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Two Distinct Osteoblast-Specific *cis*-Acting Elements Control Expression of a Mouse Osteocalcin Gene

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Osteoblasts are cells of mesodermal origin that play a pivotal role during bone growth and mineralization. The mechanisms governing osteoblast-specific gene expression are still unknown. To understand these mechanisms, we analyzed the *cis*-acting elements of mouse osteocalcin gene 2 (mOG2), the best-characterized osteoblast-specific gene, by DNA transfection experiments in osteoblastic and nonosteoblastic cell lines and by DNA-binding assays. 5′ deletion analysis of an mOG2 promoter-luciferase chimeric gene showed that a region located between −147 and −34 contained most if not all of the regulatory elements required for osteoblast-specific expression. Three different binding sites, called A, B, and C, for factors present in nuclear extracts of osteoblasts were identified in this short promoter by DNase I footprint assays. In gel retardation assays, the A element, located between bp −64 and −47, bound a factor present only in nuclear extracts of osteoblastic cell lines and nonmineralizing primary osteoblasts. The B element, located between bp −110 and −83, bound a ubiquitously expressed factor. The C element, located between bp −146 and −132, bound a factor present only in nuclear extracts of osteoblastic cell lines and nonmineralizing and mineralizing primary osteoblasts. When cloned upstream of a minimum osteocalcin promoter or a heterologous promoter, multimers of the A element strongly increased the activities of these promoters in osteoblastic cell lines at two different stages of differentiation but in no other cell line; we named this element osteocalcin-specific element 1 (OSE1). Multimers of the C element increased the activities of these promoters predominantly in a differentiated osteoblastic cell line; we named this element OSE2. This study demonstrates that two distinct *cis*-acting elements are responsible for osteoblast expression of mOG2 and provides for the first time a functional characterization of osteoblast-specific *cis*-acting elements. We speculate that these two elements may be important at several stages of osteoblast differentiation.

The mechanisms controlling osteoblast-specific gene expression are still poorly understood. This question is important considering the biological function of this cell type and the incidence of inherited or acquired bone diseases which affect osteoblast functions. Indeed, the osteoblast, a cell of mesodermal origin, plays a critical role during bone formation and mineralization (3, 40). Once it is fully differentiated, the osteoblast exits the cell cycle and produces most of the components of the bone extracellular matrix that will become mineralized. The molecular effectors controlling differentiation in the osteoblastic lineage until the stage of a mineralizing cell have not been identified. Osteoblast differentiation is marked by the sequential activation of many genes encoding bone extracellular matrix proteins like alkaline phosphatase (3), collagens (49), proteoglycans (5), bone sialoprotein (16), osteopontin (30), and osteocalcin (39) and hormone receptors such as vitamin D_3 and parathormone receptors (40). Most of these genes are expressed in a differentiation-dependent manner in osteoblasts but also in other cell types.

On the other hand, the osteocalcin gene, whose expression is regulated at the transcriptional level by hormones like vitamin D_3 (10) and growth factors like tumor necrosis factor alpha (26), is expressed at high levels only in differentiated osteoblasts and odontoblasts, the tooth counterpart of osteoblasts (39). Therefore, it is an excellent marker with which to study the mechanisms of osteoblast-specific gene expression. The *cis*-acting elements required for osteoblast-specific expression of the osteocalcin gene, although not identified, have been located in the 5′ flanking region of the gene. Two DNase I-hypersensitive sites have been found in the promoter of the rat osteocalcin gene only in osteoblast nuclei (34). In transgenic mice, 1.3 kb of the promoter of the human gene is active only in osteoblasts (22). Finally, in DNA transfection experiments, a 200-bp fragment of the rat osteocalcin promoter is active in ROS 17/2.8 osteoblastic cells (45, 47). In this 200-bp fragment, an E box, a CCAAT motif, and a putative MSX1-binding site have been identified (47). The latter two elements formed the osteocalcin box (27), a region thought to play a role in the osteoblast-specific expression of the osteocalcin gene. However, neither a mutation in the E box (45) nor forced expression of MSX1 in ROS 17/2.8 cells greatly affects the promoter activity (48). These results strongly suggest that other *cis*-acting elements, not yet characterized, are responsible for osteoblast-specific expression of the osteocalcin gene.

Several mouse or rat osteoblastic cell lines, representing various stages of osteoblast differentiation, are available, providing useful tools with which to study osteoblast-specific gene expression (29, 31, 44). Among them, the ROS cell lines derived from a rat osteosarcoma have been widely used (29). ROS 17/2.8 cells have markers of both immature and more differentiated osteoblasts (33). They express type I collagen, alkaline phosphatase, and osteocalcin genes; they have functional vitamin D_3 (14) and parathormone receptors, and they can mineralize a matrix in vivo (29) but not in tissue culture conditions. One can reproduce in these cells the pattern of activity of deletion mutants of the rat α1(I) collagen promoter

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observed in transgenic mice (24), suggesting that they are a good system with which to study osteoblast gene expression. ROS 25 cells have also some osteoblastic characteristics: they express type I collagen and alkaline phosphatase genes weakly and have few functional vitamin D_3 and parathormone receptors (14, 29). However, they express the osteocalcin gene to a much lower level than ROS 17/2.8 cells and do not mineralize a matrix in vivo (29), indicating that they most likely represent an earlier stage of osteoblastic differentiation.

With the long-standing goal of understanding the mechanisms of osteoblast-specific gene expression, we have cloned the mouse osteocalcin genes (11) and have now embarked on a systematic analysis of the regulatory elements of one of these two genes by transient DNA transfection experiments and DNA-binding assays. We report here that two *cis*-acting elements, never identified before and present within the first 147 bp of the osteocalcin promoter, are responsible for its restricted activity in osteoblasts. One of these elements, osteocalcin-specific element 1 (OSE1), binds a factor present in nuclear extracts of osteoblastic cell lines and nonmineralizing primary osteoblasts but absent from nuclear extracts of other cell lines and mouse tissues. The second *cis*-acting element, OSE2, binds a factor present only in nuclear extracts of osteoblastic cell lines and of nonmineralizing and mineralizing primary osteoblasts. Multimers of OSE1 and OSE2 strongly increase the activity of an osteocalcin minimum promoter and a heterologous promoter only in osteoblastic cell lines. Altogether, these results identify these two sequences as the first functional osteoblast-specific *cis*-acting elements characterized.

MATERIALS AND METHODS

Cell culture and DNA transfection. The ROS 17/2.8, ROS 25, and F9 cell lines were cultured in Eagle's minimal essential medium (BioWhittaker)–10% fetal bovine serum (FBS; GIBCO). The C2 myoblasts were cultured in Dulbecco's modified Eagle's medium (DMEM; GIBCO)–10% FBS. The day before transfection, the cells were plated on 10-cm-diameter dishes at a density of 5×10^5 cells per dish for the ROS 17/2.8, ROS 25, or F9 lines and 7.5×10^5 cells per dish for the C2 line. Cells were transfected by the calcium phosphate coprecipitation method as previously described (9), using 10 μg of reporter plasmid constructs and 2 μg of pRSV/β-gal. After transfection, the cells were washed twice with phosphate-buffered saline (PBS; 150 mM NaCl, 10 mM sodium phosphate [pH 7.2]), and regular medium was added for 24 h, except for C2 cells, which were placed in a differentiation medium (DMEM–2% horse serum [JRH Biosciences]) for an additional 60 h. Cells were harvested by scraping them into 0.3 ml of 0.25 M Tris-HCl (pH 7.9) and lysed by three cycles of freeze-thawing. β-Galactosidase activities present in each lysate, measured by a colorimetric enzyme assay using resorufin β-D-galactopyranoside (Boehringer Mannheim) as a substrate, were used to normalize the transfection efficiency between different experiments. Luciferase activities were assayed by using a Monolight 2010 luminometer (Analytical Luminescence Laboratory) and D-luciferin substrate (Sigma) in 100 mM potassium phosphate (pH 7.8)–5 mM ATP–15 mM $MgSO_4$–1 mM dithiothreitol. The S194 myeloma cells were grown in suspension in DMEM–10% horse serum and transfected by electroporation. Log-phase cells (10×10^6) were placed in 250 μl of medium with 25 mM HEPES (*N*-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; pH 7.3) and shocked by a gene pulser apparatus set at 960 μF and 220 V in a 0.4-cm cuvette. Cells were collected after 48 h at 37°C, washed twice with PBS, resuspended in 0.3 ml of 0.25 M Tris-HCl (pH 7.9), and lysed by three cycles of freeze-thawing. β-Galactosidase and luciferase assays were performed as described above. All transfections were repeated at least five times with two different DNA preparations.

Primary cultures of newborn mouse osteoblasts were prepared as previously described (19), with the following modifications. Mice calvaria were sequentially digested for 20, 40, and 90 min at 37°C in α modified minimal essential medium (GIBCO)–10% FBS containing 0.1 mg of collagenase P (Boehringer Mannheim) per ml and 0.25% trypsin (GIBCO). Cells of the first two digests were discarded, whereas cells released from the third digestion were plated in α minimal essential medium–10% FBS.

DNA constructions. All inserts were cloned in the p4Luc promoterless luciferase expression vector. A *Sal*I site was introduced at +13 by PCR amplification of the mouse osteocalcin gene 2 (mOG2) sequence extending from −657 to +13 (−657/+13 fragment), using as primers the oligonucleotides 5′ CCAAGACCT GGCCCAG 3′ for the 5′ side and 5′ TGGTCGACTTGTCTGT 3′ for the 3′ side. This initial fragment was used to generate p657-luc. p1316-luc was created by inserting an *Eco*RV-*Nco*I fragment of the mOG2 promoter at the 5′ end of p657-luc. p343-luc was generated by an *Nco*I-*Bgl*III deletion of p657-luc. p147-luc was obtained by an *Nco*I-*Pvu*II deletion of p657-luc. Construct integrities were confirmed by restriction analysis. p343-luc was used as the template for PCR amplification to generate further deletion mutants of the mOG2 promoter. The oligonucleotides used as 5′ primers were 5′ TTGACCCACTGAGCACAT GACCCCCAATTAGTCCTGGCAGCA 3′ for p116-luc, 5′ TCCCCTGCTCCT CCTGCTTACATCAGAGAGCACA 3′ for p74-luc, and 5′ AGGGTACCCGA TATAAATGC 3′ for p34-luc. The 3′ oligonucleotide was the same as that used to generate p657-luc in all cases. All sequences of the PCR product were verified by automatic DNA sequencing (2). Multimers (three or six copies) of the wild-type or mutated A, B, and C oligonucleotides (Table 1) were cloned in the *Sma*I site of the p34-luc construct or of pLAG5, a plasmid containing the −46 to +10 sequence of the adenovirus type 2 major late promoter fused to the luciferase reporter gene (AMLP-luc). Insert identities were confirmed by DNA sequencing (2).

Nuclear extracts. Buffers for the preparation of nuclear extracts contained the following protease inhibitors: 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, and 10 μg each of leupeptin and pepstatin per ml. Nuclear extracts from cell lines were prepared by the method of Dignam et al. (12). Aliquots of ROS 17/2.8 nuclear extracts were heated 5 min at 40 or 45°C and centrifuged for 2 min at 14,000 × *g*; the supernatant was frozen at −80°C until further use. Nuclear extracts from isolated tissues of newborn mice and of primary osteoblasts at day 2 (nonmineralizing cells) and day 10 (mineralizing cells) of culture were prepared as previously described (13, 42).

DNA binding assays. p147-luc contains a unique *Hin*dIII site and a unique *Asp* 718 site located, respectively, at the 5′ and 3′ ends of the −147/+13 mOG2 fragment. p147-luc was digested with *Hin*dIII (or *Asp* 718), dephosphorylated, labeled by using T4 polynucleotide kinase and [γ-^{32}P]ATP, and then cut with *Asp* 718 (or *Hin*dIII). The labeled fragments were purified by acrylamide gel electrophoresis. Approximately 10 fmol of the probe was added to a 50-μl reaction mixture in the presence or absence of 75 μg of nuclear extract in a buffer containing 20 mM HEPES (pH 7.9), 7.5% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol, 0.5 mM phenylmethylsulfonyl fluoride, 10 μg each of leupeptin and pepstatin per ml, and 1.5 μg of poly(dI-dC) · poly(dI-dC). Binding reaction mixtures were assembled on ice and incubated at room temperature for 15 min. DNase I (Worthington DPRF; 1 mg/ml in 50% glycerol) was diluted in cold 10 mM Tris-HCl (pH 7.5)–50 mM NaCl–62.5 mM $MgCl_2$–1 mg of bovine serum albumin per ml immediately before use. Two microliters of diluted DNase I (80 μg/ml) was added, and the binding reaction mixtures were mixed and incubated for 1 min at room temperature. Reactions were stopped by addition of 10 μl of 125 mM EDTA–5% sodium dodecyl sulfate–20 μg of yeast tRNA, extracted twice with phenol-chloroform, ethanol precipitated, washed, dried, and resuspended in a loading buffer containing 0.25 mg of bromophenol blue per ml, 0.25 mg of xylene cyanol FF per ml, 80% formamide, and 10 mM NaOH. Denatured samples were analyzed by electrophoresis on 6% polyacrylamide gels containing 7 M urea and 1× TBE (89 mM Tris-HCl, 89 mM boric acid, 8 mM EDTA). The end-labeled *Hin*dIII-*Asp* 718 fragments of p147-luc subjected to the A+G sequencing reaction of Maxam and Gilbert (32) were loaded on the same gels for identification of protected sequences. The wet gels were autoradiographed. For gel retardation assays, double-stranded oligonucleotides containing the wild-type or mutated A, B, and C regions (Table 1) were labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase, filled in with the Klenow fragment of DNA polymerase I, and purified on an acrylamide gel. Approximately 5 fmol of the probes was added to 6 μg of nuclear extracts in 10 μl of a buffer containing 20% glycerol, 50 mM Tris-HCl (pH 7.5), 50 mM NaCl, 2 mM EDTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, and 2.5 μg each of leupeptin and pepstatin per ml. Poly(dI-dC) · poly(dI-dC) (1.5 μg) and single-stranded DNA (250 fmol) were used as nonspecific competitors. After incubation at room temperature for 10 min, samples were subjected to electrophoresis on a 5% polyacrylamide gel in 0.25× TBE at 160 V for 90 min. The gels were dried and autoradiographed.

RESULTS

A −147/+13 mOG2 promoter fragment is active only in osteoblastic cells. The mouse genome contains two osteocalcin genes, mOG1 and mOG2, that are both expressed exclusively in bone (11). The DNA sequences of the promoters of these two genes are 93% identical over the first 1,000 bp upstream of the start site of transcription. They also share significant homologies with the promoters of the human and rat genes (Fig. 1). Since mOG1 and mOG2 have identical patterns of expression and very similar promoter sequences, we focused our experiments on one of them, mOG2. We first performed DNA transfection experiments by using 5′ deletion mutants of mOG2 promoter fused to the luciferase reporter gene (mOG2-

TABLE 1. Oligonucleotides used in this study

Name	Sequence[a]	Reference
A		
Wild type	GATCCTCCCCTGCTCCTCCTGCTTACATCAGAGAGCACA GAGGGGACGAGGAGGACGAATGTAGTCTCTCGTGTCTAG	This study
Mutant	GATCCTCCCCTGCTC**TTGGA**GC**A**T**G**CATCAGAGAGCACA GAGGGGACGAG**AACCT**CG**T**ACGTAGTCTCTCGTGTCTAG	This study
B		
47-bp wild type	GATCCTTGACCCACTGAGCACATGACCCCCAATTAGTCCTGGCAGCAA GAACTGGGTGACTCGTGTACTGGGGGTTAATCAGGACCGTCGTTCTAG	This study
34-bp wild type	GATCCACTGAGCACATGACCCCCAATTAGTCCTA GTGACTCGTGTACTGGGGGTTAATCAGGATCTAG	This study
34-bp mutant	GATCCACTGAG**A**ACAT**T**ACCCCCAATTAGTCCTA GTGACTC**T**TGTA**A**TGGGGGTTAATCAGGATCTAG	This study
23-bp wild type	CTAGCTGAGCACATGACCCCCAA GACTCGTGTACTGGGGGTTGATC	This study
C		
Wild type	GATCCGCTGCAATCACCAACCACAGCA GCGACGTTAGTGGTTGGTGTCGTCTAG	This study
Mutant	GATCCGCTGCAATCACCAA**GA**ACAGCA GCGACGTTAGTGGTT**CT**TGTCGTCTAG	This study
Sp1	AGGGCGGGGATTGAGGCGGG CCCGCCCCTAACTCCGCCCA	18
Muscle creatine kinase left E box	GATCCGAGCAGCCACATGTCTGG GCTCGTCGGTGTACAGACCCTAG	8
Myogenin	GATCCAACACCTGCTGCCTGAG GTTGTGGACGACGGACTCCTAG	8
CBF/NFY	AGGCAGTTCTGATTGGCTGGGGGCC CCGTCAAGACTAACCGACCCCCGG	21
C/EBP	AATTCAATTGGGCAATCAGG GTTAACCCGTTAGTCCTTAA	25
CTF/NF1	CCTTATTTTGGATTGAAGCCAATATGATAA GAATAAAACCTAACTTCGGTTATACTATTT	41
AP-1	AAAGCATGAGTCAGACACCT TTCGTACTCAGTCTGTGGAA	1

[a] The mutation substitutions introduced in the A, B, and C elements are represented in boldface.

luc) in ROS 17/2.8 osteoblastic cells, which express the osteocalcin gene at high levels (17). Our largest construct contained 1,316 bp of 5′ flanking sequence. We chose this fragment because a similar-size region of the human osteocalcin promoter confers osteoblast-specific expression to a reporter gene in transgenic mice (22). In DNA transfection experiments, successive deletions of mOG2-luc from −1316 to −147 decreased the level of expression of the reporter gene less than twofold. In contrast, a further deletion to −34 led to a 100-fold decrease, indicating that critical *cis*-acting elements are present between −147 and −34 (Fig. 2A).

To address the possible role of the DNA sequences located between −147 and −34 in mOG2 osteoblast-specific expression, we performed additional DNA transfections in ROS 25, a less differentiated osteoblastic cell line that expresses the osteocalcin gene at low levels when assayed by RNase protection assay (data not shown), and in more distant cell lines that do not express the osteocalcin gene, such as C2 mouse differentiated myotubes, F9 mouse teratocarcinoma cells, and S194 mouse myeloma cells. The p147-luc promoter was virtually inactive in C2 myotubes and in F9 and S194 cells and nearly 20-fold less active in ROS 25 cells than in ROS 17/2.8 cells (Fig. 2B). As expected, a construct containing only the first 34 bp of the mOG2 promoter was inactive in all cell lines (data not shown). These results thus establish the cell-selective activity of the mOG2 promoter in DNA transfection experiments and indicate that most if not all of the osteoblast-specific regulatory elements lie between −147 and −34.

DNase I footprint assays identify three binding sites in the −147/+13 mOG2 promoter. DNase I footprint assays were performed initially to define the sites of binding of nuclear proteins to the −147/+13 mOG2 fragment. We used as a source of proteins nuclear extracts of either ROS 17/2.8 osteoblastic cells or F9 teratocarcinoma cells. Analysis of DNA-protein interactions on both strands delineated three protected areas. The protected region designated A, lying between −64 and −47 relative to the start site of transcription in ROS 17/2.8 nuclear extracts (Fig. 3A, lanes 4 and 12), was most easily detectable on the upper strand. This region was larger when we used F9 nuclear extracts (Fig. 3A, lanes 3 and 11), raising the possibility that different factors in ROS 17/2.8 and F9 nuclear extracts bind to it. A second region, designated B and lying between −110 and −83, was protected by nuclear extracts of ROS 17/2.8 and F9 cells (Fig. 3A, lanes 3, 4, 11, and 12); a DNase I-hypersensitive site was observed only when we used F9 nuclear extracts (Fig. 3A, lane 11). The third region, designated C and lying between −146 and −132, was protected by ROS 17/2.8 nuclear extracts and by F9 nuclear extracts but to a much lower extent, suggesting that it may bind an osteoblast-specific factor (Fig. 3A, lanes 11 and 12). We noticed that several regions were protected only by F9 nuclear extracts, but they were not further studied. One of them, located 5′ of the C region, corresponds to vector sequences. The specificity of the DNA-protein complexes observed was demonstrated by competition experiments. The three footprints were competed for by a 200-fold molar excess of the unlabeled −147/+13 mOG2 fragment (Fig. 3A, lanes 5 and 13). The A footprint was com-

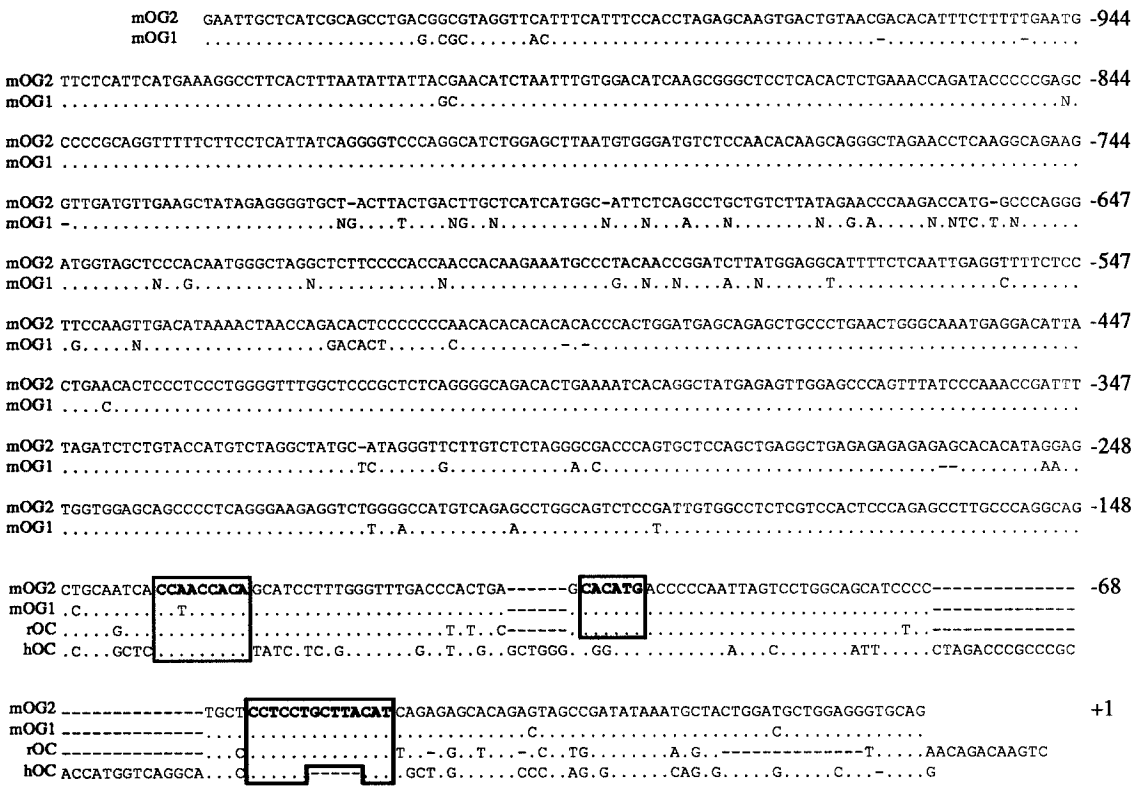


FIG. 1. Nucleotide sequence comparison of the mouse, rat, and human osteocalcin promoters. Sequences start at −1029 of mOG2, −1026 of mOG1, −143 of the rat osteocalcin gene (rOC [27]), and −176 of the human osteocalcin gene (hOC [35]) and end at +1 of each gene. The core sequences of the three binding sites described in this study are boxed. Numbers on the right correspond to the mOG2 sequence. Dots represent identical nucleotides; dashes indicate gaps introduced to maximize similarity.

peted for by a 200-fold molar excess of an A oligonucleotide, and the B and C footprints were competed for by the same molar excess of B and C oligonucleotides, respectively (Fig. 3A, lanes 6, 7, and 14), although the competition of the B region was never complete. None of these footprints were competed for by the same molar excess of an oligonucleotide containing a canonical Sp1-binding site (Fig. 3A, lanes 8 and 15).

The A region (−74 to −47) binds a nuclear factor present in osteoblastic cell lines and nonmineralizing primary osteoblasts. When we used an oligonucleotide covering the A region as a probe in a gel retardation assay and ROS 17/2.8 nuclear extract as a source of proteins, three prominent DNA-protein complexes were observed (Fig. 4A, lane 1). These three complexes were competed for by a 100-fold molar excess of the wild-type A oligonucleotide (Fig. 4A, lane 2). A double-stranded oligonucleotide containing a 6-bp mutation in the A-binding site (Table 1) did not bind any factor (Fig. 4A, lane 8) and was unable to compete for binding to the A region (Fig. 4A, lane 3). Also, double-stranded oligonucleotides containing either the B or the C region were unable to compete for binding of nuclear factors to the A oligonucleotide (Fig. 4A, lanes 4 and 5), indicating that the factors binding to the A region do not bind to the other protected regions. To determine if these three complexes were due to different nuclear factors, we performed gel retardation assays using different binding conditions. When we used heated nuclear extracts, the complexes of slower and faster mobilities were unaffected but the complex of intermediate mobility disappeared, indicating that the factor present in the latter complex was different from the factor(s) present in the former complexes (Fig. 4A, lanes 6 and 7).

To determine whether any of the factors binding to the A region had a restricted distribution, we used nuclear extracts from an array of cell lines, mouse primary osteoblasts in culture, and mouse tissues in gel retardation assays. The complexes of slower and faster mobilities were observed upon incubation of the probe with nuclear extracts of several cell lines and tissues; by contrast, the complex of intermediate mobility had a much more restricted distribution. It was observed when we used nuclear extracts of ROS 17/2.8 and ROS 25 cells (Fig. 4B, lanes 1 and 2), two cell lines representing different stages of osteoblast differentiation (29). It was also present when we used nuclear extracts of nonmineralizing primary osteoblasts but was undetectable when we used nuclear extracts of mineralizing primary osteoblasts (Fig. 4B, lanes 6 and 7). Finally, this complex was not detected when we used nuclear extracts of any other tissues (Fig. 4B, lanes 8 to 14). The DNA-protein complexes observed when we used F9 or brain nuclear extracts had different mobilities, suggesting that they contain different factors (Fig. 4B, lanes 4 and 10). In summary, these experiments indicate that at least two factors bind to the A region. One of these factors is detected only in nuclear extracts of nonmineralizing primary osteoblasts and osteoblastic cell lines, raising the possibility that this region acts as a *cis*-acting activator of mOG2 expression during early stages of osteoblast differentiation.

The B region (−110 to −83) is centered by an E box to which a ubiquitously expressed factor binds. The B region contains an E box (45), a CCAAT motif, and a putative MSX1-binding

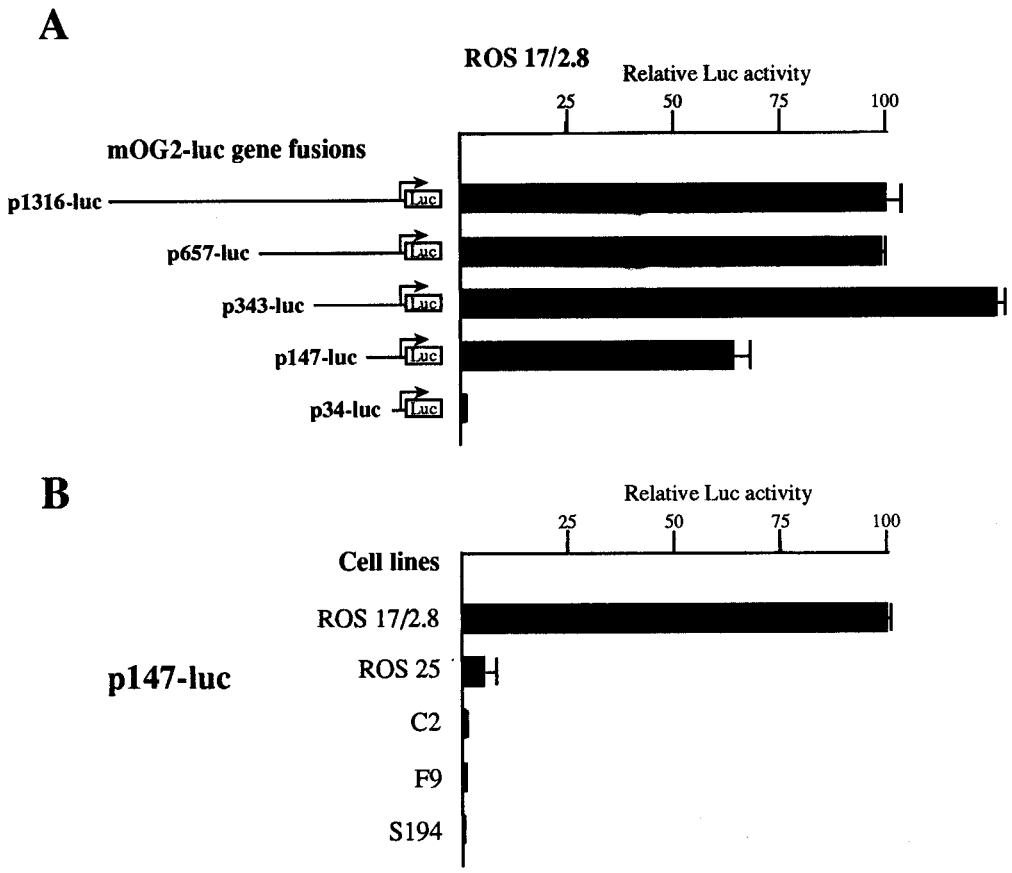


FIG. 2. Functional analysis of deletion mutants of the mOG2 promoter-luciferase chimeric gene by DNA transfection experiments. (A) Activity of deletion mutants of the mOG2-luc construct in ROS 17/2.8 cells. Deletions were generated as described in Materials and Methods; a schematic representation of each reporter construct is shown on the left. Values are relative to the activity obtained with the largest promoter fragment (p1316-luc). (B) p147-luc activity in ROS 17/2.8, ROS 25, C2, F9, and S194 cells. The results are expressed relative to those obtained in ROS 17/2.8 cells. Data represent the ratios of luciferase (Luc) to β-galactosidase activities, and values are means of 8 to 12 independent transfection experiments; error bars represent standard deviations of the means.

site (47). To determine which of these motifs could bind a factor(s) present in ROS 17/2.8 nuclear extracts, we used three different oligonucleotides (Table 1). The longest one, 47 bp long, contained the E box, the CCAAT motif, the putative MSX1-binding site and surrounding sequences. The second oligonucleotide, 34 bp long, corresponded to the protected areas observed in the DNase I footprint assay; it included the same elements but not the surrounding sequence of the MSX1-binding site. The third oligonucleotide, 23 bp long, contained the E box and only part of the CCAAT motif. When these three oligonucleotides were used as probes in a gel retardation assay, we observed in all cases identical DNA-protein complexes of slow mobility (Fig. 5A, lanes 1 to 3), suggesting that only one factor binds to the B region. This factor probably binds to the E box, since it is the only common recognizable element between the three oligonucleotides. (The fast-mobility complex present when we used the shortest oligonucleotide as a probe was not observed in every experiment.) To test this hypothesis, we performed competition experiments using as a probe the 34-bp oligonucleotide that contains both the E box and the CCAAT motif. The complex observed was competed for by an oligonucleotide containing the left E box of the muscle creatine kinase promoter (Fig. 5B, lane 4), whose sequence, CACATG, is identical to the sequence of the E box present in the B region (Table 1). In contrast, the myogenin-binding site, centered by a CACCTG E box (Table 1), could not compete for the binding of the factor to the B region (Fig. 5B, lane 5). Since a CCAAT motif is also present in the B region, we performed DNA competition experiments using as competitors oligonucleotides containing consensus binding sites for three different CCAAT-binding factors: CBF/NFY, C/EBP, and CTF/NF1 (Table 1). None of these oligonucleotides could compete for the binding of nuclear factors to the B region (Fig. 5B, lanes 6 to 8). We then introduced into the B oligonucleotide a 2-bp mutation known to abolish the binding of basic helix-loop-helix proteins to E boxes (8) (Table 1) and used this mutant oligonucleotide in direct binding and competition experiments. The binding of the factor to the B oligonucleotide was competed for by the wild-type B oligonucleotide but not by the mutant B oligonucleotide (Fig. 5B, lanes 2 and 3). This mutant B oligonucleotide did not bind any factor (Fig. 5B, lane 9). Taken together, these experiments establish that the only detectable factor binding to the B region binds to the E box and is likely to be a basic helix-loop-helix protein. This result is in agreement with a recent analysis of this region of the rat osteocalcin promoter (45). Since several E box-containing sequences are involved in cell-specific gene expression (36, 37), we tested whether the B region bound an osteoblast-specific factor. When we performed a gel retardation assay using nuclear extracts of cell lines, primary osteoblasts in culture, and mouse tissues, we observed the same DNA-pro-

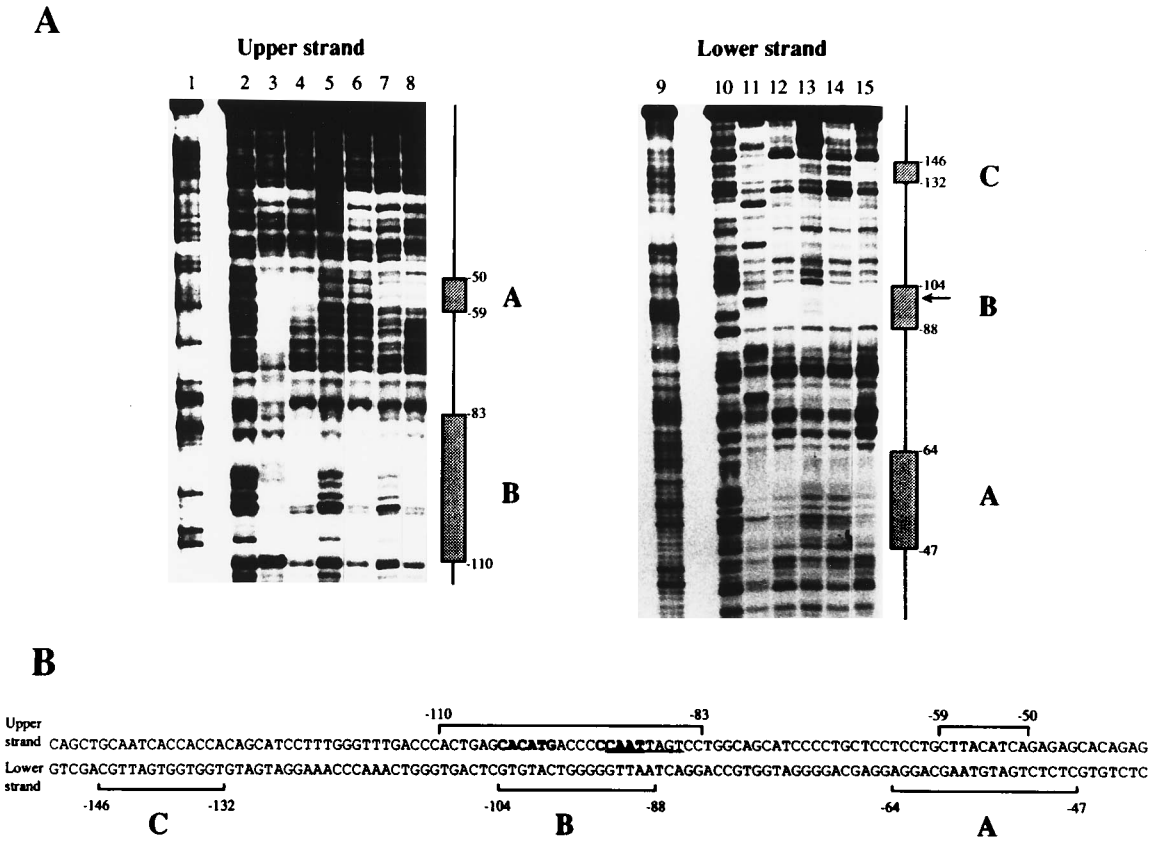


FIG. 3. Three binding sites are defined by DNase I footprint assay of the mOG2 −147/+13 promoter region. (A) ^{32}P-labeled upper strand and lower strands of the −147/+13 mOG2 promoter region were incubated in the presence or absence of nuclear extracts, digested with DNase I, and separated on a 6% sequencing gel. Protected regions observed when ROS 17/2.8 nuclear extracts were used are indicated by shaded boxes. The arrow shows the hypersensitive site present in footprint B with F9 nuclear extracts. Lanes 1 and 9, A+G Maxam-Gilbert reaction of the −147/+13 promoter fragment. Other lanes show DNase I digestion of the naked DNA incubated without nuclear extract (lanes 2 and 10), with F9 nuclear extract (lanes 3 and 11), with ROS 17/2.8 nuclear extract (lanes 4 and 12), with ROS 17/2.8 nuclear extract and a 200-fold molar excess of the −147/+13 cold fragment (lanes 5 and 13), with ROS 17/2.8 nuclear extract and a 200-fold molar excess of the A oligonucleotide (lane 6), with ROS 17/2.8 nuclear extract and a 200-fold molar excess of the B oligonucleotide (lane 7), with ROS 17/2.8 nuclear extract and a 200-fold molar excess of an Sp1 oligonucleotide (Table 1) (lanes 8 and 15), and with ROS 17/2.8 nuclear extract and a 200-fold molar excess of the C oligonucleotide (lane 14). (B) Summary of the DNase I footprint assays. The protected regions observed when ROS 17/2.8 nuclear extracts were used are indicated by open boxes. An E box and a CCAAT motif located in the B region are represented in boldface; a putative MSX1-binding site is underlined.

tein complex (Fig. 5C). Thus, our experiments indicate that the B region does not bind an osteoblast-specific factor.

The C region (−146 to −132) binds a factor present only in osteoblastic cell lines and primary osteoblasts. The C region was better protected by ROS 17/2.8 nuclear extracts in DNase I footprint assays, suggesting that it binds an osteoblast-specific factor. When we used in a gel retardation assay an oligonucleotide covering the C region as a probe, two distinct DNA-protein complexes were present in ROS 17/2.8 nuclear extracts: a prominent complex of slow mobility and a weaker complex of faster mobility (Fig. 6A, lane 1). We conducted a systematic mutational analysis of the C region to define precisely the DNA contacts made by these factors. Mutations of 1 or 2 bp introduced in the C region allowed us to identify a core sequence of eight nucleotides essential for the generation of the slower-mobility complex (Fig. 6B). The complex of faster mobility was not affected by any of the mutations, suggesting that these two complexes contain different factors. This, however, can be proven only when pure proteins become available. In DNA competition experiments, the two DNA-protein complexes were competed for by the wild-type C oligonucleotide but not by the same molar excess of either the mutant C oligonucleotide, the A oligonucleotide, or the B oligonucleotide (Fig. 7A, lanes 2 to 13). Lastly, because it has been suggested that AP-1 can bind an overlapping region of the rat osteocalcin promoter (4), we performed competition experiments using an oligonucleotide containing a consensus AP-1-binding site (Table 1). This oligonucleotide could not compete for the binding of nuclear factors to the C oligonucleotide (Fig. 7A, lanes 14 to 16).

To test whether the C region could bind an osteoblast-specific factor, we performed a gel retardation assay using nuclear extracts of the same cells and mouse tissues used previously. The factor present in the DNA-protein complex of slower mobility was present in nuclear extracts of ROS 17/2.8 cells, in mineralizing as well as nonmineralizing primary osteoblasts (Fig. 7B, lanes 1, 6, and 7), and in ROS 25 nuclear extracts when this gel was exposed for a longer period of time (data not shown). In contrast, no binding was detected in nuclear extracts of any other cell lines or mouse tissues (Fig. 7B, lanes 3 to 5 and 8 to 14). This result was not due to the quality of the nuclear extracts since the same extracts were used in the analysis of the A, B, and C regions. In conclusion, these data demonstrate that the C region binds a nuclear factor whose expression is restricted to cells of the osteoblastic lin-

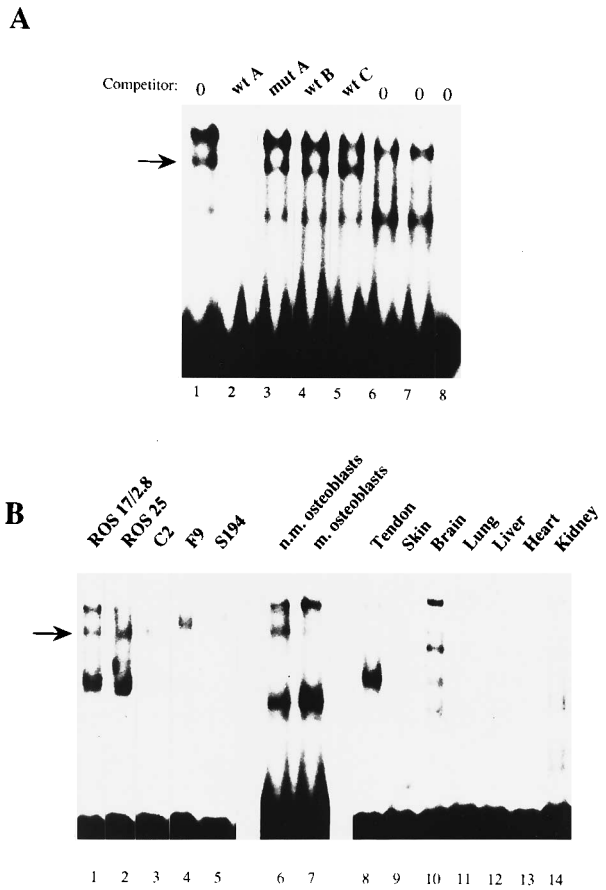


FIG. 4. The A region binds a factor present in osteoblast nuclear extracts. DNA binding was analyzed by gel retardation assay. (A) Labeled double-stranded wild-type (wt; lanes 1 to 7) and mutant (mut; lane 8) A oligonucleotides were used as probes with ROS 17/2.8 nuclear extracts. Competition experiments were performed with 100-fold molar excess of either the wild-type A oligonucleotide (lane 2), the mutant A oligonucleotide (lane 3), the B oligonucleotide (lane 4), or the C oligonucleotide (lane 5). DNA binding assays were performed with ROS 17/2.8 nuclear extracts heated for 5 min at 40°C (lane 6) or at 45°C (lane 7). (B) Equivalent amounts (6 μg) of nuclear extracts from cell lines (lanes 1 to 5), primary cultures of nonmineralizing (n.m.) and mineralizing (m) osteoblasts (lanes 6 and 7), or mouse tissues (lanes 8 to 14) were tested for binding to the A oligonucleotide. The cell-specific complex is indicated by an arrow.

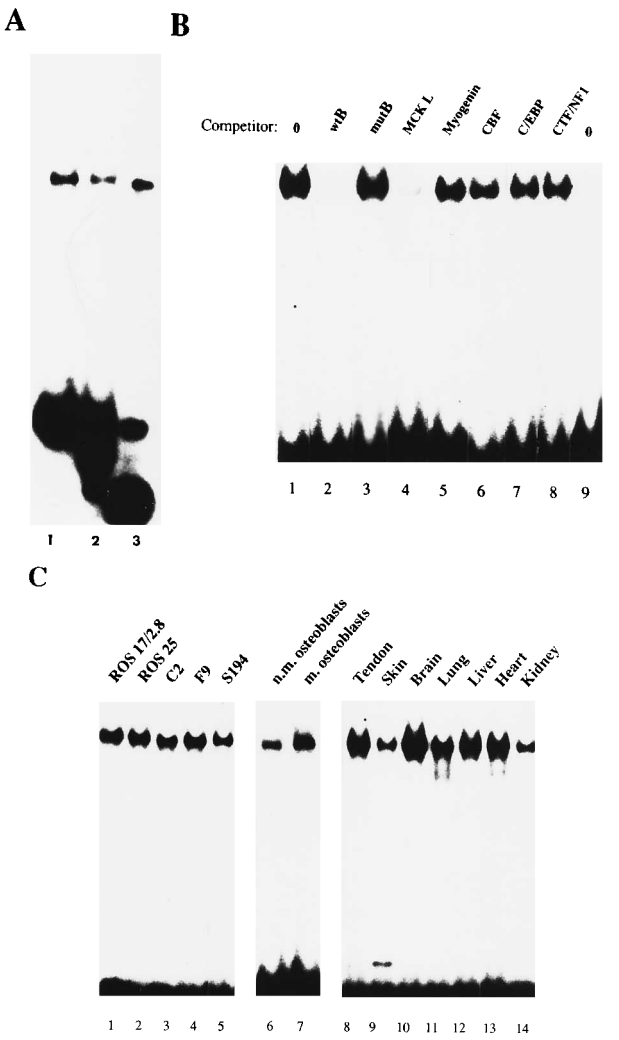
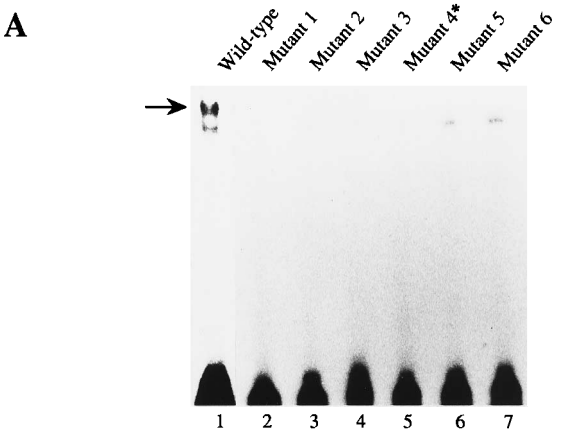


FIG. 5. The B region contains an E box to which a ubiquitously expressed factor binds. DNA binding was analyzed by gel retardation assay. (A) Three labeled wild-type oligonucleotides covering various lengths of the B region (47 [lane 1], 34, [lane 2], and 23 [lane 3] bp) (Table 1) were used as probes with ROS 17/2.8 nuclear extracts. (B) Labeled wild-type (wt B; lanes 1 to 8) and mutant (mut B; lane 9) 34-bp B oligonucleotides were used as probes with ROS 17/2.8 nuclear extracts. Competition experiments were performed with a 50-fold molar excess of oligonucleotides containing the wild-type B-binding site (lane 2) or the mutant B-binding site (lane 3) or a 200-fold molar excess of oligonucleotides containing the left E box of the muscle creatine kinase promoter (MCK L) (lane 4), the myogenin-binding site (lane 5), the CBF/NFY-binding site (lane 6), the C/EBP-binding site (lane 7), and the CTF/NF1-binding site (lane 8). (C) Equivalent amounts (6 μg) of nuclear extracts from cell lines (lanes 1 to 5), primary cultures of nonmineralizing (n.m.) and mineralizing (m) osteoblasts (lanes 6 and 7), or mouse tissues (lanes 8 to 14) were tested for binding to the B oligonucleotide.

eage, suggesting that this region is involved in the cell-specific expression of the mOG2 gene.

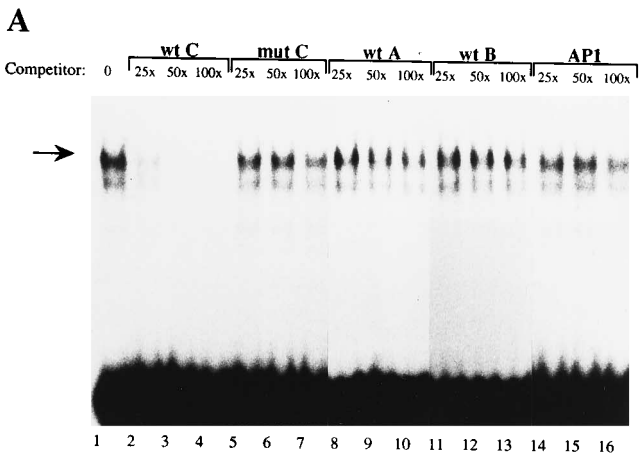
The A and C regions act as osteoblast-specific activators of transcription. To investigate the role of each of these three binding sites in mOG2 promoter cell-specific activity, we performed a series of DNA transfection experiments. Multimers of either the A, B, or C oligonucleotide were cloned upstream of the −34/+13 promoter, which contains only the TATA box as a known *cis*-acting element and has virtually no transcriptional activity (Fig. 2A). The different constructs generated were transfected in ROS 17/2.8, ROS 25, F9, and C2 cells (Fig. 8A). Six copies of the wild-type A oligonucleotide increased the activity of the −34/+13 promoter 200-fold in ROS 17/2.8 cells and 125-fold in ROS 25 cells. This result is consistent with the fact that the binding activity to the A region is represented in nuclear extracts of both cell lines (Fig. 4B). This stimulatory effect was independent of the orientation of the oligonucleotides and was specific since a mutation in the A region abolished it. In contrast, multimers of the A oligonucleotide did not increase −34/+13 promoter activity in C2 cells and had a very weak effect in F9 cells. Three copies of the B oligonucleotide, covering the OC box, increased the activity of the promoter only marginally in every cell line tested. Lastly, six copies of the wild-type C oligonucleotide increased −34/+13 promoter activity 270-fold above the basal level in ROS 17/2.8 cells, in which the binding activity to the C region is abundant, and 45-fold in ROS 25 cells, in which this binding activity is barely detectable. This effect was independent of the orientation of the oligonucleotides and was abolished by a 2-bp mutation. Multimers of the C oligonucleotide did not induce any increase in −34/+13 promoter activity in C2 or F9 cells.



B

Oligonucleotide	Sequence	Binding activity
C wild-type	AgCTgCAATCA**CCAACCAC**AgCA	+
C mutant 1	..........Ag..........	-
C mutant 2	..........gT..........	-
C mutant 3	..........gg..........	-
C mutant 4*	..........gA..........	-
C mutant 5	..........T..........	-
C mutant 6	..........g..........	-

FIG. 6. Mutational analysis of C-region bases essential for DNA-protein interaction. (A) The wild-type C oligonucleotide (lane 1) and several mutant C oligonucleotides (lanes 2 to 7) were tested in a gel retardation assay using ROS 17/2.8 nuclear extract as a source of proteins. The specific binding complex is indicated by an arrow. (B) Summary of the mutational analysis of the C-binding site. The sequence of the wild-type C oligonucleotide corresponding to the bases that were altered in mutated oligonucleotides is shown in boldface. The presence (+) or absence (−) of the specific complex in the gel retardation assay is indicated. The asterisk denotes the mutant C oligonucleotide used for subsequent DNA-binding assays and DNA transfection experiments.



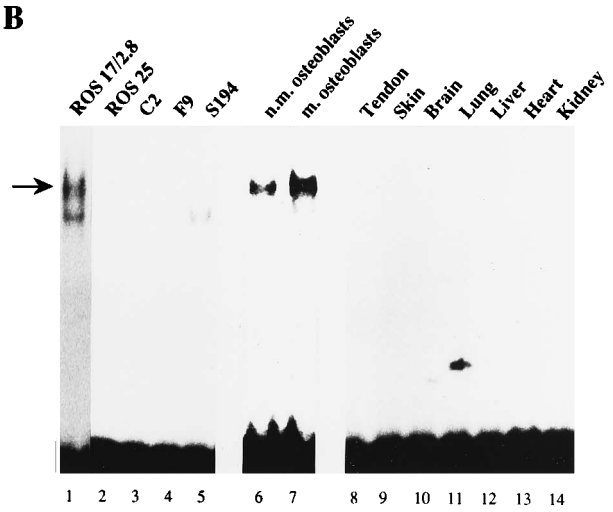


FIG. 7. The C region binds a factor present only in osteoblast nuclear extracts. DNA binding was analyzed by gel retardation assay. (A) Labeled wild-type C oligonucleotide was used as a probe with ROS 17/2.8 nuclear extracts. Competition experiments were performed with 25-, 50-, and 100-fold molar excesses of either the wild-type (wt) C oligonucleotide (lanes 2 to 4), the mutant (mut) C oligonucleotide (lanes 5 to 7), the A oligonucleotide (lanes 8 to 10), the B oligonucleotide (lanes 11 to 13), or an AP-1 oligonucleotide (lanes 14 to 16). (B) Equivalent amounts (6 μg) of nuclear extracts from cell lines (lanes 1 to 5), primary cultures of nonmineralizing (n.m.) and mineralizing (m) osteoblasts (lanes 6 and 7), or mouse tissues (lanes 8 to 14) were tested for binding to the C oligonucleotide. The specific binding complex is indicated by an arrow.

In another set of DNA transfection experiments (Fig. 8B), we cloned multimers of either the A, B, or C oligonucleotide upstream of a heterologous promoter fused to the luciferase reporter gene (AMLP-luc; see Materials and Methods). Multimers of the A oligonucleotide increased AMLP-luc activity 250-fold in ROS 17/2.8 cells and 200-fold in ROS 25 cells but not in other cell lines. Multimers of the C oligonucleotide increased the activity of AMLP-luc 80-fold in ROS 17/2.8 cells and 24-fold in ROS 25 cells but were inactive in C2 and F9 cells. Multimers of the B oligonucleotide were inactive in every cell line. In summary, the results of the transfection experiments indicate that the A and C regions act as osteoblast-specific activators of transcription, which we name OSE1 and OSE2, respectively. The B element corresponds to OCE1 (osteocalcin E-box sequence 1) present in the promoter of the rat osteocalcin gene (45); we named it mOCE1, for mouse OCE1.

Our results indicate also that OSE1 and OSE2 act differently in ROS 25 cells and lead to the hypothesis that OSE1 may be more important than OSE2 in less differentiated osteoblasts. To test this hypothesis, we generated 5′ deletion mutants of p147-luc, sequentially removing OSE2, mOCE1, and OSE1, and tested these constructs in DNA transfection experiments in ROS 17/2.8 (Fig. 9A) and ROS 25 (Fig. 9B) cells. In ROS 17/2.8 cells, a deletion from −147 to −116 removing OSE2 resulted in a fivefold decrease in promoter activity; a second deletion to −76, removing mOCE1 in addition to OSE2, did not further decrease promoter activity. The third deletion, to −34, which also removed OSE1, resulted in a further 18-fold decrease in promoter activity. In ROS 25 cells, we observed different results. A deletion of OSE2 and mOCE1 did not alter the promoter activity significantly. In contrast, when we deleted OSE1 in addition to OSE2 and mOCE1, we noted a 10-fold decrease in promoter activity. These results suggest that OSE1 and OSE2 act as transcriptional activators in ROS 17/2.8 cells and that OSE1 is the major contributor to mOG2 promoter activity in ROS 25 cells. However, this assay does not rule out the possibility that mOCE1 contributes to the mOG2 promoter activity in conjunction with OSE1 or OSE2.

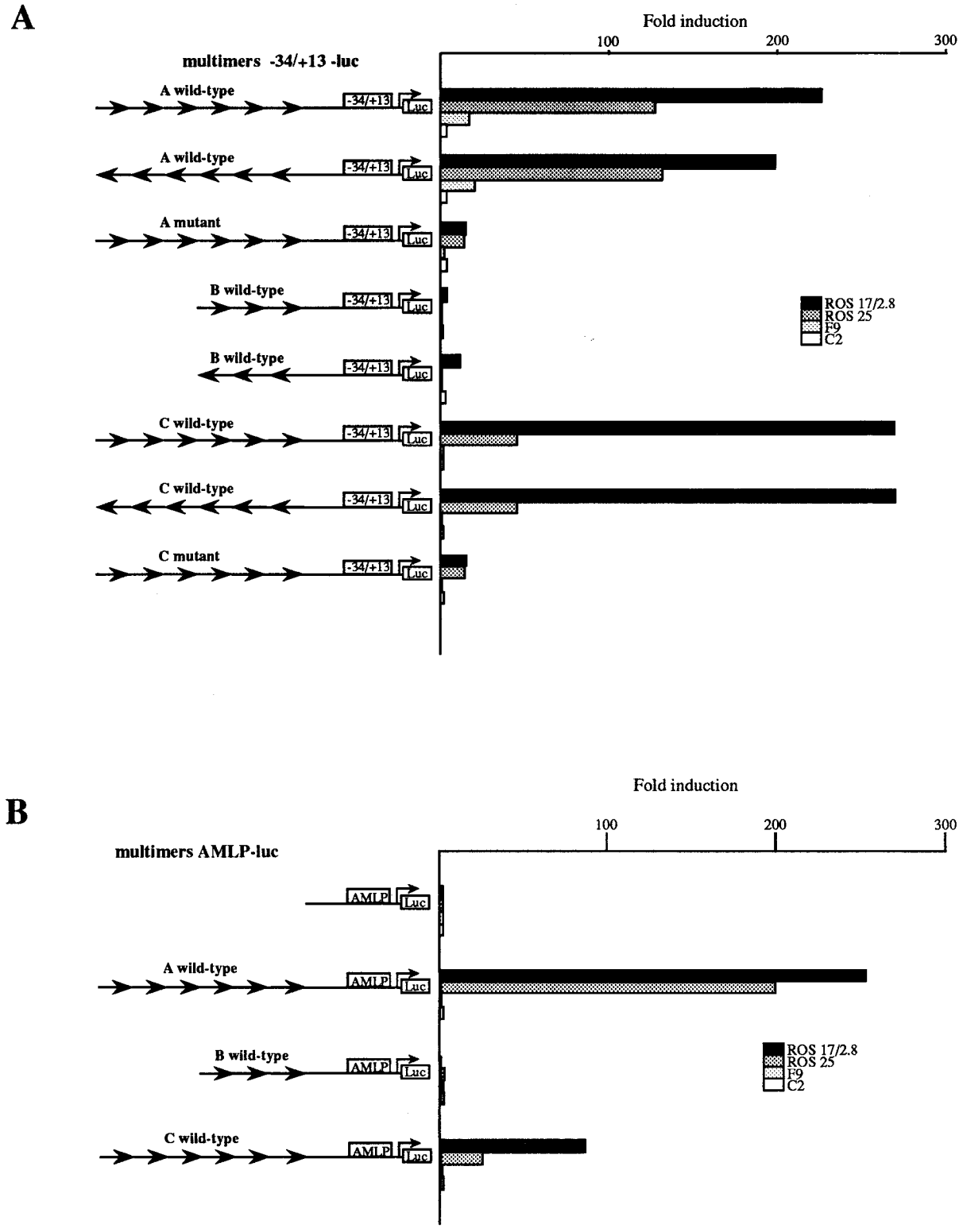


FIG. 8. Functional analysis of the A, B, and C regions by DNA transfection experiments. (A) Multimerized wild-type or mutated A, B, or C oligonucleotides fused to the −34/+13 mOG2-luc construct were assayed by transfection experiments in ROS 17/2.8, ROS 25, F9, and C2 cells. Values are relative to the activity obtained in ROS 17/2.8 cells with the −34/+13 mOG2-luc construct. (B) A multimerized wild-type A, B, or C oligonucleotide was cloned upstream of an AMLP-luc fusion construct and assayed by transfection experiments in ROS 17/2.8, ROS 25, F9, and C2 cells. Values are relative to the activity obtained in each cell line with the AMLP-luc construct. Data represent ratios of luciferase versus β-galactosidase activities, and values are means of five to nine independent transfection experiments.

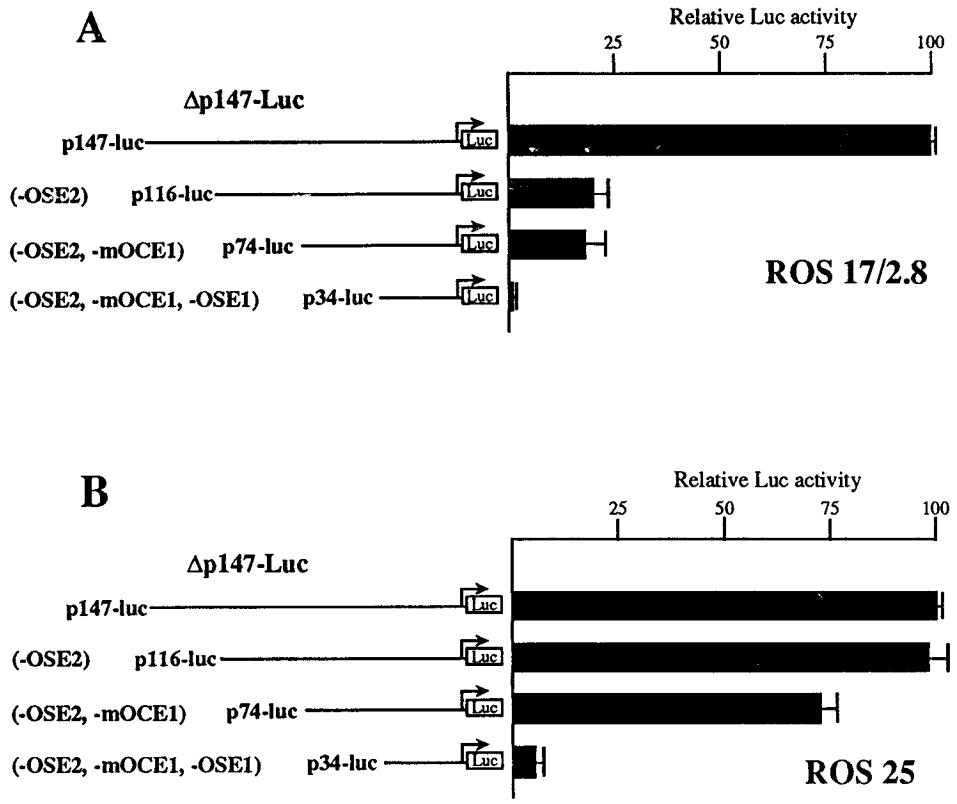


FIG. 9. Functional analysis of 5′ deletion mutants of p147-luc by DNA transfection experiments. Δp147-luc constructs were generated as described in Materials and Methods. p116-luc does not contain OSE1, p74-luc does not contain OSE1 and mOCE1, and p34-luc does not contain OSE1, mOCE1, and OSE2. Transfection experiments were performed in ROS 17/2.8 cells (A) or ROS 25 cells (B). Values are percentages of p147-luc activity. Absolute units of luciferase (Luc) activity of p147-luc were 74,246 ± 1,288 (n = 10) in ROS 17/2.8 cell and 3,242 ± 71 (n = 8) in ROS 25 cells. Data represent ratios of luciferase to β-galactosidase activities, and values are means of 8 to 12 independent transfection experiments; error bars represent standard deviations of the means.

DISCUSSION

To date, no osteoblast-specific *cis*-acting element that could (i) bind a factor present predominantly or exclusively in osteoblasts and (ii) activate transcription only in osteoblastic cells has been described. With the long-term goal of understanding the mechanisms of osteoblast-specific gene expression, we have begun to analyze the regulatory sequences of the osteocalcin gene, the best-characterized osteoblast-specific gene. In this report, we show that a short promoter fragment of mOG2 (the −147/+13 fragment) displays osteoblast-specific activity in DNA transfection experiments. This promoter fragment contains two *cis*-acting elements, which we call OSE1 and OSE2, fulfilling the definition of an osteoblast-specific activator of transcription. Considering the complexity and the size of the mouse osteocalcin gene cluster (11), it is possible that additional cell-specific regulatory elements exist elsewhere. Nevertheless, we see the careful analysis of OSE1 and OSE2 as an important step toward a better understanding of the mechanisms of osteoblast-specific gene expression.

OSE1, the first osteoblast *cis*-acting element, is located between −74 and −47. OSE1 bound a factor present in nuclear extracts of ROS 17/2.8 and ROS 25 cells. This factor was also detected in nonmineralizing primary osteoblasts but not in mineralizing osteoblasts, suggesting that OSE1-binding activity is required for the early steps of osteoblast differentiation and then becomes dispensable. The presence of this factor in ROS 17/2.8 nuclear extracts, in apparent contradiction with this hypothesis, could be explained by the fact that these cells are unable to mineralize a matrix in our culture conditions. This factor was not detectable in nuclear extracts of other cell lines or mouse tissues. Six copies or even three copies (data not shown) of OSE1 activated transcription of a minimum osteocalcin promoter or of a heterologous promoter equally well in ROS 17/2.8 and ROS 25 cells but in no other cell types tested. Results of our DNA-binding analysis as well as our transfection experiments are consistent with the hypothesis that OSE1 is an osteoblast-specific *cis*-acting activator at least as important in immature osteoblasts as in differentiated osteoblasts. The OSE1 core sequence, as defined by the limited mutational analysis that we performed and the sequence comparisons with other osteocalcin promoters, does not correspond to the binding site of any known transcription factor (15). Although this element has never been studied in the promoter of the rat or human gene, inspection of the DNA sequences of these two promoters reveals that OSE1 is present approximately at the same location in these two promoters (Fig. 1), suggesting that it could play the same role in the control of osteocalcin gene expression in these species.

mOCE1 binds a ubiquitously expressed nuclear factor. Using a combination of DNA-binding assays, competition experiments, and site-specific mutagenesis analysis, we showed that the major binding site present in the B region of the mOG2 promoter was centered by an E box. Towler et al. (47) identified a putative MSX1-binding site at the 3′ end of the rat B region to which factors present in ROS 17/2.8 and ROS 25 nuclear extracts could bind. Even when we used our longest oligonucleotide as a probe and higher amounts of nuclear extracts, we did not observe the same results, possibly because

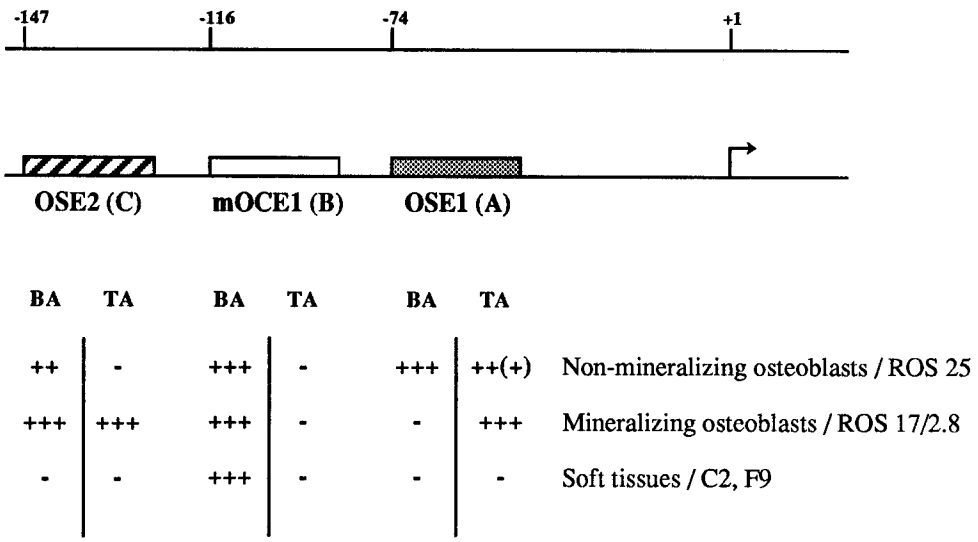


FIG. 10. Model of the functional organization of the −147/+13 mOG2 promoter. The three binding sites identified are represented by boxes. Semiquantitative representation of the binding activity (BA) present in nuclear extracts of primary osteoblasts at two stages of differentiation and of mouse tissues and semiquantitative representation of the transcription activity (TA) of multimers of each binding site as tested in DNA transfection experiments in ROS 17/2.8, ROS 25, C2, and F9 cells are shown.

of differences between the surrounding sequences and differences in assay conditions. The fact that Hoffmann et al. (20) could show binding of MSX proteins to this region only when using micromolar amounts of recombinant proteins suggests that the affinity of these proteins for this region is low. Our experiments indicate that the factor binding to mOCE1 is ubiquitously expressed and that the B region does not play a prominent role in the cell-specific expression of the gene. This hypothesis is further supported by the fact that the E box and the core sequence required for MSX1 binding (TAAT) (7) are absent in the promoter of the human gene (Fig. 1), which is also expressed only in osteoblasts.

OSE2, the second osteoblast-specific *cis*-acting element, is located between −146 and −132. OSE2 bound a factor that was present in nuclear extracts of ROS 17/2.8 cells, primary osteoblasts, and ROS 25 cells, although at very low levels, but was absent from nuclear extracts of other cell lines or mouse tissues. The nearly complete absence of OSE2-binding activity in ROS 25 nuclear extracts could explain the weak expression of p147-luc in these cells. Multiple copies of OSE2 activated transcription of a minimum osteocalcin promoter or a heterologous promoter in ROS 17/2.8 cells and, though to a lower extent, in ROS 25 cells and were inactive in other cell lines tested. The fact that OSE2 multimers increased the activity of the −34/+13 mOG2 promoter more efficiently than they increased AMLP-luc activity suggests that the factor binding to OSE2 may cooperate with as yet unidentified factors that bind to the −34/+13 mOG2 promoter. Through an extensive mutagenesis study, we defined the core sequence of OSE2, 5′ CCAACCAC 3′, and found that this 8-bp sequence is present at the same distance from the start site of transcription in the promoter of the other mouse osteocalcin gene, mOG1, where it binds the same factor. It is also present in the promoters of the rat and human genes, where it could fulfill the same function. The OSE2 sequence does not correspond to the binding site of known transcription factors (15). It has been shown that the osteocalcin genes, like other bone extracellular matrix genes, are expressed in megakaryocytes (43, 46). It will be interesting to determine whether the mOG2 promoter is active in these cells.

Recently, various promoter fragments from different genes have been implicated in osteoblast-specific gene expression. Kesterson (22) first showed that an 838-bp fragment of the human osteocalcin promoter was sufficient for osteoblast-specific expression of a reporter gene. Kesterson et al. (23) also showed that deletion of the vitamin D-responsive element abolished expression of the transgene but were unable to identify the element(s) responsible for the osteoblast-specific expression of the reporter gene. A very detailed functional analysis of the rat α1(I) collagen gene promoter has demonstrated that a 49-bp fragment located between −1719 and −1670 is responsible for the osteoblast-specific expression of the rat α1(I) collagen gene (6, 24, 38). To our knowledge, DNA-binding experiments showing that an osteoblast-specific factor binds to this element have not yet been reported, and the transcriptional activity of this sequence has not been demonstrated. Inspection of the DNA sequence of this fragment did not show any motifs similar to OSE1 and OSE2, suggesting that different osteoblast-specific *cis*-acting elements regulate the expression of the various genes expressed in osteoblasts.

Regardless of their possible involvement in the regulation of expression of other osteoblast-specific genes, OSE1 and OSE2 have two important features of osteoblast-specific activators of transcription: they bind factors present in nuclear extracts of osteoblasts and of no other cell types or tissues, and they activate transcription of minimum promoters only in osteoblastic cell lines. Our working hypothesis is that the factor binding to OSE1 may be required for the early steps of osteoblast differentiation, before the cells are able to mineralize a matrix, whereas the factor binding to OSE2 is also required in mineralizing osteoblasts, perhaps to maintain their phenotype (Fig. 10). The study of OSE1 and OSE2 function in intact animals as well as the molecular characterization of the factors binding to these elements will allow us to test this model.

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