Identification of a TAAT-containing Motif Required for High Level Expression of the COL1A1 Promoter in Differentiated Osteoblasts of Transgenic Mice*

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Milan Dodig,* Mark S. Kronenberg,a Antonio Bedalov,a,b Barbara E. Kream,c Gloria Gronowicz,d Stephen H. Clark,a,f Kristine Mack,a Yi-Hsin Liu,b Rob Maxon,b Zhong Zong Pan,b William B. Upholt,d David W. Rowe,a and Alexander C. Lichtler,a,j

From the Departments of aPediatrics, 1Medicine, 2Orthopaedic Surgery, 1BioStructure and Function, and the 2Division of Rheumatic Diseases, Department of Medicine, University of Connecticut Health Center, Farmington, Connecticut 06030, the 1Department of Veterans Affairs Medical Center, Newington, Connecticut 06111, and the 1Department of Biochemistry and Molecular Biology, Kenneth R. Norris Hospital and Institute, University of Southern California School of Medicine, Los Angeles, California 90033

Our previous studies have shown that the 49-base pair region of promoter DNA between –1719 and –1670 base pairs is necessary for transcription of the rat COL1A1 gene in transgenic mouse calvariae. In this study, we further define this element to the 13-base pair region between –1683 and –1670. This element contains a TAAT motif that binds homeodomain-containing proteins. Site-directed mutagenesis of this element in the context of a COL1A1-chloramphenicol acetyltransferase construct extending to –3518 base pairs decreased the ratio of reporter gene activity in calvariae to tendon from 3:1 to 1:1, suggesting a preferential effect on activity in calvariae. Moreover, chloramphenicol acetyltransferase-specific immunofluorescence microscopy of transgenic calvariae showed that the mutation preferentially reduced levels of chloramphenicol acetyltransferase protein in differentiated osteoblasts. Gel mobility shift assays demonstrate that differentiated osteoblasts contain a nuclear factor that binds to this site. This binding activity is not present in undifferentiated osteoblasts. We show that Msx2, a homeodomain protein, binds to this motif; however, Northern blot analysis revealed that Msx2 mRNA is present in undifferentiated bone cells but not in fully differentiated osteoblasts. In addition, cotransfection studies in ROS 17/2.8 osteosarcoma cells using an Msx2 expression vector showed that Msx2 inhibits a COL1A1 promoter-chloramphenicol acetyltransferase construct. Our results suggest that high COL1A1 expression in bone is mediated by a protein that is induced during osteoblast differentiation. This protein may contain a homeodomain; however, it is distinct from homeodomain proteins reported previously to be present in bone.

Bone mass is controlled by the balance between synthesis of bone by differentiated osteoblasts and its degradation by osteoclasts (1). Differentiation of osteoblast precursors in the peristeme to differentiated osteoblasts that reside on the osteoid surface involves a number of changes in gene expression. These include increases in type I and decreases in type III collagen synthesis (2, 3), increases in alkaline phosphatase (4), and induction of proteins characteristic of bone such as osteopontin (4), bone sialoprotein (5), and osteocalcin (4). Increased type I collagen synthesis is directly related to the structural properties of bone, as the bone matrix is made up primarily of type I collagen and hydroxyapatite.

The focus of our work has been on the expression and regulation of the COL1A1 gene in bone cells. Since type I collagen is synthesized at varying levels in many mesenchymal cell types, we propose two possible mechanisms to explain how type I collagen expression increases during osteoblast differentiation. One is that there is increased activity of common transcription factors that regulate type I collagen synthesis in many cell types. A second possibility is that one or more transcription factors that are not found in other type I collagen-producing cells are induced during osteoblast differentiation. We believe that the latter is more likely because of the many differences in regulation of type I collagen in bone compared with other tissues. For example, 1,25-dihydroxyvitamin D₃ (2, 6) and parathyroid hormone (7) inhibit type I collagen synthesis in osteoblastic cells but not periosteal cells. The cytokine interleukin-1 stimulates collagen synthesis in synovial and dermal fibroblasts (8) but decreases collagen synthesis via transcriptional mechanisms in osteoblasts (9).

Studies of transfected COL1A1 promoter constructs in fibroblasts have localized basal regulatory elements within several hundred bp of the transcription start site (10–13). In more recent studies, two motifs containing overlapping Sp1 and nuclear factor-I sites were found between –78 and –129 bp in the mouse COL1A1 gene, which mediate Sp1 inhibition and nuclear factor-I stimulation of COL1A1 collagen synthesis in NIH3T3 fibroblasts (14, 15). Although these studies identify important transcription factors, they have not elucidated the mechanism responsible for cell type specific transcription of the COL1A1 gene.

Some studies have been carried out on transcriptional regu-

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†Permanent address: University of Zagreb, School of Medicine, Salata 3b, 41000 Zagreb, Croatia.

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§To whom correspondence should be addressed: Dept. of Pediatrics, MC1515 University of Connecticut Health Center, 263 Farmington Ave., Farmington, CT 06030. Tel.: 203-679-2461; Fax: 203-679-1047; E-mail: lichtler@panda.uchc.edu.

1 The abbreviations used are: bp, base pair(s); kb, kilobases; CAT, chloramphenicol acetyltransferase; PBS, phosphate-buffered saline; FCS, fetal calf serum; CMV, cytomegalovirus.
loration of collagen and other matrix molecules in bone cells. Slack et al. (16) have reported that the region between −2.3 and −0.44 kb is required for high level expression of the human COL1A1 promoter in transgenic mouse bone. Towler et al. (17) have shown that the rat osteocalcin promoter contains an Msx1 consensus binding site that binds a nuclear factor present in ROS 17/2.8 osteosarcoma cells; mutation of this site inhibited promoter activity in transiently transfected ROS 17/2.8 and MC3T3E1 cells. In addition, they have shown that a cotransfected Msx2 expression vector modulates expression of the rat osteocalcin promoter in osteoblastic cell lines (18).

Our previous studies indicated that although the region between −3518 and −2295 bp of the COL1A1 promoter is critical for expression in cultured osteoblastic cell lines (19) and in undifferentiated primary cultures of osteoblasts derived from transgenic mice (20), it is not required for expression in whole calvariae of transgenic mice (21). Deletion of the COL1A1 promoter to −1670 bp caused a complete loss of activity in bone, thus localizing the promoter elements necessary for expression in intact bone to the 625-bp region between −2295 and −1670 bp. Further deletion mapping in transgenic mice narrowed this region to the 49 bp between −1719 and −1670 bp (22). This region contains a potential homeodomain protein binding site that is similar to but not identical to the Msx2 binding site described in the osteocalcin promoter (18).

In this study, we demonstrate that this homeodomain binding site is necessary for high level expression of the COL1A1 promoter in differentiated osteoblasts of transgenic mice. This site appears to play little or no role in COL1A1 expression in tendon or periosteum. Moreover, mouse calvarial osteoblasts cultured under conditions that promote the differentiated phenotype contain a nuclear factor that binds to the homeodomain binding site and may mediate the high level of COL1A1 transcription seen in osteoblasts. Undifferentiated cultured osteoblasts do not contain this factor. We also show that Msx2 mRNA is present in undifferentiated but not in differentiated osteoblasts and that transfected Msx2 inhibits expression of the COL1A1 promoter in osteosarcoma cells. These results suggest that although Msx2 is present in bone and binds to the homeodomain binding site, it is not the protein that is necessary for up-regulation of the COL1A1 gene in differentiated osteoblasts.

**MATERIALS AND METHODS**

Plasmids and Mutagenesis—Collagen promoter constructs (ColCAT3.6, ColCAT2.3, ColCAT1.7, and ColCAT1719), containing fragments of the COL1A1 gene linked to CAT, have been detailed previously (22). ColCAT1683 was created using a double-stranded oligonucleotide (whose sequence is ATGTTATATAGGCTCCTGCA (top strand) and CAGGTATAATTAATGGAAATTAT (bottom strand)), which contained the 13 bp of sequence between −1683 and −1670 bp, with HindIII and PsI restriction sites. This oligonucleotide was cloned into HindIII/PstI-digested ColCAT3.6.

ColCAT3.6NM1 was generated using the method of Kuncel (23) with the modification that single-stranded uracil-containing DNA was produced by M13 bacteriophage rescue using a protocol from Stratagene. Briefly, a PstI/PstI fragment of the COL1A1 promoter containing the sequence from −2400 to −1666 bp was subcloned into Bluescript (BS SK II+), transformed into CJ236 cells, and grown in the presence of the VCM13 helper phage and uridine. Single-stranded DNA was then isolated, and this template was used for mutagenesis as described (23). The Msx2-responsive osteoblasts were immortalized by introducing 0.1% colchicine (Sigma) and 0.1% collagenase P (Boehringer Mannheim), 0.2% hyaluronidase, and 0.5% phenylmethylsulfonyl fluoride. After lysis in a Dounce homogenizer, nuclei were incubated for 30 min at 4°C in buffer C (0.2 M Hepes, pH 7.9, 0.42 M NaCl, 6 mM spermidine, 0.2 mM EDTA, 0.5 mM dithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride). Extracts were desalted against buffer D (20 mM Hepes, pH 7.9, 0.1 M NaCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) using Centricron 10 concentrators (Amicon). Differentiated calvariae of transgenic mice were dissected from 6–8-day-old transgenic mice. Calvarial cells were isolated by a modification of the method of Wong and Cohn (26). After removal of sutures and adherent mesenchymal tissue, calvariae were subjected to five sequential 15-min digestions in an enzyme mixture containing 0.05% trypsin (Life Technologies, Inc.) and 0.1% collagenase P (Boehringer Mannheim) at 37°C in a rocking platform. The first two fractions were discarded, and fractions 3–5 were collected and immediately chilled by the addition of cold Dulbecco’s modified Eagle’s medium containing 10% FCS. Released cells were pooled, centrifuged, resuspended in medium, and filtered through a 100-μm Spinex filter (Millipore). An aliquot of cells was diluted 1:1 with 0.04% trypan blue in PBS, and viable cells were counted. Cells were plated at 105 cells/cm2 in six-well culture plates in Dulbecco’s modified Eagle’s medium containing 20% FCS. 24 h later the medium was changed to Dulbecco’s modified Eagle’s medium with 10% FCS (basal medium), and cells were fed again after 3 days. The cells became confluent in 1 week, after which the medium was changed to differentiation medium (−minimal essential medium containing 10% FCS, 25 μg/ml ascorbate, and 5 mM β-glycerophosphate) (22). The medium was changed daily for the duration of the experiment. Von Kossa staining to detect mineralized nodules was carried out as described (27).

Isolation of Nuclear Extracts and Gel Mobility Shift Analysis—Nuclear extracts were isolated from undifferentiated and differentiated cultured osteoblasts by the method of Dignam et al. (28), with the modification that spermine-spermidine was used in buffers in place of magnesium (29). Cells were harvested, resuspended, and incubated for 10 min on ice in buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.15 mM spermine, 0.75 mM spermidine, 0.2 mM EDTA, 0.2 mM EGTA, 0.5 mM diithiothreitol and 0.5 mM phenylmethylsulfonyl fluoride). After lysis in a Dounce homogenizer nuclei were incubated for 30 min at 4°C in buffer C (0.2 M Hepes, pH 7.9, 0.42 M NaCl, 6 mM spermidine, 0.2 mM EDTA, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride). Extracts were desalted against buffer D (20 mM Hepes, pH 7.9, 0.1 M NaCl, 0.2 mM EDTA, 20% glycerol, 0.5 mM dithiothreitol, and 0.5 mM phenylmethylsulfonyl fluoride) using Centricron 10 concentrators (Amicon). Differentiated calvariae of transgenic mice were dissected from 6–8-day-old transgenic mice. Calvarial cells were isolated by a modification of the method of Wong and Cohn (26). After removal of sutures and adherent mesenchymal tissue, calvariae were subjected to five sequential 15-min digestions in an enzyme mixture containing 0.05% trypsin (Life Technologies, Inc.) and 0.1% collagenase P (Boehringer Mannheim), 0.2% hyaluronidase (Sigma type I), 0.05% trypsin (Life Technologies, Inc.) in calcium- and magnesium-free PBS at 37°C for 30 min to remove the extracellular matrix. The cells were then resuspended in medium containing 20% FCS, centrifuged, and washed in PBS. Although enzyme treatment was not necessary for harvesting the undifferentiated cells, it was demonstrated in control experiments that treatment of undifferentiated cells with the same enzymes used for isolation of the differentiated osteoblasts did not significantly affect the gel mobility shift pattern. Mouse liver and tendon cell nuclear extracts

Transgenic Mice—Production of transgenic mice containing ColCAT3.6, ColCAT2.3, and ColCAT1719 has been described previously (19, 21, 22). New transgenic mice harboring ColCAT1683 and ColCAT3.6NM1 were produced using similar methods. Plasmids were cleaved with HindIII, which released a restriction fragment containing the test construct and about 200 bp of flanking vector sequence. After visualization on an agarose gel, the restriction fragment was isolated using SpinBind DNA purification columns (FMC). Transgenic mice were produced by microinjection of the isolated DNA into pronuclei of fertilized mouse embryos (24).

CAT Assays—Tissues were dissected from 6–8-day-old transgenic mice. To prepare tail tendon, tail skin was stripped away, and the distal tendons were harvested. Suture-free tendons, and skin were washed briefly in cold phosphate-buffed saline (PBS) and dispersed in 0.3 ml of extraction buffer (0.25 M Tris-HCl, pH 7.8, containing 0.5% Triton X-100) using a Polytron homogenizer. The homogenate was subjected to three cycles of freezing and thawing and then heated at 65°C for 15 min to inactivate endogenous deacetylases. Samples were centrifuged to remove precipitated proteins, and the supernatants were used to determine CAT activity and protein concentration. CAT activity was measured using a modified fluor diffusion assay (25). The tissue extract was incubated in a 250-μl reaction containing 0.1 M Tris-HCl, pH 7.8, 0.5 mM chloramphenicol (Sigma), and 0.1 μCi of [3H]acetylcyanomethyl A (CAT assay grade, 200 mCi/mmol, DuPont NEN), which was overlaid with a water immiscible scintillation fluid (Ultima Gold, DuPont NEN). The assay was carried out at room temperature. CAT activity was normalized to the protein content of the extract as measured by the BCA assay (Pierce). CAT assays for cultured cells were carried out in the same way, except that the cells were harvested by scraping in 0.15 M NaCl, 40 mM Tris-Cl, pH 7.4, 1 mM EDTA and were not homogenized before freeze-thawing in extraction buffer.
were isolated using the same method after digestion of the livers and tendons in the same collagenase/trypsin mixture used for isolation of calvarial osteoblasts. Protein concentrations of nuclear extracts were measured using the Coomassie Plus Protein Assay Reagent (Pierce) according to the manufacturer’s instructions. The Msx2 protein was a glutathione S-transferase-chick Msx2 fusion protein produced in Escherichia coli and purified using a gluthathione affinity column (30).

Gel mobility shift assays were carried out according to the method described in Ref. 31 with slight modifications. Gel-purified double-stranded oligonucleotide probes were end labeled using Klenow fragment to a specific activity of approximately 10,000 cpm/0.1 ng. 0.2 ng of 32P-labeled probe, 2 μg of poly(dI-dC) and nuclear extracts (5 μg of protein extraction) or Msx2 protein were incubated for 30 min at room temperature in a 20 μl reaction containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 2.5 mM CaCl2, and 5% glycerol prior to gel electrophoresis on a 5% polyacrylamide gel at 150 V for 1.5 h. Oligonucleotides used in these studies either as probes or as competitors were: (WT), AGCTGGAAACTCTATATTTTTCTTTA-TTATAGCTCTGCA (top strand) and GAGGCTAATTAGGCGGCTAGGCTTCTGGA (top strand) and GAGGGGAAATATAGGTTTCCA (bottom strand); (N1(M), AGCTTGGAAACCTTATATTTTTCTTTTCTTGA (top strand) and GAGGCTAATTAGGCGGCTAGGCTTCTGGA (top strand) and GAGGGGAAATATAGGTTTCCA (bottom strand); (N2), AGCTTGGAAACTCTATATTTTTCTTTTCTTGA (top strand) and GAGGCTAATTAGGCGGCTAGGCTTCTGGA (top strand) and GAGGGGAAATATAGGTTTCCA (bottom strand); (N3), AGCTTGGAAACTCTATATTTTTCTTTTCTTGA (top strand) and GAGGCTAATTAGGCGGCTAGGCTTCTGGA (top strand) and GAGGGGAAATATAGGTTTCCA (bottom strand); (N4), AGCTTGGAAACTCTATATTTTTCTTTTCTTGA (top strand) and GAGGCTAATTAGGCGGCTAGGCTTCTGGA (top strand) and GAGGGGAAATATAGGTTTCCA (bottom strand). Three different bands were used in this study: upper band, lower band, and middle band (Fig. 2). Gel-purified double-stranded oligonucleotide probes were end labeled using Klenow fragment to a specific activity of approximately 10,000 cpm/0.1 ng. 0.2 ng of 32P-labeled probe, 2 μg of poly(dI-dC) and nuclear extracts (5 μg of protein extraction) or Msx2 protein were incubated for 30 min at room temperature in a 20 μl reaction containing 10 mM Tris-HCl, pH 7.5, 50 mM NaCl, 1 mM dithiothreitol, 1 mM EDTA, 2.5 mM CaCl2, and 5% glycerol prior to gel electrophoresis on a 5% polyacrylamide gel at 150 V for 1.5 h. Oligonucleotides used in these studies either as probes or as competitors were: (WT), AGCTGGAAACTCTATATTTTTCTTTTCTTGA (top strand) and GAGGCTAATTAGGCGGCTAGGCTTCTGGA (top strand) and GAGGGGAAATATAGGTTTCCA (bottom strand); (N1(M), AGCTTGGAAACCTTATATTTTTCTTTTCTTGA (top strand) and GAGGCTAATTAGGCGGCTAGGCTTCTGGA (top strand) and GAGGGGAAATATAGGTTTCCA (bottom strand); (N2), AGCTTGGAAACTCTATATTTTTCTTTTCTTGA (top strand) and GAGGCTAATTAGGCGGCTAGGCTTCTGGA (top strand) and GAGGGGAAATATAGGTTTCCA (bottom strand); (N3), AGCTTGGAAACTCTATATTTTTCTTTTCTTGA (top strand) and GAGGCTAATTAGGCGGCTAGGCTTCTGGA (top strand) and GAGGGGAAATATAGGTTTCCA (bottom strand); (N4), AGCTTGGAAACTCTATATTTTTCTTTTCTTGA (top strand) and GAGGCTAATTAGGCGGCTAGGCTTCTGGA (top strand) and GAGGGGAAATATAGGTTTCCA (bottom strand). Three different bands were used in this study: upper band, lower band, and middle band (Fig. 2).

RESULTS

Identification of a 13-Base Pair Region of the COL1A1 Promoter Necessary for Activity in Osteoblasts—Our previous work revealed that the 49-bp region located between –1719 and –1670 bp is necessary for the activity of COL1A1 promoter-reporter constructs in transgenic mouse calvariae (22). To delineate further the cis-elements necessary for the high expression of the transgene in bone, a construct containing 1683 bp of 5‘ nontranscribed COL1A1 sequence fused to the CAT reporter gene was used to produce transgenic mice (Fig. 1). CAT activity in calvariae and tendon of two lines containing the new construct was compared with activity found in a previously analyzed line containing ColCAT1719 (Fig. 2). We reanalyzed the ColCAT1719 line rather than comparing our new data with previously generated results to avoid potential problems due to apparent variation among different CAT assays. The activity of ColCAT1683 in calvariae and tendon of the first line was similar to that of ColCAT1719, whereas a second ColCAT1683 line had 4–5-fold higher activity than the ColCAT1719 line. As we have shown previously, ColCAT17, extending to –1670 bp, had no activity in calvariae. The ratio of activity in bone and tendon in the ColCAT1683 line was similar to that seen previously in lines containing ColCAT2.3 or shorter constructs (21). These data suggest that the deletion from –1719 to –1683 bp does not eliminate sequences important for basal activity in the tissues analyzed and that important regulatory elements are located between –1683 and –1670 bp.

Mutation of a Homeodomain Binding Motif Strongly Affects CAT Expression in Mature Osteoblasts—Sequence analysis of the COL1A1 promoter revealed the presence of a homeodomain binding motif within the 13-bp region from –1683 to –1670 shown to be necessary for expression in calvariae. To examine the functional importance of this element we created a 6-bp replacement mutation of the homeodomain binding motif, called ColCAT3.6NM1 (Fig. 1). In this mutation the sequence TAATTA between –1683 and –1677 was converted to GCCGGC. CAT activity in calvariae and tendon from two transgenic mouse lines harboring the mutated construct was compared with the activity in a previously analyzed line (line 2)
containing wild type ColCAT3.6 (Fig. 3). The most striking aspect of these results is that although wild type ColCAT3.6 had 3-fold more activity in calvariae than tendon, a characteristic of this and all other ColCAT3.6-containing lines (21), one of the ColCAT3.6NM1 lines had equivalent activity in calvariae and tendon, while the other line had substantially lower activity in calvariae than in tendon. These results suggested that the mutation preferentially affects activity in bone.

Calvariae contain both a periosteum of fibroblasts and preosteoblasts and a layer of differentiated osteoblasts. To determine whether the mutation had a preferential effect on expression in one cell type, immunohistochemistry was performed on frozen sections of transgenic mouse calvariae using anti-CAT antibodies. ColCAT3.6 had high levels of CAT immunofluorescence in the osteoblasts and much lower but clearly detectable CAT expression in the periosteum (Fig. 4, A). Both ColCAT3.6NM1 lines, however, showed similar low level expression of CAT protein in differentiated osteoblasts and periosteum (Fig. 4, B and C). The expression of ColCAT3.6NM1 and ColCAT3.6 in periosteum was similar. Nonimmune serum gave little or no signal (Fig. 4D). These results suggested that muta
tion of the homeodomain binding site greatly reduces transgene expression in differentiated osteoblasts but not in periosteum. In summary, the results of the experiments presented in Figs. 2–4 indicate that the 13-bp region of the rat COL1A1 gene between -1683 and -1670 bp contains a motif that is necessary for high level expression in bone.

Nuclear Extracts Isolated from Murine Osteoblast Cultures Contain a Mature Osteoblast-specific Protein That Binds to the Critical Homeodomain Binding Motif—Primary mouse calvarial osteoblast cells form a mineralized collagenous matrix and express high levels of bone specific proteins when cultured under appropriate conditions (37). To determine if they could be used as an experimental model for identification and characterization of transcription factors binding to the motif present at -1677 to -1683 bp, osteoblastic cell fractions were isolated from calvariae of transgenic mice harboring ColCAT2.3. These cells were cultured until confluent (approximately 1 week) in standard medium that does not promote differentiation and were then switched to differentiation medium. Cells were harvested after 1 week in standard medium and after 4 more weeks in differentiation medium. Von Kossa staining revealed widespread formation of mineralized nodules in the 5-week cultures but no nodule formation or mineraliza
tion in early cultures.

Synthesis of high levels of type I collagen and osteocalcin is characteristic of differentiated osteoblasts in intact bone, whereas cells of the periosteum synthesize lower levels of type I collagen and do not express osteocalcin (3, 4). Northern blot analysis of our cultures showed that 1-week cultures expressed no detectable osteocalcin mRNA; however, 5-week cultures expressed osteocalcin mRNA at levels comparable to those detected in calvariae (Fig. 5). One-week osteoblast cultures expressed low levels of COL1A1 mRNA, whereas 5-week cultures expressed high levels of COL1A1 mRNA, comparable to calvariae (Fig. 5). Ethidium bromide staining of ribosomal RNA in the gel showed similar loading of RNA in all lanes.

Our previous studies showed that 1-week-old primary cultures of calvarial osteoblasts express ColCAT2.3 at a much lower level than ColCAT3.6, although the two constructs have equivalent activity in intact calvariae (20, 21). We hypothesized that 5-week cultures containing ColCAT2.3 would express the transgene at a much higher level than 1-week cul-
tures. One-week osteoblast cultures from ColCAT2.3 calvariae expressed very little CAT activity. However, there was considerable expression of the transgene at 2 weeks which was maintained at 5 weeks (Fig. 6). Our results suggest that long term osteoblastic cultures may contain cell-specific factors that bind to the motif at −1683 to −1677 bp.

To study the transcription factors that bind to this motif, nuclear proteins were extracted from both 1-week and 5-week osteoblast cultures. Mobility shift analysis using oligonucleotide probe C, containing 37 bp of the promoter sequence including the homeodomain binding motif (Fig. 7), revealed a binding activity specific to the 5-week cultures (Fig. 7, lane 1, band A). A lower mobility band was present in 1-week cultures and was also stimulated by differentiation (compare band B, lane 1 with band B, lane 3). A 6-bp replacement mutation of the homeodomain binding motif eliminated band A but did not affect band B (Fig. 7, lane 4). A shorter probe (SLA), which also contained the homeodomain binding site, produced a single shifted band with nuclear extracts from 5-week cultures which was eliminated by a 2-bp mutation of the homeodomain binding site (Fig. 7, lanes 5 and 6). Competition analysis of binding activity observed in the differentiated osteoblasts revealed that both bands could be competed by a 250-fold molar excess of unlabeled oligonucleotide C (Fig. 8, lane 2), but not with the same molar excess of the consensus TFIIID sequence, a nonspecific competitor with a T- and A-rich binding site (Fig. 8, lane 6). Furthermore, band B was competed by oligonucleotide E, which spans the 5′-two-thirds of oligonucleotide C and therefore does not include the homeodomain binding motif (lane 4). Band A was specifically competed by the Msx1 consensus sequence (38) (lane 5). Oligonucleotide A (the distal third of oligonucleotide C including the homeodomain binding motif) decreased the intensity of band A (lane 3), but competition was not as strong as was observed with the Msx1 oligonucleotide. This result is consistent with the low binding ability of oligonucleotide A probably because the probe is relatively short, and the binding domain is at its extreme end. The SLA oligonucleotide competitor, containing a 5′ extension of the oligonucleotide A sequence, also selectively competed band A (Fig. 8, lanes 7 and 8). These results indicated that band A and the single band produced by the SLA probe represent interactions with the homeodomain binding motif which we have shown to be important for transgene expression in differentiated osteoblasts. Furthermore, the binding activity to the TAAT motif appears to be relatively specific for differentiated osteoblasts as judged by the absence of a similar binding pattern from nuclear extracts of mouse liver and tendon cells (Fig. 9).

Msx-type Proteins Bind to the Homeodomain Binding Motif, but Msx2 Down-regulates Collagen Transcription—Recent studies have shown that Msx2 is present in osteoblastic cells and is capable of regulating the osteocalcin promoter (18, 39). The homeodomain binding site at −1683 to −1677 bp partially matches the Msx1 consensus derived by Catron et al. (38), which is also believed to be a consensus binding site for Msx2 because of the near identity of the homeodomains of the two
proteins (34). To determine whether Msx2 was capable of binding the COL1A1 promoter motif at -1683 to -1677 bp, we carried out gel mobility shift analysis using bacterially expressed chick Msx2 protein. As expected, both the C probe and the SLA probe were bound by Msx2 protein (Fig. 10). Next we examined the expression pattern of Msx2 mRNA during osteoblast differentiation by Northern blot hybridization of RNA extracted from mouse calvarial osteoblasts cultured under differentiating conditions for varying times (Fig. 11). Two species of Msx2 RNA of 1.4 and 2.2 kb were detected, as described previously (33). Msx2 mRNA was present at the highest levels in 1-week cultures and decreased in amount at later time points as the cells differentiated; in 5-week cultures, no Msx2 mRNA was detectable. Suture-free murine calvariae, which was scraped with a rubber policeman to remove periosteum, had no detectable Msx2 RNA (Fig. 11).

These data show that there is an inverse relationship between COL1A1 and Msx2 expression. Therefore, if Msx2 affects COL1A1 expression, it is likely to be an inhibitor. To test this hypothesis, we cotransfected ColCAT2.3, an Msx2 expression vector driven by the human CMV promoter, and the neomycin resistance gene into ROS17/2.8, an osteosarcoma cell line with many of the properties of differentiated osteoblasts. After selection in G418, stably transfected clones were pooled and assayed for CAT activity. Expression of Msx2 caused a 3-fold decrease in CAT activity compared with a control plasmid containing only the CMV promoter (Fig. 12). ROS 17/2.8 cells contain endogenous Msx2 mRNA (18); however, Northern blot analysis demonstrated increased levels of Msx2 mRNA in the cells transfected with the expression vector compared with cells transfected with the control plasmid.2

**DISCUSSION**

We showed previously that a region of the rat COL1A1 promoter between -1719 and -1670 bp is necessary for ex-
expression in transgenic mouse calvariae. In this study we have further narrowed this element to 13 bp and evaluated the importance of a homeodomain protein binding motif at −1683 to −1677 bp. Mutation of this sequence in the context of a COL1A1 promoter fragment extending to −3518 bp greatly decreases expression in differentiated calvarial osteoblasts relative to periosteal cells. We have demonstrated the presence of a protein that binds this motif in differentiated osteoblasts but not in undifferentiated osteoblasts. This factor may be a homeodomain protein, based on its binding site; however, it is also possible that this protein is a member of a different class of transcription factor which binds to a homeodomain binding site. We have shown that Msx2 binds to this site but that its mRNA is down-regulated during osteoblastic differentiation, and overexpression of Msx2 inhibits a COL1A1-CAT construct in transduced ROS 17/2.8 cells.

Our previous studies (20, 21) showed that short term cultured osteoblasts containing ColCAT2.3 had very little activity compared with lines containing ColCAT3.6, although the two lines have very similar activity in whole calvariae. We hypothesized that short term cultured osteoblasts were not fully differentiated and did not produce osteoblast-specific factors that interact with sequences downstream of −2.3 kb. In this study we show that long term culture of cells derived from ColCAT2.3 transgenic mice under conditions that allow osteoblastic differentiation have greatly increased CAT activity, suggesting that the cells produce factors that interact with sequences downstream of −2.3 kb. This result validates the use of cultured mouse osteoblasts as a source of nuclear extracts which should contain the factor that binds to the homeodomain protein binding site at −1683 to −1677 bp. We predicted that a stimulatory protein should be absent in undifferentiated mouse calvarial cells but present in differentiated cells, and evidence for such a protein was found.

Msx2 is present in osteoblastic cells (18, 39), binds to the COL1A1 promoter element between −1683 and −1670 bp, and has been shown to regulate the bone-specific osteocalcin promoter (18). For these reasons, we investigated the potential for Msx2 to regulate the COL1A1 promoter. We found that Msx2 mRNA is present at highest levels in undifferentiated osteoblast cultures, when COL1A1 mRNA levels are lowest. Msx2 levels decrease during differentiation and become undetectable in the most differentiated cultures when collagen mRNA is high. This suggested that Msx2 may inhibit COL1A1 transcription. This idea was supported by cotransfection studies in ROS 17/2.8 cells, which showed that overexpression of Msx2 inhibits transcription of ColCAT2.3.

Although our study suggests that the mutation of the homeodomain binding site had a preferential effect on expression in differentiated osteoblasts, we cannot rule out the possibility that the mutation affected expression in tendon to a lesser degree. CAT activity in tendon in the two lines containing ColCAT3.6NM1 was about 40–60% of the activity in tendon of the ColCAT3.6 line analyzed in this experiment, which is within the range of activity seen in previously analyzed lines containing nonmutated ColCAT3.6 (21). Analysis of more lines containing this construct will be necessary to address this question fully.

Our results showing that the highest levels of COL1A1 mRNA occur in differentiated osteoblast cell cultures are contrary to those of Owen et al. (40), using rat calvarial osteoblastic cells, and the studies of Quarles et al. (41), using MC3T3E1 cells. In these studies, COL1A1 mRNA levels were maximal early in the culture period when essentially all the cells were rapidly dividing and decreased steadily after this period. The results of Owen et al. and Quarles et al. appear to be inconsistent with the observation that collagen synthesis in intact mouse calvariae is highest in the osteoblast layer, which has the lowest rate of cell division (42, 43). Our results are supported by a study on cultured osteoblastic cells from fetal rat calvariae, in which COL1A1 mRNA was maximal well after the cessation of cell proliferation (44).

Similar to our results, Rossert et al. (45) recently showed that sequences between −2.3 and −0.9 kb of the mouse COL1A1 promoter are necessary for expression in transgenic mouse bone. However, they also found that sequences upstream of −2.3 kb enhanced activity in bone, unlike our previous findings (21). Similar to our studies, they found that sequences upstream of −2.3 kb were necessary for maximal expression of COL1A1 in tendon. However, they showed that a COL1A1 promoter construct extending to −900 bp had low activity in skin and tendon. In our previous studies, deletion of the rat COL1A1 promoter to −944 bp completely abolished activity in tail and tendon (21). The reasons for these discrepancies may reflect differences in the organization of the rat and mouse promoters or methodological differences. In another recent study, Sokolov et al. (46) found that 476 bp of 5′ sequence and the first intron of the human COL1A1 gene fused to the cartilage-specific COL2A1 gene were sufficient to produce appropriate expression in transgenic mouse bone. These results appear contradictory to our studies and those of Rossert et al. (45). Currently we have no explanation for this discrepancy; however, Sokolov et al. did not compare expression in differentiated and undifferentiated osteoblasts. It is possible that sequences in the COL2A1 gene can interact with the COL1A1 sequences to produce high expression in bone without the precise regulation during osteoblast differentiation characteristic of the intact COL1A1 promoter.

Our studies suggest that a protein that may contain a homeodomain is induced during osteoblast differentiation and stimulates COL1A1 transcription. There have been a few reports of the presence of homeodomain proteins in osteoblasts. These include, in addition to Msx2, Msx1 and mHox (47), whose consensus sequence closely matches the COL1A1 element. In preliminary experiments we detected mHox mRNA in cultured mouse osteoblasts; however, there was no change in levels during differentiation. Msx1 levels also do not change during osteoblast differentiation (48). Proteins binding to an Msx1 consensus site in the osteocalcin gene have been identified in ROS 17/2.8 cells (17, 49) and differentiated rat osteoblasts (49). In contrast, Ducy and Karsenty (50) found that nuclear extracts from ROS 17/2.8 cells did not bind to the analogous site.
in the the mouse osteocalcin gene, although the sequence is completely conserved between the rat and mouse genes. The reason for this discrepancy is not clear. It will be interesting to determine the relationship between the factors that bind to the osteocalcin promoter and the binding activity that we have identified.

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Note Added in Proof—While this manuscript was under review, a manuscript was published (Rossert, J. A., Chen, S. S., Eberspeacher, H., Smith, C. N., and de Crombrugghe, B. Proc. Natl. Acad. Sci. U. S. A. 93, 1027–1031) that identified an element in the mouse COL1A1 gene which is homologous to the TAAT-containing motif that we have identified. This study also demonstrated the critical role of this motif in expression in osteoblasts.

REFERENCES

1. Raisz, L. G., and Kream, B. E. (1983) N. Engl. J. Med. 309, 83–89
2. Genovese, C., Rowe, D., and Kream, B. (1984) Biochemistry 23, 6210–6216
3. Rowe, D. W., and Kream, B. E. (1982) J. Biol. Chem. 257, 8009–8015
4. Weinreb, M., Shinar, D., and Rodan, G. A. (1990) J. Bone Miner. Res. 5, 831–842
5. Chen, J. K., Shapiro, H. S., Wrana, J. L., Reimers, S., Heersche, J. N., and Sodek, J. (1991) J. Biol. Chem. 266, 13909–13915
6. Rossouw, C. M. S., Verghese, W. P., de Plooy, S. J., Bernard, M. P., Ramirez, F., and de Wet, W. J. (1991) J. Biol. Chem. 262, 15151–15157
7. Kream, B. E., Rowe, D. W., Gworek, S. C., and Raisz, L. G. (1980) J. Clin. Invest. 65, 1045–1050
8. Goldring, M. B., and Krane, S. M. (1987) J. Cell Biol. 104, 639–643
9. Kohler, T. A. (1985) Nature 317, 1421–1423
10. Cserjesi, P., Lilly, B., and Brenner, D. A. (1993) Mol. Cell. Biol. 13, 16724–16729