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Undersulfation of Proteoglycans Synthesized by Chondrocytes from a Patient with Achondrogenesis Type 1B Homozygous for an L483P Substitution in the Diastrophic Dysplasia Sulfate Transporter*

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Achondrogenesis type 1B is an autosomal recessive, lethal chondrodysplasia caused by mutations in the gene encoding a sulfate/chloride antiporter of the cell membrane (Superti-Furga, A., Hästbacka, J., Wilcox, W. R., Cohn, D. H., van der Harten, J. J., Rossi, A., Blau, N., Rimoin, D. L., Steinmann, B., Lander, E. S., and Gitzelmann, R. (1996) *Nat. Genet.* 12, 100–102). To ascertain the consequences of the sulfate transport defect on proteoglycan synthesis, we studied the structure and sulfation of proteoglycans in cartilage tissue and in fibroblast and chondrocyte cultures from a fetus with achondrogenesis 1B. Proteoglycans extracted from epiphyseal cartilage and separated on agarose gels migrated more slowly than controls and stained poorly with alcian blue. The patient's cultured cells showed reduced incorporation of [^{35}S]sulfate relative to [^{3}H]glucosamine, impaired uptake of sulfate, and higher resistance to chromate toxicity compared to control cells. Epiphyseal chondrocytes cultured in alginate beads synthesized proteoglycans of normal molecular size as judged by gel filtration chromatography, but undersulfated as judged by ion exchange chromatography and by the amount of nonsulfated disaccharide. High performance liquid chromatography analysis of chondroitinase-digested proteoglycans showed that sulfated disaccharides were present, although in reduced amounts, indicating that at least *in vitro*, other sources of sulfate can partially compensate for sulfate deficiency. A t1475c transition causing a L483P substitution in the eleventh transmembrane domain of the sulfate/chloride antiporter was present on both alleles in the patient who was the product of a consanguineous marriage. The results indicate that the defect of sulfate transport is expressed in both chondrocytes and fibroblasts and results in the synthesis of proteoglycans bearing glycosaminoglycan chains which are poorly sulfated but of normal length.

Achondrogenesis type 1B (ACG-1B)[1] (1) is an autosomal recessive, lethal chondrodysplasia with severe underdevelopment of the skeleton, extreme micromelia, and death before or immediately after birth because of thoracic hypoplasia (1–8). Both radiological and histological features differentiate ACG-1B from achondrogenesis type 1A and achondrogenesis type 2 (3). In ACG-1B, cartilage matrix contains abnormally coarse collagen fibers and stains poorly with cationic dyes which have affinity for sulfated proteoglycans (toluidine blue or alcian blue) (4, 8). Evidence of undersulfation of proteoglycans (PGs) in cartilage on the basis of histological findings and in fibroblast cultures, together with the demonstration of insufficient formation of activated sulfate metabolites, was obtained recently in one patient with ACG-1B (8). Further studies have revealed that ACG-1B is caused not by a defect in the metabolic activation of sulfate, as originally concluded (8), but by a sulfate uptake defect caused by mutations in the sulfate transporter gene (9) originally isolated as the locus responsible for the non-lethal disorder, diastrophic dysplasia (10). The pathogenesis of the severe developmental defect of the skeleton seen in ACG-1B is believed to involve undersulfation of cartilage PGs, as suggested by both the staining properties of cartilage and its markedly reduced sulfate content (9), but direct demonstration of undersulfation of individual cartilage PGs is lacking. We have obtained cartilage tissue, cultured skin fibroblasts, and epiphyseal chondrocytes maintained in alginate beads and used them to test the structure and the sulfation of PGs. The results indicate that both the large chondroitin sulfate proteoglycans (CSPGs) and the small CSPGs synthesized by ACG-1B chondrocytes bear glycosaminoglycan chains which are of normal length but not correctly sulfated.

MATERIALS AND METHODS

Case Report—The fetus studied was the product of a consanguineous marriage. Pregnancy was terminated at week 16 because of ultrasound detection of severe micromelia and macrocephaly, after appropriate parental counselling, and in accordance with legislation. X-rays showed extremely short tubular bones, missing ossification of vertebral bodies, short ribs without fractures, and crescent-shaped iliac wings characteristic for achondrogenesis type 1 (Fig. 1*A*). Histologic analysis of cartilage showed typical collagenous rings surrounding chondrocytes asso-

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[1] The abbreviations used are: ACG-1B, achondrogenesis 1B; DTDST, diastrophic dysplasia sulfate transporter; PGs, proteoglycans; GdmCl, guanidinium chloride; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; APS, adenosine 5′-phosphosulfate; PAPS, 3′-phosphoadenosine 5′-phosphosulfate; PCR, polymerase chain reaction; CSPGs, chondroitin sulfate proteoglycans; HA, hyaluronic acid; ΔDi-0S, 3–0-β(D-gluc-4-eneuronosyl)-*N*-acetylgalactosamine; ΔDi-4S and ΔDi-6S, derivatives of ΔDi-0S with a sulfate at the 4 or 6 position of the hexosamine moiety, respectively; bp, base pair; nt, nucleotide.

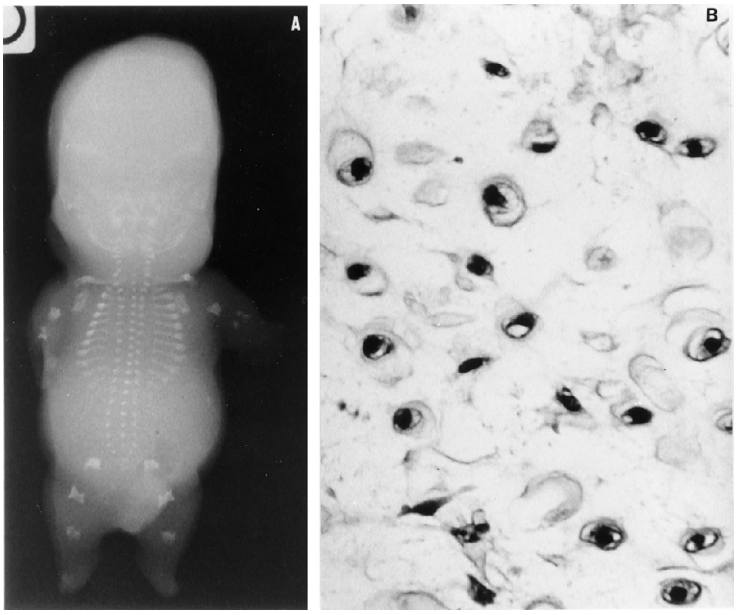

FIG. 1. **X-ray picture of the fetus showing changes typical for achondrogenesis type 1B (*left part*, see text).** Histologic section of the patient's epiphyseal cartilage showing coarse collagen fibers forming dense rings surrounding the chondrocytes (*right part*).

ciated with reduced ground substance and coarse collagen fibrils which allowed diagnosis of achondrogenesis type 1B rather than type 1A (Fig. 1*B*) (3, 4, 7, 8).

Biochemical Analysis of Cartilage—Cartilage fragments from the patient and from fetuses aborted for other causes and with no obvious signs of skeletal disorders were homogenized with a Polytron device and extracted with 4 M guanidinium chloride (GdmCl), 50 mM Tris-HCl, pH 7.4, as described previously (7). The uronic acid content of the 4 M GdmCl extract was determined by the carbazole reaction (11). Aliquots of cartilage extract were loaded on 0.8% agarose gels and submitted to electrophoresis in 0.04 M Tris-HCl buffer, pH 6.8, containing 20 mM sodium acetate. Gels were either stained with 0.1% toluidine blue in water/acetone (1/4), destained in water/acetone, and placed in water to enhance metachromasia, or stained with a solution of 0.05% alcian blue 8 GX in 0.4 M $MgCl_2$, 25 mM sodium acetate, and destained in 3% acetic acid.

Fibroblast and Chondrocyte Cultures—Cell cultures were established from the ACG-1B fetus and from fetuses with no obvious skeletal or connective tissue disorder. Skin fibroblasts were cultured in DMEM with 10% FCS and antibiotics at 37 °C in an atmosphere containing 5% CO_2. Chondrocytes were released from epiphyseal cartilage slices by sequential enzymatic digestions with hyaluronidase, trypsin, and collagenase (12). To increase the cell number, the chondrocytes were plated on plastic tissue culture flasks and expanded as monolayer in Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum (FCS). Dedifferentiated chondrocytes between the third and the fourth passage were redifferentiated by culture in alginate beads as follows: cells were resuspended in sterile alginate solution (1.25% alginate in 20 mM Hepes buffer, FMC BioProduct) at a density of 3×10^6 cells/ml. The cell suspension was aspirated in a syringe and dropped through a 22-gauge needle in a gently shaken 102 mM $CaCl_2$ solution, which resulted in gelification of the drops to beads. The beads were washed three times in sterile 0.15 M NaCl and once in DMEM. The chondrocytes encapsulated in the alginate beads were then maintained in DMEM containing 10% FCS and antibiotics at 37 °C in 5% CO_2 (13) for at least 4 weeks prior to metabolic labeling.

Analysis of Proteoglycans Synthesized by Chondrocyte Cultures—3×10^6 redifferentiated chondrocytes were labeled with 20 μCi/ml [6-^{3}H]glucosamine (DuPont NEN) and 25 μCi/ml $Na_2[^{35}S]O_4$ carrier free in fresh DMEM containing 10% FCS for 24 h at 37 °C. The medium was removed (*medium fraction)* and stored frozen after the addition of proteinase inhibitors: 10 mM EDTA disodium salt, 0.1 M 6-aminohexanoic acid, 5 mM benzamidine-HCl, 10 mM *N*-ethylmaleimide, and 1 mM phenylmethanesulfonyl fluoride (final concentrations). The alginate beads were washed twice with phosphate-buffered saline and solubilized by the addition of 3 volumes of 50 mM EDTA in phosphate-buffered saline for 10 min at room temperature. The resulting suspension was centrifuged at 2000 rpm for 10 min at 4 °C. After centrifugation, the supernatant contained the matrix deposited within some micrometers from the cell (corresponding roughly to the interterritorial matrix defined by histology) and the pellet contained the chondrocytes and the cell-associated matrix (14). To the supernatant 0.2 M GdmCl (final concentration) was added to maintain the PGs in the fluid phase. Proteinase inhibitors were added as above. This fraction was clarified by centrifugation at 12,100 × *g* for 30 min at 4 °C and then stored frozen (*alginate fraction*). The pellet was sequentially extracted for 24 h at 4 °C first with 0.2 M GdmCl in 50 mM sodium acetate, pH 5.8, in the presence of proteinase inhibitors with continuous shaking (associative conditions; *0.2 M GdmCl fraction*) and then with 4 M GdmCl in the same buffer (dissociative conditions; *4 M GdmCl fraction*) for subsequent analysis by molecular sieve chromatography, or directly with 4 M GdmCl for subsequent ion-exchange chromatography. The extracts were clarified by centrifugation at 12,100 × *g* for 30 min at 4 °C (14).

Proteoglycans Analysis in Fibroblast Cultures—Confluent skin fibroblasts in 25-cm^2 flasks were labeled for 24 h with 20 μCi/ml [6-^{3}H]glucosamine and 20 μCi/ml $Na_2[^{35}S]O_4$ in fresh DMEM without FCS. The medium was harvested as described for chondrocyte labeling and the cell-layer was extracted for 24 h with 4 M GdmCl in 50 mM sodium acetate, pH 5.8, in the presence of proteinase inhibitors. The cell extract was clarified by centrifugation at 12,100 × *g* for 30 min.

Ion-exchange Chromatography of Proteoglycans—The following protocol was used for analysis of PGs synthesized both by chondrocytes and fibroblasts. Medium and cell extract samples were dialyzed exhaustively against 50 mM sodium acetate, pH 6.0, containing 8 M urea, 0.15 M NaCl, and the proteinase inhibitors. After dialysis, 0.5% (w/v) Triton X-100 was added and the samples were applied to a DEAE-Sephacel column (Pharmacia, 1 × 9 cm, flow rate 6 ml/h) equilibrated with 50 mM sodium acetate, pH 6.0, containing 8 M urea, 0.15 M NaCl, 0.5% (w/v) Triton X-100, and the proteinase inhibitors. After loading, the column was washed with 16 ml of the same buffer, followed by elution at room temperature with a 100-ml linear gradient of 0.15–1 M NaCl in the same buffer. The gradient was controlled with an on-line conductivity monitor (Pharmacia). One-ml fractions were collected and aliquots analyzed for radioactivity. Discrimination between ^{3}H and ^{35}S decays was achieved by setting window 1 from 0 to 12 keV and window 2 from 12 to 156 keV.

Chondroitin Sulfate Disaccharide and Hyaluronic Acid Analysis—For chondroitin sulfate disaccharide analysis the PG peak from ion exchange chromatography was exhaustively dialyzed against water at 4 °C and lyophilized. PGs were then digested with 70 milliunits of both chondroitinase ABC and ACII (Seikagaku Corp., Japan) in 50 mM sodium acetate, 50 mM Tris acetate, pH 7.3, at 37 °C for 6 h. Undigested PGs were precipitated by the addition of 4 volumes of ethanol, storage at −20 °C overnight, and centrifugation, and the supernatant containing the released disaccharides was evaporated to dryness (15). Residues were solubilized in water and standard chondroitin 0-sulfate (ΔDi-0S), chondrotin 4-sulfate (ΔDi-4S), and chondrotin 6-sulfate (ΔDi-6S) were added. Disaccharides were fractionated using a Supelcosil LC-SAX (Supelco) high performance liquid chromatography column (4.6 × 250 mm) as described previously (16). The elution profile was measured at 232 nm and radioactivity in 250-μl fractions was determined. The

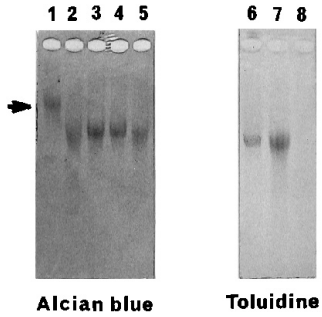

FIG. 2. **Agarose gel electrophoresis of cartilage proteoglycans.** Cartilage extracts were analyzed by agarose gel electrophoresis and stained with alcian blue or toluidine blue. An alcian blue-positive band with delayed mobility (*lane 1*, indicated by the *arrow*) which did not stain with toluidine blue (*lane 8*) was observed in the patient. *Lanes 2–7*, controls.

elution position of radiolabeled disaccharides agreed with those of the standards.

Samples of hyaluronic acid from the ion exchange fractionation were precipitated with 9 volumes of ethanol. After centrifugation, the pellet was digested with 5 units of *Streptomyces* hyaluronidase in 20 mM sodium acetate, pH 6.0, 75 mM NaCl at 60 °C overnight. Undigested products were separated from oligosaccharides by gel chromatography on a Sephadex G-50 column in 0.5 M NH_4HCO_3 (15).

Gel Filtration Chromatography—To determine their molecular sizes, PGs were prepurified by step elution (0.15–1 M NaCl) from 1-ml DEAE-Sephacel columns and chromatographed on a 0.9 × 140-cm column of Sepharose CL-2B (Pharmacia) eluted at room temperature with a dissociative buffer of 4 M GdmCl, 0.5 M sodium acetate, 50 mM sodium phosphate, pH 6.8, 0.1% (w/v) Triton X-100 and the proteinase inhibitors (10 mM EDTA, 0.1 M 6-aminohexanoic acid, 5 mM benzamidine, and 10 mM *N*-ethylmaleimide) at a flow rate of 8.25 ml/h (14). 0.9-ml fractions were collected and aliquots analyzed for radioactivity.

Activation of $^{35}SO_4^{2-}$ to APS and PAPS—All procedures were performed at 4 °C unless otherwise stated. Confluent skin fibroblasts from a 75-cm^2 flask were scraped in phosphate-buffered saline. Cells were recovered by low speed centrifugation, suspended in 0.05 M Tris-HCl, pH 8.0, and sonicated. The cell extract was clarified by centrifugation at 12,100 × *g* for 15 min and the protein content was estimated (BCA Protein Assay, Pierce) using bovine serum albumin as standard. A reaction mixture of 55 μl containing 5 μl of 0.1 M ATP, 2 μl of 10 mM cysteine-HCl, 6 μCi of carrier-free $Na_2[^{35}S]O_4$, 1 μl of 1 M $MgCl_2$, 1 μl of 0.01 M NAD^+, and 45 μl of cell extract containing 90 μg of protein in 0.05 M Tris-HCl, pH 8.0, was incubated at 37 °C (17). The reaction was allowed to proceed for 1 h. Ten-μl aliquots were then spotted onto Whatman No. 3MM paper and subjected to high voltage electrophoresis for 60 min at 2 kV (paper length, 23 cm) in H_2O/pyridine/acetic acid (493/5/2) buffer, pH 5.3, at 4 °C (18). The paper was dried and exposed to X-Omat AR films (Kodak).

Sulfate Uptake Assay—The same protocol was used for either fibroblasts or chondrocytes at the second passage as monolayer. Cells were seeded in 10-cm^2 dishes (3×10^5 cells/dish) and incubated in DMEM containing 10% FCS for 24 h at 37 °C. Sulfate uptake was performed as described previously (10, 19) using low ionic strength buffer (1 mM $MgCl_2$, 300 mM sucrose, 10 mM Tris-Hepes, pH 7.5) containing concentrations of Na_2SO_4 ranging from 2 to 250 μM. Prior to the assay, cells were washed three times with prewarmed sulfate-free low ionic strength buffer and preincubated for 2 min in the same buffer at 37 °C. The washed cells were then incubated for 1 min at 37 °C in the low ionic strength buffer containing different concentrations of Na_2SO_4 and a constant concentration of carrier-free $Na_2[^{35}S]O_4$ (0.1 μM, corresponding to 150 μCi/ml). The uptake medium was removed and the cells were washed four times with 1.5 ml of ice-cold medium containing 100 mM sucrose, 100 mM $NaNO_3$, 1 mM $MgCl_2$, and 10 mM Tris-Hepes, pH 7.5. Finally, cells were lysed in 0.8 ml of 2% SDS. Lysates were collected, heat-denatured, and centrifuged. Supernatants were assayed for radioactivity and protein content.

Chromate Sensitivity Assay—Fibroblasts were seeded in 96-microwell plates (3×10^4 cells/well unless otherwise stated) and allowed to attach overnight. Cells were then incubated in DMEM without serum and with varying concentrations of K_2CrO_4 (0–200 μM) at 37 °C for 8 days. After that period, surviving cells were fixed with 1% glutaraldehyde in phosphate-buffered saline, stained with crystal violet. Optical density in the microwells was read at 595 nm using a microplate reader (20).

TABLE I
Relative incorporation of $[^{35}S]$sulfate and $[^3H]$glucosamine in cells from the patient and a control fetus

Cells were double labeled with $[^3H]$glucosamine and $[^{35}S]$sulfate for 24 h and then the medium was harvested. Fibroblast's cell layer was extracted with 4 M GdmCl (cell fraction). Regarding chondrocytes, the alginate beads were dissolved in EDTA and the suspension was centrifuged. The cell pellet was extracted with 4 M GdmCl. The cell fraction refers to the alginate fraction plus the cell pellet. All fractions were exhaustively dialysed against 8 M urea and an aliquot was counted. In both cell strains the $^{35}S/^3H$ ratio is reduced in the patient compared to the control. This difference is less evident in the medium from chondrocytes which is consistent with the retention of PGs mainly in the alginate beads.

	Chondrocytes $^{35}S/^3H$ ratio	Fibroblasts $^{35}S/^3H$ ratio
Culture medium		
Control	1.240	0.116
Patient	1.040	0.025
Cell fraction		
Control	1.047	0.090
Patient	0.080	0.020

Amplification, Cloning, and Sequencing of Genomic DNA—Genomic DNA was extracted from confluent skin fibroblasts according to a standard protocol (21). The published sequence of the diastrophic dysplasia sulfate transporter gene (*DTDST*; Ref. 10) was used to design polymerase chain reaction (PCR) primer pairs to produce overlapping PCR products 100–400 bp long. Single strand conformation polymorphism and heteroduplex analysis was performed either on the intact PCR product or on fragments obtained by restriction digestion (9). Briefly, PCR products were denatured at 95 °C for 3 min, rapidly cooled on ice, and analyzed by electrophoresis on a 15% polyacrylamide gel (gel thickness 0.3 mm; acrylamide:piperazine diacrylamide, 85:1) containing 8% glycerol in 120 mM Tris formate buffer, pH 9.0. Top and bottom of the gels were covered with Whatman No. 3MM paper soaked with 1.04 M Tris borate buffer (2 × TBE), pH 9.0. Electrophoresis was carried out at 10 °C for 90 min with 0.8 watts/cm gel width and the gels were stained with silver nitrate. For sequence analysis, a PCR fragment spanning nt 1171 to 1523 of the *DTDST* gene was cloned using the TA cloning kit (Invitrogen) and clones were sequenced on an automatic sequencer (ALF, Pharmacia) using standard sequencing reagents and fluorescent primers (Autoread, Pharmacia).

RESULTS

Biochemical Analysis of Cartilage Extracts—Total uronic acid content of GdmCl extract of cartilage was reduced by approximately 30% in the patient as compared to controls (160 μg/mg dry weight in patient *versus* 200–250 μg/mg in controls). Equivalent amounts of uronic acid from each sample were separated on 0.8% agarose gel and stained with toluidine blue or alcian blue. The patient's sample did not stain with toluidine blue as previously observed (8), whereas staining with alcian blue revealed a band with delayed mobility (Fig. 2). Because of the electrophoretic conditions used, migration distance is a function of net charge as well as of molecular weight, and these findings were therefore consistent with the hypothesis that patient's PGs were not correctly sulfated and thus less negatively charged.

Collagen Analysis from Redifferentiated Chondrocytes—Epiphyseal chondrocytes in alginate beads were cultured for at least 4 weeks. After this period, SDS-polyacrylamide gel electrophoresis of pepsin-resistant material showed the presence of type II and type XI collagen chains with mobility similar to that of controls (data not shown). This indicated that the cells had regained a chondrocytic phenotype and at the same time ruled-out a major structural defect of collagen II, which is typical for achondrogenesis type 2 rather than type 1B (22).

Incorporation of Sulfate in Chondrocyte and Fibroblast Cultures—After labeling of chondrocytes in alginate with $[^{35}S]$sul-

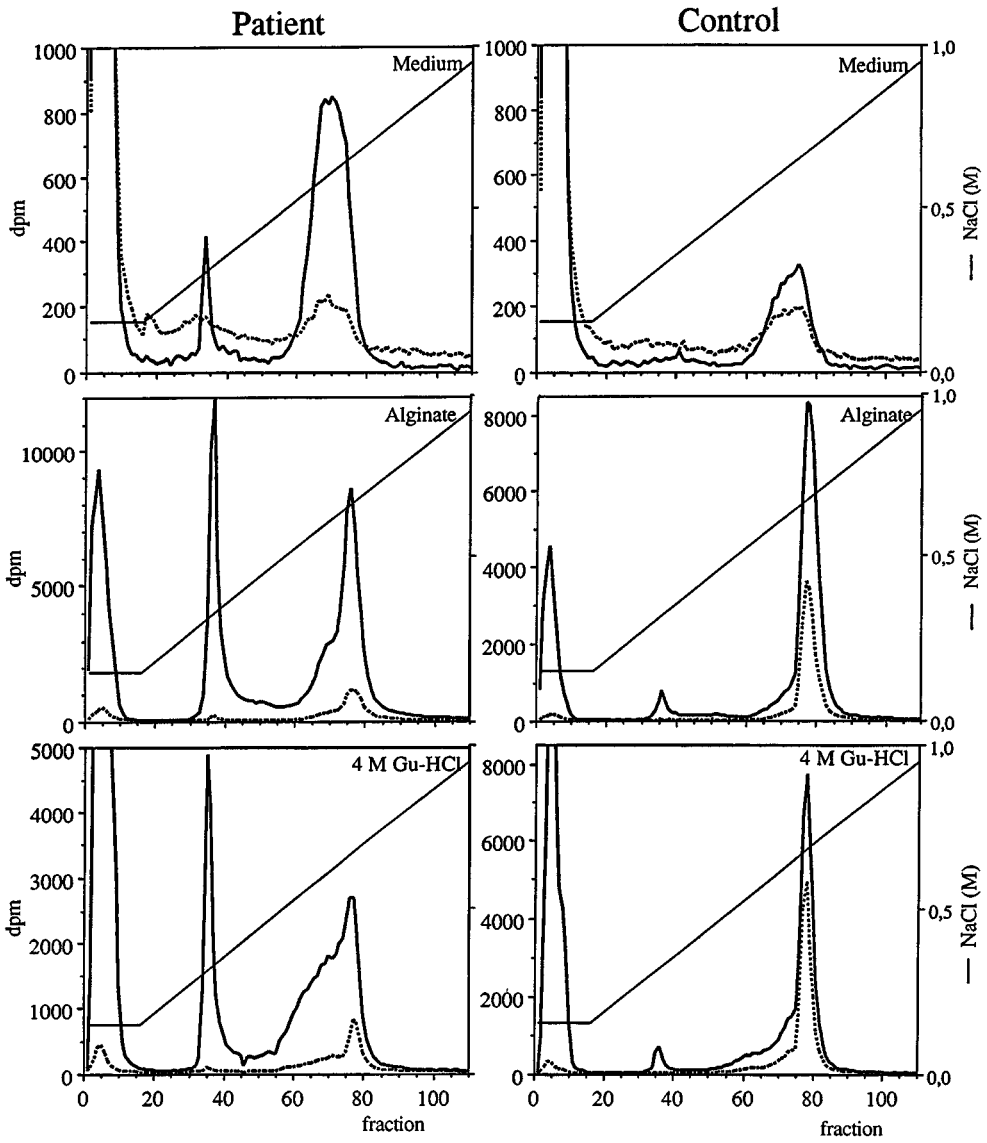

FIG. 3. **Ion exchange chromatography of labeled proteoglycans from chondrocytes.** Chondrocytes in alginate beads were double-labeled with [^{35}S]sulfate (- - - -) and [^{3}H]glucosamine (——) for 24 h. Radiolabeled PGs in the medium and two matrix pools (alginate fraction and 4 M GdmCl fraction) were analyzed by DEAE-Sephacel ion exchange chromatography using a linear gradient of NaCl (0.15–1.0 M). In all fractions from the patient's chondrocytes, ^{35}S labeling of the chondroitin sulfate proteoglycan peak (eluting at approximately 0.6 M NaCl, fractions 70–83) was markedly reduced as compared to the same peak in the control sample. Moreover, in the two matrix pools from the patient's sample this peak was broadened, some material eluting at a lower ionic strength. In the patient's samples a ^{3}H-labeled peak (fractions 32–38), corresponding to hyaluronic acid, was higher than in the control samples (see Table II).

fate for 24 h, the total amount of non-dialyzable radioactivity in the patient's cells was reduced by 30% in the medium fraction and by more than 80% in the beads fraction when compared to control. In control samples, 96% of incorporated radioactivity was recovered in the beads fraction and 4% in the medium, while in the patient's chondrocytes, 88% was found in the beads fraction and 12% in the culture medium. Having observed reduced overall sulfate incorporation, we assayed proteoglycan sulfation by double-labeling with [^{35}S]sulfate and [^{3}H]glucosamine. In the patient's sample, the ratio between non-dialyzable ^{35}S and ^{3}H activities was reduced both in the medium and the cell fraction (Table I). Similar results were obtained with fibroblasts (Table I).

Structure of Proteoglycans Synthesized by Chondrocyte Cultures—After metabolic labeling with [^{3}H]glucosamine and [^{35}S]sulfate, PGs were harvested from the culture medium and from the alginate beads using associative and dissociative conditions to yield three fractions (for ion-exchange chromatography) or four fractions (for molecular sieve chromatography; see "Materials and Methods," above). Upon ion-exchange chromatography on DEAE-Sephacel, neutral glycoproteins were eluted with the isocratic buffer in the first peak, whereas the hyaluronic acid (HA) and the polyanionic CSPGs were eluted at approximately 0.3 M and 0.6 M NaCl, respectively.

In control samples, the CSPG peak was labeled with both ^{35}S and ^{3}H, whereas in the patient's samples this peak was broadened, some material eluting already at a lower ionic strength, and its ^{35}S content was dramatically reduced (Fig. 3). This indicated that the contribution of the extracellular inorganic sulfate to PGs sulfation was impaired and that PGs synthesized by patient's chondrocytes were less negatively charged than control because of reduced sulfation. Similar results were obtained with cultured fibroblasts (data not shown). The proportion of HA relative to PGs synthesized by the patient's chondrocytes was increased when compared to controls (Table II). As undersulfated PGs could appear as species co-eluting with HA, the putative HA peak was digested with highly specific hyaluronidase and digestion products analyzed by Seph-

TABLE II
Relative amount of HA, essentially unsulfated PGs and PGs in chondrocytes

Double labeled PGs from the medium and different matrix pools were analyzed by DEAE-Sephacel chromatography (see Fig. 3). The relative amount of HA, essentially unsulfated PGs (Chn, determined as radioactivity eluting between the HA and PG peak) and PGs was calculated from ^{3}H activities. The mean values of two different experiments are reported.

	% HA	% Chn	% PGs
Culture medium			
Control	4.9 (±0.8)	2.9 (±0.6)	92.0 (±1.3)
Patient	12.4 (±3.0)	4.2 (±0.4)	83.3 (±3.3)
Alginate fraction			
Control	4.2 (±0.9)	5.2 (±1.4)	90.5 (±1.5)
Patient	20.3 (±13.0)	9.3 (±2.0)	70.0 (±12.0)
GdmCl fraction			
Control	8.0 (±2.9)	4.8 (±0.5)	86.6 (±3.3)
Patient	36.5 (±8.5)	7.5 (±0.5)	55.6 (±8.8)

adex G-50 chromatography. The relative amount of hyaluronidase resistant material in the patient was similar to the controls (2–3%).

Aliquots of the PG peak from the 4 M GdmCl fraction were digested with chondroitinase ABC and disaccharides separated by high performance liquid chromatography. In the control, the relative proportions of unsulfated (ΔDi-0S), 4-sulfate (ΔDi-4S), and 6-sulfate (ΔDi-6S) disaccharides were similar to values obtained with chick embryo epiphyseal cartilage (23). In the patient the relative amount of ΔDi-0S was higher than control (Table III); however, sulfated disaccharides with a very low $^{35}S/^3H$ ratio were present indicating that other sources of sulfate (*e.g.* sulfur containing amino acids) could partially compensate for the lack of exogenous sulfate. The higher proportion of ΔDi-4S *versus* ΔDi-6S in the patient confirmed previous observations suggesting that the biosynthetic system for sulfation at position 6 is more sensitive to sulfate availability than at position 4 (23).

To test whether PGs undersulfation changed the distribution of large and small PGs between the different matrix fractions, PGs were prepurified by step-elution from DEAE-Sephacel and subjected to molecular sieve chromatography on Sepharose CL-2B under dissociative conditions. The results (see Table IV and Fig. 4) showed that (i) undersulfation from extracellular sulfate affected both large and small proteoglycans but was slightly more pronounced in the large proteoglycans, and (ii) the degree of undersulfation was equivalent in the medium and in the different cell fractions (alginate fraction, 0.2 M GdmCl fraction, and 4 M GdmHCl fraction).

A further observation pertains to the relative proportions of large and small proteoglycans. In the cell fraction from the control (Fig. 4, *D*, *F*, and *H*) large molecular size PGs (corresponding to aggrecan) were predominant, whereas small PGs represented only 10–25% of total PGs (Table IV). Small PGs were recovered mostly in the culture medium. Conversely, in all fractions of the patient's chondrocyte cultures, the percentage of small PGs was higher, and that of large PGs lower, than in control chondrocytes (Table IV).

Finally, in the patient's cell-associated matrix (0.2 and 4 M GdmCl fraction), an ^{3}H-labeled peak free of ^{35}S (fractions 37–43) and eluting in the void volume of the column (V_0) was present (Fig. 4, *E* and *G*). Since this peak did not overlap with the proteoglycan peak, and since standard hyaluronic acid labeled with [^{3}H]glucosamine eluted in the same position (data not shown), we presumed that this peak may represent hyaluronic acid. This hypothesis was further supported by the detection, upon DEAE-Sephacel chromatography, of a large peak of HA as described above.

TABLE III
Disaccharides quantification by HPLC

After double labeling of chondrocytes the PGs peak from the 4 M GdmCl fraction purified by ion exchange chromatography was digested with chondroitinase ABC and AC II and digested product analyzed by HPLC.

	ΔDi-OS %	ΔDi-4S		ΔDi-6S	
		%	$^{35}S/^3H$	%	$^{35}S/^3H$
Control	18	30	0.374	52	0.612
Patient	29	43	0.142	28	0.223

Sulfate Activation in Cell Sonicates—The metabolic activation of inorganic sulfate requires the action of a bifunctional cytosolic enzyme, ATP sulfurylase/APS kinase, which converts sulfate first to adenosine 5′-phosphosulfate (APS) and then to PAPS using two ATP molecules (18). After translocation into the Golgi lumen through a PAPS translocase, PAPS can act as a sulfate donor for the sulfation of PGs and other glycoproteins. ATP sulfurylase and APS kinase activities were assayed in control and patient's fibroblasts by incubating cell sonicates with [^{35}S]sodium sulfate and separating newly formed, labeled APS and PAPS by paper electrophoresis (Fig. 5). APS and PAPS levels in the patient's cellular extract were in the same range as in the control extract, indicating roughly normal activities of the bifunctional enzyme, ATP sulfurylase/APS kinase. These results contradicted the original conclusion of defective metabolic activation as the cause of ACG-1B (8), which was corrected recently (9).

Defective Activity of the Sulfate Transporter in Patient's Cells—Sulfate uptake was determined in patient's fibroblasts and chondrocytes by pulse labeling with [^{35}S]sulfate for 1 min in medium containing varying concentrations of unlabeled sodium sulfate. Uptake was reduced in the patient's cells at all sulfate concentrations tested (Fig. 6). The activity of the sulfate transporter was then assayed by determining the cells sensitivity to chromate. Chromate is toxic to cells and enters them through the sulfate transporter. Therefore, sulfate transport-deficient Chinese hamster ovary cells are relatively resistant to chromate (24). Patient's and control fibroblasts were incubated in medium containing various concentrations of K_2CrO_4 for 8 days and then their viability was assessed. The LD_{50} was 2.5 μM in control cells and 40 μM in the patient's cells (Fig. 7), indicating that the sulfate transport-deficiency in the patient's cells rendered them resistant to chromate.

Identification of a Homozygous Mutation in the DTDST Gene—Genomic fragments of the *DTDST* gene were amplified by PCR reaction and subjected to single strand conformation polymorphism and heteroduplex analysis. A PCR fragment spanning nt 1171 to 1559 of the *DTDST* gene gave a single strand conformation polymorphism pattern different from that of controls. To map the sequence change more precisely, the 389-bp fragment was incubated with various restriction enzymes prior to electrophoresis. After digestion with *Scr*FI, a fragment of 148 bp seen in controls was cleaved completely in fragments of 84 and 64 bp in the patient, indicating that the patient was homozygous for a restriction site not present in controls (Fig. 8). DNA sequence analysis of clones containing this fragment identified a T to C transition at nucleotide 1475 which predicts the substitution of leucine 483 with proline within the eleventh transmembrane domain of the sulfate transporter (Fig. 9). Parental DNA was not available for study; however, the observation of the same mutation on both alleles is compatible with recessive inheritance and parental consanguinity.

TABLE IV

Percentages of large and small proteoglycans and [^{35}S]sulfate/[^{3}H]glucosamine ratio in chondrocyte cultures after molecular sieve chromatography

Double labeled PGs from the medium and different matrix pools of chondrocyte cultures were prepurified by step-elution from DEAE-Sephacel and separated by chromatography on Sepharose CL-2B (see Fig. 4); fractions were assayed for radioactivity. The percentages of large and small PGs (as calculated from ^{35}S activities) and the ^{35}S/^{3}H ratio in each peak are shown. In the patient small PGs were present in relative higher amount in all fractions; both large and small PGs are undersulfated compared to the control.

	Patient				Control			
	Large PGs		Small PGs		Large PGs		Small PGs	
	%	^{35}S/^{3}H	%	^{35}S/^{3}H	%	^{35}S/^{3}H	%	^{35}S/^{3}H
Medium	0	0	100	0.316	32	2.402	68	1.011
Alginate	31	0.199	69	0.206	74	1.141	26	0.789
0.2 M GdmHCl	64	0.189	36	0.195	89	1.611	11	0.951
4 M GdmCl	59	0.192	41	0.194	84	1.480	16	0.714

DISCUSSION

Following the identification of a sulfation defect in ACG-1B (8) and the identification of a sulfate/chloride antiporter (*DTDST*) as the gene responsible for diastrophic dysplasia (10), we have recently shown that ACG-1B is caused by homozygosity or compound heterozygosity for mutations in the *DTDST* gene causing either premature termination of the DTDST protein or structural changes mainly within transmembrane domains (9). Consistent with this notion, the patient described here was homozygous for a previously unobserved DTDST mutation predicting a non-conservative amino acid substitution in the eleventh transmembrane domain of the sulfate transporter. Based on the fact that the presence of a helix-breaking proline within a transmembrane domain can be expected to disrupt its integrity, and on the analogy with the mutations observed previously, this mutation can be assumed to be pathogenic. Accordingly, the activity of the sulfate transporter in skin fibroblasts was found to be impaired both by the sulfate uptake and by the chromate toxicity assay. The observation of a defect in sulfate uptake in cells derived from epiphyseal cartilage is direct proof that the gene is expressed also in chondrocytes.

Despite the histological and chemical evidence of defective proteoglycan sulfation in ACG-1B cartilage, including reduced or absent staining of histologic sections and tissue extracts with cationic dyes (4, 8) and markedly reduced sulfate content (9), little detail is known about the composition and the structure of PGs species synthesized by ACG-1B chondrocytes *in vivo* and *in vitro*. The alginate bead culture system allows the maintenance or the re-expression of the chondrocytic phenotype, otherwise lost in monolayer cultures (12, 14). It has been used for the study of collagens and PGs secreted by normal chondrocytes (25) as well as for the molecular characterization of mutant type II collagen in patient's affected by spondyloepiphyseal dysplasia (26) or achondrogenesis type II (22). We have used this system, together with analysis of cartilage tissue extracts, to study the structure and sulfation of PGs synthesized by chondrocytes of our patient with ACG-1B.

Preliminary analysis of cartilage extracts indicated a reduced negative charge and a reduced sulfation of PGs. Double-labeling experiments of chondrocytes cultured in alginate beads followed by ion exchange chromatography confirmed that the contribution of inorganic extracellular sulfate to PGs sulfation was markedly decreased. Even if the elution profiles of DEAE Sephacel chromatogram were similar to those of controls, the presence of ^{3}H-labeled material between the HA peak and the CSPG peak and the broadness of this latter compound in the patient indicated the presence of undersulfated material. Although enhanced degradation of PGs containing unsulfated chondroitin chains cannot be excluded, our data do not support the hypothesis of a sulfation mechanism that would produce only unsulfated and fully sulfated macromolecules (23, 27). The patient's chondrocytes synthesized higher amounts of HA com-

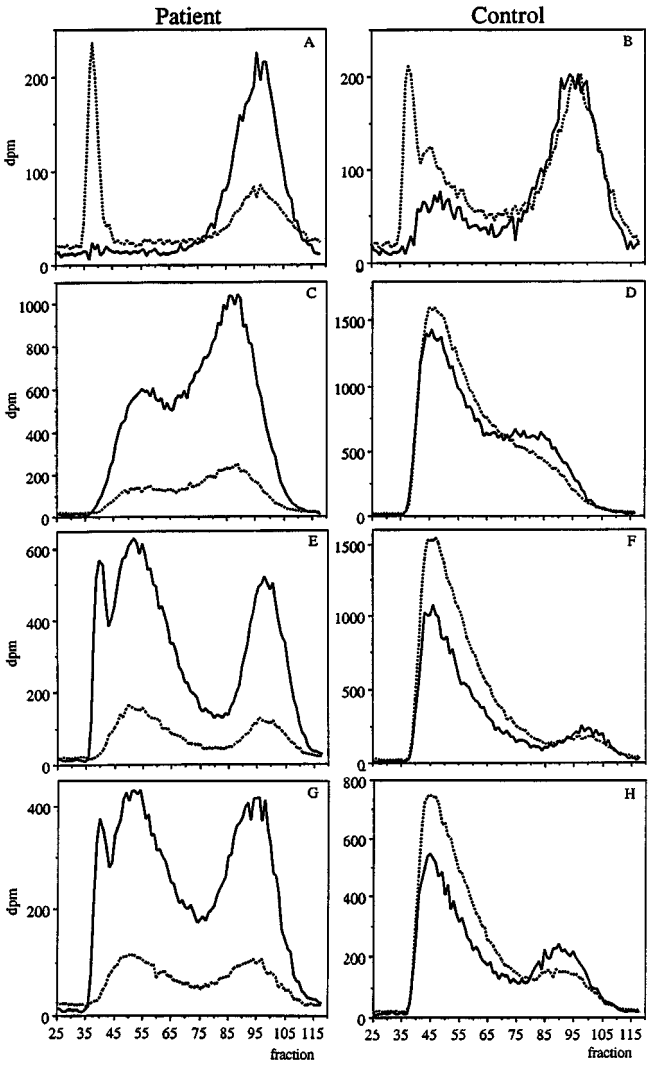

FIG. 4. **Molecular sieve chromatography in dissociative conditions of labeled proteoglycans from chondrocyte cultures.** Chondrocytes were double-labeled with [^{35}S]sulfate (- - - - -) and [^{3}H]glucosamine (——) for 24 h. The labeled medium was aspirated (medium fraction, *panels A* and *B*), the alginate gel was solubilized in EDTA (alginate fraction, *panels C* and *D*), and the residue was extracted first with 0.2 M GdmCl (0.2 M GdmCl fraction, *panels E* and *F*) and then with 4 M GdmCl (4 M GdmHCl fraction, *panels G* and *H*). PGs were prepurified by step elution from DEAE-Sephacel and subjected to molecular sieve chromatography on a Sepharose CL-2B column (see "Materials and Methods"). In the patient, both large and small PGs were undersulfated in the medium and in the matrix fractions. Moreover, the relative amount of small PGs was higher compared to the control (see also Table IV). In the 0.2 M and 4 M GdmHCl fraction (*panels E* and *G*, respectively) from the patient a ^{3}H-labeled peak (fractions 37–43) eluting at the same position as a hyaluronic acid standard was seen.

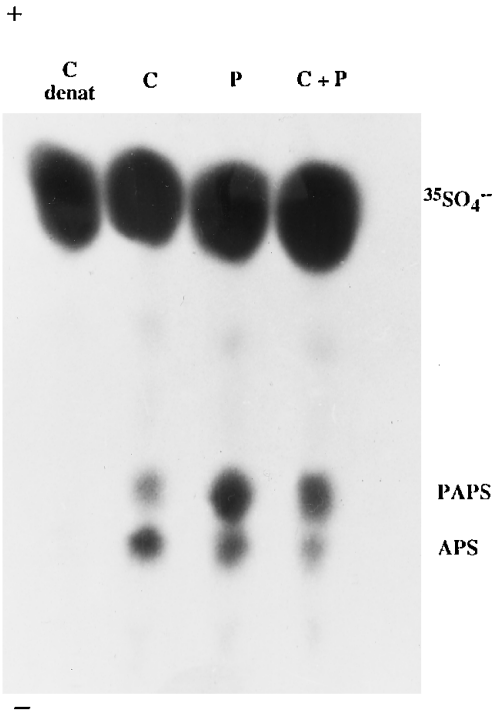

FIG. 5. **Incorporation of $^{35}S_4^{2-}$ into APS and PAPS.** [^{35}S]Sulfate incorporation in APS and PAPS was detected by paper electrophoresis after incubation of aliquots of fibroblast sonicates with 6 μCi of $Na_2[^{35}S]O_4$ at 37 °C for 1 h. APS and PAPS levels in the patient indicated that the metabolic activation of inorganic sulfate was normal. *C*, control; *P*, patient; *C denat.*, control extract denatured by boiling for 2 min before incubation with $^{35}SO_4^{2-}$; *C+P*, equal amounts of protein each from the patient and the control were mixed before incubation with $^{35}SO_4^{2-}$.

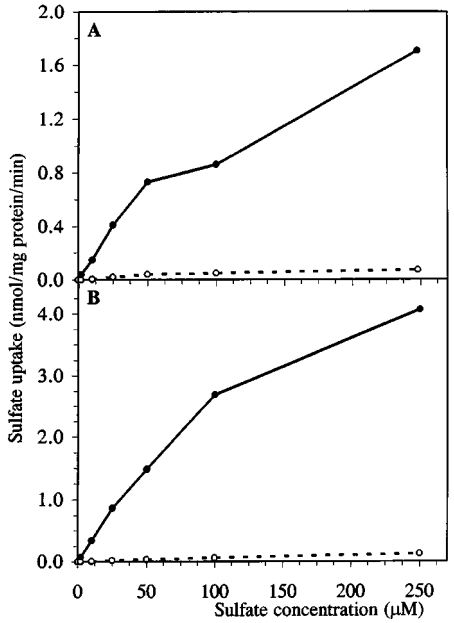

FIG. 6. **Sulfate uptake in fibroblasts and chondrocytes.** Cells were seeded in 10-cm^2 dishes and incubated in complete medium for 24 h. Sulfate uptake was performed in low ionic strength buffer containing different concentrations of Na_2SO_4 and 150 μCi/ml carrier-free $Na_2[^{35}S]O_4$ at 37 °C (see "Materials and Methods"). After 1 min incubation, cells were solubilized in 0.8 ml of 2% SDS and aliquots were used for scintillation counting and protein determination. Sulfate uptake was measured in fibroblasts (*panel A*) and chondrocytes in monolayer cultures (*panel B*). In both cell lines sulfate uptake is reduced in the patient at all sulfate concentrations considered. ●, control; ○, patient.

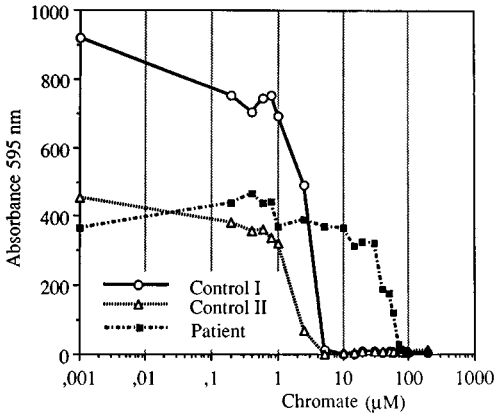

FIG. 7. **Chromate sensitivity of fibroblast cultures.** Fibroblasts were seeded in 96-microwell plates at 3×10^4 cells/well (*Control II* and *Patient*) or 6×10^4 cells/well (*Control I*) and incubated in medium containing K_2CrO_4 at the indicated concentrations for 8 days. Cells were fixed, stained with crystal violet, and optical density in the microwells was read with a multiplate reader. The LD_{50} for chromate in the patient's fibroblasts was 40 μM *versus* 2.5 μM in controls.

pared to PGs, indicating that PG undersulfation can affect not only PG metabolism but also HA synthesis. Although these components are synthesized in different cell compartments, their metabolism is highly coordinated in bovine cartilage organ cultures (28). If, as the biosynthetic studies of chondrocytes suggest, ACG-1B cartilage contains more hyaluronic acid than control cartilage, the concentration of proteoglycans may be reduced more significantly than suggested by the moderate decrease in uronic acid content. Accordingly, the poor staining of cartilage sections with chemical cationic dyes could be due not only to a lack of PGs sulfation but also to a reduced concentration of these macromolecules in the tissue.

The normal chromatographic pattern on Sepharose CL-2B provided indirect evidence that the length of glycosaminoglycan side chains of both large and small CSPGs was not significantly altered. These results correlate well with the observation of normally glycanated, albeit unsulfated, decorin in fibroblasts of a patient previously studied (8).

Disaccharide analysis of CSPGs synthesized by the patient's chondrocytes showed a higher amount of nonsulfated disaccharide (ΔDi-0S) when compared to sulfated disaccharides, thus demonstrating a reduced PGs sulfation. However, moderate amounts of sulfated disaccharides were present. Their poor labeling with ^{35}S indicated that the contribution of extracellular inorganic sulfate was low and that other sources of sulfate (possibly, sulfur containing amino acids) were able to partially compensate the lack of exogenous inorganic sulfate. These data differ from previous findings in organ culture of chick embryo cartilage showing that the contribution of other sources of sulfate was poor (23). The discrepancy between the two studies may be related to different culture conditions and/or to differences in the regulation of alternative metabolic pathways. Because of the sulfate transport defect, the patient's cells can be considered as chronically deficient in extracellular sulfate, a condition quite similar to the sulfate transport deficient mutants of Chinese hamster ovary cells in which sulfur containing amino acids provide sulfate for glycosaminoglycans synthesis (24). Additional studies performed in the patient's and control fibroblasts showed similar results (data not shown).

Other authors have demonstrated in different cell lines that sulfur containing amino acids, mainly cysteine, can provide sulfate for PGs sulfation (24, 29–31), but to our knowledge experiments on chondrocytes were restricted to the Swarm rat chondrosarcoma, where sulfur from cysteine contributed up to

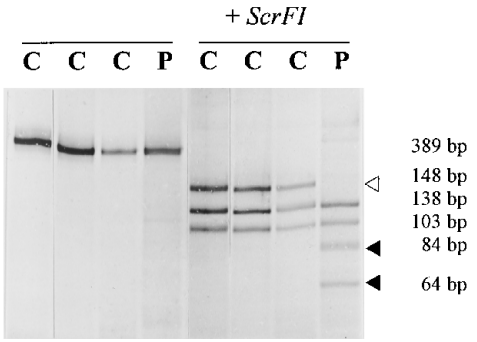

FIG. 8. **Electrophoresis of restriction fragments after *Scr*FI digestion of genomic PCR product.** A PCR fragment spanning nt 1171 to nt 1559 of the *DTDST* gene was digested with *Scr*FI and analyzed by electrophoresis on 15% polyacrylamide gels containing 8% glycerol; gel was stained with silver nitrate. The 148-bp fragment present in the controls was cleaved completely to two fragments of 84 and 64 bp in the patient, who was therefore homozygous for an additional restriction site. *C*, controls; *P*, patient.

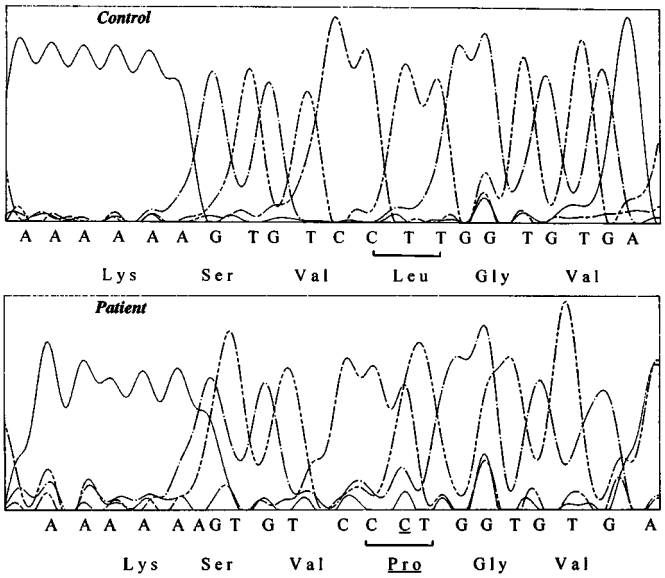

FIG. 9. **Sequences of normal and mutant DNA clones.** A 353-bp PCR fragment containing the additional restriction site for *Scr*FI was cloned and sequenced on an automatic sequencer. In the patient a T to C transition at nucleotide 1475 is present resulting in the substitution of leucine 483 by proline (*underlined*).

15% to the intracellular PAPS pool (29). No data are hitherto available regarding this metabolic pathway in cartilage, but it is likely to play a lesser role than *in vitro* due to the lower cysteine concentration in plasma (80 μM) than in culture medium (400 μM). Measurement of disaccharide content in cartilage will be required for comparison with *in vitro* findings. As chondrocytes are thought to have a greater sulfate requirement for PGs synthesis than any other tissues, cysteine metabolism in ACG-1B could be enhanced as an alternative source of sulfate.

Although the degree of undersulfation is not precisely measured in ACG-1B cartilage, it remains to be explained how decreased sulfation of PGs produces the marked histologic changes of cartilage matrix and severe bone dysplasia. We have considered four possibilities which are complementary rather than mutually exclusive. First, decreased sulfation may affect the stability of PGs in the tissue. Second, the undersulfation may change the binding properties of glycosaminoglycan chains to cationic ligands, such as specific domains of collagen II fibrils or the NC 4 peptide of collagen IX (32). There is evidence that the small chondroitin sulfate/dermatan sulfate proteoglycans, decorin and fibromodulin, bind to the surface of collagen fibrils with their core protein but bridge over to adjacent fibrils with their sulfated glycosaminoglycan chains (33). Moreover, decorin and fibromodulin can slow collagen fibril formation and reduce the diameter of fibrils *in vitro*, although it is not clear whether the core protein or the sulfated glycosaminoglycan chain mediates this effect (34, 35). Thus, a reduced concentration or changes in the binding properties of PGs might explain the coarsening of collagen fibrils typical of ACG-1B cartilage. A third hypothesis is a reduction in the hydrophilic capacity of PGs caused by undersulfation. While substantial residual water binding activity would be presumed to be associated with the galactosamine and uronic acid residues in both PGs and HA, the loss of sulfate groups might be critical in lowering the hydration state of the cartilage matrix. A fourth hypothesis invokes differences in the binding of growth factors to their cell-membrane receptors, which in several instances requires the presence of heparan sulfate, leading to a change in cell proliferation (36). Whether these or other mechanisms are at work is the aim of further studies.

The role of the large chondroitin sulfate proteoglycan, aggrecan, for the integrity of the extracellular matrix of cartilage is highlighted by the lethal chondrodysplasias associated with mutations in the aggrecan core protein leading to the absence of aggrecan in chicken (37) and in mice (38). The importance of correct sulfation of PGs had been indicated by studies in the non-lethal, recessive mouse chondrodysplasia, *brachymorphic* (*bm*). In homozygous *bm/bm* animals, who have short limbs and histological evidence of defective cartilage matrix including reduced metachromasia (39), there is a decrease in the activities of the bifunctional enzyme ATP sulfurylase/APS kinase, leading to PAPS depletion (17). The substantial residual activity of this enzyme in cartilage of affected mice may account for the non-lethal phenotype. Although the metabolic block in ACG-1B is at the level of sulfate transport rather than of sulfate activation, the severe phenotype is a further indication that PG sulfation must have a crucial role in cartilage matrix assembly. The results presented here demonstrate that chondrocytes are affected by the sulfate transport defect and synthesize PGs which are normal in size but not normally sulfated. The mechanism by which decreased sulfation is translated into the severe changes in the extracellular matrix and lastly into the lethal phenotype remains to be determined.

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Undersulfation of Proteoglycans Synthesized by Chondrocytes from a Patient with Achondrogenesis Type 1B Homozygous for an L483P Substitution in the Diastrophic Dysplasia Sulfate Transporter

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