Identification of a DNA Sequence Involved in Osteoblast-specific Gene Expression via Interaction with Helix-Loop-Helix (HLH)-type Transcription Factors

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Abstract. To elucidate regulatory mechanism(s) underlying differentiation of osteoblasts, we examined involvement of helix-loop-helix (HLH)-type transcription factors in osteoblast-specific expression of a phenotypic marker gene which encodes osteocalcin, a major noncollagenous bone matrix protein, exclusively expressed in osteoblasts. Overexpression of a dominant negative HLH protein, Id-1, decreased the activity of the 1.1-kb osteocalcin gene promoter cotransfected into rat osteoblastic osteosarcoma ROS17/2.8 cells. Analysis of deletion mutants revealed that a 264-bp fragment of osteocalcin promoter (−198 to +66) was sufficient for the Id-1-dependent suppression. Furthermore, the activity of the same promoter fragment (−198 to +66) was enhanced when antisense Id-1 expression vector was cotransfected. This osteocalcin gene promoter region contains two sites of an E-box motif, a consensus binding site for HLH proteins, which we refer to as OCE1 (CACATG, at −102) and OCE2 (CAGCTG, at −149), respectively. Mutagenesis in OCE1 but not OCE2 led to greater than 50% reduction in transcriptional activity of the osteocalcin gene promoter. Electrophoresis mobility shift assay indicated that factors in nuclear extracts prepared from ROS17/2.8 cells bound to the 30-bp oligonucleotide probe containing the E-box motif of OCE1. This binding was competed out by OCE1 oligonucleotide but neither by OCmE1 oligonucleotide in which E-box motif was mutated nor by OCE2. The OCE1-binding activity in the nuclear extracts of ROS17/2.8 cells was reduced by 70% when bacterially expressed Id-1 protein was added to the reaction mixture, suggesting the involvement of HLH proteins in the DNA/protein complex formation. In contrast to the osteoblast-like cells, OCE1-binding activity in the nuclear extracts of C3H10T1/2 fibroblasts was very low. However, when these fibroblasts were treated with recombinant human bone morphogenetic protein-2 which induced expression of osteocalcin as well as other phenotypic markers of osteoblasts, OCE1-binding activity was increased ~40-fold, indicating that OCE1 would be involved in the tissue-specific expression of the osteocalcin gene. These findings indicated for the first time that osteoblast-specific gene transcription is regulated via the interaction between certain E-box binding transcription factor(s) in osteoblasts and the OCE1 sequence in the promoter region of the osteocalcin gene.

DEVELOPMENT and differentiation of cells are under the control of various classes of transcription factors which have been identified through genetic and biochemical means. Some of these factors have been shown to play key roles during cell differentiation process. 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 expression of respective genes (for review see Stein and Lian, 1993). Osteoblasts are thought to be derived from so-called undifferentiated mesenchymal cells. Upon differentiation, osteoblasts express phenotype-related genes encoding proteins such as osteocalcin, osteopontin, alkaline phosphatase, type I collagen, parathyroid hormone receptor, and so on (for review see Rodan and Rodan, 1984). Among those, osteocalcin is considered to be the only protein expressed specifically in osteoblasts (for review see Gundberg et al., 1984).

Osteocalcin (bone γ-carboxyglutamic acid-containing protein) is the most abundant noncollagenous matrix protein in bone and is produced exclusively by relatively mature osteoblasts (for reviews see Gundberg et al., 1984; Hauschka, 1986). Serum osteocalcin level correlates well with histo-

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morphometric parameters of bone formation, and has been used clinically to estimate the level of bone turnover (for review see Lian, 1988). Osteocalcin has been suggested to control hydroxyapatite crystal growth and seems to constitute a signal for osteoclastic bone resorption. Resorption of subcutaneously implanted bone particles is impaired when the amount of osteocalcin in bone is reduced, and in vitro experiments also show that osteocalcin is a chemoattractant for monocytes, which are thought to be the precursors of osteoclasts (for reviews see Hauschka, 1986; Lian, 1988).

Cell type-specific gene expression in many types of cells are controlled at the level of transcription. Transcriptional regulation also plays a key role in osteocalcin gene expression and the gene has been used as a model in the study of cell type-specific gene expression in osteoblasts (for review see Stein and Lian, 1993). Several transcriptional regulators have been reported to bind DNA elements that are located in the 5'-flanking region of rat osteocalcin gene and to control its transcription in response to physiological stimuli. In the rat osteocalcin gene, Lian et al. (1989) previously identified in the proximal promoter region a 24-nucleotide sequence, designated as osteocalcin box (−76 to −99), which contains a CCAAT motif as a central element and is required for the basal level of osteocalcin gene transcription. A similar sequence was identified in the promoter region of human osteocalcin gene (Lian et al., 1989). Another regulatory region in rat osteocalcin gene is the TATA box (−44 to −23), which contains a consensus glucocorticoid-responsive element (Heinrichs et al., 1993). A vitamin D-responsive element and AP-1 site have been also identified in human and rat osteocalcin genes (Morrison et al., 1989; Demay et al., 1990; Ozono et al., 1990). Recently, mapping studies were undertaken to characterize the tumor necrosis factor α-responsive element [−522 to −511] in the human osteocalcin promoter (Li and Stashenko, 1993). Several other putative *cis*-acting elements involved in the transcriptional regulation were identified by computer analysis, including AP2- and NF1-binding sites and cyclic AMP-responsive element (Yoon et al., 1988; Lian et al., 1989).

Helix-loop-helix (HLH)[1] family proteins have been implicated in transcriptional regulation of cell type determination and differentiation and ~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 also referred to as an E-box motif, initially identified in the immunoglobulin heavy chain gene enhancer (for review see Murre and Baltimore, 1992). Since then, several other functional E-box sites have been identified in the 5'-flanking regions of genes expressed in a cell type-specific manner. Those include muscle creatine kinase gene (Buskin and Hauschka, 1989) as well as acetylcholine receptor α subunit gene (Piette et al., 1990) in skeletal muscle, insulin gene in pancreatic β cells (Cordle et al., 1991), secretin gene in islet cells (Wheeler et al., 1992), and proopiomelanocortin gene in pituitary cells (Therrien and Drouin, 1993). bHLH proteins can be divided into at least two classes: those that are expressed in a broad spectrum of tissues and cell types and those expressed in a tissue-restricted fashion (Murre and Baltimore, 1992). E-box sequences have not yet been implicated in osteoblast-specific expression of osteocalcin gene and so far, only ubiquitously expressed HLH genes such as E2A and Id-1 have been reported to be expressed in osteoblasts or osteoblast-like cells. Expression of Id gene in osteoblastic cells have been shown to be under the regulation of calcitrophic hormones and cytokines (Ogata and Noda, 1991; Kawaguchi et al., 1992). To elucidate the possibility of the involvement of bHLH proteins in osteoblast-specific gene expression, we examined the contribution of E-box sequences to the activity of the promoter and tissue specific expression of the osteocalcin gene in osteoblasts. In the present work, we show that E-box sequence 1 (OCE1) in rat osteocalcin gene is involved in HLH molecule-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 indicated the formation of osteoblast-specific protein/DNA complexes with this OCE1 sequence, and this binding activity was induced in C3H10T1/2 fibroblasts by recombinant human bone morphogenetic protein 2 (rhBMP-2) treatment which induces expression of osteocalcin gene in these cells. Thus, this study presents an evidence that the E-box sequence, OCE1, and transcription factors interacting with this motif are involved in osteoblast-specific osteocalcin gene transcription.

Materials and Methods

Cell Culture

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% FBS (Gibco Laboratories). C3H10T1/2 fibroblasts were obtained from RIKEN Cell Bank (Tsukuba, Japan) (Reznikoff et al., 1973). C3H10T1/2 cells were grown in DME (Gibco Laboratories) supplemented with 5% FBS. Cells were cultured in a humidified atmosphere of 95% air and 5% CO_2 at 37°C.

Construction of Plasmids

One of the promoter constructs pOC1094CAT containing a fragment (−1094 to +147) of the rat osteocalcin gene linked to the chloramphenicol acetyltransferase (CAT) gene used in this paper was previously described (Yoon et al., 1988) and was kindly provided by Dr. G. Rodan (Merck Research Laboratories). Several 5' deletion mutants of the rat osteocalcin gene promoter constructs, pOC431CAT, pOC198CAT, and pOC98CAT, were generated as follows. Promoter fragments of AB (−431 to +66) CB (−198 to +66), and DB (−99 to +66) were generated by PCR using primers (A3, C2, D2, and B3), digested with XbaI and HindIII, and then ligated into pGEM4Z-CAT (a gift from M. Young, National Institutes of Health, Bethesda, MD) after digestion of the vector with XbaI and HindIII. The PCR-oligonucleotide sequences used in deletion mutagenesis were as follows:

A2, GTAGAGAAGCTTTTCTTCCTGGGGTTTGGCTCCTGCTCTCACGG
C2, TGAACCAAGCTTCCCGGCAGCCTCTGATTGTGTCCTACCCTC
D2, TGAAAGAAGCTTCATGACCCCCAATTAGTCCTGGCAGCATCT
B3, TGGAGGTCTAGACAGCAGAGTGAGCAGAGAGAGGGTCCTCAT

Site-directed mutagenesis was performed according to the overlap extension technique using PCR as described by Ho et al. (1989). In brief, complementary oligonucleotides designed to introduce specific mutations by PCR were used to generate two DNA fragments having overlapping ends. These fragments were combined in a subsequent fusion reaction in which the overlapping ends are annealed, allowing the 3' overlap of each strand to serve as a primer for 3' extension of the complementary strand. The

1. *Abbreviations used in this paper*: BMP, bone morphogenetic protein; rhBMP, recombinant human BMP; CAT, chloramphenicol acetyltransferase; EMSA, electrophoresis mobility shift assay; GST, glutathion-S-transferase; HLH, helix-loop-helix, bHLH, basic HLH.

resulting fusion products were amplified by PCR. These mutant promoter fragments were excised with XbaI and HindIII, and then subcloned into the XbaI and HindIII sites of pGEM4Z-CAT. The oligonucleotides used in site-directed mutagenesis contain the following mutations (indicated by lowercase letters):

EM-7, TAATTGGGGGTgtaacaCGCAATAGG (mutant OCE1 site bottom strand)
EM-8, TGGTGACTGgtaacaCCGGGGCGAAG (mutant OCE2 site bottom strand)
EM-9, CTATTGCGtgttacACCCCCAATTAG (mutant OCE1 site top strand)
EM-10, TCGCCCCGGtgttacCAGTCACCAAC (mutant OCE2 site top strand)

The nucleotide sequences of each deleted and mutated promoter region were determined to confirm the absence of mutations introduced by PCR.

The expression plasmids, pE: Id (S) sense Id expression vector and pE: Id (A) antisense Id expression vector, were a gift from Dr. H. Weintraub (Fred Hutchinson Cancer Research Center, Seattle, WA) (Benezra et al., 1990).

DNA Transfection, CAT Assay, and Luciferase Assay

Transfection of plasmid DNA into ROS 17/2.8 was performed using DEAE-dextran method (Lopata et al., 1984). In brief, cells were plated 24 h before transfection at a density of 7×10^5 cells per 9.6-cm^2 culture well in modified F-12 medium supplemented with 5% FBS. The cells were transfected with 5 μg of reporter plasmid, 1 μg of pGVC (transfection efficiency control) (Promega Corp., Madison, WI), and 5 μg of expression plasmid vectors bearing Id gene as indicated in the figure legends. The cells were exposed to plasmid DNA for 3 h, treated with 10% glycerol for 90 s, and were subsequently cultured in fresh medium supplemented with 5% FBS in an atmosphere of 5% CO_2 and 95% air at 37°C for 48 h. The cells were then harvested by scraping into a buffer (150 mM NaCl, 40 mM Tris-HCl, and 1 mM EDTA, pH 7.5), lysed by three cycles of freezing and thawing followed by centrifugation. Protein concentrations of the supernatant were measured by Coomassie brilliant blue R method (Spector, 1978), and aliquots containing equivalent amounts of protein were incubated in 0.25 M Tris-HCl, pH 8.0, and 165 μg of acetyl-CoA (Sigma Chem. Co., St. Louis, MO) and 3.7 kBq of [^{14}C]chloramphenicol (1.85 GBq/mmol; Amersham Corp., Arlington Heights, IL) for 12 h at 37°C. The organic components in the reaction mixture were then extracted with ethyl acetate and separated by thin-layer chromatography using 95% chloroform 5% methanol as solvent, and were exposed to x-ray films. Amounts of acetylated chloramphenicol were quantitated by exposure to a Bioimaging plate (Fujifilm Inc., Tokyo, Japan) for 12 h and analyzed by a Bioimaging Analyzer (Fujifilm Inc.). To ensure reproducibility, experiments were repeated four to six times. To account for variation in cell transfection efficiency, CAT activity was normalized against the amount of luciferase activity (Berthold, 1990). Enzymatic assays measuring luciferase activity were performed in Autolumat LB953 (Lab. Prof. Dr. Berthold GmbH & Co., Wildbad, Germany) using Picagene System (Toyo Ink Co., Tokyo, Japan).

Preparation of Nuclear Extracts and Electrophoresis Mobility Shift Assay (EMSA)

Nuclear extracts were prepared essentially as described by Dignam et al. (1983). All steps were performed at 4°C. Confluent cells were washed with PBS twice, resuspended in 10 mM Hepes (pH 7.5), 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM DTT, and the nuclei were isolated with 10 strokes in a Dounce homogenizer. The nuclei suspension was centrifuged at 2,000 *g* for 20 min, and the pellet was resuspended in a buffer C (20 mM Hepes (pH 7.5), 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 25% (vol/vol) glycerol), and lysed as described above. The nuclear lysate was centrifuged at 12,000 *g* for 20 min, the supernatant was dialyzed against a buffer D (20 mM Hepes [pH 7.5], 100 mM KCl, 0.2 mM EDTA, 0.5 mM PMSF, 0.5 mM DTT, 20% glycerol) and centrifuged at 10,000 *g* for 5 min. Aliquots of the nuclear extracts were stored at −80°C. Oligonucleotide probes 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 $MgCl_2$. Some sequences also contained cohesive SalI sites at the ends. The annealed probes were labeled by filling-in 5′ protruding ends with [α-^{32}P]dCTP (New England Nuclear/Du Pont, Wilmington, DE) using the Klenow fragment (Pharmacia, Uppsala, Sweden) to a specific activity greater than 10^8 cpm/μg. Approximately 20,000 cpm of the probe was incubated with 5 μg of nuclear protein for 20 min at 30°C in 40 μl reaction mixture, containing buffer D, 5 μg of BSA (Sigma Chem. Co.), and 2 μg of poly (dI-dC) (Pharmacia, Piscataway, NJ). For competition experiments, competitor DNAs were incubated with the nuclear extracts for 20 min before the addition of the probe. The DNA–protein complexes were fractionated on a nondenaturing 4% polyacrylamide gel (acrylamide–bisacrylamide, 29:1) at 11 V/cm for 1.5 h in TAE buffer (0.134 mM Tris-HCl, pH 7.5, 0.66 mM CH_3COONa, and 0.2 mM EDTA·2Na) for EMSA. After electrophoresis, the gels were vacuum-dried and exposed to x-ray film using intensifying screens (New England Nuclear/Du Pont) at −80°C for 12–48 h. The oligonucleotide sequences used as probes or competitors in EMSA were as follows:

ES-19, TCGACTTGACCTATTGCGCACATGACCCCG (OCE1 top strand)
ES-20, TCGACGGGGTCATGTGCGCAATAGGTCAAG (OCE1 bottom strand)
ES-5, TCGACGCCTCCGCCCCGGCAGCTGCAGTCG (OCE2 top strand)
ES-6, TCGACGACTGCAGCTGCCGGGGCGGAGGCG (OCE2 bottom strand)
ES-7, TCGACCCAGCCAGTGCTCCAGCTGAGGCTG (OCE3 top strand)
ES-8, TCGACAGCCTCAGCTGGAGCACTGGCTGGG (OCE3 bottom strand)
ES-21, TCGACGGAGGCATTTTCTCAATTGAGGCTG (OCE4 top strand)
ES-22, TCGACAGCCTCAATTGAGAAAATGCCTCCG (OCE4 bottom strand)
ES-1, TCGACAGAACACCTGCAGCAGCTGGCAGGG (μE5+uE2 top strand)
ES-2, TCGACCCTGCCAGCTGCTGCAGGTGTTCTG (μE5+μE2 bottom strand)
ES-9, TCGACCCCCCCAACACCTGCTGCCTGAGCCG (MEF1 top strand)
ES-10, TCGACGGCTCAGGCAGCAGGTGTTGGGGGGG (MEF1 bottom strand)
ARM2S, TCGACACAATTTTCTCGATTTTCAGGTCTG (top strand)
ARM2SR, TCGACAGACGTGAAAATCGTGAAAATTGTG (bottom strand)
EM-1, TCGACTTGACCTATTGCGTGTTACACCCCG (OCmE1 top strand)
EM-2, TCGACGGGGTGTAACACGCAATAGGTCAAG (OCmE1 bottom strand)

The glutathion-S-transferase (GST) and GST-ΔNId fusion proteins were kindly provided by Dr. M. Obinata (Tohoku University, Sendai, Japan). The GST-ΔNId fusion protein was made by insertion of codons 80–176 of mouse Id-1 (Benezra et al., 1990) in frame with GST in the vector pET (Novagen Inc., Madison, WI). Purified GST or GST-ΔNId protein was added to nuclear extracts prepared from ROS17/2.8 cells and denatured by dialysis for 1 h at 4°C against 6 M guanidine HCl in buffer D. The extracts were then similarly renatured by dialysis against the same buffer without guanidine HCl and were used in EMSA as described above.

RNA Isolation and Northern Blot Analysis

Total cellular RNA was prepared at the indicated time points by the acid guanidium thiocyanate-phenol-chloroform method as described by Chomczynski and Sacchi (1987). 20 μg of the total RNA per lane was electrophoresed in 1% agarose gels containing 0.66 M formaldehyde and was transferred to a nylon filter (Hybond-N; Amersham Corp.) by electroblotting (Thomas, 1980). Filters were UV cross-linked, and prehybridized overnight at room temperature in a buffer containing 50% formamide, 5 × SSC (1 × SSC consists of 0.15 M NaCl and 10 mM sodium citrate), 5 × Denhardt's solution, 0.1% SDS, and 50 μg/ml sheared and denatured herring sperm DNA. Complementary DNA for rat osteocalcin (Yoon et al., 1987) or rat osteopontin (Oldberg et al., 1986) was excised with EcoRI and separated from vector sequences by Seaplaque gel electrophoresis and then radiolabeled by random primer method using Klenow fragment (Pharmacia) and [α-^{32}P]dCTP (New England Nuclear/Du Pont) as described by Feinberg and Vogelstein (1983). Specific radioactivity of labeled cDNA was 1 to 5 × 10^8 cpm/μg DNA. Filters were hybridized with ^{32}P-labeled cDNA probes at 42°C for 18 h in a solution containing all ingredients described above. Filters were washed in 0.1 × SSC containing 0.1% SDS for 15 min at 65°C, and were exposed to x-ray film using intensifying screens (New England Nuclear/Du Pont) at −80°C for several days. Equal loading of RNA in each lane was checked by ethidium bromide staining.

Protein

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

Results

Overexpression of Id-1 Suppresses Osteocalcin Gene Promoter Activity

We first examined the promoter sequence of rat osteocalcin gene and located five sites of E-box consensus sequence within the 1.1-kb fragment of the 5′ flanking region. These E-box sequences were designated as OCE1 to OCE5 according to the order of the distance from the transcription start site (Fig. 1). To elucidate whether the members of the HLH protein family are involved in regulating the activity of osteocalcin gene promoter in osteoblasts, we examined the effects of overexpression of the *trans*-dominant negative regulator, Id-1, which lacks the basic region adjacent to HLH domain in rat osteoblastic osteosarcoma ROS17/2.8 cells. As shown in Fig. 2 *A*, cotransfection of a sense Id-1 expression vector resulted in suppression of the promoter activity (by ~50%) of the pOC1094CAT, which contains a 1.2-kb (−1094/+147) promoter region of rat osteocalcin gene linked to a CAT reporter gene (Fig. 2 *A*, lanes *4* and *5* vs. lanes *6* and *7*). In contrast, cotransfection of another expression vector that carries Id-1 cDNA in reverse (antisense) orientation [Id1-AS] increased expression of the CAT activity (by ~60%) (Fig. 2 *A*, lanes *8* and *9*). CAT activity data were normalized against luciferase activity to correct for the transfection efficiency. Thus, the results of these cotransfection experiments suggested the involvement of HLH transcription factors in regulation of osteocalcin gene expression.

Deletion and Site-directed Mutagenesis Analyses of the 5′-flanking Region of Rat Osteocalcin Gene

To locate sequences in the 5′-flanking region of rat osteocalcin gene which mediates Id-1–dependent suppression, we constructed a series of CAT reporter constructs, which contain sequential deletion mutants of the 5′-flanking fragment placed upstream of the CAT gene (Fig. 1). The CAT activities of the two constructs in which 5′ end was deleted up to the nucleotide positions −432 and −199 (pOC431CAT and pOC198CAT, respectively) were suppressed by sense Id-1 overexpression while they were enhanced by antisense Id-1 overexpression (Fig. 2, *B* and *C*), similarly to those observed in the case of pOC1094CAT (Fig. 2 *A*). In contrast, there was neither significant suppression nor enhancement in the level of CAT activity when pOC98CAT was cotransfected with Id-1 sense or antisense expression vectors in ROS17/2.8 cells (Fig. 2 *D*). This deletion analysis implied the presence of a transcriptional machinery, which could be interfered by Id-1 protein and would regulate the transcrip-

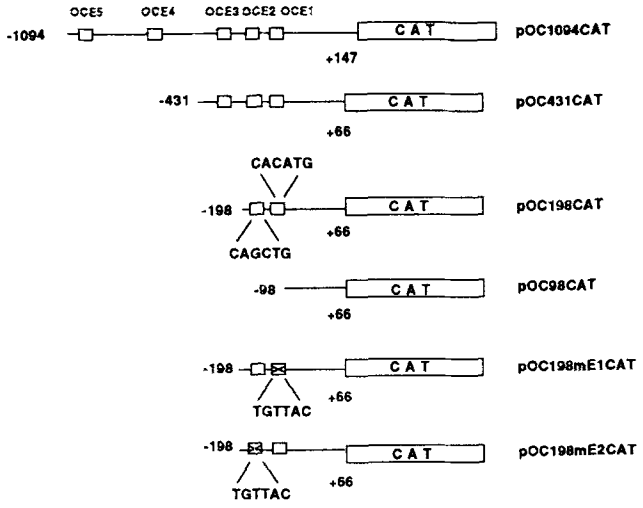

Figure 1. Construction of various 5′-deletion and site-directed mutants of rat osteocalcin gene promoter. Numbers at left represent the positions of the 5′-end nucleotides included in each construct relative to the transcription start site at position +1. E-box sequences, OCE1 to OCE5, are marked by a box.

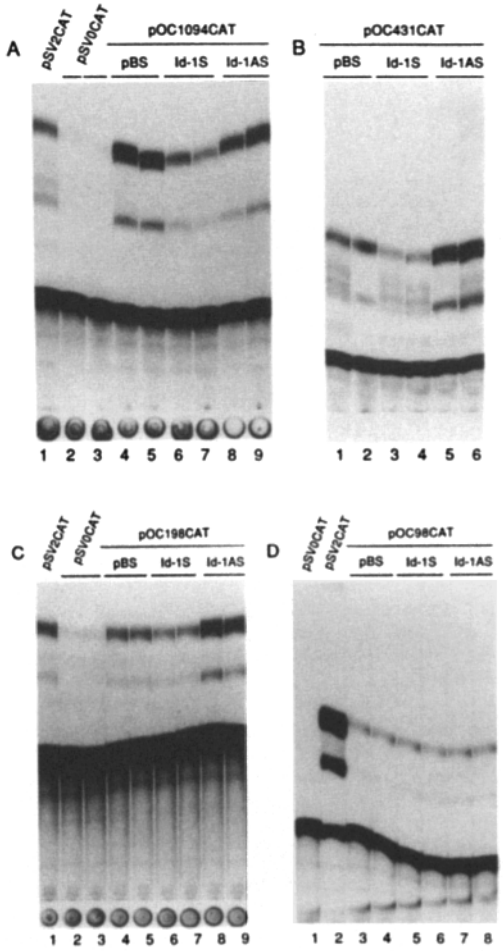

Figure 2. Suppression of rat osteocalcin promoter activity by overexpression of Id-1. CAT assay was carried out by using the lysates prepared from ROS17/2.8 cells cotransfected with 5 μg of reporter plasmid and 5 μg of expression vectors containing Id-1 cDNA in either coding (sense) [Id-1S] or inverted (antisense) [Id-1AS] orientation, or pBluescript SK(+) [pBS] as described in Materials and Methods. The DNAs for each cotransfection also include 1 μg of pGVC to estimate transfection efficiency. Cells were harvested 48 h after transfection followed by measurement of CAT and luciferase activities. Each lane represents a separate culture of experimental group performed in duplicate. pSV2CAT and pSV0CAT served as positive and negative controls, respectively. (*A*) pOC1094CAT was cotransfected with pBS, Id-1S, and Id-1AS. (*B*) pOC431CAT was cotransfected with pBS, Id-1S, and Id-1AS. (*C*) pOC198CAT was cotransfected with pBS, Id-1S, and Id-1AS. (*D*) pOC98CAT was cotransfected with pBS, Id-1S, and Id-1AS.

tional activity of the rat osteocalcin gene promoter through interaction with the promoter fragment between the nucleotide positions −198 and −100. This 99-bp region contains two E-box sequences (OCE1 and OCE2) (Fig. 1), which meet with a consensus sequence (CANNTG), reported to be recognized by HLH family proteins. These E-box sequences in OCE1 and OCE2 are CACATG and CAGCTG, respectively. These E-boxes of osteocalcin promoter might render binding sites for transcription factor(s) of HLH family. To determine whether these E-box motifs contribute to the promoter activity of the osteocalcin gene, we introduced mutations into OCE1 or OCE2 sites in pOC198CAT construct, by replacing CANNTG sequence to TGTTAC (Fig. 1). One reporter construct with site-directed mutation, pOC198-mE1CAT, in which OCE1 (−102 to −97) was replaced showed reduction of basal transcriptional activity by ~50% (Fig. 3, lanes *5* and *6*) compared to the control (Fig. 3, lanes *3* and *4*). In contrast, the transcriptional activity of another mutation construct, pOC198mE2CAT, in which OCE2 (−149 to −144) was replaced, was similar to that of pOC198CAT (Fig. 3, lanes *7* and *8*), suggesting that the OCE1 sequence but not OCE2 sequence would be necessary for basal transcriptional activity of the osteocalcin gene. The level of CAT activity of pOC198mE2CAT but not pOC198mE1CAT was suppressed by Id-1S overexpression and enhanced by Id-1AS overexpression, similarly to those observed in the case of pOC198CAT (data not shown).

Interaction of Nuclear Protein(s) with the OCE1 Motif of Osteocalcin Gene Promoter

In order to examine the binding activity of nuclear proteins which interact with OCE1 sequence, we performed EMSA using OCE1 as a probe. Incubation of the OCE1 probe with ROS17/2.8 nuclear extracts formed two major retarded bands migrating as a doublet (Fig. 4 *A*, lane *2*) which were competed by the excess amount of unlabeled OCE1 probe (lanes *3* and *4*). The inability of an unrelated ARM-2 sequence (Noda et al., 1990) to compete against the binding revealed the sequence-specific binding in the formation of these DNA/protein complexes (Fig. 4, lane *9*). Even the presence of a 500-fold molar excess of the mutant oligonucleotide OCmE1, in which TGTTAC sequence substituted for CAC-

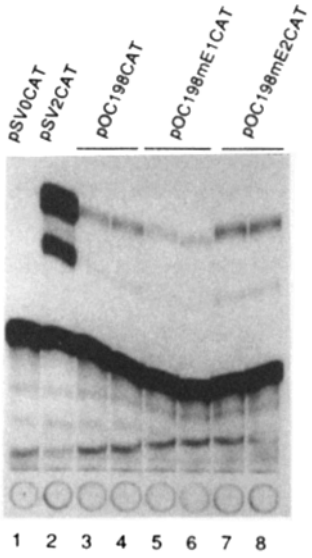

Figure 3. Effects of mutagenesis in E-box sequences on CAT activity in ROS17/2.8 cells. Substitution mutations were introduced into either E-box, OCE1 or OCE2, in pOC198-CAT to give rise to pOC198-mE1CAT and pOC198mE2-CAT, respectively, as described in the legend to Fig. 1 and Materials and Methods. These constructs were transfected into ROS17/2.8 cells, and cell extracts were prepared 48 h after transfection and were processed for CAT assays as described in Materials and Methods. Each lane, except lanes *1* and *2*, represents a separate experiment of a culture well performed in duplicate. pSV2-CAT and pSV0-CAT data are served as positive and negative controls, respectively.

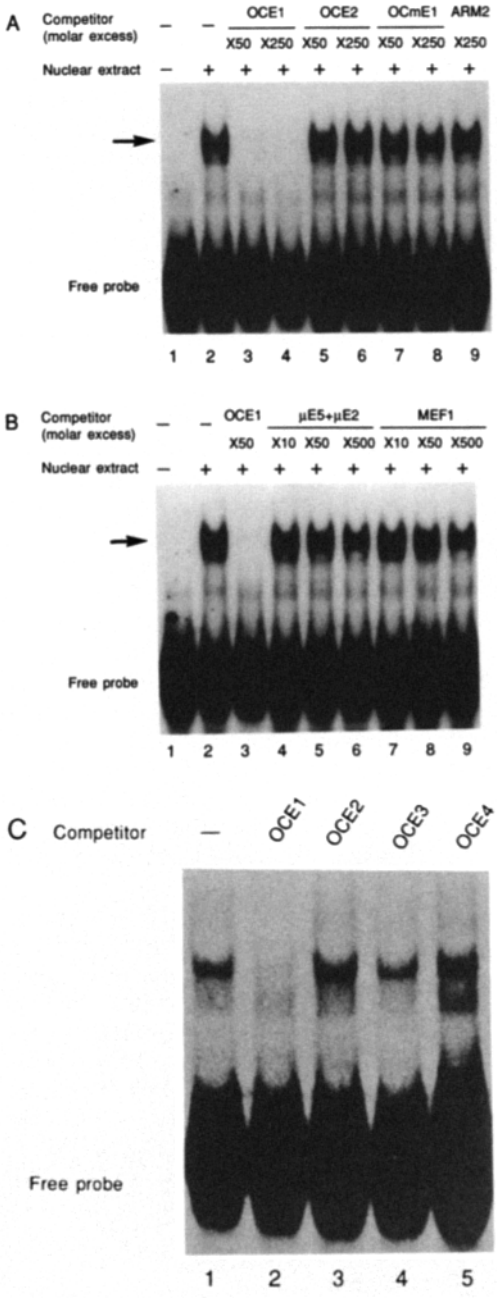

Figure 4. Electrophoresis mobility shift assay of OCE1-binding activity in ROS17/2.8 cells. ROS17/2.8 nuclear extracts were incubated with indicated amounts of unlabeled double stranded competitor oligonucleotides or vehicle (*A*, lane 2 and *B*, lane 2) in a buffer described in Materials and Methods for 20 min at room temperature. Radiolabeled OCE1 probe (20,000 cpm) was then added to these extracts, and protein-DNA complex was resolved by EMSA as described in Materials and Methods. The fold excess of the amount of competitor relative to the probe is shown above each lane (*A* and *B*). Lane *1* shows probe alone without nuclear extracts (*A* and *B*). The competitor was used at a 500-fold molar excess (*C*).

ATG, did not compete out the binding of OCE1, suggesting that of the E-box motif is crucial in the protein-DNA interaction (Fig. 4 *A*, lanes *7* and *8*). Furthermore, no binding activity in ROS17/2.8 nuclear extracts was observed when OCmE1 was used as a probe (data not shown). In order to determine whether the DNA-binding proteins in the crude nuclear extracts prepared from ROS17/2.8 cells specifically interact with OCE1 site rather than OCE2, we performed another competition assay. Competition was not observed when a 500-molar excess of unlabeled OCE2 oligonucleotide was included in the binding reaction (Fig. 4 *A*, lanes *5* and *6*), suggesting that only OCE1 is the crucial E-box sequence in the 198-bp osteocalcin gene promoter. These findings are consistent with the results of the site-directed mutation analyses, where mutation in OCE1 but not OCE2 decreased basal transcriptional activity of the 198-bp rat osteocalcin gene promoter as well as its response to Id-1 overexpression.

The specificity of the binding of the nuclear factors to OCE1 sequence was further examined in competition assays in which previously characterized binding sites for other HLH proteins were used as competitor DNAs. The DNA-protein complex formation using ROS17/2.8 nuclear extracts and the OCE1 probe was not competed out by the presence of an excess amount of a cold oligonucleotide sequence, μE5 + μE2, which consists of original binding sites for E2A gene products, E12 and E47, identified to bind immunoglobulin heavy chain enhancer (Fig. 4 *B*, lanes *4*, *5*, and *6*) (Henthorn et al., 1990). Therefore, although E12 and E47 are known to be ubiquitously expressed bHLH proteins, protein complexes which interact with OCE1 would not likely include dimers of E12/E47 proteins. Furthermore, another oligonucleotide sequence, MEF1 (muscle enhancer factor 1), which was identified as a binding site for MyoD/E12 heterodimer in muscle creatine kinase enhancer (Buskin and Hauschka, 1989), did not compete against the binding activity in ROS17/2.8 nuclear extracts to the OCE1 sequence either (Fig. 4 *B*, lanes *7*, *8*, and *9*).

Further support for OCE1 sequence-specific binding of nuclear factor(s) in ROS17/2.8 cells was provided by competition assay using other E-box sequences in the osteocalcin promoter. Competition was not observed when a 500-fold molar excess of unlabeled oligonucleotides containing OCE3 (CAGCTG at −281) or OCE4 (CAATTG at −553) was included in the binding reaction (Fig. 4 *C*). Thus, it appears that the preference for the two intervening nucleotides in the E-box in the case of OCE1 sequence could be C and A (CACATG).

The OCE1 Binding Activity Is Dependent on Osteoblastic Differentiation

To examine further the relationship between the OCE1 binding activity and osteoblastic differentiation, we performed EMSA using a cell culture model of osteoblastic differentiation. A pluripotent fibroblastic cell line, C3H10T1/2, was chosen for this experiment and was treated with rhBMP-2 to induce differentiation. As shown in Fig. 5 *A*, mRNA expression of genes encoding osteocalcin and osteopontin, two phenotype-related proteins of osteoblasts, was not detected in control C3H10T1/2 cells in Northern blot analysis (lane *1*). After treatment with rhBMP-2 (500 ng/ml) for 72 h, osteocalcin, and osteopontin mRNA expression was induced (Fig. 5 *A*, lane *2*), indicating that C3H10T1/2 fibroblasts would differentiate into osteoblasts (like-cells) in vitro. The binding activity of OCE1 in the nuclear extracts prepared from control C3H10T1/2 cells was very low (Fig. 5 *B*, lane *2*). In contrast, the OCE1-binding activity in the nuclear extracts prepared from C3H10T1/2 cells treated with rhBMP-2 was significantly increased (Fig. 5 *B*, lane *4*). The mobility

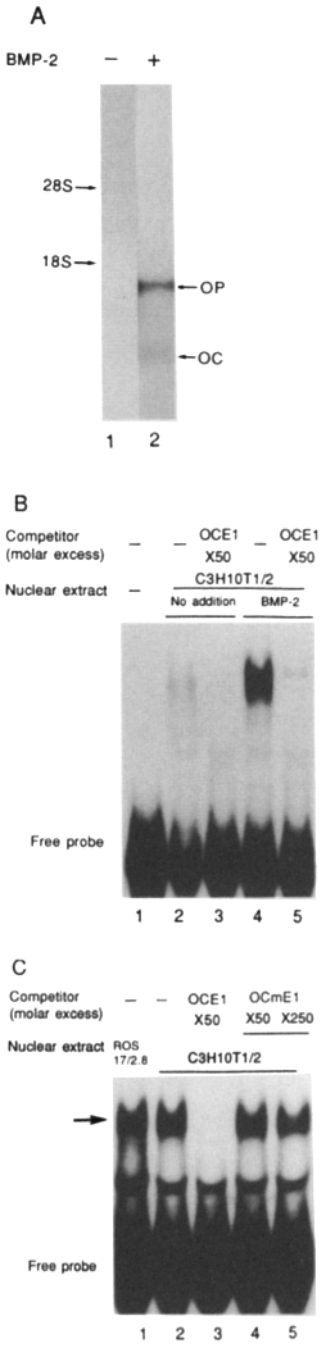

Figure 5. Effects of rhBMP-2 on osteocalcin mRNA expression and OCE1-binding activity in the nuclear extracts of C3H10T1/2 cells. (*A*) Effect of rhBMP-2 on osteocalcin and osteopontin mRNA expression in C3H10T1/2 cells. Total RNA was isolated after 72 h treatment of the cells with 500 ng/ml of rhBMP-2, and Northern blot analysis was carried out as described in Materials and Methods. Equal loading of RNA in each lane was checked by ethidium bromide staining (data not shown). Positions of osteocalcin mRNA, osteopontin mRNA, 28S, and 18S rRNA are indicated. (*B*) Effect of rhBMP-2 on OCE1-binding activity in the nuclear extracts of C3H10T1/2 cells estimated by EMSA. Nuclear extracts were prepared from C3H10T1/2 cells treated for 72 h with 500 ng/ml of rhBMP-2 (lanes *4* and *5*) and nontreated control cells (lanes *2* and *3*) and were incubated with radiolabeled OCE1 probe (20,000 cpm) followed by EMSA as described above. For competition experiments, indicated molar excess of unlabeled double stranded OCE1 competitor DNA was incubated with the nuclear extracts for 20 min prior to the addition of the probe. Lane *1* contains probe alone with no nuclear extract. (*C*) Competitive binding assay using mutant OCE1 oligonucleotide. rhBMP-2-treated C3H10T1/2 nuclear extracts (lanes *2*, *3*, *4*, and *5*) or ROS17/2.8 nuclear extracts (lane *1*) were incubated with unlabeled OCE1 (lane *3*), OCmE1 (lanes *4* and *5*) or vehicle (lanes *1* and *2*) in a buffer as described above. Radiolabeled OCE1 probe (20,000 cpm) was then added to these extracts, and protein-DNA complexes were resolved by EMSA. The fold excess of the amount of competitor is shown above each lane. Arrow indicates OCE1-specific protein-DNA complex.

of the DNA–protein complexes using C3H10T1/2 nuclear extracts was similar to those observed in the case of ROS17/2.8 nuclear extracts (Fig. 5 *C*, lane *1*). Also, similar to the ROS 17/2.8 nuclear extracts, the complex formation was competed out by an excess amount of unlabeled OCE1 (Fig. 5 *C*, lane *3*), but not OCmE1 (lanes *4* and *5*), showing the specificity of the binding. It is thus possible that similar proteins in both ROS17/2.8 cells and rhBMP-2–treated C3H10T1/2 cells would bind to OCE1. These findings suggest that high OCE1-binding activity in the nuclear extracts is present specifically in osteoblasts but not in fibroblasts and its induction is in close correlation to the differentiation of osteoblasts.

OCE1-binding Activity Can Be Blocked by GST-Id-1 Protein

To examine directly whether the DNA–protein complex formed with OCE1 contains HLH family proteins, we tested whether the DNA-binding activity can be blocked by addition of the bacterially expressed Id-1 protein to the reaction mixtures. Either GST-Id-1 fusion protein or GST alone was added to the ROS17/2.8 nuclear extracts before addition of the OCE1 probe. When GST-Id-1 protein was not denatured with guanidine-HCl, the addition of the protein did not affect formation of the DNA–protein complexes between ROS17/2.8 nuclear extracts and OCE1 (Fig. 6 *B*). On the other hand, when GST-Id-1 or GST was added to ROS17/2.8 nuclear extracts after denaturation with 6 M guanidine-HCl and renaturating, GST-Id-1 but not GST itself blocked the binding to OCE1 in a dose dependent manner (Fig. 6 *A*). These findings suggest that OCE1-binding proteins in ROS17/2.8 nuclear extracts would be heterodimers or homodimers of HLH proteins whose DNA-binding activity could be interfered by the presence of monomeric Id-1 protein.

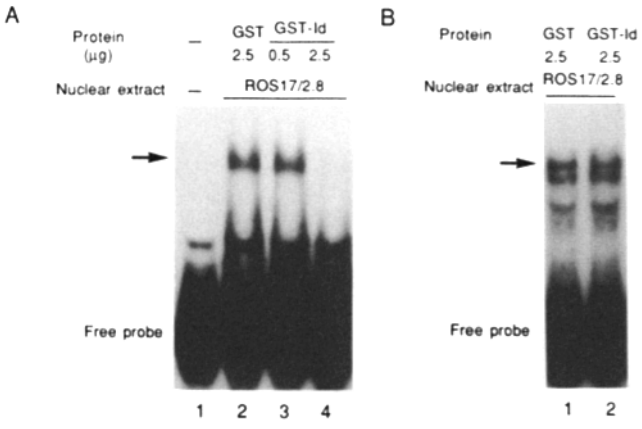

Figure 6. GST-Id-1 protein suppresses OCE1-binding activity in osteoblast-like cells. (*A*) Either bacterially expressed GST or GST-ΔNId-1 (*GST-Id-1*) proteins at the indicated doses were added to 5 μg of nuclear extracts prepared from ROS17/2.8 cells and were denatured for 1 h at 4°C by dialysis against 6 M guanidine HCl in a buffer D. The extracts were then renatured by dialysis against the same buffer without guanidine HCl. The extracts were then used for EMSA as described above. (*B*) Bacterially expressed GST or GST-ΔNId-1 (*GST-Id-1*) protein at the indicated doses were added to 5 μg of nuclear extracts prepared from ROS17/2.8 cells without denaturation and renaturation. The extracts were then subjected to EMSA as described above. Arrows indicate OCE1-specific protein–DNA complexes.

Discussion

Osteoblastic differentiation is a complex process of a series of time dependent expression of genes encoding phenotype-related proteins (for reviews see Rodan and Noda, 1991; Stein and Lian, 1993). Although responsive elements of several calcitropic regulators have been located in the promoter regions of these genes (for review see Young et al., 1993), DNA sequence(s) and the cognate transcription factor(s) involved in molecular interaction underlying osteoblastic differentiation have not yet been identified. Production of bone and bone matrix proteins, such as osteocalcin, type I collagen, osteopontin, bone sialoprotein, osteonectin and so on, is a specific function of osteoblasts and it is regulated at least in part, at transcriptional levels (Rodan and Noda, 1991). Among these proteins, osteocalcin is the only known specific product of differentiated osteoblasts. Therefore, we examined the promoter region of the osteocalcin gene in order to identify sequence(s) involved in osteoblast-specific transcription and hence required for osteoblast-specific expression. Our results presented in this report demonstrate that osteocalcin gene transcription in osteoblast is regulated via interaction between an E-box, which we call OCE1, in the promoter region and certain osteoblastic E-box–binding transcription factor(s).

bHLH proteins are involved in cell type determination and differentiation through their transcriptional activation of a repertoire of subordinate genes. bHLH proteins bind to the CANNTG motif as either homo- or heterodimers (for review see Murre and Baltimore, 1992). Dominant negative type of 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; Chiristy et al., 1991; Sun et al., 1991; Biggs et al., 1992). Id-1 appears to be expressed ubiquitously, and Id-1 mRNA expression has been observed in osteoblasts in cultures. Furthermore, Id-1 gene expression is regulated by various calcitropic agents in osteoblasts or osteoblast-like cells (Ogata and Noda, 1991; Kawaguchi et al., 1992). However, the functional significance of HLH family transcription factors have not yet been implicated in transcriptional regulation of any genes including osteocalcin gene in osteoblasts. We found that overexpression of Id-1 reduced transcriptional activity of the 1,094-bp osteocalcin promoter. Recent studies also demonstrated that similar Id-1 overexpression suppresses transcriptional activity of IgH and Igκ genes in mature B cells (Wilson et al., 1991), insulin gene in pancreatic β-cell (Cordle et al., 1991), and pro-opiomelanocorcin gene in pituitary cells (Therrien and Drouin, 1993). We also observed cotransfection of antisense Id-1 vector-enhanced transcriptional activity of the osteocalcin gene promoter. This would be due to the inactivation of the constitutively expressed Id-1 mRNA in ROS17/2.8 cells. Deletion analysis of the osteocalcin promoter region indicated that transcriptional modulation by Id-1 of rat osteocalcin gene promoter activity is mediated via a *cis*-active regulatory region between −198 and −98. Lian et al. (1989) have identified in the proximal promoter region of rat osteocalcin gene a 24-nucleotide sequence, designated as "osteocalcin box (−99 to −74)," which contains a CCAAT motif as a central element, and contributes to the basal transcriptional activity of osteocalcin gene. The promoter fragment, −198

to −98, in the rat osteocalcin gene contains two E-box sequences, OCE1 and OCE2, that suffice the consensus requirement. The introduction of mutation into OCE1 (pOC198mE1CAT) but not in OCE2 (pOC198mE2CAT) reduced the basal transcriptional activity of the osteocalcin gene. Furthermore, CAT activity of only pOC198mE2CAT but not pOC198mE1CAT was suppressed by sense Id-1 overexpression and enhanced by antisense Id-1 overexpression to a degree similar to those observed in the case of pOC198CAT (data not shown). Markose et al. (1990) reported the presence of protection in osteocalcin box juxtaposed to OCE1 by DNase I footprinting analysis. In competition binding assay, the protein complexes interacting with OCE1 site were not competed out by other E-boxes in osteocalcin gene promoter, OCE2 (CAGCTG), OCE3 (CAGCTG), or OCE4 (CAATTG). Thus, the OCE1 with CACATG type E-box motif represents a novel regulatory element of osteocalcin gene and would play a unique role in the regulation of osteocalcin promoter activity and osteoblast-specific expression of the rat osteocalcin gene. This notion is also supported by our paralleled experiments, where osteocalcin mRNA expression was suppressed significantly in ROS17/2.8 cells which were permanently transfected with sense Id-1 vector (unpublished results).

OCE1-binding activities were present in nuclear extracts prepared from ROS17/2.8 cells. The protein complexes interacting with OCE1 site were not competed out by OCE2 oligonucleotide or by the mutant oligonucleotide, OCmE1, in which TGTTAC sequence was substituted for CACATG. These results suggested that OCE1-binding activity may be required for the transcriptional activities of rat osteocalcin promoter. We and others have observed that HLH molecules, such as E12/E47 and mTwi, the murine homologue of the Drosophila twist, are expressed in osteoblasts or osteoblast-like cells (Noda, M., unpublished observation; Murray et al., 1992). However, the levels of E12/E47 mRNA expression do not appear to change during differentiation of osteoblasts. Murray et al. (1992) demonstrated that mTwi mRNA expression declined with osteoblastic maturation in mouse osteoblastic cell line MC3T3E1 like Id-1 mRNA expression (Ogata and Noda, 1991). Therefore, the protein complexes which interact with OCE1 would not likely include these known HLH molecules. In our competition assays (Fig. 4), previously characterized HLH protein binding sites were used as competitor DNAs, such as the oligonucleotide sequence, μE5 + μE2, which consists of original binding sites for E2A gene products E12 and E47 (Henthorn et al., 1990), and MEF1, which was identified as a MyoD/E12 heterodimer binding site in muscle creatine kinase enhancer (Braun et al., 1989). The failure of competition by these sequences suggest that dimers of widely distributed E12/E47 proteins or muscle-specific regulatory protein heterodimer (MyoD/E12) would not consist of the protein complexes which interact with OCE1 in ROS17/2.8 cells. We also examined the possibility that OCE1-binding proteins may share epitopes with other HLH proteins by using an antiserum against human E12 and E47 proteins (gifts from Dr. C. Murre, Whitehead Institute for Biomedical Research, Cambridge, MA) (Murre et al., 1989) in EMSA. The OCE1-binding activity in ROS17/2.8 cells was not reduced or supershifted by the presence of the antiserum (unpublished observations). Therefore, the binding complexes which interact with OCE1 would include proteins other than E12/E47 heterodimer or homodimer.

BMP-2 is sufficient by itself to induce ectopic bone formation in vivo (Wozney et al., 1988). BMP-2 was recently 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 the treatment with rhBMP-2. Our experiment indicated that the treatment with rhBMP-2 also induced osteocalcin and osteopontin mRNA expression as demonstrated by Northern blot analysis (Fig. 6 *A*). OCE1-binding activity in C3H10T1/2 nuclear extracts was very low, however it was markedly increased by the treatment with rhBMP-2. Furthermore, the size of the OCE1-binding complexes in C3H-10T1/2 nuclear extracts treated with rhBMP-2 was similar to that of the ROS17/2.8 cells nuclear extracts judging from the mobility in EMSA. These results do not contradict the idea that OCE1-binding protein(s) may be involved in osteoblast-specific transcription of osteocalcin gene.

The best-characterized members of the bHLH family involved in mammalian cell differentiation are the myogenic transcription factors including MyoD (Lassar et al., 1989), myogenin (Wentworth et al., 1991), Myf-5 (Braun et al., 1989), and MRF4 (Rhodes and Konieczny, 1989). Expression of these proteins has been implicated in both cell type determination and differentiation in skeletal muscle cells. Forced expression of these proteins converts nonmyoblastic cells such as fibroblasts into myocytes (Davis et al., 1987; Weintraub et al., 1989). "Loss of function" experiments with myogenin gene are lethal perinatally due to 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., 1992). The other bHLH protein, SCL, is expressed during early hematopoiesis and possesses a differentiation-related DNA-binding motif (Visvader and Begley, 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). Efficient osteoblast-specific expression of osteocalcin gene may require an OCE1 sequence that bind to transcription factor(s) expressed specifically in osteoblast as well as bHLH proteins which are expressed ubiquitously in many cell types. The suppression of osteocalcin promoter activity due to Id-1 overexpression and the inhibition of the OCE1-binding activity in ROS17/2.8 cells by the addition of GST-Id-1 further support the idea that HLH factors are involved in osteocalcin gene expression. Thus, in addition to the role of HLH molecules in skeletal muscle, adipocyte, pancreas, and lymphocyte, this study provides an evidence for a possibility that certain HLH factors are involved in osteoblast-specific gene transcription and osteoblastic differentiation. If such osteoblast-specific HLH transcription factors exist, these factors may be involved in osteoblast differentiation in general in addition to acting as transcription factors for osteocalcin gene. Our on-going studies are aimed at identifying these osteoblast-specific

HLH factors and exploring their roles in osteocalcin gene regulation and osteoblastic differentiation.

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