Identification of a Homeodomain Binding Element in the Bone Sialoprotein Gene Promoter That Is Required for Its Osteoblast-selective Expression*

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Bone sialoprotein is a 70-kDa extracellular matrix component that is intimately associated with biomineralization, yet the cis-acting elements of the Bsp gene that restrict its expression to mineralizing cells remain uncharacterized. To identify such elements, we analyzed a 2472-base pair fragment of the murine promoter that directs osteoblast-selective expression of a luciferase reporter gene and found that the region between –338 and –178 relative to the transcriptional start is crucial for its osteoblast-selective activity. We identified an element within this region that binds a protein complex in the nuclear extracts of osteoblastic cells and is required for its transcriptional activity. Introduction of a mutation that disrupts a homeodomain binding site within this sequence eliminates both its in vitro binding and nearly all of the osteoblastic-selective activity of the 2472-base pair promoter. We further found that the Dlx5 homeoprotein, which is able to regulate the osteoblast-specific osteocalcin promoter, can bind this element and stimulate its enhancer activity when overexpressed in COS7 cells. These data represent the first description of an osteoblast-specific element within the bone sialoprotein promoter and demonstrate its regulation by a member of a family of factors known to be involved in skeletogenesis.

Bone sialoprotein (BSP) is a 70-kDa extracellular matrix component that is selectively produced by mineralizing cell types in a pattern that correlates with the onset of mineral formation in vitro. This expression pattern, combined with evidence that BSP binds collagen (1) and can nucleate hydroxypatite formation in vitro (2), has led to the widely held theory that BSP plays a central role in bio mineralization. In osteoblasts, which produce the vast majority of BSP, expression is further restricted to those cells that have secreted, and are actively mineralizing, a type I collagen matrix (3). Thus, BSP is one of the primary markers of the terminally differentiated osteoblast, and, as such, a study of its expression is important to our understanding of the transcriptional mechanisms that mediate osteoblast differentiation.

Extensive studies of the osteocalcin gene 2, which encodes another osteoblast-specific protein, have revealed that its osteoblast-selective expression is dependent on binding sites for the Cbfa1 transcription factor in its promoter (named osteoblast-specific element 2 (OSE2)) (4). Furthermore, Cbfa1, which itself exhibits bone-restricted expression, has since been shown to be required for osteoblast development in general, as Cbfa1–/– mice lack functional osteoblasts (5). This finding prompted speculation that this factor may control the transcription of other osteoblast-related genes by binding similar OSE2 sites in their promoters. However, we recently investigated the contribution of two putative Cbfa1 binding sites to the activity of a 2.5-kb fragment of the murine Bsp promoter that exhibits osteoblast-selective expression. We found that neither site exhibited significant enhancer activity nor contributed to the activity of the 2.5-kb Bsp promoter, suggesting that other cis-acting elements must be responsible for its osteoblast-selective expression (6). Although at first this result seems surprising, it is, perhaps, less so when we consider that BSP and osteocalcin are associated with different biochemical functions in vivo. That is, whereas BSP is associated with mineral formation, osteocalcin appears to play a role in bone resorption and turnover (7, 8). Thus, it is reasonable to expect that these two genes, while both markers of the differentiated osteoblast, may be differently regulated.

In addition to Cbfa1 runt sites, a number of other known enhancer sequences have been described within the Bsp promoter, but little progress has been made toward identification of the elements necessary for its osteoblast-selective expression. Sodek and co-workers (9–11) have characterized elements of the elements necessary for its osteoblastic-selective expression. Sodek and co-workers (9–11) have characterized elements of the avian osteocalcin promoter that mediate the actions of vitamin-D, glucocorticoids, and transforming growth factor-β, and Yang and Gerstenfeld (12) reported that parathyroid hormone-responsive elements in the avian Bsp promoter are also transcriptionally active. However, none of these elements has been shown to confer tissue specificity. Kerr et al. (13) described a binding site for Ying Yang 1, a factor implicated in the transcriptional initiation of some TATA-less promoters, within intron 1 of the rat gene, which displayed higher levels of enhancer activity in UMR 106-01 osteosarcoma cells than in fibroblasts. However, since this sequence is not conserved in the corresponding region of either the human or mouse genes, and no intronic sequence is found in the –2472/+41 murine promoter, this element is clearly not essential for osteoblast-specific expression.

Because efforts to link the tissue specificity of the Bsp promoter to a variety of known transcription factor binding se-
Osteoblast Specificity of the Mouse Bsp Promoter

EXPERIMENTAL PROCEDURES

Cell Culture and Transfections—The following cell lines were used for these studies: The isolation of preosteoblastic clone 4 cells from the parent MC3T3-E1 line (14) was described by Xiao et al. (15). UMR 106-01 cells (16) were a gift from Dr. Ronald Midura (Cleveland Clinic, Cleveland, OH). C2C12 mouse myoblasts (17) were a gift from Dr. Daniel Goldman (University of Michigan, Ann Arbor, MI). ROS 17/2.8 osteosarcoma cells (18), were provided by Dr. Laurie McAuley (University of Michigan School of Dentistry). The 3T3-L1 preadipocyte (19), F9 teratocarcinoma (20), and S194 myeloma cell lines were purchased from the American Type Culture Collection (Manassas, VA). Maintenance conditions for these cell lines were previously described (6). COS7 monkey kidney cells (21) were the gift of Dr. Fred Aakari (University of Michigan) and were maintained in Dulbecco's modified Eagle's medium with 10% fetal bovine serum and 1% penicillin/streptomycin. C2C12 cells were induced to differentiate into myotubes by switching to 2% horse serum for 6 days. An adipose phenotype of lipid accumulation was induced in 3T3-L1 cells by growth in 30% fetal bovine serum for 6 days (19). F9 cells were induced to differentiate into endoderm by the addition of 0.1 μM retinoic acid and 1.0 mM dibutyryl cyclic AMP for 6 days (20). Clone 4 differentiation was accomplished by the addition of 50 μM all-trans retinoic acid (to label the positive strand) or 0.25% trypsin (Life Technologies, Inc.) for 1 h. They were then switched to the appropriate differentiation media and grown for 6 days. The cut linearized plasmid was then treated with calf intestinal alkaline phosphatase (Sigma) and was isolated by the method of Chomczynski and Sacchi (24). Total RNA from cells subjected to differentiation treatments as described above was isolated by the method of Chomczynski and Sacchi (24). 10 μg aliquots were fractionated on 1% agarose-formaldehyde gels and blotted to nylon membranes as described previously (25). The mouse BSP cDNA insert used as probe was generated from the R1 clone by digesting it with HindIII; the cDNA insert from the cosmid R1 was then treated with EcoRI and HindIII, and was labeled with [32P]dCTP using a random primer oligonucleotide (Stratagene, La Jolla, CA). Creation of p2472mut3, containing the 2472-bp Bsp promoter with a 2-bp mutation in the C element sequence, was accomplished as follows: primers Cmut3SD(+) and (−) (Table I) were used with Stratagene's Quick-Change Site-directed mutagenesis kit according to the manufacturer's instructions to introduce the desired mutation into p2472. An EcoRV XhoI fragment containing Bsp promoter sequence from −1016 to +1 was then isolated from the mutantized plasmid and ligated into the corresponding position in the unmutagenized p2472 plasmid. This strategy was used so that the resultant construct contained a much shorter length of in vitro polymerized sequence that would have to undergo confirmatory sequencing. The sequences of all in vitro synthesized constructs were confirmed by dye sequencing using a Sequenase 2.0 kit (Amersham Pharmacia Biotech). The pcDNA3-Dlx5 expression plasmid was the generous gift of Dr. Dwight Towler (Merrick, West Point, PA) and contains a sequence that encodes a FLAG epitope tag 5′ to codon 2 of a murine Dlx5 cDNA, resulting in the production of an N-terminal "FLAG-tagged" protein (23). pCMV5-β-gal was created by ligating a bacterial lacZ cDNA from pRSPV-β-gal (a gift from Dr. Daniel Wechsler, University of Michigan) into the EcoRI site of pcMV5 (Sigma).

Northern Analysis—Total RNA from cells subjected to differentiation treatments as described above was isolated by the method of Chomczynski and Sacchi (24). 10 μg aliquots were fractionated on 1% agarose-formaldehyde gels and blotted to nylon membranes as described previously (25). The mouse Bsp cDNA insert used as probe was generated from the R1 clone by digesting it with HindIII; the cDNA insert from the cosmid R1 was then treated with EcoRI and HindIII, and was labeled with [32P]dCTP using a random primer oligonucleotide (Stratagene, La Jolla, CA). Creation of p2472mut3, containing the 2472-bp Bsp promoter with a 2-bp mutation in the C element sequence, was accomplished as follows: primers Cmut3SD(+) and (−) (Table I) were used with Stratagene's Quick-Change Site-directed mutagenesis kit according to the manufacturer's instructions to introduce the desired mutation into p2472. An EcoRV XhoI fragment containing Bsp promoter sequence from −1016 to +1 was then isolated from the mutantized plasmid and ligated into the corresponding position in the unmutagenized p2472 plasmid. This strategy was used so that the resultant construct contained a much shorter length of in vitro polymerized sequence that would have to undergo confirmatory sequencing. The sequences of all in vitro synthesized constructs were confirmed by dye sequencing using a Sequenase 2.0 kit (Amersham Pharmacia Biotech). The pcDNA3-Dlx5 expression plasmid was the generous gift of Dr. Dwight Towler (Merrick, West Point, PA) and contains a sequence that encodes a FLAG epitope tag 5′ to codon 2 of a murine Dlx5 cDNA, resulting in the production of an N-terminal "FLAG-tagged" protein (23). pCMV5-β-gal was created by ligating a bacterial lacZ cDNA from pRSPV-β-gal (a gift from Dr. Daniel Wechsler, University of Michigan) into the EcoRI site of pcMV5 (Sigma).

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reactions were digested with 1.2 units of RNase free DNase I (Promega) for 60 s at room temperature. Digestion was stopped by the addition of 180 μl of stop solution (200 mM NaCl, 30 mM EDTA, 1% SDS, and 100 μg/ml yeast tRNA), followed by extraction with phenol/chloroform/isoamyl alcohol (25:24:1) equilibrated with TEB (10 mM Tris, pH 8.0, 1 mM EDTA, 15 mM β-mercaptoethanol). The samples were precipitated with 1 ml of ethanol, rinsed twice with 70% ethanol, dried, and resuspended in 5 μl of loading buffer (95% formamide, 20 mM EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol). After heating for 5 min at 95 °C, the samples were then electrophoresed on a sequencing gel of 8% acrylamide (19:1 with bis-acrylamide) in 0.05% bromphenol blue, 0.05% xylene cyanol). After heating for 5 min at 95 °C, the samples were then electrophoresed on a sequencing gel of 8% acrylamide gel. Gel mobility shift assays were performed by incubation at 4 °C for 30 min of 5 μg of nuclear extract with approximately 5 fmol of probe in a 15-μl reaction containing 2 μl of poly(dI-dC) in the same binding buffer that was used in the footprinting assays described above. For experiments including antibody, samples were then incubated for an additional 30 min with either 4 μg of anti-FLAG M2 monoclonal antibody (Stratagene) or IgG as control (Life Technologies) and, in some cases, 100 ng of FLAG peptide (Sigma). The shifted complexes were then electrophoresed on 5% acrylamide gels in 1× TGE (50 mM Tris-HCl, pH 8.5, 380 mM glycine, 2 mM EDTA, 0.2 mM β-mercaptoethanol) for 120 min at 170 V in a 4 °C cold room. Gels were dried and exposed to Kodak BioMax film at −70 °C. In order to determine positions of the protected nucleotides in these assays, the samples were run alongside aliquots of the same probes subjected to chemical sequencing reactions after the method of Maxam and Gilbert (29).

**Gel Mobility Shift Assays**—Gel shift probe was made by labeling 1.5 pmol of double-stranded C oligonucleotide (shown in Table I) with [γ-32P]ATP and T4 kinase; filling in with Klenow, cold G, A, and T nucleotides, and [γ-32P]dCTP; and purifying the labeled oligo on a 5% acrylamide gel. Gel mobility shift assays were performed by incubation at 4 °C for 30 min of 5 μg of nuclear extract with approximately 5 fmol of probe in a 15-μl reaction containing 2 μl of poly(dI-dC) in the same binding buffer that was used in the footprinting assays described above. For experiments including antibody, samples were then incubated for an additional 30 min with either 4 μg of anti-FLAG M2 monoclonal antibody (Stratagene) or IgG as control (Life Technologies) and, in some cases, 100 ng of FLAG peptide (Sigma). The shifted complexes were then electrophoresed on 5% acrylamide gels in 1× TGE (50 mM Tris-HCl, pH 8.5, 380 mM glycine, 2 mM EDTA, 0.2 mM β-mercaptoethanol) for 120 min at 170 V in a 4 °C cold room. Gels were dried and exposed to BioMax film.

**RESULTS**

**Osteoblast-selective Expression of the 2.5-kb Bsp Promoter**—In order to identify tissue-specific elements in the BSP promoter, we employed a construct containing bases −2472 to −41 of the murine gene driving expression of a luciferase reporter gene. Our decision to focus on this region was based on previous work which demonstrated that this construct was able to direct 5–10-fold higher levels of expression in osteoblastic cells than in nonbone cell lines (6), indicating that this fragment of the promoter likely contains sequence elements necessary to direct tissue-specific BSP expression. This finding is in agreement with data reported by Chen et al. (30), which showed mineralized tissue-restricted expression of a similar region of the rat BSP promoter in transgenic mice. As a prelude to a detailed analysis of the sequence elements responsible for this selective expression, a more thorough characterization of the expression of this reporter construct was performed in various osteoblastic cell lines to determine whether its regulation pattern parallels that of the endogenous BSP message.

The murine MC3T3-E1 preosteoblastic cell line produces high levels of bone-related proteins, including BSP, in response to induction of collagen matrix synthesis by ascorbic acid (31). For our studies, we employed a subclone, clone 4, which gives higher levels of bone marker transcription than the more heterogeneous parent cell line (15). Upon transfection with the p2472 construct, and treatment with ascorbic acid over a 7-day time course, clone 4 cells showed a significant induction in reporter activity—up to 40-fold over that in non-treated cells by day 7—that paralleled the increase in endogenous BSP message (Fig. 1, A and B). By way of comparison, ROS 17/2.8 osteosarcoma cells, which express high levels of osteocalcin but not of BSP (Fig. 1C), exhibited only 20% of the luciferase activity seen in clone 4 cells when transfected with the p2472 construct, comparable to those seen in C2C12 myoblasts and 3T3-L1 preadipocytes, two mesenchymal cell lines that do not express BSP (Fig. 1D). However, UMR 106-01 osteosarcoma cells, which constitutively express high levels of BSP (16), showed levels of expression of the transfected construct that are comparable to those in clone 4 cells (Fig. 2). Thus, the 2472-bp murine Bsp promoter is expressed at high levels only in cells that produce BSP and in a pattern that mirrors that of the endogenous message.

**Isolation of a Region Necessary for Osteoblast Selectivity by 5′-Deletion Analysis**—We created a series of 5′-deletions of the 2472-bp promoter construct in order to isolate regions of the fragment responsible for its osteoblast selective expression. These deletions were transfected into osteoblastic cell lines (MC3T3-E1 and UMR 106-01) and nonbone cells (C2C12 myoblasts, 3T3-L1 preadipocytes, F9 teratocarcinoma, and S194 lymphocytes) and grown under conditions that induced differentiation appropriate for each cell type (osteoblast, muscle, adipose, endoderm, and B lymphocyte, respectively; see under Experimental Procedures). The object of these treatments was to approximate a panel of different tissue types in cell culture so as to examine the tissue selectivity of the transfected Bsp promoter constructs. The results of these transfections, shown in Fig. 2, revealed three major features. (i) Deletion of the promoter sequence from −2472 to −178 caused no change in activity in the nonbone cell lines, whereas further deletion to −49 resulted in a drastic loss of activity in all cell lines. This likely means that the region from −49 to −178 contains sequences required for basal, non-tissue-specific transcription. (ii) Deletion from −2472 to −338 reduced promoter activity in clone 4 cells by approximately 60% but caused only a slight drop in UMR 106-01 cells. This segment may contain regulatory sequences that are required for high level transcription in the MC3T3-E1 cell line but not in the tumor-derived UMR line. (iii) Deletion of sequences from −338 to −178 resulted in a loss of over half of the remaining activity in both clone 4 and UMR lines. This represents the most severe drop in activity to that point and brings the activity in these cells down to that seen in the nonosteoblastic cell lines, indicating that the domain from −338 to −178 most likely contains elements crucial to the bone-selective expression of the promoter. Interestingly, we noted the presence of a sequence similar to the OSE1 site, described by Ducy and Karsenty (4) and Schinke and Karsenty (32) as important to the tissue-specific expression of osteocalcin, at −566 (TTTACATCA). However, because deletion of the region containing this sequence resulted in only a relatively small decrease in activity in clone 4 cells and none in UMR cells, and because this deletion did not abolish tissue specificity, it was not considered further.

DNase I footprint analysis was employed to determine which bases in the −338 to −178 region contacted protein complexes in the nuclear extracts of osteoblastic cells. Preincubation of a probe containing promoter sequence from −390 to −165 with increasing amounts of UMR nuclear extract resulted in the protection of three regions (Fig. 3A) on both the sense strand (lanes 2–4) and the corresponding antisense strand (lanes 6–8). The same pattern was seen when nuclear extracts from ascorbic acid-treated clone 4 cells were used (not shown). Together, these three regions, named A, B, and C, were found between −278 and −187 and are represented graphically in Fig. 3B. When compared with their corresponding sequences in the rat and human Bsp promoters (Fig. 3C), the A, B, and C elements show a high degree of sequence conservation, consistent with a potentially crucial role in regulating transcription. Both the A and B regions are AT rich and do not contain any recognizable transcription factor binding sites. The C element, however, contains a consensus binding site for engrailed-1 (33, 34), marking it as a potential binding site for a wide range of homeodomain-containing factors.

To determine whether these three elements were able to function as transcriptional enhancers, we synthesized A, B,
and C double-stranded oligonucleotides containing the sequences shown in Fig. 3C (see also Table I) and subcloned multimers of them, in both orientations, into a firefly luciferase reporter plasmid upstream of a minimal –49/–41 Bsp promoter fragment. When transfected into both the clone 4 and UMR cell lines, only the C element construct exhibited elevated reporter activity relative to the minimal promoter alone (Fig. 4A, compare rows 1, 4, and 7), with levels approximately 3-fold higher in clone 4 cells and 2-fold higher in UMR. The same pattern was observed when the three elements were placed into a similar vector containing a 34-bp minimal promoter from the murine osteocalcin gene 2, which has been shown to be enhanced by multimers of the bone-specific OSE2 element (4, 6). In this case, however, the C element displayed a 2–3-fold stimulation in both osteoblastic cell lines when placed in the forward orientation (Fig. 4B, row 4) and a 10–13-fold stimulation when in the reverse orientation (row 7). This apparent orientation dependence may result from the presence of one more copy of the C element in the reverse construct (five) than in the forward construct (four). To assess the transcriptional contributions of these elements in the context of the native promoter, we then created three reporter constructs from which either the A, B, or C element had been deleted from the –705-bp Bsp promoter and compared their activities to that of the wild type –705-bp construct when transfected into both clone 4 and UMR cell lines. The construction of these internal deletion mutants (named p705ΔA, p705ΔB, and p705ΔC) is diagrammed in Fig. 5A. The 705-bp promoter was chosen as the template for these deletions because it contains enough promoter sequence to exhibit osteoblast specificity but is short enough to make confirmatory sequencing of the resultant PCR-produced fragments feasible. As is shown in Fig. 5B, deletion of the A element did not affect promoter activity in clone 4 cells, and although deletion of the B element reduced promoter activity by approximately 35% in UMR cells, it did not affect activity in clone 4 cells. Because the B element also failed to show enhancer activity when placed in front of a minimal promoter (Fig. 4), it was not considered to be osteoblast-selective and was not further studied.

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**Fig. 1.** Osteoblast-selective activity of the 2472-bp Bsp promoter. A, time course of Bsp promoter induction. MC3T3-E1 clone 4 cells were transfected with p2472 and grown for up to 6 days with (A) or without (C) ascorbic acid. Cells were harvested at the times indicated and assayed for firefly and Renilla luciferase activities. B, inhibition of endogenous BSP mRNA in MC3T3-E1 cells. Total RNA from clone 4 cells, grown with or without ascorbic acid and harvested at the times shown, was subjected to Northern blot analysis with a labeled BSP cDNA. C, cell line specificity of BSP mRNA expression. Northern analysis was conducted on RNA from MC3T3-E1 clone 4, ROS17/2.8, C2C12, and 3T3-L1 cells grown for 6 days under differentiating conditions. D, cell line specificity of Bsp promoter activity. Clone 4, ROS17/2.8, C2C12, and 3T3-L1 cells were transfected with p2472 and grown for 6 days under the same conditions as in D. Firefly luciferase activities were normalized to Renilla luciferase activities and are shown as a percentage of the clone 4 ascorbate-treated sample.
further. In contrast to the A and B deletions, however, deletion of the C element from the 705-bp promoter caused a major loss of transcriptional activity in both cell types. And, in fact, this deletion accounted for the entire 60% drop in activity seen upon removal of the entire region from –338 to –178 in these cell lines (compare Figs. 2 and 5). The results from these deletion transfections are in agreement with the data from the multim- 
erized oligonucleotide experiments described above, and, taken together, these two complementary studies indicate that the C element is likely the only one of the three that is important for promoter activity in osteoblastic cells. Thus, we chose to focus our further investigations on this sequence element.

Identification of a Homeodomain Binding Site in the C Element that is Necessary for Osteoblast-selective Bsp Promoter Activity—Gel mobility shift assays of nuclear extracts from UMR and clone 4 cells with radiolabeled double-stranded C oligonucleotide as probe yielded a complex array of bands (Fig. 6, lane 2). All but one of these species (bands 1–5) could be competed for, to varying degrees, by the addition of 50–100-fold molar excess of unlabeled C oligonucleotide (Fig. 6, lanes 3 and 4) but not by the addition of a heterologous oligonucleotide containing the proximal OSE2 site from the Og2 promoter (data not shown), indicating that this element is able to specif-
ically bind a protein complex in the nuclear extracts of these osteoblastic cells. We synthesized a series of mutated C oligonucleotides to determine which bases in the C element were essential for protein binding. These contained the same sequence as the wild type C oligonucleotide shown in Table I, with the exception of a 2-base pair change in each. The identity of the mutated bases in each, and their effects on the binding of
the C probe when used as competitors in gel shift assays, are shown in Fig. 6. We chose a series of mutations that spanned the consensus homeodomain site because this is the only recognizable transcription factor binding site in the C sequence. Addition of a 50–100-fold molar excess of unlabeled mutant 1 or mutant 6 oligonucleotides (Fig. 6, lanes 5, 6, 15, and 16)
showed that these mutants were able to compete for protein binding nearly as well as the wild type C probe. Mutants 2–5, however, were unable to compete with the wild type sequence. Taken together, these assays show that disruption of the homeodomain binding sequence, TCAATTAA, abolishes the ability of the C element to bind protein complexes in the nuclei of osteoblastic cells.

To test whether this sequence requirement for in vitro binding also applied to the enhancer activity of the C element, the ability of the mutant oligonucleotide 3 (Cmut3) to stimulate transcription of the –49/+41 Bsp minimal promoter was compared with that of the wild type sequence described above. As expected, a construct containing three copies of the wild type sequence gave up to 3-fold higher activity in osteoblastic cells than the minimal promoter alone (Fig. 7A, row 2), but the mutant construct, which differed from the wild type by only 2 bp, showed no such stimulation. To assess the contribution of the homeodomain binding site to the osteoblast specificity of the Bsp promoter, we introduced the same 2 bp mutation, by site-directed mutagenesis using primers Cmut3SD(+) and (−) (see Table I), into the full-length 2472-bp promoter construct. Results from the transfection of this mutated construct into bone and nonbone cell lines are shown in Fig. 7B. The relative normalized activities of the wild type 2472-bp construct in these lines were the same as those shown in Fig. 2. However, in Fig. 7B, we have set the activity of the full-length construct at 100% in each cell line and expressed the activities of the site mutant and deleted constructs as a percentage of that in order to clearly illustrate the drop caused by the 2-bp change. Although the mutation had no effect on the activity of the promoter in nonosteoblastic cells, it abolished 60% of the promoter activity in the osteoblastic clone 4 and UMR cells, causing a drop nearly equivalent to the deletion of the entire sequence between –2472 and –178. Thus, the disruption of the homeodomain binding site resulted in the ablation of almost all of the osteoblast selective activity of the 2472-bp Bsp promoter.

**Dlx5 Regulation of the C Element Homeodomain Site**—We further characterized the activity of this element by examining

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**Fig. 5.** Effect of A, B, or C internal deletions on the activity of the p705 construct. A, different combinations of primers (represented as arrows) were used with p705 as a template to create PCR fragments containing sequences either upstream or downstream of the desired deletion, as described under “Experimental Procedures.” These fragments were ligated into pGL3Basic to create the p705ΔA, p705ΔB, and p705ΔC constructs represented here. The hatched bar represents Bsp promoter sequence. The open bar is pGL3 sequence. Dashed lines indicate the sequences that are deleted from the final constructs. The end points of the deleted promoter sequences are shown relative to the start of transcription above each construct. B, the deletion constructs shown in A, as well as the complete p705, were transfected into clone 4 and UMR cells. Their activities are shown as percentages of p705 activity in each cell line.
from lowest to highest mobility. Clear extract as described under "Experimental Procedures." Gel mobility shift assays were conducted with either UMR or clone 4 nucleic acid or the mutant C oligo indicated. The six shifted species observed are numbered 1–6 in order from lowest to highest mobility.

**Fig. 6. Interaction of the C element with osteoblast nuclear proteins requires an intact homeodomain binding motif.** A series of six double-stranded mutant C oligonucleotides was synthesized to span the entire homeodomain binding motif. Each oligonucleotide contains a 2-bp mutation (as denoted by the brackets) in, or adjacent to, the homeodomain consensus binding sequence. Although the corresponding mutations were made in the (–) strand, only the (+) strand is shown. The sequences of all mutants are identical to that of the wild type C oligonucleotide shown in Table I except for these 2-bp changes. Gel mobility shift assays were conducted with either UMR or clone 4 nuclear extract as described under “Experimental Procedures.” Lane 1 contains extract but no cold competitor. Lane 2 contains extract but no cold competitor. The remaining lanes contain binding reactions (with extract) incubated with a 50- or 100-fold molar excess (increasing amounts indicated by the triangles) of unlabeled wild type (w.t.) C oligo or the mutant C oligo indicated. The six shifted species observed are numbered 1–6 in order from lowest to highest mobility.

its ability to be directly regulated by the Dlx5 homeoprotein, a member of the Dlx family of *Drosophila* distalless homologues. We chose this factor as the most likely known candidate for the C element-binding protein because it has been shown to be expressed on bone and to be able to regulate the osteoblast-specific osteocalcin promoter (23, 35). Additionally, homozygous knockout of the Dlx5 gene produces mice with severe skeletal deformities, implying a crucial role in skeletogenesis (36). As represented in Fig. 8A, Dlx5 mRNA is expressed only in those cell lines that also produce BSP, thus constitutively produce BSP. One possible explanation for this observation is that region contains sequences that are necessary for the normal matrix-stimulated induction of BSP but that are inactive in osteosarcoma cells which are free from the matrix requirement. It will be interesting to identify the cis-acting elements in this region of the *Bsp* promoter that are responsible for the differences in reporter expression between these two osteoblastic cell lines. Characterization of such elements may yield additional information on the matrix-induced signaling pathways that are active in osteoblasts.

**DISCUSSION**

Of the known bone extracellular matrix constituents, bone sialoprotein is, perhaps, the most intimately associated with the primary function of the differentiated osteoblast, namely the mineralization of a collagenous matrix, yet little is known about the cis-acting elements that restrict its expression to the mineralizing osteoblast. We previously reported the isolation of a 2472-bp fragment of the murine *Bsp* promoter that is able to direct osteoblastic-selective expression of a luciferase cDNA under growth conditions which are known to support osteoblast differentiation in cell culture (6). Here we describe the results of a systematic study to identify sequences within this fragment that may direct this tissue-specific expression.

We found that the –2472/+41 base pair *Bsp* promoter, in addition to its preferential expression in osteoblastic versus nonosteoblastic cell lines, was induced by collagen matrix production in parallel with the endogenous BSP message. This up-regulation in response to matrix interaction is indicative of genes whose expression is associated with osteoblast differentiation and provides further evidence that the 2472-bp promoter contains the requisite information to appropriately regulate *Bsp* transcription during the differentiation process (37). Deletion of sequence from –2472 to –705 caused a marked drop in promoter activity in clone 4 cells, but not in UMR 106-01 cells, which constitutively produce BSP. One possible explanation for this observation is that this region contains sequences that are necessary for the normal matrix-stimulated induction of BSP but that are inactive in osteosarcoma cells which are free from the matrix requirement. It will be interesting to identify the cis-acting elements in this region of the *Bsp* promoter that are responsible for the differences in reporter expression between these two osteoblastic cell lines. Characterization of such elements may yield additional information on the matrix-induced signaling pathways that are active in osteoblasts.

This present study, however, focuses on the isolation of sequences that are important for promoter activity in osteoblastic versus nonbone cells, as these are likely to be the elements that confer bone specificity. We identified one such element, a consensus homeodomain binding site, between –199 and –192 (TCAATTAA). Disruption of this sequence caused a 60% drop in transcriptional activity only in the osteoblastic cells and accounted for almost all of the difference in activity between the bone and nonbone cell lines used in this study. To our knowledge, this is the first description of an osteoblast-specific

| wild type: | homeodomain |
|-----------|-------------|
| 2 bp mutants: | | |
| CCTTCAATTAAATC | GGGGCCCCCG CCC |

| UMR-106-01 extract | MC3T3-E1 clone 4 extract |
|-------------------|--------------------------|
| 1 | 1 |
| 2 | 2 |
| 3 | 3 |
| 4 | 4 |
| 5 | 5 |
| 6 | 6 |

| 1 | 1 |
| 2 | 2 |
| 3 | 3 |
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oligonucleotide (*lanes* 10–12). Thus, this complex is dependent on the intact homeodomain site for binding. The lower species was competed by both wild type and mutant C oligos and was also found in control nuclear extracts made from COS7 cells transfected with a β-galactosidase expression vector (*Fig. 8C, lanes* 2–4). Therefore, it does not represent homeodomain site-specific binding. To confirm that the specifically shifted species observed was the result of Dlx5 binding, we repeated the gel shift assay with a monoclonal antibody (M2) recognizing a FLAG epitope tag built into the overexpressed Dlx5. This antibody blocked the appearance of the specific species while having no effect on the lower, nonspecific band (*Fig. 8C, lane 13*). Furthermore, this blocking action was counteracted by the addition of FLAG peptide to compete for antibody binding, indicating that the protein complex observed contains Dlx5 (*Fig. 8C, lane 14*). Addition of M2 antibody had no effect in binding reactions with β-gal control extract (*Fig. 8C, lane 5*). Together, these experiments show that the Dlx5 gene is expressed in our osteoblastic cell lines, and that the Dlx5 homeoprotein is able to bind the *Bsp* C element and stimulate its transcriptional activity.
transcriptional element within the bone sialoprotein gene promoter.

There is a large body of genetic evidence implicating homeodomain proteins in the development of mineralized tissues, particularly with respect to members of the Dlx and Msx families, which are known to be expressed in tissues that give rise to skeletal structures, and mutations in which result in the display of severe skeletal phenotypes. For example, mice containing mutations in Msx1 show craniofacial abnormalities that include cleft palate and absence of specific teeth (38), while mice expressing a mutated Msx2 transgene exhibit the symptoms of craniosynostosis, a disease characterized by premature closure of the cranial sutures (39). Furthermore, both Dlx1 and Dlx2 knockout mice display abnormal formation of the skull bones derived from the first and second branchial arches (40, 41), while mice deficient in both factors show additional defects in dentition (42). In addition, a recent study by Acampora et al. (36) reported that Msx5 can bind the Bsp C element and stimulate its activity, with the p2472 w.t. activity set at 100% in each cell line in order to emphasize the percentage of drop attributed to the mutation in each cell type. Normalized luciferase activities of the wild type promoter in osteoblastic and nonosteoblastic cell lines were similar to results shown in Fig. 2.

![Fig. 7. Comparison of enhancer activities of wild type and mutant C element sequences. A, a trimer of the Cmut3 double-stranded oligonucleotide was inserted into the p49BSP vector in the forward orientation (row 2), and its transcriptional activity in clone 4 and UMR cells was compared with that of the construct containing the wild type C element (row 3). Values shown are fold induction of each construct over that of the insertless p49BSP vector (row 1). B, the 2-bp mutation corresponding to that in the Cmut3 oligonucleotide was introduced into p2472 by site-directed mutagenesis using primers Cmut3SD(+) and (−) (see Table I). The resulting p2472mut3 construct was transfected into clone 4, UMR, C2C12, 3T3-L1, and F9 cells, and its transcriptional activity was compared with that of the wild type p2472, as well as the p178 (which lacks A, B, and C elements) and p49 (minimal promoter) deletion constructs. Normalized luciferase activities are shown as a percentage of the wild type p2472 (p2472 w.t.) construct activity, with the p2472 w.t. activity set at 100% in each cell line in order to emphasize the percentage of drop attributed to the mutation in each cell type. Normalized luciferase activities of the wild type promoter in osteoblastic and nonosteoblastic cell lines were similar to results shown in Fig. 2.](http://www.jbc.org/doi/abs/10.1074/jbc.M203546200#fig7)
the mechanism of osteoblast gene expression. If it is true, as is widely held, that BSP plays a crucial role in the nucleation of mineral formation in the collagenous matrix of bone, then it is plausible to speculate that the \textit{colla}1(I) and \textit{Bsp} promoters are coordinately regulated by a shared set of osteoblast-specific transcription factors. Given the new data reported here, it is possible that one such factor may be a homeodomain protein complex that binds to both collagen and \textit{Bsp} promoters, enabling their transcription in osteoblasts, and thus coordinating both the deposition and mineralization of the collagenous ma-
 blast phenotype. Such a complex, in conjunction with other factors such as Cbfα1, might function as a central determinant of osteoblast differentiation. Our data, combined with the recent description of the Dlx5 knockout mice mentioned above, strongly support a role for Dlx5 as one such determinant. However, in considering this scenario, it is important to recognize that the results we present here, as well as the aforementioned osteocalcin promoter studies, merely demonstrate that Dlx5, or a related homeoprotein, may regulate osteoblast-specific transcription in vivo; they do not positively identify Dlx5, or any other known factor, as such a regulator. Furthermore, the apparently im-
promotes their identification merely by examination of the se-
quences to which they bind in individual promoters. Our ongo-
ing studies are aimed at identifying the factor that binds the
newly characterized osteoblast-specific element in the Bsp
promoter and at elucidating its role in the expression of the oste-
blast phenotype.

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Identification of a Homeodomain Binding Element in the Bone Sialoprotein Gene Promoter That Is Required for Its Osteoblast-selective Expression
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