Molecular Cloning and Expression of Mouse and Human cDNAs Encoding Heparan Sulfate \(\alpha\)-Glucosaminyl 3-O-Sulfotransferase*

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The cellular rate of anticoagulant heparan sulfate proteoglycan (HSPG\(\text{\textsuperscript{ACT}}\)) generation is determined by the level of a kinetically limiting microsomal activity. HSPG\(\text{\textsuperscript{ACT}}\) conversion activity, which is predominantly composed of the long sought heparan sulfate 3-glucosaminyl 3-O-sulfotransferase (3-OST) (Shwora, N. W., Fritze, L. M. S., Liu, J., Butler, L. D., and Rosenberg, R. D. (1996) J. Biol. Chem. 271, 27063–27071; Liu, J., Shwora, N. W., Fritze, L. M. S., Edelberg, J. M., and Rosenberg, R. D. (1996) J. Biol. Chem. 271, 27072–27082). Mouse 3-OST cDNAs were isolated by proteolyzing the purified enzyme with Lys-C, sequencing the resultant peptides as well as the existing amino terminus, employing degenerate polymerase chain reaction primers corresponding to the sequences of the peptides as well as the amino terminus to amplify a fragment from LTA cDNA, and utilizing the resultant probe to obtain full-length enzyme cDNAs from a \(\lambda\) Zap Express LTA cDNA library. Human 3-OST cDNAs were isolated by searching the expressed sequence tag data bank with the mouse sequence, identifying a partial-length human cDNA and utilizing the clone as a probe to isolate a full-length enzyme cDNA from a \(\lambda\) TriplEx human brain cDNA library. The expression of wild-type mouse 3-OST as well as protein A-tagged mouse enzyme by transient transfection of COS-7 cells and the expression of both wild-type mouse and human 3-OST by \textit{in vitro} transcription/translation demonstrate that the two cDNAs directly encode both HSPG\(\text{\textsuperscript{ACT}}\) conversion and 3-OST activities. The mouse 3-OST cDNAs exhibit three different size classes because of a 5' untranslated region of variable length, which results from the insertion of 0–1629 base pairs (bp) between residues 216 and 217; however, all cDNAs contain the same open reading frame of 933 bp. The length of the 3'-untranslated region ranges from 301 to 430 bp. The nucleic acid sequence of mouse and human 3-OST cDNAs are \(\approx 85\%\) similar, encoding novel 311- and 307-amino acid proteins of 35,876 and 35,750 daltons, respectively, that are 93\% similar. The encoded enzymes are predicted to be intraluminal Golgi residents, pre-suppressing their C-terminal regions with an integral membrane protein. Both 3-OST species exhibit five potential N-glycosylation sites, which account for the apparent discrepancy between the molecular masses of the encoded enzyme (\(\approx 34\) kDa) and the previously purified enzyme (\(\approx 46\) kDa). The two 3-OST species also exhibit \(\approx 50\%\) similarity with all previously identified forms of the heparan biosynthetic enzyme 3-deacetylase/N-sulfotransferase, which suggests that heparan biosynthetic enzymes share a common sulfotransferase domain.

The serine proteases of the intrinsic blood coagulation cascade are slowly neutralized by antithrombin (AT\(\text{\textsuperscript{1}}\)) (reviewed in Ref. 1). This inhibition is secondary to the generation of 1:1 enzyme-AT complexes whose formation is dramatically enhanced by the mast cell product, heparin (2). Damus et al. (3) hypothesized that endothelial cell surface heparan sulfate proteoglycans (HSPGs) function in a similar fashion to accelerate coagulation enzyme inactivation by AT and therefore are responsible for the nonthrombogenic properties of blood vessels. We initially demonstrated that perfusion of the hind limbs of normal rodents and rodents deficient in mast cells with purified thrombin and AT leads to a greatly elevated rate of thrombin-AT complex formation and that the enzyme heparitinase as well as the natural heparin antagonist platelet factor 4 suppress the above acceleration (4, 5). We subsequently showed that cultured cloned bovine macrovascular and rodent microvascular endothelial cells synthesize both anticoagulant HSPG (HSPG\(\text{\textsuperscript{ACT}}\)) and nonanticoagulant HSPG (HSPG\(\text{\textsuperscript{INACT}}\)) (6–8). HSPG\(\text{\textsuperscript{ACT}}\) bear glycosaminoglycan (GAG) chains that bind tightly to AT and accelerate thrombin-AT complex generation (6–8).

The biosynthesis of HSPG\(\text{\textsuperscript{ACT}}\) requires generation of a core protein; assembly of a linkage region of four neutral sugars on specific serine attachment sites of the core protein; elongation of a GAG backbone composed of alternating N-acetylglu-
cosamine and glucuronic acid residues; and modification of this homogeneous copolymer by partial N-deacetylation with coupled N-sulfation of glucosamine residues, partial epimerization of glucuronic acid to iduronic acid residues, partial 2-O-sulfation of uronic acid residues, and partial 6-O-sulfation and partial 3-O-sulfation of glucosamine residues (reviewed in Ref. 9). This multi-enzyme pathway generates HSPG\textsuperscript{act} with regions of defined structure that contain the primary AT binding domain sequence found in anticoagulant heparin: uronic acid → glucosamine (N-acetyl/N-sulfate) 6-O-sulfate → glucuronic acid → glucosamine N-sulfate 3-O-sulfate (6-O-sulfate) → iduronic acid 2-O-sulfate → glucosamine N-sulfate 6-O-sulfate (10–17). These reactions also produce HSPG\textsuperscript{inact} with regions of varying monosaccharide sequence that lack the primary AT-binding domain. The structure-function relationships of the AT binding domain have been elucidated with heparin/heparan sulfate oligosaccharides in association with fast reaction kinetics and equilibrium binding assays. The 6-O-sulfate group on residue 2 and the 3-O-sulfate group on residue 4 function in a thermodynamically linked fashion to supply half of the binding energy for interaction with AT and trigger a conformational event that accelerates neutralization of specific coagulation proteases (11, 12). The amino and ester sulfate groups at residues 5 and 6 as well as carboxyl groups at other sites provide the other half of the binding energy for interaction with protease inhibitor (10, 11). Furthermore, monosaccharide sequences outside the primary AT binding domain are essential in facilitating inhibition of coagulation proteases other than factor Xa (18, 19).

During the past 8 years, several biosynthetic enzymes that generate HSPG\textsuperscript{act} and HSPG\textsuperscript{inact} have been purified. These proteins include the N-acetylgalactosamine/glucuronic acid co-polymerase (20), the N-deacetylase/N-sulfotransferase (NST) (21, 22), the glucuronic acid/iduronic acid epimerase (23), the iduronic acid/glucuronic acid 2-O-sulfotransferase (24), the glucosamine 6-O-sulfotransferase (25), and the glucosamine 3-O-sulfotransferase (3-OST) (26). However, the only enzymes that have also been molecularly cloned are two structurally and functionally distinct isoforms of the N-deacetylase/N-sulfotransferase (NST-1 from liver and NST-2 from mastocytoma) (27–31). The heparan biosynthetic enzymes must function in a coordinated manner to produce the AT binding domain, because the abundance of this sequence is much greater than predicted from a random assembly of constituents (32). The postulated regulatory mechanism must direct the biosynthetic enzymes to carry out the appropriate sequence of epimerization/sulfation reactions to generate the AT binding domain (33, 34).

We have previously described a soluble cell-free system to investigate HS\textsuperscript{act} generation and developed assays for defining critical enzymatic components (35). The investigations employing the above approach define a limiting HS\textsuperscript{act} conversion activity that acts upon an excess precursor population to regulate cellular HSPG\textsuperscript{act} biosynthesis (35). The major component of the limiting HS\textsuperscript{act} conversion activity proved to be the long sought 3-OST, as documented by purification and characterization of this protein (26). The investigations utilizing the above technique also showed that HS\textsuperscript{act} precursor (35% of the HS\textsuperscript{inact} pool) is 3-O-sulfated to generate HSPG\textsuperscript{act} and that HS\textsuperscript{inact} precursor (65%) is 3-O-sulfated to produce HSPG\textsuperscript{inact}. However, only a small fraction of either substrate is modified, with the remaining precursor population exiting the Golgi apparatus to appear on the cell surface. Thus, 3-OST constitutes a rate-limiting enzymatic activity that defines the level of cellular HSPG\textsuperscript{act} generation. In contrast, the level of 3-OST activity does not appear to limit the mast cell formation of anticoagulant heparin, which contains a high proportion of molecules with the AT binding site (–30%) (36); structural and biochemical analyses indicate that the precursor of anticoagulant heparin is present in minimal amounts (37, 38).

Despite this biosynthetic difference, for both heparin and heparan, 3-O-sulfation does not guarantee the formation of anticoagulant GAG (33, 34, 39, 40). This phenomenon may be secondary to alterations in the relative concentrations of two functional forms of 3-OST that differentially act upon HS\textsuperscript{act} precursor versus HS\textsuperscript{inact} precursor or changes in the relative levels of HS\textsuperscript{act} precursor versus the HS\textsuperscript{inact} precursor. The two functional forms of 3-OST may be due to two discrete gene products, posttranslational modification of a single gene product, or the presence of a regulatory factor that directs the enzyme to modify one or the other precursor. The relative levels of HS\textsuperscript{act} precursor versus HS\textsuperscript{inact} precursor are presumably controlled by earlier biosynthetic enzymes. In the current paper, we molecularly clone as well as express murine and human 3-OST, and we show that the expressed enzyme is able to 3-O-sulfate both HS\textsuperscript{act} precursor and HS\textsuperscript{inact} precursor. Furthermore, the deduced structure of 3-OST, when compared with NST, defines a heparan sulfate sulfotransferase domain and also suggests a novel mechanism for limiting the action of the enzyme.

**EXPERIMENTAL PROCEDURES**

**Cell Lines and Cell Culture**

We have previously described the clonal L cell line LTA (35, 41), the generation of clone 33, an LTA transfectant that overexpresses the ryudcan12CA5 cDNA (33), a rapidly growing revertant of clone 33, L-33 (26), and RPPEC, an immortalized line derived from rat fat pad endothelial cells (8). Primary mouse neonatal endothelial cells from the cardiac microvasculature of day 3–5 neonates (CMVEC cells) were a generous gift from Dr. Jay Edberg (MIT/Beth Israel Hospital), whereas COS-7 cells were obtained from the ATCC. Primary human umbilical vein endothelial cells (HUVEC) were maintained according to the supplier’s (Clonetics Corp., San Diego, CA) protocol. Unless otherwise stated, all cell lines were maintained in logarithmic growth by subculturing biweekly in Dulbecco’s modified Eagle’s medium (Life Technologies, Inc.) containing 10% fetal bovine serum, 100 units/ml penicillin, and 100 units/ml streptomycin, and 100 ng/ml basic fibroblast growth factor, and also suggests a novel mechanism for limiting the action of the enzyme.

**Peptide Purification and Sequencing**

The purification of mouse 3-OST from t-33* has been previously described (26), and the final step 4 product was concentrated by reverse phase chromatography on an HP 1090 M system (Hewlett Packard) equipped with a C4 reverse phase HPLC column (250 × 2.1 mm, 300-Å pore size, 5-μm particle size) (Vydac, catalog number 214TP52) equilibrated in 60% acetonitrile, 0.1% trifluoroacetic acid (v/v). After application of the sample, the reverse phase matrix was washed with 0% acetonitrile, 0.1% trifluoroacetic acid, and bound species were eluted with 78.4% acetonitrile, 0.1% trifluoroacetic acid (v/v). After application of the sample, the reverse phase matrix was washed with 60% acetonitrile, 0.1% trifluoroacetic acid, and bound species were eluted with 78.4% acetonitrile, 0.1% trifluoroacetic acid. Samples of 1.5 or 3 μg from two independent purifications, were digested with 0.15 or 0.3 μg, respectively, of endopeptidase Lys-C (Waco) in a reaction volume of 100 μl containing 1% RTX100 (Calbiochem), 10% acetonitrile, and 100 mM Tris-HCl, pH 8.0, at 37 °C for 16 h (43). Digestion products were chromatographed on a HP 1090 M system (Hewlett Packard) equipped with the above described C4 reverse phase HPLC column equilibrated in 98% buffer A (0.1% trifluoroacetic acid (v/v)), 2% buffer B (80% acetonitrile (v/v), 0.85% trifluoroacetic acid (v/v)). After application of digestion products, the reverse phase matrix was washed with 98% buffer A, 2% buffer B, and bound species were eluted with linear gradients of buffer B increasing to 37.5% over 60 min, to 75% over 30 min, and to 98% over 15 min (44). The eluate was monitored for absorbance at 210 and 280 nm, and peptide peaks were individually collected and analyzed with a model 477A/120A Protein Sequenator (Applied Biosystems). In addition, the NH₂-terminal sequence of 1 μg of concentrated 3-OST sample was directly determined.
Isolation of Mouse 3-OST Clones

Isolation of Cytoplasmic and Poly(A)+ RNA—Cytoplasmic RNA (17.5 mg) was isolated from postconfluent cultures of LTA cells (12 flasks of 175 cm², ~1.6 x 10⁸ cells) by a modification of the procedure of Favaloro et al. (45). Monolayers were twice washed with PBS, cells were recovered by trypsinization and centrifugation (1000 x g for 2 min), and cell pellets were washed by resuspension in PBS followed by centrifugation (1300 x g for 4 min). Cells were lysed by vortexing for 30 s in 12 ml of ice-cold 0.1 M Tris-HCl, pH 7.4, 140 mM NaCl, 1% Triton X-100, 5 mM vanadyl ribonucleoside complexes (Life Technologies); samples were incubated on ice for 10 min and then vortexed for 1 min. Nuclei were pelleted by centrifugation at 6000 x g for 10 min, the supernatant was mixed with an equal volume of 200 mM Tris, pH 7.4, 300 mM NaCl, 2% SDS, 25 mM EDTA, containing 200 µg/ml proteinase K (Boehringer Mannheim), and the mixture was incubated at 65 °C for 2 h. Samples were extracted twice against an equal volume of phenol/chloroform/isooamy alcohol (25:24:1), the aqueous phase was combined with 0.7 volumes of isopropl alcohol, cytoplasmic RNA was pelleted by centrifugation at 3500 x g for 10 min, and RNA was resuspended in 3.6 ml of 10 mM Tris, pH 7.4, 1 mM EDTA. Poly(A)+ RNA (59 µg) was isolated from 16 mg of cytoplasmic RNA by two sequential purifications against 100 mg of oligo(dT)-cellulose (Life Technologies, catalog number 15839-010) according to the manufacturer’s specifications except that binding buffers contained 0.1% SDS, and LiCl was substituted for NaCl. The final eluate (1.5 ml) was extracted against 1.5 ml of phenol/chloroform/isooamy alcohol (25:24:1), the aqueous phase was then adjusted to 100 mM LiCl and 260 mM NaCl, an equal volume of isopropl alcohol was added, the mixture was centrifuged at 15,000 x g for 30 min, and the poly(A)+ RNA pellet was recovered in 40 µl of diethyl pyrocarbonate-treated water.

Construction and Generation of a Mouse 3-OST Probe—Degenerate PCR primers (Fig. 1) were obtained from Biosynthesis. First strand cDNA was generated in a 50-µl volume from 5 µg of LTA poly(A)+ RNA primed with oligo(dT) using a reverse transcriptase-PCR kit (Stratagene) according to the manufacturer’s specifications. Touchdown PCR (46, 47) reactions (50 µl) contained 1 µl of first strand cDNA, 25 pmol of each primer, 0.25 µl of AmpliTaq Gold (Perkin-Elmer), a 200 µM concentration of each dNTP, and 1 X GeneAmp PCR buffer. Two distinct sets of touchdown PCR conditions were required to obtain optimal yields of product. For amplification with primers 1S and 2A, reactions were heated to 95 °C for 9 min, subjected to 20 cycles of 94 °C for 30 s and 68 °C for 1 min with a 0.5 °C reduction per cycle, followed by 20 cycles of 94 °C for 30 s and 58 °C for 30 s with a 0.5 °C reduction per cycle and 75 °C for 30 s, and then 15 cycles of 94 °C for 30 s, 55 °C for 10 s, and ramping to 75 °C over 50 s. Alternatively, for amplification with primers 1S or 3A (or primers 2S and 3A), reactions were 20 min at 95 °C for 4 min, subjected to 47 cycles of 95 °C for 30 s and 69.5 °C for 2 min with 0.2 °C and 1 s reductions per cycle, followed by 25 cycles of 95 °C for 30 s, 60 °C for 15 s, and ramping to 75 °C over 1 min. Amplification products were purified as the retentate from centrifugal ultrafiltration against a 30,000 molecular weight cut-off membrane (Millipore, catalog number SK1P343JO), and then 200 ng of DNA was end-polished with DNA polymerase and subcloned into pCR-Script Amp SK (+)(Stratagene, catalog number 211188) according to the manufacturer’s specifications. A resulting plasmid, pNWS182, contained the 1S/3A amplification product of 779 bp, which was released by plaque-amplification by infection into Escherichia coli (end-filled with T 4 polymerase)/ Sac I insert of clone 220372 (accession numbers N90856 and W16555), and then adjusted to 100 mM LiCl and 260 mM NaCl, an equal volume of isopropl alcohol was added, the mixture was centrifuged at 15,000 x g for 30 min, and the poly(A)+ RNA pellet was recovered in 40 µl of diethyl pyrocarbonate-treated water.

Expression of 3-OST cDNAs

Construction of Expression Plasmids—The plasmid pCMV-3-OST contains the mouse 3-OST cDNA, an EcoRI/XhoI fragment from pNWS226 (Fig. 2), inserted between the CMV promoter and the bovine growth hormone polyadenylation signal of EcoRI/XhoI-digested and phosphatase-treated pcDNA3 (Invitrogen). The plasmid pCMV-ProA-3-OST was generated by ligating a BamHI/SmaI fragment, containing the protein A region from pRFK10PROTA (49), and an XhoI (end-filled with T 4 polymerase/XhoI fragment, containing most of the mouse 3-OST cDNA from pNWS226, into BamHI/XhoI-digested and phosphatase-treated pcDNA3 (Invitrogen). The in vitro transcription plasmid, pNWS237, contains a T3 promoter site 5′ of the human 3-OST cDNA and was constructed by inserting the complementary oligonucleotides 5′-AAATTATTAACCCTCACTAAAGGGAAG and 5′-AAATTCTCCCTTATGAGGAGTTAAT (Biosynthesis) into the EcoRI site of the TriplEx expression plasmid, pAL-2.

Transient Expression of the Mouse 3-OST cDNA in COS-7 Cells—For each expression construct, three 175-cm² flasks were seeded with 3.6 x 10⁶ COS-7 cells, 6 h later the medium was exchanged with DMEM containing 10% Nu-Serum (Life Technologies) with 100 µg/ml streptomycin and 100 units/ml penicillin, and cells were grown for an additional day. Monolayers were washed with PBS and then incubated at 37 °C for 2.5 h with 10 ml/flask of freshly prepared DMEM containing 235 µg/ml DEAE-dextran (molecular weight 500,000; Pharmacia Biotech Inc.), 9.5 mM Tris-HCl, pH 7.4, 0.9 mM chloroquine diphosphate (Sigma), and 3 µg/ml of the appropriate pcDNA3-based expression plasmid. Monolayers were then exposed to freshly prepared 10% MsSO in PBS for 1.5 min, washed twice with non-supplemented DMEM, and fed 30 ml/flask of DMEM containing 10% fetal bovine serum, 100 µg/ml streptomycin, and 100 units/ml penicillin; and cells were grown for an additional day. Monolayers were washed with PBS, and then cells were grown in 40 ml/flask of serum-free medium (DMEM containing 25 mg HEPES, pH 8.0, 1.0% Nutridoma SP (Boehringer Mannheim) (v/v), an additional 2 mM glutamine, 10 ng/ml biotin (Pierce), 100 µg/ml streptomycin, 100 units/ml penicillin, and 1 X a previously described Trace Metal Mix (26)) for 24 h. COS cells conditioned medium was harvested, debris was removed by centrifugation at 1,000 x g for 10 min followed by filtration through a 0.45-µm membrane, and then samples were either immediately processed or were snap frozen with liquid nitrogen and stored at −80 °C. Occasionally, conditioned medium from a second incubation of 8–24 h was also collected.

Purification of Wild-type and Protein A-tagged Mouse Recombiantly
Described previously (26). The fractions containing HS act conversion activity were pooled (approximately 4 ml), aliquoted, frozen in liquid nitrogen, and stored at −80 °C. Protein A-tagged mouse r3-OST was purified, at 4 °C, from 155 ml of previously frozen serum-free medium conditioned by COS-7 cells transfected with pCMV-3-OST. IgG-agarose beads (310 μl) of a 50/50 slurry) were gently stirred with the conditioned medium for 3 h, recovered by centrifugation at 2,000 × g for 10 min, and washed twice with 1 ml of MCG containing 250 mM NaCl to remove nonspecifically bound protein. Protein A fusion protein was eluted from the beads with two sequential 30-min incubations in 100 μl of 50 mM sodium acetate, pH 4.5, 150 mM NaCl, 0.6% CHAPS, and 1% glycerol. The pooled eluates were combined with an equal volume of 2% glycerol, and the protein concentration was adjusted to pH 8.0, mixed with an equal volume of 2% glycerol, and then aliquoted, frozen in liquid nitrogen, and stored at −80 °C.

Cell-free Synthesis of Mouse and Human r3-OST—Synthetic capped mouse and human 3-OST mRNAs were generated from NotI-linearized pNWS225 (Fig. 2) and HinDIII-linearized pNWS237, respectively, using T7 polymerase and m7G(5′-pNWS228) (Fig. 2) and m7G(5′-pNWS228) (Fig. 2), as described previously (50). Unlabeled in vitro translation reactions (25 μl) contained 0.25 μg of synthetic mRNA, 1.8 μl of canine pancreatic microsomal membranes (Promega), and 0.5 μl each of amino acid mixture minus leucine and amino acid mixture minus methionine and were performed with nucleate-treated reticulocyte lysate (Promega), according to the manufacturer's specifications.

Expression of 3-OST Enzyme (r3-OST)—Wild-type mouse r3-OST was purified, at 4 °C, from 240 ml of freshly generated serum-free medium conditioned by COS-7 cells transfected with pCMV-3-OST. The medium was adjusted to pH 8.0, mixed with an equal volume of 2% glycerol, and then loaded (25 ml/h) onto a heparin-AF Toyopearl-650M column (0.8 cm × 30 cm) (TosoHaas, Montgomeryville, PA) equilibrated in 50 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1% glycerol (v/v) (buffer C). The column was washed with 20 ml of buffer C at a flow rate of 0.8 ml/min and then with 20 ml of 150 mM NaCl, 10 mM Tris-HCl, pH 8.0, 1% glycerol (v/v) at a flow rate of 0.5 ml/min, and protein was eluted at a flow rate of 0.25 ml/min with a 20-ml linear NaCl gradient extending from 150 mM NaCl to 750 mM NaCl in buffer C. The fractions exhibiting HS act conversion activity (approximately 4 ml) were pooled, brought to a final concentration of 0.6% CHAPS (w/v) (Sigma) and dialyzed for 16 h against 4 liters of 25 mM MOPS (3-[N-morpholino]propanesulfonic acid) (Sigma), pH 7.0, 1% glycerol (v/v), 0.6% CHAPS (w/v) (MCG buffer) containing 50 mM NaCl. The dialysate was applied to a 3.5′-ADP-agarose column (0.8 × 1.2 cm, 3.7 mmol of 3′-ADP/ml of gel) (Sigma) and eluted as described previously (26). The fractions containing HSact conversion activity were pooled (approximately 4 ml), aliquoted, frozen in liquid nitrogen, and stored at −80 °C.

Identification of Enzymatic Reaction Products

32P Labeling of HS by r3-OST—32P-Labeled HS was generated by incubating the various forms of r3-OST with [32P]PAPS and unlabeled HS act, which were prepared as described previously (26, 35). Wild-type and protein A-tagged r3-OST (2500 units of HS act conversion activity) purified from COS cell-conditioned medium, were incubated in a 500-μl reaction mixture, as described previously (26), for 2 h at 37 °C, and 32P-labeled polysaccharides were purified by DEAE-Sepharose chromatography as described previously (26). For cell-free synthesized r3-OST, 32P labeling of the HS act was performed in a reticulocyte lysate-based reaction mixture (35) except that 330 μl reactions contained 100–300 units of in vitro translated r3-OST, 180 μl unlabeled HSact, 5 μl PAPS (60 × 104 cpm), and samples were incubated at 37 °C for 2 h. The reaction was quenched by the addition of 300 μl of 267 mM NaCl, 13.3 μg/ml glycogen and extraction against 600 μl of phenol/chloroform/isoamyl alcohol (25: 24:1). 32P-Labeled GAGs were ethanol-precipitated (35) and then isolated by DEAE chromatography as described previously (26). Size categorization of the HS act and HSact—The DEAE eluates containing 32P-labeled polysaccharides were vacuum-concentrated to 1/5 volume and then desalted at a flow rate of 0.9 ml/min on TSK G3000 PWXL (0.78 × 30 cm) and TSK G2500 PWXL (0.78 × 30 cm) (TosHaas) columns connected in series equilibrated in 0.1 M ammonium bicarbonate. The desalted product was then affinity-purified using AT/toncanavalin A gel to obtain HSact and HSact as described previously (26). Analysis of labeled products by treatment with GAG lyases and low pH nitric acid were performed as described previously (42). In addition, the HS act and HSact samples were each subjected to hydrazinolysis, high pH nitrous acid (pH 5.5), low pH nitrous acid (pH 1.5), and sodium borohydride reduction with the resultant disaccharides characterized on reverse phase ion pairing HPLC (RPIP-HPLC) as previously reported (33, 34). The identification of [35S]GlcA—AMN-3-O-SO3 and [35S]GlcA—AMN-3,6-O-(SO3)2 was confirmed by co-chromatography on RPIP-HPLC with the appropriate H-labeled disaccharide standards, as described in prior publications (33, 34).

Northern Blot Analysis

Total RNA from RFPEC and primary mouse CME cells was isolated by the method of Chomczynski and Sacchi (51), whereas poly(A)+ RNA was isolated from HUVEC cells as described above for LTA cells. Total RNA from the mast cell line CLMC/C57.1 (C57.1) (52) was a generous gift from Dr. Stephen J. Galli (Beth Israel Hospital). Samples were resolved on 1.2% formaldehyde-agarose gels and subjected to Northern blot analysis as described previously (50). Mouse and human samples were hybridized with mouse or human probes, respectively, and washed as described for library screening, above, except hybridizations were performed at 60 °C.

RESULTS

Peptide Sequencing and PCR Generation of a Mouse 3-O-Sulfotransferase (3-OST) Probe—The information necessary for the molecular cloning of mouse heparan sulfate d-gluocosaminy1 3-O-sulfotransferase (3-OST) was obtained by sequencing the amino terminus and Lys-C-generated peptides of the enzyme that we had previously purified from large quantities of serum-free tissue culture medium conditioned by an L cell cell line (26). These studies established the structures of 14 partially overlapping peptides which encompass 185 amino acid residues (see Fig. 3). Degenerate PCR primers were synthesized based on the sequence of the amino terminus (primer 1S) and two endopeptidase-derived fragments (primers 2S, 2A, and 3A) (Fig. 1A). When PCR was performed on an LTA first strand cDNA template, products of about 210 (primers 1S/2A), 780 (primers 1S/3A), and 610 (primers 2S/3A) bp were obtained (Fig. 1B), which suggests that all of the primer sites are contained within a single cDNA (Fig. 1C). To confirm this supposition, the two largest fragments were cloned into pCR-Script Amp SK(+) and inserts were sequenced, which revealed that the 1S/3A product is 779 bp and contains the 611-bp 2S/3A product. The 779-bp insert encodes 12 of the sequenced peptide fragments and so was 32P-labeled, as described under “Experimental Procedures,” and used as a probe for cDNA library screening.

Isolation and Characterization of Mouse 3-OST cDNAs—We constructed an amplified λ Zap Express LTA cDNA library of 1.5 × 109 primary recombinants and screened 1.3 × 109 plaques with the above described probe, which revealed 40 positives that were plaque-purified and in vitro excised into plasmids. The cDNA inserts of each plasmid were characterized to eliminate duplicated recombinants due to library amplification. Size was determined by liberating cDNA inserts with digestion at flanking EcoRI and XhoI restriction sites followed by agarose gel electrophoresis; furthermore, the sequence at both ends of each insert was obtained from flanking vector primers. This analysis revealed 25 unique primary recombinants, which predominantly contained inserts of approximately 1.7, 2.3, or 3.3 kb. These different species were considered to reflect natural size variants of the mouse message, since Northern blots of LTA poly(A)+ RNA hybridized with the 3-OST probe revealed the same three size categories of message (data not shown). The complete sequencing of 9 distinct primary recombinants, at least 2 from each size category, in conjunction with the partial sequencing of the remaining 16 clones showed that the size variants result from differences in the length of 5′-untranslated region due to the insertion of 0–1629 bp at a single common internal point, the splice variant
site. Most importantly, all clones shared identical protein coding regions, so for the sake of simplicity we present the characterization and analysis of the shortest species, the class 1 cDNA, which lacks additional sequence at the splice variant site.

Sequence data was obtained from two essentially full-length class 1 cDNAs and five partial-length cDNAs (Fig. 2A) to create a composite cDNA structure (Fig. 3) of 1685 bp, excluding the 3'-poly(A) tract. The 5'-untranslated region is 322 bp, with the splice variant site occurring between residues 216 and 217. This region contains six ATG sites that do not conform to consensus initiation sites (53) and are followed by near in-frame terminations. For all four human cDNA clones examined, only a single polyadenylation site was observed, resulting in a 3'-limit for the mouse 3-OST mRNA also exhibiting 5'-limit they are identical (both have ↓TAATTG), which raises the possibility that human 3-OST mRNA may also exhibit 5' splice variants. The first consensus ATG (with a purine occurs at –3) (53). The length of the 3'-untranslated region from all of the cDNA clones analyzed ranged from 301 to 430 bp (Fig. 2A, and data not shown). Within this terminal 129 bp, five distinct polyadenylation sites were observed, and 13–18 bp upstream from each site is a variant of the consensus polyadenylation signal (53) (Fig. 3A). Poly(A) tails were most frequently observed at the first site (position 1556; ~50% of clones).

**Isolation and Characterization of Human 3-OST cDNAs**—Three clones containing partial-length human 3-OST cDNAs were identified by expressed sequence tag data base searching (48) and were obtained from the TIGR/ATCC Special cDNAs. Three clones containing partial-length human 3-OST cDNAs—

**Predicted Protein Structures of Mouse and Human 3-OST**—

The mouse and human cDNAs encode novel 311- and 307-amino acid proteins of 35,876 and 35,750 daltons, respectively (Figs. 3 and 4), that exhibit 93% similarity. The deduced mouse and human cDNAs flanking the splice variant site on the 5'-limit are distinct (mouse, TTTAAG ↓; human, GCTCAG ↓), but on the 3'-limit they are identical (both have ↓TAATTG), which raises the possibility that human 3-OST mRNA may also exhibit 5' splice variants. The first consensus ATG (with a purine occurring at –3 and a guanosine at +4) (53) initiates an open reading frame of 921 bp. For all four human cDNA clones examined, only a single polyadenylation site was observed, resulting in a 3'-untranslated region of 266 bp, which is 26 bp less than the most frequently observed 3'-limit for the mouse cDNAs.

This analysis revealed nine distinct classes of 3-OST cDNA, which differ solely by the exact sequence present at the splice variant site. Structural analysis of mouse 3-OST genomic clones demonstrated that the nine distinct classes result from alternate splicing, which is limited to the 5'-untranslated region (N. W. Shworak, J. Liu, L. M. S. Fritz, and R. D. Rosenberg, manuscript in preparation). The functional significance of these splice variants is under investigation.

**Fig. 1. PCR isolation of a mouse 3-OST probe from LTA cDNA.** A, peptides 1, 2, and 3 are sequences that were most favorable for the design of degenerate PCR primers (displayed), and are internal regions derived from sequencing the mouse 3-OST amino terminus and Lys-C products F2–55/F1–63 and F2–4 (Fig. 3), respectively. Primers 1S and 2S are of sense orientation, whereas primers 2A and 3A are of antisense orientation. The underlined Gly and Asp of peptides 2 and 3 differ from the respectively encoded cDNA residues of Pro and Trp (Fig. 3), which exhibit low molar recoveries on peptide sequencing. B, resolution of PCR products by 1.2% agarose gel electrophoresis with ethidium bromide staining; the positions of relevant DNA size markers are indicated. First strand cDNA from LTA cells was amplified with the indicated degenerate primers as described under "Experimental Procedures." Amplification with the 1S/2A primers was independently analyzed, since distinct conditions were required to generate a PCR product. Unconsumed primers from the 1S/2A reaction are visualized as a lower band. C, deduced arrangement of primer sites on the mouse cDNA based on PCR results (B) and confirmed by sequence analysis (Fig. 3).
not flanked by cationic residues. Thus, the above stretch of 18 residues constitutes a hydrophobic leader signal, and this region is followed by a signal peptidase cleavage site between amino acids 20 and 21, as determined by the method of von Heijne (54). The possibility of signal peptidase cleavage is supported by the amino-terminal analysis of mouse 3-OST, which began with His21 (Fig. 3). Given that heparan biosynthesis is considered to occur in the \textit{trans}-Golgi, the above data suggest that the 3-OST is an intraluminal enzyme. Just past the signal peptidase cleavage site, the mouse 3-OST contains an extra 4 residues (Ala24-Pro25-Gly26-Pro27) not found in the human form. Both 3-OST proteins exhibit five potential \textit{N}\textsubscript{-}glycosylation sites, which account for the apparent discrepancy between the molecular masses of the predicted amino terminus-trimmed enzyme (\textasciitilde 34 kDa) and our previously purified enzyme (a broad band of 46 kDa was observed on SDS-polyacrylamide gel electrophoresis) (26). Only two cysteine residues are present, and these closely spaced residues are likely to

\textit{FIG. 2. Sequencing strategy for mouse and human 3-OST cDNA clones.} A schematic representation is shown of mouse class 1 (A) and human (B) 3-OST cDNA inserts contained within the indicated plasmids. The black box indicates the protein coding regions. The arrows summarize the length and direction of sequence obtained from various cDNA clones. Indicated are sequences obtained for essentially full-length clones, pNWS228 and pJL30, from vector primer sites (plain arrows) and 3-OST-specific synthetic oligonucleotides (closed circles). Also indicated are sequences of partial-length cDNAs obtained from vector primer sites (closed squares) and 3-OST-specific synthetic oligonucleotides (open squares). The location of the mouse PCR probes used for library screening is shown, whereas the human library was screened with the insert of clone 220372. The closed triangle indicates the location of the splice variant site as described under "Results."
form a disulfide bond that generates a peptide loop of 10 amino acids. Interestingly, the carboxyl-terminal 140-residue region is extremely basic (25% His, Lys, Arg; 12% Glu, Asp); however, this region does not exhibit previously recognized heparin binding motifs.

**Expression of Mouse and Human r3-OST—**Three distinct expression approaches were employed to confirm that the isolated cDNAs encode 3-OST enzyme. The resulting recombinantly expressed 3-OST enzyme was designated as r3-OST to distinguish this form from our previously purified native 3-OST enzyme. First, the vector pCMV-3-OST (a pcDNA3 derivative in which the CMV promoter transcribes the mouse 3-OST cDNA) was transiently expressed in COS-7 cells, and the resulting level of HSact conversion activity accumulated in serum-free medium over 32 h was measured, as described under “Experimental Procedures.” HSact conversion activity is a 3-OST-catalyzed reaction that requires unlabeled PAPS to convert [35S]HSinact into [35S]HSact. Before or after pcDNA3 transfection, typically COS-7-conditioned serum-free medium contained a low but detectable amount of HSact conversion activity, whereas transfection by pCMV-3-OST elevated levels 2,000-fold (Table I).

Second, to exclude the remote possibility that the expression of the mouse 3-OST cDNA indirectly induces, rather than directly encodes, HSact conversion activity, we analyzed a protein A/3-OST fusion protein. COS-7 cells were transiently transfected with pCMV-ProA3-OST, a pCMV-3-OST derivative in which the amino-terminal 26 residues of the mouse 3-OST are replaced with a protein A tag, and protein A-tagged mouse r3-OST was extracted with IgG-agarose beads from 155 ml of conditioned serum-free medium, as described under “Experimental Procedures.” The affinity purification recovered undetectable and less than 0.5% of initial HSact conversion activity from control pcDNA3 and pCMV-3-OST transfection samples, whereas 7,000 units (10% recovery) were extracted from pCMV-ProA3-OST transfection samples. Thus, the mouse 3-OST cDNA directly encodes HSact conversion activity.

Third, we examined the activities of cell-free synthesized mouse and human r3-OST. Synthetic capped mouse and human 3-OST mRNAs were generated by **in vitro** transcription and then **in vitro** translated with reticulocyte lysate in the presence and absence of canine pancreatic microsomal membranes, as described under “Experimental Procedures.” HSact conversion activity was undetectable in the control **in vitro** translation reactions that lacked mRNA template, with or

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4 Although peptide P2−22A (Fig. 3), containing Cys260, is much more hydrophobic than peptide P2−22B, containing Cys269, both were recovered from reverse-phase HPLC as a single sharp peak, which suggests coupling by a disulfide bond.
isolated mouse and human cDNAs encode HSact conversion activity of the above considerations, the above data confirm that reduced length of the human 5'-untranslated region. Independently by incubating crude or purified enzyme with [35S]PAPS and unlabeled HSinact, recovering radiolabeled GAG by DEAE chromatography and characterizing the resultant products. The HSact conversion activity secreted into medium by transfected COS-7 cells. Results are presented as the mean ± s and are derived from triplicate samples.

| Plasmid              | HSact conversion activity | units/µl |
|----------------------|---------------------------|----------|
| Nontransfected       | 0.0018 ± 0.0015           | 0.0023%  |
| pcDNA3               | 0.0024 ± 0.0024           | 0.044  |
| pCMV-3-OST           | 5.2 ± 0.18                | 0.18%   |
| pCMV-ProA3-OST       | 1.9 ± 0.15                | 0.15%   |

without microsomal membranes. A low level HSact conversion activity resulted from the addition of synthetic 3-OST mRNA templates to translation reactions lacking microsomal membranes (mouse, 0.86 ± 0.028 units/µl, n = 3; human, 2.1 ± 0.063 units/µl, n = 3); however, −15-fold greater levels occurred when microsomal membranes were included in translation reactions (mouse, 14.3 ± 0.27 units/µl, n = 3; human, 32.4 ± 2.1 units/µl, n = 3). The apparent activation of nascent r3-OST by co-translational processing within microsomes may reflect the signal peptidase cleavage, and/or a facilitation of correct protein folding. The slightly lower activity-fractionated, which revealed that in each case 35S-label was incorporated in HS act and approximately 60% of the 35S-label was incorporated in HSinact. The labeled HSact generated by the wild-type purified r3-OST were

We next examined the biochemical specificity of the HSact conversion activity generated from each expression approach by incubating crude or purified enzyme with [35S]PAPS and unlabeled HSinact, recovering radiolabeled GAG by DEAE chromatography and characterizing the resultant products. The HSact conversion activity of the wild-type mouse r3-OST produced by transfecting COS-7 cells with pCMV-3-OST (1.35 ± 0.30 units in 240 µl of conditioned serum-free medium) was first purified away from potential contaminating sulfotransferase activities by heparin-AP Toyopearl chromatography followed by 3′,5′-ADP-agarose chromatography, which yielded ~1 µg of protein containing 340,000 units (~20,000-fold purification with 25% overall recovery), whereas the IgG agarose-purified protein A-tagged r3-OST and in vitro translation reactions of mouse and human 3-OST mRNA templates were directly analyzed, as described under “Experimental Procedures.” About 0.5−1 × 106 cpm of product was generated with purified wild-type r3-OST, purified protein A-tagged r3-OST, and nonpurified in vitro translation reactions containing mouse and human r3-OST, respectively. Portions of each labeled product were incubated with purified heparitinase (0.5 units/ml) or chondroitinase ABC (0.5 units/ml), and HPLC-gel permeation chromatography analysis indicated that in all cases label was exclusively incorporated into HS. Portions of the labeled HS samples were also N-desulfated with nitrous acid at pH 1.5 and analyzed by P-2 polyacrylamide gel filtration to determine the amounts of liberated free [35S]sulfate, as described under “Experimental Procedures.” The results demonstrated no increased generation of free [35S]sulfate (data not shown). Finally, portions of the labeled samples were AT affinity-fractionated, which revealed that in each case ~40% of the [35S]-labeled product was incorporated in HSact. The labeled HSact and HSinact generated by the wild-type purified r3-OST were

5 When in vitro translation reactions lacking 3-OST mRNA templates were incubated with [35S]PAPS and unlabeled HS under otherwise standard conditions, the radioactivity recovered in DEAE eluates was 0.032 ± 0.0022% (translates without microsomes, n = 3) and 0.044 ± 0.0090% (translates with microsomes, n = 3) of initial radioactivity, whereas similar incubation of translates containing mouse or human 3-OST mRNA templates resulted in the incorporation of 1–3% of initial radioactivity. Thus, direct analysis of in vitro translates should only reflect the sulfotransferase activity of the translated r3-OST.
The abundance of each category varies with each cell line, suggesting a similar mechanism of origin. The expressed HS act conversion activities exclusively catalyze the transfer of sulfate to the 3-O position of glucosamine units in HS act and HS inact.

Northern Analysis of Rodent and Human 3-OST Expression—Northern blot analysis reveals the presence of 3-OST message in different kinds of endothelial cells as well as a mast cell line. Both cell types have previously been shown to form HS act and anticoagulant heparin, respectively (6, 8, 55). Three size categories of rodent 3-OST mRNAs (about 1.7, 2.3, and 3.3 kb) and a single size species of the human message (about 1.7 kb) (Fig. 6) are evident. As described above, the mouse forms arise from differential splicing within the 5'-untranslated region. Similar size categories are also expressed by rat (RFPEC) endothelial cells, suggesting a similar mechanism of origin. The abundance of each category varies with each cell line, which suggests that a mechanism exists to regulate such differential splicing. The immortalized mouse mast cell line, C57.1, expresses high levels of the same three size categories, which suggests that expression of a single 3-OST gene is required for the synthesis of both HS act and anticoagulant heparin.

3-OST Sequence Defines a Heparan Sulfotransferase Family—Extensive data bank searching revealed the 3-OST enzyme to be a previously unidentified protein; furthermore, the carboxyl-terminal 250 residues exhibit a low homology (30% similarity) to many previously identified sulfotransferases (which are typically ~300 residues in length) including chondroitin-, aryl-/phenol-, N-hydroxylation-, alcohol-/hydroxysteroid-, flavonol-, and nodulation factor sulfotransferases (data not shown). We also observed a slightly greater homology (40% similarity) to a functionally unidentified open reading frame of 247 amino acids from Aeromonas salmonicida (GenBank TM accession number L37077). More importantly, the 3-OST protein exhibits ~50% similarity with all previously identified forms of the heparan biosynthetic enzyme NST (representatives shown in Fig. 7). In particular, extensive homology exists across the entire 250–270 carboxyl-terminal residues of these enzymes. Thus, it appears that a common sulfotransferase structure is shared by two distinct types of heparan biosynthetic enzyme. Given that NST is a bifunctional enzyme, the above observation suggests that NST enzymes possess sulfotransferase activity within a ~270-residue carboxyl-terminal domain, whereas deacetylase activity would be contained within the remaining ~560 luminal residues. Interestingly, the region of consensus Lys302-Arg323, which encompasses the presumptive cystine-bridged peptide loop (described above), exhibits complete conservation for 12 of the 22 residues (including both cysteines) among all 3-OST and NST species.

DISCUSSION

We have previously demonstrated that the cellular rate of HSPG act generation is determined by the levels of a kinetically limiting microsomal activity, HS act conversion activity, and have proposed that 3-OST is the principal rate-limiting component of this microsomal activity (35). Based on this hypothesis, we purified the rate-limiting component of HS act conversion activity and confirmed its identity as 3-OST (26). During the current investigation, we have isolated and characterized the mouse and human 3-OST cDNAs. Three independent lines of evidence conclusively demonstrate that the murine and human cDNA clones encode the 3-OST from each species. First, the mouse cDNA exhibits all 13 sequenced peptides and the amino terminus of the purified enzyme; moreover, the primary
### 3-O-Sulfation of Heparan Sulfates

Our previous analyses of L cells in which HSact synthesis was perturbed by overexpression of tyrosin core protein or chemical mutagenesis suggest that 3-O-sulfation of HSNact versus the HSinact precursor may take place in a differential manner; however, it is unclear whether differential sulfation of the two precursors involves multiple 3-OST gene products, a posttranslational modification of a single enzyme, or the presence of accessory biosynthetic factors.

#### Comparison of putative sulfotransferase domains

| Protein | Accession Number | Consensus |
|---------|------------------|-----------|
| NST-1   | M92042           | Consensus |
| NST-2   | U52002           | Consensus |
| rNST-1  | U02304           | Consensus |
| rNST-2  | U52002           | Consensus |

**FIG. 7.** Comparison of putative sulfotransferase domains.** The program Pileup was used to align amino acid sequences from mouse 3-OST (residues 1–311), human 3-OST (residues 1–307), rat 3-deacetylase/N-sulfotransferase 1 (rNST-1, residues 516–882, accession number M92042) (26), mouse N-deacetylase/N-sulfotransferase 2 (mNST-2, residues 515–883, accession number U52004) (27), and Caenorhabditis elegans N-deacetylase/N-sulfotransferase (cNST, residues 459–819, accession number U52002) (68). Consensus residues (shaded) are indicated for each position where at least four candidates exhibit identical or similar amino acids. Numeration is given for each enzyme and for a consensus sequence.
with this difference, the carboxyl-terminal tetrapeptide residues are conserved between mouse and human 3-OST but are different from the comparable NST residues (Fig. 7). Interestingly, Cab45 and 3-OST exhibit distinct residues at this site (HEEF and FDWH, respectively) (59), which suggests localization to different Golgi compartments. The high levels of 3-OST that accumulate in tissue culture medium testify to a leaky retention mechanism, which raises the possibility that modulation of enzyme retention could serve as a mechanism to control the cellular rate of Hsac generation.

The sulfotransferases that act upon different substrates exhibit extensive structural diversity; indeed, similarity is greatest between members of this enzyme class that sulfate related substrates (62). Consistent with this observation, 3-OST and all known NST species possess a homologous carboxyl-terminal domain of ~260 residues that also exhibits homology to all known sulfotransferases. Given that this region constitutes >80% of the protein A-tagged r3-OST and so should contain the machinery for sulfation, we propose that a common domain participates in PAPS binding due to its homology with the presumptive consensus residues 311–316) similar to GGKLEKC of aryl sulfotransferase IV. This region is thought to be positioned near the catalytic site (63, 64). However, the presumptive cysteine-bridged peptide loop region contains a sequence (Fig. 7, consensus residues 311–316) similar to GGKLEKC of aryl sulfotransferase IV. This region is thought to be positioned near the active site as indicated by affinity labeling of the contained Lys and Cys with ATP dialdehyde (65). Thus, the cysteine-bridged peptide loop of 3-OST may be located near the active site, which is consistent with the above observation. In this regard, we note that integrity of the disulfide bond may be required for enzymatic activity, since 3-OST is inactivated by incubation with dithiothreitol. Interestingly, dithiothreitol also inhibits the activity of heparan sulfate 6-O-sulfotransferase but not chondroitin 4-sulfotransferase (66). We note that endothelial cell synthesis of Hsac is suppressed by exposure to the thrombogenic agent Heparin (67). Thus, it is possible that alterations in the cellular redox state may affect Hsac synthesis by disrupting a critical disulfide bond in the 3-OST. The availability of the 3-OST cDNA now provides a critical tool for determining how endothelial cells regulate HSPO4 generation and should permit an exploration of the molecular defects in this natural anticoagulant pathway that lead to vascular disorders.

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