Characterization of Osf1, an Osteoblast-specific Transcription Factor Binding to a Critical cis-acting Element in the Mouse Osteocalcin Promoters*

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The osteoblast, a cell type of mesenchymal origin, is the only cell responsible for bone matrix deposition, also called bone formation (1). Bone formation is a physiologic process of critical importance as it is involved in skeletal growth, bone remodeling, and fracture repair (2). Diseases affecting bone formation, whether they are genetically inherited like osteogenesis imperfecta, or acquired like osteoporosis, are common and very often debilitating (3–7). This underscores the biomedicole importance of elucidating the genetic basis of osteoblast-specific gene expression and osteoblast differentiation. It is likely, as it is the case in many cell lineages (8–11), that these two processes are related and that cell differentiation along the osteoblastic lineage is controlled, at least in part, by cell-specific transcription factors which need to be identified. In an effort to identify these factors we have embarked on a long-term study of the regulation of OG2, one of the two mouse osteocalcin genes, whose expression is strictly osteoblast-specific.

Previously, the analysis of the OG2 promoter defined a 147-bp fragment that confers osteoblast-specific expression to a reporter gene in vitro (12). Within this short OG2 promoter fragment we defined two osteoblast-specific cis-acting elements, termed OSE1 and OSE2 (originally designated as regions A and C, respectively), that are characterized by two important features. First, multimers of each of these elements confer osteoblast-specific activity to a heterologous promoter. Second, OSE1 and OSE2 serve as binding sites for distinct factors that are only detected in nuclear extracts of osteoblasts but of no other cell types or tissues tested (12). These osteoblast specific binding activities were named Osf1 and Osf2, respectively. Further analysis of Osf2 revealed that it is the product of the Cbfa1 gene (13). We, and others have subsequently demonstrated that Cbfa1 acts as a transcriptional activator of osteoblast differentiation in mouse and human (13–17).

To date Cbfa1 remains the only osteoblast-specific transcription factor identified (18). However, several lines of evidence suggest that other cell-specific transcription factors are involved in the control of gene expression in osteoblasts. For instance, multiple investigators have identified osteoblast-specific cis-acting elements in the promoter elements of the genes encoding 1(1) collagen, bone sialoprotein, insulin-like growth factor 1, and osteocalcin (19–25). Likewise, as mentioned above, we have shown that OSE1 binds an osteoblast-specific factor that is not Cbfa1 (12).

Although OSE1 has been poorly characterized so far, Osf1, the factor binding to it, has one important property. Osf1 is present in nuclear extracts of primary osteoblasts at the earliest stage of cellular differentiation, but is absent in nuclear extracts of differentiated osteoblasts that are surrounded by a mineralized matrix (12). Thus, Osf1 is not only an osteoblast-specific but also a stage-specific transcription factor that could conceivably act upstream of Cbfa1, provided that OSE1 has a biologic function.

The present study was aimed at defining the functional importance of OSE1 and characterizing Osf1. We first demonstrate that OSE1 is as important as OSE2 for OG2 expression in cell culture and transgenic mice. We identified an 8-bp core OSE1 sequence that is also present in the promoter of other osteocalcin genes and of the mouse Cbfa1 gene. The absence of overt homologies between OSE1 and other binding sites of
known transcription factors suggests that Osf1 may be a novel factor and led us to perform an analytical purification. This purification showed that Osf1 is a single polypeptide with an apparent molecular mass of 40 kDa. This is the first step toward identifying and eventually cloning a cDNA encoding Osf1, a second osteoblast-specific transcription factor.

**EXPERIMENTAL PROCEDURES**

**DNA Constructions**—The oligonucleotides used in this study are presented in Table I. Construction of the p1316-luc and p147-luc plasmids has been described previously (12). p1316-luc was cut out from the fragment of p4E-mOSE1-2uc (26) into pluc4, a promoterless luciferase expression vector. A 2-bp mutation was introduced into OSE1 in p1316-luc was generated by a two-step polymerase chain reaction method (27). The primers used for this mutagenesis were 5'-CTTATAAGCCACCAAGACCAGGC-3' (−674 to −652), 5'-CTCTTCTCCTGCAAATCTACAAGGC-3' (−68 to −45), 5'-GCTCTCTGATTGTGTCGGAGAGAAG-3' (−45 to −68), and 5'-TGCTGACTTGCTGTGTC-3' (from +13 to +36). p147-mOSE1-luc was generated by inserting the Pun1-SalI fragment of p1316-mOSE1-2uc into pluc4. Six copies of the wild-type or mutated OSE1 mut9 oligonucleotide were cloned into the Smal site of the p34-luc construct that has been described previously (12). Sequences of the generated plasmids were verified by automatic DNA sequencing.

**Ose1 and Osteoblast-specific Gene Expression**

**RESULTS**

**OSE1 and OSE2 Are Equally Important for Og2 Promoter Activity in Cell Culture**—To determine the relative importance of OSE1 compared with OSE2 for Og2 expression we introduced small substitution mutations into either one of these two elements in the context of a p147-luc reporter gene construct that contains the first 147 bp of the mouse Og2 promoter fused to the luciferase gene (luc) (12). These mutations abolish the DNA binding activity of Osf1 and Cbfa1 to their respective binding sites (Fig. 8B and Ref. 12). Using the wild-type and mutated p147-luc constructs we performed DNA transfection experiments in ROS17/2.8 osteoblasts (Fig. 1A). We chose ROS17/2.8 cells to perform these experiments because these cells were used in the initial characterization of OSE1 and OSE2 (12).

A 2-bp mutation introduced into OSE2 decreased the activity of p147-luc 85% confirming the critical role of OSE2 for Og2 expression (Fig. 1A). Likewise, a 2-bp mutation in OSE1 led to a 76% decrease of p147-luc activity (Fig. 1A). These results indicate that OSE1 is virtually as important as OSE2 for Og2 expression. We also introduced the same 2-bp mutation into OSE1 in the context of a longer 1316-bp Og2 promoter fragment and performed DNA transfection experiments in ROS17/2.8 cells with the wild-type and mutated p1316-luc constructs (Fig. 1B). The OSE1 mutation led to a 51% decrease of p1316-luc activity indicating that even in this larger Og2 promoter fragment the presence of OSE1 is required for maximal activity in cell culture.

**OSE1 Is Critical for Og2 Promoter Activity in Vivo**—As a further demonstration of the functional importance of OSE1 for osteocalcin expression, we generated transgenic mice carrying either the wild-type p1316-luc or the mutated p1316-luc chimeric genes. Three founder animals carrying the wild-type and two founder animals carrying the mutant chimeric gene were obtained. Transgenic mice of the F1 generation from these

15,000 × g for 15 min, dissolved in 5 ml of buffer A, and dialyzed against the same buffer for 3 h with two buffer changes. Dialyzed proteins were applied onto a 5-ml phosphocellulose column (Whatman P11). After washing the column with 5 volumes of buffer A containing 0.2 M NaCl, bound proteins were eluted in 1-ml fractions with 0.5 M NaCl in buffer A. Fractions containing OSE1 activity were pooled, dialyzed against buffer B, and incubated for 10 min with 14 μg/ml poly(dI-dC), 200 fmol/μl single-stranded OSE1 oligonucleotide, and 100 fmol/μl double-stranded mutant ΔOSE1 oligonucleotide (ΔOSE1-mut4). This mixture was applied to a 1-ml DNA affinity column containing multimerized ΔOSE1 oligonucleotide that was prepared as described by Kadonaga and Tjian (30). After a washing step at 0.2 M NaCl, the OSE1 activity was eluted with buffer A containing 0.5 M NaCl. To verify the purity of the eluted OSE1 the final EMSA was performed with both wild-type and mutant ΔOSE1 oligonucleotide in the absence of nonspecific competitor DNA.

**SDS-PAGE and Renaturation—**SDS-polyacrylamide gel electrophoresis was carried out as described by Laemmli (31). Proteins were visualized by silver staining according the method of Heukeshoven and Dernick (32). In gel slices cut out from an adjacent lane that was run on the same gel, but not stained. These gel slices were crushed with a pestle and soaked in 500 μl of elution buffer (50 mM Tris, pH 7.5, 0.1 mM EDTA, 0.1% SDS, 5 mM dithiothreitol, 150 mM NaCl, 0.5 mM phenylmethylsulfonlfuoride, 100 μg/ml bovine serum albumin) for 3 h at room temperature. Eluted proteins were precipitated with 80% acetone, dissolved in 50 μl of buffer A containing 6 μg guanidine-HCl, and denatured for 50 min. After removal of the guanidine by a 10× desalting gel spin column (Bio-Rad) the proteins were allowed to renature for 2 h at room temperature. 3 μl of renatured proteins were assayed for Osf1 binding activity by EMSA using the wild-type and mutant ΔOSE1 oligonucleotides as probes.
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founder animals were analyzed for luciferase expression in various organs at 10 days of age (Fig. 2). In all mice containing the wild-type p1316-luc transgene the relative luciferase activities in bone were between 3000 and 5000 per 100 μg of total protein (Fig. 2A). In these mice luciferase activities in other tissues were less than 1% compared with the values we measured in bone (2–30/100 μg of protein). This bone-specific luciferase expression was also observed in all mice carrying the mutated p1316-luc transgene (Fig. 2B). However, the luciferase activities in long bones of these latter mice were in average 75% decreased (700–1300/100 μg of protein) compared with what was observed in long bones obtained from mice carrying the wild-type transgene (Fig. 2B). Taken together, these data demonstrate that OSE1 is functionally important as OSE2 for OG2 expression, both in cell culture and in vivo. The functional importance of OSE1 was an incentive to better characterize this element and the factor binding to it, thereafter termed Osf1.

Osf1 Binds to an 8-bp Core OSE1 Sequence—The OSE1 oligonucleotide that was used for EMSA in our previous study covered a relatively large region of the OG2 promoter consisting of 35 bp (12). Originally, we did not find any motifs within this region sharing similarities to binding sites of known transcription factors.

To abolish Osf1 binding to DNA we originally used a large mutation affecting 6 bp (Table I). By definition this mutation of a large number of base pairs did not allow us to determine precisely the nucleotides critical for protein binding. We reasoned that by defining a shorter Osf1-binding site we may be in a better position to identify key nucleotides within OSE1 and to recognize binding sites of known transcription factors.

To achieve this goal we first assayed shorter OSE1 oligonucleotides for Osf1 binding activity by EMSA (data not shown). This led us to the definition of a shorter oligonucleotide spanning only 22 bp of the OG2 promoter that we termed ∆OSE1 (Table I). To demonstrate that the same nuclear activity is binding to the OSE1 and the ∆OSE1 oligonucleotide, direct binding and DNA competition experiments were performed by EMSA using nuclear extracts of ROS17/2.8 osteoblasts as a source of protein (Fig. 3). We previously described the presence of three protein-DNA complexes formed upon incubation of ROS17/2.8 nuclear extracts with the OSE1 oligonucleotide in EMSA (12). Osf1, defined by its osteoblast-specific presence and its heat lability, is part of the complex of intermediate mobility. Using the ∆OSE1 oligonucleotide as a probe in EMSA the same three complexes were detected (Fig. 3A, lane 1). The complex of intermediate mobility was specifically competed by the wild-type, but not by the mutant ∆OSE1 and OSE1 oligonucleotides (Fig. 3A, lanes 2–5). Likewise, when using the OSE1 oligonucleotide as a probe in EMSA the Osf1-DNA complex was specifically competed by the wild-type, but not by the mutant ∆OSE1 and OSE1 oligonucleotides (Fig. 3B, lanes 2–5). These results suggest that the same nuclear protein binds to both the OSE1 and ∆OSE1 oligonucleotides. This was further demonstrated by the osteoblast-specific nature of the factor binding to ∆OSE1 (Fig. 5A). Thus, we used the ∆OSE1 oligonucleotide as a probe for the DNA binding experiments described below.

To identify the critical base pairs within the OSE1 sequence that are required for Osf1 binding to DNA we mutagenized individually 8 bp within the ∆OSE1 oligonucleotide (Fig. 4A). The use of these various ∆OSE1 oligonucleotides in EMSA allowed us to define an 8-bp core OSE1 sequence: 5′-CTCCCCTGCTC-3′ (Fig. 4B).

OSE1 Appears to be a Unique Sequence—Analysis of the core OSE1 sequence failed to show a strong homology to any known

| Oligonucleotides used in this study |
|-----------------------------------|
| OSE1 | 5′-CTCCCCTGCTC-3′ |
| OSE1-mut | 5′-CTCCCCTGCTC-3′ |
| ∆OSE1 | 5′-CTCCCCTGCTC-3′ |
| ∆OSE1-mut | 5′-CTCCCCTGCTC-3′ |
| OSE1(Nib) | 5′-AAATCGGATACATCACACT-3′ |
| OSE1(Nib)-mut | 5′-AAATCGGATACATCACACT-3′ |

Fig. 2. OSE1 is required for OG2 promoter activity in vitro. Transgenic mice were generated carrying the wild-type (A) or mutated (B) 1316-bp OG2 promoter fused to the luciferase reporter gene. Luciferase activities were measured in tissues of 10-day-old F1 animals derived from different founders for each genotype. Luciferase activities are expressed as light units/100 μg of protein. Values are the mean of four to six transgenic mice per founder animal. Error bars represent the standard deviation.

To demonstrate that OSE1 is functionally as important as OSE2 for OG2 expression, both in cell culture and in vivo, transgenic mice were generated carrying the wild-type (OG2) and mutant (OSE1-mut) OG2 promoter that we termed ∆OSE1 (Fig. 2A). Luciferase activities derived from different founders for each genotype. Luciferase activities are expressed as light units/100 μg of protein. Values are the mean of four to six transgenic mice per founder animal. Error bars represent the standard deviation.

## Table I

| Oligonucleotides used in this study |
|-----------------------------------|
| OSE1 | 5′-CTCCCCTGCTC-3′ |
| OSE1-mut | 5′-CTCCCCTGCTC-3′ |
| ∆OSE1 | 5′-CTCCCCTGCTC-3′ |
| ∆OSE1-mut | 5′-CTCCCCTGCTC-3′ |
| OSE1(Nib) | 5′-AAATCGGATACATCACACT-3′ |
| OSE1(Nib)-mut | 5′-AAATCGGATACATCACACT-3′ |

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transcription factor-binding sites. However, a closer inspection of this 8-bp sequence showed a weak similarity with the cAMP-responsive element (CRE) whose sequence is 5'-TGACGTCA-3' (33). This led us to explore the possibility that Osf1 could be a member of the CREB (CRE-binding proteins) family of transcription factors.

The guanosine present in position 5 of the CRE is known to be critical for the binding of CREB proteins to DNA (34). Importantly, when we used a mutant D OSE1 oligonucleotide carrying an A to G mutation in position 5 of the core OSE1 sequence (D OSE1-mut5) as a probe in EMSA with ROS17/2.8 nuclear extracts, the Osf1-DNA complex was absent and replaced by a complex of slower mobility (Fig. 4, lane 6). To determine whether the factor binding to the D OSE1-mut5 oligonucleotide was osteoblast-specific, EMSA with nuclear extracts of various tissues and of primary osteoblasts was performed (Fig. 5). While Osf1, the factor binding to the D OSE1 oligonucleotide, was present only in nuclear extracts of non-mineralized primary osteoblasts (Fig. 5A, lane 8), the factor binding to the D OSE1-mut5 oligonucleotide was present in nuclear extracts of every tissue tested (Fig. 5B). Moreover, this latter factor was heat-stable (Fig. 5B, lanes 9–13) as described for CREB proteins (35), unlike Osf1, whose binding to DNA is abolished upon heating (Fig. 7 and Ref. 12). In summary, the ubiquitous nature and the heat stability of the factor binding to the D OSE1-mut5 oligonucleotide clearly distinguishes it from Osf1. Thus, Osf1 is most likely not a member of the CREB family of transcription factors, and the core OSE1 sequence appears to be unique.
to search for OSE1 sites in other genes that are expressed in osteoblasts. OSE1 is present at the same location in the promoter of OG1, the other mouse osteocalcin gene (36). We were not able to locate additional copies of OSE1 in more distal promoter regions of the mouse osteocalcin genes. When we searched the 5'-region of the mouse gene encoding the osteoblast-specific transcription factor Cbfa1 (kindly provided to us by Dr. P. Ducy), we found one OSE1 site located 3700 bp upstream of the transcriptional start site. This site is homologous, but not identical to the OSE1 site present in the OG2 promoter. In particular the surrounding sequences are totally different. Nevertheless, given the restricted expression of Cbfa1, we analyzed whether this sequence, termed OSE1<sup>Cbfa1</sup>, could act as an osteoblast-specific cis-acting element.

Six copies of the wild-type or mutant OSE1<sup>Cbfa1</sup> oligonucleotide were cloned upstream of a minimal OG2 promoter fragment in a luciferase expression vector (p34-luc). This minimal promoter fragment lacks OSE1 and OSE2 and has virtually no transcriptional activity in DNA transfection experiments (12). The multimerized wild-type OSE1<sup>Cbfa1</sup> oligonucleotide conferred osteoblast-specific activity to this promoter fragment in DNA transfection experiments as the luciferase expression was at least 30-fold higher in ROS17/2.8 cells compared with F9 teratocarcinoma cells and C2C12 myoblasts, two cell lines that do not express Cbfa1 (Fig. 6A). In contrast, multimerized mutant OSE1<sup>Cbfa1</sup> oligonucleotide failed to increase the activity of the minimum OG2 promoter.

Additionally, DNA binding and competition experiments by EMSA were performed to determine if OSE1<sup>Cbfa1</sup> serves as a binding site for Osf1. When using nuclear extracts of different tissues and non-mineralized primary osteoblasts as a source of protein, and the OSE1<sup>Cbfa1</sup> oligonucleotide as a probe, an osteoblast-specific complex migrating at the same location as the Osf1-DSE1 complex was observed (Fig. 6B). This protein-DNA complex was competed by the wild-type DSE1 and OSE1<sup>Cbfa1</sup> oligonucleotides (Fig. 6B, lanes 2 and 3). When using the DSE1 oligonucleotide as a probe in EMSA the Osf1-DNA complex was also competed by both the DSE1 and the OSE1<sup>Cbfa1</sup> oligonucleotide (Fig. 6C, lanes 2 and 3). Taken together, these functional and biochemical lines of evidence indicate that the Cbfa1 promoter contains at least one OSE1-like site whose sequence is partially identical to the one present in the OG2 promoter and that can act as an osteoblast-specific element.

Osf1 Can Be Purified to Homogeneity—The lack of overt homology of the core OSE1 sequence to any known transcription factor-binding sites excluded a polymerase-chain reaction based or low-stringency hybridization approach to cloning a cDNA encoding Osf1. Therefore we decided to purify Osf1 from osteoblast nuclear extracts in order to obtain amino acid sequence information. In a survey of various osteoblastic and mesenchymal cell lines we found one mesenchymal, pluripotent cell line, C1 (37), expressing high levels of Osf1. When performing EMSA with nuclear extracts of C1 cells, we observed only one complex, unlike what was seen with ROS17/2.8 nuclear extracts (Fig. 7, lanes 1 and 2). As it was the case for the Osf1 activity present in ROS17/2.8 nuclear extracts, this complex was heat-sensitive (Fig. 7, lane 3) and was not observed when using as probes any of the eight DSE1 oligonucleotides harboring single base pair mutations that were used to define the core OSE1 sequence (Fig. 7, lanes 4–11) indicating that this protein-DNA complex contains Osf1.

Given the higher level of Osf1 expression and the existence of a single protein-DNA complex in EMSA, C1 nuclear extracts were chosen as a source of protein for our purification approach. However, we also confirmed the results obtained with C1 cells by purifying Osf1 with the same strategy from ROS17/2.8 nuclear extracts. Fig. 8A summarizes the strategy used to purify Osf1 on an analytical scale. The presence and integrity of Osf1 was monitored at each step by EMSA using the DSE1 oligonucleotide as a probe. Enrichment of Osf1 in the purified fractions was estimated by comparing the Osf1 binding activity to the total amount of protein.

Nuclear extracts were first subjected to a 25% ammonium sulfate precipitation. Osf1 activity was only detectable in the precipitated fraction. The precipitate was dissolved in buffer A containing 0.1 M NaCl, dialyzed against the same buffer, and applied to a phosphocellulose cation exchange resin where Osf1 activity was eluted at 0.5 M NaCl. These two steps led to a 20-fold enrichment of Osf1 binding activity (Fig. 8A). Following dialysis against buffer A containing 0.1 M NaCl, the Osf1 containing fractions were applied onto a Mono Q anion exchange column. Osf1 was eluted from this column at a salt concentration between 0.35 and 0.4 M NaCl. After these three initial steps, the purified Osf1 complex was identified by EMSA using the DSE1 oligonucleotide as a probe.
purification steps we estimated that Osf1 was enriched more than 75-fold compared with nuclear extracts (Fig. 8A). The last step of the purification scheme was a DNA affinity column using the multimerized ΔOSE1 oligonucleotide coupled to a Sepharose matrix (30). The fractions containing Osf1 were dialyzed against buffer A containing 0.1 M NaCl and applied onto the DNA affinity resin. Nonspecific interactions of other proteins with this resin were significantly reduced by preincubating the protein sample with poly(dI-dC), single-stranded OSE1 oligonucleotide, and a double-stranded mutant oligonucleotide (ΔOSE1-mut4). Specific Osf1 binding activity was eluted from this column at a concentration of 0.5 M NaCl.

To demonstrate that the purified protein using this procedure was Osf1 we performed EMSA using the wild-type and mutant ΔOSE1 and OSE1<sup>Cbfa1</sup> oligonucleotides as probes in the absence of nonspecific competitor DNA (Fig. 8B). The purified protein bound specifically to the wild-type ΔOSE1 and OSE1<sup>Cbfa1</sup> oligonucleotides, but not to their mutant counterparts (Fig. 8B). Knowing the amount of radiolabeled ΔOSE1 oligonucleotide added to the binding reaction, we estimated the total amount of Osf1-DNA complex. The yield of Osf1 starting from 30 ml of nuclear extract was approximately 0.25 fmol. Thus, the four-step purification procedure led at least to a 2500-fold purification of Osf1 (Fig. 8B). The last step was Osf1 purification using nuclear extracts of C1 mesenchymal cells (lanes 2–11) as a source of protein. One unit was defined as the amount of Osf1 required to retard approximately 0.25 fmol of the radiolabeled ΔOSE1 oligonucleotide in EMSA, representing 5% retardation in our assay. Total protein concentrations were determined using the Bio-Rad Protein Assay. Footnote 1, no detectable protein concentration using 5 μl of the fraction. B, EMSA with Osf1 purified from C1 (lanes 1 to 4) or ROS 17/2.8 cells (lanes 5 and 6) using the wild-type (lanes 1 and 5) or mutant (lanes 2 and 6) ΔOSE1 oligonucleotide and the wild-type (lane 3) or mutant (lane 4) OSE1<sup>Cbfa1</sup> oligonucleotide as probes. Nonspecific competitor DNA, poly(dI-dC), was excluded in this experiment.

The Apparent Molecular Mass of Osf1 is 40 kDa—When we performed an SDS-PAGE and silver staining at this stage of the purification, we observed that the eluate of the DNA affinity resin contained two distinct polypeptides migrating at 40 and 45 kDa, respectively (Fig. 9A). To determine which of these polypeptides contains the Osf1 binding activity, a renaturation experiment was performed. Aliquots of the DNA affinity eluate fractions were subjected to SDS-PAGE, the proteins were eluted from 5-mm gel slices, precipitated by acetone, and subjected to denaturation with 6 M guanidine. After removal of the guanidine the proteins were allowed to renature and finally assayed for Osf1 binding activity by EMSA using the wild-type or mutant ΔOSE1 oligonucleotide as a probe (Fig. 9B). Specific Osf1 binding activity was recovered from the gel slice containing the polypeptide migrating at 40 kDa (Fig. 9B, gel slice B). This binding activity was only observed when using the wild-type ΔOSE1 oligonucleotide as a probe, but was absent when using the mutant ΔOSE1 oligonucleotide as a probe (Fig. 9B, lanes 5 and 6). Additionally, the protein-DNA complex migrated at the same location as the complex observed with purified Osf1 that was not subjected to an SDS-PAGE (Fig. 9B, lane 1). In contrast, a DNA binding activity that was recovered from the gel slice containing the polypeptide migrating at 45 kDa (gel slice A) differed from the Osf1 binding activity as the observed protein-DNA complex had a slower mobility in EMSA (Fig. 9B, lane 3). Other fractions of the SDS gel did not contain detectable binding activities.

**DISCUSSION**

Taken together, the results of this study indicate that OSE1 is a cis-acting element as important as OSE2 in controlling osteoblast-specific expression of osteocalcin in vivo and that it may be involved in regulating Cbfa1 expression in cells of the osteoblast lineage. We also show that the core OSE1 sequence has no overt homologies to binding sites of known transcription factors. Last, this study provides the first biochemical characterization of Osf1 and thereby expands the spectrum of regulatory elements and factors involved in osteoblast-specific gene expression.

To date only one osteoblast-specific transcription factor,
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Cbfa1, has been cloned and functionally analyzed using molecular and genetic means (13–18). All the evidence accumulated recently demonstrate that Cbfa1 acts as a differentiation factor in the osteoblast lineage by binding to the OSE2 sites that are present in the promoters of most genes expressed in osteoblasts (13). Yet, other osteoblast-specific cis-acting elements have been identified in the genes encoding α1(I) collagen, BSP, insulin-like growth factor 1, and osteocalcin (12, 19–25) suggesting the existence of additional osteoblast-specific transcription factors. OSE1, the element that we analyzed in this study, is of particular interest as it has been shown to bind a factor termed Osf1 present only in nuclear extracts of non-mineralized osteoblasts (12).

We have shown previously that OSE1 could confer osteoblast-specific activity to a heterologous promoter. However, these studies did not address the respective importance of OSE1 and OSE2 in the control of OG2 expression. This is an issue of critical importance that needed to be addressed before embarking in any further characterization of Osf1. The mutagenesis of OSE1 and OSE2 presented here demonstrates that these cis-acting elements are equally important in controlling OG2 expression. Surprisingly OSE1 appears to be more important for OG2 expression in transgenic mice than in cell culture experiments. This may reflect the genetic differences between osteoblasts in vivo and ROS17/2.8 cells.

OSE1 is also present in the promoter of other osteocalcin genes and of another gene specific for the osteoblast lineage, Cbfa1. This suggests that Osf1 may be one regulator of Cbfa1 expression in vivo and is consistent with the presence of Osf1 in nuclear extracts of non-mineralized primary osteoblasts, but not of fully differentiated osteoblasts. In contrast, OSE1 is not present in the promoter of the human osteocalcin gene (38). Several possibilities could explain this finding. We know from previous studies that the regulation of osteocalcin expression by steroid hormones such as 1,25-dihydroxyvitamin D₃ differs in mouse and human, indicating a lack of conservation of regulatory mechanisms between these two species (39, 40). We also know that the serum level of osteocalcin in humans is significantly lower than in mice (41), suggesting that the human gene is expressed at a lower level. This could well be explained by the absence of an important regulatory element such as OSE1 in the promoter of human osteocalcin. Regardless of the reasons explaining this discrepancy the functional importance of OSE1 for osteocalcin expression in mouse and the exquisite cell distribution of Osf1 are incentives to study this factor further.

To determine whether Osf1 belongs to a known family of transcription factors we defined the 8-bp core OSE1 sequence through an extensive mutagenesis approach. This sequence, 5′-TTACATCA-3′, shows no strong homology to any binding sites of known transcription factor families, but a weak similarity to the CAMP-responsive element, the binding site of CREB proteins (42, 43). However, several important biochemical characteristics distinguish Osf1 from CREB proteins. First, the CREB consensus-binding site is palindromic whereas the core OSE1 sequence is not. Second, Osf1 is heat-sensitive whereas CREB proteins are heat-stable (34). Third, and more importantly, an A to G mutation in the core OSE1 sequence that generates a better CRE-like sequence abolishes binding of Osf1 and results in the binding of an ubiquitously expressed heat-stable protein, most likely a CREB family member. The exact molecular nature of Osf1 will be defined when a cDNA becomes available.

The fact that the core OSE1 sequence shows no overt homology to known transcription factor-binding sites led us to use a classical protein purification approach to obtain amino acid sequence information. This approach has been successfully used as an initial step toward the cloning of several transcription factors (44, 45). Moreover, the significant progress in protein sequencing by nanospray mass spectrometry enables researchers to sequence unknown proteins at fentomole quantities (46). This is a critical issue as osteoblasts are adherent cells, thus precluding us from obtaining large amounts of pure protein.

Initially, Osf1 was identified in ROS17/2.8 osteoblasts (12). However, nuclear extracts of these cells also contain other factors binding to the ΔOSE1 oligonucleotide in EMSA. Although we were able to purify Osf1 from ROS17/2.8 nuclear extracts, we used C1, another osteocalcin-expressing cell line (37), as a source of protein for this purification effort because Osf1 is the only activity present in C1 nuclear extracts binding to the ΔOSE1 oligonucleotide. Moreover, Osf1 is more abundant in C1 than in ROS17/2.8 nuclear extracts. After a four-step purification, Osf1 migrated as a single band at 40 kDa in an SDS-PAGE and formed a single complex with the ΔOSE1 oligonucleotide in EMSA, even in the absence of nonspecific competitor DNA.

One important question that will need to be addressed in the future is where Osf1 resides in the genetic cascade controlling osteoblast-specific gene expression and, possibly, osteoblast differentiation. The evidence we have gathered to date can support two opposite views equally well. The presence of OSE1 in the osteocalcin promoters and our failure to detect them in the promoter of other extracellular matrix encoding genes (data not shown) may indicate that Osf1 acts downstream of Cbfa1 and is required solely for osteocalcin expression. Alternatively, the fact that an OSE1 site is present in the Cbfa1 promoter, along with the presence of Osf1 only in nuclear extracts of non-mineralized osteoblasts, would rather suggest that Osf1 acts upstream of Cbfa1. Cbfa1 and Osf1 could also regulate the expression of each other as it has been shown to be the case for transcription factors involved in adipogenesis and myogenesis (8, 47). The distinction between these possibilities will have to
await the molecular cloning of Osf1 and the analysis of its spatial and temporal expression pattern.

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REFERENCES

1. Aubin, J. E., and Liu, F. (1996) in Principles of Bone Biology (Bilezikian, J. P., Raiz, L. G., and Rodan, G. A., eds) pp. 51–67, Academic Press, San Diego, CA
2. Erlebacher, A., Filvaroff, E. H., Gitelman, S. E., and Derynck, R. (1995) Cell 80, 371–378
3. Byers, P. H., and Steiner, R. D. (1992) Annu. Rev. Med. 43, 269–282
4. Prockop, D. J., and Kivirikko, K. I. (1984) J. Biol. Chem. 259, 755–764
5. Kimmel, C. B., and Kimelman, D. (1990) Cell 62, 743–753
6. Ishida, Y., and Heersche, J. N. (1998) J. Bone Miner. Res. 13, 755–764
7. Ishida, Y., and Heersche, J. N. (1998) J. Bone Miner. Res. 13, 1822–1826
8. Otto, F., Thornell, A. P., Crompton, T., Denzel, A., Gilmour, K. C., Rosewell, I. R., Stamp, G. W. H., Beddington, R. S. P., Mundlos, S., Lindhout, D., Cole, W. G. Henn, W., Knoll, J. H. M., Owen, M. J., Mertelsmann, R., Zabel, B. U., and Olsen, B. R. (1997) Nat. Genet. 17, 1079–1090
9. Hoeffler, J. P., Meyer, T. E., Yun, Y., Jameson, J. L., and Habener, J. F. (1988) Proc. Natl. Acad. Sci. U. S. A. 85, 5889–5893
10. Laemmli, U. K. (1970) Nature 227, 680–685
11. Dignam, J. D., Lebowitz, R. M., and Roeder, R. G. (1983) Proc. Natl. Acad. Sci. U. S. A. 80, 1485–1489
12. Morrison, N. A., Shine, J., Fragonas, J. C., Verkest, V., McMenemy, M. L., and Tudor, D. A. (1997) J. Biol. Chem. 272, 786–800
13. Derycke, F., and Gannon, F. (1994) BioTechniques 16, 405
14. Goldman, B. R. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 5889–5893
15. Montminy, M. R., Sevarino, K. A., Wagner, J. A., Mandel, G., and Goodman, R. H. (1986) Proc. Natl. Acad. Sci. U. S. A. 83, 6682–6686
16. Haas, P. V., Liu, F., Allegretto, E. A., Karsenty, G., and Green, M. R. (1989) Genes Dev. 3, 2083–2090
17. Haas, P. V., Liu, F., Allegretto, E. A., Karsenty, G., and Green, M. R. (1988) Genes Dev. 2, 1216–1226
18. Redondo, C., Hogue, D. A., and Karsenty, G. (1994) J. Biol. Chem. 269, 1183–1190
19. Poliard, A., Lambin, D., Marie, P. J., Buc-Caron, M. H., and Kellermann, O. (1993) J. Cell Sci. 103, 503–511
20. Morrison, N. A., Shine, J., Pragmas, J. C., Verkest, V., McMenemey, M. L., and Eisman, J. A. (1989) Science 246, 1158–1161
21. Zhang, R., Ducy, P., and Karsenty, G. (1997) J. Biol. Chem. 272, 110–116
22. Clemens, T. L., Tang, H., Maeda, S., Kesterson, R. A., Demayo, F., Pike, J. W., and Gundberg, C. M. (1997) J. Bone Miner. Res. 12, 1570–1576
23. Hauschka, P. V., Liu, B., Cole, D. E., and Gundberg, C. M. (1989) Physiol. Rev. 69, 990–1047
24. Montminy, M. R., and Bilezikjian, L. M. (1987) Nature 328, 175–178
25. Hoefler, J. P., Meyer, T. E., Yen, Y., Jameson, J. L., and Habener, J. F. (1988) Science 242, 1430–1433
26. Landschulz, W. H., Johnson, P. F., Adashi, E. Y., Graves, B. J., and McKnight, S. L. (1988) Genes Dev. 2, 786–800
27. Edmondson, D. G., Cheng, T. C., Cserjesi, P., Chakraborty, T., and Olson, E. N. (1998) Mol. Cell. Biol. 12, 3965–3977
28. Chen, J., Thomas, H. P., Jin, H., Jiang, H., and Sodek, J. (1996) J. Bone Miner. Res. 11, 654–664
29. Umayahara, Y., J. C., Centrella, M., Rotwein, P., and McCarthy, T. L. (1997) J. Biol. Chem. 272, 31705–31800
30. Umayahara, Y., Millard, J., C., Centrella, M., McCarthy, T. L., and Rotwein, P. (1999) J. Biol. Chem. 274, 10609–10617
31. Montecino, M., Pockwinse, S., Lian, J., Stein, G., and Stein, J. (1994) Biochemistry 33, 348–353
32. Ryoo, H. M., Hoffmann, H. M., Beumer, T., Frenkel, B., Towler, D. A., Stein, G. S., Stein, J. L., van Wijnen, A. J., and Lian, J. B. (1997) Mol. Endocrinol. 11, 1681–1694
33. Breit, B., Xia, G., Fuchs, S., Franceschi, R. T., Karsenty, G., and Ducy, P. (1998) J. Biol. Chem. 273, 30509–30516
34. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A., and Struhl, K. (eds) (1995) Current Protocols in Molecular Biology, John Wiley & Sons, Inc., New York