Overexpression of Different Isoforms of Glucosaminyl *N*-Deacetylase/*N*-Sulfotransferase Results in Distinct Heparan Sulfate N-Sulfation Patterns†

Dagmar Sandbäck Pikas,‡ Inger Eriksson,§ and Lena Kjellén*,§

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

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ABSTRACT: Functional interactions of heparan sulfate (HS) with selected proteins depend on distinct saccharide sequences which are generated during biosynthesis of the polysaccharide. Glucosaminyl *N*-deacetylase/*N*-sulfotransferases (NDSTs) catalyze both the N-deacetylation and N-sulfation reactions that initiate the modification of the $(GlcNAc\text{-}GlcA)_n$ polysaccharide backbone. The *N*-acetyl/*N*-sulfate exchange is restricted to certain regions of the polysaccharide chains, and only these can be further modified by glucuronyl C5-epimerization and O-sulfation at various positions. To investigate whether NDST isoforms influenced differently the structure of HS, murine NDST-1 was overexpressed in human kidney 293 cells, and the structure of the HS produced was compared to HS from NDST-2 overexpressing cells [Cheung, W. F., Eriksson, I., Kusche-Gullberg M., Lindahl, U., and Kjellén, L. (1996) *Biochemistry 35*, 5250−5256]. The level of N-sulfation increased from 40% in control cells to 60% and 80%, respectively, in NDST-1 and NDST-2 transfected cells. Interestingly, the increase in N-sulfation was accompanied by an increased chain length, while no effect on IdoA content or O-sulfation was seen. The most extended N-sulfated domains were found in HS synthesized by NDST-2 transfected cells. Since both the *N*-deacetylase and the *N*-sulfotransferase activities were lower in these cells than in the NDST-1 overexpressing cells, we conclude that, in addition to the level of enzyme expression, the NDST isoform also is important in determining the N-sulfation pattern in HS.

Heparin and heparan sulfate (HS)[1] are structurally related glycosaminoglycans, with complex sulfate patterns (*1*−*3*). The large structural variation in HS creates many potential binding sites for different interacting proteins. The binding of biologically important proteins, such as different growth factors and enzymes, to specific heparan sulfate domains is a growing area of interest (*1*−*4*). Although several of the heparin/HS biosynthetic enzymes have recently been cloned (*3, 4*), the mechanisms for regulation of heparin and HS biosynthesis are largely unknown. While HS is produced by virtually all mammalian cells, heparin, the most highly sulfated form of heparan sulfate, is exclusively synthesized by connective tissue-type mast cells (*5*).

The formation of heparan sulfate involves a number of biosynthetic reactions (*1*−*3*), including formation of a tetrasaccharide, linking the glycosaminoglycan chain to the proteoglycan core protein. This is followed by polymerization and concomitant modification of the polysaccharide chain. The product of the polymerization reactions, catalyzed by the HS copolymerase (*6*), is a polysaccharide consisting of repeating (GlcA-GlcNAc) disaccharide units. The modification reactions are initiated by N-deacetylation and N-sulfation of a selection of GlcNAc units. These reactions are carried out by a bifunctional enzyme, the glucosaminyl *N*-deacetylase/*N*-sulfotransferase (NDST), which contains the active sites for both N-deacetylation and N-sulfation (see ref *7* and references therein). The partly N-sulfated polysaccharide is then subjected to C5 epimerization of GlcA into IdoA, 2-O-sulfation of HexA (GlcA and IdoA), 6-O-sulfation of GlcN, and, more sparsely, 3-O-sulfation of GlcN units (*3*). Since all modifications occur in the vicinity of N-sulfated GlcN units, the NDST has a key role in designing the overall structure of the heparan sulfate chain. Adding to the complexity is the variable polymer modification (*3*), where residues that satisfy the substrate specificity of a certain reaction nevertheless escape modification, creating a highly variable, heterogeneous structure of the polysaccharide chains.

To understand how the variability in heparan sulfate structure is regulated, it is important to further characterize the participating biosynthetic enzymes. A number of these have been shown to occur in several isoforms (*3*, *4*, *8*). Four NDST isoforms have so far been identified (*9*−*13*). Analysis of mRNA expression in different tissues demonstrates a wide and largely overlapping distribution of the most characterized NDST isoforms 1 and 2 (*7*), implicating a role for both of

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* To whom correspondence should be addressed.

‡ University of Uppsala.

§ Swedish University of Agricultural Sciences.

[1] Abbreviations: GlcA, D-glucuronic acid; GlcN, D-glucosamine; GlcNAc, *N*-acetyl-D-glucosamine; $GlcNSO_3$, *N*-sulfo-D-glucosamine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HexA, hexuronic acid; HS, heparan sulfate; IdoA, L-iduronic acid; $aMan_R$, 2,5-anhydro-D-mannitol; MES, 4-morpholineethanesulfonic acid; NDST, glucosaminyl *N*-deacetylase/*N*-sulfotransferase; PAPS, adenosine 3′-phosphate 5′-phosphosulfate; PBS, phosphate-buffered saline.

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these isoforms in heparan sulfate biosynthesis. However, phenotypic characterization of mice deficient in NDST-2 indicates that NDST-2 is primarily involved in heparin production in connective tissue type mast cells (*14*, *15*); NDST-2 deficient mice contain abnormal connective tissue-type mast cells and synthesize nonsulfated heparin but appear to have a normal life span (*14*, *15*). In contrast, mice deficient in NDST-1 die due to abnormal development of several vital organs (M. Ringvall, J. Ledin, I. Eriksson, L. Kjellén, and E. Forsberg, unpublished results).

Transfection of the human embryonic kidney 293 cell line with murine NDST-2 resulted in a dramatic increase in N-sulfation of the HS produced, while the other modification reactions were not affected (*16*). In this study, we have used the same cell line to study the influence of NDST-1 on HS structure. Interestingly, the two isoforms gave rise to different HS N-sulfation patterns, suggesting specific roles for the two isoforms in heparin/heparan sulfate biosynthesis.

EXPERIMENTAL PROCEDURES

Transfection of 293 Cells with NDST-1. The human embryonic kidney cell line 293 (ATCC CRL 1573) was grown at 37 °C in a CO_2 incubator in Dulbecco's modified Eagle medium/Nutrient Mix (Gibco BRL) containing 10% fetal calf serum (Sigma) and penicillin−streptomycin (100 IU/mL−100 μg/mL, Gibco BRL). Using the calcium phosphate coprecipitation method (*17*), the cells were transfected with a 2.7 kb cDNA coding for mouse NDST-1 (*7*), ligated into the expression vector pcDNA3 (Invitrogen). Control cells were transfected with pcDNA3 only. Stable clones were selected under high concentration (800 μg/mL) of geneticin (G418 sulfate, Gibco BRL) and further maintained at 400 μg/mL.

N-Deacetylase and N-Sulfotransferase Assays. Stable clones were analyzed with regard to *N*-deacetylase and *N*-sulfotransferase activity. Trypsinized cells (two-thirds of a T75 flask) were washed with cold PBS and solubilized for 30 min at 4 °C in 0.45 mL of solubilization buffer containing 50 mM Tris-HCl, pH 7.4, 1% Triton X-100, 2 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, and 10 μg/mL pepstatin. The protein concentration was determined using Bio-Rad protein assay. The solubilized cells were assayed for *N*-deacetylase and *N*-sulfotransferase activity, essentially as described (*18*). Briefly, *N*-deacetylase activity was measured using a 2.5−5 μL sample and 10 000 cpm of *N*-[^{3}H]acetyl-labeled *Escherichia coli* K5 capsular polysaccharide as substrate in a total volume of 100 μL containing 25 μg/mL Polybrene, 50 mM MES, pH 6.3, 10 mM $MnCl_2$, and 1% Triton X-100. After incubation at 37 °C for 30 min (results presented in Figure 4) or 1 h (results presented in Figure 2), the released [^{3}H]acetate was detected in a biphasic scintillation counting system. *N*-Sulfotransferase activity was analyzed by measuring incorporation of ^{35}S from the sulfate donor [^{35}S]PAPS into ∼80% N-deacetylated K5 polysaccharide. The sample (2.5−5 μL) was incubated with ∼6 μg of polysaccharide substrate and 2 μCi of [^{35}S]PAPS (specific activity 200 Ci/mol) in 50 mM HEPES, pH 7.4, 10 mM $MgCl_2$, 10 mM $MnCl_2$, 5 mM $CaCl_2$, 3.5 μM NaF, and 1% Triton X-100 in a total volume of 100 μL. After incubation for 30 min at 37 °C, the polysaccharide was precipitated by ethanol overnight. ^{35}S-Labeled polysaccharide was separated from excess [^{35}S]PAPS by centrifugating the sample through small Sephadex G-25 columns and quantified by scintillation counting.

RNA Purification and Northern Hybridization. Total RNA was isolated from control and transfected cells using the RNeasy mini-kit (Qiagen). Fifteen micrograms of denatured RNA from each clone was fractionated by 1.2% agarose gel electrophoresis in 0.02 M 4-morpholinepropanesulfonic acid, 1 mM EDTA, 5 mM sodium acetate, pH 7.0, and 7% formaldehyde. The gel was soaked in 0.05 M NaOH for 20 min and in 0.1 M Tris-HCl, pH 7.5, and 0.15 M NaCl for 30 min. The RNA was transferred to a Hybond-N^+ nylon membrane (Amersham Corp.) in 10 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate, pH 7.0) and cross-linked to the membrane by UV radiation. A probe corresponding to the most 5′-part of the mouse NDST-1 RNA [218 bp, starting at nt 1 (GenBank accession number AF049894)] was amplified by PCR using primers corresponding to nt 1−18 (sense) and nt 218−200 (antisense). The filter was hybridized with the ^{32}P-labeled probe for 80 min at 68 °C using ExpressHyb (Clontech) and then washed three times with 2 × SSC and 0.05% SDS at 20 °C and 2 × 15 min with 0.1 × SSC and 0.1% SDS at 50 °C before it finally was applied on film (Fuji) which was exposed for 7.5 h at −70 °C.

NDST-2 Expressing 293 Cells. Stable clones of 293 cells transfected with NDST-2 (clone S-2 and S-5) or vector alone (neo-3) were as described (*16*).

Metabolic Labeling of Glycosaminoglycans. Subconfluent cultures of control cells (k6 and neo-3) and cells transfected with NDST-1 (clones 6 and 11) or NDST-2 (clone S-2) in flasks (75 or 175 cm^2) were cultured as described above in the presence of 50 μCi/mL [^{3}H]GlcN (DuPont NEN) or 0.2 mCi/mL carrier-free [^{35}S]sulfate (DuPont NEN). After 24 h of incubation at 37 °C, the cells were washed with cold PBS, incubated in 3−5 mL of solubilization buffer containing 2 mM *N*-ethylmaleimide and centrifuged (800*g* for 15 min at 4 °C). Protein concentrations were determined using Quantigold (Diversified Biotech).

Heparan Sulfate Isolation. Glycosaminoglycans were isolated from the solubilized cell supernatants on DEAE-Sephacel (Amersham Pharmacia Biotech) and digested with papain as described (*16*). The samples were diluted to a final NaCl concentration of 0.15 M, and the released glycosaminoglycan chains were reisolated on 300 μL DEAE-Sephacel columns equilibrated in 0.2 M NH_4HCO_3. The columns were washed with 0.3 M NaCl followed by 0.2 M NH_4HCO_3 and finally eluted with 3 mL of 2 M NH_4HCO_3. Eluted glycosaminoglycans were pooled and lyophilized. Chondroitin sulfate was digested with 0.05 unit of chondroitinase ABC (Seikagaku) as previously described (*16*). The resultant heparan sulfate chains were recovered after gel filtration on a column (100 × 0.5 cm) of Sephadex G-50 (superfine grade, Amersham Pharmacia Biotech) in 0.2 M NH_4HCO_3.

Structural Analysis of Heparan Sulfate. Isolated [^{3}H]HS (high molecular weight material from the G-50 column) was cleaved at N-sulfated GlcN residues by deamination at pH 1.5 (*19*). The resultant ^{3}H-labeled oligosaccharides were reduced with $NaBH_4$ and separated by gel chromatography on a column (1 × 140 cm) of Bio-Gel P-10 (fine grade, Bio-Rad). Alternatively, the [^{3}H]HS was first chemically N-

deacetylated by hydrazinolysis [in 0.45 mL of hydrazine hydrate (Fluka) containing 1% (w/v) hydrazine sulfate] at 100 °C for 4 h (*20*) in the presence of 50 μg of carrier heparin. After repeated evaporation followed by desalting on a PD-10 column (Amersham Pharmacia Biotech) in 0.2 M NH_4HCO_3, polysaccharide-containing fractions were pooled and lyophilized. The [^{3}H]HS was cleaved at the generated N-unsubstituted GlcN residues by deamination at pH 3.9 (*21*) and reduced with $NaBH_4$, followed by gel chromatography on Bio-Gel P-10.

The total disaccharide composition was analyzed as follows. The isolated [^{3}H]HS was chemically N-deacetylated by hydrazinolysis for 5 h, followed by combined deamination at pH 1.5 and 3.9 (*22*) and reduction with $NaBH_4$. The resultant disaccharides, containing terminal anhydromannitol ($aMan_R$) residues, were recovered by gel filtration on a column (1 × 180 cm) of Sephadex G-15 (Amersham Pharmacia Biotech) in 0.2 M NH_4HCO_3. The disaccharides were further characterized by ion-exchange chromatography on a Partisil-10 SAX column (Whatman Inc.) eluted with a stepwise gradient of KH_2PO_4 (*23*). The proportion of GlcA-$aMan_R$ and IdoA-Man_R was analyzed by descending paper chromatography on Whatman 1MM paper in ethyl acetate/acetic acid/H_2O (3:1:1).

Isolation of HS from Intact ^{35}S-Labeled Proteoglycans. In a separate experiment, clones 6 and 11 (NDST-1), S-2 and S-5 (NDST-2), and k3 and k6 (controls) were labeled with free [^{35}S]sulfate for a shorter time. Cells were grown to subconfluency in 75 cm^2 flasks and labeled with 1 mCi of [^{35}S]sulfate for 60 min. After solubilization, the proteoglycans were isolated on DEAE-Sephacel as described (*16*) and separated from free glycosaminoglycan chains on a Superose 6 HR 10/30 column (Amersham Pharmacia Biotech) in 50 mM Tris-HCl, pH 7.5, 0.5 M NaCl, and 0.1% Triton X-100, including 1 mM EDTA, 2 mM phenylmethanesulfonyl fluoride, 2 mM *N*-ethylmaleimide, and 1 μg/mL pepstatin. Glycosaminoglycan chains were released from the core proteins by alkaline β-elimination (treatment with 0.5 M NaOH at 4 °C for 18 h). The samples were neutralized with HCl, desalted on a PD-10 column in H_2O, and treated with chondroitinase ABC for 15 h at 37 °C. HS chains were isolated on a Sephadex G-50 column (100 × 0.5 cm) in 0.2 M NH_4HCO_3 and lyophilized. The size distribution of the HS chains was analyzed by gel filtration on Superose 6 in 0.5 M NH_4HCO_3. Identical elution patterns for the HS preparations were obtained when the column was eluted with 1 M NaCl, 50 mM Tris-HCl, pH 7.4, and 0.1% Triton X-100, as used when the elution positions of saccharide standards of known molecular size were determined (*29*).

RESULTS

Transfection of the human kidney 293 cells with NDST-1 resulted in stable clones expressing high levels of the 2.7 kb mRNA (Figure 1). The mRNA expression correlated with an up to 10-fold increase in *N*-deacetylase and *N*-sulfotransferase activity (Figure 2). The stable NDST-1 expressing cell lines were metabolically labeled with [^{3}H]GlcN or [^{35}S]-sulfate. Radioactively labeled glycosaminoglycans were isolated from solubilized cells by DEAE ion-exchange chromatography, and incorporation of ^{3}H or ^{35}S/mg of protein was calculated. The ratio of incorporated ^{35}S over ^{3}H was increased from 1.4 in the control clone to 2.2 in NDST-1 clone 11, indicating an increased sulfation of glycosaminoglycans in the NDST-1 transfected cells. Human kidney 293 cells expressing NDST-2 were previously generated and

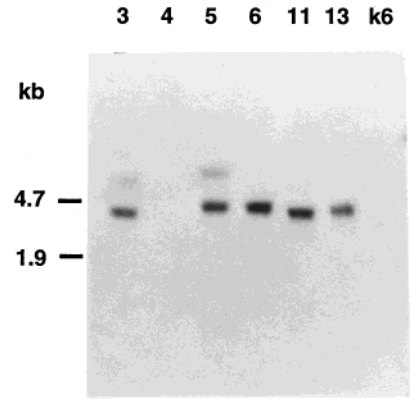

FIGURE 1: NDST-1 expression in transfected and control cells. Total RNA from each clone (k6 = control) was separated by agarose electrophoresis, blotted to a nylon membrane, and hybridized with a 218 nt ^{32}P-labeled probe hybridizing with the 5′-region of NDST-1. The positions of 28S (4.7 kb) and 18S (1.9 kb) rRNA are indicated.

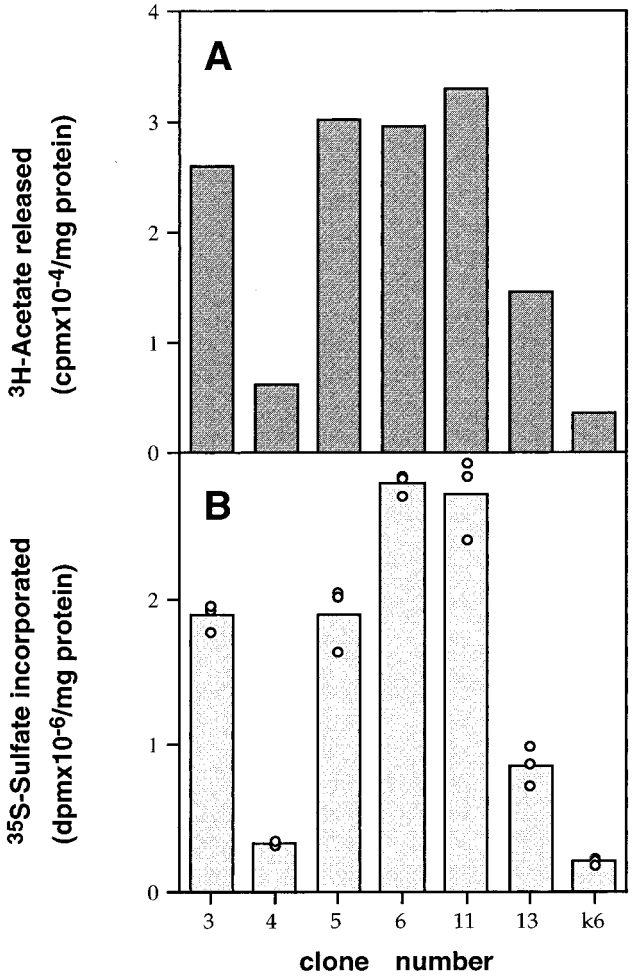

FIGURE 2: *N*-Deacetylase and *N*-sulfotransferase activities in NDST-1 transfected and control cells. Cells from different clones were solubilized, and release of [^{3}H]acetate (A) and incorporation of [^{35}S]sulfate (B) were measured in 5 μL samples in separate assays as described in Experimental Procedures. The *N*-sulfotransferase activity shown is the mean of triplicate values, while the *N*-deacetylase activity was measured on single samples.

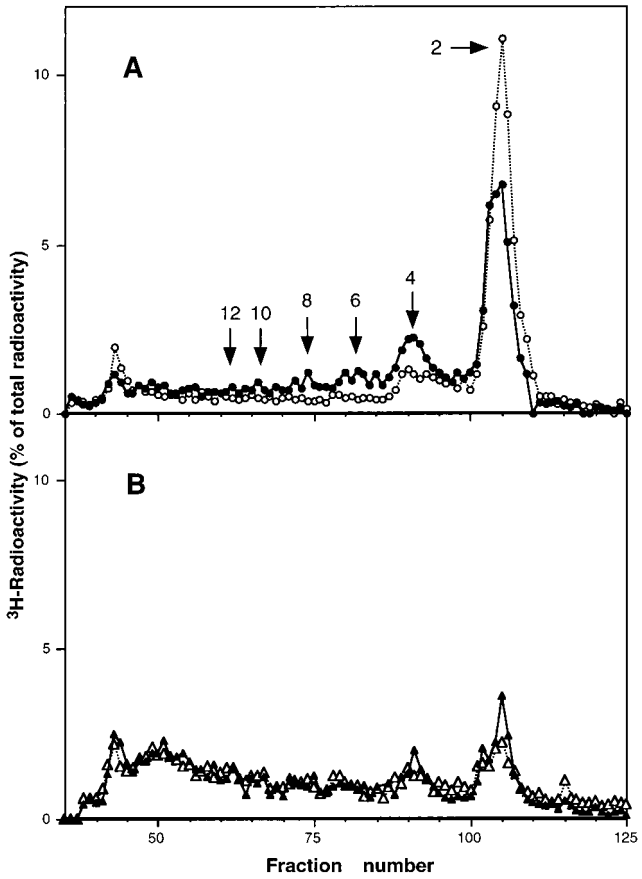

FIGURE 3: Gel chromatography of HS cleaved at N-sulfated glucosamine residues. [^{3}H]Glucosamine-labeled HS from cells transfected with NDST-1 (filled circles) or NDST-2 (open circles) (A) and their respective control cells (B) were treated with nitrous acid at pH 1.5, followed by gel chromatography on a Bio-Gel P-10 column. Fractions of 0.8 mL were collected at a flow rate of 1.7 mL/h and analyzed by scintillation counting. The positions of HS standard oligomers (di- to dodecasaccharides) are indicated by arrows.

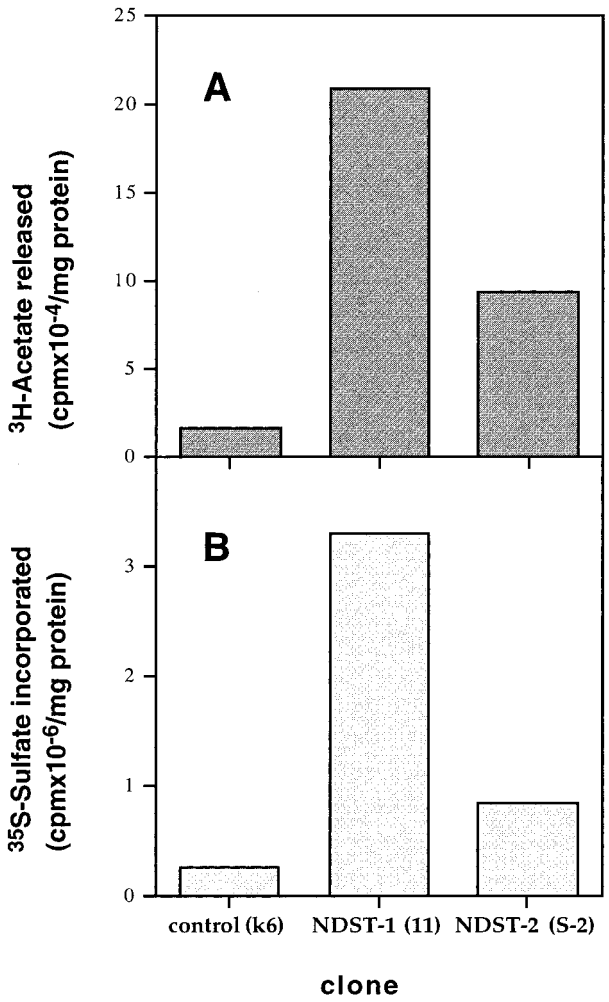

FIGURE 4: Comparison of *N*-deacetylase and *N*-sulfotransferase activities in NDST-1 and NDST-2 clones used for HS structural analysis. *N*-Deacetylase (A; single samples) and *N*-sulfotransferase (B; duplicate samples) activities were measured in solubilized cells (2.5 μL samples) from the 293 cell clones used for HS structural analyses.

characterized (*16*). Also, in these cells, the ratio of ^{35}S over ^{3}H was increased compared to the ratio in control cells (*16*). The NDST-2 expressing cells were used in this study together with the cells stably transfected with NDST-1 to compare the effect of the two isoenzymes on heparan sulfate structure. The transfected cells, as well as the corresponding control cells, were metabolically labeled with [^{3}H]GlcN, and ^{3}H-labeled glycosaminoglycans were isolated from solubilized cells by DEAE ion-exchange chromatography followed by papain digestion. The heparan sulfate chains (resistant to chondroitinase ABC; ~60% of the ^{3}H-labeled material) were isolated after gel chromatography on Sephadex G50.

Increased N-Sulfation of HS in NDST Overexpressing Cells. Information regarding the overall degree of N-sulfation, as well as the length of N-acetylated domains, was obtained by cleaving the heparan sulfate at N-sulfated glucosamine units with nitrous acid at pH 1.5, followed by gel chromatography on Bio-Gel P-10. Overexpression of NDST-1 and NDST-2 resulted in both increased HS N-sulfation, as illustrated by the increased proportion of disaccharides (Figure 3), and a decreased level of extended acetylated domains (see also ref *16*). However, HS from NDST-1 clones (repeated experiments from two different clones; all data not shown) contained fewer *N*-sulfate groups in N-sulfated domains than HS from the NDST-2 clone, as shown by the lower proportion of disaccharides. Calculations based on peak areas also indicated a higher degree of N-sulfation in the NDST-2 clone (80%) compared to the NDST-1 clone (60%), while ≈40% of the GlcN units in control cells were N-sulfated.

Enzyme Activities in NDST Overexpressing Cells. To see whether the difference in N-sulfation reflected different levels of *N*-deacetylase and ***N***-sulfotransferase activity in the clones, these activities were measured in Triton X-100 solubilized cells using the appropriate assays (Figure 4). Surprisingly, the clone producing the most highly sulfated HS, due to overexpression of NDST-2, had lower *N*-deacetylase and *N*-sulfotransferase specific activities than the NDST-1 over-expressing clone, indicating that in addition to the level of NDST expression, the NDST isoform is important for the N-sulfation pattern.

Isoenzyme-Specific N-Sulfation. To further investigate the distribution of *N*-sulfate groups along the polysaccharide chains, another approach was used. The isolated [^{3}H]HS was chemically N-deacetylated and cleaved at the resulting N-unsubstituted amino groups, leaving the N-sulfated domains intact. The lengths of the N-sulfated domains were

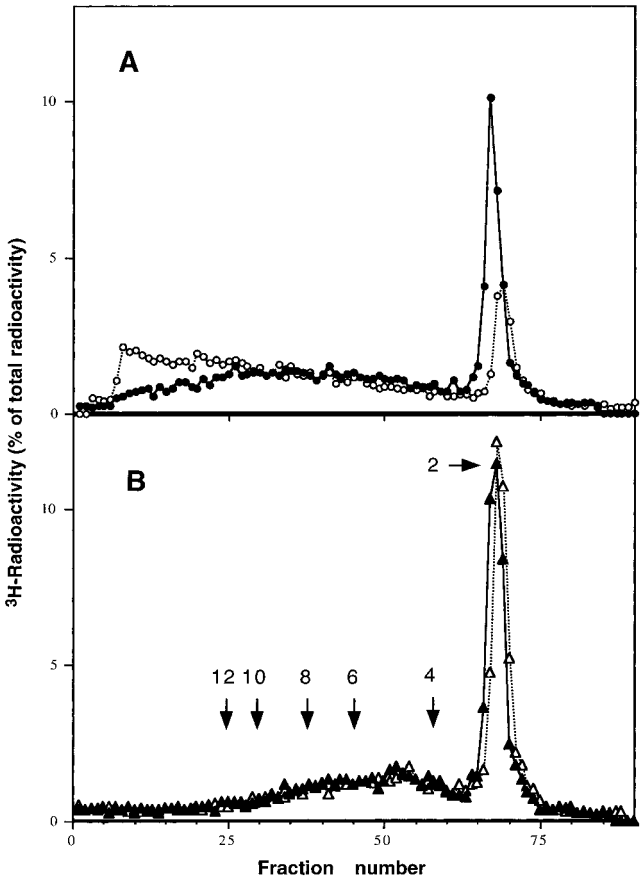

FIGURE 5: Gel chromatography of HS cleaved at non-N-sulfated glucosamine residues. [^{3}H]Glucosamine-labeled HS from cells transfected with NDST-1 (filled circles) or NDST-2 (open circles) (A) and their respective control cells (B) were treated with hydrazine followed by nitrous acid treatment at pH 3.9. The resultant oligosaccharide fragments were separated by gel chromatography on Bio-Gel P-10. Fractions of 0.8 mL were collected at a flow rate of 1.7 mL/h and analyzed by scintillation counting. The positions of HS standard oligomers (di- to dodecasaccharides) are indicated by arrows.

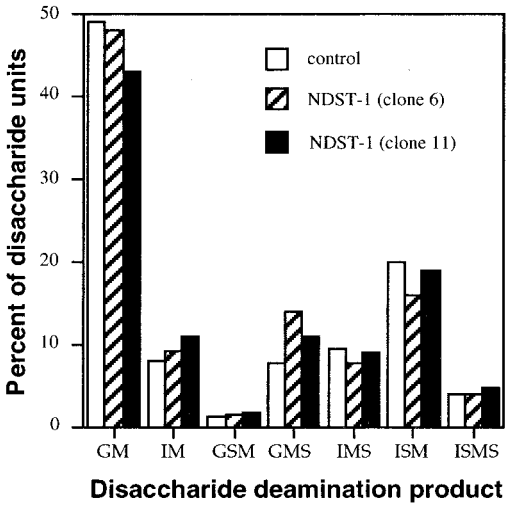

FIGURE 6: Disaccharide composition of HS from NDST-1 overexpressing and control cells. [^{3}H]Glucosamine-labeled HS from control cells and cells transfected with NDST-1 (clones 6 and 11) were isolated and degraded into disaccharides by treatment with hydrazine followed by combined deaminative cleavage at pH 1.5 and 3.9. The reduced deamination products were analyzed by ion-exchange chromatography and paper chromatography. The diagram illustrates the combined results from these analyses. Abbreviations: GM, GlcA-aMan$_R$; IM, IdoA-aMan$_R$; GSM, GlcA(2-OSO$_3$)-aMan$_R$; GMS, GlcA-aMan$_R$(6-OSO$_3$); IMS, IdoA-aMan$_R$(6-OSO$_3$); ISM, IdoA(2-OSO$_3$)-aMan$_R$; ISMS, IdoA(2-OSO$_3$)-aMan$_R$(6-OSO$_3$).

determined by gel chromatography (Figure 5). HS from both control clones showed an identical size distribution of N-sulfated domains, with a dominance of N-sulfated fragments in the size of tetra- to decasaccharides (Figure 5B). The NDST-1 overexpressing clone synthesized HS with an increased number of N-sulfated fragments in the size of 8−18-mers (Figure 5A). The most dramatic effect was obtained in the NDST-2 clone, where the HS contained a high proportion of the most extended N-sulfated domains (Figure 5A).

Disaccharide Composition. Previous characterization of the disaccharide composition of HS synthesized in NDST-2 overexpressing cells showed surprisingly that the increased N-sulfation did not affect the subsequent HS modifications, GlcA C5 epimerization and O-sulfation at various positions (*16*). Similar experiments were now performed with the NDST-1 overexpressing cells (clones 6 and 11). Metabolically labeled [^{3}H]HS was degraded into disaccharides after chemical N-deacetylation followed by combined deamination at pH 1.5 and 3.9. The resultant disaccharides were separated by ion-exchange chromatography. The nonsulfated disaccharides containing either GlcA or IdoA could not be resolved by this method and were therefore separated by paper chromatography. The GlcA/IdoA content and the *O*-sulfate substitution patterns of the disaccharides were similar in control and NDST-1 transfected cells (Figure 6).

Increased N-Sulfation Affects Polysaccharide Chain Elongation. Since it has been suggested that the HS copolymerase and NDST act in concert during chain elongation (*24*), it was of interest to investigate if the chain length was altered in NDST transfected cells. NDST-1 or NDST-2 overexpressing clones as well as control clones were labeled for a shorter period (60 min) with [^{35}S]sulfate, and labeled macromolecules were isolated be DEAE ion-exchange chromatography. After isolation of the proteoglycans by gel chromatography, the protein core bound glycosaminoglycan chains were released by alkali treatment and treated with chondroitinase ABC. The length of the remaining HS chains was determined by gel chromatography on Superose 6 (Figure 7). The results revealed a significant increase in chain length in cells overexpressing NDST. Control cells produced HS chains with an average M_r of $\sim 20 \times 10^3$, corresponding to $\sim$48 disaccharides (considering an average M_r for disaccharides of 420, based upon the degree of N- and O-sulfation). NDST-2 cells had a major peak at M_r $\sim 45 \times 10^3$, corresponding to $\sim$105 disaccharides (M_r average 430), while NDST-1 cells produced HS chains of intermediate length. Thus, the doubling of N-sulfation in HS from NDST-2 cells, compared to control cells, correlated with a dramatic increase in chain length ($\sim$120%). Repeated experiments with other clones (NDST-1; clone 6, NDST-2; clone S-5; and control clone k3) resulted in similar data (data not shown). Thus, the increase in chain length correlated with the type of NDST isoform that was overexpressed and was not likely to be due to unspecific differences between the different clones.

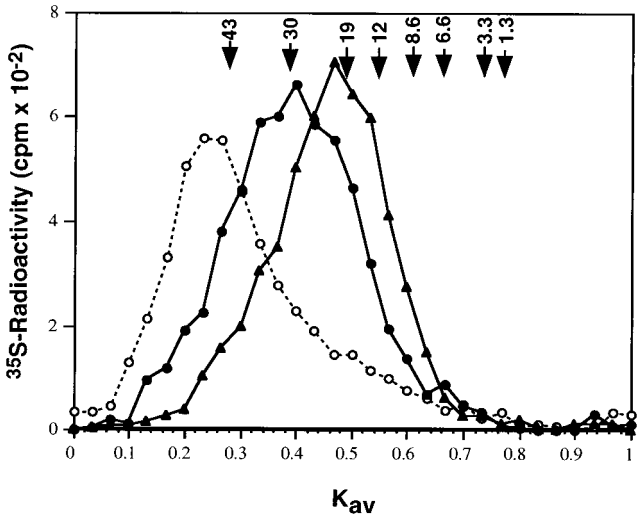

FIGURE 7: Size determination of HS synthesized by NDST-1 or -2 overexpressing cells and control cells. [^{35}S]Sulfate-labeled HS from control cells (triangles) and cells transfected with NDST-1 (filled circles) or NDST-2 (open circles) were isolated from intact proteoglycans as described in Experimental Procedures. The size distribution was analyzed by gel chromatography on a Superose 6 column in 0.5 M NH_4HCO_3. Fractions of 0.5 mL were collected at a flow rate of 0.5 mL/min and analyzed by scintillation counting. The positions of heparin fragments (1.3, 3.3, 6.6, and 8.6 kDa) and hyaluronan fragments (11.9, 18.9, 30, and 43 kDa), according to ref *29*, are indicated by arrows.

DISCUSSION

The aim of this study was to investigate the influence of the NDST-1 and NDST-2 isoforms on HS structure. Since NDST-2 had previously been expressed in 293 cells with a dramatic effect on N-sulfation of the HS synthesized (*16*), we decided to investigate if overexpression of NDST-1 in the same cell line had similar effects on HS structure or if the NDSTs influenced HS structure in an isoform-specific manner. In a paper by Cheung et al. (*16*), it was concluded on the basis of Northern blotting that the 293 cells expressed NDST-1, while no transcript for NDST-2 could be detected. A reexamination of this result using RT-PCR demonstrated that NDST-2 is actually also expressed by the cells, although at a low level (I. Eriksson and L. Kjellén, unpublished results). In contrast, high expression of NDST mRNA was seen after transfection of NDST-1 (Figure 1) and NDST-2 (*16*), respectively. On the basis of *N*-deacetylase activity, the increase in expression was on average ≈8-fold in the NDST-2 transfected cells (*16*) and up to 10-fold in the NDST-1 expressing cells (Figure 2). Structural analyses showed that the HS produced in NDST-1 and NDST-2 transfected cells had an increased degree of N-sulfation (Figure 3), demonstrating the importance of the enzyme concentration/amount for N-sulfation. However, HS from NDST-2 cells had a higher degree of N-sulfation (80%) than HS from NDST-1 cells (60%) despite lower *N*-deacetylase/*N*-sulfotransferase activity in solubilized NDST-2 cells (Figure 4). Thus, the NDST isoform also is important for the extent of N-sulfation.

Interestingly, the increase in N-sulfation correlated with an increase in the average polysaccharide chain length (Figure 7). A coupling between the polymerization and the sulfation processes has been suggested earlier on the basis of studies in a heparin-producing mouse mastocytoma microsomal system (*25*). In this study, the polysaccharide chain length increased when the sulfate donor PAPS was added to the incubations. These data can be explained by a decrease in K_m for the GlcA-transfer activity, which has a preference for substrates that are N-sulfated at the penultimate GlcN residue, toward the nonreducing end (*24*, *26*). Our results suggest that this lowered K_m has an impact on the heparin/HS biosynthesis in the intact cell and that the N-deacetylation/N-sulfation process may adjust the polysaccharide chain length in vivo.

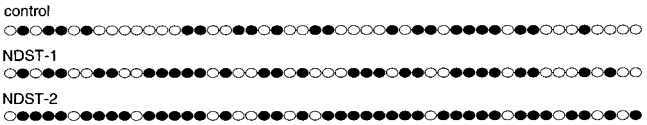

FIGURE 8: Tentative N-sulfation pattern of HS from NDST-1 and NDST-2 overexpressing clones. The figure illustrates possible distribution of N-sulfated glucosamine units in a HS polysaccharide of 50 disaccharide units that are either N-acetylated (open symbol) or N-sulfated (filled symbol). The reducing end is to the right. Data are based on the results presented in Figures 3 and 5.

On the basis of the results presented in Figures 3 and 5, tentative heparan sulfate chains with N-sulfation patterns characteristic for control 293 cells, NDST-1 and NDST-2 overexpressing clones, respectively, were constructed (Figure 8). The data will be biased due to the presence of fragments which have been cleaved at the reducing end by degradative enzymes in the cell (and by papain during the preparation) rather than by the deamination reagent. Therefore, the structures are not absolute but may serve to illustrate some typical features of HS synthesized by different isoforms of NDST. In HS isolated from normal cells and tissues the distribution of *N*-sulfate groups is not uniform. Instead, domains with consecutive N-sulfated GlcN units, domains with alternating $GlcNSO_3$ and GlcNAc units, and domains with consecutive N-acetylated GlcN units can be identified (*3*). This was also the case in untransfected 293 cells, as demonstrated by the size distribution of oligosaccharides obtained after deaminative cleavage of the HS chains (Figures 3B and 5B). The HS of these cells contains preferentially short N-sulfated domains, most of them containing only two consecutive *N*-sulfate groups, represented by a hexasaccharide after nitrous acid cleavage at pH 3.9 (Figure 5B). The overexpression of NDSTs influenced the N-sulfation pattern so that the number of more extended N-sulfated domains was increased at the expense of N-acetylated domains. The number of alternating $GlcNSO_3$ units (present as tetrasaccharides after deamination at pH 3.9) was constant or decreased after overexpression of either of the two isoforms (Figure 5A). Hence, none of the two NDST isoforms seem to be exclusively devoted to produce the alternating sequences mentioned above. Rather, overexpression of NDST-1 increases the number of domains with 3−6 consecutive $GlcNSO_3$ (8−14-mers in Figure 5A), while NDST-2 in addition increases the number of long domains (>6 $GlcNSO_3$ in a row). This is consistent with the high levels of NDST-2 in heparin-producing mast cells (*7*). It is also characteristic for heparin that single N-acetylated GlcN units occur interspersed between N-sulfated GlcN units. This pattern was also seen in the NDST-2 overexpressing clone, where sequences with only one N-acetylated GlcN unit (tetrasaccharides) was most abundant, while two or more

consecutive N-acetylated GlcN units (≥hexasaccharides) were relatively rare (Figure 3A).

Rat NDST-1 has previously been expressed in COS cells and in a COS cell mutant with reduced NDST levels (*27*). In this system, overexpression of the enzyme in the mutant cell increased the synthesis of fully N-sulfated oligosaccharides in the size classes of decasaccharides or greater, consistent with our results. However, transfection of the wild-type COS cells with NDST-1 had little effect on N-sulfation. These authors, who studied the biosynthesis of the basic FGF binding domain of heparan sulfate, hypothesized that the reason for the lack of effect of NDST-1 on wild-type cells was that the heparan sulfate of these cells already contained sufficient levels of N-sulfated domains with high affinity for basic FGF. Apparently, the 293 cells used in this study could tolerate highly N-sulfated heparan sulfate, as synthesized by both NDST-1 and NDST-2 transfected cells.

Other biosynthetic modifications such as GlcA C5 epimerization and O-sulfation occur in or in the vicinity of N-sulfated regions (*2*, *3*). The distribution and extension of *N*-sulfate groups would therefore be expected to affect these modifications. However, overexpression of NDST-2 did not influence the degree of further modifications (*16*). In this paper it was discussed whether this was due to inappropriate positioning of NDST-2 in the Golgi compartment in relation to the other heparan sulfate modifying enzymes which normally would cooperate with NDST-1. Since our results with NDST-1 showed a similar conservation of the IdoA ond *O*-sulfate content, this explanation appears less likely. Perhaps the epimerase and the different *O*-sulfotransferases are present in limiting amounts without any capacity to modify the increased amounts of substrate saccharide generated by the transfected NDST. This hypothesis could possibly be tested by cotransfecting an NDST isoform with another modification enzyme and investigating how this would affect the structure of the endogenous HS. The cDNA for the D-glucuronyl C5-epimerase would be a good choice since this enzyme is responsible for the first modification reaction following N-deacetylation/N-sulfation (*28*).

The finding of this paper, that overexpression of NDST-1 and -2 gives rise to HSs with different N-sulfation patterns and chain lengths, suggests that controlled expression of the different NDST isoforms is an important way to regulate HS biosynthesis. It will be interesting to investigate if the recently identified third and fourth NDST isoforms (*12*, *13*) differ from NDST-1 and -2 in their processing of the substrate, creating other N-sulfation patterns.

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REFERENCES

1. Lindahl, U., Lidholt, K., Spillmann, D., and Kjellén, L. (1994) *Thromb. Res. 75*, 1−32.
2. Salmivirta, M., Lidholt, K., and Lindahl, U. (1996) *FASEB J. 10*, 1270−1279.
3. Lindahl, U., Kusche-Gullberg, M., and Kjellén, L. (1998) *J. Biol. Chem. 273*, 24979−24982.
4. Rosenberg, R. D., Shworak, N. W., Liu, J., Schwartz, J. J., and Zhang, L. (1997) *J. Clin. Invest. 99*, 2062−2070.
5. Kjellén, L., and Lindahl, U. (1991) *Annu. Rev. Biochem. 60*, 443−475.
6. Lind, T., Tufaro, F., McCormick, C., Lindahl, U., and Lidholt, K. (1998) *J. Biol. Chem. 273*, 26265−26268.
7. Kusche-Gullberg, M., Eriksson, I., Pikas, D. S., and Kjellén, L. (1998) *J. Biol. Chem. 273*, 11902−11907.
8. Shworak, N. G., 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.
9. Hashimoto, Y., Orellana, A., Gil, G., and Hirschberg, C. B. (1992) *J. Biol. Chem. 267*, 15744−15750.
10. Orellana, A., Hirschberg, C. B., Wei, Z., Swiedler, S. J., and Ishihara, M. (1994) *J. Biol. Chem. 269*, 2270−2276.
11. Eriksson, I., Sandbäck, D., Ek, B., Lindahl, U., and Kjellén, L. (1994) *J. Biol. Chem. 269*, 10438−10443.
12. Aikawa, J., and Esko, J. D. (1999) *J. Biol. Chem. 274*, 2690−2695.
13. Aikawa, J., and Esko, J. D. (1999) in *15th International Symposium on Glycoconjugates*, p S40, Glycoconjugate Journal, Tokyo, Japan.
14. Humphries, D. E., Wong, G. W., Friend, D. S., Gurish, M. F., Qiu, W.-T., Huang, C., Sharpe, A. H., and Stevens, R. L. (1999) *Nature 400*, 769−772.
15. Forsberg, E., Pejler, G., Ringvall, M., Lunderius, C., Tomasini-Johansson, B., Kusche-Gullberg, M., Eriksson, I., Ledin, J., Hellman, L., and Kjellén, L. (1999) *Nature 400*, 773−776.
16. Cheung, W. F., Eriksson, I., Kusche-Gullberg, M., Lindahl, U., and Kjellén, L. (1996) *Biochemistry 35*, 5250−5256.
17. Graham, F. L., and van der Eb, A. J. (1973) *Virology 52*, 456−467.
18. Pettersson, I., Kusche, M., Unger, E., Wlad, H., Nylund, L., Lindahl, U., and Kjellén, L. (1991) *J. Biol. Chem. 266*, 8044−8049.
19. Shively, J. E., and Conrad, H. E. (1976) *Biochemistry 15*, 3932−3942.
20. Guo, Y., and Conrad, H. E. (1989) *Anal. Biochem. 176*, 96−104.
21. Shaklee, P. N., and Conrad, H. E. (1984) *Biochem. J. 217*, 187−197.
22. Riesenfeld, J., Höök, M., and Lindahl, U. (1982) *J. Biol. Chem. 257*, 421−425.
23. Maccarana, M., Sakura, Y., Tawada, A., Yoshida, K., and Lindahl, U. (1996) *J. Biol. Chem. 271*, 17804−17810.
24. Lidholt, K., and Lindahl, U. (1992) *Biochem. J. 287*, 21−29.
25. Lidholt, K., Kjellén, L., and Lindahl, U. (1989) *Biochem. J. 261*, 999−1007.
26. Lidholt, K., Fjelstad, M., Jann, K., and Lindahl, U. (1994) *Carbohydr. Res. 255*, 87−101.
27. Ishihara, M., Guo, Y., Wei, Z., Yang, Z., Swiedler, S. J., Orellana, A., and Hirschberg, C. B. (1993) *J. Biol. Chem. 268*, 20091−20095.
28. Li, J., Hagner-McWhirter, Å., Kjellén, L., Palgi, J., Jalkanen, M., and Lindahl, U. (1997) *J. Biol. Chem. 272*, 28158−28163.
29. Petersen, F., Brandt, E., Lindahl, U., and Spillman, D. (1999) *J. Biol. Chem. 274*, 12376−12382.

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