Increased incidence of unsulphated and 4-sulphated residues in the chondroitin sulphate linkage region observed by high-pH anion-exchange chromatography

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We report the isolation, characterization and quantification of five octasaccharides, four hexasaccharides and two tetrasaccharides, derived from the chondroitin sulphate (CS) linkage region of 6–8-year-old bovine articular cartilage aggrecan, following digestion with chondroitin ABC endolyase. Using a novel high-pH anion-exchange chromatography (HPAEC) method, in conjunction with one- and two-dimensional ^{1}H-NMR spectroscopy, we have identified the following basic structure for the CS linkage region of aggrecan: ΔUA(β1–3)GalNAc[0S/4S/6S]-(β1–4)GlcA(β1–3)GalNAc[0S/4S/6S](β1–4)GlcA(β1–3)Gal[0S/6S](β1–3)Gal(β1–4)Xyl, where ΔUA represents 4,5-unsaturated hexuronic acid, and 4S and 6S represent an O-ester sulphate group on C-4 and C-6 respectively. The octa-, hexa- and tetrasaccharide linkage region fragments were used to develop a HPAEC fingerprinting method, with detection at $A_{232\,nm}$, and a linear response to approx. 0.1 nmol of substance. The sulphation patterns of CS linkage regions, of up to octasaccharide in size, from articular and tracheal cartilage aggrecan were examined. The results show that in articular cartilage, for the majority (53 %) of octasaccharides the 2-deoxy-2-*N*-acetyl amino-D-galactose (GalNAc) residues closest to the linkage region are both 6-sulphated; however, in a significant portion (34 %), one or more of these GalNAc residues are unsulphated, and in 8 % both are unsulphated. Approximately 10–18 % of the chains have a 4-sulphated GalNAc in the first disaccharide, and 12 % have a sulphated linkage region Gal residue. No evidence was found for uronic acid sulphation. These data show that there is a significant increase in the incidence of unsulphated and 4-sulphated GalNAc residues adjacent to the linkage region compared with the rest of the chain. Bovine tracheal cartilage linkage regions displayed very similar sulphation profiles to those from articular cartilage, despite the presence of a higher level of GalNAc 4-sulphation within the repeat region of the main CS chain.

Key words: chondroitin ABC lyase/endolyase, chondroitin 6-sulphate, HPAEC fingerprinting.

INTRODUCTION

The sulphated glycosaminoglycan (GAG) chondroitin sulphate (CS) is an abundant component of the extracellular matrix of articular cartilage. Its functions are not fully elucidated, but it has a role in contributing osmotic swelling pressure, and hence load-bearing properties, to articular cartilage. Other data suggest an involvement in molecular interactions; the expression of some CS epitopes in the rodent is developmentally regulated [1,2], and chondroitin 4-sulphate has been shown to have a role in the cytoadherence of malaria-infected red blood cells [3], the adherence of the malaria parasite to human placenta [4,5], and the regulation of neurite outgrowth [6]. There is evidence that a CS proteoglycan, appican, has a role in Alzheimer's disease [7], and a chondroitin 6-sulphate-containing epitope of the proteoglycan DSD-1 is reported to influence neurite outgrowth [8]. The pattern of sulphation in GAGs is clearly of importance in identifying sites of interaction and antibody epitopes [9].

CS chains comprise a linkage region, a chain cap and a repeat region. The latter consists of a repeating disaccharide [–4)GlcA-(β1–3)GalNAc(β1–]$_n$, which may be sulphated on C-4 and/or C-6 of 2-deoxy-2-*N*-acetyl amino-D-galactose (GalNAc), and C-2 of glucuronic acid (GlcA). CSs with 3-sulphated GlcA residues [10] or fucose branches [11] have also been identified. The sulphation pattern of CS is not random: 2-sulphated uronic acids are only found between a 4-sulphated GalNAc (GalNAc4S) on the non-reducing side, and a 6-sulphated GalNAc (GalNAc6S) on the reducing side [12,13].

The major capping residue in CS from swarm rat chondrosarcoma aggrecan [14] is a sulphated GalNAc (85–90 % GalNAc4S or GalNAc4,6S, where 4S and 6S represent an O-ester sulphate group on C-4 and C-6 respectively). However, in human articular cartilage aggrecan, non-reducing terminal GalNAc4,6S, which is almost absent in foetal tissue, represents 60 % of caps in the normal adult [15], but only 30 % in osteoarthritic cartilage [16]. The GalNAc4,6S structure is, in contrast, very rare within the repeat region.

CS is O-linked to a serine of a protein core. The linkage region has the general structure GlcA(β1–3)Gal(β1–3)Gal(β1–4)Xyl(β1-O-)Ser [17]. There are reports of galactose (Gal) sulphation in CS isolated from aggrecan of shark cartilage [18,19] and bovine nasal septum [20,21], but not in that from swarm rat chondrosarcoma [22].

The tetrasaccharide linkage region described above is common to CS, dermatan sulphate (DS), heparan sulphate (HS) and heparin, but the mechanisms whereby these different polysaccharides are elaborated on this structure are unknown. Sugahara et al. [23] have identified a DS-specific linkage region

Abbreviations used: B(A/T)C, bovine (articular/tracheal) cartilage; CS, chondroitin sulphate; 1/2D, one-/two-dimensional respectively; DS, dermatan sulphate; EAC, equine articular cartilage; GAG, glycosaminoglycan; Gal, galactose; GalNAc, 2-deoxy-2-*N*-acetyl amino-D-galactose; GlcA, glucuronic acid; HPAEC, high-pH anion-exchange chromatography; HS, heparan sulphate; KS, keratan sulphate; SEC, size-exclusion chromatography; ΔUA, 4,5-unsaturated hexuronic acid (4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic acid); Δdi-6S, ΔUAβ(1–3)GalNAc6S-ol.

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in aorta containing iduronic acid (IdoA), i.e. IdoA(α1–3)-Gal(β1–3)Gal(β1–4)Xyl. Several studies on HS have shown that the amino acid sequence of the protein core may have an effect upon the GAG synthesized [24,25]. The detailed structure of the priming oligosaccharide is also important in the elaboration of GAGs [26,27].

Chondroitin lyases are eliminases that cleave the GalNAc-(β1–4)GlcA bond in CS. A recent study of the specificity of commercial chondroitin ABC lyases [28] revealed that most contained an endo- and an exo-lyase, but only the exolyase is able to cleave a tetrasaccharide into two disaccharides. {The authors [28] found that 'protease-free' chondroitin ABC lyase, as produced by Seikagaku Corp. (Chuo-Ku, Tokyo, Japan), was exclusively the endolyase form.}

In the present study, five octasaccharide, four hexasaccharide and two tetrasaccharide linkage region fragments derived from 6–8-year-old bovine articular cartilage (BAC) aggrecan have been isolated and characterized by ^{1}H-NMR spectroscopy. A high-pH anion-exchange chromatography (HPAEC) fingerprinting method for CS linkage regions has been developed and used to examine aggrecan samples derived from BAC, equine articular cartilage (EAC) and bovine tracheal cartilage (BTC).

MATERIALS AND METHODS

A Mono-Q 10/10 column and Sepharose CL-6B resin were purchased from Pharmacia (Uppsala, Sweden), the Spherisorb S5 SAX column was from Phase Separations Ltd. (Deeside, Clwyd, U.K.), the Toyapearl HW-40s resin was from Anachem (Luton, Herts., U.K.) and the Bio-Gel P2 resin was from Bio-Rad (Watford, Herts., U.K.). Diphenyl carbamyl chloride-treated trypsin (bovine pancreas, EC 3.4.21.4), guanidinium chloride (practical grade) and mixed whale and shark CS were purchased from the Sigma Chemical Co. (Poole, Dorset, U.K.). Chondroitin ABC endolyase (Protease-free) (*Proteus vulgaris*, EC 4.2.2.4), keratanase II and chondroitin ACII lyase (*Arthrobacter aurescens*, EC 4.2.2.5) were obtained from the Seikagaku Corporation via ICN Biomedicals Ltd. (High Wycombe, Bucks. U.K.). Caesium chloride was from Fluka Chemicals (Gillingham, Dorset, U.K.), lithium perchlorate (ACS grade) and piperazine were from Aldrich Chemical Co. (Gillingham, Dorset, U.K.) and sodium hydroxide (A. R. 46/48 %) was from Fisons Scientific Equipment (Loughborough, Leics., U.K.). All other chemicals were of analytical grade.

Preparative isolation of CS linkage region fragments from aggrecan

The large aggregating proteoglycan, aggrecan, was isolated from BAC femoral heads (6–8-year-old animals) as described previously [29]. Briefly, the diced cartilage was extracted into 4 M guanidinium chloride/50 mM sodium acetate in the presence of a protease inhibitor cocktail (100 mM 6-aminohexanoic acid/10 mM EDTA/5 mM benzamidine hydrochloride, pH 6.8) for 48 h at 4 °C. The extract was taken to associative conditions by dialysis against the protease inhibitor cocktail, and solid CsCl was added to achieve a density of 1.6 g/ml. Following density-gradient centrifugation at 100000 ***g*** and 12 °C for 48 h the proteoglycan aggregate fraction A1 ($\rho > 1.5$) [30] was pooled, dialysed exhaustively against water, and then freeze-dried.

The A1 fraction was resuspended in a minimum volume of 0.1 M Tris/HCl, pH 8.0, and digested with chondroitin ABC endolyase (1 unit/100 mg at 37 °C for 24 h), and then in the same buffer with diphenyl carbamyl chloride-treated trypsin (2 mg/100 mg at 37 °C for 5 h). The peptidoglycan fragments thus generated were subjected to size-exclusion chromatography (SEC) on a column of Sepharose CL-6B (152 cm × 3.2cm) eluted with 0.5 M sodium acetate/10 mM EDTA, pH 6.8, at 16 ml/h. Fractions (8 ml) were assayed for GAGs by the 1,9-dimethyl-Methylene Blue assay [31], and those representing excluded material (6B0) together with keratan sulphate (KS)-rich 6B1 and CS-rich 6B2 material [32,33] were pooled separately, and dialysed exhaustively against water before freeze-drying.

The GAGs in the 6B2 fraction were released from the peptide by β-elimination with 1 M sodium borohydride in 0.05 M NaOH at 45 °C for 24 h [34], the reaction being terminated by the careful addition of acetic acid. The released CS oligosaccharides and any O-linked KS chains present were separated both from each other and from peptides by ion-exchange chromatography on a Mono-Q 10/10 column (see Figure 1). The oligosaccharides were pooled as shown, and desalted by chromatography on a Bio-Gel P-2 column before freeze-drying.

Individual CS oligosaccharides were isolated for structural characterization and calibration of the HPAEC fingerprinting method by 'strong' anion-exchange chromatography. A 10-mg aliquot of each Mono-Q pool was resuspended in 500 μl of 2 mM lithium perchlorate, pH 5.0, and chromatographed on a Spherisorb S5 SAX column (25 cm × 1 cm). Bound material was eluted at a flow speed of 2 ml/min by a linear gradient of 0.002–0.25 M lithium perchlorate, pH 5.0. The column eluate was monitored on-line at 232 nm, and fractions (2 ml) were pooled (results not shown), desalted as described above, and then freeze-dried. The structure of these linkage regions was determined both by one- and two-dimensional (1D and 2D) ^{1}H-NMR spectroscopy, and by analysis of the products of chondroitin lyase digestion [35].

HW-40s SEC analysis

The oligosaccharides and the products of their digestion were subjected to SEC on a column (50 cm × 1 cm) of Toyapearl HW-40s eluted in 0.5 M ammonium acetate at 0.4 ml/min [35]. The eluate was monitored on-line by measuring A_{232}.

Isolation of linkage region tetrasaccharide

The linkage region tetrasaccharide ΔUA(β1–3)Gal(β1–3)-Gal(β1–4)Xylitol (Xyl-ol) [where ΔUA represents 4–5-unsaturated hexuronic (or 4-deoxy-α-L-*threo*-hex-4-enepyranosyluronic) acid] was isolated from BAC aggrecan CS, prepared as described above, by an exhaustive digestion with chondroitin ACII lyase, followed by Toyapearl HW-40s SEC to separate the resistant tetrasaccharide from disaccharides derived from the repeat region.

Chondroitin lyase digestion of linkage regions

Oligosaccharides were resuspended separately (1 nmol amounts, as determined by measuring A_{232} [36]) in 10 μl of 0.1 M ammonium acetate, pH 8, and then digested with 1 m-unit of chondroitin ABC endolyase or ACII lyase, in a total volume of 15 μl. The oligosaccharides were digested at 37 °C for 15 min before the enzyme was inactivated by heating at 100 °C for 1 min, and the oligosaccharides were reduced by the addition of 5 μl of 100 mM sodium borohydride.

HPAEC analysis

The reduced linkage region oligosaccharides, purified as described above, and the products of their digestion were examined by HPAEC on a Dionex chromatography system using a method described previously for CS repeat region disaccharides [37], and extended to allow fingerprinting of di-, tetra- and hexa-

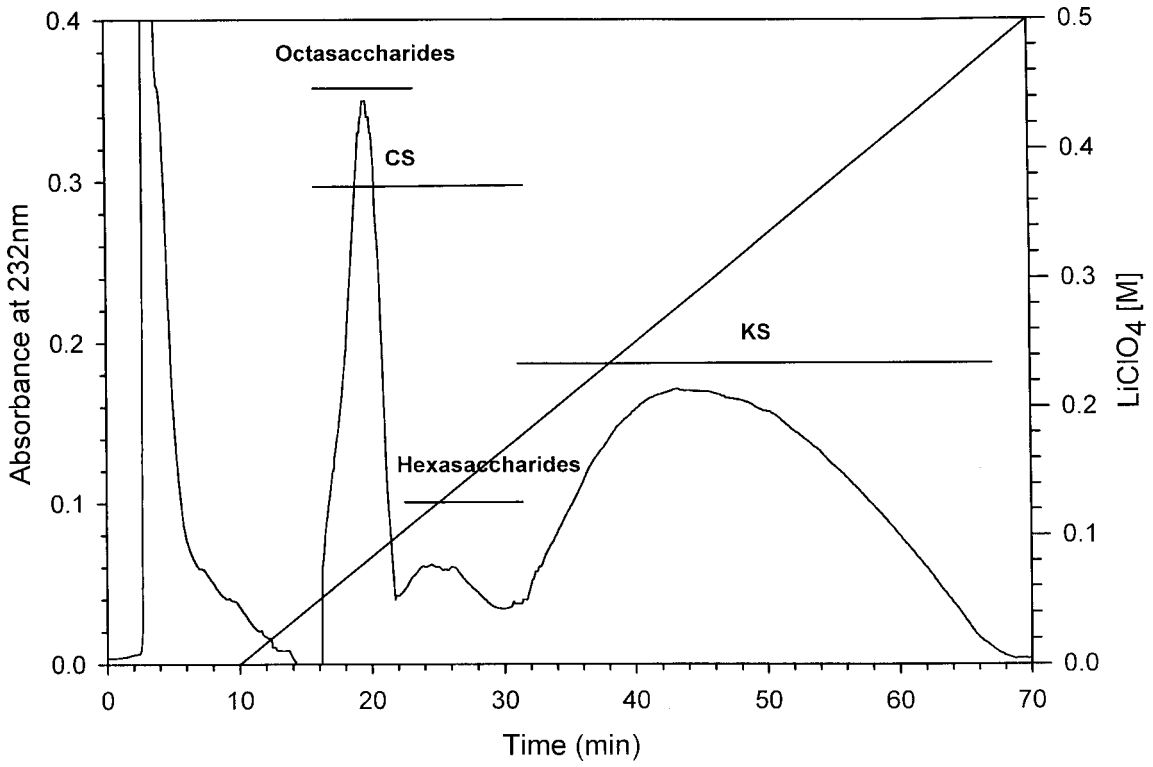

Figure 1 Mono-Q ion-exchange chromatography of CS oligosaccharides

The oligosaccharides released from the 6B2 fraction following alkali borohydride release were chromatographed on a Mono-Q 10/10 column and eluted at 2 ml/min with a linear gradient of 0.002–0.5 M lithium percholate/10 mM piperazine, pH 5.0, over 60 min, with on-line monitoring by measurement of A_{232}, and 2-ml fractions were collected.

saccharides (see Figure 4) [38]. The Carbopac PA1 column was initially calibrated with reduced repeat region di- and tetrasaccharides that were derived from mixed-whale and -shark CS by chondroitin ABC endolyase digestion, as described elsewhere [38]. The structure and purity of these standard oligosaccharides were confirmed by HPAEC analysis and both 1D and 2D ^{1}H- and ^{13}C-NMR spectroscopy.

Following subsequent calibration for linkage region octa-, hexa- and tetra-saccharides, the fingerprinting method was used to examine the structure of CS linkage regions from BAC, EAC and BTC samples, each isolated from an individual animal. A simplified protocol was employed for the preparation of such linkage regions for analysis.

The linkage regions were released by β-elimination from 6B2, isolated as described above, and the oligosaccharides desalted before digestion of KS with 1 m-unit of keratanase II in a minimum volume of 10 mM sodium acetate, pH 6.5, for 24 h at 37 °C, the enzyme being inactivated by heating at 100 °C for 1 min. Aliquots of these octasaccharides were resuspended in a minimum volume of 0.1 M ammonium acetate, pH 8, and digested with chondroitin ABC endolyase to generate hexasaccharides. Following inactivation of the enzyme by boiling, the oligosaccharides were reduced by the addition of 0.25 vol. of 100 mM sodium borohydride. The linkage regions were separated from smaller KS and CS oligosaccharides and amino acids by SEC on an HW-40s column, as described above. The linkage regions were pooled, and the volatile ammonium acetate was removed by repeated freeze-drying and resuspension in distilled water, before examination using the HPAEC fingerprinting method described above. The relative abundance of each oligosaccharide identified was determined by estimation of the peak area and the application of a response factor equivalent to the inverse of the molar absorption coefficient [36].

NMR spectroscopy

Samples (0.1–1 mg) were dissolved in 0.5 ml of 99.8% $^{2}H_2O$, buffered to pH 7 with 10 mM sodium phosphate, and referenced with sodium 3-trimethylsilyl[$^{2}H_4$]propionate as an internal standard. After micro-filtration through 0.45-μm nylon filters, samples were freeze-dried and exchanged several times with 0.5 ml of 99.8% $^{2}H_2O$, followed once with 0.5 ml of 99.96% $^{2}H_2O$, before a final dissolution into 0.7 ml of 99.96% $^{2}H_2O$.

Very-high-field ^{1}H-NMR spectra at 600 MHz were obtained using a Varian Unity INOVA spectrometer equipped with a 5-mm probe capable of field-gradient experiments. 1D spectra, together with 2D gradient COSY-45 correlation spectra, were run at 43 °C. All chemical shifts are quoted relative to internal sodium 3-trimethylsilyl[$^{2}H_4$]propionate at 0.0 p.p.m. The 1D spectra were acquired using a 7000-Hz spectral width and approx. 28 K complex points, and then processed by application of a 0.1-Hz exponential window function and Fourier transformation into 64 K complex points. For the 2D gradient COSY-45 spectra, the spectral width was 1750.7 Hz, and 4 (Hexa-I), 48 (Hexa-II), 4 (Hexa-III) and 56 (Hexa-IV) acquisitions for each of 512 increments were sampled into 1 K complex points. The arrays were zero-filled to 2 K × 2 K complex points, and transformed in each dimension after application of a sinebell window function (offset 10%). 1D and COSY-45 spectra were reprocessed for measurement and presentation using the software package Gifa

V4.2 [39], obtained from Dr. M.-A. Delsuc (University of Montpellier, France).

RESULTS

Two oligosaccharide pools have been isolated from the CS-rich BAC aggrecan fraction 6B2 [32,33] by Mono-Q ion-exchange chromatography (Figure 1). The oligosaccharide pools were examined by SEC, where it was revealed that the components of the first-eluting peak were approximately octasaccharide in size, whereas those of the second were principally hexasaccharide in size.

The ^{1}H-NMR spectra and the structure of the five octasaccharides (Octa-I–Octa-V; see Table 2) present in the first Mono-Q peak (Figure 1) have been described previously [29,35]. The hexasaccharides present in the second peak were isolated by Spherisorb S5 ion-exchange chromatography, and then examined by 600 MHz ^{1}H-NMR spectroscopy. The full ^{1}H-NMR spectra of oligosaccharides Hexa-I–Hexa-IV were obtained (Table 1), and Hexa-IV was identified as having a previously unreported structure.

Table 1 Proton chemical shifts for linkage region hexasaccharides

Spectra were obtained at 600 MHz and 43 °C. Shifts from internal sodium 3-trimethylsilyl-[$^2H_4$]propionate are given as δ values in p.p.m. Those marked with an asterisk are derived from 1D data. –, not assigned.

		Hexasaccharide		
Residue	Proton	Hexa-II	Hexa-III	Hexa-IV
ΔUA (**F**)	1	5.191*	5.268*	5.191*
	2	3.796	3.840	3.794
	3	4.112*	3.951	4.109*
	4	5.883*	5.965*	5.881
GalNAc (**E**)	1	4.601	4.641	4.600
	2	4.039*	4.082	4.036
	3	3.955*	4.158	3.953
	4	4.181*	4.632	4.182
	5	≈ 4.018	–	≈ 4.007
	6	≈ 4.223	–	≈ 4.22
	6′	≈ 4.246	–	≈ 4.24
	CH_3	2.061*	2.100*	2.062*
GlcA (**D**)	1	4.689*	4.688*	4.695*
	2	3.483*	3.468*	3.481*
	3	3.640*	3.642*	3.637*
	4	3.778	3.796	3.781
	5	–	–	3.747
Gal (**C**)	1	4.683*	4.683	4.666
	2	3.771	3.768	3.782
	3	3.812	3.820	3.832
	4	4.161*	4.164	4.196
	5	≈ 3.707	≈ 3.712	3.938
	6	–	–	~ 4.196
	6′	–	–	~ 4.196
Gal (**B**)	1	4.626	4.624	4.628
	2	3.744	3.740	3.732
	3	3.851	3.851	3.827
	4	4.197*	4.198	4.271*
	5	≈ 3.735	~ 3.735	3.728
	6	–	–	~ 3.794
	6′	–	–	~ 3.794
Xyl-ol (**A**)	1	3.679	3.676	3.678
	1′	3.757	3.755	3.756
	2	3.898	3.898	3.899
	3	3.789	3.788	3.789
	4	3.996	3.994	4.002
	5	3.801	3.801	3.799
	5′	3.862	3.859	3.859

The 600 MHz ^{1}H-NMR spectra for the hexasaccharides Hexa-I–Hexa-IV show strong similarities to each other, and to the octasaccharide linkage regions Octa-I–Octa-V [29]; clearly they are closely related. Partial 1D-NMR signals for Hexa-IV are shown in Figure 2, and a 2D-gradient COSY-45 spectrum for the 3.44–4.74 p.p.m. region of this fragment is shown in Figure 3.

Each oligosaccharide yields responses corresponding to a single *N*-acetyl methyl group in the range 2.06–2.1 p.p.m. (omitted for clarity from all Figures), and also a pair of signals, each representing single protons, at approx. 5.2 and 5.9 p.p.m., arising respectively from H-1 and H-4 in a Δ(4,5)-unsaturated residue derived from glucuronate via enzymic cleavage by chondroitin ABC endolyase. The latter are inset on the 1D spectrum (Figure 2), but omitted from the 2D spectrum (Figure 3).

A response at approx. 3.9 p.p.m. derived from H-2 of a reduced and ring-opened chain terminal sugar may be observed in Figure 3 (labelled A2). This and other connected signals compare closely with the xylitol data reported by Huckerby et al. [29] for octasaccharide linkage regions Octa-I–Octa-V.

Six saccharide units are present in Hexa-I–Hexa-IV, but since only a single GalNAc residue is observed, along with signals for two Gal residues, one GlcA and a xylitol residue, these data clearly confirm that these are hexasaccharide linkage region fragments with the basic structure:

ΔUA(β1–3)GalNAc(β1–4)GlcA(β1–3)Gal(β1–3)Gal(β1–4)Xyl-ol
F **E** **D** **C** **B** **A**

The sulphation status of the non-reducing terminal disaccharide may be determined by comparison with Huckerby et al. [29], which reports that the signals of ΔUA (**F**) are perturbed according to the sulphation status of GalNAc (**E**); H-1 and H-4 signals lie at approx. 5.191 and 5.882 p.p.m. when residue **E** is 6-sulphated (Hexa-II and Hexa-IV), but at approx. 5.268 and 5.965 p.p.m. when it is 4-sulphated (Hexa-III). In GalNAc (**E**), responses also reflect sulphation; the methyl shifts to approx. 2.100 p.p.m. and H-4 shifts by more than 0.4 p.p.m. down-field upon 4-sulphation in Hexa-III.

Within the linkage region significant differences in signals derived from both Gal residues may be observed in oligosaccharide Hexa-IV when compared with Octa-I–Octa-V [29] and Hexa-I–Hexa-III. Specifically in Gal (**C**), signals from H6 and H6′ may be seen at approx. 4.196 p.p.m., whereas in an unsulphated residue they are observed at approx. 3.75 p.p.m. Clearly Gal (**C**) is sulphated on C-6.

Hexasaccharides Hexa-I–Hexa-IV have sulphation patterns as summarized in Table 2, and the almost complete chemical-shift values for Hexa-II, Hexa-III and Hexa-IV are summarized in Table 1. The structure of oligosaccharide Hexa-IV is thus:

ΔUA(β1–3)GalNAc6S(β1–4)GlcA(β1–3)-
Gal6S(β1–3)Gal(β1–4)Xyl-ol

Development of a fingerprinting methodology

Octasaccharide linkage regions Octa-I–Octa-V [29], hexasaccharides Hexa-I–Hexa-IV and the tetrasaccharide linkage regions (Tetra-I and Tetra-II), derived from these hexasaccharides via digestion with chondroitin ACII lyase, were used to establish a fingerprinting method for tetra-, hexa- and octasaccharide CS linkage regions; the elution times for each are shown in Table 2, and a composite chromatogram showing the elution of each octa- and hexa-saccharide is shown in Figure 4.

Oligosaccharides Octa-I and Hexa-I are eluted very early,

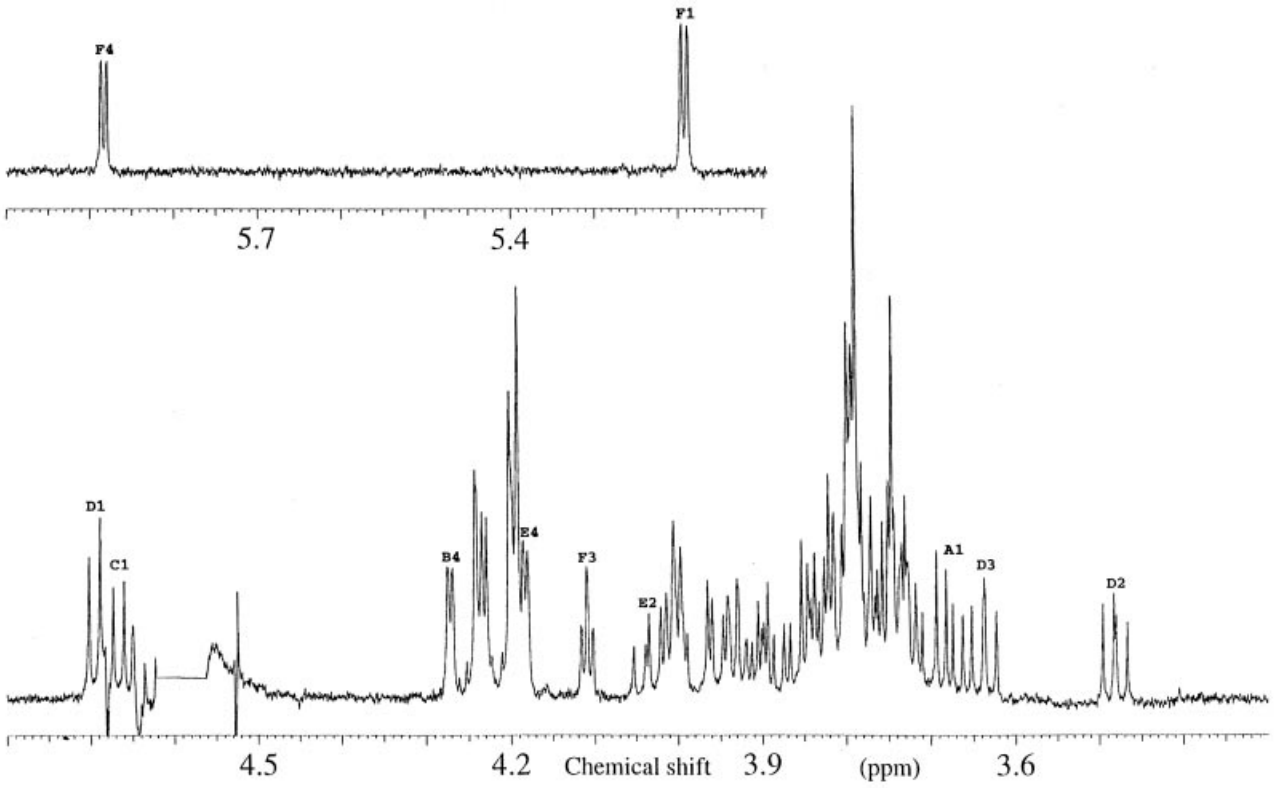

Figure 2 Partial 600 MHz ^{1}H-NMR spectrum of hexasaccharide Hexa-IV at 43 °C

The oligosaccharide has the structure:

ΔUA(β1–3)GalNAc6S(β1–4)GlcA(β1–3)Gal6S(β1–3)Gal(β1–4)Xylitol

F E D C B A

significantly in advance of all monosulphated disaccharides [38], with only a slight retardation when compared with an unsulphated disaccharide. Oligosaccharides Octa-II, Octa-III, Hexa-II and Hexa-III are eluted very closely to each other, and also very closely to ΔUAβ(1–3)GalNAc6S-ol (Δdi-6S), whereas Octa-IV, Octa-V and Hexa-IV are eluted very closely together at the start of a region in which a series of disulphated tetrasaccharides are eluted, but significantly in advance of a disulphated disaccharide.

Following digestion of Hexa-IV by chondroitin ACII lyase, a novel oligosaccharide is observed eluting at approx. 32 min: the sulphated tetrasaccharide linkage region fragment ΔUA(β1–3)-Gal6S(β1–3)Gal(β1–4)Xyl-ol.

For the analytical isolation of CS linkage regions from 6B2 following their release by β-elimination, a simple single-step isolation procedure using SEC on a column of Toyapearl HW-40s was adopted. The most significant component, as judged by measuring A_{232}, is an octa- or hexa-saccharide-sized peak, found upon analysis by HPAEC to comprise oligosaccharides that are eluted at positions expected for the previously characterized CS linkage region octa- or hexa-saccharides.

Sulphation pattern within the linkage region

The CS linkage regions of 6–8-year old BAC ($n = 17$), EAC ($n = 2$) and BTC ($n = 2$) 6B2 were examined by HPAEC; the results are summarized in Figure 5 and Table 2.

There is only a slight species-related difference in the abundance of linkage region structures between EAC and BAC samples. The dominant octasaccharide was Octa-IV, representing 52 % of the linkage regions. In contrast, 5–8 % of the chains (Octa-I) have both GalNAc residues unsulphated. Octasaccharide Octa-III, in which the GalNAc adjacent to the linkage region alone (GalNAc; **E**) is unsulphated, varied from 8 ± 0.6 % in equine to 16 ± 2 % in the bovine samples, whereas oligosaccharide Octa-II, in which the GalNAc sulphation is reversed, varied from 10 ± 4 % in bovine to 5 ± 0.4 % in equine samples. The oligosaccharide for which the greatest species-related difference was observed was Octa-V, representing 32 ± 3 % of the equine linkage regions, but only 12 ± 2 % in bovine samples.

Separate analysis of the hexasaccharide linkage region fragments for these samples confirmed that from both species the first repeat region disaccharide was 6-sulphated upon GalNAc (**E**) in the majority of cases, and was associated with an unsulphated linkage region in 53–59 % of chains, and a sulphated linkage region in 12 %. In samples from equine cartilage, oligosaccharide Hexa-III [4-sulphated on GalNAc (**E**)] represented 18 ± 2 %, but was slightly less abundant (10 ± 5 %) from bovine sources, corroborating the data relating to the incidence of 4-sulphated GalNAc residues observed in the octasaccharides. On the other hand, an unsulphated disaccharide was found on a greater percentage of bovine (24 ± 8 %) than equine (11 ± 4 %) linkage regions.

The hexasaccharide linkage regions from BTC have a sul-

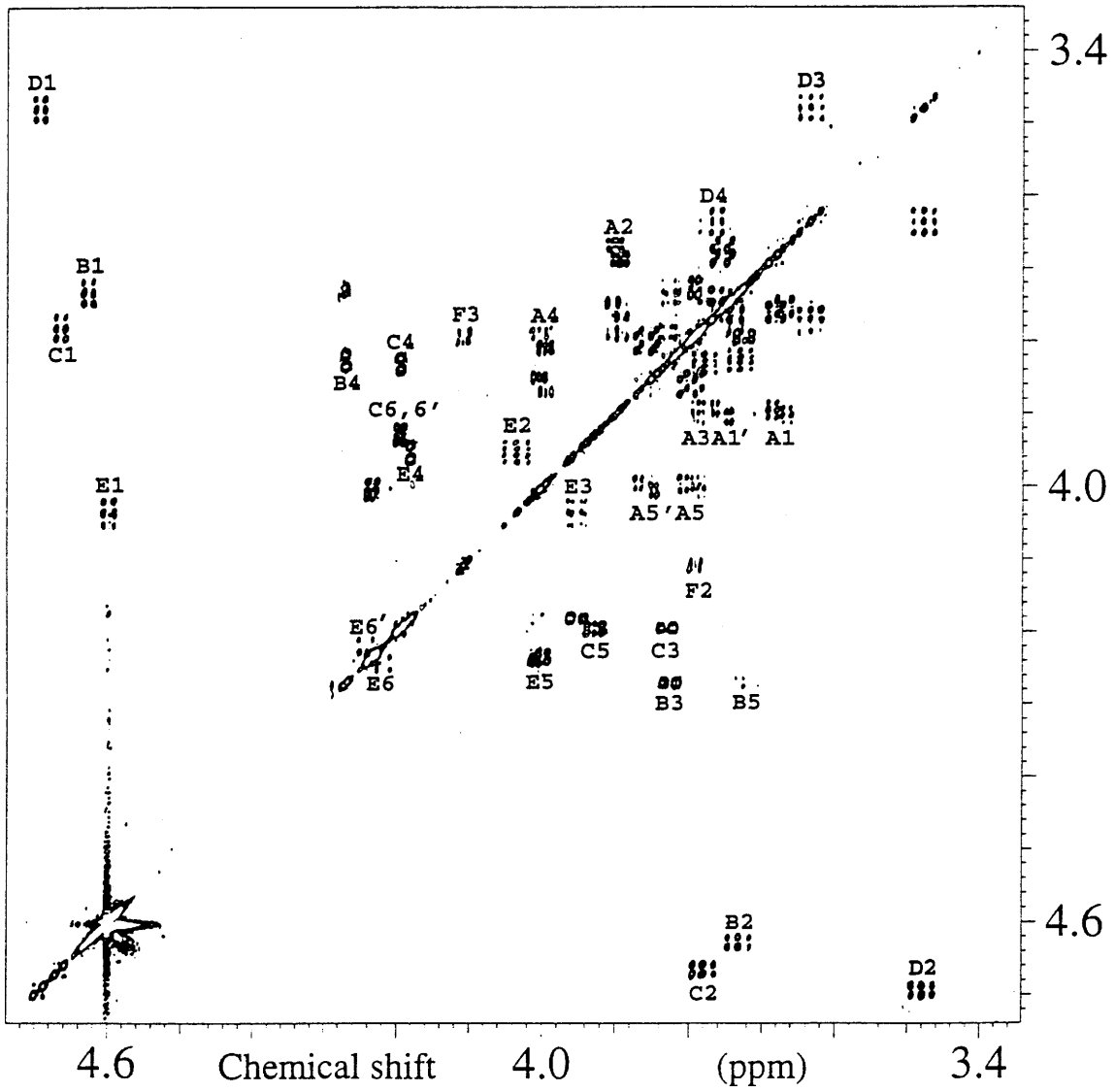

Figure 3 Partial 600 MHz gradient COSY-45 spectrum of hexasaccharide Hexa-IV at 43 °C

The oligosaccharide has the structure:

ΔUA(β1–3)GalNAc6S(β1–4)GlcA(β1–3)Gal6S(β1–3)Gal(β1–4)Xyl-ol

F E D C B A

phation pattern that is very similar to that of the articular cartilage. The majority have a sulphated GalNAc (**E**), and levels of 4- and 6-sulphation, along with Gal sulphation, are similar to those found for BAC aggrecan CS.

Action of chondroitin ABC endolyase

In the present study chondroitin ABC endolyase has been used to remove the disaccharide present at the non-reducing terminus of the linkage region octasaccharides, generating a resistant hexasaccharide. In order to examine the size restriction reported recently upon the action of this enzyme [28], previously isolated tetrasaccharides [38] were digested with chondroitin ABC endolyase as described above; in each case, the enzyme failed to cleave, although this enzyme can cleave the non-reducing terminal disaccharides from octasaccharides Octa-I–Octa-V.

DISCUSSION

We have isolated and characterized, by ^{1}H-NMR spectroscopy and HPAEC, five octasaccharides, four hexasaccharides and two tetrasaccharides derived from the CS linkage region of BAC aggrecan. This is the first study to isolate CS linkage region oligosaccharides of this length from articular cartilage aggrecan, and to examine the sulphation pattern of the repeat region tetrasaccharide adjacent to the core linkage region tetrasaccharide.

Linkage region fingerprinting method

Linkage regions with a constant sulphation level, but increasing size, exhibit a tendency for slightly more retarded elution. Clearly, the level of sulphation dominates the elution position of the oligosaccharides (see Table 2).

Table 2 HPAEC elution position and abundance of linkage region oligosaccharides along with standards

The elution of the reduced oligosaccharides examined in the present study was determined by HPAEC on a CarboPac PA1 column, monitored on-line by measuring A_{232} and compared with that of standards, as described in the legend to Figure 4. The abundance of each oligosaccharide is shown as a percentage (means ± S.D.). –, oligosaccharide not studied.

Oligosaccharide abbreviation	Oligosaccharide	% Abundance (mean ± S.D.) BAC	EAC	BTC	Elution time (min)
Δdi-0S	ΔUAβ(1–3)GalNAc-ol	≈ 1		≈ 1	21.3
Δdi-4S	ΔUAβ(1–3)GalNAc4S-ol	≈ 5		≈ 44	29.7
Δdi-6S	ΔUAβ(1–3)GalNAc6S-ol	≈ 94		≈ 55	36.3
	ΔUAβ(1–3)GalNAc6Sβ(1–4)GlcAβ(1–3)GalNAc6S-ol				53.4
	ΔUAβ(1–3)GalNAc6Sβ(1–4)GlcAβ(1–3)GalNAc4S-ol				47.8
	ΔUAβ(1–3)GalNAc4Sβ(1–4)GlcAβ(1–3)GalNAc4S-ol				49.6
Tetra-I	ΔUAβ(1–3)Galβ(1–3)Galβ(1–4)Xyl-ol	87 ± 5	88 ± 1	83 ± 1	20.4
Tetra-II	ΔUAβ(1–3)Gal6Sβ(1–3)Galβ(1–4)Xyl-ol	12 ± 5	12 ± 1	17 ± 1	31.6
Hexa-I	ΔUAβ(1–3)GalNAcβ(1–4)GlcAβ(1–3)Galβ(1–3)Galβ(1–4)Xyl-ol	24 ± 8	11 ± 4	15 ± 4	24.1
Hexa-II	ΔUAβ(1–3)GalNAc6Sβ(1–4)GlcAβ(1–3)Galβ(1–3)Galβ(1–4)Xyl-ol	53 ± 2	59 ± 3	56 ± 3	35.3
Hexa-III	ΔUAβ(1–3)GalNAc4Sβ(1–4)GlcAβ(1–3)Galβ(1–3)Galβ(1–4)Xyl-ol	10 ± 5	18 ± 2	12 ± 2	36.3
Hexa-IV	ΔUAβ(1–3)GalNAc6Sβ(1–4)GlcAβ(1–3)Gal6Sβ(1–3)Galβ(1–4)Xyl-ol	13 ± 5	12 ± 1	17 ± 1	48.0
Octa-I	ΔUAβ(1–3)GalNAcβ(1–4)GlcAβ(1–3)GalNAcβ(1–4)GlcAβ(1–3)Galβ(1–3)Galβ(1–4)Xyl-ol	8 ± 2	5 ± 3	—	26.0
Octa-II	ΔUAβ(1–3)GalNAcβ(1–4)GlcAβ(1–3)GalNAc6Sβ(1–4)GlcAβ(1–3)Galβ(1–3)Galβ(1–4)Xyl-ol	10 ± 4	5 ± 0.4	—	36.1
Octa-III	ΔUAβ(1–3)GalNAc6Sβ(1–4)GlcAβ(1–3)GalNAcβ(1–4)GlcAβ(1–3)Galβ(1–3)Galβ(1–4)Xyl-ol	16 ± 2	8 ± 0.6	—	35.6
Octa-IV	ΔUAβ(1–3)GalNAc6Sβ(1–4)GlcAβ(1–3)GalNAc6Sβ(1–4)GlcAβ(1–3)Galβ(1–3)Galβ(1–4)Xyl-ol	53 ± 5	51 ± 1	—	47.7
Octa-V	ΔUAβ(1–3)GalNAc4Sβ(1–4)GlcAβ(1–3)GalNAc6Sβ(1–4)GlcAβ(1–3)Galβ(1–3)Galβ(1–4)Xyl-ol	12 ± 2	32 ± 3	—	49.3

Many of these linkage region oligosaccharides are almost co-eluted. Thus Hexa-II, Hexa-III, Octa-II, Octa-III and Δdi-6S are all eluted between 35.3 min and 36.3 min. Furthermore, Octa-IV, Octa-V and Hexa-IV are all eluted close to a region in which disulphated tetrasaccharides are found [38]. Thus, for linkage region fingerprinting, it is important to perform a prior SEC experiment to separate oligosaccharides into pools of differing sizes and to exclude any repeat region disaccharides that may interfere with the analysis.

This simple fingerprinting methodology, utilizing the absorbance at 232 nm of the ΔUA and requiring no chemical modification for detection, enables analytical quantification of biological samples to determine the sulphation pattern close to the linkage region with sensitivity of approx. 0.1 nmol. Allied with recent work by Plaas et al. [15], who have developed a HPAEC fingerprinting method for the non-reducing termini of CS and fingerprinting methodologies that are focused on the repeat region [38], it is now possible to perform a detailed analysis of complete CS chains at the nanomolar level. The method for linkage region analysis that we report here utilizes the same chromatographic conditions as those employed for the analysis of di-, tetra- and hexa-saccharides from the repeat region [38], simplifying the complete analysis of CS chains.

In BAC, EAC and BTC aggrecan, approximately 75–90 % of the CS linkage region oligosaccharides are sulphated on GalNAc **E** (Figure 5). These data are in agreement with those of Cheng et al. [21], who found that this residue was sulphated in almost 100 % of chains from old human articular cartilage.

This study extends these observations; we report that the majority (approximately 65–83 %) of the chains examined have both GalNAc residues closest to the linkage region sulphated. In about 52–54 % of the oligosaccharides (Octa-IV) both are 6-sulphated, whereas in bovine and equine tissue approximately 12 % and 32 % of the chains respectively are 6-sulphated on GalNAc (**E**) and 4-sulphated on GalNAc (**G**). In BAC, about 10 % of the linkage regions are 4-sulphated on GalNAc (**E**) (Hexa-III), whereas 18 % have this structure in EAC. Within the repeat region, 4-sulphation would be expected to account for less than approximately 5 % of the total GalNAc sulphates [38]. Clearly, in CS from BAC and EAC, there is a preferential localization of 4-sulphated GalNAc residues close to the linkage region, being most pronounced in the equine samples.

We have found that linkage region fragments (Octa-I) representing about 5–8 % of those examined are completely unsulphated and, in total, 26 % of BAC and 13 % of EAC linkage regions were found to lack sulphation on GalNAc (**E**). The average abundance of unsulphated GalNAc residues in the repeat region would not be expected to be greater than approximately 1 % [38]. These data suggest a biosynthetic pattern in which there is also a very significant preferential localization of unsulphated GalNAc residues towards the linkage region. In about 5–10 % of the oligosaccharides isolated (Octa-II), GalNAc (**E**) was sulphated without sulphation of GalNAc (**G**). The sulphation partner of this oligosaccharide, in which the GalNAc sulphation is alternated (Octa-III), is significantly more abundant, representing approximately 8 % or 16 % of the linkage region octasaccharides observed in this study. Thus the preferential localization of unsulphated disaccharides towards the linkage region is observed even within the first two disaccharides of the repeat region. The observation of altered sulphation at the linkage region has also been reported for HS [40], heparin [41] and KS [42].

The newly reported oligosaccharide, Hexa-IV, is the first observation of sulphation of linkage region Gal (**C**) (see Figure 5) in articular cartilage. The general observation of linkage region Gal sulphation is in agreement with other workers who have identified sulphated Gal residues in shark cartilage aggrecan [18,19] and bovine nasal septum aggrecan [20,21]. However, Shibata et al. [22] and Cheng et al. [21] failed to find any Gal sulphation in swarm rat chondrosarcoma or human articular cartilage aggrecan.

None of the linkage region oligosaccharides examined had any uronic acid sulphation, although, on average, 3–5 % of the uronic acids within the repeat region of BAC aggrecan CS

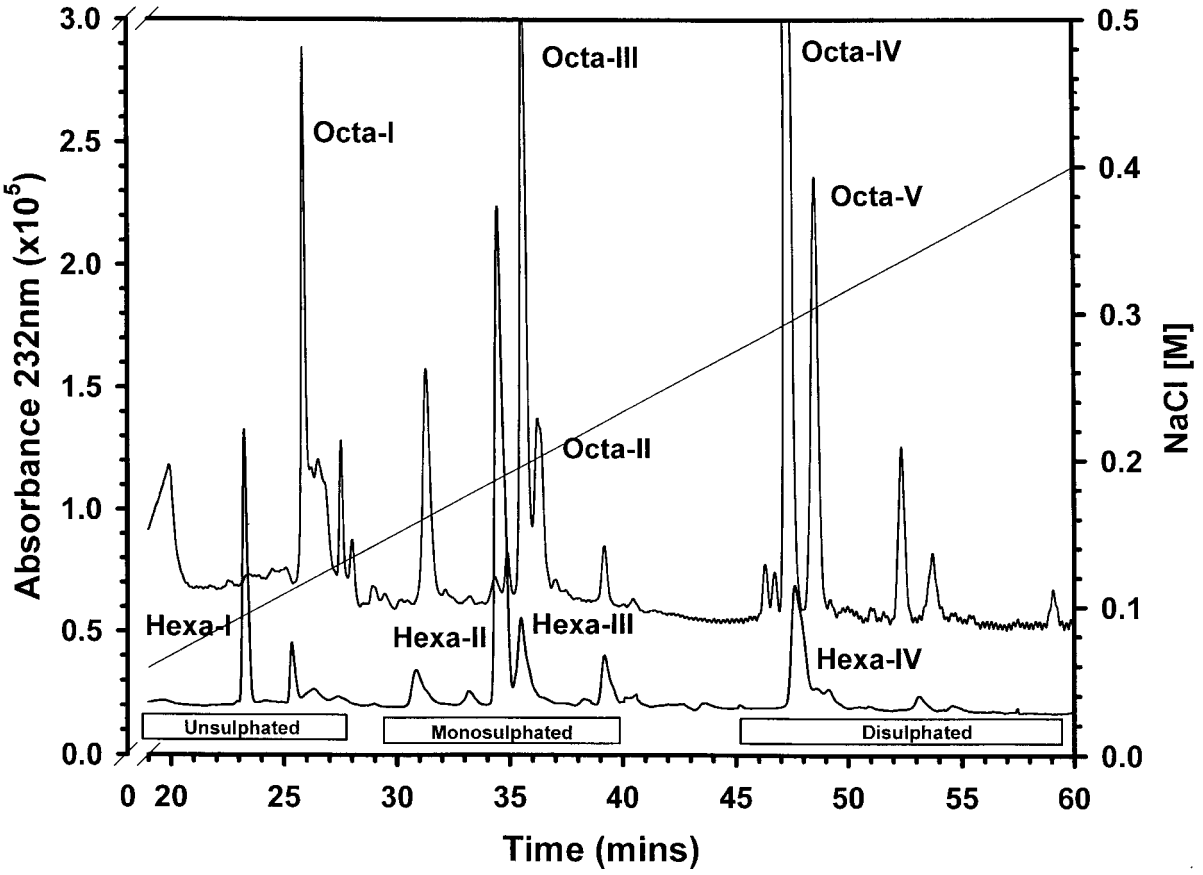

Figure 4 HPAEC analysis of the octa- and hexa-saccharide pools

The oligosaccharide pools from Mono-Q ion-exchange chromatography were chromatographed on a CarboPac PA1 column (250 mm × 4 mm) with a PA1 guard column (50 mm × 4 mm) maintained at 30 °C, and were eluted at 1ml/min. A 12-min isocratic period of 98% eluent A (0.1 M NaOH)/2% eluent B (1.3 M NaCl in 0.1 M NaOH) was followed by a linear gradient of 2–46% eluent B over 50 min; 46–87% eluent B over 8 min; 87–100% eluent B over 6 min; followed by a 4-min isocratic phase of 100% eluent B. The eluted oligosaccharides were monitored on-line by measuring A_{232}. Note that the oligosaccharide nomenclature is on the basis of elution order during preparative chromatography using a Spherisorb 5B SAX column; however, during analytical HPAEC, Octa-II is eluted after Octa-III.

are sulphated [38]. Thus these data suggest a reduced level of GlcA sulphation close to the linkage region. However, recent data point towards 2-sulphated uronic acids only occurring between a 4-sulphated GalNAc on the non-reducing side and a 6-sulphated GalNAc residue on the reducing side [12,13]. Of the oligosaccharides examined in this work, this environment is seen only in the novel tetrasaccharide repeat region extension of oligosaccharide Octa-V, representing approximately 12% or 32% of CS linkage regions from BAC and EAC respectively. It is not known if a sulphotransferase exists that is capable of sulphating a GlcA in this position relative to the linkage region. Uronic acid sulphation close to the linkage region, if present, is at a very low level; indeed, there are no reports of sulphated uronic acids occurring close to the linkage region.

Samples of BTC aggrecan were also examined by this fingerprinting method; however, only an abundance of hexasaccharide linkage region was observed. These data show that the linkage region of CS from this tissue is very similar to that of BAC. CS from BTC is highly 4-sulphated, although 6-sulphation might account for over 50% of GalNAc sulphation. Previously, Cheng et al. [43] have shown that the sulphation pattern from this tissue is non-random and that the majority of the 6-sulphated GalNAc residues are located towards the non-reducing end. The preliminary data reported here suggest that there may also be a preferential localization of 6-sulphated GalNAc residues at the linkage region. It is clear that these chains show the same preferential localization of unsulphated residues as seen in articular cartilage aggrecan CS linkage region.

Chondroitin ABC endolyase can digest a linkage region octasaccharide

We have confirmed the report [28] that chondroitin ABC endolyase cannot digest a CS tetrasaccharide into two disaccharides. However, we have shown that this enzyme can cleave off the non-reducing terminal disaccharide attached to an octasaccharide linkage region, irrespective of its sulphation profile.

The mechanism whereby the octasaccharides reported both here and by Huckerby et al. [29] remain intact when chondroitin ABC endolyase digestion is performed before their release, but are converted into hexasaccharides if it is performed afterwards, is currently unclear. It is possible that the protein core presents a barrier to the enzyme, which is then unable to gain access to cleave the final disaccharide.

In conclusion, it is clear from these data that the pattern of

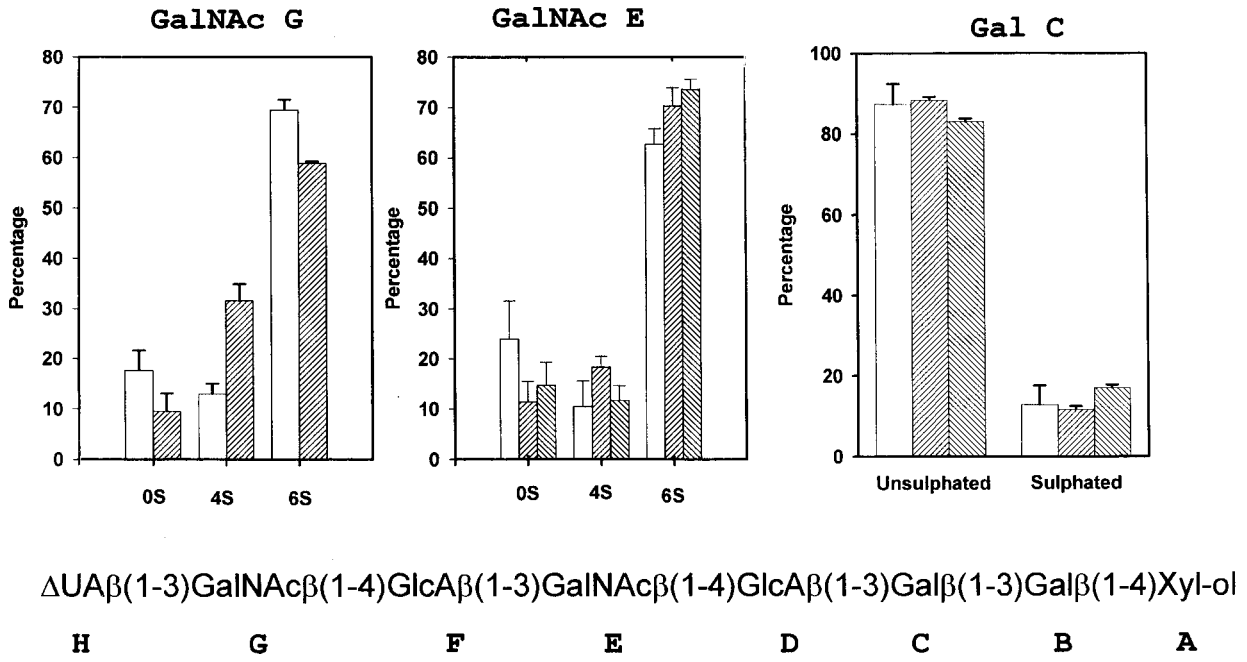

Figure 5 General structure of CS linkage regions, together with the incidence and position of sulphation

BAC is shown by the open bars, EAC by the bars with vertically ascending hatching, and BTC by the bars with descending hatching. 0S, 4S and 6S represent no O-ester sulphate groups, O-ester sulphate group on C-4, and O-ester sulphate group on C-6 respectively.

sulphation close to the linkage region of CS chains differs markedly from that seen in the repeat region. In particular, there is a decreased incidence of GalNAc 6-sulphation and a concomitant increase in the abundance of unsulphated and 4-sulphated residues.

We thank the Arthritis Research Campaign for support (Grant no. N0511). The Engineering and Physical Sciences Research Council is acknowledged for support in respect of use of their 600 MHz Edinburgh NMR Centre facilities, and the Biotechnology and Biological Sciences Research Council is acknowledged for support and travel funding. H. G. Morris is thanked for technical support.

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Received 3 September 1999/3 December 1999; accepted 4 February 2000