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Cloning and Characterization of the 5′-Flanking Region of the Mouse Diastrophic Dysplasia Sulfate Transporter Gene

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Dyastrophic dysplasia sulfate transporter (DTDST) plays an important role in proteoglycan synthesis in the extracellular matrix of bone and cartilage. Recently, we found that the mouse DTDST gene was induced in pluripotent C3H10T1/2 cells during differentiation by bone morphogenetic protein-2 (BMP-2). To clarify the transcriptional regulation of the DTDST gene, we have cloned the 5′-flanking region of the mouse DTDST gene by the PCR based gene walking method. Sequence analysis revealed the presence of the TATA box followed by GC rich sequences containing two Sp-1 binding sites and a CBFA1 binding site. Transient transfection assays demonstrated that the basal transcriptional activity in osteoblastic MC3T3-E1 cells was mainly present between −309 and −275 bp upstream of the transcription start site (Segment −309/−275) which contained the consensus sequence for the xenobiotic-responsible element (XRE). Nuclear proteins from MC3T3-E1 cells and C3H10T1/2 cells could bind to this short segment *in vitro.* BMP-2 increased the promoter activity as well as the nuclear protein binding to the sequence in C3H10T1/2 cells. The present data suggest that the DTDST gene expression in osteoblasts and differentiating precursor cells to osteoblast/chondrocyte lineage would be mainly regulated by undetermined XRE binding transcription factors. © 1997 Academic Press

The extracellular matrix of bone and cartilage contains a large amount of sulfate, which is incorporated into glycosaminoglycan chains of proteoglycans (1). Proteoglycans produced by osteoblasts (2-4), possess a variety of functions to actively regulate bone metabolism (5,6). Hästbacha *et al.* reported that diastrophic dysplasia, a congenital skeletal dysplasia was caused by genetic mutations of the sulfate transporter which they named distrophic dysplasia sulfate transporter (DTDST) (7). The defect of DTDST gene is supposed to cause undersulfation of proteoglycans in the extracelluar matrix of bone and cartilage.

Bone morphogenetic protein-2 (BMP-2), a member of transforming growth factor-β (TGF-β) superfamily was reported to induce osteoblastic and chondrocytic phenotypes in pluripotent immature fibroblastic C3H10T1/2 cells (8). Using this model, we previously cloned the mouse homolog of DTDST cDNA as a gene induced during differentiation by BMP-2. The expression of the mouse DTDST gene was relatively abundant in osteoblastic MC3T3-E1 cells but very weak in C3H10T1/2 cells and its expression in C3H10T1/2 cells was induced by BMP-2 (9). Clarifying the regulatory mechanism of the DTDST gene expression would give a clue for better understanding of both the differentiation of osteoblast/chondorocyte lineage and BMP-2 action on their progenitor cells. For this aim, we have cloned and characterized the 5′-flanking region of the mouse DTDST gene.

MATERIALS AND METHODS

Materials and cell culture. MC3T3-E1 cells, established as an osteoblastic cell line from normal newborn mouse calvaria, were

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The nucleotide sequence data reported in this paper will appear in the GSDB, DDBJ, EMBL, and NCBI nucleotide sequence databases under Accession No. AB004666.

Abbreviations used: BMP-2, bone morphogenetic protein-2; bHLH, basic-helix-loop-helix; CBFA1, core binding factor α 1; EMSA, electrophoresis mobility shift assay; NF-1, nuclear factor-1; PCR, polymerase chain reaction; RACE, rapid amplifications of cDNA ends; Sp-1, specificity protein 1; TGF-β, transforming growth factor-β; XRE, xenobiotic responsible element.

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kindly gifted from Dr. H. Kodama (Ohu University, Koriyama, Japan). Mouse embryonic fibroblastic C3H10T1/2 cells were supplied by Riken Cell Bank (Wako, Japan). MC3T3-E1 cells and C3H10T1/2 cells were maintained in alpha modified minimal essential medium Eagle (α-MEM), basal medium Eagle (BME), respectively, supplemented with 10% fetal calf serum (FCS) in a humidified atmosphere of 5% CO_2 in air at 37°C.

Recombinant human bone morphogenetic protein-2 (BMP-2) was a kind gift from Dr. Wozney (Genetics Institute, MA) (10).

Cloning of the 5′-flanking region of the mouse DTDST gene. We have cloned the 5′-flanking region of the mouse DTDST gene using the PCR based method for DNA walking (LA PCR *in vitro* cloning Kit, Takara Shuzo, Shiga, Japan). Mouse genomic DNA was extracted from MC3T3-E1 cells using Genomix (Talent, Trieste Italy), digested with restriction enzymes (*Bam* HI, *Eco* RI, *Hind* III, *Pst* I, *Xba* I and *Sal* I) and ligated to cassette adaptors with the cohesive ends. The 5′-flanking region of the mouse DTDST gene was amplified from the above generated genomic libraries with two rounds of consecutive PCR using the common primers (C1 and C2) corresponding to the cassette sequence and the gene specific 3′-end primers (S1 5′-TACACGGGCTCC-GCGGCGCGTCCT-3′, nt 41-64 and S2 5′-TCC-CGCAAGACTGGGCTGCGGGAT-3′, nt 17-41). The gene specific oligonucleotide primers were synthesized based on the cDNA sequence that we previously determined (9) (DDBJ accession number: D42049) The first PCR amplification was performed using S1 and C1. The reaction was incubated at 95°C for 10 min followed by 30 cycles, each at 95°C/30 s and 70°C/5 min, with a final elongation step at 72°C/8 min. The PCR products were appropriately diluted, then subjected to the secondary PCR using the internal primers, S2 and C2. PCR products were subcloned into pGEM-T Easy vector (Promega, Madison, WI), and were sequenced with a DNA sequencer (373A, Perkin-Elmer, CA).

Determination of the transcription start site. The 5′ end of the mouse DTDST cDNA was determined by the rapid amplification of cDNA ends (RACE) method (5′-AmpliFINDER RACE Kit, Clontech, Palo Alto, CA). Briefly, an antisense DTDST specific primer, 5′-CAG-CACATGTCCTGGAATTGA-3′ (nt +127 to +147) was used to extend towards the 5′-end with poly A (+) RNA from MC3T3-E1 cells with AMV reverse transcriptase. The resulted single strand cDNA was ligated with a single strand anchor adaptor by T4 RNA ligase and amplified with another gene specific antisense primer, 5′-ATTTTA-CGCCTTAACGCTCCG-3′ (nt +73 to +93) and anchor adaptor specific primer. The amplified fragment were cloned into the pGEM-T vector (Promega) and sequenced.

DNA transfection and luciferase assay. A mouse DTDST 5′-flanking region fragment (−852 to +41) was subcloned into a reporter plasmid, pGL3 basic vector (Promega) to be fused to the luciferase gene. Deletion mutant plasmids were generated by taking advantage of the internal restriction enzyme sites (−401Luc, −309Luc, −275Luc and −172Luc) and by PCR (−85Luc) using a high fidelity thermostable DNA polymerase (LA PCR kit ver.2, Takara Shuzo). Cells were transfected by the liposome mediated method (LipofectAce, GIBCO/BRL). After trasfection, the cell were grown in a normal growth medium for 24 h with or without 100 ng/ml of BMP-2. Luciferase activity was determined in Luminophotometer TD-4000 (NDS, Tokyo Japan) using Luciferease assay system (Promega).

Nuclear extraction and electrophoresis mobility shift assay (EMSA). Nuclear extract from cell lines was prepared by the method previously described (11). Cells reaching confluence with or without stimulation with 100 ng/ml BMP-2 for 24h were used. All steps were performed at 4 °C. Cells were washed with phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM sodium phosphate [pH 7.2]) twice, resuspended in a buffer A (60 mM KCl, 15 mM NaCl, 0.15 M spermine, 0.5 mM spermidine, 15 mM HEPES [pH 7.8], 14 mM 2-mercaptoethanol, 0.5 mM PMSF, 0.5 mM DTT) containing 0.3 M sucrose and the nuclei were isolated with 10 strokes in a Dounce homogenizer. The homogenate was layered on a buffer A containing 0.9 M sucrose and centrifuged at 2,000g for 15 min. The pellet was subjected to the same procedure again. The nuclei were lysed by incubating in a buffer (0.4 M KCl, 0.5 mM EDTA, 20 mM Tris-HCl [pH 7.9], 25 mM NaCl, 0.5 mM PMSF, 0.5 mM DTT) on ice for 30 min, microcentrifuged at the maximal speed for 10 min. The supernatant containing nuclear proteins was stored at −80°C until use. Radiolabeled double stranded DNA probes were generated as follows: Complementary oligonucleotides with an extra dGTP at the each 5′ end were annealed by heating to 95 °C for 5 min followed by cooling slowly to room temperature in the presence of 50 mM Tris-HCl (pH 8.0) and 10 mM $MgCl_2$, then the protruded 5′ ends were filled with [α-32P] dCTP (Amersham, UK) using Klenow fragment. The −309 to −275 fragment generated by digesting with *Bam* HI and *Sac* I was similarly labeled with [α-32P] dCTP and cold dNTPs. Approximately 20,000 cpm of the probe was incubated with 5 μg of crude nuclear extract for 20 min at 30 °C in 15 μl reaction mixture containing 10 mM Tris-HCl (pH 7.5), 50 mM NaCl, 1 mM EDTA, 1 mM DTT, 25% glycerol, 2 mg of poly (dI-dC) (Pharmacia, NJ). For competition experiments, competitor DNA was incubated with the nuclear extract for 20 min before the addition of the probe. The DNA-protein complexes were fractionated on a non-denaturing 4.5% polyacrylamide gel at 11V/cm for 1.5 h in 0.25 × TBE (1 × TBE; 89 mM Tris-HCl, 89 mM boric acid, 8 mM EDTA). After electrophoresis, the gels were vacuum-dried and exposed to x-ray film at −80 °C for 48 h.

RESULTS

Cloning of the 5′-flanking region of the mouse DTDST gene. We obtained a PCR product expanding to the first *Hind* III site in the upstream of the 5′-end of mouse DTDST cDNA (Fig. 1). The 5′-end of cDNA of the DTDST gene was determined by the RACE method. The obtained PCR product by RACE was single-banded on an agarose gel electrophoresis (data not shown), which was subcloned into plasmids and sequenced. Based on the 5′-end sequence, we determined the transcription start site at 245 bp upstream from the ATG start codon as shown in Fig. 1. A presumed TATA box was located at 34 bp upstream of the transcription start site followed by a GC rich region including two GGG-CGGG sequences at position −54 and −197, which constituted a consensus binding site for the transcription factor Sp-1 (12). A CAAT box for NF-1 binding was seen at position −166 (13,14). The consensus binding sequence for CBFA1 which was recently reported as the master gene to determine ostoblast differentiation (15-17) was located at position −89. E-boxes, CANNTG were seen at position −819, −620, −569 and −146.

Functional analysis of the DTDST 5′-flanking region. The 852 bp fragment and constructed deletions of the fragment were transfected into osteoblastic MC3T3-E1 cells. The transcriptional activity was markedly different between −309Luc and −275Luc (Fig. 2B). Thus, the region of −309 to −275 (Segment −309/−275) was considered to be important in the basal promoter activity. The promoter activity was also determined in C3H10T1/2 cells. Although the expression of DTDST gene is weak in C3H10T1/2 (9), the −852Luc and other deletion constructs had promoter activities at the basal level in C3H10T/2 cells de-

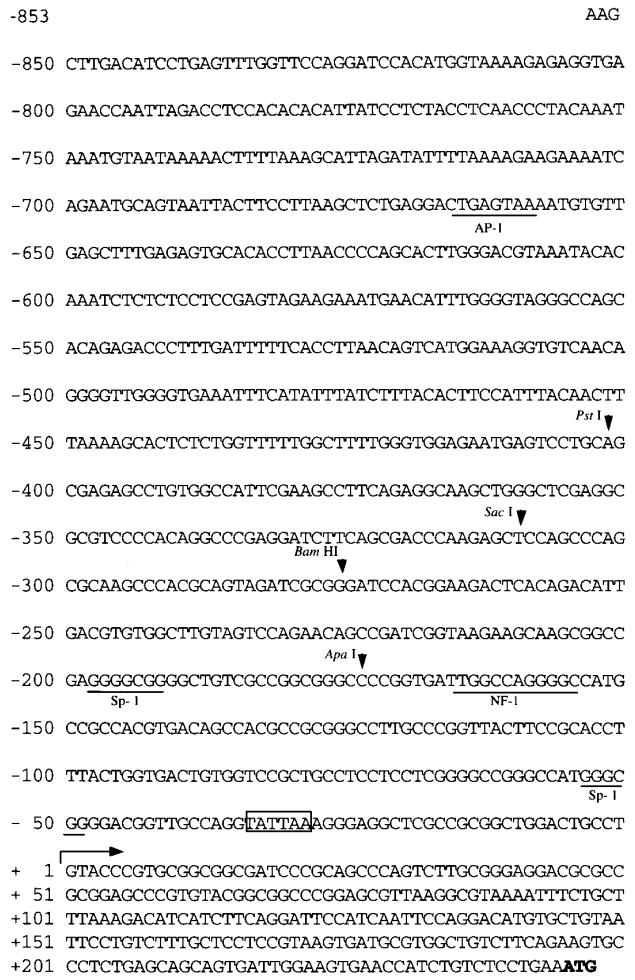

FIG. 1. Nucleotide sequence of the 5′-flanking region of the mouse DTDST gene. The 852 bp fragment was obtained by PCR amplification of a mouse genomic library and sequenced. The transcription start site, as indicated by +1 and an arrow, was determined by the 5′-RACE method using poly A+ RNA from mouse osteoblastic MC3T3-E1 cells. The start codon for the gene is shown in bold characters. The TATA box is boxed. The binding motives for Sp-1, NF-1, and AP-1 are underlined. The restriction enzyme sites used for deletion mutants were indicated by arrowheads.

pending on the length of the promoter sequence as seen in MC3T3-E1 cells (Fig. 2C). Moreover, BMP-2 enhanced the promoter activity in C3H10T1/2 cells by up to 50 % in −309Luc and −275Luc (Fig. 2C).

Nuclear protein binding to the DTDST gene promoter region. An EMSA was performed to examine whether Segment −309/−275 could bind nuclear proteins. Shifted bands were observed when the radiolabeled probe was incubated with crude nuclear extract from MC3T3-E1 cells (Fig. 3A). Since Segment −309/−275 contains 6 bp-tandem repeat sequences with a 5 bp-insertion (nt −307 to −291) and a xenobiotic-responsible element (XRE) consensus sequence, CACGCW (18) (nt −290 to −285) as shown in Fig. 2A, a competition analysis was performed using three DNA fragments as competitors; Segment −308/−288 containing the 6 bp-direct repeat sequence but not the whole XRE site, Segment −290/−272 covering the rest of Segment −309/−275 but also lacking the complete XRE site and Segment −309/−275 itself. As shown in Figure 3-A, neither of the competitors, Segment −308/−288 nor Segment −290/−272 inhibited the binding of Segment −309/−275 to the nuclear extract, but Segment −309/−275 did. When each of Segment −308/−288 and Segment −290/−272 was radiolabeled and their binding to the nuclear proteins was examined, no band shift was observed (data not shown).

Next, we examined the effect of BMP-2 on the nuclear protein binding to Segment −309/−275 in C3H10T1/2 cells. The nuclear protein binding to the segment at the basal level was weak but the binding was increased by treating cells with 100 ng/ml BMP-2 for 24h (Fig. 3B).

Since Sp-1 is a ubiquitous protein that promote basal transcription in various genes (19), we examined the nuclear protein binding to the down stream Sp-1 site, 5′-CCATGGGCGGGGAC-3′ (nt −58 to −44). The radiolabeled probe however, could not bind any nuclear proteins either in MC3T3-E1 cells or C3H10T1/2 cells with or without BMP-2 stimulation (data not shown).

DISCUSSION

The DTDST gene, identified as the gene responsible for diastrophic dysplasia, is considered to play a crucial role in proteoglycan synthesis (7). We previously reported that the expression of the DTDST gene was one of the features of osteoblastic cells and that was induced during their differentiation by bone morphogenetic protein-2 (BMP-2), a member of TGF-β superfamily (9). The present study was intended to clarify the mechanisms of the transcriptional regulation of the DTDST gene in osteoblastic cells and their precursor cells in differentiation by BMP-2.

The 5′-flanking region of the DTDST gene contained several motives commonly seen in the promoter regions of various gene: Sp-1 site and CAAT box are considered to bind ubiquitously present transcription factors to promote transcription (12-14). The consensus sequence for CBFA1 was also located. A series of recent reports concerning CBFA1 indicates that CBFA1 is the key molecule to determine osteoblast differentiation (15-17): CBFA1 knock-out mouse could not develop bone tissue and lacked of osteoblasts. Chondrocytes were preserved but enchondrial ossification was mostly depressed. CBFA1 therefore acts in the osteoblast precursor cells to induce differentiation into osteoblasts but not chondrocytes. Since chondrocytes uptake sulfate to synthesize proteoglycans as well as osteoblasts, DTDST gene expression would be regulated by other *trans-*

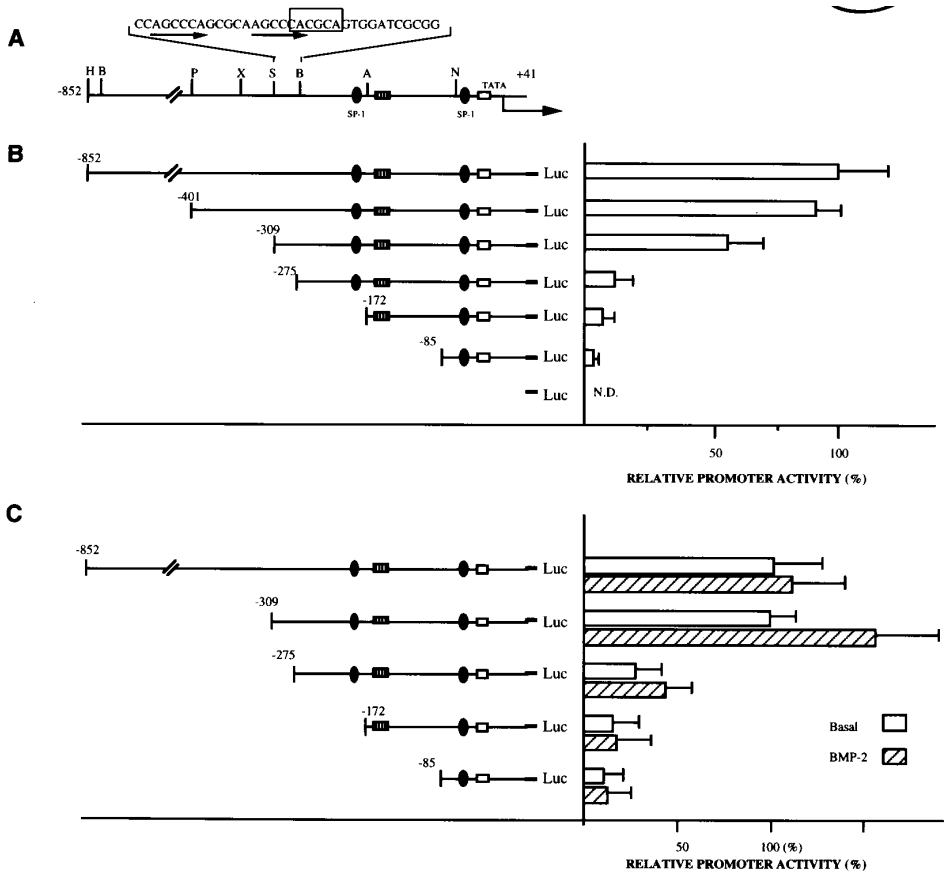

FIG. 2. Promoter activity of the 5′-flanking sequence of the mouse DTDST gene. (**A**) Restriction map of the 5′-flanking sequence. The transcription start site is indicated by an arrow. The positions of the sites for *Hind* III (H), *Bam* HI (B), *Pst* I (P), *Xho* I (X), *Sac* I (S), *Apa* I (A), and *Nco* I (N) are shown. The locations of the consensus sequences for the binding of transcription factors and TATA box binding proteins Sp-1 and NF-1 (CAAT box) are indicated as an open rectangle, semi-closed oval, and a striped rectangle, respectively. The nucleotide sequence of Segment −309/275 is shown. A tandem 6 bp-repeat is indicated by arrows. A core sequence for XRE is boxed. (**B**) Schematic representation of the plasmid constructs containing various lengths of the 5′-flanking region of the mouse DTDST gene. Promoter activity in MC3T3-E1 cells was measured as light intensity/μg of total protein, and data are expressed as relative activity with respect to the mean activity of −852Luc, which has given a value of 100%. Activity values represent the mean ± S.E.M. of three independent experiments. (**C**) Promoter activity in C3H10T1/2 cells treated with or without BMP-2. The representations are similar to B.

acting factors that appear in an earlier developmental stage than CBFA1. In this study, however, his sequence located at −89 did not seem to contribute much to the DTDST transcription. Similarly, Sp-1 site reported as the effective *cis*-acting element for other osteoblastic phenotypes, α1(I) collagen (20) and α2(I) collagen (21) gene did not have a strong promoter activity. From the results of the luciferase assay experiments, the main basal promoter activity would be existent between position −309 and −275 (Segment −309/−275). The segment contained 6 bp-direct repeat sequences and a consensus sequence for XRE. The electrophoresis mobility shift assay using two kinds of competitor DNA showed that the Segment −309/−275 binding nuclear proteins seemed to recognize a sequence standing on both Segment −308/−288 and Segment −290/−272. That is, the region containing the XRE site but not the 6 bp-direct repeat sequence might bind the nuclear proteins. XRE was identified as the *cis*-acting element that responded to xenobiotic stimulation in cytochrome p-450c gene promoter (18) and is considered to bind the nuclear proteins such as aryl hydrocarbon receptor (AHR) and aryl hydrocarbon receptor nuclear translocater (ARNT) (22). These proteins both contain bHLH motives, though most bHLH-bearing transcription factors recognize E-box, CANNTG, which seems not so important for the promoter activity of the 5′-flanking region of the DTDST gene in this study. Tamura *et al.* reported the importance of bHLH transcription factors in the transcription of the rat osteocalcin gene (23). Thus the XRE site in Segment −309/−275 might bind unknown bHLH type transcritption factor characteristic to osteoblasts. Because XRE is not found in the promoter sequences of osteocalcin (24), alkaline phosphatase

Under such circumstances, the transfected luciferase-chimera gene might bind *trans*-acting factors in the cytoplasm that are essentially inactive before its delivery to the nucleus. Genomic DNA is generally has many modifications such as nucleic acid-methylation and interaction between nuclear matrix proteins, which restrict the binding to

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