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Developmental Regulation of Osteocalcin Expression in MC3T3-E1 Osteoblasts: Minimal Role of the Proximal E-Box *cis*-Acting Promoter Elements

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Abstract Osteoblasts undergo a temporal sequence of development characterized by transcriptional upregulation of osteoblast-specific genes. Basic helix-loop-helix (bHLH) transcription factors may control this developmental process through binding to E-box *cis*-acting elements in developmentally regulated genes. To investigate the role of bHLH proteins in MC3T3-E1 osteoblasts, which undergo a developmental sequence in vitro, we analyzed the transcriptional control of osteocalcin gene expression by stable transfection of an osteocalcin promoter–luciferase chimeric gene (p637OC-luc) and assessed the role of E-box *cis*-acting elements in osteocalcin promoter by DNA binding assays. We compared our findings in MC3T3-E1 osteoblasts with transient DNA transfections and DNA binding experiments in Ros 17/2.8 osteoblasts. We found that the activity of 637-OC luciferase promoter was low in undifferentiated 5-day-old cultures but increased in parallel with endogenous osteocalcin message expression in mature MC3T3-E1 osteoblasts, consistent with developmental stage-specific transcriptional upregulation of the osteocalcin gene. We identified two putative E-box elements in the proximal osteocalcin promoter, E-box 1 (CACATG) at −102 and E-box 2 (CAGCTG) at position −149. In gel mobility shift assays, factors present in nuclear extracts derived from differentiated osteoblast bound to oligonucleotide probes containing the E-box 1 and E-box 2 elements. Binding to the E-box 2 probe was not specific for the core CAGCTG element, whereas the CACATG site in E-box 1 oligonucleotide was required for specific binding of these nuclear factors. Stable transfection of p637OC-luc containing a mutant E1 site (p637OC-luc E1m), however, did not alter the developmental upregulation of osteocalcin promoter activity in MC3T3-E1 osteoblasts. Moreover, the E-box 1 mutation had no effect on either basal or vitamin D–stimulated activity of the osteocalcin promoter in Ros 17/2.8 osteoblasts in transient transfection experiments. These data suggest that osteoblasts contain undefined factors that bind to the E-box 1 CACATG site in the proximal osteocalcin promoter; however, this E-box element does not play a significant role in the developmental stage-specific regulation of the osteocalcin gene in MC3T3-E1 osteoblasts. J. Cell. Biochem. 65:11–24.

Key words: basic helix-loop-helix proteins; E-box; differentiation; transcription; transfection; osteocalcin; Id

Osteoblasts undergo a temporal sequence of development characterized by the initial proliferation of undifferentiated cells followed by a step-wise postmitotic expression of maturation associated genes [Owen et al., 1990; Tonna and Cronkite, 1961; Aubin et al., 1982]. Recent efforts have focused on osteocalcin to define the factors responsible for transcriptional control of osteoblastic osteoblast-specific gene expression [Yoon et al., 1988; Desbois et al., 1994; Bortell et al., 1993]. Most analysis has employed transient transfection experiments to compare osteocalcin promoter function in nonosteoblastic cell lines with malignant osteosarcoma cells, such as Ros 17/2.8 osteoblasts. Such investigations have identified putative cis-acting elements in the proximal osteocalcin promoter and trans-activating factors, such as MSX-2 [Towler et al., 1994; Hoffman et al., 1994], PEBP2 alpha/AML-1 [Banerjee et al., 1996; Geoffroy et al., 1995], and members of the basic-helix-loop helix (bHLH) family of transcription factors [Glackin et al., 1992; Murray et al., 1992; Ogata and Noda, 1991; Tamura and Noda, 1994], that regulate osteoblast-specific gene expression. Though comparison of osteoblastic with nonosteoblastic cell lines provides some insights into the developmental process,

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such studies do not permit the examination of temporal changes in gene expression within the osteoblast lineage. The *cis*-acting elements and transcriptional factors that control the temporal pattern of gene expression may differ from transcriptional elements that determine osteoblast specificity.

The study of transcriptional control of osteoblast development requires assessment of promoter activity at different stages of the normal developmental sequence. Information regarding the transcriptional control of development is difficult to obtain from malignant transformed osteoblasts because these cells lack a temporal sequence of differentiation. In addition, osteosarcoma cells lack the precise coordination of cell proliferation and differentiation that characterize the development of normal diploid osteoblasts [Pardee et al., 1978]. Finally, stable transfection and genomic integration of promoter segments, rather than transient transfection of small promoter fragments, may be necessary to define the factors responsible for the transcriptional control of development [Frenkel et al., 1996]. Consequently, many questions remain about the precise molecular mechanisms that control stage-specific expression of the osteocalcin gene during the temporal sequence of osteoblast development.

A better understanding of the differential utilization of *cis*-acting elements and corresponding changes in trans-acting factors during the multistepped process of differentiation would be facilitated by an in vitro model system of osteoblast development. Normal diploid osteoblasts, which undergo development in culture, are not ideal for promoter studies due to cell heterogeneity and difficulties related to creating stable transfection in primary cultures. A possible alternative is the clonal MC3T3-E1 osteoblast cell line, a nontransformed clonal cell line that resembles primary osteoblasts cultures [Sudo et al., 1983; Quarles et al., 1992]. In this regard, MC3T3-E1 cells in culture undergo growth arrest, form a mineralizable extracellular matrix, and express differentiation markers such as alkaline phosphatase and osteocalcin in a developmental stage-specific fashion [Quarles et al., 1992]. Moreover, MC3T3-E1 cells can be stably transfected without losing their capacity to differentiate in vitro [Phillips et al., 1994].

Recent studies indicate that members of the superfamily of bHLH transcriptional factors may control the temporal sequence of differentiation in MC3T3-E1 osteoblasts. bHLH proteins, which regulate development in a variety of tissues, consist of tissue specific factors (*e.g.*, myoD in muscle) and ubiquitous non-cell-specific bHLH proteins (*e.g.*, E12) that form heterodimers [Edmonson and Olson, 1993; Cordle et al., 1991; Wilson et al., 1991; Kreider et al., 1992]. These heterodimers activate gene transcription by binding to consensus E-box domains ("CANNTG") in the promoter/enhancer region of target genes [Lassar et al., 1991; Tapscott and Weintraub, 1991]. In addition, HLH proteins lacking the basic DNA binding domain, such as Id (inhibitor of differentiation), act as negative regulatory elements by forming heterodimers with bHLH proteins that are incapable of DNA binding [Pesce and Benezra, 1993; Barone et al., 1994; Sun et al., 1992]. With regard to osteoblasts, Id-1 is abundantly expressed in proliferating undifferentiated preosteoblasts and decreases prior to osteoblast-specific gene expression [Ogata and Noda, 1991; Murray et al., 1992]. In addition, many factors which promote osteoblast differentiation, such as vitamin D, are associated with decrements in Id [Yoon et al., 1988] and increased binding of nuclear factors to a generic E-box motif [Kawaguchi et al., 1992]. Moreover, overexpression of Id-1 prevents the temporal sequence of development in the MC3T3-E1 osteoblasts [Murray et al., 1992], consistent with competition between Id and putative osteoblast-specific bHLH factors [Benezra et al., 1990]. Finally, a putative E-box *cis*-acting element, E-box 1 (which consists of a CACATG motif located at −102 relative to the transcription initiation site) has been identified in the rat osteocalcin promoter. This site has been shown to bind putative transcription factors and regulate expression of a minimal osteocalcin promoter in Ros17/2.8 osteosarcoma cells [Tamura and Noda, 1994], consistent with the presence of E-box–dependent gene transcription in osteoblasts.

Though these findings implicate bHLH nuclear binding proteins in osteoblast differentiation, the role of E-box elements in regulating the developmental stage-specific expression of osteoblast specific genes has not been investigated. In the current investigations we examined MC3T3-E1 osteoblasts for expression of maturation-associated bHLH proteins by DNA binding assays and investigated the role of

proximal E-box promoter elements in the transcriptional upregulation of osteocalcin during osteoblast development by stable DNA transfection experiments.

MATERIALS AND METHODS

Materials

All chemicals used were of reagent grade or molecular biology reagent grade. 1,25 dihydroxy vitamin D was a generous gift of Dr. Milan Uskokovic (Hoffman-La Roche Inc., Nutley, NJ); recombinant human bone morphogenic protein-2 (rhBMP-2) and the rat osteocalcin probe were obtained from Genetics Institute (Cambridge, MA). The Id-1 probe was a gift of Dr. Weintraub (Fred Hutchinson Cancer Research Center, Seattle, WA). pGEX-Id was a gift of Dr. Benezra (Memorial Sloan Kettering Cancer Center, New York, NY). The rat p637OC-luc was a gift from Dr. Towler and Dr. Rodan (Merck, West Point, PA). The E12 rabbit polycolonal IgG antibody (H-208) was obtained from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Cell culture media and fetal bovine serum (FBS) were obtained from Gibco (Gaithersberg, MD) and Hyclone (Logan, UT) laboratories.

Cell Culture

Ros 17/2.8 osteoblasts were grown in one-to-one mixture of Dulbecco's modified Eagle's medium and Nutrient Mixture F-12 (DMEM-F12) supplemented with 10% (V/V) FBS. MC3T3-E1 cells were grown in α minimum essential medium (αMEM) supplemented with 10% (V/V) FBS, 0.13 mM of ascorbic acid, 5 mM β-glycerol phosphate, and 100 units/ml penicillin and streptomycin. All cell cultures were incubated in a humidified incubator with 5% CO_2 at a temperature of 37°C.

Transient Transfections

Ros 17/2.8 cells were transfected by modifications of the DEAE-Dextran protocol [Lopata et al., 1984]. Briefly, cells were plated at a density of 8×10^5 cells/60 mm culture dish 1 day prior to transfection. Cells were transfected in duplicate or triplicate, and transfection efficiencies were monitored with parallel transfection with a Rous sarcoma virus LTR (RSV) promoter/luciferase reporter construct (RSV luc). We used 5 μg/ml of OC luc constructs in each transfection. DNA was added to cells in 1.0 ml of working solution containing DMEM-F12, 50 mM Tris-HCl (pH 7.3), 20 mM HEPES (pH 7.2), and 200 μg/ml DEAE-Dextran. Twenty minutes after the addition of DNA, the transfection solution was diluted by the addition of serum-free DMEM-F12 medium, and the cells were incubated for an additional 20 min. Following aspiration of the media, the cells were subjected to DMSO shock by treatment with 1 ml of 10% DMSO in a Hepes-buffered sodium phosphate solution (21 mM Hepes, pH 7.1, 5 mM KCl, 0.7 mM Na_2HPO_4, 137 mM NaCl, and 6 mM dextrose) for 2 min at room temperature. Next, we removed the DMSO solution, washed the cells twice with calcium- and magnesium-free phosphate buffered saline (pH 7.1), and then incubated the cells in fresh complete media for the specified times. Luciferase activity was measured using the Luciferase assay system (Promega, Madison, WI) as per the manufacturer's protocol using a BioOrbit 1251 luminometer (Turku, Finland).

Creation of Stably Transfected MC3T3-E1 Osteoblasts With Rat OC Promoter/Luciferase Reporter Constructs

Stable transfection of MC3T3-E1 was performed by a pooled protocol as previously described [Phillips et al., 1994]. MC3T3-E1 cells were plated at a density of 100,000 cells/60 mm dish the day before transfection. The following day the cells were cotransfected using Lipofectin reagent with 9 μg of the rat osteocalcin promoter luciferase promoter construct, p637OC-luc plasmid [Towler et al., 1994], which consists of a genomic sequence from nucleotides −637 to the start site of transcription, and 1 μg of the plasmid pSV2-neo, a plasmid in which the expression of the *neomycin* resistance gene is controlled by regulatory signals from the SV40 early region. After 48 h of incubation, the transfected cells were detached and plated on a single 100 mm diameter dish in MEM containing 10% FBS. Transfectants were selected in the presence of the selection antibiotic G418 (500 μg/ml).

Site-Directed Mutagenesis

Site-directed mutagenesis was performed according to modifications of the overlap extension technique using polymerase chain reaction (PCR) by modifications of previously described methods [Ho et al., 1989]. Complementary oligonucleotide primers were designed to introduce TGTTAC for the CANNTG site of E-box 1 and

E-box 2 of the 6370C-luc promoter. The primers used for PCR were as follows:

E1F 5′-CCTATTGCGTGTTACACCCCCAATTAGTCC-3′

E1R 5′-ATTGGGGGTGTAACACGCAATAGGTCAAAC-3′

E2F 5′-TCGCCCCGGTGTTACCAGTCACCAACCACA-3′

E2R 5′-TGGTGACTGGTAACACCGGGGCGAAGGCTG-3′

VF 5′-CATAACCCGGGAGGTACC-3′

VR 5′-CTTAGATCTCGAGCTAGC-3′.

Initially, we used primer pairs VF (vector forward) and E1R and VR (vector reverse) and E1F to amplify from 637OC-luc two DNA fragments having overlapping ends. These fragments were purified by agarose gel electrophoresis and then combined in a subsequent fusion reaction in which the overlapping ends were annealed, allowing the 3′ overlap of each stand to serve as a primer for 3′ extension of the complementary strand. The resulting fusion product was amplified by PCR using the VF and VR primers to generate a full-length segment containing the mutation. The PCR product was ligated into the KpnI/Mlui-digested promoterless pGL2-Basic GeneLight Plasmid (Promega) containing the luciferase reporter gene. Using a similar strategy, we also mutated the E-box 2 site in p637OC-luc to TGTTAC by PCR-mediated site-directed mutagenesis. The mutations in both constructs were confirmed by sequencing.

Nuclear Extract Preparation

Nuclear extracts were prepared by a modification of a previously described method [Dignam et al., 1983]. Confluent cultures of MC3T3-E1 and Ros 17/2.8 were treated for 48 h with either vitamin D at a final concentration of 2×10^{-8} M or recombinant human bone morphogenic protein-2 (rhBMP-2) at a final concentration of 10 ηg/ml. Nuclear extracts were prepared from give confluent plates in each treatment group as follows. After induction of differentiation, cells were washed with ice-cold phosphate-buffered saline containing 0.5 mM dithiothretol (DTT), 0.2 mM phenylmethylsulfonyl fluoride (PMSF), 2 μM leupeptin, 5 mM NaF, and 1 mM Na_3VO_4. The cells were sedimented by centrifugation at 500*g* at 4°C for 5 min and incubated in 7 ml of hypotonic buffer containing 10 mM Tris, pH 7.9, 10 mM KCl, 1.5 mM $MgCl_2$, 5 mM NaF, 0.5 mM Na_3VO_4, 0.5 mM DTT, 2 μM leupeptin, and 0.2 mM PMSF for 10 min and then homogenized with ten strokes of a Dounce glass homogenizer. The resulting nuclei were collected by centrifugation at 2,300*g* for 5 min at 4°C, resuspended in 0.2 ml of extraction buffer containing 20 mM Tris pH 7.9, 0.5 M KCl, 1.5 mM $MgCl_2$, 5 mM NaF, 0.5 mM Na_3VO_4, 1 mM DTT, 2 μM leupeptin, 0.5 mM PMSF and 20% glycerol, and gently mixed by rotating the suspension for 30 min at 4°C. The nuclear extracts were obtained by centrifuging the resulting mixture at 15,000*g* for 15 min at 4°C and collecting the supernatant. The supernatant was dialyzed against dialysis buffer (20 mM Tris, pH 7.9, 50 mM KCl, 5 mM NaF, 0.1 mM Na_3VO_4, 0.2 mM EDTA, 1 mM DTT, 0.5 mM PMSF, and 20% glycerol), cleared by centrifugation at 15,000*g* for 15 min at 4°C, and stored at −70°C for later use.

Gel Mobility Shift Assay

To determine the presence of E-box binding factors in osteoblast nuclear extracts, we used a 30 base pair double-stranded oligonucleotide containing either the E-box 1 or E-box 2 site from the osteocalcin promoter [Yoon et al., 1988] in gel mobility shift assays. In addition, we used oligonucleotides in which the E-box site was mutated. The E-box oligonucleotides were synthesized on a DNA synthesizer (Applied Biosystems International, Foster City, GA). The sequences of these oligonucleotides (the putative E-box binding domain and the mutated sequence are underlined) used in all binding reactions are as follows:

E1 5′TCGACTTGACCTATTGCGCACATGACCCCG

E1m 5′TCGACTTGACCTATTGCGTGTTACACCCCG

E2 5′GATCGCCCCGGCAGCTGCAGTCACCAACCA

E2m 5′GATCGCCCCGGTTTTTTCAGTCACCAACCA.

The double-stranded oligonucleotide was labeled using the large fragment of DNA polymerase and [α-^{32}P] dATP 3,000 Ci/mmol (New England Nuclear, Boston MA). The unincorporated label was separated from the labeled oligonucleotide using a Sephadex G-50 gravity flow column (Pharmacia, Uppsala, Sweden). The DNA binding assay was performed as follows. Nuclear protein fraction (10 µg) from osteoblastic cells was preincubated for 5 min at room temperature in a final volume of 20 µl containing 10 mM Tris-HCl, pH 8.0, 50 mM NaCl, 1 mM EDTA, 5% Glycerol, 4 mM dithiothretol, and 2 µg poly (dI-dC). This was followed by the addition of 1 µl of the radiolabeled oligonucleotide (100,000 cpm) and incubation of the mixture for an additional 20 min at room temperature. When antibodies were used in the reaction, the final mixture was incubated for 20 min at 30°C. The mixture was subjected to electrophoresis using a 4% acrylamide gel in 0.25× TBE (0.1 M Tris, pH 8.3, 0.1 M boric acid, 20 µM EDTA) at 150 v. The gels were then dried and analyzed by autoradiography. Quantifying of band intensity was accomplished by scanning densitometry (GS 300 Transmittance Scanning Densitometer; Hoefer Scientific Instruments, San Francisco, CA).

RNA Extractions

Total cellular RNA was isolated by the guanidine isothiocyanate method [Chomczynski and Sacchi, 1987]. Cells were lysed by the addition of 1 ml of a denaturing solution containing 4 mM guanidine thiocyanate, 25 mM sodium citrate, pH 7.0, 0.5% sarcosyl, and 0.1 M 2-mercaptoethanol. The cell lysate was transferred to a 15 ml polypropylene centrifuge tube (Corning, Corning, NY), and the following were added to the lysate in the specified order: 0.1 ml of 2 M sodium acetate, pH 4.0, 1 ml of TE (pH 8) saturated phenol, and 0.2 ml of chloroform: isoamyl alcohol (49:1). The resulting solution was centrifuged at 10,000*g* for 20 min at 4°C and the upper aqueous phase collected. The RNA was precipitated by addition of 1 ml of isopropanol to the aqueous phase, followed by incubation at −20°C for 2 h and centrifugation at 10,000*g* for 20 min at 4°C. The supernatant was discarded, and the RNA pellet was washed with cold 70% ethanol. The washed RNA pellet was dissolved in sterile water and stored at −70°C for future use.

Northern Blot Hybridizations

For Northern blot hybridization, 15 µg of total RNA was loaded on a 1.2% formaldehyde agarose gel and electrophoresed in 1× MOPS buffer (0.02 M MOPS, pH 7.0, 5 mM sodium acetate, 1.0 mM EDTA). The RNA was transferred to nytran membrane (Schleicher & Schuell, Keene, NH) by overnight capillary transfer in 10× SSC (1.5 M sodium chloride, 0.15 M sodium citrate, pH 7.0) and the RNA immobilized on the membrane by UV crosslinking using a Stratalinker (Stratagene, La Jolla, CA). Northern analysis was carried out as described [Sambrook et al., 1989]. Briefly, the blot was prehybridized at 42°C in a solution containing 50% deionized formamide, 2.5× SSC, 50 mM sodium phosphate, pH 6.5, 1× Denhardt's solution, 0.5% SDS, and 100 µg salmon sperm DNA. The blot was hybridized overnight at 42°C in the prehybridization solution containing 10% Dextran sulphate and 2×10^6 cpm/ml of the random labeled osteocalcin probe. The blot was washed 4× 5 min at room temperature in a solution containing 2× SSC and 0.1% SDS, followed by washing at 50°C in a solution containing 0.1× SSC and 0.1% SDS. The blot was air-dried and bands visualized by autoradiography.

Protein Purification and Determination

Fusion protein pGEX-Id and the pGEX protein was bacterially expressed and purified according to prior techniques (Ausubel, 1993). Proteins analysis was performed with a Bio-Rad Protein assay kit (Bio-Rad Laboratories, Hercules, CA) based on the Bradford method using bovine serum albumin as the standard [Bradford, 1976].

Data Analysis

Analysis of variance was performed on data derived from transfection assays with the Statgraphics software package (Statistical Graphics Corp., Inc., Princeton, NJ) using a Northgate Elegance "ZXP" (Minneapolis, MN).

RESULTS

Developmental Stage-Specific Transcriptional Upregulation of the Osteocalcin Gene in MC3T3-E1

We initially assessed the effects of maturation stage on the expression of osteocalcin message and osteocalcin gene transcription in MC3T3-E1 osteoblasts. Different stages of

maturation were accomplished by growing MC3T3-E1 cells in media containing serum supplemented with ascorbic acid and β-glycerol phosphate for various time periods. We observed time-dependent increases in endogenous osteocalcin mRNA levels during osteoblastic differentiation in MC3T3-E1 cells (Fig. 1A), consistent with developmental regulation of this gene. Indeed, osteocalcin message, identified as a 0.6 kb transcript, was not detected in phenotypically immature 5-day-old cells but was expressed at high levels in differentiated 17-day-old MC3T3-E1 osteoblasts (Fig. 1A). The time of osteocalcin expression varied between different experiments, but significant amounts of osteocalcin message was not observed prior to day 10 of culture in most of our experiments.

To document that the increase in osteocalcin expression was due to developmental dependent increase in gene transcription, we analyzed the 5′-flanking region of the osteocalcin gene in MC3T3-E1 osteoblasts. For these studies we stably transfected MC3T3-E1 cells with p637OC-luc, a chimeric construct containing the proximal 637 bp region of the rat osteocalcin promoter linked to a luciferase reporter gene [Towler et al., 1994]. The 637 bp region of the osteocalcin promoter contains four putative E-box elements as well as several other cis-acting elements required for osteoblast specific gene expression (Fig. 2) [Yoon et al., 1988; Desbois et al., 1994]. We found that promoter activity (expressed as either as luciferase activity per microgram of protein (Fig. 3A) or per DNA content [Fig. 3B]) increased as a function of culture duration in parallel with upregulation of the endogenous OC message (Fig. 1A). Analysis of endogenous osteocalcin message expres-

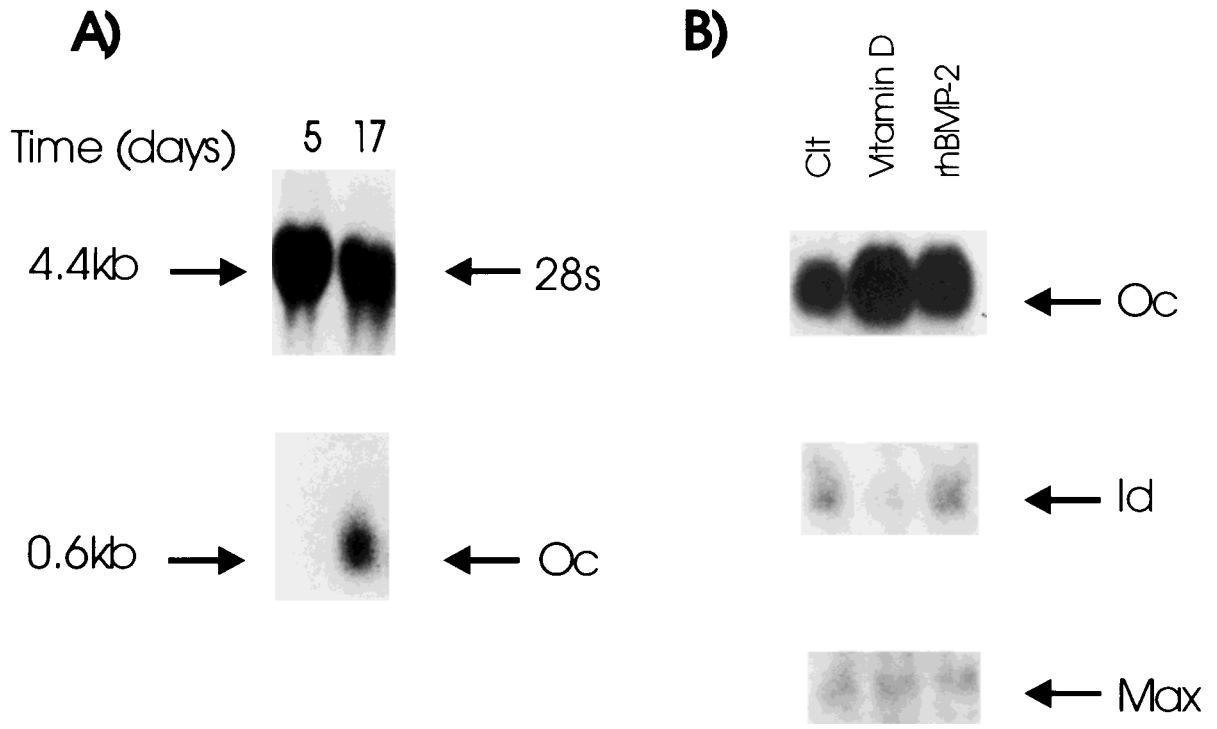

Fig. 1. Northern analysis of osteocalcin message during development in MC3T3-E1 osteoblasts and after 1,25 dihydroxyvitamin D and rhBMP-2 treatment in Ros 17/2.8 osteoblasts. **A:** Total RNA (15 μg), isolated from MC3T3-E1 osteoblasts grown for 5 and 17 days in the presence of ascorbic acid and β-glycerol phosphate, was hybridized with an osteocalcin or 28S probe as indicated. Five-day-old cultures displayed no detectible osteocalcin message, whereas by day 17 MC3T3-E1 osteoblasts demonstrated marked increases in osteocalcin mRNA levels shown as a 0.6 kb transcript. 28S, represented by a 4.4 kb band, was included to show that differences in OC mRNA levels are not explained by RNA loading. **B:** Total cellular RNA was isolated from Ros 17/2.8 cells stimulated to differentiate by treatment with either 2×10^{-8} M 1,25 dihydroxyvitamin D or 10 ηg/ml rhBMP-2 for 48 h and 15 μg used for Northern blot analysis by hybridizing with radiolabeled cDNA probes for OC or Id. Treatment with either vitamin D or rhBMP-2 for 48 h notably increased osteocalcin mRNA levels in Ros 17/2.8 cells compared to unstimulated controls. Id mRNA, detected as a 1.1 kilobase band, was present at high levels in undifferentiated cells but decreased following vitamin D treatment and did not change in response to rhBMP-2. The constitutively expressed β-zip transcription factor, Max, identified as a 2.0 kilobase band, was included as a control for RNA loading.

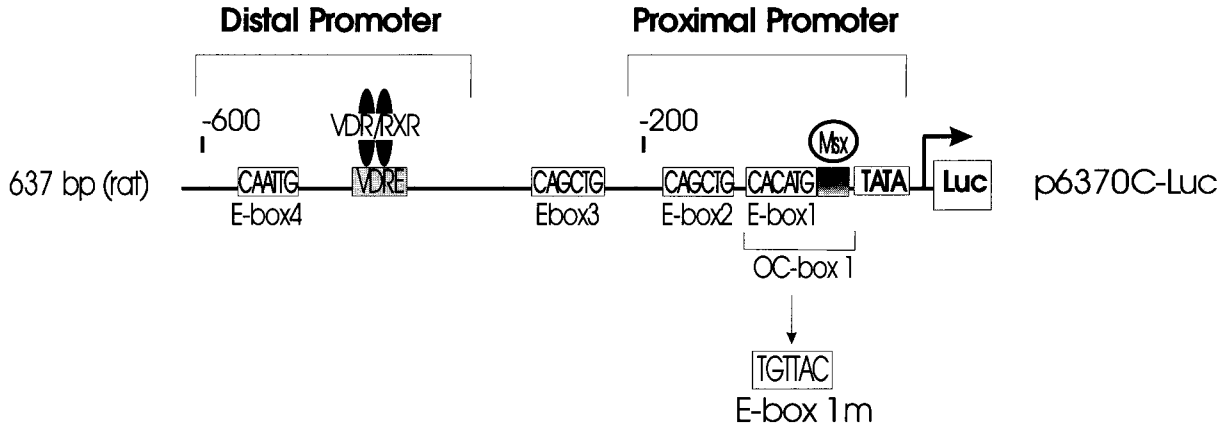

Fig. 2. Location of putative E-box elements in the osteocalcin promoter luciferase chimeric construct. The p637OC-luc construct contains 637 bp of the rat osteocalcin gene promoter 5′ to the transcription start site subcloned into pGL2-Basic Gene Light Plasmid (Promega) containing the luciferase (Luc) gene. Numbers represent the relative positions of the 5′ nucleotides in each *cis*-acting element. Four putative E-box elements (CANNTG) are located within the first 600 bps of the OC promoter. Site-directed mutants of E-box 1 (CACATG) to E-box 1m (TGTTAC) indicated by the *arrow* was performed using an overlap extension technique using PCR as described in Materials and Methods. The tissue-specific OC-1 box (shown in *brackets*), the vitamin D hormone responsive element, and the MSX homeodomain binding site are also shown for reference.

sion in stably transfected MC3T3-E1 demonstrated developmental stage-dependent increases similar to that observed in untransfected controls (data not shown). These findings indicate that MC3T3-E1 osteoblasts are a suitable model to study stage-specific regulation of the osteocalcin gene.

Interaction of Nuclear Proteins Derived From MC3T3-E1 Osteoblasts With Putative E-Box Domains in the Osteocalcin Promoter

The presence of HLH proteins in differentiated osteoblasts was investigated by electrophoretic mobility shift assays (EMSA) with oligonucleotides encoding two potential E-box *cis*-acting elements (CACATG and CAGCTG) located at −149 to −144 and −102 to −97 of the transcription start site of the osteocalcin promoter, respectively [Yoon et al., 1988] (Fig. 2). We evaluated these two elements because they are located in the proximal (−199 to 32) of the promoter that is involved in expression of osteocalcin in osteoblast cell lines [Towler et al., 1994].

We first performed gel shift analysis using oligonucleotide probes containing either the E-box 1 or E-box 2 site and nuclear extracts derived from mature MC3T3-E1 osteoblasts (Fig. 4A,B). Incubation of the E1 probe with MC3T3-E1 nuclear extracts obtained from 14-day-old MC3T3-E1 cells formed a predominant retarded band (denoted by the arrow) which was completed by excess amount of unlabeled E1 probe (Fig. 4A; compare lanes 1 and 2). Competition with the oligonucleotide in which the E-box binding element CACATG was mutated to TGTTAC (E1m) revealed that this band has specific binding for the E-box 1 motif (Fig. 4A, lanes 1,3). Vitamin D treatment had little effect on binding to this E-box 1 in MC3T3-E1 osteoblasts (data not shown). Preincubation of the gel shift complex derived from MC3T3-E1 osteoblasts with an antibody recognizing E12 failed to shift the observed binding (Fig. 4A; compare lanes 4 and 5 with lane 1). In addition, nuclear extracts from 5-day-old cultures revealed a similar binding pattern with E-box 1 oligonucleotide (data not shown).

Next, we tested osteoblastic nuclear extracts for the presence of factors that bind to an oligonucleotide probe containing the E-box 2 site (CAGCTG) in the osteocalcin promoter (Fig. 2). We observed several retarded bands following incubation with nuclear extracts derived from MC3T3-E1 (Fig. 4B). These bands were competed by excess cold E2 (Fig. 4B; compare lanes 2 and 3). In addition, we observed an increase in binding of these bands in response to vitamin D and rhBMP-2 stimulation (Fig. 4B; compare lanes 2 with lanes 4 and 5).

E-Box Binding Activity in Ros 17/2.8 Osteoblasts

We compared these results in MC3T3-E1 osteoblasts to osteocalcin message expression and E-box binding activity in Ros 17/2.8 osteosarcoma cells. Unlike MC3T3-E1 cells, Ros 17/2.8

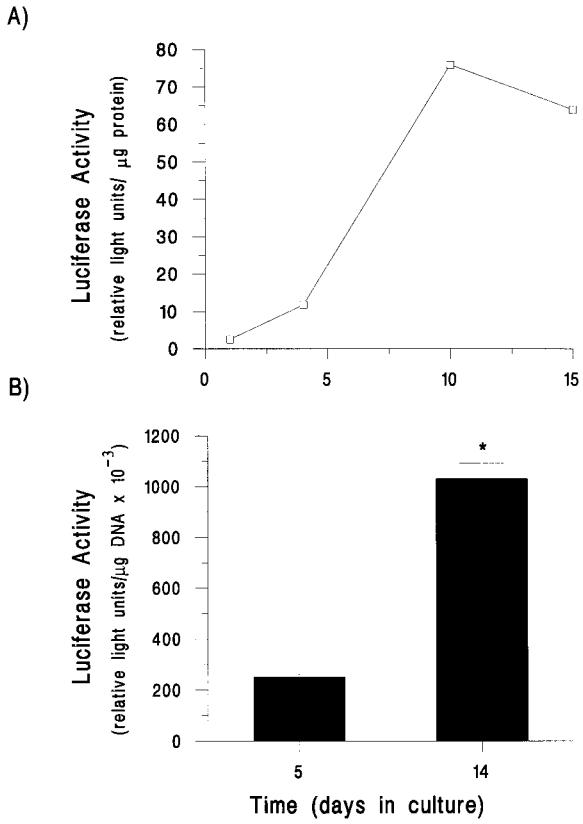

Fig. 3. Developmental regulation of OC promoter-luciferase chimeric constructs stably transfected into MC3T3-E1 osteoblasts. MC3T3-E1 cells were stably transfected with p637 OC-luc as described in Materials and Methods. Luciferase enzymic activity was analyzed in transfected cells at various times during a 14 day culture period under optimal differentiating conditions. The transcriptional activity of the osteocalcin promoter corrected for total protein (**A**) or cell number (**B**) increased as a function of culture duration and in parallel with the observed developmental-dependent increase in osteocalcin mRNA (Fig. 1). Values in B represent the mean ± SEM of three separate determinations.

osteoblasts constitutively express high levels of osteocalcin message as well as Id-1 mRNA levels (Fig. 1B). Treatment of Ros 17/2.8 osteoblasts with 1,25 dihydroxy vitamin D (2×10^{-8} M), however, further increased osteocalcin message and decreased expression of Id-1 (Fig. 1B). This reciprocal relationship between Id and osteocalcin expression is similar to that observed in mature MC3T3-E1 osteoblasts [Ogata and Noda, 1991; Murray et al., 1992]. Thus, Ros17/2.8 osteosarcoma cells provide another model to study the role of bHLH proteins in regulating osteocalcin gene expression.

Ros 17/2.8 nuclear extracts displayed binding to the E1 oligonucleotide (Fig. 4C, lane 1). Vitamin D treatment, however, had little effect on binding to this E-box 1–specific site (Fig. 4C; compare lanes 1 and 2) in Ros 17/2.8 osteoblasts. The observed gel shift complex in Ros 17/2.8 osteoblasts was competed by addition of cold unlabeled E-box 1 oligonucleotide (100-fold excess) (Fig. 4C; compare lanes 1 and 3), whereas addition of similar amounts of an oligonucleotide containing the mutated E-box 1 site (Em1) did not perturb the observed gel shift complex (Fig. 4C; compare lanes 1 and 4), indicating specific binding to the CANNTG element. Preincubation of the gel shift complex derived from Ros 17/2.8 with an antibody recognizing E12 failed to shift the observed binding (Fig. 4C, compare lanes 1 and 5), suggesting that the ubiquitous E12 bHLH protein may not be the partner to this putative bHLH protein. Nevertheless, these observations in MC3T3-E1 and Ros 17/2.8 osteoblasts demonstrate that nuclear factors are present in differentiated osteoblast that display specific binding to the CACATG E-box 1 element in the osteocalcin promoter.

We also tested Ros 17/2.8 nuclear extracts for the presence of factors that bind to an oligonucleotide probe containing the E-box 2 site (CAGCTG) in the osteocalcin promoter (Fig. 2). Similar to MC3T3-E1 osteoblasts, we observed several retarded bands following incubation with nuclear extracts derived from Ros 17/2.8 with the E-box 2 oligonucleotide probe (Fig. 4D). In addition, we observed an increase in binding of these bands in response to vitamin D and rhBMP-2 stimulation (Fig. 4D). Binding of Ros 17/2.8 nuclear extracts to E-box 2 probe was competed by excess cold E2 (Fig. 5A). However, additional studies indicate that the observed binding was not specific for the CAGCTG site in the E-box 2 probe. Indeed, we found that incubation with excess of an unlabeled E-box 2 oligonucleotide in which the CAGCTG site was mutated to TTTTTT competed the binding as effectively as excess cold E-box 2 oligonucleotide without the mutated E-box site (Fig. 5A). However, we found that preincubation with purified pGEX-Id protein (0.4 µg) with nuclear extracts from differentiated Ros 17/2.8 osteoblasts disrupted the binding to the E-box 2 oligonucleotide (Fig. 5B). These findings suggest that in vitro disruption of bHLH heterodimeric complex formation by excessive amounts of purified Id protein is nonspecific under these study conditions. In addition, the E-box 2 probe

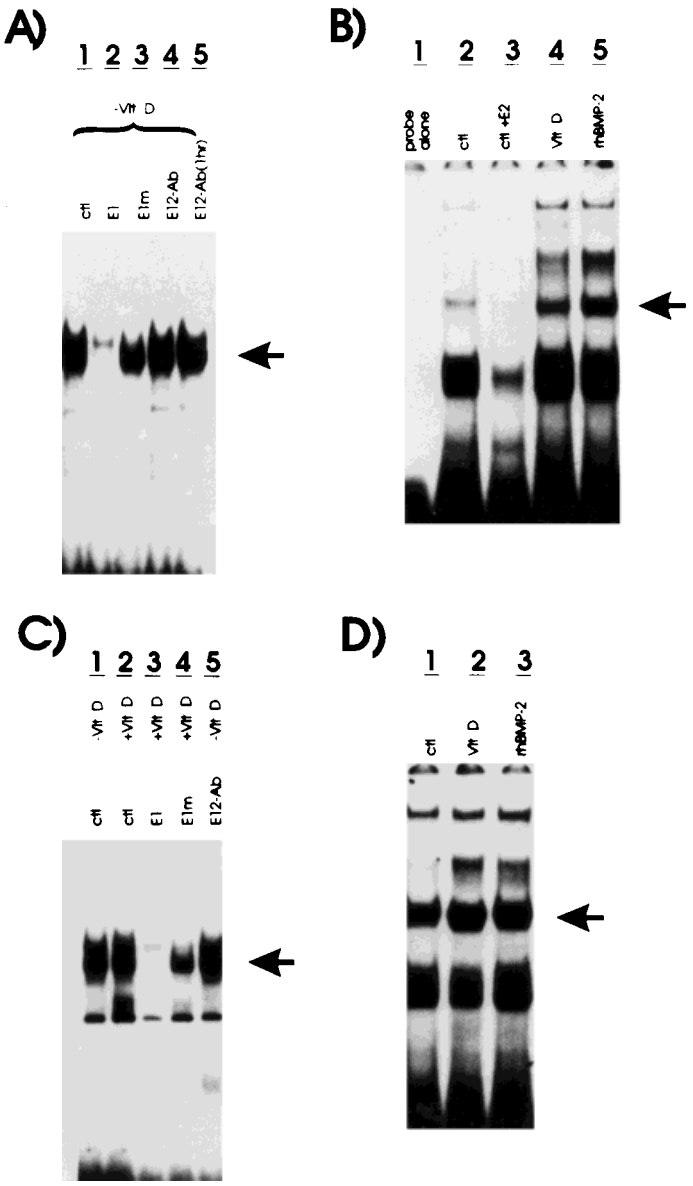

Fig. 4. Gel mobility shift assay of E-box 1 and 2 binding in osteoblasts. Electrophoretic mobility shift assays were carried out using 10 µg of nuclear protein from mature MC3T3-E1 cells grown for 14 days in the presence of differentiation medium and absence of vitamin D treatment (A,B) or 10 µg of nuclear protein from Ros 17/2.8 cells treated with 2×10^{-8} M vitamin D or vehicle for 48 h (C,D). **A:** Specific binding to the E-box 1 site in the osteocalcin promoter of nuclear extracts derived from MC3T3-E1 osteoblasts. The position of the E-box 1–specific band is represented by the *arrow* (*lane* 1). Cold wild-type oligonucleotide (E1) competed the retarded band (compare lanes 1 and 2), whereas competition with the mutated oligonucleotide (E1m) failed to disrupt binding (*lane 3*), consistent with the presence of nuclear factors with specificity for the CACATG site. An antibody to E12 (E12-Ab) failed to shift the observed band (*lanes 4,5*), suggesting that E12 may not part of the protein:DNA complex. Addition of probe alone resulted in no retarded bands (not shown). **B:** Binding of MC3T3-E1 osteoblast nuclear extracts to the E-box 2 oligonucleotide derived from the osteocalcin promoter. Probe alone (*lane 1*). Incubation of an oligonucleotide probe containing the putative E-box 2 site CAGCTG with nuclear extracts from 14-day-old MC3T3-E1 osteoblasts resulted in retardation of several bands that were competed by 100-fold excess of unlabeled E2 oligonucleotide probe (compare *lanes 2 and 3;* the most prominent band is indicated by the *arrow*). Vitamin D or rhBMP-2 treatment for 48 h increased the binding intensity of several bands to E-box 2 in MC3T3-E1 osteoblasts (compare lanes 2 with *4 and 5*). **C:** Specific binding to the E-box 1 site in the osteocalcin promoter of nuclear extracts derived from Ros 17/2.8 in the presence and absence of vitamin D. The position of the E-box 1–specific band is represented by the *arrow.* Cold wild-type oligonucleotide (E1) competed the retarded band (Compare *lanes 1 and 3*), whereas competition with the mutated oligonucleotide (E1m) failed to disrupt binding (*lane 4*), consistent with the presence of nuclear factors with specificity for the CACATG site. An antibody to E12 (E12-Ab) failed to shift the observed band (*lane 5*). Vitamin D had little effect on E-box 1 binding activity in Ros 17/2.8 osteoblasts (compare lanes 1 and 2). **D:** Gel mobility shift analysis was performed with nuclear extracts obtained from Ros 17/2.8 cells treated with 2×10^{-8} M vitamin D for 48 h and the E-box 2 oligonucleotide probe. In contrast to MC3T3-E1 osteoblasts, vitamin D and rhBMP-2 had minimal effects on E-box 2 binding in Ros 17/2.8 osteoblasts (compare *lane 1* with *lanes 2 and 3*).

appears to have binding sites for factors other than bHLH heterodimers. Thus, our results demonstrate that the "CANNTG" site within E-box 1 but not E-box 2 binds factors in nuclear extracts of mature MC3T3-E1 and Ros 17/2.8 osteoblasts.

Site-Directed Mutagenesis Analysis of the E-Box 1 Element in MC3T3-E1 and Ros17/2.8 Osteoblasts

To analyze the role of the E-box 1 site in regulating the temporal expression of osteocalcin in osteoblasts, we created a chimeric construct containing the osteocalcin promoter in which the E-box 1 site was mutated from CACATG to TGTTAC (p637OC-luc E1m). MC3T3-E1 cells were stably transfected with either the chimeric plasmid, p637OC-luc E1m, containing the mutated E-box 1 site or the wild-type plasmid p637OC-luc. Stage-specificexpression of osteocalcin was achieved by growth of cells in differentiation media for 14 days similar to above. Surprisingly, we found that mutagenesis of the E-box 1 site had no effect on transcriptional activity of the osteocalcin gene promoter during the developmental upregulation of osteocalcin gene transcription in MC3T3-E1 osteoblasts (Fig. 6A). We also evaluated the effect of mutagenesis in E-box 1 and E-box 2 in Ros 17/2.8 cells. We observed no differences in luciferase activity in Ros 17/2.8 cells transiently transfected with either the wild-type or chimeric constructs containing the E-box 1 and 2 mutation either in the absence or presence of 1,25 dihydroxy vitamin D treat-

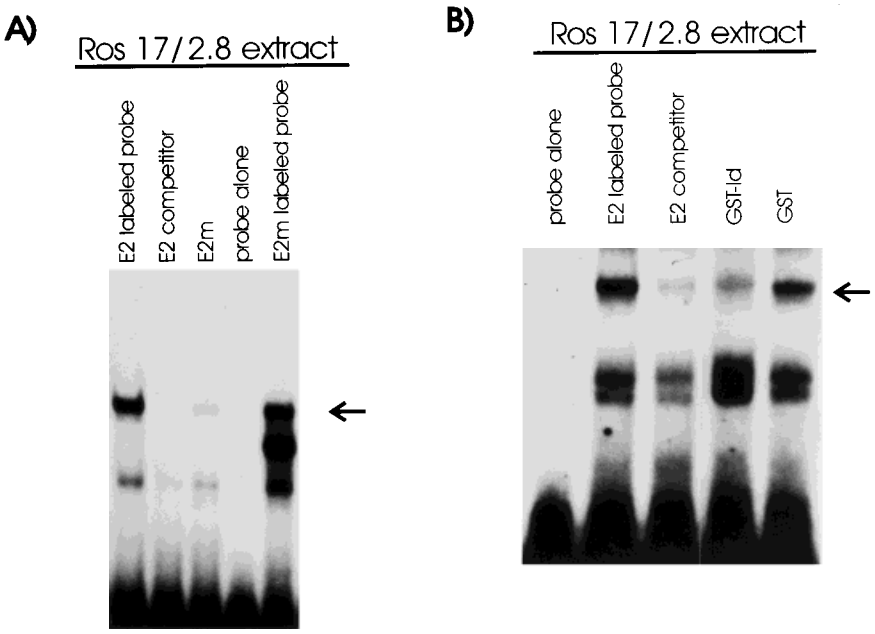

Fig. 5. Nonspecificity of E-box 2 binding activity in osteoblasts. **A:** Gel mobility shift analysis was performed with nuclear extracts obtained from Ros 17/2.8 cells treated with 2×10^{-8} M vitamin D for 48 h and the E-box 2 oligonucleotide probe. Retardation of two major bands was observed when nuclear extracts were incubated with the E2-labeled probe; the most predominant band, which corresponds to that depicted in Fig. 4C, is labeled by an *arrow.* Competition studies were performed using 100-fold excess unlabeled E-box 2 oligonucleotide (E2 competitor) or an oligonucleotide in which the E-box 2 site had been mutated from CACATG to TTTTTT (E2m). Both E2 and E2m competed the bands, indicating that binding is not specific for the E-box 2 site. Gel mobility shift assays with osteoblast nuclear extracts and the labeled E2m oligonucleotide probe that lacks the E-box site (E2m-labeled probe) also demonstrated the same retarded band. Probe alone resulted in no retarded bands. **B:** Id inhibits binding of nuclear extract proteins to E-box 2 probe from the osteocalcin promoter. The migration of the free probe in the absence of nuclear extracts is indicated by probe alone. The E2-labeled probe retarded bands derived from incubation of nuclear extracts derived from vitamin D–treated Ros 17/2.8 cells. The *arrow* denotes the same band depicted in A and Fig. 4D. Competition of the binding by the unlabeled E-box 2 oligonucleotide diminished binding. Binding nuclear extracts preincubated with 0.4 μg of purified pGEX-Id protein with the E-box 2 oligonucleotide (GST-Id) inhibited binding, whereas preincubation of nuclear extract with an equivalent amount of purified GST protein had no effect. In spite of failure to show specificity for the CANNTG site of the oligonucleotide, excess Id fusion protein reduced the intensity of the retarded band. This suggests that the high concentrations of Id may have nonspecific effects in vitro.

ment (Fig. 6B). Thus, the mutated E-box 1 and E-box 2 sites in the osteocalcin promoter had no effect on transcriptional activity of this promoter in the osteoblast cell lines that we studied.

DISCUSSION

Our current studies reexamine the role of proximal E-box elements and bHLH proteins in the regulation of osteoblast development. We identified specific binding of nuclear extracts from differentiated osteoblasts to a consensus E-box element (E-box 1) in the proximal osteocalcin promoter (Fig. 4A,C), which is consistent with the presence of bHLH proteins in osteoblasts that bind to the CACATG site in the proximal osteocalcin promoter. Though gel mobility shift analysis and Id-1–mediated reduction of E-box 2 activity also suggested binding of an osteoblast factor to E-box 2 (Figs. 4B,D, 5B), mutations of the core CAGCTG element in the E-box 2 probe failed to abolish binding of the observed nuclear factor (Fig. 5A), indicating that binding to E-box 2 is not specific for this site. In addition, overexpression of osteocalcin promoter containing a mutated E-box 2 site failed to modify osteocalcin transcription in Ros 17/2.8 osteoblasts (Fig. 6B). On the other hand, in spite of specific binding to the E-box 1 site, we also were unable to demonstrate that E-box 1 regulates either the developmental stage-specific expression of osteocalcin in stably transfected MC3T3-E1 osteoblasts (Fig. 6A) or osteocalcin gene transcription in transiently transfected Ros 17/2.8 osteoblasts (Fig. 6B). Thus, neither the E-box 1 nor E-box 2 site is of functional significance in osteoblasts when examined in the context of the 637 bp osteocalcin promoter.

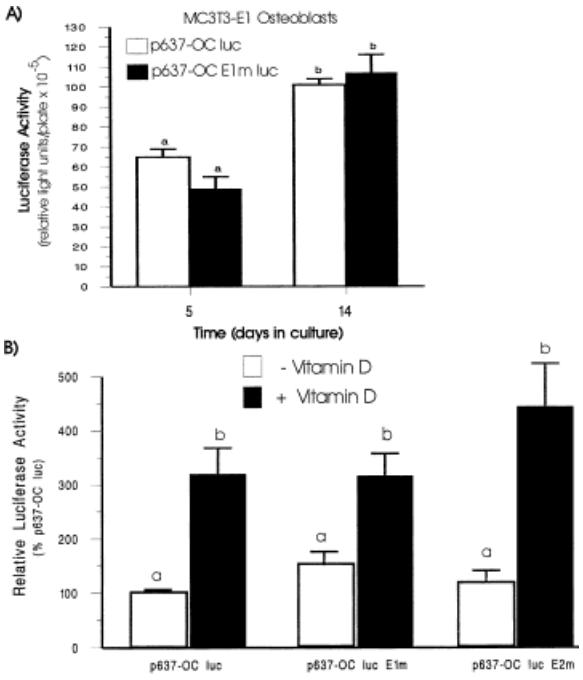

Fig. 6. Effects of E-box mutations on osteocalcin gene transcription in osteoblasts. **A:** Effects of E-box 1 mutation on developmental regulation of p637OC-luc stably transfected into MC3T3-E1 osteoblasts. MC3T3-E1 cells were stably transfected with p637OC-luc or a promoter containing a mutated E-box 1 site (p637OC-luc-E1) and grown in differentiation media for 14 days. Luciferase activity increased during development in both the constructs, suggesting that the E-box 1 site is not functionally important. **B:** Effects of E-box 1 mutation on vitamin D–stimulated osteocalcin gene transcription in Ros 17/2.8 osteoblasts. Ros 17/2.8 were transiently transfected with p637OC-luc or the same promoter construct in which either the E-1 site (p637OC-luc E1m) or the E-2 site (p637OC-luc E2m) had been mutated. Neither the E1m nor the E2m affected basal or vitamin D–stimulated osteocalcin promoter activity. Values represent the mean ± SEM of three separate determinations. a and b superscripts represent significant differences. Columns sharing the same superscript are not different at $p < 0.05$.

The observation that E-box 2 is not functional is in agreement with other investigations, whereas our data regarding the role of E-box 1 differ from findings by others [Tamura and Noda, 1994]. Indeed, prior studies using a −198 bp rat osteocalcin promoter transfected into Ros 17.2.8 osteoblasts found a 50% reduction in transcriptional activity in osteocalcin promoter constructs in which the E-box 1 site had been mutated [Tamura and Noda, 1994]. In contrast, similar to our results, investigation of the mouse osteocalcin promoter identified a factor from Ros 17/2.8 and mouse primary osteoblastic nuclear extracts that binds to the E-box 1 site but found by deletional analysis that the E-box 1 element did not contribute significantly to osteocalcin gene expression in Ros 17/2.8 osteoblasts [Ducy and Karsenty, 1995]. The disparities between these various studies may be due to differences in model systems, promoter fragments (−637 vs. −198), assay conditions, and/or transfection protocols. Distinctions due to MC3T3-E1 and Ros 17/2.8 osteoblasts and/or stable vs. transient transfections, however, do not explain these discrepancies, since we also found no evidence of E-box 1 function in transient transfections of the −637 bp osteocalcin

promoter in Ros 17/2.8 osteoblasts (Fig. 6B). More likely, elements in the longer 637 bp promoter construct, which results in its higher basal level of expression, may have obscured our ability to detect a small E-box 1 effect. Nonetheless, our studies suggest that E-box 1 does not play a significant role in regulation of either the temporal upregulation or the osteoblastic expression of osteocalcin in MC3T3-E1 and Ros 17/2.8 osteoblasts.

In spite of our inability to demonstrate function of the E-box 1 site, we did identify a factor in osteoblasts that binds specifically to the CACATG core site in the osteocalcin promoter. The identity of this factor remains unknown. While it is possible that it may regulate osteoblast function other than osteocalcin, this factor showed no significant hormonal or developmental regulation. Moreover, this putative bHLH factor was not shifted by an antibody to E12, a ubiquitous bHLH binding partner. Consequently, its role in the developmental and/or osteoblast-specific expression remains to be established.

Though previous studies have shown that excessive amounts of bacterially expressed Id-1 will disrupt binding of a similar osteoblastic factor to the E-box 1 site of the osteocalcin promoter [Tamura and Noda, 1994], the physiological relevance of this is uncertain, in view of our findings that excessive amounts of Id may have nonspecific effects in vitro. Indeed, the amount of Id needed to inhibit HLH DNA binding to E-box 2 in our studies as well as to E-box 1 in studies by Tamura [Tamura and Noda, 1994] was a least fortyfold greater than that required to disrupt bHLH:bHLH interactions in myotubules, where Id is known to have a physiological role [Jen et al., 1992].

Finally, the current studies are one of the first to analyze the osteocalcin promoter by stable transfection in a clonal cell line that undergoes a temporal sequence of development. This is important from several perspectives. First, our studies highlight the usefulness of the MC3T3-E1 osteoblast cell line as a model to study the developmental upregulation of the osteocalcin gene. Like primary cultures, MC3T3-E1 osteoblasts proliferate and express low levels of osteocalcin early in culture; and the onset of differentiation is associated with cessation of replication, formation of extracellular matrix, and upregulation of markers of differentiation such as (liver/bone/kidney) alkaline phosphatase and expression of osteocalcin. We demonstrated that, unlike primary cultures in which promoter analysis is difficult, MC3T3-E1 osteoblast can be stably transfected without altering the temporal sequence of osteocalcin gene expression. Moreover, our studies show that the increase in osteocalcin is due to transcriptional upregulation in developing MC3T3-E1 osteoblasts. In addition, we demonstrate that the stably integrated 637 bp rat osteocalcin promoter contains sufficient elements to direct the temporal upregulation of osteocalcin gene transcription during osteoblast development. Thus, MC3T3-E1 cells are a suitable model for the study of developmental upregulation of the osteocalcin gene.

In conclusion, the MC3T3-E1 osteoblast model permits assessment of the temporal regulation of osteocalcin gene transcription in vitro. Using this model we were unable to define a role for E-box 1 in the developmental expression of osteocalcin. Except for binding of nuclear factors to the E-box element, we have no direct data to support bHLH involvement in osteoblast development. Further studies are need to establish a role of bHLH proteins in osteoblast development. These may include identification of osteoblast-specific bHLH proteins and examination of the role of more distal E-box elements in regulating osteoblast development.

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