Distinct Effects on Heparan Sulfate Structure by Different Active Site Mutations in NDST-1[†]

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ABSTRACT: Heparan sulfate polymerization and modification take place in the Golgi compartment. The modification reactions are initiated by glucosaminyl *N*-deacetylase/*N*-sulfotransferase (NDST), a bifunctional enzyme that removes *N*-acetyl groups from selected *N*-acetyl-D-glucosamine units followed by N-sulfation of the generated free amino groups. Four isoforms of NDST have been identified. NDST-1 and -2 have a wide and largely overlapping tissue distribution, but it is not known if they can act on the same heparan sulfate chain. We have introduced point mutations into NDST-1 cDNA, which selectively destroy the *N*-deacetylase or *N*-sulfotransferase activity of the enzyme [Wei, Z., and Swiedler, S. J. (1999) *J. Biol. Chem. 274*, 1966−70 and Sueyoshi, T., et al. (1998) *FEBS Lett. 433*, 211−4]. Stable 293 cell lines expressing the NDST-1 mutants were then generated. Structural analyses of heparan sulfate synthesized by these cells and by cells overexpressing wild-type NDST-1 demonstrate that the N-deacetylation step is not only prerequisite but also rate-limiting, determining the degree of N-sulfation. Transfection of mutant NDST-1 lacking *N*-deacetylase activity had no effect on heparan sulfate sulfation, while cells expressing wild-type enzyme or NDST-1 lacking *N*-sulfotransferase activity both resulted in the production of oversulfated heparan sulfate. Since no increase in the amount of N-unsubstituted glucosamine residues was seen after transfection of the mutant lacking *N*-sulfotransferase activity, the results also suggest that two different enzyme molecules can act on the same glucosamine unit. In addition, we show that oversulfation of heparan sulfate produced by cells tranfected with wild-type NDST-1 or the mutant lacking *N*-sulfotranferase activity results in decreased sulfation of chondroitin sulfate.

Heparan sulfate (HS)[1] and the structurally related polysaccharide heparin consist of repeating glucuronic acid (GlcA) and *N*-acetylglucosamine (GlcNAc) units (*1*). HS proteoglycans are found on the cell surface of all adherent mammalian cells and in basement membranes. The major cell surface proteoglycans belong to the syndecan or glypican families, while perlecan, agrin, and collagen XVIII are basement membrane components (*2*). The variation in polysaccharide structure is large with complex sulfate patterns that create potential binding sites for different interacting proteins (*3*, *4*). During biosynthesis of the polysaccharide in the Golgi compartment, the growing chain is modified by sulfotransferases and a GlcA C5 epimerase. The first modification reaction is catalyzed by glucosaminyl *N*-deacetylase/*N*-sulfotransferase (NDST), which removes acetyl groups from GlcNAc residues, which are then sulfated through the *N*-sulfotransferase activity. Further modification steps include C5 epimerization of GlcA into IdoA, 2-*O*-sulfation of GlcA/IdoA, 6-*O*-sulfation of GlcN, and (more sparsely) 3-*O*-sulfation of GlcN units (*5*).

Many of the modification enzymes exist in several isoforms. Four isoforms of NDST (NDST-1−4) have been identified. NDST-1 and -2 have a wide and largely overlapping tissue distribution, while the expression of NDST-3 and -4 appears to be more restricted (*6*). It is not known if a given polysaccharide chain is modified by more than one NDST isoform, but the colocalization of NDST-1 and -2 in many tissues at least suggests that it is possible. Mutation of lysine 614 to an alanine and mutation of cysteine 486 to a tryptophan have previously been shown to completely destroy NDST-1 *N*-sulfotransferase and *N*-deacetylase activity, respectively (*7*, *8*). We have introduced these point mutations into NDST-1 cDNA, followed by overexpression of the altered cDNA in 293 cells. Structural analyses of HS produced by stable cell lines expressing the NDST-1 mutants indicate that the deacetylase of one enzyme and the sulfotransferase of another can act on the same glucosamine unit.

EXPERIMENTAL PROCEDURES

Generation of NDST-1 Mutants. Point mutations were introduced into mouse NDST-1 cDNA (Accession no.: NM_008306) using PCR. To destroy the *N*-deacetylase avtivity (MdAc), a PCR was performed using as a template NDST-1 cDNA cloned into a pCDNA3 expression vector

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[1] Abbreviations: HS, heparan sulfate; GlcA, D-glucuronic acid; GlcNAc, *N*-acetyl-D-glucosamine; NDST, glucosaminyl *N*-deacetylase/*N*-sulfotransferase; MdAc, NDST with a mutation in the *N*-deacetylase active site; MNS, NDST with a mutation in the *N*-sulfotransferase active site; dmut, NDST with mutations in both active sites; PBS, phosphate-buffered saline; PAPS, adenosine 3′-phosphate 5′-phosphosulfate; CS, chondroitin sulfate.

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(Invitrogen) (*9*), with sense primer ccggtaccgccgtggctttatccacaatggcatcatggtcctccctcggcagacctg**gg**gcc (IE31, nt 1401−1462; altered nucleotide in bold) and upstream, antisense primer atctggttggcatgaaaggcg (H216, corresponding to nt 2318−2298). The PCR product was cleaved with KpnI, with cleavage sites corresponding to nt 1407 and 2142 in NDST-1, and the cleavage product was used to replace the corresponding wild-type sequence of NDST-1 in pCDNA3. To destroy the *N*-sulfotransferase activity (MNS), a PCR was performed using the same template with sense primer cccaaagcttcttatcattggcccccag**gc**aacaggc (IE 32, nt 1812−1848; altered nucleotide in bold) and upstream, antisense primer ccaagcttgtgctggtaccaggagt (H11, corresponding to nt 2156−2132). The PCR product was cleaved using the 2142 restriction site of KpnI and a cleavage site for HindIII at nt 1816 and ligated into the pCDNA3 vector containing NDST-1 devoid of the corresponding wild-type fragment. A construct containing both point mutations (dmut) was also made by replacing the KpnI fragment (nt 1407−2142) of MNS with that from MdAc.

Transfection of Human Embryonic 293 Cells with the NDST-1 Mutants. The 293 cells were grown at 37 °C in a CO_2 incubator in Dulbecco's modified Eagle Medium/F12 nutrient mix (GIBCO BRL) containing 10% fetal calf serum (SIGMA), fungizone (2.5 IU/mL), and penicillin−streptomycin (100 IU/mL to 100 μg/mL GIBCO BRL). Transfections with constructs of MdAc, MNS, and dmut were made using the calcium phosphate coprecipitation method (*10*). Stable clones were selected under high concentration of Geneticin (800 μg/mL, G418 sulfate, SIGMA) and were further maintained at a G418 concentration of 400 μg/mL. Cells previously transfected with the NDST-1 and pcDNA3 vector only were used as controls (*9*).

Enzyme Assays. Cells in a T 25 flask were trypsinized, washed with cold phosphate-buffered saline (PBS), and solubilized for 30 min at 4 °C in 0.15 mL solubilization buffer (1% Triton X-100, 50 mM Tris-HCl (pH 7.4), 2 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, and 10 μg/mL pepstatin). Protein concentration was measured using the Bio-Rad protein assay. *N*-deacetylase and *N*-sulfotransferase activities were analyzed as described previously (*11*).

Northern Blot. Total RNA was isolated from the different clones using the RNeasy mini-kit (Qiagen). Denaturated total RNA from each clone (25 μg) was separated on a 0.9% agarose gel, and the RNA was blotted on a Hybond-N^+ membrane (Amersham). An NDST-1 probe corresponding to nucleotides 1−435 in NDST-1 cDNA (Accession no.: NM_008306) and a β-actin probe included in the Northern-Max-Gly kit were labeled with [^{32}P]dATP using Strip-Ez DNA Probe Synthesis and Removal kit (Ambion). The filter was first hybridized with the NDST-1 probe in ULTRAhyb solution for 2 h at 42 °C. After a 10 min wash in Low Stringency Wash Solution (Ambion) at 42 °C and a 2 × 15 min wash in High Stringency Wash Solution (Ambion) at 42 °C, the filter was applied on film (Kodak), which was exposed for 16 h. After stripping, the filter was hybridized with the β-actin probe.

SDS−PAGE and Western Blot. Trypsinized cells in T75 flasks were treated with 0.2 mL solubilization buffer containing 2 mM *N*-ethylmaleimide. Thirty μL of the supernatant obtained after centrifugation at 2500*g* for 15 min was denatured in loading dye at 96 °C. The solubilized proteins were separated on a 10% SDS−PAGE gel at 120 V in a Laemmli system under reducing conditions and electroblotted (Bio-Rad) at 225 mA for 1.5 h to a Hybond ECL nitrocellulose membrane (Amersham). The membrane was blocked in 5% milk before incubation for 1 h with anti-NDST-1 (DAST 1A; see below), diluted 1:500. After washing in TBS buffer 3 × 5 min, the membrane was incubated for 1 h with anti-rabbit-HRP antibody diluted 1:3000. After washing with TBS as described above, the filter was developed in an ECL system (RPN 2106, Amersham) according to the manufacturers protocol and exposed to a film (Fuji) for 1 min.

NDST-1 Antibody. The peptide KYFQIFSEEKDPL, corresponding to amino acids 569−581 in the mouse NDST-1 protein (Accesion no.: NP 032332), was selected for antibody production performed by Genosys Biotechnologies, UK. A keyhole limpet hemocyanin conjugate of the peptide was used as antigen for the generation of polyclonal rabbit antibodies. The resulting antiserum DAST 1A was used in Western blotting at a dilution of 1:500 in 5% milk.

Metabolic Labeling of Glycosaminoglycans. Subconfluent cultures of cells from stable clones expressing NDST-1, MNS, and MdAc or cells transfected with empty vector were cultured in T75 flasks as described above in low glucose D-MEM (GibcoBRL), 5% fetal calf serum, in the presence of 50 μCi/mL [^{3}H]GlcN (DuPont NEN) or in D-MEM F12 mix with 0.2 mCi/mL carrier-free [^{35}S]sulfate (DuPont NEN). After 24 h of incubation with [^{3}H]GlcN or 60 min incubation with [^{35}S]sulfate at 37 °C, the cells were washed with cold PBS, incubated in 2 mL of solubilization buffer, and centrifuged at 800*g* for 15 min at 4 °C. The supernatant containing radiolabeled macromolecules was recovered.

Heparan Sulfate Preparation. Metabolically ^{3}H-labeled proteoglycans and polysaccharide chains were purified on DEAE Sephacel (Amersham Pharmacia Biotech) as described previously (*12*). After papain (Sigma) digestion, the ^{3}H-labeled glycosaminoglycans were recovered after desalting on a PD-10 column. Lyophilized ^{3}H-glycosaminoglycans were then digested with 0.06 unit of chondroitinase ABC (Seikagaku) as previously described (*12*), followed by gel filtration on a Sephadex G-50 column (90 × 0.5 cm, superfine grade, Amersham Pharmacia Biotech) in 0.2 M NH_4HCO_3. ^{3}H-Labeled HS eluting in the void volume of the column was recovered.

Isolation of HS Chains from Intact ^{35}S-Labeled Proteoglycans. ^{35}S-Labeled proteoglycans and free polysaccharide chains obtained after solubilization of the cells were isolated on DEAE-Sephacel as previously described (*12*). The ^{35}S-labeled proteoglycans were recovered after gel filtration on a Superose 6 10/30 column (Amersham Pharmacia Biotech) in 50 mM Tris-HCl, 0.5 M NaCl, 0.1% Triton X-100, 2 mM EDTA, 1 mM phenylmethanesulfonyl fluoride, 2 mM *N*-ethylmaleimide, and 10 μg/mL pepstatin. To release the polysaccharide chains, the proteoglycans were treated with 0.5 M NaOH for 18 h at 4 °C, followed by neutralization with HCl and desalting on a PD-10 column. After treatment with chondroitinase ABC, the resulting free ^{35}S-labeled HS chains were reisolated and analyzed for size distribution on the Superose 6 column eluted with 0.5 M NH_4HCO_3.

Structural Analysis of Heparan Sulfate. ^{3}H-Labeled HS was cleaved with nitrous acid at pH 1.5 (*13*) or at pH 3.9 (*14*). The generated oligosaccharides were separated by gel

Table 1: *N*-Deacetylase and *N*-Sulfotransferase Activities[a]

overexpressing clones	*N*-deacetylase activity (^{3}H-acetate released; cpm)	*N*-sulfotransferase activity (^{35}S-sulfate incorporated; cpm × 10^{-3})
MNS	358	14
MdAc	32	686
NDST-1	266	660
vector only	32	61

[a] Cells were trysinized and solubilized in Triton X-100 containing buffer as described in Experimental Procedures. The extracts from the different cells were used for determination of *N*-deacetylase and *N*-sulfotransferase activity (triplicate samples; 80 μg of total protein analyzed). The mean values are given in the table.

chromatography on a column of Bio-Gel P-10 (1 × 140 cm; fine grade, Bio-Rad) eluted in 0.5 M NH_4HCO_3 (pH 1.5 samples) or on a Superose 6 10/30 column in 0.5 M NH_4HCO_3 (pH 3.9 samples).

Quantitation of ^{3}H-Glucosamine and ^{35}S-Sulfate Incorporation. The different cell lines were incubated for 10 h as described above, with 50 μCi/mL [^{3}H]GlcN or with 0.2 mCi/mL carrier-free [^{35}S]sulfate. The 10 h incubation time was chosen after a preliminary time course experiment, when NDST-1 overexpressing cells and control cells were incubated in the presence of ^{35}S-sulfate, and the amount of ^{35}S-labeled glycosaminoglycans synthesized was determined. The radiolabeled glycosaminoglycans were isolated by DEAE ion exchange chromatography, and the amounts of ^{3}H and ^{35}S was measured and related to protein concentration (determined using a Bio-Rad protein assay). After treatment with 0.5 M NaOH for 18 h at 4 °C followed by neutralization, the labeled glycosaminoglycans were digested with chondroitinase ABC and subjected to gel chromatography on a Sephadex G-50 column. The relative amount of ^{3}H-labeled and ^{35}S-labeled HS and chondroitin sulfate (CS) were calculated based on peak areas.

Anion Ion-Exchange Chromatography. ^{35}S-Labeled glycosaminoglycans (10 000 cpm) were applied to a Mono Q column (Amersham Pharmacia Biotech) equilibrated in 50 mM Tris-HCl (pH 8.0), 0.3 M NaCl before and after treatment with nitrous acid at pH 1.5. The column was eluted in the same buffer with a gradient ranging from 0.3 to 1.5 M NaCl.

RESULTS

Point mutations were introduced into NDST-1 cDNA to create enzyme molecules devoid of either *N*-deacetylase or *N*-sulfotransferase activity. After transfection, stable clones were selected expressing the *N*-deacetylase mutant enzyme (MdAc) or the *N*-sulfotransferase mutant enzyme (MNS). These cell lines and the previously generated cell lines transfected with wild-type NDST-1 or empty vector (*9*) were analyzed for *N*-deacetylase and *N*-sulfotransferase activity (Table 1). The enzyme activities of cells transfected with empty vector are very low as compared to those of NDST-1 transfected cells. However, it should be noted that this level is sufficient for the 293 cells to produce N-sulfated heparan sulfate (see below and ref *9*). Cells transfected with the *N*-sulfotransferase mutant enzyme (MNS) retain their *N*-deacetylase activity, while cells expressing the *N*-deacetylase mutant enzyme (MdAc) have lost their *N*-deacetylase activity but display high levels of *N*-sulfotransferase activity.

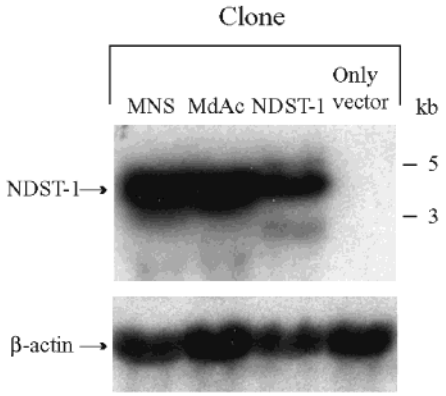

FIGURE 1: mRNA expression. Total RNA from each clone was separated by agarose electrophoresis, blotted to a Hybond$^+$ membrane, and hybridized with NDST-1 probe corresponding to nucleotides 1−435 of NDST-1 cDNA. After stripping, the membrane was hybridized with a β-actin probe. Positions of standard RNA of known size are indicated (RNA Millennium, Ambion).

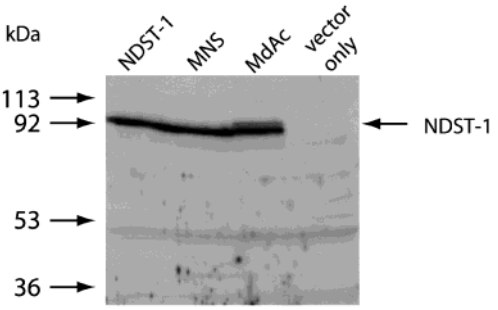

FIGURE 2: Protein expression. Cells were trypsinized and solubilized in Triton X-100 containing buffer, and an aliquot was separated by SDS−PAGE electrophoresis under reducing conditions. The separated proteins were blotted to a nitrocellulose membrane. NDST-1 was detected using the ECL system (Amersham) after incubation with anti-NDST-1 antibody, followed by incubation with a secondary antibody, conjugated to horseradish peroxidase. Positions of LMW standard proteins are indicated (Bio-Rad).

Northern blot analysis demonstrated that similar amounts of MNS and MdAc mRNA were present in the two cell lines, slightly exceeding the level of NDST-1 mRNA (Figure 1). Expression at the protein level was also equal in the different cell lines, as demonstrated by Western blotting (Figure 2). All four cell lines examined showed similar background because of unspecific binding of the antibody.

No Increase in HS Free Amino Groups as a Result of MNS Expression. To investigate how the presence of the mutant enzymes within the cells influenced the structure of HS synthesized, ^{3}H-labeled HS isolated after incubation of the cells with ^{3}H-glucosamine was characterized. Since a larger proportion of the HS glucosamine residues could be expected to remain deacetylated, but devoid of sulfate in cells overexpressing MNS, the ^{3}H-labeled HS preparations were treated with nitrous acid at pH 3.9, which results in the cleavage of the polysaccharide at N-unsubstituted glucosamine units. As shown in Figure 3, the treatment has only minor effects on the elution behavior on Superose 6 of HS from both MNS (Figure 3A) and NDST-1 overexpressing cells (Figure 3B). The low molecular weight peak appearing after the treatment in both samples indicates the presence of some N-unsubstituted glucosamine residues, but no increase is seen in HS from MNS-expressing cells as compared to HS synthesized by the cells overexpressing NDST-1. Similar

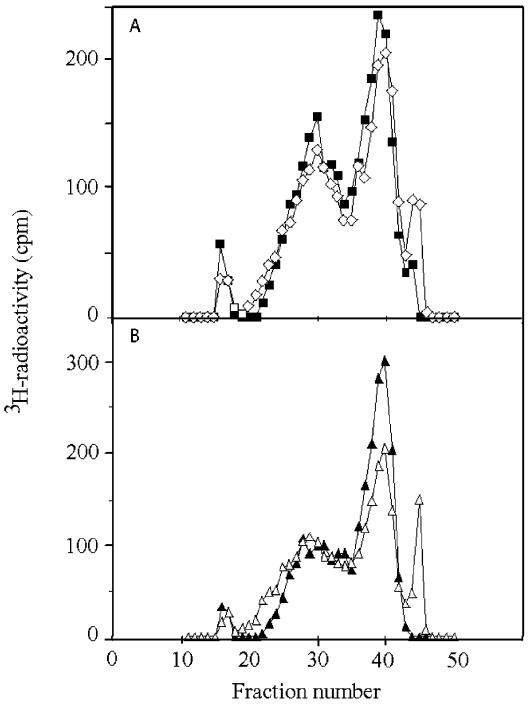

FIGURE 3: Gel chromatography of HS cleaved at N-unsubstituted GlcN residues. [^{3}H]HS was treated with nitrous acid at pH 3.9 followed by gel chromatography on a Superose 6 column at a flow rate of 1 mL/min. Fractions of 0.5 mL were collected. (A) HS from cells overexpressing the NDST-1 mutant MNS and (B) HS from cells overexpressing NDST-1. Filled and open symbols represent samples before and after nitrous acid treatment, respectively.

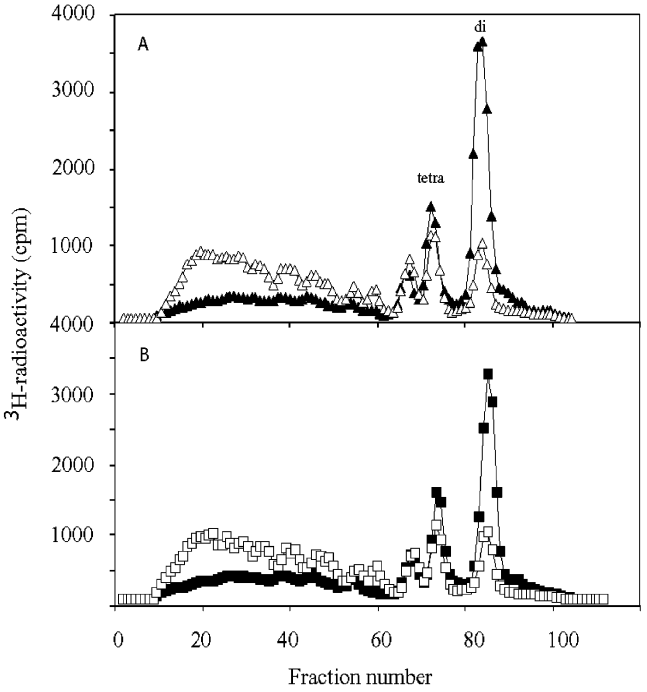

FIGURE 4: Gel chromatography of HS cleaved at N-sulfated GlcN residues. [^{3}H]HS was treated with nitrous acid at pH 1.5 followed by gel chromatography on a Bio-Gel P-10 column. Fractions of 0.8 mL were collected at a flow rate of 1.6 mL/h. (A) HS from cells expressing NDST-1 (▲) and cells transfected with only vector (×) and (B) HS from cells overexpressing the NDST-1 mutants MNS (■) and MdAc (□), respectively. The elution positions for di- and tetrasaccharides are indicated.

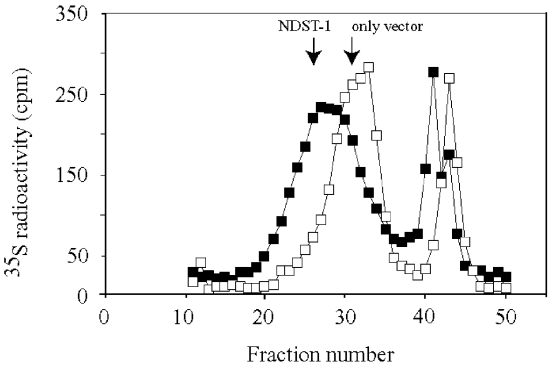

FIGURE 5: Size determination of HS synthesized by the different clones. [^{35}S]Sulfate labeled polysaccharide chains released from isolated proteoglycans of cells overexpressing MNS (■) and MdAc (□), respectively, were digested with chondroitinase ABC and analyzed for chain length by gel chromatography on a Superose 6 column. Fractions of 0.5 mL were collected at a flow rate of 0.5 mL/min. The elution positions of HS from cells expressing NDST-1 and empty vector are indicated.

results were obtained for HS from cells transfected with MdAc or vector alone (data not shown).

Increased N-Sulfation with the Mutant Lacking N-Sulfotransferase Activity. To study the degree of N-sulfation, the ^{3}H-labeled HS preparations were treated with nitrous acid at pH 1.5, followed by gel chromatography on Bio-Gel P-10 (Figure 4). During nitrous acid treatment, the polysaccharide chain is cleaved at N-sulfated glucosamine units; the extent of depolymerization thus correlates with the degree of N-sulfation. HS produced by cells overexpressing NDST-1 and MNS both showed increased N-sulfation as compared to HS from cells transfected with empty vector. In contrast, no effect on N-sulfation was seen in HS from MdAc-expressing cells. The degree of N-sulfation, based on calculations of peak areas, was 64% in HS from cells overexpressing NDST-1 and 59% in HS from MNS-overexpressing cells, while no effect on N-sulfation as compared to the control was seen in HS from MdAc-expressing cells (33% N-sulfation).

Increased Chain Length in Cells Overexpressing NDST-1 and MNS. ^{35}S-Labeled HS chains obtained from isolated proteoglycans after alkali treatment and chondroitinase ABC digestion were analyzed for chain length by gel chromatography on a Superose 6 column (Figure 5). While cells overexpressing MdAc produce HS with the same chain length as cells transfected with empty vector ($M_r \approx 20 \times 10^3$; ref *9*), HS from cells transfected with MNS had an increased chain length. The size of these chains was similar to that of HS from cells overexpressing NDST-1 ($M_r \approx 30 \times 10^3$; ref *9*; Figure 5). The retarded peaks seen in Figure 5 are the chondroitinase ABC degradation products. The double peak found after digestion of ^{35}S-labeled glycosaminoglycans from MNS expressing cells was reproducible and present also after digestion of ^{35}S-labeled glycosaminoglycans from NDST-1 expressing cells (see also below).

Decreased CS Sulfation in Cells Overexpressing NDST-1 and MNS. Overexpression of NDST-1 and its mutants could possibly affect the organization of the biosynthesis machinery and influence, for example, the amount of polysaccharides synthesized. However, when the different cell lines previously described, along with a cell line expressing an NDST-1 mutant lacking both enzyme activities (dmut), were incubated with [^{3}H]GlcN for 10 h and the amounts of ^{3}H-labeled HS and CS produced were quantitated, they all seemed to

Table 2: [^{3}H]GlcN and [^{35}S]Sulfate Incorporation into Glycosaminoglycans Synthesized by the Different Clones[a]

clone	^{35}S-GAG/^{3}H-GAG	^{35}S-HS/^{3}H-HS	^{35}S-CS/^{3}H-CS
MNS	1.4	2.0	0.8
MdAc	0.9	0.8	1.2
dmut	0.8	0.8	1.2
NDST-1	1.2	1.5	0.4
only vector	0.9	0.9	1.2

[a] Cells were metabolically labeled with [^{3}H]GlcN and [^{35}S]sulfate separately, and radiolabeled glycosaminoglycans were isolated. The total amount of ^{3}H- and ^{35}S-labeled glycosaminoglycans per mg of protein was determined. The relative amount of radiolabeled CS and HS was determined after chondroitinase ABC treatment of the isolated glycosaminoglycans followed by gel chromatography on Sephadex G50.

produce similar amounts of polysaccharides (data not shown). All cell lines were also incubated with ^{35}S-sulfate and the ratio between incorporated ^{35}S- and ^{3}H-radioactivity was determined (Table 2). As expected, the ^{35}S/^{3}H ration was higher for HS from MNS and NDST-1 cell lines than for HS synthesized by the other cell lines (Table 2). In addition, these clones also synthesized CS with a decreased ^{35}S/^{3}H ratio (Table 2).

When analyzed by ion-exchange chromatography, it was noted that a portion of the CS from cells overexpressing NDST-1 eluted before the main peak, which had an elution position similar to the CS synthesized by cells transfected with empty vector (Figure 6). CS synthesized by cells overexpressing MNS was similar to that produced by NDST-1 overexpressing cells, while cells expressing MdAc or dmut synthesized CS with the same elution pattern as cells transfected with empty vector (data not shown). Tentatively, the altered elution properties of the chondroitinase ABC degradation products in Figure 5 could be due to the production in MNS and NDST-1 expressing cells of undersulfated CS.

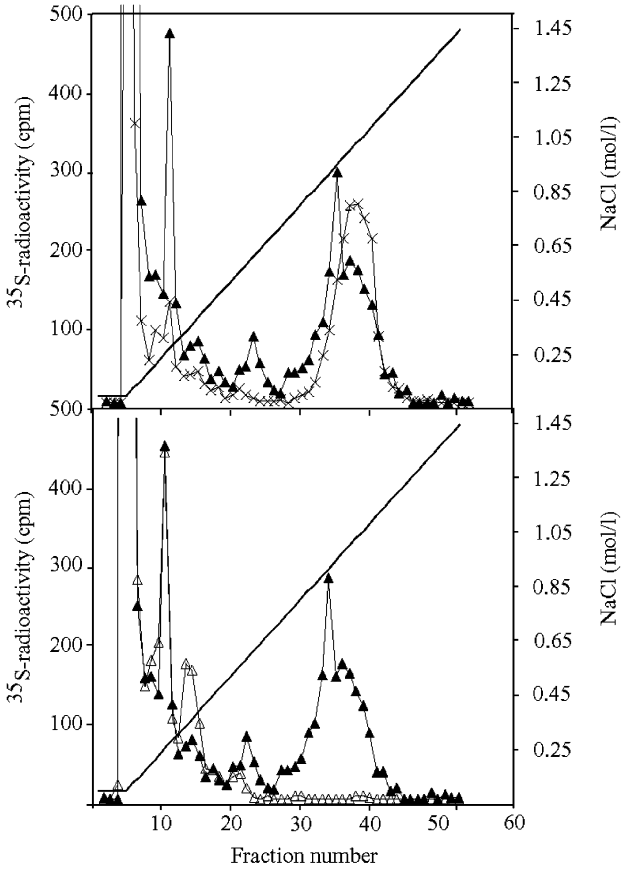

FIGURE 6: Chondroitin sulfate charge density. Cells overexpressing NDST-1 (▲) and cells transfected with only vector (×) were metabolically labeled with [^{35}S]sulfate, and isolated glycosaminoglycans were treated with nitrous acid at pH 1.5. The digests were analyzed by MonoQ anion exchange chromatography as described in Experimental Procedures. [^{35}S]Sulfate labeled glycosaminoglycans from cells overexpressing NDST-1 were also digested with chondroitinase ABC after nitrous acid treatment and analyzed on the column (△).

DISCUSSION

Overexpression of NDST-1 as well as NDST-2 in human embryonic 293 cells have previously been shown to result in increased N-sulfation of heparan sulfate produced by the cells (*9*, *12*). Apparently, the transfected NDST is transported to the Golgi compartment where it encounters the endogenous heparan sulfate synthesized by the cells. It has previously been suggested that different NDST isoforms may work together in the same cell (*4*). Tentatively, two (or more) NDST isoforms could be located to different Golgi compartments and act on the polysaccharide at different stages of maturation of the chain.

In this paper, we have characterized HS produced by 293 cells after transfection of NDST-1 lacking *N*-deacetylase activity, *N*-sulfotransferase activity, or both. Similar amounts of the wild type and mutant NDSTs appeared to be expressed by the cells (Figures 1 and 2), and the difference in enzyme activities in extracts of the transfected cells suggested that the two different mutations had been successful (Table 1). Enzyme activity assays on an isolated truncated His-tagged variant of MNS support this notion (data not shown). Several of the results obtained are puzzling. No increase in N-unsubstituted glucosamine residues was found in HS produced by cells overexpressing MNS (Figure 3). In addition, the level of N-sulfation of HS synthesized by cells expressing MNS is the same as that for HS produced by cells transfected with NDST-1 (Figure 4). Several explanations to these results are possible, one being that the MNS mutant still retains *N*-sulfotransferase activity. On the basis of the results presented in Table 1 and the lack of *N*-sulfotransferase activity in isolated His-tagged MNS (see above), this alternative seems unlikely. Another possibility could be that the overexpressed molecules only indirectly influence the biosynthesis, increasing the activities of endogenous NDST enzymes. However, since the effects are clearly linked to the enzyme activities of the overexpressed molecules, this explanation is also less satisfacory. Instead, we favor the third possibility that MNS has acted as an *N*-deacetylase, and an endogenous *N*-sulfotransferase has transferred sulfate groups to the free amino groups; 293 cells have previously been shown to express both NDST-1 and -2 (*9*). Such a mechanism would explain why HS structure and chain length are similarly influenced by MNS and wild-type NDST-1. Obviously, N-deacetylation is not only prerequisite but also rate-limiting in HS N-sulfation since the presence of large amounts of *N*-sulfotransferase in MdAc did not lead to any increase in N-sulfation.

Another finding is that the mutants do not influence the amount of ^{3}H-HS produced by the cells, but the polysac-

charide chain length is increased from $M_r \approx 20 \times 10^3$ in control cells to $M_r \approx 30 \times 10^3$ in cells overexpressing NDST-1 or MNS (Figure 5). Thus, the polymerization reactions appear to occur at the same rate in all cells, but the presence of NDST-1 or MNS influences by unknown mechanisms the machinery to make fewer but longer chains. Interestingly, the D-glucuronosyltransferase reaction has been shown to prefer an N-sulfated substrate (*15*), and chain elongation in a mastocytoma microsomal system is promoted by concomitant N-sulfation (*16*).

Finally, oversulfation of HS in the NDST-1 and MNS overexpressing cells results in decreased sulfation of chondroitin sulfate (Table 2, Figure 6). One explanation to this result could be reduced availability of the sulfate donor PAPS for the chondroitin sulfate sulfotransferases, which instead is used for HS N-sulfation. However, the elution profile on Mono Q (Figure 6) may suggest that the lowered sulfation is more selective since a large portion of the labeled chondroitin sulfate synthesized by the NDST-1 overexpressing cells elutes at the same position as chondroitin sulfate produced by control cells.

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