Identification of an Osteoblastic Silencer Element in the First Intron of the Rat Osteocalcin Gene†

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ABSTRACT: The osteocalcin gene has been used as a model for studying the regulation of gene expression by 1,25-dihydroxyvitamin D_3, as well as for examining factors which contribute to osteoblast-specific regulation of gene expression. Most of these studies have focused on transactivation. We report the identification of a sequence in the first intron of the rat osteocalcin gene which suppresses the expression of osteocalcin−CAT fusion genes approximately 10-fold in ROS 17/2.8 and UMR 106 osteosarcoma cells. Mutation of a TTTCTTT motif in the first intron abolishes this suppression. The silencing effect of this motif is also observed after bone morphogenic protein-2 (BMP-2)-induced expression of the osteoblastic phenotype in the MLB13MYC clone 17 cell line. Mutation of the splice donor site does not affect suppression by these sequences in ROS 17/2.8 cells. When multimerized and placed upstream of the native osteocalcin promoter, these sequences retain their ability to mediate transcriptional repression. Electrophoresis mobility shift analysis demonstrates a specific protein−DNA interaction with the TTTCTTT motif in nuclear extracts from ROS 17/2.8, UMR 106, and MLB13MYC clone 17 cells but not those from COS-7 kidney cells. The mutation of this motif, which abolishes suppressing activity in the native context, also abolishes binding. The presence and activity of this suppressor in cells of the osteoblast lineage suggest that it is expressed with other cell-specific transcriptional regulators of the osteocalcin gene, coordinately regulating expression of this gene in bone cells.

The osteocalcin gene is thought to be expressed only in mature osteoblasts. Recently, however, several investigators have identified osteocalcin transcripts in extraosseous tissues including chondrocytes, megakaryocytes, liver, and brain (Lian et al., 1993; Fleet & Hock, 1994; Thiede et al., 1994; Neugebauer et al., 1995), bringing into question the previous observations of cell-specific expression of this gene. Two osteocalcin genes have been isolated from the mouse genome, and a third osteocalcin-related gene has also been described (Rahman et al., 1993; Desbois et al., 1994). Expression of the osteocalcin genes was shown to be truly osteoblast-specific whereas the related gene was found to be expressed in several extraosseous tissues (Desbois et al., 1994), suggesting that the transcripts found by other investigators in extraosseous tissues might correspond to transcripts of the related gene. Osteocalcin is not found in all cells of the osteoblast lineage, but only in the most mature cells on the bone-forming surface.

The marked tissue-specific and developmental expression of the osteocalcin gene suggests that its transcription is tightly regulated both by positive and by negative factors. It has been shown that the sequences in the 5′ flanking region of the osteocalcin gene play an important role in its bone-specific expression (Towler et al., 1994; Ducy & Karsenty, 1995). The sequences between −121 and −64 of the rat osteocalcin gene (Towler et al., 1994) and those between −147 and +13 of the mouse osteocalcin gene-2 (Ducy & Karsenty, 1995) have been shown to be sufficient for differential expression of osteocalcin−luciferase fusion genes in bone cells *versus* non-bone cells. A sequence containing part of the coding region of the first exon and the 5′ end of intron I (+24 to +151) of the rat osteocalcin gene has been shown to markedly decrease transcription of reporter genes in the native context, as well as in an orientation-dependent fashion when placed 3′ to the chloramphenicol acetyltransferase (CAT)[1] reporter gene in osteocalcin−CAT and heterologous promoter−CAT fusion genes (Frenkel et al., 1993, 1994). The data of Frenkel et al. differ from the data presented herein in several respects: the sequences from +87 to +151 were only able to suppress transcription 2-fold, the silencers they describe were unable to repress transcription when placed 5′ to the native osteocalcin promoter, and they act as repressors in both osteoblastic and nonosteoblastic cells.

We have identified a sequence in the first intron of the rat osteocalcin gene that silences expression of osteocalcin−CAT fusion genes 10-fold in two osteosarcoma cell lines (ROS 17/2.8 cells, which express the endogenous osteocalcin gene, and UMR 106 cells, which do not). Mutation or deletion of these sequences abolishes the silencing activity. When multimerized, these sequences are able to suppress transcription when placed upstream of the native osteocalcin

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[1] Abbreviations: CAT, chloramphenicol acetyltransferase; RSV, Rous sarcoma virus; SV40, simian virus 40; CMV, cytomegalovirus; BMP-2, bone morphogenic protein-2; EMSA, electrophoresis mobility shift analysis.

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promoter. A specific protein−DNA complex that interacts with these sequences is present in cells of the osteoblast lineage, but not in COS-7 kidney cells. Studies in an *in vitro* model of osteoblast differentiation demonstrate that both the function of the silencer and the expression of proteins that bind to it are induced as the cells acquire osteoblastic markers (including expression of the endogenous osteocalcin gene) in response to bone morphogenic protein-2.

MATERIALS AND METHODS

Construction of Fusion Genes. To create the ***A****va*I−***B****am*HI ***C***AT construct (ABC), the *Bam*HI−*Ava*I fragment (−1750 to +147) of rat genomic osteocalcin DNA (Yoon & Rutledge, 1988; Demay et al., 1989) was blunt-ended using the large fragment of DNA polymerase I and subcloned into the blunt-ended *Sal*I site of puOCAT (Demay et al., 1989). The sequences between +95 and +147 were deleted using *Bal*31 exonuclease digestion to create ***B****am*HI−***B****al*31 ***C***AT (BBC). Site-directed mutagenesis (U.S.E. Mutagenesis Kit, Pharmacia) was carried out in POC-1 which contains genomic DNA containing the 5′ regulatory region and the entire coding region of the rat osteocalcin gene (Demay et al., 1989). A unique *Nsi*I site was substituted for the CCTCCT (atgCaT) at +106 to +111 to generate the t1 mutant, and for TTCTTT (aTgcaT) at +119 to +124 to generate the t2 mutant. The *Ava*I site was blunt-ended to permit subcloning of the *Sac*I−*Ava*I (−306 to +147) fragment into *Sac*I−*Sma*I M13mp18 for sequencing. The sequence of the mutated fragment was confirmed, following which the *Sac*I−*Sal*I fragment was excised from M13mp18 and subcloned into puOCAT (Demay et al., 1989). The *Sac*I−*Sac*I (−1750 to −306) fragment of BBC was then inserted in its native context to generate t1ABC and t2ABC. This subcloning strategy resulted in the introduction of stop codons into all three frames between the 3′ end of the osteocalcin sequences and the initiator ATG of the CAT gene. Site-directed mutagenesis was also carried out to mutate the splice donor site in ABC ($^{+99}$ATCC^{+102} to tatg) to generate (sd-)ABC. As with the engineering of t1- and t2ABC, the *Sac*I−*Ava*I fragment was sequenced to confirm the introduction of this mutation and the fidelity of the other bases from −306 to +147. This fragment was substituted for the same region in an ABC fusion gene that had not been subjected to mutagenesis to circumvent the possibility that additional mutations in other areas of the plasmid or fusion gene had occurred during the mutagenesis procedure.

For the experiments addressing the effect of the 5′ end of intron I placed upstream of the native osteocalcin promoter, an oligonucleotide adaptor containing a *Bam*HI site (GGGATCCGGTACCG) (with 3′ overhangs to permit ligation into the *Sac*I site) was inserted into the *Sac*I site of BSC (*Bal*31 to *Sac*I-CAT; −306 to +94-CAT). Four copies of the wild-type sequences corresponding to the bases from +95 to +142 were introduced in the reverse orientation into the *Bam*HI site to generate wtS (wild-type suppressor)-BSC. A similar copy number and similar orientation of the same sequences containing the t2 mutation in the TTTCTTT motif were also introduced into this site to generate t2mS-BSC. The sequence and the orientation of the subcloned oligonucleotides were confirmed by the dideoxynucleotide chain termination method using Sequenase (United States Biochemicals) after subcloning into M13.

Cell Culture and Transfections. ROS 17/2.8 cells were maintained in Ham's F-12 medium with L-glutamine supplemented with 10% fetal bovine serum (GIBCO), penicillin, and streptomycin. Transfection was carried out by calcium phosphate precipitation as previously described (Demay et al., 1989). UMR 106 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with L-glutamine, 10% fetal bovine serum (GIBCO), penicillin, and streptomycin. Transfections were carried out by 24 h lipofection (Lipofectin, GIBCO-BRL). COS-7 kidney cells were grown in DMEM supplemented with L-glutamine, 10% fetal bovine serum, penicillin, and streptomycin and were transfected by the DEAE-dextran method (Ausubel et al., 1994). Osteocalcin−chloramphenicol acetyltransferase (CAT) fusion genes were cotransfected with Rous sarcoma virus (RSV)−luciferase, and CAT activity was normalized for luciferase activity. For experiments examining the relative promoter activity of the BBC plasmid in the ROS 17/2.8 and UMR 106 cells, cotransfection experiments were also performed with pGL-2-CMV (a kind gift of Dr. P. Aftring, Massachusetts General Hospital, Boston) and pGL-3-Control (Promega, Madison WI) which have the luciferase gene under the control of the CMV and SV40 promoters, respectively. MLB13MYC clone 17 (C17) cells (Rosen et al., 1994) were maintained in DMEM supplemented with L-glutamine, 10% heat-inactivated Nu-Serum (Becton Dickinson Labware), penicillin, and streptomycin. Transfection was carried out by the calcium phosphate precipitation method. After transfection, 200 ng/mL recombinant human bone morphogenic protein-2 (rhBMP-2) was added. Medium containing fresh rhBMP-2 was added daily for 72 h. For all reporter gene assays, at least three individual transfections were performed in triplicate using at least two different plasmid DNA preparations. At 72 h posttransfection, cells were harvested and lysed, and CAT assays were performed as described (Demay et al., 1989). CAT activity was quantitated by densitometric scanning of TLC plate autoradiograms and normalized for transfection efficiency using luciferase activities in the ROS 17/2.8, UMR 106, and COS-7 cells. Luciferase activity was measured using a Monolight 2010 luminometer (Analytical Luminescence Laboratory) and D-luciferin (Sigma, St. Louis MO) in 15 mM glycylglycine (pH 7.8), 10 mM $MgSO_4$, 2.5 mM EGTA, 12 mM KPO_4 (pH 7.8), 1 mM DTT, and 2 mM ATP. Because of promoter squelching by viral promoter−luciferase plasmids in the C17 cells, relative induction of CAT activity by rhBMP-2 was determined by the ratio of signal intensity (+rhBMP-2/−rhBMP-2) on autoradiograms of TLC plates normalized for the protein content of the cell lysates (Biorad Protein Assay Buffer, Biorad, Hercules, CA).

Electrophoresis Mobility Shift Analysis (EMSA). Oligonucleotides were synthesized corresponding to the sequences of interest, with GATC overhangs to permit subcloning into a *Bam*HI site. Double-stranded oligonucleotides were labeled with [α-^{32}P]dATP by filling in recessed ends with the large fragment of DNA polymerase I. Nuclear extracts were prepared by the method of Dignam et al. (1983). Nuclear extracts were equilibrated for 30 min at room temperature in a buffer containing 110 mM KCl, poly(dI-dC)·poly(dI-dC) (0.1 μg/μg of extract protein), with or without unlabeled competitor DNAs, and then incubated with [α-^{32}P]dATP-labeled DNA probes for 15 min at room temperature. The protein−DNA complex was electrophoresed on a 4% poly-

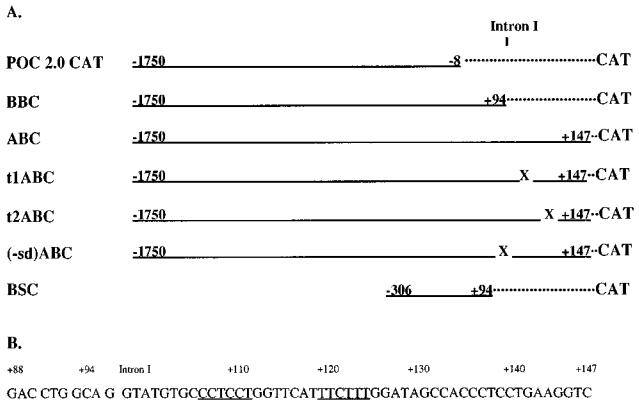

FIGURE 1: (A) Schematic representation of the osteocalcin−CAT fusion genes. The numbers represent the position relative to the transcription start site. X represents the introduction of a substitution mutation. (B) Sequences at the 3′ end of the first exon and the 5′ end of the first intron of the rat osteocalcin gene are shown. The 3′ end of BBC is indicated by +94 and the 3′ end of ABC by +147. The bases mutated in the t1ABC (CCTCCT) and t2ABC (TTCTTT) substitution mutations are underlined.

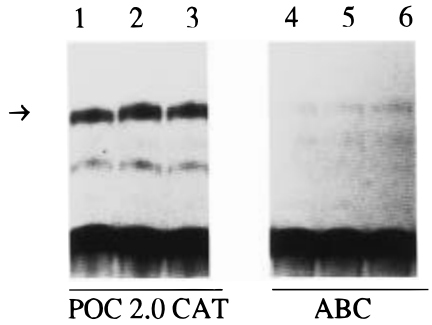

FIGURE 2: Relative CAT activity of POC 2.0 CAT and ABC in UMR 106 cells. Following transfection of these two osteocalcin−CAT fusion genes, cell lysates were normalized for protein content prior to CAT assays. Three separate experiments were performed with two different plasmid preparations. Representative results are shown above. The first three lanes represent the basal CAT activity of three independent transfections of POC 2.0 CAT into UMR 106, cells, and lanes 4−6 represent that of ABC. The arrow indicates the major acetylated chloramphenicol derivative. The intensity of these bands reflects relative CAT activity.

acrylamide gel in 2.5% glycerol, 190 mM glycine, 1 mM EDTA, and 25 mM Tris (pH 8.5) at 4 °C and subjected to autoradiography after drying.

RESULTS

The osteocalcin gene is expressed in mature osteoblasts and has been used as a marker of osteoblast differentiation. The gene is expressed in the rat osteosarcoma cell line, ROS 17/2.8, which exhibits properties of mature osteoblasts, but not in the UMR106 osteosarcoma cell line, which expresses some features of differentiated osteoblasts, including parathyroid hormone receptors and alkaline phosphatase activity (Partridge et al., 1983). To perform investigations directed at examining the developmental expression of the rOC gene, we pursued the original observations of Yoon et al. (1988) that a fusion gene containing the sequences from −1097 to +147 of the rOC gene, fused to a CAT reporter, was expressed in ROS 17/2.8 cells, but not in UMR 106 cells. Our previous studies have demonstrated that a fusion gene containing the rOC sequences from −1750 to −8 (POC 2.0 CAT) (Figure 1) could drive expression of a CAT reporter in both ROS 17/2.8 cells and UMR 106 cells [Demay et al. (1989) and Figure 2]. To address whether the sequences between −8 and +147 of the rOC gene contained sequences responsible for silencing the osteocalcin gene in UMR 106 cells, a rOC−CAT fusion gene was engineered containing the sequences from −1750 to +147 (ABC). Transfection of POC 2.0 CAT into UMR 106 cells resulted in detectable CAT activity whereas with ABC CAT activity was markedly attenuated (Figure 2). This suggested that the sequences responsible for silencing ABC in UMR 106 cells were present in the first exon or the first intron. The bases from +95 to +147 were, therefore, deleted by *Bal*31 exonuclease digestion, and the expression of this fusion gene, BBC (see Figure 1), was compared to that of ABC in UMR 106 cells (Figure 3A). The expression of BBC was 15-fold greater than that of ABC in UMR 106 cells, confirming that the sequences in the first intron contain significant silencing activity. To examine whether this silencing was also present in the ROS 17/2.8 cells which express the endogenous osteocalcin gene, ABC and BBC were transfected into these cells as well. As shown in Figure 3A, BBC expression was 8-fold higher than that of ABC in the ROS 17/2.8 cells. These data demonstrate that the first intron of the osteocalcin gene contains sequences capable of suppressing CAT activity in both ROS 17/2.8 and UMR 106 cells.

To identify the bases in the sequences from +95 to +147 of the rOC gene responsible for this silencing, site-directed mutagenesis was undertaken to abolish two motifs in the intron: the first (t1) of two CCCTCCCT repeats (+105 to +111) and a T-rich sequence (t2) TTTCTTT (+118 to +124) (Figure 1).

As shown in Figure 3A, the t1 substitution mutant had little effect on the basal expression level of ABC in either cell line. In contrast, the t2 substitution mutant markedly increased the basal expression level of ABC in both in ROS17/2.8 cells and UMR 106 cells, rescuing the suppressive effect of the intron I sequences.

Both the ABC construct and the BBC construct contain the sequences thought to be responsible for the bone-specific expression of the osteocalcin gene (Towler et al., 1994; Ducy & Karsenty, 1995). Because BBC is well expressed in the UMR 106 cells relative to ABC, we examined the relative contributions of the upstream sequences and of the TTTCTTT motif to silencing of osteocalcin−CAT fusion genes in the UMR 106 cells. The basal activity of BBC was compared between ROS17/2.8 cells and UMR 106 cells. The RSV promoter−, CMV promoter−, and SV40 promoter−luciferase fusion genes were used as controls for transfection efficiency in three separate experiments for each luciferase fusion gene. There was no substantial difference between the expression of these luciferase fusion genes in the ROS 17/2.8 and UMR 106 cells. As shown in Figure 3B, the transcriptional activity of BBC in UMR 106 cells was approximately 10% of that observed in ROS 17/2.8 cells. In COS-7 cells, the expression of all osteocalcin−CAT fusion genes (corrected with RSV−luciferase cotransfection) was less than 0.5% of BBC expression in the ROS 17/2.8 cells. This suggests that silencing of the OC gene occurs by several mechanisms. The silencing that we have observed in the UMR 106 cells is significant but not as dramatic as that in the COS-7 cells. This result suggests the possibility that UMR 106 cells have acquired some of the osteoblastic transcription factors necessary for the expression of the

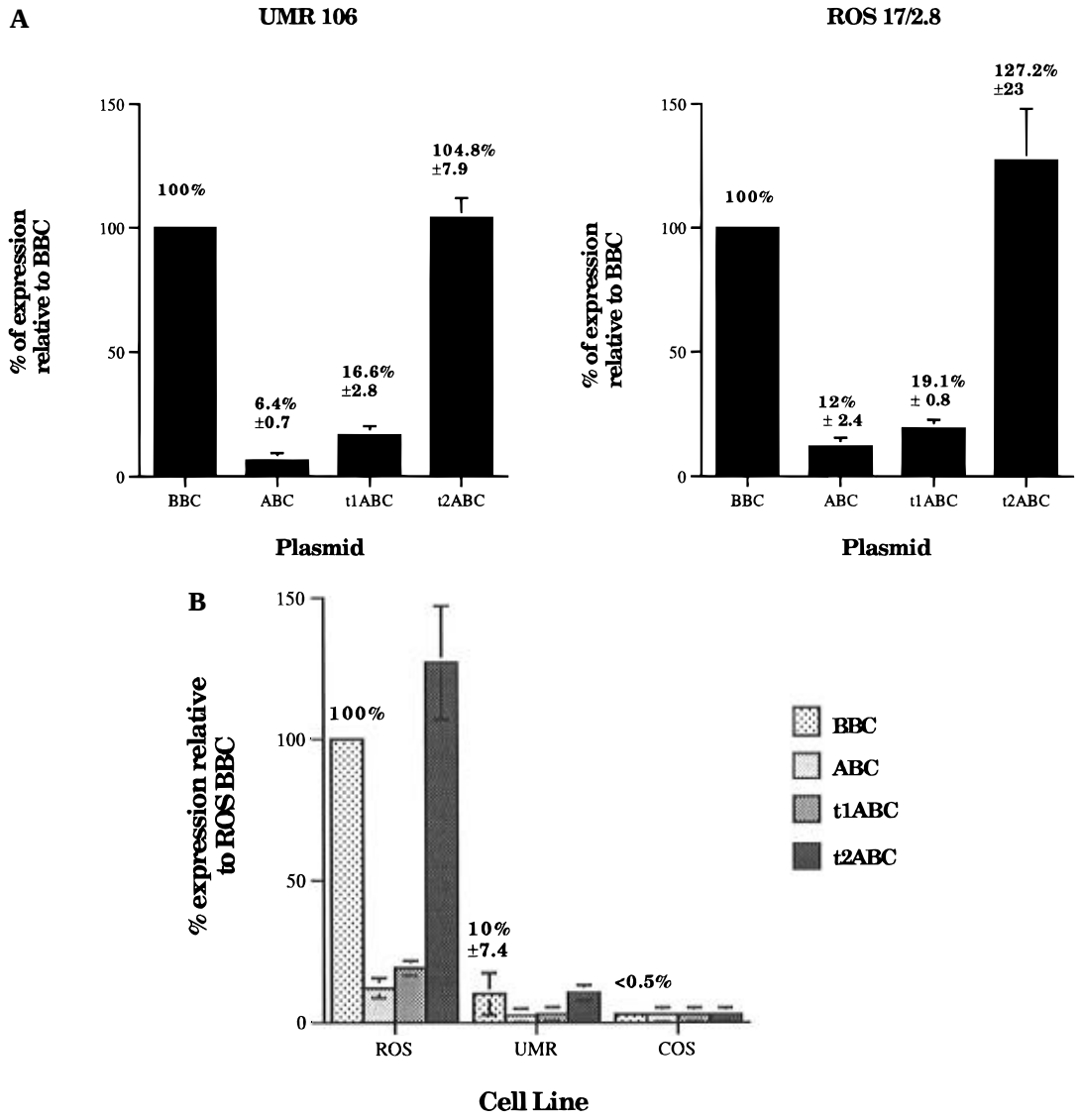

FIGURE 3: (A) Relative expression of osteocalcin−CAT fusion genes in UMR 106 and ROS 17/2.8 cells. The values above the bars represent the mean and SEM of at least three independent transfections performed in triplicate with at least two different plasmid preparations. All values are normalized to BBC expression (CAT activity) and corrected for transfection efficiency using RSV−luciferase as a control. (B) Relative expression of osteocalcin−CAT fusion genes in UMR 106, ROS 17/2.8, and COS-7 cells. The basal expression of BBC was compared among three cell lines using cotransfected luciferase fusion genes in independent experiments for normalization. Each comparison represents the mean and SEM of three independent transfections performed in triplicate with at least two plasmid preparations. No detectable CAT activity was observed with any of the osteocalcin−CAT fusion genes in the COS-7 cells. The relative expression of BBC in the ROS 17/2.8, and UMR 106 cells was normalized, in independent transfections, using luciferase reporter genes driven by the RSV, SV40, and CMV promoters. The normalized BBC expression in the UMR 106 cells reflects normalization based on three independent transfections for each of the three luciferase fusion genes (total of nine experiments).

osteocalcin gene in bone cells. The lack of BBC and t2ABC activity in COS-7 cells suggests that they have more powerful silencers or that they lack the transcriptional regulators necessary for the cell-specific induction of osteocalcin gene expression.

To address whether silencing by the first intron was observed only in cells committed to the osteoblastic lineage, ABC and BBC were transfected into the prechondrocytic cell line MLB13MYC clone 17 (C17). This latter cell line is capable of acquiring markers of a mature osteoblast, including the expression of the endogenous osteocalcin gene, when treated with BMP-2 (Rosen et al., 1994). The C17 cells demonstrated very low level CAT activity after transfection with both ABC and BBC. Interestingly, after treatment with BMP-2, BBC expression increased 5-fold whereas the expression of ABC did not change (Figure 4). To examine whether mutations at the t2 position rescued the suppressive effects of ABC in C17 cells, the expression of t2ABC was compared to that of ABC and BBC in this cell line. As shown in Figure 4, mutation of the TTTCTTT motif partially rescued the suppressive effect of ABC in the rhBMP-2-treated C-17 cells. These data suggest that the sequences in the first intron of the rOC gene contain a silencer that is active in osteoblast-like cells (ROS 17/2.8, UMR 106, and rhBMP-2-treated C17 cells), but that additional sequences are responsible for the low level of expression of this gene in cells not committed to the osteoblast lineage (COS-7 and untreated C17 cells).

Because of the remote possibility that the suppression observed with ABC was secondary to a splicing artifact not observed with t2ABC, the splice donor site in the first intron

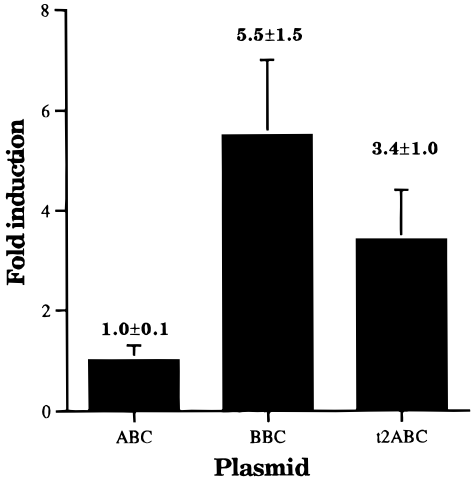

FIGURE 4: Relative induction of osteocalcin−CAT fusion genes in BMP-2-treated C17 cells. The C17 cells were treated, or not, with BMP-2 for 72 h posttransfection. For each osteocalcin−CAT fusion gene, the fold induction by BMP-2 reflects the mean and SEM of three independent transfections performed 3 times with two different plasmid preparations.

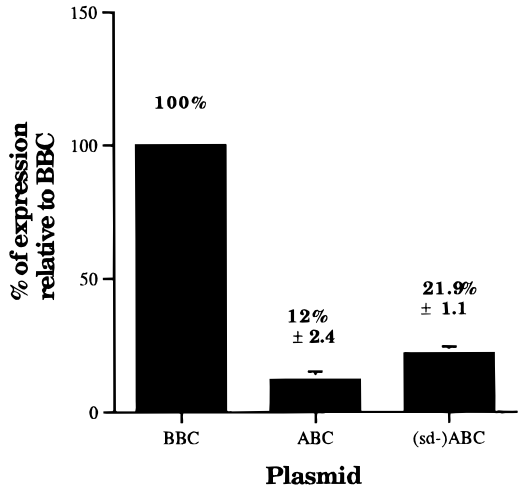

FIGURE 5: Effect of mutation of the splice donor site of ABC on basal expression. The splice donor site of ABC was mutated to generate (sd-)ABC, and the expression of this osteocalcin−CAT fusion gene was compared to that of ABC and BBC after transfection into ROS 17/2.8 cells. The values above the bars represent the mean and SEM of at least three independent transfections performed in triplicate with at least two different plasmid preparations. All values are normalized to BBC expression and corrected for transfection efficiency using RSV−luciferase as a control.

of the osteocalcin gene in ABC was mutated by site-directed mutagenesis. The expression of the resultant fusion gene, (sd-)ABC, was suppressed relative to BBC (Figure 5), confirming that silencing by the sequences in the first intron did not require splicing.

To address whether the sequences in the first intron of the rat osteocalcin gene could suppress the expression of the native promoter without being transcribed, they were placed upstream of BSC (Figure 1). As shown in Figure 6, four copies of the sequences between $^{+}$95 and $^{+}$142 suppressed expression of the native promoter 3-fold in ROS 17/2.8 cells (wtS-BSC). A similar fusion gene containing four copies of these sequences with substitution mutations in the TTTCTTT motif resulted in a 2-fold decrease in expression (t2mS-BSC). This latter result suggests that, in

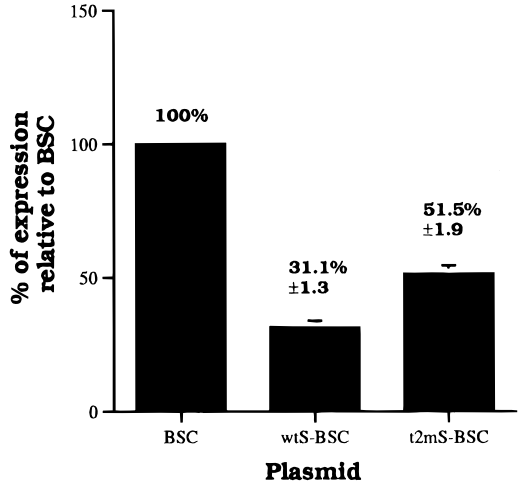

FIGURE 6: Sequences between +95 and +142 mediate repression when multimerized and placed upstream of the native osteocalcin promoter. Oligonucleotides containing four copies of the wild-type suppressor (wtS) sequences, or those containing the t2 substitution mutant (t2mS), were ligated into BSC (−306 to +94 CAT) to examine whether they could suppress transcription when placed upstream of the native osteocalcin promoter. The values above the bars represent the mean and SEM of at least three independent transfections performed in triplicate with at least two different plasmid preparations. All values are normalized to BSC expression and corrected for transfection efficiency using RSV−luciferase as a control.

addition to this motif, other sequences in this region may contribute to transcriptional silencing of the osteocalcin gene when multimerized or placed upstream to the native promoter.

To examine whether the cell lines in which the sequences between $^{+}$95 and $^{+}$147 suppress CAT activity contain a nuclear factor which specifically binds to the TTTCTTT motif, a 21 bp oligonucleotide ($^{+}$112 to $^{+}$132) containing this sequence was synthesized. In addition, a second oligonucleotide was synthesized with a substitution mutation in the TTTCTTT motif, identical to that introduced into t2 ABC (t2m). When the wild-type oligonucleotide was incubated with nuclear extracts from ROS 17/2.8 and UMR 106 cells, a high molecular weight DNA−protein complex (Figure 7, closed arrowhead) was observed. This complex was specifically competed for by 10- and 100-fold molar excess of the wild-type (wt) unlabeled oligonucleotide. Competition was markedly attenuated when the mutant oligonucleotide (t2m) was used. In contrast to the nuclear extracts from osteoblastic cells, nuclear extracts from COS-7 cells generated a more rapidly migrating DNA−protein complex (Figure 7 open arrow) which was competed for by both the wild-type and mutant oligonculeotides, suggesting that the protein responsible for complex formation was not interacting with the TTTCTTT motif. When nuclear extracts isolated from untreated and rhBMP-2-treated C17 cells were incubated with the wild-type oligonucleotide, a similar complex was observed. Interestingly, rhBMP-2 treatment markedly increased the intensity of the complex observed (Figure 8, closed arrow). An intense more rapidly migrating complex was observed in all four cell lines; however, competition with the wild-type and mutant oligonucleotides was variable among cell lines and did not correlate well with the functional data obtained.

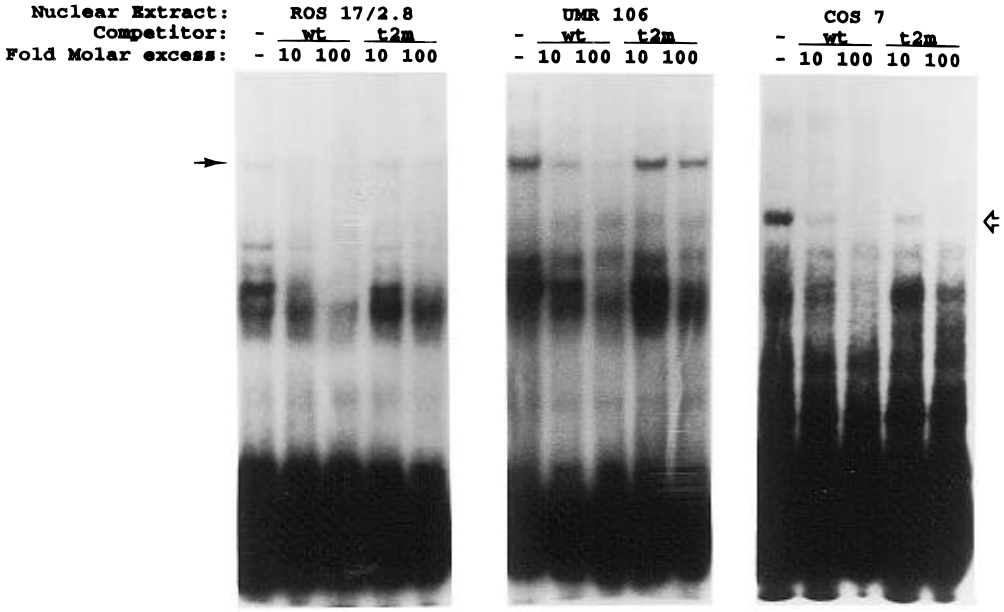

FIGURE 7: Interaction of the TTTCTTT motif with nuclear extracts from ROS 17/2.8, UMR 106, C17, and COS-7 cells. A double-stranded oligonucleotide containing the sequences from +112 to +132 of the rat osteocalcin gene was used as a radioactive probe and incubated with nuclear extracts from ROS 17/2.8, UMR 106, and COS-7 cells. The specificity of the complexes was evaluated by competition with 10- and 100-fold molar excess of the unlabeled double-stranded wild-type (wt) oligonucleotide. The role of the TTTCTTT motif was evaluated by examining competition by a double-stranded oligonucleotide with the t2 substitution mutation (t2m).

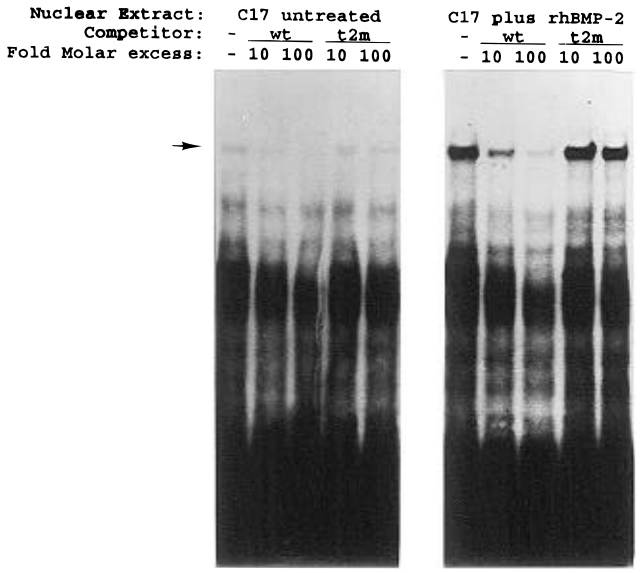

FIGURE 8: Interaction of the TTTCTTT motif with nuclear extracts from untreated and rhBMP-2-treated C17 cells. A double-stranded oligonucleotide containing the sequences from +112 to +132 of the rat osteocalcin gene was used as a radioactive probe and incubated with nuclear extracts from untreated C17 cells and cells which had been treated with rhBMP-2 for 72 h prior to extract preparation. The specificity of the complexes was evaluated by competition with 10- and 100-fold molar excess of the unlabeled double-stranded wild-type (wt) oligonucleotide. The role of the TTTCTTT motif was evaluated by examining competition by a double-stranded oligonucleotide with the t2 substitution mutation (t2m).

DISCUSSION

We have demonstrated that the sequences in the first intron of the rOC gene, including a TTTCTTT motif, contain a silencer element. Mutation or deletion of these sequences results in a 10-fold increase in expression of the osteocalcin−CAT fusion genes in two osteoblastic cell lines: ROS 17/2.8, which express the endogenous osteocalcin gene, and UMR 106 cells, which do not. The absence of detectable CAT activity in the COS-7 cells, with all of the osteocalcin−CAT fusion genes tested, suggests that these cells do not have the transcriptional regulators necessary for osteocalcin gene induction, or that they contain more powerful silencers than the ROS 17/2.8 or UMR 106 cells. However, the EMSA data demonstrate that COS-7 cells do not contain a nuclear protein that specifically recognizes the TTTCTTT motif, whereas nuclear extracts from ROS 17/2.8 and UMR 106 cells do. This suggests that the protein that binds to this motif is expressed in conjunction with cell-specific transcriptional activators of the osteocalcin gene, thereby coordinately regulating the OC gene in bone cells. Other sequences in the 5′ flanking region of the osteocalcin gene have been shown to be important in promoting osteoblast-specific OC gene expression (Towler et al., 1994; Ducy & Karsenty, 1995). Similar to the suppression of osteocalcin−luciferase fusion gene expression observed by Towler et al. in ROS 25/1 cells (which do not express osteocalcin) relative to ROS 17/2.8 cells, we observed a 10-fold suppression of BBC expression in UMR 106 cells (which do not express osteocalcin) relative to ROS 17/2.8 cells. The suppression of BBC expression in the cell lines which do not express osteocalcin (UMR 106, COS-7, and untreated C17 cells) is likely due to multiple regulatory regions of the osteocalcin gene, including those upstream elements identified by Towler (Towler et al., 1994) and Ducy (Ducy & Karsenty, 1995).

To explore the hypothesis that this silencer becomes "activated" during osteoblast development, the role of the silencer was examined in rhBMP-2-treated C17 cells, an *in vitro* model of osteoblastic differentiation. Treatment of these C17 cells with rhBMP-2 results in a marked increase in the protein−DNA complex generated by this sequence, paralleled by an increase in expression of osteocalcin−CAT fusion genes with mutations or deletions of the TTTCTTT motif and induction of endogenous osteocalcin mRNA

synthesis. The observation that mutation of the TTTCTTT motif does not completely rescue wild-type expression suggests that other sequences in exon I may contribute to silencing of the osteocalcin gene as the C17 cells switch from the chondrocytic to the osteoblastic lineage and acquire the osteoblastic phenotype. Notable in this respect is the observation that when sequences from the first intron are multimerized and placed upstream of the native osteocalcin promoter, mutations in the TTTCTTT motif do not completely rescue suppression in ROS 17/2.8 cells. Therefore, it is possible that other sequences in this region of the first intron have a suppressive effect which is only apparent in ROS 17/2.8 cells when these sequences are present in multiple copies. Notable in this respect are the CCTCCT motifs which are present in the first intron. As demonstrated in Figure 3A, mutagenesis of one of these motifs modestly decreases suppression by the sequences in the first intron. It is possible that the interaction of both CCTCCT motifs with the TTTCTTT motif, especially when present as multimers, may synergistically suppress osteocalcin gene transcription. Elucidation of the exact role of the TTTCTTT motif in repression of osteocalcin−CAT activity will require cloning and functional characterization of the nuclear protein that binds to this DNA sequence.

Regulatory sequences have been isolated from the coding region of the osteocalcin gene (Frenkel et al., 1993, 1994) and intronic sequences of numerous other genes. In addition to 5′ regulatory elements (Pavlin et al., 1992; Krebsbach et al., 1993; Rossert et al., 1995), the sequences in the first intron of the α1(I) collagen gene have been shown to be required for the bone-specific expression of this gene (Bornstein & McKay, 1988). Intronic sequences have also been shown to be important for expression of the tenascin gene in cell lines which express the endogenous gene (Gherzi et al., 1995). Silencers have been described in the intronic sequences of several genes including the developmentally regulated Keratin 18 (Pankov et al., 1994) and platelet-derived growth factor A chain (Wang et al., 1990, 1994) genes as well as the α1(I) collagen gene (Bornstein & McKay, 1988). None of these silencers recognize a TTTCTTT motif. However, in *Drosophila*, a cofactor of the developmentally regulated *dorsal* gene binds to a sequence containing an identical TTTCTTT motif (Jiang et al., 1993; Kirov et al., 1993). This protein, along with *dorsal*, mediates *dorsal*-specific suppression of *zerknullt*, *decapentaplegic* (the *Drosophila* homologue of BMP-2), and *tolloid* (the *Drosophila* homologue of BMP-1). This *Drosophila* protein cooperates with *dorsal* for suppression of target genes. If the bone cell protein which binds to the TTTCTTT motif is related to the *dorsal* cofactor, it may require cooperativity with another transcriptional regulator to mediate repression of the osteocalcin gene. Interestingly, the TTTCTTT motif is found in multiple other sites in the rOC gene, including the 5′ regulatory region, as well as a second copy at the 3′ end of the first intron. This motif is also present in the mouse osteocalcin genes and in the 5′ regulatory regions of other bone cell genes including those encoding mouse osteopontin (Craig & Denhardt, 1991), mouse PTH/PTHrP receptor (Karperien), and alkaline phosphatase (Zernik et al., 1990) in the rat. This suggests that the protein which interacts with this silencer motif may have a more protean role in regulating the expression of several genes in the osteoblast.

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