

# Biosynthesis of heparan sulphate with diverse structures and functions: two alternatively spliced forms of human heparan sulphate 6-*O*-sulphotransferase-2 having different expression patterns and properties

Hiroko HABUCHI\*, Goichiro MIYAKE\*, Ken NOGAMI\*, Asato KUROIWA†‡, Yoichi MATSUDA†‡, Marion KUSCHE-GULLBERG§, Osami HABUCHI||, Masayuki TANAKA¶ and Koji KIMATA\*<sup>1</sup>

\*Institute for Molecular Science of Medicine, Aichi Medical University, Nagakute, Aichi 480-1195, Japan, †Laboratory of Cytogenetics, Division of Bioscience, Graduate School of Environmental Earth Science, Hokkaido University, North 19 West 8, Kita-ku, Sapporo 060-0810, Japan, ‡Chromosome Research Unit, Faculty of Science, Hokkaido University, North 19 West 8, Kita-ku, Sapporo 060-0810, Japan, §Department of Medical Biochemistry and Microbiology, Uppsala University, S-75123 Uppsala, Sweden, ||Department of Life Science, Aichi University of Education, Igaya-cho, Kariya, Aichi 448-8542, Japan, and ¶Seikagaku Corporation, Nihonbashi-Homcho 2-9, Japan

Heparan sulphate 6-*O*-sulphotransferase (HS6ST) catalyses the transfer of sulphate from adenosine 3'-phosphate, 5'-phosphosulphate to the 6th position of the *N*-sulphoglucosamine residue in HS. We previously described the occurrence of three isoforms of mouse HS6ST, mHS6ST-1, -2, and -3 [Habuchi, Tanaka, Habuchi, Yoshida, Suzuki, Ban and Kimata (2000) *J. Biol. Chem.* **275**, 2859–2868]. In the present study, we have characterized HS6ST-2 and HS6ST-1 human isoforms, including their chromosomal localizations. In the process of their cDNA cloning, we found two forms of HS6ST-2: the original (hHS6ST-2) and a short form (hHS6ST-2S) with 40 amino acids deleted. Both hHS6ST-2 and hHS6ST-2S catalysed the same sulphation reaction, but their preferences for sulphation sites in HS substrates

were different. Dot-blot analysis of the two forms showed that the original form was exclusively expressed in adult and foetal brain tissues, whereas the short form was expressed preferentially in ovary, placenta and foetal kidney, suggesting that the expression of two forms of hHS6ST-2 is strictly regulated to yield tissue-dependent differences in the fine structure of HS. A refined analysis of their reaction products has led us to another finding, that HS6STs could also transfer sulphate to *N*-sulphoglucosamine residues located at the non-reducing terminal of HS with high affinity.

**Key words:** cDNA cloning, GlcNSO<sub>3</sub>-6-*O*-sulphation, glycosaminoglycan.

## INTRODUCTION

Heparan sulphate proteoglycans (HSPGs) are present ubiquitously on the cell surface, extracellular matrix and basement membranes [1,2]. The HS chains in HSPGs interact with huge numbers of ligands such as growth factors, morphogens, proteases, viral glycoproteins and basement membrane components [3–5]. These interactions in *in vitro* and *in vivo* systems have been shown to be implicated not only in various cell behaviours such as cell growth, differentiation, adhesion and migration, but also in tissue morphogenesis during development [6–9]. Furthermore, HS is involved in various patho-physiological phenomena such as inflammation, blood coagulation, tumour cell malignancy and microbial infection [10,11]. Recent genetic studies on *Drosophila*, *Caenorhabditis elegans*, Zebrafish and mice are uncovering these functions of HSPGs [12].

Not all but most interactions between HS and ligand molecules occur in regions of the HS with specific monosaccharide sequences and sulphation positions [13]. These sequences are generated by complex but strictly regulated modification reac-

tions during the biosynthesis of HS [14]. To clarify the biological function of the sophisticated structure of HSs, it is important to reveal the regulation mechanism of the biosynthesis of HS. At the first step in the biosynthesis of HS, xylose (Xyl) assembles at specific serine residues on the core protein, and after the completion of the linkage structure GlcA-Gal-Gal-Xyl (where GlcA stands for glucuronic acid), transfer of the first *N*-acetylglucosamine (GlcNAc) residue is followed by the elongation of repeating units composed of GlcA and GlcNAc residues [15]. These precursor HS chains undergo a series of modification reactions [11,16,17]: *N*-deacetylation and *N*-sulphation of GlcNAc residue, conversion of GlcA into iduronic acid (IdoA), 2-*O*-sulphation of IdoA residue, 6-*O*-sulphation of *N*-sulphoglucosamine (GlcNSO<sub>3</sub>) residue and 3-*O*-sulphation of GlcNSO<sub>3</sub>/glucosamine residue. The extent to which the modifications proceed and the order in which each modification occurs generate the divergent structures in the HS. In vertebrates, each modification reaction, except for GlcA C-5 epimerase [18,19] and HS 2-*O*-sulphotransferase [20], is catalysed by isoforms that are derived from different but related genes and constitute a gene

Abbreviations used: CDSNS-heparin, completely desulphated and *N*-resulphated heparin; CHO, Chinese-hamster ovary; ΔDi-NS, 2-deoxy-2-sulphamino-4-*O*-(4-deoxy- $\alpha$ -L-threo-hex-4-enopyranosyluronic acid)-D-glucose; ΔDi-(N,2)diS, 2-deoxy-2-sulphamino-4-*O*-(4-deoxy-2-*O*-sulpho- $\alpha$ -L-threo-hex-4-enopyranosyluronic acid)-D-glucose; ΔDi-(N,6)diS, 2-deoxy-2-sulphamino-4-*O*-(4-deoxy- $\alpha$ -L-threo-hex-4-enopyranosyluronic acid)-6-*O*-sulpho-D-glucose; ΔDi-(N,6,2)tris, 2-deoxy-2-sulphamino-4-*O*-(4-deoxy-2-*O*-sulpho- $\alpha$ -L-threo-hex-4-enopyranosyluronic acid)-6-*O*-sulpho-D-glucose; ΔDi-OS, 2-acetamide-2-deoxy-4-*O*-(4-deoxy- $\alpha$ -L-threo-hex-4-enopyranosyluronic acid)-D-glucose; ΔDi-6S, 2-acetamide-2-deoxy-4-*O*-(4-deoxy- $\alpha$ -L-threo-hex-4-enopyranosyluronic acid)-6-*O*-sulpho-D-glucose; FISH, fluorescence *in situ* hybridization; GAG, glycosaminoglycan; GlcA, glucuronic acid; GlcNAc, *N*-acetylglucosamine; GlcNSO<sub>3</sub>, *N*-sulphoglucosamine; HexA, hexuronic acid; HS, heparan sulphate; HS6ST, HS 6-*O*-sulphotransferase; HSPG, heparan sulphate proteoglycan; IdoA, iduronic acid; NS-heparosan, deacetylated and *N*-sulphated heparosan; PAPS, adenosine 3'-phosphate 5'-phosphosulphate; RT, reverse transcriptase.

<sup>1</sup> To whom correspondence should be addressed (e-mail Kimata@amugw.aichi-med-u.ac.jp).

The nucleotide sequences reported in the present study have been submitted to the DDBJ/GenBank®/EMBL DataBank with accession nos AB067776 (human HS6ST-2 mRNA) and AB067777 (human HS6ST-2S mRNA).

family; N-deacetylation and N-sulphation of GlcNAc residue by four HS N-deacetylases and N-sulphotransferases [21–25], 6-O-sulphation of GlcNSO<sub>3</sub> residue by three heparan sulphate 6-O-sulphotransferases (HS6STs) [26,27], and 3-O-sulphation of GlcNSO<sub>3</sub>/glucosamine residue by at least five HS3-O-STs [28–30]. However, in invertebrates such as *Drosophila* and *C. elegans*, only one HS N-deacetylase and N-sulphotransferase and one HS6ST appear to be involved in the N-sulphation and 6-O-sulphation respectively [31,32]. The occurrence of multiple isoforms of the enzymes may indicate that the biosynthesis of HS is much more strictly regulated in vertebrates than in invertebrates. We described three isoforms of mouse HS6ST encoded by distinct genes previously. The expression patterns, substrate specificities and catalytic properties of these isoforms were quite different from each other. *Drosophila* has only one HS6ST that shows intermediate substrate specificity between mouse (m)HS6ST-1 and mHS6ST-2 [32]. We have also suggested that *Drosophila* HS6ST plays a pivotal role in tracheal development by affecting signal transduction via fibroblast growth factor receptor (*breathless*). The apparent requirement of *Drosophila* HS6ST in the tracheogenesis may correspond to the observation in vertebral systems that 6-O-sulphated glucosamine residues as well as 2-O-sulphated IdoA residues in HS are unequivocally needed for fibroblast growth factor 2 signalling [33–36]. The *Drosophila* system seems to offer valuable information on human diseases, because *Drosophila* tracheogenesis shares many features with the angiogenesis that is disordered in various human diseases [32].

We have investigated human isologues of HS6Ts to determine whether or not the properties of the human enzymes are different from those of other animal enzymes and to clarify the relationship between the fine structures of HS and human disease. We described the complete sequence of human HS6ST-1 previously and studied some of the enzyme properties as the first report on HS6ST [26]. Although we were isolating the cDNA of human isologues of HS6T-2, we found two forms generated by alternative splicing. In the present study, we show the occurrence of HS6ST-2 and its alternatively spliced form, HS6ST-2S, in humans and their different substrate specificities and restricted expression patterns in various tissues. We have also characterized human HS6ST-1 further and found a sharp difference in the expression pattern between humans and mice. Furthermore, we have examined the chromosomal localizations of genes for these enzymes for clues to the possibility that abnormal or defective expression of these genes may cause some diseases in humans. Overall, HS6STs could be important determinative factors for the tissue- and species-dependent differences in the fine structure of HS.

## EXPERIMENTAL

### Materials

H<sub>2</sub><sup>35</sup>SO<sub>4</sub> was purchased from PerkinElmer Life Science (Branchburg, NJ, U.S.A.); Hybond N<sup>+</sup> from Amersham Pharmacia Biotech; Human Multiple Tissue Expression Array from ClonTech (Palo Alto, CA, U.S.A.); HiLoad 16/60 Superdex 30 pg and Fast Desalting column HR 10/10 from Amersham Pharmacia Biotech; and a PAMN column from YMC (Kyoto, Japan). Heparitinases I (EC 4.2.2.8), II and III (EC 4.2.2.7), chondroitin sulphate A, chondroitin sulphate C, completely desulphated and N-resulphated heparin (CDSNS-heparin), 6-O-desulphated heparin (6ODS-heparin), 2-O-desulphated heparin (2ODS-heparin), HS from pig aorta and an unsaturated glycosaminoglycan (GAG) disaccharide kit were obtained from Seikagaku Corp. (Tokyo, Japan). Heparin from porcine intestinal mucosa, unlabelled adenosine 3'-phosphate 5'-phosphosulphate

(PAPS), N-acetylglucosamine 6-sulphate and anti-FLAG M2 agarose were purchased from Sigma. Deacetylated and N-sulphated heparosan (NS-heparosan) was prepared by chemical deacetylation and N-sulphation from N-acetyl heparosan, which was prepared from *Escherichia coli* K5 by Dr Terumi Saito (Kanagawa University, Japan). [<sup>35</sup>S]PAPS was prepared as described previously [37].

### Isolation of cDNAs encoding human HS6ST-2

Human counterparts to mouse HS6ST-2 were searched in DNA databases using the TBLASTN program. We found two cDNA genes (GenBank<sup>®</sup> accession nos. AL049679 and AA315705) and two genomic DNAs (Z86064 and Z81365) covering the predicted coding region. The cDNA containing the whole coding region were amplified from the cDNA prepared from human brain RNA (ClonTech) by PCR using Q buffer (Qiagen, Valencia, CA, U.S.A.) and 5'-site primer (nt -14 to 6, CCAGCGTCGGGAACATGGAT; 1 stands for the first nucleotide of the putative coding region) and 3'-site primer [nt 1507–1526, GCCATTTAACGCCATTTCTC]. The PCR products were purified and separated by 1% (w/v) agarose-gel electrophoresis. These purified products were amplified again by *pfu* Taq polymerase (Stratagene) using 5'-primer with *Eco*RI site and 3'-primer with *Kpn*I site. They were then digested with *Eco*RI and *Kpn*I and cloned into *Eco*RI–*Kpn*I site of pBluescript II KS.

### A trial for molecular cloning of cDNAs encoding human HS6ST-3

Human counterparts of mouse HS6ST-3 were searched from the database using the TBLASTN program. We found two genomic DNAs (GenBank<sup>®</sup> accession nos. AL158192 and AL138816) covering the predicted coding regions corresponding to exon 1 and exon 2 respectively. The two expected PCR fragments were amplified from human genomic DNA (ClonTech) using primer 1 (5'-site primer: nt 3–25, ACCATGGATGAAAGGTTCAACAAGTGGC; 1 implies the first nucleotide of putative coding region) and primer 2 (3'-site primer: nt 680–704, CTGGTGTG-GCTGTGGTTGCGGGGAC) and using primer 3 (5'-site primer: nt 705–732, GAATTTCTATTACATCAATGTTACGG) and primer 4 (3'-site primer: nt 1392–1416, AGGTCAC-CATCTCACCACCTGGCTG) respectively. We then tried to amplify cDNAs from human brain RNA and human liver RNA (ClonTech) by reverse transcriptase (RT)-PCR using three sets of primers: 1 and 2, 3 and 4, and 1 and 4. The set of primers 3 and 4 yielded the cDNA, but the other sets have never brought about the amplification.

### DNA sequence analysis

The subcloned cDNAs were sequenced on both strands by the dideoxy chain-termination method using Taq polymerase (dye terminator cycle sequencing; PerkinElmer) with a DNA sequencer (Applied Biosystems PRISM 310, Foster, CA, U.S.A.). The DNA sequences thus obtained were compiled and analysed using GENETYX-MAX computer programs (Software Development Co., Tokyo, Japan). The nucleotide and deduced amino acid sequences were compared with other sequences in the nucleic acid and protein databases of GenBank<sup>®</sup>, DDBJ, dSTS and dbEST.

### Construction of the expression vectors pFLAG-CMV2-hHS6ST-2, -2S and -1

For the construction of the expression vectors, pFLAG-CMV2-hHS6ST-2 and -2S, cDNA fragments containing open reading frames of 1497 bp for hHS6ST-2 and 1377 bp for hHS6ST-2S

were excised from pBluescript KS II with *EcoRI* and *KpnI* and ligated into the *EcoRI-KpnI* site of the pFLAG-CMV2 expression vector (Eastman Kodak Co., New Haven, CT, U.S.A.) respectively. The inserted sequences were confirmed on a single strand as described above. pFLAG-CMV2-hHS6ST-1 was constructed as described previously [26].

#### Transfection of cDNAs and transient expression of HS6ST in COS-7 cells

COS-7 cells ( $5.5 \times 10^5$ ) precultured for 48 h in a 60-mm culture dish were transfected with 5  $\mu\text{g}$  of pFLAG-CMV2-hHS6ST-1, -2 and -2S respectively, or pFLAG-CMV2 alone. The transfection was performed using TransFast according to the manufacturer's recommended instructions (Promega, Madison, WI, U.S.A.). After incubation in Dulbecco's modified Eagle's medium containing 10% (v/v) foetal calf serum and antibiotics for 72 h, the cell layers were washed with Dulbecco's modified Eagle's medium alone, scraped and homogenized in 1 ml of 10 mM Tris/HCl (pH 7.2), 0.5% (w/v) Triton X-100, 0.15 M NaCl, 20% (v/v) glycerol, 10 mM  $\text{MgCl}_2$  and 2 mM  $\text{CaCl}_2$ . The homogenates were subjected to stirring for 1 h and then centrifuged at 10000 g for 30 min. FLAG fusion proteins in the supernatant (cell extract) were isolated by anti-FLAG M2 affinity chromatography according to the method described by the manufacturer, and the activities of HS6STs in the supernatants and FLAG-bound fractions were measured as described below.

#### Assay for sulphotransferase activities

Sulphotransferase activities were determined as described previously. Briefly, the standard reaction mixture (50  $\mu\text{l}$ ) contained 2.5  $\mu\text{mol}$  of imidazole-HCl (pH 6.8), 3.75  $\mu\text{g}$  of protamine chloride, 25 nmol (500  $\mu\text{M}$  as hexosamine) of acceptor GAGs, 50 pmol of [ $^{35}\text{S}$ ]PAPS (approx.  $5 \times 10^5$  c.p.m.; 1  $\mu\text{M}$ ) and enzyme. After incubation for 20 min at 37 °C, the reaction was stopped by heating at 100 °C for 1 min. Carrier chondroitin sulphate A (0.1  $\mu\text{mol}$  as GlcA) was added to the reaction mixture, and the  $^{35}\text{S}$ -labelled polysaccharides were isolated by precipitation with ethanol containing 1.3% (w/v) potassium acetate and 0.5 mM EDTA, followed by gel chromatography on a Fast Desalting column to remove [ $^{35}\text{S}$ ]PAPS and its degradation products. The amounts of enzymes added to the reaction mixture were chosen so as to obtain a linear incorporation of [ $^{35}\text{S}$ ]sulphate. We measured  $^{35}\text{S}$  incorporation into various GAGs with each purified recombinant enzyme under the conditions. One unit of enzyme activity was defined as the amount required to transfer 1 pmol of sulphate/min to CDSNS-heparin.

#### Measurement of the relative specific activity of FLAG-tagged recombinant human HS6ST-2 and HS6ST-2S proteins

The FLAG fusion proteins were purified as described above. The relative amounts of the FLAG-tagged proteins were determined by Western blotting using anti-FLAG antibody. Immune complexes were detected using the ECL<sup>®</sup> detection system (Amersham Pharmacia Biotech) and the density of the protein bands were quantified by NIH image software (version 1.57).

#### Analysis of enzymic reaction products

Analysis of the reaction products was performed by HPLC as described previously [38] with some modifications. Briefly,  $^{35}\text{S}$ -labelled products were digested with a mixture of 10 m-units of heparitinase I, 5 m-units of heparitinase II and 10 m-units of heparitinase III in 40  $\mu\text{l}$  of 50 mM Tris/HCl (pH 7.2), 1 mM  $\text{CaCl}_2$  and 4  $\mu\text{g}$  of BSA at 37 °C for 2 h. The digests were

subjected to gel chromatography on Superdex 30 pg equilibrated with 0.2 M  $\text{NH}_4\text{HCO}_3$ . The  $^{35}\text{S}$ -labelled disaccharide fractions were injected on to a PAMN column together with standard unsaturated disaccharides. Fractions of 0.6 ml were collected and their radioactivities were measured. Unsaturated hexuronic acid (HexA) residues of the unsaturated disaccharides were cleaved by treatment with mercuric acetate [39,40]. Unsaturated disaccharide fraction (100  $\mu\text{l}$ ) was added to 100  $\mu\text{l}$  of 70 mM mercuric acetate adjusted to pH 5.0 and the mixture was left at room temperature (25 °C) for 30 min. To remove mercuric acetate, 60  $\mu\text{l}$  of a 50% slurry of Dowex 50  $\text{H}^+$  resin was added to the reaction mixture and then the resin was removed by filtration. The resin was washed with water. The pass-through fractions and wash fractions were combined, neutralized with 2 M NaOH and then subjected to PAMN column chromatography.

#### Northern-blot analysis

Human multiple tissue Northern-blot filters, on which 2  $\mu\text{g}$  of polyadenylated RNAs from various human tissues were blotted, were prehybridized in ExpressHyb Hybridization Solution<sup>™</sup> (ClonTech) at 68 °C for 30 min and then hybridized in the same solution containing  $^{32}\text{P}$ -labelled probes ( $1 \times 10^6$  c.p.m./ml) at 68 °C for 1 h. The probes used were a 1203-base fragment at positions 280–1482 of the hHS6ST-1 cDNA (probe 1), a 1377-base fragment at the coding region of the hHS6ST-2S cDNA (probe 2, cross-hybridization for both hHS6ST-2cDNA and hHS6ST-2ScDNA) and a 120-base fragment at positions 510–629 of the hHS6ST-2 cDNA (probe 3, specific hybridization for hHS6ST-2) respectively. Probes 1 and 2 were labelled with [ $\alpha$ - $^{32}\text{P}$ ]dCTP by random oligonucleotide priming (Ready-to-Go DNA labelling kit; Amersham Pharmacia Biotech). Probe 3 was labelled at the 5'-end with [ $\gamma$ - $^{32}\text{P}$ ]ATP using T4 polynucleotide kinase. The membranes were washed several times with  $2 \times \text{SSC}$  and 0.05% SDS at room temperature and subsequently with  $0.1 \times \text{SSC}$  and 0.1% SDS at 50 °C twice. The membranes were exposed to X-ray film with an intensifying screen at  $-80$  °C.

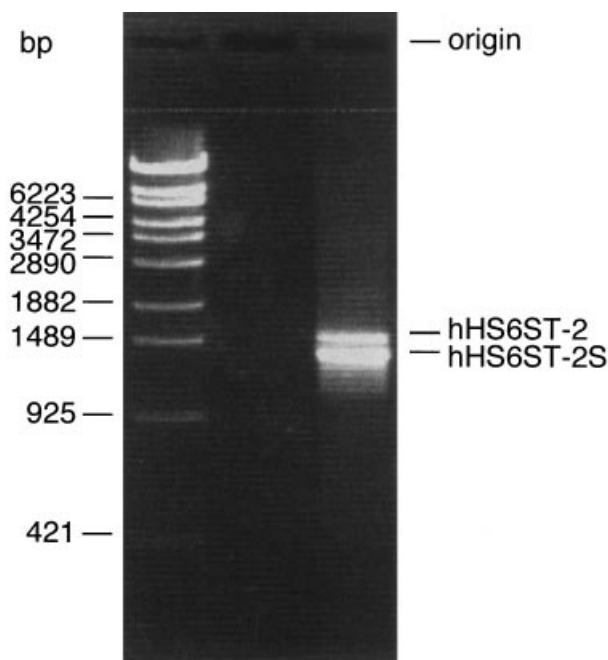
#### Chromosome preparation and *in situ* hybridization

The direct R-banding fluorescence *in situ* hybridization (FISH) method was used for the chromosomal assignment of the human *HS6ST-1* and human *HS6ST-2* genes. The preparation of R-banded chromosomes and FISH were performed as described by Matsuda et al. [41]. The 2.0 and 1.5 kb human cDNA fragments inserted in the *EcoRI* site of pBluescript were used for chromosomal mapping of *HS6ST-1* and *HS6ST-2* genes respectively. The cDNAs were labelled by nick translation with biotinylated 16-dUTP (Roche Diagnostics, Branchburg, NJ, U.S.A.) following the standard procedures. The hybridized biotinylated probes were reacted with goat anti-biotin antibodies (Vector Laboratories, Burlingame, CA, U.S.A.), and then stained with fluoresceinated anti-goat IgG (Nordic Immunology, Oslo, Norway). FISH images were observed under Nikon fluorescence microscope using Nikon filter sets B-2A and UV-2A. Kodak Ektachrome ASA100 films were used for microphotography.

## RESULTS

### Molecular cloning of human isologues of mouse HS6STs and the occurrence of HS6ST-2S, alternative spliced form of HS6ST-2 in human

We cloned human HS6ST-1 previously and characterized this enzyme [26]. In the present study, we aimed to investigate human



**Figure 1** RT-PCR amplifications of two forms of human HS6ST-2

The cDNA prepared from human brain RNA was amplified using a 5'-site primer (nt -14 to 6, CCAGCGTCGGGAACATGGAT; 1 stands for the first nucleotide of putative coding region) and 3'-site primer (nt 1507-1526, GCCATTTAAGCCATTTC). The PCR products were subjected to 1% agarose-gel electrophoresis.

counterparts to the other two isoforms of mouse HS6ST. For cloning HS6ST-2, we searched DNA databases using the TBLASTN program, and found two cDNA genes (GenBank® accession nos AL049679 and AA315705) and two genomic DNAs (Z86064 and Z81365) covering the predicted coding region. The cDNA prepared from human brain RNA was amplified using 5'- and 3'-site primers as described in the Experimental section. Agarose-gel electrophoresis of the PCR products revealed two bands (Figure 1); the size of one band was 1500 bp that was a length expected from mHS6ST-2, and the size of another band was approx. 1400 bp. The predicted amino acid sequence deduced from the nucleotide sequences of the longer PCR product showed 92% identity with the sequence of mHS6ST-2, and also indicated the presence of one hydrophobic segment in the N-terminal region and nine putative N-glycosylation sites as observed in mHS6ST-2 (Figure 2). On the other hand, the nucleotide sequence of the shorter PCR product was identical with the sequence of hHS6ST-2, except for the loss of 120 bases that resulted in the deletion of 40 amino acids from position 171-210 in hHS6ST-2 (enclosed by a box in Figure 2). Since the protein deduced from the shorter PCR product still had two consensus sequences for PAPS-binding sites, a 5'-phosphosulphate-binding (5'-PSB) site and a 3'-phosphate-binding (3'-PB) site (Figure 2), it was expected to have sulphotransferase activity. Therefore we named this novel form as hHS6ST-2S. A genome analysis using Z81365 and Z86064 showed that the human *HS6ST-2* gene consists of four exons and spans more than 156 kb from the first ATG to the poly (A) signal. Also the mHS6ST-2 genome comprises four exons (H. Habuchi and K. Kimata, unpublished work). Comparison of the nucleotide sequences of *hHS6ST-2S* with those of Z81365 and Z86064 revealed that *hHS6ST-2S* was a spliced form lacking exons 2 and

3 (Figure 3). To examine if other spliced variants lacking either exon 2 or 3 may occur, we performed the second PCR using the cDNA products from human brain RNA by RT-PCR as template and a set of primers (the forward primer, nt 382-406 and the reverse primer, nt 710-734). Only two DNA bands of approx. 330 and 210 bp were detected, which were assigned to the products from hHS6ST-2 and from hHS6ST-2S respectively. None of the DNA bands for the other spliced variants lacking either exon 2 or 3 were detected. The results suggested that the other hypothetical spliced variants are not expressed, at least in the brain. For cloning the human isoform of mHS6ST-3, we also searched DNA databases and found two genomic DNAs covering this region. However, several attempts to isolate the complete cDNA using brain and liver cDNA libraries did not succeed, although the 3'-site of the predicted coding region, nucleotides 705-1416, was amplified. The failure in the amplification may be caused by the presence of differently spliced forms, because Northern-blot analysis of mHS6ST-3 revealed the presence of many spliced forms [27].

#### Expression of hHS6ST-2 and hHS6ST-2S cDNAs in COS-7 cells and enzyme properties of the expressed proteins of human HS6STs

The cDNAs were inserted into a mammalian expression vector, pFLAG-CMV2, and COS-7 cells were transfected with either pFLAG-CMV2-hHS6ST-2 or pFLAG-CMV2-hHS6ST-2S, or with the vector alone as a control. Sulphotransferase activity in the cell extracts from these transfected cells was assayed using CDSNS-heparin as a substrate. The total activity of sulphotransferases in the cells transfected with pFLAG-CMV2-hHS6ST-2 and with pFLAG-CMV2-hHS6ST-2S was increased approx. 12- and 7-fold respectively, over the control (results not shown), suggesting that both the isolated cDNAs encoded proteins having HS6ST activity. The activity of sulphotransferase in the transfectants with pFLAG-CMV2-hHS6ST-1 was also increased as reported previously [26]. The recombinant enzymes were purified by anti-FLAG monoclonal antibody affinity chromatography and the relative amounts of the purified proteins were estimated by measuring the staining intensities of the FLAG-tag bands using anti-FLAG antibody as described in the Experimental section. The relative specific activities of those recombinant hHS6STs were almost equal to each other (results not shown). Their sulphotransferase activities were examined towards various heparin derivatives, HSs from different sources or other GAGs at a concentration of 500  $\mu$ M (as hexosamine) (Table 1). The differences in substrate structure among the heparin derivatives and HS samples were characterized by disaccharide composition analysis (Table 2). All enzyme proteins tested were able to transfer sulphate to CDSNS-heparin, 6ODS-heparin, NS-heparosan, heparin and HS preparations; the activity towards heparin was relatively low. We then examined the effects of concentration of CDSNS-heparin, NS-heparosan and HS on the activity of these enzymes to characterize substrate specificities of hHS6ST-2, -2S and hHS6ST-1 (Figure 4). The activities of the recombinant hHS6ST-2, hHS6ST-2S and hHS6ST-1 towards NS-heparosan and HS were increased with the increasing concentrations of the substrates. The activity of hHS6ST-1 towards CDSNS-heparin also increased with the increasing concentration, whereas those of hHS6ST-2 and hHS6ST-2S towards CDSNS-heparin, on the other hand, showed a maximum at 100  $\mu$ M and decreased at the higher concentrations, indicating that the substrate preference of hHS6ST-2 appeared to be similar to that of hHS6ST-2S, but not to hHS6ST-1. Such concentration-dependent differences in sub-

-14	CCAGCGTCGGGAACATGGATGAGAAATCCAACAGCTGCTGCTAGCTTTGGTGATGCTCTTCTATTGCCGATCGCTCCCAATACG	76
	M D E K S N K L L L A L V M L F L F A V I V L Q Y V	26
77	TGTGCCCCGGCACAGAATGCCAGCTCCTCCGCTGCAGGCGTTTCAGCTCCCCGGTGCCGGACCCGTACCCTCGGAGGATGAGAGCTCCG	166
	C P G T E C Q L L R L Q A F S S P V P D P Y R S E D E S S A	56
167	CCAGGTTCTGTCGGCCGCTACAATTTACCCGCGGACCTCCTGCGCAAGGTAGACTTCGACATCAAGGGCGATGACCTGATCGTGTTC	256
	R F V P R Y N F T R G D L L R K V D F D I K G D D L I V F L	86
257	TGCACATCCAGAAGACCGGGGACCCTTTGCGCCGCACTTGGTGCCTAACATCCAGCTGGAGCAGCCGTGCGAGTGCCGCGTGGGTC	346
	H I Q K T G G T T F G R H L V R N I Q L E Q P C E C R V G Q	116
347	AGAAGAAATGCACCTTGCACCGGGCGGTAAGCGGAAACCTGGCTCTTCTCCAGGTTCTCCACGGGCTGGAGCTGCGGGTTGCACGCCG	436
	K K C T C H R P G K R E T W L F S R F S T G W S C G L H A D	146
437	ACTGGACCGAGCTCACCAGCTGTGTGCCCTCCGTGGTGGACGGCAAGCGCAGCCAGGCTGAGACCGTCCAGCTGGAGGATTTTTCAGA	526
	W T E L T S C V P S V V D G K R D A R L R P S R W R I F Q I	176
527	TTCTAGATGCAGCAAGTAAGGATAAACGGGTTCTCCAAACACTAACGCAGGCCCAACTCTCCGTCATCCACAAAGACCCGGAACACAT	616
	L D A A S K D K R G S P N T N A G A N S P S S T K T R N T S	206
617	CTAAGAGTGGGAAGAACTTCCACTACATCCACCTCCTCCGAGACCCAGTGTCCCGGTAAGTGTGAGTGGAGGCATGTCAGAGAGGGG	706
	K S G K N F H Y I T I L R D P V S R Y L S E W R H V Q R G A	236
707	CAACATGGAAGCATCCCTGCATGTCTGCGATGGAAGGCCCTCCAACCTCCGAAGAGCTGCCAGCTGCTACACTGGCGATGACTGGTCTG	796
	T W K A S L H V C D G R P P T S E E L P S C Y T G D D W S G	266
797	GCTGCCCCCTCAAAGAGTTTATGGACTGTCCCTACAATCTAGCCAACAACCGCCAGGTGCGCATGCTCTCCGACCTGACCTGGTAGGCT	886
	C P L K E F M D C P Y N L A N N R Q V R M L S D L T L V G C	296
887	GCTACAACCTCTCTGTCATGCCTGAAAAGCAAAGAAACAAGGTCCTTCTGGAAAGTGCCAAGTCAAATCTGAAGCAGATGGCGTTCTTCG	976
	Y N L S V M P E K Q R N K V L L E S A K S N L K H M A F F G	326
977	GCCTCACTGAGTTTCAGCGGAAGACCAATATCTGTTTGAGAAAACCTCAACATGAACCTTATTTCCGCAATTACCAGTATAATACCA	1066
	L T E F Q R K T Q Y L F E K T F N M N F I S P F T Q Y N T T	356
1067	CTAGGGCCTCTAGTGTAGAGATCAATGAGGAAATCAAAGCGTATTGAGGGACTGAATTTCTGGATATGGAGTTGTACAGCTATGCCA	1156
	R A S S V E I N E E I Q K R I E G L N F L D M E L Y S Y A K	386
1157	AAGACCTTTTTTTCAGAGGTATCAGTTTATGAGGCAGAAAAGAGCATCAGGAGGCCAGCGAAAGCGTCAGGAACAACGAAATTTCTGA	1246
	D L F L Q R Y Q F M R Q K E H Q E A R R K R Q E Q R K F L K	416
1247	AGGGAAGGCTCCTTCAGACCCATTTCCAGAGCCAGGGTCAGGGCCAGAGCCAGAATCCGAATCAGAATCAGAGTCAGAACCCAAATCCGA	1336
	G R L L Q T H F Q S Q G Q G Q S Q N P N Q N Q S Q N P N P N	446
1337	ATGCCAATCAGAACCTGACTCAGAATCTGATGCAGAATCTGACTCAGAGTTTGGACCAGAAGGAGAACCAGGAAAGCCCGAAGCAGAACT	1426
	A N Q N L T Q N L M Q N L T Q S L S Q K E N R E S P K Q N S	476
1427	CAGGCAAGGAGCAGAATGATAACACCAGCAATGGCCACCAACGACTACATAGGCAGTGTAGAGAAATGGCGTTAA	1500
	G K E Q N D N T S N G T N D Y I G S V E K W R *	499

**Figure 2** Nucleotide sequences of the human HS6ST-2 and -2S cDNA, and the predicted amino acid sequences

The predicted amino acid sequence is shown below the nucleotide sequence. The putative transmembrane hydrophobic domain is underlined. Potential N-linked glycosylation sites are shown by black dots. The deleted portion in human HS6ST-2S is shown within a box. The putative PAPS-binding sites are shown by white box for 5'-phosphosulphate and black boxes for 3'-phosphate in PAPS.

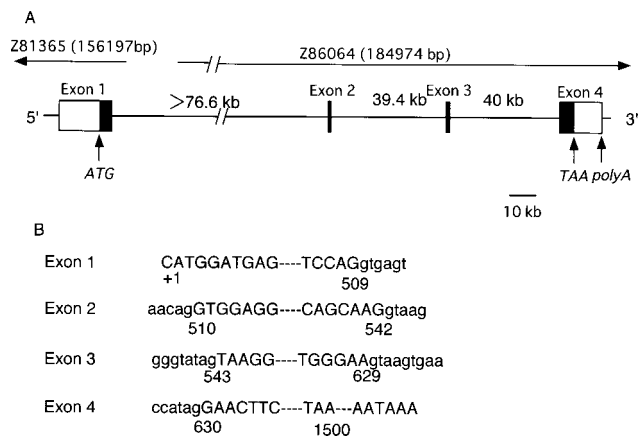
strate preferences between hHS6ST-1 and hHS6ST-2 appeared to be similar to those between corresponding mouse isoforms reported previously [28].

The mechanism for the decreasing activities of hHS6ST-2 and hHS6ST-2S at the higher concentrations of CDSNS-heparin is not clear at the moment. However, we found that 2ODS-heparin, which has a structure corresponding to the products from CDSNS-heparin by hHS6ST-2 and hHS6ST-2S, strongly inhibited both the hHS6ST-2 and hHS6ST-2S activities (results not shown), suggesting that the two forms of hHS6ST-2 may have products that lead to inhibition of the enzyme reaction (so-called product inhibition). As shown in Table 2, 1.4% of GlcNSO<sub>3</sub> units in CDSNS-heparin used for the acceptor substrate still harbour 6-O-sulphate residue. Therefore the observed biphasic concentration dependency of their activities when

CDSNS-heparin was used as acceptor substrate could be due to both these enzyme properties and the contaminating products in the substrate. However, the decrease in the activity of hHS6ST-2 and hHS6ST-2S was not observed when NS-heparosan and HS (pig aorta) were used as the acceptors. Therefore one may consider that high IdoA content of CDSNS-heparin may also contribute to its inhibitory activity.

#### Analysis of sulphation sites of acceptor substrates

We examined the sulphation positions of the acceptor substrates by analysing the structures of the <sup>35</sup>S-labelled products obtained from HS (pig aorta) or CDSNS-heparin on incubation with each recombinant enzyme and [<sup>35</sup>S]PAPS. The <sup>35</sup>S-labelled products were digested with a mixture of heparitinases I, II and III, and



**Figure 3 Putative genomic organization of HS6ST-2**

Human genomic clones (accession nos. Z81365 and Z86064) located at Xq26 containing the nucleotide sequence of human HS6ST-2 cDNA indicated that the human HS6ST-2 gene was composed of at least four exons. (A) Exons are indicated by boxes and introns are indicated by lines. Closed boxes represent the coding region and open boxes represent the 5'- and 3'-untranslated regions. The lengths of lines are roughly proportional to the number of nucleotides. ATG, TAA and poly (A) indicate the initiation codon, termination codon and the presumptive polyadenylation signal respectively. (B) Nucleotide sequences of the exon-intron junctions. Nucleotide sequences at the boundary of exons and introns are indicated by upper- and lower-case letters respectively. Numbers under sequences represent the nucleotide numbers indicated in Figure 2.

**Table 1 Acceptor substrate specificities of the recombinant sulphotransferases purified by anti-FLAG antibody affinity column chromatography**

Sulphotransferase activities were assayed using various glycosaminoglycans (500  $\mu$ M) as acceptors. Sulphotransferase fractions were prepared from COS-7 cells transfected with pFLAG-CMV2-hHS6ST-1, pFLAG-CMV2-hHS6ST-2 and pFLAG-CMV2-hHS6ST-2S respectively, as described in the Experimental section. Recombinant proteins were purified on the anti-FLAG antibody column.

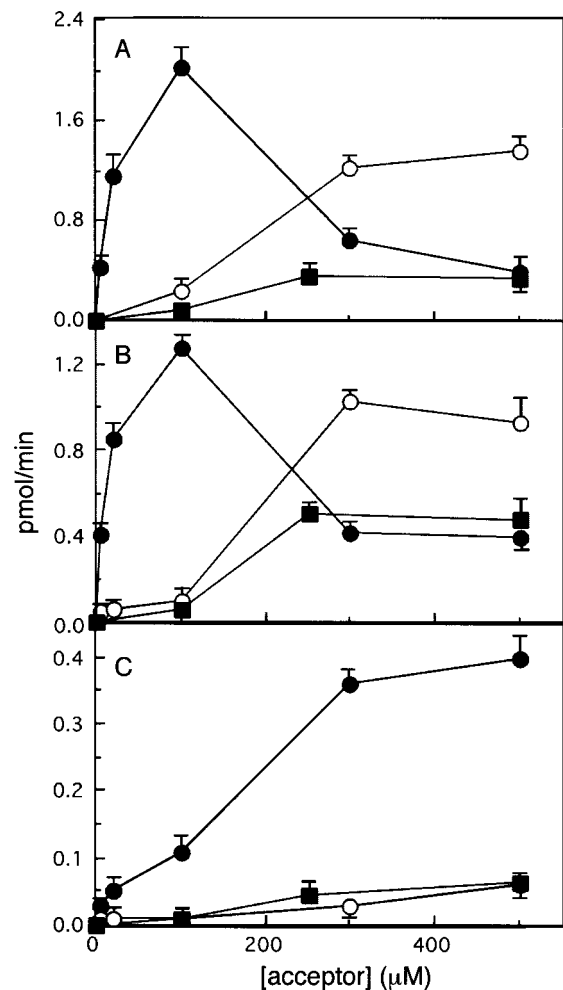
Substrate	Relative activities of sulphotransferases* (%)		
	hHS6ST-2	hHS6ST-2S	hHS6ST-1
CDSNS-heparin	100 $\pm$ 1.0	100 $\pm$ 3.2	100 $\pm$ 5.6
NS-heparosan	202 $\pm$ 6.0	189 $\pm$ 12	11 $\pm$ 2.0
Heparin	2.4 $\pm$ 0.9	2.1 $\pm$ 0.4	0.2 $\pm$ 0.1
6ODS-heparin	18 $\pm$ 1.0	10 $\pm$ 1.2	1.0 $\pm$ 0.3
Heparan sulphate pig aorta	84 $\pm$ 5.0	119 $\pm$ 9.4	16 $\pm$ 1.0

\* The values indicate the relative rate of the incorporation into various substrates to that into CDSNS-heparin. The amount of each enzyme used was designed to give the same rate of  $^{35}$ S incorporation into CDSNS-heparin (0.4 pmol/min). The values given are the means  $\pm$  S.D. of determinations from three independent experiments.

**Table 2 Disaccharide compositions of heparin, modified heparin, N-sulphated heparosan and HSs used as substrates**

The unsaturated disaccharide compositions of various glycosaminoglycans are expressed as percentages of total disaccharides.

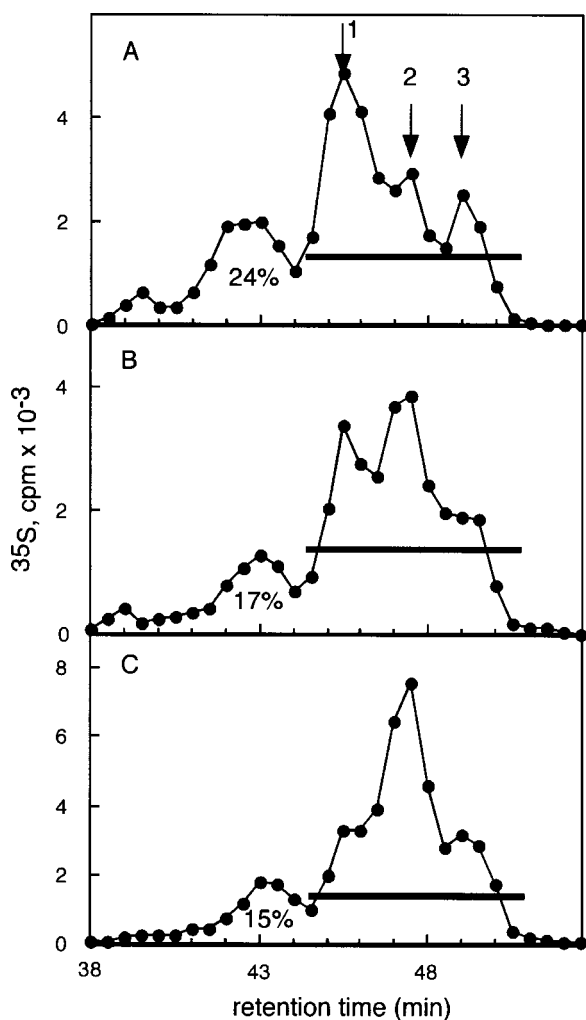
Glycosaminoglycans	Unsaturated disaccharides (% of total)					
	$\Delta$ Di-OS	$\Delta$ Di-6S	$\Delta$ Di-NS	$\Delta$ Di-(N,6)diS	$\Delta$ Di-(N,2)diS	$\Delta$ Di-(N,6,2)triS
CDSNS-heparin	16.4	0.4	78.6	1.4	1.1	0
NS-heparosan	39.6	0	58.1	0	0	0
Heparin	11.5	3.3	3.6	10.6	7.4	61.8
6ODS-heparin	8.2	1.8	13.6	4.2	70.1	2.1
Heparan sulphate pig aorta	63.9	6.0	18.5	2.3	4.4	3.8



**Figure 4 Effects of variable concentrations of acceptor substrates on the activities of hHS6ST-2 (A), hHS6ST-2S (B) and hHS6ST-1 (C)**

The sulphotransferase activity was measured using 0.4 unit of recombinant enzyme as described in the Experimental section, except that various amounts of CDSNS-heparin (●), NS-heparosan (○) or HS (pig aorta) (■) were added. Values represent means of triplicate independent experiments.

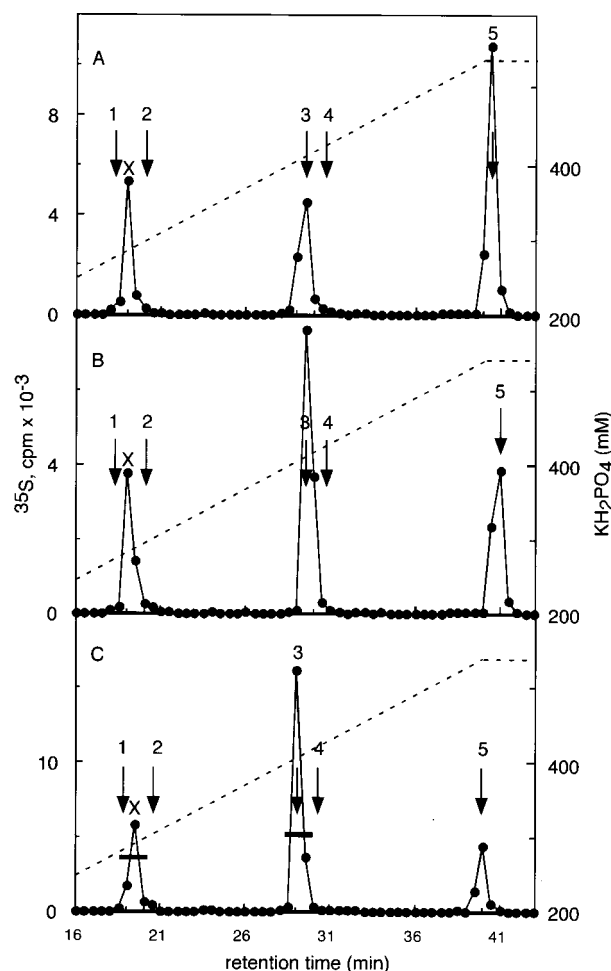
then subjected to gel chromatography on Superdex 30 pg and subsequently to PAMN column chromatography. More than 95% of the digests of the  $^{35}$ S-labelled products from CDSNS-heparin after incubation with each recombinant enzyme were eluted in the disaccharide fractions from the Superdex 30 column,



**Figure 5** Superdex 30 pg chromatography of the heparitinase digests of  $^{35}\text{S}$ -labelled products derived from HS (pig aorta) by incubation with  $^{35}\text{S}$ PAPS and with the recombinant purified hHS6ST-2 (A), hHS6ST-2S (B) and hHS6ST-1 (C)

The recombinant purified enzymes were prepared as described in the Experimental section. (A)–(C) Products labelled by sulphotransferase reactions were digested with a mixture of heparitinases and then subjected to Superdex 30 pg column chromatography as described in the Experimental section. The disaccharide fractions indicated by horizontal bars were collected, freeze-dried and used for the next step. Percentages of tetrasaccharide fractions to digests of  $^{35}\text{S}$ -labelled products are shown in the Figures. The arrows indicate the elution positions of unsaturated disaccharides used as internal standards:  $\Delta\text{Di}-(\text{N},6,2)\text{triS}$  (1),  $\Delta\text{Di}-(\text{N},6)\text{diS}$  (2) and  $\Delta\text{Di}-(\text{N},6)\text{triS}$  (3).

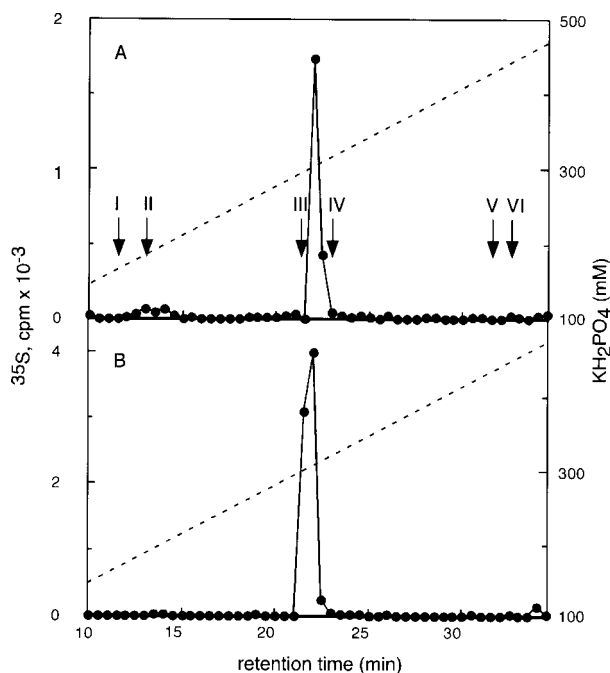
and most of the radioactivity in the disaccharide fractions was eluted at the position of 2-deoxy-2-sulphamino-4-*O*-(4-deoxy- $\alpha$ -*L*-threo-hex-4-ene-pyranosyluronic acid)-6-*O*-sulpho-D-glucose [ $\Delta\text{Di}-(\text{N},6)\text{diS}$ ] by the subsequent PAMN column chromatography (results not shown). In contrast, the digests of the  $^{35}\text{S}$ -labelled products from HS (pig aorta), when applied to Superdex 30 pg column, were eluted in the tetrasaccharide fractions (molecular mass was approx. 950 Da) as well as in the disaccharide fractions, and both the elution profiles of the disaccharide fractions and the proportions of tetrasaccharide to disaccharide fractions were very different among the three recombinant enzymes (Figure 5). The subsequent PAMN



**Figure 6** HPLC on PAMN columns of the disaccharide fractions obtained by heparitinase digestion of  $^{35}\text{S}$ -labelled products derived from HS (pig aorta) by incubation with  $^{35}\text{S}$ PAPS and with the recombinant purified hHS6ST-2 (A), hHS6ST-2S (B) and hHS6ST-1 (C)

The disaccharide fractions indicated by horizontal bars in Figure 5 were subjected to PAMN column chromatography as described in the Experimental section. The elution was carried out with a linear gradient of 40–550 mM  $\text{KH}_2\text{PO}_4$  for 40 min and then at 550 mM  $\text{KH}_2\text{PO}_4$  for 10 min. The dotted lines indicate concentrations of  $\text{KH}_2\text{PO}_4$  used for the elution. The closed circles show the elution patterns of the products derived from HS (pig aorta). The fractions indicated by horizontal bars were collected and desalted by Superdex 30 pg gel chromatography and freeze-dried. These samples were used for the next step. The arrows indicate the elution positions of  $\Delta\text{Di}-(\text{N},6)\text{diS}$  (1),  $\Delta\text{Di}-(\text{N},6)\text{triS}$  (2),  $\Delta\text{Di}-(\text{N},6)\text{diS}$  (3), 2-deoxy-2-sulphamino-4-*O*-(4-deoxy- $\alpha$ -*L*-threo-hex-4-ene-pyranosyluronic acid)-*D*-glucose [ $\Delta\text{Di}-(\text{N},2)\text{diS}$ ] (4) and  $\Delta\text{Di}-(\text{N},6,2)\text{triS}$  (5).

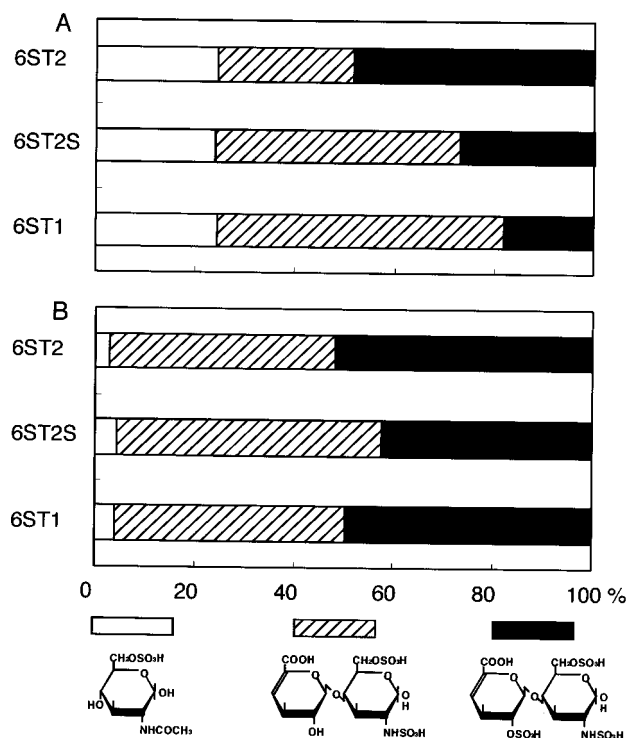
column chromatography of the disaccharide fractions (indicated by horizontal bars in Figure 5) showed an elution profile consisting of three peaks corresponding to  $\Delta\text{Di}-(\text{N},6)\text{diS}$ , 2-deoxy-2-sulphamino-4-*O*-(4-deoxy-2-*O*-sulpho- $\alpha$ -*L*-threo-hex-4-ene-pyranosyluronic acid)-6-*O*-sulpho-D-glucose [ $\Delta\text{Di}-(\text{N},6,2)\text{triS}$ ] and an unidentified compound (peaks 3, 5 and X in Figure 6 respectively). Since peak X eluted between 2-acetamido-2-deoxy-4-*O*-(4-deoxy- $\alpha$ -*L*-threo-hex-4-ene-pyranosyluronic acid)-6-*O*-sulpho-D-glucose ( $\Delta\text{Di}-(\text{N},6)\text{diS}$ ) and 2-deoxy-2-sulphamino-4-*O*-(4-deoxy- $\alpha$ -*L*-threo-hex-4-ene-pyranosyluronic acid)-*D*-glucose ( $\Delta\text{Di}-(\text{N},2)\text{diS}$ ) from the PAMN column (Figure 6), and near 2-acetamido-2-deoxy-4-*O*-(4-deoxy- $\alpha$ -*L*-threo-hex-4-ene-pyranosyl-



**Figure 7** HPLC on PAMN columns of  $^{35}\text{S}$ -labelled peak X (A) and  $\Delta\text{Di}$ -(N,6)diS (B) treated with mercuric acetate

The peak X fraction and  $\Delta\text{Di}$ -(N,6)diS fraction in Figure 6(C) were collected and treated with mercuric acetate as described in the Experimental section. The reaction products were chromatographed again on the PAMN column. The elution was carried out with a linear gradient of 10–540 mM  $\text{KH}_2\text{PO}_4$  for 40 min. The eluate was monitored by measuring the absorbance at 215 nm. The arrows in (A) indicate the elution positions of the internal standards:  $\Delta\text{Di}$ -OS (I),  $\text{GlcNAc-6SO}_4$  (II),  $\Delta\text{Di-6S}$  (III),  $\Delta\text{Di-NS}$  (IV),  $\Delta\text{Di}$ -(N,6)diS (V) and  $\Delta\text{Di}$ -(N,2)diS (VI).

uronic acid)-D-glucose ( $\Delta\text{Di}$ -OS) on Superdex 30 gel chromatography (results not shown), and, in addition, a disulphated monosaccharide, *N*-sulphoglucosamine 6-sulphate [ $\text{GlcNSO}_3(6\text{SO}_4)$ ], had been found to be released from the non-reducing end of HS after the heparitinase II digestion [42], we assumed peak X to be a sulphated monosaccharide. To confirm that peak X corresponded to  $\text{GlcNSO}_3(6\text{SO}_4)$ ,  $^{35}\text{S}$ -labelled peak X and  $^{35}\text{S}$ -labelled  $\Delta\text{Di}$ -(N,6)diS were treated with mercuric acetate to remove unsaturated HexA residues [39,40], and the reaction products were again chromatographed on the PAMN column (Figure 7). Most of the radioactivity of the treated peak X was recovered at the position of the starting materials (Figure 7A), whereas the radioactivity of the reaction product from  $\Delta\text{Di}$ -(N,6)diS, which was expected to be  $\text{GlcNSO}_3(6\text{SO}_4)$ , was eluted at the same position as peak X (Figure 7B). Furthermore, considering that  $\text{GlcNAc}(6\text{SO}_4)$  is eluted at peak 2 (13.2 min) by PAMN column chromatography, peak X probably corresponds to  $\text{GlcNSO}_3(6\text{SO}_4)$ . Percentages of the  $^{35}\text{S}$  radioactivity incorporated into  $\text{GlcNSO}_3(6\text{SO}_4)$ ,  $\Delta\text{Di}$ -(N,6)diS and  $\Delta\text{Di}$ -(N,6,2)triS are shown in Figure 8(A). The disaccharide fractions of the  $^{35}\text{S}$ -labelled products from 6ODS-heparin were likewise analysed. The major products were  $\text{GlcNSO}_3(6\text{SO}_4)$ ,  $\Delta\text{Di}$ -(N,6)diS and  $\Delta\text{Di}$ -(N,6,2)triS, and their percentages of  $^{35}\text{S}$  radioactivity are shown in Figure 8(B). There was a considerable difference in the relative incorporation into the major three products not only between the two substrates but also among the three enzymes. For  $\text{GlcNSO}_3(6\text{SO}_4)$  derived from the non-reducing end, the value was 24% from pig aorta HS and below 4% from 6ODS-



**Figure 8** The ratio of  $\text{GlcNS}_6\text{S}$ ,  $\Delta\text{Di}$ -(N,6)diS and  $\Delta\text{D}$ -(N,6,2)triS derived from heparitinase digestion of  $^{35}\text{S}$ -labelled products derived from pig aorta HS (A) and 6ODS-heparin (B)

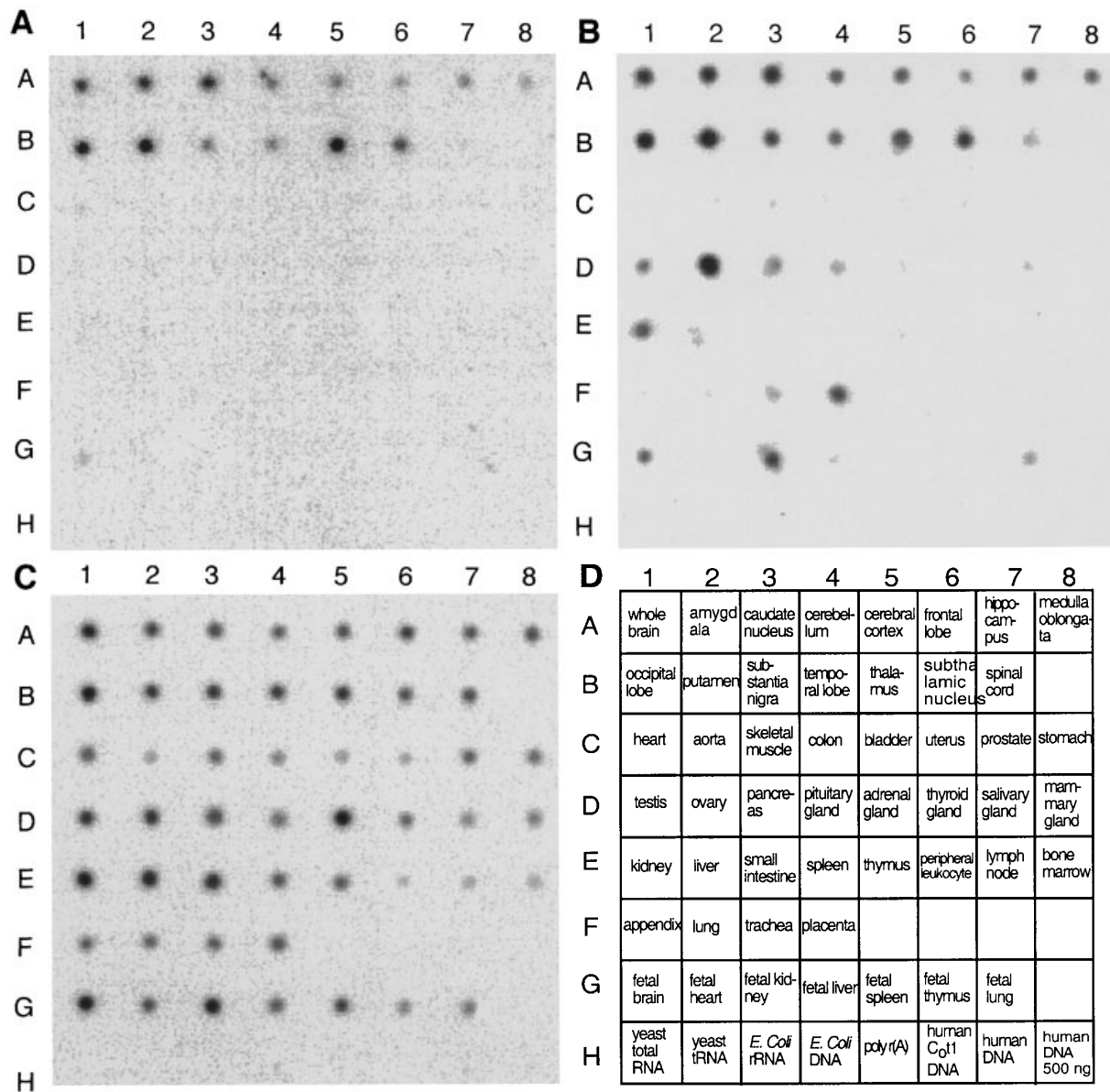
The percentages of  $^{35}\text{S}$ -labelled  $\text{GlcNSO}_3(6\text{SO}_4)$  (open bar),  $\Delta\text{Di}$ -(N,6)diS (hatched bar) and  $\Delta\text{Di}$ -(N,6,2)triS (black bar) in heparitinase digests of  $^{35}\text{S}$ -labelled products were calculated from Figure 6(A), Figure 6(B) and Figure 6(C) respectively. The percentages from  $^{35}\text{S}$ -labelled 6ODS-heparin were calculated in a similar way.

heparin, and the relative incorporation into  $\Delta\text{Di}$ -(N,6)diS and  $\Delta\text{Di}$ -(N,6,2)triS derived from internal  $\text{GlcNSO}_3$  residues in HS was quite different among the three enzymes. The tetrasaccharide fractions from the Superdex 30 column may contain 3-*O*-sulphated residues or receive further modifications, as judged from the reported heparitinase substrate specificities [43], but it was difficult to determine the structures at the radioisotope level. Considering the results together, hHS6ST-2 appeared to have a preference to transfer sulphate to position 6 of  $\text{GlcNSO}_3$  residues in IdoA 2-*O*-sulphate [ $\text{IdoA}(2\text{SO}_4)\text{-GlcNSO}_3$ ] unit or highly sulphated regions, and hHS6ST-1 to transfer sulphate to position 6 of  $\text{GlcNSO}_3$  residues in HexA- $\text{GlcNSO}_3$  unit. Interestingly, hHS6ST-2S had a preference that was somewhere in between. These preferences were clearer in pig aorta HS.

#### Expression of transcripts in various tissues

The tissue-dependent expression was compared among the two forms of HS6ST-2 and HS6ST-1 by dot-blot analysis using the Human Multiple Tissue Expression (MTE<sup>TM</sup>) Array (Figure 9; ClonTech). When probe 3 was used for the detection of the original form of hHS6ST-2 (Figure 9A), positive signals were only detected in tissues related to brain, particularly in the thalamus, putamen, occipital lobe, amygdala and caudate nucleus. On the other hand, when probe 2, which is capable of hybridizing both hHS6ST-2 and hHS6ST-2S was used, positive signals were observed in ovary, foetal kidney, placenta and





**Figure 9** Dot-blot analysis of hHS6ST-2 (A), hHS6ST-2 and hHS6ST-2S (B) and hHS6ST-1 (C) transcripts in various human tissues

The sources of the polyadenylated [poly(A)<sup>+</sup>] RNA are indicated in (D). The same membrane was hybridized sequentially with <sup>32</sup>P-labelled probes 1, 2 and 3 respectively, as described in the Experimental section.

kidney in addition to the tissues detected with probe 3 (Figure 9B), which suggested that the short form (hHS6ST-2S) was expressed mainly in ovary, placenta and foetal kidney. hHS6ST-1 was expressed rather ubiquitously in various tissues, although a higher level of expression was observed in adrenal gland, kidney, liver, intestine, foetal brain and foetal kidney (Figure 9C).

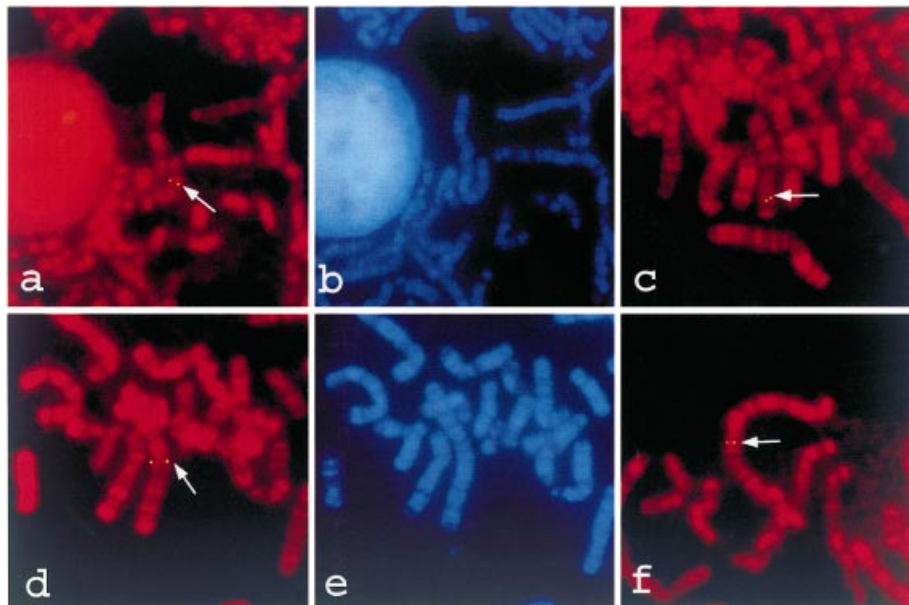
#### Assignment of the human *HS6ST-1* and *HS6ST-2* genes by FISH

Human *HS6ST-1* and *HS6ST-2* genes are found at chromosome (Chr) 2q21 and Xq25 respectively, in the DNA database. To investigate more closely the loci of these genes to know if they are related to human diseases, we applied the directed R-banding

FISH as described in the Experimental section. *HS6ST-1* and *HS6ST-2* were localized to Chr 2q21.1 and Chr Xq26.1 respectively (ISCN, 1985; Figure 10). Interestingly, the genes for glypicans 3 and 4, which are glycosylphosphatidylinositol-anchored cell-surface HSPGs, are on chromosome Xq26.1, close to the hHS6ST-2 gene.

#### DISCUSSION

In this study, we have investigated the human counterparts of mouse HS6ST isoforms, which we reported previously [27], and found that human HS6ST-2 is present in two forms: the original long form (hHS6ST-2) and a short form (hHS6ST-2S). The latter was generated by alternative splicing in the coding region of the



**Figure 10** Chromosomal localization of the human *HS6ST-2* and *HS6ST-1* genes

The 1.5 and 1.3 kb fragments from human *HS6ST-2* and *HS6ST-1* cDNA were used as biotinylated probes. Arrows indicate the hybridization signals. The *HS6ST-2* gene was localized to Xq26.1 (a)–(c) and the *HS6ST-1* gene to 2q21.1 (d)–(f). R- and G-banded patterns are demonstrated in (a), (c), (d) and (f), and (b) and (e) respectively.

*HS6ST-2* gene, and lacked 40 amino acids encoded by exons 2 and 3. In spite of this deletion, *HS6ST-2S* still had two PAPS-binding sites which are present in exons 1 and 4 respectively. It catalysed the transfer of sulphate from PAPS to position 6 of the  $\text{GlcNSO}_3$  residue in HS, and its specific activity was not significantly different from that of *HS6ST-2*. The activity profiles of h*HS6ST-2* and h*HS6ST-2S* towards various substrates were somewhat similar to each other, but quite different from those of h*HS6ST-1* (Table 1). Structural analysis of the  $^{35}\text{S}$ -labelled products formed from HSs with the recombinant enzymes also suggested a difference in the way h*HS6ST-2* showed a preference to transfer sulphate to position 6 of  $\text{GlcNSO}_3$  residues in  $\text{IdoA}(2\text{SO}_4)\text{-GlcNSO}_3$  unit or highly sulphated regions, whereas h*HS6ST-1* transferred sulphate to position 6 of  $\text{GlcNSO}_3$  residues in  $\text{HexA-GlcNSO}_3$  unit.

Detailed analysis for the structures of the  $^{35}\text{S}$ -labelled products formed from HSs revealed that h*HS6ST-1*, h*HS6ST-2* and h*HS6ST-2S* were all capable of transferring sulphate not only to position 6 of internal  $\text{GlcNSO}_3$  residue of the HS as described above, but also to position 6 of  $\text{GlcNSO}_3$  residues at the non-reducing end of the acceptor substrates. One may wonder here if  $\text{GlcNSO}_3(6\text{SO}_4)$  could be formed from the unsaturated disaccharides during the digestion with heparitinases, due to the possible contamination of  $\beta$ -glucuronidase acting on unsaturated uronic acid or chemical instability. However, this is unlikely, because little  $\text{GlcNSO}_3(6\text{SO}_4)$  monosaccharide was detected in the digests of the  $^{35}\text{S}$ -labelled products from CDSNS-heparin, in which  $\Delta\text{Di}(\text{N},6)\text{diS}$  was a major component. As observed with  $\text{GalNAc4S-6ST}$  (where  $\text{GalNAc}$  stands for *N*-acetylgalactosamine) purified from squid cartilage, which catalysed the 6-sulphation of both the internal and non-reducing terminal  $\text{GalNAc4SO}_4$  residues of chondroitin sulphate [44,45], it is probable that all of these *HS6STs* can transfer sulphate to position 6 of  $\text{GlcNSO}_3$  residues located not only in the internal repeating units, but also at the non-reducing end in HS.  $\text{GlcNSO}_3(6\text{SO}_4)$  may actually be present at the non-reducing end

of native HS, because a previous study [42] described that digestion of HS from the lung and pancreas with heparitinase II yielded the release of 5 and 4%  $\text{GlcNSO}_3(6\text{SO}_4)$  monosaccharide relative to the total product respectively. Recently, it has been suggested that a  $\text{GlcNAc-6-sulphatase}$  secreted into the extracellular space contributes to the regulation of HS-dependent Wnt signalling by the modification of the structure of HS [46]. Based on the homology in amino acid sequence between the extracellular  $\text{GlcNAc-6-sulphatase}$  and the lysosomal  $\text{GlcNAc-6-sulphatase}$  that hydrolyses 6-*O*-sulphate on the non-reducing terminal  $\text{GlcNAc}$  and  $\text{GlcNSO}_3$  [47], the extracellular  $\text{GlcNAc-6-sulphatase}$  could cleave 6-*O*-sulphate at the non-reducing terminal  $\text{GlcNAc}$  and  $\text{GlcNSO}_3$ . If this is the case, it may be that sulphation and desulphation of the  $\text{GlcNSO}_3(6\text{SO}_4)$  residues located at the non-reducing end of HS modify the binding of Wnt to HS.

We detected only a trace of activity to transfer sulphate to position 6 of  $\text{GlcNAc}$  residues in HS with the recombinant h*HS6ST-1*, h*HS6ST-2* or h*HS6ST-2S* under the reaction conditions used in the present study, although HSs are rich in  $\text{GlcNAc}$  residues. In contrast with these results, Chinese-hamster ovary (CHO) cell transfectants that were overexpressing m*HS6ST-1* or m*HS6ST-2* or m*HS6ST-3* produced HS containing much more  $\text{HexA-GlcNAc}(6\text{SO}_4)$  when compared with wild-type CHO-K1 cells (H. Habuchi and K. Kimata, unpublished work). Zhang et al. [48] also have reported that CHO cells transfected with *HS6ST-1* generated HS containing more  $\text{HexA-GlcNAc}(6\text{SO}_4)$  than the original cells. They claimed that *HS6ST-1* could sulphate  $\text{GlcNAc}$  residues in AT III-binding sites *in vitro* [48]. Considering our observations on the weak activity of the recombinant *HS6STs* towards  $\text{GlcNAc}$  residues *in vitro*, the following explanations are equally possible: (1) the acceptor substrates used in the present study do not contain structural domains required for the sulphation of position 6 of  $\text{GlcNAc}$  residues; (2) there are Golgi-localized factors that stimulate the 6-*O*-sulphation of  $\text{GlcNAc}$  in the cell; and (3) as other  $\text{GlcNAc 6-O-sulphotransferases}$  transfer

sulphate to position 6 of terminal GlcNAc residues in keratan sulphate and oligosaccharides [17], *in vivo* 6-O-sulphation of GlcNAc residues in HS may be performed in a similar way, i.e. the sulphation could take place when the chain is elongating.

Dot-blot analysis showed that each HS6ST isoform has its own characteristic pattern of expression. The original hHS6ST-2 was expressed characteristically in brain-related tissues. It is intriguing that alternative splicing of hHS6ST-2 strictly occurred in a tissue-dependent manner. Comparison in the expression pattern between Figures 9(A) and 9(B) revealed that the short form (hHS6ST-2S) was preferentially expressed in ovary, placenta and foetal kidney. Human HS6ST-1 was expressed rather constitutively in various tissues in sharp contrast with our previous findings that mouse HS6ST-1 was expressed preferentially in liver [27]. The observations suggest that isologues of HS6STs have similar enzymic properties but differ in their expression in certain tissues, depending on the animal species, and may be partly responsible for some of the species-specific tissue functions.

We have mapped more precisely the HS6ST-2 gene to Xq26.1 and the HS6ST-1 gene to 2q21.1 respectively, using directed R-banding FISH. We then examined any inherited disorders related to these chromosomal sites using the Online Mendelian Inheritance-in-Man database. No such disease was mapped to 2q21.1 where HS6ST-1 is localized, whereas several diseases were mapped to Xq26 where HS6ST-2 was localized. The genes responsible for some of these diseases have already been identified, but the genes responsible for premature ovarian failure, Charcot-Marie-tooth disease and Panhypopituitarism disease have not. It is uncertain at this stage whether or not the HS6ST-2 gene is related to any of these disorders.

Our preliminary results have suggested that the short form of HS6ST-2 is also expressed in mice. In addition, we have detected this form in chicken. However, interestingly, to the best of our knowledge, the long form has not been detected (K. Nogami, H. Suzuki, H. Habuchi, H. Iwata and K. Kimata, unpublished work), suggesting that the short form is in fact the original from an evolutionary perspective. Considering that no isoform for HS6ST was detected in *Drosophila* [32], it is interesting to speculate that higher animals with more isoforms may have the capacity to create a more diverse and finer structure for HS, which could reflect more functional diversity.

As observed in mouse HS6ST, both the expressed proteins from human HS6ST-2 and HS6ST-2S cDNA were secreted into the culture medium. The secretion of HS6ST seems to occur under physiological conditions, because the HS6ST activity was detected in human serum. However, at this time it remains to be studied how the secreted HS6STs differ in function from those present inside the cells and whether one or both of the isoforms of HS6ST are present in serum. Several heparin-binding cell growth factors such as vascular endothelial growth factor and fibroblast growth factor 2 are involved in the angiogenesis that often takes place in malignant tumour tissues [49]. It is now known that HSPGs, especially 6-O-sulphated HSPGs, play essential roles in signalling [33–35]. The determination of HS6ST activities in sera from patients with different tumours will provide information on the correlation between these activities and disease.

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## REFERENCES

- 1 Iozzo, R. V. (2001) Heparan sulfate proteoglycans: intricate molecules with intriguing functions. *J. Clin. Invest.* **108**, 165–167
- 2 Bernfield, M., Gotte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J. and Zako, M. (1999) Functions of cell surface heparan sulfate proteoglycans. *Annu. Rev. Biochem.* **68**, 729–777
- 3 Conrad, H. E. (1998) Heparinoid/protein interactions. In *Heparin-Binding Proteins* (Conrad, H. E., ed.), pp. 183–202, Academic Press, New York, NY
- 4 Lindahl, U., Kusche-Gullberg, M. and Kjellen, L. (1998) Regulated diversity of heparan sulfate. *J. Biol. Chem.* **273**, 24979–24988
- 5 Chang, Z., Meyer, K., Rapraeger, A. C. and Friedl, A. (2000) Differential ability of heparan sulfate proteoglycans to assemble the fibroblast growth factor receptor complex *in situ*. *FASEB J.* **14**, 137–144
- 6 Baeg, G. H. and Perrimon, N. (2000) Functional binding of secreted molecules to heparan sulfate proteoglycans in *Drosophila*. *Curr. Opin. Cell Biol.* **12**, 575–580
- 7 Perrimon, N. and Bernfield, M. (2000) Specificities of heparan sulphate proteoglycans in developmental processes. *Nature (London)* **404**, 725–728
- 8 Ornitz, D. M. (2000) FGFs, heparan sulfate and FGFRs: complex interactions essential for development. *Bioessays* **22**, 108–112
- 9 Selleck, S. B. (2000) Proteoglycans and pattern formation: sugar biochemistry meets developmental genetics. *Trends Genet.* **16**, 206–212
- 10 Tumova, S., Woods, A. and Couchman, J. R. (2000) Heparan sulfate proteoglycans on the cell surface: versatile coordinators of cellular functions. *Int. J. Biochem. Cell Biol.* **32**, 269–288
- 11 Rosenberg, R. D., Shworak, N. W., Liu, J., Schwartz, J. J. and Zhang, L. (1997) Heparan sulfate proteoglycans of the cardiovascular system. Specific structures emerge but how is synthesis regulated? *J. Clin. Invest.* **99**, 2062–2070
- 12 Forsberg, E. and Kjellen, L. (2001) Kjellen, heparan sulfate: lessons from knockout mice. *J. Clin. Invest.* **108**, 175–180
- 13 Gallagher, J. T. and Lyon, M. (2000) Heparan sulfate: molecular structure and interactions with growth factors and morphogen. In *Proteoglycans* (Iozzo, R. V., ed.), pp. 27–60, Marcel Dekker, New York
- 14 Esko, J. D. and Lindahl, U. (2001) Molecular diversity of heparan sulfate. *J. Clin. Invest.* **108**, 169–173
- 15 Sugahara, K. and Kitagawa, H. (2000) Recent advances in the study of the biosynthesis and functions of sulfated glycosaminoglycans. *Curr. Opin. Struct. Biol.* **10**, 518–527
- 16 Habuchi, H., Habuchi, O. and Kimata, K. (1998) Biosynthesis of heparan sulfate and heparin. *Trends Glycosci. Glycotechnol.* **10**, 65–80
- 17 Habuchi, O. (2000) Diversity and functions of glycosaminoglycan sulfotransferases. *Biochim. Biophys. Acta* **1474**, 115–127
- 18 Crawford, B. E., Olson, S. K., Esko, J. D. and Pinhal, M. A. (2001) Cloning, golgi localization, and enzyme activity of the full-length heparin/heparan sulfate-glucuronic acid C5-epimerase. *J. Biol. Chem.* **276**, 21538–21543
- 19 Li, J. P., Gong, F. E. L., Darwish, K., Jalkanen, M. and Lindahl, U. (2001) Characterization of the  $\alpha$ -glucuronyl C5-epimerase involved in the biosynthesis of heparin and heparan sulfate. *J. Biol. Chem.* **276**, 20069–20077
- 20 Kobayashi, M., Habuchi, H., Yoneda, M., Habuchi, O. and Kimata, K. (1997) Molecular cloning and expression of Chinese hamster ovary cell heparan-sulfate 2-sulfotransferase. *J. Biol. Chem.* **272**, 13980–13985
- 21 Hashimoto, Y., Orellana, A., Gil, G. and Hirschberg, C. B. (1992) Molecular cloning and expression of rat liver *N*-heparan sulfate sulfotransferase. *J. Biol. Chem.* **267**, 15744–15750
- 22 Orellana, A., Hirschberg, C. B., Wei, Z., Swiedler, S. J. and Ishihara, M. (1994) Molecular cloning and expression of a glycosaminoglycan *N*-acetylglucosaminyl *N*-deacetylase/*N*-sulfotransferase from a heparin-producing cell line. *J. Biol. Chem.* **269**, 2270–2276
- 23 Kusche-Gullberg, M., Eriksson, I., Sandback, D. and Kjellen, L. (1998) Identification and expression in mouse of two heparan sulfate glucosaminyl *N*-deacetylase/*N*-sulfotransferase genes. *J. Biol. Chem.* **273**, 11902–11907
- 24 Aikawa, J. and Esko, J. D. (1999) Molecular cloning and expression of a third member of the heparan sulfate/heparin GlcNAc *N*-deacetylase/*N*-sulfotransferase family. *J. Biol. Chem.* **274**, 2690–2695
- 25 Aikawa, J., Grobe, K., Tsujimoto, M. and Esko, J. D. (2001) Multiple isozymes of heparan sulfate/heparin GlcNAc *N*-deacetylase/GlcNAc *N*-sulfotransferase. *J. Biol. Chem.* **276**, 5876–5882
- 26 Habuchi, H., Kobayashi, M. and Kimata, K. (1998) Molecular characterization and expression of heparan-sulfate 6-sulfotransferase. *J. Biol. Chem.* **273**, 9208–9213
- 27 Habuchi, H., Tanaka, M., Habuchi, O., Yoshida, K., Suzuki, H., Ban, K. and Kimata, K. (2000) The occurrence of three isoforms of heparan sulfate 6-O-sulfotransferase having different specificities for hexuronic acid adjacent to the targeted *N*-sulfoglucosamine. *J. Biol. Chem.* **275**, 2859–2868

- 28 Shworak, N. W., Liu, J., Fritze, L. M., Schwartz, J. J., Zhang, L., Logeart, D. and Rosenberg, R. D. (1997) Molecular cloning and expression of mouse and human cDNAs encoding heparan sulfate  $\alpha$ -glucosaminyl 3-O-sulfotransferase. *J. Biol. Chem.* **272**, 28008–28019
- 29 Shworak, N. W., Liu, J., Petros, L. M., Zhang, L., Kobayashi, M., Copeland, N. G., Jenkins, N. A. and Rosenberg, R. D. (1999) Multiple isoforms of heparan sulfate  $\alpha$ -glucosaminyl 3-O-sulfotransferase. *J. Biol. Chem.* **274**, 5170–5184
- 30 Liu, J., Shworak, N. W., Sinay, P., Schwartz, J. J., Zhang, L., Fritze, L. M. and Rosenberg, R. D. (1999) Expression of heparan sulfate  $\alpha$ -glucosaminyl 3-O-sulfotransferase isoforms reveals novel substrate specificities. *J. Biol. Chem.* **274**, 5185–5192
- 31 Toyoda, H., Kinoshita-Toyoda, A., Fox, B. and Selleck, S. B. (2000) Structural analysis of glycosaminoglycans in *Drosophila* and *Caenorhabditis elegans* and demonstration that *tou-velu*, a *Drosophila* gene related to EXT tumor suppressors, affects heparan sulfate *in vivo*. *J. Biol. Chem.* **275**, 21856–21861
- 32 Kamimura, K., Fujise, M., Villa, F., Izumi, S., Habuchi, H., Kimata, K. and Nakato, H. (2001) *Drosophila* heparan sulfate 6-O-sulfotransferase (dHS6ST) gene. *J. Biol. Chem.* **276**, 17014–17021
- 33 Pye, D. A., Vives, R. R., Turnbull, J. E., Hyde, P. and Gallagher, J. T. (1998) Heparan sulfate oligosaccharides require 6-O-sulfation for promotion of basic fibroblast growth factor mitogenic activity. *J. Biol. Chem.* **273**, 22936–22942
- 34 Guimond, S., Maccarana, M., Olwin, B. B., Lindahl, U. and Rapraeger, A. C. (1993) Activating and inhibitory heparin sequences for FGF-2 (basic FGF). *J. Biol. Chem.* **268**, 23906–23914
- 35 Pellegrini, L., Burke, D. F., von Delft, F., Mulloy, B. and Blundell, T. L. (2000) Crystal structure of fibroblast growth factor receptor ectodomain bound to ligand and heparin. *Nature (London)* **407**, 1029–1034
- 36 Schlessinger, J., Plotnikov, A. N., Ibrahim, O. A., Eliseenkova, A. V., Yeh, B. K., Yayon, A., Linhardt, R. J. and Mohammadi, M. (2000) Crystal structure of a ternary FGF–FGFR–heparin complex reveals a dual role for heparin in FGFR binding and dimerization. *Mol. Cell* **6**, 743–750
- 37 Delfelt, D. M. and Conrad, H. E. (1985) Preparation and high-performance liquid chromatography of 3'-phosphoadenosine-5'-phospho[ $^{35}$ S]sulfate with a predetermined specific activity. *Anal. Biochem.* **148**, 303–310
- 38 Habuchi, H., Suzuki, S., Saito, T., Tamura, T., Harada, T., Yoshida, K. and Kimata, K. (1992) Structure of a heparan sulphate oligosaccharide that binds to basic fibroblast growth factor. *Biochem. J.* **285**, 805–813
- 39 Ludwigs, U., Elgavish, A., Esko, J. D., Meezan, E. and Roden, L. (1987) Reaction of unsaturated uronic acid residues with mercuric salts. *Biochem. J.* **245**, 795–804
- 40 Calabro, A., Benavides, M., Tammi, M., Hascall, V. C. and Midura, R. J. (2000) Microanalysis of enzyme digests of hyaluronan and chondroitin/dermatan sulfate by fluorophore-assisted carbohydrate electrophoresis (FACE). *Glycobiology* **10**, 273–281
- 41 Matsuda, Y., Harada, Y. N., Matsuume-Sakai, S., Lee, K., Shiomi, T. and Chapman, V. M. (1992) Location of the mouse complement factor H gene (cfh) by FISH analysis and replication R-banding. *Cytogenet. Cell Genet.* **61**, 282–285
- 42 Nader, H. B., Porcionatto, M. A., Tersariol, I. L., Pinhal, M. A., Oliveira, F. W., Moraes, C. T. and Dietrich, C. P. (1990) Purification and substrate specificity of heparitinase I and heparitinase II from *Flavobacterium heparinum*. *J. Biol. Chem.* **265**, 16807–16813
- 43 Desai, U. R., Wang, H. M. and Linhardt, R. J. (1993) Substrate specificity of the heparin lyases from flavobacterium heparinum. *Arch. Biochem. Biophys.* **306**, 461–468
- 44 Ito, Y. and Habuchi, O. (2000) Purification and characterization of *N*-acetylgalactosamine 4-sulfate 6-O-sulfotransferase from the squid cartilage. *J. Biol. Chem.* **275**, 34728–34736
- 45 Ohtake, S., Ito, Y., Fukuta, M. and Habuchi, O. (2001) Human *N*-acetylgalactosamine 4-sulfate 6-O-sulfotransferase cDNA is related to human B cell recombination activating gene-associated gene. *J. Biol. Chem.* **276**, 43894–43900
- 46 Dhoot, G. K., Gustafsson, M. K., Ai, X., Sun, W., Standiford, D. M. and Emerson, Jr, C. P. (2001) Regulation of Wnt signaling and embryo patterning by an extracellular sulfatase. *Science* **293**, 1663–1666
- 47 Freeman, C. and Hopwood, J. J. (1992) Human glucosamine-6-sulphatase deficiency. *Biochem. J.* **282**, 605–614
- 48 Zhang, L., Beeler, D. L., Lawrence, R., Lech, M., Liu, J., Davis, J. C., Shriver, Z., Sasisekharan, R. and Rosenberg, R. D. (2001) 6-O-sulfotransferase-1 represents a critical enzyme in the anticoagulant heparan sulfate biosynthetic pathway. *J. Biol. Chem.* **276**, 42311–42321
- 49 Iozzo, R. V. and San Antonio, J. D. (2001) Heparan sulfate proteoglycans: heavy hitters in the angiogenesis arena. *J. Clin. Invest.* **108**, 349–355

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