Cbfa1/RUNX2 Directs Specific Expression of the Sclerosteosis Gene (SOST)*

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Loss-of-function mutations in the sclerosteosis gene (SOST) cause a rare sclerosing bone dysplasia characterized by skeletal overgrowth. Cbfa1/RUNX2 is a key transcriptional regulator of osteoblast function. Here we link these two pathways by demonstrating, via gel shift and transient transfection analyses, that Cbfa1 binding to the proximal SOST promoter contributes to differential SOST expression in two osteosarcoma cell lines. Additionally, an E-box binding motif in the 1.8-kb proximal SOST promoter appears to be functional in SAOS-2 cells, but does not account for SAOS-specific expression of SOST. The regulation of SOST expression by Cbfa1 suggests a potential role for the sclerosteosis gene in homeostatic regulation of osteoblast differentiation and function. Furthermore, the juxtaposition of Cbfa1, E-box, and C/EBP binding sites in the SOST proximal promoter bears an intriguing resemblance to the promoter for osteocalcin, another osteoblast-specific gene with a loss-of-function phenotype of bone overgrowth.

Sclerosteosis is a rare, progressive disorder characterized by general skeletal overgrowth (1). Symptoms of sclerosteosis include gigantism, entrapment of cranial nerves, increased intracranial pressure due to widening of the calvarium of the skull, and increased thickness and density of both trabecular and cortical bone (2-4). The disease is inherited in an autosomal recessive manner (5), and has been mapped to null mutations in sclerostin, or SOST (6, 7). The SOST gene is expressed in osteoblast cells and encodes a secreted 213 amino acid polypeptide with homology to the DAN family. Because DAN family proteins are secreted TGF-β antagonists (8, 9), SOST may repress bone growth by antagonizing TGF-β or BMP function.

Cbfa1/RUNX2 is a sequence-specific DNA-binding protein whose consensus element RACCRCW (10) is found in the promoters of a variety of genes related to osteoblast differentiation or function, including osteocalcin, osteopontin, bone sialoprotein, and type I collagen (11-16). Homozygous deletion of mouse Cbfa1 leads to a complete absence of bone formation due to an arrest in osteoblast maturation that prevents endochondral ossification (17, 18). Mice heterozygous for Cbfa1 develop bone abnormalities strikingly similar to those of patients suffering from the heritable genetic disorder cleidocranial dysplasia (CCD), an observation, which led to the discovery that Cbfa1 haploinsufficiency is the cause of CCD (17, 19, 20). Although Cbfa1 was originally implicated in osteoblast differentiation, a further role for Cbfa1 in mature bone was demonstrated by creation of a transgenic mouse line expressing a dominant negative form of Cbfa1 using the bone-specific osteocalcin promoter. The inactivity of this promoter in pre-osteoblasts allowed these animals to develop a normal skeleton, but they later developed an osteopenic phenotype due to decreased bone formation, indicating that Cbfa1 plays a role in postnatal osteoblast function as well (21). Surprisingly, an osteopenic phenotype also developed in transgenic mice overexpressing wild-type Cbfa1 from the pro-α (1) collagen promoter. This phenotype, caused by a late stage blockage of osteoblast maturation that resulted in a dramatic decrease in the number of osteocytes, suggests that Cbfa1 negatively regulates a late stage of osteoblast/osteocyte development (22).

Among the target genes of Cbfa1/RUNX2 is osteocalcin, an osteoblast-specific gene that is the most highly expressed non-collagenous protein in bone (23). Mice in which both osteocalcin genes were deleted showed an increased rate of bone formation, implicating osteocalcin as a negative regulator of bone growth, although the mechanism has not been fully defined (24). The osteocalcin promoter features three Cbfa consensus motifs, and studies have shown Cbfa1 to be important for both basal and vitamin D-regulated osteocalcin transcription (10). A C/EBP binding motif in the proximal osteocalcin promoter also has been shown to be functional, and the C/EBPβ protein has been shown to synergistically cooperate with Cbfa1 at this promoter through a direct interaction (25). E-box consensus binding sites are present in the osteocalcin proximal promoter as well, and one particular E-box appears functionally important, with mutations reducing transcriptional activity by greater than 50% in the rat ROS17/2.8 osteoblastic osteosarcoma cell line (26).

To gain insight into the pathway by which the newly discovered SOST gene might inhibit osteoblast differentiation and/or activity, we use a comparative genomics approach to identify conserved elements in the mouse and human noncoding sequences near the SOST genomic locus. A 1.8-kb fragment of the proximal promoter is well conserved between humans and mice and is active in the SAOS-2 but not the MG-63 human osteosarcoma cell line, paralleling the pattern of SOST expression in these cells. Promoter deletion analysis demonstrates that a 140-bp element just upstream of the SOST gene accounts for this transcriptional activity. The active region contains two E-boxes, a C/EBP binding site, and a Cbfa1 binding site. Mutational and gel mobility shift analyses demonstrate that transactivation by Cbfa1 accounts for the difference in SOST expression between the two cell lines. These observations are further supported by the expression of Cbfa1 in SAOS-2 cells but not MG-63, and the ability of transfected Cbfa1 to stimulate a SOST reporter in MG-63 cells. An upstream E-box is functional and may be a target of MyoD regulation, but does

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¶ The abbreviations used are: TGF-β, transforming growth factor β; CCD, cleidocranial dysplasia; CSE, conserved sequence element; BMP, bone morphogenetic protein; EMSA, electrophoretic mobility shift assay.

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not account for the difference in SOST expression between these two cell lines. Our results identify SOST as a novel target gene for Cbfα1, and suggest a possible feedback inhibition role for the SOST protein in maintaining Cbfα1 at levels appropriate for osteoblast function and/or development.

MATERIALS AND METHODS

SOST Genomic Sequence Analysis—Approximately 200 kb of human genomic sequence from chromosome 17q12-q21 containing SOST and bounded proximally by MEOX1 and distally by DUSP3, was compared with the syntenic region from the mouse genome6 in an attempt to identify conserved sequence elements (CSEs) in non-coding sequence that could serve as a starting point for the identification of factors that affect the regulation of SOST. A CSE is defined as a 100 bp or greater sequence with minimum 50% identity. Both the human and mouse sequences were masked for repeats prior to comparison using RepeatMasker.7 Alignment of the human and mouse sequences was estimated using BLASTn (29) with default parameters. The alignment was scanned with a 100-bp window, and the percent identity within the window was calculated and plotted using VISTA (30, 31). A 15-kb region was calculated and plotted using VISTA (30, 31). A 15-kb region was determined using Repeatmasker.genome.washington.edu/cgi-bin/RepeatMasker.

Reported and Expression Constructs—The SOST promoter sequence selected for further study spans nucleotides −2000 to −190 of the SOST gene locus relative to the position (+1) of the initiation methionine for the SOST open reading frame (6). The sequence was amplified from human genomic DNA (Promega) by PCR using the following 5′ and 3′ primers, respectively: 5′-TCTCCCGGGGTGTTGATCTTTAGAAGTTCAAG-3′ and 5′-GCCCCTAGATATCCAAAGACTTCTCTCTAGACTGCTC-3′. The resulting PCR product was digested with SmaI and BglII and inserted into the pGL2-Basic luciferase reporter vector (Promega) digested with the same restriction enzymes. PCR was carried out using HotStarTag master mix (Qiagen) with the following conditions: 95 °C for 1 min, followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min, and 72 °C for 3 min.

Large-scale deletion mutations of the SOST promoter were constructed by digestion at native restriction sites (MluI, HpaI, SpH1, HindIII, BamHI, EcoRV), treatment with Klenow or T4 DNA polymerase to fill in any overhangs, digestion with BglII, and insertion into pGL2-Basic (Promega) digested with SmaI and BglII. Finer-scale deletions and point mutations involving the SOST promoter fragment between the EcoRV and BglII sites were generated using a PCR-based approach with oligonucleotides spanning the ends of the desired promoter sequence and bearing BglII, EcoRV, or SmaI restriction sites. Oligonucleotides used to generate mutations in transcription factor binding sites were as follows (mutated nucleotides are underlined): upstream E-box mutant primer: 5′-GCTCCTCGGGGCAGGACTAAACCGAGGTCCGCGGACGGACG-3′. Downstream E-box mutant primer (reverse orientation): 5′-GCGGAAGATCTCCTGCTTTACACCCAGAGGAGGGGAGCAGAGCAGGACTAAACCGAGGTCCGCGGACGGACG-3′. Cbfα1 site mutant primer: 5′-GCTCCTAGATATCCAAAGACTTCTCTCTAGACTGCTC-3′. Site mutants with longer flanking 5′ and 3′ sequences were used to mutate the Cbfα1 and Ebox sites in the full-length SOST-luc promoter via PCR-based mutagenesis.

Expression constructs for human MyoD1 and Cbfα1/RUNX2 were created using the mammalian expression vector pCD409, which has been previously described (32). MyoD1 cDNA was generated by PCR using 5′-CGTTAACCAGGTCGCGATTAGCACATCGGCCCCACC-3′ as a forward primer and 5′-CGGCTAGATATCTGCTAGACAGACCTGTTATAATCGG-3′ as a reverse primer, using HotStarTag (Qiagen) as template. Cbfα1 cDNA was generated by PCR using Saos-2 cDNA as a template with 5′-CGACAGGTGCACACCACCTGGGATTTCCGTAGATATCGG-3′ as a forward primer and 5′-CGCGGAATATAGTACAGCCTGCCCCAACATGTT-3′ as a reverse primer, using HotStarTag (Qiagen) as template. Cbfα1 cDNA was then inserted into the pCD409 vector using BglII and XhoI restriction sites to generate the plasmid pCD409-Cbfα1.

Cell Treatment, RNA Harvest, and Semi-Quantitative RT-PCR—SAOS-2 and MG-63 cells were plated at 104 cells/plate on 10-cm plates (Corning) and treated with either 12.5- OH2 vitamin D3 (Calbiochem) at 10−5 M, Osteogenesis Induction Medium (OIM, see below), both vitamin D3 and OIM, or neither for 72 h prior to harvest. OIM consisted of 0.1 μM dexamethasone (Sigma), 50 μM ascorbic acid 2-phosphate (Sigma), and 10 mM β-glycerophosphate (Sigma). RNA was prepared using the RNeasy Midi kit (Qiagen) according to the manufacturer's instructions, and its quality was evaluated using an Agilent 2100 Bioanalyzer. Double-stranded cDNA was prepared with a Super Script Choice Kit (Invitrogen) using 1 μg of input RNA and 50 ng of random hexamers. RT-PCR of MyoD family members was performed using the following intron-spanning primers: for MyoD, 5′-AAGTAAATAGGCGCCCTTTAGGACAC-3′ and 5′-CGATGTCGACAGGACTTGCTG-3′ for Myogenin, 5′-AGTGCCATCAGCTACTCAGGAG-3′ and 5′-TGCTTAACTTCTTACATTGAGATG-3′ for Myf5, 5′-AATCTATAGCTGCTGGGATGG-3′ and 5′-GATGCAGCTGTCTACAGC-3′ for Myf6. PCR reactions were carried out for 40 cycles using the Advantage GC PCR kit (Clontech) with 1 μM Gt reagent as described above. For positive control samples, cDNA from human heart (Clontech) and human fetal skeletal muscle (Clontech) libraries was combined.

Real-Time Quantitative RT-PCR—RNA samples were treated with DNase (Ambion, Austin, TX), and reverse-transcribed using TaqMan Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) according to the manufacturer's instructions using random hexamers. Samples were distributed on plates at either 5 or 20 ng per well and run in triplicate. TaqMan primer and probe sets were optimized and determined to be 300, 900, and 300, 50 nM each; the forward and reverse primer concentrations for human SOST and Cbfα1 were 300, 900, and 300, 50 nM each; the VIC-labeled TaqMan probe was used at 200 nM. Multiplex PCR reactions were run in 25-μl volumes with TaqMan Universal PCR Master Mix on an Applied Biosystems Prism 7700 Sequence Detection System. Threshold cycle values (Ct) were determined using Sequence Detector software version 1.7a (Applied Biosystems) and transformed to 2−ΔΔCt for relative expression compared with Hprt1 for 1 min, and 72 °C for 1 min. The VAH plaques were stained for alkaline phosphatase using the Leukocyte Alkaline Phosphatase Kit (Clontech) and assayed using a MicroBeta Trilux luminescence counter (Wallace). Luciferase values were normalized to SEAP data to correct for well-to-well variations in transfection efficiency. Each construct was tested in a minimum of two experiments with consistent results.

Nuclear Extract Preparation and Electrophoretic Mobility Shift Analysis—Nuclear extracts were prepared from SAOS-2 and MG-63 cells growing on 10-cm plates at a density similar to that used in transfection experiments (2.1×106 cells/plate) using a Nuclear Extract Kit (Active Motif, Carlsbad, CA) according to the manufacturer's instructions. Protein concentrations of nuclear extracts were determined using the Coomassie Protein Assay reagent (Pierce), and extracts were aliquoted and stored at −80 °C until use. Probes for the electrophoretic mobility shift assay (EMSA) were end-labeled using 2 μl of Redivue 32P]ATP (10 μCi/ml) (Amersham Biosciences) and 1 μl of T4 polynucleotide kinase (New England Biolabs) and purified over G-5 Sephadex Quick
Cas designated in Fig. 3. Gel Extraction kit (Qiagen). Smaller probes encompassing regions A
purified by agarose gel electrophoresis and recovered using the Qiaex II
nucleotides in a solution of 50 mM Tris-HCl, pH 8, and 10 mM MgCl2 for
in this study is indicated with a bar. The upstream promoter region analyzed
in SAOS-2 and MG-63 cells relative
to the
sion in SAOS-2 and MG-63 cells relative
to the
housekeeper gene using TaqMan PCR as described under “Materials and Methods.” Prior to RNA harvest,
cells were treated 72 h with Vit D3, Osteogenesis Induction Medium (OIM), Vit D3, + OIM, or were left untreated. OIM
contained dexamethasone (0.1 μM), ascorbic acid 2-phosphate (50 μM), and β-glyceroxophosphate (10 mM). D, SAOS-2 and
MG-63 cells were transiently transfected with the SOST-luciferase reporter vector (1 μg) or with the parent vector, pGL2-
Basic (Promega, 1 μg) and analyzed 48 h after transfection. Luciferase data were
normalized to the activity of cotransfected secreted alkaline phosphatase (BD Biosciences, 20 ng) following chemilumines-
cent analysis of supernatants for SEAP.

Fig. 1. SOST genomic locus in human and mouse, and characterization of SOST promoter activity in
SAOS-2 and MG-63 cells. A, sequence conservation between human and mouse in a 10-kb region containing the SOST
gene. Percent identity is calculated in a 100-bp window. Regions with a minimum of 50% identity are shown. The alignment of the SOST transcript (NM_025337.1/gi/
13376845) with the genomic sequence is represented above the plot with a horizontal arrow indicating the direction of tran-
scription. Peaks that correspond to exons from the aligned transcript are shaded blue. The first CSE 5' of SOST spans 783
bp and has an average of 71% identity. The upstream promoter region analyzed
in this study is indicated with a bar. B, alkaline phosphatase staining (red) of growth phase untreated MG-63 and
SAOS-2 cell cultures. Cells were counter-
Stained with hematoxylin. C, quantitative real-time PCR analysis of SOST expres-
sion in SAOS-2 and MG-63 cells relative
to the
expression of HPRT
clidean min at 4°C. Antibodies used were the PEBP2α/Cbfa1 (S-19) goat polyclonal IgG (Santa Cruz Biotechnology) or the Integrinβ2 K-19 goat polyclonal IgG (Santa Cruz Biotechnology).

RESULTS
SOST Proximal Promoter Cloning and Function in SAOS-2
versus MG-63 Cells—To search for homology between noncoding sequences in the SOST genomic locus, we used the VISTA pro-
gram to compare mouse and human sequences deposited in the respective Celera databases (Fig. 1A) (30, 31). Human and mouse
noncoding sequences showed greater than 50% homology in regions immediately upstream of the SOST open reading frame, in
the gene’s first intron, immediately downstream of the tran-
scribed sequence, and in several more distant 3'-regions.
Because noncoding sequences immediately upstream of genes may contain important proximal control elements, we
decided to focus on the well-conserved 1.8-kb sequence directly
upstream of the SOST gene. This region, referred to hereafter as the SOST promoter fragment, contained ~780 bp of highly
conserved sequence in its 5'-end and a shorter well-conserved region just upstream of the SOST open reading frame (Fig. 1A).
We inserted the 1.8-kb sequence into the pGL2-basic luciferase reporter
vector (Promega) and tested the ability of this pro-
moter fragment to drive luciferase expression in the SAOS-2
and MG-63 osteosarcoma cell lines.
By several criteria, SAOS-2 cells appear to represent a more differentiated osteoblast cell type than MG-63 cells. SAOS cells stain intensely positive for alkaline phosphatase and appear more rounded, whereas MG-63 cells are only weakly positive for alkaline phosphatase activity and have a spindly fibroblast-like appearance (Fig. 1B). When we examined both cell lines for SOST expression using Taqman PCR, basal SOST expression was readily detected in SAOS-2 but not MG-63 cells (Ct cut-off = 36), consistent with the previously reported expression of SOST in differentiated osteoblast cells (Fig. 1C) (6). SOST expression appeared to be up-regulated by vitamin D in SAOS-2 cells, and could be weakly induced by a combination of vitamin D and OIM (Osteogenesis Induction Medium) in MG-63 cells, but was not detected in untreated MG-63 cells.

To test whether our SOST promoter fragment contained elements necessary to recapitulate the differences in SOST expression between SAOS-2 and MG-63 cells, we transfected the reporter construct into both cell types. The SOST-luc reporter consistently showed a 5-fold increase in luciferase activity compared with empty vector in SAOS-2 cells but no such increase in MG-63 cells, in agreement with the expression pattern of the SOST protein in these cell lines (Fig. 1D). This result suggested that SAOS-2 cells endogenously express a specific factor(s) that could bind this promoter element to up-regulate the expression of SOST.

Deletion Analysis of the SOST Proximal Promoter Fragment—To delineate the promoter element(s) required for SAOS-specific SOST expression, we generated a series of deletion constructs using the promoter region's native restriction sites. Because we particularly wished to test the function of a large block of well-conserved (82% identity) sequence in the 5′-region of the promoter, we deleted promoter elements in a 5′ to 3′ fashion (Fig. 2A).

Deletion of 5′-promoter elements encompassing the well-conserved region did not decrease luciferase activity, but instead revealed an −2-fold repressive effect of this region. Because this upstream element was dispensable for SAOS-specific SOST activity, we chose not to characterize it further. Further deletions to the 5′-boundary of the EcoRV restriction site, leaving only 140 nt of 3′-SOST promoter sequence, also failed to reduce SAOS-specific SOST activity, suggesting that the remaining 140 nt contained SAOS-specific regulatory positive elements. To confirm this observation, we tested a construct containing all SOST pro-

\[\text{ATCTGAA} \text{AACCACA} \text{GCCGCGAGCAGTGGAGTGC} -331\]
\[\text{CGGGAGCACGGCTTGGG} \text{CTTGCCCTCA} \text{CACGCCGCC} -360\]
\[\text{TCTCTCTGGGT} \text{CACTGGGAGTGGCCAACAGCAATT} -224\]
\[\text{GGAAGTTTGCTAGCTAGGAGAGAGTCTTGGG} -190\]

\[\text{AACCACA} = \text{Chfl binding site} \quad \text{CACGTG} = \text{Upstream E box}\]
\[\text{CTTGCCCTCA} = \text{C/EBP binding site} \quad \text{CACCTG} = \text{Downstream E box}\]

4 B. R. Sevetson and P. Yang, unpublished data.
moter sequences except those downstream (3') of the EcoRV site. This construct had activity similar to that of empty vector alone (Fig. 2A, bottom construct), confirming that important promoter elements were contained in this 140-nt sequence.

Finer scale deletion analysis of the EcoRV fragment (Fig. 2B, EcoRV-1744-1836) suggested that its 5'-sequence encompassed important regulatory elements. Sequence examination revealed consensus binding sites for a number of transcription factors in this region, including two E-box elements and single binding sites for Cbfa1/RUNX2 and C/EBP proteins (Fig. 2C).

Electrophoretic Mobility Shift Analysis of Active SOST Promoter Fragment—Because SOST is expressed in SAOS-2 but not MG-63 cells, we reasoned that nuclear extracts from these two osteosarcoma lines would bind differentially to the active (s) of the SOST promoter, permitting isolation of the factor(s) responsible for SAOS-specific expression. Further deletion analysis within the EcoRV promoter fragment demonstrated that 5'-sequences could not be deleted without a loss of activity, but that some 3'-sequences, including the downstream E-box, could be eliminated without affecting promoter activity (Fig. 3A). Thus, a minimal region of 75 nt retained maximal transcriptional activity and was chosen as a probe in gel mobility shift assays. The lack of contribution of the downstream E-box was also confirmed by site-directed mutagenesis experiments (Fig. 6A).

Nuclear extracts were prepared from untreated SAOS-2 and MG-63 cells and incubated with the 32P-labeled probe containing the minimal active SOST element, followed by gel electrophoresis. Both SAOS-2 and MG-63 nuclear extracts produced a slow-migrating band of essentially equal mobility (Fig. 3B, top arrow), but SAOS-2 extracts also produced a faster migrating band that was not detected in MG-63 cells (Fig. 3B, bottom arrow). To identify the region of the probe responsible for the faster migrating SAOS-specific band, we tested the nuclear extracts using 32P-labeled annealed oligonucleotide pairs representing the portions of the full-length probe designated A, B, C, and D (Fig. 3B). Probes B and D weakly bound factors present in both MG-63 and SAOS-2 cells, whereas probe A strongly and specifically bound to a factor present only in SAOS-2 cells (Fig. 3B, lane 2).

The importance of region A, which contained the Cbfa1 binding site, was confirmed using unlabelled annealed oligonucleotides representing regions A–D as competitors to the full-length SOST gel shift probe (Fig. 3C) in the presence of SAOS-2 nuclear extracts. The unlabeled full-length probe was an effective competitor for both shifted bands at all concentrations used. Unlabeled probe A efficiently competed the fast-migrating SAOS-specific band but not the slower migrating shared band, whereas probes B and D eliminated the band present in both cell lines (Fig. 3D). A version of probe B bearing a mutated E-box consensus site was an ineffective cold competitor, indicating that specific binding to E-box sequences was required for successful competition by the sequences in region B (Fig. 3C).

Interaction of Cbfa1 with Its Binding Site in the SOST Proximal Promoter—By employing mutation-bearing oligonucleotides in further competition experiments similar to those described above, we defined a 9-nt region including the Cbfa1 consensus site as the SAOS-specific element (data not shown). To confirm the specificity of Cbfa1 binding to its cognate element in the SOST proximal promoter, we constructed a series of competitor probes bearing individual mutations in each of these 9 nucleotides (Fig. 4A) as well as oligonucleotides in which all nucleotides outside the 9-nucleotide core were changed (lane 3, Fig. 4A). Each mutant oligonucleotide pair was annealed and tested for its ability to block binding between the SAOS-2 nuclear extract and the 32P-labeled region A probe.

The lack of signal in lane 3 indicated that only the central 9 nt are necessary for effective competition. Lanes 4 through 12 (Fig. 4A) represent oligonucleotide competitors containing the indicated base substitution within the 9-nt core. The most effective mutations, and therefore the poorest competitors, were those that changed the two central cytosine nucleotides to adenine, consistent with the requirement for cytosine residues at these positions in the Cbfa1 consensus. Mutation of adenine
to guanine generally had little effect, a result attributable to the ability of Cbfa1 to recognize either A or G at these positions. Overall, the results were in good agreement with the identification of the SAOS-specific band in these assays as Cbfa1.

To further confirm that Cbfa1 participated in the binding of SAOS-2 nuclear extracts to probe A, we performed a gel supershift analysis using antibodies directed against either Cbfa1 or a control protein (integrin α2/H9252). Addition of the Cbfa1 antibody to a binding reaction containing SAOS-2 nuclear extract and probe A resulted in a supershifted band of decreased mobility, whereas incubation with the control antibody had no effect (Fig. 4B). This result confirmed that Cbfa1 is present in the SAOS-specific complex that binds to probe A.

**Cbfa1 Expression and Transfection Analysis**—If Cbfa1 is a regulator of SOST expression, then differential expression of Cbfa1 between SAOS-2 and MG-63 cells might account for the specific expression of SOST in SAOS-2 cells. We used Taqman PCR analysis to quantitatively compare Cbfa1 expression between the two cell lines (Fig. 5A). Cbfa1 expression was undetectable in untreated MG-63 cells, but was robust in SAOS-2 cells under all conditions tested, consistent with a role for Cbfa1 in SOST promoter regulation in SAOS-2 cells. In both cell lines, Cbfa1 could be up-regulated by treatment with a combination of vitamin D3 and osteogenesis induction medium (OIM), but Cbfa1 expression remained higher in SAOS-2 cells under all conditions tested.

To determine whether transfected Cbfa1 could drive further increases in the transcriptional activity of the SOST promoter, we recovered the Cbfa1 gene from an SAOS-2 cDNA library and inserted it into a mammalian expression vector. When overexpressed in SAOS-2 cells, wild-type Cbfa1 increased activity of
both the EcoRV fragment and the 1.8-kb SOST promoter, further confirming that SOST is a novel Cbfa1 target gene (Fig. 5B). Furthermore, overexpression of wild-type Cbfa1 in the MG-63 cell line resulted in an ∼10-fold increase in activity using the EcoRV fragment of the SOST promoter, which in the absence of Cbfa1 was no more active than a control plasmid (Fig. 5C).

In addition to wild-type Cbfa1, SAOS-2 cells were found to express a splice variant lacking exon 4 (data not shown), the expression of which has been previously reported (34). Semi-quantitative RT-PCR analysis showed that both full-length and ΔExon4 forms of Cbfa1 were expressed at similar levels and were not differentially regulated (data not shown). Both wild-type and ΔExon4 splice forms of Cbfa1 were active in transfection assays, with the splice variant showing an ∼2-fold increase in activity compared with wild-type protein (data not shown). It is not clear whether the ΔExon4 form of Cbfa1 is an inherently more potent transactivator than the wild-type protein or whether the observed differences were due to altered protein stability and/or expression levels.

Role of MyoD Transactivation in the SOST Promoter—Deletion and site-directed mutagenesis experiments showed that while the downstream E-box element is dispensable for SOST promoter activity in SAOS-2 cells, the upstream E-box appears to be functional (Fig. 6A). Deletion or point mutation of this E-box element resulted in a consistent 3-fold decrease in SOST promoter activity. Additionally, as previously described, oligonucleotides bearing a mutated E-box were unable to compete for binding of the slower-mobility band found in both SAOS-2 and MG-63 nuclear extracts (Fig. 3C), whereas the wild-type E-box made an effective competitor.

To ascertain whether any members of the MyoD family might be expressed in SAOS-2 and/or MG-63 cells and could therefore transactivate this promoter, we performed semi-quantitative RT-PCR analysis on both cell types. Although intron-spanning primers for myogenin, Myf5, and Myf6 produced bands of the expected size from positive control cDNAs from a mixture of heart and fetal skeletal muscle libraries, these three genes were not detected in SAOS-2 or MG-63 cells (Fig. 6B). In contrast, MyoD was expressed in both cell lines and therefore could be responsible for E-box-dependent transcription. MyoD was expressed at similar levels in both SAOS-2 and MG-63 cells, consistent with our prior observation that the MyoD binding site was bound by factors present in both SAOS-2 and MG-63 nuclear extracts (Fig. 3B).

Transient expression of MyoD in SAOS-2 cells resulted in robust dose-dependent activation of both the 1.8-kb SOST promoter and the 140-nt EcoRV promoter fragment (Fig. 6C). These data, taken together with the MyoD expression data, and the results of E-box mutagenesis described above, suggest that MyoD may contribute to SOST transcription through the upstream E-box.

Examination of Potential Cbfa1 and MyoD Cooperativity—Because both Cbfa1 and MyoD were able to independently transactivate the EcoRV SOST promoter fragment, we coexpressed the two proteins to examine whether their potential interaction would result in a synergistic effect. In SAOS-2 cells transfected with the EcoRV fragment, Cbfa1 caused a 8–9-fold increase in luciferase activity, whereas MyoD expression increased the activity by ∼4-fold (Fig. 7A). When the two factors were coexpressed, luciferase activity increased ∼20-fold above that of reporter alone. In MG-63 cells, Cbfa1 caused a 6-fold increase and MyoD a 2–3-fold increase in luciferase activity, with a combined increase of ∼10-fold during coexpression (Fig. 7B). This relatively modest increase in promoter activity during coexpression of Cbfa1 and MyoD suggests a non-coopera-

Fig. 6. Role of E-box, MyoD expression in SOST proximal promoter expression. A, SAOS-2 cells were transiently transfected (1 μg) with the indicated deletions or mutations of the SOST proximal promoter and assayed after 48 h. Activity of the EcoRV fragment (−331 to −190) was defined as 100%. Upstream (CACCTG) and downstream (CACTTG) E-boxes were changed to GTAAAC by primer-directed mutagenesis. SEAP (20 ng) was cotransfected and luciferase data were normalized to SEAP activity. Data represent average ± S.D. B, semi-quantitative RT-PCR analysis (40 cycles) was performed on SAOS-2 and MG-63 cDNA from cells treated with vitamin D, osteogenesis induction medium, Vit D + OIM, or neither. Primer pairs for each gene spanned at least one intron. Positive control bands were generated using a mixture of human heart (Clontech) and human fetal skeletal muscle (Clontech) cDNA samples. C, SAOS-2 cells were transiently transfected with the SOST proximal promoter (−2000 to −190; 500 ng) or the EcoRV fragment (−331 to −190; 500 ng); a mammalian expression plasmid for MyoD1 (50, 200, or 500 ng for 1.8-kb promoter; 300 ng for EcoRV fragment); and secreted alkaline phosphatase (SEAP). Cells were harvested and assayed for luciferase and SEAP activity 48 h after transfection, and SEAP data were used to normalize luciferase values.
FIG. 7. Effects of coexpressing Cbfa1 and MyoD on the SOST EcoRV fragment. SAOS-2 (A) or MG-63 (B) cells were transfected with the EcoRV promoter fragment (600 or 1600 ng, respectively) and the indicated combinations of Cbfa1 (100 or 200 ng, respectively) and/or MyoD (100 or 200 ng, respectively) mammalian expression plasmids, along with a plasmid expressing secreted alkaline phosphatase (SEAP). Cells were harvested and assayed for luciferase and SEAP activity 48 h after transfection, and SEAP data were used to normalize luciferase values. The difference in scale between the two graphs reflects the generally consistent with those derived from smaller promoter fragments (Fig. 6A) in that mutation of both sites is required to eliminate promoter activity. The inability of the E-box mutation alone to affect transcription leaves open the possibility of an interaction between these two binding sites.

To further assess the role of Cbfa1 and MyoD in SOST transcription, we mutated the Cbfa1 and E-box binding sites, both separately and together, in the context of the full-length SOST promoter (Fig. 7C). Mutation of the Cbfa1 site from AACCACA to GGAAGAG resulted in an ~80% decrease in promoter activity, whereas mutation of the E-box (CACGTG to GTAAAC) by itself had little apparent effect. However, simultaneous mutation of both the Cbfa1 site and the E-box eliminated all remaining promoter activity. These results are generally consistent with those derived from smaller promoter fragments in that mutation of both sites is required to eliminate promoter activity. The inability of the E-box mutation alone to affect transcription leaves open the possibility of an interaction between these two binding sites.

FIG. 8. Feedback inhibition model for role of SOST in modulating Cbfa1 expression. The Cbfa1 and MyoD transcription factors stimulate the SOST proximal promoter, leading to SOST up-regulation and presumed inhibition of TGF-β/BMP targets; the dotted line between MyoD and SOST indicates that their coexpression has not been established in animal tissues. BMP stimulation leads to up-regulation of Cbfa1 (35–37) and Id-1 (44). In addition to up-regulating bone-related target genes (11–16), Cbfa1 can down-regulate transcription of MyoD (43). In turn, Cbfa1 is down-regulated during myogenic differentiation (43) (not indicated in graphic). Studies differ on whether Cbfa1 is autostimulatory (21) or autoinhibitory (38) with regard to its own transcription.

DISCUSSION

The molecular cloning of SOST as the underlying cause of sclerosteosis established the importance of this novel gene in human osteoblast function and/or differentiation (6, 7). By studying promoter elements responsible for specific expression of the SOST gene in the SAOS-2 human osteosarcoma cell line, we have uncovered a connection between SOST gene expression and Cbfa1, a key regulator of osteoblast growth and differentiation. The differential expression of Cbfa1 in SAOS-2 and MG-63 cells, taken together with the delineation of a functional Cbfa1 consensus element in the SOST proximal promoter via transfection and gel shift analyses, identifies Cbfa1 as a major determinant of SAOS-2-specific SOST expression. As both of these genes are expressed in mature osteoblasts (6, 13), we anticipate that Cbfa1 may influence SOST expression in this context as well.

The SOST gene encodes a secreted polypeptide believed to inhibit bone growth through antagonism of the TGF-β/BMP family of secreted signaling proteins. Loss of SOST function in sclerosteosis leads to skeletal dysplasia marked by massive bone overgrowth and gigantism. In contrast, Cbfa1 is a transcription factor necessary for osteoblast differentiation. Mutations in Cbfa1 have been linked to cleidocranial dysplasia, a condition of bone abnormalities featuring clavicle hypoplasia and short stature (17, 19, 20). The strikingly disparate outcomes of deficiencies in SOST and Cbfa1 might appear to suggest that Cbfa1 would be predicted to antagonize SOST expression rather than stimulate it. One possible explanation of this seeming disparity is that SOST may function in an inhibitory circuit to maintain Cbfa1 expression at appropriate levels (Fig. 8).
through autoregulation of the Cbfa1 promoter, which contains multiple Cbfa1 binding sites. Both positive (21) and negative (38) Cbfa1 autoregulation have been described, perhaps reflecting the ability of Cbfa1 to act, within certain contexts, as a transcriptional repressor (39, 40). Our results suggest that Cbfa1 regulation of SOST may form another component of this regulatory circuit, conceivably by countering direct Cbfa1 autostimulation, although the contributions of the SOST pathway and direct Cbfa1 autoregulation to the transgenic phenotypes described above is unclear. An examination of Cbfa1 and SOST expression levels in transgenic mice misexpressing SOST and/or Cbfa1 would help to clarify the inter-relationship of these pathways.

Consideration of MyoD adds a new layer of potential complexity to this regulatory circuit (Fig. 8). Although MyoD is not responsible for SAOS-specific SOST expression in our system, mutation of the upstream E-box element resulted in a 3-fold drop in SOST promoter expression, and transiently transfected MyoD up-regulated the SOST promoter reporter. These results suggest that MyoD may contribute to SOST expression and thus, indirectly, to inhibition of Cbfa1. Osteoblasts and myoblasts derive from common progenitor cell populations, including mesenchymal stem cells and muscle satellite cells (41, 42), and antagonistic interactions between the myogenic and osteogenic pathways are well established in other systems. Cbfa1 overexpression in C2C12 cells leads to a block in myogenic differentiation and a repression of MyoD transcription (43), while BMP-2 treatment of osteoblast precursors results in up-regulation of Id1, a MyoD antagonist (44, 45), along with the previously mentioned up-regulation of Cbfa1 (35–37). Conversely, Cbfa1 is down-regulated in C2C12 cells undergoing myogenic differentiation (43), and forced expression of myogenin, a MyoD family member, can suppress Cbfa1 expression during myogenesis (42). MyoD up-regulation of SOST expression would therefore be consistent with the antagonistic relationship between the osteogenic and myogenic pathways reported in these various systems; however, confirmation of a link between MyoD and SOST awaits verification of their co-expression in vivo. The failure we observed of MyoD and Cbfa1 to show synergistic cooperativity when transiently coexpressed with the SOST proximal promoter (Fig. 7) is consistent with the antagonistic nature of these signaling pathways.

In addition to SOST, many other osteoblast-related genes are up-regulated by Cbfa1. Among these, osteocalcin bears intriguing parallels to SOST both in its loss-of-function phenotype and its proximal promoter. Like human patients with sclerosteosis, mice lacking osteocalcin develop increased bone thickness and mass (24). The proximal promoters of both genes contain binding sites for Cbfa1, E-box-binding proteins such as MyoD and C/EBP family members (10, 25, 26). Although our mutation of the upstream E-box element resulted in a 3-fold repressive effect (Fig. 2A), presumably due to recruitment of one or more transcriptional repressors. Additionally, vitamin D appears to up-regulate SOST expression in SAOS-2 cells (Fig. 1C), although our analysis did not reveal vitamin D receptor consensus binding elements. The importance of distal SOST gene regulation is further underscored by the existence of van Buchem disease, a disorder which shares many of the characteristics of sclerosteosis but lacks gigantism and fused digits (33, 52), and is not associated with mutations in the SOST coding sequence. Two recent reports have attributed van Buchem disease to the deletion of a 52-kb region downstream of the SOST locus (27, 28), suggesting that this genomic region contains elements important for the regulation of SOST expression. This region contains two segments of 157 and 480 bp, which are highly conserved between human and mouse, suggesting the presence of important regulatory elements (55) in this distal region, which lay outside the scope of our genomic analysis.

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REFERENCES

1. Truswell, A. S. (1958) J. Bone Joint Surg. 40, 208–218
2. Beighton, P. (1988) J. Med. Genet. 25, 203–209
3. Epstein, S., Hamersma, H., and Beighton, P. (1979) S. Afr. Med. J. 55, 1105–1110
4. Shera, S., Witkop, c., Hill, S., Fallon, M., Vierstein, L., Gauer, G., McKeever, P., Long, S., Altmann, J., Miller, N. H., Testelbaum, S. L., and Schlesinger, S. (1983) Neurology 33, 267–277
5. Beighton, P., Davidson, J., Durr, L., and Hamersma, H. (1977) Clin. Genet. 11, 1–7
6. Balemans, W., Ebeling, M., Patel, N., Van Hul, E., Olson, P., Dosszegi, M., Laczko, C., Wuyt, V., Van Den Ende, J., Willems, P., Paes-Alves, A. F., Hill, S., Pauwels, T., Tacconi, P., Dikkers, F. G. Stratakis, C., Lindpaintner K., Vickery, B., Poensrner, D., and Van Hul, W. (2001) Hum. Mol. Genet. 10, 537–543
7. Bruckwold, M. E., Gardner, J. C., Van Ness, J., Pauper, B. W., Koveacevich, B. R., Proff, S., Skonier, J. E., Zhao, L., Sabo, P. J., Fa, Y., Alsch, R. S., Gillett, L., Colbert, T., Tacconi, P., Galas, D., Hamersma, H., Beighton, P., and Mulligan, J. (2001) Am. J. Hum. Genet. 68, 577–589
8. Pearce, J. J., Penny, G., and Rossant, J. (1999) Dev. Biol. 209, 98–110
9. Hsu, D. R., Economides, A. N., Wang, X., Eimon, P. M., and Harland, R. M. (1998) Mol. Cell 1, 673–683
10. Javed, A., Gutierrez, S., Montecino, M., van Wijnen, A. J., Stein, J. L., Stein, G. S., and Lian, J. B. (1999) Mol. Cell. Biol. 19, 7491–7500
11. Mertens-Manuguerra, H. L., van Wijen, A. J., Hielbert, S., Bidwell, J. P., Fey, E., Lian, J., Stein, J., and Stein, G. S. (1999) Biochemistry 34, 13125–13132
12. Geoffroy, V., Ducy, P., and Karsenty, G. (1995) J. Biol. Chem. 270, 30973–30979
13. Ducy, P., Zhang, B., Geoffroy, V., Hidal, A. L., and Karsenty, G. (1997) Cell 89, 747–754
14. Ducy, P., and Karsenty, G. (1995) Mol. Cell. Biol. 15, 1858–1869
15. Bernot, C., Hiebert, S. W., Little, L., Lian, J. B., and Stein, G. S. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 4968–4973
16. Harada, H., Tagashira, S., Fujiwara, M., Ogawa, S., Katsumata, T., Yamaguchi, A., Komori, T., and Nakatsuka, M. (1999) J. Biol. Chem. 274, 6972–6978
17. Otto, F., Thornell, A. P., Crompton, T., Denzel, A. G., Gilmour, K. C., Rosewell, I. R., Stamp, G. W., Beddington, R. S., Mundlos, S., Olsen, B. R., Selby, P. R., and Owen, M. J. (1997) Cell 89, 763–775
18. Komori, T., Yagi, H., Nomura, S., Yamaguchi, A., Sasaki, K., Deguchi, K., Shimizu, Y., Brunson, R. T., Gao, Y. H., Insada, M., Sato, M., Okamoto, R., Marui, Y., Yoshida, S., and Kishimoto, T. (1997) Cell 89, 755–764
19. Lee, B., Thirunavukkaras, S., Zhu, L., Pastore, L., Baldwin, A., Hecht, J., Geoffroy, V., Ducy, P., and Karsenty, G. (1997) Nat. Genet. 16, 307–310
20. Mundlos, S., Otto, F., Mundlos, K. J., Ayleworth, S. A., Albrecht, S., Lindahl, D., Cole, W. G., Henn, W., Knudt, J. H., Owen, M. J., Mertelsmann, R., Zabel, B. U., and Olsen, B. R. (1997) Cell 89, 773–779
21. Ducy, P., Starbeck, M., Priemel, M., Shen, J., Finero, G., Geoffroy, V., Ameling, M., and Karsenty, G. (1999) Genes Dev. 13, 1025–1036
22. Liu, W., Tooyosawa, S., Furuchi, T., Kanatani, M., Yoshida, C., Liu, Y., Himeno, M., Narai, S., Yamaguchi, A., and Komori, T. (2001) J. Cell Biol. 155,
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