# Expression of Heparan Sulfate D-Glucosaminyl 3-O-Sulfotransferase Isoforms Reveals Novel Substrate Specificities\*

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From the ‡Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139, the ¶Department of Medicine, Harvard Medical School, Beth Israel Hospital, Boston, Massachusetts 02215, and the ¶Département de Chimie, Ecole Normale Superieure, 75231 Paris Cédex 05, France

The 3-O-sulfation of glucosamine residues is an important modification during the biosynthesis of heparan sulfate (HS). Our previous studies have led us to purify and molecularly clone the heparan sulfate D-glucosaminyl 3-O-sulfotransferase (3-OST-1), which is the key enzyme converting nonanticoagulant heparan sulfate (HS<sup>inact</sup>) to anticoagulant heparan sulfate (HS<sup>act</sup>). In this study, we expressed and characterized the fulllength cDNAs of 3-OST-1 homologous genes, designated as 3-OST-2, 3-OST-3<sub>A</sub>, and 3-OST-3<sub>B</sub> as described in the accompanying paper (Shworak, N. W., Liu, J., Petros, L. M., Zhang, L., Kobayashi, M., Copeland, N. G., Jenkins, N. A., and Rosenberg, R. D. (1999) J. Biol. Chem. 274, 5170-5184). All these cDNAs were successfully expressed in COS-7 cells, and heparan sulfate sulfotransferase activities were found in the cell extracts. We demonstrated that 3-OST-2, 3-OST-3<sub>A</sub>, and 3-OST-3<sub>B</sub> are heparan sulfate D-glucosaminyl 3-O-sulfotransferases because the enzymes transfer sulfate from adenosine 3'-phosphophate 5'-phospho-[<sup>35</sup>S]sulfate ([<sup>35</sup>S]PAPS) to the 3-OH position of glucosamine. 3-OST- $3_A$  and 3-OST- $3_B$ sulfate an identical disaccharide. HSact conversion activity in the cell extract transfected by 3-OST-1 was shown to be 300-fold greater than that in the cell extracts transfected by 3-OST-2 and 3-OST-3<sub>A</sub>, suggesting that 3-OST-2 and 3-OST-3<sub>A</sub> do not make HS<sup>act</sup>. The results of the disaccharide analysis of the nitrous aciddegraded [<sup>35</sup>S]HS suggested that 3-OST-2 transfers sulfate to GlcA2S-GlcNS and IdoA2S-GlcNS; 3-OST-3A transfers sulfate to IdoA2S-GlcNS. Our results demonstrate that the 3-O-sulfation of glucosamine is generated by different isoforms depending on the saccharide structures around the modified glucosamine residue. This discovery has provided evidence for a new cellular mechanism for generating a defined saccharide sequence in structurally complex HS polysaccharide.

The cell surface and extracellular matrix contain heparan sulfate proteoglycans which consist of a core protein and single

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or multiple heparan sulfate (HS)<sup>1</sup> side chains. The highly charged HS side chains are the copolymers of glucuronic acid (GlcA)/iduronic acid (IdoA) and N-acetylated glucosamine (GlcNAc) with various sulfations. HS binds to specific proteins such as antithrombin and several growth factors, thereby regulates various biological processes including anticoagulation and angiogenesis (1). The unique sulfation pattern of a monosaccharide sequence within HS is believed to be critical for binding to the target protein. The biosynthesis of HS involves the formation of a polysaccharide backbone followed by serial sulfation and epimerization reactions. The HS backbone is elongated by HS copolymerase-dependent (2) transfer of GlcA and GlcNAc to the tetrasaccharide linkage region present on core proteins. This process creates a polysaccharide backbone of approximately 100 repeating disaccharide units (1). This polysaccharide is structurally altered at GlcNAc to form Nsulfated glucosamine (GlcNS) by heparan sulfate N-deacetylase/N-sulfotransferase (3–5). The heparan sulfate  $C_5$  epimerase subsequently converts occasional GlcA to IdoA within the polysaccharide (6). Heparan sulfate (uronosyl) 2-O-sulfotransferase transfers sulfate from PAPS to IdoA to form 2-O-sulfated iduronic acid (IdoA2S) (7), and heparan sulfate (D-glucosaminyl) 6-O-sulfotransferase transfers sulfate to the 6-OH position of glucosamine to form 6-O-sulfated glucosamine (8). Although this mechanism provides a scheme for generating the average structure of HS, it is unable to explain how HS with defined monosaccharide sequences are produced.

We have delineated a mechanism for generating HS with a specific monosaccharide sequence based on our investigation of the biosynthesis of anticoagulant active HS (HS<sup>act</sup>). Our results show that the synthesis of HS<sup>act</sup> requires a limiting factor, as well as a precursor with a defined polysaccharide structure which can be modified by the limiting factor. HS<sup>act</sup>, normally present as 1–10% of total HS, contains a structurally defined antithrombin-binding pentasaccharide with a sequence of -GlcNSorAc6S-GlcA-GlcNS3S $\pm$ 6S-IdoA2S-GlcNS6S- (9, 10). Our previous work demonstrated the presence of a specific pathway for the biosynthesis of HS<sup>act</sup> in L cells. Overexpression

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<sup>&</sup>lt;sup>1</sup> The abbreviations used are: HS, heparan sulfate; PAPS, adenosine 3'-phosphosphate 5'-phosphosulfate; GlcA, D-glucuronic acid; GlcNAc, N-acetyl-D-glucosamine; 3-OST, heparan sulfate D-glucosaminyl 3-Osulfotransferase; GlcNS, N-sulfo-D-glucosamine; IdoA, L-iduronic acid; HS<sup>act</sup>, anticoagulant heparan sulfate; HS<sup>inact</sup>, nonanticoagulant heparan sulfate; IdoA2S, L-iduronic acid 2-O-sulfate; GlcA2S, D-glucuronic acid 2-O-sulfate; GlcNR'6S, N-acetyl-D-glucosamine 6-O-sulfate or Nsulfo-D-glucosamine 6-O-sulfate; GlcNS3S±6S, N-sulfo-D-glucosamine 3-O-sulfate or N-sulfo-D-glucosamine 3,6-O-bisulfate; HPLC, high performance liquid chromatography; SAX-HPLC, strong anion exchange-HPLC; RPIP-HPLC, reversed phase ion pairing HPLC; AnMan, 2,5anhydro-D-mannitol; AnMan3S, AnMan6S, and AnMan3S6S, 2,5anhydro-D-mannitol 3-O-sulfate, 6-O-sulfate and 3,6-O-bisulfate, respectively; AT, antithrombin; Mes, 4-morpholineethanesulfonic acid.

of syndecan-4 core protein or chemical mutagenesis in wildtype L cells suggest that  $HS^{act}$  generation requires a limiting factor, and the limiting factor was identified as heparan sulfate 3-O-sulfotransferase (3-OST-1, EC 2.8.2.23) (11–15).

We recently proposed that HS biosynthetic enzymes are present in multiple isoforms, and each isoform has slightly different substrate specificities and recognizes the monosaccharide sequences around the modification sites (1). Indeed, the available data support this hypothesis (16-18). As documented by several researchers including ourselves, 3-O-sulfation of glucosamine is a rare modification that constitutes the last step in the biosynthesis of HS (14, 15, 19, 20). We have shown that 3-OST-1 requires an appropriate HS precursor with defined monosaccharide sequence for sulfation to occur and make HS<sup>act.2</sup> It is likely that 3-O-sulfated HS are specifically involved in other HS-related biological processes. If this is the case, studies of 3-O-sulfation should facilitate the identification of new biologically active HS-binding proteins. The 3-OST-1, which we have previously purified and molecularly cloned, only sulfates the glucosamine in GlcA-GlcNS±6S. However, there are at least two other types of 3-O-sulfated glucosamine that were previously characterized. For example, the HS isolated from glomerular basement membrane contains substantial amounts of an IdoA2S-GlcNS3S disaccharide unit (21, 22). Pejler et al. (23) also reported that a heparin isolated from the clams Anomalocardia brasiliana and Tivela mactroides contained IdoA-GlcNS3S6S disaccharide (23). It is apparent that neither the IdoA2S-GlcNS3S nor the IdoA-GlcNS3S6S is the product of 3-OST-1. These observations suggest the possible existence of different heparan sulfate 3-O-sulfotransferases, which specifically sulfate the 3-OH positions of glucosamine residues that are linked to various sulfated/nonsulfated uronic acid residues at the nonreducing termini. In this article, we report the expression and characterization of new heparan sulfate 3-O-sulfotransferase isoforms, designated as 3-OST-2,  $3\text{-}\mathrm{OST}\text{-}3_\mathrm{A}\text{,}$  and  $3\text{-}\mathrm{OST}\text{-}3_\mathrm{B}$  that were isolated as described in the accompanying article (24). We have found that 3-OST-2 and 3-OST-3<sub>A</sub> catalyze the 3-O-sulfation of glucosamine in GlcA2S-GlcNS and IdoA2S-GlcNS, respectively, and 3-OST-3<sub>A</sub> and 3-OST-3<sub>B</sub> sulfate an identical disaccharide. The HS modified by 3-OST-2 and 3-OST-3  $_{\rm A}$  do not contain anticoagulant activity. Our studies reveal the presence of different isoforms, each of which sulfates the sugar residue in unique disaccharides or possibly in oligosaccharide sequences. Thus, we provide a new mechanism for the biosynthesis of HS with specific monosaccharide sequences and different potential biological functions.

# EXPERIMENTAL PROCEDURES Materials

Plasmids pJL2.7, pJL3.3, and pJL3.6 containing 3-OST-2, 3-OST-3<sub>A</sub> and 3-OST-3<sub>B</sub> were isolated from human brain and liver cDNA libraries (24). The [ $^{35}$ S]PAPS ( $\approx$ 150 Ci/mmol) was prepared by incubating 0.4 mCi/ml [ $^{35}$ S]Na<sub>2</sub>SO<sub>4</sub> (ICN) and 16 mM ATP with 5 mg/ml dialyzed yeast extract (Sigma) as described previously (14). Iduronate 2-sulfatase,  $\beta$ -glucuronidase, and  $\alpha$ -iduronidase were purified from bovine liver (10). The following [ $^{3}$ H]disaccharide standards were prepared from antithrombin-binding heparin octasaccharide (a gift from Dr. Lun H. Lam, Glycomed) (12): GlcA-[ $^{3}$ H]AnMan6S, IdoA2S-[ $^{3}$ H]AnMan6S, and GlcA-[ $^{3}$ H]AnMan3S6S.

### Expression of 3-OST Isoforms

The expression plasmids for 3-OST-2 and 3-OST-3<sub>A</sub> were constructed by inserting *EcoRI/XbaI* cleaved fragments from pJL 2.7 and pJL 3.3 into pcDNA3 (Invitrogen) which was digested with *EcoRI/XbaI* followed by phosphatase treatment. The expression plasmid for 3-OST-3<sub>B</sub> was similarly constructed by inserting the *HindIII/NotI* cleaved fragment from pJL 3.6 into pcDNA3 which was digested with *HindIII/NotI*. The 3-OST-1 expression construct has been previously described (15). The expression plasmids were transfected into exponentially growing COS-7 cells by using DEAE dextran and dimethyl sulfoxide (15).

# Measurement of 3-OST-2 and 3-OST-3<sub>A</sub>/-3<sub>B</sub> Activities

The crude enzymes were extracted from transiently transfected COS-7 cell pellets by vortexing  $2.5 \times 10^5$  cells in 200 µl of ice-cold 0.25 M sucrose with 1% Triton X-100 (v/v), and the insoluble residue was removed by spinning at 10,000 imes g for 10 min. To determine HS sulfotransferase activity, the reaction contained  $10-40 \ \mu g$  of cell extract protein, 200 nM unlabeled HS<sup>inact</sup> isolated from 33 cells (14), 60  $\times$ 10<sup>6</sup> cpm of [<sup>35</sup>S]PAPS, 50 mM MES (Sigma), pH 7.0, 10 mM MnCl<sub>2</sub>, 5 mM  $\rm MgCl_2,~75~\mu g/ml$  protamine chloride (Sigma), 120  $\mu g/ml$  bovine serum albumin, and 1% Triton X-100 (v/v) in a final volume of 50 µl. The reaction mixture was incubated at 37 °C for 2 h, guenched by heating to 100 °C for 2 min and spun at 14,000  $\times\,g$  for 2 min to remove insoluble material. The sample was then subjected to a 200-µl DEAE-Sepharose column to isolate [35S]HS (14, 25). One unit of HS sulfotransferase activity was defined as the transfer of 1 fmol ( $\times 10^{-15}$  mol) of sulfate from PAPS to heparan sulfate under standard assay conditions. The procedure for determining HS<sup>act</sup> conversion activity was based upon our previous report (14). One unit of the HSact conversion activity was defined as 0.5% increase of HS<sup>act</sup> per 20 min under standard conditions.

## Degradation of <sup>35</sup>S-Labeled HS

Low pH Nitrous Acid Degradation of [ $^{35}S$ ]HS—Purified [ $^{35}S$ ]HS modified by 3-OST-2, 3-OST-3<sub>A</sub>, and 3-OST-3<sub>B</sub> (2  $\times$  10<sup>4</sup> to 4  $\times$  10<sup>6</sup> cpm) was mixed with 20  $\mu$ g of porcine kidney HS (ICN), treated on ice with HNO<sub>2</sub>, at pH 1.5 for 30 min, and then reduced under alkaline conditions with 0.5  $\,\rm M$  NaBH\_4 (26). The resultant  $^{35}S$ -disaccharides were mixed with IdoA2S-[^3H]AnMan6S (2  $\times$  10<sup>4</sup> to 2  $\times$  10<sup>6</sup> cpm) and desalted on a Bio-Gel P-2 gel column (0.75  $\times$  200 cm) which was equilibrated with 0.1 M ammonium bicarbonate at a flow rate of 4 ml/h.

High Performance Liquid Chromatography—Separation and characterization of  $^{35}$ S-labeled disaccharides were carried out by HPLC using either a C $_{18}$ -reversed phase column (0.46  $\times$  25 cm) (RPIP-HPLC) (Vy-dac) or a Partisil-5 strong anion exchange column (0.46  $\times$  25 cm) (SAX-HPLC) (Whatman). The RPIP-HPLC was eluted with 4.2% acetonitrile for 45 min followed by 9% acetonitrile for 15 min and then by 10.2% acetonitrile for 120 min in 10 mM ammonium dihydrophosphate and 1 mM tetrabutylammonium dihydrophosphate (Sigma) at a flow rate of 0.5 ml/min (11, 12, 27); the SAX-HPLC was eluted with 30 mM KH\_2PO\_4 for 80 min followed by a linear gradient from 30 to 400 mM KH\_2PO\_4 over 120 min at a flow rate of 0.8 ml/min (11,12).

#### Determination of the Structures of Peak-X and Peak-Y

Preparation of <sup>3</sup>H-Labeled Di- and Monosaccharide Standards-Disaccharide IdoA3S-GlcNS3SOMe (methyl-(3-O-sodium sulfonato-sodium  $\alpha$ -L-idopyranosyluronate)-(1 $\rightarrow$ 4)-2-deoxy-2-sodium sulfonatamido-3-Osodium-sulfonato- $\alpha$ -D-glucopyranoside) and GlcA3S-GlcNS3SOMe (methyl-(3-O-sodium sulfonato-sodium  $\beta$ -D-glucopyranosyluronate)- $(1\rightarrow 4)$ -2-deoxy-2-sodium sulfonatamido-3-O-sodium sulfonato- $\alpha$ -D-glucopyranoside) were chemically synthesized (28). The structures of these two compounds were confirmed by <sup>1</sup>H NMR (<sup>2</sup>H<sub>2</sub>O, 250 Mhz).<sup>4</sup> IdoA3S-[3H]AnMan3S and GlcA3S-[3H]AnMan3S were prepared by low pH nitrous acid treatment of the corresponding chemically synthesized disaccharide followed by a reduction with sodium [<sup>3</sup>H]borohydride. GlcA2S-[3H]AnMan6S was prepared by epimerization of IdoA2S-[<sup>3</sup>H]AnMan6S in the presence of hydrazine and hydrazine sulfate. The products were confirmed by chemical desulfation in the presence of 0.5 M sulfuric acid followed by  $\beta$ -glucuronidase digestion. [<sup>3</sup>H]AnMan3S was prepared from  $\beta$ -D-glucosamine 3-O-sulfate (Sigma) by nitrous acid treatment at pH 1.5 followed by NaB<sup>3</sup>H<sub>4</sub> reduction. [<sup>3</sup>H]AnMan6S was prepared from β-glucuronidase-treated GlcA-[<sup>3</sup>H]AnMan6S.

Epimerization and Chemical Desulfation of  ${}^{35}S$ - and  ${}^{3}H$ -Labeled Disaccharides— ${}^{35}S$ -Labeled disaccharides (5–10 × 10<sup>4</sup> cpm) were

<sup>&</sup>lt;sup>2</sup> Zhang, L., Yoshida, K., Liu, J., and Rosenberg, R. D. (1999) J. Biol. Chem. **274**, in press.

<sup>&</sup>lt;sup>3</sup> All <sup>3</sup>H-labeled disaccharide and monosaccharide standards used in the experiments were labeled at the C<sub>1</sub> position of 2,5-anhydromannitol. The structures of di- and oligosaccharides were presented in an abbreviated format omitting D-, L-  $\alpha$ 1 $\rightarrow$ 4, and  $\beta$ 1 $\rightarrow$ 4 for each sugar residue in order to conserve space and improve the clarity. -GlcA-, -GlcNR', and -IdoA- represents the linkage of  $\rightarrow$ 4)- $\beta$ -D-GlcA(1 $\rightarrow$ ,  $\rightarrow$ 4)- $\alpha$ -D-GlcNR'(1 $\rightarrow$ , and  $\rightarrow$ 4)- $\alpha$ -L-IdoA-(1 $\rightarrow$ , respectively.

<sup>&</sup>lt;sup>4</sup> S. Tripathy, J.-M. Mallet, M. Petitou, and P. Sinaÿ, manuscript in preparation.

FIG. 1. Bio-Gel P-2 fractionation of depolymerized [<sup>35</sup>S]HS. [<sup>35</sup>S]HS generated by incubating [<sup>35</sup>S]PAPS with unlabeled HS<sup>inact</sup> and cell extracts prepared from COS-7 cells transfected with control vector, 3-OST-2, or 3-OST-3, were depolymerized with low pH nitrous acid (pH 1.5). The depolymerized [<sup>35</sup>S]HS was mixed with IdoA2S- [3H]AnMan6S as an internal standard and chromatographed on a column (0.75  $\times$  200 cm) of Bio-Gel P-2 equilibrated with 0.1 M ammonium bicarbonate. The *three panels* represent the profiles of the products of [<sup>35</sup>S]HS prepared with 40  $\mu$ g of cell extract of COS-7 cells transfected with vector alone (A), 3-OST-2 (B), and 3-OST-3<sub>A</sub> (C). Fractions of one-half ml were collected, analyzed for <sup>3</sup>H and <sup>35</sup>S, and then were pooled as indicated by the solid bar. The arrows indicate the elution positions of hexa-, tetra-, and disaccharide.



mixed with appropriate <sup>3</sup>H-labeled disaccharide standards (1–5 × 10<sup>5</sup> cpm) in 50 µl of anhydrous hydrazine (Aldrich) containing 10 mg/ml hydrazine sulfate and incubated at 100 °C in a sealed 1.0-ml vacuum hydrolysis tube (Pierce) for 16 h followed by HIO<sub>3</sub> oxidation as described by Shaklee and Conrad (29). The partial acid hydrolysis of <sup>35</sup>S-labeled disaccharide (2 × 10<sup>4</sup> cpm) was performed in 0.5 M H<sub>2</sub>SO<sub>4</sub> and incubated at 100 °C for 15 min as described elsewhere (30). The partially desulfated [<sup>35</sup>S]disaccharide became susceptible to α-iduronidase or β-glucuronidase which permitted the establishment of the structure of the disaccharide as well as the position of [<sup>35</sup>S]sulfate (14,31).

β-Glucuronidase and α-Iduronidase Digestion—<sup>35</sup>S-Labeled disaccharide (1 × 10<sup>4</sup> cpm) was mixed with GlcA-[<sup>3</sup>H]AnMan6S or GlcA-[<sup>3</sup>H]AnMan3S6S in 50 μl of 50 mM NaAcO, pH 4.5, and 100 μl of purified β-glucuronidase (5,000 units) and incubated at 37 °C for 36 h. For α-iduronidase digestion, the <sup>35</sup>S-labeled disaccharide was mixed with IdoA-[<sup>3</sup>H]AnMan6S (1–10 × 10<sup>4</sup> cpm) in 50 μl of buffer containing 250 mM sodium formate and 400 mM NaCl, pH 3.55, and 100 μl of α-iduronidase (8 units) and incubated at 37 °C for 36 h.

Iduronate 2-Sulfatase Digestion-Because iduronate 2-sulfatase was extremely sensitive to phosphate (IC<sub>50</sub> = 25-100  $\mu$ M) (32), the disaccharide samples were desalted extensively by using a combination of DEAE chromatography, P-2 gel, and descending paper chromatography. Briefly, the RPIP-HPLC purified [35S]disaccharides were diluted 5-10-fold with water to bring the concentration of phosphate to less than 5 mM, neutralized with 10 M NaOH and charged to a 1-ml DEAE-Sephacel column (Sigma), which was equilibrated with 10 mM triethylammonium bicarbonate buffer (Sigma), pH 8.0. The column was washed with  $10 \times 1$  ml of 100 mM triethylammonium bicarbonate, pH 8.0, and eluted with 400 mM triethylammonium bicarbonate, pH 8.0. The resultant disaccharides were loaded on a P-2 gel column (0.75 imes200 cm) equilibrated and eluted with 0.1 M ammonium bicarbonate at a flow rate of 4 ml/h. The disaccharides were then loaded on 3MM paper (Whatman) which was developed in ethyl acetate/formic acid/acetic acid/water = 36:2:6:1 for 2 h (12, 13), and the disaccharides were recovered by eluting from the 3MM paper with  $3 \times 0.2$  ml of water. The condition for iduronate 2-sulfatase digestion followed the procedures reported by Bielicki et al. (32).

#### RESULTS

Determination of [<sup>35</sup>S]Sulfation Sites within HS—In the accompanying paper (24), we reported the isolation of 3-OST-2,  $3-OST-3_A$ , and  $3-OST-3_B$  cDNAs with about 60% similarity to human 3-OST-1 and about 45% similarity to two forms of human heparan sulfate N-deacetylase/N-sulfotransferase in the proposed sulfotransferase domain (24). Because of such high homology, it seemed likely that these cDNAs encode heparan sulfate sulfotransferase activities. To test this hypothesis, we incubated 40  $\mu$ g of cell extracts protein from COS-7 cells transfected with 3-OST-2, 3-OST-3<sub>A</sub>, 3-OST-3<sub>B</sub>, or control vector with known amounts of [35S]PAPS and 200 nm of various unlabeled glycosaminoglycans including HS, heparin, chondroitin sulfate A, B, and C, and keratan sulfate. As expected, the amount of [35S]HS and was increased by 2.5-3-fold compared with the control, whereas the remaining glycosaminoglycans failed to show a detectable increase in [35S]sulfate incorporation, suggesting that these cDNAs encode enzymes that specifically transfer [<sup>35</sup>S]sulfate from [<sup>35</sup>S]PAPS to heparan sulfate. Furthermore, the [<sup>35</sup>S]HS was depolymerized by a mixture of heparitinase-I to IV, and the product migrated as a tetrasaccharide on Bio-Gel P-2 and anion exchange-HPLC (data not shown). It is worthwhile to note that 3-OST-2 and 3-OST-3<sub>A</sub> activities were not found in the medium unless the transmembrane domains were removed.

The specific sites at which HS is sulfated by 3-OST-2, 3-OST- $3_A$ , or 3-OST- $3_B$  were determined by incubating [ $^{35}S$ ]PAPS and unlabeled HS with cell extracts from COS-7 cells transfected with expression constructs containing the appropriate cDNAs. Because 3-OST- $3_A$ - and 3-OST- $3_B$ -modified [ $^{35}S$ ]HS yielded an identical  $^{35}S$ -labeled disaccharide after the nitrous acid degradation, we only present the data of 3-OST- $3_A$ . The [ $^{35}S$ ]HS prepared with transfected cell extracts were depolymerized with low pH (pH 1.5) nitrous acid followed by sodium borohydride reduction and analyzed by Bio-Gel P-2 column (Fig. 1). The resultant gel filtration chromatograms demonstrated that the majority of the depolymerized [ $^{35}S$ ]HS (90 ± 2%) eluted at positions ranging from disulfated disaccharide to free sulfate



FIG. 2. RPIP-HPLC of <sup>35</sup>S-labeled HS disaccharides. The disaccharides obtained by gel filtration (pooled fractions in Fig. 1) were mixed with various <sup>3</sup>H-labeled disaccharide standards as internal standards and separated on RPIP-HPLC as described under "Experimental Procedures." The *three panels* represent the chromato-grams of <sup>35</sup>S-labeled disaccharides derived from [<sup>35</sup>S]HS prepared with the cell extracts of COS-7 cells transfected with vector alone (A), 3-OST-2 (B), and 3-OST-3<sub>A</sub> (C). The arrows indicate the elution positions of <sup>3</sup>H-labeled disaccharide standards, where 2 represents IdoA2S-[<sup>3</sup>H]AnMan, 3 represents GlcA-[3H]AnMan6S, 4 represents IdoA-[3H]AnMan6S, 5 represents GlcA-[3H]AnMan3S6S, and 6 represents IdoA2S-[3H]AnMan6S.

(Fig. 1). Compared with control cell extracts (Fig. 1A), 3-OST-2 (Fig. 1B) and 3-OST-3<sub>A</sub> cell extracts (Fig. 1C) generated 2.4-fold greater amounts of  $^{35}\text{S}\text{-disulfated}$  disaccharides.

The chromatographic fractions containing <sup>35</sup>S-labeled counts were pooled and then analyzed on RPIP-HPLC to determine the nature of the labeled disaccharides (Fig. 2). The control cell extracts exhibited [<sup>35</sup>S]sulfate and small amounts of <sup>35</sup>S-labeled IdoA2S-AnMan6S, which is a common disaccharide of heparan sulfate (Fig. 2A). The 3-OST-2 cell extracts possessed  $^{35}$ S-labeled Peak-X and Peak-Y (Fig. 2B), which accounted for 60-80% of  $[^{35}S]SO_4$  incorporated into HS by the expressed enzyme. The separation between Peak-Y and GlcA-[<sup>3</sup>H]AnMan3S6S was improved on SAX-HPLC (data not shown). The 3-OST- $3_A$  cell extracts contained Peak-X (Fig. 2C), which accounted for about 70% of  $[^{35}S]SO_4$  incorporated into HS by the expressed enzyme. Neither Peak-X nor Peak-Y coeluted with known <sup>3</sup>H-labeled heparin disaccharides. It is of interest to note that nearly 80% of the degraded  ${}^{35}S$ -labeled material was eluted as tetrasaccharides and hexasaccharides on Bio-Gel P-2 column when 3-OST-2- and 3-OST-3<sub>A</sub>-modified [<sup>35</sup>S]HS were treated by hydrazinolysis followed by high pH (pH 5.5), low pH (pH 1.5) nitrous degradation and sodium borohydride reduction. We suspected that this unexpected result was caused by a high degree of ring contraction during the degradation of the [<sup>35</sup>S]HS.

We obtained secreted forms of 3-OST-2 and 3-OST-3<sub>A</sub> by truncating the putative transmembrane domains and expressing in Sf9 insect cells. These secreted enzymes sulfate the same disaccharides as those of the cell extracts from the COS-7 cells transfected with 3-OST-2 and 3-OST-3<sub>A</sub>. Furthermore, 3-OST-3<sub>A</sub> was successfully purified from the insect cell serum-free medium by using Heparin-Toyopearl 650M and 3',5'-ADP-agarose chromatography and exhibited the expected molecular weight

on SDS gel.<sup>5</sup> Taken together, these results suggest that 3-OST-2 and 3-OST-3<sub>A</sub> are directly responsible for the HS sulfotransferase activities.

Determination of the Structures of Peak-X and Peak-Y—To determine the structure of Peak-X, it was mixed with IdoA2S-[<sup>3</sup>H]AnMan6S (Fig. 3A) and exhaustively digested with iduronate 2-sulfatase. A mono-[<sup>35</sup>S]sulfated disaccharide and IdoA-[<sup>3</sup>H]AnMan6S (Fig. 3B) were generated. The shift in retention time of the <sup>35</sup>S-labeled peak on SAX-HPLC suggested that Peak-X contained a 2-O-sulfated iduronic acid residue. The mono-[<sup>35</sup>S]sulfated disaccharide was then digested with  $\alpha$ -iduronidase which generated a mono-[<sup>35</sup>S]sulfated monosaccharide (Fig. 3C), coeluting with [<sup>3</sup>H]AnMan3S but not with [<sup>3</sup>H]AnMan6S on RPIP-HPLC (Fig. 3C, inset). This latter result shows that [<sup>35</sup>S]sulfate was located at the 3-OH position of the 2,5-anhydro-D-mannitol residue. Therefore, the data suggest that Peak-X has the structure of IdoA2S-[<sup>35</sup>S]AnMan3S.

The structure of Peak-Y was determined by a combination of partial chemical desulfation followed by  $\beta$ -glucuronidase digestion (Fig. 4). The sulfate was removed from the glucuronic acid residue by incubating Peak-Y and GlcA-[<sup>3</sup>H]AnMan3S6S with 0.5 M sulfuric acid, and analyzing the desulfated products on RPIP-HPLC. The GlcA-[<sup>3</sup>H]AnMan3S6S, which serves as an internal standard, allowed us to monitor the extent of the chemical desulfation and determine the eluting position of monosulfated disaccharides on RPIP-HPLC. The Peak-Y yielded a partially desulfated mono-[<sup>35</sup>S]sulfated disaccharide, which coeluted with GlcA-[<sup>3</sup>H]AnMan3S on RPIP-HPLC (Fig. 4). The structure of the partially desulfated Peak-Y was confirmed to be GlcA-[<sup>35</sup>S]AnMan3S by digesting with  $\beta$ -glucuronidase and

<sup>&</sup>lt;sup>5</sup> J. Liu, Z. Shriver, P. Blaiklock, K. Yoshida, R. Sasisekharan, and R. D. Rosenberg, manuscript in preparation.



FIG. 3. SAX-HPLC analysis of the iduronate 2-sulfatase and  $\alpha$ -iduronidase digested Peak-X. Panel A shows the elution positions of Peak-X and IdoA2S- [<sup>3</sup>H]AnMan6S on SAX-HPLC; Panel B shows the elution positions of IdoA-[3H]AnMan6S and mono- [35S]sulfated disaccharide yieled by the iduronate 2-sulfatase digested Peak-X; Panel C shows the elution positions of [3H] AnMan6S and mono-[35S]sulfated disaccharide digested with  $\alpha$ -iduronidase (80) units/ml). The *inset* represents the chro-matography of the <sup>35</sup>S-labeled monosaccharide on RPIP-HPLC, which was eluted at a flow rate of 0.5 ml/min with 1.2% acetonitrile in 1 mM tetrabutvlammonium dihydrophosphate and 10 mM ammonium dihydrophosphate. Under these conditions, maximum resolution was obtained for [<sup>3</sup>H]AnMan6S and [<sup>3</sup>H]AnMan3S.

showing that the elution position of the <sup>35</sup>S-labeled peak was shifted from 55 to 9 min on SAX-HPLC (Fig. 4, *inset*). Thus, the data suggest that the structure of Peak-Y is GlcA2S-[<sup>35</sup>S]AnMan3S. It should be noted that Peak-Y prior to partial acid desulfation was resistant to the action of  $\beta$ -glucuronidase (data not shown).

Our data suggested that the structural difference between Peak-X and Peak-Y is at the position of the proton on  $C_5$  of the uronic acid residue. To confirm this hypothesis, Peak-Y and Peak-X were epimerized by incubating samples with hydrazine containing 1% hydrazine sulfate at 100 °C for 16 h followed by HIO<sub>3</sub> oxidation. The product from Peak-X coeluted with Peak-Y, and the product from Peak-Y coeluted with Peak-X on RPIP-HPLC. Furthermore, we have also found that the epimerized Peak-Y became susceptible to iduronate 2-sulfatase. In contrast, Peak-Y is totally resistant to the digestion of iduronate 2-sulfatase (data not shown).

To eliminate the possibility that Peak-X or Peak-Y contains a 3-O-sulfated uronic acid residue, we compared the elution positions of Peak-X and IdoA3S-[<sup>3</sup>H]AnMan3S and Peak-Y and GlcA3S-[<sup>3</sup>H]AnMan3S, respectively. As expected, Peak-X (retention time = 95.0 min) did not coelute with IdoA3S-[<sup>3</sup>H]AnMan3S (retention time = 97.4 min), suggesting that Peak-X does not contain 3-O-sulfated iduronic acid residue. Likewise, Peak-Y contains a 2-O-sulfated glucuronic acid residue, because Peak-Y (retention time = 108.0 min) did not coelute with GlcA3S-[<sup>3</sup>H]AnMan3S (retention time = 115.0 min).

Comparison of  $HS^{act}$  Conversion Activities among 3-OST Isoforms—We previously reported that 3-OST-1 was a key enzyme for generating  $HS^{act}$ , and the activity was determined by the  $HS^{act}$  conversion assay (13, 14). We, therefore, compared the HS sulfotransferase and HS<sup>act</sup> conversion activities with fixed amount of COS-7 cell extracts (10 µg of protein) containing the 3-OST isoforms. First, we determined the HS sulfotransferase activities by mixing unlabeled HS and [35S]PAPS with the COS-7 cell extract transfected with empty vector (control), 3-OST-1, 3-OST-2, and 3-OST-3 $_{\rm A}$  respectively, as described under "Experimental Procedures." 3-OST-1, 3-OST-2, and 3-OST-3<sub>A</sub> showed a similar increase of the HS sulfotransferase activity compared with the control. This suggested that the three isoforms were expressed at similar level, as well as that the substrate in the reaction has sufficient amount of [<sup>35</sup>S]sulfate acceptor sites for each isoform (Fig. 5A). Second, we assayed for the HS<sup>act</sup> conversion activity on these cell extracts by mixing with <sup>35</sup>S-labeled HS<sup>inact</sup> and unlabeled PAPS as described under "Experimental Procedures." 3-OST-1 elevated the level of HS<sup>act</sup> conversion activity by more than 300-fold above the detection limit (Fig. 5B). On the contrary, the cell extracts transfected with control, 3-OST-2, and 3-OST-3<sub>A</sub> did not show any detectable HS<sup>act</sup> conversion activity (detection limit = 0.5 units) (Fig. 5B).

## DISCUSSION

The cDNAs, which have approximately 60% similarity to the earlier cloned 3-OST-1 in the sulfotransferase domain, were demonstrated to encode heparan sulfate 3-*O*-sulfotransferase isoforms. The strategy used to characterize these cDNAs is the following: 1) expression of the cDNAs in COS-7 cells by transient transfection; 2) preparation of <sup>35</sup>S-labeled HS by mixing the [<sup>35</sup>S]PAPS and unlabeled HS with the COS-7 cell extracts transfected with 3-OST-2, 3-OST-3<sub>A</sub>, and 3-OST-3<sub>B</sub>; 3) determination of the <sup>35</sup>S labeling site within the [<sup>35</sup>S]HS by degrading the HS with nitrous acid at low pH (pH 1.5). A similar



**Retention Time (min)** 

FIG. 4. HPLC analysis of chemically desulfated Peak-Y. Peak-Y and GlcA-[<sup>3</sup>H]AnMan3S6S standard was subjected to partial chemical desulfation and analyzed on RPIP-HPLC. The *inset* shows the elution position of the partially desulfated Peak-Y digested with β-glucuronidase (50,000 units/ml) on SAX-HPLC. The arrow in the inset represents the elution position of the partially desulfated Peak-Y.

approach has been previously used to characterize 3-OST-1, heparan sulfate (D-glucosaminyl) 6-O-sulfotransferase, and heparan sulfate (uronosyl) 2-O-sulfotransferase (7, 8, 14). Our data suggest that 3-OST-2, 3-OST-3\_A, and 3-OST-3\_B are heparan sulfate 3-O-sulfotransferases because they transfer [<sup>35</sup>S]sulfate from [<sup>35</sup>S]PAPS to the 3-OH position of the glucosamine residue, and 3-OST- $3_A$  and 3-OST- $3_B$  sulfates an identical disaccharide.

To our knowledge, the activities of 3-OST-2 and 3-OST-3<sub>A</sub> have not been previously reported. The substrate specificities of 3-OST-2 and 3-OST- $3_A$  at the disaccharide level were identified by determining the structures and [<sup>35</sup>S]sulfate labeling sites of two <sup>35</sup>S-labeled disaccharides designated as Peak-X and Peak-Y. These two disaccharides are the products of low pH nitrous acid-treated [<sup>35</sup>S]HS prepared by 3-OST-2 and 3-OST-3<sub>A</sub>. Peak-X was identified to be IdoA2S-[<sup>35</sup>S]AnMan3S, and Peak-Y was identified to be GlcA2S-[<sup>35</sup>S]AnMan3S. The structure GlcA2S-AnMan3S has not been identified previously in naturally occurring heparin and heparan sulfate. However, because of the potential side reactions during chemical degradation of [35S]HS, the substrate specificities of 3-OST-2 and 3-OST-3<sub>A</sub> have not been conclusively determined. Such side reactions can only be controlled by using chemically defined oligosaccharides with expected modifications.

The differences between the isoforms and 3-OST-1 are as follows: 3-OST-1 generates HS<sup>act</sup>, while 3-OST-2 and 3-OST-3<sub>A</sub> do not. In addition, the isoforms transfer sulfate to uronylglucosamine disaccharides with different sulfated uronic acid residues. For example, 3-OST-1 transfers sulfate to the 3-OH position of the glucosamine within GlcA-GlcNS  $\pm$  6S; 3-OST-2 transfers sulfate to the 3-OH position of the glucosamine within GlcA2S-GlcNS and IdoA2S-GlcNS; 3-OST-3<sub>A</sub> transfers

sulfate to the 3-OH position of the glucosamine within IdoA2S-GlcNS. However, we cannot rule out the possibility that 3-OST-2 and 3-OST- $3_A$  sulfate the 3-OH position of the glucosamine within GlcA2S-GlcNH<sub>2</sub> and IdoA2S-GlcNH<sub>2</sub>, respectively.<sup>6</sup> Furthermore, 3-OST-1 is a secretary protein, because substantial amounts of the activity were detected in the cell medium and no putative membrane spanning region occurs within the primary sequence of this enzyme (14, 15). We found that 3-OST-2 and 3-OST-3<sub>A</sub> are membrane-bound proteins, because their activities were detected only in cell extracts and putative membrane-spanning regions occur within their primary sequences (24).

The unique substrate specificities of 3-OST-2 and  $3-OST-3_A$ suggest that their products possess distinct biological functions. Since Peak-X could be released from the 3-OST-3<sub>A</sub>-modified [<sup>35</sup>S]HS by direct low pH nitrous treatment, it became evident that this disaccharide is derived from -GlcNS6S(?)-IdoA2S-GlcNS3S- sequence. It is very interesting to note that -GlcNS6S(?)-IdoA2S-GlcNS3S- has been identified to be present in substantial amounts in the glomerular basement membrane HS (21). This substance is believed to regulate the permeability of glomeruli (33, 34). Indeed both  $3-OST-3_A$  and 3-OST- $3_{\rm B}$  are expressed in human kidney (24).

It is also very interesting to note that 3-OST-2 mRNA colocalizes with HS that contains a high level of GlcA2S in human brain. Although 3-OST-2 modifies the substrate HS con-

 $<sup>^{6}</sup>$  We have recently found that purified 3-OST-3  $_{\rm A}$  sulfates the glucosamine residue with free amino group. The conclusion was based on the results of the structural analysis of two <sup>35</sup>S-labeled tetrasaccharides, which were isolated from heparin lyases degraded the [35S]HS [35S]sulfated by purified 3-OST-3<sub>A</sub>.



FIG. 5. Comparison of the capability of generating HSact among 3-OST isoforms. Panel A shows the HS sulfotransferase activity in the extract of COS-7 cell transfected with empty vector (control), 3-OST-1, 3-OST-2, and 3-OST-3<sub>A</sub>. The sulfotransferase activity was determined by mixing unlabeled HS (200 nM) with [ $^{35}S$ ]PAPS (60  $\times$  $10^6$  cpm, 150 Ci/mmol) and cell extracts (10  $\mu g)$  in 50  $\mu l$  of reaction buffer as described under "Experimental Procedures." The <sup>35</sup>S-labeled HS was extracted by using DEAE chromatography and eluted with 1 M sodium chloride. One unit of HS sulfotransferase activity was defined as the transfer of 1 fmol ( $\times 10^{-15}$  mol) sulfate from PAPS to heparan sulfate under standard assay conditions. Panel B shows the HSact conversion activity in the extract of COS-7 cells transfected with empty vector (control), 3-OST-1, 3-OST-2, and 3-OST-3<sub>A</sub>. The HS<sup>act</sup> conversion activity was determined by mixing [<sup>35</sup>S]HS<sup>inact</sup> (1  $\times$  10<sup>5</sup> cpm), cell extracts (10  $\mu$ g), and unlabeled PAPS (500  $\mu$ M) in 50  $\mu$ l of reaction buffer containing protamine. The amount of converted [<sup>35</sup>S]HS<sup>act</sup> was determined by measuring the <sup>35</sup>S-labeled counts bound to ConA/AT affinity chromatography. One unit of the  $\mathrm{HS}^{\mathrm{act}}$  conversion activity was defined as 0.5% increase of HS<sup>act</sup> per 20 min under standard conditions. The presented data are the average of duplicate determinations, and the error bar indicates the range.

taining both IdoA2S- and GlcA2S-linked glucosamine, the enzyme likely prefers to sulfate the disaccharide containing GlcA2S because the product contains higher level of Peak-Y when a low concentration of substrate was used in the enzymatic reaction. For example, we have found that the ratio between Peak-Y (GlcA2S-[<sup>35</sup>S]AnMan3S) and Peak-X (IdoA2S-[<sup>35</sup>S]AnMan3S) in the 3-OST-2-modified HS was increased from 0.5 to 1.5 as the concentration of substrate was decreased from 200 to 20 nm.<sup>7</sup> Furthermore, 3-OST-2 should sulfate the sequence containing GlcA2S with much higher efficiency because the population of a sulfated glucuronic acid is approximately 5-50-fold less frequent than that of a sulfated iduronic acid in any given heparan sulfate (35-38). Our Northern analysis has demonstrated that 3-OST-2 is predominantly expressed in human brain as described in the accompanying article (24). Interestingly, the level of GlcA2S in HS isolated from various sections of human brain was reported to be 5-8fold higher than that in the HS isolated from kidney and aorta,

<sup>7</sup> J. Liu, unpublished data.

respectively (39). This suggests a special biological function of the HS modified by 3-OST-2 in human brain.

We have previously demonstrated that the antithrombinbinding site on heparan sulfate is synthesized in a two-step process in which six copies of a precursor structure are generated per polysaccharide chain that possesses the correct positioning of all critical groups except for the absence of the 3-O-sulfate group. In the final step of the reaction, the amount of 3-OST-1 is up-regulated at a transcriptional level which completes the formation of the antithrombin-binding site and controls the supply of this site present on the cell surface (40). It appears likely that parallel reaction pathways might exist in which other precursor structures, of different monosaccharide sequences, are generated. These precursors may be recognized by sulfating enzymes distinct from 3-OST-1 but homologous to it. This would result in multiple, distinct HS end products with different biologic functions. In this situation the regulation of the levels of enzymes homologous to 3-OST-1 might be expected to control the production of non-anticoagulant HS.

In summary, our investigation has revealed a new cellular mechanism for generating defined monosaccharide sequences within the structurally complicated HS polysaccharide. The classical model of the HS biosynthetic machinery involves serial sulfation and epimerization of HS. However, this model is unable to explain the formation of specific saccharide structures or the regulation of their levels. Our results demonstrate that specific disaccharides containing 3-O-sulfated glucosamine can be generated by specialized isoforms of heparan sulfate 3-O-sulfotransferase, although whether these isoforms recognize disaccharide or oligosaccharide sequences remains to be determined. These isoforms are also expressed at different levels in different human tissues. If this is the case for all classes of HS biosynthetic enzymes, the individual isoforms of a particular enzyme could play a key role in generating tissuespecific HS having defined saccharide sequence with unique biological functions. Therefore, the isolation and characterization of the cDNAs encoding specialized isoforms should allow us to understand how a specific monosaccharide sequence is generated. Furthermore, manipulation of the level of the special isoform within a cell or animal tissue may provide an excellent approach for studying the specific biological effects of HS

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