Functional Analysis of Diastrophic Dysplasia Sulfate Transporter
ITS INVOLVEMENT IN GROWTH REGULATION OF CHONDROCYTES MEDIATED BY SULFATED PROTEOGLYCANS

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Mutations in the diastrophic dysplasia sulfate transporter (DTDST) gene constitute a family of recessively inherited osteochondrodysplasias including achondrogenesis type 1B, atelosteogenesis type II, and diastrophic dysplasia. However, the functional properties of the gene product have yet to be elucidated. We cloned rat DTDST cDNA from rat UMR-106 osteoblastic cells. Northern blot analysis suggested that cartilage and intestine were the major expression sites for DTDST mRNA. Analysis of the genomic sequence revealed that the rat DTDST gene was composed of at least five exons. Two distinct transcripts were expressed in chondrocytes due to alternative utilization of the third exon, corresponding to an internal portion of the 5'-untranslated region of the cDNA. Injection of rat and human DTDST cRNA into Xenopus laevis oocytes induced Na⁺-independent sulfate transport. Transport activity of the expressed DTDST was markedly inhibited by extracellular chloride and bicarbonate. In contrast, canalicular Na⁺-independent sulfate transporter Sat-1 required the presence of extracellular chloride in the cRNA-injected oocytes. The activity profile of sulfate transport in growth plate chondrocytes was studied in the extracellular presence of various anions and found substantially identical to DTDST expressed in oocytes. Thus, sulfate transport of chondrocytes is dominantly dependent on the DTDST system. Finally, we demonstrate that undersulfation of proteoglycans by the chloride treatment of chondrocytes significantly impaired growth response of the cells to fibroblast growth factor, suggesting a role for DTDST in endochondral bone formation.

In animals, most of the bone initially forms as cartilage (cartilaginous bone rudiments), which is later replaced by bone (endochondral ossification) (1). In adults, cartilage is left in particular body portions such as rib, auricle, and joints and functions as a load-bearing tissue. Thus, cartilage is essential for growth and maintenance of animal skeletal systems. Biological functions of cartilage are mostly dependent on the properties of its extracellular matrix, whose major components are cartilage-specific collagens and sulfated proteoglycans (2). Recently, three human congenital chondrodysplasias, i.e. diastrophic dysplasia, atelosteogenesis type II, and achondrogenesis type 1B (ACG-1B), have been demonstrated to be caused by mutations in a single gene (3–5). The gene, the diastrophic dysplasia sulfate transporter (DTDST), is presumed to encode a Na⁺-independent sulfate transporter on the basis of its structural similarity with rat hepatocanalicular sulfate transporter (Sat-1) and human intestine-specific sulfate transporter (DRA; down-regulated in adenoma) (3, 6).

Rossi (7) recently demonstrated that chondrocytes isolated from a ACG-1B patient synthesized chondroitin sulfate proteoglycans that bore glycosaminoglycan chains that were of normal size but were undersulfated. The oocyte expression system has been proved to be a powerful tool for functional analysis of the DRA and Sat-1 gene products (6, 8). In the present study, we directly characterized the sulfate transport activity of rat DTDST by injection of its cRNA into Xenopus oocytes and compared it to that of normal rat costal chondrocytes. The activity profile of the gene product showed an ion dependence of transport distinct from that of Sat-1, suggesting that DTDST was a sulfate/chloride antipporter. Hästbacka and co-workers (3) demonstrated a ubiquitous expression of DTDST mRNA in the body. Histological observations of diastrophic dysplasia patients would suggest cartilage to be the most relevant tissue to examine. However, they did not include cartilage in their study. The present Northern blot analysis clearly indicated that the level of DTDST mRNA was particularly high in cartilage and intestine. Only a marginal level of the transcript was detected in other tissues. A unique ion dependence of sulfate transport in culture chondrocytes further supported the notion that cartilage is predominantly dependent on the DTDST system for its sulfate utilization.

MATERIALS AND METHODS

Cell Culture—Chondrocytes were isolated from growth plate cartilage of ribs of young Wistar rats (weighing 90–110 g), as described (9). After removal of peripheral muscular tissues and perichondrium from whole ribs, a portion of the growth plate cartilage was isolated and

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3 The abbreviations used are: ACG-1B, achondrogenesis type 1B; DIDS, 4,4'-disothiocyanato-2,2'-disulfonic acid stilbene; DTDST, diastrophic dysplasia sulfate transporter; FGF, fibroblast growth factor; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; RACE, rapid amplification of cDNA ends; PCR, polymerase chain reaction; RT-PCR, reverse transcription-PCR; PBS, phosphate-buffered saline.
minced. Cells were isolated by sequential treatment of cartilage with EDTA, trypsin, and collagenase. The isolated cells were plated at a density of 10^4 cells/well in 24-multiwell plates, or 2 x 10^4 cells/well in 48-multiwell plates, or 1 x 10^4 cells/well in 96-multiwell plates in DMEM/F-12 medium containing 5% FBS. Cells were grown to confluence in 37 °C under 5% CO2 in air. The culture medium was renewed every other day. Rat UMR-106 osteosarcoma cells were maintained in DMEM containing 10% FBS as described (10).

The cDNA from UMR-106 was used to amplify rat DTDST cDNA by PCR with the specific primer set (primer 3 and 4). The other set was a combination of the 5'- and 3'-regions of rat DTDST genomic sequence were amplified using the Marathon cDNA amplification kit (CLONTECH) and was used to amplify DTDST genomic DNA sequences. The 5'-untranslated region of rat DTDST gene was amplified by PCR with sense primer 11 and antisense primer 12. An approximately 1.3-kb genomic DNA fragment was amplified. Further 5'-regions of rat DTDST genomic sequence were amplified using the GenomeWalker kit (CLONTECH) by two steps of PCR reactions with the gene-specific antisense primer (rDTD-GSP2) and the nested gene specific antisense primer (rDTD-NGSP3), corresponding to the sequence in exon I. The PCR products were cloned into pCRII vectors by TA cloning for sequence analysis.

**Functional Analysis of DTDST**

### Summary of PCR primers used in this study

| Primers | Nucleotide sequences |
|---------|----------------------|
| 5'-GTGAAAACATATGCTCC-3' | 5'-CATGACATTTGTCTCTAC-3' |
| 5'-ATGCTCTAGTTAGAATAAG-3' | 5'-ATTAGCAAGAGAAGCAGC-3' |
| 5'-TTGCAAACAAGGAAGAC-3' | 5'-TCTACTCTAAGCTACCAG-3' |
| 5'-GCAGTTGAAAGAGGGTTGATG-3' | 5'-CCGGCCAAATCTCAGGAGC-3' |
| 5'-AGACATCATTCTGGCTCTC-3' | 5'-AGGTCTTCCATGAGGTCTC-3' |
| 5'-ATACGATTCTACTGGAAGTGTC-3' | 5'-AACAGACACACATCATTTGAGG-3' |

**Claoning of DTDST cDNA and Sat-1 cDNA**—The oligonucleotide primers used in this study are summarized in Table I. Human placenta cDNA (Quick-Clone cDNA) was purchased from CLONTECH (Palo Alto, CA) and was used to amplify human DTDST cDNA by polymerase chain reaction (PCR) with Ex Taq DNA polymerase (Perkin-Elmer) and specific primers to the 5'- and 3'-ends of the entire coding sequence in human DTDST cDNA: primer 1 and primer 2. Further 5'-regions of human DTDST cDNA were amplified by rapid amplification of cDNA ends (RACE) using the Marathon cDNA amplification kit (CLONTECH) with the gene-specific antisense primer (hDTD-GSP1) and the nested gene-specific antisense primer (hDTD-NGSP1). The cDNA from rat UMR-106 cells and rat kidney were reverse transcribed into cDNA with oligo(dT) primer using the cDNA synthesis Superscript preamplification system (Life Technologies, Inc.). The cDNA from rat kidney was used to amplify an entire coding sequence rat Sat-1 cDNA by PCR with the specific primer set (primer 3 and 4). The resulting cDNA was cloned into pCRII vector.

**Cloning of Rat DTDST Genomic DNA**—Rat genomic DNA was purchased from CLONTECH and used to amplify DTDST genomic DNA sequences. The 5'-untranslated region of rat DTDST gene was amplified by PCR with sense primer 11 and antisense primer 12. An approximately 1.3-kb genomic DNA fragment was amplified. Further 5'-regions of rat DTDST genomic sequence were amplified using the GenomeWalker kit (CLONTECH) by two steps of PCR reactions with the gene-specific antisense primer (rDTD-GSP2) and the nested gene specific antisense primer (rDTD-NGSP3), corresponding to the sequence in exon I. The PCR products were cloned into pCRII vectors by TA cloning for sequence analysis.

**Nucleotide Sequence Analysis**—The DTDST cDNA and genomic sequences were determined by dye terminator cycle-sequencing reactions using specific primers and the purified PCR fragments or cloned plasmids as templates and an automated sequencer (Applied Biosystems model 373A, Perkin-Elmer). Sequences were analyzed using DNASIS software (Hitachi Software Engineering) and the computer programs BLAST (11) and MACAW (12).

**Northern Blot Hybridization**—Total RNA was prepared from various rat tissues by a single step method according to Chomczynski and Sacchi (13). Isolated total RNA (20 μg/ lane) was separated by electrophoresis on a 1% agarose/formaldehyde gel and transferred onto Hybond-N nylon membrane (Amersham Pharmacia Biotech). The cloned rat DTDST and Sat-1 cDNA fragments were labeled by the BcaBEST labeling kit (TAKARA, Ohtu, Japan) and [α-32P]dCTP (3,000 Ci/mmole; Amersham Pharmacia Biotech). Human β-actin cDNA was similarly labeled and used as a control probe. The blots were hybridized overnight at 42 °C with a probe (2 x 10^6 cpm/ml) in 50% formamide, 5 x saline/sodium phosphate/EDTA, 2 x Denhardt's solution, 2% SDS, and 100 μg/ml denatured salmon testis DNA (Sigma). The filters were washed twice at room temperature for 15 min in 2 x SSC, 0.05% SDS and then washed twice at 50 °C for 40 min in 0.1 x SSC, 0.1% SDS and exposed to Hyperfilm-MP x-ray films (Amersham Pharmacia Biotech) at -80 °C with an intensifying screen.

Detection of Splice Variants of DTDST mRNA by RT-PCR—Total RNAs (2.5 μg each) isolated from UMR-106 cells and rat growth plate chondrocytes were reverse transcribed into cDNA with random hexamer using the cDNA synthesis Superscript preamplification system. One-fifth or one-hundredth of the cDNAs were used to amplify two alternative spliced transcripts of the DTDST gene by PCR. PCR amplification was performed by using AmpliTaq DNA polymerase (Perkin-Elmer) and sense primer 11 and antisense primer 12 (Table I). Thermal cycling was carried out for 28 cycles (30 s at 94 °C, 30 s at 55 °C, and 30 s at 72 °C). Aliquots of the PCR products were resolved on 5% polyacrylamide gel along with molecular size markers, and the amplified products were stained with ethidium bromide.

In Vitro Transcription and Injection into Oocytes—Plasmids harbor-
ing cloned sulfate transported cDNAs were linearized with appropriate restriction enzymes that have a cleavage site immediately downstream of the cDNA insert. Capped cRNAs were synthesized by T7 RNA polymerase, T3 RNA polymerase, or SP6 RNA polymerase using the mCAP RNA capping kit (Stratagene). Unincorporated nucleotides were removed with Quick Spin columns (Boehringer Mannheim, Germany), and cRNA was recovered by ethanol precipitation and resuspended in water for injection into oocytes.

**Sulfate Transport Assay—**The handling of Xenopus laevis oocytes and the transport assay were carried out as reported by Bissig (8). 

**[35S]Sulfate (carrier-free) was purchased from NEN Life Science Products. Oocytes were washed twice in 0.1 M NaCl and incubated in 100 µl of uptake solution per oocyte (200 mM sucrose or 100 mM various salts, 1 mM [35S]sulfate (40 µCi/µl), 10 mM HEPES/Tris, pH 7.5) for 5 min at 37 °C. Uptake was stopped by washing the oocytes three times with ice-cold washing solution containing 5 mM K2SO4. Each oocyte was then dissolved in 0.2 ml of 10% SDS, and the oocyte-associated radioactivity was determined in a liquid scintillation spectrometer after the addition of scintillation fluid (ACS II, Amersham Pharmacia Biotech).

Sulfate uptake was measured as described by Håstbacka (3) with slight modifications. Confluent chondrocytes cultured in 24-multiwell plates were washed three times in ion-free washing solution (300 mM sucrose, 10 mM HEPES/Tris, pH 7.5). The cells were then incubated in 500 µl of uptake solution (300 mM sucrose or 149 mM various salts), 1 mM [35S]sulfate (40 µCi/ml), 10 mM HEPES/Tris, pH 7.5) for 5 min at 37 °C. Uptake was stopped by washing the cells four times with ice-cold washing solution containing 5 mM K2SO4. The cells were then dissolved in 0.3 ml of DMEM/F-12 medium or sulfate-free DMEM/F-12 medium containing 5% FBS with 0.3–2.5 mM (15). In this study, serum sulfate concentration and a contaminant. Sulfate concentration in serum was also reported to be 0.01% H2O2 for 7 min. Nuclei were stained with methyl green. Then the sections were dehydrated in ethanol, cleared in xylene, and mounted in Permount (Fisher).

**Thymidine Incorporation and Proteoglycan Synthesis in Growth Plate Chondrocytes—**For determination of thymidine incorporation, rat growth plate chondrocytes were grown to confluency in DMEM/F-12 medium or sulfate-free DMEM/F-12 medium containing 5% FBS with or without sodium chloride (Sigma) in 96-multiwell plates. Sulfate-free DMEM/F-12 medium, in which all of the sulfate salts in the standard formula were substituted by chloride salts, was specially prepared and obtained from Nikken Biomedical Lab (Osaka, Japan). Antibiotics, which are the significant sources of sulfate, were also omitted. Humphries et al. (14) noted that the antibiotic-sulfate-free medium thus prepared may still contain 0.01 mM sulfate as a possible minor contaminant. Sulfate concentration in serum was also reported to be 0.3–2.5 mM (15). In this study, serum sulfate concentration and a sulfate contaminant in the sulfate-free DMEM/F-12 were assumed to be 2.5 and 0.01 mM, respectively. Cells were preincubated in the same medium containing 0.3% FBS for 24 h. The medium was replaced by the same medium containing 0.3% FBS with human recombinant FGF-2 (R & D Systems, Minneapolis, MN) in the presence or absence of 10 µg/ml heparin (Wako Pure Chemical, Osaka, Japan). Cells were incubated for another 26 h and labeled with 2 µCi/ml [3H]thymidine (Amersham Pharmacia Biotech) for the last 4 h. Radioactivity incorporated into DNA was determined as described previously (16).

For determination of proteoglycan synthesis, chondrocytes were grown to confluency in 48-multiwell plates. The medium was replaced with 0.3 ml of DMEM/F-12 medium or sulfate-free DMEM/F-12 medium containing 0.3% FBS with or without sodium chloride and incubated for 48 h. Then the cultures were labeled with 5 µCi/ml [35S]sulfate or 10 µCi/ml [3H]glucosamine for another 24 h. After incubation, the medium was collected, and the cell layer was rinsed with phosphate-buffered saline (PBS). Proteoglycans recovered in the medium and PBS rinse were combined. [35S]Sulfate and [3H]glucosamine incorporated into proteoglycans were determined after Pronase E digestion and precipitation by 1% cetylpyridinium chloride in the presence of chondroitin sulfate, as described (16).

**Immunohistochemistry—**Developing bovine tails were collected from 5-month-old fetuses and fixed overnight at 4 °C in periodate/lysine/paraformaldehyde solution in 0.01 M PBS (pH 7.4). The caudal vertebrae were dissected out, dehydrated in a graded series of ethanol, and embedded in paraffin. Longitudinal serial sections were cut at 6 µm. Deparaffinized sections were treated with 1% H2O2 in methanol for 30 min to reduce endogenous peroxidase activity and washed in PBS.

**RESULTS**

**Structure of Rat DTDST Gene and Tissue Distribution of Its Transcripts—**Physicochemical properties of extracellular matrix macromolecules profoundly affect growth and differentiation of cell types in animals. Bone and cartilage produce a large amount of extracellular matrix components, which include sulfated proteoglycans. Thus, these tissues are assumed to develop an efficient transport system for sulfate anion to meet their demand for sulfate during proteoglycan synthesis. To study the properties of the sulfate transporting system and its role in the growth
regulation, we attempted to clone rat DTDST cDNA. Using RT-PCR with human DTDST-specific primers 5 and 6 (Table I), we amplified rat cDNA from total RNA isolated from rat UMR-106 osteoblastic cells. Then the entire coding region and the 5' and 3'-regions of the cDNA were isolated by RT-PCR and RACE reactions. In the coding region, the cloned cDNA sequence had a higher similarity to human DTDST cDNA (73% identical) than that of rat hepatocanalicular sulfate transporter Sat-1 cDNA (43% identical) (3, 8). We therefore identified the cloned cDNA as rat DTDST cDNA. For comparison, we also cloned human DTDST cDNA from human placenta cDNA by PCR using primers 1 and 2 and then 5'-RACE (Table I). The resultant RACE fragments contained the 5'-flanking sequence (277 to 21) of the putative initiator ATG of human DTDST cDNA (3). Comparison of the cDNA sequence with the previously reported genomic sequence revealed the presence of 3'-splice acceptor and an exon/intron junction at 226/225 (3). Rat Sat-1 cDNA was also amplified from total RNA isolated from rat kidney and sequenced (8).

Interestingly, two forms of rat DTDST cDNA that differ in their 5'-untranslated region were isolated by RACE reactions. One contained an additional 130-base pair insert that was absent from the other. To explore the cause of the differences between the two cDNA clones, we analyzed the corresponding genomic sequences by PCR reactions using GenomeWalker kit. Comparison of the genomic sequence with the cDNA sequence revealed that the 5'-untranslated region of rat DTDST sequence is encoded by four exons (exons I–IV), as shown in Fig. 1A. These exons were separated by intron I (8 kilobase pairs), intron II (512 base pairs), and intron III (1 kilobase pair). We also confirmed the presence of an approximately 1.8-kilobase pair after codon 233, the position of which is identical to that previously reported in human DTDST (3). Furthermore, it was shown that two forms of cDNA were generated by alternative utilization of exon III (Fig. 1B). As shown in Fig. 1C, RT-PCR using sense primer 11 and antisense primer 12 revealed the presence of these two alternative transcripts in UMR-106 osteoblastic cells and growth plate chondrocytes. These transcripts were also detected in articular cartilage (data not shown). The nucleotide sequence and the deduced amino acid sequence of rat DTDST cDNA are shown in Fig. 2.

Functional Analysis of Rat DTDST Expressed in Oocytes—To determine the anion transport function of DTDST gene product unequivocally, we injected cRNAs of human and rat DTDST into Xenopus oocytes and compared them to cRNA prepared from rat Sat-1 cDNA. First, we examined endogenous sulfate transport in Xenopus oocytes using water-injected oocytes and found that water-injected oocytes displayed only low [35S]sulfate uptake (data not shown). In contrast, human or rat DTDST cDNA-injected oocytes displayed significant uptake of [35S]sulfate in the outside sulfate pool in the presence of sodium gluconate outside (Fig. 3). The level of [35S]sulfate uptake in the presence of sodium glucuronate was nearly the same as that in the sucrose-containing medium without sodium glucuronate.
(data not shown), indicating a Na\(^+\)-independent sulfate transport of DTDST. The presence of outside chloride anion, however, resulted in a significant inhibition of the DTDST-directed sulfate uptake. In contrast to DTDST, Sat-1 cRNA-injected oocytes displayed a chloride-dependent sulfate uptake as previously reported (8), demonstrating that DTDST constitutes a unique transport system distinct from that of Sat-1 (Fig. 3).

We then studied the cis-inhibition pattern of DTDST-directed sulfate transport activity (Fig. 4). Human DTDST-directed sulfate uptake was sensitive to thiosulfate and oxalate. 4,4'-Disothiocyanostilbene-2,2'-disulfonic acid (DIDS; 1 mM) completely blocked the sulfate transport activity of DTDST. In agreement with a previous report (8), Sat-1-directed sulfate uptake displayed an identical cis-inhibition pattern. Inhibition of sulfate transport by oxalate was also reported in sulfate uptake directed by DRA, the gene of which is intestinal-specific sulfate transporter and has a significant homology with DTDST (18). Moreover, DRA has been demonstrated to transport oxalate (6). Thus, the results may suggest that both DTDST and Sat-1 also transport oxalate.

Properties of the Sulfate Transport System in Growth Plate Chondrocytes—To explore the nature of sulfate transport in chondrocytes, we cultured growth plate chondrocytes isolated from rat ribs in DMEM/F-12 medium containing 5\% FBS (9). Confluent cultures of chondrocytes were rinsed three times with ion-free solution containing 300 mM sucrose and 10 mM

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**Fig. 2—continued**
HEPES/Tris (pH 7.5). Then cells were incubated with 1 mM [35S]sulfate for 5 min. [35S]Sulfate uptake was measured by the method reported by Hästbacka (3). In the presence of sodium gluconate, chondrocytes exhibited the active inward transport of sulfate anion (Fig. 5). However, the presence of extracellular chloride and bicarbonate markedly inhibited sulfate uptake of the cells. This Na\textsuperscript{+}-independent transport profile of chondrocytes was almost identical to that of the DTDST cRNA-injected oocytes (Figs. 3 and 5). The presence of extracellular chloride markedly facilitated sulfate efflux from chondrocytes in which [35S]sulfate had previously been loaded (Fig. 6). Bicarbonate had no effect on efflux of sulfate anion, suggesting that the cells possess a chloride/sulfate antiport system.

By Northern blot analysis, we characterized the tissue distribution of DTDST mRNA using total RNA isolated from various rat tissues including bone and cartilage (Fig. 7). Since the hybridization signals with β-actin probe were significantly weaker in rib cartilage than in other tissues, the result clearly indicated that cartilage was one of the major expression sites of DTDST mRNA (approximately 8 kilobase pairs in size). Compared with cartilage, the level of DTDST mRNA in bone was very low. Unexpectedly, DTDST mRNA was also clearly detected in small intestine where intestine-specific sulfate transporter DRA is present (6). Sat-1 mRNA was expressed in liver and kidney as previously reported (8). Taken together, these results suggested that chondrocytes evidently possess the DTDST sulfate/chloride antiport system.

Growth Response of Growth Plate Chondrocytes in Sulfate-
deficient Culture—Undersulfation of proteoglycans is a major consequence of impairment of the DTDST transport system in chondrocytes (7). Thus, we studied the growth factor response of chondrocytes associated with undersulfated proteoglycans. Rat growth plate chondrocytes in confluent culture were maintained in sulfate-free DMEM/F-12 medium containing 0.3% FBS for 72 h. We first measured [35S]sulfate and [3H]glucosamine incorporated into proteoglycans for the last 24 h of incubation. Sulfate concentration in serum was reported to be 0.3–2.5 mM, depending on the species and lots of serum (15). Sulfate incorporation into proteoglycans in sulfate-free medium containing 0.3% FBS was estimated to be reduced to approximately 25% of the control cultures maintained in the standard DMEM/F-12 medium containing 0.3% FBS, assuming the sulfate concentration in FBS to be 2.5 mM (15) and a minor sulfate contamination in sulfate-free medium to be 0.01 mM (14). However, glucosamine incorporation was not significantly affected by the culture in sulfate-free medium compared with that in standard medium, suggesting that there was no significant change in the rate of proteoglycan synthesis in chondrocytes under the low sulfate culture conditions. Treatment of cells with sodium chlorate, a reversible inhibitor of glycosaminoglycan sulfation, resulted in further reduction of sulfate incorporation to several percent of the level in the cultures main- tained in sulfate-free DMEM/F-12 medium containing 0.3% FBS alone (Table II), whereas [3H]glucosamine incorporation was not significantly affected. FGF-2 is known to be a potent mitogen for chondrocytes (19). As shown in Fig. 8, human recombinant FGF-2 stimulated [3H]thymidine incorporation in chondrocytes cultured in the standard DMEM/F-12 in a dose- dependent manner. The addition of 10 μg/ml heparin potentiated the growth response of the cells to FGF-2. When chondrocytes, however, were maintained in undersulfation conditions, the growth response of cells to FGF-2 was significantly abolished. Cellular responsiveness to FGF-2 could not be recovered by the addition of heparin in the severely undersulfated culture treated with sodium chlorate.

Localization of FGF-2 in Epiphyseal Cartilage of Developing Bone—FGF-2 is expressed in virtually all cell types in developing bone, which includes cartilage, perichondrium and periosteum, bone, and the surrounding connective tissues (20, 21). It is usually hard to demonstrate extracellular localization of growth factors in bone and cartilage by immunohistochemistry, since epitopes of the minor proteins are masked by the abundant extracellular matrix components. After unmasking the FGF-2 epitope with the hyaluronidase treatment, we demonstrated extracellular localization of FGF-2 in the developing bovine bone for the first time in detail by using a specific monoclonal antibody against bovine FGF-2 (bFM-1) (17). All of the epiphyseal cartilage zones and bone at the primary ossification center were clearly stained by bFM-1 (Fig. 9A), whereas preimmune IgG showed no positive staining (Fig. 9B). At higher magnification, the cell surface or pericellular space was shown to be stained intensely in the proliferating cartilage zone (Fig. 9C) as well as in the resting cartilage zone. Interestingly, virtually no immunoreactivity was detected in the interterritorial space (Fig. 9C). However, as cartilage differentiation progressed toward the lower hypertrophic and calcified zone, immunoreactivity gradually diffused out from the pericellular to the interterritorial space of cartilage matrix (Fig. 9, A and D). In the lowest layers of chondrocytes, although the cell surface staining persisted, cells appeared to be separated by an FGF-2-negative pericellular space from the intensely stained interterritorial matrix. In addition, the extracellular FGF-2 immunoreactivity was also noted in osteoblasts (Fig. 9D, arrows).

**DISCUSSION**

The DTDST gene has been implicated in the pathogenesis of at least three human chondrodysplasias (22) and encodes a membrane transporter with 12 membrane-spanning domains, which is homologous to the hepatocanalicular sulfate transporter Sat-1 (3, 8). However, the anion transport activity of the gene product has not been directly determined. In the present study, we have clearly demonstrated that the gene product indeed functions as a Na"-independent sulfate transporter by the use of an oocyte expression system (Fig. 3). DTDST-directed sulfate transport was markedly inhibited by thiocyanate and oxalate, but not by succinate. DIDS completely blocked DTDST-directed sulfate uptake (Fig. 4). Thus, DTDST exhibited a cis-inhibition pattern identical to that of Sat-1.

Despite a striking sequence similarity to Sat-1, DTDST-directed sulfate uptake was significantly repressed by outside chloride anion (Fig. 3). In contrast, Sat-1-directed uptake was greatly facilitated by the presence of outside chloride anion but repressed by outside bicarbonate. These findings were compat-

### TABLE II

| Concentration of sodium chlorate | [35S]Sulfate incorporation (cpm/well) | [3H]Glucosamine incorporation (cpm/well) |
|---------------------------------|-------------------------------------|------------------------------------------|
| 0 mM                            | 225,000 ± 52,000 (100%)             | 7,270 ± 900 (100%)                       |
| 10 mM                           | 16,500 ± 10,500 (7%)                | 9,780 ± 3,660 (134%)                    |
| 25 mM                           | 9,580 ± 2,040 (4%)                  | 9,470 ± 720 (130%)                      |

Growth plate chondrocytes grown in 96-multiwell plates were metabolically labeled with [35S]sulfate or [3H]glucosamine in the presence of indicated concentrations of sodium chlorate in sulfate-free DMEM/F-12 medium containing 0.3% FBS. The labeled proteoglycans were recovered from the culture by coprecipitation with cold chondroitin sulfate and 0.1% CPC. Radioactivities in the precipitates were determined. Values (cpm/well) are the means ± S.D. for triplicate wells. Numbers in parentheses represent percentages of control values.

**Fig. 8. Effect of sodium chlorate on DNA synthesis in rat growth plate chondrocytes stimulated by FGF-2.** Rat growth plate chondrocytes were grown to confluency in DMEM/F-12 medium or sulfate-free DMEM/F-12 medium containing 5% FBS with or without sodium chlorate in 96-multiwell plates. They were then preincubated in the same medium containing 0.3% FBS for 24 h. The medium was replaced by the same medium containing 0.3% FBS with FGF-2 in the presence or absence of 10 μg/ml heparin. Cells were incubated for another 26 h and labeled with 2 μCi/ml [3H]thymidine for the last 4 h. Values are the means ± S.D. for triplicate wells.
ible with the notion that canalicular transporter Sat-1 is a sulfate/bicarbonate antiporter (8, 23). Hästbacka et al. (3) presumed DTDST to encode a sulfate/chloride antiporter on the basis of their observation that skin fibroblasts from a DTD patient lacked a sulfate/chloride antiport activity previously characterized in human lung fibroblasts (IMR-90) (24). The fact that DTDST-directed sulfate uptake depended on outside chloride gives further support to the notion that DTDST functions as a sulfate/chloride antiporter.

Previously, Hästbacka (3) reported by Northern blot analysis using poly(A)⁺ RNA that expression of DTDST mRNA could be detected widely in multiple tissues in humans. However, taking into consideration that mutations in the gene participate in the pathogenesis of osteochondrodysplasia, cartilage and bone must be important target tissues to examine. In the present study, Northern blot analysis using total RNA revealed that DTDST mRNA is predominantly expressed in cartilage (Fig. 7). No obvious hybridization signal was detected in bone. Since cartilage synthesizes a large amount of sulfated proteoglycans and deposits them in their extracellular matrix to maintain its biological functions, cartilage-specific expression of the DTDST gene must reflect a greater requirement of cartilage for proteoglycan sulfation via the DTDST transport system. Interestingly, we also found that small intestine was one of the major expression sites of DTDST mRNA (Fig. 7). Mutations in the intestine-specific sulfate transporter gene DRA are implicated in congenital chloride diarrhea (25). Congenital chloride diarrhea is characterized by large watery stools with a high chloride concentration, possibly due to a defect of the chloride/bicarbonate exchange system in the ileum and colon (26, 27). Thus, DTDST together with DRA may participate in the intestinal chloride/bicarbonate exchange system. However, the physiological functions of DTDST expressed in the intestines remain to be elucidated.

Since carrier-mediated sulfate transport at the plasma membrane is rate-limiting for macromolecular sulfation (24), proper DTDST functions must be critical for sulfation of proteoglycans and matrix organization in cartilage. In the course of our study, we for the first time established the structure of DTDST transcripts in cartilage and found that there exist two types of transcripts derived from an alternative splicing of the third exon in the 498-base pair 5' -untranslated region (Figs. 1 and 2). It is not known at present what the functional significance is of these alternative transcripts in cartilage. However, common ion dependence of sulfate transport in DTDST cRNA-injected oocytes and cultured chondrocytes strongly indicated that the DTDST transport system plays a crucial role in cartilaginous sulfate uptake (Figs. 3 and 5). Induction of sulfate efflux by outside chloride further suggested the presence of DTDST-like sulfate/chloride antiporter (Fig. 6). Although the possibility that some unknown sulfate transport systems are responsible for sulfate uptake into chondrocytes cannot be ruled out, epiph-

**Fig. 9.** Immunohistochemical localization of FGF-2 in developing caudal vertebrae. A, anti-FGF-2 monoclonal antibody, bFM-1, revealed localization of FGF-2 in epiphyseal cartilage of bovine developing bone. B, no positive staining was observed when mouse preimmune IgG was used as a negative control. A high magnification shows intense staining for FGF-2 on the cell surface (or pericellular space) of chondrocytes in the resting and proliferating cartilage zones (C). FGF-2 is also noted in both the cell membrane of hypertrophic chondrocytes and the extracellular matrix of the lower hypertrophic and calcified cartilage zones (D). Osteoblasts at the bone-forming front are indicated by arrowheads. Bars, 50 μm.
yseal cartilage cells isolated from an ACG-1B fetus completely lacked sulfate transport activity (7). The cells produced severely undersulfated proteoglycans in culture. Rossi et al. have shown that this particular patient bore a mutation in the coding region of the DTDST gene causing one amino acid substitution in the transport protein (7). These results suggested that the DTDST sulfate transporter is a major sulfate transporter in chondrocytes. The tissue distribution of DTDST mRNA (Fig. 7) was also compatible with this notion.

Achondrogenesis type IB is a lethal chondrodysplasia characterized by severe underdevelopment of the skeleton and extreme micromelia. Histologic sections of the patient's epiphyseal cartilage showed collagenous rings surrounding chondrocytes with coarse collagen fibrils (7). Skeletal underdevelopment and micromelia in the ACG-1B patients imply a causative role of undersulfated proteoglycans during development.

Fibroblast growth factors are known to be a potent mitogen for chondrocytes (28) and participate in limb morphogenesis (29). Sulfated proteoglycans are also important for transmission of growth signals (30). We prepared cultures of rat growth plate chondrocytes associated with undersulfated proteoglycans by treatment of the cells with sodium chloride in sulfate-free medium (Table II). Our sulfate-free culture conditions did not affect the rate of proteoglycan synthesis of the cells as estimated by [3H]glucosamine incorporation, but [35S]sulfate incorporation into proteoglycans was greatly reduced. Under the normal culture conditions, FGF-2 clearly stimulated DNA synthesis in quiescent chondrocytes in confluency. Under the normal culture conditions, FGF-2 clearly stimulated DNA synthesis in quiescent chondrocytes in confluence. FGF-2 in the severely undersulfated culture, depending on the dose of chlorate (Fig. 8). It is known that extracellular space in cartilage has a highly organized structure due to the complex territorial matrix (33, 34). As shown in Fig. 9, territorial matrix (33, 34). As shown in Fig. 9, the distribution pattern of FGF in cartilage. Isolated chondrocytes are capable of reconstructing these extracellular structures in vitro (33). We speculate that polyanionic heparin molecules could not be readily accessible to the signal transmission machinery in the pericellular microenvironment through the barrier of abundant extracellular matrix in the chondrocyte cultures. Thus, unlike endothelial cells, the proper organization of sulfated proteoglycans in the cartilage matrix may be an important requirement for chondrocytes to grow. The DTDST-mediated sulfate transport system in chondrocytes may be of crucial importance to endochondral bone development.

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