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

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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 isologues, 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\(^2\), 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 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 reactions 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\(^2\)) residue and 3-O-sulphation of GlcNSO\(^2\)/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-a-L-threo-hex-4-enepyranosyluronic acid)-o-glucose; ΔDi-(N-2)diS, 2-deoxy-2-sulphamino-4-O-(4-deoxy-a-L-threo-hex-4-enepyranosyluronic acid)-o-glucose; ΔDi-(N,6)diS, 2-deoxy-2-sulphamino-4-O-(4-deoxy-a-L-threo-hex-4-enepyranosyluronic acid)-6-O-sulpho-c-glucose; ΔDi-(N,6,2)triS, 2-deoxy-2-sulphamino-4-O-(4-deoxy-2-O-sulpho-a-L-threo-hex-4-enepyranosyluronic acid)-6-O-sulpho-c-glucose; FISH, fluorescence in situ hybridization; GAG, glycosaminoglycan; GlcA, glucuronic acid; GlcNAc, N-acetylglucosamine; GlcNSO\(^2\), N-sulphoglucosamine; HexA, hexuronic acid; HS, heparan sulphate; HS6ST, HS 6-O-sulphotransferase; HSFP, heparan sulphate proteoglycan; IdoA, iduronic acid; NS-heparosan, deacetylated and N-sulphated heparosan; PAPS, adenosine 3’-phosphate 5’-phosphosulphate; RT, reverse transcriptase.

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The nucleotide sequences reported in the present study have been submitted to the DDBJ/GenBank\& EMI 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 residue by three heparan sulphate 6-O-sulphotransferases (HS6STs) [26,27], and 3-O-sulphation of GlcNSO/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-sulphatated IdooA 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 HS6STs 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 HS6ST-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

H₂¹³SO₄ was purchased from PerkinElmer Life Science (Branchburg, NJ, U.S.A.); Hybond N+ 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-acetylglycosamine 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). [³⁵S]PAPS was 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* 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, CCACGGTCCGGGA-ACATGGAT; 1 stands for the first nucleotide of the putative coding region) and 3′-site primer (nt 1507–1526, GCCATTACAC-CCGATTTTCTC). 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 EcoRI site and 3′-primer with KpnI site. They were then digested with EcoRI and KpnI and cloned into EcoRI-KpnI 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* 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 the putative coding region) and primer 2 (3′-site primer: nt 680–704, CTGGTGTGGCTGTGGTGGTGG; 14 stands for the first nucleotide of putative coding region) and primer 3 (5′-site primer: nt 705–732, GAATTTCTATACATACATAATGTTAACGG) and primer 4 (3′-site primer: nt 1392–1416, AGGTCTACATCTCACCACCTGGCTG) 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®, 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 × 10^4) precultured for 48 h in a 60-mm culture dish were transfected with 5 μg of pFLAG-CMV2-hHS6ST-T1, -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 %, (v/v) Triton X-100, 0.15 M NaCl, 20 %, (v/v) glycerol, 10 mM MgCl2 and 2 mM CaCl2. 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 μl) contained 2.5 μmol ofimidazole-HCl (pH 6.8), 3.75 μg ofprotamine chloride, 25 nmol (500 μM as hexosamine) ofacceptor GAGs, 50 pmol of [35S]PAPS (approx. 5 × 10^4 c.p.m.; 1 μM) 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 μmol as GlcA) was added to the reaction mixture, and the 35S-labelled polysaccharides were isolated by precipitation with ethanol containing 13 %, (v/v) potassium acetate and 0.5 mM EDTA, followed by gel chromatography on a Fast Desalting column to remove [35S]PAPS and its degradation products. The amounts of enzymes added to the reaction mixture were chosen so as to obtain a linear incorporation of 35S-sulphate. We measured 35S 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® 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, 35S-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 μl of 50 mM Tris/HCl (pH 7.2), 1 mM CaCl2 and 4 μg of BSA at 37 °C for 2 h. The digests were subjected to gel chromatography on Superdex 30 pg equilibrated with 0.2 M NH4HCO3. The 35S-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 μl) was added to 100 μl 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 μl of a 50 %, slurry of Dowex 50 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 μg of polyadenylated RNAs from various human tissues were blotted, were prehybridized in ExpressHyb Hybridization Solution® (Clontech) at 68 °C for 30 min and then hybridized in the same solution containing [32P]-labelled probes (1 × 10^6 c.p.m./μl) 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 [α-32P]dCTP by random oligonucleotide priming (Ready-to-Go DNA labelling kit; Amersham Pharmacia Biotech). Probe 3 was labelled at the 5'-end with [γ-32P]ATP using T4 polynucleotide kinase. The membranes were washed several times with 2 × SSC and 0.05 %, SDS at room temperature and subsequently with 0.1 × 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

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revealed that sequences of Kimata, unpublished work). Comparison of the nucleotide genome comprises four exons (H. Habuchi and K. human genome analysis using Z81365 and Z86064 showed that the activity. Therefore we named this novel form as hHS6ST-2S. A sequence deduced from the nucleotide sequences of the longer of another band was approx. 1400 bp. The predicted amino acid 1500 bp that was a length expected from mHS6ST-2, and the size products revealed two bands (Figure 1); the size of one band was 330 and 210 bp were detected, which were assigned to the 3'-site primer, nt 710–734). Only two DNA bands of approx. 300 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 isologue 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 sulpho-transferases 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 μM (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 CDSNC-heparin, on the other hand, showed a maximum at 100 μM 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-

![Figure 1 RT-PCR amplifications of two forms of human HS6ST-2](image-url)

The cDNA prepared from human brain RNA was amplified using a 5'-site primer (nt — 14 to 6, CCAGGGTCGGAACAGGGAT; 1 stands for the first nucleotide of putative coding region) and 3'-site primer (nt 1507–1526, GCCATTTAAGCCATTTCCTC). The PCR products were subjected to 1% agarose-gel electrophoresis.
substrate preferences between hHS6ST-1 and hHS6ST-2 appeared to be similar to those between corresponding mouse isologues 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$_2$ 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.

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.

Analysis of sulphation sites of acceptor substrates

We examined the sulphation positions of the acceptor substrates by analysing the structures of the $^{35}$S-labelled products obtained from HS (pig aorta) or CDSNS-heparin on incubation with each recombinant enzyme and $[^{35}$S]PAPS. The $^{35}$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 μM) 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.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Relative activities of sulphotransferases* (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>hHS6ST-2</td>
</tr>
<tr>
<td>CDSNS-heparin</td>
<td>100 ± 1.0</td>
</tr>
<tr>
<td>NS-heparosan</td>
<td>202 ± 6.0</td>
</tr>
<tr>
<td>Heparin</td>
<td>2.4 ± 0.9</td>
</tr>
<tr>
<td>6ODS-heparin</td>
<td>18 ± 1.0</td>
</tr>
<tr>
<td>Heparan sulphate pig aorta</td>
<td>84 ± 5.0</td>
</tr>
</tbody>
</table>

* 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 35S incorporation into CDSNS-heparin (0.4 pmol/min). The values given are the means ± 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.

<table>
<thead>
<tr>
<th>Glycosaminoglycans</th>
<th>ΔD(-1)-OS</th>
<th>ΔD(-1)-6S</th>
<th>ΔD(-1)-NS</th>
<th>ΔD(-1)-(N,6)ds</th>
<th>ΔD(-1)-(N,2)ds</th>
<th>ΔD(-1)-(N,6,2)triS</th>
</tr>
</thead>
<tbody>
<tr>
<td>CDSNS-heparin</td>
<td>16.4</td>
<td>0.4</td>
<td>78.6</td>
<td>1.4</td>
<td>1.1</td>
<td>0</td>
</tr>
<tr>
<td>NS-heparosan</td>
<td>39.6</td>
<td>0</td>
<td>58.1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Heparin</td>
<td>11.5</td>
<td>3.3</td>
<td>3.6</td>
<td>10.6</td>
<td>7.4</td>
<td>61.8</td>
</tr>
<tr>
<td>6ODS-heparin</td>
<td>8.2</td>
<td>1.8</td>
<td>13.6</td>
<td>4.2</td>
<td>70.1</td>
<td>2.1</td>
</tr>
<tr>
<td>Heparan sulphate pig aorta</td>
<td>83.9</td>
<td>6.0</td>
<td>18.5</td>
<td>2.3</td>
<td>4.4</td>
<td>3.8</td>
</tr>
</tbody>
</table>
Figure 5  Superdex 30 pg chromatography of the heparitinase digests of 
35S-labelled products derived from HS (pig aorta) by incubation with
[35S]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 
35S-labelled products are shown in the Figures. The arrows indicate the elution positions of unsaturated disaccharides used as internal standards: ΔD-(N,6,2)triS (1), ΔD-(N,6)diiS (2) and ΔD-OS (3).

and most of the radioactivity in the disaccharide fractions was
eluted at the position of 2-deoxy-2-sulphamino-4-O-(4-deoxy-
α-l-threo-hex-4-enepyranosyluronic acid)-6-O-sulpho-D-glucose [
ΔD-(N,6)diiS] by the subsequent PAMN column chromatography (results not shown). In contrast, the digests of the 
35S-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 column chromatography of the disaccharide fractions (indicated by horizontal bars in Figure 5) showed an elution profile consisting of three peaks corresponding to ΔD-(N,6)diiS, 2-deoxy-2-sulphamino-4-O-(4-deoxy-
α-l-threo-hex-4-enepyranosyluronic acid)-6-O-sulpho-D-glucose [ΔD-(N,6,2)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-α-l-threo-hex-4-enepyranosyluronic acid)-6-O-sulpho-D-glucose (ΔD-6S) and 2-deoxy-2-sulphamino-4-O-(4-deoxy-
α-l-threo-hex-4-enepyranosyluronic acid)-D-glucose (ΔD-NS) from the PAMN column (Figure 6), and near 2-acetamido-2-deoxy-4-O-(4-deoxy-α-l-threo-hex-4-enepyranosyl-
The peak X fraction and \( \Delta \text{Di}-(N,6)\text{diS} \) 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 K\( \text{H}_2\text{PO}_4 \) for 40 min. The eluate was monitored by measuring the absorbance at 215 nm.

For \( \text{GlcNSO}_6S \) derived from pig aorta HS and below 4 from 6ODS-heparin, and the relative incorporation into \( \Delta \text{Di}-(N,6)\text{diS} \) and \( \Delta \text{Di}-(N,6,2)\text{triS} \) derived from internal \( \text{GlcNSO}_6S \) 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 \( \text{GlcNSO}_6S \) derived in IdoA 2-O-sulphate [IdoA(2SO\( \text{D} \))] unit or highly sulphated regions, and hHS6ST-1 to transfer sulphate to position 6 of \( \text{GlcNSO}_6S \) residues in HexA-GlcNSO\( \text{D} \)). 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\textsuperscript{TM}) 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...
kinetic 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 HSPIGs, 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

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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 GlcNSO residue in HS, and its specific activity was not significantly different from that of HS6ST-2. The activity profiles of hHS6ST-2 and hHS6ST-2S towards various substrates were somewhat similar to each other, but quite different from those of hHS6ST-1 (Table 1). Structural analysis of the 35S-labelled products formed from HSs with the recombinant enzymes also suggested a difference in the way hHS6ST-2 showed a preference to transfer sulphate to position 6 of GlcNSO residues in IdoA(2SO3)-GlcNSO unit or highly sulphated regions, whereas hHS6ST-1 transferred sulphate to position 6 of GlcNSO residues in HexA-GlcNSO unit.

Detailed analysis for the structures of the 35S-labelled products formed from HSs revealed that hHS6ST-1, hHS6ST-2 and hHS6ST-2S were all capable of transferring sulphate not only to position 6 of internal GlcNSO residue of the HS as described above, but also to position 6 of GlcNSO residues at the non-reducing end of the acceptor substrates. One may wonder here if GlcNSO(6SO3) could be formed from the unsaturated disaccharides during the digestion with heparitinases, due to the possible contamination of β-glycuronidase acting on unsaturated uronic acid or chemical instability. However, this is unlikely, because little GlcNSO(6SO3) monosaccharide was detected in the digests of the 35S-labelled products from CDSNS-heparin, in which ADi-(N,6)DiS was a major component. As observed with GalNAc4S-6ST (where GalNAc stands for N-acetylgalactosamine) purified from squid cartilage, which catalysed the 6-sulphation of both the internal and non-reducing terminal GalNAc4SO3G residues of chondroitin sulphate [44,45], it is probable that all of these HS6STs can transfer sulphate to position 6 of GlcNSO residues located not only in the internal repeating units, but also at the non-reducing end in HS. GlcNSO(6SO3) 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% GlcNSO(6SO3) monosaccharide relative to the total product respectively. Recently, it has been suggested that a GlcNAc-6-sulphotransferase 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 GlcNAc-6-sulphotransferase and the lysosomal GlcNAc-6-sulphotransferase that hydrolyses 6-O-sulphate on the non-reducing terminal GlcNAc and GlcNSO [47], the extracellular GlcNAc-6-sulphotransferase could cleave 6-O-sulphate at the non-reducing terminal GlcNAc and GlcNSO. If this is the case, it may be that sulphation and desulphation of the GlcNSO(6SO3) 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 GlcNAc residues in HS with the recombinant hHS6ST-1, hHS6ST-2 or hHS6ST-2S under the reaction conditions used in the present study, although HSs are rich in GlcNAc residues. In contrast with these results, Chinese-hamster ovary (CHO) cell transfectants that were overexpressing mHS6ST-1 or mHS6ST-2 or mHS6ST-3 produced HS containing much more HexA-GlcNAc(6SO3) 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 HexA-GlcNAc(6SO3) than the original cells. They claimed that HS6ST-1 could sulphate GlcNAc residues in AT III-binding sites in vitro [48]. Considering our observations on the weak activity of the recombinant HS6STs towards GlcNAc residues in AT III-binding sites 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 GlcNAc residues; (2) there are Golgi-localized factors that stimulate the 6-O-sulphation of GlcNAc in the cell; and (3) as other 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 4q21.1 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 4q21.1 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 Panhydropituitary 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. 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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