Bone formation and resorption are tightly coupled under normal conditions, and the interaction of osteoclast precursors with cells of the osteoblast lineage is a prerequisite for osteoclast formation. Cbfa1 is an osteoblast-specific transcription factor that is essential for osteoblast differentiation and bone formation. At present, it is not known whether Cbfa1 regulates any of the osteoblast-derived factors involved in the bone resorption pathway. Osteoprotegerin (OPG) is an osteoblast-secreted glycoprotein that functions as a potent inhibitor of osteoclast differentiation and bone resorption.

Cloning and computer analysis of a 5.9-kilobase human OPG promoter sequence revealed the presence of 12 putative Cbfa1 binding elements (osteoblast-specific element 2 (OSE2)), suggesting a possible regulation of OPG by Cbfa1. We cloned the promoter upstream of the β-galactosidase reporter gene (pOPG5.9gal) and evaluated whether Cbfa1 could regulate its expression in transient transfection assays. The 5.9-kilobase promoter directed increased levels of reporter gene expression, reminiscent of OPG protein levels in osteoblastic cell lines (BALC and U2OS) as compared with the nonosteoblastic cell line COS1. Cotransfection of a Cbfa1 expression construct along with pOPG5.9gal reporter construct led to 39-, 7-, and 16-fold increases in β-galactosidase activity in COS1, BALC, and U2OS cells, respectively. Removal of all the putative OSE2 elements led to an almost complete loss of transactivation. Mutational analysis demonstrated that the proximal OSE2 element contributes to a majority of the effects of Cbfa1, and Cbfa1 bound to the proximal element in a sequence-specific manner. Further, overexpression of Cbfa1 led to a 54% increase in OPG protein levels in U2OS cells. These results indicate that Cbfa1 regulates the expression of OPG, thereby further contributing to a molecular link between bone formation and resorption.

Bone growth, development, and maintenance in mammals is a highly regulated process. The level of bone mass is dependent on the balance between bone formation and resorption. At the cellular level, this balance involves the coordinate regulation and interaction of the component cell types: bone-forming osteoblasts and bone-resorbing osteoclasts. Osteoblasts are derived from mesenchymal stem cells, and their formation and function are under the control of an osteoblast-specific transcription factor known as core binding factor a1 (Cbfa1) or osteoblast specific factor 2 (Osf2) (1–3). Cbfa1 has been shown to regulate the expression of genes that characterize the osteoblast phenotype, including osteocalcin, osteopontin, type I collagen, bone sialoprotein, and collagenase-3, by binding to DNA sequence elements called OSE2 that are present in the control regions of these genes (1, 4–6). Cbfa1 knock out animals completely lack bones because of a maturational arrest of osteoblasts and die soon after delivery (2, 3). Further, haploinsufficiency of the Cbfa1 gene product in mice and humans heterozygous for the Cbfa1 gene results in a condition called cleidocranial dysplasia that is characterized by delayed ossification (2, 3, 7–10).

Osteoclasts that are primarily involved in bone resorption are multinucleated cells derived from hematopoietic precursors of the monocyte/macrophage series. It has been known for a long time that osteoclast precursors need to interact with cells of the osteoblast lineage to differentiate into mature osteoclasts (11–14). Recently, proteins involved in this interaction have been identified and are now being extensively characterized (15–18). They include RANK (receptor activator of NF-κB) ligand (15, 18), also known as osteoclast differentiation factor or TRANCE (tumor necrosis factor-related activation-induced cytokine) (19). RANK ligand is a membrane-bound protein of the tumor necrosis factor ligand family that is expressed on the osteoblast cell surface and has been shown to play a major role in osteoclast differentiation along with macrophage colony-stimulating factor (20). RANK ligand binds to its receptor RANK (16) on hematopoietic cells and initiates a cascade of signaling events that leads to osteoclast differentiation. Furthermore, stromal/osteoblastic cells also secrete a glycoprotein called osteoprotegerin (OPG) (17), a soluble member of the tumor necrosis factor receptor superfamily that acts as a decoy receptor for RANK ligand and prevents its interaction with the cognate receptor RANK (21, 22). OPG has been shown to be a potent inhibitor of osteoclast differentiation, survival and function in vitro and bone resorption in vivo (23–25). OPG knock out mice show severe early onset osteoporosis (24, 26), whereas transgenic animals overexpressing OPG are osteopetrotic (17).

While the role of Cbfa1 in osteoblast differentiation and bone formation is fairly well understood, it is not known whether it can modulate the expression of regulators of osteoclast differentiation.
entiation and function, namely RANK ligand and OPG, that are generated by cells of the osteoblast lineage. To search for any potential role of Cbfα1 in regulating osteblast formation, we directly examined the effect of Cbfα1 on OPG gene transcription by cloning a 5.9-kb region of the human OPG promoter and analyzed its transcriptional activity in cotransfection experiments. We present evidence that Cbfα1 increases OPG gene transcription in osteoblastic and nonosteoblastic cells, via sequence-specific binding and transactivation mediated by the OSE2 elements and that Cbfα1 overexpression enhances OPG protein levels in osteoblastic cells.

**EXPERIMENTAL PROCEDURES**

**Cloning of the Human OPG Promoter**—To clone the human OPG promoter, two approaches were combined to identify and isolate an approximately 5.9-kb genomic fragment located immediately 5' to the coding region of the OPG gene. The first approach involved using the Genome Walker kit (CLONTECH, Palo Alto, CA). This is a PCR-based method used to "walk" genomic DNA adjacent to known sequences. Gene-specific primers were designed from the published OPG sequence (GenBankTM accession number U94332) (27) as follows: GSP1 5'–ggg gat gtc cag aaa cag gag cgc g-3' and nested GSP2 5'-cag aac aac tgt ttc att ggg gta-3'. These primers are specific to the Genome Walker library adapter to amplify 1874-, 1874–1875-, and 391-bp fragments of DNA upstream of the OPG coding region. Sequence analysis demonstrated that these were overlapping proximal fragments of the 5'-flanking region of the OPG gene.

To clone larger promoter fragments containing more distal sequences we next screened a conventional genomic library. To do this, the full-length OPG CDNA (GenBankTM accession number U94332) was sent to Genome Systems (St. Louis, MO) and used to screen a human P1 library. The screen yielded three positive clones, each containing 70–100 kb of genomic DNA. To determine which P1 clone contained the promoter, each clone was digested with a panel of restriction enzymes and then Southern blotted with the 1874-bp promoter fragment described above. Clone 19401 digested with SstI and EcoRI yielded a 5936-bp band that hybridized to the 1874-bp OPG promoter fragment. These primers were used in combination with the promoter specific to the Genome Walker library adapter to amplify 1874-, 1874–1875-, and 391-bp fragments of DNA upstream of the OPG coding region. Sequence analysis demonstrated that these were overlapping proximal fragments of the 5'-flanking region of the OPG gene.

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**Plasmid Construction**—All plasmid constructs containing 5' deletions in the OPG promoter were generated using standard cloning procedures. The 5936-bp SstI-EcoRI fragment of the human OPG gene, containing sequences from −5917 to +19 relative to the transcriptional start site, was cloned into the 3xOSE-pGL2 vector by digesting with BglII and subcloning the 0.2-kb insert into the BglII site of pGL2-Basic (Promega Corporation, Madison, WI) and designated pOPG5.9. The region of the promoter corresponding to the OSE2 core element (AACCTCA) (OSE2 element 1, +129 to +136) was PCR amplified from pCMV5-Osf2 (obtained from Dr. Gerard Karsenty, Houston, TX) and subcloned into the pCMV5 cloning site of pGL2-Basic vector that was digested with StuI and NcoI, releasing about 2.3 kb of the 5' end of the OPG promoter. The promoter segment covering the −252 to +19 region of the human OPG gene was generated by digesting with BglII and subcloning the 0.2-kb insert into the BglII site of pGL2-Basic. pOPG5.9, pOPG3.6, pOPG1.9, pOPG0.9, pOPG0.4, and pOPG0.2 were digested with KpnI and BglII, and the promoter fragments were subcloned into the KpnI/BglII sites of pPGal-basic (CLONTECH, Palo Alto, CA), to generate pOPG5.9gal, pOPG3.6gal, pOPG1.9gal, pOPG0.9gal, pOPG0.4gal, and pOPG0.2gal, respectively.

**Sequence Analysis**—The sequence of the 5.9-kb OPG promoter was analyzed for the presence of consensus transcription factor binding sites, using OSE2 (Cbfα1-binding site) and OSE-like elements using the GCG Wisconsin Package, Genetics Computer Group, Inc. (Madison, WI).

**Cell Culture and DNA Transfection**—The monkey kidney cell line COS-1 was obtained from American Type Culture Collection (ATCC CRL 1650) and was grown in Dulbecco's modified Eagle medium supplemented with 10% fetal bovine serum (FBS) (HyClone Laboratories, Logan, UT) and 2 mM L-glutamine (Life Technologies, Inc.). COS1 cells were chosen for use in transient transfection studies owing to their ability to initiate replication and generate multiple copies of transfected plasmids that contain the SV40 origin of replication, eventually resulting in the synthesis of substantial amounts of the protein of interest (30). BALB/c mouse calvaria-derived stromal/osteoblastic cell line (31) was obtained and cultured with 5% CO2 in MEM medium supplemented with 15% FBS and 2 mM L-glutamine; the human osteoblast-like osteosarcoma cell line U2OS was grown in McCoy's 5A medium supplemented with 10% FBS and 2 mM L-glutamine; and R172/28 osteoblast-like osteosarcoma cells were grown in Ham's F-12 medium supplemented with 10% FBS. All cultures were maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO2. For plasmid transfection studies, 1 × 106 cells were plated per well in 6-well plates and incubated for 24 h. Cells were then transfected with 1 μg each of the reporter (OPG promoter constructs linked to βgal or the control reporter vector pPGal-basic) and the effectors (pEF-Cha1 or the control expression vector pEF/myc/cyto), using Fugene transfection reagent (Roche Molecular Biochemicals). The constructs (1 μg each in a total volume of 20 μl in Tris-EDTA buffer, pH 8.0) were mixed with diluted Fugene reagent (154 μl of serum-free medium + 6 μl of Fugene) and incubated for 15 min at room temperature. The DNA-Fugene mix was then added dropwise to the plates, and the cells were incubated for an additional 48 h. Following transfection, the plates were washed twice with PBS (Life Technologies, Inc.) and then lysed with 100 μl of lysis buffer provided with the βgal reporter gene assay kit (Roche Molecular Biochemicals). The cell extracts were then heated to 95 °C for 2 min and 1 μl of each extract was added to a microtiter plate containing 20 μl of the supernatant was transferred to white microtiter plates, and the βgal activity was measured as per the manufacturer's instructions, using an automated injection MLX Luminometer (Dynex Corporation, Chantilly, VA). As a positive control and to verify transfection efficiency, separate plates were transfected with a plasmid containing a βgal expression plasmid (pPGal-Promoter, CLONTECH, Palo Alto, CA).
that has the βgal reporter gene coding region under the control of the SV40 early promoter (referred to as SV40-βgal in the text and in Fig. 4). This was done to avoid possible squelching of factors that could arise when cotransfecting multiple plasmids (32, 33). The luciferase reporter vector was not used because of our recent finding that cryptic enhancer elements in the luciferase reporter vector pGL3-Basic could mediate transactivation by Cbfa1, leading to spurious background luciferase expression (34). All the experiments were repeated at least three times in triplicate wells, using different plasmid preparations. The βgal activities represent the integral value of light emitted over a period of two seconds and are expressed as fold induction over basal (control vector-transfected) levels. Results were analyzed using Student’s t test, and probability (p) values of less than 0.05 were considered statistically significant.

To analyze the effect of Cbfa1 transfection on OPG secretion, U2OS cells were plated at 1 × 10^5 cells/well in 6-well plates and allowed to grow for 24 h. The culture medium was then removed, cells were washed with 1 × PBS (Life Technologies, Inc.) and replaced with medium containing 0.1% FBS and incubated overnight. The next morning, cells were transfected with 1 μg of either empty vector (pEF/myc/cyto) or pEF-Cbfa1 using Fugene reagent as described above. 5.5 h after transfection, the medium was removed, plates were washed with 1 × FBS, and complete medium was added and incubated for 4 h. The cells were then serum-starved (in medium with 0.1% FBS) and incubated further. 48 h after transfection, the supernatant was collected, and the amount of secreted OPG was quantified in an ELISA as described below.

**RESULTS**

Cloning of the 5.9-kb OPG Promoter and Analysis of Putative Transcription Factor Binding Sites in the Promoter—We cloned and sequenced a 5.9 kb fragment of the 5′-flanking region of the human OPG gene (−5917 to +19) as outlined under “Experimental Procedures.” Computer analysis of the 5.9-kb sequence schematically summarized in Fig. 1 revealed the presence of a putative TATA box (at position −27), three CCAAT box sequences (at positions −669, −752, and −826), and consensus binding sites for a variety of transcription factors, including AP1, AP2, and SPI, as described in the previously published 1.1-kb promoter sequence (27). In addition, 12 putative binding sites for the osteoblast-specific transcription factor Cbfa1 (OSE elements) were also identified (Fig. 1 and Table 1) (35, 36). These putative OSE elements were identical or similar to the ones found in the regulatory regions of several known targets of Cbfa1 that are expressed abundantly in osteoblasts, namely osteocalcin, osteopontin, type I collagen, and bone sialoprotein (1, 6).

The basal transcriptional activity directed by the 5.9-kb region was analyzed by its ability to drive the expression of a βgal reporter gene after transient transfection into the following cell lines: U2OS, a human osteoblast-like osteosarcoma cell line; BALC, a mouse calvaria-derived stromal/osteoblastic cell line that is known to efficiently support osteocalcin differentiation (31, 39); and the nonbone cell line COS1, a SV40 immortalized monkey kidney cell line (30). As shown in Fig. 2A, the 5.9-kb promoter fragment directed a high level of expression of the βgal reporter gene in U2OS cells. The expression level was highest (16-fold higher than that of the promoter-less reporter vector pβgal-Basic) in U2OS cells and was very low in COS1 and BALC cells. To further ensure that promoter expression reflects endogenous OPG expression, we evaluated the amount of OPG protein produced (and secreted into the medium) in these cell lines using an α-OPG antibody-based ELISA. As

**FIG. 1.** Schematic representation of the 5.9-kb OPG promoter showing the location of consensus basal transcription regulatory elements and the OSE elements. The 5.9-kb fragment was cloned upstream of the βgal coding sequence in the vector pβgal-Basic (CLONTECH) for use in functional studies using transient transfection assays. The OSE elements are numbered 12 through 1, from the distal to the proximal end of the promoter. The scale on top shows the approximate location of the OSE elements in the promoter. The arrow represents the transcription start site in the OPG promoter sequence (27).
Table I

Location and sequence of putative OSE₂ and OSE₂-like elements in the 5.9-kb OPG promoter

| OSE₂ element | Sequence | Position relative to transcription start site |
|--------------|----------|-----------------------------------------------|
| 12           | 5'-TGGGCT-3' | -5909 to -5903 |
| 11           | 5'-AGCCCA-3' | -5784 to -5778 |
| 10           | 5'-ACCCCTC-3' | -5599 to -5593 |
| 9            | 5'-CACCCTCA-3' | -5572 to -5566 |
| 8            | 5'-GACCTCA-3' | -3520 to -3514 |
| 7            | 5'-ACCCACA-3' | -3409 to -3403 |
| 6            | 5'-GGCCACA-3' | -2526 to -2520 |
| 5            | 5'-GCCCAACA-3' | -2398 to -2392 |
| 4            | 5'-AACCTCA-3' | -2139 to -2133 |
| 3            | 5'-ACCCACA-3' | -1614 to -1608 |
| 2            | 5'-GACCACA-3' | -990 to -984 |
| 1            | 5'-AACCTCA-3' | -309 to -303 |

![Graph A](image1.png)

**Fig. 3.** Transactivation of the 5.9-kb OPG promoter by Cbfa1 in osteoblastic and nonosteoblastic cells. A, immunoblot analysis of Cbfa1 expression in transfected COS1 cells. COS1 cells were transfected with either empty vector (pEF/myc/cyto) or pEF-Cbfa1, and the expression of the Cbfa1-Myc fusion protein was detected by immunoblot analysis using a monoclonal α-Myc antibody. B, the 5.9-kb OPG-βgal construct was cotransfected into COS1, BALC, and U2OS cells, along with either the Cbfa1 expression construct (pEF-Cbfa1) or the empty vector (pEF/myc/cyto) using Fugene6 transfection reagent. Values represent the fold-induction of β-gal activity (means ± S.E.) in Cbfa1-transfected cell extracts compared with that in empty vector-transfected cell extracts. The fold induction value observed in each cell line is indicated on top of the bars.

shown in Fig. 2B, high levels of OPG were detected in the culture medium in U2OS cells, and only very low levels were present in BALC and COS1 cell media.

Transactivation of the 5.9-kb OPG Promoter by Cbfa1—The presence of several putative OSE₂ elements in the 5.9-kb OPG promoter, combined with the essential role of Cbfa1 in osteoblast development and the physiologically observed link between cells of the osteoblast lineage and osteoclastogenesis, prompted us to analyze whether OPG is one of the targets of Cbfa1. To test the ability of Cbfa1 to transactivate the OPG promoter, COS1, BALC, and U2OS cells were cotransfected with pOPG5.9-βgal construct along with either the Cbfa1 expression construct (pEF-Cbfa1) or the empty vector (pEF/myc/cyto). At first, we determined whether the Cbfa1 protein was synthesized from pEF-Cbfa1, by performing immunoblot analysis of transfected COS1 cell extracts using a monoclonal α-Myc antibody. As shown in Fig. 3A, substantial amounts of Cbfa1 protein was detected in cells transfected with pEF-Cbfa1, and the protein was capable of binding to the OSE₂ element (see Fig. 9, lane 8). Cotransfection of pEF-Cbfa1 and pOPG5.9-βgal led to a 39-fold increase in OPG promoter activity in COS1 cells, a 7-fold increase in BALC cells, and a 16-fold increase in U2OS cells compared with pEF/myc/cyto control (Fig. 3B). This transactivation of the OPG promoter by Cbfa1 is consistent with the presence of functional consensus OSE₂ sites in the OPG promoter, or it could reflect an indirect effect of Cbfa1 via other sites in the promoter.

Effect of 5' Deletions in the OPG Promoter on Base-line Expression—To assess the relative contribution of the different OSE₂ elements, we made sequential 5' deletions of the promoter and obtained six different deletion constructs that pres-
ent a sequential decrease in the number of consensus OSE2 elements they contain. The deletion constructs (3.6-, 1.9-, 1.5-, 0.9-, 0.4-, and 0.2-kb OPGβgal) were analyzed for basal expression in BALC and U2OS cells. βgal activity was measured in cell extracts (from three independent transfection experiments performed in triplicate) and is indicated as the percentage of change compared with that of the 5.9-kb OPGβgal construct. The SV40-βgal construct was used as a positive control and for comparison of the relative strength of the OPG promoter.

Sequential 5′ Deletions of the OPG Promoter Result in a Decrease in Cbfa1 Transactivation—To obtain further evidence of a role for the OSE2 elements in OPG promoter expression, we performed cotransfection experiments in COS1 cells, which are nonosteoblastic and do not express endogenous Cbfa1 (40) or OPG (Fig. 2B). As shown in Fig. 4, in both the cell lines, in comparison with the 5′ deletion constructs, the 5.9-kb promoter directed relatively low levels of expression. Sequential deletions led to a progressive increase in base-line expression, suggesting the presence of negative regulatory elements in the distal region of the promoter. In U2OS, highest expression (2–3-fold) was observed with constructs containing 0.9- and 0.4-kb promoter sequences that include just the proximal OSE2 element. Similarly, in BALC, the 0.9-kb promoter directed the highest level of expression, but in contrast to U2OS, additional 5′ deletions (0.4 and 0.2 kb) resulted in decreased basal expression (Fig. 4). As a control, the SV40 promoter-driven βgal construct (SV40-βgal) was used, and it directed very high levels of βgal expression compared with any of the OPG promoter-βgal constructs.

Sequential 5′ Deletions of the OPG Promoter Result in a Decrease in Cbfa1 Transactivation—To obtain further evidence of a role for the OSE2 elements in OPG promoter expression, we performed cotransfection experiments in COS1 cells, which are nonosteoblastic and do not express endogenous Cbfa1 (40) or OPG (Fig. 2B). Analysis of the promoter deletions demonstrate that sequential removal of the distal OSE2 elements (elements 12 through 2; Table I and Fig. 1) led to a decrease in Cbfa1 inducibility of the OPG promoter (Fig. 5). The removal of all the putative OSE2 elements led to a near complete loss of transactivation, from 25-fold in the 0.4-kb OPGβgal construct to only 4-fold in the 0.2-kb OPGβgal construct (Fig. 5), suggesting that the OSE2 elements may indeed contribute to the observed effects of Cbfa1. To evaluate whether the effects of Cbfa1 observed in COS1 would have any relevance in the stromal/osteoblastic cell line BALC, and in the human osteosarcoma cell line U2OS, identical cotransfections were performed in BALC and U2OS cell lines using the 5′ deletion constructs. As shown in Fig. 5, compared with the 7-fold induction of the
5.9-kb promoter in BALC cells, sequential 5’ deletions led to a decrease and eventually resulted in a complete loss of transactivation. Similarly, in U2OS cells, the 16-fold induction in βgal activity directed by the 5.9-kb OPG promoter was nearly lost with the removal of regions harboring the OSE2 elements. Interestingly, deletion of a 183-bp region (−372 to −190) containing the most proximal OSE2 resulted in either a complete loss of transactivation or marginal responsiveness in all three cell lines (Fig. 5; compare 0.4- and 0.2-kb fragments), suggesting that it plays an important role in mediating Cbfa1 transactivation.

Substitution or Deletion of the Proximal OSE2 Element Results in a Decrease in Cbfa1-mediated Transactivation—To further validate the role of the proximal OSE2 element, we next asked whether mutations in this element affect promoter activity. A substitution mutation within this element in the 0.4-kb OPG βgal construct and an additional deletion mutant that lacks the proximal OSE2 element (0.3-kb OPG βgal) were evaluated for Cbfa1 responsiveness. There was no significant change in the basal expression levels directed by these mutant constructs in COS1 cells, but a 45–65% decrease in basal promoter activity was observed in U2OS osteoblastic cells (Fig. 5). Because the endogenous OPG levels are high in U2OS cells, this suggests that the addition of OSE2 sequences to the 0.2-kb OPG minimal promoter should rescue or maintain full Cbfa1 inducibility in the absence of a functional proximal element. For these studies, we mutated the proximal element in the 5.9-kb promoter (Fig. 7A) and evaluated its responsiveness to Cbfa1 in cotransfection experiments. There was no change in base-line expression directed by the wild type and mutant promoter fragments. However, mutation of the proximal element in the 5.9-kb promoter resulted in a 60% decrease in Cbfa1-mediated transactivation (Fig. 7B). Collectively, these results substantiate the relative importance of the proximal OSE2 element in mediating Cbfa1 effects on OPG promoter expression in an osteoblastic cell line.

Addition of OSE2 Sequences to the 0.2-kb OPG Minimal Promoter Restores Cbfa1 Responsiveness—We then tested whether the addition of OSE2 elements could restore Cbfa1 inducibility to the 0.2-kb OPG minimal promoter. For this, either one (1OSE-0.2kb-OPG-βgal) or three copies (3xOSE-0.2kb-OPG-βgal) of a canonical OSE2 sequence were linked to the 0.2-kb minimal promoter, and the constructs were cotransfected into COS1 cells, along with either pEF-Cbfa1 or the empty vector pEF/myc/cyto. Addition of one copy of the OSE2 element resulted in a 2-fold increase in activity of the minimal promoter (Fig. 8), consistent with a 2-fold (50%) decrease in activity of the 0.4-kb promoter fragment that had a substitution mutation in the proximal OSE2 element (0.4-kb OSEmut-βgal) or when the element was deleted (0.3 kb), as shown in Fig. 6. Interestingly, addition of three copies of the OSE2 element resulted in a 17-fold increase in Cbfa1 transactivation as compared with the 3.6-fold induction of the 0.2-kb minimal promoter fragment.

Cbfa1 Binds in a Sequence-specific Manner to the Proximal OSE2 Element In Vitro—The core sequence of the proximal OSE2 element is identical to the OSE2 element in the osteopontin promoter that has been shown to specifically bind Cbfa1 in vitro (1). To assess the ability of Cbfa1 to interact with the proximal OSE2 element, electrophoretic mobility shift assays were performed with nuclear extracts from ROS 17/2.8 osteoblast-like osteosarcoma cells that express large amounts of Cbfa1 (36, 41–43). The native proximal OSE2 element, the mutated proximal OSE2 element (containing the substitution
mutation that resulted in a 2-fold decrease in Cbfa1-mediated transactivation. A, schematic representation of the 5.9-kb OPG promoter region containing either wild type or mutated proximal OSE2 element. B, effect of substitution mutations in the proximal OSE2 element on Cbfa1 transactivation of the 5.9-kb OPG promoter. U2OS cells were transfected with pOPG5.9-βgal or pOPG5.9prox.OSEmut-βgal construct, along with either pEF/myc/cyto or pEF-Cbfa1, in triplicate. The βgal activity in cell extracts was measured 48 h after transfection and is expressed as the fold increase in Cbfa1 transfected cells compared with empty vector transfected cells.

Fig. 8. Addition of OSE2 sequences to the 0.2-kb OPG minimal promoter restores Cbfa1 responsiveness. Constructs containing either one or three copies of the consensus OSE2 element linked to the 0.2-kb OPG minimal promoter and the wild type 0.2-kb OPGβgal construct were cotransfected into COS1 cells, along with pEF-Cbfa1 or pEF/myc/cyto. The fold induction in βgal activity directed by each promoter construct in the presence of Cbfa1, compared with its own control (in the presence of pEF/myc/cyto) is shown. Extracts from cells transfected with the promoter-less reporter vector pβgal-Basic served as a control.

Fig. 7. Substitution mutation in proximal OSE2 element in the 5.9-kb OPG promoter significantly decreases Cbfa1-mediated transactivation. A, schematic representation of the 5.9-kb OPG promoter region containing either wild type or mutated proximal OSE2 element. B, effect of substitution mutations in the proximal OSE2 element on Cbfa1 transactivation of the 5.9-kb OPG promoter. U2OS cells were transfected with pOPG5.9-βgal or pOPG5.9prox.OSEmut-βgal construct, along with either pEF/myc/cyto or pEF-Cbfa1, in triplicate. The βgal activity in cell extracts was measured 48 h after transfection and is expressed as the fold increase in Cbfa1 transfected cells compared with empty vector transfected cells.

Overexpression of Cbfa1 Increases Endogenous OPG Protein Levels in U2OS Cells—Because overexpression of Cbfa1 results in a very strong stimulation of OPG promoter activity, we analyzed whether Cbfa1 could increase OPG protein levels in U2OS cells, using the OPG ELISA. As shown in Fig. 10, cells transfected with the Cbfa1 expression construct had a 54% increase in the levels of OPG protein secreted into the culture medium, compared with empty vector transfected cells (20 pg/10^3 cells versus 13 pg/10^3 cells).

DISCUSSION

It has been speculated that Cbfa1 through its effects on osteoblast lineage commitment and function could also, in part, directly or indirectly regulate the bone resorption process. In the present work, we provide evidence that OPG, a negative regulator of osteoclast formation and function that is secreted by stromal/osteoblastic cells, is a target of Cbfa1. We have cloned and characterized a 5.9-kb human OPG promoter and shown that it is robustly transactivated by Cbfa1 in a sequence-specific manner in both osteoblastic (BALC, U2OS) and nonosteoblastic (COS1) cells. Additionally, we demonstrate that this promoter is sufficient to direct osteoblast-specific expression, reminiscent of endogenous OPG protein expression.

The initial indication that Cbfa1 could regulate the expression of OPG came from the observation that there are 12 putative OSE2 elements in the OPG promoter. The complexes formed with nuclear extracts from Cbfa1-transfected COS1 cells that has no endogenous Cbfa1 expression (40), and those formed with nuclear extracts from ROS 17/2.8 cells had identical gel migration patterns suggesting that they are Cbfa1-OSE2 complexes. Also, a similar Cbfa1-OSE2 complex was seen in nuclear extracts from U2OS cells, but the intensity was much lower (Ref. 4 and data not shown).
FIG. 9. Sequence-specific binding of Cbfa1 to the proximal OSE2 element in vitro. Electrophoretic mobility shift assays were performed with nuclear extracts from ROS 17/2.8 cells or transfected COS1 cells. In lanes 1–5, complexes formed using ROS 17/2.8 nuclear extracts and various labeled OSE2 probes are shown. Lane 1, free probe; lane 2, wild type osteocalcin OSE2 (OC wt); lane 3, mutant osteocalcin OSE2 (OC mut); lane 4, wild type OPG proximal OSE2 (OPG wt); lane 5, mutant OPG proximal OSE2 (OPG mut). The arrow represents the Cbfa1-specific complex. The consensus OSE2 element from the osteocalcin promoter served as a positive control. The exposure time for the middle panel (lanes 4 and 5) was longer than that for the left panel (lanes 1–3). Complexes formed using nuclear extracts from transfected COS1 cells are shown in lanes 6–11. The wild type OPG proximal OSE2 element (OPG wt, lanes 6–8) and the mutant OPG proximal OSE2 element (OPG mut, lanes 9–11) were used as probes.

FIG. 10. Overexpression of Cbfa1 increases endogenous OPG protein levels in U2OS cells. U2OS cells were transfected with either pEF/myc/cyto or pEF-Cbfa1. 48 h after transfection the OPG protein levels in U2OS cells were quantified using an OPG ELISA. Data represent the means ± S.E. of three independent experiments. * significantly different from control (p < 0.005).

The removal of the proximal OSE2 element almost completely abolished Cbfa1 effects in all three cell lines. This loss of transactivation was restored by addition of either one or three copies of OSE2 element to a minimal 0.2-kb OPG promoter (Fig. 8). Electrophoretic mobility shift assays performed with nuclear extracts from ROS 17/2.8 cells and Cbfa1-transfected COS1 cells, showed that Cbfa1 binds to the proximal OSE2 element in a sequence-specific manner. Interestingly, in transactivation studies, residual effects of Cbfa1 were still observed after deletion of the most proximal OSE2 element, suggesting that a full complement of Cbfa1 effects on OPG promoter may be mediated in part via noncanonical OSE2 element(s) present in this region. Also, a substitution or deletion mutation in the proximal OSE2 element led to a 45–65% decrease in basal promoter activity in U2OS osteoblastic cells and a 40–70% decrease in Cbfa1-transfected COS1 and U2OS cells, suggesting that additional factors besides Cbfa1 are involved in regulating expression of the OPG promoter. It is conceivable that Cbfa1 induces the expression of another factor(s) that in turn binds to an element(s) in the promoter and induces expression. Cbfa1 could also interact with other promoter binding factors that regulate OPG promoter activity. Precedence for interaction between Cbfa1 and AP-1 has been suggested in the mediation of parathyroid hormone regulation of the mouse (5) and rat collagenase-3 promoter (33), and Cbfa1 and ETS1 have been shown to enhance osteopontin promoter activity in a synergistic manner (6). Collectively, these results suggest that Cbfa1 regulates the OPG promoter in a sequence-specific manner via contributions from one or more of the OSE2 elements.

This paper provides the first direct demonstration that Cbfa1 regulates the expression of OPG, as reflected by changes in transcription and protein levels. The fact that mutation of the proximal OSE2 element decreases OPG promoter activity in U2OS cells suggests that Cbfa1 plays a major role in OPG expression in osteoblastic cells. We have shown that Cbfa1 overexpression results in a 54% increase in the level of OPG protein that is secreted into the culture medium in U2OS cells. Because the only known functions of OPG in bone are inhibition of osteoclast formation and function, these results, at least in part, favor a role for Cbfa1 in inhibiting bone resorption. However, it remains to be determined whether Cbfa1 overexpression results in an increase in OPG protein levels in vivo, and the consequence, if any, on osteoclast formation/function and bone resorption is still undetermined.
In a recent study, it has been shown that OPG mRNA is expressed in the fibroblastic connective tissues that are present in the calvarial region of Cbfa1−/− mice embryos (44). However, based on the fact that Cbfa1−/− animals lack functional osteoblasts (2, 3) and that OPG is expressed abundantly in fibroblasts (45), it would be difficult to unequivocally determine whether Cbfa1 regulates the expression of OPG in osteoblasts in vivo using this system. A previous report in abstract form has also indicated the presence of potential OSE sites in the promoter for RANK ligand, the cognate ligand for OPG (46), but there is as yet no evidence that Cbfa1 overexpression specifically increasing the production of OPG, which in turn promotes the differentiation of mesenchymal stem cells to form osteoblasts (45), it would be difficult to unequivocally determine whether Cbfa1 regulates the expression of OPG in osteoblasts (2, 3) and that OPG is expressed abundantly in fibroblasts (45).

In summary, in addition to the known role of Cbfa1 in promoting the differentiation of mesenchymal stem cells to form mature osteoblasts, expression of Cbfa1 appears capable of specifically increasing the production of OPG, which in turn could interfere with the interaction of RANK ligand with its receptor, RANK, on osteoclast precursors (15–18, 25), thereby leading to an inhibition of osteoclast differentiation (Fig. 11). Our data suggest the exciting possibility that the net effects of Cbfa1 on bone could result from an increase in osteoblast formation/activity and inhibition of osteoclast formation/activity.

Acknowledgments—We thank Drs. Gerard Karsenty and Patricia Ducy (Baylor College of Medicine, Houston, TX) for helpful discussions and Drs. Venkatesh Krishnan, Andrew Geiser, and Charles Frolik (Lilly Research Labs, Indianapolis, IN) for critical review of the manuscript and for valuable suggestions.

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Regulation of OPG by Chfα1

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