

## Expression of heparan sulphate L-iduronyl 2-O-sulphotransferase in human kidney 293 cells results in increased D-glucuronyl 2-O-sulphation

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Functionally important interactions between heparan sulphate and a variety of proteins depend on the precise location of O-sulphate groups. Such residues occur at C-2 of L-iduronic (IdoA) and D-glucuronic acid (GlcA) units, and at C-3 and C-6 of D-glucosamine (GlcN) units. Stable transfection of human embryonic kidney 293 cells with a cDNA encoding mouse mastocytoma IdoA 2-O-sulphotransferase resulted in an approx. 6-fold increase in O-sulphotransferase activity, compared with control cells, as determined using O-desulphated heparin as an acceptor. Structural analysis of endogenous heparan sulphate in the transfected cells, following metabolic labelling with either [<sup>3</sup>H]GlcN or [<sup>35</sup>S]sulphate, showed appreciable formation of

-GlcA(2-OSO<sub>3</sub>)-GlcNSO<sub>3</sub>- disaccharide units (6% of total disaccharide units; 17% of total O-sulphated disaccharide units) that were essentially absent from heparan sulphate from control cells. The increase in GlcA 2-O-sulphation was accompanied by a decrease in the amount of IdoA formed, whereas overall 2-O-sulphation or 6-O-sulphation remained largely unaffected. These findings indicate that 2-O-sulphation of IdoA and GlcA residues is catalysed by the same enzyme in heparan sulphate biosynthesis.

**Key words:** D-glucuronyl C-5 epimerization, D-glucuronyl 2-O-sulphotransferase, N-sulphation, O-sulphation, 6-O-sulphation.

### INTRODUCTION

Heparan sulphate (HS) proteoglycans, which are ubiquitous on cell surfaces and in the extracellular matrix, are composed of extended polysaccharide (glycosaminoglycan) chains covalently attached to various core proteins. HS has been implicated in diverse biological processes, such as assembly of extracellular matrices, control of cellular growth and differentiation, regulation of blood coagulation, viral infection, etc. [1–4]. Most of the functional roles of HS appear to depend on interactions of specific polysaccharide structures with selected proteins. Such structures are generated in HS biosynthesis, through the co-ordinated action of several enzymes. A precursor polysaccharide composed of alternating D-glucuronic acid (GlcA) and N-acetyl-D-glucosamine (GlcNAc; 2-deoxy-2-acetoamido-D-glucose) units thus is modified through a series of reactions that include, in consecutive order, N-deacetylation and N-sulphation of GlcN residues, C-5 epimerization of GlcA to L-iduronic acid (IdoA), 2-O-sulphation of uronic acid residues, and finally 6-O- and 3-O-sulphation of GlcN residues [2–4]. The modification reactions are variously constrained, through mechanisms that are only partly understood, but clearly depend on the substrate specificities of the corresponding enzymes. Regulated polymer modification yields HS species with different structural properties, as required for interactions with distinct proteins. The structurally related heparin may be conceived as an extensively modified, highly sulphated HS variant that is synthesized exclusively by con-

nective-tissue-type mast cells [5]. Current attempts to unravel the regulatory mechanisms in HS/heparin biosynthesis aim at the molecular cloning and characterization of the enzymes involved in the process. Such studies have shown that several of the enzymes occur as distinct isoforms that, in some cases at least, show distinct substrate specificities [6].

6-O-Sulphated GlcN and 2-O-sulphated IdoA residues are the major O-sulphated units in both heparin and HS, whereas 2-O-sulphated GlcA and 3-O-sulphated GlcN [2] are rare constituents. Nevertheless, such rare units may be functionally important, as shown by the obligatory requirement for a 3-O-sulphate group in the interaction of heparin/HS with the blood anticoagulant protein antithrombin [2,4]. The consistent occurrence of 2-O-sulphated GlcA units in HS from some tissues, such as human cerebral cortex [7], points to a specific functional role also for this component. It would therefore be of interest to define the mechanism of GlcA 2-O-sulphation, and to clarify the regulation of this reaction.

A 2-O-sulphotransferase (2-OST) catalysing the transfer of sulphate groups from adenosine 3'-phosphate 5'-phosphosulphate (PAPS) to IdoA residues in O-desulphated heparin was purified from Chinese hamster ovary (CHO) cells [8]. A corresponding cDNA was cloned from a CHO cell [9], and subsequently from a mouse embryo cDNA library [10]. In the present study a highly similar cDNA was isolated from a mouse mastocytoma cDNA library. Transfection of this cDNA into human embryonic kidney (HEK) 293 cells resulted, unexpectedly,

Abbreviations used: HEK, human embryonic kidney; HS, heparan sulphate; 2-OST, 2-O-sulphotransferase; CHO, Chinese hamster ovary; GlcA, D-glucuronic acid; IdoA, L-iduronic acid; GlcNAc, N-acetyl-D-glucosamine (2-deoxy-2-acetoamido-D-glucose); PAPS, adenosine 3'-phosphate 5'-phosphosulphate; HexA, unspecified hexuronic acid; G3PHD, glyceraldehyde-3-phosphate dehydrogenase; aMan<sub>n</sub>, 2,5-anhydro-D-mannitol (formed by reduction of terminal 2,5-anhydromannose residues with NaBH<sub>4</sub>); NSO<sub>3</sub>, N-sulphate group; OSO<sub>3</sub>, O-sulphate, ester sulphate group (the locations of O-sulphate groups are indicated in parentheses).

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The nucleotide sequence data reported will appear in the GenBank, EMBL and DDBJ Nucleotide Sequence Databases under accession number AF169243.

in the formation of a HS with decreased amounts of 2-O-sulphated IdoA units compared with control cells. Instead, appreciable 2-O-sulphation of GlcA residues was detected.

## MATERIALS AND METHODS

### Cloning and cDNA analysis of mouse mastocytoma 2-OST

A  $\lambda$ gt 11 mouse mastocytoma cDNA library [11] was screened by hybridization with an ~ 800 bp cDNA fragment of the CHO cell 2-OST transcript (nt -23 to 790; see Figure 2A of [9]). The probe was labelled with [ $\alpha$ - $^{32}$ P]dCTP (DuPont NEN) using Ready-To-Go DNA labelling beads (Pharmacia Biotech). After screening of about  $1 \times 10^6$  plaques, positive clones were plaque-purified and the phage DNA was isolated using the Lambda Midi kit (Qiagen). cDNA fragments obtained from the phage DNA after digestion with *EcoRI* were subcloned into the plasmid vector pUC 19 and sequenced using Cy5-labelled primers and the Cy5 AutoRead Sequencing kit (Pharmacia Biotech). Sequences were read by the ALF express system (Pharmacia Biotech) and analysed with the aid of the Lasergene software package (DNA-Star Inc.). Of the analysed clones, six contained cDNA encoding 2-OST. One of the clones, 3Ea1, was shown to contain the whole open reading frame. This clone was characterized further and used in subsequent studies.

### Expression of mouse 2-OST

To obtain an expression construct, clone 3Ea1 was digested with *Apal* and *Eco52I*, and the resultant cDNA fragment was purified and then digested with *BsrB1*. A fragment of 1723 bp thus generated, containing the open reading frame, was purified and ligated into the *EcoRV/NotI* sites of the pcDNA3 expression vector (Invitrogen). HEK 293 cells, grown as reported previously [12], were transfected with 5  $\mu$ g of pcDNA3-2-OST expression plasmid or with pcDNA3 vector alone using LipofectAMINE (Life Technologies Inc.) according to the protocol of the manufacturer. Stable clones were selected at a high concentration (800  $\mu$ g/ml) of Geneticin (G418; Gibco/BRL) and further maintained in normal growth medium containing 400  $\mu$ g/ml Geneticin. HEK cell clones containing the mouse enzyme were further selected by O-sulphotransferase assay using crude enzyme preparations (essentially as described in [13]).

### RNA preparation and Northern blot analysis

Total RNA was isolated from control transfected and 2-OST-transfected HEK cells using the RNeasy Total RNA kit (Qiagen). Denatured total RNA was fractionated on a 1.2% (w/v) agarose gel, blotted to a nylon membrane as described previously [12] and hybridized on two occasions with probes labelled with [ $\alpha$ - $^{32}$ P]dCTP, as described above. The probes used were a 217 bp *SacI* cDNA fragment recognizing the coding region of mouse 2-OST, and a 1.1 kb cDNA clone (Clontech) recognizing human glyceraldehyde-3-phosphate dehydrogenase (G3PHD) mRNA. The filters were hybridized at 65 °C in ExpressHyb solution (Clontech), first with the 217 bp *SacI* fragment and subsequently with human G3PHD cDNA.

### Metabolic labelling of glycosaminoglycans in HEK 293 cells

Subconfluent cultures of HEK cells were labelled at 37 °C for 24 h with 100  $\mu$ Ci/ml Na $^{35}$ SO $_4$  or [6- $^3$ H]glucosamine/HCl (both from DuPont NEN). After incubation the culture medium was removed, and the cell layer was washed twice with cold PBS and then detached with trypsin/EDTA solution. After addition of

trypsin inhibitor and centrifugation at 200 g for 15 min, the cell pellet was collected and incubated at 4 °C for 30 min with solubilization buffer containing 0.05 M Tris/HCl (pH 7.4), 1% Triton X-100 and protease inhibitors (2 mM EDTA, 1 mM PMSF, 2 mM *N*-ethylmaleimide and 10  $\mu$ g/ml pepstatin). After centrifugation at 400 g for 15 min, the supernatant was collected and an aliquot (100  $\mu$ l) was removed for protein determination using the Bio-Rad protein assay kit (Bio-Rad Laboratories). The remaining supernatant was adjusted to 0.15 M NaCl and proteoglycans were isolated by DEAE ion-exchange chromatography followed by papain digestion to obtain glycosaminoglycans, essentially as described in [12]. Following papain digestion, radioactive glycosaminoglycans were recovered by re-chromatography on a DEAE-Sephacel column eluted with 2.0 M NH $_4$ HCO $_3$ .

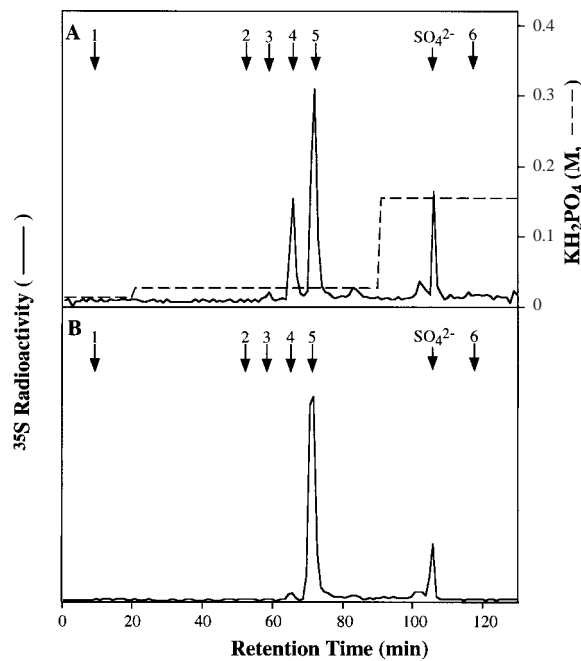
### Purification and structural analysis of HS

Galactosaminoglycans were eliminated by chondroitinase ABC digestion [12], and resistant HS chains were recovered after gel chromatography on a column (1 cm  $\times$  180 cm) of Sephadex G-25 in 0.2 M NH $_4$ HCO $_3$ . The product was desalted by lyophilization. Cleavage of the HS chains at N-sulphated GlcN residues was achieved by treatment with nitrous acid at pH 1.5 [14]. The resulting oligosaccharides were separated on a Bio-Gel P-10 Fine (Bio-Rad) column (1.5 cm  $\times$  150 cm) in 0.5 M NH $_4$ HCO $_3$  and effluent fractions were analysed for radioactivity. The resultant chromatograms were used to calculate the proportions of N-sulphate groups in the intact polysaccharides. Alternatively, the deamination was followed by reduction with NaBH $_4$  and the labelled deamination products were fractionated by gel chromatography on Sephadex G-15 equilibrated with 0.2 M NH $_4$ HCO $_3$ . Fractions corresponding to disaccharides were collected, lyophilized repeatedly and separated by anion-exchange HPLC (see below). For essentially complete depolymerization to disaccharides, labelled HS was chemically N-deacetylated by treatment with 70% (w/v) aqueous hydrazine (Fluka) containing 1% (w/v) hydrazine sulphate at 96 °C for 4 h, and the product was treated with nitrous acid at pH 1.5 and at pH 3.9 [15], followed by reduction with NaBH $_4$ . Labelled disaccharides were analysed by anion-exchange HPLC using a Whatman Partisil 10-SAX column [16], as described in the legend to Figure 4. The non-sulphated disaccharides GlcA-aMan $_R$  and IdoA-aMan $_R$  (where aMan $_R$  is 2,5-anhydro-D-mannitol, formed by reduction of terminal 2,5-anhydromannose residues with NaBH $_4$ ) were resolved by descending paper chromatography as described in [12]. HexA-[ $^3$ H]aMan $_R$  disaccharides (where HexA denotes unspecified hexuronic acid), with and without O-sulphate groups in different positions, used as reference compounds were as described in [17]; in addition, a synthetic GlcA(2-OSO $_3$ )-[ $^3$ H]aMan $_R$  standard was generated as reported in [18].

## RESULTS

### cDNA cloning and expression of mouse 2-OST

Mouse mastocytoma 2-OST cDNA was isolated following screening of a cDNA library with a cDNA probe corresponding to parts of the coding region of the CHO cell cDNA transcript (see the Materials and methods section). Several positive clones were isolated and subsequently sequenced. One clone was shown to encompass an open reading frame of 1068 bp, and contained in addition 26 bp of 5'- and ~ 0.6 kb of 3'-untranslated sequence (GenBank accession no. AF169243). The open reading frame encodes a protein of 356 amino acid residues which has 98% identity with the published hamster HS 2-OST [9] and 97%

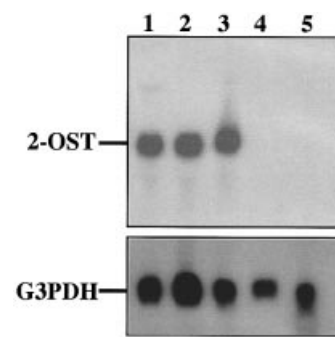


**Figure 1** Anion-exchange HPLC of disaccharides isolated after deaminative cleavage of  $^{35}\text{S}$ -labelled heparin

De-O-sulphated heparin with the predominant structure  $(\text{-IdoA-GlcNSO}_3)_n$  was incubated with cell extracts from (A) control HEK cells or (B) 2-OST-transfected HEK cells plus [ $^{35}\text{S}$ ]PAPS essentially as described in [13]. After incubation, labelled polysaccharides were recovered by gel filtration, lyophilized and treated with nitrous acid, pH 1.5, followed by reduction with  $\text{NaBH}_4$ . The resulting  $^{35}\text{S}$ -labelled disaccharides were analysed on a Partisil-10 SAX column eluted at a rate of 1 ml/min with  $\text{KH}_2\text{PO}_4$  solutions of stepwise increasing concentration (indicated by the broken line). Non-sulphated disaccharides were eluted with 0.012 M, monosulphated disaccharides with 0.026 M and disulphated disaccharides with 0.15 M  $\text{KH}_2\text{PO}_4$ . The elution positions of standard disaccharides are indicated by arrows: 1, non-sulphated HexA-aMan $_6$ ; 2, GlcA(2-OSO $_3$ )-aMan $_6$ ; 3, GlcA-aMan $_6$ (6-OSO $_3$ ); 4, IdoA-aMan $_6$ (6-OSO $_3$ ); 5, IdoA(2-OSO $_3$ )-aMan $_6$ ; 6, IdoA(2-OSO $_3$ )-aMan $_6$ (6-OSO $_3$ ).  $\text{SO}_4^{2-}$  indicates inorganic sulphate.

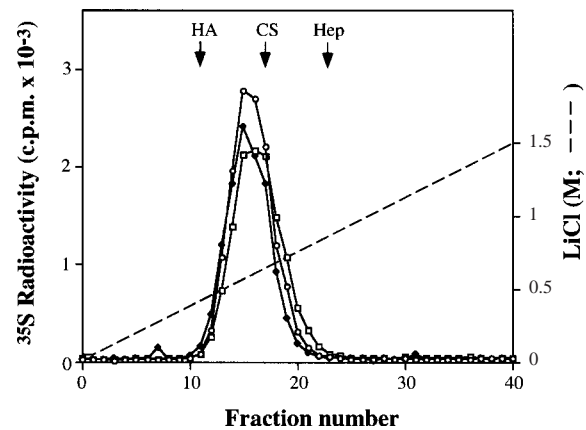
identity with the corresponding human enzyme. Of the four amino acid changes between the hamster and the mouse enzymes, three are conservative substitutions. Except for a single nucleotide, the sequence of the mouse mastocytoma enzyme is identical to that of the 2-OST cDNA recently published by Bullock et al. [10]. Exchange of this nucleotide converts a valine residue (Figure 1, unit 271, in [10]) into a phenylalanine residue.

The cDNA coding for the mouse mastocytoma 2-OST was stably transfected into the HEK cell line. Control HEK cells were transfected with the vector alone. A total of 25 2-OST-transfected clones and eight control transfected clones were isolated and analysed for O-sulphotransferase activity, using O-desulphated heparin as a sulphate acceptor. The 2-OST-transfected HEK cell clones displayed a 3–6-fold increase in O-sulphotransferase activity compared with that of control clones. The O-sulphotransferase activities of the HEK clones used in this study were  $70 \pm 8$  pmol/min per mg of protein ( $n = 3$ ) and  $75 \pm 4$  pmol/min per mg ( $n = 3$ ) for the 2-OST-transfected clones (C3 and 2B2 respectively), and  $13 \pm 1$  pmol/min per mg ( $n = 3$ ) for the control clone (cB2). This increase in activity was correlated with 2-O-sulphation of IdoA residues in the exogenous acceptor polysaccharide. Lysates of control HEK cell clones thus transferred both IdoA 2-O- and GlcN 6-O-sulphate groups to the exogenous substrate, whereas lysates of the transfected HEK cell clones catalysed predominantly IdoA 2-O-sulphation (Figure 1).



**Figure 2** Expression of mouse mastocytoma 2-OST in HEK cells

Northern blot analysis of total RNA isolated from stably 2-OST-transfected cells (clones C3, 2A3 and 2B2; lanes 1–3), and control transfected cells (clones cB1 and cB2; lanes 4 and 5). Total RNA (10  $\mu\text{g}$ ) was separated by electrophoresis, blotted on to a nylon membrane and hybridized, first with a  $^{32}\text{P}$ -labelled cDNA fragment specific for mouse 2-OST and subsequently with a probe recognizing human G3PDH (see the Materials and methods section).



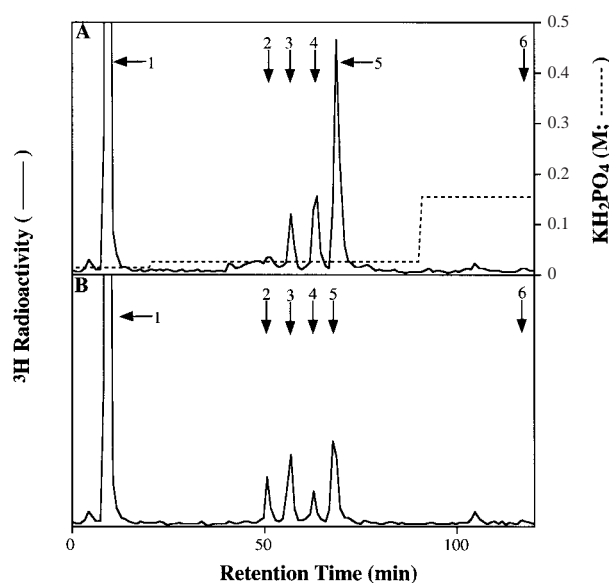
**Figure 3** Ion-exchange chromatography of  $^{35}\text{S}$ -labelled HS isolated from control transfected or 2-OST-transfected HEK cells

$^{35}\text{S}$ -Labelled HS from control transfected (cB2,  $\blacklozenge$ ) or 2-OST-transfected (2B2,  $\circ$ ; C3,  $\square$ ) HEK cell clones was isolated as described in the Materials and methods section. Samples of  $^{35}\text{S}$ -labelled HS (15000 c.p.m.) were mixed with unlabelled standards of hyaluronan (HA), chondroitin sulphate (CS) and heparin (Hep) and were applied to a 1 ml column of DEAE-Sephacel equilibrated with 0.05 M LiCl in 0.05 M acetate buffer, pH 4.0. The column was eluted with a gradient from 0.05 to 1.5 M LiCl in the above acetate buffer. Fractions of 0.5 ml were collected and analysed for radioactivity by scintillation counting, and for hexuronic acid by the m-phenylphenol colorimetric assay [33].

Figure 2 shows the results of Northern blot analysis using a mouse mastocytoma 2-OST cDNA probe that was hybridized to total RNA isolated from 2-OST-transfected (lanes 1–3) and control transfected (lanes 4 and 5) cells. RNA from the transfected HEK cells showed a strongly hybridizing component of the expected size (1.7 kb) for the expressed construct. In contrast, no signal was detected in control cells that had been transfected with the vector alone. The increased 2-OST mRNA levels were correlated with the O-sulphotransferase activities of the transfected cells.

#### Effects of transfection on HS structure

Two 2-OST-transfected HEK cell clones (C3 and 2B2; lanes 1 and 3 respectively in Figure 2) and one control HEK cell clone (cB2; lane 5 in Figure 2) were metabolically labelled with



**Figure 4** Anion-exchange HPLC of disaccharides obtained on deamination of  $^3\text{H}$ -labelled HS isolated from (A) control transfected (cB2) or (B) 2-OST-transfected (2B2) HEK cells

Labelled polysaccharide was prepared as described in the Materials and methods section, and was degraded after N-deacetylation by combined deamination at pH 1.5 and pH 3.9 followed by reduction of the products. Samples of isolated disaccharides were analysed on a Partisil-10 SAX column as described in the legend to Figure 1. The  $^3\text{H}$ -labelled standards indicated by arrows 1–6 are: 1, non-sulphated HexA-aMan<sub>R</sub>; 2, GlcA(2-OSO<sub>3</sub>)-aMan<sub>R</sub>; 3, GlcA-aMan<sub>R</sub>(6-OSO<sub>3</sub>); 4, IdoA-aMan<sub>R</sub>(6-OSO<sub>3</sub>); 5, IdoA(2-OSO<sub>3</sub>)-aMan<sub>R</sub>; 6, IdoA(2-OSO<sub>3</sub>)-aMan<sub>R</sub>(6-OSO<sub>3</sub>).

[ $^{35}\text{S}$ ]sulphate or [ $^3\text{H}$ ]glucosamine. The yields of [ $^3\text{H}$ ]glucosamine-labelled polysaccharides differed only slightly between 2-OST-transfected cells ( $290 \times 10^3$  and  $260 \times 10^3$  c.p.m./mg of protein

for the two clones) and control cells ( $230 \times 10^3$  c.p.m./mg); for the  $^{35}\text{S}$ -labelled material the difference was even smaller ( $190 \times 10^3$ ,  $210 \times 10^3$  and  $200 \times 10^3$  c.p.m./mg respectively). However, the proportion of HS, i.e. material resistant to digestion by chondroitinase ABC, increased from 79% of the  $^3\text{H}$ -labelled glycosaminoglycans in the control HEK cell clones to 88% and 89% in the two 2-OST-transfected HEK cell clones; for the  $^{35}\text{S}$ -labelled polysaccharides this increase was from 54% to 65% and 70% respectively. No apparent difference in total net charge density was noted on anion-exchange chromatography of HS from transfected and control cells (Figure 3).

The N-substitution patterns of  $^3\text{H}$ -labelled HS from 2-OST-transfected clone 2B2 and control cB2 cells were analysed by treatment of the polysaccharides with nitrous acid at pH 1.5 (this results in cleavage of the chains at the sites of N-sulphated GlcN units) followed by gel chromatography of the products on Bio-Gel P10 (results not shown). Calculations based on the distribution of radiolabel between oligosaccharides of different sizes [19] indicated that ~30% and ~40% of the GlcN units in HS from 2-OST-transfected and control cells respectively were N-sulphated. Introduction of the 2-OST enzyme thus led to considerably decreased N-sulphation of the polysaccharide.

The overall disaccharide composition of HS from transfected and control HEK cells was determined following N-deacetylation and complete deaminative cleavage of the samples. Separation of the products by anion-exchange HPLC yielded a major peak of non-sulphated disaccharide, a group of different mono-O-sulphated disaccharides and only minor amounts of di-O-sulphated disaccharide (Figure 4). The non-O-sulphated disaccharides were separated into GlcA-aMan<sub>R</sub> and IdoA-aMan<sub>R</sub> species by paper chromatography. Analysis of the relative proportions of the various components revealed that some structural parameters remained unaffected by the 2-OST transfection, whereas others were dramatically affected (Table 1). The overall relationship between non-O-sulphated and O-sulphated disaccharide units thus was essentially unchanged, as were

**Table 1** Effect of 2-OST transfection on the composition of HS produced by HEK cells

[ $^{35}\text{S}$ ]sulphate- or [ $^3\text{H}$ ]GlcN-labelled samples from control cells (clone cB2) and cells transfected with 2-OST (clones 2B2 and C3) were completely degraded to disaccharides that were analysed by anion-exchange HPLC (see the Materials and methods section). Individual preparations from each clone were analysed; the values for the  $^3\text{H}$ -labelled control clone cB2 represent averages of two preparations (which varied by  $\leq 2\%$ ). ND, not determined. The values in parentheses relate to total mono-O-sulphated  $^3\text{H}$ -labelled disaccharides.

Deamination products	$^3\text{H}$ -labelled (% of total [ $^3\text{H}$ ]disaccharides)			$^{35}\text{S}$ -labelled (% of mono-O- [ $^{35}\text{S}$ ]sulphated disaccharides)*		
	2B2	C3	cB2	2B2	C3	cB2
GlcA-aMan <sub>R</sub>	63†	ND	53			
IdoA-aMan <sub>R</sub>	2†	ND	10			
GlcA(2-OSO <sub>3</sub> )-aMan <sub>R</sub>	6 (18)	6 (19)	1 (4)	16	17	4
GlcA-aMan <sub>R</sub> (6-OSO <sub>3</sub> )	9 (27)	6 (20)	5 (14)	18	15	10
IdoA-aMan <sub>R</sub> (6-OSO <sub>3</sub> )	5 (15)	3 (9)	8 (23)	9	9	21
IdoA(2-OSO <sub>3</sub> )-aMan <sub>R</sub>	13 (40)	15 (52)	21 (59)	57	59	65
IdoA(2-OSO <sub>3</sub> )-aMan <sub>R</sub> (6-OSO <sub>3</sub> )	2	5	1			
Totals						
IdoA-containing disaccharides	22	ND	42			
Non-sulphated disaccharides	65	65	63			
2-O-sulphated disaccharides	21	26	23	73	76	69
Containing GlcA(2-OSO <sub>3</sub> )	6	6	1			
Containing IdoA(2-OSO <sub>3</sub> )	15	20	22			
6-O-sulphated disaccharides	16	14	14	27	24	31

\* The di-O-sulphated  $^{35}\text{S}$ -labelled disaccharide was not resolved from unknown components.

† The amounts of the two non-sulphated disaccharide species derived from  $^3\text{H}$ -labelled transfected HEK cells were determined in two separate experiments using the 2B2 clone. The results differed by  $\leq 2\%$  from those shown.

the proportions of total 2-O- and 6-O-sulphated disaccharides. The most striking difference was the appearance in the transfected HEK cells of 2-O-sulphated GlcA units, which were barely detectable in the HS from control cells (Figure 4; Table 1). The sulphated GlcA units were recovered as GlcA(2-OSO<sub>3</sub>)-aMan<sub>R</sub> disaccharide, which amounted to almost 20% of the mono-O-sulphated disaccharide units of HS from transfected cells. This particular disaccharide species was identified by its co-migration with a synthetic reference compound [18] on anion-exchange HPLC and on high-voltage paper electrophoresis, by its resistance to digestion with  $\beta$ -D-glucuronidase and by its partial conversion into IdoA(2-OSO<sub>3</sub>)-aMan<sub>R</sub> upon treatment with hydrazine/hydrazine sulphate (results not shown). The formation of -GlcA(2-OSO<sub>3</sub>)-GlcNSO<sub>3</sub>- units (recovered as the corresponding aMan<sub>R</sub>-containing derivatives after deamination) was corroborated by analysis of <sup>35</sup>S-labelled HS samples. The compositions of HS <sup>35</sup>S-labelled disaccharides derived from two distinct 2-OST-transfected HEK cell clones thus were strikingly similar, with the proportion of the GlcA(2-O-<sup>35</sup>SO<sub>3</sub>)-aMan<sub>R</sub> component being the same as that of GlcA(2-OSO<sub>3</sub>)-[<sup>3</sup>H]aMan<sub>R</sub> in the HS from [<sup>3</sup>H]GlcN-labelled 2-OST-transfected cells (Table 1). Again, this <sup>35</sup>S-labelled disaccharide species was barely detectable in HS from control HEK cells. Formation of the <sup>3</sup>H-labelled GlcA(2-OSO<sub>3</sub>)-aMan<sub>R</sub> disaccharide was not dependent on complete depolymerization of the HS chain, but was also observed following direct low-pH deaminative cleavage (results not shown), thus indicating that at least part of the 2-O-sulphated GlcA residues were located in the contiguous N-sulphated domains of the polymer (see [15]).

Another major difference between HS species from 2-OST-transfected and control HEK cells was the decrease in IdoA content, from ~40% of total hexuronic acid in control HS to only ~20% following transfection (Table 1). This difference was expressed through all types of IdoA-containing disaccharide units, and was particularly apparent for the non-O-sulphated sequences, where IdoA was virtually absent in HS from transfected cells. A larger proportion of the total IdoA residues in HS from 2-OST-transfected cells were 2-O-sulphated (70%, compared with ~55% in control HS).

## DISCUSSION

The structural changes in HS induced by transfection of HEK cells with 2-OST were highly distinctive. While the overall extent of O-sulphation, including 2-O-sulphation, remained more or less unaffected, 2-O-sulphated GlcA residues, essentially absent from control cells, increased in 2-OST-transfected cells, with a concomitant decrease in 2-O-sulphated IdoA units. The most reasonable interpretation of these findings is that 2-OST recognizes both GlcA and IdoA residues as substrates. This conclusion is supported by the finding that recombinant 2-OST will sulphate GlcA residues in an exogenous (-GlcA-GlcNSO<sub>3</sub>)<sub>n</sub> acceptor polysaccharide of the appropriate structure (J. Rong, H. Habuchi, K. Kimata, U. Lindahl and M. Kusche-Gullberg, unpublished work). Similar findings were recently reported for a 2-OST capable of sulphating both GlcA and IdoA units in chondroitin/dermatan sulphate [20]. Notably, a mutant CHO cell line deficient in 2-OST activity was found to lack both IdoA(2-OSO<sub>3</sub>) and GlcA(2-OSO<sub>3</sub>) units [21]. Although we cannot exclude the presence of additional genes (see below), our results strongly suggest that a single enzyme sulphates both IdoA and GlcA residues in HS (and heparin?) biosynthesis.

These findings raise the question of how sulphation of GlcA residues is normally regulated in HS biosynthesis. The HS synthesized by the 2-OST-transfected HEK cells appears similar

in composition to HS isolated from aged human cerebral cortex [7]. Moreover, both polysaccharides contain 2-O-sulphated GlcA units in contiguous N-sulphated domains. A HS preparation obtained from neonatal brain lacked this particular component, suggesting that sulphation of GlcA, relative to IdoA, residues is subject to regulation. While mere overproduction of 2-OST, as achieved in the present study of enzyme transfection – albeit artificial – is apparently sufficient to induce a compositional change mimicking the effects of such regulation, the regulatory mechanisms operating in the unperturbed cell are likely to be more refined. Conceivably, such mechanisms may change the site of enzyme action along the biosynthetic assembly line, so that the 2-O-sulphation reaction is initiated after or along with N-sulphation but before GlcA C-5 epimerization. Studies of polymer modification using a microsomal fraction from (a heparin-producing) mouse mastocytoma showed that 2-O-sulphation of GlcA residues took place before 6-O-sulphation of GlcN units [22]. Moreover, back-epimerization of IdoA(2-OSO<sub>3</sub>) to GlcA(2-OSO<sub>3</sub>) residues was found not to occur [22]; in fact, the presence of O-sulphate groups either on the potential target hexuronic acid or on adjacent GlcN units will preclude epimerization [23].

The shift in O-sulphation pattern induced by 2-OST transfection was accompanied by additional structural changes in cellular HS, namely considerable decreases in N-sulphation as well as total IdoA formation. Several explanations to be considered include irregular positioning of the enzyme in the Golgi complex, leading to perturbation of the appropriate interaction between the different biosynthetic enzymes. While the mechanisms behind the observed effects remain unclear, it is notable that HS produced by mutant CHO cells deficient in 2-O-sulphation was more extensively N-sulphated than the corresponding wild-type polysaccharide [21]. These findings suggest that the N- and 2-O-sulphation reactions may be mutually interdependent, such that N-sulphation is not only prerequisite to O-sulphation [2], but also restricted by the latter reaction(s). The inhibition of IdoA formation in the 2-OST-transfected HEK cells may be secondary to the decreased N-sulphation (since N-sulphate groups are required for substrate recognition by the GlcA C-5 epimerase [2]), but also to the 2-O-sulphation of GlcA residues, which will thus be withdrawn from C-5 epimerization.

Most of the enzymes involved in the biosynthesis of HS have now been purified and their cDNAs cloned. Interestingly, each of a number of these enzymes, including the 'polymerase' catalysing chain elongation [24], the GlcN N-deacetylase/N-sulphotransferase [25–28], the GlcN 6-O-sulphotransferase [2] and the GlcN 3-O-sulphotransferase [4,29,30], appears to occur as several genetically distinct isoforms that may differ with regard to substrate specificity. Indeed, the present study was partly initiated because of our previous finding of a protein fraction, partially purified from mouse mastocytoma tissue, that contained IdoA 2-O- as well as GlcN 6-O-sulphotransferase activities (presumably committed to the biosynthesis of heparin) [13]. Our present results do not support the notion of a mast-cell-specific enzyme with dual O-sulphotransferase activities, since the mastocytoma 2-OST was found to be strikingly similar to the enzyme species implicated previously in HS biosynthesis [9]. So far, no 2-OST isoforms have been found in vertebrates. Notably, however, mouse 2-OST was found to be differentially expressed during embryonic development [10]. Mice deficient in 2-O-sulphation display renal agenesis as well as eye and skeletal defects, and die before birth [10]. It is not known whether these defects reflect a total lack of 2-O-sulphate groups in HS, or only partial loss due to elimination of one out of two or more 2-OST isoforms. Two isoforms of a protein in *Drosophila melanogaster* were recently described, both with ~50% amino acid similarity (~30%

identity) relative to vertebrate 2-OST [31]. These proteins differ from the previously known segregation distorter protein in *Drosophila* [32], now recognized as a 2-OST homologue with 75% similarity to vertebrate 2-OST (50% identity on the amino acid level). Whether these *Drosophila* proteins have 2-OST activity similar to that of the vertebrate enzyme remains to be investigated.

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