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Effects of primer-concentration on uronosyl-epimerization and sulfation patterns in *p*-hydroxyphenyl-*O*-*β*-D-xylopyranoside-primed galactosaminoglycans produced by skin fibroblasts

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By supplying skin fibroblasts with different concentrations of the galactosaminoglycan chain-primer *p*-hydroxyphenyl-*O*-*β*-D-xylopyranoside we have produced and recovered glycan-chains that were subsequently radio-iodinated in the hydroxyphenyl group and subjected to sequence analysis by using graded enzymic treatment followed by a combination of gel chromatography and electrophoresis. Fragments extending from the tagged reducing end to the cleavage-point were identified and quantified. Degradation by chondroitin B lyase of chains primed at 0.1 or 0.5 mM xyloside gave profiles indicating a periodic and wave-like distribution of iduronate-containing repeats, with high incidence around positions 2, 5 and onwards, whereas in chains produced at 1.0 mM xyloside the incidence of iduronate was similar in positions 1–4 and then declined. Degradation by chondroitin AC lyase indicated a high incidence of glucuronate in or near the linkage-region. There was a relatively uniform degree of sulfation in chains primed at low xyloside concentration, whereas chains primed at 1.0 mM xyloside gave very heterogeneous charge-patterns in all segments of the chain, including the linkage-region, giving the impression that adequate sulfation, probably at C-4 and at the first opportunity, is necessary to obtain an ordered and periodic epimerization pattern.

***Keywords*: Dermatan sulfate, epimerization, glycosaminoglycan, sulfation, xylosides**

***Abbreviations*: CS, chondroitin sulfate; DS, dermatan sulfate; GAG, glycosaminoglycan; Gal, D-galactose, GalNAc, *N*-acetyl-D-galactosamine; GlcUA, D-glucuronic acid; GlyUA, glycuronic acid; ΔGlyUA, 4,5-unsaturated glycuronic acid; IdoUA, L-iduronic acid; Xyl-Phe-OH, *p*-hydroxyphenyl-*O*-*β*-D-xylopyranoside; Xyl, D-xylose**

Introduction

Proteoglycans constitute a family of proteins that are characterized by the presence of covalently attached glycosaminoglycan (GAG) side-chains and are found in almost all mammalian tissues, inside cells, at the cell-surface, and in pericellular and extracellular matrices. Proteoglycans are structurally diverse; core proteins may vary in size from 10 to 400 kDa and they can contain only a single GAG chain, or well over 100. The GAG chains are linear polymers of repeating disaccharides containing hexosamine and hexuronic acid (or, in the case of keratan sulfate, galactose). The hexuronic acid-containing GAGs are bound to serine residues in the core protein via the common tetrasaccharide structure –4GlcUA*β*1-3Gal*β*1-3Gal*β*1-4Xyl*β*1– forming the so-called GAG-protein linkage region. This structure serves as the primer for chain elongation, to form either $(–4GlcUA\beta1\text{-}4GlcNAc\alpha1–)_n$, the core polymer in heparan sulfate/heparin, or $(–4GlcUA\beta1\text{-}3GalNAc\beta1–)_n$, the corresponding one in chondroitin sulfate (CS) and dermatan sulfate (DS). As both types of polymers share the same linkage-region, a hitherto unknown mechanism selects certain core-protein primers for, *eg* CS/DS-synthesis. Subsequent modifications of the galactosaminoglycan core by C-5 epimerization of GlcUA to IdoUA and different kinds of *O*-sulfation all in an incomplete and, as it appears, sporadic manner, generates a bewildering complexity. Theoretically such sequences can encode considerable information, provided that mechanisms for deciphering that information exist. It is becoming increasingly evident that GAG chains contain a variety of sites that are recognized by various

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0282–0080

extracellular matrix proteins, cytokines, growth factors, enzymes and inhibitors (for reviews, see [1–5]).

Methods for sequence analysis of GAGs have only recently become available. Strategies can be based on oligosaccharide mapping [6], mass-spectrometry [7], or on labelling of the reducing end with radio-iodinatable [8] or fluorescent compounds [9, 10]. We have used radio-iodination, followed by graded enzymic degradation and separation of the products by high-resolution gradient polyacrylamide gel electrophoresis (PAGE). Fragments extending from the radiolabelled reducing end to the point of cleavage have been identified by blotting and autoradiography [8]. This procedure has been applied to CS from the proteoglycan aggrecan [11] and to CS/DS from the proteoglycans decorin and biglycan [12]. These analyses revealed non-random positioning of 4- and 6-*O*-sulfated GalNAc in CS and a periodic, wave-like and tissue-specific distribution pattern for both the different ester-sulfates and for the IdoUA- and GlcUA-containing repeats in DS.

The sugars in the GAG-to-protein linkage-region (see above) can also be esterified to some extent. This includes 2-*O*-phosphate on the Xyl [13], 6-*O*-sulfate on the first Gal [14], and/or 4-*O*-sulfate or 6-*O*-sulfate on the second Gal in CS [14, 15] and IdoUA instead of GlcUA in DS [16]. The significance (if any) of this microheterogeneity in the linkage-region, e.g. for regulation of the sulfation and epimerization patterns during biosynthesis is unknown.

Biosynthesis of proteoglycans commences with the formation of the core protein on ribosomes attached to the endoplasmic reticulum. The Ser residue in a consensus sequence such as D/EGSGD/E is recognized by xylosyltransferase, a Xyl-Ser bond is formed followed by step-wise addition of the other sugars (all from UDP-sugar donors) to form first the linkage-region (see above) then the chain of repeating disaccharides, concomitant with transport through the secretory pathway. The assembly of the glycan chain is finally achieved by epimerization of GlcUA to IdoUA and 2-*O*-sulfation of IdoUA (partial), in DS, and 4-*O*- or 6-*O*-sulfations of GalNAc, in CS and DS (for review, see [1]).

Aryl and alkyl *O*-*β*-D-xylopyranosides can penetrate cellular membranes and act as artificial chain initiators of CS/DS biosynthesis forming free glycan-chains that are secreted into the medium [17, 18]. In skin fibroblasts, addition of xyloside competes with xylosylated core protein and at 1 mM xyloside, no proteoglycan is secreted. Furthermore, galactosaminoglycan chains primed on exogenous xyloside contain progressively more GlcUA and 6-*O*-sulfate with increasing xyloside concentration [19]. By using *p*-hydroxyphenyl-*O*-*β*-D-xylopyranoside (Xyl-Phe-OH) as primer, CS/DS-chains recovered from cell culture media can be radio-iodinated in the reducing terminal adduct and more detailed sequence analysis can be performed [20]. In the present study, we have used this approach to examine whether the uronosyl-epimerization and sulfation patterns of primed CS/DS-chains are affected by the xyloside concentration.

Materials and methods

Materials

DS, Xyl-Phe-OH, radioactively-labelled precursors, cells, culture media, enzymes, separation media, and electrophoresis equipment were the same as described earlier [8, 11, 12, 20, 21]. Chondroitin C lyase (no assigned EC number) which cleaves GalNAc-GlcUA bonds when the GalNAc is un- or 6-*O*-sulfated was from The Seikagaku Corp., Tokyo, Japan. Chondro-glycuronidase (no assigned EC number) which acts on a *Δ*GlyUA1-3GalNAc linkage when the GalNAc is 6-*O*-sulfated or unsulfated and glycuronate 2-*O*-sulfatase (no assigned EC number) which removes sulfate from 2-*O*-sulfated terminal *Δ*GlyUA was prepared from *F. heparinum* as described [22]. The glycuronidase preparation was free of *β*-galactosidase and 2-*O*-sulfatase activity. ^{125}I-Labelled Xyl-PheOH was prepared as decribed [21].

Preparation of ^{125}I labelled CS/DS

Human skin fibroblasts in confluent culture (75–175 cm^2 dishes) were incubated with D-[1-^{3}H]galactose or D-[6-^{3}H] glucosamine (10–20 $\mu Ci\,ml^{-1}$) in the presence of Xyl-Phe-OH for 24 h. Radioactively-labelled xyloside-primed CS/DS-chains were recovered from the medium by ion exchange-chromatography and subsequently separated from contaminating proteoglycans by hydrophobic interaction chromatography and from hyaluronan by gel chromatography. The isolated xyloside-primed CS/DS-chains were labelled in the Phe-OH moiety with ^{125}I using chloramine T. All steps have been described in detail elsewhere [20, 21].

Sequence analysis

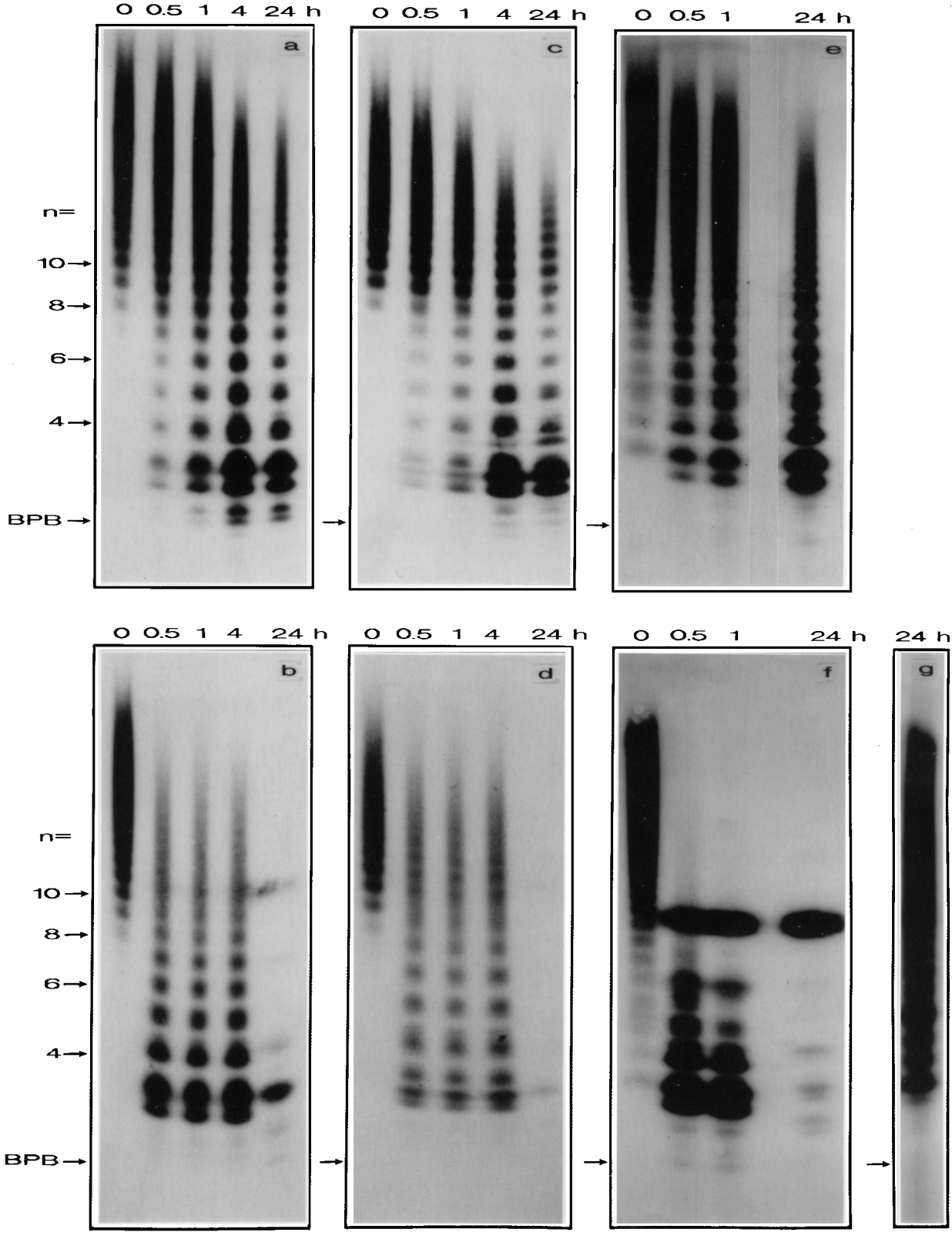
Xyloside-primed and radio-iodinated CS/DS-chains were digested with chondroitin B lyase to cleave GalNAc-IdoUA bonds, with chondroitin AC-I or AC-II lyase to cleave GalNAc-GlcUA bonds, and chondroitin C lyase to cleave GalNAc-GlcUA bonds when the GalNAc was 6-*O*-sulfated or unsulfated, all under the conditions specified either by the manufacturer or as described previously [11, 12]. Degradation products were either separated by gel chromatography or by electrophoresis and radioactively-labelled components were detected by *γ*-spectrometry or by autoradiography after blotting, respectively [8, 11, 12]. Non-reducing terminal ΔGlyUA linked to unsulfated or 6-*O*-sulfated GalNAc was removed by treatment with 1–2 mU of chondro-glycuronidase in 20 mM $Ca(OAc)_2$, 2 mM NaOAc (pH 7.0) at 37 °C over-night (1 unit cleaves 1 μmole of substrate min^{-1}). Sulfate was removed from terminal ΔGlyUA(-2-*O*-sulfate) by treatment with 1 mU of glycuronate 2-*O*-sulfatase in 20 mM NaOAc (pH 6.5) at 37 °C overnight (1 unit cleaves 1 μmole of substrate min^{-1}).

Results

Skin fibroblasts were incubated with 0.1, 0.5 or 1.0 mM Xyl-Phe-OH using [^{3}H]galactose, which is also incorporated into GlcUA [20], or [^{3}H]glucosamine to trace the products formed. In accordance with previous results [20] the yield of secreted [^{3}H]CS/DS-chains was approximately the same at 0.5 and 1.0 mM xyloside but only half as much was obtained at 0.1 mM. The CS/DS-chains were recovered, radioiodinated and subjected to partial cleavage with either chondroitin B lyase or by chondroitin AC-I lyase to identify the location of IdoUA- and GlcUA-containing repeats, respectively (Figure 1). The degradation products included a number of oligosaccharide fragments some comprising the linkage-region and others derived from various segments of the repetitive chain structure. Fragments comprising the linkage-region have the general carbohydrate back-bone structure ΔGlyUA-(GalNAc-GlyUA)$_n$-Gal-Gal-Xyl-Phe-OH, where $n = 0, 1, 2$ etc. and are selectively labelled in the Phe-OH adduct with ^{125}I. Only these fragments were detected using the present method. Previous studies [20] have shown that the Xyl moiety of the xyloside-primed chains remains non-phosphorylated. When the Gal residues are also unsubstituted the Gal-Gal-Xyl-Phe-OH portion is uncharged and fragments of the type $n = 0$ and 1 migrate more slowly than $n = 2$ and 3 on electrophoresis [20]. In fact, unsubstituted $n = 0$ appears in approximately the same position as a regular, normally sulfated $n = 8$ (see Figure 1f). Also fragments $n = 1$–3 behave anomalously, but from $n = 4$ and upwards they form a regular ladder-like pattern (see also Figures 2 and 3 below). The variation in the intensity of the bands suggested that chains primed at 0.1 and 0.5 mM xyloside (Figure 1a, c) contained relatively little IdoUA in positions 4–6 and, conversely, more GlcUA in the same section (Figure 1b, d). IdoUA appeared to be common in positions 10–12 (Figure 1c). Undegraded chains (zero-time lanes in Figure 1) were very polydisperse and ranged in size from $n = 10$ to $n = 25$ (*ie* M_r 5000–12 500). Chains primed at 1.0 mM xyloside (Figure 1e, f) had the greatest size-distribution and sometimes only three repeats had been added. These chains also had a distinctly different degradation pattern indicating that there was no wave-like distribution of IdoUA (Figure 1e). Exhaustive digestion with the AC enzyme (Figure 1f) generated almost exclusively a low-mobility, low-charged saccharide with the same mobility as regular $n = 8$–10, presumably the saccharide ΔGlyUA-Gal-Gal-Xyl-Phe-OH (see further below). Exhaustive digestion with chondroitin C lyase (Figure 1g) generated a series of fragments extending from intact chains down to position 3, indicating that 6-*O*-sulfation, or non-sulfation, was relatively scarce and that no particular position was preferred.

The shorter saccharide fragments ($n = 0$–3) generated by graded enzymic treatment of GAG-Xyl-[^{125}I]Phe-OH were not well resolved by electrophoresis (see [20] and Figure 1) and were therefore subjected to gel chromatography on Bio-Gel P-6 (Figure 2). This procedure separated saccharide fragments with $n = 0$, 1, 2, 3, 4 and sometimes 5 repeats. Those with more disaccharide repeats were, when applicable, subsequently resolved by electrophoresis (see inserts in Figure 2a, c). Degradation by chondroitin B lyase of CS/DS-chains primed at 0.1 or 0.5 mM xyloside (Figure 2a, c) gave saccharide profiles indicating a periodic, wave-like distribution of IdoUA-containing repeats, with high incidence around positions 2, 5 and onwards. The chains produced at 1.0 mM xyloside were different. The incidence of IdoUA was similar in positions 1–4 and then declined (Figure 2e). Regular CS-chains (totally resistant to the B-enzyme) were not detected (see also Figure 1e). Degradation by chondroitin AC lyase of CS/DS-chains primed at 0.1 or 0.5 mM xyloside (Figure 2b, d) indicated a high incidence of GlcUA in or near the linkage-region which was even more pronounced in chains primed at 1.0 mM xyloside (Figure 2f). As the AC-enzyme releases the first disaccharide repeat, the incidence of GlcUA in position 1 may be significantly underestimated, as for example in chains primed at 0.1 mM xyloside (Figure 2b).

The linkage-region and the first two or three disaccharide repeats may be unsulfated or sulfated to a variable degree, resulting in charge differences between saccharides within the same size-group ($n = 0$, 1, 2, etc.). This was examined by electrophoresis (Figure 3) of the various size-pools obtained after partial enzymic degradations (Figure 2). There were indeed a great number of charge-variants within each saccharide-pool, especially of the shorter ones ($n = 0$–3). The patterns also varied between chains generated at different xyloside concentrations. The shortest linkage-region fragments were those obtained after cleavage with chondroitin AC lyase ($n = 0$ in Figure 3b, d, f) having the tetrasaccharide back-bone ΔGlyUA-Gal-Gal-Xyl-Phe-OH. These included two charge-variants (**I** and **II**). The low-mobility variant (**I**) was the predominant product from chains primed at 1.0 mM xyloside (Figure 3f). As shown above (Figure 1f) it was also the almost exclusive product obtained after exhaustive digestion with AC lyase indicating that an IdoUA-Gal-Gal sequence was not present in these chains. Component **I** had the same mobility as a corresponding fragment previously isolated from cells treated with chlorate (a sulfation inhibitor) [20]. Treatment of **I** with glycuronidase (Figure 4, lanes 1 and 2) generated a radiolabelled product with the same electrophoretic mobility (essentially zero) as radioiodinated Xyl-Phe-OH. Furthermore, the glycuronidase-generated product could only be visualized by direct autoradiography of the gel. It could not be detected after blotting to a cationic membrane. These results indicate that component **I** was unsubstituted ΔGlyUA-Gal-Gal-Xyl-Phe-OH. As it was the exclusive product of exhaustive digestion with AC lyase but not particularly prominent after C lyase treatment (see Figure 1f, g), the succeeding GalNAc should be largely 4-*O*-sulfated (see also Table 1). The high-mobility variant (**II**) of ΔGlyUA-Gal-Gal-Xyl-Phe-OH, which was a minor product from chains primed at 1.0 mM xyloside, was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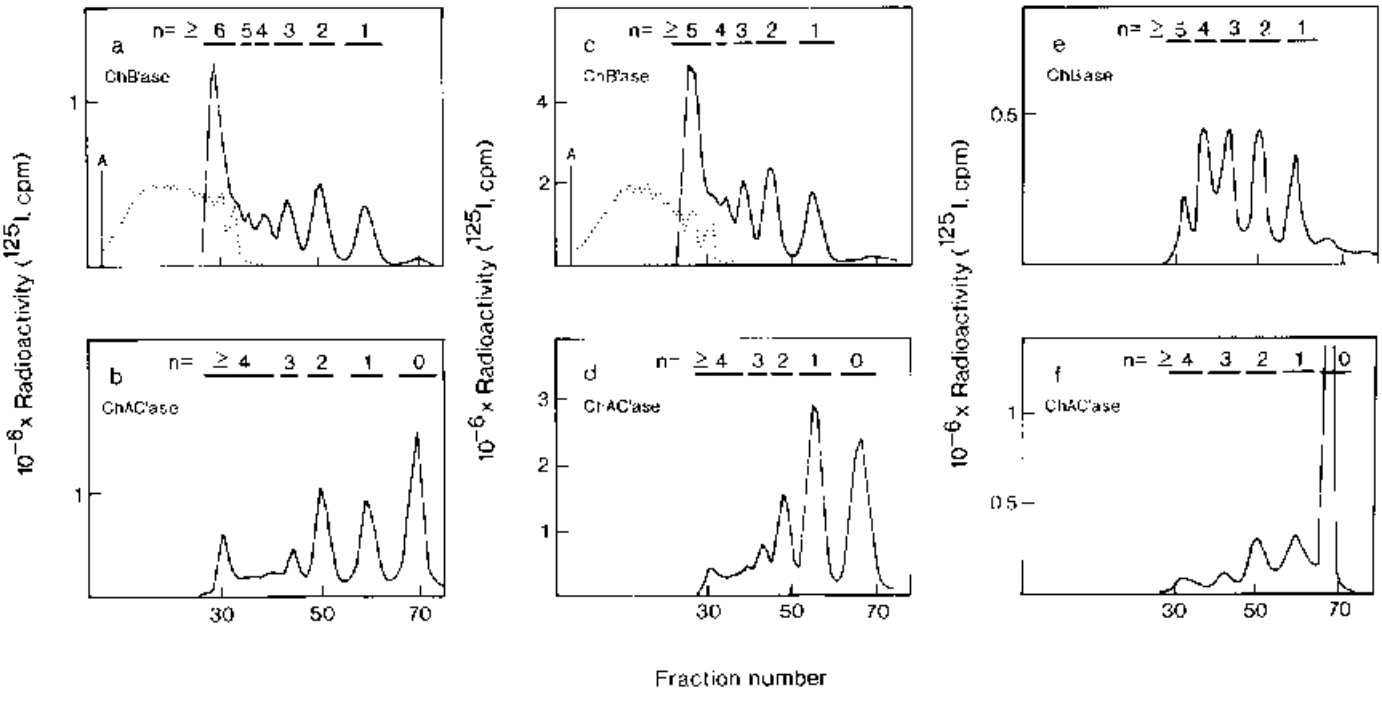

Figure 2. Gel chromatography on Bio-Gel P-6 of radio-iodinated HO-Phe-Xyl-primed CS/DS-chains subjected to partial enzymatic cleavage. Chains were produced at (a, b) 0.1 mM, (c, d) 0.5 mM and (e, f) 1 mM HO-Phe-Xyl, recovered from the medium and radio-iodinated as described in the Materials and Methods section. Samples (5×10^7 c.p.m. of ^{125}I (a, b), 9×10^7 c.p.m. of ^{125}I (c, d), and 1×10^7 c.p.m. of ^{125}I (e, f) supplemented with carrier DS (400 μg) were treated with (a, c and e) chondroitin B lyase (ChB'ase, 1.65 mU) or (b, d and f) chondroitin AC-I lyase (ChAC'ase, 16.5 mU) for 15 h and then chromatographed. The degradation products have the general carbohydrate back-bone structure ΔGlyUA-(GalNAc-GlyUA)$_n$-Gal-Gal-Xyl-Phe-OH, where $n = 0$, 1, 2 etc. Fractions were pooled as indicated by the bars. Saccharide pools $n = 6$ or higher in (a) and $n = 5$ or higher in (c) were subjected to electrophoresis to separate higher saccharides. Tracks were scanned by densitometry and the results are shown as inserts (dotted line).

presumably sulfated. Also this component was sensitive to glycuronidase (Figure 4, lanes 1 and 2) indicating that the GlcUA was not preceded by a 4-*O*-sulfated Gal, but rather by a 6-*O*- or unsulfated Gal. The corresponding linkage-region tetrasaccharide generated by AC lyase from chains primed at the two lower xyloside concentrations ($n = 0$ in Figure 3b, d) were dominated by variants that had a mobility similar to that of **II**. Also after exhaustive digestion with AC lyase (see Figure 1b, d) a component migrating like variant **II** of $n = 0$ (Figure 3b, d) was the major product. Thus, an IdoUA-Gal-Gal sequence could not be detected in chains primed at any xyloside concentration.

Hexasaccharides comprising the linkage region plus the first disaccharide repeat (back-bone structure ΔGlyUA-Gal-NAc-GlcUA-Gal-Gal-Xyl-Phe-OH) were obtained after degradation with either the B lyase or the AC lyase ($n = 1$ in Figure 3). A hexasaccharide pool generated by AC lyase from chains with GlcUA in the first repeat and primed at 1.0 mM xyloside ($n = 1$ in Figure 3f) contained mainly one low-mobility (**III**) and one high-mobility component (**IV**). A minor

Figure 1. Electrophoresis of radio-iodinated HO-Phe-Xyl-primed CS/DS-chains subjected to graded enzymatic cleavage. Skin fibroblast cultures were incubated with D-[1-^{3}H]galactose or D-[6-^{3}H]glucosamine in the presence of (a, b) 0.1 mM, (c, d) 0.5 mM and (e–g) 1 mM HO-Phe-Xyl and CS/DS-chains were isolated from the medium and ^{125}I-labelled in the HO-Phe group as described in the Materials and Methods section. Samples ($1–10 \times 10^6$ c.p.m. of ^{125}I) supplemented with carrier DS (80 μg) were treated with (a, c and e) chondroitin B lyase (0.3 mU) or (b, d and f) chondroitin AC-I lyase (6.6 mU) or (g) chondroitin C lyase (3.3 mU) for the time-periods indicated above each lane. The 24-h time-points in b, d, f, and g were obtained after the addition of an extra amount of enzyme to ensure complete cleavage. Incubations were stopped by boiling for 2 min and aliquots were electrophoresed. Banding patterns were visualized by blotting and autoradiography. The direction of migration is from the top to the bottom. BPB, BromoPhenolBlue marker. The fragments observed have the general carbohydrate back-bone structure ΔGlyUA-(GalNAc-GlyUA)$_n$-Gal-Gal-Xyl-PheOH[^{125}I] with possible sulfations at GalNAc and sometimes also Gal and GlyUA. Fragments with $n = 4$ or higher migrate in a regular fashion, i.e. smaller ones have a higher mobility than larger ones; their positions are indicated on the left side. As the segment Gal-Gal-Xyl-PheOH is frequently unsubstituted and uncharged, small fragments ($n = 0–3$) have a lower average charge density than longer ones, migrate more slowly (between the positions of BPB and $n = 4$) and are not well resolved. Unsulfated fragments of the type $n = 0$ migrate extremely slowly (like $n = 8$) [20]. See also Figure 3.

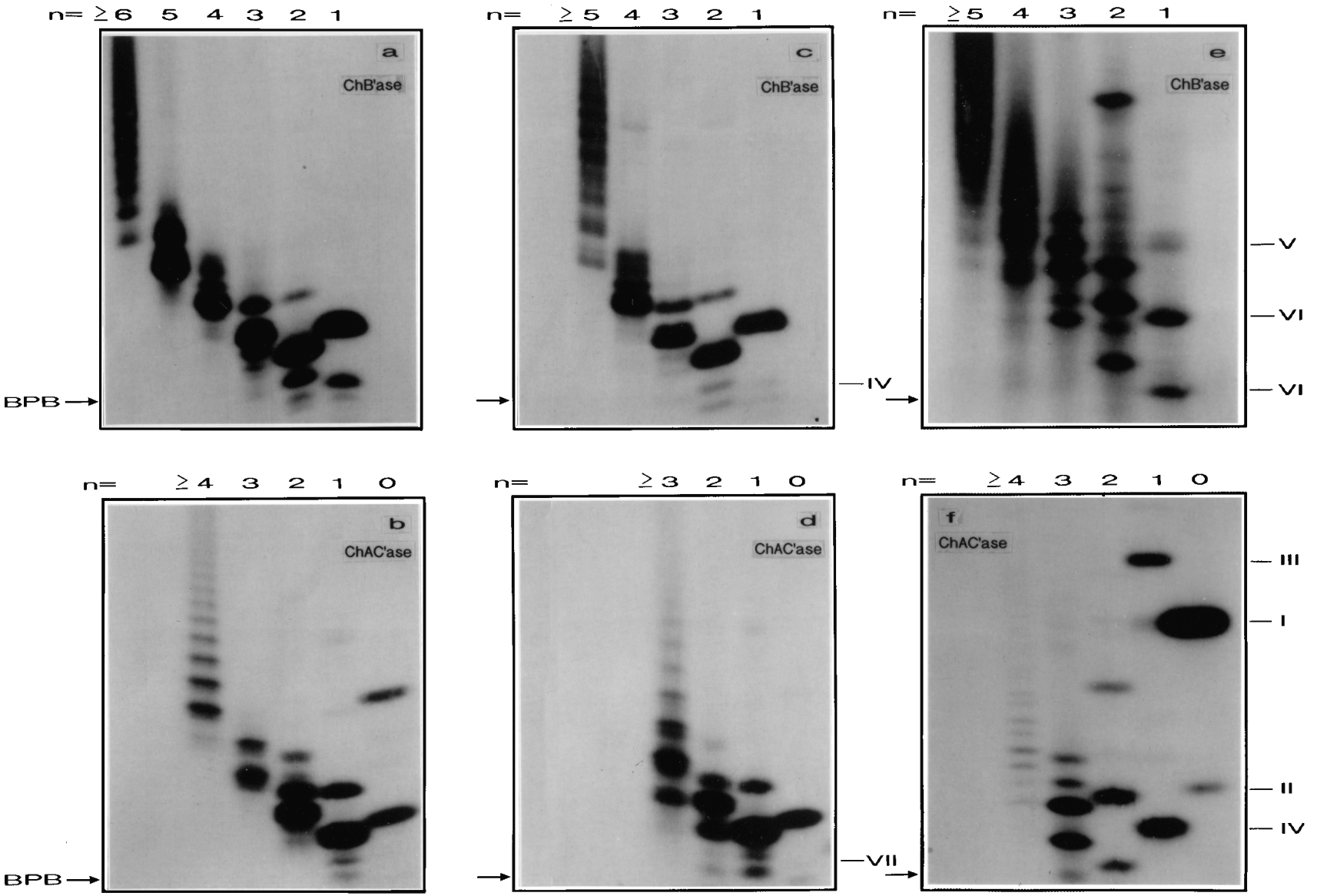

Figure 3. Electrophoresis of oligosaccharide pools derived from radio-iodinated HO-Phe-Xyl-primed CS/DS-chains subjected to partial enzymatic cleavage and gel chromatography on Bio-Gel P-6 as described in Figure 2. Chains were produced at (a, b) 0.1 mM, (c, d) 0.5 mM and (e, f) 1 mM HO-Phe-Xyl, degraded with (a, c and e) chondroitin B lyase (ChB'ase) or (b, d and f) chondroitin AC-I lyase (ChAC'ase), separated into saccharide pools $n = 1, 2, 3, 4, 5$ etc. which were individually electrophoresed. For identification, see above each track. BPB, BromoPhenolBlue marker. Components discussed in the text are marked with **I-VII.**

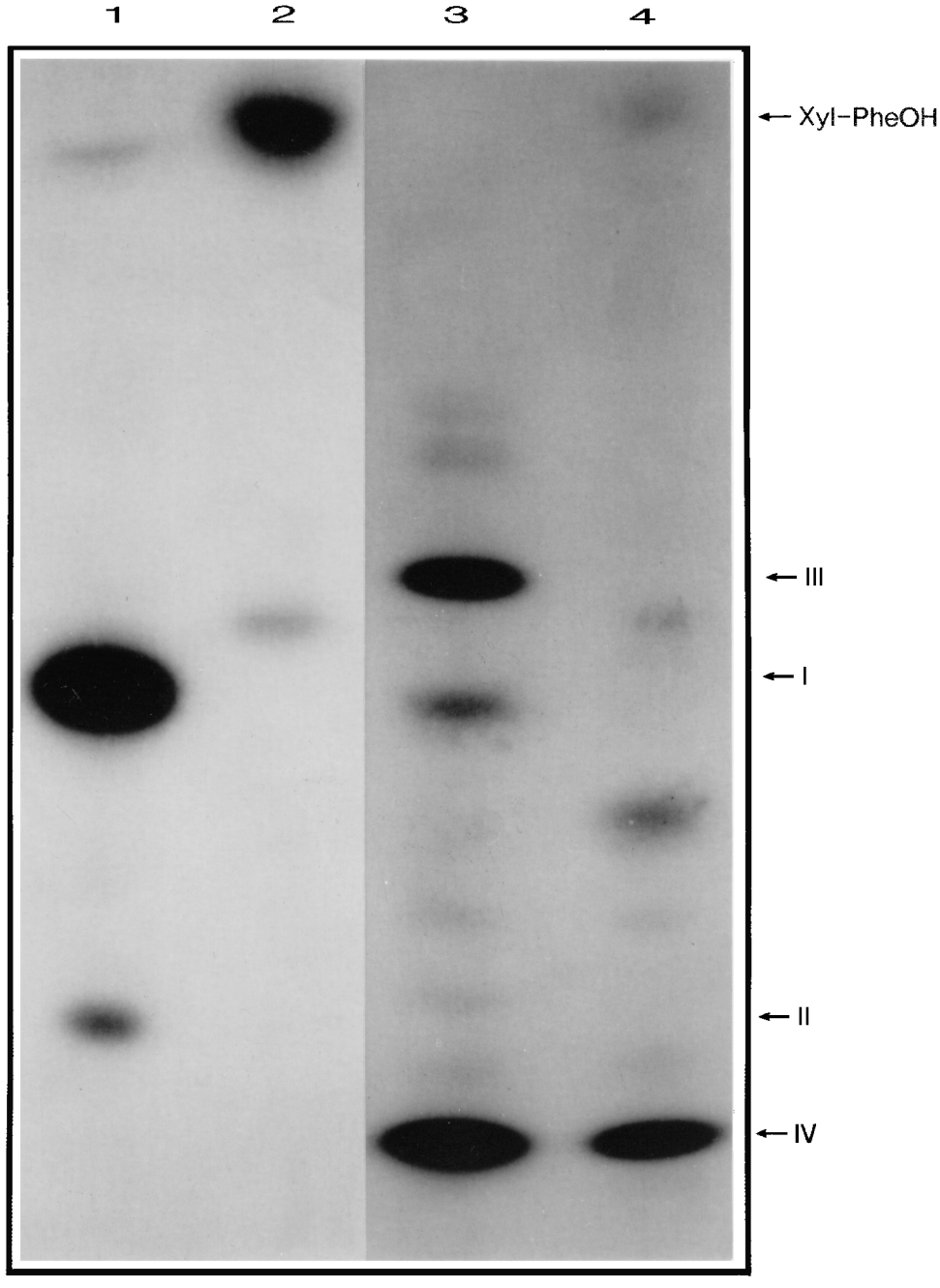

Figure 4. Electrophoresis of various saccharides of the type ΔGlyUA-(GalNAc-GlyUA)$_n$-Gal-Gal-Xyl-Phe-OH before and after treatment with glycuronidase. Lane 1, saccharide pool with $n = 0$ and where ΔGlyUA was originally GlcUA ($n = 0$ in Figure 3f); Lane 2, the same saccharide after enzymic treatment. In these cases, the gel was autoradiographed directly, omitting the blotting step. Lane 3, saccharide pool with $n = 1$ and where ΔGlyUA was originally GlcUA ($n = 1$ in Figure 3f); Lane 4, the same saccharide after enzymic treatment; Components discussed in the text are marked with **I-IV**.

band (in the same position as **I**) was also seen. As component **I** was unsulfated and component **III** migrated even more slowly, the latter should also be unsulfated. The great difference in electrophoretic mobility between components **III** and **IV** suggested that the latter carried many sulfate groups, most likely 4. To examine the terminal disaccharide repeat more closely, the hexasaccharide pool was treated with glycuronidase (Figure 4). The two major charge variants (**III** and **IV** in Figure 3f) behaved differently (Figure 4, lanes 3 and 4). The slow-moving component **III**, and also he minor one

Table 1. Proposed structures of linkage-region sequences identified via saccharide components obtained from CS/DS primed with 1.0 mM xyloside.

Component	*Derived from* ***sequence***
I	GalNAc(4S)-**GlcUA-Gal-Gal-Xyl**
II	**-GlcUA-Gal-Gal-Xyl (diS)**
III	**-GlcUA-GalNAc-GlcUA-Gal-Gal-Xyl**
IV	**-GlcUA-GalNAc-GlcUA-Gal-Gal-Xyl (tetraS)**

S, sulfate (position not specified); diS, disulfated; tetraS, tetrasulfated; 4S, 4-*O*-sulfate.

moving like component **I**, were cleaved by glycuronidase, indicating that the penultimate GalNAc was unsulfated (or 6-*O*-sulfated). However, the fast-moving component **IV** was partially resistant to glycuronidase. The resistant variant should carry a terminal ΔGlyUA-GalNAc-4-*O*-sulfate repeat and the sensitive variant a 6-*O*-sulfated one. Component **IV** was resistant to 2-*O*-sulfatase (results not shown). Chains primed at the two lower xyloside concentrations yielded two AC-generated linkage-region hexasaccharide variants with the same mobility at components **II** and **IV** (Figure 3b, d). Their relative migration rates suggested that they were tri- and tetra-sulfated, respectively.

Hexasaccharides with the back-bone ΔGlyUA-GalNAc-GlcUA-Gal-Gal-Xyl-Phe-OH and generated by B lyase from chains with IdoUA in the first repeat (Figure 3a, c, e) comprised four charge variants (marked **IV**, **V**, **VI** and **VII**). Component **VI** was the predominant product at all xyloside concentrations. Chains primed at 0.1 mM xyloside also generated a faster-moving band in the position of component **IV** (Figure 3a), whereas chains primed at 0.5 mM xyloside only generated component **VI** (Figure 3c). In corresponding saccharides generated from chains primed at 1.0 mM xyloside (Figure 3e), this component was accompanied by two others, one lower-charged (**V**) and one higher-charged variant (**VII**). These three variants were probably di-, tri- and tetra-sulfated, respectively.

The longer saccharides ($n = 2$, 3 and 4) derived from chains primed at the lower xyloside concentrations displayed relatively similar and more uniform charge-patterns after degradation with either lyase (Figure 3a–d). In contrast, chains primed at 1.0 mM xyloside gave very heterogeneous charge-patterns also in the longer saccharide pools (Figure 3e, f). For example, a fragment comprising the linkage-region with two disaccharide repeats and released from a chain with IdoUA in the second repeat contained five major variants ($n = 2$ in Figure 3e), one of which was extremely slow-moving. A similar saccharide-pool comprising the linkage-region with 2 disaccharide repeats but released from a chain with GlcUA in the second repeat ($n = 2$ in Figure 3f) contained three well-spaced charge-variants.

Discussion

Past [20] and present studies indicate that Xyl-Phe-OH can prime synthesis of IdoUA-containing galactosaminoglycan chains, *ie* DS, in skin fibroblasts. Other workers, using 4-methylumbelliferyl β-D-xyloside as initiator, have also demonstrated DS-synthesis in the same type of cells [23]. The present approach using radio-iodination was sufficiently sensitive to permit more detailed analysis of the relatively small amounts of material obtained from biosynthesis experiments. As short fragments could not be resolved by electrophoresis alone, a combination of gel chromatography and electrophoresis had to be used. The presence of an additional negatively charged group at the reducing end would have facilitated electrophoretic separation, but, unfortunately, a charged xyloside cannot penetrate cellular membranes [18]. Nevertheless, the present procedure revealed a periodic and wave-like distribution pattern for GlcUA and IdoUA-containing repeats in chains primed at 0.1 or 0.5 mM xyloside. This wave-pattern was similar to that obtained with DS derived from tissues [8, 24] or from the proteoglycans decorin and biglycan [12]. However, the wave-pattern was not present in chains primed at 1.0 mM xyloside. There was also a lower overall degree of C-5 uronosyl epimerization, probably connected with insufficient and/or incorrect sulfation in agreement with earlier observations [19].

Dermatan sulfate is produced by both vertebrates and invertebrates and a variant with IdoUA(-2-*O*-sulfate)-GalNAc(-6-*O*-sulfate) has been found in a Tunicate species [26]. However, in mammalian tissues efficient and extensive C-5 epimerization of GlcUA to IdoUA seems to be absolutely dependent on 4-*O*-sulfation of an adjacent GalNAc [27]. Overall, IdoUA is generally associated with fully sulfated segments of a chain. Therefore, it is not surprising that fragments derived from chains primed at 0.1 and 0.5 mM xyloside had a minimal charge variation. For example, saccharides $n = 1$, 2 and 3 in Figure 3c, which represent segments like –IdoUA-(GalNAc-GlyUA)$_n$-Gal-Gal-Xyl-Phe-OH, had mobilities in keeping with a high and uniform degree of sulfation. In contrast, saccharides representing –GlcUA-(GalNAc-GlyUA)$_n$-Gal-Gal-Xyl-Phe-OH (Figure 3d) were much more variably charged. In chains primed at 1.0 mM xyloside, IdoUA was present in the first four positions and then declined. This may reflect the processivity in the mode of action of the epimerase, which was noted in *in vitro* experiments where chondroitin-chains, (GlcUA-GalNAc)$_n$, were incubated with crude epimerase from fibroblasts [28].

CS/DS-chains primed on xyloside do not become phosphorylated on the Xyl moiety [20, 23, 25]. Also DS derived from tissues [8, 16, 24] or proteoglycans [12] lack this type of substitution. However, the two Gal residues of the linkage-region and the first 1–2 disaccharide repeats may be variously substituted [12, 16]. In CS/DS primed with

Xyl-Phe-OH many variants were detected. At the highest xyloside concentration, when the degree of epimerization was lowest, the sequence –GlcUA-Gal-Gal-Xyl was mainly unsubstituted and often followed by a 4-*O*-sulfated GalNAc (component **I** in Table 1). At lower xyloside concentrations, the sequence –GlcUA-Gal-Gal-Xyl was generally more charged and most likely sulfated at one or both of the Gal residues (component **II** in Table 1). The position of possible sulfate groups in type **II** components from chains primed at the lower xyloside concentrations was not determined. In chains primed at 1.0 mM xyloside the segment –GlcUA-GalNAc-GlcUA-Gal-Gal-Xyl-Phe-OH must sometimes be totally unsulfated (component **III**) or excessively oversulfated (component **IV**). The segment –IdoUA-GalNAc-GlcUA-Gal-Gal-Xyl-Phe-OH displayed three charge variants (components **V**, **VI** and **VII**).

The general impression is that correct sulfation, i.e. at C-4 of the first GalNAc, or possibly at C-4 of the second Gal residue or, most likely, at both residues [16], is necessary to obtain an ordered and periodic epimerization pattern. When the primer concentration is raised, sulfation becomes imprecise and scattered, which may disturb the polymerization/epimerization process. The present results indicate that priming with iodinatable xylosides is useful in obtaining information about the interplay between elongation, sulfation and epimerization during DS biosynthesis. The present findings should also be taken into account when proteoglycans are expressed at high levels by cDNA-transfection techniques.

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