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ARTICLES

Identification of DERMO-1 as a Member of Helix-Loop-Helix Type Transcription Factors Expressed in Osteoblastic Cells

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Abstract Several members of the basic helix-loop-helix (bHLH) type of transcription factors have now been reported, and almost every member of this class has been implicated in transcriptional regulation in cell type determination and differentiation. Previously, we reported that dominant negative HLH proteins are involved in osteoblastic phenotype expression, such as osteocalcin, and hence differentiation (Tamura and Noda [1994] J. Cell Biol. 126:773–782). In this work, we used degenerate PCR cloning in order to identify cDNA clones encoding bHLH proteins expressed in osteoblastic osteosarcoma ROS17/2.8 cells. Sequence analyses of the 47 clones revealed that 11 clones encoded products with a characteristic motif of the bHLH transcription factor family. Of these clones, sequences in the amplified region of seven clones were homologous to the mouse twist, and three clones were homologous to the mouse twist–related HLH protein, Dermo-1. To confirm Dermo-1 mRNA expression in osteoblastic cells, we performed reverse transcription polymerase chain reaction (RT-PCR) analysis using mRNA from ROS17/2.8 cells and MC3T3-E1 cells by Dermo-1 specific primers and Northern blot analysis. These analyses demonstrated that Dermo-1 mRNA was expressed in these osteoblast-like cell lines. Nucleotide sequence analysis of the partial rat Dermo-1 cDNA cloned from ROS17/2.8 library revealed that it has the highest degree of homology with the mouse Dermo-1 cDNA, and the partial amino acid sequence deduced from the obtained rat Dermo-1 was identical with the corresponding region of the mouse Dermo-1 amino acid sequence. To further examine the role of Dermo-1 in the regulation of osteoblastic differentiation, we examined mRNA levels of Dermo-1 and twist in C3H10T1/2 cells treated with recombinant human bone morphogenetic protein (rhBMP)-2. Using the RT-PCR method, the mRNA levels of Dermo-1 and twist were found to be decreased by the treatment with rhBMP-2 in C3H10T1/2 cells. We also observed that the mRNA level of Dermo-1 was decreased about fourfold by the treatment with rhBMP-2 in C3H10T1/2 cells by Northern blot analysis. Moreover, Dermo-1 mRNA was detected at lower levels in 21-day-old differentiated MC3T3-E1 cells compared with 3-day-old undifferentiated MC3T3-E1 cells. These results suggested that Dermo-1 could be involved in the osteoblastic differentiation in a negative manner. J. Cell. Biochem. 72:167–176, 1999.

Key words: dermo-1; osteoblast; transcription factor

Elucidation of molecular mechanisms controlling differentiation of osteoblasts is one of the major subjects in bone biology. Differentiation of cells is controlled at the level of transcription by various classes of transcription factors that have been identified through biochemical and genetic means. Extracellular signals including hormones, growth factors, cytokines, or extracellular matrix components as well as their intracellular mediators regulate cell differentiation or expression of phenotypes by modulating the activities of transcription factors involved in the expression of respective target genes.

Helix-loop-helix (HLH) family proteins have been implicated in transcriptional regulation of cell type determination and differentiation, and nearly 40 members have been reported to belong to this class. These members of basic HLH (bHLH) family proteins bind to a core sequence, CANNTG, which is referred to as an E-box motif, initially identified in the immunoglobulin heavy chain gene enhancer. Since then, sev-

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eral other functional E-box sites have been identified in the 5′-flanking regions of genes that are expressed in a cell type–specific manner. Basic HLH proteins can be divided into at least two classes: those that are expressed in a broad spectrum of tissues and cell types (class A) and those expressed in a tissue-restricted fashion (class B) [for review, see Murre and Baltimore, 1992]. Class A bHLH family members are the E2A gene products (E12 and E47) [Henthorn et al., 1990], HEB (BETA1 and HTF4), and ITF2. Class B members are MyoD [Lassar et al., 1989], myogenin [Wright et al., 1989], Myf-5 [Braun et al., 1989], MRF4 [Rhodes and Konieczny, 1989], acute-scute [Villares and Cabrera, 1987], MASH-1, MASH-2 [Johnson et al., 1990], SCL [Aplan et al., 1992], lyl-1 [Mellentin et al., 1989], neuro-D [Lee et al., 1995], twist [Thisse et al., 1988], Dermo-1 [Li et al., 1995], and so on. bHLH proteins bind to the CANNTG motif as either homo- or hetero-dimers [Murre et al., 1989]. The best-characterized members of the bHLH transcription factor family involved in mammalian cell differentiation are the myogenic transcription factors including MyoD, myogenin, Myf-5, and MRF4. Expression of these proteins has been implicated in both cell type determination and differentiation in skeletal muscle cells. Forced expression of these proteins converts non-myoblastic cells such as fibroblasts into myocytes [Davis et al., 1987; Weintraub et al., 1989]. "Loss of function" experiment with myogenin gene is perinatally lethal because of the major defects in skeletal muscle as demonstrated using homologous recombination techniques [Hasty et al., 1993; Nabeshima et al., 1993]. In central and peripheral nervous systems, MASH proteins (mammalian homologues of achaete-scute gene) have been suggested to play critical roles in neurogenesis [Johnson et al., 1990]. The other bHLH protein, SCL, is expressed during early hematopoiesis and possesses a differentiation-related DNA binding motif [Visvader and Begley, 1991]. On the other hand, dominant negative HLH proteins such as Id-1, Id-2, HLH462, and emc, which lack the basic DNA-binding domain, have been shown to heterodimerize with bHLH proteins to make them unable to bind to DNA [Benezra et al., 1990; Biggs et al., 1992; Chiristy et al., 1991; Sun et al., 1991]. The observation that myeloid differentiation can be inhibited by overexpression of Id-1 implies an important role for the bHLH factors in this lineage of cells as well [Kreider et al., 1992].

In our previous work, the E-box sequence 1 (OCE1) in the rat osteocalcin gene was implicated in HLH factor-mediated expression of the gene in osteoblasts by deletion and site-directed mutagenesis analyses of the promoter region. Furthermore, electrophoresis mobility shift assay (EMSA) experiments using this OCE1 sequence indicated the formation of osteoblast-specific protein/DNA complexes, and this binding activity was induced in C3H10T1/2 fibroblasts by recombinant human bone morphogenetic protein 2 (rhBMP-2) treatment, which also induces expression of osteocalcin gene in these cells. Thus, a previous study presented evidence that the E-box sequence, OCE1, and transcription factors interacting with this motif are involved in osteoblast-specific osteocalcin gene transcription [Tamura and Noda, 1994].

However, the nature of the bHLH proteins involved in the regulation of osteoblastic differentiation has not been clarified. In this study, to identify cDNA clones encoding bHLH proteins expressed in osteoblasts, we designed partially degenerate oligonucleotides by taking advantage of the conserved amino acid sequences located in the basic and the second helix regions of class B bHLH proteins and performed polymerase chain reaction (PCR) cloning. Furthermore, we examined the expression of these clones in osteoblastic cells and investigated the possible involvement of these clones in regulation of osteoblastic function.

MATERIALS AND METHODS

Cell Cultures

Rat osteoblastic osteosarcoma ROS17/2.8 cells [Majeska et al., 1980] were kindly provided by Dr. G. Rodan (Merck Research Laboratories, West Point, PA) and were maintained in modified F-12 medium (Gibco Laboratories, Grand Island, NY) supplemented with 5% fetal bovine serum (FBS) (Gibco Laboratories). C3H10T1/2 fibroblasts were obtained from RIKEN Cell Bank (Tsukuba, Japan) [Reznikoff et al., 1973]. C3H10T1/2 cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Gibco Laboratories) supplemented with 5% FBS. MC3T3-E1 cells were provided by Dr. Kodama (Ohu University, Koriyama, Japan) [Kodama et al., 1981] and were grown in alpha modified Eagle's medium (α-MEM) (Gibco Laboratories) supplemented with 5% FBS. To induce differentiation

of MC3T3-E1 cells, cultures were grown in α-MEM supplemented with 5% FBS for up to 21 days. Cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Protein

Recombinant human BMP-2 [Wang et al., 1990] was kindly provided by Dr. J. Wozney (Genetics Institute, Cambridge, MA).

RNA Isolation and First-Strand cDNA Synthesis

Total cellular RNA was prepared according to the acid guanidium thiocyanate-phenol-chloroform method as described by Chomczynski and Sacchi [1987]. cDNA was prepared from 5 µg of total RNA of ROS17/2.8 cells, MC3T3-E1 cells, and 10T1/2 cells using First Strand cDNA Synthesis kit (Pharmacia Co., Gaithersburg, MD) according to the manufacturer's instructions.

Degenerate PCR

The entire cDNA preparation was used for PCR under the following conditions: 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 15 mM $MgCl_2$, 1 µg of each primer, and 2.5 U of AmpliTaq DNA polymerase (Perkin Elmer Co.) in a 100-ml final volume. The primer sequences were

5′-CAGCATGC(CA)GGGAGCGC(CA)(GA)(GC)(CA)G-3′

and 5′-CCAAGCTTCAGG(CA)(TAC)(GC)T(GC)GATGTA-3′

with the *Sph*I and *Hind*III cloning sites underlined (Fig. 1). The cDNA was amplified with PCR profile of 95°C for 50 sec, 48°C for 1 min 30 sec, 72°C for 1 min repeated for 3 cycles, then 94°C for 50 sec, 50°C for 1 min, 72°C for 1 min repeated for 25 cycles, followed by 7 min at 72°C. The amplified DNA was fractionated by polyacrylamide gel electrophoresis, and bands corresponding to the predicted size of 148 bp were recovered from the gel using the QIAEXII DNA extraction kit (Qiagen Co., Chatsworth, CA), then reamplified under the same PCR condition. The bands corresponding to the predicted size of 148 bp were recovered from the gel and subcloned into pCRScript AmpSK(+) using pCR-ScriptAmpSK(+) cloning kit (Stratagene Co., La Jolla, CA).

Sequencing

Sequencing was carried out with Cycle Sequencing FS ready reaction kits (Applied Biosystems Co., Foster City, CA) and ABI373 DNA sequencer according to the manufacturer's instructions.

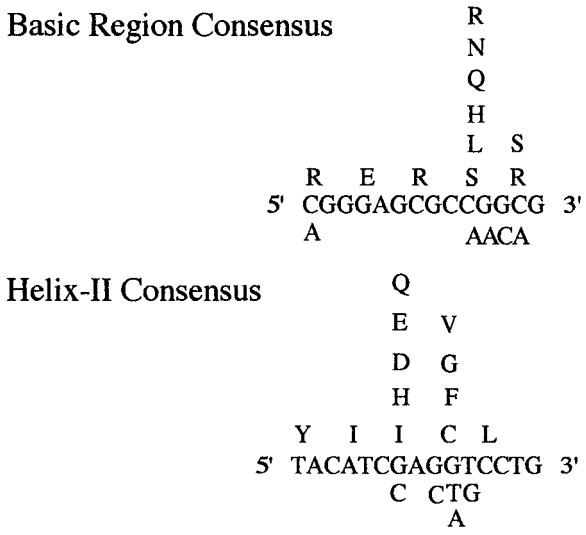

Fig. 1. Primer design strategy for PCR amplification of the basic HLH type of transcription factor in ROS 17/2.8 cells. The location of the upstream (top) and downstream (bottom) primer binding sites are shown in relation to the basic HLH domain. The possible encoded amino acid sequences are shown above.

RT-PCR

Total RNAs from ROS17/2.8 cells, C3H10T1/2 cells, and MC3T3-E1 cells were used in quantitative PCR designed to work the linear (exponential) range of amplification. The quantity of cDNA synthesized in each RT reaction was first normalized by means of PCR amplification using mouse beta-actin primers (sequences 5′-TAAAGACCTCTATGCCAAACAC and 5′-CTCCTGCTTGCTGATCCACAT; 950 through 970 and 1143–1163, respectively) [Tokunaga et al., 1986]. Identical amounts of cDNA from each RT reaction were used in PCR using Dermo-1 and twist-specific primers as described below. PCR condition was 95°C for 2 min, then 25 cycles of 50 sec at 94°C, 1 min at 57°C, 1 min at 72°C, and a final elongation of 7 min at 72°C. Amplification products were separated though 1.2% agarose gels and quantitated by autodensitometry (Bio-Profile, Vilber Loumat Co., Paris, France). For sequencing of rat Dermo-1 cDNA, we subcloned the amplification product (DEL5-DEL4) of ROS17/2.8 cells into pCR-Scripts vector (Stratagene Co.). For Northern blot analysis, the amplification products (DEL2-DEL4) of

C3H10T1/2 cells were subcloned into pCR-Scripts vector.

DEL2 5′-ATGGAGGAGGGCTCCAGC-3′
DEL4 5′-CTAGTGGGAGGCGGACAT-3′
DEL5 5′-TGGAGAGGCAGCCCAAGCGC-3′
TWI3 5′-CTAGTGGGACGCGGACATGG-3′
TWI4 5′-GCCCGGCCCAGGGCAAGCGC-3′

Northern Blot Analysis

Thirty micrograms of total RNA was fractionated by electrophoresis in 1% agarose gels containing 0.66 M formaldehyde and were transferred to a nylon filter (Nytran; Schleicher & Schuell, Keene, NH). Filters were UV crosslinked by Stratalinker (Stratagene) and prehybridized in QuickHyb hybridization solution (Stratagene) at 68°C for 10 min. A cDNA fragment for human glyceraldehyde-3-phosphate-dehydrogenase (GAPDH) was provided by S. Sakiyama (Chiba Cancer Center Research Institute and Hospital, Chiba, Japan). A cDNA fragment for mouse Dermo-1 was excised with *Ban*II and *Eco*RII, and cDNA fragment for GAPDH was by *Eco*RI. They were separated from vector DNA by SeaPlaque (FMC Co., Philadelphia, PA) gel electrophoresis and then radiolabeled by random primer method using Prime-It II labeling kit (Stratagene) and [α-^{32}P]dCTP. Specific radioactivity of the radiolabelled cDNA was 1–5 $\times$ 10^8 cpm/mg DNA. The hybridization was carried out at 63°C for 1 h in QuickHyb hybridization solution and 0.5 mg/ml salmon sperm DNA with 1 $\times$ 10^6 cpm/ml of ^{32}P-labeled cDNA probes. Filters were washed in 0.1 $\times$ SSC (1 $\times$ SSC = 150 mM NaCl, 15 mM trisodium citrate, pH 7.0) containing 0.1% sodium dodecyl sulfate (SDS) for 30 min at 60°C and were exposed to Molecular Dynamics Storage Phosphor Screen (Molecular Dynamics, Sunnyvale, CA). Bands were quantitated with a Molecular Imager (Molecular Dynamics). Equal loading of RNA in each lane was checked by hybridization with ^{32}P-labeled GAPDH cDNA.

RESULTS

Basic HLH proteins are involved in cell type determination and differentiation through their transcriptional activation of a repertoire of subordinate genes. We designed partially degenerate oligonucleotides as primers to identify cDNA clones encoding bHLH proteins expressed in osteoblasts (Fig. 1). The upstream primer was designed to correspond to the RER-R consensus sequence found in the basic region of all bHLH transcription factors. The downstream primer was designed to be specific for class B bHLH transcription factors [Peyton et al., 1996]. These oligonucleotides were used in PCRs to amplify cDNAs prepared from the mRNA of ROS17/2.8 cells as described in Materials and Methods. Amplified products were fractionated by polyacrylamide gel electrophoresis. Sequencing of the 47 clones was done, and then similarities of their DNA sequence were searched by BLAST program of Genbank. These analyses revealed that about ¼ of the clones (11 clones) encoded products with a characteristic motif of bHLH transcription factor family. Of these clones, DNA sequences in the amplified region of the seven clones were homologous to the mouse twist [Wolf et al., 1991], and the three clones were homologous to the mouse twist–related HLH protein, Dermo-1. According to the BLAST search of Genbank, other clones (36 clones) did not encode bHLH protein motif.

To confirm the Dermo-1 mRNA expression in osteoblastic cells, we performed reverse transcription (RT)-PCR analysis using total RNA from ROS17/2.8 cells and MC3T3-E1 cells by Dermo-1 specific primers: DEL-4 and DEL-5. The predicted size of amplified products using DEL-4 and DEL-5 primers is 418 bp. Figure 2 indicated that Dermo-1 mRNA was expressed

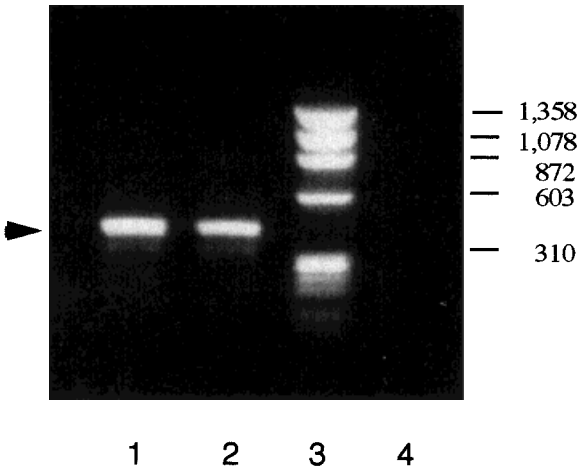

Fig. 2. Dermo-1 mRNA expression in ROS17/2.8 cells and MC3T3-E1 cells. Total RNAs from ROS17/2.8 cells and MC3T3-E1 cells were reverse transcribed and PCR-amplified with Dermo-1 specific primers, DEL-4 and DEL-5, for 25 cycles. DNA fractionated on the gel was stained with ethidium bromide. **Lane 1:** MC3T3-E1 cells. **Lane 2:** ROS17/2.8 cells. **Lane 3:** phiX-HaeIII DNA size marker. The size of the amplified band of Dermo-1 is 418 bp. No product was observed in the absence of reverse transcriptase in ROS17/2.8 cells (**lane 4).**

in the two osteoblast-like cell lines, ROS17/2.8 cells and MC3T3-E1 cells. Then, we subcloned this amplification product (DEL5-DEL4) of ROS17/2.8 cells into pCR-Scripts vector and sequenced this plasmid. The cDNA sequence of rat Dermo-1 and its sequence similarity to mouse Dermo-1 are shown in Figure 3A. The predicted amino acid sequence of rat Dermo-1 from the cDNA sequence and sequence similarity to amino acid sequence of mouse Dermo-1 are shown in Figure 3B. The cDNA sequence of Dermo-1 derived from MC3T3-E1 cells was also identical with already reported mouse Dermo-1 cDNA sequence [Li et al., 1995] (data not shown).

Next, we performed Northern blot analysis using mouse Dermo-1 cDNA (198 bp, from 9 to 207) as a probe. We detected a single Dermo-1 mRNA band of an apparent molecular size of 1.4 kb in MC3T3-E1 cells (Fig. 5, lane 1) and ROS17/2.8 cells (Fig. 5, lane 5).

To elucidate the regulation of Dermo-1 and twist expression, we examined mRNA levels of Dermo-1 and twist in C3H10T1/2 cells treated with rhBMP-2. Total RNA was isolated after 72 h treatment of the cells with 500 ng/ml of rhBMP-2 and quantitative RT-PCR was carried out as described in Materials and Methods. The levels of Dermo-1 and twist mRNA expression were decreased by the treatment with rhBMP-2 in C3H10T1/2 cells (Fig. 4). In addition, we also observed that Dermo-1 mRNA expression was decreased about fourfold by the treatment with rhBMP-2 in C3H10T1/2 cells (Fig. 5, lanes 3 and 4) by Northern blot analysis. To confirm the negative correlation between Dermo-1 mRNA expression and osteoblastic differentiation, we examined the mRNA expression of Dermo-1 in proliferating MC3T3-E1 cells vs. differentiated MC3T3-E1 cells. The level of Dermo-1 mRNA expression in 21-day-old differentiated MC3T3-E1 cells (Fig. 5, lane 2) was lower (23%) than that in 3-day-old undifferentiated MC3T3-E1 cells (Fig. 5, lane 1).

DISCUSSION

Using partially degenerate oligonucleotides as primers to identify cDNA clones encoding bHLH proteins expressed in osteoblasts, we cloned Dermo-1 and twist cDNA from the ROS17/2.8 cell library. Dermo-1 is one of the bHLH transcription factors and was first reported by Li et al. [1995]. Using the yeast two-hybrid system to screen for tissue-specific bHLH proteins, which dimerize with the ubiquitous bHLH protein E12, they cloned a novel bHLH protein and named it Dermo-1. Within its bHLH region, Dermo-1 shares extensive homology with twist, which is expressed in embryonic mesoderm.

During mouse embryogenesis, the expression pattern of Dermo-1 is similar to, but distinct from, that of twist. Dermo-1 is expressed at a low level in the sclerotome and dermatome of the somites and in the limb buds at day 10.5 p.c. It accumulates predominantly in dermatome, prevertebrae, and the derivatives of the branchial arches by day 15.5 p.c. As differentiation of the prechondial cells proceeds, Dermo-1 expression becomes restricted to the perichondrium. Expression of Dermo-1 increases continuously in the dermis through day 17.5 p.c. and is also detected in the dermis of neonates, but is downregulated in adult tissues [Li et al., 1995]. Our results indicate for the first time that osteoblastic cells express Dermo-1 mRNA (Figs. 2, 3).

Twist is one of the bHLH transcription factors and was originally reported in *Drosophila* as an essential molecule for the early establishment of the mesoderm [Thisse et al., 1988]. In the presumptive mesoderm of the cellular blastoderm embryo, twist is initially activated by dorsal. There expression pattern suggests that twist and Dermo-1 may regulate different target genes [Gitelman, 1997]. Recently, it was reported that the chromosomal locations of the twist and Dermo-1 genes are distinct on the human genome [Perrin-Schmitt et al., 1997]. Possible targets of twist include msh-2 [Bodmer et al., 1990], PS2 [Leptin, 1991], Zfh-1 [Lai et al., 1993], DFR1 [Shishido et al., 1993], and D-mef2 [Lilly et al., 1995], since mesodermal expression of these genes is disrupted in twist mutant embryos of *Drosophila*. In vertebrates, twist may be involved in the negative regulation of cellular determination and differentiation of several linages [Spicer et al., 1996]. Hamamori et al. [1997] reported that twist physically interacts with the myogenic bHLH proteins in vitro as well as in vivo and that this interaction is required for the inhibition of Myo-D by twist through the basic domain. Recently, Ghouzzi et al. [1997] reported 21-bp insertions and non-sense mutation of the twist gene in seven Saethre-Chotzen syndrome (acrocephalo-syndactyly type III, ACS III) probands. We and others have observed that mouse twist,

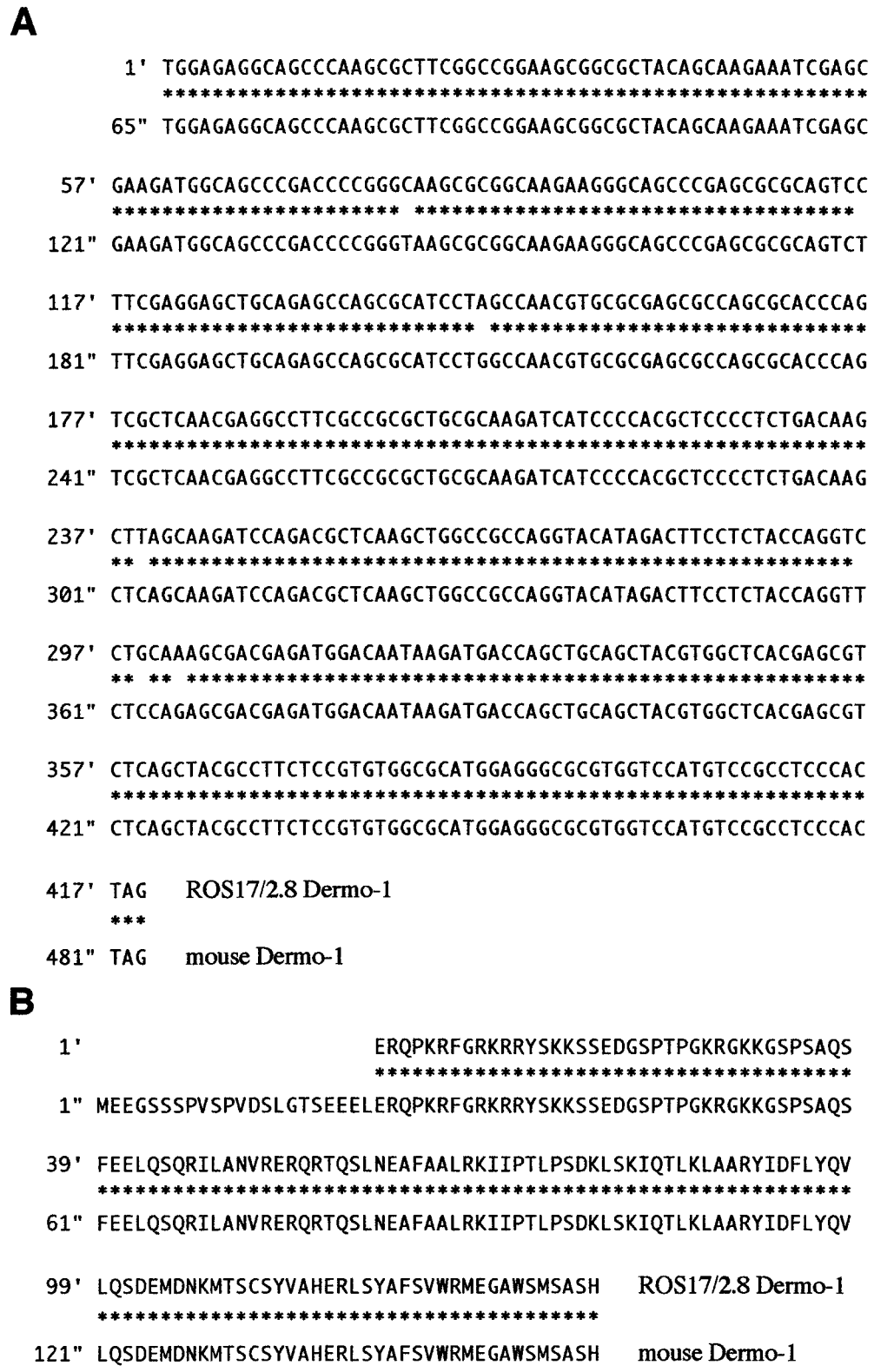

Fig. 3. **A:** Partial Dermo-1 cDNA sequence obtained from ROS17/2.8 cells (upper) and sequence similarity to mouse dermo-1 cDNA (lower). PCR-amplified DNA obtained by using Dermo-1 specific primers, DEL-4 and DEL-5, was subcloned into pCRScript AmpSK(+) and was sequenced by autosequencer. **B:** Deduced partial Dermo-1 amino acid sequence based on cDNA obtained from ROS17/2.8 (upper) and sequence similarity to mouse Dermo-1 (lower).

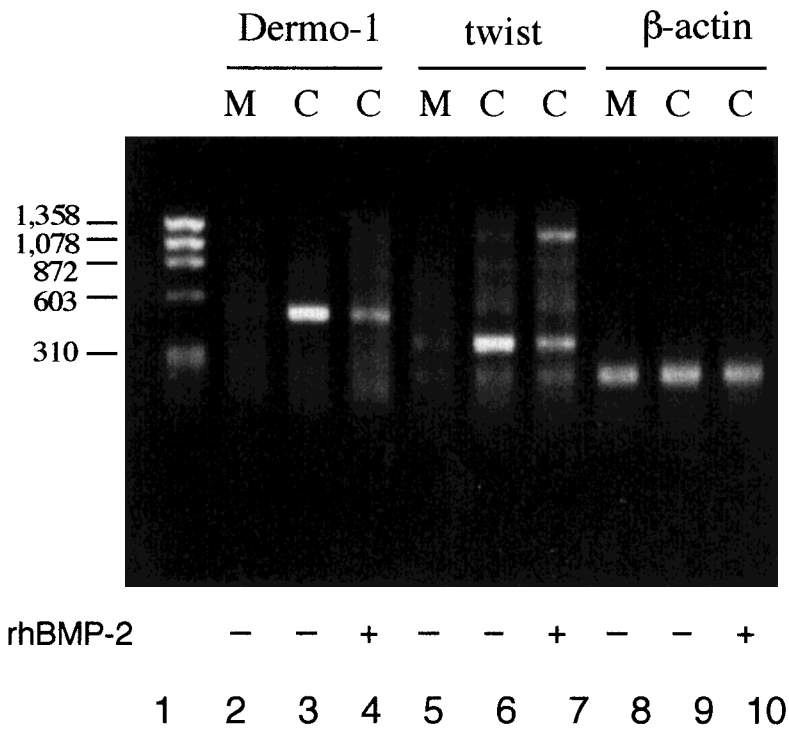

Fig. 4. Effect of rhBMP-2 on Dermo-1 and twist mRNA expression in C3H10T1/2 cells. Total RNA was isolated after 72 h treatment of the C3H10T1/2 cells (C) with 500 ng/ml of rhBMP-2 (**lane 4, 7, 10**) or vehicle (**lanes 3, 6, 9**), and was reverse transcribed. Total RNA was isolated from MC3T3-E1 cells (M) (**lanes 2, 5, 8**), and reverse transcribed. Then PCR was carried out with Dermo-1 primer (DEL2 and DEL4) (lanes 2–4) and twist primer (TWI3 and TWI4) (lanes 5–7) as described in Materials and Methods. Equality in the amount of cDNA in each lane was checked by PCR using β-actin primers (lanes 8–10). DNA was stained with ethidium blomide on the gel. **Lane 1** is phiX-HaeIII DNA size marker. The sizes of the amplified bands of Dermo-1, twist and β-actin are 482 bp, 422 bp, and 213 bp, respectively.

the murine homologue of the *Drosophila* twist, is expressed in osteoblasts or osteoblast-like cells. Murray et al. [1992] demonstrated that twist mRNA expression declined with osteoblastic maturation in mouse osteoblastic cell line MC3T3-E1 as observed in the case of Id-1 mRNA expression [Ogata and Noda, 1991].

BMP-2 induces ectopic bone formation in vivo [Wozney et al., 1988]. BMP-2 was reported to induce osteoblastic differentiation and to upregulate the expression of most of the genes encoding osteoblastic phenotype-related proteins in vitro. Katagiri et al. [1990] reported that the level of alkaline phosphatase mRNA expression and enzyme activity were increased in C3H10T1/2 cells by treatment with rhBMP-2. Our previous studies indicated that treatment with rhBMP-2 also induced osteocalcin and osteopontin mRNA expression as demonstrated by Northern blot analysis [Tamura and Noda, 1994]. Here, we show that rhBMP-2 reduced Dermo-1 and twist mRNA expression in C3H10T1/2 cells by RT-PCR (Fig. 4). We also observed that Dermo-1 mRNA expression was decreased about fourfold by treatment of rhBMP-2 in C3H10T1/2 cells using Northern blot analysis (Fig. 5). MC3T3-E1 cells are a clonal osteoblastic cell line that undergo a temporal sequence of development characterized by the downregulation of proliferation and stepwise upregulation of osteoblastic markers such as alkaline phosphatase activity during cultures [Quarles et al., 1992]. The level of Dermo-1 mRNA expression was decreased by long-term culture of MC3T3-E1 cells (Fig. 5). These results suggested that Dermo-1 could be involved in the osteoblastic differentiation in a negative manner.

Using the promoter fragment −198 to −98 of the rat osteocalcin gene that contains two E-box sequences, OCE1 and OCE2, we demonstrated that the OCE1 with CACATG type E-box motif represents a novel regulatory element of the osteocalcin gene and could play a unique role in the regulation of osteocalcin promoter activity and osteoblast-specific expression of the rat osteocalcin gene [Tamura and Noda, 1994]. In our preliminary experiments using co-transfection of pOC198-luciferase reporter plasmid and mammalian expression plasmid carrying Dermo-1 or twist did not affect CAT activities (data not shown). Therefore, the binding complexes that interact with OCE1 would include proteins other than Dermo-1 or twist. This notion is also supported by another observation. Namely, OCE1-binding activity in C3H10T1/2 nuclear extracts was very low; however, it was markedly increased by treatment

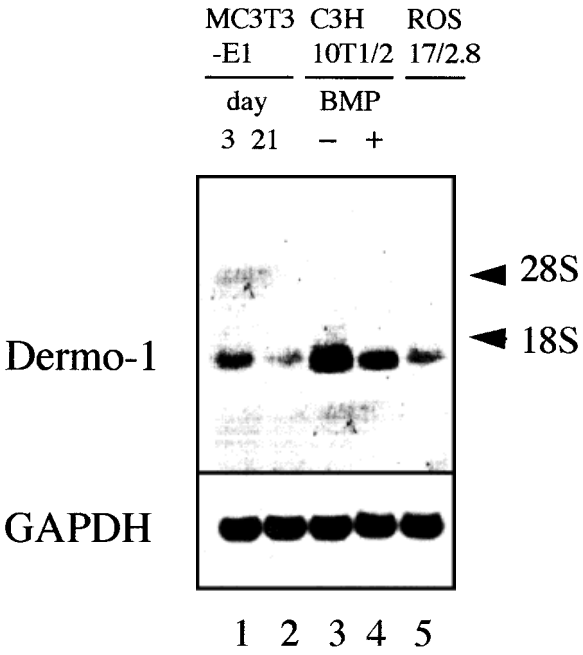

Fig. 5. Northern blot analysis of Dermo-1 mRNA expression in MC3T3-E1 cells, C3H10T1/2 cells and ROS17/2.8 cells. Total cellular RNA was isolated from 3-day-old MC3T3-E1 cells (**lane 1**), 21-day-old MC3T3-E1 cells (**lane 2**), and ROS17/2.8 cells (**lane 5**). Total cellular RNA was isolated after 72 h treatment of C3H10T1/2 cells with 500 ng/ml of rhBMP-2 (+, **lane 4**) and vehicle (−, **lane 3**). Northern blot analysis (30 μg of total cellular RNA per lane) using ^{32}P-labeled rat Dermo-1 cDNA as a probe was carried out as described in Materials and Methods (upper). Equal loading in lanes was checked by hybridization with ^{32}P-labeled GAPDH as a probe (lower). Arrows indicated the positions of 28S and 18S ribosomal RNA.

with rhBMP-2. On the other hand, Dermo-1 or twist mRNA expressions were decreased by treatment with rhBMP-2 (Fig. 4).

Recently Ducy et al. [1997] identified Osf2/Cbfa1 as an osteoblast-specific transcription factor and as a regulatory of osteoblastic differentiation. Osf2/Cbfa1 binds an osteoblast-specific cis-acting element, termed OSE2, located between −146 and −132 in the osteocalcin promoter. It is still to be elucidated how these differentiation-related transcription factors, i.e., Osf2/Cbfa1 and Dermo-1, coordinately regulate the functions of osteoblasts.

In conclusion, our results presented in this report demonstrate that Dermo-1 mRNA is expressed in osteoblasts. We also found that the expression levels of Dermo-1 as well as twist are downregulated by treatment of BMP-2, which is involved in osteoblastic differentiation. Moreover, Dermo-1 mRNA was detected at lower levels in differentiated MC3T3-E1 cells compared with undifferentiated MC3T3-E1 cells. Therefore, Dermo-1 may be a negative regulator of the differentiation of osteoblast. Our further studies are aimed at identifying target genes of Dermo-1 and twist and exploring their roles in osteoblastic differentiation.

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