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Bone Tissue–Specific Transcription of the Osteocalcin Gene: Role of an Activator Osteoblast–Specific Complex and Suppressor Hox Proteins That Bind the OC Box

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Abstract Bone-specific expression of the osteocalcin gene is transcriptionally controlled. Deletion analysis of osteocalcin promoter sequences by transient transfection of osseous (ROS 17/2.8) and nonosseous (R2 fibroblast) cells revealed that the most proximal 108 nucleotides are sufficient to confer tissue-specific expression. By gel mobility shift assays with wild-type and mutated oligonucleotides and nuclear extracts from several different cell lines we identified a novel transcription factor complex which exhibits sequence-specific interactions with the primary transcriptional element, the OC box (nt −99 to −76). This OC box binding protein (OCBP) is present only in osteoblast-like cells. Methylation interference demonstrated association of the factor with OC box sequences overlapping the Msx homeodomain consensus binding site. By assaying several mutations of the OC box, both in gel shift and transient transfection studies using ROS 17/2.8, we show the following. First, binding of OCBP correlates with osteocalcin promoter activity in ROS 17/2.8 cells. Increased binding leads to a 2–3-fold increase in transcription, while decreased binding results in transcription 30–40% of control. Second, homeodomain protein binding suppresses transcription. However, Msx expression is critical for full development of the bone phenotype as determined by antisense studies. Last, we show that one of the mutations of the OC box permits expression of osteocalcin in non-osseous cell lines. In summary, we demonstrate association of at least two classes of tissue-restricted transcription factors with the OC box element, the OCBP and Msx proteins, supporting the concept that these sequences contribute to defining tissue specificity.

Key words: osteocalcin, transcriptional regulation, homeodomain protein, Msx, bone-specific, OC box

Understanding the precise molecular events controlling commitment and development of the bone cell phenotype is central to defining normal bone tissue formation as well as causes of bone-related diseases of unknown etiology. A viable approach to study the mechanisms that drive osteoblast differentiation from the stromal–mesenchymal progenitor is to characterize factors that regulate expression of osteoblast-specific genes. Progression through the osteoblast lineage is characterized by the temporal expression of genes that reflect stages of growth and maturation [Owen et al., 1990]. The osteocalcin gene is expressed in progenitor-like cells after induction of osteoblast differentiation [Shalhoub et al., 1992; Katagiri et al., 1994; Rosen et al., 1994], in postproliferative osteoblasts *in vitro* and *in vivo*, and is upregulated at the onset of mineralization of the bone extracellular matrix *in vitro* and *in vivo* [reviewed in Stein and Lian, 1993]. Osteocalcin is also expressed in several osteosarcoma or transformed cell lines exhibiting properties (e.g., hormone responsiveness and ability to form a mineralized matrix) of the mature osteoblast phenotype [Rodan and Noda, 1991; Rodan et al., 1993]. This developmental regulation and tissue specificity provides an opportunity to search for factors which control transcription of osteocalcin that may also control osteoblast differentiation.

Regulatory cascades of transcription factors control differentiation of multiple distinct cell types and tissues. In the development of skeletal myoblasts from mesodermal progenitors, a fam-

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ily of myogenic bHLH proteins activate the myogenic program and are involved in maintenance of the myoblastic phenotype [Olson and Klein, 1994]. In hepatocytes, the transcription factor families C/EBP, HNF-1, HNF-3, and HNF-4 are involved in controlling hepatocyte-specific transcription [Lai and Darnell, 1991] and also control coordinate expression of each other [Kuo et al., 1992]. In adipocytes, C/EBP α, β, and δ regulate adipocyte differentiation, and ectopic expression of C/EBP α [Umek et al., 1991; Freytag et al., 1994] and C/EBP β [Yeh et al., 1995] can induce fibroblasts to differentiate into adipocytes. All of these factors and their cognate regulatory elements are key determinants in regulating tissue-specific gene expression and dictating development of the differentiated phenotype.

The bone-expressed osteocalcin promoter has been extensively studied for regulatory elements contributing to transcriptional activity in osteoblasts. Several regulatory domains have been identified in the rat and human OC promoters that bind both ubiquitous and tissue-restricted transcription factors. Contributors to modulated levels of OC expression include Fos/Jun family–related proteins which bind selectively to several AP-1 sites [McCabe et al., submitted]; a TGFβ response element [Banerjee et al., in press]; a multisubunit CP1-NFY/CBF-like CAAT factor complex [Towler and Rodan, 1995]; a vitamin D response element (VDRE) [Demay et al., 1990; Kerner et al., 1989; Markose et al., 1990] which is flanked by two tissue-specific nuclear matrix protein binding sites [Bidwell et al., 1993; Merriman et al., 1995]; and several glucocorticoid response elements [Aslam et al., 1995]. Two osteoblast-specific elements were reported in studies using the mouse promoter [Ducy and Karsenty, 1995]; one is equivalent to the site in the rat promoter (−147 to −132) which binds a tissue-specific nuclear matrix complex [Bidwell et al., 1993; Merriman et al., 1995]; the other lies in a proximal domain (nt −64 to −47).

Also within the proximal promoter domain of the osteocalcin gene is the OC box (−99 to −76), a highly conserved 24 nucleotide sequence that represents a multipartite protein/DNA interaction site. The OC box is required for basal transcription [Heinrichs et al., 1993; Towler et al., 1994a] and earlier studies suggested its involvement in tissue-specific expression [Heinrichs et al., 1995].

Recently, homeodomain proteins from the Msx family, which are important regulators of development [Gehring et al., 1994; Krumlauf, 1994], were shown to bind with sequence specificity to the OC box [Hoffmann et al., 1994; Towler et al., 1994b]. Furthermore, we showed Msx-2 expression is developmentally regulated during osteoblast differentiation *in vitro* [Hoffmann et al., 1994]. A role for Msx-1 and Msx-2 in craniofacial and cranial development has also been demonstrated [Satokata and Maas, 1994; Jabs et al., 1993], suggesting that these homeodomain binding proteins are important transcriptional factors involved in bone development. Point mutations of the homeodomain consensus binding site in the context of 1,097 nucleotides of the osteocalcin promoter lead to a decrease in activity of this promoter [Hoffmann et al., 1994; Towler et al., 1994b], but a role for these sequences in tissue-specific expression of the osteocalcin gene has not yet been demonstrated.

The objective of the present study was to further characterize the contribution of the OC box and cognate binding activities in regulation of tissue-specific transcription of the osteocalcin gene. We demonstrate that, in addition to the association of homeodomain proteins (Hox), the OC box interacts with a non-homeodomain protein, designated OCBP (OC box binding protein). The OCBP factor is restricted to cells of osteoblast origin and appears to act as a positive regulator of osteocalcin transcription.

MATERIALS AND METHODS

Cell Culture

Rat osteosarcoma cells (ROS 17/2.8) [Majeska et al., 1980] were grown in F-12 medium (GIBCO) supplemented with 5% fetal calf serum. Rat UMR-106 [Partridge et al., 1980] and R2 fibroblast cells [Topp, 1981] were cultured in minimal essential medium (MEM: GIBCO) supplemented with 10% fetal calf serum. HeLa S3 cells [Puck et al., 1956] were grown in Joklik-modified minimal essential medium (GIBCO) supplemented with 5% fetal calf serum and 5% horse serum. Mouse MC3T3-E1 cells [Kodama et al., 1981] were grown in α-minimal essential medium (GIBCO) plus 10% fetal calf serum. Human osteosarcoma cell line Saos-2 (obtained from ATCC) was grown in Dulbecco-modified Eagle medium (DME: GIBCO) supplemented with 10% fetal calf serum. H4 rat hepatoma cells (obtained from ATCC) were grown in MEM (GIBCO) 5% fetal calf serum and 5% horse se-

rum. FRTL-5 cells [Vitti et al., 1982; Ambesi-Impiombato and Perrild, 1989] were grown in Coon's modified F12 medium supplemented with 5% calf serum and 1.7% 6H.

Normal osteoblasts obtained from calvariae of fetal rats of 21 day gestation were isolated and subjected to sequential digestions of 20, 40, and 90 min at 37°C in 2 mg/ml collagenase A (Boehringer-Mannheim, Indianapolis, IN) with 0.25% trypsin (Gibco, Grand Island, NY). Cells released in initial digests were discarded, and those released from the third digestion were plated at a density of 4×10^5 cells/100 mm dish. Cells were fed every 2 days with minimal essential medium (MEM; Gibco) supplemented with 25 μg/ml ascorbic acid. All subsequent feedings contained medium supplemented with 10% fetal *calf* serum, 50 μg/ml ascorbic acid, and 10 mM β-glycerol phosphate [Aronow et al., 1990; Bellows et al., 1986].

Plasmids and Constructs

The construct pOCZCAT [Schepmoes et al., 1991] which contains the rat osteocalcin 5′ promoter fragment (−1,097–+23), the cloramphenicol acetyl transferase (CAT) gene, and the pGEM 7zf(+) backbone was used in the generation of all constructs described in this paper. The −1,727 deletion construct was created by isolating a 630 bp fragment of the upstream osteocalcin promoter (Baker et al., 1992) and ligating it upstream of the osteocalcin promoter sequences in pOCZCAT. The −1,097 construct is pOCZCAT. The remainder of the deletion constructs were formed by isolating restriction fragments of the osteocalcin promoter from the pOCZCAT construct and placing them upstream of the CAT gene in a XhoI/HindIII—digested pGEM-7ZF(+) plasmid containing the chloramphenicol acetyltransferase gene in the multiple cloning site. The −724 construct was formed from an NcoI/HindIII fragment, −531 from an HincII/HindIII fragment, −348 from a BglII/HindIII fragment, and −108 from an AosI/HindIII fragment.

pOCZCAT was used as a template for PCR reactions to form point mutations of the osteocalcin promoter. Point mutations were introduced into the promoter region using oligonucleotides of the OC box region which contain the specific mutation of interest.

Transfection Experiments

Cells plated at a density of $4–5 \times 10^5$ cells/100 mm plate were used for transient transfection experiments. ROS 17/2.8, R2, and HeLa cells were transfected by the DEAE-dextran method [Ausubel et al., 1989]. The total amount of exogenous DNA was maintained at 20 μg/plate consisting of 10 μg experimental construct and 10 μg salmon sperm DNA. All plasmid DNA stocks were checked for supercoiled structure and compared with expression of plasmids of similar quality. Cells were shocked with glycerol 3 hr post-transfection, then incubated in F12 medium supplemented with 5% fetal calf serum. UMR and MC3T3 cells were transfected by the calcium phosphate coprecipitation method [Ausubel et al., 1989]. Exogenous DNA totaled 15 μg/100 mm plate and was kept on the cells overnight. All cells were harvested using 125 μl/plate of 1× promoter lysis buffer (0.25 M Tris-HCl, pH 8.0, 0.1% Triton X-100, Promega) 65 hr after transfection, and CAT activity determined as an indicator of *in vitro* expression of the transfected plasmid. Relative transfection efficiencies of the different cell lines were determined by comparing the CAT activity of the positive control plasmid pSV2CAT [Gorman et al., 1982]. In this study we observed variability in expression of the full-length pOCZCAT construct compared to previous publications [Aslam et al., 1995; Frenkel et al., 1996; Heinrichs et al., 1993]. The relative expression of the shorter deletion constructs reported here and in prior publications is consistent. The difference is related to cell passage number and serum lots. Comparisons are drawn between cells transfected on the same day under identical conditions. Thus any variations observed do not affect the conclusions drawn here or in other reports [Aslam et al., 1995; Frenkel et al., 1996; Heinrichs et al., 1993].

CAT Assays

Chloramphenicol acetyltransferase (CAT) activity was determined as previously described by Ausubel et al. [1989]. The samples were incubated with 0.25 μCi (1 Ci = 37 GBq) of ^{14}C-chloramphenicol (Dupont) for 4–12 hr. After ethyl acetate extraction, the samples were separated by chromatography on TLC plates (Whatman). Radioactivity on the TLC plates was quantitated using a β-scope 603 blot analyzer from Betagen (Mountain View, CA).

Gel Mobility Shift Assay

Nuclear extracts were prepared as described by Bortell et al. [1992]. Protein–DNA interactions were characterized using a 45 nucleotide probe (nt −120 to −76) or a 24 nucleotide probe

(nt −99 to −76) encoding the OC box of the osteocalcin promoter. Probes were prepared by 5′ end labeling, using ^{32}P ATP and T4 polynucleotide kinase. For each binding reaction containing the 45 nucleotide probe, 40 fmol probe and 5 μg nuclear extract was used in a reaction containing 5 μg BSA (fraction V, Sigma), 0.1% NP40, 10 mM DTT, 4 μg poly(dI-dC), 25% glycerol, 25% sucrose, 37 mM $MgCl_2$, 50 mM Tris-HCl, pH 7.5, 250 mM NaCl, and 1 mM EDTA. The suspension was incubated for 10 min at room temperature. Protein–DNA complexes were separated on a 4% (40:1) nondenaturing polyacrylamide 0.5× TBE gel. The gel was electrophoresed in 0.5× TBE buffer for 3 hr at 250 V. Gels were dried and autoradiographed on Kodak XAR film, using an intensifying screen, at −70°C.

Gel shift assays of the 24 nucleotide probes (nt −99 to −76) were performed as described above except they had a total volume of 10 μl and contained only 1 μg poly(dI-dC). In addition, the complexes formed were separated on a 6.5% PAGE gel containing 0.5× TBE. Competitions were performed in reactions as described above with the addition of 100–200× molar excess competitors.

Methylation Interference

For methylation interference assays, a 45 nucleotide probe (nt −120 to −76) was used which encodes the OC box of the osteocalcin promoter. Probes were 5′ end labeled using ^{32}P ATP and T4 polynucleotide kinase. Two hundred and fifty femtomoles of probe was methylated for 4 min at 23°C in 1× cacodylic acid using 10 μl dimethylsulfate. The reaction was stopped by adding 50 μl stop buffer [1.5 M sodium acetate, pH 7, 1 M b-mercaptoethanol, 5 μg poly(dI-dC)]. Gel mobility shift assays were performed loading four binding reactions on a 4% (40:1) nondenaturing polyacrylamide 0.5× TBE gel. Gels were electrophoresed for 3 hr at 250 V, and exposed to Kodak XAR film at 4°C.

After localization of bands using the autoradiogram, protein-bound probe and free probe were cut out of the gel and electro-eluted from the gel using an elutrap [Schleicher and Schuell] for at least 2 hr at 200 V in 0.25× TBE buffer. Eluates were cleaved by adding 10 μl piperidine for protein-bound probe and free probe, followed by incubation for 30 min at 90°C, and transfer of tubes to dry ice. Reaction products were lyophilized using a speed vac concentrator (Savant). Lyophilization was repeated thrice by dissolving pellets with 100, 50, and 25 μl dH_2O, respectively. Radioactivity was measured using a scintillation counter (Beckman LS9800), and equal counts of bound probe, free probe, and G-ladder were dissolved in appropriate amounts of formamide loading dye (80% formamide, 50 mM TBE, 1 mM EDTA, 0.1% Bromophenol blue, 0.1% xylene cylanol). Samples were counted again and boiled for 5 min before loading on an 8% denaturing polyacrylamide gel. The gel was electrophoresed at 50 W, dried, and autoradiographed on Kodak XAR film. This experiment was repeated three times.

Antisense Treatment

Antisense phosphorothionate–modified (at 3′ end) oligonucleotides were directed against the 5′ UTR and start codon of Msx-1 (GGCCGACG-ACGATACTGAAG) and Msx-2 (GCCGCGTC-TTTCAGTACCGA). Every 24 hr, beginning on day 1, 1–2 μM of gel and column-purified antisense oligonucleotides was added along with fresh media containing 10% serum to osteoblasts plated in 24 well dishes. Cell viability was not affected at these concentrations. Oligonucleotides with a scrambled sequence served as a control for the effect of the oligonucleotide treatment. Treatments were carried out until day 18, at which point cultures were fixed and stained for alkaline phosphatase to show phenotypic development. Secreted osteocalcin levels were determined throughout the course of the experiment and nodules were counted in each well (> 3 wells were counted per condition per experiment).

Biochemical and Histochemical Analyses

Medium from cell layers was analyzed for osteocalcin by radioimmunoassay as previously described [Gundberg et al., 1984]. Cells were washed 2× with PBS on ice, fixed for 10 min with 2% paraformaldehyde, and then rinsed 2× with 0.1 M cacodylic buffer. For alkaline phosphatase, fixed cell layers were incubated for 30 min at 37°C with 20 mg/ml naphthol AS-MX phosphate disodium salt with 40 mg/ml Fast red TR salt (Sigma Chemical Co., St. Louis, MO) in a pH 8.4 Tris buffer.

RESULTS

The Proximal 108 Nucleotides of the Osteocalcin Promoter are Sufficient for Cell Type–Specific Expression

A series of deletion mutations of the rat osteocalcin gene promoter (Fig. 1A) was constructed

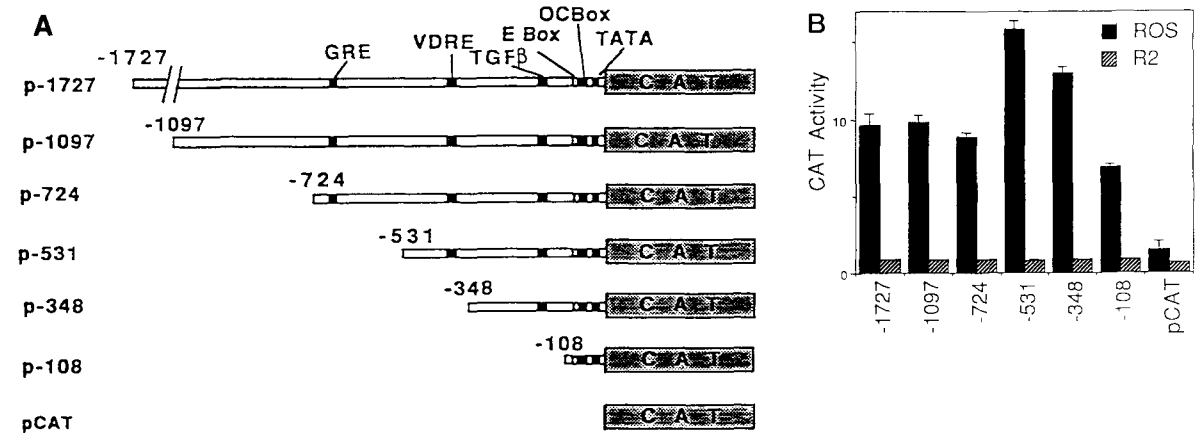


Fig. 1. Deletion analysis of the 5′ flanking sequences of the rat osteocalcin promoter. **A:** Schematic diagram of the deleted promoter constructs. Constructs are named for the number of the 5′-most nucleotide of the osteocalcin promoter sequences that they contain. Promoter sequences are represented by unfilled lines with promoter regulatory elements indicated by small boxes. The 3′ end is constant for all constructs at position +24 of the osteocalcin gene. Promoter sequences are upstream of the chloramphenicol acetyl transferase (CAT) gene which was used to assay activity of the promoter. pCAT is a promoterless construct. All constructs have a pGEMzf(+) backbone. The osteocalcin regulatory elements labeled in construct p-1730 include the glucocorticoid response elements (GRE), vitamin D response element (VDRE), transforming growth factor β binding site (TGFβ), E box, OC box, and TATA box. **B:** ROS 17/2.8 and R2 fibroblast cells were transiently transfected with the deletion constructs represented in A. CAT activity was assayed and the activity of the truncated promoters is reported here as percent acetylation of chloramphenicol. Standard deviation is represented by a line above the large bars. Standard deviation for all of the R2 values is ± 0.02 and is not discernible on the histogram. Data are representative of the mean values obtained from triplicates in two independent experiments.

to assay for sequences involved in tissue-specific expression. Activity of these constructs was assayed by transient transfection in an osseous cell line, ROS 17/2.8, rat osteosarcoma cells, and a nonosseous cell line, R2, rat fibroblasts (Fig. 1B). Truncations of the promoter lead to some variation in level of expression in ROS cells as a result of removal of different regulatory elements, such as the upstream negative glucocorticoid response element, vitamin D response element, and transforming growth factor β binding sites (Fig. 1A,B). However, as few as 108 nucleotides of promoter sequences are sufficient to maintain basal and cell type–specific expression of the osteocalcin gene; the −108 construct is expressed only in the ROS 17/2.8, but not in the nonosseous R2 cells. These results confirm those recently reported by Towler et al. [1994a] which demonstrate that the nucleotides between −121 and −64 are necessary and sufficient for basal, tissue-specific expression of the rat promoter in ROS 17/2.8, ROS 25/1, C2C12 mouse myoblast, and NRK rat fibroblast cells. Similar results were also reported for nucleotides −147 to −34 of the mouse osteocalcin promoter in ROS 17/2.8, ROS 25/1, C2, F9, and S194 cells [Ducy and Karsenty, 1995].

Nuclear proteins associating with this basal domain of the osteocalcin promoter were investigated using gel mobility shift assays (Fig. 2). Sequences used as probes and competitors in these assays are summarized in Table I. Two previously identified binding sites are located within these sequences. The first is an E-box motif which is a consensus binding site for helix-loop-helix (HLH) proteins [Tamura and Noda, 1994], and the second is an Msx-consensus binding site located within the highly conserved OC box sequence [Heinrichs et al., 1993; Hoffmann et al., 1994; Towler et al., 1994b]. Complexes formed on the 45 nucleotide probe (−120 to −76) used in this assay that are due to the association of HLH and Msx-related proteins were identified by competition analysis (Fig. 2A, lanes 3–6). Competition with the 5′ 32 nucleotides of the 45 nucleotide probe, containing the HLH binding site but not the homeodomain consensus sequence, decreased the association of the two slowest-migrating complexes, labeled HLH (Fig. 2A, lane 3). Competition with the Hox binding site consensus (Fig. 2A, lane 6) decreases the association of the fastest-migrating group of complexes. These competition studies revealed that another protein–DNA complex, labeled OCBP for the OC box binding protein, associates with the 3′ 24 nucleotides of the 45 nucleotide probe. These 24 nucleotides represent the OC box sequence and do not contain the

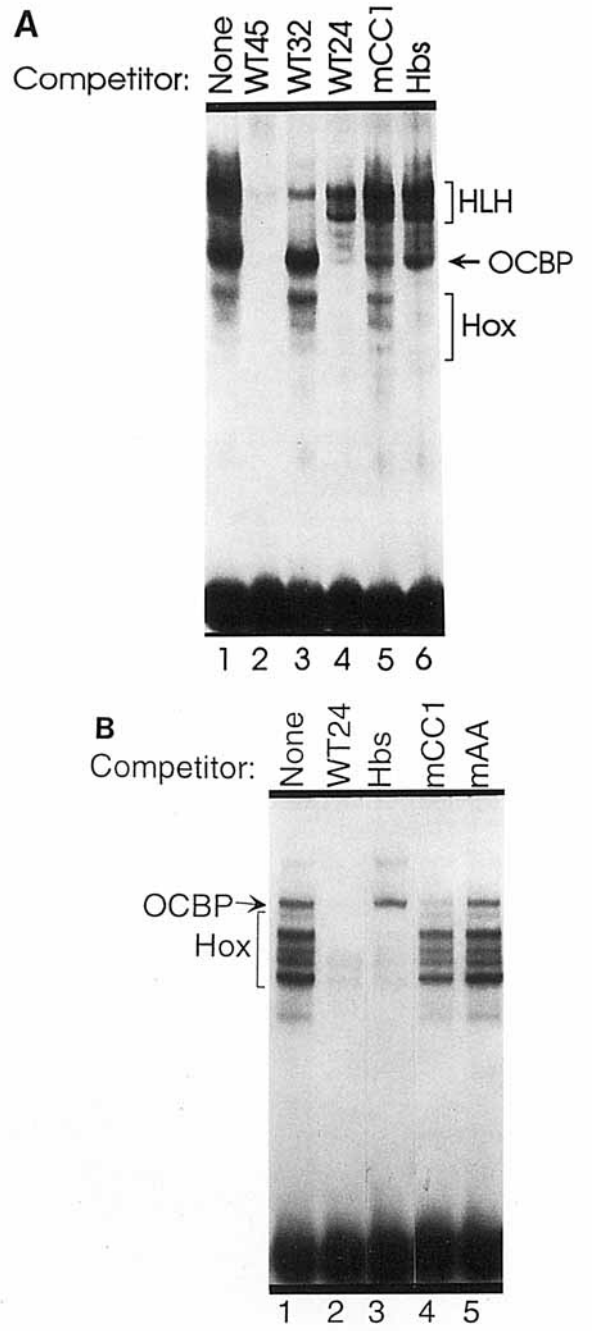


Fig. 2. Competition of proteins associating with osteocalcin promoter sequences. Gel mobility shift assays with either ^{32}P-end-labeled WT45 or WT24 DNA, and 5 μg ROS 17/2.8 rat osteosarcoma nuclear extracts were performed. Control assays labeled "none" did not contain competitor DNA. The remaining reactions include competitor as noted above each lane containing the sequences detailed in Table I. Protein–DNA complexes that are disrupted by oligonucleotides containing the E box sequences are labeled HLH, those disrupted by competition with the Msx consensus sequences are labeled Hox, and the OC box binding protein is labeled OCBP. **A:** ROS nuclear proteins were bound to the WT45 sequences (detailed in Table I) and competed with 200-fold excess unlabeled competitor. **B:** ROS nuclear proteins were bound to the WT24 probe and competed with 100-fold excess unlabeled competitor.

HLH binding site (Fig. 2A, lane 4). Because this complex is not strongly competed by either the HLH (WT32) or Hox (Hbs) binding sites, it represents a third class of factor(s) binding to this region of the osteocalcin promoter.

To further characterize OCBP binding to the OC box, and the relationship of OCBP binding to the Hox protein binding site, an oligonucleotide with a mutation in the Hox consensus DNA binding site (mCC1, ggAATTAG) was used as competitor (Fig. 2A, lane 5). mCC1 sequences compete the OCBP complex more efficiently than the Hox complexes, indicating that the mCC1 mutant oligonucleotide associates more strongly with OCBP than with the Msx family of proteins.

A Nonhomeodomain Protein Binds With Sequence Specificity to a Site Which Overlaps the Hox Binding Site

To explore OCBP DNA binding interactions in greater detail, a 24 nucleotide OC box probe was used in gel mobility shift assays under conditions which allow higher resolution of the complexes (Fig. 2B). A series of wild-type and mutant oligonucleotides were used as competitors. Five Msx-related complexes have previously been described [Hoffmann et al., 1994] and are labeled "Hox" in Figure 2B. The OCBP is clearly discernible in these gels and is unrelated to the Hox proteins as demonstrated by competition with the Hox binding site (lane 3). Binding of OCBP in the presence of Hox competitor (Hbs) indicates that this protein/DNA complex is formed independently of homeodomain complexes. In addition, antibody to the Msx-1 homeodomain does not affect the formation of this complex *in vitro* [Hoffmann et al., 1994]. The mCC1 (ggAATTAG) mutant which alters sequences that flank the core of the Msx consensus binding site does not effectively compete the Msx complexes, but does displace the OCBP complex (lane 4), further confirming that OCBP is distinct from the homeodomain protein class. Interestingly, the mAA mutant (CCggTTAG) which alters the core of the Hox DNA binding domain consensus does not effectively compete any of the OC box–associated complexes, either Hox complexes or the OCBP (Fig. 2B, lane 5), suggesting that AA are critical nucleotides for interaction of both classes of transcription factors.

Close proximity of OCBP binding to the homeodomain site is thus suggested by competition studies. Therefore, the precise nucleotide

TABLE I. Oligonucleotide Sequences Used for Probes and Competitors*

	E-box Msx
WT45	−120 TGGGTTTGACCTATTGCGCACATGACCCCCAATTAGTCCTGGCAG −76
	OC box
WT32	−120 TGGGTTTGACCTATTGCGCACATGACCCCCAA −89
WT24	−99 ATGACCCCCAATTAGTCCTGGCAG −76
Hbs	GCCTCCAATTAGTGT
mCC1	ATGACCCggAATTAGTCCTGGCAG
mCC2	ATGACCCgtAATTAGTCCTGGCAG
mAA	ATGACCCCCggTTAGTCCTGGCAG

*Wild-type osteocalcin promoter sequences are named WT and numbered according to the length of the oligonucleotide. The osteocalcin promoter location of these sequences is numbered at each end of the oligonucleotides. WT45 contains the first 32 nucleotides of the basal −108 promoter plus an additional 12 nucleotides upstream. The locations of the OC box, E-box, and Msx-consensus are marked on these sequences. WT24 is the OC box sequence. The oligonucleotides containing the Msx-consensus DNA binding site are designated Hbs for Hox binding site. The rat OC box was mutated at several locations as indicated by the nucleotides designated in lowercase letters. Oligonucleotides containing mutations of the OC box have been named according to the nucleotides which have been mutated. CAT constructs containing these mutations in the osteocalcin promoter (p-1097) used in transient transfections are designated with the same names as the oligonucleotides containing these mutations within the OC box.

contacts of the OCBP with OC box sequences were determined. The pattern of methylation interference is shown in Figure 3A and compared to the methylation interference pattern of the Msx group of proteins [Heinrichs et al., 1993] which bind to the same domain, shown in Figure 3B. Interference patterns were confirmed by densitometric analysis. These data demonstrate that the OCBP complex binds sequences that flank the Msx consensus core (ATTA). As contact points are found on both sides of the Msx consensus binding motif, binding of OCBP and Msx-related proteins may be mutually exclusive. The OCBP binding site is near a previously identified AP-1 recognition site [Owen et al., 1990]; however, OCBP is not AP-1 related because it is not competed by an AP-1 consensus binding site, and antibodies directed against Fos and Jun family members do not affect formation of this complex (data not shown).

Binding of the OCBP Functionally Correlates to Upregulation of Osteocalcin Gene Transcription

Protein–DNA interactions of each OC box mutant were analyzed in relation to transcriptional activity in ROS 17/2.8 cells (Fig. 4). Expression of OC promoter–CAT constructs carrying the same mutations used in gel mobility shift assays was analyzed following transient transfection. Two mutations, mCC1 and mCC2, which increased association of OCBP, resulted in a 252% and 287% increase in expression, respectively. Interestingly, these mutations have opposite effects on the binding of Hox complexes; mCC1 decreases binding and mCC2 increases binding. The increase in transcriptional activity is therefore correlated with formation of the OCBP complex, not the Hox complex. By comparing mCC1 and mCC2 sequences (Table 1) it is apparent that altered complex formation is a consequence of a single nucleotide change, nucleotide −91 from cytosine to guanine. In three mutations (mTT, mAA, and mAG) decreased promoter activity is reflected in the relative representation of OCBP binding (Fig. 4, lanes 2, 4, 5). The same binding patterns were observed using binding conditions of lower stringency (data not shown). Taken together, these findings suggest that the OCBP complex acts as a positive regulator of osteocalcin transcription.

The OCBP Expression is Restricted to Osteoblast-Like Cells

Since binding of OCBP correlated to increased promoter activity of the osteocalcin gene in ROS 17/2.8 cells, nuclear extracts from the osseous cell lines ROS 17/2.8, UMR, Saos, and MC3T3, and the nonosseous cell lines, HeLa, FRTL-5, R2, and H4 were used to investigate cell type–specific formation of the OCBP complex with OC box sequences (Fig. 5). Using the OC box wild-type sequence as probe (Fig. 5A), osseous cell extracts form OCBP complexes as well as subsets of the Msx-related complexes found in ROS cells. Nonosseous cells do not contain the OCBP as demonstrated by binding patterns with both the wild-type probe (Fig. 5A) and the mCC1 probe (Fig. 5B). The mCC1 mutant probe retains binding of significant levels of the OCBP

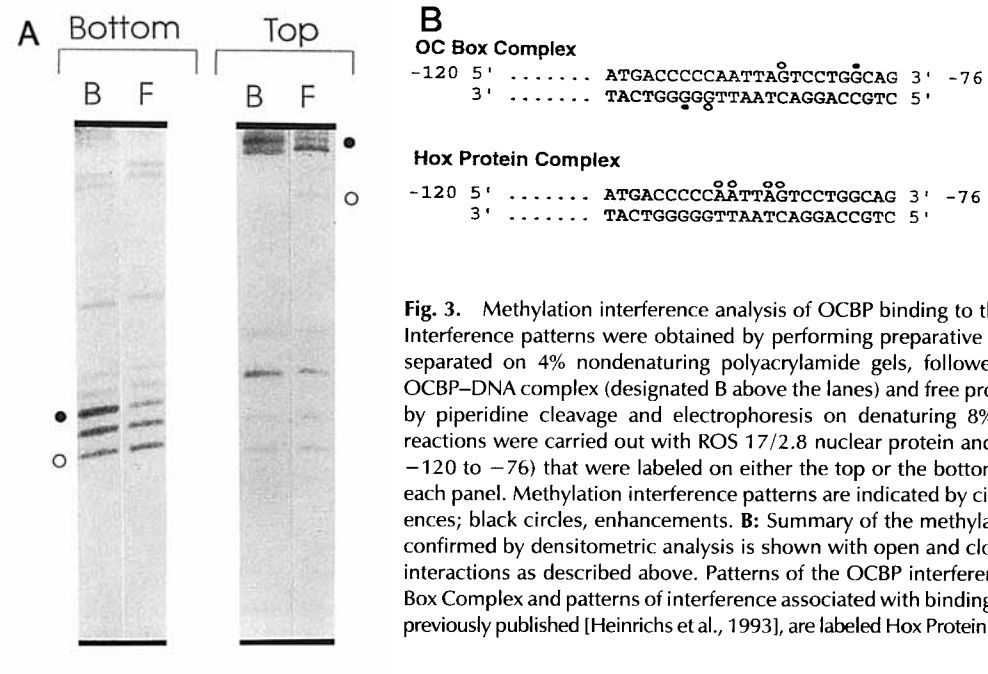


Fig. 3. Methylation interference analysis of OCBP binding to the osteocalcin promoter. **A:** Interference patterns were obtained by performing preparative gel shift assays which were separated on 4% nondenaturing polyacrylamide gels, followed by electroelution of the OCBP–DNA complex (designated B above the lanes) and free probe (designated F), and then by piperidine cleavage and electrophoresis on denaturing 8% sequencing gels. Binding reactions were carried out with ROS 17/2.8 nuclear protein and methylated probes (OC nt −120 to −76) that were labeled on either the top or the bottom strand as indicated above each panel. Methylation interference patterns are indicated by circles. Open circles, interferences; black circles, enhancements. **B:** Summary of the methylation interference pattern as confirmed by densitometric analysis is shown with open and closed circles used to indicate interactions as described above. Patterns of the OCBP interference analysis are labeled OC Box Complex and patterns of interference associated with binding of the Msx-related proteins, previously published [Heinrichs et al., 1993], are labeled Hox Protein Complex.

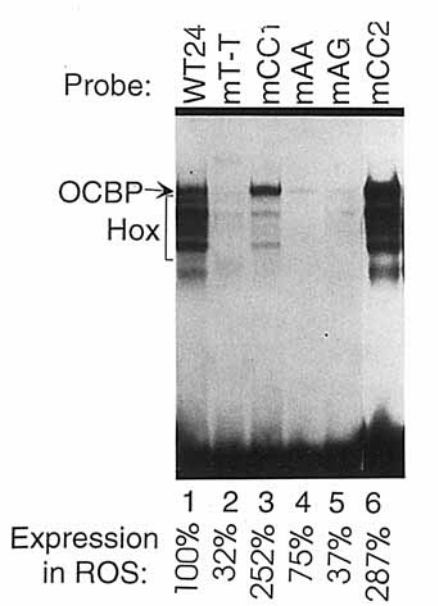


Fig. 4. Binding of ROS 17/2.8 nuclear proteins to wild-type and mutated OC box sequences. Radiolabeled oligonucleotides containing the sequences indicated above each lane and detailed in Table II were incubated with 5 μg ROS 17/2.8 nuclear protein and separated on a 6.5% polyacrylamide gel. Equal amounts of radioactivity were loaded in each lane. Identity of the Msx-related complexes and OCBP were determined by competition analysis (data not shown) and are labeled to the left of the gel. Expression of p-1097 osteocalcin promoter–CAT constructs containing the same mutations as the oligonucleotides used in the gel mobility shift assays shown here was assayed by monitoring CAT activity in transiently transfected ROS 17/2.8 cells. The percent acetylation was normalized to wild-type p-1097 expression and is represented at the bottom of each lane as relative values of expression. Transfection data represent the mean value of at least five independent experiments with 2–6 samples per experiment.

compared with binding of the Hox complexes. Relative association of the different complexes with wild-type and mutant sequences is more clearly seen in Figure 4, where probes of equal specific activity were used to compare complex formation. However, the data in Figure 6 illustrate that the OCBP is not detectable in nonosseous cells even with the mCC1 probe, and that the mCC1 mutation does not bind factors which did not bind to the wild-type probe but rather alters the relative ratio of complexes forming with the sequences.

OCBP Formation is Ion Dependent and Heat Labile

To further characterize properties of the OCBP, we determined if the addition of chelating agents to the gel mobility shift reactions prevented formation of this complex (Fig. 6A). EDTA and EGTA did not diminish OCBP formation even at 25 mM concentration (Fig. 6A, lanes 6, 7). However, 1,10-phenanthroline (PNT) did decrease OCBP formation as shown by titration of this chelator from 0 to 20 mM concentrations (Fig. 6A, lanes 1–4). These data demonstrate that the OCBP requires the presence of an ion for formation.

In addition, complexes associating with the WT24 OC box probe were tested for their temperature lability (Fig. 6B). The OCBP and a subset of the Hox complexes associating with the OC box appear to be temperature sensitive. Heating of ROS 17/2.8 nuclear extracts at 45°C

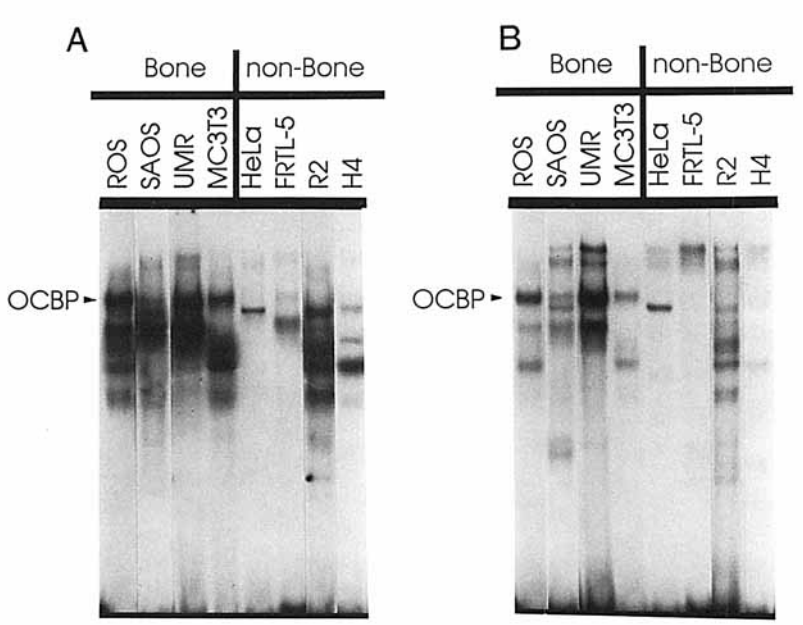


Fig. 5. Association of nuclear proteins from osteoblastic (bone) and nonosseous (non-Bone) cell lines with OC box sequences and the mCC1 mutant. Radiolabeled oligonucleotides containing (**A**) wild-type OC box sequences or (**B**) mCC1 sequences (detailed in Table II) were incubated with 5 μg nuclear protein from different cell lines as indicated. Equal amounts of radioactivity were used in each reaction and the protein/DNA interactions were separated on a 6.5% polyacrylamide gel. Msx-related complexes and OCBP were determined by competition analysis (data not shown). Migration of the OCBP is indicated to the left of the gels.

for 5 minutes diminished formation of four of the six complexes associating with the OC box sequences. This heat lability is more pronounced than previously reported using a 45 nucleotide probe [Heinrichs et al., 1993] because of the better resolution of the multiple Hox complexes in this study.

Abrogation of Tissue-Specific OC Expression by Mutated OC Box Sequences

Transcriptional activity of osteocalcin promoter/CAT constructs containing wild-type and mutated OC box sequences was explored in different cell lines (Table II). The wild-type OC promoter construct is expressed only in osteoblasts (ROS 17/2.8 and MC3T3). For the four mutations which resulted in decreased promoter activity in ROS 17/2.8 cells, no expression was detected in other cell lines. Notably, both the mCC1 and mCC2 mutants exhibited increased expression in ROS 17/2.8 cells (Table II, Fig. 4), but the mCC2 mutant was not expressed in nonosseous cells. Only the mCC1 mutation resulted in a loss of cell type–specific expression leading to activity in UMR, HeLa, and R2 cells (Table II). Characteristic expression of the wild-type and mCC1 OC box mutation in R2 fibroblast cells is shown in Figure 7, demonstrating the dramatic loss of cell-type specificity exhibited by mCC1. Absence of expression of the mCC2 mutant, which differs by only one nucleotide from mCC1, may relate to the finding that mCC2 does not alter the ratio of Hox OCBP complex formation relative to wild-type OC box sequences, (Fig. 4, lanes 1 and 6), and therefore remains specific to osteoblasts (Table II). In contrast, the mCC1 mutant nearly abrogates Hox protein binding. This finding suggests that in nonosseous cells, Hox protein binding contributes to suppression of OC transcription and further supports the concept that the OC box controls tissue-specific expression.

Repression of Osteoblast Differentiation by Msx Antisense Treatments

The mCC1 mutant retains binding of the OCBP but increases activity of the osteocalcin promoter in cells that do not express the OCBP. It is possible therefore that this change in tissue specificity is due to altered association of Hox-related factors which are found in nonbone cell lines. To further investigate the influence of Hox proteins on repression of osteocalcin expression, antisense experiments were performed in cultures of fetal rat calvarial–derived osteoblasts which undergo differentiation *in vitro*. In

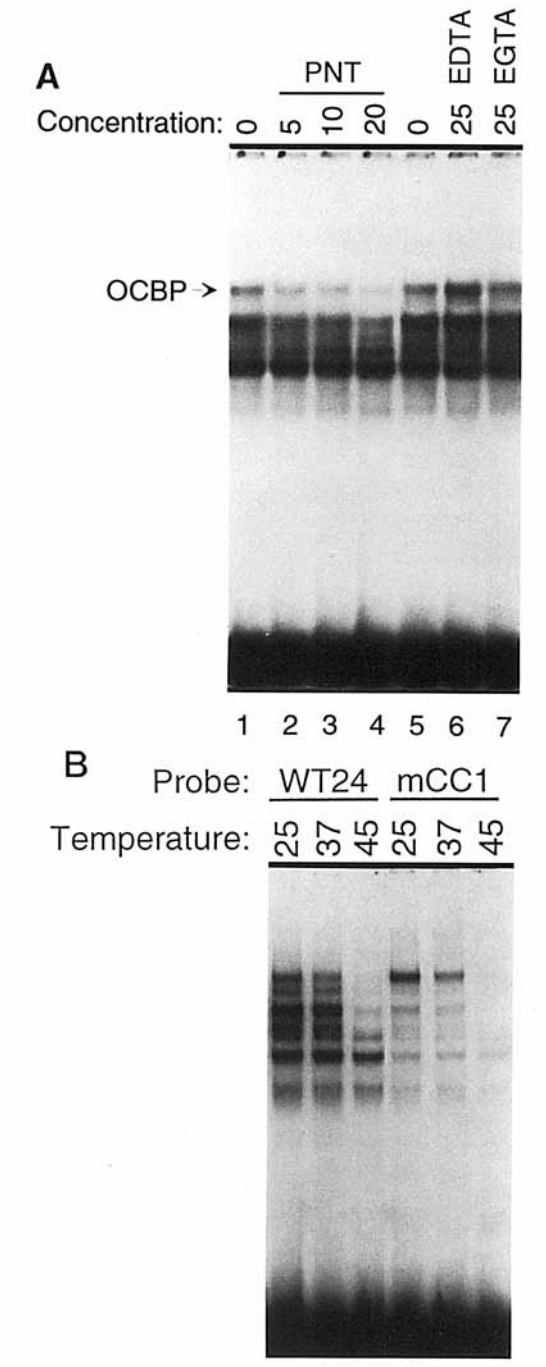


Fig. 6. The OCBP requires a metal ion to associate with the OC box and is temperature sensitive. **A:** Various concentrations of the chelating agents EDTA, EGTA, or 1,10-phenanthroline (PNT) were added to gel mobility shift assays containing the WT24 probe and 5 μg ROS 17/2.8 nuclear extract. The chelating agent and the millimolar concentration used is indicated above each lane. **B:** Five micrograms of ROS 17/2.8 nuclear extract which had been incubated for 5 min at the temperatures (°C) indicated above each lane was used for binding reactions with the WT24 probe or the mCC1 mutant.

the growth period, Msx-2 is expressed at maximal levels while Msx-1 mRNA levels are constitutive [Hoffmann et al., 1994]. Osteocalcin is expressed in these cultures only in the postproliferative period.

Osteoblast cultures were incubated with antisense oligonucleotides directed against Msx-1, Msx-2, or a scrambled sequence. Antisense oligonucleotides were added to medium daily throughout the osteoblast developmental sequence, beginning in the growth period. No increase in secreted osteocalcin (measured by radioimmunoassay) was observed in the early culture periods (data not shown). Rather, treatment with antisense to either Msx message, but not with scrambled sequence, resulted in decreased nodule formation (Fig. 8). Although small, immature nodules were present in all cultures, mature, large, nodules were less abundant in Msx-1 and Msx-2 antisense-treated cultures (Fig. 8c, d, and e). This suppression of osteoblast differentiation was paralleled by a decrease in osteocalcin levels (Fig. 8e).

DISCUSSION

In this study we have demonstrated that the proximal 108 nucleotides of the rat osteocalcin promoter are sufficient to retain tissue-specific expression. The proximal promoter (−108 to +24) contains three transcriptional regulatory elements, the E box, OC box and TATA box, which were defined by mutational analysis and sequence-specific protein–DNA interactions. The E box interacts with an HLH-related factor [Tamura and Noda, 1994], the OC box is recognized by homeodomain proteins [Hoffmann et al., 1994; Towler et al., 1994a,b], and the TATA box interacts with TFIID and associated factors [Dynlacht et al., 1991; Weinmann, 1992]. Although analysis of mutations of these sequences in nonosseous cell lines has not previously been reported, it has been shown that mutation of either the E box or the OC box leads to a reduction in expression of the osteocalcin promoter in ROS 17/2.8 cells [Tamura and Noda, 1994; Hoffmann et al., 1994; Towler et al., 1994a]. However, Msx-related proteins appear to represent negative regulators of osteocalcin promoter activity as suggested from our expression studies and gel mobility shift analysis in ROS 17/2.8 [this study], the pattern of Msx-2 expression during osteoblast development [Hoffmann et al., 1994], and overexpression studies by Towler et al. [1994b]. In fact, a recently cloned rHox pro-

TABLE II. Sequences of Wild-Type and OC Box Mutant Constructs and Their Expression in Different Cell Lines*

Construct	Sequence	Expression ROS 17/2.8	UMR	MC3T3	HeLa	R2
WT	−99 ATGACCCCCAATTAGTCCTGGCAG **−76**	+	−	+	−	−
mAA	ATGACCCCCggTTAGTCCTGGCAG	+/−	−	ND	−	−
mT	ATGACCCCCAAaTAGTCCTGGCAG	+/−	−	ND	−	−
mT-T	ATGACCCCCAAaTAGcCCTGGCAG	+/−	−	ND	−	−
mAG	ATGACCCCCAATTtcTCCTGGCAG	+/−	−	ND	−	−
mCC1	ATGACCCggAATTAGTCCTGGCAG	++	+	++	+	+
mCC2	ATGACCCgtAATTAGTCCTGGCAG	++	−	+	−	−

p-1097 (detailed in Fig. 1A) containing OC box sequences indicated here were transiently transfected into ROS 17/2.8, UMR, MC3T3, Hela, or R2 cells. Promoter activity was assayed by measuring the cellular extracts for acetylation of chloramphenicol. The presence of activity is represented by a + and no activity is represented by a −. Respectively, +/− and ++ represent a decrease or increase, of expression relative to WT. ND designates data that were not determined. Transfection efficiencies were monitored by assessing the activity of a transiently transfected construct containing an SV40 promoter. ROS data represents four independent experiments with at least duplicate samples in each experiment. R2 data represents triplicate samples from two independent experiments. HeLa data represents 3–9 samples. UMR and MC3T3 data represent three independent experiments with 3–6 samples each.

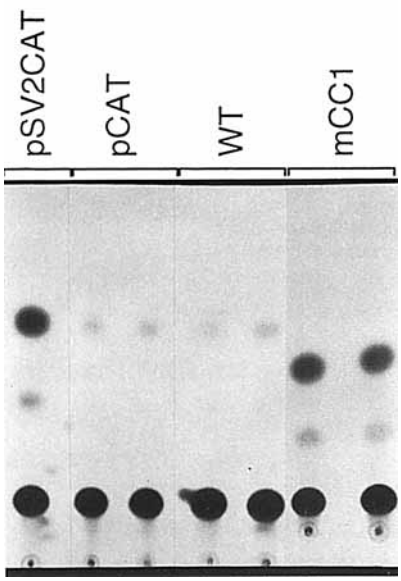
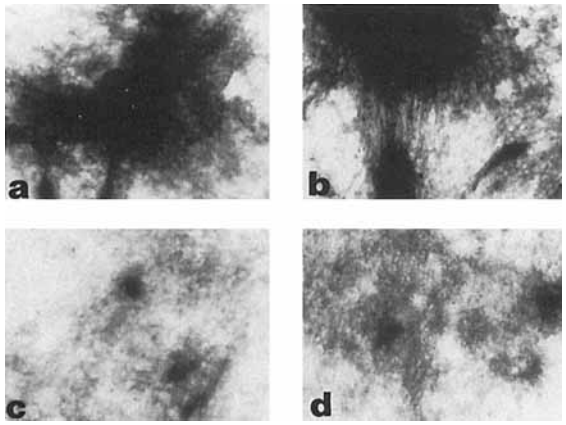


Fig. 7. The loss of cell-type specificity by the mCC1 construct. Representative CAT activity in R2 fibroblast cells. pSV2CAT construct contains an SV40 promoter upstream of the CAT gene and is used as a positive control for activity. pCAT is a promoterless construct used as a negative control. Representative activity of the osteocalcin promoter (p-1097) containing wild-type OC box sequences (WT) or the mCC1 (mCC1) mutant of these sequences is shown here for R2 cells. mCC1 samples were separated simultaneously on a TLC plate separate from the other samples.

tein also downregulates osteocalcin transcription [Hu et al., 1995], and Msx-1 activity has been shown to have a negative effect on transcription in general [Catron et al., 1995]. Thus, a second class of factors that function as positive regulators must associate with these sequence elements to account for transcriptional activity of the OC box. Here, our results demonstrate an additional regulatory factor binds at the OC box, the OC box binding protein (OCBP). OCBP is unrelated to homeodomain proteins and as an enhancer of promoter activity may contribute to control of the tissue-specific activity of the osteocalcin promoter.

Recent observations by Ducy and Karsenty [1995] also demonstrated that a region from −147 to −34 contains other sequence elements required for osteoblast-specific expression of the mouse OC gene. However, they dismiss the contribution of OC box sequences in regulating tissue specificity based on a lack of osteoblast-specific protein–DNA interaction with the OC box in their nuclear extracts. In contrast, data we present here and earlier studies [Heinrichs et al., 1993, 1995; Hoffmann et al., 1994; Towler et al., 1994a,b] indicate the OC box plays a key role in tissue-specific expression of the osteocalcin gene.

The OCBP we identified interacts with the same sequence element as the homeodomain (Hox) proteins *in vitro,* but binds independently of the Hox proteins, as indicated by the competition studies using the homeodomain binding site and mutated OC box sequences. The binding of OCBP is positively correlated with osteocalcin transcriptional activity in osteoblasts. The mCC1 mutation both increases the association of OCBP and also reduces association of the Hox proteins, leading to a significant 2.5-fold in-



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	% NODULES LARGE	% NODULES SMALL	% SECRETED OSTEOCALCIN
CONTROL	100	100	100
SCRAMBLE	100 ± 7	71 ± 9	71 ± 20
MSX - 1	29 ± 6	88 ± 11	32 ± 6
MSX - 2	37 ± 6	97 ± 6	41 ± 19

Fig. 8. Msx-1 or Msx-2 antisense suppresses osteoblast differentiation. Fetal rat calvaria–derived primary cultures were fed every 24 hr with fresh media containing 10% serum (untreated cells, **a**) and 1 μM phosphorothionate-modified 20mer oligonucleotides directed against Msx-1 (**c**), Msx-2 (**d**), or a scrambled mRNA sequence (**b**). Treatment began on day 5 and continued until day 18. Cultures were then fixed and stained for alkaline phosphatase activity and mineralization to show phenotypic development. (**e**) Summary of relative nodule formation and secreted osteocalcin levels. Nodules were counted in four or more wells for each condition assayed and expressed as a percentage of nodules found in control, untreated plates (100% = 30 or more nodules). Early stage focal nodules were scored as "SMALL," while later stage, fully developed nodular areas are termed "LARGE." Secreted osteocalcin levels represent three individual assays per well, four individual wells/condition, and are determined by RIA.

crease in promoter activity. However, since the mCC2 mutation, which increases binding of all factors, stimulates promoter activity 2.9-fold, the positive effect on transcription is contributed to by enhanced OCBP binding. This is further substantiated by the mutations that decrease OCBP binding (e.g., mAA, mAG, and mT-T) which result in a significant loss of promoter activity, thus supporting a role for the OCBP as an activator of osteocalcin transcription. Notably, OCBP binding with mCC2 is greater than with mCC1 although the increase in transcriptional activity relative to the wild-type promoter is in the same range; however, mCC2 retains binding of the homeodomain complexes which may repress further activation by the OCBP. Also, promoter activity is twofold greater in mAA (75%) compared to mutants which partially retain Hox interactions (mT-T 32%, and mAG 37%), again suggesting repressor activity of Hox proteins. There is no evidence from our studies that a new binding site has been created by the mCC1 or mCC2 mutation because the OCBP complex is formed with WT sequences; the mutations simply preserve and enhance OCBP binding.

The change in pattern of OCBP and homeodomain complex formation when the OC box is mutated can be attributed to a single nucleotide change at position −91, which is contiguous to the core nucleotides for Hox protein–DNA binding. Studies which determined the optimum binding site for Msx proteins demonstrated that these proteins prefer thymine at this position in the consensus sequence over guanine [Catron et al., 1993]. This is demonstrated in our gel mobility shift experiments which show mCC1 has a reduced affinity for homeodomain proteins, while mCC2 maintains association. The nucleotide at position −91 is a contact point for the OCBP as indicated by methylation interference studies reported in this paper. However, the change to guanine at this position increases the formation of the OCBP. These observations have two possible explanations. First, although the wild-type cytosine at this position may be acceptable for formation of the OCBP, associating proteins may actually prefer guanine as found in mCC1 or thymine as found in mCC2. Alternatively, an increase in OCBP formation in osteoblasts may be facilitated by the decrease in affinity of the Hox family proteins for these sequences when the sequence at position −91 is changed.

The mutation mCC1 increases expression of the OC promoter in ROS and MC3T3-E1 cells, and allows expression in the nonosseous cell lines (R2 and HeLa cells) and in an osteoblastic cell line, UMR106, which does not normally express osteocalcin. Thus, the OC box sequence is involved in tissue-specific expression, and this nucleotide appears critical to such regulations. The guanine substitution (in mCC1) results in an increase in OCBP binding and a decrease in Hox protein association in osteoblasts, which may reflect the basis for its effectiveness in permitting expression in nonosseous cells. The mutation may result in decreased binding of a suppressor Hox-related protein present in nonosseous cells or binding of an unknown factor. However, gel binding assays do not reflect the appearance of new complexes but rather changes

in the relative ratios of complexes observed with the wild-type sequences. The observation that minimal promoter sequences effect expression is not unprecedented. The biological importance of small promoter sequences in controlling tissue specificity in retinal photoreceptor cells has recently been demonstrated [Bobola et al., 1995].

The role of Msx proteins as supressors of transcriptional activity of the osteocalcin promoter was explored using antisense analysis in cultures of primary rat osteoblasts. The results demonstrate that the role of Msx proteins on osteoblast development is more complex than simple interaction with the osteocalcin promoter. Indeed, Msx proteins regulate collagen expression [Dodig et al., 1995], which is a critical component of the bone extracellular matrix and osteoblast differentiation [Lynch et al., 1995]. Our data demonstrate that a decrease in Msx protein production diminishes the differentiation of bone cells. As a consequence of this lack of differentiation, osteocalcin production is decreased rather than increased.

The OCBP is absent from the nonosseous cell lines studied and is found in all osteoblast cells we examined, including those that do not express OC. Therefore, formation of the OCBP complex may reflect a broader functional role in osteoblast differentiation. This is suggested by the detection of OCBP in UMR cells which do not express osteocalcin. Recently it was demonstrated that a UMR subclone could produce a mineralized matrix in culture under appropriate conditions, reflecting the mature osteoblast phenotype [Stanford et al., 1995]. The OCBP is clearly not the only determinant of osteocalcin expression, and other required factors may be lacking in osteoblasts having OCBP but not expressing osteocalcin. It is becoming increasingly evident that multiple sequences contribute to tissue specificity by sequence-specific interactions with both positive and negative regulatory factors [Cuif et al., 1992; Liu et al., 1992].

In summary, we have presented several lines of evidence for the involvement of the OCBP complex in directing bone tissue–specific expression of the osteocalcin gene. OCBP functions as a key positive regulatory factor in transcriptional control; the data also supports a role for the Hox proteins in contributing to tissue-specific suppression of osteocalcin. It is apparent that multiple classes of regulatory factors bind to a very short and highly conserved segment of the proximal OC gene promoter to control tissue-specific expression *in vitro*. The extent to which these factors act independently or synergistically to regulate osteocalcin gene transcription remains to be established.

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