Undersulfated Heparan Sulfate in a Chinese Hamster Ovary Cell Mutant Defective in Heparan Sulfate N-Sulfotransferase*

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Heparan sulfate N-sulfotransferase catalyzes the transfer of sulfate groups from adenosine 3'-phosphate,5'-phosphosulfate to the free amino groups of glucosamine residues in heparan sulfate. We have identified a Chinese hamster ovary cell mutant, designated pgsE-606, which is 3-5-fold defective in N-sulfotransferase activity. The residual enzyme activity is indistinguishable from the wild-type enzyme with respect to K_m values for adenosine 3'-phosphate,5'-phosphosulfate and N-desulfoheparin, pH dependence, Arrhenius activation energy, and thermal lability. The mutation is recessive, and mixing experiments indicate that the mutant does not produce soluble antagonists of N-sulfotransferase. Inspection of the heparan sulfate chains from the mutant showed that the extent of N-sulfation is reduced about 2-3-fold. The addition of sulfate to hydroxyl groups on the chain is reduced to a similar extent, suggesting that N-sulfation and O-sulfation are normally coupled. Nitrous acid fragmentation of the chains showed that N-sulfated glucosamine residues are spaced much less frequently than in heparan sulfate from wild-type cells. The close correlation of enzyme activity to the number and position of N-sulfate groups indicates that N-sulfotransferase plays a pivotal role in determining the extent of sulfation of heparan sulfate.

Heparan sulfate and heparin are synthesized by polymerization of N-acetylgalactosamine and glucuronic acid residues. The chains are synthesized while attached to a core protein through the linkage tetrasaccharide, -D-GlcUApβ1→3-D-Galpβ1→4-D-Xypβ1→[L-Ser] (1-4). After chain polymerization, the repeating disaccharides are modified by a series of enzyme-catalyzed reactions thought to occur in the trans-Golgi (5). N-Acetyl-D-glucosamine residues are deacetylated, and sulfate is transferred from PAPS to the resulting free amino groups. Adjacent glucuronic acid residues on the reducing side of N-sulfated glucosamine residues may epimerize to iduronic acid and subsequently undergo 2-O-sulfation. Glucosamine residues near the sites of N-sulfation are sulfated at C-6, and occasionally sulfate is added to N-sulfated glucosamine residues at C-3 (1-3). Recent studies indicate that xylose in the linkage tetrasaccharide of heparan sulfate chains is phosphorylated at C-2 (6).

Studies of heparin biosynthesis in mastocytoma microsomes indicate that N-deacetylation and N-sulfation begin chain modification and that the reactions take place sequentially in the order described above (1, 4, 7). Thus, modified sugars tend to be clustered along the chain (1, 4). The overall level of polymer modification seems to be genetically determined since different cell lines maintain a characteristic sulfation pattern through numerous cell generations (3, 8). Over 80% of N-acetylgalactosamine residues in mast cell heparin are N-sulfated, whereas 40-50% of the N-acetylgalactosamine residues in heparan sulfate are N-sulfated (3, 8). Lindahl and co-workers (1) proposed that the polymer modification reactions may be organized like an assembly line in which core proteins bearing unmodified chains encounter fixed "enzyme stations" arranged in the order in which the modifications take place. This model suggests that the extent of modification and the position of the modified sugars are determined by the quantity and distribution of the biosynthetic enzymes.

One can address how the modification reactions are regulated by studying mutants defective in heparan sulfate biosynthesis. Several CHO cell mutants with defects in glycosaminoglycan biosynthesis are available, including strains defective in xylosyltransferase (9), galactosyltransferase I (10), sulfate transport (11), and heparan sulfate and chondroitin sulfate synthesis (12). In this report, we describe a new CHO cell mutant, designated pgsE-606, which is defective in heparan sulfate N-sulfotransferase activity. Characterization of the heparan sulfate made by the mutant suggests that O-sulfation is coupled to N-sulfation and that the specific activity of heparan sulfate N-sulfotransferase determines the extent of polymer modification.

EXPERIMENTAL PROCEDURES

Cell Culture—Chinese hamster ovary cells (CHO-K1) were obtained from the American Type Culture Collection (CCL-61; Rockville, MD). Mutant 606 was identified by 35S-colony autoradiography as described (9), and the purity of the strain was verified by resolation from cultures containing only mutant colonies. Cell hybrid mutants defective in glycosaminoglycan synthesis were previously designated by numbers. Because they define unique complementation groups, the strains will be indexed in this and future publications according to the three letter code, pgs, an acronym for proteoglycan synthesis. The phenotypes of the complementation groups are: pgsA, xylosyltransferase-deficient; pgsB, galactosyltransferase I-deficient; pgsC, sulfate transport-deficient; pgsD, heparan sulfate-deficient; pgsE, N-sulfotransferase-deficient. The individual strain numbers are separated from the three letter code by a dash. For example, mutant 745, defective in xylosyltransferase, is designated pgsA-745.

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1 The abbreviations used are: PAPS, adenosine 3'-phosphate,5'-phosphosulfate; CHO, Chinese hamster ovary; HPLC, high performance liquid chromatography; MES, 2-(N-morpholino)ethanesulfonic acid; Me2SO, dimethyl sulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; CR, Chinese hamster ovary; HEPES, high performance liquid chromatography; Me2SO, dimethyl sulfoxide; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; MES, 2-(N-morpholino)ethanesulfonic acid.
bridization studies indicate that mutant 606 belongs to a new complementation group designated pgsE. Cells were maintained in Ham’s F-12 medium (Meditech, Washington, DC) supplemented with 7.5% (v/v) fetal bovine serum (HyClone Laboratories, Logan, UT), 100 μg/ml streptomycin sulfate, and 100 units/ml penicillin G. Low glucose (1.0 mM) medium was used for all radioassay experiments and was prepared as described previously (11). Cells were grown at 37 °C ± 0.2 °C in an atmosphere of 5% CO2 in air and 100% relative humidity. They were subcultured every 3–4 days with 0.125% trypsin, and after 15–20 passages, fresh cells were revived from frozen stocks stored in liquid nitrogen.

A thionine-resistant, ouabain-resistant clone (OT-1) of wild-type CHO cells was isolated by first obtaining a spontaneous mutant resistant to 10 μM 6-thioguanine in hypoxanthine-free F-12 medium supplemented with dialyzed fetal bovine serum. The mutant was treated with mutagen (13), and an ouabain-resistant clone was selected in F-12 medium containing 1 μM ouabain. Cell hybrids were generated by cotransferring 2 × 10^6 mutant or wild-type cells with 2 × 10^5 OT-1 cells in 24-well culture dishes at 33 °C. The next day, the medium was removed, and the cells were washed and incubated for 1 min with 0.2 ml of 50% (w/v) polyethylene glycol prepared in F-12 medium without serum (14). The treated cells were well treated and incubated overnight at 33 °C in regular growth medium. Pulsed cells were treated with trypsin, resuspended in 2 ml of growth medium, and 0.1, 0.5, or 1.0 ml of the cell suspension was plated at 37 °C in medium containing 1 mM ouabain and 10 μM aminopterin. Under these conditions, only cell hybrids survive because wild-type and pgsE-606 cells are killed by ouabain, and OT-1 cells are resistant to aminopterin. The cultures were overlaid with Whatman No. 42 filter paper discs (13) and incubated for 10 days. The growth medium was replenished once. Five colonies from a fusion of pgsE-606 cells with OT-1 cells were picked and recloned under selective conditions to ensure their purity.

Isolation of Radiolabeled Heparan Sulfate—CHO cell monolayers were labeled with 10 μCi/ml Na[35]SO4, (25–40 Ci/mg) or 20 μCi/ml of 0.16–0.18% glucosamine (40Ci/mmol) for 3 days (10). The cells were washed three times with cold phosphate-buffered saline without calcium or magnesium (15) and solubilized with 0.1 M NaOH at 25 °C for 15 min. Two 50-μl aliquots of each alkaline extract were removed for protein determination according to the method of Lowry et al. (16) with bovine serum albumin as standard. The remainder of the cell extracts was adjusted to pH 5.5 with 0.1 M acetic acid, combined with the medium, and treated with 2 mg/ml nonspecific protease (Boehringer Mannheim) at 40 °C (10). Five mg of shark chondroitin sulfate (Sigma) was included as carrier to ensure efficient recovery of radiolabeled glycosaminoglycan. After incubation, the reaction mixture was diluted 5-fold with water to reduce the salt concentration to 0.1 M. The solution was applied to a 0.5-ml column of DEAE-Sephacel prepared in disposable polypropylene pipette tips plugged with glass wool. The columns were washed with 15 ml of 0.1 M NaCl, 20 mM Tris-HCl, pH 7.4, to remove SO4. The glycosaminoglycans were eluted with 2 ml of 0.1 M NaCl, 20 mM Tris-HCl, pH 7.4, and precipitated with 4 volumes of ethanol at 4 °C. The precipitate was dissolved in 1 ml of 0.5 M sodium acetate at 10 °C and precipitated with 4 volumes of ethanol at 4 °C. The purified glycosaminoglycans were analyzed by anion-exchange HPLC as described (11) except the salt gradient started at 50 mM instead of 0.2 M NaCl, and all buffers contained 0.2% 2-mercaptoethanol (Cibi-schem). Detergent was included to increase the resolution of the HPLC column and did not affect the elution of glycosaminoglycan. The lower salt concentration permitted the isolation of undersulfated glycosaminoglycan chains. Structural Studies of Purified Heparan Sulfate—To examine the amount and distribution of N-sulfate groups on heparan sulfate, glycosaminoglycans isolated by DEAE-Sephacel chromatography were reapplied to DEAE-Sephacel. Elution with 0.7 M NaCl, 20 mM Tris-HCl, pH 7.4, separated radioactive CHO cell glycosaminoglycans from the shark cartilage chondroitin sulfate carrier. Labeled glycosaminoglycans were eluted at a flow rate of 0.1 ml/min, and 0.5-ml fractions were collected. The elution of sulfate and heparin from the column was measured by barbital reaction (19).

Nitrous acid deamination of heparan sulfate was performed according to the low pH method of Shively and Condor (24). A portion of purified 35S-H-heparan sulfate was lyophilized to dryness and resuspended in 45 μl of water containing 50 μg of heparin 150 μl of nitrous acid, pH 1.5, was added, and the mixture was incubated for 10 min at room temperature. The sample was neutralized with 15 μl of 2 M Na2CO3 and adjusted to 0.5 M with 0.5 M pyridine acetate containing 100 μg of heparin and a small amount of sucrose. Nitrous acid was dissolved in 250 μl of 0.5 M pyridine acetate, and a small amount of sucrose was added to determine the Vc of the column. Free sulfate was separated from intact chains by chromatography on Sephadex G-50 (25 × 1.0 cm) with 0.5 M pyridine acetate, pH 5.0, as the eluate. The column was run at a flow rate of 0.1 ml/min, and 0.5-ml fractions were collected. Sucrose eluted close to the position of the heparan sulfate disaccharides.

Heparan Sulfate N-Sulfotransferase Assay—CHO cells were grown to confluence in 150-mm diameter dishes, rinsed three times with cold phosphate-buffered saline (15), and detached with a rubber policeman in 1 ml of 0.25 M sucrose buffer containing 50 mM Tris-HCl, pH 7.5, 1 μg/ml leupeptin, 0.5 μg/ml peptatin, and 0.2 mM EDTA. The cells were sedimented at 5 min at 4 °C in a clinical centrifuge, resuspended in sucrose buffer, and stored at −20 °C. The cell suspension was frozen and thawed at least twice to generate homogenates.

N-Sulfotransferase activity was measured by incubating 1 mg/ml crude CHO cell homogenate protein at 37 °C with 1 mg/ml N-desulfoheparin, 50 μM [35S]PAPS (200–800 cpm/pmol), 10 mM MgCl2, 1 mM MnCl2, 1% Triton X-100 (v/v), and 50 mM HEPES, pH 7.4, in a total volume of 25 μl. The reaction was stopped by the addition of 475 μl of 0.1 M EDTA, pH 7.4, containing 0.25 mg/ml of heparin. The reaction mixture was applied to a 0.25-ml DEAE-Sephacel column prepared in disposable polypropylene pipette tip plugged with glass wool. The column was washed with 7 ml of 0.25 M NaCl, and 35S-labeled product was eluted with 1 ml of 1 M NaCl. Standard chondroitin sulfate (0.8 mg) was added as carrier, and 35S-labeled product was precipitated overnight at 4 °C with 4 volumes of ethanol. After centrifugation (5 min at 10000 rpm in a clinical centrifuge), the ethanol was aspirated. The pellet was resuspended in 0.5 ml of water and 2.5 ml of Patterson and Green scintillation fluid (21), and radioactivity was determined by liquid scintillation spectrometry. A mixture of 25 mM HEPES and 25 mM MBS was used to study the pH dependence of the reaction. The pH of the reaction mixture was determined with a pH meter. Over 90% of the radioactive sulfate transferred to N-desulfoheparin was liberated by solvolysis in 95% MeSO4, 5% H2O (v/v) as described above. Preparation of Substrates—[35S]PAPS was prepared following the procedure of Renostro and Segel (22) using a yeast homogenate (Saccharomyces cerevisiae) as described by Robbins (23). One mg of 35S was incubated with 16 mM ATP, 32 mM MgCl2, 120 mM Tris-HCl, pH 8.5, and 3 mg/ml yeast extract protein for 1 h at 37 °C. The reaction was stopped by heating the reaction mixture for 2 min at 100 °C. Precipitated protein was sedimented at 12,500 rpm for 15 min at 4 °C in a microcentrifuge, washed once with buffer (100 mM Tris-HCl, pH 8.1) and resuspended in buffer (100 mM Tris-HCl, pH 8.1) and resuspended in buffer (100 mM Tris-HCl, pH 8.1) and resuspended in buffer (100 mM Tris-HCl, pH 8.1) and resuspended in buffer (100 mM Tris-HCl, pH 8.1). Purified [35S]PAPS was lyophilized to dryness, resuspended to 5 × 105 cpm/ml, and stored at −20 °C. [35S]PAPS was stable for at least 2 months. An aliquot was chromatographed on polyethyleneimine cellulose (EM 2-(0.23 M NaCl).2 The fractions containing heparan sulfate were pooled, diluted with water, and reapplied to a DEAE-Sephacel column. The heparan sulfate was eluted with 1.0 M NaCl, desalted by gel filtration chromatography, and lyophilized.

N-Sulfate groups were removed from heparan sulfate by solvolysis according to the method of definebo (25). N-desulfated heparin (Sigma) and 50 μCi of [35S]PAPS were desalted by gel chromatography on a Bio-Gel P-10 column (75 × 1.5 cm) with 0.5 M pyridine acetate, pH 5.0, as the eluate. The column was run at a flow rate of 50 μl/min, and 1-ml fractions were collected. Sucrose eluted close to the position of the heparan sulfate disaccharides.
Science) in 0.75 M Tris base, 0.45 M HCl, and 0.5 M LiCl (pH 6.7) to remove residual protein and nucleic acids. One hundred mg of purified heparin was N-desulfated by solvolysis at 60 C for the quantitation of N-(35S)SO4 groups. The reaction mixture was applied to a PD10 column in 0.5 M pyridinium acetate, pH 5.0, to remove Me2SO and free sulfate groups. The eluate was lyophilized to dryness, resuspended in water, and rechromatographed on a 5-ml DEAE-Sephasel column. N-Desulfoheparin was rechromatographed on a 3-ml DEAE-Sephasel column. N-Desulfoheparin was prepared from porcine intestinal heparin (Sigma) after purification by anion-exchange chromatography on DEAE-Sephasel to remove residual protein and nucleic acids. N-Desulfoheparin was eluted with 0.5 M NaCl, precipitated with 4 volumes of ethanol, resuspended to 100 mg/ml in water, and stored at −20°C. Reaction of N-desulfoheparin with carbazole (19) and 2,4,6-trinitrobenzene-sulfonic acid (25) indicated that solvolysis increased the number of free amino groups from <0.01 equivalents/mole of uronic acid to 0.16 equivalents/mole of uronic acid. Although the reaction with 2,4,6-trinitrobenzene-sulfonic acid depended on the concentration of N-desulfoheparin, no attempt was made to ensure that it was quantitative.

**RESULTS**

**Mutant Identification**—We described previously a replicating technique that permitted the identification of proteoglycan-deficient mutants of CHO cells (9). In this procedure, mutagen-treated cells were cloned on plastic tissue culture dishes and replica plated to polyester cloth. Colonies on the replica were incubated with 35S to allow incorporation of label into newly synthesized proteoglycans. The radioactive proteoglycans were precipitated with trichloroacetic acid, and the amount of 35S-labeled proteoglycans present in each colony was measured by autoradiography. Colonies that gave a reduced autoradiographic signal compared with wild-type colonies were considered mutant strains. One colony that incorporated 2-3-fold less sulfate than wild-type cells was designated mutant 606. This strain complements existing mutants in proteoglycan synthesis, and therefore it defines a new complementation group, pgsE-2.3.

Wild-type and pgsE-606 cells were incubated with [6-3H]glucosamine and [35S]SO4, and the labeled glycosaminoglycans were analyzed by anion-exchange HPLC (Fig. 1). 35S-Labeled glycosaminoglycans from wild-type cells resolved into two peaks eluting at 0.29-0.54 M and 0.56-0.64 M NaCl (Fig. 1A, closed symbols). Enzymatic digestion with heparitinase and chondroitinase ABC indicated that the first peak was heparan sulfate, while the second was chondroitin 4-sulfate (11). The heparan sulfate peak was more heterogeneous than observed in previous studies because the glycosaminoglycans were bound to DEAE-Sephasel and washed with 0.1 M instead of 0.3 M NaCl prior to elution (Refs. 10-12; see "Experimental Procedures").

The 35S-labeled glycosaminoglycans from pgsE-606 cells also resolved into two peaks (Fig. 1B). Some material eluted in the same position as chondroitin 4-sulfate from wild-type cells, and quantification of the 35S counts indicated that the mutant made as much chondroitin 4-sulfate as wild type (1300 ± 200 cpm/µg of cell protein versus 1300 ± 600 cpm/µg of cell protein, respectively; n = 5 determinations). Analysis by paper chromatography of the chondroitin sulfate disaccharides liberated with chondroitinase ABC confirmed that both mutant and wild-type cells contained over 95% chondroitin 4-sulfate (data not shown). In contrast, the amount of 35S-labeled heparan sulfate from pgsE-606 was reduced compared with wild type (1100 ± 300 cpm/µg of cell protein versus 3700 ± 1200 cpm/µg of cell protein, respectively). When the 3H-labeled heparan sulfate from pgsE-606 cells was examined (Fig. 1B), the majority of the glycosaminoglycan eluted at the leading edge of the 35S-heparan sulfate (0.29-0.47 M NaCl) and near the position where hyaluronic acid elutes (about 0.3 M NaCl), suggesting that the heparan sulfate from the mutant was less negatively charged. However, the amount of 3H-labeled heparan sulfate in mutant and wild-type cells was comparable (3000 ± 1200 cpm/µg of cell protein versus 3100 ± 1100 cpm/µg of cell protein, respectively). This finding suggested that the mutant synthesized heparan sulfate chains but did not add sulfate to them to the same extent as wild type. Since the level of 35S-chondroitin sulfate in mutant and wild-type cells was comparable, the decrease in sulfation of

![Fig. 1. Anion-exchange HPLC of glycosaminoglycans from wild-type, pgsE-606, and hybrid cells.](image)
heparan sulfate was not due to a mutation in sulfate transport or activation to PAPS (10).

Characterization of Heparan Sulfate—Another portion of 35S,3H-labeled glycosaminoglycans was treated with chondroitinase ABC, and heparan sulfate was purified by anion-exchange HPLC (see "Experimental Procedures"). The position of sulfates on the chains was examined by solvolysis in 95% Me2SO, 5% water, which preferentially releases sulfate from amino groups with little loss of O-sulfate (18). Treatment of 35S,3H-heparan sulfate liberated 50% ± 2% of 35S counts from wild-type heparan sulfate and 47% ± 3% from mutant heparan sulfate (n = 3 determinations), indicating that the ratio of N-sulfate to O-sulfate groups was not altered in the mutant. Since heparan sulfate synthesized by pgsE-606 cells is about 3.5-fold less sulfated than that made by parental cells, the heparan sulfate from the mutant contained about one-third as many N-sulfated glucosamine residues and O-sulfate groups. The location of O-sulfate groups has not been determined.

To examine the frequency of N-sulfated glucosamine residues along the chains, heparan sulfate was treated with nitrous acid at pH 1.5, and the products generated were analyzed by gel filtration chromatography (Fig. 2). At low pH, only N-sulfated glucosamine residues are susceptible to cleavage; N-acetylated and unsubstituted residues are resistant (29). Therefore, the size and relative amounts of the oligosaccharides generated by this treatment give the frequency of N-sulfated glucosamine residues along the chain. The cleavage patterns of the 3H-heparan sulfate were very different for the two strains (Fig. 2, open symbols). Nearly 50% of wild-type heparan sulfate was cleaved into small oligosaccharides (2-, 4-, and 6-mers, Fig. 2A), while only 17% of the oligosaccharides generated from mutant heparan sulfate were of this size (Fig. 2B). Instead, most of the mutant chains remained as large oligosaccharides (68% of the 3H counts were in oligosaccharides greater than 14-mers in the mutant versus 28% in the wild type). The oligosaccharides that eluted near the void volume of the column (Fractions 59–68) were smaller than the starting heparan sulfate chains based on gel filtration HPLC (data not shown), indicating that all the chains from pgsE-606 cells contained at least 1 N-sulfated glucosamine residue.

When we calculated the number of N-sulfated glucosamine residues, we found that 40% of glucosamine residues in heparan sulfate chains from wild-type cells were N-sulfated, whereas only 21% of the glucosamine residues in the heparan sulfate chains from pgsE-606 cells were N-sulfated. Since the majority of the nitrous acid-generated oligosaccharides from pgsE-606 cells ran near the void volume of the column, the calculation of N-sulfated glucosamine residues was an overestimate. The actual decrease in N-sulfated glucosamine residues between mutant and wild type was probably closer to the 3-fold reduction observed when the chains were treated with 95% Me2SO, 5% water. The cleavage pattern of 35S-heparan sulfate was independent of the strain except that the amount of label was reduced in pgsE-606 cells (Fig. 2, closed symbols). This finding suggested that the pattern of O-sulfation around N-sulfation sites was similar in both wild-type and pgsE-606 heparan sulfate.

**pgsE-606 Cells Lack N-Sulfotransferase—**Characterization of the purified heparan sulfate suggested that pgsE-606 cells might be deficient in N-sulfotransferase activity. To test this hypothesis, an assay for the N-sulfotransferase was optimized in crude CHO cell homogenates. Enzyme activity in wild-type and pgsE-606 was proportional to time, but pgsE-606 homogenates had only 20% of wild-type activity (Fig. 3). Wild-type and pgsE-606 homogenates were mixed in ratios of 1:1, 1:2, 1:5, or 1:10 and assayed for N-sulfotransferase activity. When the activity was plotted against the percent of mutant extract in the mixture (Fig. 3, inset), the values fell on a line which intersected the y axis at the experimentally measured value obtained when wild type was assayed alone. These results suggested that pgsE-606 cells did not contain a soluble inhibitor.

The number of N-sulfated glucosamine residues was calculated first by determining the percentage of 3H counts in each oligosaccharide. This was achieved by assuming that each peak was symmetric and by extension of the ascending and descending aspects of the peak to the base line. Photo reproductions of the graphs were made, and each peak was excised, weighed, and expressed as a percentage of the total. The total 3H counts in heparan sulfate were multiplied by the fractional weight of each peak to obtain the percentage of 3H counts in each peak. Next, we assumed that for each oligosaccharide containing n sugars, there were n/2 glucosamine residues, and 1 reducing terminal anthranosamine residue derived from cleavage of an N-sulfated glucosamine residue. The percentage of 3H counts in each oligosaccharide was divided by n/2 to obtain the fraction of 3H counts which was attributable to N-sulfated glucosamine residues. For example, the tetrasaccharide in the n = 4 peak in Fig. 2A originally contained 2 glucosamine residues, one of which had been N-sulfated. This peak represented 22% of the total 3H counts. Therefore, n/2 = 2, and N-sulfated glucosamine residues accounted for 22%/2 = 11% of the 3H counts. The regions of the chromatogram where oligosaccharides >20-mers migrated were treated as though they were 22-mers. The values were summed to estimate the total number of glucosamine residues that had been N-sulfated.
of N-sulfotransferase activity or lack a soluble activator present in wild-type cells.

To examine if the mutation in mutant 606 was due to altered kinetic properties of N-sulfotransferase, wild-type and mutant 606 homogenates were assayed with varying concentrations of PAPS or N-desulfoheparin. Double-reciprocal plots were constructed, and the \( K_a \) and \( V_{max} \) for the reaction were determined graphically (Fig. 4). Wild-type N-sulfotransferase had a \( K_a \) for PAPS of 4.5 \( \mu \)M, a \( K_a \) for N-desulfoheparin of 0.35 mg/ml, and an average \( V_{max} \) of 25 pmol of sulfate transferred/min/mg of cell protein. N-sulfotransferase activity was also assayed over a pH range of 5.5-8.5 to examine if the decrease in activity in pgsE-606 cells was caused by a change of ionizable groups on the enzyme. Both enzymes had similar pH profiles with optimal activity at pH 6.8-7.0 (Fig. 5A).

To test if the decrease in N-sulfotransferase activity in pgsE-606 cells reflected an alteration in the activation energy required for the reaction to occur, N-sulfotransferase activity was assayed at 16, 24, 27, 33, and 37 °C. An Arrhenius plot of the initial rates was constructed, and the activation energies for the enzyme reactions were determined from the slope (Fig. 5B). No significant difference in activation energy between wild-type enzyme (14.5 kcal/mol) or pgsE-606 enzyme (16 kcal/mol) was observed. To examine whether N-sulfotransferase in the mutant was more sensitive to thermal inactivation than in the wild type, homogenates were preincubated at 45 °C for various times before being assayed for enzyme activity at 37 °C (Fig. 5C). The time course of enzyme inactivation was similar in both cell types (\( t_{90} = 4.8 \) min in the mutant versus 4.5 min in the wild type).

Expression of N-Sulfotransferase—Cell hybrids of pgsE-606 cells and clone OT-1 were prepared to examine whether the decrease in sulfation and N-sulfotransferase activity in mutant 606 cells was dominant or recessive to the wild-type phenotype. The proteoglycan composition of clone OT-1 is identical to that of the wild type (data not shown) and bears appropriate drug selection markers for the counterselection of parental cells after fusion (see “Experimental Procedures”). N-Sulfotransferase activity in five hybrids prepared from OT-1 and pgsE-606 cells was not restored to the level seen in wild-type cells (15 ± 1 pmol/min/mg in the hybrids versus 20 ± 4 pmol/min/mg in the wild type). However, the activity was greater than that observed in pgsE-606 cells (8 ± 1 pmol/min/mg). Enzyme activity in hybrids prepared between wild-type cells and clone OT-1 was approximately the same as that found in parental wild-type cells (22 ± 4 pmol/min/mg of cell protein), and in a separate experiment unfused OT-1 and wild-type cells had the same amount of enzyme activity (34 ± 1 pmol/min/mg). Thus, the intermediate amount of activity found in hybrids of OT-1 and pgsE-606 cells was due to altered activity in OT-1 cells. Heparan sulfate isolated from the hybrids was sulfated to the same extent as heparan sulfate from parental wild-type cells (Fig. 1C). Together, these results indicated that the mutation in 606 is recessive.

**DISCUSSION**

**pgsE-606 May Be Heterozygous for N-Sulfotransferase—** In previous studies we showed that the incorporation of \( ^{35} \text{SO}_4 \) into macromolecules is a reliable measure of proteoglycan synthesis, and we designed an autoradiographic screening method to detect mutants defective in proteoglycan assembly (9). Most of the mutants identified in this way display gross defects in \( ^{35} \text{SO}_4 \) incorporation (9-11), but some of the mutants defective in sulfate transport (pgsC-604) and galactosyltransferase I (pgsB-650) still incorporated some sulfate, presumably because the mutations in these strains did not completely inactivate the gene (10, 11). We reasoned that mutations in specific sulfotransferases might also appear as partially defective mutants by colony autoradiography since numerous sulfotransferases exist which transfer sulfate residues to different positions along the glycosaminoglycan chains. Using colony autoradiography, we found mutant pgsE-606 which is partially defective in sulfate incorporation due to a deficiency in heparan sulfate N-sulfotransferase.

Unlike mutants defective in galactosyltransferase I (pgsB), sulfate transport (pgsC), and heparan sulfate synthesis (pgsD), mutants deficient in N-sulfotransferase are relatively rare (10-12). Strain 606 was the only mutant found defective
in N-sulfotransferase among about 10° mutagen-treated cells. The residual enzyme activity is identical to the activity in wild-type cells (Figs. 3-5), and cell hybridization studies showed that the N-sulfotransferase-deficient phenotype is recessive. These findings suggest that the mutation affects expression of N-sulfotransferase rather than its structure. The pgsE locus may define a regulatory gene that modulates the expression of N-sulfotransferase. Alternatively, the pgsE locus may encode N-sulfotransferase, and if the locus is diploid in CHO, then the 606 allele may inactivate one copy of the gene. Several diploid genes have been found in CHO cells, including adenosine phosphoribosyltransferase (26, 27), dihydrofolate reductase (28), and the low density lipoprotein receptor (29). Interestingly, the residual activity in heterozygous mutants is typically 30-70% of that found in wild-type cells (26-29). The residual N-sulfotransferase activity found in pgsE-606 cells falls within this range, suggesting that N-sulfotransferase may also be diploid in CHO cells.

N-Sulfotransferase Determines the Extent of Sulfation of Heparan Sulfate—Characterization of the sulfation pattern of heparan sulfate synthesized by the mutant showed a close correlation of enzyme activity with the extent of sulfation. These findings show that N-sulfotransferase activity measured in cell extracts is responsible for N-sulfation of amino sugars in heparan sulfate in vivo. Moreover, they show that the extent of sulfation of the chains depends on the specific activity of the enzyme. Thus, N-sulfotransferase is a rate-limiting enzyme in heparan sulfate synthesis. Other polymer modification enzymes, including N-deacetylase, uronosyl epimerase, and O-sulfotransferases, may also be rate limiting. Because N-sulfation is dependent upon the removal of the acetyl groups from N-acetylglucosamine residues, the N-deacetylase presumably determines the overall extent of polymer modification. Additional mutants defective in these enzymes should reveal if the extent of deacetylation, uronic acid epimerization, and O-sulfation depends on the activity of the individual enzymes.

If the amount of N-sulfotransferase determines the extent of N-sulfation, then the variation in N-sulfation of heparan sulfates among cells (8) may reflect differences in the specific activity of heparan sulfate N-sulfotransferase. In crude liver homogenates, N-sulfotransferase has a specific activity similar to that measured in wild-type CHO cells (30 pmol/min/mg, Ref. 30), and the heparan sulfate characterized from an established liver cell line (8) or freshly prepared hepatocytes (31, 32) contains as many N-sulfated glucosamine residues as heparan sulfate from CHO cells (40-50%). In contrast, a cell line derived from the Furth mastocytoma tumor produces heparin chains containing 70-80% N-sulfated glucosamine residues, and the cells have 100 pmol/min/mg of N-sulfotransferase activity. Although the enzyme activity measured in cell extracts may not accurately reflect the activity found in intact cells, the extent of sulfation correlates reasonably well with the measured enzyme activity.

Lindahl et al. (1) proposed from a series of elegant studies of heparin biosynthesis in mouse mastocytoma microsomes that the modification enzymes may be arranged topographically in the order in which the reactions proceed. Newly made chains still attached to the core protein were envisioned to pass through enzyme stations that catalyze the various sulfate addition and sugar epimerization reactions. This model predicts that the quantity of each biosynthetic enzyme would determine the extent of the respective modification. Our observation that decreasing the activity of N-sulfotransferase reduces the number of N-sulfated residues is consistent with the model, although our findings do not prove that the enzymes are localized in a specific topographic arrangement. Nevertheless, our studies establish that N-sulfotransferase...

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Footnote:

6 R. L. Montgomery, K. J. Bame, and J. D. Esko, unpublished results.
occupies a key position in sulfation of heparan sulfate. Additional studies are needed to establish if other enzymes, including the N-deacetylase, uronosyl epimerase, and various O-sulfotransferases, are affected in the mutant. The decrease in O-sulfation parallels the decrease in N-sulfation, suggesting that the O-sulfotransferases and N-sulfotransferase may be coordinately regulated or exist in a functional complex. The coupling of O-sulfation to N-sulfation may also reflect the fact that O-sulfotransferases, are affected in the mutant. The decrease in O-sulfation parallels the decrease in N-sulfation, suggesting that N-sulfotransferase may not form strong associations with other enzymes in the pathway. Thus, N-sulfotransferase may act independently of other modification enzymes.

The heparan sulfate from pgsE-606 cells differs from that produced by wild-type cells in several ways. Analysis of the oligosaccharides generated by nitrous acid deamination indicates that about 20% of the glucosamine residues in heparan sulfate from the mutant are N-sulfated compared with 40% in the wild type. In addition, the most frequent spacing between N-sulfated glucosamine residues increases from one to four disaccharides in wild-type cells to greater than seven disaccharides in the mutant. The extent of O-sulfation is also reduced in pgsE-606 cells, but the distribution of O-linked sulfate among nitrous acid depolymerization products is similar in mutant and wild-type cells. It is interesting to note that the heparan sulfate generated from heparan sulfate of both wild-type and pgsE-606 cells are more extensively O-sulfated than other oligosaccharides (except disaccharides). This unusual distribution of O-sulfate groups was noted previously in CHO cell heparan sulfate but has not been observed in other cell types (8). It is possible that the O-sulfotransfase(s) in CHO cells preferentially act on hexasaccharide regions flanked by N-sulfated residues.

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REFERENCES