6-O-Sulfotransferase-1 Represents a Critical Enzyme in the Anticoagulant Heparan Sulfate Biosynthetic Pathway*

Using recombinant retroviral transduction, we have introduced the heparin/heparan sulfate (HS) 3-O-sulfotransferase 1 (3-OST-1) gene into Chinese hamster ovary (CHO) cells. Expression of 3-OST-1 confers upon CHO cells the ability to produce anticoagulantly active HS (HSact). To understand how 6-OST and other proteins regulate HSact biosynthesis, a CHO cell clone with three copies of 3-OST-1 was chemically mutagenized. Resulting mutants that make HS but are defective in generating HSact were single-cell-cloned. One cell mutant makes fewer 6-O-sulfated residues. Modification of HS chains from the mutant with pure 6-OST and 3'-phosphoadenosine 5'-phosphosulfate increased HSact from 7% to 51%. Transfection of this mutant with 6-OST-1 created a CHO cell line that makes HS, 50% of which is HSact. We discovered in this study that (i) 6-OST-1 is a limiting enzyme in the HSact biosynthetic pathway in vitro when the limiting nature of 3-OST-1 is removed; (ii) HS chains from the mutant cells serve as an excellent substrate for demonstrating that 6-OST-1 is the limiting factor for HSact generation in vitro; (iii) in contradiction to the literature, 6-OST-1 can add 6-O-sulfate to GlcNAc residues, especially the critical 6-O-sulfate in the antithrombin binding motif; (iv) both 3-O- and 6-O-sulfation can be the final step in HSact biosynthesis in contrast to prior publications that concluded 3-O-sulfation is the final step in HSact biosynthesis; (v) in the presence of HS interacting protein peptide, 3-O-sulfate-containing sugars can be degraded into disaccharides by heparitinase digestion as demonstrated by capillary high performance liquid chromatography coupled with mass spectrometry.

Heparin/heparan sulfate (HS) is a linear polymer covalently attached to the protein cores of proteoglycans, which are abundantly and ubiquitously expressed in almost all animal cells. HS is assembled by the action of a large family of enzymes that catalyze chain polymerization (alternating addition of GlcNAc and GlcUA residues), GlcNAc N-deacetylation and N-sulfation, glucuronic acid (GlcUA) epimerization to L-iduronic acid (IdoUA), 2-O-sulfation of uronic acid residues, and 3-0- and 6-O-sulfation of glucosaminyl residues. Tissue-specific and developmentally regulated expression of the biosynthetic enzymes and enzyme isoforms produces HS chains with distinct sequences (1–3). These different sequences enable interactions to occur with a broad array of protein ligands that modulate a wide range of biological functions in development, differentiation, homeostasis, and bacterial/viral entry (reviewed in Refs. 4–11).

The specificity of any HS and protein interaction is largely dictated by arrangements of sulfates along the chain. For example, the pentasaccharide sequence, GlcNAcNS6S-GlcUA-GlcNSS3S-6S-IDUA2S-GlcNS6S, represents the minimum sequence for antithrombin (AT) binding, where the 3S (3-O-sulfate) and 6S (6-O-sulfate) groups constitute the most critical elements involved in the interaction (12–16). To delineate the biosynthetic pathway that regulates anticoagulant heparan sulfate (HSact) biosynthesis, our laboratory has purified as well as molecularly cloned 3-OST-1 (17,18). We have demonstrated that 3-OST-1, usually existing in limited amounts, acts upon HSact precursor to produce HSact and upon HSinact precursor to produce 3-O-sulfated HSinact (17, 19). When 3-OST-1 is no longer limiting, the capacity for HSact generation is determined by the abundance of HSact precursors (20). In this case, the limitation in HSact production is the presence of the critical 6-O-sulfate groups and/or the availability of proper epimerization/sulfation patterns in the precursors.

In most cases, the critical 6S in the AT binding oligosaccharides sequenced and characterized in heparin is attached to GlcNAc residues (a summary table for heparin is presented in Refs. 21, 22). We previously reported that the critical 6S in heparan sulfate: HSact, total heparan sulfate; CHAPS, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; GAG, glycosaminoglycan; PAPS, 3'-phosphoadenosine 5'-phosphosulfate; AT, antithrombin; 3-OST-1, glucosaminyl 3-O-sulfotransferase-1; 6-OST-1, -2, -3, glucosaminyl 6-O-sulfotransferase-1, -2, -3; GlcUA, glucuronic acid; IdUA, iduridonic acid; 2S, 2-O-sulfate; 3S, 3-O-sulfate; 6S, 6-O-sulfate; NS, N-sulfate; PBS, phosphate-buffered saline; CHO, Chinese hamster ovary; HIP, heparin/heparan sulfate interacting protein; IPRP-HPLC, ion pairing reverse phase-high pressure liquid chromatography; ESI-MS, electrospray ionization-mass spectrometry; FGF, fibroblast growth factor; FGFR, FGF receptor; RT-PCR, reverse transcriptase-polymerase chain reaction; MES, 4-morpholinoethanesulfonic acid; bp, base pair(s); UTR, untranslated repeat; nt, nucleotide(s); CDSNS-heparin, N-, O-desulfated, re-N-sulfated heparin.

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HS\textsuperscript{act} from F9 cells is exclusively attached to GlcNAc residues (20). To date, three 6-O-sulfotransferases have been cloned, but reported substrate specificities indicate that none of them can put a 6-O-sulfate group on GlcNAc residues (2). To understand how specific 6-O-sulfotransferases and other factors regulate HS\textsuperscript{act} biosynthesis, we created cell mutants that are defective in the formation of HS\textsuperscript{act} precursors. We chose Chinese hamster ovary (CHO) cells, because a series of HS biosynthetic mutants have been successfully made in this cell line (23–28). However, CHO wild-type cells do not generate 3-O-sulfated residues and therefore do not make any HS\textsuperscript{act}. Using recombinant retroviral transduction, we have introduced the HS 3-O-sulfotransferase-1 (3-OST-1) gene into CHO cells (29). 3-OST-1 expression gives rise to CHO cells with the ability to produce HS\textsuperscript{act}. A cell line was chosen that has three copies of 3-OST-1 as determined by Southern analysis (29). After chemical mutagenesis of this cell line, mutant cells that were positive for FGF-2 binding but negative for AT binding were FACS sorted and cloned. The scheme for making HS\textsuperscript{act} precursor mutants is outlined in Fig. 1. The advantage of having multiple copies of 3-OST-1 is that other upstream genes that are responsible for generating specific HS precursor structures can be sought after chemical mutagenesis without concern for the loss of 3-OST-1. FGF-2 selection is employed to ensure that the mutant cells still make HS. By using this scheme, we obtained a 6-O-sulfate-defective mutant. After correcting the mutant with 6-O-sulfotransferase-1 transfection, we created a cell line that makes HS, 50% of which is HS\textsuperscript{act}. This represents the highest percentage of HS\textsuperscript{act} production by any cell line reported so far.

The approach used in this study includes placing multiple copies of a downstream enzyme in CHO cells, mutating the cells to obtain mutants deficient in upstream enzymes that are part of the pathway, and then characterizing the mutants. This technique may constitute a general approach for defining and obtaining the components of biosynthetic pathways once the terminal biosynthetic enzyme has been obtained and proteins that recognize the final product of the pathway are available.

EXPERIMENTAL PROCEDURES

Cell Culture—Wild-type Chinese hamster ovary cells (CHO-K1) were obtained from the American Type Culture Collection (CCL-61, ATCC, Rockville, MD). Wild-type and mutant cells were maintained in Ham’s F-12 medium supplemented with 10% fetal bovine serum (HyClone), penicillin G (100 units/ml), and streptomycin sulfate (100 \mu g/ml) at 37 °C under an atmosphere of 5% CO\textsubscript{2} in air and 100% relative humidity. The cells were passaged every 3–4 days with 0.125% (w/v) trypsin and 1 mM EDTA, and after 10–15 cycles, fresh cells were revived from stocks stored under liquid nitrogen. Low-sulfate medium was composed of Ham’s F-12 medium supplemented with penicillin G (100 units/ml) and 10% fetal bovine serum that had been dialyzed 200-fold against phosphate-buffered saline (PBS) (30). Low-glucose Ham’s F-12 medium contained 1 mM glucose supplemented with penicillin G (100 units/ml), streptomycin sulfate (100 \mu g/ml), and fetal bovine serum that had been dialyzed 200-fold against PBS (30). All tissue culture media and reagents were purchased from Life Technologies, Inc. (Gaithersburg, MD) unless otherwise indicated.

3-OST-1 Recombinant Retroviral Transduction—The method of 3-OST-1 recombinant retroviral transduction into CHO cells was described in detail in our previous publication (29).

Antithrombin and FGF-2 Labeling—The same procedure has been described as used previously (29).

Cell Sorting—Nearly confluent monolayers of 3-OST-1-transduced CHO-K1 cells were detached by adding 10 ml of 2 mM EDTA in PBS containing 10% fetal bovine serum and centrifuged. The cell pellets were placed on ice, 50 %v/v each of fluorescein-AT and Alexa 594-FGF-2 were added. After 30 min, the cells were washed once and resuspended in 1 ml of 10% fetal bovine serum in PBS containing 2 mM EDTA. Flow cytometry and cell sorting were performed on FACScan and FACStar instruments (Becton Dickinson) using dual color detection filters. Cells positive for AT and FGF-2 binding were sorted and subsequently single-cell-cloned into a 96-well plate. The single-cell clones were expanded and frozen for further analysis.

Twelve 3-OST-1-transduced CHO-K1 clones were obtained as described above. The number of copies of 3-OST-1 in the individual clones was determined by Southern analysis. Genomic DNA (10 \mu g) was digested with 40 units of EcoRI overnight at 37 °C, electrophoresed on a 0.7% (w/v) agarose gel, transferred to GeneScreen Plus (PerkinElmer Life Sciences), and probed with 3-OST-1 cDNA labeled with the Megaprime labeling kit (Amersham Pharmacia Biotech). Blots were hybridized in ExpressHyb solution (CLONTECH) containing 3-OST-1 probe (2 \times 10\textsuperscript{5} cpm/ml), followed by autoradiography. The cell clone with three copies of 3-OST-1 was expanded and frozen for further studies.

Mutant Screening—Wild-type CHO cells with three copies of 3-OST-1 were mutagenized with ethylmethane sulfonate as described in the literature (31) and frozen under liquid nitrogen. A portion of cells was thawed, propagated for 3 days, and labeled with both Alexa 594-FGF-2 and fluorescein-AT. The labeled cells were sorted, and FGF-2-positive and AT-negative cells were collected. Approximately 1 \times 10\textsuperscript{4} sorted cells were collected into 1 ml of complete F-12 Ham’s media then plated in T-75 flasks. Sorted cell populations were maintained in complete F-12 Ham’s medium for 1 week, then the cells were labeled and sorted again as described above. After five rounds of sorting, FGF-2-positive and AT-negative cells were single-cell-sorted into a 96-well plate. The single-cell clones were expanded and frozen for further analysis. The sorting profiles of CHO-K1 with three copies of 3-OST-1, mutant, and the 6-OST-1 correctant of the mutant were shown by dual-color fluorescence flow cytometric analysis (see Fig. 2).

HS Preparation and Analysis—The method was the same as one published previously (29).

cDNA Cloning and Expression of CHO 6-OST-1—Sequences coding for CHO 6-OST-1 were amplified from a CHO-K1/cDNA quick-clone library (CLONTECH). The reaction mixture contained 2 units of Pfu polymerase (Stratagene), 1 ng of cDNA, and 100 pmol of the primers. The sense primer has an added BglI site (5’-GGACATGCTGCGAGGACCATGGTTGAGCGCGCCAGCAAGTTC-3’) and the antisense primer has an added XbaI site (5’-GGTCTTAGACTACCCTTCAATGTTGGCCTC-3’). The 6-OST-1 primer sequences are derived from the human 6-OST-1 cDNA sequence (from residues 240 to 264) and to the complement of this sequence (from residues 1147 to 1172) as reported (32). After 30 thermal cycles (1 min of denaturation at 94 °C, 2 min of annealing at 55 °C, 3 min of extension at 72 °C), the amplification products were analyzed in 1% agarose gels and detected by ethidium bromide staining. The amplification products were excised from the gel and cleaned by Gel Extraction kit (Qiagen). The PCR product was treated with BglI and XbaI, ligated into XbaI- and BamHI-digested plnD/Hydrog phosph (CLONTECH), and transformed into Escherichia coli. Transfected cells from each transformant were used to prepare DNA. The PCR reactions were sequenced and found to be identical. plnD/Hydrog 6-OST-1 transplanted was transfected into the CHO mutant cells. Cells positive for AT and FGF-2 binding were sorted and subsequently single-cell-cloned into a 96-well plate. The single-cell clones were expanded and frozen for further analysis.

6-O-Sulfation of HS in Vitro—The standard reaction mixture contained 50 mM MES (pH 7.0), 1% (w/v) Triton X-100, 5 mM MgCl\textsubscript{2}, 2.5 mM MnCl\textsubscript{2}, 0.075 mg/ml protease chloride, 1.5 mg/ml bovine serum albumin, either metabolically labeled [35S]HS or non-radioactive HS chains, cold PAPS (0.5 mM) or [35S]PAPS (25 \mu M, 2 \times 10\textsuperscript{7} cpm), and 70 ng of purified baculovirus-expressed human 6-OST-1. The reaction was carried out at 37 °C for 1 h. After ethanol precipitation, the pellets were washed with 75% ethanol, dried briefly under vacuum, and dissolved in water for further analysis.

Separation of HS\textsuperscript{act} and HS\textsuperscript{act} by AT-affinity Chromatography—The procedure is identical to that described previously (29).

Disaccharide Analysis of HS—Heparinase I (EC 4.2.2.8), heparinase II (no EC number), and heparinase III (EC 4.2.2.7) were obtained from Seikagaku, and heparitinase IV was a gift from Dr. Yoshida, Seikagaku Corp., Tokyo. Heparinase I recognizes the sequences GlcNAc/NS-O-S-NS\textsuperscript{act}-NS\textsuperscript{act} and heparinase II (EC 4.2.2.7) was obtained from Pure Enzymes (heparinase III) and heparinase IV recognizes the sequences GlcNAc/NS\textsuperscript{act}-NS\textsuperscript{act}-NS\textsuperscript{act}. Heparinase IV was used to obtain the disaccharides. The reaction products and references can be found in Refs. 33 and 34. The digestion of HS\textsuperscript{act} was carried out in 100 \mu l of 40 mM ammonium acetate (pH 7.0) containing 3.5 mM CaCl\textsubscript{2} with 1 milliunit (mU) of heparitinase I or 1 mU of each heparitinase I, heparitinase II, heparitinase IV, and heparinase III.
rinase (heparitinase III). The digestion was incubated at 37 °C overnight unless otherwise indicated.

For low pH nitrous acid degradation, radiolabeled HS samples were mixed with 10 μg of bovine kidney HS (ICN) and digested (35).

Disaccharides were purified by Bio-Gel P2 chromatography and resolved by ion-pairing 30 × 0.78-cm stainless-steel columns with 0.5 mM ammonium bicarbonate. Bio-Gel P2 samples (200 μl) were mixed with dextran blue (5 μg) and phenol red (5 μg) and loaded onto the column. The samples were eluted at a flow rate of 4 ml/h with collection of 0.5-ml fractions. The desired fractions were dried under vacuum, individually or pooled, to remove ammonium bicarbonate.

\[ \Delta UA-GlcNS3S Disaccharide Structure Determination by Capillary IPRR-HPLC Coupled with Mass Spectrometry \]

It has been reported that heparin molecules exhibit a high affinity for a synthetic peptide (CRPKAKAKAKAKDQTK) mimicking a heparin-binding domain of heparin interacting protein (HIP) also show an extremely high affinity for AT (37). We expected that inclusion of this small peptide in the heparitinase digestion solution would protect 3-O-sulfated HS disaccharides eluted right before HIP peptide-protected, AT-binding HS oligosaccharides. However, in the presence of the HIP peptide, all the 3-O-sulfate-labeled disaccharides were degraded into disaccharides instead of oligosaccharides or tetrasaccharides. As a solution, we eluted our prep-PAP labeled DNA was purified by capillary gel electrophoresis and loaded on to the column. The samples were eluted at a flow rate of 4 ml/h with collection of 0.5-ml fractions. The desired fractions were dried under vacuum, individually or pooled, to remove ammonium bicarbonate.

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The samples were eluted at a flow rate of 4 ml/h with collection of 0.5-ml fractions. The desired fractions were dried under vacuum, individually or pooled, to remove ammonium bicarbonate.
HSact-defective Mutant Makes Less 6-O-Sulfate-containing Disaccharides—Because HS from the mutant has similar charge density to that of the wild-type, the decrease in AT binding in the mutant should correlate with a change in the structure of the HS chains.

We checked the synthesis of GAGs in the mutant by biosynthetic labeling studies with [35S]sulfate. The 3-OST-1-expressing wild-type and mutant cells produced the same amount of [35S]HS and contained ~70% HS and ~30% chondroitin sulfate (data not shown), which is consistent with the result shown in Fig. 4 (68% of HS in the mutant versus 66% of HS in the 3-OST-1-expressing wild-type). [35S]Sulfate-labeled HS chains from the 3-OST-1-expressing wild-type and mutant cells were then digested with a mixture of heparitinases. The resulting disaccharides (~93% of total [35S]sulfate counts) were separated on a Bio-Gel P2 column and then further resolved by IPRP-HPLC with appropriate internal standards (Table I). The mutant cells make decreased amounts of 6-O-sulfated disaccharides, including ΔUA-GlcNAc6S, ΔUA-GlcNS6S, and ΔUA2S-GlcNS6S (Table I).

HSmut-defective Mutant Does Not Contain a Point Mutation in 6-OST-1 Coding Region—Because Northern and RT-PCR analyses indicate that the mutant and wild-type have the same level of 6-OST-1 mRNA, this observation raises the possibility that the mutant might have point mutation(s) in the 6-OST-1 amino acid coding region that cause decreased 6-O-sulfotransferase activities. The coding regions of 6-OST-1 RT-PCR products from the mutant were double-strand-sequenced. No point mutation was observed compared with wild-type (data not shown).

HSmut-defective Mutant Has Decreased 6-O-Sulfotransferase Activities—The mutant cells are defective in AT binding (Fig. 2E). Disaccharide compositional studies indicate that the mutant makes less 6-O-sulfated residues (Table I); however, there was no change in either the level of mRNA expression nor the mutation of the coding sequence of 6-OST-1 (data not shown). These results suggest that the mutant might have a defect in 6-OST-1 activities. To test this hypothesis, sulfotransferase activity assays were conducted using HS from wild-type CHO cells, N-0-desulfated, re-N-sulfated heparin (CDSNS-heparin) and 6-O-desulfated heparin (a generous gift from Dr. Jeffrey Esko) as substrates and crude cell homogenates from the mutant and the wild-type, respectively. The results confirm the Northern analysis that CHO cells express three isoforms of 6-OST activities. To test this hypothesis, sulfotransferase activity assays were conducted using HS from wild-type CHO cells, N-0-desulfated, re-N-sulfated heparin (CDSNS-heparin) and 6-O-desulfated heparin (a generous gift from Dr. Jeffrey Esko) as substrates and crude cell homogenates from the mutant and the wild-type, respectively. The results confirm the Northern analysis that CHO cells express three isoforms of 6-OST activities. To test this hypothesis, sulfotransferase activity assays were conducted using HS from wild-type CHO cells, N-0-desulfated, re-N-sulfated heparin (CDSNS-heparin) and 6-O-desulfated heparin (a generous gift from Dr. Jeffrey Esko) as substrates and crude cell homogenates from the mutant and the wild-type, respectively. The results confirm the Northern analysis that CHO cells express three isoforms of 6-OST activities. To test this hypothesis, sulfotransferase activity assays were conducted using HS from wild-type CHO cells, N-0-desulfated, re-N-sulfated heparin (CDSNS-heparin) and 6-O-desulfated heparin (a generous gift from Dr. Jeffrey Esko) as substrates and crude cell homogenates from the mutant and the wild-type, respectively.
mutants. 2-O-Sulfotransferase activity was similar in mutant cells (118 ± 3 pmol/min/mg) and the wild-type cells (122 ± 2 pmol/min/mg) when CDSNS-heparin was used as substrate. However, 30–39% reduction of 6-O-sulfotransferase activities in the mutant was observed with all three substrates (Table II).

In Vivo 6-OST-1-corrected Mutant (Correctant) Makes HS, 50% of Which Is HSact—Decreased 6-OST-1 activity in the mutant might be responsible for its deficiency in AT binding. To test this idea, the CHO 6-OST-1 coding region was successfully amplified and sequenced from the CHO-K1 quick-clone cDNA library by PCR, because only partial 6-OST-1 coding sequence from CHO cells has been reported (32). The CHO 6-OST-1 sequence has been deposited in GenBank® (accession number AB006180; the differences in the amino acid residues from the previously reported partial sequence of the same cDNA (32) are commented upon). CHO 6-OST-1 cDNA was then expressed in the mutant. The stable transfectants were labeled with fluorescein-AT and Alexa 594-FGF-2 and subjected to dual-color FACS (“Experimental Procedures”).

In Vitro 6-OST-1 Sulfation Generates Three Kinds of 6-O-containing Disaccharides—To explain the difference between 6-OST-1 substrate specificity observed in vivo and the published data (2), we expressed and purified 6-OST-1 in bacteria and baculovirus. [35S]Sulfate metabolically labeled mutant HS chains were treated with baculovirus-expressed pure 3-OST-1, 6-OST-1, or both plus cold PAPS and AT-affinity purified. HSact was isolated and HSact% was quantitated (Table III). For the HS chain from mutant, 3-OST-1 modification alone increases HSact% from 7% to 12%, 6-OST-1 modification alone increases HSact% from 7% to 51%, both 3-OST-1 and 6-OST-1 modification increases HSact% from 7% to 64%. For the HS chain from wild-type with three copies of 3-OST-1 genes, 3-OST-1 modification alone increases HSact% from 26% to 40%, 6-OST-1 modification alone increases HSact% from 26% to 64%, both 3-OST-1 and 6-OST-1 modification increases HSact% from 26% to 70%. These results indicate both 3-OST-1 and 6-OST-1 are critical enzymes involved in HSact production. The yield of
HS^act by 6-OST-1 treatment of mutant HS chain is similar to that of the wild-type even though 6-O-sulfation is severely decreased in the mutant (Table I, Fig. 5). To locate where 6-OST-1 adds 6S residues along the HS chains, equal amounts of HS from 3-OST-1-expressing wild-type and mutant cells were in vitro labeled with purified baculovirus expressed FIG.3.

Structure determination by capillary IPRP-HPLC coupled with mass spectrometry. Cold HS-chain form wild-type CHO cells were labeled with 3-OST-1 plus PAP34S. Purified HS was digested with a combination of 1 mU of each heparitinase I, heparitinase II, heparitinase IV, and heparinase in the presence of 0.5 mg/ml heparin/heparan sulfate interacting protein (HIP) peptide. 0.5 µg of digested HS was injected into capillary IPRP-HPLC coupled with MS. A, UV tracer of capillary IPRP-HPLC from 35.85 to 39.71 min; peak B contains both ΔUA-GlcNS6S and ΔUA-GlcNS3S, and peak D contains ΔUA2S-GlcNS; B, negative polarity MS spectra from 37.44 to 38.17 min, which equals the UV peak from 36.64 to 37.37 min; C, amplification of the m/z 494.0–501.0 region from panel B; D, negative polarity MS spectra from 38.17 to 39.06 min, which equals the UV peak from 37.37 to 38.26 min; E, amplification of the m/z 494.0–501.0 region from panel D.

FIG.4. HPLC anion-exchange chromatography of GAGs. [3H]GlcN-labeled GAG chains from wild-type and mutant were isolated by protease digestion and β-elimination (see "Experimental Procedures"). Samples were analyzed by HPLC anion-exchange chromatography (see "Experimental Procedures"). Solid tracer, mutant; broken tracer, wild-type. The broken line indicates the concentration gradient of sodium chloride.
6-OST-1 and [35S]PAPS either for 20 min or overnight. Only ∼1/3 as much radioactivity was incorporated into wild-type HS as compared with the mutant. [35S]Sulfate-labeled HSs were isolated and digested with a mixture of heparitinases. The resulting disaccharides (∼94% of [35S]sulfate counts) were separated on a Bio-Gel P2 column and were then further resolved by IPRP-HPLC with appropriate internal standards. In duplicate experiments, the values varied by <±20% of those shown.

| Name          | Mutant | Wild-type | Correctant | %    |
|---------------|--------|-----------|------------|------|
| ΔUA-GlcNS     | 35     | 30        | 27         |      |
| ΔUA-GlcNac6S  | 5      | 7         | 9          |      |
| ΔUA-GlcNS6S   | 4      | 9         | 13         |      |
| ΔUA2S-GlcNS   | 36     | 20        | 22         |      |
| ΔUA2S-GlcNS6S | 19     | 34        | 29         |      |

Contributions of 6-OST-1 in Generating HSact Oligosaccharides—To further locate the 6-O-sulfate addition in AT-binding HSact oligosaccharides, cold mutant HS chains were treated with purified baculovirus-expressed 6-OST-1 with [35S]PAPS overnight. After heparitinase I digestion, HSact oligosaccharides were affinity-purified (7% of 6-O-[35S]sulfate-labeled HS[total]). The HSact oligosaccharides were then treated with low pH nitrous acid that cleaves N-sulfated residues, and a combination of heparitinases that cleaves 3-O-sulfate-containing sugar into tetrasaccharides and all other sugars into disaccharides. Treated and untreated HSact oligosaccharides were run on Bio-Gel P6 columns (Fig. 7). Di- and tetrasaccharides were collected from enzyme and low pH nitrous-treated samples as indicated. The tetrasaccharides resistant to a combination of heparitinases I and II and heparinase digestion represented the 3-O-sulfate-containing tetrasaccharides as reported earlier (20, 33). The presence of similar amounts of tetrasaccharides from both nitrous and enzyme degradation suggests the 3-O-containing tetrasaccharides have the structures, UA-2S-GlcNAc6S-GlcUA-GlcN3S3S-6S. To prove this, the tetrasaccharides (Fig. 8A) collected from enzyme digestion (Fig. 7C) were further digested into disaccharides (Fig. 8B) with heparitinase I in the presence of HIP peptide (the same method as shown in Fig. 3). IPRP-HPLC profiles of 6-O-sulfate-tagged HSact di- and tetrasaccharides from Fig. 7C were shown in Fig. 8. Table IV summarizes the 6-O-[35S]sulfate-labeled disaccharide compositions calculated based on the HPLC data (Fig. 8). In HSact oligosaccharides, 6-OST-1 adds 6-sulfates not only at GlcUA/IdoUA-GlcNAc, GlcUA-GlcNAc, and IdoUA2S-GlcNS, but also at GlcUA-GlcNS3S. These results show that 6-OST-1 is the enzyme that not only puts the critical 6-sulfate group in HSact oligosaccharides but also other 6-sulfate groups in HSact oligosaccharides as well. 3-OST-1 and 6-OST-1 are, therefore, the critical enzymes for the generation of HSact.

# DISCUSSION

A new approach for generating HSact biosynthetic mutants and a novel method for characterizing 6-O-sulfotransferase substrate specificity have been developed in this study. This approach includes placing multiple copies of a downstream enzyme into CHO cells and then mutagenizing these cells. Mutants defective in enzymes that are part of the anticoagulant HS biosynthetic pathway were then sorted by FACS. A mutant was then characterized both in vivo and in vitro to delineate enzymes involved in generating anticoagulant HS. The advantage of sorting is that it targets GAG synthesis selectively and very large populations of cells can be screened, which make it possible to detect specific mutations in effecting distinct HS structures. This technique may constitute a general approach for defining, obtaining, and characterizing the components of a biosynthetic pathway once the terminal biosynthetic enzyme is obtained and proteins that recognize the final product of the pathway are available.

It has been reported that a type of size exclusion chromatography coupled with mass spectrometry is effective for compositional analysis of chondroitin sulfate oligosaccharides. Mass spectrometric detection produces far more information than conventional UV or fluorescent detectors and allows the monosaccharide composition of individual components to be determined (39). Introducing the stable isotope PAP34S into the 3-O-position of HS by pure 3-OST-1 as described in Fig. 3, a 3-O-sulfate-containing disaccharide with a unique mass, which has not been reported before, was readily identified by a combination of capillary IPRP-HPLC coupled with mass spectrometry. The method developed in this paper consumes 0.5 μg of total HS for separating and detecting different HS disaccharides. It will serve as a practical way of accomplishing HS disaccharide analysis of general HS samples from cells or tissues without radioisotope labeling. Furthermore, we could treat biologically

### Table I

**Disaccharide composition of HS from mutant, wild-type, and 6-OST-1 correctant cells**

| Substrates       | 6-O-sulfotransferase activities | Wild-type | Mutant |%  |
|------------------|---------------------------------|-----------|--------|---|
| HS (CHO-K1)      | 5.8 ± 0.3                       | 3.9 ± 0.4 | 70%    |
| 6-O-Desulfated heparin | 4.4 ± 0.3                        | 2.7 ± 0.5 | 61%    |
| CDSNS-heparin  | 11 ± 2                          | 7 ± 1     | 62%    |

* Numbers in parentheses represent percentage of wild-type activity.

### Table II

**Decreased 6-OST-1 activities in HSact defective mutant**

Sulfotransferase activities were assayed using three different substrates and crude cell homogenates from wild-type and mutant CHO cells as a source of enzyme.

| Substrates       | 6-O-sulfotransferase activities | Wild-type | Mutant |%  |
|------------------|---------------------------------|-----------|--------|---|
| ΔUA-GlcNS        | 35                              | 30        | 27     |   |
| ΔUA-GlcNac6S     | 5                               | 7         | 9      |   |
| ΔUA-GlcNS6S      | 4                               | 9         | 13     |   |
| ΔUA2S-GlcNS      | 36                              | 20        | 22     |   |
| ΔUA2S-GlcNS6S    | 19                              | 34        | 29     |   |

### Table III

**6-OST-1 limits the anticoagulant HS generation**

Treated with baculovirus-expressed pure 3-OST-1, 6-OST-1, or both plus cold PAPS, the modified [35S]sulfate metabolic-labeled mutant and wild-type HS chains were AT-affinity purified. HSact was isolated and HS[act] was quantitated. In different experiments, the values varied by <±2% of those shown.

### Table IV

**Disaccharide composition of individual components to be determined**

| Control       | +3-OST-1 | +6-OST-1 | +3 & 6-OST-1 |%  |
|---------------|----------|----------|---------------|---|
| Mutant        | 24       | 40       | 64            |   |
| Wild-type     | 26       | 40       | 64            |   |

It has been reported that a type of size exclusion chromatography coupled with mass spectrometry is effective for compositional analysis of chondroitin sulfate oligosaccharides. Mass spectrometric detection produces far more information than conventional UV or fluorescent detectors and allows the monosaccharide composition of individual components to be determined (39). Introducing the stable isotope PAP34S into the 3-O-position of HS by pure 3-OST-1 as described in Fig. 3, a 3-O-sulfate-containing disaccharide with a unique mass, which has not been reported before, was readily identified by a combination of capillary IPRP-HPLC coupled with mass spectrometry.
inactive HS oligosaccharides in vitro with different pure sulfotransferases plus stable sulfur isotopes of PAPS (PAP$^{33}$S and PAP$^{34}$S). Labeled oligosaccharides should regain biological function. The different stable isotope-tagged, biologically active oligosaccharides could then be sequenced by a combination of capillary IPRP-HPLC for separation and mass spectrometry for detection. In this manner, biologically critical regions can be pinpointed and sequenced.

Because in vitro 3-O-sulfation can transform anticoagulant inactive HS chain into anticoagulant active HS chain, it has been concluded that 3-O-sulfation is the final modification step during HS biosynthesis. This study shows in vitro 6-O-sulfation can transform 3-O-sulfate-containing anticoagulant inactive HS chain into anticoagulant active HS chain as well. It shows that it is still unclear whether 6-O-sulfation occurs before or after 3-O-sulfation during biosynthesis.

Three 6-OST genes have been cloned and shown to be expressed in a tissue-specific pattern. Furthermore, the individual isozymes appear to differ in substrate specificity. We do not know how 6-OST-2 and 6-OST-3 contribute in HS$^{act}$ generation in vivo. However, by comparing baculovirus-expressed and purified human 6-OST-1, 6-OST-2, and 6-OST-3, we found...
6-OST-1 is the most potent enzyme in generating HS act by the in vitro assay we describe in this report. It has been shown that the critical 6- and 3-sulfates in HS act oligosaccharides function in a thermodynamically linked fashion and work as a pincer in terms of AT binding. Previously we showed that there were multiple 3-O-sulfation sites in F9 HS act and that 3-O-sulfates are either added to all the sites or none of the sites. In other words, 3-OST-1 works in a processive mode during biosynthesis by an unknown mechanism. Because CHO wild-type makes HS act precursor with the critical 6S in the absence of 3-OST-1 and the mutant makes HS act precursor with the critical 3S in the lower level of 6-OST-1, it suggests that 6-OST-1 and 3-OST-1 might not be physically coupled in a processive mode in making HS act during biosynthesis.

It has been observed that two different sulfated domains are present in HS, the NS domain, and NAc/NS domains (40, 41). The NS domains consist of contiguous iduronosyl N-sulfoglucosamine units, whereas the NAc/NS domain consists of alternating N-acetylated and N-sulfated disaccharides. From the acceptor specificities of 6-OST-1, 6-OST-2, and 6-OST-3 using N-sulfated heparosan and desulfated re-N-sulfated heparin as substrate, it has been suggested that sulfation of position 6 of the N-sulfoglucosamine residues in the NS domain may be catalyzed by 6-OST-1, whereas sulfation of position 6 of the N-sulfoglucosamine residues in the NA/NS domain may be catalyzed by 6-OST-2 and 6-OST-3 (2). Because there is no 6-OST-2 and 6-OST-3 in CHO cells, our results indicate that 6-OST-1 makes all 6-O-sulfated residues in CHO cells (Tables I and IV, Figs. 5 and 6). Furthermore, 30% reduction in 6-OST-1 activities in the mutant results in 3.7-fold reduction in HS act production, which suggests that in vivo 6-O-sulfation of GlcNAc residues might be very sensitive to the level of 6-OST-1 activity. Indeed, overexpression of 6-OST-1 in the mutant results in the greatest increase in GlcNAc6S-containing disaccharide (Fig. 5). In vitro 6-O-sulfation at different time intervals (Fig. 6) also demonstrated that IdoUA2S-GlcNS is the preferred substrate for 6-O-sulfation. The in vitro studies (Fig. 6) may explain why GlcNAc6S-containing disaccharides were not observed in previous publications (2, 32, 42).

The mutant characterized in this manuscript is defective in AT binding (Fig. 2E), because it has decreased 6-O-sulfotransferase activities (Table II) and makes decreased amounts of 6-O-sulfated residues (Table I). The defect in this mutant can be corrected by both in vivo and in vitro 6-O-sulfations (Tables III and IV, Figs. 5–7). All the results show that 6-O-sulfotransferase-1 represents a critical enzyme in the anticoagulant HS biosynthetic pathway in CHO cells. However, the cause of decreased 6-O-sulfotransferase activities in the mutant is unclear. Because 6-OST-1 enzyme functions in vitro, a defect effecting an auxiliary protein is unlikely. The possibility remains that a defect in either mRNA stabilization or transla-

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2 L. Zhang, D. L. Beeler, and R. D. Rosenberg, unpublished observation.

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**Fig. 7. Bio-Gel P6 fractionation of digested HS.** 6-O-[35S]sulfate-tagged [3H]HS from mutant were digested with 1 mU of heparitinase I for 1 h. HS act oligosaccharides were obtained by AT-affinity chromatography (see "Experimental Procedures"). HS act oligosaccharides were treated with low pH nitrous acid and then either NaBH₄-reduced or treated with heparitinase I, II, and heparinase was analyzed by Bio-Gel P6 chromatography ("Experimental Procedures"). The fractions indicated were pooled for further analysis. A, 6-O-[35S]sulfate-tagged mutant HS act oligosaccharides; B, 6-O-[35S]sulfate-tagged mutant HS act oligosaccharides treated with low pH nitrous acid and NaBH₄; C, 6-O-[35S]sulfate-tagged mutant HS act oligosaccharides digested with heparitinases. n = the number of monosaccharide units in each peak.
from Fig. 7. In vitro 6-O-sulfated and AT affinity-purified [3H]HS<sup>smt</sup> oligosaccharides were digested with a mixture of heparitinases. The resulting di- and tetrasaccharides were separated on a Bio-Gel P6 column (see Fig. 7C). A, tetrasaccharides collected from Fig. 7C, peak 1: ΔUA-GlcNAc<sup>635S</sup>-GlcUA-GlcNS<sup>3S</sup>, peak 2: ΔUA-GlcNAc<sup>635S</sup>-GlcUA-GlcNS<sup>635S</sup>6<sup>35S</sup>; B, disaccharides of the digested tetrasaccharides in the presence of HIP peptide; peak 1: ΔUA-GlcNAc<sup>635S</sup>, peak 2: ΔUA-GlcNS<sup>635S</sup>6<sup>35S</sup>; C, disaccharides collected from Fig. 7C, peak 1: ΔUA-GlcNS<sup>635S</sup>6<sup>35S</sup>, peak 2: ΔUA-GlcNS<sup>635S</sup>6<sup>35S</sup>. The broken line indicates the gradient of acetonitrile.

In Fig. 8, IPRP-HPLC of 6-O-sulfate-tagged HS<sup>smt</sup> di- and tetrasaccharides. 6-OST-1 and 6-OST-3 are not present in CHO cells, CHO wild-type cells and our 6-OST-1-defective mutant cells are ideal for studying the in vivo and in vitro substrate specificity for 6-OST-2 and 6-OST-3 in terms of AT-binding, FGF-FGFR-activating, and other biologically relevant sequence generations.

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**Table IV**

| Name                  | Percentage of total |
|-----------------------|---------------------|
| ΔUA-GlcNS<sup>635S</sup> | 6                   |
| ΔUA2S-GlcNS<sup>635S</sup> | 50                  |
| ΔUA-GlcNAc<sup>635S</sup>  | 29                  |
| ΔUA-GlcNS<sup>635S</sup>6<sup>35S</sup> | 15                  |

**FIG. 8.** IPRP-HPLC of 6-O-sulfate-tagged HS<sup>smt</sup> di- and tetrasaccharides.
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6-O-Sulfotransferase-1 Represents a Critical Enzyme in the Anticoagulant Heparan Sulfate Biosynthetic Pathway

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