Human Serum Contains a Novel β1,6-*N*-Acetylglucosaminyltransferase Activity That Is Involved in Midchain Branching of Oligo(*N*-acetyllactosaminoglycans)†

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ABSTRACT: Incubation of UDP-GlcNAc and radiolabeled GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc (**1**) with human serum resulted in the formation of the branched hexasaccharide GlcNAcβ1–3Galβ1–4GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAc (**2**) in yields of up to 22.2%. The novel reaction represents midchain branching of the linear acceptor; the previously known branching reactions of oligo(*N*-acetyllactosaminoglycans) involve the nonreducing end of the growing saccharide chains. The structure of **2** was established by use of appropriate isotopic isomers of it for degradative experiments. The hexasaccharide **2** was cleaved by an exhaustive treatment with jack bean β-*N*-acetylhexosaminidase, liberating two GlcNAc units and the tetrasaccharide Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc (**3**). Endo-β-galactosidase from *Bacteroides fragilis* cleaved **2** at one site only, yielding the disaccharide GlcNAcβ1–3Gal (**4**) and the branched tetrasaccharide GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAc (**5**). The structure of **5** was established by partial acid hydrolysis and subsequent identification of the disaccharide GlcNAcβ1–6Gal (**6**), together with the trisaccharides GlcNAcβ1–6Galβ1–4GlcNAc (**7**) and GlcNAcβ1–3(GlcNAcβ1–6)Gal (**8**) among the cleavage products. Galactosylation of **2** with bovine milk β1,4-galactosyltransferase and UDP-[6-^{3}H]Gal gave the octasaccharide [6-^{3}H]Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAcβ1–3([6-^{3}H]-Galβ1–4GlcNAcβ1–6)[U-^{14}C]Galβ1–4GlcNAc (**17**), which could be cleaved with endo-β-galactosidase into the trisaccharide [6-^{3}H]Galβ1–4GlcNAcβ1–3Gal (**18**) and the branched pentasaccharide GlcNAcβ1–3-([6-^{3}H]Galβ1–4GlcNAcβ1–6)[U-^{14}C]Galβ1–4GlcNAc (**19**). Partial hydrolysis of **2** with jack-bean β-*N*-acetylhexosaminidase gave the linear pentasaccharide **1** and the branched pentasaccharide Galβ1–4GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAc (**20**). The serum β1,6-GlcNAc transferase catalyzed also the formation of GlcNAcβ1–3Galβ1–4GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4Glc (**11**) from UDP-GlcNAc and GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4Glc (**10**). The pentasaccharide Galα1–3Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc (**16**), too, served as an acceptor for the enzyme. The trisaccharide GlcNAcβ1–3Galβ1–4GlcNAc (**9**), and the tetrasaccharide GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Gal (**15**) were poor acceptors, confirming that the enzyme does not cause branching at the nonreducing area of the acceptors. The poor acceptor properties of the tetrasaccharide **15** imply that the GlcNAc residue at the reducing end side of the branching galactose in the pentasaccharide **1** plays a role in the reaction.

Poly- and oligo(*N*-acetyllactosaminoglycans) (Koenderman, 1987), which contain repeating [Galβ1–4GlcNAcβ1–3][1] units, occur in keratan sulfates (Hirano et al., 1961; Bray et al., 1967; Greiling & Scott, 1989) and in O-linked (Lloyd & Kabat, 1968) and in N-linked (Finne et al., 1978; Järnefelt et al., 1978; Irimura et al., 1981; Fukuda, 1985) saccharides of glycoproteins, as well as in glycolipids (Gardas, 1976; Koscielak et al., 1976). Branches are often present in the linear *N*-acetyllactosamine chains, at position 6 of some of the galactose residues. In many poly(*N*-acetyllactosaminoglycans) the 1→6-linked branches are believed to consist of only one β-linked *N*-acetyllactosamine unit, often with a branch terminating "cap" consisting of sialic acid, fucose, α-linked galactose, or α-linked *N*-acetylgalactosamine (Fukuda, 1985; Dabrowski et al., 1988). However, larger and more complex branches are also known (Bruntz et al., 1988).

The linear poly(*N*-acetyllactosaminoglycans) carry i determinants, and the branched ones carry I determinants (Feizi et al., 1978; Watanabe et al., 1979) of the human blood group Ii system (Wiener et al., 1956). The i antigens can be converted into I structures when β1,6-GlcNAc transferases are expressed. These enzymes have been described in human serum (Zielenski & Koscielak, 1983a,b), Novikoff tumor cells ascites fluid (Van den Eijnden et al., 1983; Koenderman et al., 1987), hog gastric mucosa (Piller et al., 1984; Brockhausen et al., 1986; Seppo et al., 1990), and mouse lymphoma cells (Basu & Basu, 1984)). The known β1,6-GlcNAc transferases can bring a GlcNAc to the backbone of poly- and oligo(*N*-acetyllactosaminoglycans), at the galactose of the nonreducing terminus (Van den Eijnden et al., 1983; Zielenski & Koscielak, 1983a,b; Basu & Basu, 1984) or at the penultimate galactose residues that are substituted with a β1–3-linked *N*-acetylglucosamine (Piller et al., 1984; Brockhausen et al., 1986; Koenderman et al., 1987; Seppo et al., 1990). No β1,6-GlcNAc transferases have been reported so far, adding a GlcNAc branch to the more internally located galactose

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[1] Abbreviations: Gal, D-galactose; GalNAc, 2-acetamido-2-deoxy-D-galactose; Glc, D-glucose; GlcNAc, 2-acetamido-2-deoxy-D-glucose; β1,3-GlcNAc transferase, *N*-acetyllactosaminide β1,3-*N*-acetylglucosaminyltransferase; β1,6-GlcNAc transferase, *N*-acetyllactosaminide β1,6-*N*-acetylglucosaminyltransferase; HPLC, high-performance liquid chromatography; Lac, lactose; MH, maltoheptaose; MP, maltopentaose; MT, maltotriose; MTet, maltotetraose; WGA, wheat germ agglutinin.

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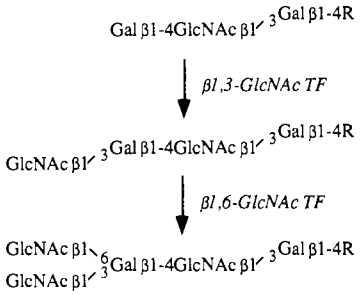
residues of poly(*N*-acetyllactosaminoglycan) backbones. Furthermore, no branching reactions appear to be known where a GlcNAcβ1–6Galβ1–4R backbone sequence would react with β1,3-GlcNAc transferase forming a branch. In conclusion, only one pathway appears to be known for the formation of branches to the backbones of poly(*N*-acetyllactosaminoglycans), and it is intimately connected to the elongation process of the main chain as shown below:



In the present report we describe the synthesis of suitable linear acceptors, and show that human serum contains a β1,6-GlcNAc transferase that forms middle-chain branches by transferring GlcNAc units from UDP–GlcNAc in β1,6 linkage to internal galactose residues in certain oligo(*N*-acetyllactosaminoglycans). A preliminary report of these findings has been presented (Leppänen et al., 1989).

Materials and Methods

Oligosaccharides. Radiolabeled GlcNAcβ1–6Gal (**6**), GlcNAcβ1–6Galβ1–4GlcNAc (**7**), GlcNAcβ1–3Galβ1–4GlcNAc (**9**), GlcNAcβ1–3Gal (**4**), and Galβ1–4GlcNAc were obtained from metabolically labeled embryonal carcinoma cells by partial acid hydrolysis of the isolated poly(*N*-acetyllactosaminoglycans) as described (Renkonen et al., 1988). The trisaccharide [U-^{14}C]Galβ1–4GlcNAcβ1–3[U-^{14}C]Gal (**18**) was isolated from endo-β-galactosidase digests of radiolabeled poly(*N*-acetyllactosaminoglycans) from embryonal carcinoma cells (Renkonen, 1983); the trisaccharide probably originated from nonreducing termini of the intact glycan. Radiolabeled GlcNAcβ1–3(GlcNAcβ1–6)Gal (**8**) and GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAc (**5**) were also isolated from embryonal carcinoma cells (Seppo et al., 1990).

GlcNAcβ1–3[6-^{3}H]Galβ1–4Glc (**14**) was prepared by incubating [6-^{3}H]Galβ1–4Glc (Amersham, UK) with UDP–GlcNAc (Sigma, St. Louis, MO) and human serum essentially as described by Yates and Watkins (1983). GlcNAcβ1–6Galβ1–4Glc (**13**) was purchased from Sigma. The branched tetrasaccharide GlcNAcβ1–3(GlcNAcβ1–6)[6-^{3}H]Galβ1–4Glc (**12**) was prepared by incubating the trisaccharide **14** and UDP–GlcNAc with the β1,6-GlcNAc transferase of pig stomach microsomes (Brockhausen et al., 1986). The product identification involved partial digestions with jack bean β-*N*-acetylhexosaminidase, which gave GlcNAcβ1–6[6-^{3}H]-Galβ1–4Glc (**13**), GlcNAcβ1–3[6-^{3}H]Galβ1–4Glc (**14**), and [6-^{3}H]Galβ1–4Glc. The cleavage products were separated and identified by use of prolonged paper chromatography with solvent B (O. Renkonen et al., unpublished data). Galβ1–4GlcNAcβ1–3Galβ1–4Glc was obtained by galactosylating the trisaccharide **14** with UDP–Gal and β1,4-galactosyltransferase. The product was isolated by use of paper chromatography with solvent A (R_{MTet} = 0.89, R_{MP} = 1.37).

The tetrasaccharide [U-^{14}C]Galβ1–4GlcNAcβ1–3[U-^{14}C]Galβ1–4GlcNAc (**3**) was obtained from embryonal carcinoma cells as described (Renkonen et al., 1989). Galβ1–4GlcNAcβ1–3[U-^{14}C]Galβ1–4GlcNAc was obtained by galactosylating GlcNAcβ1–3[U-^{14}C]Galβ1–4GlcNAc of teratocarcinoma cells with UDP–galactose and bovine milk β1,4-galactosyltransferase. [6-^{3}H]Galβ1–4GlcNAcβ1–3[U-^{14}C]Galβ1–4GlcNAc was prepared from GlcNAcβ1–3[U-^{14}C]Galβ1–4GlcNAc and UDP–[6-^{3}H]Gal with bovine milk galactosyltransferase.

The pure biantennary pentasaccharide marker Galβ1–4GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAc (**20**) was constructed by partial β1,4-galactosylation of the tetrasaccharide **5** as described by Renkonen et al. (submitted to *Biochem. Cell Biol.* for publication).

A 1:1 mixture of the isomeric pentasaccharides Galβ1–4GlcNAcβ1–3(GlcNAcβ1–6)[U-^{14}C]Galβ1–4GlcNAc and GlcNAcβ1–3(Galβ1–4GlcNAcβ1–6)[U-^{14}C]Galβ1–4GlcNAc was prepared by partial treatment of the hexasaccharide Galβ1–4GlcNAcβ1–3(Galβ1–4GlcNAcβ1–6)[U-^{14}C]Galβ1–4GlcNAc with *Escherichia coli* β-galactosidase (Renkonen et al., 1990). The mixture was α1→3-galactosylated by incubating it with UDP–Gal:*N*-acetyllactosaminide α1,3-galactosyltransferase from calf thymus essentially as described by Blanken and Van den Eijnden (1985). Paper chromatography (solvent A) of the product revealed only one major peak; the acceptor pentasaccharides were not present, implying that both isomers had reacted completely.

Enzymatic in Vitro Synthesis of Key Acceptors. The radiolabeled tetrasaccharide GlcNAcβ1–3[U-^{14}C]Galβ1–4GlcNAcβ1–3[U-^{14}C]Gal (**15**) was obtained by *N*-acetylglucosaminylating [U-^{14}C]Galβ1–4GlcNAcβ1–3[U-^{14}C]Gal with UDP–GlcNAc and β1,3-GlcNAc transferase from human serum (Seppo et al., 1990; Yates & Watkins, 1983; Piller & Cartron, 1983; Hosomi et al., 1984); the tetrasaccharide **15** was purified by paper chromatography. Characterization of the tetrasaccharide included cleavage with jack bean β-*N*-acetylhexosaminidase into [U-^{14}C]Galβ1–4GlcNAcβ1–3[U-^{14}C]Gal (**18**). The linkage position of the newly inserted GlcNAc residue was identified as follows. The tetrasaccharide **15** was galactosylated with bovine milk β1,4-galactosyltransferase and UDP–Gal into a pentasaccharide that proved to be Galβ1–4GlcNAcβ1–3[U-^{14}C]Galβ1–4GlcNAcβ1–3[U-^{14}C]Gal. This was established when the pentasaccharide was cleaved with endo-β-galactosidase into the trisaccharide Galβ1–4GlcNAcβ1–3[U-^{14}C]Gal (**18**) and the disaccharide GlcNAcβ1–3[U-^{14}C]Gal (**4**); the products were separated by paper chromatography (not shown). The trisaccharide represented the nonreducing tail of the substrate pentasaccharide; its cleavage with jack bean β-galactosidase released the GlcNAcβ[U-^{14}C]Gal species that occupied the nonreducing end of the tetrasaccharide **15**. This disaccharide migrated in paper chromatography like the authentic GlcNAcβ1–3Gal marker but unlike the GlcNAcβ1–6Gal marker. Moreover, periodate oxidation of the disaccharide, followed by acid hydrolysis (Hough, 1965), gave [U-^{14}C]lyxose (37% yield) but not [^{14}C]threose, as shown by paper chromatography (data not shown). This indicated the presence of a 3-substituted galactose and the absence of a 4-substituted galactose. Moreover, the loss of C-1 from the [U-^{14}C]galactose residue of the disaccharide during the oxidation implies that the Gal residue was unsubstituted at position 2. Hence, our data establish that the newly added GlcNAc unit of the tetrasaccharide **15** was β1,3-linked.

The different versions of the pentasaccharide **1**, GlcNAcβ1–3[6-^{3}H]Galβ1–4GlcNAcβ1–3[U-^{14}C]Galβ1–4GlcNAc, GlcNAcβ1–3[U-^{14}C]Galβ1–4GlcNAcβ1–3[U-^{14}C]Galβ1–4GlcNAc and GlcNAcβ1–3Galβ1–4GlcNAcβ1–3[U-^{14}C]Galβ1–4GlcNAc, were obtained by incubating UDP–GlcNAc (Sigma) and the appropriately labeled versions

of the tetrasaccharide Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc (**3**) with the β1,3-GlcNAc transferase of human serum. The pentasaccharide product was separated from the tetrasaccharide acceptor by paper chromatography (see Table I); a minor impurity was finally removed from **1** by chromatography on agarose-linked wheat germ agglutinin (WGA) (Renkonen et al., 1988). Structural analysis of the pentasaccharide **1** was carried out by three experiments. (1) Enzymic galactosylation of one version of the pentasaccharide **1** gave the hexasaccharide Galβ1–4GlcNAcβ1–3[U-^{14}C]-Galβ1–4GlcNAβ1–3[U-^{14}C]Galβ1–4GlcNAc (R_{MP} = 0.71, R_{MH} = 1.49). The latter was cleaved with endo-β-galactosidase (*Escherichia freundii*) into a 1:1 mixture of Galβ1–4GlcNAcβ1–3[U-^{14}C]Gal (**18**) and GlcNAcβ1–3[U-^{14}C]Gal (**4**). The trisaccharide product was cleaved with jack bean β-galactosidase into GlcNAcβ1–3[U-^{14}C]Gal, and the latter was oxidized with periodate into a product that was cleaved with acid into radiolabeled lyxose (32% yield based on the amount of the disaccharide). This experiment established that the GlcNAc unit at the nonreducing end of **1** was linked at position 3 of the penultimate galactose. (2) The tritium-containing version of pentasaccharide **1**, GlcNAcβ1–3[6-^{3}H]Galβ1–4GlcNAcβ1–3[U-^{14}C]Galβ1–4GlcNAc, was treated with jack bean β-galactosidase: Only 0.7% of the [6-^{3}H]Gal was liberated as the free monosaccharide, suggesting that the pentasaccharide **1** was rather pure and that it contained only 0.7% of the branched pentasaccharide [6-^{3}H]Galβ1–4GlcNAcβ1–3(GlcNAcβ1–6)[U-^{14}C]Galβ1–4GlcNAc (**20**). (3) A treatment with endo-β-galactosidase (*Bacteroides fragilis*) cleaved GlcNAcβ1–3[6-^{3}H]Galβ1–4GlcNAcβ1–3[U-^{14}C]Galβ1–4GlcNAc (**1**) almost totally, yielding GlcNAcβ1–3[6-^{3}H]Gal, GlcNAcβ1–3[U-^{14}C]Gal, and a small amount (8% of total [U-^{14}C]) of GlcNAcβ1–3-[U-^{14}C]Galβ1–4GlcNAc.

GlcNAcβ1–3[6-^{3}H]Galβ1–4GlcNAcβ1–3Galβ1–4[1-^{14}C]-Glc (**10**) was obtained by *N*-acetylglucosaminylating [6-^{3}H]Galβ1–4GlcNAcβ1–3Galβ1–4[1-^{14}C]Glc with UDP–GlcNAc and β1,3-GlcNAc transferase of human serum as described above.

Galα1–3[6-^{3}H]Galβ1–4GlcNAcβ1–3[U-^{14}C]Galβ1–4GlcNAc (R_{MTet} = 0.81, R_{MP} = 1.26) was obtained from UDP–Gal and [6-^{3}H]Galβ1–4GlcNAcβ1–3[U-^{14}C]Galβ1–4GlcNAc, by incubating them with UDP–Gal:*N*-acetyllactosaminide α1,3-galactosyltransferase from calf thymus essentially as described by Blanken and Van den Eijnden (1985).

β1,6-GlcNAc Transferase Reaction with Serum. The β1,6-GlcNAc transferase reaction was carried out by incubating radioactive acceptor saccharides (1–20 000 pmol) with 4.5–14.7 μmol of UDP–GlcNAc, 5 μmol of Tris-HCl, pH 7.5, 0.8 μmol of NaN_3, 1 μmol of $MnCl_2$, 0.05 μmol of ATP, and 100 μL of serum, or centrifugally ultrafiltrated serum (the macromolecules being concentrated about 2-fold), from freshly drawn human blood of healthy donors. The incubations were carried out in tightly capped tubes at 37 °C for specified periods.

Some tests were performed to optimize the reaction conditions: increasing yields of the hexasaccharide **2** were consistently obtained from the pentasaccharide **1** with increasing incubation times with serum; the maximal yields were obtained in 12–18 days. No significant change in the yield of the hexasaccharide **2** was observed in the presence of 3 μmol of GlcNAc in the reaction mixture. The yield of the hexasaccharide **2** increased 40% when the concentration of not complexed Mn^{2+} increased from 0 to 15 mM.

β1,4-Galactosyltransferase Reaction. β1,4-Galactosyltransferase reactions were performed by incubating the labeled acceptor saccharides with β1,4-galactosyltransferase (EC 2.4.1.22) from bovine milk (Sigma) and with UDP–Gal (Sigma) or with UDP–[6-^{3}H]Gal (Amersham, U.K.) or with UDP–[U-^{14}C]Gal (Amersham), essentially as described by Brew et al. (1968).

Chromatographic Methods. Paper chromatographic runs of desalted saccharides were performed on Whatman III Chr paper with the upper phase of 1-butanol/acetic acid/water (4:1:5 v/v; solvent A) or with 1-butanol/ethanol/water (10:1:2 v/v; solvent B). Appropriate marker mixtures of unlabeled saccharides, generally of GlcNAc, galactose, lactose, and malto-oligosaccharides, were run on both sides of each sample lane. After completed chromatographic runs the marker lanes were cut out from the paper sheet and stained with aniline–phthalate or with silver nitrate. Finally, the distribution of radioactivity in the sample lanes was counted as is described (Renkonen et al., 1989).

HPLC runs were carried out with use of system I of Blanken et al. (1985).

Affinity chromatography on agarose-linked wheat germ agglutinin (WGA) was carried out as described by Renkonen et al. (1988). In some experiments columns of the same size, but of a higher WGA content (9.6 mg of WGA/mL of gel), were used, and the elution was carried out with use of only the sugar-free buffer. The WGA columns were calibrated monthly with radiolabeled galactose, which is eluted at void volume. The results are presented as a difference $\Delta = V_e - V_0$, where V_e is the peak position of the saccharide and V_0 is the void volume.

Cleavage Reactions. Digestions with jack bean β-galactosidase (EC 3.2.1.23) (Sigma, St. Louis, MO) were carried out as in Renkonen et al. (1988). Incubations with jack bean β-*N*-acetylhexosaminidase (EC 3.2.1.52) (Sigma) were carried out for 16 h (unless otherwise indicated) at 37 °C in 0.05 M sodium citrate (pH 4.0), containing 55 mM γ-galactonolactone and 150 milliunits of the enzyme. α-Galactosidase (EC 3.2.1.22) from green coffee beans (Sigma) was used essentially as described by Leppänen et al. (1986).

Cleavage with endo-β-galactosidase from *B. fragilis* (Boehringer) was carried out in 40-μL reaction mixtures containing 0.25 unit/mL of the enzyme, 50 mM sodium acetate buffer, pH 5.8, containing 0.2 mg/mL bovine serum albumin, and 0.5 mg/mL sodium azide. Incubations were performed at 37 °C for 24 h. Under these conditions radiolabeled trisaccharide GlcNAcβ1–3Galβ1–4GlcNAc was completely cleaved. Treatment with endo-β-galactosidase from *E. freundii* (Seikagaku, Tokyo, Japan) was carried out as described in Renkonen et al. (1989).

Partial acid hydrolysis was performed according to Renkonen et al. (1989), with use of aqueous 0.1 M trifluoroacetic acid at 100 °C for 40 min. Periodate oxidation of disaccharides and subsequent acid hydrolysis were carried out as described in Renkonen et al. (1989).

All enzyme reactions were terminated in a boiling water bath; the digests were desalted by ion exchange (Renkonen et al., 1989) and analyzed by paper chromatography.

Results

The structures of the key saccharides are collected in Table I. In most of the experiments to be described the analysis was carried out by paper chromatography; therefore, the chromatographic mobilities are also shown in Table I.

Incubation of UDP–GlcNAc and Pentasaccharide GlcNacβ1–3Galβ1–4GlcNAcβ1–3[U-^{14}C]Galβ1–4GlcNAc (**1**)

Table I: Structural Formulas of the Key Saccharides of the Present Experiments[a]

No	Saccharide	R_{Gal}	R_{Lac}	R_{MT}	R_{MTet}	R_{MP}	R_{MH}
1	GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc				0.89	1.41	
2	GlcNAcβ1-6(GlcNAcβ1-3)Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc				0.56	0.90	1.80
3	Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc			0.79	1.27		
4	GlcNAcβ1-3Gal	0.73	1.23				
5	GlcNAcβ1-6(GlcNAcβ1-3)Galβ1-4GlcNAc			0.88	1.41		
6	GlcNAcβ1-6Gal	0.61	1.04	1.40			
7	GlcNAcβ1-6Galβ1-4GlcNAc		0.87	1.19			
8	GlcNAcβ1-6(GlcNAcβ1-3)Gal			0.97	1.56		
9	GlcNAcβ1-3Galβ1-4GlcNAc	0.63	1.06	1.43			
10	GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4Glc				0.62	0.98	2.05
11	GlcNAcβ1-6(GlcNAcβ1-3)Galβ1-4GlcNAcβ1-3Galβ1-4Glc					0.67	1.42
12	GlcNAcβ1-6(GlcNAcβ1-3)Galβ1-4Glc			0.66	1.06	1.66	
13	GlcNAcβ1-6Galβ1-4Glc			0.85	1.36		
14	GlcNAcβ1-3Galβ1-4Glc		0.74	1.01	1.64		
15	GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Gal			0.68	1.09	1.75	
16	Galα1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc				0.81	1.26	
17	Galβ1-4GlcNAcβ1-6(Galβ1-4GlcNAcβ1-3)Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc					0.28	0.56
18	Galβ1-4GlcNAcβ1-3Gal		0.74	0.95	1.52		
19	Galβ1-4GlcNAcβ1-6(GlcNAcβ1-3)Galβ1-4GlcNAc				0.80	1.21	
20	GlcNAcβ1-6(Galβ1-4GlcNAcβ1-3)Galβ1-4GlcNAc				0.79	1.21	

[a] Note: R_{Gal}, R_{Lac}, R_{MT}, R_{MTet}, R_{MP}, and R_{MH} give our best values for the mobilities of the marker saccharides in relation to galactose, lactose, maltotriose, -tetraose, -pentaose, and -heptaose, respectively, in solvent A. Also shown are the values for the saccharides synthesized for the first time during this study. The individual data obtained in the present experiments are given in the text.

with Human Serum. UDP-GlcNAc (14.7 μmol) and GlcNAcβ1-3Galβ1-4[U-^{14}C]GlcNAcβ1-3Galβ1-4GlcNAc (3702 cpm, 84.2 nmol) (**1**) were incubated with human serum that had been concentrated 2-fold by ultrafiltration. Paper chromatography (Figure 1A) revealed that most of the original pentasaccharide (peak 2; R_{MTet} = 0.93, R_{MP} = 1.48; 2777 cpm; 63.1 nmol) had remained intact. However, 16.1% of the pentasaccharide had been converted into a slower moving compound (peak 1; R_{MTet} = 0.62, R_{MP} = 0.97; 549 cpm; 12.5 nmol), representing obviously a hexasaccharide. A small amount of label migrating like Galβ1-4GlcNAcβ1-3[U-^{14}C]Galβ1-4GlcNAc (peak 3; R_{MT} = 0.83, R_{MTet} = 1.35; 96 cpm; 2.2 nmol) was also present in the reaction mixture; it was probably formed by the action of endogenous β-*N*-acetylhexosaminidase. The identity of the slow component was established as the hexasaccharide GlcNAcβ1-3Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)[U-^{14}C]Galβ1-4GlcNAc (**2**) in the experiments described below. The hexasaccharide **2** was formed also when UDP-GlcNAc (4.5 μmol) and picomolar amounts of other labeled versions of the pentasaccharide **1**, GlcNAcβ1-3[U-^{14}C]Galβ1-4GlcNAcβ1-3[U-^{14}C]Galβ1-4GlcNAc or GlcNAcβ1-3[6-^{3}H]Galβ1-4GlcNAcβ1-3[U-^{14}C]Galβ1-4GlcNAc were incubated with human serum (data not shown).

Exhaustive β-N-Acetylhexosaminidase Digestion of the Hexasaccharide **2**. The hexasaccharide **2**, formed in a reaction similar to that in Figure 1A, was cleaved by β-acetylhexosaminidase into a product (R_{MT} = 0.79, R_{MTet} = 1.32) that chromatographed on paper like the tetrasaccharide Galβ1-4GlcNAβ1-3[U-^{14}C]Galβ1-4GlcNAc (**3**) (Figure 1B). This suggested that two *N*-acetylhexosamine residues were released; one of them represented the β-GlcNAc unit at the nonreducing end of the original pentasaccharide acceptor, while the other was the newly inserted β-*N*-acetylhexosamine residue.

The hexasaccharide **2** was constructed in pure form also from UDP-[U-^{14}C]GlcNAc and GlcNAcβ1-3Galβ1-4[U-^{14}C]GlcNAcβ1-3Galβ1-4GlcNAc. The cleavage of the resulting hexasaccharide **2** "version" with β-*N*-acetylhexosaminidase released [^{14}C]GlcNAc that was isolated in a good yield by paper chromatography with solvent A and subsequently identified with chromatography on borate-containing paper, using a solvent system that separates GlcNAc and GalNAc (Rasilo & Renkonen, 1982) (data not shown). Hence, the hexasaccharide **2** was biantennary and carried at the nonreducing end two terminal β-linked GlcNAc units. The notion that there are two nonreducing *N*-acetylglucosamine residues in the hexasaccharide **2** was confirmed by the easy galactosylation of the two branches with UDP-Gal and the β1,4-galactosyltransferase of human milk (see below); in our hands β1,3-linked GalNAc is not galactosylated by this enzyme.

Partial β-N-Acetylhexosaminidase Digestion of the Hexasaccharide **2**. The hexasaccharide **2**, formed in a reaction similar to that in Figure 1A, was digested partially with β-*N*-acetylhexosaminidase, and the digest was chromatographed on paper with solvent A for 11 days (Figure 1C). The uncleaved hexasaccharide (peak 1) contained 65% of the radioactivity; in addition, two different pentasaccharides were formed: peak 2 (22% of the radioactivity; R_{MTet} = 0.79, R_{MP} = 1.21) chromatographed like the biantennary pentasaccharide marker Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)[U-^{14}C]Galβ1-4GlcNAc (**20**) while peak 3 (12% of the radioactivity; R_{MTet} = 0.87, R_{MP} = 1.34) chromatographed like the linear pentasaccharide GlcNAcβ1-3Galβ1-4GlcNAcβ1-3[U-^{14}C]-Galβ1-4GlcNAc (**1**). Endo-β-galactosidase (*B. fragilis*) treatments confirmed the chromatographic data: peak 2 resisted the action of endo-β-galactosidase, while 94% of peak 3 was cleaved, releasing GlcNAcβ1-3[U-^{14}C]Gal (**4**) (88%; R_{Gal} = 0.74, R_{Lac} = 1.21) and GlcNAcβ1-3[U-^{14}C]Galβ1-4GlcNAc (**9**) (5.4%; R_{Gal} = 0.64, R_{Lac} = 1.05, R_{MT} = 1.39) (data not shown).

Endo-β-galactosidase Treatment of the Hexasaccharide (**2**). The hexasaccharide **2**, formed in the enzymatic *N*-acetylglucosaminylation of the pentasaccharide GlcNAcβ1-3[6-^{3}H]Galβ1-4GlcNAcβ1-3[U-^{14}C]Galβ1-4GlcNAc with UDP-GlcNAc and serum, was cleaved almost completely by a treatment with endo-β-galactosidase from *B. fragilis* (Figure 1D) into a disaccharide (peak 2) and a tetrasaccharide (peak 1). All of the ^{3}H radioactivity of the digest was found in the disaccharide fraction, migrating like GlcNAcβ1-3Gal (**4**) in paper chromatography with solvents A (R_{Gal} = 0.72, R_{Lac} = 1.24) and B (R_{Lac} = 1.46, R_{MT} = 3.05); 95% of the apparent ^{14}C label was present in the tetrasaccharide fraction (R_{MT} = 0.87, R_{MTet} = 1.44), chromatographing like authentic GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4GlcNAc (**5**).

Characterization of the Disaccharide, Liberated in the Endo-β-galactosidase Digestion. The disaccharide fraction, liberated in the endo-β-galactosidase digestion in Figure 1D, was cleaved completely by jack bean β-*N*-acetylhexosaminidase, liberating [^{3}H]Gal (data not shown). Together with the structure of the pentasaccharide acceptor **1**, this establishes the identity of the peak 2 material as GlcNAcβ1-3[6-^{3}H]Gal, originating from the nonreducing end of the hexasaccharide **2**.

WGA-Agarose Chromatography of the Tetrasaccharide, Liberated in the Endo-β-galactosidase Digestion. The tetrasaccharide, liberated in the endo-β-galactosidase hydrolysis in Figure 1D, was identified in WGA-agarose chromatography by use of the column of high lectin content. The tetrasaccharide was eluted from the column as one peak (Figure 2A; Δ = 55.7) almost exactly like the synthetic tetrasaccharide GlcNAcβ1-3(GlcNAcβ1-6)[U-^{14}C]Galβ1-4GlcNAc marker (Figure 2B; Δ = 56.2). The tetrasaccharide migrated much more slowly in the experiments of Figure 2 than in our previous experiments (Δ = 3.75), which were carried out by use of a column of low lectin content (Seppo et al., 1990). A number of oligosaccharides that are only slightly retarded and poorly separated in the column of low lectin content (Renkonen et al., 1991a) were remarkably well separated in the column of high lectin content (see arrows in Figure 2B).

Mild Acid Hydrolysis of the Tetrasaccharide from the Endo-β-galactosidase Digest of the Hexasaccharide **2**. The tetrasaccharide, liberated in an endo-β-galactosidase digestion similar to that in Figure 1D, was cleaved partially with acid. Prolonged paper chromatography of the digest with solvent B separated clearly five peaks from each other (Figure 1E). The "fingerprinting" pattern revealed in the chromatogram was very similar to that obtained from the synthetic GlcNAcβ1-3(GlcNAcβ1-6)[U-^{14}C]Galβ1-4GlcNAc by Seppo et al. (1990). Peak 1 represented the starting tetrasaccharide, peak 3 was GlcNAcβ1-3[U-^{14}C]Galβ1-4GlcNAc (**9**), peak 4 was shown to be pure disaccharide GlcNAcβ1-3[U-^{14}C]Gal (**4**), and peak 5 represented pure [U-^{14}C]-Galβ1-4GlcNAc. The saccharides carrying information about the linkage of the newly introduced GlcNAc unit of the hexasaccharide **2** were the peak 2 and the two minor components visible as shoulders in the front edges of peaks 1 and 3.

Fractions 3-7 of Figure 1E were pooled to give a mixture of peak 1 and the minor component (1b) running in front of

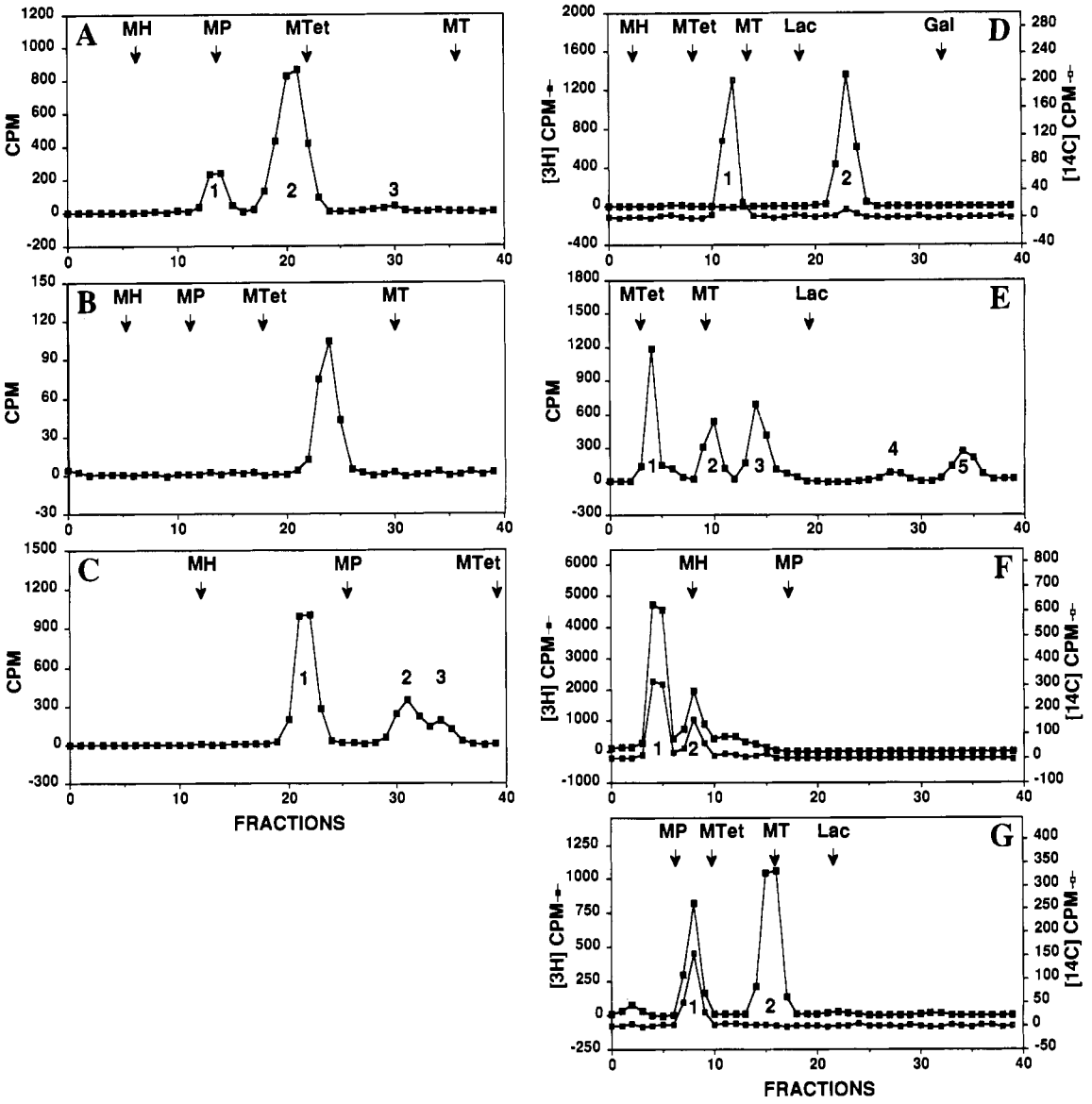


FIGURE 1: Paper chromatograms related to the formation and structural analysis of the hexasaccharide **2**. (Panel A) Formation of the branched hexasaccharide **2**. The pentasaccharide GlcNAcβ1–3Galβ1–4GlcNAcβ1–3[U-^{14}C]Galβ1–4GlcNAc (**1**) (84.2 nmol) and UDP–GlcNAc (14.7 μmol) were incubated for 12 days at 37 °C with 100 μL of concentrated human serum, and the desalted reaction mixture was chromatographed on paper with solvent A for 137 h. Peak 1 is the hexasaccharide formed, peak 2 is the pentasaccharide **1**, and peak 3 occupies the position of radiolabeled Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc marker. Unlabeled markers are MT, maltotriose; MTet, maltotetraose; MP, maltopentaose; and MH, maltoheptaose. (Panel B) Exhaustive β-*N*-acetylhexosaminidase digestion of the hexasaccharide **2**. The hexasaccharide GlcNAcβ1–3Galβ1–4GlcNAcβ1–3(GlcNAcβ1–6)[U-^{14}C]Galβ1–4GlcNAc (**2**), obtained as in Figure 1A, was digested for 8 h with 150 milliunits of jack bean β-*N*-acetylhexosaminidase in a reaction mixture of 45 μL that contained 0.05 M sodium citrate/citric acid, pH 4.0, and 441 μg of γ-galactonolactone. The desalted digest was chromatographed on paper with solvent A for 143 h. The only peak visible occupies the position of the Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc marker. Unlabeled markers are as in panel A. (Panel C) Partial β-*N*-acetylhexosaminidase digestion of the hexasaccharide **2**. The hexasaccharide GlcNAcβ1–3Galβ1–4GlcNAcβ1–3(GlcNAcβ1–6)[U-^{14}C]Galβ1–4GlcNAc (**2**), obtained as in Figure 1A, was digested for 5 min with 150 milliunits of jack bean β-*N*-acetylhexosaminidase in a reaction mixture (45 μL) that contained 0.05 M sodium citrate/citric acid, pH 4.0, and 441 μg of γ-galactonolactone. The desalted digest was chromatographed for 260 h with solvent A. Peak 1 represents the original hexasaccharide **2**; peak 2 is the pentasaccharide Galβ1–4GlcNAcβ1–3(GlcNAcβ1–6)[U-^{14}C]Galβ1–4GlcNAc (**20**); peak 3 represents the linear pentasaccharide GlcNAcβ1–3Galβ1–4GlcNAcβ1–3[U-^{14}C]Galβ1–4GlcNAc (**1**). Unlabeled markers are as in panel A. (Panel D) Endo-β-galactosidase digestion of the hexasaccharide GlcNAcβ1–3[6-^{3}H]Galβ1–4GlcNAcβ1–3(GlcNAcβ1–6)[U-^{14}C]Galβ1–4GlcNAc (**2**). The doubly labeled hexasaccharide was treated with endo-β-galactosidase, and the digest was chromatographed on paper for 61.5 h with solvent A. The original hexasaccharide was not visible in the digest. Peak 1 represented the tetrasaccharide GlcNAcβ1–3(GlcNAcβ1–6)[U-^{14}C]Galβ1–4GlcNAc (**5**), and peak 2 was the disaccharide GlcNAcβ1–3[6-^{3}H]Gal. Unlabeled markers are Gal, galactose; Lact, lactose; MT, maltotriose; MTet, maltotetraose; and MH, maltoheptaose. (Panel E) Partial acid hydrolysis of the tetrasaccharide (**5**) obtained by endo-β-galactosidase cleavage of the hexasaccharide **2**. The tetrasaccharide was hydrolyzed partially with acid (0.1 M trifluoroacetic acid, 100 °C for 40 min), and the digest was chromatographed on paper with solvent B for 269 h. Markers are as in panel D. (Panel F) Enzymatic β1,4-galactosylation of the hexasaccharide **2**. The hexasaccharide GlcNAcβ1–3[6-^{3}H]Galβ1–4GlcNAcβ1–3(GlcNAcβ1–6)[U-^{14}C]Galβ1–4GlcNAc (2 pmol) was incubated with UDP–[6-^{3}H]Gal (50 pmol) and β1,4-galactosyltransferase (50 mU), and the reaction mixture was chromatographed on paper with solvent A for 3 days (not shown). The material migrating between the starting line and the maltopentaose marker was collected, extracted from the paper, desalted, and rechromatographed with solvent A for 8 days. Peak 1 represents an octasaccharide fraction, while peak 2 is a heptasaccharide fraction. Unlabeled markers on the chromatogram: MP, maltopentaose; MH, maltoheptaose. (Panel G) Endo-β-galactosidase cleavage of the octasaccharide from panel F. The octasaccharide from panel F was treated exhaustively with endo-β-galactosidase (*B. fragilis*) as described under Materials and Methods, and the digest was chromatographed on paper with solvent A for 63.5 h. Peak 1 represents the pentasaccharide GlcNAcβ1–3([6-^{3}H]Galβ1–4GlcNAcβ1–6)[U-^{14}C]Galβ1–4GlcNAc (**19**), while peak 2 is the trisaccharide [6-^{3}H]Galβ1–4GlcNAcβ1–3[6-^{3}H]Gal (**18**). Unlabeled markers are as in panel D.

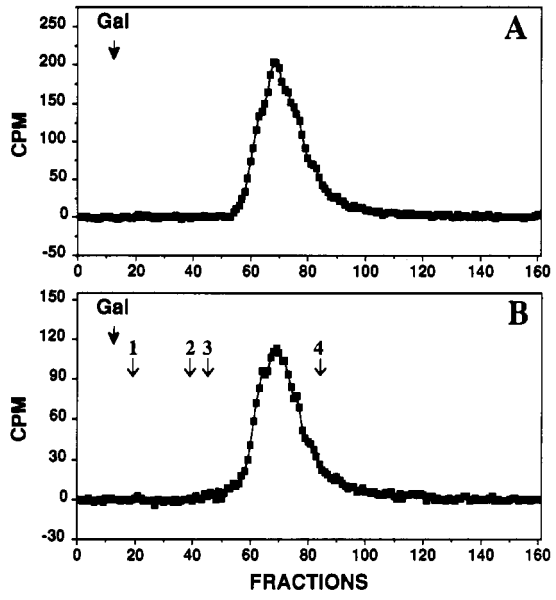


FIGURE 2: WGA–agarose chromatograms. (Panel A) WGA–agarose (high lectin content) chromatography of the tetrasaccharide **5** obtained by endo-β-galactosidase cleavage of the hexasaccharide **2**. (Panel B) WGA–agarose (high lectin content) chromatography of the GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAc marker. The arrow under Gal shows the position of the galactose marker; the arrows 1–4 show the positions of radiolabeled GlcNAcβ1–3Gal (**4**), GlcNAcβ1–3Galβ1–4GlcNAc (**9**), Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc (**3**), GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc (**1**), respectively; the hexasaccharide GlcNAcβ1–3Galβ1–4GlcNAcβ1–3(GlcNAcβ1–6)Galβ1–4GlcNAc (**2**) emerged after fraction 160, approximately at fraction 210. Only the sugar-free buffer was used in all experiments of both panels.

it, and the mixture was resolved completely by a new 24-day run with solvent B (data not shown). In the same way, fractions 13–18 of Figure 1E were pooled to give a mixture of peak 3 and the minor component (3b) running ahead of it, and the mixture was resolved in a new 24-day run with solvent B (data not shown). The structural characterization of the important cleavage products was carried out as follows.

Component 1b chromatographed like the GlcNAcβ1–3-(GlcNAcβ1–6)Gal marker in HPLC, on paper with solvents A (R_{MT} = 0.95, R_{MTet} = 1.53) and B (R_{MT} = 0.67, R_{MTet} = 2.02), and also in a WGA–agarose column of low lectin content (Δ_2 = 40.8) (data not shown). It had the appearance of a pure component in the other chromatography experiments, but in WGA–agarose chromatography it revealed the presence of a nonbinding impurity of 24%. Periodate oxidation, subsequent acid hydrolysis, and paper chromatography with solvent A gave 56% radiolabeled lyxose and 44% radiolabeled galactose (data not shown). All these data are compatible with the notion that the major component of 1b was GlcNAcβ1–3(GlcNAcβ1–6)[U-^{14}C]Gal (**8**).

Peak 2 of Figure 1E chromatographed in HPLC and on paper with solvents A (R_{Lac} = 0.86, R_{MT} = 1.18) and B (R_{Lac} = 0.51, R_{MT} = 1.05) like authentic GlcNAcβ1–6Galβ1–4GlcNAc (**7**). Even in the WGA–agarose column of low lectin content, the saccharide of peak 2 (Δ = 9.0) behaved like the GlcNAcβ1–6Galβ1–4GlcNAc marker (Δ = 10.1). Endo-β-galactosidase (*B. fragilis*) did not cleave the peak 2 saccharide, but β-*N*-acetylhexosaminidase cleaved 92% of it, releasing [^{14}C]Galβ1–4GlcNAc (data not shown). We have found that the GlcNAcβ1–6Galβ1–4GlcNAc markers of biological and synthetic origin, too, resist the action of endo-β-galactosidase from *B. fragilis* (O. Renkonen et al., unpublished observations). Hence, all these findings support the view that peak 2 of Figure 1E represents GlcNAcβ1–6[^{14}C]Galβ1–4GlcNAc (**7**).

Component 3b chromatographed in HPLC, on paper with solvents A (R_{Gal} = 0.61, R_{Lac} = 1.03, R_{MT} = 1.38) and B (R_{Lac} = 0.90, R_{MT} = 1.86), and also in the WGA–agarose column of low lectin content (Δ_2 = 40.6) like authentic GlcNAcβ1–6Gal (Δ_2 = 40.4). It had the appearance of a pure component in the other chromatography experiments, but in WGA–agarose chromatography it revealed the presence of a nonretained impurity of 20%. The HPLC experiment excludes the presence of GlcNAcβ1–4Gal, because the GlcNAcβ1–3Gal marker migrated faster than the GlcNAcβ1–6Gal marker and GlcNAcβ1–4Gal is known to run faster than GlcNAcβ1–3Gal (Blanken et al., 1985). All these data are compatible with the notion that component 3b represents GlcNAcβ1–6Gal (**6**). Hence, the data on all cleavage products found in the partial acid hydrolysate of Figure 1E support the conception that the most probable structure of the tetrasaccharide **5** is GlcNAcβ1–3(GlcNAcβ1–6)[U-^{14}C]Galβ1–4GlcNAc.

Enzymatic β1,4-Galactosylation of the Hexasaccharide **2**. The hexasaccharide **2**, GlcNAcβ1–3[6-^{3}H]Galβ1–4GlcNAcβ1–3(GlcNAcβ1–6)[U-^{14}C]Galβ1–4GlcNAc (2 pmol) was incubated with bovine milk β1,4-galactosyltransferase and UDP–[6-^{3}H]Gal (50 pmol) for 24 h, and the products were analyzed by prolonged paper chromatography with solvent A (Figure 1F). Two components had been formed; the chromatographic mobility of peak 1 corresponded to that of an octasaccharide (R_{MP} = 0.26, R_{MH} = 0.57), while peak 2 appeared to represent a mixture of heptasaccharides (R_{MP} = 0.47, R_{MH} = 1.03); the hexasaccharide **2** had reacted completely.

The structural analysis of the octasaccharide of peak 1 was carried out by cleaving the material with endo-β-galactosidase (*B. fragilis*). Paper chromatography of the digest revealed two cleavage products (Figure 1G). Peak 1 (R_{MTet} = 0.81, R_{MP} = 1.27; 1285 cpm ^{3}H; 237 cpm ^{14}C) represented the pentasaccharide GlcNAcβ1–3([^{3}H]Galβ1–4GlcNAcβ1–6)-[U-^{14}C]Galβ1–4GlcNAc (**19**), while peak 2 (R_{Lact} = 0.72, R_{MT} = 0.97, R_{MTet} = 1.59; 2457 cpm ^{3}H) was the trisaccharide [^{3}H]Galβ1–4GlcNAcβ1–3[^{3}H]Gal (**18**). It was reassuring to see that the two products were formed in equimolar amounts, as judged from their tritium contents. The identification of the two products is based on the paper chromatographic mobilities (cf. Table I). In addition, the saccharide of peak 1 was cleaved completely by a treatment with β-*N*-acetylhexosaminidase into a tetrasaccharide that migrated in paper chromatography with solvent A (R_{MT} = 0.65, R_{MTet} = 1.06, R_{MP} = 1.67) like the synthetic marker of [^{3}H]Galβ1–4GlcNAcβ1–6[U-^{14}C]Galβ1–4GlcNAc. The identity of the tetrasaccharide was confirmed by chromatographing it on the WGA–agarose column of low lectin content, from which it emerged at the same position (Δ = 16.5) as the synthetic Galβ1–4GlcNAcβ1–6Galβ1–4GlcNAc marker (Δ = 17.5). These data establish the structure of the peak 1 saccharide of Figure 1F as [6-^{3}H]Galβ1–4GlcNAcβ1–3[6-^{3}H]Galβ1–4GlcNAcβ1–3([6-^{3}H]Galβ1–4GlcNAcβ1–6)[U-^{14}C]Galβ1–4GlcNAc (**17**).

The β1,6-GlcNAc Transferase of Serum Acts Also on GlcNAcβ1–3[6-^{3}H]Galβ1–4GlcNAcβ1–3Galβ1–4[1-^{14}C]Glc (**10**). When the pentasaccharide GlcNAcβ1–3[6-^{3}H]Galβ1–4GlcNAcβ1–3Galβ1–4[1-^{14}C]Glc (**10**) (12.4 pmol) and UDP–GlcNAc (14.7 μmol) were incubated for 11 days together with human serum that had been concentrated 2-fold by ultrafiltration, a hexasaccharide (**11**) was formed in an 8.3% yield (Figure 3A; R_{MP} = 0.66, R_{MH} = 1.34). A parallel experiment carried out with 12.3 pmol of the pentasaccharide

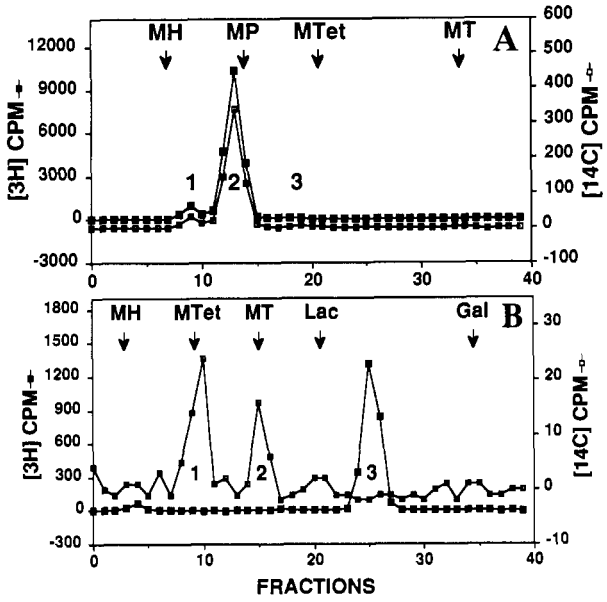


FIGURE 3: Paper chromatograms related to the formation and structural analysis of the hexasaccharide **11**. (Panel A) Formation of the branched hexasaccharide GlcNAcβ1-3Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4Glc (**11**). The pentasaccharide GlcNAcβ1-3[6-^{3}H]Galβ1-4GlcNAcβ1-3Galβ1-4[1-^{14}C]Glc (**10**) (12.4 pmol) and UDP-GlcNAc (4.5 μmol) were incubated for 20 days at 37 °C with 100 μL of normal human serum, and the reaction mixture was chromatographed on paper with solvent A for 139 h. Peak 1 represents the hexasaccharide **11** (8.3% of the total tritium label), while peak 2 is the starting pentasaccharide (**10**); peak 3 occupies the position expected for Galβ1-4GlcNAcβ1-3Galβ1-4Glc. Markers are as in Figure 1A. (Panel B) Endo-β-galactosidase cleavage of the hexasaccharide (**11**). The digest was chromatographed on paper with solvent A for 51 h. Peak 1 occupies the position of the GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4Glc marker; peak 2 resembles closely the GlcNAcβ1-3Galβ1-4Glc marker; and peak 3 migrates like the GlcNAcβ1-3Gal marker. Unlabeled markers are as in Figure 1D.

1 gave the hexasaccharide **2** in a 20.8% yield. Hence, the reaction rate of **10** was 41% of the rate of **1**.

The hexasaccharide (**11**) from an experiment similar to that of Figure 3A was digested with endo-β-galactosidase (*B. fragilis*) (Figure 3B). In the digest 96% of the 6-^{3}H radioactivity was found in a disaccharide fraction (peak 3; R_{Gal} = 0.73, R_{Lac} = 1.23), which chromatographed like authentic GlcNAcβ1-3Gal (**4**) on paper. Of the 1-^{14}C radioactivity, 62% (peak 1; R_{MT} = 0.64, R_{MTet} = 1.03, R_{MP} = 1.62) chromatographed like the tetrasaccharide GlcNAcβ1-3(GlcNAcβ1-6)[1-^{14}C]Galβ1-4Glc marker (**12**) and 32% (peak 2; R_{Lac} = 0.74, R_{MT} = 1.01, R_{MTet} = 1.62) migrated like trisaccharide GlcNAcβ1-3Galβ1-4Glc marker (**14**). These data suggest that the major component of the hexasaccharide in Figure 3A was GlcNAcβ1-3[6-^{3}H]Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)Galβ1-4[1-^{14}C]Glc (**11**). The formation of the trisaccharide (**14**) in the endo-β-galactosidase treatment of Figure 3B probably reflects the action of a contaminating β-*N*-acetylhexosaminidase on the tetrasaccharide **12** and/or on the hexasaccharide **11**; alternatively, the substrate hexasaccharide **11** was contaminated by the pentasaccharide **10**. We do not know whether the endo-β-galactosidase used contained any β-*N*-acetylhexosaminidase activity, but we know that partial hydrolysis of **12** effected by β-*N*-acetylhexosaminidase of jack bean releases the 1→6-linked GlcNAc residue much faster than the 1→3 linked one (Renkonen et al., 1991b). The data of Figure 3B may not be incompatible with those of Figure 1D, because the putative contaminating β-*N*-acetylhexosaminidase may cleave **12** faster than **5**. We know that jack bean β-*N*-acetylhexosaminidase acts in this manner (Renkonen et al., 1991b).

The β1,6-GlcNAc Transferase of Serum Acts Also on Galα1-3[6-^{3}H]Galβ1-4GlcNAcβ1-3[U-^{14}C]Galβ1-4GlcNAc, but the Product Has Been Only Partially Characterized. When the pentasaccharide Galα1-3[6-^{3}H]Galβ1-4GlcNAcβ1-3[U-^{14}C]Galβ1-4GlcNAc (6.6 pmol, 3299 cpm ^{14}C, 15 837 cpm ^{3}H) and UDP-GlcNAc (14.7 μmol) were incubated for 11 days with human serum that had been concentrated 2-fold by ultrafiltration, a hexasaccharide-like peak was formed in a yield of 8.5%. Its chromatographic mobility (R_{MP} = 0.83, R_{MH} = 1.73) was almost identical with that of a marker mixture of the hexasaccharides Galα1-3Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)[U-^{14}C]Galβ1-4GlcNAc and GlcNAcβ1-3(Galα1-3Galβ1-4GlcNAcβ1-6)[U-^{14}C]Galβ1-4GlcNAc (R_{MP} = 0.83, R_{MH} = 1.67). A parallel experiment carried out with 6.6 pmol of pentasaccharide **1** gave the hexasaccharide **2** in a yield of 22.2%, implying that the reaction rate of Galα1-3[6-^{3}H]Galβ1-4GlcNAcβ1-3[U-^{14}C]Galβ1-4GlcNAc was 38% of the rate of **1**.

The hexasaccharide Galα1-3[6-^{3}H]Galβ1-4GlcNAcβ1-3-(GlcNAcβ1-6)[U-^{14}C]Galβ1-4GlcNAc was degraded completely by a treatment with α-galactosidase from green coffee beans, releasing the pentasaccharide [6-^{3}H]Galβ1-4GlcNAcβ1-3(GlcNAcβ1-6)[U-^{14}C]Galβ1-4GlcNAc (**20**) (R_{MTet} = 0.79, R_{MP} = 1.24; solvent A). The pentasaccharide in turn was cleaved completely by a treatment with jack bean β-galactosidase, releasing the tetrasaccharide GlcNAcβ1-3-(GlcNAcβ1-6)[U-^{14}C]Galβ1-4GlcNAc (**5**) (R_{MT} = 0.88, R_{MTet} = 1.42; solvent A) and [^{3}H]galactose, which were identified by paper chromatography.

The β1,6-GlcNAc Transferase of Serum Acts Poorly on GlcNAcβ1-3Galβ1-4[U-^{14}C]GlcNAcβ1-3Gal (**15**). The tetrasaccharide GlcNAcβ1-3Galβ1-4[U-^{14}C]GlcNAcβ1-3Gal (**15**) (1.4 pmol, 1020 cpm) was incubated with UDP-GlcNAc (4.5 μmol) and human serum for 18 days. The digest was chromatographed on paper with solvent A, which revealed that a pentasaccharide was formed in a 0.5% yield. A parallel experiment carried out with 1.4 pmol of the pentasaccharide **1**, gave the hexasaccharide **2** in an 8.9% yield, indicating that the reaction rate of the tetrasaccharide **15** was only 6% of the rate of **1**.

Serum β1,6-GlcNAc Transferase Acts Very Poorly on GlcNAcβ1-3[U-^{14}C]Galβ1-4GlcNAc (**9**). The trisaccharide GlcNAcβ1-3[U-^{14}C]Galβ1-4GlcNAc (**9**) (7.6 pmol, 5000 cpm) was incubated with UDP-GlcNAc (4.5 μmol) and human serum for 8 days, and the mixture was desalted and chromatographed on paper. A product migrating at the position of the branched tetrasaccharide GlcNAcβ1-3-(GlcNAcβ1-6)[U-^{14}C]Galβ1-4GlcNAc was found only in questionable trace amounts (0.18% yield). A parallel experiment with the pentasaccharide **1** (7.6 pmol, 5000 cpm) gave the hexasaccharide **2** in a yield of 7.3%, indicating that the reaction rate of the trisaccharide **9** was only 2% of the rate of **1**.

Serum from Different Adult Individuals Contains the β1,6-GlcNAc Transferase. Sera from three different persons were tested in a parallel fashion for the presence of β1,6-GlcNAc transferase by using the pentasaccharide **1** as acceptor and UDP-GlcNAc as donor. The reaction products were chromatographed on paper with solvent A, which revealed that the hexasaccharide **2** was formed in all three reactions, the relative yields varying between 71 and 100%.

Discussion

Our present data show that human serum contains a novel type of β1,6-GlcNAc transferase, which catalyzes the transfer of a GlcNAc unit from UDP-GlcNAc to the position 6 of the

second glycosyl unit (counting from the reducing end), rather than to the fourth glycosyl unit, of the linear pentasaccharide GlcNAcβ1–3Galβ1–4GlcNAcβ1–3Galβ1–4GlcNAc (**1**). The well-known β1,6-GlcNAc transferase of hog stomach microsomes catalyzes analogous branch formation at the penultimate galactose unit at the nonreducing end of linear structures but not at the midchain galactoses (Brockhausen et al., 1986).

The structural analysis of the key product, the hexasaccharide **2**, consisted of several elements. First, endo-β-galactosidase cleaved **2** into a disaccharide and a tetrasaccharide, the former representing the nonreducing end of the original acceptor. Hence, the conversion of the pentasaccharide **1** into the hexasaccharide **2** could not involve the nonreducing area of the acceptor. The second set of data identify the newly inserted monosaccharide as a βGlcNAc unit and show that it is transferred to the pentasaccharide **1** at the second glycosyl unit, which is a galactose, at position 6. The newly formed GlcNAcβ1–6Gal linkage was identified by a thorough analysis of the products formed by partial acid hydrolysis of the tetrasaccharide that was liberated by endo-β-galactosidase: all three molecules containing the new linkage could be isolated and identified among the cleavage products of the tetrasaccharide. Finally, the newly formed GlcNAcβ1–6Gal linkage was identified also by galactosylating the hexasaccharide **2** into the octasaccharide **17** and by cleaving this with endo-β-galactosidase into the pentasaccharide **19** and subsequently cleaving with β-N-acetylhexosaminidase into Galβ1–4GlcNAcβ1–6Galβ1–4GlcNAc. The latter has been synthesized in our laboratory by converting the branched tetrasaccharide GlcNAcβ–3(GlcNAcβ1–6)-Galβ1–4GlcNAc (**5**) (Seppo et al., 1990) into the trisaccharide GlcNAcβ1–6Galβ1–4GlcNAc (**7**) by a partial β-*N*-acetylhexosaminidase treatment (O. Renkonen et al., submitted for publication), followed by enzymatic β1,4-galactosylation of the trisaccharide **7**. The synthetic Galβ1–4GlcNAcβ1–6Galβ1–4GlcNAc, characteristically, binds quite strongly to immobilized wheat germ agglutinin, and so does the linear derivative obtained from octasaccharide **17**.

The data show that the trisaccharide **9** is a very poor acceptor in comparison to the pentasaccharide **1**. Therefore, it is natural to ask whether the intermediary tetrasaccharide **3** is a good or a poor acceptor. We do not have the answer at present, but our data show that the incubation of the tetrasaccharide with UDP–GlcNAc and human serum yields almost pure pentasaccharide **1**, and only trace amounts of the pentasaccharide **20** (See the Materials and Methods section, under Enzymatic in Vitro Synthesis of Key Acceptors). At the first sight this suggests that the β1,6-GlcNAc transferase does not act on the tetrasaccharide **3**, but this view may not be true: the incubation was optimized for the action of β1,3-GlcNAc transferase of serum, not for the β1,6-GlcNAc transferase, and the integrated data obtained from the experiment indicated that the products included pentasaccharide **1** (46 600 cpm), the hexasaccharide **2** (2150 cpm), and the pentasaccharide **20** (350 cpm). It is quite possible that the latter, too, was converted to the hexasaccharide **2** during the incubation. The finding that the pentasaccharide **16** appears to be a reasonably good acceptor, in contrast to the trisaccharide **9**, suggests that the βGal residue at the nonreducing area of **16** may participate in enhancing the reactivity of **9**. Obviously, the β1,6-GlcNAc transferase and the β1,3-GlcNAc transferase of serum must be separated, before the substrate properties of tetrasaccharide **3** for the β1,6-GlcNAc transferase can be reliably defined.

Our results show that jack bean β-*N*-acetylhexosaminidase cleaves the β1,3-linked GlcNAc of the biantennary hexasaccharide **2** faster than the β1,6-linked GlcNAc, suggesting that the outer nonreducing end of the long 1→3-linked chain is more freely exposed to the outside world than the 1→6-linked chain. In contrast, the tetrasaccharides **5** and **12** are cleaved faster in the β1,6-linked GlcNAc (O. Renkonen et al., submitted for publication). The partial cleavage of **2** represents a route to the pure pentasaccharide **20**, uncontaminated by the isomeric pentasaccharide **19**.

The reducing end GlcNAc of the acceptor pentasaccharide **1** appears to be important for the β1,6-GlcNAc transferase reaction: the tetrasaccharide **15** reacted much more slowly than the pentasaccharide **1**. Also the reducing end Glc of the pentasaccharide **10** was important for the reaction: the pentasaccharide **10** is a better acceptor than the tetrasaccharide **15**. It is noteworthy that the reducing end GlcNAc of the hexasaccharide Galβ1–4GlcNAcβ1–3(Galβ1–4GlcNAcβ1–6)Galβ1–4GlcNAc exerts a retarding effect on enzymatic cleavage of the β-galactosidic linkage of the 1–6 branch; in the pentasaccharide Galβ1–4GlcNAcβ1–3(Galβ1–4GlcNAcβ1–6)Gal this linkage is cleaved 10 times faster than in the hexasaccharide by *E. coli* β-galactosidase (Renkonen et al., 1990). Even the β-*N*-acetylhexosaminidase cleavage of branched trisaccharide **8** is markedly more branch-specific than that of the tetrasaccharide **5**, although both are cleaved faster at the 1–6 than at the 1–3 linkage (Renkonen et al., 1991b). It seems possible that analogous spatial relationships may exist between the reducing end GlcNAc and the GlcNAc of the 1–6 branch in these oligosaccharides and between the reducing end GlcNAc and the incoming GlcNAc in the β1,6-GlcNAc transferase reactions.

The origin of the novel enzyme remains unclear. However, the red blood cell forming tissue can probably be excluded as a main source of this enzyme. Our preliminary analyses showed that two samples of fetal serum contained distinct, albeit reduced, amounts of this enzyme (data not presented). Fetal red blood cells contain largely nonbranched poly(*N*-acetyllactosaminoglycans) in their glycoproteins (Fukuda et al., 1979). Accordingly, the proerythroblasts in fetal bone marrow should be devoid of the β1,6-GlcNAc transferase, but the lack of expression of the enzyme does not result in a total absence of the enzyme in the fetal serum.

The novel β1,6-GlcNAc transferase reaction appears to offer some advantages for the in vitro construction of oligo(*N*-acetyllactosaminoglycans) that consist of a long linear chain of β1,3-linked Galβ1–4GlcNAc units in combination with short β1,6-linked side chains consisting of only one Galβ1–4GlcNAc unit. It has been suggested that this type of backbone prevails in many natural poly(*N*-acetyllactosaminoglycans) (Fukuda, 1985).

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