Position and Orientation-Selective Silencer in Protein-Coding Sequences of the Rat Osteocalcin Gene†

Baruch Frenkel,‡ Jolanda Mijnes,‡,§ Michael A. Aronow,‖ Gerard Zambetti,‡,⊥ Chaitali Banerjee,‡ Janet L. Stein,‡ Jane B. Lian,*,‡ and Gary S. Stein*,‡

Departments of Cell Biology and Orthopedic Surgery, University of Massachusetts Medical Center, Worcester, Massachusetts 01655

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ABSTRACT: Osteocalcin (OC) is a bone-specific protein which is expressed postproliferatively by osteoblasts during late stages of differentiation. We have found that a silencer element is present within the rat OC gene (between nt +39 and +104), overlapping the OC signal prepropeptide-coding sequence. The presence of this sequence in OC promoter-CAT reporter constructs suppresses promoter activity in transiently transfected proliferating osteoblasts, which do not express OC, by up to 50-fold. This is the first demonstration of contribution from protein-coding sequences to silencing of animal genes. The element appears to be bipartite; silencer activity requires both the protein-coding sequence +39 to +63 and the +93 to +104 exon 1/intron 1 border region. Both of these domains contain sequences highly similar to silencer motifs in several other genes, including chicken lysozyme as well as rat collagen type II, insulin, and growth hormone. OC silencer activity is fully retained when the element is placed outside the RNA-coding region, 3′ but not 5′ of the OC-CAT fusion gene. Repression activity is orientation independent in the native position but requires the native orientation when located in 3′ extragenic positions. The silencer does not inhibit the activity of the heterologous SV40 early promoter. These results suggest interaction between the transcribed silencer and specific OC promoter element(s) residing farther upstream. The OC transcribed silencer may contribute to developmental control of OC expression.

Positive and negative transcriptional regulatory elements generally reside outside RNA-coding regions; most are located at various distances upstream of the transcription start site. However, some regulatory elements are found 3′ of the transcription start site, commonly outside protein-coding regions (Glass et al., 1988; Levine & Manley, 1989; Drummond et al., 1992; Kadesch et al., 1986; Vuorio & de Crombrugghe, 1990; Pierce et al., 1991; Atchison et al., 1989; Hurt et al., 1989; Yang et al., 1986). A functional significance for intragenic location of silencers has been initially proposed in prokaryotic (Mandal et al., 1990) and later in eukaryotic (Drummond et al., 1992; TenHarmsel et al., 1993) systems, suggesting a requirement for interaction between elements upstream and downstream of the transcription start site. The downstream elements in these cases reside outside protein-coding regions.

Here we report the first identification of a functional silencer within protein-coding sequences of a gene in animal cells. We present the initial characterization of a strong transcriptional silencer overlapping the first exon (encoding the signal prepropeptide) of the rat osteocalcin (OC) gene. This nucleotide sequence suppresses OC gene promoter activity in proliferating osteoblasts, suggesting a molecular mechanism for the tissue-specific developmental regulation of OC expression.

Osteocalcin is a bone-specific protein which is expressed postproliferatively in mature osteoblasts and is upregulated with the onset of extracellular matrix mineralization both *in vivo* and *in vitro* (McKee et al., 1993; Owen et al., 1993; Stein et al., 1990). Transcription of the OC gene is developmentally regulated and responsive to steroid hormones including vitamin D and glucocorticoids [reviewed by Lian and Stein (1992)]. Many studies have addressed the molecular basis for hormonal regulation of OC gene expression, and steroid response elements have been identified within the 5′ promoters of the human (Ozono et al., 1990; Pike 1990; Stromstedt et al., 1991) and the rat (Demay et al., 1990; Markose et al., 1990; Yoon et al., 1988) genes. However, our understanding of basal OC expression during osteoblast differentiation is limited.

Basal OC gene transcription is a prerequisite for vitamin D responsiveness, reflecting enhancer rather than inducer activity (Owen et al., 1991, 1993). Basal transcription may be determined directly by positive *trans*-acting factors binding to proximal promoter elements, such as the TATA domain and the CCAAT-containing OC box (Heinrichs et al., 1993a; Markose et al., 1990; Owen et al., 1990; Stromstedt et al., 1991). In addition, OC transcription during proliferation may be prevented by negative regulation, either by occupancy of these basal promoter elements by inhibitory factors (Heinrichs et al., 1993b; Owen et al., 1990; Stromstedt et al., 1991) or by activity of independent negative *cis*-acting elements [see Levine and Manley (1989) for review].

Shortly after genomic cloning of the rat OC gene (Lian et al., 1989), we made the expected observation using a reporter transfection system that the OC promoter (nt −1097/+151) failed to support efficient expression in proliferating normal diploid rat calvarial osteoblasts, not expressing OC. Other laboratories reported that similar constructs exhibited efficient OC gene promoter activity in rat osteosarcoma cells, expressing

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* Address correspondence to these authors at Department of Cell Biology, University of Massachusetts Medical Center, 55 Lake Ave. North, Worcester, MA 01655. Tel: 508-856-5625. Fax: 508-856-6800.

‡ Department of Cell Biology.

§ Present address: Department of Immunology and Infectious diseases, Utrecht University, 3508TD Utrecht, The Netherlands.

‖ Department of Orthopedic Surgery.

⊥ Present address: Department of Biochemistry, St. Jude Children's Research Hospital, Memphis, TN 38101.

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OC while proliferating (Klein-Nulend et al., 1991; Terpening et al., 1991; Yoon et al., 1988). Expression in normal diploid osteoblasts was obtained when the OC RNA-coding sequence +24 to +151 was removed from the reporter construct. This observation initiated a study to determine whether the deleted sequence contained a negative regulatory element, operative in proliferating normal rat calvarial osteoblasts.

MATERIALS AND METHODS

Plasmid Constructions. OC-CAT fusion genes used in this report were constructed using standard protocols (Ausubel et al., 1989) and are illustrated in the respective figures. The *Hin*dIII–*Bam*HI fragment of pSV2CAT (Gorman et al., 1982) was the source for CAT-coding DNA. OC promoter sequence −1097/+151 (*Eco*RI/*Ava*I; Lian et al., 1989) was used to construct pOCACAT (Figure 1, construct no. 2). In this construct, the residual OC-RNA coding sequence and the CAT-coding sequence are in reading frame. A *Hin*dIII restriction site was introduced at position +23 by site-directed mutagenesis, to construct pOCZCAT (Figure 1, construct no. 1). Both constructs were cloned in pUC 19 and assayed for promoter activity as described below. In addition, OCZCAT was cloned in pGEM-7Zf(+) (Promega, USA), and this plasmid served as a parent vector for the construction of all the following OC-CAT plasmids.

Constructs 3–7 (Figure 1A) were obtained by inserting the respective sequences (see below) in the unique pOCZCAT-*Hin*dIII site, at the junction of the OC promoter with the CAT reporter gene. Construct no. 8 (and no. 8r) (Figure 2A) was obtained by inserting the fragment +24/+151 (see below) in the unique pOCZCAT-*Xho*I site, immediately 5′ of the *Eco*RI site at −1097. Constructs 9,10 (Figure 2A), and 11–14 (Figure 4A) were made by inserting the respective fragments (see below) in the unique pOCZCAT-*Bam*HI site 3′ of the CAT-polyadenylation signals.

Each of constructs 3–5 and 7–9 was made by a single cloning step, where inserts were obtained as follows: a synthetic double-stranded oligonucleotide, 5′-AGACACCATAAGGA-3′ (upper strand) with *Hin*dIII overhangs was used as an insert for construct no. 3. The insert for construct no. 5 was similarly obtained as a double-stranded oligonucleotide (+104/+151 OC sequence) with *Hin*dIII overhangs, where only the +104 end would restore a *Hin*dIII recognition site. The inserts for constructs 4 and 7–9 were obtained as the respective restriction fragments filled-in by Klenow fragment (see Figure 1 for restriction endonucleases used). Construct no. 6 was obtained by blunt-end ligation of the restriction fragment +39/+92 with *Hin*dIII-linearized and blunt-ended pOCZΔ(104/151)-CAT (no. 5).

Inserts for constructs 10–14 were obtained by using an intermediate plasmid, pUCOC(24/151), in which the filled-in +24/+151 OC restriction fragment (*Hin*dIII/*Ava*I) was cloned in the *Sma*I site of pUC 19 (orientation: 5′ *Eco*RI > *Kpn*I > (+24/+151) > *Bam*HI > *Xba*I > *Hin*cII 3′). The fragments +24/+104 and +87/+151 were then obtained by PCR using pUCOC(24/151) as template; M13 (forward and reverse, respectively) primers and oligonucleotides corresponding to the OC +87/+104 sequence (lower and upper strand, respectively) were used to prime the PCR reactions. The two PCR products, as well as the +64/+104 fragment, obtained by digesting the +24/+104 fragment with *Sau*96I (see Figure 1A), were cloned in pOCZCAT to yield constructs 11, 12, and 14, respectively (Figure 4A). The insert for making constructs 10 and 10r (Figure 2A) was obtained as the *Pvu*II-*Hin*cII restriction fragment of pUCOC(24/151), containing the +24/+151 OC sequence and the 5′ contiguous 108 bp vector sequence. The insert for construct no. 13 (Figure 4A) was obtained as the *Ava*II/*Hin*cII fragment of pUCOC(+24/+151).

pOC(24/151)SV-CAT and pOC(151/24)SV-CAT (Figure 3A) were obtained by cloning the filled-in OC +24/+151 fragment 5′ of the SV40-CAT fusion gene in the unique *Bgl*II site of pCATcontrol (Promega, USA). pSV2CATOC(24/151), pSV2CATOC(151/24), pSV2CATOC(24/151)$_2$, and pSV2CATOC(24/151)$_4$ (Figure 3B) were similarly obtained by inserting the OC +24/+151 fragment in pSV2CAT (Gorman et al., 1982) at the unique *Bam*HI site, 3′ of the CAT domain. Fusion areas of all plasmids were sequenced by the dideoxynucleotide method.

Cell Culture, Transfections, and CAT Assays. Fetal rat calvarial osteoblasts were isolated as previously described and maintained in MEM supplemented with 10% FCS (Owen et al., 1993). Cells were trypsinized after reaching confluence (day 8) and replated at a density of 3.5×10^5 cells per 100-mm plate. DEAE-dextran mediated transient transfection (Ausubel et al., 1989) was carried out with 20 μg of cation-exchange-purified plasmid DNA (Qiagen, CA). In addition, concentration curves were performed for most constructs. Following transfection, cells were fed with fresh medium containing 10^{-8} M vitamin D and harvested 48 h later. CAT activity in cell lysates was measured as the acetylation of [^{14}C]chloramphenicol; the substrate and products were separated using TLC (Ausubel et al., 1989) and directly counted (Betascope 603 analyzer, Betagen, MA). The expression level of each construct was evaluated as percent conversion relative to control using 2–4 independent plasmid preparations.

RESULTS

Evidence for a Negative Regulatory Element in the OC Gene RNA-Coding Region. Expression levels of two constructs, pOCACAT and pOCZCAT, consisting of the initial ~1 kb of the rat OC promoter sequences fused to the chloramphenicolacetyltransferase (CAT) reporter gene and differing in the 3′ ends of the OC portion, were compared (Figure 1A,B). pOCACAT contains 128 base pairs of transcribed sequence, including the first exon and part of the first intron, that are not present in pOCZCAT. The CAT activity obtained following the transfection of osteoblasts with pOCACAT was 8–50-fold lower than that of pOCZCAT. The low transcriptional activity from pOCACAT suggested a negative regulatory element within the osteocalcin RNA-coding sequence, operative in proliferating osteoblasts.

For other genes, inclusion of RNA coding regions in reporter constructs (Atchison et al., 1989; Hurt et al., 1989; Yang et al., 1986) resulted in increased, rather than decreased, transcriptional activity, indicating that the presence of RNA-coding sequence does not necessarily inhibit expression. However, unlike the interpretation of standard 5′ deletion analyses, it was necessary to establish that the decreased CAT activity of pOCACAT did not result from the presence of the residual splicing site (+97), interfering with RNA processing, or the residual OC-ATG start codon (+34), which, although in reading frame with the start codon of the CAT gene, could cause a decrease in the measured activity of the OC-CAT fusion protein.

To address these possibilities, three partial segments (+24/+51, +39/+92, and +104/+151), spanning most of the +24/+151 sequence, were individually introduced back into pOCZCAT (Figure 1A, constructs 3–5) and tested for ability

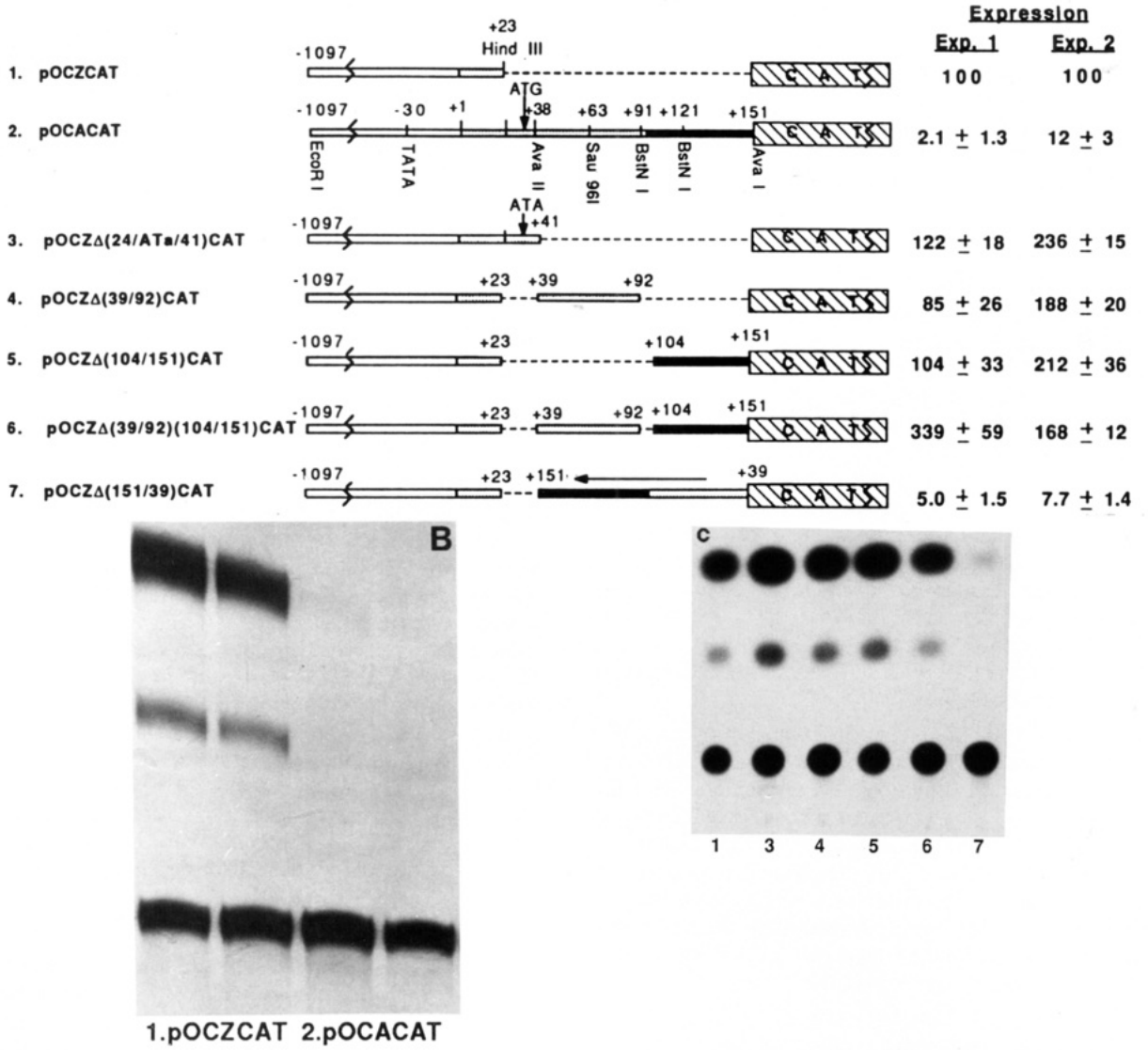

FIGURE 1: Deletion of the OC +24/+151 sequence increases expression of an OC promoter-CAT reporter construct. (A, top) Schematic illustration and expression levels of pOCZCAT (no. 1) and derivative constructs containing OC RNA-coding sequences. Constructs are named both numerically and generically. In pOCZCAT, the sequence +24/+151 is deleted relative to pOCACAT (no. 2), and various segments of this sequence are cloned back to generate constructs 3–7. Broken lines indicate deletions relative to pOCACAT (no. 2) and the horizontal arrow in construct 7 indicates reverse orientation. In construct 3 the ATG start codon is mutated as shown. Open bars designate OC 5′ promoter sequences, stippled areas represent OC mRNA-coding sequences, and solid bars show intronic sequences. Chloramphenicolacetyltransferase (CAT)-coding sequence is also shown. Details of gene constructions are described under Materials and Methods. Osteoblasts were transfected with 20 μg of the respective plasmids and cells were harvested 48 h later for CAT assay. Expression levels of two independent plasmid preparations are given for each construct and designated as experiments (Exp.) 1 and 2. Values are mean ± SD (3–4 plates) relative to pOCZCAT, defined as 100 units. (B) CAT assay comparing the expressions of pOCZCAT and pOCACAT are shown. (C) Representative CAT assay comparing pOCZCAT (no. 1) to constructs 3–7. Lanes are designated by the numbers of the constructs assayed.

to restore the inhibitory activity exhibited by pOCACAT (Figure 1B). The sequence +93/+103, containing the splicing signal, was excluded, and the OC ATG start codon in construct no. 3 was mutated to ATA. As Figure 1A,C indicates, none of the three segments in constructs 3–5 restored the inhibitory activity exhibited by the entire +24/+151 sequence.

The inability of these three segments to independently restore the inhibitory effect suggested that the putative negative regulatory element contained in the +24/+151 domain was truncated in each of the three constructs. To test this possibility, pOCZΔ(39/92)(104/151)CAT and pOCZΔ(151/39)CAT were constructed (Figure 1). In the former, most of the exonic and the intronic sequences are present, but the splicing region is still missing. In the latter, the splicing region is present but nonfunctional, since the +151/+39 sequence is cloned in the reverse orientation. The sequence +24/+38 was eliminated from this construct because it would have introduced an ATG potential start codon in either orientation. As shown in Figure 1, the expression of pOCZΔ(39/92)-(104/151)CAT was 2–3-fold higher than that of pOCZCAT (although up to 7-fold activation was occasionally observed). In contrast, pOCZΔ(151/39)CAT exhibited 15-fold lower activity compared to pOCZCAT, restoring the inhibitory effect initially observed with pOCACAT (no. 2). This result suggests that a negative regulatory element is indeed present within the +39/+151 transcribed sequence of the rat OC gene. Furthermore, this element can function in both orientations. In addition, we conclude that the +93/+103 sequence at the exon 1/intron 1 boundary, missing in pOCZ(39/92)(104/151)CAT, may be essential for the inhibitory activity.

The Intragenic OC Gene Regulatory Element Is a Transcriptional Silencer. As the OC +24/+151 domain is a normally transcribed sequence, the question arises as to whether the observed negative effect is directly on the promoter

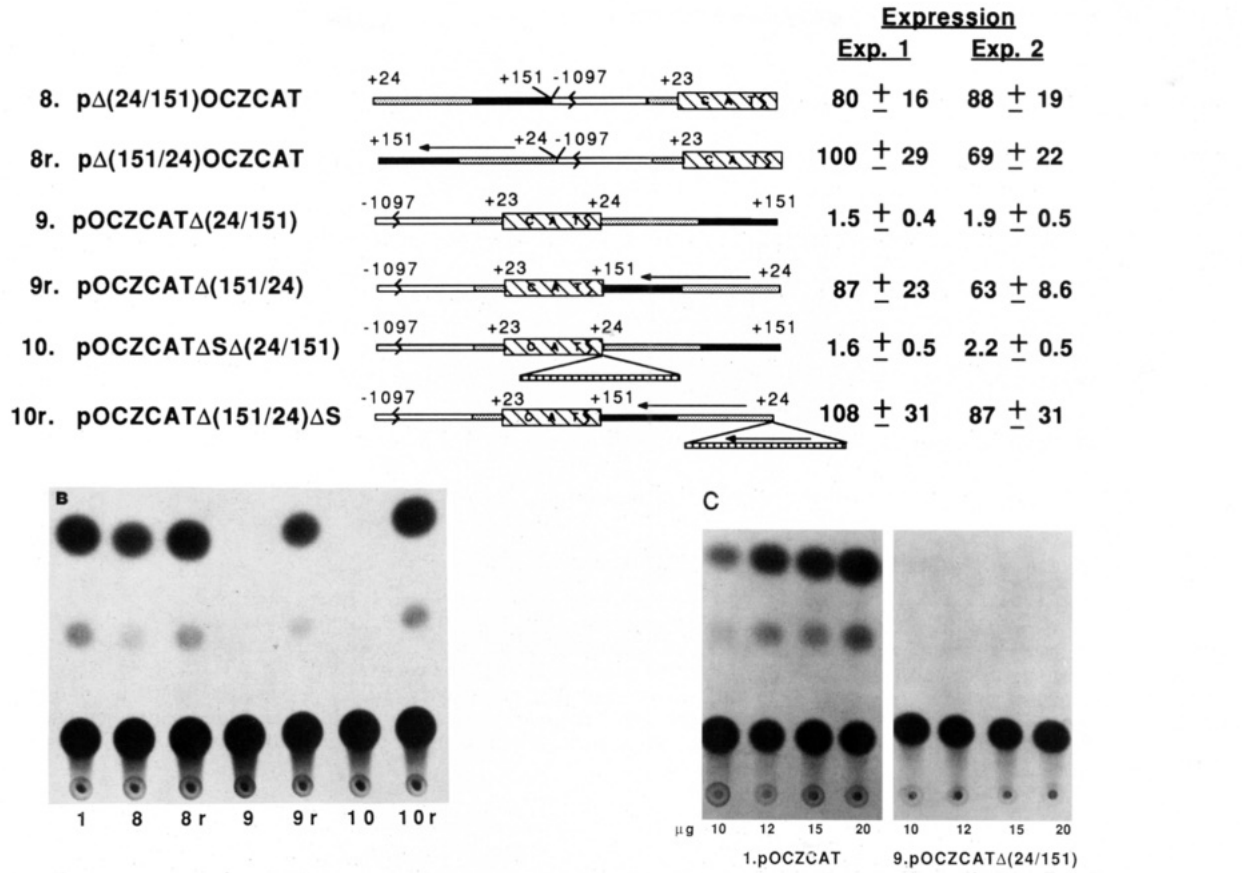

FIGURE 2: OC silencer can operate from an extragenic position. (A, top) The OC +24/+151 sequence, either alone (constructs 8, 8r, 9, 9r) or with a 108 bp nonspecific sequence (striped bars in nos. 10 and 10r), was cloned in pOCZCAT (no. 1 in Figure 1) as illustrated, either in the native (8–10) or the reverse (8r-10r) orientation. Two independent plasmid preparations of each construct were assayed for CAT activity as described in Figure 1, and values are given relative to pOCZCAT, defined as 100 units (mean ± SD, n = 3). (B) Representative CAT assay. Lanes are designated by the numbers of the constructs. (C) Concentration curve for construct 9 is shown. The amounts of DNA used for transfection are indicated.

or is mediated by either premature termination, poor efficiency of RNA processing, or decreased mRNA stability. To address this question, the +24/+151 sequence was cloned again in pOCZCAT, but this time outside the RNA-coding region, either 5′ of the OC promoter or 3′ of the CAT-polyadenylation signals. Both orientations were tested in each of these two locations. As shown in Figure 2A,C complete repression of promoter activity by the +24/+151 sequence was obtained at the 3′position, in the native orientation, suggesting that this sequence exerts its inhibitory activity at the transcriptional level. However, the inhibitory activity at this position was completely abolished in the reverse orientation, as well as in both orientations at the 5′ position (Figure 2A,B). This element therefore satisfies the criteria of a silencer since it represses promoter activity over a large distance. However, the element lacks the absolute position and orientation independence characteristic of classical enhancers and silencers. The position and orientation selectivity of the OC silencer may have resulted from the presence of a serendipitous sequence 5′ of the downstream site where the +24/+151 sequence was introduced that contributed to restoration of inhibitory activity. To test this possibility, we introduced again the +24/+151 sequence downstream of the OC-CAT fusion gene, but with a nonspecific 108 base-pair spacer (Figure 2A,B). However, silencer activity was completely retained in this construct (no. 10), thus requiring an alternative explanation for the position and orientation selectivity of the silencer (see Discussion).

The OC Silencer Does Not Affect a Heterologous Promoter. In our initial experiments examining the capacity of the +24/+151 OC sequence to inhibit the activity of a heterologous promoter, we used the plasmid pCATcontrol (Promega), an SV40 promoter-CAT reporter construct in which the tested sequence can be cloned contiguous to the SV40 promoter (Figure 3A). Subsequently, when the possible importance of a 3′ location for silencer activity became evident, the +24/+151 sequence was cloned in pSV2CAT, downstream of the CAT gene (Figure 3B), thereby providing a context for the +24/+151 sequence similar to that in pOCZCATΔ(24/151) (no. 9). However, as summarized in Figure 3, the SV40 promoter was not affected by the +24/+151 OC fragment, introduced at either the 5′ or the 3′ positions in either orientation. Furthermore, increasing the copy number of the insert resulted in a limited enhancement rather than an inhibitory effect (Figure 3, bottom two lanes).

Location of the OC Silencer within Exon 1. To further localize the silencer sequence, we utilized the ability of the OC +24/+151 sequence to suppress OC gene promoter activity from the 3′ extragenic position (Figure 2) as the basis for a mapping assay, where no contribution from mRNA structure or processing (e.g., splicing) could occur. The exonic +39/+92 and the intronic +104/+151 sequences, which did not inhibit promoter activity either independently or together (Figure 1), were each extended to include the splicing region and were each cloned in pOCZCAT, 3′ of the CAT-polyadenylation signals. As shown in Figure 4A,B the inhibitory activity was completely restored by the +24/+104 sequence, while the +87/+151 sequence only had a minor effect. Similar to the whole +24/+151 sequence, the partial +24/+104 sequence was functional only in its native orien-

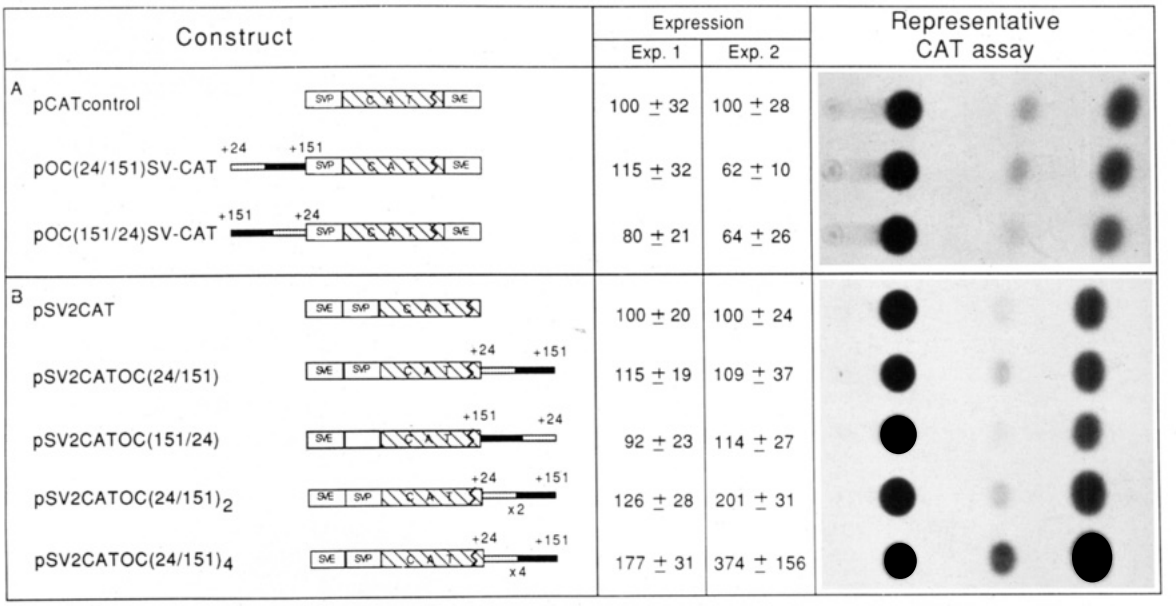

FIGURE 3: OC +24/+151 fragment does not repress SV40 promoter. The +24/+151 OC fragment was cloned in either pCATcontrol or in pSV2CAT at the positions and orientations as illustrated. pCATcontrol and pSV2CAT both contain the same SV40 sequences but differ with respect to the position of the enhancer (SVE) relative to the promoter (SVP). The constructs pSV2CATOC(24/151)$_2$ and pSV2CATOC-(24/151)$_4$ contain two and four copies of the OC +24/+151 sequence, respectively. Each construct was tested for promoter activity in transfected osteoblasts as described under Materials and Methods, using two independent plasmid preparations. Expression values are the mean ± SD of CAT activity in three plates, where 100 units are defined as the activity of either pCATcontrol (A) or pSV2CAT (B). Representative CAT assays are shown.

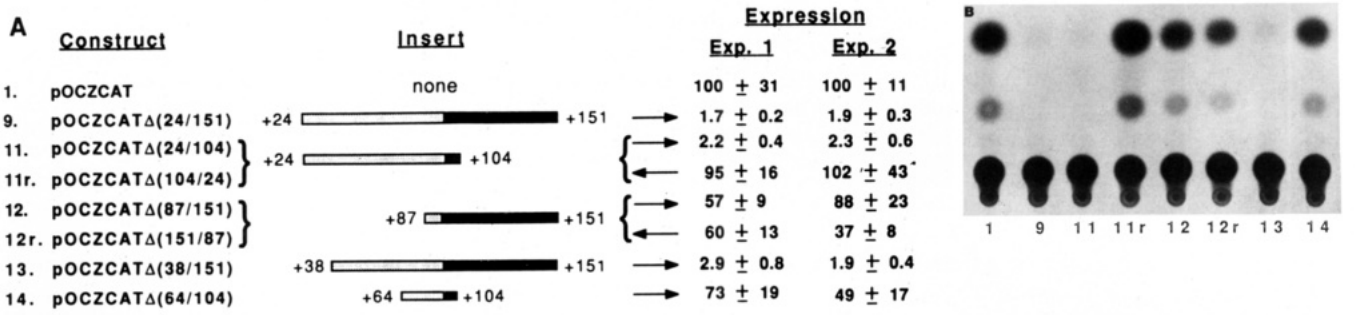

FIGURE 4: Both the +38/+64 exonic domain and the +93/+104 exon 1/intron 1 junction of the OC gene are required for silencer activity. (A) Various segments of the +24/+151 sequence were cloned in pOCZCAT (Figure 1, construct 1) outside the RNA coding region, 3′ of the CAT polyadenylation signals (see Materials and Methods). Inserts are illustrated as in Figure 1, and their orientation relative to the OC-CAT fusion gene is indicated by an arrow. Osteoblasts were transfected with 20 μg of each plasmid and CAT activity assayed 48 h later. Expression values relative to pOCZCAT are mean ± SD of at least three plates. (B) Representative CAT assay is shown. Lanes are designated by the numbers of the constructs assayed.

tation. As shown in Figure 5A,B the +93/+104 region, which is required for silencer activity (Figure 4), restored a 5-bp perfect interrupted palindrome (+89/+99), sharing similarity with one or both arms of palindromes in silencer elements of other genes. The palindrome in the OC silencer overlaps the canonical splicing signal supporting cleavage at the 5′ end of the first intron (see the hatched box in Figure 5A). This region (+93/+104) is, however, insufficient to reconstitute a functional silencer, as demonstrated by the relatively high transcriptional activity of pOCZCATΔ(87/151) containing this sequence (Figure 4). Extending the palindromic sequence upstream only to position +64 was still insufficient to restore silencer activity (Figure 4, construct no. 14). This suggests that similar to the well-characterized N-2.4 kb silencer of the chicken lysozyme gene (Baniahmad et al., 1990) and others (Kondo et al., 1992; Drummond et al., 1992), the OC transcribed silencer is bipartite, consisting of one component at the exon 1/intron 1 boundary, and an additional component upstream of position +64. The most 5′ possible end of the silencer appears to be at nt +38, because inhibitory activity was restored by the +38/+151 sequence (Figures 1 and 4, constructs 7 and 13, respectively). Interestingly, the AC-CCTCTCT motif (+40), previously suggested as a consensus silencer sequence (Baniahmad et al., 1987), was identified within the +38/+64 domain. A summary of negative regulatory elements containing this or similar motifs is presented in Figure 5C.

DISCUSSION

While several silencers have been identified within RNA-coding regions of genes in animal cells, none have been previously described within protein-coding sequences. The OC transcribed silencer reported here overlaps the first exon (97 nt) of the rat OC gene (Figure 5A), encoding the signal prepropeptide (Lian et al., 1989). The unique location is one distinguishing feature of this negative regulatory element.

Silencers, by analogy to enhancers, were initially defined as autonomous *cis*-acting negative regulatory elements that can act in a position and orientation independent manner (Brand et al., 1985). However, the increasing evidence for silencers that do not entirely fulfill these criteria suggests a relaxation of the original definition; examples include silencers

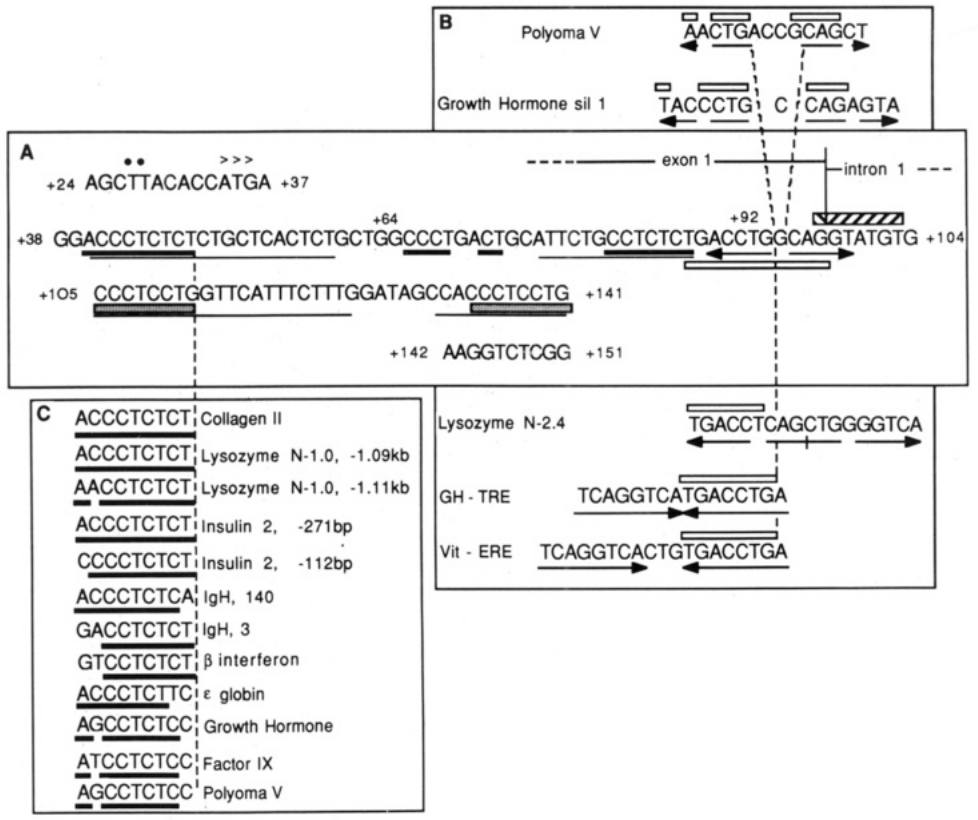

FIGURE 5: Sequence similarity of the OC +24/+151 silencer with other genes. (A) The OC +24/+151 sequence is shown as four continuous lines. Two thymidine residues introduced to generate an artificial *Hin*dIII site (Figure 1, construct 1) are marked by "••" (+27) and the ATG initiation codon (+34) is designated by ">>>". The exon 1/intron 1 junction is indicated and the canonical splicing signal is marked by a hatched bar. The sequence is pyrimidine-rich; four TC-rich stretches are underlined. The silencer motif ACCCTCTCT (Baniahmad et al., 1987) at +40, and two similar exonic sequences (+65, +81) are heavily underlined. Eight base pair direct repeats within the intronic sequence (+105, +134), sharing similarity with the exonic motif (+40), are underlined by stippled bars. Note the 5 bp interrupted palindrome (+89), overlapping the splicing signal. The open bar (+87/+97) focuses on the part of the palindrome and the contiguous bases which exhibit similarity to palindromes in other regulatory elements. (B) Palindromes identified in silencers of the polyoma virus (Furukawa et al., 1990), rat growth hormone (Roy et al., 1992), and chicken lysozyme (Baniahmad et al., 1990) genes are aligned with the respective OC silencer sequence. Also shown are sequence similarities to the thyroid and estrogen-response half elements of the rat growth hormone and the *Xenopus* vitellogenin A2 genes, respectively (Glass et al., 1988). (C) Sequence similarity between the exonic ACCCTCTCT motif (+40) and silencer sequences associated with genes encoding rat collagen type II (Savagner et al., 1990), chicken lysozyme, rat insulin, mouse immunoglobulin heavy chain, human β-interferon (Baniahmad et al., 1987), human ε-globin (Cao et al., 1989), rat growth hormone (Baniahmad et al., 1987), human factor IX (Salier et al., 1990), and the polyoma virus (Baniahmad et al., 1987). Broken lines throughout panels are drawn to designate appropriate alignment.

which are promoter-specific (Jackson et al., 1992), position-dependent (Baniahmad et al. 1987; Vacher & Tilghman, 1990), or orientation-dependent (Kondo et al., 1992; Nakamura et al., 1989). The OC transcribed silencer described in this study does not comply with the classical definition because (a) it does not affect a heterologous promoter (Figure 3), (b) it is functional following relocation to 3′ positions but not to a 5′ position (Figure 2), and (c) it is orientation-dependent at 3′ positions (Figure 2).

Silencers (and enhancers) are often composed of multiple elements (Baniahmad et al., 1990; Frish & Morisaki, 1990; Pierce et al., 1991). In cases where cooperativity between elements occurs, silencer activity may be lost following the deletion of sequences which are separated by as much as 100 bp (Baniahmad et al., 1990). In our study, silencer activity was lost either following the deletion of the sequence +93/+104 (construct 6 *vs* 7; construct 4 *vs* 11 and 13), or following the deletion of the sequence +38/+63 (construct 14 *vs* 13 and 11). These results suggest that the OC transcribed silencer is bipartite, similar to other regulatory elements such as silencers associated with the rat epoxide hydrolase (Kondo et al., 1992) and the chicken lysozyme (Baniahmad et al., 1990) genes.

The +93/+104 OC sequence contains a palindrome sharing similarity with silencers identified in the chicken lysozyme gene, the rat growth hormone gene, and the polyoma virus (Figure 5B). Together with the 5′ flanking two base pairs, the left arm of this palindrome exhibits a 6/6 match with that of the chicken lysozyme N-2.4 silencer and a 7/7 match with thyroid and estrogen response half elements (Figure 5B). Transcriptional repression mediated by thyroid (Baniahmad et al., 1992) and steroid (McDonnell et al., 1992) hormone receptors has recently been described.

The +38/+63 OC sequence contains the consensus silencer motif (Baniahmad et al., 1987) ACCCTCTCT (+40), identified in *cis*-negative regulatory elements of genes encoding human collagen type II (Savagner et al., 1990), chicken lysozyme, rat insulin, mouse immunoglobulin heavy chain, human β-interferon (Baniahmad et al., 1987), human ε-globin (Cao et al., 1989), rat growth hormone (Baniahmad et al., 1987), human factor IX (Salier et al., 1990), the polyoma virus (Baniahmad et al., 1987) (Figure 5C), and rat glutathione transferase P (Imagawa et al., 1991). In some of these cases, including OC, the motif is found more than once, with minor modifications (Figure 5A,C). The motif is also found in the rat CR1 repetitive sequences (Savagner et al., 1990). Repetitive sequences, including the rat CR1, have been implicated

in negative transcriptional regulation (Laimins et al., 1986; Saffer & Thurston, 1989). The significance of these sequence similarities remain to be explored.

While silencers often inhibit heterologous promoters (Baniahmad et al., 1987, 1990; Farrell et al., 1990; Jackson et al., 1992; Nakamura et al., 1989; Roy et al., 1992; Li et al., 1993) including that of SV40 (Colantuoni et al., 1987; Saffer & Thurston, 1989; Imagawa et al., 1991), the OC transcribed silencer does not (Figure 3). This may be explained by a requirement for an interaction between the identified OC silencer and other OC promoter element(s). In addition, the inability of the OC +24/+151 element to act from the 5′ position (Figure 2) may be explained by a requirement for the basal promoter to be located between the potential 5′ and the identified 3′ silencer components, similar to the recently characterized silencers of the human insulin-like growth factor II (Drummond et al., 1992) as well as the *Drosophila* alcohol dehydrogenase (Benyajati et al., 1992) and *Ultrabithorax* (TenHarmsel et al., 1993) genes. The requirement for interaction between multiple silencer elements may also provide an explanation for the strong orientation dependence of the transcribed silencer sequence at the 3′ position (Figures 2 and 4); however, the concept that upstream and downstream elements should be correctly oriented requires further explanation for the repression activity observed with the +151/+38 fragment (reverse orientation) in its native position (Figure 1, construct no. 7). Interestingly, orientation dependent cross-talk between positive OC promoter elements has been recently described (Terpening et al., 1991).

Silencers identified in eukaryotic genes are generally involved in transcriptional extinction in nonexpressing tissues (Baniahmad et al., 1990; Brand et al., 1985; Kadesch et al., 1986; Li et al., Pierce et al., 1991; Roy et al., 1992; Savagner et al., 1990). In a limited number of cases, silencers have been proposed to play a role in the developmental control of gene expression (Drummond et al., 1992; Emerson et al., 1989; Furukawa et al., 1990; Vacher & Tilghman, 1990). Since normal osteoblasts express OC only postproliferatively (Stein et al., 1990), the OC silencer may be specifically operative in nondifferentiated osteoblasts. Loss of this inhibitory activity during osteoblast differentiation could provide a molecular mechanism for postproliferative onset of OC gene transcription. This mechanism is further supported by several reports, in which constructs containing the OC transcribed silencer were efficiently expressed in ROS 17/2.8 osteosarcoma cells that, in contrast to normal osteoblasts, express the OC gene while proliferating (Klein-Nulend et al., 1991; Terpening et al., 1991; Yoon et al., 1988). This observation demonstrates that expression of transiently transfected plasmids that, similar to pOCACAT, contain the transcribed silencer, may indeed occur in the appropriate context of *trans-acting* factors. Normal diploid osteoblasts at postproliferative stages of differentiation may represent an analogous situation. While our studies were in progress, Klein-Nulend et al. (1991) reported that, similar to proliferating normal osteoblasts used in our studies, UMR 106 osteosarcoma cells, not expressing OC, do not support transcription from an OC promoter-CAT reporter fusion gene containing the +24/+151 RNA-coding sequence. This further supports the proposed contribution by the silencer to the developmental control of the OC gene. Direct evidence from postproliferative normal diploid osteoblasts is required, however, to support the proposed developmental switch in OC silencer activity.

Limited enhancement of expression mediated by the OC +24/+151 sequence has occasionally been observed (Figures 1 and 3). This observation should be considered within the developing concept of association between positive and negative transcriptional control mediated by the same (Baniahmad et al., 1992) or contiguous elements. Examples include the immunoglobulin kappa (Pierce et al., 1991), the chick β-globin (Emerson et al., 1989), the rat prolactin (Jackson et al., 1992) genes, and the polyoma virus (Furukawa et al., 1990). The latter silencer (sharing sequence similarity with the OC silencer, see Figure 5) is located between two enhancer elements, and the binding of F9 cell differentiation-related positive factors to the enhancers seems to overcome the inhibition mediated by negative factors constitutively bound to the silencer (Furukawa et al., 1990). Similar to the polyoma virus silencer in F9 cells, OC silencer activity may also be alleviated during osteoblast differentiation, by the binding of positive factors that are absent during proliferation. Alternatively, silencer activity may be offset simply by a decrease in the representation/potency of negative regulatory factors during osteoblast differentiation. Ongoing protein–DNA interaction studies using nuclear extracts from proliferating and differentiated osteoblasts seem to favor the first possibility.

In summary, our results suggest that an element overlapping the first exon of the rat osteocalcin gene functions as a negative regulatory element in proliferating osteoblasts. This element is a transcriptional silencer although its activity is strongly dependent on the nature of and the position/orientation relationship with the affected transcriptional unit. Molecular mechanisms remain to be established by which this element, together with 5′ OC promoter domains, mediates the tissue-specific developmentally regulated transcription of the OC gene.

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