

# Heparan Sulfate 3-O-Sulfotransferase Isoform 5 Generates Both an Antithrombin-binding Site and an Entry Receptor for Herpes Simplex Virus, Type 1\*

Received for publication, April 30, 2002, and in revised form, June 28, 2002  
Published, JBC Papers in Press, July 23, 2002, DOI 10.1074/jbc.M204209200

Guoqing Xia<sup>‡</sup>, Jinghua Chen<sup>§</sup>, Vaibhav Tiwari<sup>¶</sup>, Wujian Ju<sup>§</sup>, Jin-Ping Li<sup>||</sup>, Anders Malmström<sup>‡</sup>, Deepak Shukla<sup>¶</sup>, and Jian Liu<sup>§\*\*</sup>

From the <sup>‡</sup>Cell and Molecular Biology, Biomedical Center C13, Lund University, Lund S-22184, Sweden, the <sup>§</sup>Division of Medicinal Chemistry and Natural Products, University of North Carolina, School of Pharmacy, Chapel Hill, North Carolina 27599, the <sup>¶</sup>Departments of Ophthalmology and Visual Sciences and Microbiology and Immunology, University of Illinois, Chicago, Illinois 60612, and the <sup>||</sup>Department of Medical Biochemistry and Microbiology, Uppsala University, S-75123 Uppsala, Sweden

**Heparan sulfate 3-O-sulfotransferase transfers sulfate to the 3-OH position of a glucosamine residue of heparan sulfate (HS) to form 3-O-sulfated HS. The 3-O-sulfated glucosamine residue contributes to two important biological functions of HS: binding to antithrombin and thereby carrying anticoagulant activity, and binding to herpes simplex viral envelope glycoprotein D to serve as an entry receptor for herpes simplex virus 1. A total of five HS 3-O-sulfotransferase isoforms were reported previously. Here we report the isolation and characterization of a novel HS 3-O-sulfotransferase isoform, designated as HS 3-O-sulfotransferase isoform 5 (3-OST-5). 3-OST-5 cDNA was isolated from a human placenta cDNA library and expressed in COS-7 cells. The disaccharide analysis of 3-OST-5-modified HS revealed that 3-OST-5 generated at least three 3-O-sulfated disaccharides as follows: IdoUA2S-AnMan3S, GlcUA-AnMan3S6S, and IdoUA2S-AnMan3S6S. Transfection of the plasmid expressing 3-OST-5 rendered wild type Chinese hamster ovary cells susceptible to the infection by herpes simplex virus 1, suggesting that 3-OST-5-modified HS serves as an entry receptor for herpes simplex virus 1. In addition, 3-OST-5-modified HS bound to herpes simplex viral envelope protein glycoprotein D. Furthermore, we found that 3-OST-5-modified HS also bound to antithrombin, suggesting that 3-OST-5 also produces anticoagulant HS. In summary, our results indicate that a new member of 3-OST family generates both anticoagulant HS and an entry receptor for herpes simplex virus 1. These results provide a new insight regarding the mechanism for the biosynthesis of biologically active HS.**

Heparan sulfates (HSs)<sup>1</sup> are highly sulfated polysaccharides, present on the surface of mammalian cells and in the extracel-

lular matrix in large quantities. HSs play critical roles in a variety of important biological processes, including assisting viral infection, regulating blood coagulation and embryonic development, suppressing tumor growth, and controlling the eating behavior of mice by interacting with specific regulatory proteins (1–5). HS polysaccharides carry negative charges under physiological pH, and the disaccharide repeating units consist of 1→4-linked sulfated glucosamine and uronic acid. The unique sequences determine to which specific proteins HSs bind, thereby regulating biological processes.

The biosynthesis of HS occurs in the Golgi apparatus. It is initially synthesized as a copolymer of glucuronic acid and *N*-acetylated glucosamine by *D*-glucuronyl and *N*-acetyl-*D*-glucosaminyltransferase, followed by various modifications (6). These modifications include *N*-deacetylation and *N*-sulfation of glucosamine, C<sub>5</sub> epimerization of glucuronic acid to form iduronic acid residues, 2-*O*-sulfation of iduronic and glucuronic acid, as well as 6-*O*-sulfation and 3-*O*-sulfation of glucosamine. Several enzymes that are responsible for the biosynthesis of HS have been cloned and characterized (see review by Esko and Lindahl (7)). These enzymes have become essential tools for investigating the relationship between the structures and functions of HS.

What is still unknown is the detailed mechanism for regulating the biosynthesis of HS with a defined saccharide sequence. A recent report (8) suggests that the expression levels of various HS biosynthetic enzyme isoforms contribute to the synthesis of specific saccharide sequences in specific tissues. HS *N*-deacetylase/*N*-sulfotransferase, 3-*O*-sulfotransferase, and 6-*O*-sulfotransferase are present in multiple isoforms. Each isoform is believed to recognize a saccharide sequence around the modification site in order to generate a specific sulfated saccharide sequence (8–10). For instance, HS *D*-glucosaminyl 3-*O*-sulfotransferase (3-OST) isoforms generate 3-*O*-sulfated glucosamine residues that are linked to different sulfated uronic acid residues. 3-OST-1 transfers sulfate to the 3-OH position of an *N*-sulfated glucosamine residue that is linked to a glucuronic acid residue at the nonreducing end

\* This work was supported by National Institutes of Health Grants AI50050-01 (to J. L.) and AI053836-01 (to D. S.), a grant from Pharmacy Foundation of North Carolina (to J. L.), and a grant from Swedish Research Council 7479 (to A. M.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

The nucleotide sequence(s) reported in this paper has been submitted to the GenBank™/EBI Data Bank with accession number(s) AF503292.

\*\* To whom correspondence and reprint requests should be addressed: Rm. 309, Beard Hall, CB7360, University of North Carolina, Chapel Hill, NC 27599. Tel.: 919-843-6511; Fax: 919-843-5432; E-mail: jian\_liu@unc.edu.

<sup>1</sup> The abbreviations used are: HS(s), heparan sulfate(s); PAPS, 3'-phosphoadenosine 5'-phosphosulfate; HSV-1, herpes simplex virus,

type 1; gD, herpes envelope glycoprotein D; AT, antithrombin; HS<sup>act</sup>, anticoagulant-active or antithrombin-binding HS; HS<sup>inact</sup>, non-antithrombin-binding HS; 3-OST, HS *D*-glucosaminyl-3-*O*-sulfotransferase; IdoUA2S, L-iduronic acid 2-*O*-sulfate; AnMan, AnMan3S, AnMan6S, and AnMan3S6S, 2,5-anhydromannitol, 2,5-anhydromannitol 3-*O*-sulfate, 6-*O*-sulfate, and 3,6-*O*-disulfate; ConA, concanavalin A; CHO, Chinese hamster ovary; RPIP-HPLC, reverse-phase ion-pairing HPLC; MES, 2-(*N*-morpholino)ethanesulfonic acid; X-gal, 5-bromo-4-chloro-3-indolyl-β-*D*-galactopyranoside.

(GlcUA-GlcNS $\pm$ 6S). However, 3-OST-3 transfers sulfate to the 3-OH position of an *N*-unsulfated glucosamine residue that is linked to a 2-*O*-sulfated iduronic acid at the nonreducing end (IdoUA2S-GlcNH $_2$  $\pm$ 6S) (11). The difference in the substrate specificity of 3-OSTs results in distinct biological functions. For example, the HS modified by 3-OST-1 binds to antithrombin (AT) and has anticoagulant activity (12). However, the HS modified by 3-OST-3 (3-OST-3A and 3-OST-3B)<sup>2</sup> binds to glycoprotein D (gD) of herpes simplex virus, type 1, thus mediating viral entry (13).

The HS- and heparin-regulated anticoagulation mechanisms have been studied extensively. It is now known that HS and heparin interact with AT, a serine protease inhibitor, to inhibit the activities of thrombin and factor Xa in the blood coagulation cascade (2). Anticoagulant-active HS (HS<sup>act</sup>) and heparin contain one or multiple AT-binding sites per polysaccharide chain. This binding site contains a specific pentasaccharide sequence with a structure of -GlcNS(or Ac)6S-GlcUA-GlcNS3S( $\pm$ 6S)-IdoUA2S-GlcNS6S-. The 3-*O*-sulfation of glucosamine for generating GlcNS3S( $\pm$ 6S) residue, which is carried out by 3-OST-1 (EC 2.8.2.23), is the critical modification for the synthesis of HS<sup>act</sup> (12, 14).

Cell surface HS assists herpes simplex viral infection (15). A recent report (13) suggests that a specific 3-*O*-sulfated HS is involved in assisting HSV-1 entry. The 3-*O*-sulfated HS is generated by 3-OST-3 but not by 3-OST-1. In addition, the 3-*O*-sulfated HS provides binding sites for HSV-1 envelope glycoprotein D, which is a key viral protein involved in the entry of HSV-1 (13). Because 3-OST-3-modified HS is rarely found in HS from natural sources, the study suggests that HSV-1 recognizes a unique saccharide structure. Indeed, the result from the structural characterization of a gD-binding octasaccharide revealed that the octasaccharide possesses a specific saccharide sequence (16). In addition, the binding affinity of the 3-*O*-sulfated HS for gD is about 2  $\mu$ M (13). This affinity is similar to that reported for the binding of gD to the protein receptors, suggesting that HSV-1 utilizes both protein and HS cell surface receptors to infect target cells (17, 18). It is believed that the interaction between gD and the 3-*O*-sulfated HS or the protein entry receptors somehow triggers the fusion between the virus and the cell in the presence of other viral envelope proteins, including gB, gH, and gL (19). A study of the co-crystal structure of gD and herpes entry receptor HveA suggests that the binding of HveA to gD induces conformational changes in gD (20).

It is known that the enzymes for synthesizing HS<sup>act</sup> and an entry receptor for HSV-1 belong to two 3-OST isoforms. These two isoforms generate the 3-*O*-sulfated glucosamine residue located in different saccharide sequences as described above. In this article, we report that a 3-OST homologous protein, designated as HS 3-*O*-sulfotransferase isoform-5 (3-OST-5), possesses the activities for generating both HS<sup>act</sup> and HSV-1 entry receptor (or gD-binding HS). Our results suggest that the biosynthesis of HS<sup>act</sup> and the gD-binding HS is regulated by several enzymes. The newly identified enzyme provides an additional tool for both understanding the mechanism for the biosynthesis of the biologically active HS and for investigating the relationship between the saccharide sequences and the biological functions of HS.

## EXPERIMENTAL PROCEDURES

### Materials

Recombinant human 3-OST-3A and mouse 3-OST-1 enzymes were expressed in Sf9 cells using a baculovirus expression system. The enzymes were purified by heparin-Toyopearl and 3',5'-ADP-agarose chromatographies as described previously (11, 21). [<sup>35</sup>S]PAPS was prepared by incubating 0.4 to 2 mCi/ml [<sup>35</sup>S]Na<sub>2</sub>SO<sub>4</sub> (carrier-free, ICN) and 16 mM ATP with 5 mg/ml dialyzed yeast extract (Sigma) (12). HS was either isolated from 33-cells, an L-cell variant, or from Chinese hamster ovary (CHO) cells as described previously (12). The concentrations of the unlabeled HS were determined by a method reported by Björnsson (22). Preparation of metabolically <sup>35</sup>S-labeled HS from CHO cells was described elsewhere (23). Human AT is from Cutter Biological (Berkeley, CA). A truncated form of herpes simplex virus, type 1, glycoprotein D, gD-1(306t), and monoclonal anti-gD (DL6) were generous gifts of Drs. Cohen and Eisenberg of the University of Pennsylvania (24). The <sup>3</sup>H-labeled disaccharide standards, GlcUA-AnMan3S6S and IdoUA2S-AnMan6S, were prepared from <sup>3</sup>H-labeled HS (gifts from Dr. Rosenberg, Massachusetts Institute of Technology) (25). The <sup>35</sup>S-labeled disaccharide standards, IdoUA2S-AnMan3S and IdoUA2S-AnMan3S6S, were prepared from low pH (pH 1.5) nitrous acid-degraded HS that was modified by purified 3-OST-3 enzyme as described by Liu *et al.* (11).

### Isolation of 3-OST-5 cDNA

GenBank™ data base was probed with the amino acid sequence of 3-OST-1 using tBlastn. By using Genscan, we predicted a 1,041-bp open reading frame that encodes a homologous protein from a genomic clone RP11-112L15 with GenBank™ accession number AL355498. The open reading frame sequence, designated as 3-OST-5, was located in two exons. Both 5'- and 3'-primers were synthesized based upon the sequence of exon 1 and exon 2, respectively. The following are the sequences of the two specific primers: 5'-GGAGGGCC ATG CTA TTC AAA CAG-3' (5'-primer), and 5'-TTA GGG CCA GTT CAA TGT CCT-3' (3'-primer). We cloned the cDNA using PCR from a human placenta cDNA library with the two specific primers. The resultant PCR product (about 1.0 kb) was inserted into the pGEM-T-easy vector (pGEM-T-3OST5) and sequenced for both strands. The isolated cDNA has an identical sequence that was predicted from the genomic clone. 5'-Rapid amplification of cDNA ends was performed but failed.

### Cell Culture

Both COS-7 and wild type CHO cells were from Dr. R. Jude Samulski (University of North Carolina). COS-7 cells and CHO cells were maintained in logarithmic growth by subculturing biweekly at 37 °C under 6% CO<sub>2</sub> humidified atmosphere. COS-7 cells and CHO cells were grown in Dulbecco's modified Eagle's medium (Invitrogen) and in F-12 medium (Invitrogen) supplemented with 10% fetal bovine serum (JRH Biosciences), respectively.

### Expression of 3-OST-5

3-OST-5 expression plasmid was constructed by inserting the open reading frame of 3-OST-5 into a pcDNA3.1 (Invitrogen) expression vector. The open reading frame was obtained by reamplifying the sequence from pGEM-T-3OST5 by PCR using the following primers: 1) the 5'-specific primer, 5'-TCA AAGCTT GCCACC ATG CTATTCAAA-CAGCA-3', contains an *Hind*III site (underlined), the consensus Kozak sequence (italicized) and a start codon; and 2) the 3'-specific primer, 5'-GC TCTAGA TTAGGGCCAGTTCAATGTCCT-3', contains an *Xba*I site (underlined). PCRs were carried out using Advantage-2 PCR kit (CLONTECH) with the initial denaturation for 2 min at 94 °C, followed by 30 cycles of a reaction consisting of 45 s for denaturation at 94 °C, 45 s for annealing at 62 °C, and 90 s for elongation at 72 °C. The PCR fragment was subcloned into pcDNA3.1 (Invitrogen) using *Hind*III/*Xba*I sites. The coding region of the construct was completely sequenced on both strands, and the construct was designated pcDNA3.1-3OST5. pcDNA3.1-3OST5 or pcDNA3 plasmid was transfected into exponentially growing COS-7 cells using LipofectAMINE 2000 (Invitrogen).

### Measurement of 3-OST-5 Activity

*Preparation of [<sup>35</sup>S]HS*—The crude enzyme was extracted from transfected COS-7 cells. The cells were harvested 72 h after transfection. Approximately 3  $\times$  10<sup>6</sup> cells were mixed with 100  $\mu$ l of cold 0.25 M sucrose containing 1% Triton X-100 (v/v) and incubated on ice for 30 min. The insoluble residues were removed after centrifuging at 10,000  $\times$  g for 10 min. The HS sulfotransferase activity was determined by incubating 40  $\mu$ g of cell extract with 1  $\mu$ g of unlabeled HS (from

<sup>2</sup> Human 3-OST-3A and 3-OST-3B have nearly identical amino acid sequences in the proposed sulfotransferase domain. Both enzymes sulfate identical disaccharides and have the activity in assisting HSV-1 entry. For clarity, we used 3-OST-3 to represent both 3-OST-3A and 3-OST-3B unless specified otherwise.



33-cells),  $4 \times 10^7$  cpm of [ $^{35}\text{S}$ ]PAPS, in 50  $\mu\text{l}$  of a buffer containing 50 mM MES, 10 mM  $\text{MnCl}_2$ , 5 mM  $\text{MgCl}_2$  and 1% Triton X-100 (pH 7). The reaction was incubated at 37 °C for 2 h, quenched by heating at 100 °C for 1 min, and centrifuged at  $10,000 \times g$  for 1 min to remove insoluble materials. The sample was then subjected to a 200- $\mu\text{l}$  DEAE-Sepharose column to purify [ $^{35}\text{S}$ ]HS (12).

**Disaccharide Analysis of [ $^{35}\text{S}$ ]HS**—The [ $^{35}\text{S}$ ]HS modified by 3-OST-5 was mixed with 20  $\mu\text{g}$  of unlabeled HS (from ICN) and degraded with nitrous acid at pH 1.5 followed by reduction with sodium borohydride (26). The resultant  $^{35}\text{S}$ -labeled disaccharides were mixed with a  $^3\text{H}$ -labeled disaccharide standard, IdoUA2S-[1- $^3\text{H}$ ]AnMan6S, and desalted on a Bio-Gel P-2 column (0.75  $\times$  200 cm) that was equilibrated with 0.1 M ammonium bicarbonate at a flow rate of 4 ml/h. The  $^3\text{H}$ -labeled disaccharide standard was used to locate the elution position of the disaccharides from the Bio-Gel P-2 column.

The disaccharides were resolved by a  $\text{C}_{18}$ -reversed phase column (0.46  $\times$  25 cm) (Vydac) under the reverse-phase ion-pairing HPLC (RPIP-HPLC) condition (11). Briefly, the column was eluted with acetonitrile as follows: 8% for 30 min followed by 15% for 15 min and followed by 19.5%, in a solution containing 38 mM ammonium phosphate monobasic, 2 mM phosphoric acid, and 1 mM tetrabutylammonium phosphate monobasic (Fluka) at a flow rate of 0.5 ml/min.

#### Characterization of 3-OST-5-modified HS

**Preparation of 3-OST-1 and 3-OST-3-modified HS**—To prepare 3-OST-3-modified HS, HS (1  $\mu\text{g}$  from 33-cells) was mixed with 30 ng of purified 3-OST-3A enzyme and [ $^{35}\text{S}$ ]PAPS ( $4 \times 10^7$  cpm) in a buffer containing 50 mM MES, 1% Triton X-100, 1 mM  $\text{MgCl}_2$ , 2 mM  $\text{MnCl}_2$ , 150 mM NaCl, and 168  $\mu\text{g}/\text{ml}$  bovine serum albumin (pH 7), in a final volume of 50  $\mu\text{l}$ . The reaction was incubated at 37 °C for 2 h and was then heated at 100 °C for 2 min. The resultant product was centrifuged at 14,000 rpm for 1 min to remove insoluble materials. The supernatant was loaded onto a 200  $\mu\text{l}$  DEAE-Sepharose column, and the [ $^{35}\text{S}$ ]HS was eluted from the column with 1000 mM NaCl (12). To prepare 3-OST-1-modified HS, we utilized 70 ng of purified enzyme and followed nearly identical procedures except for omitting the 150 mM NaCl.

**Demonstration of 3-OST-5-modified HS Binding to AT**—The binding of the 3-OST-5-modified HS to AT was determined using an AT/concanavalin A (ConA)-Sepharose approach (12). Briefly, HS (10,000–100,000 cpm) was incubated in 150  $\mu\text{l}$  of a buffer, which contains 10 mM Tris-HCl, 150 mM NaCl, 1  $\mu\text{M}$  dextran sulfate, 1 mM  $\text{Ca}^{2+}$ ,  $\text{Mg}^{2+}$ , and  $\text{Mn}^{2+}$ , and 0.1 mg/ml AT (pH 7.5), at room temperature for 30 min. The solution was mixed with the prewashed ConA-Sepharose (60  $\mu\text{l}$  of 1:1 slurry) and agitated at room temperature for 1 h. The gel was then washed with  $3 \times 1$  ml of a buffer containing 10 mM Tris-HCl, 0.0004% Triton X-100, and 150 mM NaCl (pH 7.5). The HS was eluted from the gel by 1 ml of a buffer containing 10 mM Tris-HCl, 1000 mM NaCl, and 0.0004% Triton X-100 (pH 7.5).

**Determination of the Binding of 3-OST-5-modified HS to gD**—The assay for determining the binding of 3-O-sulfated HS to gD was carried out by an immunoprecipitation procedure using anti-gD monoclonal antibody (13). The enzyme-modified HS (100,000–200,000 cpm) was incubated in 50  $\mu\text{l}$  of a buffer containing 50 mM Tris-HCl, 150 mM NaCl, and 0.01% Triton (pH 7) (binding buffer), and 2 mg/ml of gD at room temperature for 30 min. The anti-gD monoclonal antibody DL6 (5  $\mu\text{l}$ ) was added and incubated at 4 °C for 1 h followed by the addition of protein A-agarose gel (80  $\mu\text{l}$  of 1:1 slurry) and agitated at 4 °C for an additional hour. The HS was eluted from the gel with 1 ml of 1000 mM NaCl in the binding buffer.

**Determination of HS<sup>act</sup> Conversion Activity**—The assay was specifically designed to determine the HS biosynthetic activity that generates HS<sup>act</sup> (12). Briefly, cell extract was incubated with metabolically  $^{35}\text{S}$ -labeled nonanticoagulant HS (from wild type CHO cells) and unlabeled PAPS. The resultant [ $^{35}\text{S}$ ]HS was subjected to the AT-binding assay as described above. The increase in the percentage of the [ $^{35}\text{S}$ ]HS that binds to AT correlated to the amount of HS<sup>act</sup> conversion activity in the cell extract.

#### Herpes Simplex Viral Entry Assay

The conditions for growing cells and different HSV strains were described previously (13). The viral infectivity assay is based on visualization of the cells carrying  $\beta$ -galactosidase activity (25). CHO cells were transfected in 6-well dishes, using LipofectAMINE (Invitrogen) with pcDNA3.1-3OST5 plasmid or control plasmid (pcDNA3) at 1.5–2.0  $\mu\text{g}$  per well in 1 ml. At about 36 h post-transfection, cells were exposed to recombinant HSV-1 (HSV-1(KOS)gL86) (a gift from Dr. Spear, Northwestern University) that expresses  $\beta$ -galactosidase upon viral

entry. At 6 h post-infection, the cells were fixed in PBS containing 2% formaldehyde and 0.2% glutaraldehyde, permeabilized in 2 mM  $\text{MgCl}_2$  containing 0.01% deoxycholate and 0.02% Nonidet P-40, and incubated with buffered X-gal (0.5 mg/ml). Three hours later the infected cells were visible in blue due to the action of  $\beta$ -galactosidase on X-gal. The transfection efficiency for CHO cells was determined by transfecting a plasmid expressing  $\beta$ -galactosidase as a reporter gene.

#### Northern Blot Analysis

The coding sequence of 3-OST-5 was labeled with [ $\gamma$ - $^{32}\text{P}$ ]dCTP in a reaction with Klenow enzyme (Roche Molecular Biochemicals) and used as a probe to hybridize the Human Multiple Tissue Northern (MTN<sup>®</sup>) blot (CLONTECH). The hybridization was carried out in ExpressHyb Hybridization Solution (CLONTECH) at 60 °C for 1 h, and the blot was washed with  $0.1 \times$  SSC containing 0.5% SDS at 60 °C for 40 min (where  $1 \times$  SSC contains 150 mM NaCl and 15 mM sodium citrate, pH 7.0). The membrane was exposed to an x-ray film for 4 days.

#### RESULTS

**Isolation of the cDNA Encoding 3-OST-5**—Probing the non-redundant data base of National Center for Biotechnology Information with the deduced amino acid sequence of human 3-OST-1 (accession number AF033827), we identified a 165-kb genomic clone with GenBank<sup>™</sup> accession number AL355498. An open reading frame was found to be 1041 bp. The predicted open reading frame was located in two exons that were gapped by a 4.5-kb intron. The open reading frame of this protein, assigned as 3-OST-5, was amplified from a human placenta cDNA library using specific 5'- and 3'-primers as described under "Experimental Procedures." 3-OST-5 cDNA sequence and the predicted amino acid sequence are shown in Fig. 1. We attempted to obtain further upstream 3-OST-5 sequence by using 5'-rapid amplification of cDNA ends but failed. The isolated 3-OST-5 cDNA contains 1041 bp, and the deduced peptide of 346 amino acid residues predicts a type II membrane-bound protein. The protein has four potential N-glycosylation sites with the predicted molecular mass of 40,407 Da. The deduced amino acid sequence of 3-OST-5 has 71 and 58% homology to 3-OST-1 and 3-OST-3 in the sulfotransferase domains, respectively (Fig. 2). Putative PAPS-binding sites were also found in 3-OST-5 based upon the PAPS-binding consensus sequences (27, 28) (Fig. 2). The genomic BAC clone RP11-112L15, which contains 3-OST-5 gene, was annotated to be mapped on human chromosome 11q22.2. Alignment of the genomic sequence with 3-OST-5 cDNA revealed that exon 1 and exon 2 contain 107 and 934 bp of the open reading frame, respectively.

**Determination of the  $^{35}\text{S}$ -Labeled Sulfation Site of 3-OST-5-modified HS**—Because the cloned 3-OST-5 has high homology to 3-OST-1 and 3-OST-3, we tested the hypothesis for 3-OST activity. The plasmid expressing 3-OST-5 was transiently transfected into exponentially growing COS-7 cells. The cells were solubilized with detergent. HS sulfotransferase activity was determined by incubating with unlabeled HS and [ $^{35}\text{S}$ ]PAPS as described under "Experimental Procedures." The resultant [ $^{35}\text{S}$ ]HS was subjected to nitrous acid degradation at pH 1.5 followed by sodium borohydride reduction to prepare  $^{35}\text{S}$ -labeled disaccharides. This approach has been employed to successfully characterize the sulfation sites of 3-OST-2 and 3-OST-3 (8, 13).

The  $^{35}\text{S}$ -labeled disaccharides were resolved on RPIP-HPLC, and the chromatograms are shown in Fig. 3. Comparing the profiles of the degraded [ $^{35}\text{S}$ ]HS that was modified by pcDNA3-transfected cells (Fig. 3A), we found additional  $^{35}\text{S}$ -labeled disaccharides in the 3-OST-5-modified HS (Fig. 3B). By co-eluting with the appropriate disaccharide standards on RPIP-HPLC, we identified that three of the  $^{35}\text{S}$ -labeled disaccharides were 3-O-sulfated disaccharides with the structures of IdoUA2S-AnMan3S (eluted at 38.5 min), GlcUA-AnMan3S6S (eluted at 55.5 min), and IdoUA2S-AnMan3S6S (eluted at 70.3

```

ATGCTATTCAAACAGCAGCGCTGGCTGAGACAGAAGCTCCTGGTCTGGGAAGCCTTGCCTTGGGAGTCTCCTGTATCTAGTGGCCAGAGTGGGAGCTTGGATAGGCTACAACCCATT 120
M L F K Q Q A W L R Q K L L V L G S L A V G S L L Y L V A R V G S L D R L Q P I 40
TGCCTCCATTGAAGGTCAGCTGGTGGAGCCCGCACTCAGGCTGAATCCCACTTGGCCCTGCAGTTAAGCGTGGCTGCTGCAGAGTCCGGAAGGGCAACGCTTCCAAGGAGCAG 240
C P I E G R L G G A R T Q A E F P L R A L Q F K R G L L H E F R K G N A S K E Q 80
GTTCCCTCCATGACCTGGTCCAGCAGCTCCCAAGGCCATTATCATGGGGTGGAGAAAGGAGGACAAGGGCCCTGCTGAAATGCTGAACTACATCCGGCAGTAGTCAAAGCCTCT 360
V R L H D L V Q Q L P K A I I I G V R K G G T R A L L E M L N L H P A V V K A S 120
CAAGAAATCCACTTTTTGATAATGATGAGAATTATGGTAAGGGCATTGAGTGGTATAGAAAAGATGCCTTTTTCTACCCCTCAGCAAAATCACAATTGAAAAGAGCCAGCATATTT 480
Q E I H F F D N D E N Y G K G I E W Y R K K M P F S Y P Q Q I T I E K S P A Y F 160
ATCACAGAGGAGGTTCCAGAAAGGATTACAAAATGAACATCCATCAAGTTGTGATCATTGTCCAGGAGCCAAACCAAGAGCTATTTCTGATTATACTCAGGTGCTAGAGGGGAAG 600
I T E E V P E R I Y K M N S S I K L L I I V R E E P T T R A I S D Y T Q V L E G K 200
GAGAGGAAGAACAAAACCTTATTAACAAGTTGAGAAGCTGGCCATAGACCCCTAATACATCGCAAGTGAACACAAAATACAAGCAGTAAAGACCAGCTCTACCAAAACATCTGGAAAGG 720
E R K N K T Y Y K F E K L A I D P N T C E V N T K Y K A V R T S I Y T K H L E R 240
TGGTTGAAATCTTTCCAATTGAGCAATTCATGTCGTGAGAGATCGCCTCATCAGGAACTCTGCGCAAGCTCAGCTCGTGGAGAAGTTCTCTAAATCTGCCTCCCAAGGATAAGT 840
W L K Y F P I E Q F H V V D G D R L I T E P L P E L Q L V E K F L N L P P R I S 280
CAATACAATTTATACTTCAATGCTACAGAGGGTTTTACTGCTTGGCTTTAATATATCTTTAATAAGTGCCTGGCGGCGAGCAAGGGCGCATTTCATCCAGAGTGGACCCCTCTGTC 960
Q Y N L Y F N A T R G F Y C L R F N I I F N K C L A G S K G R I H P E V D P S V 320
ATTACTAAATTCGCAAAATCTTTTCATCCTTTAATCAAAAATTTTACCAGATCACTGGGAGGACATTGAACTGGCCCTAA 1041
I T K L R K F F H P F N Q K F Y Q I T G R T L N W P * 346
    
```

Fig. 1. The nucleotide and deduced amino acid sequences of 3-OST-5 (GenBank™ accession AF503292). The single predicted membrane spanning domain and four potential N-linked glycosylation sites are indicated by the *doubled underline* and by the *underlines* with a ● below the glycosylated Asn, respectively.

```

h3ST5 -----MLPKQQ-----AWLQKLLVNLGSAVNGSLY---LVARVGSLD---RQOPIC-----RIGSRGGARTAEFPLRL-----QFKRE--- 66
h3ST1 -----MLPKQQ-----AWLQKLLVNLGSAVNGSLY---LVARVGSLD---RQOPIC-----RIGSRGGARTAEFPLRL-----QFKRE--- 39
h3ST3A MAP--PGPASALSTSABPLSR-----SIFRKFPLMLCSLTSLYVF-YCAERQTLGSPVVGSGGEBEAGAPGGVLAGGERELAWPAAARKRLLDLPQWRRRRPPAPRDEGEEA 111
h3ST3B MGQRLSGGRSCLDVPGRLLPQPPPPPPVPRKRLRFLFAMLVWLYMLFSLCAGSCAAAPG---LVLGSGSRA-----AHDSPALATAPDGTPPRLPPEAP-----PATPLASGKEM 104
                                     [...]5'-PSB-]
h3ST5 -----LLHEFRKGNASKEQVRLHDLVQCFHAILIIGVKGOTRALLELNHEHAWKASQEHFFNDENYKCHENYKMKMFPSPYQOITHEKSEYFHTBEHFERI 169
h3ST1 -----LVRDG-----VAPNGSAGQLPFIIGVKGOTRALLELNHEHAWKASQEHFFNDENYKCHENYKMKMFPSPYQOITHEKSEYFHTBEHFERI 133
h3ST3A AWEESFPLSGGGPQGGAGSTVABAPPTLALLDEGSKQLPQAIIGVKGOTRALLELNHEHAWKASQEHFFNDENYKCHENYKMKMFPSPYQOITHEKSEYFHTBEHFERI 229
h3ST3B AEGAASP-----EQSPFVPPSPSPSIFSGSGSKQLPQAIIGVKGOTRALLELNHEHAWKASQEHFFNDENYKCHENYKMKMFPSPYQOITHEKSEYFHTBEHFERI 214
                                     [...]3'-PSB-]
h3ST5 YKNSISIKLLIIVREFTTRAI SDYTCVLEGKERNKNTYKPELDAIDPNTC-EVATKIKAVETSIVYKHLBNLKYFPPIEFHVVDGRLIIEFPELQIWFNPLNPPRSQYNYLFNA 288
h3ST1 YSNPSPISIKLLIIVREFTTRAI SDYTCVLEGKERNKNTYKPELDAIDPNTC-EVATKIKAVETSIVYKHLBNLKYFPPIEFHVVDGRLIIEFPELQIWFNPLNPPRSQYNYLFNA 250
h3ST3A SANSKDTKLIWVRDFTTRAI SDYTCVLEGKERNKNTYKPELDAIDPNTC-EVATKIKAVETSIVYKHLBNLKYFPPIEFHVVDGRLIIEFPELQIWFNPLNPPRSQYNYLFNA 345
h3ST3B SANSKDTKLIWVRDFTTRAI SDYTCVLEGKERNKNTYKPELDAIDPNTC-EVATKIKAVETSIVYKHLBNLKYFPPIEFHVVDGRLIIEFPELQIWFNPLNPPRSQYNYLFNA 330
h3ST5 TKGFCFLRFNLI FNR--CLAGSKGRVHPEDVPSVITLREHFFEFNKFYCTGRTLNWP- 346
h3ST1 TKGFCFLR-DSGRDR--CLHESKGRVHPEDVPSVITLREHFFEFNKFYCTGRTLNWP- 307
h3ST3A TKGFCFLKKAEGSSRPHCCKTKGRTHPEIDREVVRRRLREFYRFPNLFKYQTGHDEGWDG 406
h3ST3B TKGFCFLKKAEGSSRPHCCKTKGRTHPEIDREVVRRRLREFYRFPNLFKYQTGHDEGWDG- 390
    
```

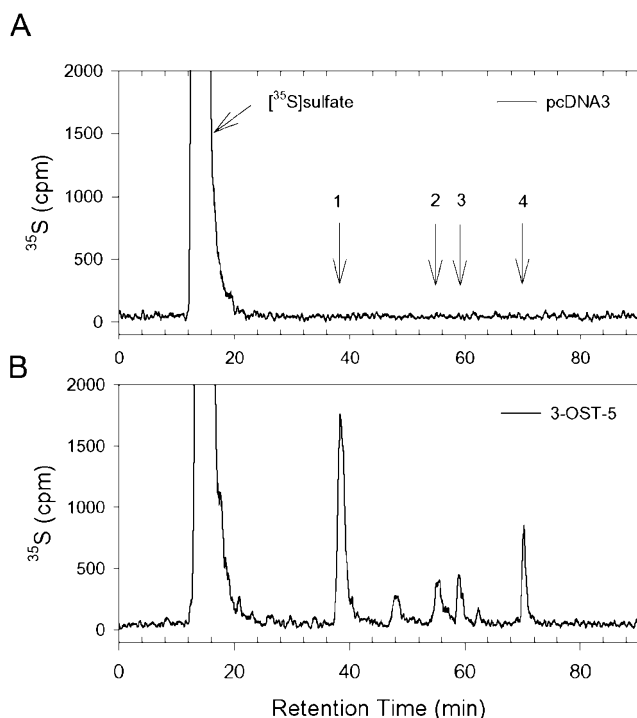
Fig. 2. Multiple amino acid sequence alignment of human 3-OST-5 with human 3-OST-1, 3-OST-3A, and 3-OST-3B. The alignment was performed by using the program BioEdit. Introduced gaps are shown as *hyphens*, and aligned amino acids are *boxed* and *shaded with black* for identical residuals and *dark gray* for similar residuals. 5'-PSB represents the putative domain that binds to 5'-phosphate of PAPS, and 3'-PSB represents the putative domain that binds to 3'-phosphate of PAPS.

min). A small peak of <sup>35</sup>S-labeled disaccharide with a structure of IdoUA2S-AnMan6S was also observed (eluted at 59.6 min). The IdoUA2S-AnMan6S was observed in HS modified with pcDNA3-transfected COS-7 cells (data not shown), although the level of this disaccharide varied between experiments. The presence of IdoUA2S-AnMan6S, which is a common disaccharide in HS, was unlikely to be associated with the activity of 3-OST-5. We also detected a minor <sup>35</sup>S-labeled peak at 48 min. The identity of this <sup>35</sup>S-labeled peak was unknown. Taken together, these results suggest that the expressed 3-OST-5 has the anticipated 3-OST activity. It is important to note that IdoUA2S-AnMan3S and IdoUA2S-AnMan3S6S are characteristic disaccharides of 3-OST-3-modified HS (13), whereas GlcUA-AnMan3S6S is a characteristic disaccharide of 3-OST-1-modified HS (12).

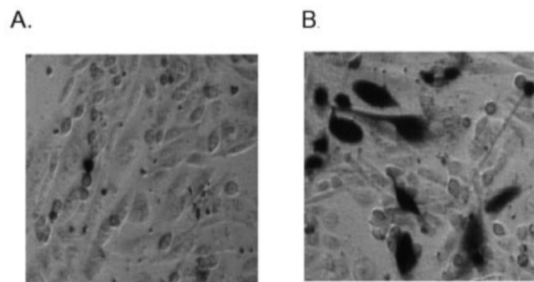
*The Activity of 3-OST-5 in Assisting HSV-1 Entry*—A previous study (13) demonstrated that 3-OST-3-modified HS serves as a receptor for herpes simplex virus, type 1, entry. Because we detected the disaccharides of 3-OST-3-modified HS, it appeared logical that 3-OST-5-modified HS might also generate an entry receptor for HSV-1. Therefore, we decided to test this possibility using an approach that was previously published (13). A recombinant β-galactosidase-expressing HSV-1 strain

(HSV-1(KOS)gL86) was used for the entry assay. This recombinant virus expresses β-galactosidase from an insert in the viral genome immediately upon entry into cells. As shown in Fig. 4A, the mock-transfected CHO cells are resistant to HSV-1 entry (no dark cells) as reported previously (29). In contrast, a significant number of CHO cells transfected with pcDNA3.1-3OST5 were rendered susceptible (Fig. 4B, *dark cells*) to the entry of HSV-1. We estimated that about 20% of the cells were susceptible to HSV-1 infection, which coincided with the transfection efficiency. In separate sets of experiments, we also found that 3-OST-5-modified HS does not generate receptors for other alphaherpesviruses. The viruses examined for entry via 3-OST-5-modified HS included wild type HSV-2, HSV-1 Rid mutants (24), bovine herpesvirus, and pseudorabies virus (data not shown).

Furthermore, we examined whether 3-OST-5-modified HS bound to gD. The results showed that 3-OST-5-modified HS has about a 2-fold increase in the binding to gD compared with the control, suggesting that 3-OST-5-modified HS generates gD-binding sites (Table I). We noted that the gD-binding percentage of 3-OST-5-modified HS (9.0%) was less than that of 3-OST-3-modified HS (23.1%). Such deviation is likely due to the fact the 3-OST-5 enzyme was in a mixture containing other HS



**FIG. 3. RPIP-HPLC chromatograms of the disaccharide analysis of 3-OST-5-modified HS.** Cell extracts from COS-7 cells transfected with the empty plasmid vector pcDNA 3 (A) or with the plasmid expressing 3-OST-5 (B) were incubated with unlabeled HS and [ $^{35}\text{S}$ ]PAPS to prepare the [ $^{35}\text{S}$ ]HS. The resultant [ $^{35}\text{S}$ ]HS was depolymerized by nitrous acid at pH 1.5 followed by sodium borohydride reduction. The resultant [ $^{35}\text{S}$ ]labeled disaccharides were resolved on RPIP-HPLC. The elution positions of the disaccharide standards are indicated by arrows, where arrow 1 represents IdoUA2S-AnMan3S; arrow 2 represents GlcUA-AnMan3S6S; arrow 3 represents IdoUA2S-AnMan6S; and arrow 4 represents IdoUA2S-AnMan3S6S.



**FIG. 4. Entry of HSV-1 into CHO-K1 cells and transfected CHO-K1 cells.** CHO-K1 cells were transfected with control plasmid (A) or with pcDNA3.1-3OST5 (B). At 36 h after transfection, the cells were exposed to KOS-gL86 at 100 plaque-forming units/cell. Six hours later, the cells were washed, fixed, and incubated with X-gal to identify infected cells (dark cells).

sulfotransferases, whereas purified 3-OST-3A enzyme was employed to prepare 3-OST-3-modified HS. Taken together, our results suggest that 3-OST-5 is capable of assisting the entry of HSV-1 by generating a receptor for gD. In addition, it is apparent that the mechanism for 3-OST-5-assisted HSV-1 infection is very similar to what was previously characterized for 3-OST-3. However, we could not conclude whether or not the saccharide sequences of the gD-binding site in 3-OST-5-modified HS and the sequence in 3-OST-3A-modified HS were identical.

**Demonstration of 3-OST-5-modified HS Binding to AT—**From the result of the disaccharide analysis of 3-OST-5-modified HS as described above, we found that 3-OST-5-modified HS also contains GlcUA-AnMan3S6S. This observation

**TABLE I**  
The binding of 3-OST-5-modified HS to gD and AT

	Binding to gD <sup>a</sup> (%)	Binding to AT <sup>b</sup> (%)
Control <sup>c</sup>	5.0 ± 0.4 (n = 2)	0.68 ± 0.24 (n = 4)
3-OST-5 modified HS	9.0 ± 0.1 (n = 2)	4.3 ± 1.8 (n = 4)
3-OST-5 modified HS <sup>actd</sup>	Not determined	34.7 (n = 1)
3-OST-1 modified HS <sup>e</sup>	6.6 ± 0.3 (n = 2)	37.0 ± 3.3 (n = 4)
3-OST-3 modified HS <sup>e</sup>	23.1 ± 3 (n = 2)	1.4 ± 0.7 (n = 4)

<sup>a</sup> The binding of the HS and gD was determined by incubating modified [ $^{35}\text{S}$ ]HS with gD followed by immunoprecipitation using anti-gD monoclonal antibody (DL6) to precipitate the complex of [ $^{35}\text{S}$ ]HS and gD. Data are presented as the mean ± S.D., where *n* represents the number of determinations.

<sup>b</sup> The binding of the HS to AT was determined by incubating modified [ $^{35}\text{S}$ ]HS and AT by using AT/ConA-Sepharose gel as described under "Experimental Procedures."

<sup>c</sup> Control was the [ $^{35}\text{S}$ ]HS that was prepared by incubating HS with the cell extract transfected with pcDNA3.

<sup>d</sup> 3-OST-5-modified HS<sup>act</sup> was prepared by AT-affinity fractionation from 3-OST-5-modified HS as described under "Experimental Procedures."

<sup>e</sup> 3-OST-1- and 3-OST-3-modified HS were prepared by incubating unlabeled HS (from 33 cells), [ $^{35}\text{S}$ ]PAPS, and purified 3-OST-1 (70 ng) and 3-OST-3 (35 ng), respectively.

prompted us to determine whether or not 3-OST-5-modified HS bound to AT. The results for the binding of [ $^{35}\text{S}$ ]HS to AT are shown in Table I. As expected, 37.0% of 3-OST-1-modified HS bound to AT, whereas only 1.4% of 3-OST-3-modified HS bound to AT. Those results were consistent with the previous reports (8, 12). Comparing the percentages of HS<sup>act</sup> between 3-OST-5-modified HS and control, we found that the binding of 3-OST-5-modified HS to AT was 6.3-fold higher than the control sample. The result suggests that 3-OST-5-modified HS binds to AT. The conclusion was further strengthened by the results as presented below.

We also determined the HS<sup>act</sup> conversion activity of the cell extract from 3-OST-5-transfected cells, as this assay was specifically designed to measure the enzymatic activity that generates anticoagulant HS (12). We incubated nonanticoagulant [ $^{35}\text{S}$ ]HS with cell extract and unlabeled PAPS. The resultant [ $^{35}\text{S}$ ]HS was subjected to AT/ConA-affinity gel. We found that about 4.8% of the [ $^{35}\text{S}$ ]HS bound to AT/ConA-affinity gel after incubation with 3-OST-5-transfected cell extract, whereas only 0.12% of the [ $^{35}\text{S}$ ]HS bound to AT/ConA-affinity gel after incubation with the control cell extract (transfected with pcDNA3). Our results demonstrated that the HS<sup>act</sup> conversion activity in 3-OST-5-transfected cells was elevated by 40-fold. The data were consistent with the conclusion that 3-OST-5 has the activity in synthesizing HS<sup>act</sup>.

We compared the composition of the  $^{35}\text{S}$ -labeled disaccharides from 3-OST-5-modified HS<sup>act</sup> and from 3-OST-5-modified HS<sup>inact</sup> (nonantithrombin-binding HS). The HS<sup>inact</sup> and HS<sup>act</sup> were separated by the AT/ConA-affinity approach as described under "Experimental Procedures." Nearly 35% of 3-OST-5-modified HS<sup>act</sup> bound to AT-affinity gel (Table I), suggesting that the fractionation had significantly enriched HS<sup>act</sup>. Both the HS<sup>inact</sup> and HS<sup>act</sup> were degraded with nitrous acid at pH 1.5 followed by sodium borohydride reduction, and the resultant  $^{35}\text{S}$ -labeled disaccharides were analyzed by RPIP-HPLC (Fig. 5). As shown in Fig. 5B,  $^{35}\text{S}$ -labeled GlcUA-AnMan3S6S was the major disaccharide in the 3-OST-5-modified HS<sup>act</sup>. It is noteworthy that GlcUA-AnMan3S6S is a characteristic disaccharide of 3-OST-1-modified HS and is part of the AT-binding site (12). We also noted that GlcUA-AnMan3S6S was still observed in HS<sup>inact</sup>. Two possible reasons might contribute to this



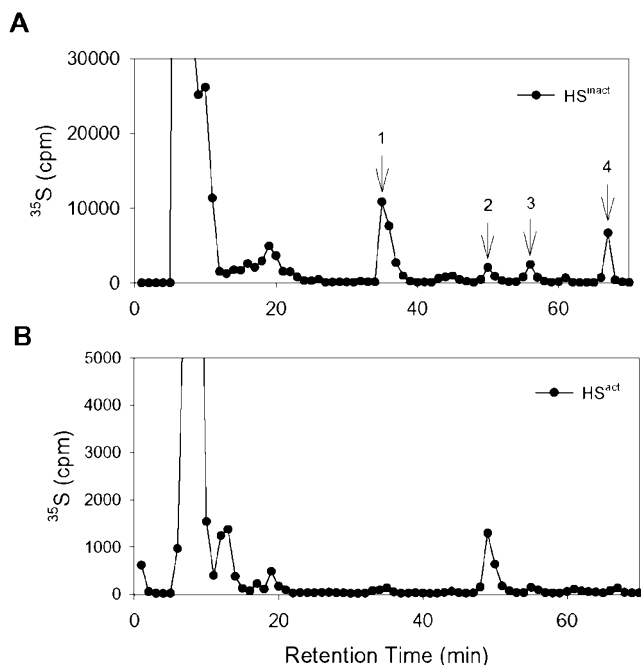


FIG. 5. RPIP-HPLC chromatograms of the disaccharide analysis of 3-OST-5-modified  $\text{HS}^{\text{inact}}$  and  $\text{HS}^{\text{act}}$ .  $\text{HS}^{\text{act}}$  was prepared from 3-OST-5-modified HS using an AT-affinity column.  $\text{HS}^{\text{inact}}$  was the fraction that does not bind to AT-affinity column.  $\text{HS}^{\text{act}}$  was eluted from the AT-affinity column with 1 M NaCl. Both  $\text{HS}^{\text{inact}}$  and  $\text{HS}^{\text{act}}$  were degraded with nitrous acid (pH 1.5) followed by sodium borohydride reduction. The arrows indicate the elution positions of disaccharide standards as described in Fig. 3.

observation. First, the AT-affinity fractionation was incomplete. Second, it is known that  $\text{HS}^{\text{inact}}$  contains GlcUA-AnMan3S6S (12). Taken together, our results demonstrated that 3-OST-5 enzyme generated an AT-binding site, and the  $\text{HS}^{\text{act}}$  contained the disaccharide, GlcUA-AnMan3S6S. In conclusion, our results indicated that 3-OST-5 synthesized both  $\text{HS}^{\text{act}}$  and gD-binding HS (8).

Of particular interesting is that two  $^{35}\text{S}$ -labeled disaccharides, IdoUA2S-AnMan3S and IdoUA2S-AnMan3S6S, were absent in 3-OST-5-modified  $\text{HS}^{\text{act}}$  (Fig. 5B). Those two disaccharides are believed to be parts of the gD-binding site in 3-OST-5-modified HS, which contribute to the activity in assisting HSV-1 entry. The data suggested that 3-OST-5 enzyme sulfates two subpopulations of HS substrates. One population is  $\text{HS}^{\text{act}}$  precursor, which becomes  $\text{HS}^{\text{act}}$  after 3-OST-5 modification. Another population is gD-binding HS precursor, which becomes gD-binding HS after 3-OST-5 modification. The data supported the conclusion from the previous reports (23, 30), which demonstrated that the biosynthesis of  $\text{HS}^{\text{act}}$  is regulated by the availability of the  $\text{HS}^{\text{act}}$  precursors for 3-O-sulfation.

**Tissue Distribution of 3-OST-5**—Northern analysis was carried out on a human Northern multiple tissue blot using 3-OST-5 open reading frame as a probe. It appears that 3-OST-5 is predominantly expressed in skeletal muscle with a size of  $\sim 2.4$  and  $\sim 3.8$  kb (Fig. 6, top panel). The distribution of 3-OST-5 is distinct from those of 3-OST-1 and 3-OST-3A and 3-OST-3B as reported by Shworak and colleagues (28).

#### DISCUSSION

HS is composed of sulfated glucosamine and glucuronic/idoic acid residues. The 3-O-sulfated glucosamine is a rare constituent in the HS from natural sources and plays critical roles in binding to AT (31), herpes simplex virus, type 1, envelope protein gD (13), growth factor receptor (32), and fibroblast growth factor 7 (33). Although we know that the saccharide

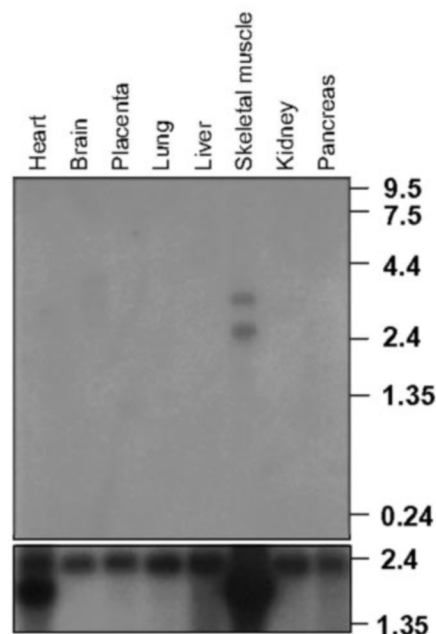


FIG. 6. Expression of 3-OST mRNA in human tissues. A human multiple tissue Northern blot was hybridized with human 3-OST-5 (upper panel) and  $\beta$ -actin (lower panel) cDNA probes labeled with [ $^{32}\text{P}$ ]dCTP under the conditions described under "Experimental Procedures."

sequences of the gD-binding site and AT-binding sites are distinct (13), it still remains to be investigated whether or not the sequences for the bindings of fibroblast growth factor 7 and fibroblast growth factor receptor are identical to the AT-binding site. The 3-O-sulfated glucosamine residue is biosynthesized by 3-OST. At least five different isoforms of 3-OST isoforms have been identified (34). The characteristic disaccharides of the 3-OST-modified HS and the biological functions of the modified HS are summarized in Table II.

In this study, we report a new member of 3-OST family. From our data, we were unable to conclude that the ATG of 3-OST-5 represents the genuine initiation codon because we did not obtain a further 5'-upstream cDNA sequence. It should be noted that 5'-untranslated regions play an important role in determining the translational efficiency of heparan sulfate *N*-deacetylase/*N*-sulfotransferase isoforms in different tissues (35). Nevertheless, the isolated 3-OST-5 contains a transmembrane domain, which predicts a type II membrane-bound protein, and a functional sulfotransferase domain. Those domains are found in the previously characterized full-length 3-OST-2 and 3-OST-3 (28), although the full-length 3-OST-1 lacks transmembrane domain (14). If an additional sequence were present in 3-OST-5 coding region, we would expect that it would encode an additional cytosolic domain. It is known that the cytosolic domains of previously characterized 3-OST do not contribute to the enzymatic activities<sup>3</sup> (11, 14).

Amino acid homology search revealed that 3-OST-5 has 58% homology to 3-OST-3 and 72% homology to 3-OST-1, respectively, in the predicted sulfotransferase domain. The data suggest that the amino acid sequence of 3-OST-5 is closer to 3-OST-1. However, we failed to identify a domain in 3-OST-5 that is specifically homologous to 3-OST-1 or to 3-OST-3, implying that the substrate specificities of 3-OST isoforms are

<sup>3</sup> A truncated 3-OST-3A maintains its activity by removing its intracellular and transmembrane domains. 3-OST-1 enzyme that was originally purified from L-cell media was a truncated form, which lacks the first 20 amino acid residues from the N terminus.

TABLE II  
Summary of the products and biological functions of 3-OST isoform-modified HS

The information for 3-OST-1, 3-OST-2, 3-OST-3A, and 3B was taken from Ref. 34.

3-OST isoforms	The characteristic disaccharides of enzyme modified HS <sup>a</sup>	Biological functions of the enzyme modified HS
3-OST-1	GlcUA-AnMan <b><u>3S</u></b> ±6S <sup>b</sup>	AT-binding HS
3-OST-2	GlcUA2S-AnMan <b><u>3S</u></b> and IdoUA2S-AnMan <b><u>3S</u></b>	Unknown <sup>c</sup>
3-OST-3A	IdoUA2S-AnMan <b><u>3S</u></b> ±6S	Entry receptor for HSV-1
3-OST-3B	IdoUA2S-AnMan <b><u>3S</u></b> ±6S	Entry receptor for HSV-1
3-OST-4	Unknown	Unknown <sup>c</sup>
3-OST-5	GlcUA-AnMan <b><u>3S6S</u></b> and IdoUA2S-AnMan <b><u>3S</u></b> ±6S	AT-binding HS and entry receptor for HSV-1

<sup>a</sup> The disaccharides were prepared by subjecting the enzyme-modified HS to the degradations of nitrous acid.

<sup>b</sup> The 3-O-sulfate group is shown in boldface and underlined to emphasize the modification.

<sup>c</sup> A recent review by Shukla and Spear (19) indicated that 3-OST-2 and 3-OST-4 generate entry receptors for HSV-1.

determined by the three-dimensional structures of the enzymes. We noted that 3-OST-1 is a secreted protein because it lacks a transmembrane domain (14), whereas 3-OST-5 is likely a membrane-bound protein with a predicted transmembrane domain. Indeed, we failed to detect 3-OST-5 activity in the media.<sup>4</sup> A recent report (36) demonstrated that the transmembrane domain of the 3-OST had no effect on their substrate specificities. Based upon their results, we concluded that the additional transmembrane domain of 3-OST-5 is unlikely to provide profound effect on the activities in synthesizing HS<sup>act</sup> and gD-binding HS.

The substrate specificity of 3-OST-5 was characterized by determining the identities of the <sup>35</sup>S-labeled disaccharides from low pH nitrous acid-degraded 3-OST-5-modified HS. We found that the enzyme generates at least three 3-O-sulfated disaccharides as follows: IdoUA2S-AnMan**3S**, GlcUA-AnMan**3S6S**, and IdoUA2S-AnMan**3S6S**.<sup>5</sup> Among these disaccharides, IdoUA2S-AnMan**3S** and IdoUA2S-AnMan**3S6S** are the characteristic disaccharides of 3-OST-3-modified HS, whereas GlcUA-AnMan**3S6S** is a characteristic disaccharide of 3-OST-1-modified HS (11, 12). Thus, we concluded that 3-OST-5 possesses both the activities of 3-OST-1 and 3-OST-3. It is known that 3-OST-1 generates HS<sup>act</sup>, whereas 3-OST-3 generates entry receptor for HSV-1 (12, 13). As expected, 3-OST-5-modified HS binds to AT and gD. In addition, transfection of the plasmid expressing 3-OST-5 rendered the susceptibility of HSV-1 to wild type CHO cells. Because both 3-O-sulfated glucosamine N-sulfate (GlcNS3S±6S) and 3-O-sulfated N-unsubstituted glucosamine (GlcNH<sub>2</sub>3S±6S) are susceptible to low pH nitrous acid degradation (8), we could not conclude whether or not 3-OST-5 sulfates N-unsubstituted glucosamine or N-sulfated glucosamine. Such a conclusion will become possible when a purified 3-OST-5 enzyme is available. Regardless of the limitations in the disaccharide analysis, our results indicate that 3-OST-5 has both 3-OST-1- and 3-OST-3-activities. It should be noted that two additional isoforms of 3-OST, 3-OST-2 and 3-OST-4, were reported (28). It is known that 3-OST-2 does not generate HS<sup>act</sup> (8).

Our Northern analysis suggests that 3-OST-5 is predominantly expressed in the human skeletal muscle tissue. A previous report demonstrated that 3-OST-1 and 3-OST-3 (including 3-OST-3A and 3-OST-3B) were widely expressed in numerous human tissues, but low in skeletal muscle (28). The tissue distribution of 3-OST-5 suggests that 3-OST-5-modified

HS may provide a unique biological function in this tissue. It is very interesting to note that a unique subset of HS was identified in human skeletal muscle tissues by a set of antibodies that bind to HS (37). An elegant study was recently published (38) to determine the binding sequences of these antibodies. However, it still remains to be investigated whether or not the unique subset of HS found in the skeletal muscle tissue is associated with 3-OST-5 modification.

Clinical manifestations of herpes simplex virus are typically seen in two types of cells as follows: mucosal epithelium with characteristic herpetic lesions, and in a few cases, neuronal cells, causing life-threatening encephalitis. Acute viremia exists but is very rarely seen in infected patients. Based on the current knowledge, it is unlikely that infection of HSV in skeletal muscle has long term pathogenic effects. It is very interesting to note that recent studies (39, 40) show that herpes simplex virus, type 1, can be utilized as a viral vector to deliver genes to the human skeletal muscle. For example, a specially engineered herpes simplex virus 1 was used for Duchenne muscular dystrophy (39). The advantages for using herpes simplex virus, type 1, vector include high transduction efficiency and the capability to pack a large gene (40). Given the facts that 3-OST-5-modified HS serves as an entry receptor for HSV-1 and it is expressed in the human skeletal muscle, it is possible that the 3-OST-5-modified HS could serve as a receptor in skeletal muscle tissues for HSV-1 infection. If this is the case, further investigation of the mechanism for the 3-OST-5-assisted HSV-1 entry will be beneficial for not only understanding/modulating the pathogenic effects of the virus but also for its improved use as an important gene therapy vector for treating muscular dystrophy.

**Acknowledgments**—We thank Drs. Gary Cohen and Roselyn Eisenberg (University of Pennsylvania) for providing purified recombinant gD and anti-gD monoclonal antibody (DL6), Dr. Robert Rosenberg (Massachusetts Institute of Technology) for providing the <sup>3</sup>H-labeled disaccharide standards, and Dr. Pat Spear (Northwestern University) for providing recombinant HSV-1 (HSV-1(KOS)gL86). We also thank Drs. Jude Samulski and Rihe Liu (University of North Carolina) for insightful discussions.

#### REFERENCES

- Liu, J., and Thorp, S. C. (2002) *Med. Res. Rev.* **22**, 1–25
- Rosenberg, R. D., Showrak, N. W., Liu, J., Schwartz, J. J., and Zhang, L. (1997) *J. Clin. Invest.* **99**, 2062–2070
- Bernfield, M., Gotte, M., Park, P. W., Reizes, O., Fitzgerald, M. L., Lincecum, J., and Zako, M. (1999) *Annu. Rev. Biochem.* **68**, 729–777
- Alexander, C. M., Reichsman, F., Hinkes, M. T., Lincecum, J., Becker, K. A., Cumberledge, S., and Bernfield, M. (2000) *Nat. Genet.* **25**, 329–332
- Reizes, O., Lincecum, J., Wang, Z., Goldberger, O., Huang, L., Kaksonen, M., Ahima, R., Hinkes, M. T., Barsh, G. S., Rauvala, H., and Bernfield, M. (2001) *Cell* **106**, 105–116
- Lindahl, U., Kusche-Gullberg, M., and Kjellen, L. (1998) *J. Biol. Chem.* **273**, 24979–24982
- Esko, J. D., and Lindahl, U. (2001) *J. Clin. Invest.* **108**, 169–173
- Liu, J., Showrak, N. W., Sinay, P., Schwartz, J. J., Zhang, L., Fritze, L. M. S., and Rosenberg, R. D. (1999) *J. Biol. Chem.* **274**, 5185–5192
- Aikawa, J.-I., Grobe, K., Tsujimoto, M., and Esko, J. D. (2001) *J. Biol. Chem.* **276**, 5876–5882
- Habuchi, H., Tanaka, M., Habuchi, O., Yoshida, K., Suzuki, H., Ban, K., and Kimata, K. (2000) *J. Biol. Chem.* **275**, 2859–2868

<sup>4</sup> 3-OST-1 is a secreted enzyme. The activity was readily detected in the media (14). The failure for detecting 3-OST-5 activity in the media also suggests that it was unlikely that the transfection of the plasmid expressing 3-OST-5 activates the expression of previously known 3-OST-1 in COS-7 cells.

<sup>5</sup> We noted that 3-OST-1-modified HS also generates GlcUA-AnMan**3S**. We did not investigate whether 3-OST-5 generates GlcUA-AnMan**3S** because we utilized the RPIP-HPLC condition that is specifically designed to resolve disulfated or trisulfated disaccharides. Thus, we still do not know if 3-OST-5 generates GlcUA-AnMan**3S**.

11. Liu, J., Shriver, Z., Blaiklock, P., Yoshida, K., Sasisekharan, R., and Rosenberg, R. D. (1999) *J. Biol. Chem.* **274**, 38155–38162
12. Liu, J., Shworak, N. W., Fritze, L. M. S., Edelberg, J. M., and Rosenberg, R. D. (1996) *J. Biol. Chem.* **271**, 27072–27082
13. Shukla, D., Liu, J., Blaiklock, P., Shworak, N. W., Bai, X., Esko, J. D., Cohen, G. H., Eisenberg, R. J., Rosenberg, R. D., and Spear, P. G. (1999) *Cell* **99**, 13–22
14. Shworak, N. W., Liu, J., Fritze, L. M. S., Schwartz, J. J., Zhang, L., Logeart, D., and Rosenberg, R. D. (1997) *J. Biol. Chem.* **272**, 28008–28019
15. WuDunn, D., and Spear, P. G. (1989) *J. Virol.* **63**, 52–58
16. Liu, J., Shriver, Z., Pope, R. M., Thorp, S. C., Duncan, M. B., Copeland, R. J., Raska, C. S., Yoshida, K., Eisenberg, R. J., Cohen, G., Linhardt, R. J., and Sasisekharan, R. (2002) *J. Biol. Chem.* **277**, 33456–33467
17. Willis, S. H., Rux, A. H., Peng, C., Whitbeck, C., Nicola, A. V., Lou, H., Hou, W., Salvador, L., Eisenberg, R. J., and Cohen, G. (1998) *J. Virol.* **72**, 5938–5947
18. Krummenacher, C., Rux, A. H., Whitbeck, J. C., Ponce-de-Leon, M., Lou, H., Baribaud, I., Hou, W., Zou, C., Geraghty, R. J., Spear, P. G., Eisenberg, R. J., and Cohen, G. H. (1999) *J. Virol.* **73**, 8127–8137
19. Shukla, D., and Spear, P. G. (2001) *J. Clin. Invest.* **108**, 503–510
20. Carfi, A., Willis, S. H., Whitbeck, J. C., Krummenacher, C., Cohen, G. H., Eisenberg, R. J., and Wiley, D. C. (2001) *Mol. Cell* **8**, 169–179
21. Hernaiz, M., Liu, J., Rosenberg, R. D., and Linhardt, R. J. (2000) *Biochem. Biophys. Res. Commun.* **276**, 292–297
22. Bjornsson, S. (1993) *Anal. Biochem.* **210**, 282–291
23. Zhang, L., Yoshida, K., Liu, J., and Rosenberg, R. D. (1999) *J. Biol. Chem.* **274**, 5681–5691
24. Nicola, A. V., Willis, S. H., Naidoo, N. N., Eisenberg, R. J., and Cohen, G. H. (1996) *J. Virol.* **70**, 3815–3822
25. Shworak, N. W., Shirakawa, M., Collic-Jouault, S., Liu, J., Mulligan, R. C., Birinyi, L. K., and Rosenberg, R. D. (1994) *J. Biol. Chem.* **269**, 24941–24952
26. Shively, J. E., and Conrad, H. E. (1976) *Biochemistry* **15**, 3932–3942
27. Negishi, M., Pedersen, L. G., Petrotchenko, E., Shevtsov, S., Gorokhov, A., Kakuta, Y., and Pedersen, L. C. (2001) *Arch. Biochem. Biophys.* **390**, 149–157
28. 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
29. Shieh, M.-T., WuDunn, D., Montgomery, R. I., Esko, J. D., and Spear, P. G. (1992) *J. Cell Biol.* **116**, 1273–1281
30. Zhang, L., Beeler, D. L., Lawrence, R., Lech, M., Liu, J., Davis, J. C., Shriver, Z., Sasisekharan, R., and Rosenberg, R. D. (2001) *J. Biol. Chem.* **276**, 42311–42321
31. Lindahl, U., Backstrom, G., Thunberg, L., and Leder, I. G. (1980) *Proc. Natl. Acad. Sci. U. S. A.* **77**, 6551–6555
32. McKeehan, W. L., Wu, X., and Kan, M. (1999) *J. Biol. Chem.* **274**, 21511–21514
33. Ye, S., Luo, Y., Lu, W., Jones, R. B., Linhardt, R. J., Capila, I., Toida, T., Kan, M., Pelletier, H., and McKeehan, W. L. (2001) *Biochemistry* **40**, 14429–14439
34. Liu, J., and Rosenberg, R. D. (2002) in *Handbook of Glycosyltransferases and Their Related Genes* (Taniguchi, N., and Fukuda, M., eds) pp. 475–483, Springer-Verlag, Tokyo
35. Grobe, K., and Esko, J. D. (2002) *J. Biol. Chem.* **277**, 30699–30706
36. Yabe, T., Shukla, D., Spear, P. G., Rosenberg, R. D., Seeberger, P. H., and Shworak, N. W. (2001) *Biochem. J.* **359**, 235–241
37. Jenniskens, G. J., Oosterhof, A., Brandwijk, R., Veerkamp, J. H., and van Kuppevelt, T. H. (2000) *J. Neurosci.* **20**, 4099–4111
38. Dennissen, M. A. B. A., Jenniskens, G. J., Pieffers, M., Versteeg, E. M. M., Petitou, M., Veerkamp, J. H., and van Kuppevelt, T. H. (2002) *J. Biol. Chem.* **277**, 10982–10986
39. Akkaraju, G. R., Huard, J., Hoffman, E. P., Goins, W. F., Pruchnic, R., Watkins, S. C., Cohen, J. B., and Glorioso, J. C. (1999) *J. Gene Med.* **1**, 280–289
40. Wang, Y., Fraefel, C., Protasi, F., Moore, R. A., Fessenden, J. D., Pessah, I. N., Difrancesco, A., Breakefield, X., and Allen, P. D. (2000) *Am. J. Physiol.* **278**, C619–C626