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Characterization of a silencer element in the first exon of the human osteocalcin gene

Yi-Ping Li*, Wei Chen and Philip Stashenko

Department of Cytokine Biology, Forsyth Dental Center, 140 The Fenway, Boston, MA 02115, USA

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ABSTRACT

Osteocalcin, the major non-collagenous protein in bone, is transcribed in osteoblasts at the onset of extracellular matrix mineralization. In this study it was demonstrated that sequences located in the first exon of the human osteocalcin gene possess a differentiation-related osteocalcin silencer element (OSE). Osteocalcin was rendered transcribable in UMR-106 cells and proliferating normal osteoblasts after deletion of the –3 to +51 region. Site-specific mutagenesis of this region revealed that a 7 bp sequence (TGGCCCT) (+29 to +35) is critical for silencing function. Mobility shift assays demonstrated that a nuclear factor bound to the OSE. The OSE binding protein was present in proliferating normal pre-osteoblasts and in UMR-106 and ROS 17/2.8 osteosarcoma cells, but was absent from post-proliferative normal osteoblasts. The binding protein was inhibited by fragments containing the +29/+35 sequence, but not by other promoter fragments or by the consensus oligomers of unrelated nuclear factors AP-1 and Sp1. DNase I footprinting demonstrated that the OSE binding-protein protected the +17 to +36 portion of the first exon, consistent with the results of mapping studies and competitive mobility shift assays. It is hypothesized that this silencer is activated by complexing of the OSE binding protein to the OSE during the osteoblast proliferation stage and that the OSE binding protein is down-regulated at the onset of extracellular matrix mineralization.

INTRODUCTION

The expression of osteoblast phenotype-related genes is precisely regulated during the differentiation process. A temporal sequence of gene expression associated with development of the osteoblastic phenotype has been defined *in vivo* (33,53) and *in vitro* (1,57). Early osteoblast differentiation stages are characterized by cell proliferation and high levels of associated markers, such as H4 histone and type I collagen (34). Once cell division declines, cellular nodules are formed and the expression of alkaline phosphatase peaks. This is followed by osteopontin and osteocalcin expression, which is coincident with extracellular matrix mineralization.

Osteocalcin, which is predominantly expressed in mature osteoblasts, constitutes the most abundant non-collagenous protein in bone (15,40). Serum levels of osteocalcin are used as a marker of bone turnover (8,28,58). Osteocalcin gene expression is tightly regulated at multiple levels, which apparently accounts for variations in the activity and inducibility of the gene during the osteoblast developmental sequence (6,16,29,36). The regulation of osteocalcin expression is clearly complex and involves the action and interaction of both enhancer and suppressor control elements. In contrast to the effects of hormonal regulation, the molecular mechanisms for regulation of basal osteocalcin expression during osteoblast differentiation are poorly understood.

Recently, using a human osteocalcin promoter–CAT fusion gene, we demonstrated that constructs containing portions of the first exon and first intron are expressed less well in UMR-106 osteosarcoma cells than constructs lacking this region, suggesting the presence of a silencer element (26). Similarly, Yoon *et al.* (56) and Klein-Nulend *et al.* (21) demonstrated that rat osteocalcin gene constructs containing the regulatory region, the first exon and a 50 bp portion of the first intron are well expressed in ROS 17/2.8 cells, but not in UMR-106 cells. Recently Frenkel *et al.* (11) reported that a repressor element may be present within these sequences in the rat osteocalcin gene.

In the present work we have undertaken mapping studies to characterize the *cis*-acting OSE element in the human osteocalcin gene. Our results demonstrate that this element is present at coordinates +29 to +35 of the first exon. Furthermore, an OSE binding protein is present in rat osteosarcoma cell lines ROS 17/2.8 and UMR-106 and in proliferating normal osteoblasts, but not in post-proliferative osteoblasts. This interaction may be responsible, at least in part, for restricting expression of the osteocalcin gene during early stages of osteoblast development.

MATERIALS AND METHODS

Cell culture

The rat osteosarcoma cell line ROS 17/2.8 was maintained in F-12 medium supplemented with L-glutamine (4 mM), 1% penicillin/streptomycin and 5% fetal bovine serum (FBS). UMR-106 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% FBS. Normal rat osteoblasts (ROB) were obtained by sequential enzymatic digestion of fetal rat calvaria as described (4). In brief, 21 day fetal rat calvaria were

* To whom correspondence should be addressed

removed and digested with trypsin (2.5 mg/ml) and collagenase A (2.0 mg/ml). The initial 20 and 30 min digests were discarded. The cells from the third 60 min digest were collected, centrifuged and resuspended and plated in α-MEM supplemented with L-glutamine, 50 mg/ml gentamycin, 10 mM Hepes buffer, 1% penicillin/streptomycin and 10% FBS at 5×10^5 cells/100 mm plate. After the cells reached confluence (days 5–7) the medium was switched from MEM to BGJb to which 50 μg/ml ascorbic acid and 10 mM β-glycerol phosphate were added. For second passage ROB (SubROB) experiments cells were obtained by trypsinization of ROB on day 6.

DNA transfection and CAT assay

Transfection of plasmid DNA into ROS 17/2.8, UMR-106 and ROB was performed using the calcium phosphate co-precipitation technique as described (25). In brief, UMR-106, ROS 17/2.8, ROB and SubROB cells were plated at 3×10^5 cells/60 mm culture dish. A construct which carries the β-galactosidase gene downstream of the LTR of the Rous sarcoma virus was co-transfected in all experiments and served as an internal control for transfection efficiency. Constructs were tested in duplicate or triplicate using independent plasmid preparations. CAT assays were performed on lysates as described by Neumann *et al.* (32). CAT activity in transfected cultures was standardized by normalization to β-galactosidase activity and to the protein concentration of cell extracts using a protein assay kit (BioRad, Richmond, CA).

Osteocalcin gene constructs

An osteocalcin promoter–CAT fusion gene (pHOCCAT) of the human osteocalcin 5′-flanking region was used as a starting point for deletion analysis (–1700 to +299, numbered relative to the cap site) (25). pHOCCAT was first subjected to partial digestion with *Hin*dIII and *Bam*H1, followed by addition of a *Kpn*I linker to the *Sal*I site to produce pOCAT-20.1. For deletion pOCAT-20.1 DNA was digested with *Kpn*I and *Bam*H1 and the product was subjected to controlled 3′→5′ digestion with *Exo*III as described (14,26). The series of 3′ deletions generated were religated with a *Xho*I linker and their sizes were determined by electrophoresis in a 3:1 NuSieve–agarose gel and DNA sequence analysis using the dideoxy chain termination method (46).

For generation of OSE–heterologous SV40 promoter constructs the filled in *Hin*dIII–*Hha*I DNA fragment from construct pOCAT-20.4 (–19 to +61) was cloned into the *Sma*I sites of pESV-CAT. pESV-CAT contains an SV40 promoter and SV40 enhancer and was produced by insertion of a polylinker into the pCAT-Control (Promega, Madison, WI) *Bgl*II site. Fusion areas of all plasmids were sequenced by the dideoxy chain termination method.

Oligonucleotide-directed mutants

A 2.0 kb *Bam*HI fragment (–1700/+299) containing the putative OSE from pHOCCAT was cloned into M13mp18 and was subjected to oligonucleotide-directed mutagenesis as described by Kunkel (23). The mutagenic oligomers utilized included:

5′-AGTGTGAGGGCAAGCTTGGTGTCTCGGG-3′ (+1 to +6 mutagenesis),
5′-GGCGAGGAGTAAGCTTGCTCTCATGGTGT-3′ (+9 to +15),
5′-AGGGCCAATAGAAGCTTGAGTGTGAGGG-3′ (+20 to +26),
5′-AAGTGCGGCCAAGCTTAATAGGGCGAGG-3′ (+30 to +35) and
5′-GCCAGCGATGCAAAGCTTGGTGTCTCGGGT-3′ (+1 to +46).

A *Hin*dIII restriction site was thereby introduced into the first exon of osteocalcin at the indicated locations (Table 3). The 2.0 kb *Bam*HI fragment from the mutant gene was isolated and cloned into the identical site in pOCCAT to produce pOCCATm1–6, pOCATm9–15, pOCATm20–26, pOCATm30–35 and pOCATm1–46.

Northern blotting

Total RNA from day 4, 14 and 28 ROB cells and transfected UMR-106 cells in the presence of 10^{-8} M vitamin D was isolated by the method of Chomczynski and Sacchi (61). RNA was blotted onto nylon membranes after formaldehyde–agarose gel electrophoresis and hybridized with radiolabeled probes. The 0.6 kb *Eco*RI rat osteocalcin cDNA fragment (25), 1.6 kb *Hin*dIII–*Bam*HI CAT fragment (60) and 2 kb human β-actin cDNA (Clontech) were used as probes. Probes were radiolabeled using a random primer labeling kit (Stratagene) with [α-^{32}P]dCTP. Hybridization was performed as described previously (25). Films were scanned with a densitometer and band intensities normalized to the standard β-actin.

Electrophoretic mobility shift assays

For preparation of nuclear extracts, 10^8 ROS 17/2.8, UMR-106 and ROB cells at different stages of differentiation were cultured. Nuclear extracts were produced according to the method of Dignam *et al.* (9). Gel retardation assays were carried out as described (27). A ^{32}P-labeled *Hin*dIII–*Hha*I DNA fragment from construct pOCAT-20.4 (–19 to +61) was utilized as a probe. The fragments were end-labeled with [^{32}P]dATP using Klenow fragment and were isolated following electrophoresis in 3:1 NuSieve–agarose. The number of labeled bands with reduced mobility indicated OSE binding by nuclear protein(s). In gel retardation competition experiments a 10–100-fold excess of unlabeled specific or non-specific competitor fragments were added during the pre-incubation step.

DNase I footprint analysis

Footprint analysis was carried out as described (7,26). In brief, the construct pOCAT-20.4 produced above containing the OSE was digested with *Hin*dIII, generating a 3′-recess which was filled in with [α-^{32}P]dCTP-labeled nucleotides. The DNA was precipitated and subsequently digested with *Apa*I to generate a 270 bp DNA fragment labeled only at the 3′-end on the sense strand. The labeled fragments were separated on an agarose gel and electroeluted. Nuclear extract (40 μg) was incubated with 2 μg poly(dI–dC) as the non-specific competitor and labeled fragment (15 000 c.p.m.) for 30 min at 15°C. DNase I was added (2 U/reaction) and the digestion was allowed to proceed for 1 min at 20°C. Digestion was halted by addition of 200 ml 0.3% SDS, 150 mM NaCl. Proteins were extracted with phenol/chloroform and the DNA precipitated. Samples were heated for 3 min at 90°C before loading onto an 8% polyacrylamide sequencing gel to obtain the sequences of the protected portions of DNA.

Table 1. Presence of a silencer element in the first exon of the human osteocalcin gene

Construct	Relative CAT activity in cell type (%)			
	ROS 17/2.8	UMR-106	ROB	Sub-ROB
(A) Basal				
pOCAT-20.8(–3)	100	100	100	100
pOCAT-20.1(+299)	53 ± 14[a]	31 ± 3.5	35 ± 7.5	18 ± 2.8
Fold suppression	1.8	3.3	2.9	5.6
(B) Vitamin D stimulated				
pOCAT-20.8(–3)	100	100	100	100
pOCAT-20.1(+299)	49 ± 1.1[a]	19 ± 7.0	22 ± 5.6	5.4 ± 3.0
Fold suppression	2.0	5.2	4.5	18.5

[a]Mean ± SD of three independent experiments.

RESULTS

In vitro transcription of deletion mutants of the osteocalcin gene

In order to determine if a silencer element lies within the first exon or first intron of the human osteocalcin gene deletion, pOCAT-20.8, in which the entire first exon and first intron was removed, was produced by *Exo*III digestion. pOCAT-20.8 (–1700 to –3), as well as the long construct pHOCCAT (–1700 to +299). These were transiently transfected into rat osteosarcoma cell lines ROS 17/2.8 and UMR-106, ROB and SubROB cells and were tested for their ability to express CAT activity. The CAT activity of the –3 construct was arbitrarily set at 100% and the relative activities of the different constructs were determined for each cell (Table 1A). The data shown are the mean ± SD of three independent experiments, each done in triplicate, normalized to the expression of RSV *lacZ* as an internal standard. The basal expression of pOCAT-20.1(+299) was suppressed 1.8-fold in ROS 17/2.8, 3.2-fold in UMR-106, 2.9-fold in ROB and 5.6-fold in SubROB cells relative to pOCAT-20.8(–3). In our previous work (25) we demonstrated that vitamin D had an inductive effect in the long construct pHOCCAT. The short construct pOCAT-20.8 had the same vitamin D inductive pattern as construct pHOCCAT. Very similar results were obtained in the presence of 10^{-8} M vitamin D, with suppression of 2.0-, 5.2-, 4.5- and 18.5-fold in ROS 17/2.8, UMR-106, ROB and SubROB respectively (Table 1B). Under both conditions it was noted that the strength of suppression was greater in UMR-106, ROB and SubROB than in ROS17/2.8. Taken together these data therefore indicate that a silencer element is present within the sequence –3 to +299.

The proportion of transfected cells was also investigated. X-Gal stained ROS cells comprised ~15–20% of the entire population after transfection with pSVLacZ and 72 h incubation (data not shown). The relative levels of β-galactosidase activity between ROS, ROB, SubROB and UMR were ~4, 3, 2.5 and 1 respectively. The high transfection levels indicated that the cells which are successfully transfected may be representative of the entire population, including normal non-transformed cells.

Mapping studies of the osteocalcin silencer element (OSE)

Since the characteristics of the –3/+299 silencer in UMR-106 were manifested similarly to normal diploid osteoblasts, we used the UMR-106 system to further characterize and map the silencer element. An additional series of 3′ deletion mutants were made and were transiently transfected into UMR-106 with or without vitamin D stimulation to assay silencing activity. Five of these deletions from +205 to +51 (constructs 20.2–20.6) showed a pattern of silencing activity similar to construct 20.1(+299) (Table 2A) under basal conditions. In contrast, silencing activity was partially lost with the +33 deletion (pOCAT-20.7) and was further abrogated with two shorter constructs (–3 and –15). An identical pattern of response was observed in the presence of vitamin D (Table 2B). These findings strongly suggest that a silencer element (OSE) is present in the –3 to +51 region of the first intron of the human osteocalcin gene.

Table 2. Deletion analysis of the first exon and intron for localization of the osteocalcin silencer element

Construct	Relative CAT activity in cell type (%)		
	UMR-106	ROB	Sub-ROB
(A) Basal			
pOCAT-20.8(–3)	100	100	100
pOCAT-20.9(–15)	89 ± 11[a]	NT	NT
pOCAT-20.7(+33)	55 ± 5.2	75 ± 5.4	61 ± 6.4
pOCAT-20.6(+51)	28 ± 6.0	35 ± 4.3	27 ± 5.3
pOCAT-20.5(+52)	25 ± 4	20 ± 4.9	20 ± 4.6
pOCAT-20.4(+61)	28 ± 5.7	NT	NT
pOCAT-20.3(+151)	30 ± 3.4	NT	NT
pOCAT-20.2(+205)	26 ± 3.2	NT	NT
pOCAT-20.1(+299)	28 ± 5.0	35 ± 7.5	18 ± 2.8
(B) Vitamin D stimulated			
pOCAT-20.8(–3)	100	100	100
pOCAT-20.9(–15)	75 ± 8.7[a]	NT	NT
pOCAT-20.7(+33)	40 ± 5.1	60 ± 7.4	60 ± 4
pOCAT-20.6(+51)	18 ± 3.8	16 ± 6.1	12 ± 7
pOCAT-20.5(+52)	15 ± 2.9	15 ± 3.5	11.3
pOCAT-20.4(+61)	16 ± 4.3	NT	NT
pOCAT-20.3(+151)	16 ± 2.4	NT	NT
pOCAT-20.2(+205)	18 ± 2.5	NT	NT
pOCAT-20.1(+299)	18 ± 5.0	18 ± 2.8	7.9 ± 2.3

[a]Mean ± SD of three independent experiments.

Table 3. Effect of site-specific mutagenesis of the first exon

	+1 20 40	Relative CAT activity (%)	n^a
Wild-type	ACACCATGAGAGCCCTCACACTCCTCGCCCTATTGGCCCTGGCCGCACTTT	1.00	
pOCATm1–6	ACACCAAGCTTGCCCTCACACTCCTCGCCCTATTGGCCCTGGCCGCACTTT	1.16 ± 0.09[b]	3
pOCATm9–15	ACACCATGAGAGCAAGCTTACTCCTCGCCCTATTGGCCCTGGCCGCACTTT	1.54 ± 0.05	2
pOCATm20–26	ACACCATGAGAGCCCTCACACTCAAGCTTCTATTGGCCCTGGCCGCACTTT	1.56 ± 0.12	3
pOCATm30–35	ACACCATGAGAGCCCTCACACTCCTCGCCCTATTAAGCTTGGCCGCACTTT	5.61 ± 0.78	5
pOCATm1–46	ACACC AAGCTT T	4.90 ± 0.85	4

[a]*n*, mean number of determinations.
[b]Mean ± SD.

Site-directed mutagenesis

In order to directly assess possible sites of silencing activity site-specific mutagenesis of the –3/+51 region was carried out. The wild-type sequences +1/+6, +10/+16, +20/+26, +30/+35 and +1/+46 were modified by replacement with a *Hin*dIII restriction site by oligonucleotide-directed mutagenesis, to yield constructs pOCATm1–6, pOCATm9–15, pOCATm20–26, pOCATm30–35 and pOCATm1–46 respectively. The osteocalcin ATG start codon in construct pOCATm16 was mutated to AAG. The results, summarized in Table 3, clearly demonstrate that mutagenized constructs pOCATm30–35 and pOCATm1–46 completely lost silencing activity. Mutagenesis of other sequences, including +1/+6, +9/+15 and +20/+26 within the +1 to +46 region had no effect on silencing activity. These findings therefore identify the GC-rich sequence at +29/+35 as important for osteocalcin silencing function in osteoblastic cells.

Northern blotting

The silencer element is located downstream of the RNA start site and could affect polymerase elongation, RNA processing, stability or translation, in addition to transcription. In order to provide direct evidence that the element acts at the transcriptional level we performed Northern blot analysis using 15 µg total RNA isolated from UMR-106 cells transfected with constructs pOCAT-20.1, pOCAT-20.6, pOCAT-20.9 and pOCAT-20.8 respectively in the presence of 10^{-8} M vitamin D. Hybridization to the CAT probe was standardized to an internal control, β-actin. As shown in Figure 1, the steady-state levels of CAT mRNA from different constructs changed in parallel and to the same extent as the CAT activities (compare with Table 2B).

The OSE does not inhibit a heterologous promoter

The OSE was next tested for its capacity to inhibit the activity of an SV40 heterologous promoter. However, as summarized in Figure 2, the SV40 promoter was not affected by the –19/+61 OC fragment introduced 5′ of the SV40 promoter in either orientation or with multiple copies of the insert.

Mobility shift analysis of the OSE-containing region

The functional identification of this silencer led us to characterize an OSE binding protein(s) present in UMR and other osteoblastic cells. Mobility shift assays were performed using a ^{32}P-labeled *Hin*dIII–*Hha*I fragment (–19/+61) from construct pOCAT20.4 as

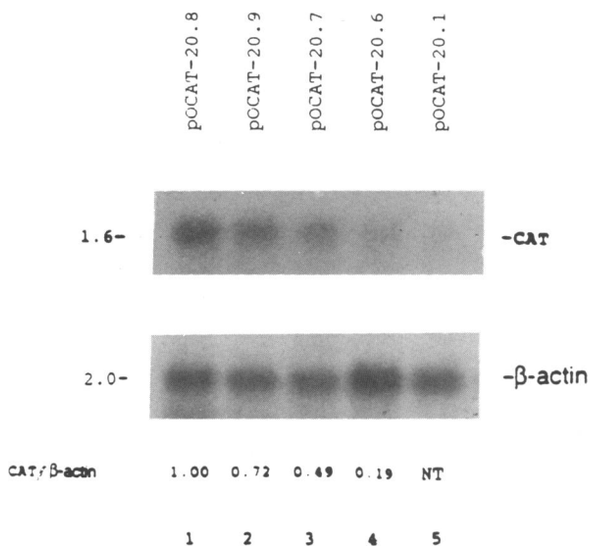

Figure 1. Expression of mRNA from UMR-106 cells transfected with constructs pOCAT-20.8 (lane 1), pOCAT-20.9 (lane 2), pOCAT-20.7 (lane 3), pOCAT-20.6 (lane 4) and pOCAT-20.1 (lane 5) respectively. The blot had 15 µg total RNA in each lane and was hybridized to the CAT probe (60). A probe for human β-actin was used as a control. CAT/β-actin, hybridization to CAT probe standardized to β-actin. ND, not determined since the band was too weak to detect by densitometry.

a probe with nuclear extracts from ROS 17/2.8 and UMR-106 cells. As shown in Figure 3, OSE binding activity was detected in nuclear extracts derived from UMR-106 (lane 3) and to a lesser extent ROS 17/2.8 (lane 2). Binding activity of the UMR-106 extract was competed for by excess unlabeled *Hin*dIII–*Hha*I fragment –17/+61 and by +24/+54, but not by an irrelevant *Apa*I–*Hha*I fragment (–237 to –17) or by +1/+28. Nuclear extracts from normal ROB on day 4 after isolation from calvaria also expressed the silencer binding protein (Fig. 4, lane 2), whereas extracts from days 14 (lane 3) and 28 (lane 4) ROB lacked binding activity. The OSE binding protein in extracts of day 4 ROB, ROS 17/2.8 and UMR-106 cells was again inhibited by the +24/+54 fragment, but was unaffected by –237/–17 or by unrelated Sp1 and AP-1 consensus sequences. These data suggest an identity between the OSE binding protein present in normal ROB and osteosarcomas UMR-106 and ROS 17/2.8.

Of interest, the endogenous osteocalcin mRNA level also changed on these different days, e.g. there was no osteocalcin

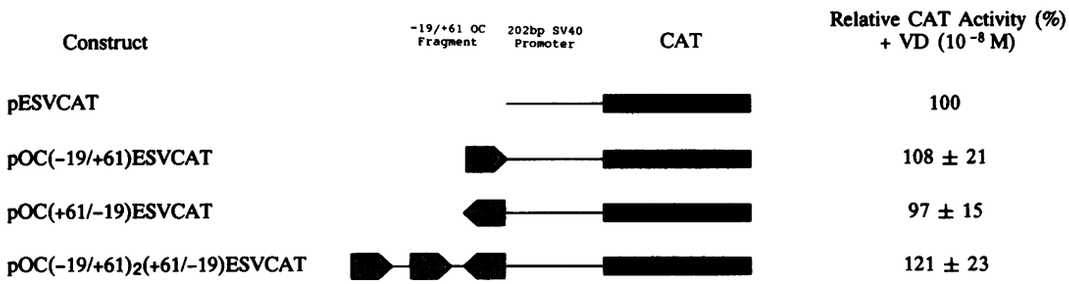

Figure 2. Effect of OSE-containing sequences on a heterologous SV40 promoter. The 5′→3′ orientation is indicated as ▶. The data represent the mean ± SD of three independent experiments.

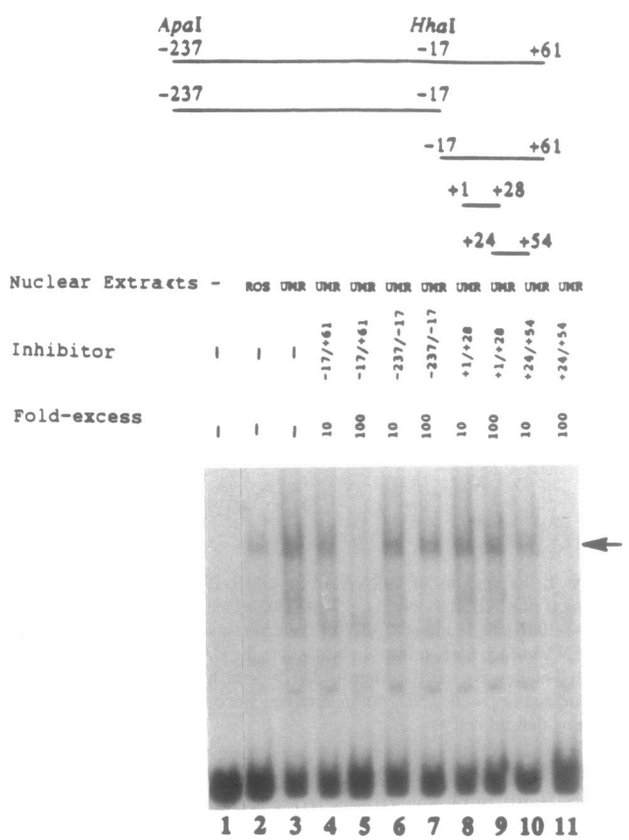

Figure 3. Electrophoretic mobility shift assay of DNA binding proteins from nuclear extracts of ROS 17/2.8 and UMR-106 osteoblastic cells. A ^{32}P-labelled *Hin*dIII–*Hha*I fragment from construct pOCAT-20.4 (–17 to +61) was end-labelled and utilized as a probe. The labelled bands with reduced mobility indicate OSE binding by nuclear protein(s). Lane 1, probe; lane 2, ROS 17/2.8 extract, no inhibitor; lanes 3–11, UMR-106 extract; lane 3, no inhibitor; lanes 4 and 5, fragment –17/+61 as inhibitor; lanes 6 and 7, –237/–17 as inhibitor; lanes 8 and 9, +1/+28 as inhibitor; lanes 10 and 11, +24/+54 as inhibitor. For each pair of lanes the inhibitor was used at 10- (first lane) and 100-fold excess (second lane). The OSE–protein complex is indicated by the arrow.

gene transcription during the proliferation stage of ROB (day 4), whereas osteocalcin was transcribed on day 14 and at high levels on day 28 (Fig. 5).

Finally, the *Hin*dIII–*Hha*I fragments (–19/+299) were isolated from constructs pHOCCAT, pOCATm1–6, pOCATm9–15, pOCATm20–26 and pOCATm30–35. These were used in competitive mobility shift assays with nuclear extracts from UMR-106 cells. As shown in Figure 6, the OSE binding factor was completely inhibited by a 100-fold excess of homologous *Hin*dIII–*Hha*I fragment (lanes 3 and 4), as well as by *Hin*dIII *Hha*I fragments from mutant 1–6 (lanes 5 and 6) and mutant 9–15 (lanes 7 and 8). The fragment from mutant 20–26 (lanes 9 and 10) showed only slightly reduced inhibition. In contrast, the fragment from mutant 30–35 (lanes 11 and 12) had nearly complete loss of inhibition, indicating an alteration of nucleotides which were essential for binding. Thus the core sequence TGGCCCT appears to be of importance for OSE factor binding.

DNase I footprinting

DNase I footprinting was carried out to further define the region of contact between the OSE binding proteins and the osteocalcin gene. As shown in Figure 7, UMR-106 nuclear proteins protected a region of the probe (~+17 to +36) which included the OSE (+29/+35). Somewhat greater protection occurred on residues +29/+35, with weaker protection of +17/+28. The area of protected nucleotides was therefore consistent with the inhibition patterns observed in competitive mobility shift assays described above.

DISCUSSION

A temporal sequence of gene expression associated with development of the osteoblastic phenotype has been defined *in vivo* (33,53). Similar results have been obtained *in vitro* during the development of mineralizing nodules from calvaria-derived osteoblastic cells (1,57). In the present study two cell culture models have been used. For calvaria-derived osteoblastic cell cultures the early osteoblast differentiation stages (proliferating preosteoblasts) are characterized by cell proliferation and high levels of associated markers, such as histone H4 and expression of type I collagen (34). Once cell division declines cellular nodules are formed and alkaline phosphatase (ALP) is expressed. This is followed by extracellular matrix mineralization and osteopontin and osteocalcin expression. For the second model transformed osteosarcoma-derived cell lines are assumed to represent 'frozen' stages of differentiation (59). They can also be assigned a position in the temporal sequence, based on the markers which they express. For example, UMR-106, which expresses ALP but not osteocalcin, and ROS 17/2.8, which

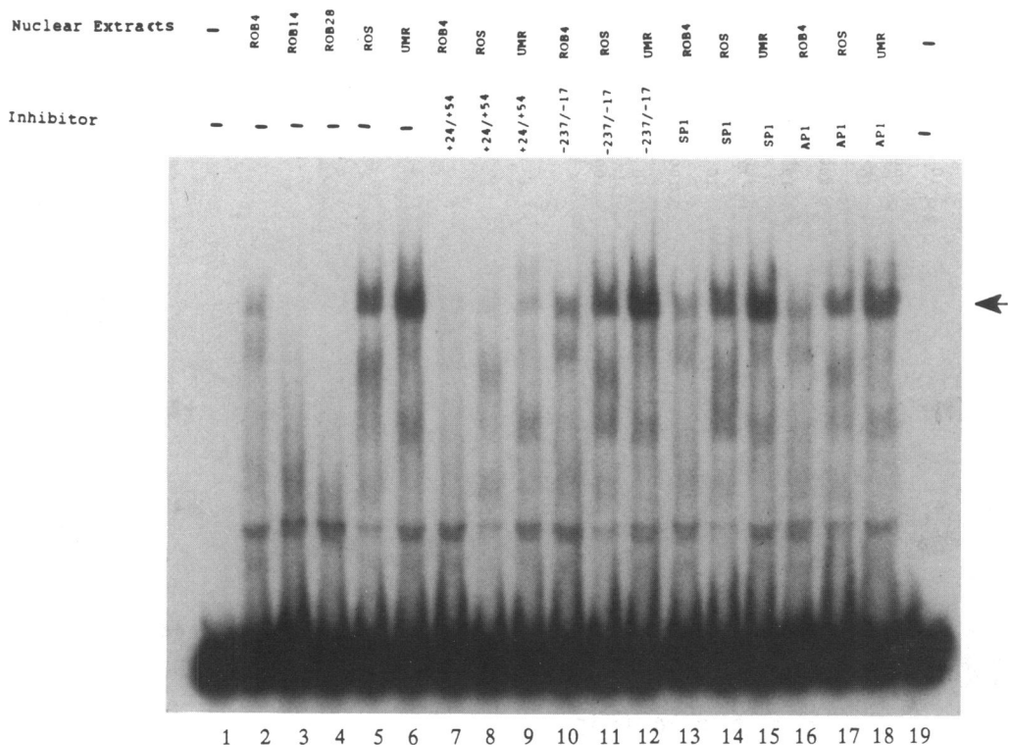

Figure 4. Electrophoretic mobility shift assay of DNA binding proteins from nuclear extracts of normal rat osteoblasts and osteosarcoma cell lines. A ^{32}P-labelled *Hin*dIII–*Hha*I fragment from construct pOCAT-20.4 (–17/+61) was utilized as a probe. Lanes 1 and 19, probe; lanes 2, 7, 10, 13 and 16, ROB day 4 extract; lane 3, ROB day 14 extract; lane 4, ROB day 28 extract; lanes 5, 8, 11, 14 and 17, ROS 17/2.8 extract; lanes 6, 9, 12, 15 and 18, UMR-106 extract; lanes 2–6, no inhibitor; lanes 7–9, +24/+54 as inhibitor; lanes 10–12, –237/–17 as inhibitor; lanes 13–15, 22mer SP1 consensus sequence 5′-ATTCGATCGGGGCGGGGCGAGC-3′ as inhibitor; lanes 16–18, 21mer AP-1 consensus sequence 5′-CGCTTGATGAGTCAGCCGGAA-3′ as inhibitor. All inhibitors in 100-fold excess versus probe.

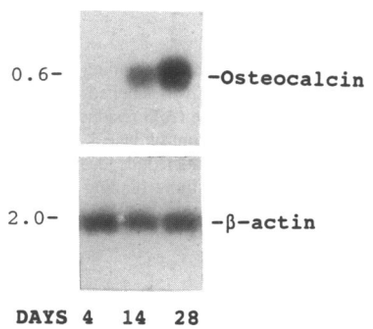

Figure 5. Northern blot analysis of endogenous osteocalcin mRNA expression in normal rat osteoblasts (ROB) following *in vitro* culture. Hybridization was carried out with ^{32}P-labelled probes for osteocalcin and human β-actin as a control. Transcript sizes are indicated in kilobases. In actively proliferating ROB cells (day 8) osteocalcin mRNA is undetectable, whereas osteocalcin was rendered transcribable on day 14 and at high levels on day 28.

expresses osteocalcin (25), were used as transformed counterparts of relatively immature and mature osteoblasts respectively.

The goal of these studies was to elucidate one of the molecular mechanisms by which the osteocalcin gene is rendered transcribable as osteoblastic cells differentiate and matrix mineralization commences. Our results have demonstrated that a GC-rich sequence (TGGCCCT) located at +29/+35 in the first exon of the human osteocalcin gene possesses a development-related silencing function. The presence of this sequence in osteocalcin promoter–CAT reporter constructs suppressed promoter activity up to 18-fold in transiently transfected proliferating osteoblasts and ~5–6-fold in UMR-106 cells, the latter appearing to represent an early stage of osteoblast differentiation (38,50). Moreover, a DNA binding protein was present in nuclear extracts from osteosarcomas UMR-106 and ROS 17/2.8, as well as in normal proliferating osteoblasts (day 4), which bound to a region of the first exon which includes the OSE. This binding activity was absent when mineralization commenced (day 14) and in the late mineralization stage (day 28). The interaction between the OSE and its binding protein therefore represents a plausible mechanism by which transcription of the osteocalcin gene is inhibited in less mature osteoblastic cells, but is permitted in well-differentiated osteoblasts. To our knowledge the OSE described herein is not identical to any other previously described inhibitory element, although similar GC-rich motifs do represent a common feature of many silencers (47).

Two other possibilities to explain the observed silencing activity were that the low transcriptional activity of pOCAT-20.1 resulted from the presence of the exon–intron splice site, which interfered with RNA processing, or that the osteocalcin ATG start codon could cause a decrease in activity of the osteocalcin–CAT fusion protein. However, the constructs pOCAT-20.5 and 20.6, which lack the splice site, retained full silencing activity (Table 2). Similarly, construct pOCATm1–6, in which the ATG codon was mutated to AAG, had no change in activity (Table 3). Thus neither of these explanations for silencing activity are

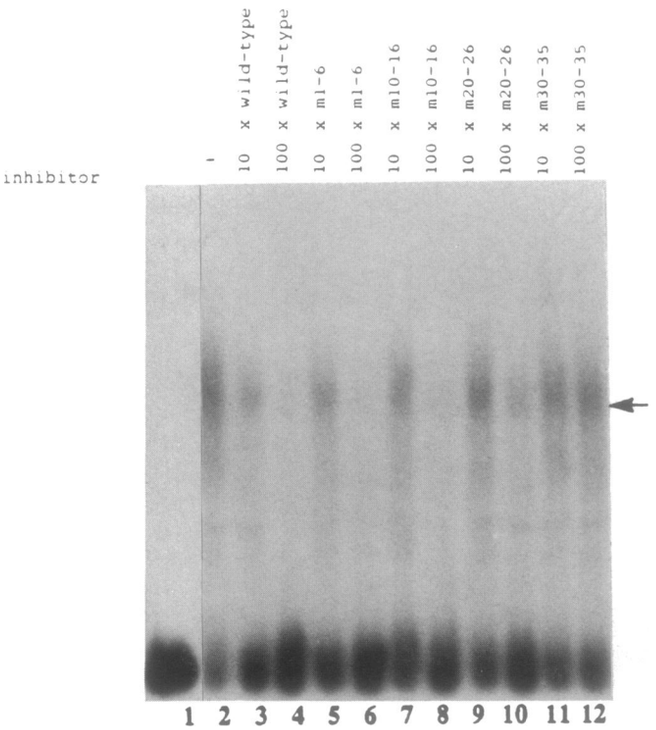

Figure 6. Competitive mobility shift assays to localize the OSE binding protein site. Four *Hin*dIII–*Hha*I fragments (–17/+299) with one internal site mutagenized by site-directed mutagenesis (see Table 2) were used in competitive mobility shift assays with nuclear extract from UMR-106 cells. Lane 1, probe; lane 2, no inhibitor; lanes 3 and 4, wild-type pHOCCAT fragment as inhibitor; lanes 5 and 6, pOCCATm1–6 as inhibitor; lanes 7 and 8, pOCATm9–15 as inhibitor; lanes 9 and 10, pOCATm20–26 as inhibitor; lanes 11 and 12, pOCATm30–35 as inhibitor. For each pair of lanes the inhibitor was used at 10- (first lane) and 100-fold excess (second lane). The OSE–protein complex is indicated by the arrow.

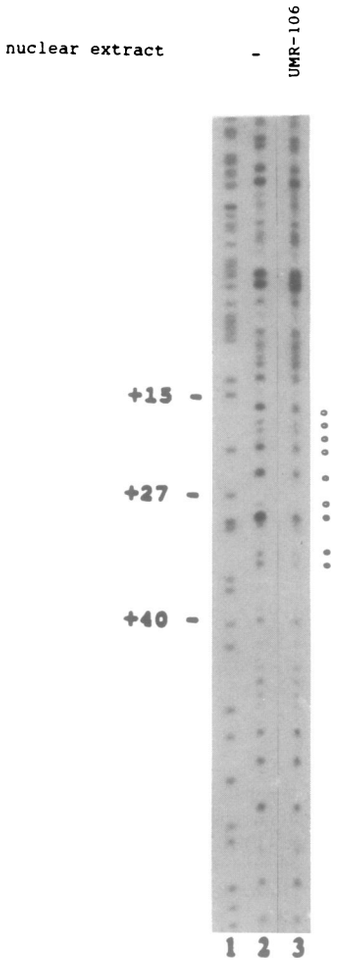

Figure 7. DNase I footprinting of the OSE binding protein. The *Apa*I–*Hin*dIII fragment of pHOCCAT (–237/+61) was labelled at the 3′-end on the sense strand. Lane 1, degradative sequence (A/G residues); lane 2, incubation with bovine serum albumin (40 μg) as a control; lane 3, 40 μg nuclear extract from UMR-106. Numbers on left indicate nucleotide location in the osteocalcin gene first exon. Solid dot (●) indicates strong binding activity and open dot (○) indicates weak binding activity.

tenable. In order to provide direct evidence that the element acts at the transcriptional level we carried out Northern blot analyses, which confirmed that the steady-state level of CAT mRNA changed to the same extent as CAT activity (Fig. 1).

While this manuscript was in preparation, Frenkel *et al.* (12) also reported that a silencer element is present in the first exon of the rat osteocalcin gene. Based on functional transfection studies it was concluded that the rat element is bipartite, with one portion located at coordinates +7 to +15 and the other at +56 to +66 in the exon–intron border region (numbering according to the human sequence). No DNA–protein interaction data were provided to confirm the transfection results. In the rat the proximal region contains an ACCCTCTCT motif (+7/+15), previously suggested as a consensus silencer sequence (2). Interestingly, this sequence is absent from the human osteocalcin gene (Fig. 8). Moreover, in our studies the +7/+15 region was not protected in footprinting assays (Fig. 6) nor was OSE binding activity competed for by excess +1/+28 fragment (Fig. 3). Modification of the +10/+16 region by site-specific mutagenesis had no effect on silencing activity (Table 3). The distal portion of the bipartite element in the rat gene truncates a 5 bp interrupted palindrome (+56/+66) which exhibits similarity to silencers of polyoma virus (13), rat growth hormone (45) and chicken lysozyme (3). This sequence was also absent from the human osteocalcin gene (Fig. 8). Constructs pOCAT-20.4, 20.5 and 20.6, which removed the +61/+299, +52/+299 and +51/+299 regions of the human gene respectively, had no effect on silencing activity (Table 2), implying a lack of a silencing element in the +52/+299 region. Taken together these data strongly indicate that the human osteocalcin gene lacks functional negative elements in either the +7/+15 or +60/+71 regions. There may thus be species differences in regulation of the osteocalcin gene. It is interesting to note, however, that the human osteocalcin silencer motif TGGCCCT also occurs in the first exon of the rat osteocalcin gene (Fig. 8).

Contrary to our expectations, the OSE binding protein was also present in ROS 17/2.8, which constitutively expresses the osteocalcin gene and which may represent a late stage of differentiation (6,44). Although the reason for this is unclear, it is possible that expression of OSE inhibitory activity may require cooperation between the OSE complex and an unknown transcription factor or cofactor (31,41,42), which may not be present in ROS 17/2.8 due to the late differentiation stage of this

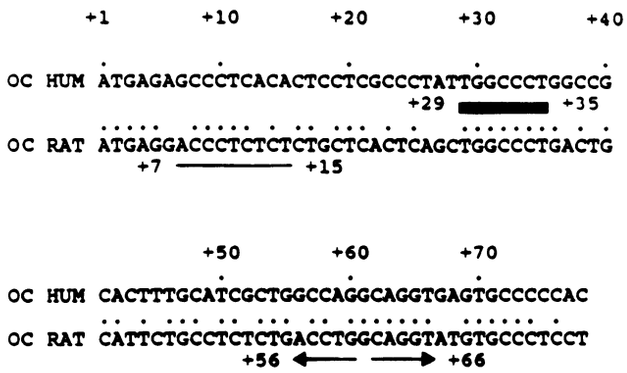

Figure 8. DNA sequence comparison between the first exon of the human and rat osteocalcin genes.

cell or to a transformation-associated modification (6). This possibility is supported by several observations. First, the OSE binding protein only slightly suppresses osteocalcin promoter activity in ROS 17/2.8, even though expression of the OSE binding protein is higher in ROS 17/2.8 than in normal proliferating osteoblasts (Fig. 4). Second, the OSE fails to inhibit a heterologous promoter, suggesting that there are other promoter elements which must interact with the OSE which are absent from the SV40 promoter region (Fig. 2). Finally, in ROS 17/2.8 cells, compared with normal osteoblasts, the absence of a stringent requirement for cessation of proliferation to allow osteocalcin transcription is reflected in modifications in transcription factor binding at the two primary basal regulatory elements (6).

Constitutive silencers have been demonstrated in many genes (24,43), including c-*myc* (18), c-*fos* (30), lysozyme (3), insulin (5), growth hormone (37), MHC class I (55) immunoglobulin κ (39) and type II collagen (48). Inducible silencers like the TIE element of transin/stromelysin have also been characterized (20). Most silencers locate to the 5′-promoter regulatory region, although a few are present in introns (10,51) or the 3′-untranslated region (22). Silencers have been found, in other systems, to control tissue-specific gene expression during development and differentiation. For example, the negative element in the lysozyme gene acts as a silencer in fibroblasts or promacrophages, but is inactive in mature macrophages (3). The expression and silencing of the ε-globin gene during development may be modulated by the interactions of a regulatory protein with the *cis*-acting DNA silencer (52). At present the exact nature of the mechanism by which silencers exert negative transcriptional control is not known. Silencers may involve a conformational change, such as DNA looping, to disorganize the transcriptional initiation complex (17,43) or binding of a specific protein to the DNA silencer sequence to 'lock' the transcriptional start site (19).

Osteocalcin is expressed post-proliferatively at the onset of extracellular matrix mineralization (1,34). Schule *et al.* (49) described an AP-1 site within the vitamin D responsive element (VDRE) of the human osteocalcin gene promoter and indicated that Jun–Fos binding can down-regulate osteocalcin gene transcription. Owen *et al.* (35) also presented evidence that AP-1 sites residing within two promoter elements of the osteocalcin gene, the 'osteocalcin box', which contains a CCAAT motif as a central element, as well as the VDRE, bind the Jun–Fos protein complex. Gel electrophoretic mobility shift analysis demonstrated high AP-1 binding activity in proliferating osteoblasts and a dramatic decrease in this activity concomitant with slowed proliferation of confluent primary cultures of normal osteoblasts (36). From these results a model has been proposed in which coordinate occupancy of the AP-1 sites in the VDRE and osteocalcin box in proliferating osteoblasts may suppress both basal and vitamin D-enhanced osteocalcin gene transcription, as well as transcription of other genes associated with osteoblast differentiation. This model has been referred to as 'phenotype suppression' (35).

Whereas phenotype suppression may explain why osteocalcin is not synthesized in proliferating osteoblasts, a key unresolved question has been the mechanism by which the osteocalcin gene is rendered transcribable and vitamin D responsive only following the onset of extracellular matrix mineralization. No osteocalcin expression is seen after completion of proliferation until the onset of mineral accumulation. In cultures in which mineralization is delayed the induced expression of osteocalcin is also delayed (34). This observation indicates that other factors besides Jun–Fos must also be involved in the regulation of osteocalcin gene expression. The present findings demonstrate the existence of at least one such additional regulatory mechanism, i.e. the OSE, which operates in the presence and absence of vitamin D and hence independently of the VDRE and which also appears to be distinct from Jun–Fos control. Our study is the first to definitively localize the OSE, by site-directed mutagenesis, gel shift assays and DNase I footprinting. Further verification of its functionality must await development of transgenic animals to demonstrate that the OSE is active *in vivo*.

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