In Vitro Biosynthesis of a Decasaccharide Prototype of Multiply Branched Polylactosaminoglycan Backbones[†]

Anne Leppänen,[‡] Heidi Salminen,[‡] Ying Zhu,[‡] Hannu Maaheimo,[‡] Jari Helin,[‡,§] Catherine E. Costello,[§] and Ossi Renkonen*[,‡]

Institute of Biotechnology and Department of Biosciences (Division of Biochemistry), University of Helsinki, Biocenter 1A, P.O. Box 56, FIN-00014 Helsinki, Finland, and Mass Spectrometry Resource, Department of Biophysics, Boston University School of Medicine, 80 East Concord Street, Boston, Massachusetts 02118

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ABSTRACT: Multiply branched polylactosaminoglycans are expressed in glycoproteins and glycolipids of many cells. Interest in their biology stems from their abundant expression in early embryonal cells and from their ability to carry multiple lectin-binding determinants, which makes them prominent ligands and antagonists of cell adhesion proteins. A prototype of their backbones is represented by the decasaccharide LacNAcβ1−3′(LacNAcβ1−6′)LacNAcβ1−3′(LacNAcβ1−6′)LacNAc (**5**), where LacNAc is the disaccharide Galβ1−4GlcNAc. Here, we describe *in vitro* biosynthesis of glycan **5**. Incubation of the linear hexasaccharide LacNAcβ1−3′LacNAcβ1−3′LacNAc (**1**) with UDP-GlcNAc and a midchain β1,6-GlcNAc transferase activity (GlcNAc to Gal), present in rat serum [Gu, J., Nishikawa, A., Fujii, S., Gasa, S., & Taniguchi, N. (1992) *J. Biol. Chem. 267,* 2994−2999], gave the doubly branched octasaccharide LacNAcβ1−3′(GlcNAcβ1−6′)LacNAcβ1−3′(GlcNAcβ1−6′)LacNAc (**4**). The latter was converted to **5** by enzymatic β1,4-galactosylation. In the initial branching reaction of **1**, two isomeric heptasaccharide intermediates, LacNAcβ1−3′LacNAcβ1−3′(GlcNAcβ1−6′)LacNAc (**2**) and LacNAcβ1−3′(GlcNAcβ1−6′)LacNAcβ1−3′LacNAc (**3**), were formed first at comparable rates. Later, both intermediates were converted to **4**, revealing two distinct pathways of the reaction: **1** → **2** → **4** and **1** → **3** → **4**. These data suggest that, regardless of their chain length, linear polylactosamines similar to **1** contain potential branching sites at each of the internal galactoses. The enzyme-binding epitope of **1** is probably LacNAcβ1−3′LacNAc, because the trisaccharides GlcNAcβ1−3′LacNAc and LacNAcβ1−3Gal as well as the tetrasaccharide GlcNAcβ1−3′LacNAcβ1−3Gal were poor acceptors, while LacNAcβ1−3′LacNAc was a good one. Midchain β1,6-GlcNAc transferase activities present in serum of several mammalian species, including man, resembled closely the rat serum activity in their mode of action and in their acceptor specificity. We suggest that analogous membrane-bound Golgi enzymes are involved in the biosynthesis of multiply branched polylactosamines *in vivo*.

Polylactosaminoglycans consist of repeating *N*-acetyllactosamine (LacNAc)[1] units linked together by β(1−3′) linkages to linear backbone chains. In branched polylactosaminoglycans, some of the 3-substituted galactose residues within the primary chains are substituted also at position 6 by additional backbone elements. The backbones are often decorated distally by various α-linked sugar residues and/or by sulfate groups. Linear and branched polylactosaminoglycan backbones, respectively, represent the blood group i and I antigens (Niemann et al., 1978; Feizi et al., 1979; Watanabe et al., 1979), which are developmentally regulated in the course of murine embryonic development (Kapadia et al., 1981), in embryonal carcinoma cells (Muramatsu et al., 1978; Kapadia et al., 1981), and in human erythrocytes (Fukuda et al., 1979, 1984a,b).

Multiply branched polylactosaminoglycan backbones are present for example in glycoproteins of human red blood cells (Fukuda et al., 1984b), in murine (Muramatsu et al., 1978) and human embryonal carcinoma cells (Rasilo & Renkonen, 1982; Fukuda et al., 1985), as well as in the parasitic protozoan *Trypanosoma brucei* (Zamse et al., 1991). They are also expressed in glycolipids from several types of human and animal cells (Gardas, 1976; Koscielak et al., 1976; Dabrowski et al., 1984; Dabrowski et al., 1988; Hanfland et al., 1988; Nudelman et al., 1989; Barnett & Clark, 1992). Additional examples are likely to be found as experimental recognition of these structures develops further. Many of the multiply branched backbones probably represent long chains of β1,3′-bonded LacNAc units, which bear short LacNAc branches.

The present report concerns *in vitro* biosynthesis of the decasaccharide LacNAcβ1−3′(LacNAcβ1−6′)LacNAcβ1−3′(LacNAcβ1−6′)LacNAc (**5**), the smallest possible prototype of multiply branched poly-*N*-acetyllactosamine backbones. Our interest stems from the adhesion properties of

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* To whom correspondence should be addressed. Telephone: 358-9-708-59375. Fax: 358-9-708-59563. E-mail: ossi.renkonen@helsinki.fi.

[‡] University of Helsinki.

[§] Boston University School of Medicine.

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[1] Abbreviations: Gal, D-galactose; GlcNAc, *N*-acetyl-D-glucosamine; HPAEC-PAD, high-pH anion exchange chromatography with pulsed amperometric detection; HPLC, high-performance liquid chromatography; Lac, lactose (Galβ1−4Glc); LacNAc, *N*-acetyllactosamine (Galβ1−4GlcNAc); MALDI-TOF-MS, matrix-assisted laser desorption-ionization mass spectrometry with time-of-flight detection; ManNAc, *N*-acetylmannosamine; MH, maltoheptaose [Glcα1−4(Glcα1−4)$_5$Glc]; MP, maltopentaose [Glcα1−4(Glcα1−4)$_3$Glc]; MT, maltotriose (Glcα1−4Glcα1−4Glc); MTet, maltotetraose [Glcα1−4(Glcα1−4)$_2$Glc]; NMR, nuclear magnetic resonance; sLex, Neu5Acα2−3Galβ1−4(Fucα1−3)-GlcNAc; WGA, wheat germ agglutinin.

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multiply branched backbones, carrying distal LacNAc units that are decorated by α2,3′-bonded *N*-acetylneuraminic acid and α1,3-linked fucose. The resulting multivalent sialyl Lewis X sequences have proven to be nanomolar L-selectin antagonists, which inhibit adhesion of lymphocytes to the activated endothelium of rejecting organ transplants *in vitro* (Turunen et al., 1995; Seppo et al., 1996; Renkonen et al., 1997). These observations suggest that the multivalent sLex polylactosamines should be evaluated as potential anti-inflammatory agents also in *in vivo* experiments. This, in turn, is possible only when the antagonists can be synthesized enzymatically or chemoenzymatically in relatively large amounts.

In vitro biosynthesis of polylactosaminoglycans containing a *single* backbone branch has been repeatedly documented. Distally acting β1,6-GlcNAc transferases (GlcNAc to Gal) have been described, which catalyze typically the reaction GlcNAcβ1−3Galβ1−4GlcNAc + UDP-GlcNAc → GlcNAcβ1−3(GlcNAcβ1−6)Galβ1−4GlcNAc + UDP (Piller et al., 1984; Brockhausen et al.; 1986, Koenderman et al., 1987; Seppo et al., 1990; Ropp et al., 1991; Gu et al., 1992). In the linear pentasaccharide GlcNAcβ1−3′LacNAcβ1−3′LacNAc, the enzyme catalyzes the branch formation at the distal branching site, generating the hexasaccharide GlcNAcβ1−3′(GlcNAcβ1−6′)LacNAcβ1−3′LacNAc but not the isomer GlcNAcβ1−3′LacNAcβ1−3′(GlcNAcβ1−6′)-LacNAc (Helin et al., 1995). *Midchain* β1,6-GlcNAc transferases (GlcNAc to Gal) have been also described, which catalyze typically the reaction LacNAcβ1−3′LacNAc + UDP-GlcNAc → LacNAcβ1−3′(GlcNAcβ1−6′)LacNAc + UDP (Leppänen et al., 1991; Gu et al., 1992; Niemelä et al., 1995b; Maaheimo et al., 1997). The two types of β1,6-GlcNAc transferases appear to possess surprisingly distinct substrate specificities. The distally acting enzymes act only on acceptors that contain the GlcNAcβ1−3Galβ1−4GlcNAcβ1-R sequence at the nonreducing terminus, transferring only to the galactose residue close to the nonreducing end of linear acceptors. On the contrary, the midchain β1,6-GlcNAc transferases accept both GlcNAc- and Gal-terminated substrates, but they work only at midchain galactoses of the acceptor and do not transfer a GlcNAc to the penultimate Gal of linear acceptors of the GlcNAcβ1−3Galβ1−4GlcNAcβ1-R type (Leppänen et al., 1991).

In contrast to those of the singly branched molecules, the pathways leading to biosynthesis of multiply branched poly-*N*-acetyllactosamine backbones are poorly understood. It has been suggested that the distally acting β1,6-GlcNAc transferase is responsible for multiple branching, acting in a concerted fashion with β1,4-galactosyl transferase and β1,3-*N*-acetylglucosaminyltransferase in repeated cycles at sites close to the growing ends of the polylactosamine (Fukuda et al., 1984b; Fukuda, 1985). These enzymes are indeed capable of generating multiply branched polylactosamine backbones *in vitro* (Seppo et al., 1995, 1996; Turunen et al., 1995), but structures containing branched branches are obtained, rather than naturally occurring backbones featuring a long main chain with several short branches. Synthesis of the latter type of backbones by the distally acting enzymes would require that the elongation reactions catalyzed by β1,3-GlcNAc and β1,4-Gal transferases be branch-specific, working at the main chain LacNAc unit but not at the branch LacNAc units. However, this is not the case; in contrast, elongation of the main chain as well as the β1,6-bonded branch is observed when the biantennary hexasaccharide LacNAcβ1−3′(LacNAcβ1−6′)LacNAc is treated with UDP-GlcNAc and the β1,3-GlcNAc transferase of human serum (Vilkman et al., 1992). β1,4-Gal transferase in turn transfers faster to the β1,6-linked GlcNAc than to the β1,3-bonded GlcNAc in the trisaccharide GlcNAcβ1−3(GlcNAcβ1−6)-Gal (Blanken et al., 1982) and in the tetrasaccharide GlcNAcβ1−3(GlcNAcβ1−6)Galβ1−4GlcNAc (Renkonen et al., 1992). Taken together, the reported data suggest that the distally acting β1,6-GlcNAc transferases do not generate the natural type of multiply branched polylactosamine backbones. On the other hand, the midchain enzymes appeared to us to be good candidates for initiating the pathways leading to naturally occurring polylactosamines of multiple branches.

Here, we show that the activities of midchain β1,6-GlcNAc transferase(s) present in the blood serum of rat and other mammalian species, including man, initiate *in vitro* biosynthesis of a prototype of multiply branched backbones of polylactosamines by transferring β1,6-bonded GlcNAc units from UDP-GlcNAc to both of the internal galactoses of the linear hexasaccharide LacNAcβ1−3′LacNAcβ1−3′LacNAc (**1**). The resulting doubly branched octasaccharide LacNAcβ1−3′(GlcNAcβ1−6′)LacNAcβ1−3′(GlcNAcβ1−6′)-LacNAc (**4**) was subsequently converted into the mature backbone decasaccharide LacNAcβ1−3′(LacNAcβ1−6′)-LacNAcβ1−3′(LacNAcβ1−6′)LacNAc (**5**) by *in vitro* incubation with UDP-Gal and β1,4-galactosyltransferase.

The successful synthesis of glycans **4** and **5** has opened up an enzymatic synthesis route to a tetravalent sialyl Lewis X saccharide that proved to represent a nanomolar inhibitor of L-selectin-mediated adhesion of lymphocytes to activated endothelium (Renkonen et al., 1997). In addition, the successful *in vitro* synthesis of glycan **4**, which represents a naturally occurring sequence, suggests that the midchain β1,6-GlcNAc transferase activities present in mammalian blood serum are closely related to the enzymes that are actually responsible for the *in vivo* biosynthesis of multiply branched polylactosamine backbones in Golgi membranes.

EXPERIMENTAL PROCEDURES

Acceptor Oligosaccharides. The unlabeled pentasaccharide GlcNAcβ1−3′LacNAcβ1−3′LacNAc (**6**) was synthesized enzymatically from GlcNAc by stepwise β1,4-galactosylation and β1,3-*N*-acetylglucosaminylation reactions as described in Leppänen et al. (1991). The radiolabeled **6**, GlcNAcβ1−3Galβ1−4GlcNAcβ1−3[^{14}C]Galβ1−4GlcNAc (paper chromatography, $R_{MTet} = 0.90$, $R_{MP} = 1.40$; solvent A), was obtained in the same way, but UDP-[^{14}C]-Gal was used instead of unlabeled UDP-Gal in the first β1,4-galactosylation step.

The unlabeled hexasaccharide LacNAcβ1−3′LacNAcβ1−3′LacNAc (**1**) was obtained by enzymatic β1,4-galactosylation of unlabeled **6** as described in Brew et al. (1968). The doubly labeled **1**, Galβ1−4GlcNAcβ1−3[^{3}H]Galβ1−4GlcNAcβ1−3[^{14}C]Galβ1−4GlcNAc (paper chromatography, $R_{MP} = 0.71$, $R_{MH} = 1.59$; solvent A), was prepared otherwise like the unlabeled hexasaccharide; but UDP-[^{14}C]-Gal was used instead of unlabeled UDP-Gal in the first β1,4-galactosylation step, where GlcNAc served as the acceptor, and UDP-[^{3}H]Gal was used in the second β1,4-galactosylation step, where GlcNAcβ1−3[^{14}C]Galβ1−4GlcNAc served

as the acceptor. The tritium-labeled isotopomer, [^{3}H]Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAc, was obtained by using UDP-[^{3}H]Gal in the galactosylation of unlabeled glycan **6**.

Marker Saccharides. Galβ1−4GlcNAcβ1−3[^{14}C]Galβ1−4GlcNAcβ1−3[^{14}C]Gal was obtained by β1,4-galactosylating GlcNAcβ1−3[^{14}C]Galβ1−4GlcNAcβ1−3[^{14}C]Gal (Leppänen et al., 1991) enzymatically, using UDP-Gal and bovine milk β1,4-galactosyltransferase. In paper chromatography, the product migrated close to maltopentaose marker (R_{MTet} = 0.60, R_{MP} = 0.94, R_{MH} = 2.00; solvent A), and an endo-β-galactosidase treatment cleaved it completely, releasing equal amounts of Galβ1−4GlcNAcβ1−3[^{14}C]Gal and GlcNAcβ1−3[^{14}C]Gal. Radiolabeled Galβ1−4GlcNAcβ1−3Gal was obtained as in Leppänen et al. (1991). Radiolabeled tetrasaccharide GlcNAcβ1−3(GlcNAcβ1−6)Galβ1−4GlcNAc was synthesized as described in Seppo et al. (1990). Radiolabeled GlcNAcβ1−3(GlcNAcβ1−6)Galβ1−4GlcNAcβ1−3Gal was obtained by incubating radiolabeled GlcNAcβ1−3Galβ1−4GlcNAcβ1−3Gal with UDP-GlcNAc and hog gastric mucosal microsomes known to contain distally acting β1,6-GlcNAc transferase (Piller et al., 1984; Seppo et al., 1990). The pentasaccharide product migrated close to maltopentaose marker in paper chromatography (R_{MTet} = 0.69, R_{MP} = 1.09, solvent A). Radiolabeled Galβ1−4GlcNAcβ1−3(GlcNAcβ1−6)Galβ1−4GlcNAc was synthesized by partial β-galactosidase treatment of branched hexasaccharide Galβ1−4GlcNAcβ1−3(Galβ1−4GlcNAcβ1−6)Galβ1−4GlcNAc (Renkonen et al., 1992).

Serum Samples. Bovine, equine, and ovine blood serum samples from freshly drawn blood of healthy adult animals were provided by S. Sankari (Veterinary Faculty, University of Helsinki). Serum of pregnant rats was obtained from H. Rauvala (Institute of Biotechnology, University of Helsinki).

Enzyme-Catalyzed Transferase Reactions. β1,6-GlcNAc transferase reactions with the serum samples were performed essentially as described for human serum (Leppänen et al., 1991). The reactions were carried out in the presence of 20 mM EDTA, which inhibits the serum β1,3-GlcNAc transferase activity (Yates & Watkins, 1983); 200 mM D-galactose and 60 mM γ-galactonolactone were occasionally added to inhibit β-galactosidase activity. All reaction mixtures were desalted by passing them through a mixed bed of Dowex AG 1 (AcO^-) and Dowex AG 50 (H^+) and subsequently lyophilized. When radiolabeled, the products were separated from the acceptors by paper chromatography and identified using degradative endo-β-galactosidase and β-galactosidase treatments as well as partial acid hydrolysis. When unlabeled, the products were separated by gel filtration and HPAEC-PAD and structurally analyzed by using MALDI-TOF mass spectrometry and one-dimensional ^{1}H-NMR spectrometry at 500 MHz.

Relative reactivities of different oligosaccharides as acceptors for the β1,6-GlcNAc transferase in rat serum were measured in parallel experiments with equimolar samples of the reference pentasaccharide **6**. The reactions were carried out using 5.2 pmol of radiolabeled acceptors, 6.8 μmol of UDP-GlcNAc, and 50 μL of rat serum. D-Galactose (20 mM) was present in the reaction mixtures, and the samples were incubated at 37 °C for 2 d.

Galactosylation with bovine milk β1,4-galactosyltransferase (EC 2.4.1.90) from Sigma (St. Louis, MO) was carried out essentially as described by Brew et al. (1968).

Chromatographic Methods. Paper chromatographic runs of desalted radiolabeled saccharides were performed on Whatman III Chr paper with 1-butanol/ethanol/water (10:1:2 v/v, solvent E) or the upper phase of 1-butanol/acetic acid/water (4:1:5 v/v, solvent A). Radioactivity was monitored as in Renkonen et al. (1989), using Optiscint (Wallac, Turku, Finland) as the scintillant. Marker lanes, containing appropriate unlabeled maltooligosaccharides and lactose on both sides of the sample lane, were stained with silver nitrate.

Affinity chromatography on immobilized wheat germ agglutinin (WGA) was carried out as described (Renkonen et al., 1991c).

Gel permeation chromatography on a column of Bio-Gel P-4 (Bio-Rad, Richmond, CA) (1 × 145 cm) was carried out with water, collecting fractions of 1.7 mL and monitoring UV absorbance at 214 nm. Gel permeation chromatography on a column of Superdex 75 HR (10 × 300 mm) (Pharmacia, Uppsala, Sweden) was performed as in Maaheimo et al. (1994).

HPAE chromatography with PAD detection was carried out with a Dionex Series 4500i HPLC system (Dionex, Sunnyvale, CA) equipped with a CarboPac PA-1 column (4 × 250 mm) preceded by a CarboPac PA-1 quard column, at a flow rate of 1 mL/min. The column was equilibrated with 100 mM NaOH, and the run was performed by increasing the sodium-acetate concentration by 1 mM/min in 100 mM NaOH. The samples were chromatographed in small batches (less than 120 nmol) due to the limited capacity of the column. The eluted samples were rapidly neutralized with 0.4 M acetic acid and desalted in a Dowex AG 50 (H^+) column (0.7 × 4 cm).

Degradative Experiments. Partial acid hydrolysis of a doubly labeled sample of the octasaccharide **4** (51 000 cpm ^{3}H, 33 000 cpm ^{14}C) was carried out using 0.1 M trifluoroacetic acid at 100 °C essentially as described in Seppo et al. (1990).

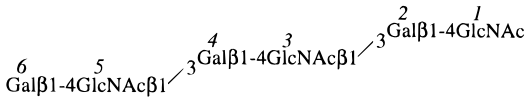
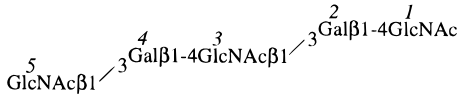
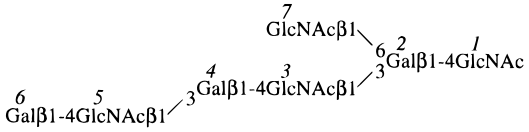
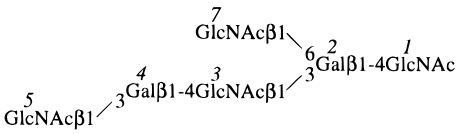
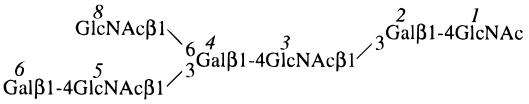
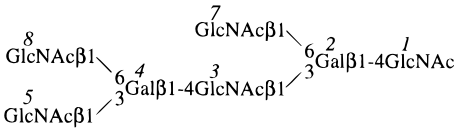
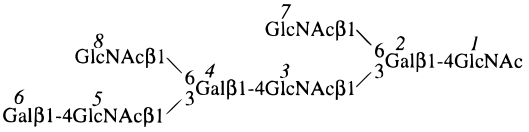
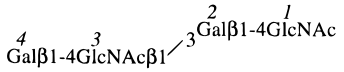
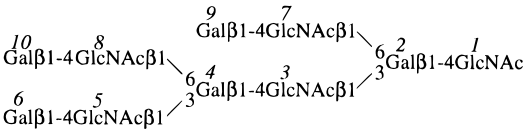
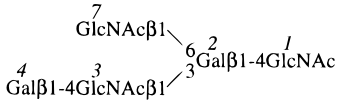
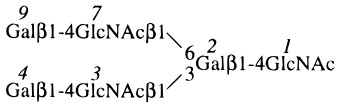
Digestions with endo-β-galactosidase from *Bacteroides fragilis* (EC 3.2.1.103) (Boehringer Mannheim, Germany) were performed according to Leppänen et al. (1991). Parallel control digestions cleaved GlcNAcβ1−3Galβ1−4GlcNAc completely.

Exhaustive digestions with jack bean β-*N*-acetylhexosaminidase (EC 3.2.1.30) (Sigma) and jack bean β-galactosidase (EC 3.2.1.23) (Sigma) were carried out as described in Leppänen et al. (1991) and Renkonen et al. (1991a), respectively. Partial hydrolysis with β-*N*-acetylhexosaminidase was performed as described in Renkonen et al. (1991b).

NMR Spectroscopy. One-dimensional ^{1}H-NMR spectroscopy of the oligosaccharides was performed in ^2H_2O at 500 MHz on a Varian Unity 500 spectrometer. Prior to NMR experiments, the saccharides were twice lyophilized from ^2H_2O and then dissolved in 600 μL of ^2H_2O (99.996 at. %, Cambridge Isotope Laboratories, Woburn, MA). The spectra were recorded at 23 °C using a modification of the WEFT sequence for water suppression as described in Hård et al. (1992). The ^{1}H chemical shifts were referenced to internal acetone, 2.225 ppm.

Matrix-Assisted Laser Desorption-Ionization Mass Spectrometry. Matrix-assisted laser desorption-ionization (MALDI) mass spectrometry was performed with the Vision 2000 reflectron time-of-flight (TOF) instrument (Finnigan MAT, Hemel, Hempstead, U.K.). It was operated in the positive ion mode, with an accelerating voltage of 5 kV, with

Table 1: Structures of the Key Saccharides and Denotation of Monosaccharide Residues

No	Saccharide	No	Saccharide
1		**6**	
2		**7**	
3		**8**	
4		**9**	
5		**10**	
		11	

irradiation at 337 nm (3 ns pulse width) from a nitrogen laser (Laser Science Inc., Newton, MA). The signal from the secondary electron multiplier was acquired at 500 MHz. The matrix was 2,5-dihydroxybenzoic acid (10 g/L) in water. For each experiment, an aliquot of the sample solution corresponding to 2−5 pmol of oligosaccharide was mixed with 1.5 μL of the matrix solution on the stainless steel sample probe and air-dried. External calibration was used; this method has an accuracy of ±0.1% (±1 unit at *m/z* 1000).

For decasaccharide **5** and pentasaccharide **6**, MALDI-TOF analysis was performed with a BIFLEX mass spectrometer (Bruker-Franzen Analytik, Bremen, Germany). Experimental procedures identical to those described above were employed. Dextran standard 5000 from *Leuconostoc mesentroides* (Fluka Chemica-Biochemica) was used as external calibrant.

RESULTS

Table 1 shows the structures of the oligosaccharides of the present experiments and the numbering of the monosaccharide residues.

*Branching Reaction of Hexasaccharide **1** Catalyzed by β1,6-GlcNAc Transferase Activity Present in Rat Serum*. The linear radiolabeled hexasaccharide [^{3}H]Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAc (**1**) gave two isomers of heptasaccharides with single branches (**2** and **3**), together with a doubly branched octasaccharide (**4**), when it was incubated with UDP-GlcNAc and the β1,6-GlcNAc transferase activity present in rat serum under conditions promoting a partial reaction (Figure 1A). Endo-β-galactosidase hydrolysis was used initially to establish the presence of the branches of **2** and **3**; this enzyme does not cleave internal β-galactosidic bonds of LacNAc units bearing branches at position 6′, although it cleaves internal β-galactosidic linkages of most other LacNAc residues of polylactosamines (Scudder et al., 1984). The hydrolysate of the heptasaccharide mixture of Figure 1A contained two radiolabeled cleavage products, the hexasaccharide [^{3}H]Galβ1−4GlcNAcβ1−3(GlcNAcβ1−6)Galβ1−4GlcNAcβ1−3Gal and the trisaccharide [^{3}H]Galβ1−4GlcNAcβ1−3Gal, which were identified by paper chromatography (Figure 1B). The results suggested that 43% of the heptasaccharide mixture consisted of **2** and 57% of **3**. ^{3}H-labeled terminal galactose was released as free [^{3}H]Gal when **2** + **3** and **4** were separately incubated with β-galactosidase, confirming that terminal galactose residue in **1** had not accepted a β1,6-GlcNAc unit during the reaction (data not shown).

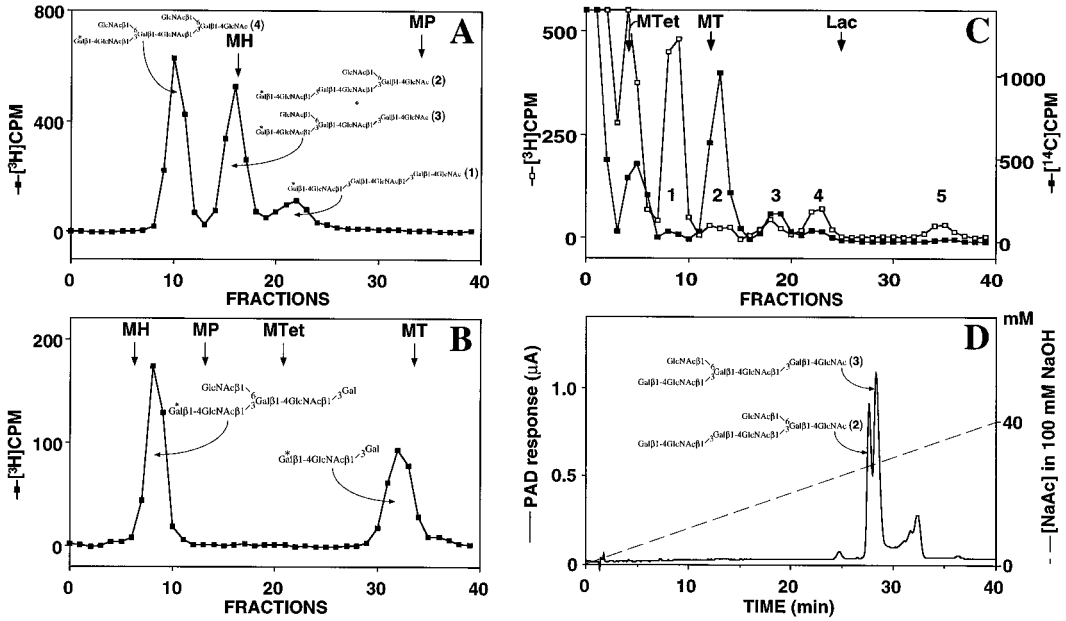


FIGURE 1: Experiments related to the branching reaction of hexasaccharide **1**, catalyzed by rat serum β1,6-GlcNAc transferase activity. (A) Paper chromatography of the products from a partial reaction of radiolabeled hexasaccharide **1** [solvent A, 329 h; unlabeled markers MP (maltopentaose) and MH (maltoheptaose)]. The asterisk shows the position of the radiolabel. (B) Paper chromatography (solvent A for 122 h; unlabeled markers MT, MTet, MP, and MH are maltotriose, -tetraose, -pentaose, and -heptaose, respectively) of an endo-β-galactosidase digest of the radiolabeled heptasaccharides **2** and **3** (from panel A). The asterisk shows the position of the radiolabel. The identity of the branched hexasaccharide [^{3}H]Galβ1−4GlcNAcβ1−3(GlcNAcβ1−6)Galβ1−4GlcNAcβ1−3Gal was confirmed in a separate experiment, where it was converted by a β-*N*-acetylhexosaminidase treatment into a product cochromatographing with the radiolabeled Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAcβ1−3Gal marker. (C) Paper chromatography [solvent E, 14 d; unlabeled markers as in panel B, and additionally Lac (lactose)] of a partial acid hydrolysate of the doubly labeled octasaccharide Galβ1−4GlcNAcβ1−3(GlcNAcβ1−6)[^{3}H]-Galβ1−4GlcNAcβ1−3(GlcNAcβ1−6)[^{14}C]Galβ1−4GlcNAc (**4**). Peak 1 contained Galβ1−4GlcNAcβ1−3[^{3}H]Gal and GlcNAcβ1−3(GlcNAcβ1−6)[^{3}H]Gal together with GlcNAcβ1−3(GlcNAcβ1−6)[^{14}C]Gal. Peak 2 represented GlcNAcβ1−6[^{14}C]Galβ1−4GlcNAc, the most prominent single cleavage product of small size. Peak 3 represented GlcNAcβ1−3[^{14}C]Galβ1−4GlcNAc and GlcNAcβ1−3[^{3}H]-Galβ1−4GlcNAc. Peak 4 was GlcNAcβ1−6[^{14}C]Gal and GlcNAcβ1−6[^{3}H]Gal. Peak 5 contained GlcNAcβ1−3[^{14}C]Gal and GlcNAcβ1−3[^{3}H]Gal. (D) Preparative scale separation of the isomeric heptasaccharides **2** and **3** on a CarboPac PA-1 column in a HPAEC-PAD chromatographic experiment. The dashed line indicates the rise of the sodium acetate concentration in the eluant. The heptasaccharide **2** at 27.6 min and the heptasaccharide **3** at 28.3 min were separated surprisingly clearly. The peak at 24.7 min represented the linear hexasaccharide **1**, while the peaks eluting between 30 and 33 min contained the octasaccharide **4** as well as the reducing-end ManNAc epimers of **2** and **3**. The latter ones were probably formed nonenzymatically under the alkaline conditions prevailing during the HPAE chromatographic run.

The presence of GlcNAcβ1−6Gal linkages at both internal galactoses of **4** was proven by synthesizing a doubly labeled isotopomer of glycan **1** (Galβ1−4GlcNAcβ1−3[^{3}H]Galβ1−4GlcNAcβ1−3[^{14}C]Galβ1−4GlcNAc) and by converting it into the doubly branched octasaccharide **4** [Galβ1−4GlcNAcβ1−3(GlcNAcβ1−6)[^{3}H]Galβ1−4GlcNAcβ1−3(GlcNAcβ1−6)[^{14}C]Galβ1−4GlcNAc], which was subjected to partial acid hydrolysis. The hydrolysate was fractionated by paper chromatography as shown in Figure 1C. It revealed identifiable small cleavage products, which contained GlcNAcβ1−6[^{14}C]Gal and GlcNAcβ1−6[^{3}H]Gal sequences. To further confirm the identification of the diagnostic cleavage products, the mixed disaccharide isotopomers of GlcNAcβ1−6[^{14}C]Gal and GlcNAcβ1−6[^{3}H]Gal were subjected to affinity chromatography on a column of immobilized wheat germ agglutinin (WGA), which retarded both isotopomers in a way that is highly characteristic of GlcNAcβ1−6Gal (Renkonen et al., 1991c) (not shown). Even the trisaccharide isotopomers GlcNAcβ1−3(GlcNAcβ1−6)[^{14}C]Gal and GlcNAcβ1−3(GlcNAcβ1−6)[^{3}H]Gal cochromatographed in the WGA−agarose column with authentic GlcNAcβ1−3(GlcNAcβ1−6)Gal marker in a highly characteristic manner (not shown). GlcNAcβ1−6[^{14}C]-Galβ1−4GlcNAc, in turn, was converted completely to [^{14}C]-Galβ1−4GlcNAc by a treatment with jack bean β-*N*-acetylhexosaminidase (not shown). The ^{14}C-labeled products of partial acid hydrolysis established the presence of a β1,6-linked GlcNAc residue at the reducing end LacNAc unit of the octasaccharide, while the ^{3}H-labeled cleavage products established the presence of another similar residue at the middle LacNAc unit. Together with the absence of other radiolabeled cleavage products of small size, the data establish firmly the structure of the octasaccharide as Galβ1−4GlcNAcβ1−3(GlcNAcβ1−6)[^{3}H]Galβ1−4GlcNAcβ1−3(GlcNAcβ1−6)[^{14}C]Galβ1−4GlcNAc (**4**).

In a preparative scale experiment with UDP-GlcNAc and rat serum, an unlabeled sample of 438 nmol of the linear hexasaccharide acceptor **1** yielded 254 nmol of the doubly branched octasaccharide **4** and 78 nmol of a mixture of the singly branched heptasaccharides **2** and **3**. The products were isolated by gel filtration followed by HPAEC-PAD (Figure 1D). To assign the heptasaccharide peaks, an analogous branching reaction was performed with 22 nmol of ^{3}H-labeled acceptor **1**, the resulting heptasaccharide peaks were isolated and were cleaved separately with endo-β-galactosidase, and the hydrolysates were analyzed by paper chromatography as in Figure 1B.

In the MALDI-TOF mass spectrum of the octasaccharide **4** (not shown), the measured value for the monoisotopic ^{12}C species of the molecular ion $(M + Na)^+$ *m/z* was 1542.1 (calcd *m/z* of 1542.6). No major impurity signals were visible in the mass range appropriate for oligosaccharide molecular ions. When examined on a MALDI-TOF instrument, oligosaccharide $(M + Na)^+$ ions with masses greater

Table 2: MALDI-TOF-MS Data[a]

saccharide	assignment	calcd *m/z*	obs *m/z*
1	$(M + Na)^+$	1136.4	1136.2
2	$(M + Na)^+$	1339.5	1339.2
3	$(M + Na)^+$	1339.5	1339.1
4	$(M + Na)^+$	1542.6	1542.1
5	$(M + Na)^+$	1867.7	1868.2
6	$(M + Na)^+$	974.9	974.8
7	$(M + Na)^+$	1177.4	1177.1
8	$(M + Na)^+$	1381.3	1381.3

[a] All values are monoisotopic except those for saccharides **5**, **6**, and **8** which represent average molecular masses.

than 1000 Da exhibit similar signal strengths irrespective of structure (Naven & Harvey, 1996). Hence, our data confirmed that octasaccharide **4** was highly pure. MALDI-TOF mass spectra (not shown) for the heptasaccharides **2** and **3** revealed that they, too, were pure and of the expected size (Table 2).

The 500 MHz ^{1}H-NMR spectra of the acceptor **1** and the products **2**−**4** fully confirmed the structures predicted from the degradative tracer experiments (Table 3, Figure 2). The H-1 proton doublets of the two β1,6-GlcNAc residues of **4** were distinct and could be assigned via the corresponding resonances of the singly branched heptasaccharides **2** and **3**. The chemical shift of H-1 of residue 7 in octasaccharide **4** (4.585 ppm) was identical to that of the corresponding residue in pentasaccharide **10**, while the H-1 of residue 8 resonated at a somewhat lower field at 4.593 ppm. The H-1 signals of the analogous (=underlined) GlcNAc units in the hexasaccharide GlcNAcβ1−3(GlcNAcβ1−6)Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAc (J. Helin et al., unpublished experiments) and in the decasaccharide GlcNAcβ1−3(GlcNAcβ1−6)Galβ1−4GlcNAcβ1−3[GlcNAcβ1−3(GlcNAcβ1−6)Galβ1−4GlcNAcβ1−6]Galβ1−4GlcNAc (Seppo et al., 1995), too, resonate at 4.593 ppm. In comparison to glycan **1**, the H-1 and H-4 resonances of the branch-bearing galactose residues of glycans **2**−**4** revealed consistent upfield shifts, similar to those of the GlcNAcβ1−3(GlcNAcβ1−6)Galβ1−4GlcNAc sequence (Koenderman et al., 1987). Marked downfield shifts of the H-1 resonances of the reducing end GlcNAc were regularly associated with the transfer of a β1,6-bonded GlcNAc to galactose residue 2.

Both heptasaccharide isomers **2** and **3** were separately converted into **4** in experiments involving a repeated incubation of **2** and **3** with UDP-GlcNAc and rat serum (data not shown). This establishes that **4** was generated via two different pathways, **1** → **2** → **4** and **1** → **3** → **4**, as shown in Scheme 1.

*β1,4-Galactosylation of the Octasaccharide **4**.* A sample (84 nmol) of the branched octasaccharide **4** was β1,4-galactosylated with bovine milk β1,4-galactosyltransferase. The oligosaccharides of the reaction mixture (65 nmol) were isolated by gel permeation chromatography on a Superdex 75 HR column. The MALDI-TOF mass spectrum of the oligosaccharide products revealed that octasaccharide **4** had been completely converted into the decasaccharide **5** (Figure 3, Table 2). The ^{1}H-NMR spectrum of **5** revealed a doublet of two protons at 4.465 ppm (Table 3, Figure 2), which are assigned to H-1's of galactose residues 9 and 10 in accordance with the analogous H-1 resonances of residue 9 in glycan **11** (Niemelä et al., 1995a; Maaheimo et al., 1997). The H-1's of β1,6-bonded GlcNAc residues 7 and 8 in decasaccharide **5** again revealed distinct chemical shifts at 4.624 and 4.639 ppm, respectively. They were assigned by comparison with the H-1 resonance of GlcNAc 7 in glycan **11** and with the H-1 resonance of the ^{6,3}GlcNAc unit (underlined) in the tetradecamer Galβ1−4GlcNAcβ1−3(Galβ1−4GlcNAcβ1−6)Galβ1−4GlcNAcβ1−3[Galβ1−4GlcNAcβ1−3(Galβ1−4GlcNAcβ1−6)Galβ1−4GlcNAcβ1−6]Galβ1−4GlcNAc (Seppo et al., 1995).

*Branching Reactions of the Hexasaccharide **1** Catalyzed by β1,6-GlcNAc Transferase Activities Present in Serum of Other Mammalian Species.* Treatment of the linear hexasaccharide **1** with UDP-GlcNAc and the blood serum of man, cow, horse, or sheep yielded also the branched products **2**−**4**, which were identified by paper chromatography and by incubations with exo- and endo-β-galactosidases. In a typical reaction, radiolabeled hexasaccharide **1** was subjected to the branching reaction with human serum that had been concentrated 2.6-fold by ultrafiltration. Paper chromatography of

Table 3: ^{1}H Chemical Shifts of Saccharides **1**−**11** at 23 °C in ^2H_2O

		saccharide										
reporter group	residue[a]	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**[b]	**10**[b]	**11**[c]
H-1	1	5.204(α) 4.719(β)	5.211α 4.730β	5.204α 4.720β	5.210α 4.730β	5.209α 4.726β	5.204α 4.720β	5.211α 4.730β	5.212α 4.730β	5.205α 4.721β	5.212α 4.731β	5.208α 4.725β
	2[d]	4.464	4.452	4.465	4.456	4.458	4.467	4.452	4.456	4.465 4.462	4.454	4.457
	3[d]	4.702	4.702 4.697	4.710 4.703	4.703 4.696	4.702 4.696	4.703 4.698	4.697 4.692	4.703 4.696	4.707 4.703	4.701 4.696	4.700 4.695
	4	4.464	4.466	4.456	4.456	4.458	4.467	4.468	4.456	4.480	4.481	4.480
	5	4.702	4.702	4.695	4.696	4.696	4.680	4.681	4.676	–	–	–
	6	4.479	4.480	4.480	4.480	4.480	–	–	–	–	–	–
	7[d]	–	4.585	–	4.585	4.624	–	4.584	4.585	–	4.585	4.624 4.618
	8	–	–	4.593	4.593	4.639	–	–	4.593	–	–	–
	9[d]	–	–	–	–	4.465	–	–	–	–	–	4.464 4.467
	10	–	–	–	–	4.465	–	–	–	–	–	–
H-4	2	4.157	4.144	4.155	4.148	4.148	4.154	4.146	4.143	4.159	4.149	4.151
	4	4.157	4.157	4.149	4.148	4.148	4.154	4.153	4.143	nd	nd	3.924
	9	–	–	–	–	nd[e]	–	–	–	–	–	3.924

[a] For saccharide numbers and residue denotation, see Table 1. [b] Niemelä et al. (1995b). [c] Niemelä et al. (1995a). [d] The two values given correspond to the two anomers of the oligosaccharide. [e] nd, not determined.

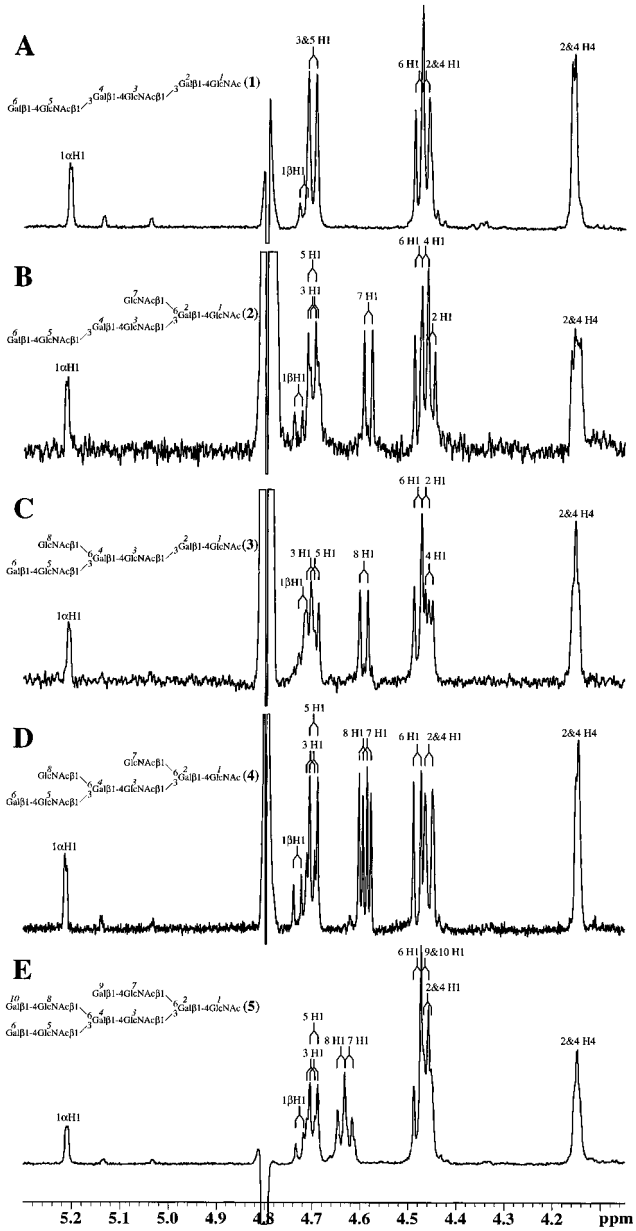


FIGURE 2: Anomeric proton regions of 500 MHz ^{1}H-NMR spectra at 23 °C in ^2H_2O: (A) hexasaccharide **1** (400 nmol), (B) heptasaccharide **2** (26 nmol), (C) heptasaccharide **3** (26 nmol), (D) octasaccharide **4** (254 nmol), (E) decasaccharide **5** (66 nmol).

the reaction mixture revealed the presence of **4** in 7.3% yield and a mixture of **2** and **3** in 36.6% yield (data not shown). Analysis of the mixture of **2** and **3** by endo-β-galactosidase cleavage and subsequent paper chromatography revealed that 46% of the mixture consisted of **2** and 54% of **3**. The experiments conducted with serum from cow, horse, and sheep gave analogous results. Taken together, this series of experiments establishes the presence of midchain β1,6-GlcNAc transferase activity in serum of all mammalian species tested.

*Branching Reaction of Pentasaccharide **6** Catalyzed by β1,6-GlcNAc Transferase Activity Present in Rat Serum.* The linear pentasaccharide GlcNAcβ1−3Galβ1−4GlcNAcβ1−3[^{14}C]Galβ1−4GlcNAc (**6**) gave the singly branched hexasaccharide **7** and a small amount of the doubly branched heptasaccharide **8** upon incubation with UDP-GlcNAc and rat serum (Figure 4A). Structural characterization of **7** was performed with endo-β-galactosidase hydrolysis (Figure 4B). A single labeled product was formed; it was identified as the tetrasaccharide GlcNAcβ1−3(GlcNAcβ1−6)[^{14}C]Galβ1−4GlcNAc, indicating that only the hexasaccharide **7** was present in the hexasaccharide fraction. The pentasaccharide GlcNAcβ1−3(GlcNAcβ1−6)Galβ1−4GlcNAcβ1−3[^{14}C]-Gal (expected position in solvent A paper chromatography, $R_{MTet} = 0.69$, $R_{MP} = 1.09$) was not seen in the experiment of Figure 4B; its absence indicated that, during the synthesis of **7**, the isomeric hexasaccharide GlcNAcβ1−3(GlcNAcβ1−6)Galβ1−4GlcNAcβ1−3[^{14}C]Galβ1−4GlcNAc was not formed. Hence, the β1,6-GlcNAc transferase activity present in rat serum generated from glycan **6** the very same hexasaccharide **7** that was formed by the midchain β1,6-GlcNAc transferase activity present in human serum (Leppänen et al., 1991).

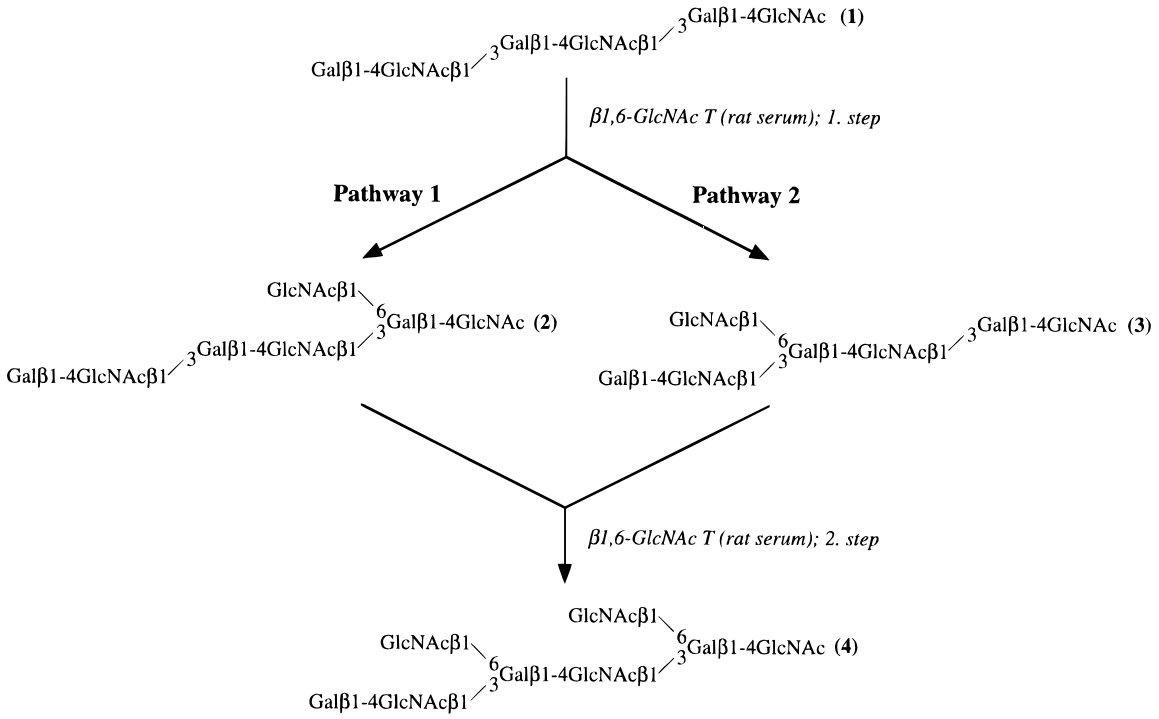
The doubly branched heptasaccharide **8** was formed also from isolated **7** upon reincubation with UDP-GlcNAc and rat serum (not shown); it was resistant to endo-β-galactosidase. Glycans **7** and **8** were obtained also in a preparative scale branching reaction of **6** and were isolated using gel filtration or HPAEC (not shown). The one-dimensional ^{1}H-NMR spectra of the acceptor **6** and the products **7** and **8** (Figure 5, Table 3) fully confirmed the structures predicted from the degradative tracer experiments. In MALDI-TOF mass spectra of **6**−**8**, the observed *m/z* values for major peaks, assigned to $(M + Na)^+$, were quite close to the calculated values (Table 2). As evaluated from the MALDI-TOF mass spectra, the purity of **6**−**8** was more than 90%.

It appears that the GlcNAc branch in glycan **7** induced some reactivity of the galactose residue 4 (see Table 1 for denotation of the monosaccharide residues), making possible the partial reaction **7** → **8** that was repeatedly observed, while the analogous reaction **6** → GlcNAcβ1−3′(GlcNAcβ1−6′)-LacNAcβ1−3′LacNAc was not detected. However, it is not clear at present whether the reactions **6** → **7** and **7** → **8** were catalyzed by one or several activities present in rat serum.

*Branching Reactions of the Pentasaccharide **6** Catalyzed by β1,6-GlcNAc Transferase Activities Present in Serum of Other Mammalian Species.* Branching reactions of the linear radiolabeled pentasaccharide GlcNAcβ1−3Galβ1−4GlcNAcβ1−3[^{14}C]Galβ1−4GlcNAc (**6**) catalyzed by bovine, equine, and ovine blood serum gave also the singly branched glycan **7** as the only hexasaccharide product. This was established in each case by paper chromatography of the product and its endo-β-galactosidase digestion, followed by chromatographic identification of the sole cleavage product as the tetrasacccharide GlcNAcβ1−3(GlcNAcβ1−6)[^{14}C]Galβ1−4GlcNAc (not shown).

*Branching Reaction of Tetrasaccharide **9** Catalyzed by β1,6-GlcNAc Transferase Activity Present in Rat Serum.* The radiolabeled tetrasaccharide **9** (Galβ1−4GlcNAcβ1−3[^{14}C]-Galβ1−4GlcNAc) gave the branched pentasaccharide Galβ1−4GlcNAcβ1−3(GlcNAcβ1−6)[^{14}C]Galβ1−4GlcNAc (**10**) upon incubation with UDP-GlcNAc and rat serum (Figure 6). β-Galactosidase cleaved **10** into the tetrasaccharide GlcNAcβ1−3(GlcNAcβ1−6)[^{14}C]Galβ1−4GlcNAc, showing that terminal galactose residue in **9** had not accepted a β1,6-GlcNAc unit during the branching reaction (not shown). Glycan **10** resisted the action of endo-β-galactosidase, which confirmed the presence of the β1,6-bonded GlcNAc branch (Scudder et al., 1984). The NMR spectra of **9** and **10**, which have been reported elsewhere (Niemelä et al., 1995b; Maaheimo et al., 1997), revealed that the presence of the β1,6-bonded GlcNAc at the reducing end LacNAc caused a

Scheme 1: Two Equally Prominent Pathways, Leading from 1 to 4, Observed during *in Vitro* Incubation of UDP-GlcNAc, **1**, and Rat Serum[a]



[a] The β1,6-GlcNAc transferase reactions along the two pathways were also catalyzed by serum of other mammalian species, including man. It is not clear whether one or several distinct enzymes are responsible for the reactions shown.

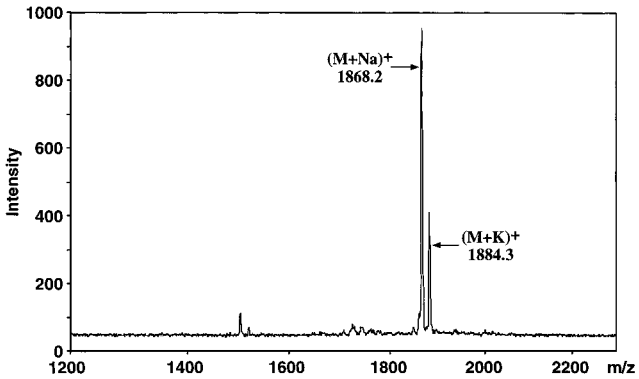


FIGURE 3: MALDI-TOF mass spectrum of the decasaccharide **5**. The two major signals are assigned to molecular ions $(M + Na)^+$ (calculated *m/z* for $Gal_5GlcNAc_5$ = 1867.7) and $(M + K)^+$ (calculated *m/z* = 1883.8). A relatively pure sample is indicated by the scarcity of other signals.

distinct change in the chemical shifts of the H-1 proton of GlcNAc residue 1 (Table 3). This suggests the presence of some interaction between GlcNAc residues 1 and 7 in glycan **10** and likewise in glycans **2**, **4**, **7**, and **8** (see Table 3).

Relative Reactivites of Acceptor Saccharides with Rat Serum. To define the minimal sequence capable of efficient backbone branching, a number of radiolabeled saccharides were compared as acceptors in the reaction catalyzed by rat serum that is rich in midchain β1,6-transferase activity. Table 4 shows that the relative reactivity of GlcNAcβ1−3Galβ1−4GlcNAc was much smaller than that of the tetrasaccharide Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAc. Even the trisaccharide Galβ1−4GlcNAcβ1−3Gal and the tetrasaccharide GlcNAcβ1−3Galβ1−4GlcNAcβ1−3Gal were poor acceptors. Taken together, the data of Table 4 suggest that the tetrasaccharide Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAc (**9**), comprised of two complete LacNAc residues, is the optimal

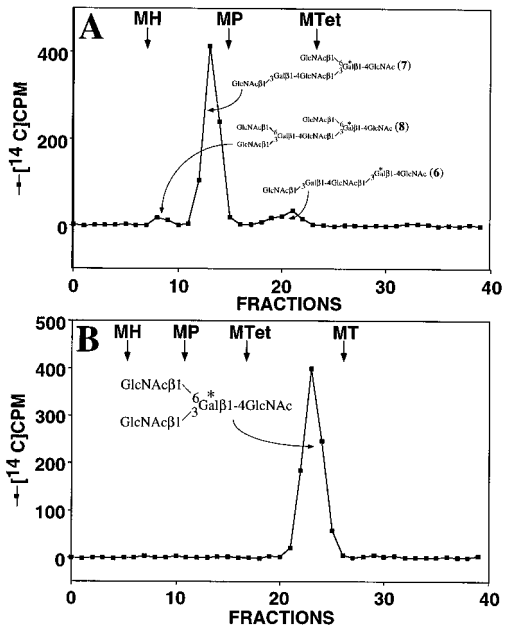


FIGURE 4: Experiments related to the branching reaction of pentasaccharide **6**, catalyzed by rat serum β1,6-GlcNAc transferase activity. (A) Paper chromatography of the products from a reaction of radiolabeled pentasaccharide **6** (solvent A for 115 h; unlabeled markers as in Figure 1B). The asterisk shows the position of the radiolabel. (B) Paper chromatography (solvent A, 69 h; unlabeled markers as in Figure 1B) of the products from an endo-β-galactosidase digestion of the radiolabeled hexasaccharide **7** from panel A. The radiolabeled product cochromatographed with an authentic GlcNAcβ1−3(GlcNAcβ1−6)Galβ1−4GlcNAc marker and gave the diagnostic trisaccharides GlcNAcβ1−6[^{14}C]Galβ1−4GlcNAc and GlcNAcβ1−3[^{14}C]Galβ1−4GlcNAc upon a separate cleaving experiment involving partial hydrolysis of the tetrasaccharide with jack bean β-*N*-acetylhexosaminidase (not shown).

small substrate. The relative reactivities of the two midchain galactoses of the linear hexasaccharide Galβ1−4GlcNAcβ1−

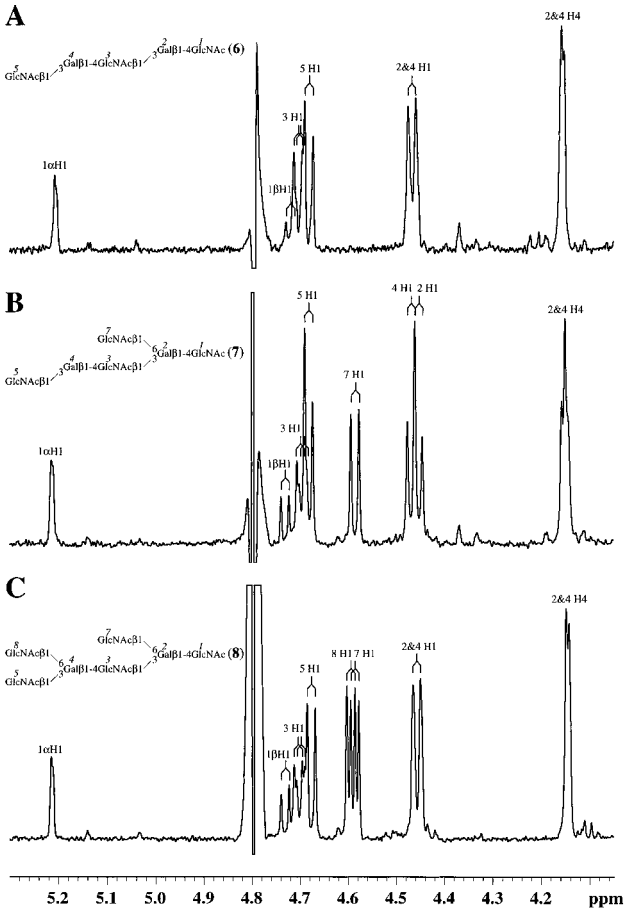


FIGURE 5: Anomeric proton regions of 500 MHz ^{1}H-NMR spectra at 23 °C: (A) pentasaccharide **6** (314 nmol), (B) hexasaccharide **7** (96 nmol), (C) heptasaccharide **8** (39 nmol).

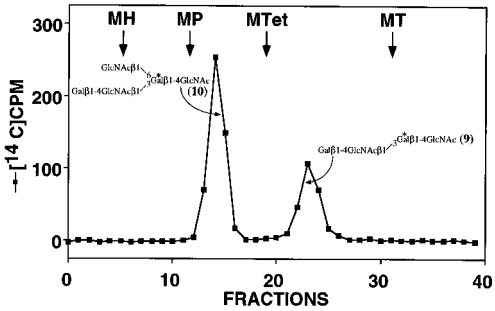


FIGURE 6: Paper chromatography (solvent A for 133 h; unlabeled markers as in Figure 1B) of the products from a partial branching reaction of the radiolabeled tetrasaccharide **9**, catalyzed by rat serum β1,6-GlcNAc transferase activity. The pentasaccharide product cochromatographed with an authentic sample of **10**.

3Galβ1−4GlcNAcβ1−3Galβ1−4GlcNAc (**1**) were 60−75% of that in the tetrasaccharide **9**. β1,6-GlcNAc transferase activity present in human serum resembled closely that of rat serum in substrate specificity.

DISCUSSION

The present data show that β1,6-GlcNAc transferase activity present in rat serum catalyzed the formation of two adjacent branches in the linear hexasaccharide LacNAcβ1−3′LacNAcβ1−3′LacNAc (**1**), generating eventually the doubly branched octasaccharide product LacNAcβ1−3′-(GlcNAcβ1−6′)LacNAcβ1−3′(GlcNAcβ1−6′)LacNAc (**4**) along two distinct pathways (Scheme 1). Primarily, two isomeric heptasaccharides (**2** and **3**) were formed, which

Table 4: Relative Reactivities of Different Oligosaccharides during β1,6-GlcNAc Transferase Reactions

Primer and acceptor site (*)	Relative reactivity [a]	
	Rat serum	Human serum
GlcNAcβ1-3(*)Gal	0	-
GlcNAcβ1-3(*)Galβ1-4GlcNAc	5	2 [b]
Galβ1-4GlcNAcβ1-3(*)Gal	20	-
Galβ1-4GlcNAcβ1-3(*)Galβ1-4GlcNAc	200	160
GlcNAcβ1-3Galβ1-4GlcNAcβ1-3(*)Galβ1-4GlcNAc	**100**	**100** [b]
GlcNAcβ1-3(*)Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc	0	0 [b]
GlcNAcβ1-3Galβ1-4GlcNAcβ1-3(*)Gal	12 [c]	6 [b,c]
Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAcβ1-3(*)Galβ1-4GlcNAc	120 [d]	120 [e]
Galβ1-4GlcNAcβ1-3(*)Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc	150 [d]	140 [e]

[a] Relative reactivities of the oligosaccharides were compared in parallel experiments with equimolar samples of the reference pentasaccharide GlcNAcβ1-3Galβ1-4GlcNAcβ1-3Galβ1-4GlcNAc, which reacts solely at the reducing end LacNAc unit with rat serum (the present data) as well as with human serum (Leppänen et al., 1991). [b] Data from Leppänen et al. (1991). [c] The location of the branch was not proven. [d,e] The data were calculated by taking into account the fact that the octasaccharide derived from the branching experiment carried branches at galactose 2 and galactose 4, while in the heptasaccharides, the branches were present at either galactose 2 ([d]43%, [e]46%) or galactose 4 ([d]57%, [e]54%) (see Table 1 for the numbering of the monosaccharide residues).

carried the β1,6-bonded GlcNAc branch at different sites of the acceptor chain. During the second stage of the reaction, the two heptasaccharides were separately converted into the doubly branched octasaccharide **4**.

It appears that the first stage of the branching reaction, giving rise to two distinct products, involved two different modes of binding of the hexasaccharide acceptor to the catalytic site of the transferase. In both ways of binding, a complete tetrasaccharide LacNAcβ1−3′LacNAc sequence of the acceptor was probably involved, as shown in Figure 7. This notion is supported by the poor reactivity of the trisaccharides GlcNAcβ1−3′LacNAc and LacNAcβ1−3Gal as well as that of the tetrasaccharide GlcNAcβ1−3′LacNAcβ1−3Gal and by the good reactivity of the tetrasaccharide LacNAcβ1−3′LacNAc (Table 4). Even the pentasaccharide GlcNAcβ1−3′LacNAcβ1−3′LacNAc (**6**) appeared to bind effectively to the catalytic site of the enzyme by the LacNAcβ1−3′LacNAc sequence, but not by the GlcNAcβ1−3′LacNAc sequence at the nonreducing end; the primary branch formation occurred solely at galactose 2 of glycan **6** (for numbering of the monosaccharide residues, see Table 1).

β1,6-GlcNAc transferase activities present in human, bovine, equine, and ovine blood serum were similar to that

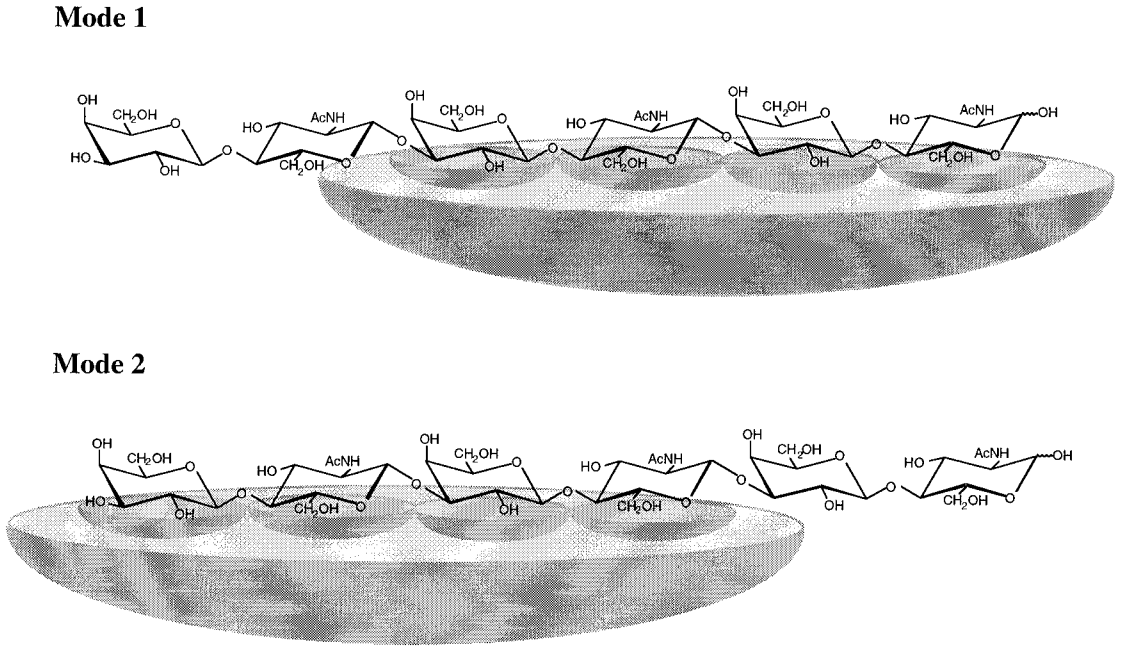


FIGURE 7: Postulated bimodal binding of the hexasaccharide acceptor LacNAcβ1−3′LacNAcβ1−3′LacNAc (**1**) to the catalytic site of the midchain β1,6-GlcNAc transferases present in mammalian blood serum. The binding mode 1 leads to branch formation at galactose residue 2, while the binding mode 2 yields a branch at galactose 4. It is not clear whether one type of enzyme or two distinct enzymes are involved.

in rat serum. They all catalyzed the transfer of a β1,6-bonded GlcNAc to both internal galactose residues of the hexasaccharide **1**, as well as the selective transformation of the pentasaccharide **6** into the singly branched hexasaccharide **7**. In addition, the human serum activity resembled strikingly the rat serum activity in substrate specificity (Table 4); both worked well with the tetrasaccharide LacNAcβ1−3′LacNAc but poorly with GlcNAcβ1−3′LacNAc, LacNAcβ1−3Gal, and GlcNAcβ1−3′LacNAcβ1−3Gal. Hence, our data suggest that the midchain β1,6-GlcNAc transferases of all five mammalian sera bind hexasaccharide **1** in the bimodal manner depicted in Figure 7.

In the β1,6-GlcNAc transferase reactions catalyzed by rat and human serum, the primary branch formation at either of the acceptor sites of **1** was not excessively inhibited in comparison to that of the tetrasaccharide LacNAcβ1−3′LacNAc (see Table 4). Nor did the first GlcNAc branch transferred to **1** prevent the secondary branching reactions, which led to the doubly branched octasaccharide **4**. These data suggest that even longer polylactosamine acceptors will probably react with midchain β1,6-GlcNAc transferase(s) in the same way as the hexasaccharide **1**, paving a general route to more highly branched backbone structures of polylactosamines.

On the basis of our data, it is not clear whether one or several midchain β1,6-GlcNAc transferase activities are present in rat serum, for example. It is conceivable that certain reactions among the observed transformations **1** → **2** → **4** and **1** → **3** → **4** may be catalyzed by different enzymes. However, in the present context, it is not important to know whether one or several activities participate in these reactions. What really counts is that the midchain β1,6-GlcNAc transferases are able, either as a single enzyme or as a group of enzymes, to catalyze the conversion of linear polylactosamine backbones to multiply branched chains analogous to glycan **4**.

After the generation of two GlcNAc branches to the main chain of the acceptor **1**, the complete *in vitro* biosynthesis of a prototype of mature multibranched polylactosamine backbones became possible. The octasaccharide **4** was successfully β1,4-galactosylated in the present experiments by bovine milk β1,4-galactosyltransferase at both β1,6-bonded GlcNAc branches to yield the decasaccharide **5**. In separate experiments described elsewhere, glycan **4** has been transformed even further, to the complete tetravalent sialyl Lewis X glycan sLexβ1−3′(sLexβ1−6′)LacNAcβ1−3′-(sLexβ1−6′)LacNAcβ1−3′(sLexβ1−6′)LacNAc, where sLex represents the tetrasaccharide Neu5Acα2−3Galβ1−4(Fucα1−3)GlcNAc (Renkonen et al., 1997). Remarkably, this tetravalent sLex glycan has proved to be a nanomolar inhibitor of L-selectin-mediated lymphocyte adhesion to inflammation-activated endothelium of blood vessel walls in the Stamper−Woodruff binding assay (Renkonen et al., 1997). As the lymphocyte−endothelium adhesion represents the first step in lymphocyte extravasation, these data suggest that the tetravalent sialyl Lewis X glycan can be used to downregulate inflammations caused by lymphocyte infiltration. In view of this and other recent data implying multiply branched polylactosaminoglycans as high-affinity inhibitors of cell-to-cell adhesion processes and as putative cross-linkers of receptors (Litscher et al., 1995; Maaheimo et al., 1995; Seppo et al., 1995, 1996; Turunen et al., 1995), the enzymatic synthesis as well as the chemical synthesis (Matsuzaki et al., 1993; Shimizu et al., 1996) of these glycans gains in significance.

In the NMR spectrum of the octasaccharide **4**, the two β1,6-bonded GlcNAc units (residues 7 and 8 of Table 3) were characterized by distinct H-1 resonances. This was the case also in the decasaccharide **5**, where GlcNAc residues 7 and 8 were β1,4-galactosylated; in addition, there was a marked galactosylation-associated downfield shift in the H-1 resonances of residues 7 and 8 in glycan **5**. Analogous observations have been reported for the two GlcNAc H-1 resonances even in an analogous sialooligosaccharide where the distal galactoses were also carrying α2,3-bonded Neu5Ac residues (Renkonen et al., 1997). These features should provide possibilities for monitoring the progress of glyco-

syltransferase and glycosidase reactions at distinct sites of glycans **4** and **5** as well as sialylated **5**.

The present data concern only *in vitro* experiments with midchain β1,6-GlcNAc transferase activity (GlcNAc to Gal) present in soluble form in mammalian blood serum, but it appears likely that similar membrane-bound Golgi enzymes are involved in the biosynthesis of singly and multiply branched polylactosaminoglycans *in vivo*. Supporting this notion, we find a remarkable structural analogy between the products of the present synthesis experiments and the naturally expressed polylactosamine backbones of multiple branches. In addition, we have actually discovered (A. Leppänen et al., unpublished experiments) that lysates of human embryonal carcinoma cells of line PA1, which abundantly express multiply branched polylactosamines (Rasilo & Renkonen, 1982; Fukuda et al., 1985), catalyze reactions similar to those of the present experiments.

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