Overexpression of Heparan Sulfate 6-O-Sulfotransferases in Human Embryonic Kidney 293 Cells Results in Increased N-Acetylglucosaminy1 6-O-Sulfation* 

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Heparan sulfate (HS), a linear polysaccharide covalently attached to various core proteins, is expressed ubiquitously in animal cells and found at the cell surface and in the extracellular matrix (1). HS chains are known to interact with a broad array of proteins such as extracellular matrix molecules, growth factors, morphogens and their receptors, and thereby modulate their biological functions. Therefore, HS plays important roles in a variety of developmental, physiological, and pathological processes (2, 3). The actions of HS largely depend on the amount and distribution of its sulfate groups that provide specific binding sites for proteins. Especially the spacing of sulfate groups plays important roles in generating biologically active HS.

Assembly of HS is a multistep process involving many different endoplasmic reticulum and Golgi resident enzymes (3). The enzymes catalyze a series of reactions including chain polymerization (alternating addition of glucuronic acid (GlcUA) and N-acetylgalactosamine (GlcNAc) residues), N-deacylation and N-sulfation of GlcNAc residues, epimerization of GlcUA to iduronic acid (IdoUA), 2-O-sulfation of uronic acid residues and 6-O- and 3-O-sulfation of GlcN residues (2–4). The end product is highly heterogeneous containing complex and distinct sequences based on variable patterns of N-acetyl, N-sulfate, and O-sulfate groups (2–4). Partial N-deacylation/N-sulfation of GlcNAc units allow approximately half of the GlcNAc residues to escape N-sulfation, limiting the extent of epimerization and O-sulfation, reactions that are essentially confined to regions containing N-sulfates. Essentially three different domain structures are found in HS chains: NS, NA/NS, and NA domains. The NS domains consist of contiguous N-sulfated regions containing both IdoUA and GlcUA and a variable number of O-sulfates, the less sulfated NA/NS domains are mixed sequences of alternating N-acetylated and N-sulfated disaccharide units that are believed to flank the NS domains and separating them from the unsulfated NA domains that consist of consecutive N-acetylated regions with GlcUA as the only hexuronic acid moiety (5). The regulation of the biosynthesis, as required to generate specific domain structures, is poorly understood, but it apparently involves arrays of enzyme isoforms that differ with regard to substrate specificities and kinetic properties. The N-deacylase/N-sulfotransferase (NDST) (6), 6-O-sulfotransferase (6-OST) (7), and the 3-O-sulfotransferase (3-OST) (8–10) families each contain several members, whereas only one GlcUA C5 epimerase (11, 12) and one hexuronic acid 2-O-sulfotransferase (2-OST) (13, 14) have been described.

Recent studies have indicated the importance of 6-O-sulfation in developmental processes. In Drosophila, 6-O-sulfation is catalyzed by a single 6-OST that is expressed specifically in mesodermal and tracheal cells (15). RNA interference-mediated disruption of its single 6-OST resulted in severe changes in fibroblast growth factor signaling (15). Morpholino-mediated functional knock-downs of 6-OSTs in zebrafish led to disturbed muscle development and angiogenesis (16, 17). Moreover, Caenorhabditis elegans animals with null mutations in the single ortholog of the 6-OST exhibit defects in the nervous system development (18). In Xenopus 6-O-sulfation of HS is important for retinal axon guidance (19). In contrast to Xenopus and Drosophila, more complex organisms have multiple 6-OST enzymes. Two 6-OSTs have been described in zebrafish and chicken, and three in mouse and human (7,
The expression pattern of each 6-OST transcript varies during development (21, 23). In the early mouse embryo, 6-OST1 mRNA is expressed predominantly in epithelial and neural derived tissues, whereas 6-OST2 is more highly expressed in mesenchymal derived tissues. 6-OST3 transcript appears later than the other two and in a more restricted manner (23). In the adult mouse 6-OST1 is highly expressed in liver, 6-OST2 is preferentially expressed in the brain and spleen, whereas 6-OST3 has a diffuse expression in all tissues examined (7).

Specific recognition between proteins and HS may require defined sequences within the HS chain. The length and position of NS domains as well as 2-O- and 6-O-sulfation patterns are likely factors to influence binding (4). Very little is known about how different 6-OST-sulfation patterns are created. 6-O-Sulfate groups occur on glucosamine residues mainly in -GlcUA/IdoUA-GlcNS6S-, -GlcUA-GlcNS3S6S-, -GlcUA/IdoUA-GlcNAc6S- and in -GlcUA2S/IdoUA2S-GlcNS6S-disaccharide units. The three mouse 6-OST enzymes are all capable of sulfating all potential target structures in vitro, although with different efficiency (24). Yet, the roles of each individual 6-OST in generating specific HS protein-binding sequences and their functions in vivo remain unknown. A strict control of the expression of 6-OSTs may be one of the means for the cell to regulate HS structure and function, and alteration in the expression levels of the three 6-OSTs might influence HS structure we have characterized HS synthesized by human embryonic kidney (HEK) 293 cells after stable transfection of mouse 6-OST1, 6-OST2, and 6-OST3 enzymes. HS in the transfected cells showed increased formation of -GlcUA-GlcNS6S- and -GlcUA-GlcNAc6S-disaccharide units. The different isoforms gave rise to similar 6-O-sulfation patterns, but the level of expression had a dramatic influence on the structural properties of the polysaccharide, suggesting a need for strict regulation of 6-OST levels in HS biosynthesis.

**EXPERIMENTAL PROCEDURES**

Expression of Mouse 6-O-Sulfotransferases—A full-length mouse cDNA clone coding for 6-OST1 was amplified from mouse liver total RNA (a gift from D. Gullberg) using Superscript™ II One-step reverse transcription-PCR (Invitrogen) with the sense primer 5′-ACC ATG GTT GAG CGC GCC AGC AAG TTC GT-3′ and the antisense primer 5′-TGC CCC TGA CAC TAC TAC CA-3′. The amplified product was cloned into TOPO TA plasmid (Invitrogen). Insertion of the entire coding region for 6-OST1 was confirmed by sequence analysis. The insert was excised with EcoRI and subcloned into the corresponding site of the pcDNA3 plasmid vector (Invitrogen). The 6-OST2 and 6-OST3 expression constructs have been described previously (25). The different expression plasmids were transfected into HEK 293 cells using Lipofectamine (Invitrogen). Stably transfected cell clones expressing 6-OST1, -2, or -3, and control clones transfected with vector alone, were selected as described previously (26). Cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% (v/v) fetal calf serum (Invitrogen), 100 µg/ml streptomycin sulfate, 100 units/ml penicillin G, and 400 µg/ml Geneticin (G418 sulfate, Invitrogen).

O-Sulfotransferase Assay—HEK cell clones containing mouse 6-OSTs were further selected by measuring the incorporation of $^{35}$S from $[^{35}S]PAPS$ into HS after a 16-hour pulse with fresh medium (27). The standard reaction mixture for measuring O-sulfotransferase activity contained 50 mM HEPES (pH 7.4), 1% (v/v) Triton X-100, 100 mM MnCl$_2$, 5 mM CaCl$_2$, 3.5 µM NaF, 100 µM unlabeled PAPS, 2 µCi of $[^{35}S]PAPS$, 20 µg of de-O-sulfated heparin, 5 µl of cell lysate in a final volume of 50 µl. After incubation for 30 min at 37 °C, the reaction was stopped by heating at 100 °C for 3 min, and 0.2 mg of heparin added as a carrier. After ethanol precipitation, the pellets were dissolved in water, and $^{35}$S-labeled polysaccharides were separated from unreacted $[^{35}S]PAPS$ by centrifugation through Sephadex G-25 (27). The eluent, containing the $^{35}$S-labeled polysaccharides, was quantified by scintillation counting. All assays were made in duplicate.

Isolation of Metabolically Radiolabeled HS—Subconfluent cultures of 6-OST1-, 6-OST2-, and 6-OST3-overexpressing cells and control cells were labeled with 200 µCi/ml Na$_2$[35SO$_4$]$_2$ (1494 Ci/mmol; PerkinElmer Life Sciences) or 50 µCi/ml [6-$^3$H]glucosamine HCl (24 Ci/mmol; Amersham Biosciences) at 37 °C for 24 h. After incubation, the culture medium was removed and frozen at −20 °C for further use. The cell layer was washed twice with cold phosphate-buffered saline and solubilized at 4 °C for 60 min in 0.05 M Tris/HC1 (pH 7.4), 1% (v/v) Triton X-100, and protease inhibitors (2 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 2 mM N-ethylmaleimide, and 10 µg/ml pepstatin A). After centrifugation at 400 × g for 15 min, the supernatant was collected, and a 100-µl aliquot was removed for protein determination using the BCA protein determination kit (Pierce). The remaining supernatant was used for purification of the labeled polysaccharides. The sample was adjusted to 0.15 M NaCl and applied to a 300-µl DEAE-Sephalocolumn (Amersham Biosciences) equilibrated in 0.15 M NaCl, 0.05 M Tris/HC1 (pH 7.4), 0.1% (v/v) Triton X-100. The column was washed with the equilibration buffer, followed by 0.15 M NaCl, 0.05 M acetate buffer (pH 4.0), 0.1% (v/v) Triton X-100. The pH was increased to 7.4 with 0.15 M NaCl Tris/HC1 (pH 7.4), 0.1% (v/v) Triton X-100, and finally the labeled macromolecules were eluted with 2 M NaCl. After desalting by gel chromatography using PD-10 columns (Sephadex G-25, Amersham Biosciences) in 10% ethanol, the polysaccharide chains were released from the core protein by treatment with 0.5 M NaOH overnight at 4 °C. After completed B-elimination the samples were neutralized with 4 M HCl and desalted by gel chromatography using PD-10 columns eluted with 0.2 M NH$_4$HCO$_3$ followed by lyophilization. Nucleic acids were digested with Benzonase (Merck) and galactosaminoglycans eliminated by digestion with chondroitinase ABC (Seikagaku). Resistant HS chains were recovered after gel chromatography on a column (1 × 180 cm) of Sephadex G-25 superfine in 0.2 M NH$_4$HCO$_3$ and desalted by lyophilization.

Anionic Properties of Labeled HS—Labeled HS samples were applied to a 1-ml column of DEAE-Sephalocolumn (Amersham Biosciences) equilibrated with 0.2 M NaCl, 0.05 M Tris/HC1 (pH 7.5). The column was washed with >10 ml of the equilibration buffer and was then eluted with a 50-ml linear gradient, ranging from 0.2 to 1.5 M NaCl in 0.05 M Tris/HC1 (pH 7.5) at a flow rate of 0.5 ml/min. Fractions of 1 ml were collected. In analytical runs, the labeled HS samples were mixed with internal standards of Escherichia coli K5 capsular polysaccharide (0.6 mg) and chondroitin sulfate A (0.5 mg). A 50-µl aliquot from each effluent fraction was subjected to uronic acid determination by the carbazol method (28). The remaining material was monitored for radioactivity.

Preparative DEAE anion-exchange chromatography of labeled HS was performed using the same column without added internal standards.

Domain Structure Analysis of Labeled HS—Isolated $[^{35}S]$-labeled HS or $[^{35}S]/[^{3}H]$GlcN double-labeled HS chains were cleaved at N-sulfated GlcN residues by deamination with nitrous acid at pH 1.5 followed by reduction with NaBH$_4$ (29). The resultant oligosaccharides were separated by gel chromatography on a column (1 × 185 cm) of Bio-Gel P-10.
Heparan Sulfate 6-O-Sulfation

TABLE 1

Glycosaminoglycan synthesis in control and transfected HEK 293 cells

HEK 293 cells were transfected with vector alone (mock) or with plasmids containing 6-OST1, 6-OST2, or 6-OST3 cDNA. Cells were metabolically labeled with [35S] or [3H]GlcN for 24 h. The labeled glycosaminoglycans were extracted from the cell layer and the medium (see “Experimental Procedures”) and quantified. The values are given from single incubations or as the means from two to four independent incubations ± mean deviation.

| Clone          | Level of overexpression | Glycosaminoglycan produced                                      |
|---------------|------------------------|----------------------------------------------------------------|
|               |                        | 35S-Labeled cell lysate | 35S-Labeled cell medium | 3H-labeled cell lysate |
|               |                        | HS                 | CS              | HS                 | CS              | HS                 | CS              |
|               |                        | %                  | %               | %                  | %               | %                  | %               |
| HEK 293**     | –                      | 67 ± 5             | 33 ± 5          | 40 ± 4             | 60 ± 4          | ND**               | ND              |
| Mock          | –                      | 64 ± 8             | 36 ± 10         | ND                 | ND              | ND                 | ND              |
| 6-OST1        | +                     | 63 ± 4             | 37 ± 4          | 44                 | 56              | 59                 | 41              |
| 6-OST2        | +                     | 64 ± 5             | 36 ± 5          | 48                 | 52              | 55                 | 45              |
| 6-OST3        | + +                   | 58                 | 42              | ND                 | ND              | ND                 | ND              |
|               | + ++                  | 56                 | 44              | 45                 | 55              | ND                 | ND              |
|               | + + ++                | 61                 | 39              | ND                 | ND              | ND                 | ND              |
|               | + + + + +             | 64 ± 8             | 36 ± 8          | ND                 | ND              | ND                 | ND              |
|               | + + + + + +           | 61 ± 9             | 39 ± 9          | 33 ± 3             | 67 ± 3          | ND                 | ND              |

*6-OST overexpression resulted in increased O-sulfotransferase activity measured on a protein basis (cpm/mg of protein), which varied between low (+), medium (++), high (+++), and very high (++++) in different cellular clones. + corresponds to a 1–7-fold increase and ++++ to a 29-fold increase, respectively. See also the “Results.”

**Material resistant to chondroitinase ABC.

†Material susceptible to chondroitinase ABC.

‡Untransfected 293 cells.

ND, not determined.

(fine grade, Bio-Rad) in 0.2 M NH₄HCO₃. Alternatively, the [35S]-labeled chains were first chemically N-deacetylated by treatment with 70\% (w/v) aqueous hydrazine (Fluka) containing 1\% (w/v) hydrazine sulfate at 96 °C for 4 h (30), and the product was treated with nitrous acid at pH 3.9. The oligosaccharides thus obtained by cleavage at the generated N-unsulfated GlcN units were reduced with NaBH₄ (29) and fractionated by gel filtration on the Bio-Gel P-10 column.

Compositional Analysis of Labeled HS—Labeled HS chains were depolymerized with nitrous acid at pH 1.5 (as described above). The labeled deamination products were fractionated by gel chromatography on Sephadex G-15 (1 × 180 cm) in 0.2 M NH₄HCO₃. Fractions corresponding to oligo- and disaccharides were collected and desalted by lyophilization. For total depolymerization to disaccharides, labeled HS chains were chemically N-deacetylated as above, and the products were treated with nitrous acid at pH 1.5 and at 3.9 (29) followed by reduction with NaBH₄. Labeled disaccharides were analyzed by anion-exchange HPLC using a Whatman Partisil 10-SAX column eluted with aqueous KH₂PO₄ of stepwise increasing concentration at a rate of 1 ml/min (24).

Disaccharide Analysis of Unlabeled HS—Unlabeled HS for reversed phase ion pairing HPLC analysis was prepared from selected 6-OST-1, 6-OST-2, and 6-OST-3-overexpressing cell clones. Cells were cultured as described above. For harvest, cells were washed with phosphate-buffered saline and detached by scraping with 5 ml of cold phosphate-buffered saline, collected after centrifugation at 16,000 × g for 5 min and lyophilized. HS was prepared from lyophilized cell pellets, digested with heparin lyases, and analyzed as described in Ref. 31.

RESULTS

HEK 293 cells were stably transfected with cDNAs corresponding to the coding regions of mouse 6-OST1, 6-OST2, and 6-OST3, respectively. Approximately 20 individual clones each of 6-OST1-, 6-OST2-, and 6-OST3-transfected cells were analyzed for mRNA levels and O-sulfotransferase activity. Northern blot analyses resulted in hybridization signals of different intensities, indicating that the transfections had resulted in clones with different expression levels of 6-OST1, 6-OST2, or 6-OST3 mRNA (not shown). In contrast, no signal was obtained from mock (vector-transfected) or wild type (nontransfected) 293 cells. The overexpression resulted in increased O-sulfotransferase activity (measured as described under “Experimental Procedures”) that varied among different cell clones from 2- to 7-fold increase (low, +) to ≥29-fold increase (very high, +++++) in 6-OST1 and 6-OST3 transfecants, whereas for 6-OST2 transfecants, the increased activity ranged from 2- to 7-fold (low, +) to an ~22-fold increase (high, ++ +). The increase in O-sulfotransferase activity essentially correlated well with the observed differences in mRNA levels.

Selected stable cell clones were metabolically labeled with [3H]GlcN or [35S]Labeled glycosaminoglycans were isolated from the cell layer and the culture medium and quantified. No apparent differences were noted in the yields of [3H]GlcN- or [35S]-labeled glycosaminoglycans (calculated as cpm/mg of cellular protein or as cpm/culture flask, data not shown) nor in the relative proportions of labeled glycosaminoglycans (chondroitin sulfate (CS) versus HS) between 6-OST-transfected and control cell clones (Table 1). Thus, 6-OST transfections did not appear to affect the amounts nor the sulfation degree of the glycosaminoglycans synthesized. Notably, HEK 293 cells overexpressing HS NDST-2 or 2-OST showed, in contrast to the 6-OST-overexpressing cells, 10–25% increased proportion of [35S]HS, i.e. material resistant to digestion by chondroitinase ABC (26, 32).

Effect of 6-OST Overexpression on HS Fine Structure—For structural analysis the various 35S-labeled samples were treated with chondroitinase ABC to degrade any galactosaminoglycans present in the samples. Chondroitinase ABC-resistant labeled polysaccharides were depolymerized with nitrous acid at pH 1.5 and reduced with NaBH₄ resulting in cleavage of the chains at N-sulfated GlcN residues and conversion of GlcNS units to aMan₉. Consecutive N-sulfated disaccharides will be recovered as disaccharides, whereas the alternating NA/NS sequences will yield tetrasaccharides. Regions of unmodified NA domains yield oligosaccharides of at least 6-mer size. Separation of the resulting deamination products by gel chromatography on Sephadex G-15 showed that the control HS had been converted to a major 35S-labeled disaccharide peak (~80–90%) and in addition smaller peaks of tetra- and longer oligosaccharides (Fig. 1A). In contrast, deamination of HS produced by the transfected cells resulted in increased formation of longer 35S-labeled oligosaccharides resistant to deamination (Fig. 1, B and C). The amount of such deamination-resistant sulfated oligosaccharides increased with the level of 6-OST expression and was isoform-independent, although more noticeable in HS isolated from 6-OST3-overexpressing cells. Labeled oligosaccharides (~6, pooled as indicated
in Fig. 1C) were resistant to further degradation with chondroitinase ABC or low pH deamination and were thus not the result of incomplete digestion and/or deaminative cleavage (data not shown). They were however completely degraded to disaccharides by treatment with hydrazine followed by combined deamination at pH 1.5 and 3.9 and then by reduction of products with NaBH4. The resulting labeled disaccharides were analyzed by anion-exchange HPLC (see “Experimental Procedures”). The values are given from single analyses or as the means from two independent experiments ± mean deviation.

| Clone | Level of overexpression | GlcUA-aMan6S | IdoUA-aMan6S | IdoUA2S-aMan6S | IdoUA2S-aMan6S | GlcUA-aMan6S | IdoUA-aMan6S | IdoUA2S-aMan6S | IdoUA2S-aMan6S |
|-------|-------------------------|--------------|--------------|----------------|----------------|--------------|--------------|----------------|----------------|
| HEK 293 | —                       | 15 ± 1       | 8 ± 1        | 49 ± 1          | 28 ± 2         | 21 ± 6       | 28 ± 2       | 34 ± 5         | 17 ± 1         |
| Mock  | —                       | 23 ± 1       | 12 ± 1       | 46 ± 5          | 19 ± 6         | 21 ± 5       | 27           | 23 ± 13        | 13             |
| 6-OST1 | +                       | 22           | 10           | 41             | 27             | 37           | 27           | 23 ± 13        | 13             |
|       | ++                      | 40 ± 2       | 18 ± 7       | 21 ± 9         | 21 ± 5         | 37           | 27           | 23 ± 13        | 13             |
|       | +++                     | 51           | 23           | 7              | 19             | 58           | 26           | 10 ± 6         | 6              |
| 6-OST2 | ++++                    | 76           | 11           | 4              | 9              | 47           | 28           | 12 ± 13        | 13             |
|       | + (+)                   | 45 ± 3       | 11 ± 2       | 26 ± 2         | 18 ± 3         | 65           | 19           | 8              | 8              |
| 6-OST3 | +++                     | 69           | 10           | 12             | 9              | 47           | 28           | 12 ± 13        | 13             |
|       | ++++                    | 51 ± 5       | 12 ± 1       | 16 ± 1         | 21 ± 6         | 65           | 19           | 8              | 8              |

* As in Table 1.
* Untransfected 293 cells.

The increase in GlcUA-aMan6S was proportional to the level of 6-OST overexpression and not isoform-dependent (see Table 2). Very high levels of 6-OST activity resulted in the formation of ~80% GlcUA-aMan6S, indicating that high 6-OST expression resulted in N-sulfated domains composed essentially of -GlcUA-GlcNS6S-disaccharide units in the intact HS. The overall disaccharide composition of HS from transfected and control cells was determined after N-deacetylation and complete deaminative cleavage of [3H]GlcN-labeled samples to disaccharides. Again, the most striking effect induced by 6-OST overexpression was the increase in GlcUA-aMan6S-disaccharides (Table 2). In the complete deaminative cleavage process both GlcNS and GlcNAc residues are converted to aMan6S residues, and therefore the relative amounts of GlcNAc6S and GlcNS6S were not determined. To characterize fully the
Heparan Sulfate 6-O-Sulfation

TABLE 3
Disaccharide composition of unlabeled HS from mock- or 6-OST-transfected HEK 293 cells

Total cellular HS (unlabeled) was degraded to disaccharides (digestion with heparin lyases I, II, and III) that were analyzed by reversed phase ion pairing HPLC (see “Experimental Procedures”). The values are given from single analyses or as means from two independent experiments ± mean deviation.

| Clones       | Level of overexpression | ΔHexUA-GlcNAc | ΔHexUA-GlcNS | ΔHexUA-GlcNAc6S | ΔHexUA-GlcNS6S | ΔHexUA2S-GlcNS | ΔHexUA2S-GlcNS6S | Total 6-O-sulfation |
|--------------|-------------------------|---------------|--------------|-----------------|----------------|-----------------|-------------------|---------------------|
|              |                         | % of total disaccharides |
| Mock         | −                       | 51.5 ± 0.5    | 12.5 ± 3.5   | 17.5 ± 2       | 4.5 ± 1.5      | 8.5 ± 1.5       | 5.5 ± 0.5         | 27 ± 2              |
| 6-OST1       | ++                      | 46 ± 2        | 9.5 ± 5.5    | 23 ± 3         | 9 ± 3          | 7.5 ± 1.5       | 5 ± 1             | 37 ± 7              |
| 6-OST2       | ++                      | 36 ± 6        | 13 ± 1.5     | 31 ± 3         | 13 ± 2         | 2 ± 1           | 5 ± 1             | 49 ± 14             |
| 6-OST3       | +++                     | 39 ± 4        | 8 ± 2        | 33.5 ± 0.5     | 11.5 ± 4.5     | 4.5 ± 2.5       | 3.5 ± 0.5         | 48.5 ± 5.5          |
| 6-OST2       | +++                     | 45.5 ± 2.5    | 9 ± 3        | 27.5 ± 3.5     | 10 ± 4         | 4 ± 2           | 4 ± 0.5           | 41 ± 5.8            |
| 6-OST3       | +++                     | 43 ± 3        | 10 ± 3       | 29 ± 0         | 8.5 ± 3.5      | 5.5 ± 3.5       | 4 ± 1             | 41.5 ± 4.5           |

*As in Table 1.

- 3H/H ratio of HS fragments obtained after pH 1.5 deamination.

- The 35S/3H ratio of different oligosaccharides after low pH nitrous acid treatment of HS from wild type (open circles) and 6-OST3 (closed circles) was calculated as 35S dpm/3H dpm from the elution profiles shown in Fig. 2. The 35S-disaccharides include inorganic 35S derived from labeled H-sulfate groups (see Table 4).

- FIGURE 3. 35S/3H ratio of HS fragments obtained after pH 1.5 deamination. The 35S/3H ratio of different oligosaccharides after low pH nitrous acid treatment of HS from wild type (open circles) and 6-OST3 (closed circles) was calculated as 35S dpm/3H dpm from the elution profiles shown in Fig. 2. The 35S-disaccharides include inorganic 35S derived from labeled H-sulfate groups (see Table 4).

- FIGURE 2. Gel chromatography of 35S/3H double-labeled products obtained after cleavage of HS at N-sulfated glucosamine residues. 35S/3H-Labeled HS chains isolated from solubilized cell layers from wild type (A) and 6-OST3 (++) overexpressing cells (B) were depolymerized by low pH nitrous acid treatment followed by reduction with NaBH4, and the resultant fragments were separated on a Bio-Gel P-10 column. C is a magnification of B. The numbers above the various peaks in A indicate the number of monosaccharide units in each saccharide species.

Composition of HS from the 6-OST-transfected and control cells, total cellular unlabeled HS chains from cells overexpressing low to medium levels of 6-OSTs were isolated and subjected to complete lyase depolymerization using heparin lyases (see “Experimental Procedures”). The lyase-cleaved products were analyzed using reversed phase ion-pairing chromatography (31). The transfection did not dramatically change the total amount of N-sulfated disaccharides but significantly increased the amounts of total O-sulfated disaccharides. The percentage composition revealed that ΔHexUA-GlcNAc6S (derived from -GlcUA-GlcNAc6S- in intact HS) and ΔHexUA-GlcNS6S (derived from -GlcUA-GlcNS6S- in HS) were considerably increased compared with HS from control cells (Table 3). The increased 6-O-sulfation was accompanied by a reduction of the proportion of nonsulfated units and 2-O-sulfated disaccharide units. No detectable amounts of ΔHexUA2S-GlcNAc6S-disaccharides (representing -IdoUA2S-GlcNAc6S-disaccharide units in HS) were observed in any of the samples analyzed.

Effect of 6-OST Overexpression on HS Domain Structure—To determine the organization of the N-sulfated domains, metabolically [35S]/[3H]GlcN double-labeled HS samples from cells and media were purified and subjected to low pH HNO3 treatment followed by Bio-Gel-P-10 gel chromatography. The length of the resultant 3H-labeled fragments reflects the spacing of N-sulfated GlcN units. The elution pattern of HS from control cells was characteristic of most HS, with similar amounts of GlcN-labeled di- and tetrasaccharides and a range of differently sized nitrous acid-resistant oligosaccharides with most of the 35S recovered in di- and tetrasaccharides (Fig. 2A). In contrast, HS from one of the most highly overexpressing 6-OST clones showed a reduction in the relative proportions of 3H-labeled di- and tetrasaccharides and had a more diffuse oligosaccharide pattern (Fig. 2B). The most dramatic difference was, however, the increased proportions of longer oligosaccharides in the high 6-OST-overexpressing cells. The overall ratio of incorporated 35S over 3H was similar in mock-transfected and 6-OST-overexpressing cells. The 35S/3H ratio decreased dramatically with increasing oligosaccharide size in HS from mock-transfected cells but not in HS from the overexpressing cells (Fig. 3). Substantial amounts of inorganic 35S are released upon deamination (pH 1.5) of labeled polysaccharides. The...
TABLE 4

Relative proportions of $^{35}$S and $^3$H radioactivity in the different oligosaccharides obtained after deamination at pH 1.5 shown in Fig. 2

$^{35}$SO$_4$ represents inorganic $^{35}$S derived from labeled N-sulfate groups.

| Oligosaccharide | Mock | 6-OST3 (++++++) | Mock | 6-OST3 (++++++) |
|-----------------|------|----------------|------|----------------|
|                  | % of total $^{35}$S radioactivity | % of total $^3$H radioactivity | |
| V$_o$ (V$_o$) 12-mers | ND$^a$ | 16.5 | 9 | 30 |
| 12-mers | ND | 15 | 18 | 25 |
| 10-mers | ND | 1.5 | 5 | 3.5 |
| 8-mers | 3.5 | 5 | 5 | 4 |
| 6-mers | 11 | 6 | 11 | 6 |
| 4-mers | 84 | 6 | 18 | 5 |
| 2-mers and $^{35}$SO$_4$ | 46 | | 27.5 | 18.5 |

$^a$ ND, not detected.

released inorganic $^{35}$S that elutes together with the disaccharides represents N-$^{35}$S groups in the intact polysaccharide. In HS from mock-transfected cells ~95% of total $^{35}$S radioactivity and 45% of total $^3$H radioactivity were recovered in the di- and tetrasaccharide fractions. Corresponding values for the high 6-OST-overexpressing clones were 52 and 24% for $^{35}$S and $^3$H radioactivity, respectively. In the control samples ~5% $^{35}$S was detected in fragments $\geq$ 6-mers (Table 4). In contrast, the high 6-OST-overexpressing clones incorporated ~48% of total $^{35}$S radioactivity in fragments $\geq$ 6-mers (Table 4). Thus, introduction of high amounts of 6-OST led to an altered domain structure and a redistribution of O-sulfate groups with increased 6-O-sulfation of nitrous acid-resistant sequences, presumably on both N-acetylated and N-sulfated GlcN units.

The elution patterns shown in Fig. 2 indicated that HS from the highly overexpressing cells was less N-sulfated in contrast to the low and medium overexpressing clones (Table 3). Therefore, we estimated the amounts of GlcNS units in the two HS preparations, from the proportion of $^3$H counts in each oligosaccharide fraction corrected for the number of internal GlcNAc residues. By this method HS from mock-

To gain more information concerning the changes in HS structure, $^{35}$S-labeled HS chains from the culture media of nontransfected HEK 293 cells, high 6-OST1, and high 6-OST3-overexpressing cells were chemically N-deacetylated and depolymerized by treatment with nitrous acid at pH 3.9. These conditions cleave HS at GlcNAc residues, and the resulting oligosaccharides represent the lengths of the N-sulfated domains. Disaccharides are formed from regions with at least two consecutive N-acetylated disaccharides. Oligosaccharides, 6-mers and longer, essentially correspond to long N-sulfated sequences. Analysis of HS from control cells by gel chromatography on Bio-Gel P-10 showed a distribution of differently sized NS domains (Fig. 5A).
The majority of the N-sulfated oligosaccharides were distributed as tetra- to decasaccharides, with ~17, 32, and 51% of total radioactivity recovered as di-, tetra-, and longer than tetrasaccharides, respectively. 6-OST overexpression resulted in a different pattern with a dominance of di- and tetrasaccharides (Fig. 5B). The disaccharide content was significantly higher (39–42% in HS from two different clones) than that of the control HS. The amount of tetrasaccharides, 33%, was similar to that of the mock-transfected cells.

**Effect of 6-OST Overexpression on HS Size and Charge Density**—To correlate the effects on HS fine structure with overall charge density, 35S-labeled intact HS chains from transfected and control cells were analyzed by anion-exchange chromatography (Fig. 6). HS from control cells emerged as one distinct peak just before the CS standard (Fig. 6A). The HS chains from cells expressing low to medium levels of either of the 6-OSTs eluted as much broader peaks containing two subpopulations, one that eluted as HS from control cells and one less retarded subfraction (Fig. 6, B and C). With an increasing level of 6-OST expression a new population of highly charged HS appeared after the elution position of CS standard (Fig. 6, D–F). The elution profiles of 3H-labeled HS chains were similar to those for the 35S-labeled chains (data not shown). Again, the effect of transfection on the polyanionic properties of HS was largely an effect of the level of overexpression rather than isoform expressed, although it was most pronounced for HS from 6-OST3-overexpressing cells.

To define the structural properties of the different DEAE populations generated by 6-OST overexpression, two DEAE subfractions were isolated by preparative ion-exchange chromatography of HS from a highly 6-OST3-overexpressing clone (Fig. 6F). The macromolecular properties of the less retarded pool (P1 in Fig. 6F) and the more retarded pool (P2 in Fig. 6F) were analyzed by gel chromatography on Superdex 30 (Fig. 7). The 35S-labeled P1 material was heterogeneous and contained both longer and shorter chains ranging from 6-mers to fragments ≥ 20-mers, sufficiently long to be excluded from the Superdex 30 column (Fig. 7A). In contrast, P2 material eluted as a fairly distinct peak near the void volume of the column (Fig. 7B). Deamination (pH 1.5) of P1 material converted a smaller portion (25%), mainly shorter oligosaccharides to disaccharides (Fig. 7C), indicating a high content of resistant sulfated N-acetylated poly- and oligosaccharides, whereas deamination of P2 resulted largely in shorter oligosaccharides (40%) and disaccharides (40%) (Fig. 7D). Digestion with heparin lyases completely degraded the 35S-labeled P1 and P2 material into disaccharides, showing that the nitrous acid-resistant material was indeed HS (data not shown).

Finally, we investigated the polyanionic properties of the low pH nitrous acid-resistant HS fragments recovered in the excluded volume of the G-15 column (pool ≥ 6 in Fig. 1C) by DEAE anion-exchange chromatography. The 35S-labeled N-acetylated HS fragments eluted as a rather broad peak before and overlapping with the elution position of
the CS standard similar to P1 in Fig. 6f (data not shown), lacking the more highly charged chains.

Taken together, these findings suggest that 6-OST overexpression induces synthesis of two structurally different HS sequences. The less retarded DEAE fraction (P1) contains a major fraction of relatively highly sulfated oligomers representing sequences of ≥2 consecutive GlcNAc residues interspersed between GlcNS residues in the intact HS chain, whereas the more retarded P2 fraction contains mainly N-sulfated GlcN domains.

**DISCUSSION**

The distribution of 6-O-sulfate groups is important to differential protein binding by HS chains. Therefore, the expression patterns and enzyme specificities of HS 6-OSTs may be critical for generating different protein binding domains in HS chains. The aim of this study was to examine the influence of 6-OST1, 6-OST2, and 6-OST3 expression levels on HS structure.

The 6-O-sulfate groups in HS occur in different structural contexts. Approximately half the total 6-O-sulfate in HS is found in contiguous N-sulfated domains, often in juxtaposition to IdoUA2S (5). In most HS species other 6-O-sulfates are located mainly in NA/NS domains, which largely lack 2-O-sulfate groups (5). In a few HS samples significant 6-O-sulfation was observed in NA domains (recovered together with a modified GlcNS residue, aManR, at the reducing end), but no attempt was made to define the position of the sulfate groups (5). It is tempting to speculate that 6-O-sulfation of different target sequences would be catalyzed by different 6-OST isoforms. Because expression of other HS biosynthetic enzymes (HS 2-OST and HS NDST1 and 2 (32, 33)) in HEK 293 cells has given important information regarding their roles in HS biosynthesis we decided to express the three murine isoforms of 6-OST in the same system. The structural changes induced by overexpression of 6-OSTs in HEK 293 cells were distinctive but not isoform-specific. However, it should be noted that even at a relatively low expression level, the changes were most extreme in 6-OST3-expressing cells. Overexpression of 6-OSTs resulted in increased 6-O-sulfation of both GlcNS and GlcNAc units accompanied by a decrease in total IdoUA units and 2-O-sulfation, resulting in formation of heavily 6-O-sulfated GlcUA-rich NS domains. In addition, there was a change in the distribution of N-sulfated domains and a formation of extended 6-O-sulfated regions composed primarily of consecutive N-acetylated disaccharide units. Our findings suggest a gradual change in HS structure from an intermixed distribution of NA, NA/NS, and NS domains to more extended regions of 6-O-sulfated NA and NS domains, which was dependent on the level of expression and most marked in 6-OST3-expressing cells.

Furthermore, overexpression of 6-OSTs dramatically changed both the polyanionic properties (Fig. 6) and the chain length of HS (Fig. 7). Cell surface HS proteoglycans are either shed into the medium or taken up by endocytosis and degraded to oligosaccharides by cellular lyses in the lysosomes. Because the labeled HS chains were isolated from solubilized cell layers, it is not clear whether the shorter chains are degradation products or if they are derived from intact proteoglycans.

Formation of specific protein binding sequences, dependent on the distribution of 6-O-sulfate groups, is believed to be a result of strictly controlled HS biosynthesis. HS polymerization proceeds from the reducing toward the nonreducing end, and modifications of the -GlcUA-GlcNAc-polymer to HS begin with the N-deacetylation/N-sulfation of selected GlcN residues. All subsequent modifications are believed to occur within or in close vicinity to GlcNS residues (34). Current information regarding the biosynthesis indicates that 6-O-sulfotransferase reactions are controlled by the extent of N-sulfation and that no 6-O-sulfate group is found more than one disaccharide away from the nearest N-sulfate group. The majority of GlcNAc 6-O-sulfate groups are expected to be found predominantly in alternating sequences (NA/NS domains) within -GlcNS-(HexUA-GlcNAc6S-GlcUA-GlcNS)- sequences (5, 35). Thus, such sequences will appear as tetrasaccharides in Fig. 5. For longer NA domains, it has been reported that 6-O-sulfation of GlcNAc residues is confined to the GlcNAc adjacent to the nonreducing terminal uronic acid residue of NA domains and that fewer than 10% of the reducing terminal GlcNS residues are 6-O-sulfated (Fig. 8) (35, 36). Thus, oligosaccharides resistant to low pH deamination are expected to have the general structure HexUA-GlcNAc ± 6S-GlcUA-[GlcNAc-GlcUA]-GlcNAc-GlcUA-aManR or, but more rarely, HexUA-GlcNAc ± 6S-GlcUA-[GlcNAc-GlcUA]-GlcNAc6S-GlcUA-aManR ± 6S (35, 37). Structural studies of HS from mock-transfected and 6-OST-overexpressing cells after N-deacetylation and cleavage at the resulting free amino groups indicate that both mock-transfected and 6-OST-overexpressing HEK 293 cells synthesize HS chains with 6-O-sulfated GlcNAc residues adjacent to the reducing terminal GlcUA acid residue (Fig. 8). Such residues would appear as disaccharides in Fig. 5. An intriguing finding concerns the increase in the amount of sulfated disaccharides in HS from 6-OST-overexpressing cells (Fig. 5b). These results suggest the formation of extensive O-sulfation of the GlcNAc adjacent to the reducing terminal uronic acid residue, and/or alternatively, but maybe less likely, within contiguous N-acetylated sequences (Fig. 8).

Enzyme overexpression differs from physiological conditions and
may not reflect normal regulation of 6-O-sulfation, which may depend on regulatory proteins affecting the availability of the enzyme’s substrate. However, it may still give valuable information of the enzyme properties. The increase in the level of any of the 6-OSTs resulted in changes in HS structure which in several aspects resemble HS synthesized by mice lacking either the 2-OST or the C5-epimerase (38, 39). All three polysaccharides contain highly 6-O-sulfated NS domains with fewer alternating sequences. In both 2-OST and C5-epimerase mutant HS 6-O-sulfated HexUA-GlcNS units are dramatically increased (31, 38, 40). However, neither the 2-OST nor the C5-epimerase mutant HS showed increased formation of GlcNAc6S disaccharide units. On the other hand, mouse embryonic stem cells deficient in two of four existing enzymes that carry out the coupled N-deacetylation/N-sulfation of the HS chains (NDST1 and NDST2) synthesize 6-O-sulfated N-acetylated HS chains (41). This result indicates that the 6-O-sulfotransferases are not strictly dependent on N-sulfate groups for substrate recognition.

The finding that overexpression of any of the three different 6-OSTs had similar effects on HS structure was both interesting and unexpected. The transcript levels of 6-OST isoforms vary in different tissues and during development (7, 20, 22), and it is generally believed that the fine structure of HS is determined through a developmentally regulated and tissue-specific expression of biosynthetic enzyme isoforms (2). So far only a few studies have correlated changes in HS structural motifs with changes in enzyme transcription levels (20, 42). Even less is known about the relative levels of the different enzyme proteins in vivo and how the amount of each enzyme and/or enzyme activity levels will affect HS structure in vivo. The finding in this paper, that not the specific HS 6-OST isoform but the level of expression gives rise to HS with different sulfation patterns, suggests that one way to control HS structure is by the amount of active enzymes.

The individual roles of 6-OST isoenzymes in HS biosynthesis may be better understood by the generation of mice lacking either one of the 6-OSTs. Transgenic animals will undoubtedly be of invaluable help to understand the individual roles of 6-OSTs in HS biosynthesis, but the complexity of HS-protein interactions constitutes a problem. Conditional knock-outs of HS biosynthetic enzymes are probably required to be able to separate one effect from another. Finally, the actions of the recently discovered extracellular HS 6-O-endosulfatases (Sulf enzymes) show that cells have other means to modulate HS structure and protein binding functions than exclusively by altering HS biosynthesis (43–46).

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