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# Assessment of glycosaminoglycan-protein linkage tetrasaccharides as acceptors for GalNAc- and GlcNAc-transferases from mouse mastocytoma

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**Two glycosaminoglycan-protein linkage tetrasaccharide-serine compounds, GlcA$\beta$1-3Gal$\beta$1-3Gal$\beta$1-4Xyl$\beta$1-*O*-Ser and GlcA$\beta$1-3Gal(4-*O*-sulfate)$\beta$1-3Gal$\beta$1-4Xyl$\beta$1-*O*-Ser, were tested as hexosamine acceptors, using UDP-[$^3$H]GlcNAc and UDP-[$^3$H]GalNAc as sugar donors, and solubilized mouse mastocytoma microsomes as enzyme source. The nonsulfated Ser-tetrasaccharide was found to function as an acceptor for a GalNAc residue, whereas the Ser-tetrasaccharide containing a sulfated galactose unit was inactive. Characterization of the radio-labelled product by digestion with $\alpha$-*N*-acetylgalactosaminidase and $\beta$-*N*-acetylhexosaminidase revealed that the [$^3$H]GalNAc unit was $\alpha$-linked, as in the product previously synthesized using serum enzymes, and not $\beta$-linked as found in the chondroitin sulfate polymer. Heparan sulfate/heparin biosynthesis could not be primed by either of the two linkage Ser-tetrasaccharides, since no transfer of [$^3$H]GlcNAc from UDP-[$^3$H]GlcNAc could be detected. By contrast, transfer of a [$^3$H]GlcNAc unit to a [GlcA$\beta$1-4GlcNAc$\alpha$1-4]$_2$-GlcA$\beta$1-4-aMan hexasaccharide acceptor used to assay the GlcNAc transferase involved in chain elongation, was readily detected. These results are in agreement with the recent proposal that two different *N*-acetylglucosaminyl transferases catalyse the biosynthesis of heparan sulfate. Although the mastocytoma system contains both the heparan sulfate/heparin and chondroitin sulfate biosynthetic enzymes the Ser-tetrasaccharides do not seem to fulfil the requirements to serve as acceptors for the first HexNAc transfer reactions involved in the formation of these polysaccharides.**

***Keywords*: Biosynthesis/GalNAc transferase/GlcNAc transferase/glycosaminoglycan/proteoglycan**

## Introduction

Heparin/heparan sulfate (HS) and chondroitin sulfate (CS) are synthesized as proteoglycans, the glycosaminoglycan chains being attached to serine units in the core protein moieties via a common GlcA$\beta$1-3Gal$\beta$1-3Gal$\beta$1-4Xyl tetrasaccharide linkage region [1]. Formation of the polysaccharide chain is initiated by the subsequent addition, to the nonreducing-terminal GlcA residue, of either a GlcNAc or a GalNAc unit. GlcNAc substitution is followed by the addition of (-4GlcA$\beta$1-4GlcNAc$\alpha$1-)$_n$ disaccharide units and generation of a heparin/HS precursor chain, whereas the addition of the first GalNAc unit induces the formation of (-4GlcA$\beta$1-3GalNAc$\beta$1-)$_n$ chondroitin sequences. While the addition of the first *N*-acetylhexosamine unit is thus a committing step in relation to the subsequent polymerization process, the mechanism in control of this step, promoting the incorporation of either a GlcNAc or a GalNAc unit, is not properly understood. Additional substituents on the tetrasaccharide linkage region include phosphate groups at C2 of the Xyl unit and sulfate groups at C6 of both Gal units, and at C4 of the GlcA-linked Gal unit [2–9]. Notably, the sulfated linkage tetrasaccharides were found to be associated with CS-type chains only, and were not present in heparin or HS, suggesting that these particular sulfate groups might be involved in the differential chain initiation.

A murine mast-cell tumour originally described by Furth *et al.* [10] has been extensively used in studies of heparin biosynthesis [11–14]. The tumour also produces significant amounts of CS which occurs bound to the same serglycin core protein as the heparin chains, apparently to the same regions of Ser-Gly repeats [15]. This tissue should thus provide a suitable experimental system for attempts to probe the regulation of CS *vs* heparin chain initiation. The

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present study was undertaken in order to establish such a system, using synthetic carbohydrate-serine compounds as glycosyl acceptors in the appropriate GalNAc- and GlcNAc-transferase reactions. Remarkably, the mastocytoma microsomal enzymes failed to substitute the tetrasaccharide linkage region with either β-linked GalNAc or α-linked GlcNAc residues, but instead were found to catalyse the addition of a GalNAc unit in α configuration.

## Materials and methods

### Materials

A microsomal fraction was prepared as described [16] from a transplantable mouse mastocytoma [10]. UDP-[6-$^3$H]GlcNAc (27 Ci mmol$^{-1}$) and UDP-[1-$^3$H]GalNAc (6.3 Ci mmol$^{-1}$) were obtained from New England Nuclear. Unlabelled UDP-GlcA and UDP-GlcNAc were from Sigma. D-[1-$^{14}$C]-Galactosamine (60 mCi mmol$^{-1}$) and D-[1-$^{14}$C] glucosamine (309 mCi mmol$^{-1}$) were from Amersham International Ltd, UK. β-*N*-Acetylhexosaminidase (Jack bean) and α-*N*-acetylgalactosaminidase (Acremonium sp.) were obtained from Seikagaku, Japan. Linkage tetrasaccharide-serine compounds, GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-*O*-Ser and GlcAβ1-3Gal(4-*O*-sulfate)β1-3Galβ1-4Xylβ1-*O*-Ser were chemically synthesized as described by Goto and Ogawa [17]. The hexasaccharide with the structure [GlcAβ1-4GlcNAcα1-4]$_2$-GlcAβ1-4-aMan was recovered by gel chromatography on Sephadex G-50 from an oligosaccharide mixture generated by partial *N*-deacetylation (hydrazinolysis) of *E. coli* K5 polysaccharide [18] (provided by Italfarmaco S.p.A., Milan, Italy), followed by deaminative cleavage at the resulting N-unsubstituted GlcN units [14]. Oligosaccharides of the type [GlcA-GalNAc]$_n$-GlcA-aTal, containing the same alternating sugar residues as chondroitin sulfate, were derived from the *E. coli* K4 capsular polysaccharide [19] (provided by Italfarmaco S.p.A., Milan, Italy). The K4 polysaccharide was first subjected to acidic treatment (pH 1.5 at 80° for 30 min) to remove fructose units from the chondroitin backbone and was then degraded in essentially the same manner as the K5 polysaccharide [14]. Oligosaccharides ⩾10 monosaccharide units were recovered by gel chromatography.

### Enzyme incubations

Transfer of GlcNAc and GalNAc units to oligosaccharide acceptors was assessed essentially as described [14]. Samples contained ~100 μM acceptor saccharide (the K4 oligosaccharides ⩾10 were considered to contain, on average 18 monosaccharide units), 1 μCi UDP-[$^3$H]GlcNAc (400 mCi mmol$^{-1}$), or 1 μCi UDP-[$^3$H]GalNAc (400 mCi mmol$^{-1}$), and 1 mg of mastocytoma microsomal protein, in a total volume of 100 μl of 10 mM $MnCl_2$, 10 mM $MgCl_2$, 5 mM $CaCl_2$, 1% Triton X-100, 50 mM Hepes, pH 7.2. Following incubation at 37 °C for 30 min, the reactions were stopped by the addition of 100 μl of 10% trichloroacetic acid; the precipitated protein was pelleted by centrifugation, and the supernatant was neutralized with NaOH. After the addition of 0.5 mg of carrier heparin (from pig intestinal mucosa), the samples were applied to a column (197 × 1 cm) of Sephadex G-25 (Pharmacia), equilibrated with 1 M NaCl, 0.1% Triton X-100, 50 mM Hepes, pH 7.2. Effluent fractions of 2 ml were collected at a rate of 4 ml h$^{-1}$ and analysed for radioactivity.

### Enzyme digestions

Enzymatically labelled oligosaccharide product recovered by gel chromatography was desalted on a PD-10 column (Pharmacia). Digestions of the product with β-*N*-acetylhexosaminidase or α-*N*-acetylgalactosaminidase were performed essentially according to the instructions from the manufacturer. Samples of ~2500 cpm were incubated at 37 °C overnight with 208 mIU of α-*N*-acetylgalactosaminidase or 1 IU of β-*N*-acetylhexosaminidase in a total volume of 25 μl of 50 mM sodium citrate, pH 4.5.

### Characterization of incorporated hexosamine residue

Labelled desalted oligosaccharide (~50 000 c.p.m.) was hydrolysed in 1.5 ml of 8 M HCl at 100 °C for 3 h, and the hydrolysate was evaporated to dryness. Samples (~10 000 cpm) of the $^3$H-labelled product were mixed with 10 000 cpm each of $^{14}$C-labelled GlcN and GalN standards in 200 μl of 0.01 M HCl, and subjected to cation-exchange chromatography on an HPLC column (0.45 × 25 cm) of Aminex A-5 (Bio-Rad). The column was equilibrated in 0.14 M potassium phosphate buffer, pH 7, with 50 ml of methanol added to 1 l of buffer, and was eluted with the same buffer at a rate of 0.12 ml min$^{-1}$. Fractions of 0.48 ml were collected. Radioactivity in aliquots was measured in a Beckman LS 6000IC scintillation counter.

## Results

Two synthetic tetrasaccharide-serine compounds (in the following denoted Ser-tetrasaccharides) were tested as acceptors for GlcNAc and GalNAc residues in glycosyltransferase reactions catalysed by a solubilized microsomal fraction from mouse mastocytoma. One of these compounds had the structure, GlcAβ1-3Galβ1-3Galβ1-4Xylβ1-*O*-Ser, whereas the other one carried a single sulfate group on one of the Gal units, GlcAβ1-3Gal(4-$OSO_3$)β1-3Galβ1-4Xylβ1-*O*-Ser.

Incubation of solubilized microsomal fraction with UDP-[$^3$H]GalNAc in the absence of any added acceptor compound resulted in the formation of labelled material that was largely excluded from Sephadex G-25 (Figure 1C). This material was susceptible to digestion by chondroitinase AC (data not shown), suggesting that [$^3$H]GalNAc had been incorporated into endogenous, chondroitin-related

polysaccharide [13]. Similar incubation, in addition containing the nonsulfated Ser-tetrasaccharide, yielded a distinct peak of labelled material that emerged at the elution position expected for a pentasaccharide (Figure 1A). The microsomal preparation thus contains a GalNAc-transferase capable of adding a GalNAc unit to the Ser-tetrasaccharide sequence. In contrast, no labelled oligosaccharide product above the background incorporation observed in control incubations was detected in the presence of the sulfated Ser-tetrasaccharide compound (Figure 1B), indicating that the sulfated linkage region will not serve as an acceptor for the GalNAc transferase. The addition of ~100 μM chondroitin oligosaccharides, with nonreducing-terminal GlcA units, to incubations of UDP-[$^3$H]GalNAc and mastocytoma microsomal fraction, led to a 20–30% increase in the labelled fraction excluded from Sephadex G-25 (data not shown), presumably due to elongation of the oligosaccharides with a single labelled monosaccharide unit. However, such an addition, along with that of the nonsulfated Ser-tetrasaccharide at a similar concentration, did not appreciably affect the formation of $^3$H-labelled Ser-pentasaccharide (data not shown). Conversely, the increase in labelled excluded material was observed regardless of the presence or absence of Ser-tetrasaccharide. It therefore seems reasonable to conclude that the transfer of GalNAc units to chondroitin oligosaccharides and to the Ser-tetrasaccharide is catalysed by two distinct enzymes that do not compete for the same substrates.

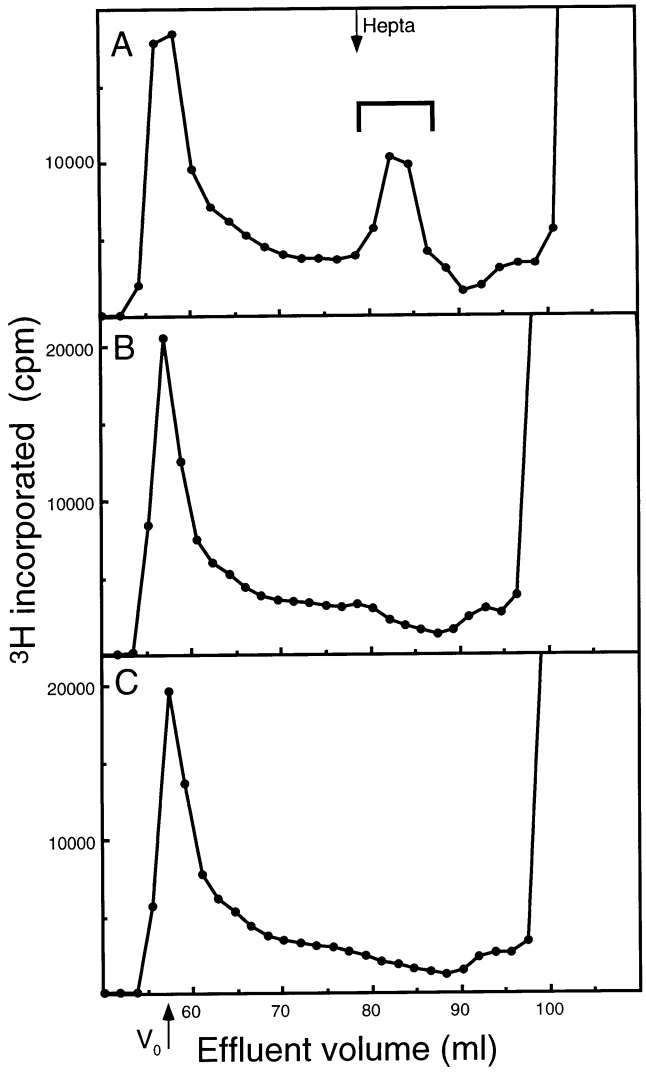


**Figure 1.** Gel chromatography of products formed during incubations of mastocytoma microsomal fraction with UDP-[$^3$H]GalNAc. Mastocytoma microsomal fraction, solubilized in 1% Triton X-100, was incubated with UDP-[$^3$H]GalNAc and (A) nonsulfated Ser-tetrasaccharide (GlcA$\beta$1-3Gal$\beta$1-3Gal$\beta$1-4Xyl$\beta$1-*O*-Ser); (B) sulfated Ser-tetrasaccharide (GlcA$\beta$1-3Gal(4-*O*-sulfate)$\beta$1-3Gal$\beta$1-4Xyl$\beta$1-*O*-Ser); (C) no exogenous acceptor (control). The samples were separated by gel chomatography on Sephadex G-25. The *arrow* indicates the position of a nonsulfated heptasaccharide derived from *E. coli K5* polysaccharide (GlcNAc-GlcA)$_3$-aMan. Fractions indicated by the *bar* were pooled and desalted for further analysis.

In order to ascertain the identity of the hexosamine unit transferred to the Ser-tetrasaccharide, the $^3$H-labelled Ser-pentasaccharide product was subjected to strong acid hydrolysis, and the hydrolysate was analysed by cation-exchange HPLC as described in Methods. The released [$^3$H]hexosamine comigrated exclusively with a $^{14}$C-labelled GalN standard, and was clearly separated from [$^{14}$C] glucosamine (data not shown), thus confirming that the Ser-pentasaccharide contained a labelled GalNAc unit.

To define the anomeric linkage configuration of this [$^3$H]GalNAc the labelled Ser-pentasaccharide was digested with $\beta$-*N*-acetylhexosaminidase. No degradation could be detected on subsequent analysis of the digest by gel chromatography (Figure 2). In contrast, quantitative degradation was observed after digestion with $\alpha$-*N*-acetyl-galactosaminidase (Figure 2), demonstrating that the radiolabelled monosaccharide unit was $\alpha$-linked, and not $\beta$-linked as expected for CS.

Incubation of the microsomal preparation with UDP-[$^3$H]GlcNAc, under conditions otherwise similar to those with UDP-[$^3$H]GalNAc, again yielded a labelled macromolecular material, albeit in relatively low quantity (Figure 3B). However, addition of Ser-tetrasaccharide, sulfated or nonsulfated, to such incubations failed to induce any formation of labelled Ser-pentasaccharide (Figure 3A). No transfer of [$^3$H]GlcNAc to Ser-tetrasaccharides was detectable, even at a five-fold increased UDP-[$^3$H]GlcNAc concentration (data not shown). In contrast, substitution of a [GlcA-GlcNAc]$_2$-GlcA-aMan hexasaccharide, derived from *E. coli* K5 capsular polysaccharide, for the Ser-tetrasaccharides, resulted in the ample formation of labelled heptasaccharide (Figure 3C). This reaction was previously implicated in elongation of the actual polysaccharide chain [20].

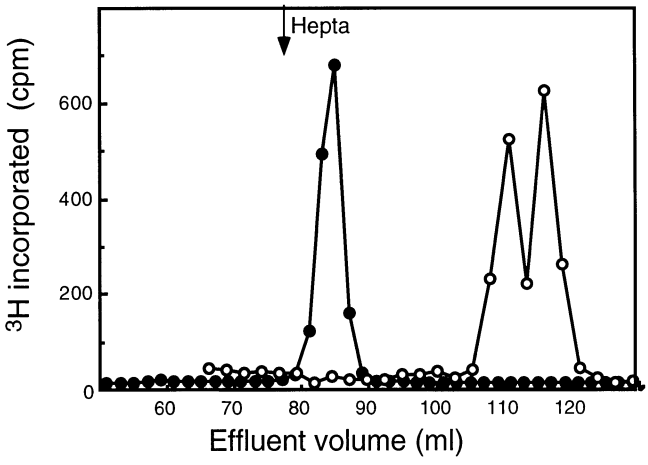


**Figure 2.** Exo-hexosaminidase digestion of [$^3$H]GalNAc containing product. [$^3$H]GalNAc-labelled Ser-pentasaccharide was recovered by gel chromatography as shown in Figure 1A. The product was digested with *β-N*-acetylhexosaminidase (●) or with *α-N*-acetylgalactosaminidase (○) and applied to a column (197 × 1 cm) Sephadex G-25 equilibrated with 1 M NaCl, 0.1% Triton X-100, 50 mM Hepes, pH 7.2. The arrow indicates the elution position of a nonsulfated heptasaccharide [(GlcNAc-GlcA)$_3$-aMan] derived from *E. coli* K5 polysaccharide.

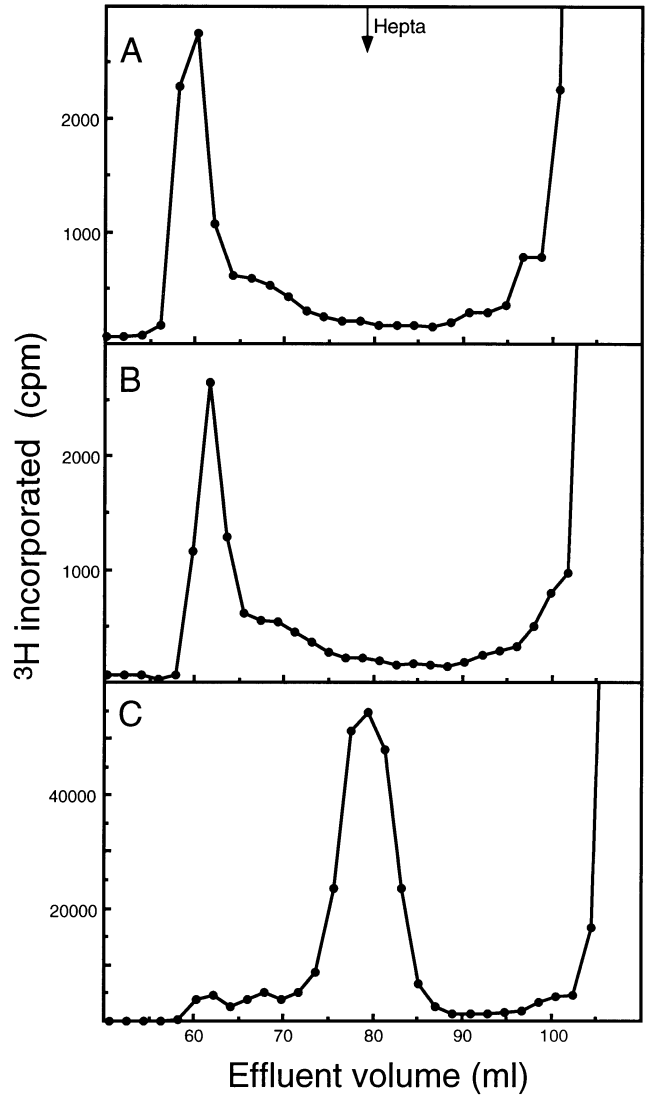


**Figure 3.** Gel chromatography of products formed during incubations of mastocytoma microsomal fraction with UDP-[$^3$H]GlcNAc. Mastocytoma microsomal fraction, solubilized in 1% Triton X-100, was incubated with UDP-[$^3$H]GlcNAc and (A) nonsulfated or sulfated Ser-tetrasaccharide; (B) no exogenous acceptor (control); (C) hexasaccharide [GlcA-GlcNAc]$_2$-GlcA-aMan. The samples were separated by gel chomatography on Sephadex G-25. The arrow indicates the position of a nonsulfated heptasaccharide derived from *E. coli* K5 polysaccharide (GlcNAc-GlcA)$_3$-aMan. Heptasaccharide standard is as in Figure 1.

## Discussion

The mechanisms that control whether a putative core protein will be substituted with CS or with heparin/HS type GAG chains are poorly understood. *β*-D-Xylosides, used as primers for GAG formation in different cells have generally induced CS chains, but no significant amounts of HS [21–30]. It has therefore been proposed that chondroitin formation involves a default pathway, whereas heparin/HS biosynthesis would require some additional, specifying factors. Notably, priming of HS biosynthesis could be achieved by modulating the aglycone structure of the *β*-D-xylosides, such that HS constituted up to 50% of the total glycosaminoglycan initiated using 2-naphthol-*β*-D-xyloside as a primer [31, 32]. Morover, transfection of cells with chimeric core proteins showed that the type of GAGs found depended on defined amino acid residues close to the GAG-substituted serine units [33]. These results point to the role of the peptide sequence in directing the protein core into the appropriate biosynthetic assembly line. However, they are not readily applicable to serglycin proteoglycans of the type synthesized by the mastocytoma used in the present study. These proteoglycans contain extended sequences of ser-gly repeats, in which the serine residues may be substituted by either CS or heparin chains.

The dual biosynthetic capacity of the mastocytoma tissue is known from earlier studies [13, 34], and was here verified by the finding that both labelled GlcNAc and GalNAc were incorporated, from their UDP-derivatives, into microsomal macromolecular material. Moreover, addition of the appropriate oligosaccharide acceptors resulted in the predicted transfer of hexosamine units. The system thus would appear well suited for studies of mechanisms in the control of GAG structure during proteoglycan biosynthesis. The first distinguishing step in the process is presumably the addition of the first GalNAc or GlcNAc unit to the serine-bound GlcA*β*1-3Gal*β*1-3Gal*β*1-4Xyl*β*1-linkage tetrasaccharide sequence. The information available regarding the initial hexosamine transfer reactions is partly contradictory.

Rohrmann *et al.* [35] reported a GalNAc-transferase from calf arterial tissue that was claimed to catalyse transfer of a β-linked GalNAc unit to the linkage trisaccharide (GlcAβ1-3Galβ1-3Gal). By contrast, more recent analysis implicated an α-linked GalNAc unit, incorporated at the corresponding site during incubation with an enzyme preparation from fetal calf serum [36]. In both cases, the assignment of configuration was based on susceptibility to exo-hexosaminidases of known specificity; however, an α-linked GalNAc residue was also demonstrated by $^1$H NMR spectroscopy [37]. These discrepancies remain to be clarified.

The results of the present study indicate that mouse mastocytoma cells also contain a GalNAc-transferase, capable of adding an α-linked GalNAc unit to the linkage tetrasaccharide.

By contrast, neither β-linked GalNAc nor α-linked GlcNAc units were transferred to this position, indicating that the Ser-tetrasaccharide compounds were unable to serve as acceptors of the first HexNAc unit transferred in either CS or HS biosynthesis. Nevertheless, the mastocytoma microsomal fraction was shown to contain both HS- and CS-synthesizing enzyme systems. Unconventional mechanisms may be considered to account for the formation of the proper HexNAc units. Theoretically, β-linked GalNAc could thus be formed by inversion at C1 of an already α-linked GalNAc residue. However, the incorporated GalNAc unit was quantitatively released by digestion with α-*N*-acetylgalactosaminidase. Further, all incorporated radioactivity could be accounted for as GalNAc, following release of the monosaccharide by acid hydrolysis, thus excluding any significant conversion of GalNAc into α-linked GlcNAc by inversion at C4. In fact, Fritz *et al.* [38] demonstrated transfer of α-linked GlcNAc to a GlcAβ1-3Galβ1-*O*-naphtalenemethanol acceptor. It seems reasonable, at present, to assume the occurrence of specific α-GlcNAc- and β-GalNAc-transferases, which differ from the corresponding enzymes involved in major chain elongation [20, 38]. Further work should be aimed at better defining the acceptor recognition requirements of these enzymes, that apparently were not satisfied by the Ser-tetrasaccharides used in the present study.

The functional significance of the α-GalNAc transfer reaction remains obscure. Conceivably, the α-GalNAc substitution of the linkage tetrasaccharide sequence could reflect the lack of specificity of an enzyme that normally utilizes other acceptor structures. However, it is also possible that the reaction may serve as a stop signal that prevents further chain elongation. Since 4-*O*-sulfation of the adjacent Gal unit was found to prevent α-GalNAc transfer, the occurrence of this sulfate group (which has been found in CS-substituted linkage regions; see Introduction), might in fact promote GAG chain formation. Clearly, more information is needed in order to evaluate these alternatives.

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