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Initiation of poly-*N*-acetyllactosamine chain biosynthesis occurs preferentially on complex multiantennary asparagine-linked oligosaccharides*

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ABSTRACT

An *N*-acetyl-β-D-glucosaminyltransferase activity involved in the initiation of poly-*N*-acetyllactosamine chain biosynthesis can be solubilized from Ehrlich ascites tumor cell microsomal membranes. The ability of this enzyme to act on linear and branched acceptor substrates has been studied. The results indicate that complex-type tri- and tetra-antennary oligosaccharides exhibiting the branching pattern β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-[β-D-Gal*p*-(1→4)-β-D-Glc*p*-NAc-(1→2)]-D-Man are the preferred substrates for the enzyme, and therefore, may represent the structures upon which the generation of poly-*N*-acetyllactosamine chains proceeds more efficiently.

INTRODUCTION

N-Acetyl-β-D-glucosaminyltransferases are enzymes that catalyze the incorporation of *N*-acetylglucosamine (D-GlcNAc) residues from the donor molecule UDP-D-GlcNAc into suitable acceptor substrates[1,2]. During the assembly of the *N*-linked carbohydrate chains in glycoproteins, these enzymes are involved in three major steps which occur at distinct intracellular compartments along the pathway: (*i*) Synthesis of the lipid-linked precursor intermediate dolichol-diphosphate-D-GlcNAc$_2$ by sequential incorporation of two D-GlcNAc units, which takes place in the endoplasmic reticulum, ultimately results in the transfer *en bloc* of the oligosaccharide moiety of the precursor, D-Glc*p*NAc$_2$-D-Man*p*$_9$Glc$_3$, into an appropriate asparagine (Asn) residue of a nascent polypeptide chain[3,4]. (*ii*) Processing of the high-mannose sugar chains found frequently, but not always, in immature glycoproteins to generate either complex-type or hybrid-type oligosaccharides, and further conversion of the complex-type chains into bi-, tri-, and tetra-antennary structures, are all steps that occur within the stacks of the Golgi apparatus[3–6]. (*iii*) Formation of long repeating units of the disaccharide *N*-acetyllactosamine, especially on complex-type multiantennary structures, which exhibit either a

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linear or a branched configuration, are often referred to as blood group i and I antigenic substances, respectively[7–12].

A distinct biological function for poly-*N*-acetyllactosamine chains has yet to be defined; however, their involvement in a variety of processes has been documented. In addition to being subject to developmental regulation during erythrocyte maturation[7,9,11] and in the early mouse embryo[13,14], poly-*N*-acetyllactosamine chains act as receptors for cell-surface (1→4)-β-D-galactosyltransferase of embryonal carcinoma cells[15], and thus may participate in such cell–cell interactions as neural crest-cell migration[16], late morula compaction[17], and sperm-binding to the egg zona pellucida[18]. These chains have also been implicated in the binding of murine natural killer cells to susceptible tumor targets[19].

In recent years, our laboratory has been studying in detail the structure and biosynthesis of cell-membrane glycoconjugates from Ehrlich ascites carcinomas. One of the striking findings was the observation that the majority of cell-surface glycoproteins was terminated in α-D-galactopyranosyl groups at the non-reducing position[20–22], as opposed to the more common sialic acid residues. In addition, Asn-linked oligosaccharides exhibited a multiantennary structure consisting of variable repeats of the disaccharide unit *N*-acetyllactosamine[22]. Interestingly, poly-*N*-acetyllactosamine chains were originally described on membrane glycolipids[8,9,12] and glycoproteins[23–28] from human erythrocytes, and there have since been found in a host of cell types of tumorigenic origin[29–35].

The initial step in the biosynthesis of poly-*N*-acetyllactosamine chains is the transfer of a D-GlcNAc residue in (1→3)-β-linkage to the D-galactose moiety of *N*-acetyllactosamine-terminated glycoconjugates. An enzymatic activity that carries out the reaction indicated above has been detected in microsomal extracts of Ehrlich ascites tumor cells[36], as well as in other cell types and tissues[37–42]. However, little is known about the biochemical nature and acceptor specificity of the Ehrlich tumor cell enzyme. In contrast, the corresponding (1→3)-α-D-galactosyltransferase responsible for the expression of terminal non-reducing α-D-Gal*p* residues on Ehrlich ascites cell-membrane glycoproteins has been recently purified to homogeneity, and its substrate specificity has been studied in detail[43–45].

The present communication describes the specificity of a (1→3)-*N*-acetyl-β-D-glucosaminyltransferase activity from Ehrlich ascites tumor cells involving the use of bi-, tri-, and tetra-antennary substrates. Our results indicate that, as the number of antennae increases, so does the ability of oligosaccharides to serve as more efficient acceptors for the enzyme.

RESULTS AND DISCUSSION

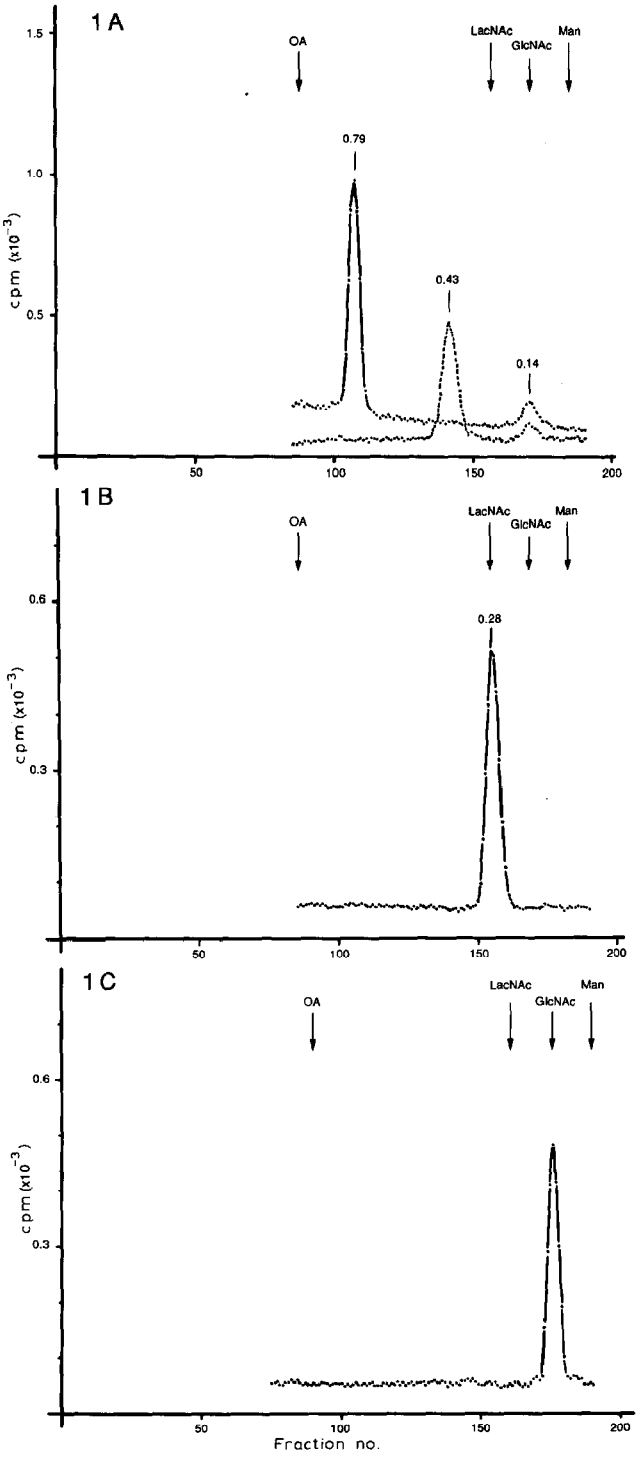
Incorporation of [^{14}C]-D-GlcNAc into linear and branched oligosaccharides. — All the carbohydrate structures listed in Table I share the sequence motif β-D-Gal*p*-(1→4)-D-GlcNAc at their terminal, non-reducing ends. These disaccharide units are also found in poly-*N*-acetyllactosamine chains. Previously, we have shown that Ehrlich ascites

TABLE I

Oligosaccharide Substrates

Oligosaccharide	
1. β-D-Gal*p*-(1→4)-D-GlcNAc	
2. β-D-Gal*p*-(1→3)-D-GlcNAc	
3. β-D-Glc*p*NAc-(1→3)-D-Gal	
4. β-D-Glc*p*NAc-(1→6)-D-Gal	
5. β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-D-Man	TRI-2
6. β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)-D-Man	TRI-6
7. β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→4)- β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)- D-Man	PENTA-2,4
8. β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)- β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)- D-Man	PENTA-2,6
9. β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→6)- β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→3)- D-Man	HEPTA
10. β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→6)- β-D-Gal*p*-(1→4)-βDGlc*p*NAc-(1→4)- α-D-Man*p*-(1→3)- D-Man β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-	NONA-I
11. β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)- α-D-Man*p*-(1→6)- β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)- D-Man β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-α-D-Man*p*-(1→3)-	NONA-II
12. β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→6)- α-D-Man*p*-(1→6) β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)- D-Man β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→4)- α-D-Man*p*-(1→3) β-D-Gal*p*-(1→4)-β-D-Glc*p*NAc-(1→2)-	UNDECA

tumor cells express a (1→3)-*N*-acetyl β-D-glucosaminyltransferase which is responsible for the initiation of poly-*N*-acetyllactosamine chain biosynthesis[36]. In order to study the specificity of this transferase towards linear and branched structures, detergent extracts of Ehrlich tumor cells[36] were incubated with radioactive UDP-D-GlcNAc and a series of


1A
OA
LacNAc
GlcNAc
Man
0.79
0.43
0.14
cpm (x10⁻³)
1.5
1.0
0.5
50
100
150
200
1B
OA
LacNAc
GlcNAc
Man
0.28
cpm (x10⁻³)
0.6
0.3
50
100
150
200
1C
OA
LacNAc
GlcNAc
Man
cpm (x10⁻³)
0.6
0.3
50
100
150
200
Fraction no.

oligosaccharide acceptors (see Table I). Fig. 1A shows the elution profile obtained when PENTA-2,6 (Table I, and Fig. 1A solid line) was incubated with UDP-[^{14}C]-D-GlcNAc in the presence of Ehrlich tumor-cell detergent extracts, followed by separation of the reaction products on a Bio-Gel P-2 gel filtration column. Likewise, the dotted line shows the results of a separate run using LacNAc as the acceptor in a similar incubation. For these two substrates, appropriate studies were conducted to ensure that incorporation of radioactivity into the products (see below) was linear with respect to the amount of protein used, within the range of concentrations tested (data not shown). Two peaks of radioactivity exhibiting widely different mobilities were apparent: the slower fraction co-migrated with an authentic sample of [^{3}H]-D-GlcNAc, while the faster moving peak showed greater mobility than the parent compound. When the latter fraction was digested with bovine kidney *N*-acetyl-β-D-glucosaminidase, all the label was released as [^{14}C]-D-Glc*p*NAc (data not shown).

*Initiation of poly-*N*-acetyllactosamine chain synthesis by Ehrlich tumor cell extracts.* — The fraction of radioactivity corresponding to a high molecular weight oligosaccharide (see Fig. 1A) was pooled, lyophilized, and subjected to digestion with *E. freundii* endo-β-D-galactosidase[46] in order to demonstrate the initiation of poly-*N*-acetyllactosamine chain synthesis. Fig. 1B shows the elution profile resulting from the digestion of [^{14}C]-β-D-Glc*p*NAc-PENTA-2,6 with *E. freundii* endo-β-D-galactosidase and subsequent gel filtration analysis on a column of Bio-Gel P-2 (identical results were obtained in the case of [^{14}C]-β-D-Glc*p*NAc-LacNAc). The single peak of radioactivity eluted with a mobility which was much slower than that of the undigested compound. From the elution profile, the compound appeared to be similar in molecular weight to the disaccharide standards. Further treatment of this fraction with bovine kidney *N*-acetyl-β-D-glucosaminidase yielded [^{14}C]-D-GlcNAc exclusively (Fig. 1C).

In similar fashion, the experimental approach outlined above was utilized to examine the ability of linear and branched carbohydrate structures to support the initiation of poly-*N*-acetyllactosamine chain synthesis. The results shown in Table II express acceptor potency as a ratio of nmol of [^{14}C]-D-GlcNAc incorporated per nmol of oligosaccharide substrate used in the incubation. Because of saturation kinetics exhibited by enzymes, these ratios cannot be compared in a strict sense. However, we previously determined that substrate concentration was saturating for bi-, tri-, and tetra-antennary oligosaccharides, and near saturating (above the K_m) for linear struc-

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Fig. 1. Chromatographic analysis of poly-*N*-acetyllactosamine chains synthesized by Ehrlich ascites tumor cell extracts. (*1A*) Separation on a Bio-Gel P-2 column of the reaction products obtained by incubation of PENTA-2,6 (solid line) or LacNac (dotted line) with UDP-[^{14}C]-D-GlcNAc in the presence of detergent extracts of Ehrlich tumor cells (see "Experimental Procedures" for details). (*1B*) Gel-filtration analysis of radioactive oligosaccharides released by digestion of [^{14}C]-D-GlcNAc covalently bound to PENTA-2,6 with *E. freundii* endo-β-D-galactosidase (see text). Identical results were obtained with the other structures listed in Table I. (*1C*) Chromatography of the labeled carbohydrate fraction shown in 1B after treatment with beef kidney β-*N*-acetyl-D-glucosaminidase. Standards employed on the Bio-Gel P-2 gel filtration column were: *OA*, ovalbumin; LacNAc, *N*-acetyllactosamine; GlcNAc, *N*-acetyl-D-glucosamine; Man, D-mannose.

TABLE II

Initiation of Poly-*N*-acetyllactosamine Chain Synthesis by Ehrlich Ascites Tumor-Cell Extracts

Oligosaccharide	*Conc. (mM)*	[^{14}C]-D-GlcNAc (nmol)[a]	Ratio ($\times 10^4$)[b]
1. β-D-Gal*p*-(1→4)-D-GlcNAc	24.0	0.27	1.1
2. β-D-Gal*p*-(1→3)-D-GlcNAc	16.0	0.14	0.9
3. β-D-Glc*p*NAc-(1→3)-D-Gal	8.0	<0.01	<0.1
4. β-D-Glc*p*NAc-(1→3)-D-Gal	8.0	<0.01	<0.1
5. TRI-2	1.2	0.08	6.7
6. TRI-6	1.2	0.08	6.7
7. PENTA-2,4	1.1	0.07	6.4
8. PENTA-2,6	1.2	0.31	25.8
9. HEPTA	1.2	0.10	8.3
10. NONA-I	0.48	0.18	37.5
11. NONA-II	0.48	0.26	54.2
12. UNDECA	0.24	0.21	87.5

[a] The values listed above represent the average of two separate experiments (actual variation was <10%.
[b] Incorporation of [^{14}C]-D-GlcNAc (nmol) per nmol of oligosaccharide substrate tested.

tures (not shown). Therefore, the ratios listed in Table II may underestimate the true efficiency of multiantennary acceptors relative to linear oligosaccharides, and thus direct comparisons may represent a lower limit of relative acceptor potency.

All the structures terminating in the disaccharide β-D-Gal*p*-(1→4)-D-GlcNAc incorporated [^{14}C]-D-GlcNAc residues efficiently when incubated with Ehrlich ascites tumor cell extracts (Table II). On the other hand, two disaccharides exhibiting terminal non-reducing D-GlcNAc units (entries 3 and 4) failed to serve as acceptor substrates. Among linear oligosaccharides, TRI-2 and TRI-6 showed a similar degree of radioactive D-GlcNAc incorporation which appeared to be six times higher, on a percentage basis, than that of LacNAc (entry 1). Interestingly, the (1→3)-positional isomer (entry 2) was almost as good an acceptor as LacNAc. With regard to branched oligosaccharides, PENTA-2,4 as well as the biantennary structure HEPTA (see Table I) did not differ much in their ability to act as substrates from their linear counterparts TRI-2 and TRI-6, while PENTA-2,6 exhibited increased efficiency in [^{14}C]-D-GlcNAc incorporation. In fact, triantennary and tetraantennary structures containing the branching pattern present in PENTA-2,6 (NONA-II and UNDECA in Table I, respectively) were the best acceptors tested. Another triantennary oligosaccharide, NONA-I, showing a different branching pattern, was slightly inferior in acceptor potency.

Our laboratory has previously demonstrated the presence of an *N*-acetyl-β-D-glucosaminyltransferase in Ehrlich ascites tumor cell extracts which elaborates the sequence of β-D-Glc*p*NAc-(1→3)-β-D-Gal*p*-(1→4)-D-GlcNAc, and thus is responsible for the initiation of poly-*N*-acetyllactosamine chain synthesis[36]. The objective of the present study was to examine the specificity of the above transferase towards linear and branched oligosaccharides terminating in the acceptor sequence β-D-Gal*p*-(1→4)-D-GlcNAc. The experimental approach involved the use of the *E. freundii endo*-β-D-

galactosidase, which specifically hydrolyzes poly-*N*-acetyllactosamine chains by splitting the glycosidic bond between an internal D-Gal*p* and D-Glc*p*NAc residue[46,47]. Initially, radioactive D-GlcNAc was incorporated into appropriate substrates by incubation with UDP-[^{14}C]-D-GlcNAc in the presence of transferase-containing extracts from Ehrlich tumor cells[36]. After separation of the oligosaccharides possessing covalently bound [^{14}C]-β-D-GlcNAc units by gel filtration, they were digested with *E. freundii* endo-β-D-galactosidase, and the products were analyzed by chromatography on Bio-Gel P-2. The enzymatic release of labeled oligosaccharides with the expected size was confirmed by comparison with known standards.

The results presented in Table II suggest that the Ehrlich tumor cell *N*-acetyl-β-D-glucosaminyltransferase requires a branched structure such as the one found in PENTA-2,6 in order for poly-*N*-acetyllactosamine chain synthesis to proceed efficiently. Thus, tri- and tetra-antennary oligosaccharides containing this branching pattern (NONA-II and UNDECA, respectively) are the best acceptors tested. In contrast, PENTA-2,4 and NONA-I, which exhibit a different branching pattern, are less efficient substrates, while the biantennary chain HEPTA behaves as a relatively poor acceptor. Our results are in agreement with recent observations reported by van den Eijnden *et al.*[42]. Also consistent with these conclusions is the fact that several investigators have shown that *N*-acetyllactosamine repeating units occur more commonly in tri- and tetra-antennary Asn-linked oligosaccharides and constitute a relatively small proportion in biantennary chains[22,32,34,48,49]. Furthermore, poly-*N*-acetyllactosamine units exhibiting increasing lengths are differentially distributed among individual antennae, and, in those carbohydrates for which structural information is available, the location of longer chains appears to correlate with the presence of a β-D-Glc*p*NAc-(1→6) antenna attached to the α-D-Man-(1→6) residue of the oligosaccharide core[32,34].

In addition to the (1→3)-*N*-acetyl-β-D-glucosaminyltransferase activity discussed above, Ehrlich cell carcinomas express a separate enzyme, (1→3)-α-D-galactosyltransferase[43–45], which also utilizes non-reducing *N*-acetyllactosamine as an acceptor substrate. This poses the question of whether competition between the two transferases for available oligosaccharide substrates may take place inside the cell. Analysis of the Asn-linked carbohydrate chains present on membrane glycoproteins of Ehrlich ascites tumor cells indicate that most poly-*N*-acetyllactosamine chains are terminated in α-D-galactopyranosyl groups[21,22]. Therefore, the conclusion is that biosynthesis of polylactosamine chains precedes incorporation of α-D-Gal residues during the processing and maturation of Asn-linked oligosaccharides. Thus, it appears likely that the (1→3)-*N*-acetyl-β-D-glucosaminyltransferase and the (1→3)-α-D-galactosyltransferase may be localized in distinct intracellular compartments along the *N*-glycosylation pathway, *i.e.*, the former may be lodged in the *cis* or *medial* Golgi, whereas the latter may be localized in the *trans* Golgi complex[4,6].

EXPERIMENTAL

Materials. — UDP-[U-^{14}C]-D-GlcNAc (297 mCi.mmol^{-1}) was purchased from Du Pont/New England Nuclear Research Products. Unlabeled UDP-D-GlcNAc, Triton X-100, 2-(*N*-morpholino)ethanesulfonic acid (MES), and ovalbumin were obtained from Sigma Chemical Co. Bio-Gel P-2 and P-4 resins were products of Bio-Rad Laboratories. Beef kidney *N*-acetyl-β-D-glucosaminidase (EC 3.2.1.30) was purchased from Boehringer Mannheim Biochemicals, and *E. freundii* endo-β-D-galactosidase[46] was kindly donated by Dr. Y.-T. Li, Tulane University. *N*-Acetyllactosamine[50], and β-D-Gal*p*-(1→3)-D-GlcNAc were synthesized in this laboratory by Nike Plessas. All the other oligosaccharides listed in Table I were a generous gift of Dr. J. Lönngren, University of Stockholm[51].

Preparation of oligosaccharides terminated in [^{14}C]-β-D-GlcNAc. — Incorporation of [^{14}C]-labeled β-D-GlcNAc residues into oligosaccharide acceptors was carried out in a final volume of 0.1 mL and contained the following components: 20mM MES, pH 6.1; 2mM $MnCl_2$; 0.5% (v/v) Triton X-100; 2mM 2-mercaptoethanol; 0.3mM UDP-[^{14}C]-D-GlcNAc (0.2 μCi); 0.24–24mM oligosaccharide (see Table II), and 0.3–0.4 mg of transferase-containing Ehrlich tumor cell detergent extract, prepared as previously described[36]. After a 4-h incubation period at 37°, the entire reaction mixture was spotted on a strip of Whatman 3MM paper and subjected to high-voltage electrophoresis in 1.24M pyridine–0.064M acetic acid, pH 6.4 at 3000 V for 30 min, in order to remove unreacted nucleotide sugar. The radioactive material remaining at the origin of the electrophoretogram was eluted from the paper by soaking with water, and the product was lyophilized, dissolved in H_2O (0.3 mL) containing ovalbumin and D-Man as internal standards, and subsequently applied to a column of Bio-Gel P-2 (100 × 1.6 cm) equilibrated with 0.04% NaN_3 in H_2O. The peak of radioactivity corresponding to a high molecular weight oligosaccharide was pooled and lyophilized for further characterization.

Glycosidase digestions. — The incorporation of [^{14}C]-D-GlcNAc residues in the β-linkage was confirmed by digesting radioactive oligosaccharides prepared as described above with beef kidney *N*-acetyl-β-D-glucosaminidase. Briefly, labeled sugars were treated with 0.25 units of the glycosidase in 50mM citrate, pH 5.0, for 1 h at 37° (one unit is defined as the amount of enzyme that releases 1 μmol of terminal non-reducing β-linked D-GlcNAc from the corresponding carbohydrate chain per minute). The resulting radioactive products were analyzed by gel filtration on Bio-Gel P-2 (see below), and their migrations were compared to that of an authentic sample of [^{3}H]-D-GlcNAc.

Linear and branched oligosaccharides exhibiting labeled poly-*N*-acetyllactosamine chains were subjected to digestion with *E. freundii* endo-β-D-galactosidase[46] in a reaction mixture that contained 1.6 units of enzyme in 50mM NaOAc at pH 5.7. Incubations were carried out for 18 h at 37° and were stopped by heating the sample for 5 min at 100°. Subsequently, the release of small oligosaccharides containing [^{14}C]-D-GlcNAc was assessed by gel filtration analysis on a column of Bio-Gel P-2 (see below).

Analysis of [^{14}C]-GlcNAc-containing oligosaccharides by gel filtration. — Separation of [^{14}C]-labeled oligosaccharides released from *in vitro* synthesized poly-*N*-acetyllactosamine chains by glycosidase digestion was performed on a column of Bio-Gel P-2 (100 × 1.6 cm) equilibrated with 0.04% NaN_3 in H_2O. Samples contained ovalbumin and D-Man as internal calibration standards for the purpose of comparing separate runs. The column was developed with 0.04% NaN_3 in H_2O, and fractions (0.7 mL) were collected and analyzed for radioactivity by liquid scintillation. The elution positions of ovalbumin and D-Man were determined by measuring optical density at 280 nm and by the phenol–sulfuric acid colorimetric method[52], respectively.

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