Expression of the Mouse Mastocytoma Glucosaminyl N-Deacetylase/N-Sulfotransferase in Human Kidney 293 Cells Results in Increased N-Sulfation of Heparan Sulfate†

Wing-Fai Cheung,‡,§ Inger Eriksson,§ Marion Kusche-Gullberg,‡ Ulf Lindahl,‡ and Lena Kjellén*,§

Department of Veterinary Medical Chemistry, The Swedish University of Agricultural Sciences, and Department of Medical and Physiological Chemistry, University of Uppsala, The Biomedical Center, Box 575, S-751 23 Uppsala, Sweden

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ABSTRACT: The biosynthesis of heparin and heparan sulfate involves a series of polymer-modification reactions that is initiated by N-deacetylation and subsequent N-sulfation of *N*-acetylglucosamine residues. These reactions are catalyzed by a combined N-deacetylase/N-sulfotransferase. Proteins expressing both activities have previously been purified from mouse mastocytoma, which generates heparin, and from rat liver, which produces heparan sulfate. In the present study, the mouse mastocytoma enzyme has been expressed in the human kidney cell line, 293, to investigate whether it could promote modification of the endogenous heparan sulfate precursor polysaccharide into a heparin-like molecule. The N-deacetylase activity of the stably transfected cell clones was ∼8-fold higher, on a cell-protein basis, than that of control cells, while the N-sulfotransferase activity was increased ∼2.5-fold. The amounts of glycosaminoglycans synthesized were the same in control and transfected cells, measured as incorporation of [^{3}H]-glucosamine, whereas ^{35}S-labeled glycosaminoglycans were ∼50% increased in transfected cells, with an increased relative content of heparan sulfate. Structural analysis demonstrated that the glucosamine units of the "heparan sulfate" from transfected cells were almost exclusively N-sulfated, as expected for heparin, whereas more than half of the glucosamine units of the control polysaccharide remained N-acetylated. Notably, the increased N-sulfation was not accompanied by increased O-sulfation, nor by C-5 epimerization of D-glucuronic to L-iduronic acid units. The implications of these findings are discussed with regard to the regulation of the biosynthetic process.

Heparan sulfate (HS)[1] proteoglycans, occurring on cell surfaces and in extracellular matrices, have been attributed a multitude of biological functions (Kjellén & Lindahl, 1991; Lindahl et al., 1994). Most of these effects are due to interactions between the polysaccharide chains and proteins and depend on the charge density and/or the fine structure of the HS (Spillmann & Lindahl, 1994). While various proteoglycan core proteins may carry HS side chains (David, 1993; Timpl, 1993; Lindahl et al., 1994), the structural properties of these chains do not seem to correlate with the type of core protein but rather with the type of cell producing the proteoglycan (Schmidtchen & Fransson, 1992; Kato et al., 1994; Shworak et al., 1994).

Heparin is structurally related to HS but is more heavily N- and O-sulfated and has a higher L-iduronic acid/D-glucuronic acid (IdoA/GlcA) ratio (Lindahl, 1989; Lindahl et al., 1994). It is synthesized as part of the proteoglycan serglycin, produced by connective tissue-type mast cells. Formation of the polysaccharide chain in heparin/HS biosynthesis is catalyzed by a GlcA/GlcNAc transferase, a single protein with two catalytic activities (Lidholt et al., 1992; Lind et al., 1993) that assembles D-glucuronic acid (GlcA) and *N*-acetyl-D-glucosamine (GlcNAc) units in alternating sequence. The resultant $(GlcA\text{-}GlcNAc)_n$ polymers are then modified through a series of consecutive reactions that presumably occurs concomitantly with chain elongation (Lidholt & Lindahl, 1992) and which involves the addition of sulfate groups at various positions and the conversion of GlcA into IdoA units [see Lindahl (1989) and Lindahl et al. (1994) for reviews]. The first modification step is N-deacetylation and subsequent N-sulfation of GlcNAc residues. Since all subsequent modifications occur in the vicinity of N-sulfate groups, N-deacetylation/N-sulfation has a key role in determining the extent of modification of the polysaccharide chain. In fact, this step determines whether the final product will be considered a HS (approximately equal amounts of N-acetylated and N-sulfated GlcN units) or a heparin (generally >80% N-sulfated GlcN units) (Gallagher & Walker, 1985).

Proteins with glucosaminyl N-deacetylase/N-sulfotransferase activity have been isolated from rat liver (Brandan &

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* To whom correspondence should be addressed.

‡ Department of Medical and Physiological Chemistry, University of Uppsala.

§ Department of Veterinary Medical Chemistry, The Swedish University of Agricultural Sciences.

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[1] Abbreviations: CS, chondroitin sulfate; GlcA, D-glucuronic acid; GlcNAc, 2-acetamido-2-deoxy-D-glucose (*N*-acetyl-D-glucosamine); $aMan_R$, 2,5-anhydro-D-mannitol (formed by reduction of terminal 2,5-anhydromannose residues with $NaBH_4$); HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HPLC, high-performance liquid chromatography; HS, heparan sulfate; MES, 4-morpholinoethanesulfonic acid; PMSF, phenylmethanesulfonyl fluoride; $-OSO_3$, *O*-sulfate, ester sulfate group (the locations of *O*-sulfate groups are indicated in parentheses).

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Hirschberg, 1988; Ishihara et al., 1993; Wei et al., 1993) and from a mouse mastocytoma (Pettersson et al., 1991). The cDNA sequences and deduced amino acid sequences showed that while large portions of the proteins were highly similar, the N-terminal regions showed significant differences (Hashimoto et al., 1992; Eriksson et al., 1994; Orellana et al., 1994). Moreover, the enzymes from the two sources differed strikingly with regard to the size of mRNA transcripts, which was ~4 kb for the mastocytoma enzyme but ~8 kb for the liver enzyme (Eriksson et al., 1994; Orellana et al., 1994). Indeed, characterization of mouse genomic clones indicate that two different genes encode the mastocytoma and liver enzymes.[2] These findings raised the possibility that the rat liver protein is designed to catalyze N-deacetylation/N-sulfation in HS biosynthesis, whereas the mastocytoma protein promotes the same reactions in heparin biosynthesis (Hashimoto et al., 1992; Eriksson et al., 1994; Orellana et al., 1994). In the present study we investigated this possibility by transfecting the mastocytoma enzyme into a heparan sulfate-synthesizing cell.

MATERIALS AND METHODS

Cell Culture, Expression Vector, and DNA Transfection. The human embryonic kidney cell line 293 (ATCC CRL 1573) was maintained in Dulbecco's modified Eagle's medium (DMEM-H) with 10% fetal calf serum (Gibco/BRL). Full-length cDNA of mouse mastocytoma N-deacetylase/N-sulfotransferase [3.3-kb *Eco*RI fragment; see Eriksson et al. (1994)] was ligated into the expression vector pCMV5 (Andersson et al., 1989). The insert is under the control of the early gene promoter of human cytomegalovirus (Thomsen et al., 1984). The kidney 293 cells were transfected with the construct together with pSV2-neo (Southern & Berg, 1982) using the calcium phosphate coprecipitation method (Graham & van der Eb, 1973). Control cells were transfected with pSV2-neo only. Stable clones were selected under high concentration (800 μg/mL) of Geneticin (G418, Gibco/BRL). Cell clones containing the mouse enzyme cDNA were further selected by PCR using two oligonucleotides, E21, 5′ ggC-CggAggTACCCAgAC 3′, and E29, 5′ gTAggCATTgA-CAggTA 3′, the resultant fragment corresponding to nucleotides 2651−3134 (Eriksson et al., 1994). Southern blot hybridization was also performed to confirm that the mouse cDNA was stably integrated in the host cell genome (data not shown).

RNA Purification and Northern Hybridization. Total RNA was isolated from control and transfected cells by the LiCl/urea/SDS method (Sambrook et al., 1989). Denatured total RNA was fractionated on a 1.2% agarose gel containing formaldehyde (Lehrach et al., 1977). After electrophoresis, the gel was soaked in 0.05 M NaOH for 30 min and rinsed with water before capillary transfer of the RNA to a nylon membrane, Hybond N^+ (Amersham Corp.) in 10× SSC (1× SSC is 0.15 M NaCl and 0.032 M sodium citrate, pH 7.0). The membrane was hybridized with the full-length cDNA fragment at 68 °C in a solution containing 6× SSC, 5× Denhardt's solution (Denhardt, 1966), 0.5% SDS, and 100 μg/mL denatured salmon sperm DNA for 18 h. The membrane was washed twice with 2× SSC and 0.1% SDS for 15 min at room temperature and then twice with 0.1× SSC and 0.1% SDS for 15 min at 60 °C. Autoradiography was done at −70 °C overnight on Kodak Scientific Imaging film X-OMAT.

N-Acetylglucosaminyl Deacetylase and N-Sulfotransferase Assays. Confluent cultured control and transfected cells were lysed in 0.5 mL of solubilization buffer containing 0.05 M Tris-HCl, 1% Triton X-100, and 0.001 M phenylmethanesulfonyl fluoride (PMSF), pH 7.5. Cleared supernatants were recovered after centrifugation at 12 000 rpm for 15 min at 4 °C. Protein concentration was measured by the modified Bradford dye-binding method using the Bio-Rad protein assay kit. Enzyme activities were determined as described previously (Pettersson et al., 1991). Briefly, N-deacetylase activity was measured by using *N*-[^{3}H]acetyl-labeled *Escherichia coli* K5 capsular polysaccharide as a substrate. Samples of enzyme protein and 10 000 cpm of substrate were mixed with 0.05 M MES, pH 6.3, 0.01 M $MnCl_2$, and 1% Triton X-100 in a total volume of 200 μL. After incubation at 37 °C for 60 min, reactions were terminated by addition of 200 μL of "stop solution" (1 M monochloroacetic acid, 0.5 M NaOH, and 2 M NaCl). The released [^{3}H]acetate was determined by scintillation counting (Beckman LS 3800) in a biphasic system obtained by adding 5 mL of toluene, containing 0.5% 2,5-diphenyloxazole, 0.03% 1,4-bis[2-(5-phenyloxazolyl)]benzene, and 10% isoamyl alcohol, to the assay mixtures. N-Sulfotransferase activity was measured by using N-deacetylated *E. coli* K5 capsular polysaccharide as a sulfate acceptor. Incubations containing 3.5 μg of acceptor, 2 μCi of [^{35}S]PAPS, and enzyme protein in a total volume of 100 μL of 0.05 M HEPES, pH 7.4, 0.01 M $MgCl_2$, 0.005 M $CaCl_2$, 0.01 M $MnCl_2$, 3.5 μM NaF, and 1% Triton X-100 were incubated at 37 °C for 30 min. The reactions were terminated by the addition of 400 μL of ethanol, containing 1.3% sodium acetate along with 0.5 mg of carrier heparin, and the samples were kept at −20 °C overnight. After centrifugation at 13 000 rpm for 10 min, the supernatants were discarded and the pellets were dissolved in 100 μL of water. The ^{35}S-labeled polysaccharide was separated from residual unincorporated label by centrifugation through syringes packed with Sephadex G-25 in 0.2 M NH_4HCO_3, as described (Pettersson et al., 1991). Labeled polysaccharides were recovered in the effluents and were analyzed by scintillation counting.

Metabolic Labeling of Glycosaminoglycans in 293 Cells. Subconfluent cultures of control and transfected cells were grown in T25 flasks (Falcon) and incubated with 5 mL of DMEM-H in the presence of 10% fetal calf serum and 200 μCi/mL [^{35}S]sulfate (Du Pont NEN) or 50 μCi/mL [^{3}H]GlcN (Du Pont NEN). After 24 h of incubation at 37 °C, the culture medium was removed and the cells were rinsed twice with 5 mL of ice-cold phosphate-buffered saline (PBS). The cell monolayer was then lysed by incubation at 4 °C for 30 min with 3 mL of solubilization buffer containing in addition the protease inhibitors 0.001 M PMSF (Sigma), 0.002 M *N*-ethylmaleimide (Aldrich), 0.002 M EDTA, and 10 μg/mL pepstatin A (Sigma). After centrifugation at 2000 rpm for 15 min, the supernatant was collected, adjusted to 0.15 M with regard to NaCl, and applied onto a 300-μL column of DEAE-Sephacel (Pharmacia), equilibrated with 0.05 M Tris-HCl, pH 7.5, 0.15 M NaCl, 0.1% Triton X-100, and protease inhibitors (the same as above). The column was first washed with equilibration buffer and subsequently with

[2] M. Kusche-Gullberg, I. Eriksson, H. Wlad, and L. Kjellén, unpublished results.

0.05 M acetate buffer, pH 4.0, containing 0.15 M NaCl, 0.1% Triton X-100, and protease inhibitors. The proteoglycans were eluted with the acetate buffer containing 2 M NaCl.

Isolation and Analysis of Glycosaminoglycans. For structural analysis of HS, the proteoglycans were digested with papain (Sigma) for 18 h at 56 °C in 0.05 M acetate buffer, pH 5.5, containing 2 M NaCl, 0.01 M EDTA, and 0.01 M cysteinium chloride. After dialysis, the polysaccharide chains were digested with 0.05 unit of chondroitinase ABC (Seikagaku Corp.) in a total volume of 250 μL and in the presence of 0.05 M Tris-HCl, pH 8.0, 0.03 M sodium acetate, and 100 μg/mL BSA. The heparan sulfate chains were recovered after gel chromatography on a column (100 × 0.5 cm) of Sephadex G-50 in 0.05 M Tris-HCl and 0.2 M NaCl, pH 7.5. Effluent fractions of 0.6 mL were analyzed for radioactivity. Fractions containing the (undigested) radioactive heparan sulfate were pooled, dialyzed against water, and subsequently freeze-dried. For compositional analysis and analytical ion-exchange chromatography, the polysaccharide chains were released from the proteoglycan core protein by treatment with 0.5 M NaOH for 24 h at room temperature followed by neutralization with 4 M HCl. The proportions of HS and CS in labeled polysaccharide preparations were calculated from the proportions of degradation products determined by gel chromatography on Sephadex G-50 following treatment with HNO_2 at pH 1.5 (Shively & Conrad, 1976) and chondroitinase ABC, respectively.

For analytical anion-exchange chromatography, ^{35}S-labeled HS (∼13 000 cpm) was applied to a 2-mL DEAE-Sephacel column which had been equilibrated with 0.05 M Tris−HCl and 0.15 M NaCl, pH 7.5. After the column was washed with 10 mL of 0.05 M acetate buffer and 0.05 M NaCl, pH 4.0, the column was eluted with a 60-mL salt gradient ranging from 0.05 to 1.5 M NaCl in 0.05 M acetate buffer, pH 4.0. Fractions of 1 mL were collected every 20 min. The NaCl concentration of the fractions was estimated by measuring the conductivity.

Structural Analysis of Heparan Sulfate. Information regarding the proportions of N-sulfated and N-acetylated GlcN units was obtained by deamination [pH 1.5; see Shively and Conrad (1976)] of [^{3}H]GlcN-labeled HS, followed by separation of the resultant oligosaccharides by gel chromatography. Samples of HS (20 μL; ∼24 000 cpm of ^{3}H) were mixed with 200 μL of HNO_2 reagent and were left at room temperature for 10 min. The reaction was stopped by adding 30 μL of 2 M Na_2CO_3. The samples were then applied to a column (200 × 1 cm) of Sephadex G-25, which was eluted with 0.2 M NH_4HCO_3. Fractions of 2 mL were collected every 30 min and analyzed for radioactivity by scintillation counting. The overall N-sulfate/N-acetyl ratios were calculated from peak areas as described (Jacobsson et al., 1979; Riesenfeld et al., 1982).

For analysis of disaccharide composition, isolated [^{3}H]-GlcN-labeled HS was treated with HNO_2 as described above, followed by reduction with $NaBH_4$ (Pejler et al., 1987). Deaminated and neutralized samples (control cells, ∼180 000 cpm of ^{3}H; transfected cells, ∼90 000 cpm of ^{3}H) were treated with 100 μL of 1% $NaBH_4$ (freshly prepared in 1 mM NaOH) for 4 h at room temperature. The excess $NaBH_4$ was eliminated by acidification of the samples (pH ∼4) with 4 M acetic acid followed by neutralization with 4 M NaOH (Bienkowski & Conrad, 1985). The resultant ^{3}H-labeled degradation products were separated by gel chromatography on Sephadex G-25, as described above, and the HexA-[^{3}H]-$aMan_R$ disaccharides were pooled and lyophilized. They were then analyzed by anion-exchange HPLC (Bienkowski & Conrad, 1985), as described in more detail in the legend to Figure 4. The nonsulfated disaccharides, GlcA-$aMan_R$ and IdoA-$aMan_R$, were not adequately resolved in this procedure and were instead separated by descending paper chromatography on Whatman 1MM paper in ethyl acetate/acetic acid/H_2O (3:1:1), following isolation by preparative paper electrophoresis (80 V/cm) in 0.083 M pyridine/0.05 M acetic acid, pH 5.3. Paper strips were cut into 1-cm segments that were eluted with H_2O, and the aqueous extracts were subjected to scintillation counting. The HexA-[1-^{3}H]-$aMan_R$ disaccharides, with or without O-sulfate groups in various positions, that were used as reference compounds in the separation procedures were as described (Pejler et al., 1987).

RESULTS

The aim of the present study was to transfect a HS-producing recipient cell with cDNA corresponding to the mastocytoma N-deacetylase/N-sulfotransferase 4-kb transcript, in order to determine the effects of such transfection on the structure of the endogenous polysaccharide. Northern blot analysis of total RNA from human embryonic kidney cells (293) using a ∼400-bp rat cDNA probe for the 8-kb transcript [nucleotides 1634−2020; see Hashimoto et al. (1992)] and a 3.3-kb mouse cDNA probe for the 4-kb transcript [nucleotides 1−3306; see Eriksson et al. (1994)] showed only the 8-kb transcript, at a low level of expression, in these cells (data not shown).[3] The kidney 293 cell line was therefore considered appropriate for the study.

Expression of the Transfected Mastocytoma N-Deacetylase/N-Sulfotransferase. The kidney 293 cells were transfected with the 3.3-kb cDNA that contains the coding region of the 4-kb mouse mastocytoma N-deacetylase/N-sulfotransferase transcript, and, in addition, some 5′ and 3′ noncoding sequence (Eriksson et al., 1994). Total RNA from control and cDNA-transfected cells was analyzed by Northern blotting, using the 3.3-kb cDNA as a probe. A strong hybridizing band corresponding to 3.3 kb appeared in RNA from the transfected cells, the expression being more pronounced than in mastocytoma cells (Figure 1). In contrast, no signal was obtained from the control cells.

The overexpression of the mastocytoma transcript in the cDNA transfected cells correlated with increased N-deacetylase and N-sulfotransferase catalytic activities (Table 1). Both activities were higher in the transfected cells than in the control cells, in support of the conclusion that the cDNA encodes an enzyme that catalyzes both reactions. Notably, the increase in N-deacetylase activity was greater (8-fold) than that of the N-sulfotransferase activity (2.5-fold).

Effect of Transfection on Glycosaminoglycan Biosynthesis. Transfected cells and control cells were incubated with [^{3}H]-GlcN and with [^{35}S]sulfate, and radiolabeled glycosaminoglycans were isolated. As shown in Table 2, the [^{3}H]GlcN incorporation remained unchanged after transfection, suggesting that the total glycosaminoglycan production is similar

[3] The two probes have previously been used to detect 8- and 4-kb transcripts in human tissues under medium-stringency washing conditions (2× SSC and 0.1% SDS at 65 °C for 30 min; unpublished observation).

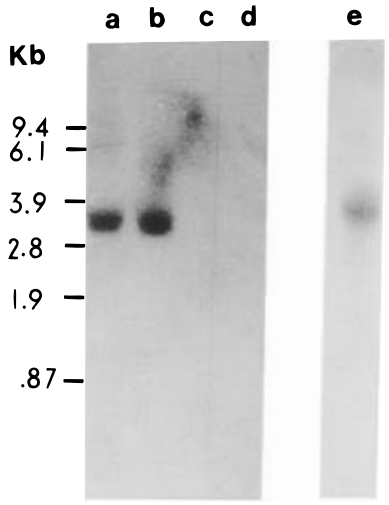

FIGURE 1: Expression of the mouse mastocytoma transcript in 293 cells. Twenty micrograms of total RNA from transfected cells, clones S-1 and S-5 (lane a and b); control cells, clones neo-5 and neo-8 (lanes c and d); and mouse mastocytoma tissue (lane e) were separated on a formaldehyde-containing agarose gel and hybridized with ^{32}P-labeled full-length mouse cDNA probe (see Materials and Methods). Sizes of RNA markers are indicated in kilobases. Lanes a−d were exposed for 24 h; lane e was exposed for 5 days.

Table 1: N-Deacetylase and N-Sulfotransferase Activity in Control and Transfected 293 Cells

clone	N-deacetylase activity [cpm of ^{3}H ($\times10^{-3}$)/ mg of protein]	N-sulfotransferase activity [cpm of ^{35}S ($\times10^{-3}$)/ μg of protein]
neo[a]	2.0	2.8
neo-3	2.3	2.6
neo-4	1.8	2.2
neo-8	2.2	2.7
S[b]	16.9	6.6
S-1	17.6	9.3
S-2	16.0	6.0
S-5	20.6	7.7

[a] Averaged results from 8 independent control clones; neo-3, -4, and -8 indicate values for individual clones. [b] Averaged results from 6 independent transfected clones; S-1, -2, and -5 indicate values for individual clones.

in control and transfected cells. In contrast, the amount of [^{35}S]sulfate incorporated into glycosaminoglycans was ∼50% increased in the transfected cells. Analysis of the composition of the ^{35}S-labeled polysaccharide showed that cells harboring the mouse enzyme produced relatively more HS (∼85% of total glycosaminoglycans, compared to ∼65% for the control cells) and less CS than control cells (Table 2).

To investigate whether the increased level of ^{35}S-labeled HS in the transfected cells was due to production of HS with a higher sulfate density, the isolated HS was subjected to anion-exchange chromatography. As shown by the elution patterns in Figure 2, the HS produced by the transfected cells consistently emerged 2−3 fractions later than the control HS. The salt concentration required for the elution of HS from transfected clones was ∼0.68 M (corresponding to the peak fraction of the distribution), compared to ∼0.61 M for HS from control clones.

Structural Analysis of Heparan Sulfate. Control and transfected 293 cells were incubated with [^{3}H]GlcN and labeled HS was isolated as described in Materials and Methods. Samples were treated with HNO_2 at pH 1.5, which results in cleavage of the chains at the sites of N-sulfated

Table 2: Glycosaminoglycan Synthesis in Control and Transfected 293 Cells

	glycosaminoglycans produced			
		[^{35}S]sulfate labeled		
clone	[^{3}H]glucosamine labeled [cpm ($\times10^{-5}$)/flask]	total [cpm ($\times10^{-5}$)/flask]	HS[a] (%)	CS[b] (%)
neo[c]	1.6	6.7	65	40
neo-3	1.6	6.7	62	42
neo-4	1.7	6.1	65	39
neo-8	1.4	6.1	63	43
S[d]	1.6	9.5	85	15
S-1	1.3	8.1	86	15
S-2	2.2	10.5	86	13
S-5	1.4	10.4	85	13

[a] Material susceptible to HNO_2, pH 1.5, as determined by gel chromatography (see Materials and Methods). [b] Material susceptible to chondroitinase ABC, as determined by gel chromatography (see Materials and Methods). [c] Averaged results from 8 independent control clones; neo-3, -4, and -8 indicate values for individual clones. [d] Averaged results from 6 independent transfected clones; S-1, -2, and -5 indicate values for individual clones.

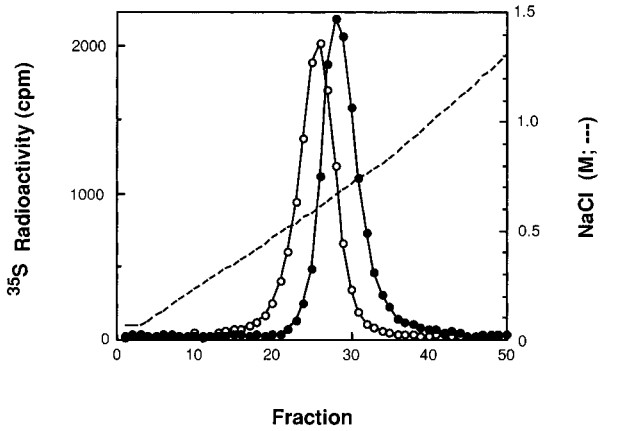

FIGURE 2: Charge density of heparan sulfate produced by control and transfected cells. [^{35}S]Sulfate-labeled HS from control cells (neo-3, ○) or transfected cells (S-2, ●) was loaded onto a 2-mL DEAE-Sephacel column that was eluted with a gradient from 0.05 to 1.5 M NaCl in 0.05 M acetate buffer, pH 4.0. Fractions of 1 mL were collected and analyzed for radioactivity.

GlcN units while N-acetylated units remain intact (Shively & Conrad, 1976). Separation of the products by gel chromatography showed that HS from the control cells had been converted into the mixture of di- and tetrasaccharides and larger oligosaccharides that is typical for HS (Figure 3). Calculations based on peak areas indicated that ∼40% of the GlcN units of the intact polysaccharide had been N-sulfated. By contrast, the HS produced by the transfected cells was extensively degraded, with 86% of the ^{3}H-radioactivity appearing in the disaccharide fraction (compared to 22% disaccharide from control HS). The N-sulfate content of HS thus was drastically increased through transfection of the cells with the mastocytoma N-deacetylase/N-sulfotransferase and amounted to ∼90% of the total N-substituents.

N-Sulfation is prerequisite to the subsequent polymer-modification reactions in the biosynthetic process, and it was therefore of interest to further characterize the N-sulfated disaccharide units of the HS isolated from control and transfected cells. Such structures were recovered as HexA-[^{3}H]aMan$_R$ disaccharides following HNO_2/$NaBH_4$ treatment of the [^{3}H]GlcN-labeled polysaccharides. Anion-exchange HPLC of the disaccharides derived from control HS again

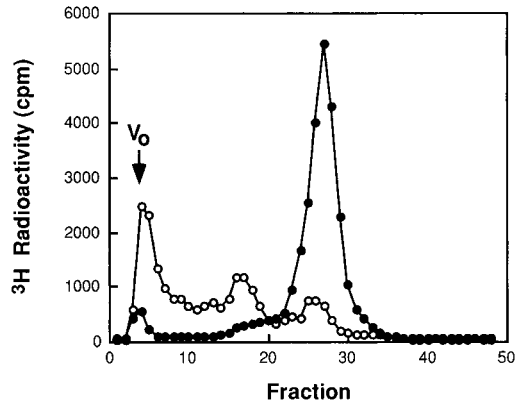

FIGURE 3: Deaminative cleavage of ^{3}H-labeled heparan sulfate. [^{3}H]-Glucosamine-labeled HS from control cells (neo-3, ○) or transfected cells (S-2, ●) was treated with nitrous acid at pH 1.5 followed by gel chromatography on a Sephadex G-25 column. Fractions of 2 mL were collected and assayed for radioactivity. The peak emerging between fractions 23 and 30 corresponds to disaccharides.

revealed a typical pattern, with the mono-O-sulfated disaccharide, IdoA(2-OSO_3)-aMan$_R$, being the predominant component (47% of the total disaccharide products), followed by the di-O-sulfated species, IdoA(2-OSO_3)-aMan$_R$(6-OSO_3) (26% of the total disaccharides) (Figure 4A). Unexpectedly, the disaccharides relating to the HS from transfected cells turned out to be 80% nonsulfated, the remainder appearing as mono-O-sulfated components (Figure 4B). The identity of the nonsulfated disaccharides was confirmed by high-voltage paper electrophoresis at pH 5.3 (Figure 5), where they comigrated with a mixture of GlcA-aMan$_R$ and IdoA-aMan$_R$ standards (migration due to the carboxylate group of the HexA units). Further identification of the nonsulfated disaccharides was afforded by paper chromatography, which indicated ~75% GlcA-aMan$_R$ and ~25% IdoA-aMan$_R$ (data not shown).

DISCUSSION

HS preparations from different cells or tissues display structural distinctions [see, *e.g.*, Kato et al. (1994) and Lindahl et al. (1995)] that are presumably introduced at the level of biosynthesis. Previous studies of heparin/heparan sulfate biosynthesis, mainly using a microsomal preparation from a heparin-producing mouse mastocytoma [see Lindahl (1989) and Lindahl et al. (1994) for reviews], have provided information on the various enzymes involved and their primary substrate specificities, but so far they have not revealed the mechanisms that are responsible for the modulation of polysaccharide structure. A current model of the biosynthetic apparatus predicts that these enzymes are translocated in a processive fashion along their polysaccharide substrate (Lidholt & Lindahl, 1992); transitions between modified and unmodified saccharide sequences thus would require an on−off mode of action. While different cells would be expected to differentially regulate such a process, it has been tacitly assumed that the enzymes involved are basically similar from one cell type to another.

The initial polymer-modification reactions, N-deacetylation and N-sulfation of GlcN units, of pivotal importance to the overall structure of the final product, are obviously subject to differential regulation. Members of the heparin/HS family of polysaccharides range from ~40% (proportion of total GlcN units) to virtually complete N-sulfation, as seen for

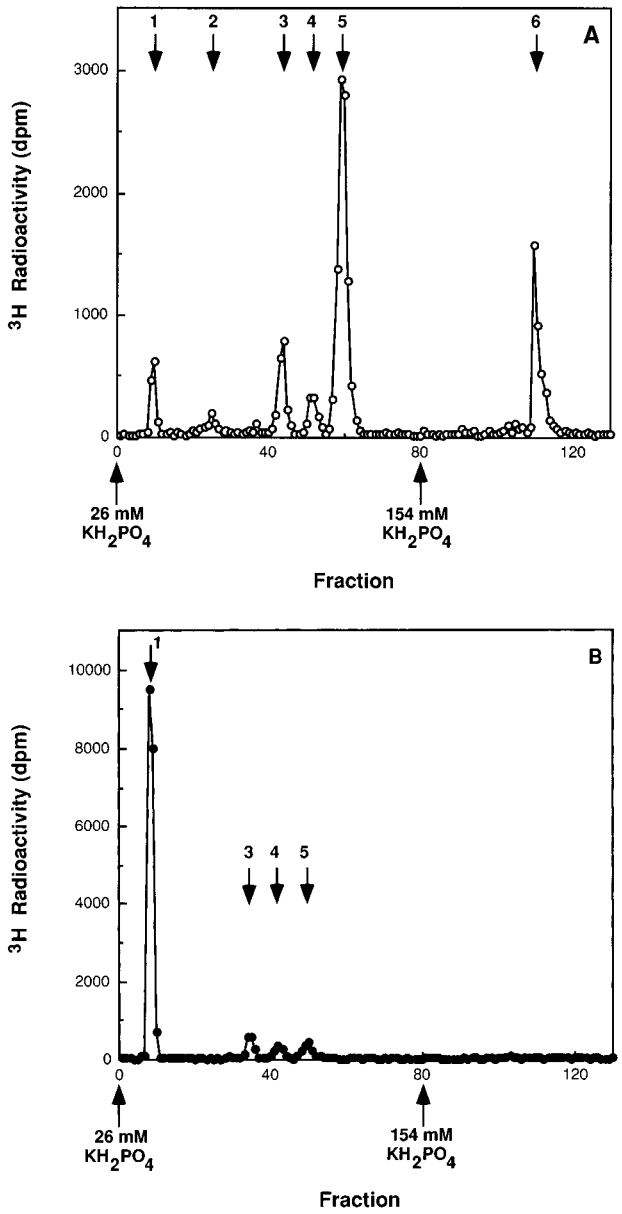

FIGURE 4: Anion-exchange HPLC of HexA-[^{3}H]aMan$_R$ disaccharides derived from heparan sulfate. HS from [^{3}H]GlcN-labeled (A) control cells (pooled neo-3, -4, and -8); and (B) transfected cells (pooled S-1, -2, and -5) was deaminated at pH 1.5 and reduced with $NaBH_4$ (see Materials and Methods). The products were separated by gel chromatography and the disaccharide-containing fractions were pooled and lyophilized. Labeled disaccharides were analyzed on a Whatman Partisil-10 SAX column (4.6 × 250 mm), eluted at a rate of 1 mL/min with KH_2PO_4 solutions of stepwise-increasing concentration, 26 mM for elution of nonsulfated and monosulfated disaccharides, and 154 mM for elution of disulfated disaccharides. Fractions of 1 mL were collected and analyzed for radioactivity by liquid scintillation counting. The elution positions of standard disaccharides are indicated by arrows: 1, nonsulfated HexA-aMan$_R$ (containing either GlcA or IdoA units); 2, GlcA(2-OSO_3)-aMan$_R$; 3, GlcA-aMan$_R$(6-OSO_3); 4, IdoA-aMan$_R$(6-OSO_3); 5, IdoA(2-OSO_3)-aMan$_R$; 6, IdoA(2-OSO_3)-aMan$_R$(6-OSO_3).

selected HS and heparin preparations, respectively. The distribution of polysaccharides within this range of N-sulfation is considered to be discontinuous, such that individual species tend to fall into one of two relatively distinct groups of HS (<60% N-sulfation) and heparin (>80% N-sulfation) (Gallagher & Walker, 1985; Lyon et al., 1994). The finding of two distinct N-deacetylase/N-sulfotransferases, encoded by separate genes, raised the

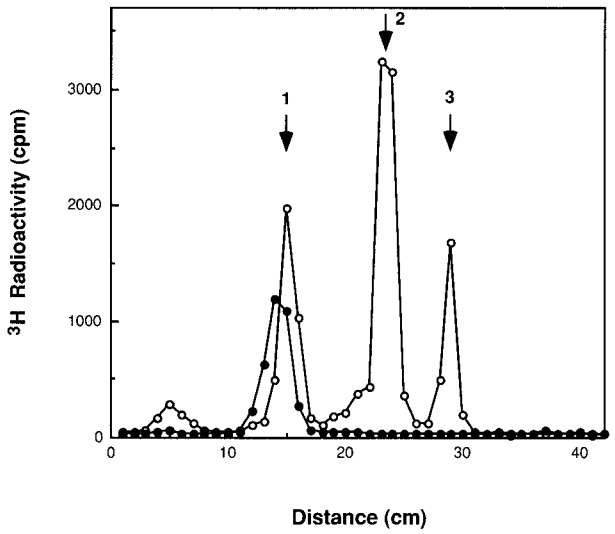

FIGURE 5: High-voltage paper electrophoresis of disaccharides. Nonsulfated disaccharides recovered after anion-exchange HPLC of deaminated [^{3}H]GlcN-labeled HS from transfected cells (●) were separated by paper electrophoresis (pH 5.3) as described in Materials and Methods. Superimposed is an electrophoretogram of standard disaccharides (○) run on a parallel paper strip: 1, mixture of GlcA-aMan$_R$ and IdoA-aMan$_R$; 2, GlcA-aMan$_R$(6-OSO$_3$) (not separated from other mono-O-sulfated disaccharides); 3, IdoA(2-OSO$_3$)-aMan$_R$(6-OSO$_3$).

possibility that one of these enzymes would be committed to heparin, the other to HS biosynthesis. Preliminary *in situ* hybridizations do not support this notion, since the 4-kb enzyme was found not to be restricted to heparin-producing cells.[4] The two enzymes may nevertheless differ in capacity to introduce N-sulfate groups in the growing polymer. It was therefore considered essential to assess whether introduction of the mastocytoma-type enzyme into a cell expressing the liver-type enzyme would change the structure of newly synthesized, endogenous polysaccharide.

Introduction of cDNA corresponding to the mastocytoma transcript into kidney 293 cells resulted in overexpression of the cognate mRNA (Figure 1). The appreciable increase in enzyme activity demonstrated that the transcript was translated into an active protein. The differential increment, ~8-fold for the N-deacetylase as compared to ~2.5-fold for the N-sulfotransferase activity (Table 1), may possibly pertain to differences in the catalytic properties of the two N-deacetylase/N-sulfotransferase enzymes, as described (Orellana et al., 1994). While overexpression of the mastocytoma enzyme, as expected, did not affect the total amount of [^{3}H]-GlcN-labeled glycosaminoglycans, it led to an enhanced formation of [^{35}S]sulfate-labeled polysaccharide, with a higher relative content of HS than seen for the control cells (Table 2). A change in HS structure was further indicated by the more pronounced polyanion character of the transfected cell product, as compared to that of control cells, demonstrated by ion-exchange chromatography (Figure 2). Indeed, compositional analysis of [^{3}H]GlcN-labeled material revealed a dramatic change in N-substitution, from that of a typical, or even low-sulfated, HS, with less than half of the GlcN units being N-sulfated, to a typical "heparin" pattern with almost exclusively N-sulfated GlcN residues. This result may indicate that involvement of the mastocytoma-type enzyme is prerequisite to the extensive N-deacetylation/N-sulfation seen in heparin biosynthesis. Alternatively, it is possible that the effect observed may be due to the increased amounts of the enzyme in the cell, as a result of overexpression. If so, N-sulfation could be directly controlled by the level of expression of the N-deacetylase/N-sulfotransferase enzymes. However, it was reported that similar overexpression of the liver-type enzyme had no apparent effect on the structure of the endogenous HS (Ishihara et al., 1993).

Since N-deacetylation/N-sulfation is the first of the biosynthetic polymer-modification reactions, and the subsequent steps depend on the presence of N-sulfate groups (Lindahl, 1989; Lindahl et al., 1994), it was of interest to determine whether also these later modifications (GlcA C-5 epimerization, O-sulfation) would occur at higher frequency in the polysaccharide synthesized by the transfected cells. When N-sulfation is diminished, as in the CHO cell mutant defective in N-deacetylase/N-sulfotransferase, all subsequent modification reactions are impeded (Bame et al., 1991). Conversely, one would expect the increased N-sulfation of the novel polysaccharide to trigger the subsequent modification reactions. Identification of the disaccharides isolated after low-pH deaminative cleavage (thus derived from the potentially modifiable N-sulfated regions) clearly demonstrated that this was not the case. While the disaccharides contained significant amounts of IdoA residues and O-sulfate groups, the proportions of these constituents were much lower than in the corresponding control fraction, such that ~20% of the disaccharides obtained from HS produced by the transfected cells carried O-sulfate groups, compared to 90% for control HS (Figure 4).[5] The increase in the initial, N-deacetylation/N-sulfation, polymer-modification reaction thus was not pursued through the subsequent epimerization and O-sulfation steps. Indeed, the polysaccharide synthesized by the transfected cells differs in structure from any previously described naturally occurring glycosaminoglycan.

A number of explanations for this finding may be considered. The enzymes that catalyze these reactions in the Golgi compartment may be rate-limiting and are present in insufficient amounts to efficiently process the increased amounts of substrate saccharide generated by the transfected N-deacetylase/N-sulfotransferase. Also, more subtle mechanisms may apply. It thus has been suggested that normal control of HS formation depends on regulatory proteins that ascertain the appropriate interaction between the biosynthetic enzymes and their polysaccharide substrate (Shworak et al., 1994). Introduction of the transfected enzyme may somehow perturb such a regulatory device. Further, the mastocytoma N-deacetylase/N-sulfotransferase may be inappropriately positioned in relation to the enzymes (presumably a membrane-bound complex) that catalyze the subsequent reactions. The two N-deacetylase/N-sulfotransferase enzymes may even be confined to different Golgi subcompartments, as suggested by the low level of structural homology between the 80 most N-terminal amino acids of the proteins (Eriksson et al., 1994;

[4] M. Durbeej, M. Kusche-Gullberg, I. Eriksson, and L. Kjellén, unpublished results.

[5] It is notable that the overall degree of O-sulfation does not appear to be altered after transfection. Since the disaccharide fraction of HS from transfected cells contained 86% of the total disaccharides, while the corresponding fraction from control HS contained only 22% of total disaccharides (see Figure 3), the identified O-sulfated disaccharides would represent 17% (0.20 × 0.86) and 20% (0.90 × 0.22) of the total disaccharides in HS from transfected cells and control cells, respectively.

Orellana et al., 1994). This region, which includes a short N-terminal cytoplasmic tail, a membrane-spanning domain, and a "stem region", is essential for Golgi retention, and presumably also for the specific location of the protein within the Golgi apparatus (Shaper & Shaper, 1992). Indeed, also the enzymes (GlcA C-5 epimerase, O-sulfotransferases) catalyzing the subsequent polymer-modification reactions may occur in different forms that preferentially associate with one or the other of the two N-deacetylase/N-sulfotransferase enzymes.

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