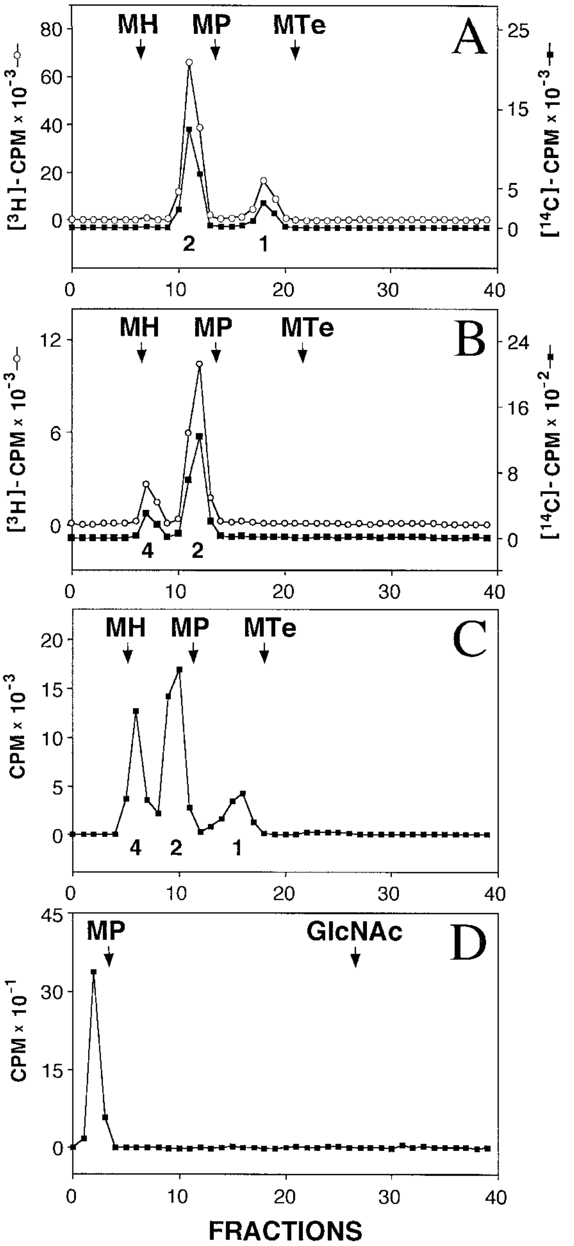

Fig. 1. Paper chromatography of synthetic polylactosamines in solvent A. A: Glycans from incubation of GlcNAcβ1-3[^{3}H]Galβ1-4GlcNAcβ1-3[^{14}C]Galβ1-4GlcNAc (**1**) with UDP-GlcNAc and hog gastric mucosal microsomes. Peak 1 represents glycan **1** and peak 2 is a hexasaccharide-like product. B: Oligosaccharides from incubation of GlcNAcβ1-3(GlcNAcβ1-6)[^{3}H]Galβ1-4GlcNAcβ1-3[^{14}C]Galβ1-4GlcNAc (**2**) with UDP-GlcNAc and human serum. Peak 2 represents glycan **2** and peak 4 is a heptasaccharide-like product. C: Oligosaccharides from a prolonged incubation of GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4[^{14}C]GlcNAc (**1**) with UDP-GlcNAc and hog gastric mucosal microsomes. Peak 4 represents glycan **4**, while peak 2 is believed to contain glycan **2** and related hexasaccharides, and peak 1 probably represents a mixture of glycan **1** and the isomeric GlcNAcβ1-6Galβ1-4GlcNAcβ1-3Galβ1-4[^{14}C]GlcNAc [27]. D: Oligosaccharides from an endo-β-galactosidase digest of the doubly branched glycan **4**, GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4[^{14}C]GlcNAc. The heptasaccharide preserved its integrity during the enzyme treatment, and no [^{14}C]GlcNAc was released.

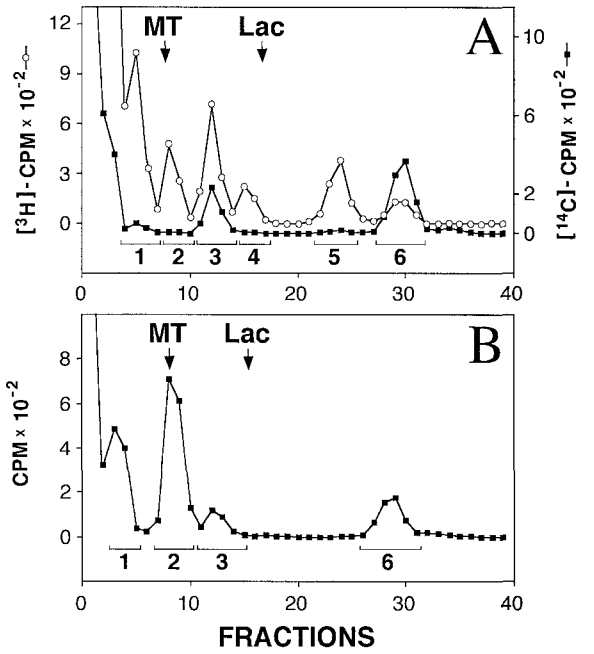

Fig. 2. Paper chromatography (Solvent E) of degradation products from branched polylactosamines. A: Partial acid hydrolysate of the doubly labeled glycan GlcNAcβ1-3(GlcNAcβ1-6)[^{3}H]Galβ1-4GlcNAcβ1-3[^{14}C]Galβ1-4GlcNAc (**2**) of Fig. 1A. Among the hydrolysis products, peak 1 contained GlcNAcβ1-3(GlcNAcβ1-6)[^{3}H]Galβ1-4GlcNAc and GlcNAcβ1-3(GlcNAcβ1-6)[^{3}H]Gal, peak 2 represented GlcNAcβ1-6[^{3}H]Galβ1-4GlcNAc, peak 3 contained GlcNAcβ1-3[^{3}H]Galβ1-4GlcNAc and GlcNAcβ1-3[^{14}C]Galβ1-4GlcNAc, peak 4 was GlcNAcβ1-6[^{3}H]Gal, peak 5 was GlcNAcβ1-3[^{3}H]Gal, while peak 6 represented [^{3}H]Galβ1-4GlcNAc and [^{14}C]Galβ1-4GlcNAc. B: Partial acid hydrolysate of GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4[^{14}C]GlcNAc (**4**) of peak 4 in Fig. 1C. Here, peak 1 represents GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4[^{14}C]GlcNAc, peak 2 GlcNAcβ1-6Galβ1-4[^{14}C]GlcNAc, peak 3 GlcNAcβ1-3Galβ1-4[^{14}C]GlcNAc and peak 6 Galβ1-4[^{14}C]GlcNAc.

ppm, [19]). Even the H-1 resonances, and likewise the H-4-signals, of the two galactoses in glycan **2** were distinct, while in the parent pentasaccharide **1** they were not.

The MALDI-TOF mass spectrum of glycan **2** revealed a major peak of monoisotopic m/z 1177.1 (not shown), that was assigned to $(M+Na)^+$ of $GlcNAc_4Gal_2$ (calculated m/z 1177.4).

3.2. Enzyme-assisted synthesis of hexasaccharide **4**

Hexasaccharide **2** is a positional isomer of hexasaccharide **3**, GlcNAcβ1-3Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc, which is formed from pentasaccharide **1** and UDP-GlcNAc by the activity of a β1,6-GlcNAc transferase present in human serum [15]. We anticipated that the serum enzyme would transfer the branch to galactose-2 even in hexasaccharide **2**. Actual incubation of hexasaccharide **2** with UDP-GlcNAc and human serum gave a product that chromatographed like a heptasaccharide (Fig. 1B). The branching rate of **2** was comparable to that of **1**.

A doubly branched heptasaccharide-like product was generated also in a prolonged incubation of the pentasaccharide GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4[^{14}C]GlcNAc (**1**) with UDP-GlcNAc and the gastric mucosal microsomes (Fig. 1C). Partial acid hydrolysis of the heptasaccharide-like product (peak 4) released GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4[^{14}C]GlcNAc and GlcNAcβ1-6Galβ1-4[^{14}C]GlcNAc (Fig. 2B); both cleavage products were converted quantitatively into Galβ1-4[^{14}C]GlcNAc by β-*N*-acetylhexosaminidase (not shown). These data establish that peak 4 in Fig. 1C contains the reducing end sequence GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4[^{14}C]GlcNAc. Supporting this, peak 4 glycan resisted completely a treatment with endo-β-galactosidase (Fig. 1D), while glycan **2**, GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAcβ1-3Galβ1-4[^{14}C]GlcNAc was almost completely cleaved at the reducing end LacNAc, releasing [^{14}C]GlcNAc in a parallel experiment (not shown). Branching is known to make glycans of this type resistant to endo-β-galactosidase [28]. Hence, peak 4 in Fig. 1C represents the doubly branched saccharide GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4[^{14}C]GlcNAc (**4**), formed primarily along the pathway **1**→**2**→**4**.

Preparative scale incubation of the branched hexasaccharide **2** (320 nmol) with UDP-GlcNAc and gastric mucosal microsomes, followed by gel filtration gave 8 nmol of the heptasaccharide **4**. Its ^{1}H-NMR-spectrum (Fig. 3B and Table 2) was identical to the spectrum of another sample of glycan **4**, synthesized recently in our laboratory along the pathway **1**→**3**→**4** [19]. In particular, two one-proton signals from β1,6-linked GlcNAc-units were observed in the present spectrum of **4**. One of these (δ = 4.592 ppm) is assigned to GlcNAc-7 because it is identical with the corresponding signal of hexasaccharide **2**. The other one (δ = 4.584 ppm) is assigned to H-1 of GlcNAc-6 because of its similarity with the analo-

Table 2
Chemical shifts of reporter groups in glycans **1-4**. For glycan numbers and denotation, see Table 1

Reporter group	Residue	Saccharides			
		1[a]	**2**	**3**[a]	**4**
H-1	*1*	5.204(α)	5.206(α)	5.211(α)	5.211(α)
		4.720(β)	4.721(β)	4.730(β)	4.730(β)
	2	4.467	4.465	4.452	4.455
	3[b]	4.703/4.698	4.708/4.704	4.697/4.692	4.703/4.697
	4	4.467	4.456	4.468	4.455
	5	4.680	4.675	4.681	4.675
	6	-	-	4.584	4.584
	7	-	4.592	-	4.592
H-4	*2*	4.154	4.155	4.146	4.143
	4	4.154	4.145	4.153	4.143

[a]Data from [19].
[b]The two values given correspond to the two anomers of the oligosaccharide.

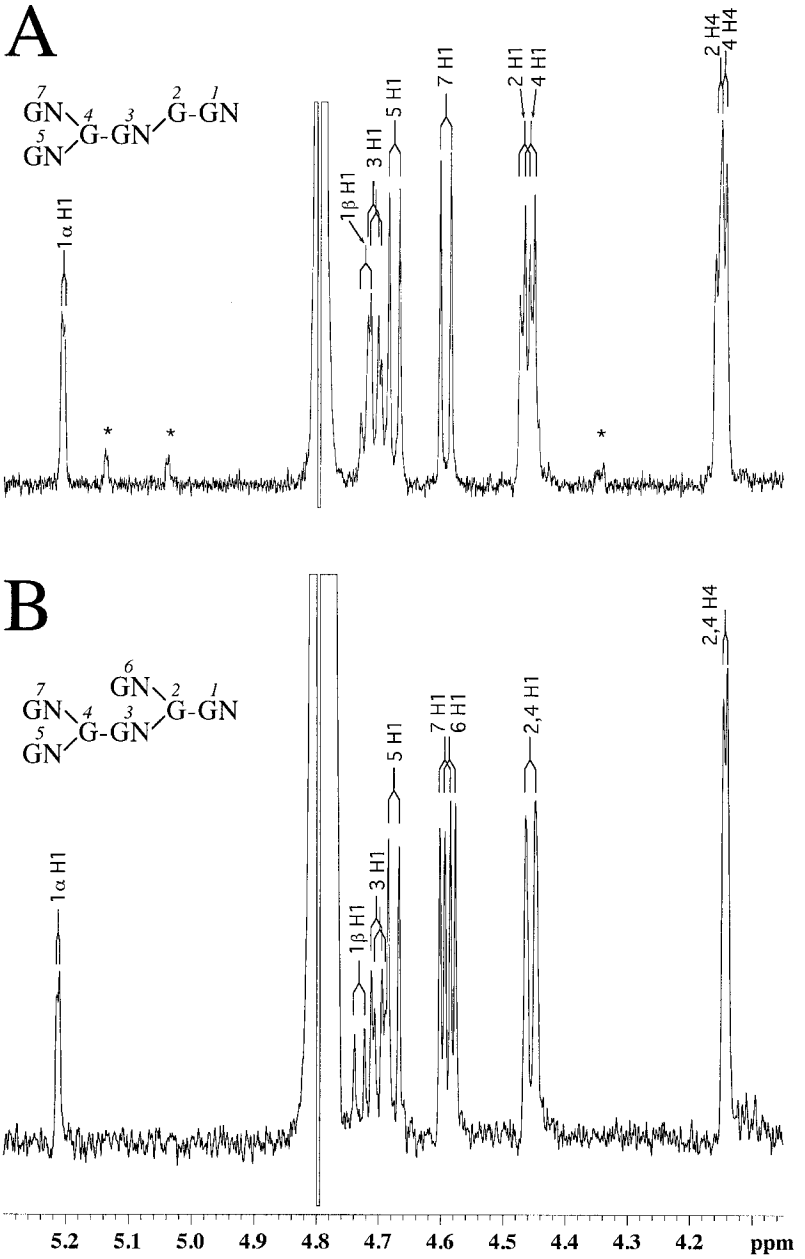

Fig. 3. Anomeric regions of ^{1}H-NMR spectra of oligosaccharides synthesized in the present experiments A: Hexasaccharide **2**. The signals marked by an asterisk (*) probably arise from the reducing-end ManNAc epimer of **2**. (Similar resonances belonging to the reducing end monosaccharide were observed in an isomeric contaminant of a synthetic sample of GlcNAcβ1-3Galβ1-4GlcNAc. The contaminant trisaccharide was isolated chromatographically and degraded by a stepwise treatment with β-*N*-acetylhexosaminidase and β-galactosidase. In Dionex chromatography, the reducing end monosaccharide co-migrated with the ManNAc marker.) B: Heptasaccharide **4**.

gous resonance of glycan **3** [13]. The conversion of **1**→**4** involved equal upfield shifts of 0.012 ppm on the H-1 signals of both galactoses, implying that both of them became branch-bearing in the process. The conversion induced analogous shifts even at H-4 signals of the two galactoses.

4. Discussion

Here, we have found that the β1,6-GlcNAc transferase activity present in hog gastric mucosal microsomes converts the pentasaccharide acceptor GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc (**1**) to the hexasaccharide GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc (**2**), rather than to the isomeric GlcNAcβ1-3Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc (**3**). The present experiments involved (i) the use of synthetic acceptor molecules that were radiolabeled in appropriate monosaccharide residues and (ii) degradation as well as ^{1}H-NMR spectroscopy of the branch-bearing products. The data extend the observations of Piller et al. [20], who had shown that the hog gastric mucosal microsomes transfer from UDP-[^{14}C]GlcNAc to β-methylglycoside of glycan **1**, but had not established the structure of the product.

It is now clear that the gastric mucosal activity transfers β1,6-bonded GlcNAc units to the trisaccharide GlcNAcβ1-

3**Gal**β1-4GlcNAc [20,21] as well as to the pentasaccharide GlcNAcβ1-3**Gal**β1-4GlcNAcβ1-3Galβ1-4GlcNAc (**1**) at the galactose units in bold print. The present data, showing that the primary reaction with glycan **1** *site-specifically* does not involve galactose-2, suggest that the primary reaction is overwhelmingly restricted to distal GlcNAcβ1-3**Gal**β1-4GlcNAc determinants in polylactosamines. This conclusion is supported by the data of Piller et al. [20] who did not find any transfer to the methyl glycoside of Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc. Accordingly, we call the gastric mucosal activity as a *distal* β1,6-GlcNAc transferase, in short dIGnT6, to emphasize its site-specific action and the fact that it generates precursors of blood group I type polylactosamines.

The hexasaccharide **2** represents a positional isomer of hexasaccharide **3**. The latter one has been synthesized previously from pentasaccharide **1** by using the activity of another type of β1,6-GlcNAc transferase, which is present in blood serum of mammals [15,19]. The serum activity transfers in the first place only to galactose residues that belong to other than the most distal LacNAc unit of the acceptor. Examples of good acceptors are glycan **1** and the tetrasaccharide Galβ1-4GlcNAcβ1-3**Gal**β1-4GlcNAc at galactose-2 [17,18] as well as the hexasaccharide Galβ1-4GlcNAcβ1-3**Gal**β1-4GlcNAc β1-3**Gal**β1-4GlcNAc at galactose-2 and galactose-4 [19]. We call this activity as *central* β1,6-GlcNAc transferase (cIGnT6) to emphasize its *area*-specific action on polylactosamine chains.

In a complementary experiment, advantage was taken of the alternative site-specificities of dIGnT6 and cIGnT6. The dIGnT6 activity of hog gastric mucosal microsomes was first used to convert the pentasaccharide **1** into the branch-bearing hexasaccharide **2**, and the latter was then converted into the doubly branched heptasaccharide **4** by the cIGnT6 activity of serum. In analogous complementary experiments described elsewhere [13], the nonasaccharide GlcNAcβ1-3′LacNAcβ1-3′(GlcNAcβ1-6′)LacNAcβ1-3′(GlcNAcβ1-6′)LacNAc with two 'central' branches was generated with the aid of serum cIGnT6 activity; subsequently the dIGnT6 activity of hog gastric mucosal microsomes was used to transfer the distal branch, generating the triply branched decasaccharide GlcNAcβ1-3′(GlcNAcβ1-6′)LacNAcβ1-3′(GlcNAcβ1-6′)LacNAcβ1-3′(GlcNAcβ1-6′)LacNAc. In the future, the use of the two IGnT6 activities of complementary site-specificities, together with improving availability of chemically synthesized long-chain polylactosamines [29], will probably pave synthetic routes to multiply branched backbones similar to those believed to be expressed in glycoproteins [3,4].

Besides the strong dIGnT6 activity, we find also the presence of a weak cIGnT6 activity in hog gastric mucosal microsomes, catalyzing the conversion **2**→**4**. A little surprisingly, this activity did not catalyze detectable **1**→**3** conversion. Perhaps, the formation of glycan **4** was more easily detected in our experiments than the generation of glycan **3**; alternatively, the conversion **2**→**4** was faster than the conversion of **1**→**3**. Presently, it is not clear whether the activities of dIGnT6 and cIGnT6 in the gastric mucosal microsomes are due to one or several enzymes.

Taken together, the present data represent an accurate description of in vitro biosynthesis of a long polylactosamine chain carrying two short branches that are transferred to the primary chain sequentially and site-specifically by using two distinct types of polylactosamine β1,6-GlcNAc transferase activities. The dIGnT6 activity present in hog gastric mucosal microsomes transfers preferentially to one single site close to the distal area of the acceptor, requiring the non-reducing-end sequence GlcNAcβ1-3Gal-R. By contrast, the cIGnT6 activity present in mammalian serum and in several rat tissues [15,16,19] transfers preferentially to multiple central sites of the backbone acceptor, tolerating GlcNAcβ1-3Gal-R as well as Galβ1-4GlcNAc-R sequences at the non-reducing end of the acceptor.

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