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G/C Element Contributes to the Cell Line–Specific Expression of the Proximal Osteocalcin Promoter

Daniella Goldberg, Edith Gardiner, Nigel A. Morrison, and John A. Eisman

Bone and Mineral Research Division, Garvan Institute of Medical Research, St. Vincent's Hospital, Sydney, New South Wales 2010, Australia

Abstract Sequential activation of cell type–specific genes occurs during osteoblast development. The promoter of one such gene, osteocalcin, has been widely studied, but the DNA sequences that govern osteoblast-specific expression have not been defined. The proximal osteocalcin promoter linked to pTKCAT directs strong promoter activity in osteoblast-like ROS17/2.8 cells and comparatively weak promoter activity in nonosteoblastic NIH3T3 cells. To identify sequences important in conferring cell-specific expression of the osteocalcin gene, a deletion series of the human proximal promoter was constructed and the activities assessed in ROS17/2.8 and NIH3T3 cells. These studies identified a 30 bp sequence within the proximal promoter (osteocalcin repressor element-1 [ORE-1]) which is responsible for repressing the transcriptional activity in NIH3T3 cells. In electrophoretic mobility shift assays from both NIH3T3 and ROS17/2.8 cells, a protein complex bound to the ORE-1 that was related to a complex which binds the G/C-rich repressor element in the collagen type I (α1) promoter. In addition, there was a second complex from NIH3T3 cells but not ROS17/2.8 cells that bound the ORE-1 fragment. The presence of this additional factor in NIH3T3 cells parallels the observation that constructs carrying the ORE-1 sequence have repressed promoter activity relative to the analogous constructs lacking the ORE-1 when transfected into NIH3T3 and suggests that the NIH3T3-specific factor is a repressor. These data indicate that the G/C element in the ORE-1 contributes to the repression of osteocalcin gene transcription in a nonosteoblast cell line. The high homology between the ORE-1 sequence and a related sequence in the collagen type I (α2) proximal promoter suggests that homologous regions in other osteoblast-expressed genes may function similarly.

Key words: osteocalcin promoter, G/C element, collagen type I (α1) promoter, osteoblast, ORE-1

The osteocalcin gene, which is specifically expressed in bone tissue by osteoblasts, has been used to examine the molecular mechanisms governing osteoblast-specific expression. The mechanisms governing cell-specific expression are complex. DNA elements contributing to cell-specific expression have been identified in various systems; for example, in myoblasts, certain muscle specific genes have a common MyoD site through which MyoD and other nuclear factors control muscle specific expression [Weintraub et al., 1991]. A similar mechanism may exist in the osteoblast.

Osteocalcin is synthesised in the final stages of osteoblast development during bone mineralisation [Hauschka et al., 1989], but the precise function of the osteocalcin protein remains unknown. Specific steroid hormone, growth factor, and basal regulatory DNA regions have been identified in the promoters of the human and rat osteocalcin genes [Heinrichs et al., 1993a,b; Kerner et al., 1989; Li and Stashenko, 1993; Morrison et al., 1989; Pike, 1990; Stromstedt et al., 1991; Towler et al., 1994]. However, the sequences of basal elements contributing to the activation of these osteoblast-cell-specific gene promoters have not been defined.

The proximal osteocalcin promoter exhibits high basal activity in ROS17/2.8 cells, an osteoblast-like cell line that expresses osteocalcin, but comparatively low activity in the fibroblast-like cell line NIH3T3 [Pike, 1990]. In this study a series of osteocalcin promoter deletion constructs was used to identify regulatory sequences that contribute to basal activity. A 30 bp osteocalcin repressor element-1 (ORE-1) which confers transcriptional repression in NIH3T3 cells was identified and examined by

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Address reprint requests to Daniella Goldberg, Bone and Mineral Research Division, Garvan Institute of Medical Research, St. Vincent's Hospital, Sydney, NSW 2010, Australia.

electrophoretic mobility shift (EMS) analyses and by transfection of promoter reporter constructs into osteoblast and nonosteoblast cell lines. This osteocalcin regulatory region is highly homologous to promoter sequences in the osteoblast-expressed collagen type I (α1) and (α2) genes and, we suggest, may contribute to osteoblast-specific regulation.

MATERIALS AND METHODS

Cell Culture

All media and serum were obtained from ICN Flow Laboratories (Costa Mesa, CA). The ROS17/2.8 osteosarcoma cell line, a kind gift from S. Rodan (Merck, Sharpe & Dohme Research Laboratories, West Point, PA) was cultured in Ham's F12 medium supplemented with 7.5% fetal calf serum (FCS), 14 mM sodium bicarbonate, 1.5 mM glutamine (Cytosystems, Sydney, Australia), 8 mM $CaCl_2$, 10 μg/ml gentamicin (David Bull Laboratories, Mulgrave, Australia), and 28 mM HEPES (Commonwealth Serum Laboratories, Melbourne, Australia). ROS17/2.8 cells were subcultured 1:3 every fourth day. The NIH3T3 mouse fibroblast cell line was obtained from American Type Culture Collection (Rockville, MA) and was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, 4 mM glutamine, 0.2 U/ml human insulin (David Bull Laboratories), 20 μg/ml gentamicin, 25 IU/ml penicillin, 25 μg/ml streptomycin, 14 mM sodium bicarbonate, and 20 mM HEPES. NIH3T3 cells were subcultured 1:9 every fourth day.

DNA Constructs

The human osteocalcin promoter-CAT unidirectional deletion constructs were derivatives of pOSCAT2 which contains 1.3 kb of the human osteocalcin promoter driving the chloramphenicol acetyl transferase (CAT) gene [Morrison et al., 1989]. These deletions included Δ-353, Δ-199, Δ-169, Δ-41, referenced by their 5′ endpoints. pOSCAT1, which contains 344 bp of the human proximal osteocalcin promoter, was described previously [Morrison et al., 1989]. Thirty base pair, double-stranded, blunt-ended oligonucleotides (Macromolecular Resources, Colorado State University, Fort Collins, CO) were synthesized and used in EMS assays and in heterologous promoter reporter constructs:

ORE-1: 5′-CCC AGG GGC CCC TGG CCC AGC AGC CGC AGC-3′
COLL: 5′-GTT CCA AAT TGG GGG CCG GGC CAG GCA GTT-3′.

Single and triple copies of the ORE-1 in forward orientation were ligated into the blunted HindIII site of the pTKCAT (pTK) promoter kindly provided by R.J. Miksicek (German Cancer Research Center, Heidelberg, Germany). A region (−226 to −163) was deleted from pOSCAT1 by PCR mutagenesis to create pOSCAT1 without the ORE-1. Plasmid preparations used in transfection studies were prepared by alkaline lysis and CsCl equilibrium ultracentrifugation.

Transient Transfection

Transient transfection assays were performed as previously described using calcium phosphate–DNA precipitation [Morrison et al., 1989]. To reduce variability, DNA calcium phosphate precipitates were prepared in bulk for use with different cell lines. Cells in exponential growth phase were cotransfected with 20 μg of CAT construct and pCH110 (5 μg) (Promega, Sydney, Australia) or pRSV-βgal (10 μg) [Gorman et al., 1982] as a control for transfection efficiency. ROS17/2.8 and NIH3T3 cell lines were transfected at 1.7 and 1.2×10^4 cells/cm^2, respectively. Cells in DMEM with 2% charcoal stripped FCS were exposed to DNA for 16 h, and the DNA was then removed from the cells by washing monolayers with 1× phosphate buffered saline. Cells were then cultured in the usual medium until harvesting after a total transfection period of 40–48 h, and cell lysates were prepared by freeze/thawing. The β-galactosidase activity was assayed by cleavage of ONPG (Sigma, St. Louis, MO) and spectrophotometeric determation at 420 nm. A nonchromatographic CAT extraction assay was used [Morrison et al., 1989], and CAT activity was expressed in the linear range of the assay. CAT activity was normalised for transfection efficiency with respect to β-galactosidase activity after correcting for background CAT and β-galactosidase values derived from mock transfections. The activity of the heterologous promoter constructs was expressed relative to the activity of the pTK construct control transfection in the cell line tested.

Electrophoretic mobility shift (EMS) assays

Nuclear extracts were made using a modification of Dignam [Dignam et al., 1983]. Nuclear extracts prepared from 10^7 cells were stored in buffer C (20 mM HEPES, pH 7.9, 0.42 M NaCl, 3 mM $MgCl_2$, 0.5 mM DTT, and 0.5 mM PMSF) in 50 μl aliquots at −70°C. All double-stranded oligonucleotides used in EMS analyses were labelled with T4 polynucleotide kinase (Promega, Sydney, Australia) and [γ-^{32}P]ATP (Redivue, 3,000 Ci/mmol; Amersham, Sydney, Australia) and purified on 15% polyacrylamide gels. The binding reaction was based on Sagami [Sagami et al., 1986]. The labelled fragments (20 fmol) were combined with saturating quantities of nonspecific DNA (poly dI-dC, 10 μg) (Pharmacia, Uppsala, Sweden), and 5× binding buffer at final concentrations: 10 mM HEPES, pH 7.9, 50 mM NaCl, 0.5 mM EDTA, 10% glycerol, 2 mM $MgCl_2$, and 1 mM DTT. In competition experiments, unlabelled competitor oligonucleotide was then added. Nuclear extract protein (1–5 μg) was added last to a total volume of 40 μl, and the reaction was incubated at room temperature for 20 min. Reaction products were resolved by polyacrylamide gel electrophoresis on 4.5% gels containing 5% glycerol at 30 mA for 3–4 h in 0.5× Tris Borate EDTA running buffer at 4°C. The dried gel was autoradiographed with intensifying screens at −70°C for 12 and 72 h. Scatchard analysis of the sequence-specific competition experiment was derived from Cerenkov counts of the bound and free probe bands cut from the dried gel. Densitometric analysis of the EMS banding pattern was carried out with the PhosphorImager SI system (Molecular Dynamics, Melbourne, Australia) analyzer. Phosphorimage analysis was used to determine intensities of bands within a given lane. Each band was expressed as a fraction of the total bound counts within a lane. This fraction was used to evaluate cell line differences.

RESULTS

DNA Sequences Contribute to Differential Expression of the Human Proximal Osteocalcin Promoter

Transfection of a series of unidirectional deletion constructs of the osteocalcin promoter into osteoblastic ROS17/2.8 and fibroblastic NIH3T3 cells identified regions that contributed to differential expression of the proximal promoter (Fig. 1). The shortest construct, Δ-41, displayed strong

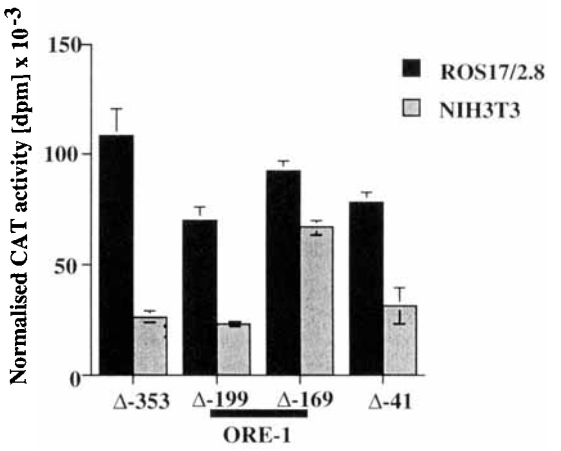

Fig. 1. Activity of human osteocalcin proximal promoter deletion constructs in NIH3T3 and ROS17/2.8 cells. The normalised promoter activity of unidirectional deletion constructs spanning the human osteocalcin promoter (−353 to +31) in NIH3T3 and ROS17/2.8 cells. The 5′ end points of the constructs are indicated by their names. The results are the mean ± SEM of normalised CAT activity from a representative of two transient transfection experiments into the two cell lines. The consistently strong promoter activity of the deletions in ROS17/2.8 cells contrasts with the repressed promoter activity in NIH3T3 cells. The 30 bp sequence between Δ-199 and Δ-169 that contributes to the repression in NIH3T3 cells was termed ORE-1.

transcriptional activity in ROS17/2.8 cells and only half this activity in NIH3T3 cells. The strongest promoter activity in NIH3T3 cells was shown by the Δ-169 construct. The inclusion of the 30 bp region between Δ-199 and Δ-169 conferred 2.9 ± 0.2–fold repression to the osteocalcin promoter in NIH3T3 cells. The addition of further sequences between Δ-353 and Δ-199 did not alter activity in the NIH3T3 cells, although it did increase activity in ROS17/2.8 cells.

Osteocalcin and Collagen Type I (α1) Sequences Bind a Common Complex

EMS analysis of the functional sequence between −199 and −169 (ORE-1) showed a complex of ROS17/2.8 nuclear proteins that bound in a sequence-specific manner (Fig. 2). Scatchard analysis, generated from the measured amount of bound and free probe in each lane in Figure 2 (inset), revealed two high affinity protein-DNA interactions with dissociation constants of 3×10^{-12} M and 1×10^{-8} M (Fig. 2).

Within the ORE-1 sequence is a G/C-rich region similar to the G/C-rich inhibitory element in the collagen type I (α1) promoter sequence (COLL) (Fig. 3A). The COLL shift gener-

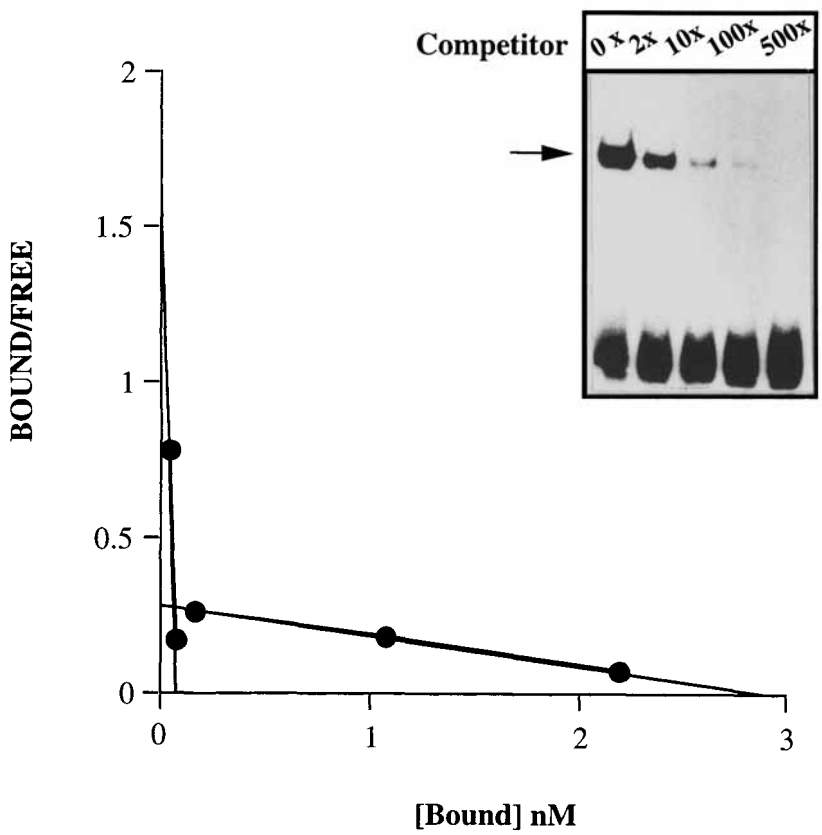

Fig. 2. Electrophoretic mobility shift analysis with ROS17/2.8 nuclear proteins. ORE-1 was incubated with no competitor or a 2-, 10-, 100-, and 500-fold molar excess of unlabelled ORE-1 competitor. The arrow indicates the single complex bound to ORE-1 (**inset**). Scatchard analysis of the protein-DNA interactions was calculated from Cerenkov counts of the bound and free bands. The Scatchard plot indicates two high affinity sites with dissociation constants of approximately 3×10^{-12} M and 1×10^{-8} M.

ated a more complex EMS banding pattern (Fig. 3B, lanes 1–4) than the ORE-1 shift (Fig. 3B, lanes 8–9), but the migration rates of the bands suggested that the complex bound to the ORE-1 could be analogous to complex C in the COLL shift. Further evidence that a related factor bound to the G/C elements in COLL and ORE-1 was shown in EMS cross-competition assays in which the unlabelled ORE-1 competed complex C and partially competed complex B from the labelled COLL oligonucleotide (Fig. 3B, lanes 5–7). In agreement, the converse experiment showed the unlabelled COLL cross-competed the ORE-1 bound complex (data not shown). These data indicate that complex C in the COLL shift is related to the complex bound to the 30 bp ORE-1 sequence. Since ORE-1 has repressive function in the transfection studies and binds a protein common to the COLL repressor element, it was termed the osteocalcin repressor element.

Cell Line Specificity of ORE-1

In NIH3T3 cell extracts the ORE-1 EMS assays showed a band that comigrated with the complex previously detected in ROS17/2.8 extracts as well as a second, more slowly migrating complex (Fig. 4A, lanes 1,2). The similarity between the COLL complex C and the ORE-1 complex in ROS17/2.8 shifts (Fig. 3B) was also evident in the NIH3T3 shifts, since the COLL banding pattern is similar in the two cell types (Fig. 4A, lanes 3,4). The COLL shift in NIH3T3 extracts differs slightly from the ROS17/2.8 shift in the fact that the intensity of complex C is reduced relative to bands A and B. This observation was confirmed by phospor-image analysis of the within-lane ratios of band intensities.

ORE-1 function in ROS17/2.8 and NIH3T3 cells was assayed by transfection of constructs carrying single or triple copies of the ORE-1 upstream of the promoter in pTK (Fig. 4B).

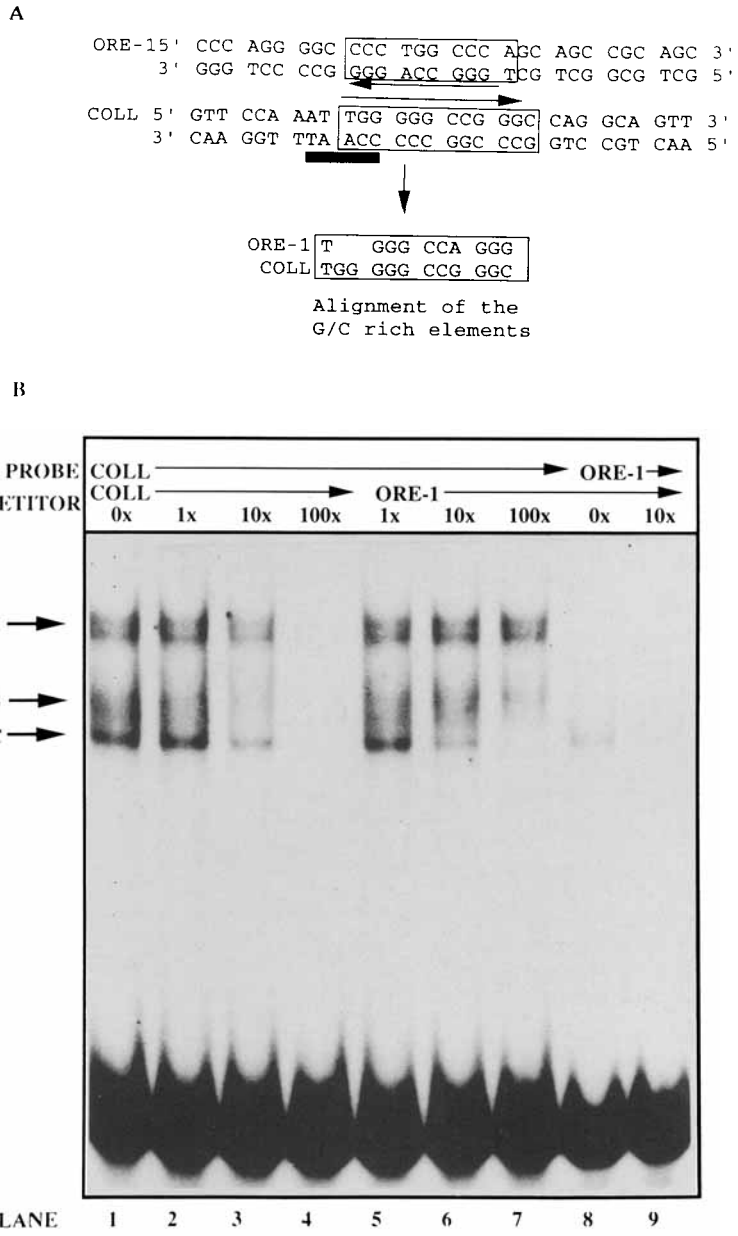

Fig. 3. Electrophoretic mobility shift comparisons of the ORE-1 and a collagen type I (α1) promoter sequence (**A, B,** and **C,** arrows). **A:** The osteocalcin promoter repressor element-1 (ORE-1) and the similar G/C-rich sequence in the collagen type I (α1) promoter (COLL) are aligned. Note the G/C element in the ORE-1 and COLL are in different orientations relative to the transcription start site. The reverse CCAAT motif in COLL is underlined in bold. **B:** EMS cross-competition analysis of the ORE-1 and COLL sequence. The labelled ORE-1 and COLL oligonucleotides were incubated with ROS17/2.8 nuclear extract. COLL exhibited three bands, each of which was specifically competed by 1-, 10-, and 100-fold molar excess unlabelled COLL competitor (*lanes 1–4*). Cross-competition of the labelled COLL with unlabelled ORE-1 at 1-, 10-, and 100-fold molar excess competitor (*lanes 5–7*) showed that only the lower band C was competed specifically by the ORE-1. The ORE-1 formed a single complex (*lanes 8, 9*) which migrated at the same rate as band C.

Although a single copy of the ORE-1 did not cause a significant effect (data not shown), three copies significantly reduced the activity of the construct relative to the pTK construct to 0.47 ± 0.01 ($P < 0.001$, n = 3) in the NIH3T3 cells. In contrast, a modest decrease in the ROS17/2.8 cells to 0.81 ± 0.11 was not statistically significant ($P = 0.176$, n = 4).

To determine whether ORE-1 showed this repressor activity in NIH3T3 cells, we deleted 63 bp including the ORE-1 sequence from the native promoter context in pOSCAT1, a con-

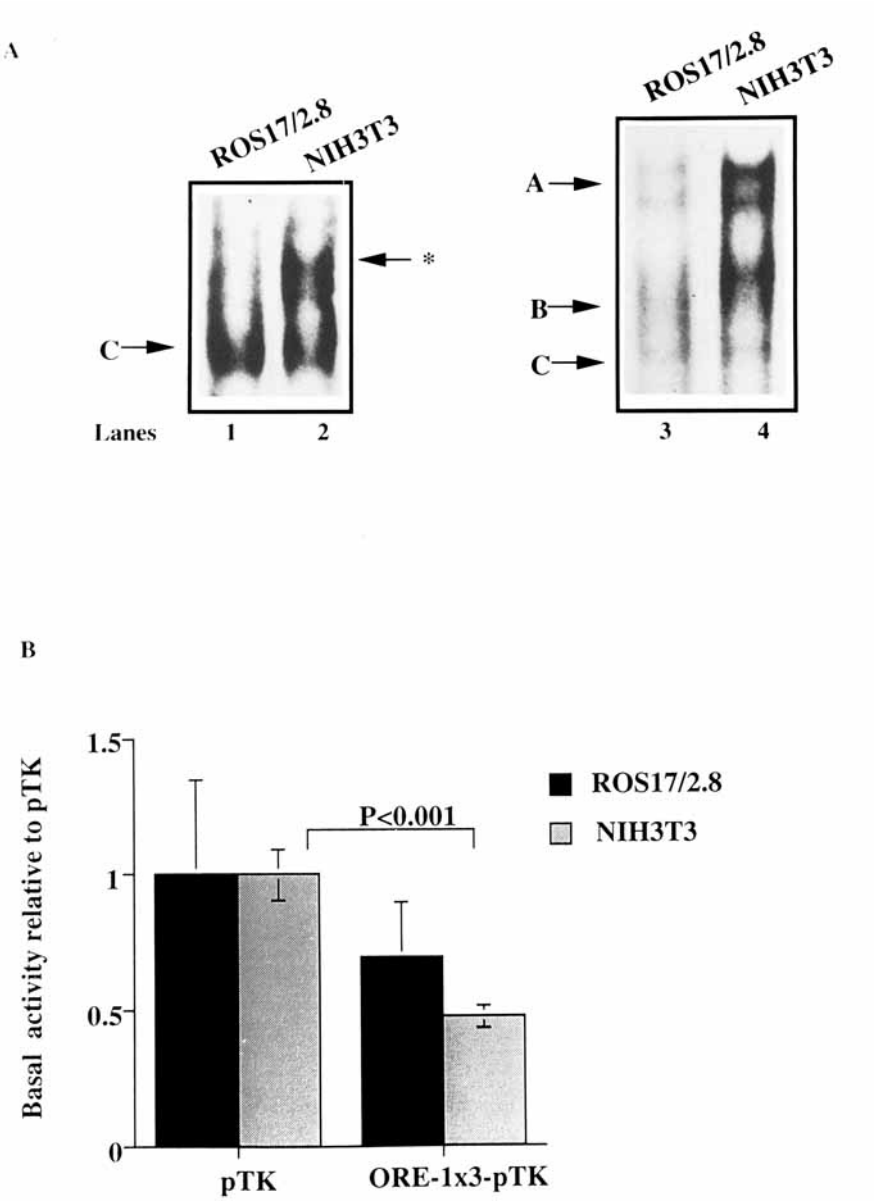

Fig. 4. Electrophoretic mobility shift and functional analyses of the ORE-1 in NIH3T3 and ROS17/2.8 cells. **A:** ORE-1 and COLL EMS assays in NIH3T3 and ROS17/2.8 cells. The labelled oligonucleotides were incubated with NIH3T3 and ROS17/2.8 nuclear extracts. The labelled unbound oligonucleotide is not shown. In the ORE-1 shifts (*lanes 1, 2*), complex C is a common band, whereas the NIH3T3-specific band is indicated by an asterisk. In the COLL shifts (*lanes 3, 4*) complexes, A, B, and C are identified as in Fig. 3. The relatively stronger signal in bands A and B of the NIH3T3 shift was reproducible. **B:** NIH3T3 and ROS17/2.8 cells were transfected with pTK constructs containing three copies of the ORE-1 upstream of the TK promoter. The promoter activity relative to the activity of pTK in each cell line is the mean ± SEM of three independent transfections, and the statistical significance was determined using the Student's *t*-test.

struct that contains 344 bp of the proximal osteocalcin promoter. Consistent with previous data (Fig. 1), the intact pOSCAT1 directed different levels of transcriptional activity in NIH3T3 and ROS17/2.8 cells. With the ORE-1 deleted, however, the levels of transcriptional activity in the two cell types were similar, thus indicating that the ORE-1 sequence causes a threefold transcriptional repression in NIH3T3 cells (Fig. 5). The differing activities of pOSCAT1 with and without the ORE-1 in ROS17/2.8 cells is addressed in the Discussion below.

DISCUSSION

The differential basal activities of the proximal osteocalcin promoter in osteoblastic ROS17/2.8 cells and nonosteoblastic NIH3T3 cells may be attributed to a defined sequence of basal

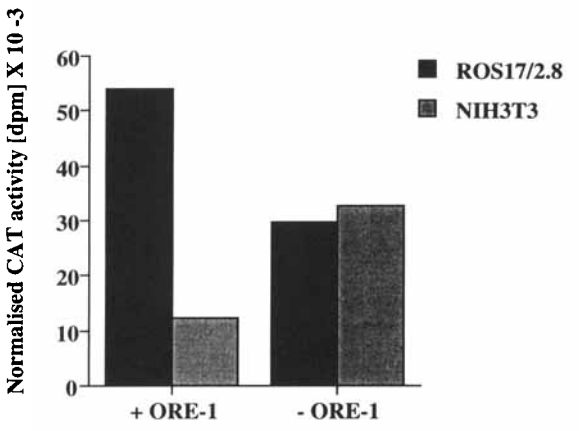

Fig. 5. Differential activity of pOSCAT1 involves a complex of proteins. NIH3T3 and ROS17/2.8 cells were transiently transfected with pOSCAT1 constructs that contain (+ ORE-1) or do not contain (− ORE-1) the ORE-1 sequence. The adjacent sequences 5′ to the ORE-1 were also deleted in these constructs (see Discussion). The basal normalised CAT activity corrected for transfection efficiency is a representative of two independent transient transfections.

regulatory elements. We identified ORE-1, a 30 bp sequence between −199 and −169 that caused a significant threefold repression of promoter activity in the NIH3T3 cells but no significant repression in the ROS17/2.8 cells. Further transfection studies using reporter constructs carrying heterologous and homologous promoters with or without this 30 bp sequence (Figs. 2, 5), confirmed the NIH3T3-specific repressive function of the ORE-1. In agreement, EMS analysis demonstrated that the complex bound to the ORE-1 was related to a complex formed by a G/C-rich repressor element in the collagen type I (α1) promoter sequence. Comparison of the ORE-1 shifts in the two cell lines demonstrated an NIH3T3-specific complex which may represent the DNA-protein interaction responsible for the observed repression. This combination of transfection and EMS experiments indicated that the ORE-1 binds a factor that, at least in part, mediates transcriptional repression of the osteocalcin promoter in NIH3T3 cells.

Three complexes bound to the 30 bp collagen type I (α1) oligonucleotide, and mutational analysis showed that only one complex was related to the G/C element. The presence of other complexes suggests that other DNA binding sites exist in the COLL sequence. Analysis of the COLL sequence shows that there is a reverse CCAAT binding site (Fig. 3A), which has been shown to be bound by the CCAAT binding factor (CBF) and nuclear factor 1 (NF1) [Karsenty and de Crombrugghe, 1990; Nehls et al., 1992]. Although we have not defined such interactions in this study, it is likely that binding to one or both of these factors may generate at least one of the other complexes in the COLL shift.

Previous investigators have purified the inhibitory factor-2 (IF-2), which binds to the G/C-rich element (−124 to −113) in the collagen type I (α1) promoter. The IF-2 consisted of two polypeptides with very similar migration rates, each capable of independently binding the COLL sequence [Karsenty et al., 1991]. Similarly, our Scatchard analysis of the complex bound to the ORE-1 indicated that there are two high affinity protein-DNA interactions, supporting the concept that the same factor consisting of two polypeptides binds to the similar G/C-rich elements in the collagen type I (α1) and osteocalcin promoters (Fig. 2). In additional EMS experiments on high resolution gels, the ORE-1 shift displayed a doublet banding pattern (data not shown).

The ORE-1 shift with NIH3T3 cells demonstrated a second more slowly migrating complex that was not present in the ROS17/2.8 shift (Fig. 4). It is possible that this additional factor is a repressor, responsible for the repressive effect of the ORE-1 seen in NIH3T3 cells. Phospor-image analysis of the COLL shift in NIH3T3 cell extracts showed that the intensity of bands A and B was increased, whereas band C was constant in the two cell extracts. In the present analysis of the ORE-1 element we have not addressed how these different intensities in the COLL shift may relate to the G/C element. Interestingly, in other studies from our laboratory, mutation of the COLL G/C element significantly reduced band A, suggesting that it, not band C, relates to the G/C element [Goldberg et al., submitted]. These apparently contradictory findings with the COLL sequence have not yet been reconciled; however, this does not diminish the fact that the EMS data, in agreement with the transfection studies, suggest that there is an extra protein from NIH3T3 cells which binds the G/C element in the ORE-1 sequence.

Removing from the osteocalcin promoter the region from −226 to −163, which encompasses the ORE-1, caused a threefold increase in relative transcriptional activity in NIH3T3 cells. This supports the concept that the G/C element functions as an inhibitory sequence in NIH3T3 cells but raises the issue of whether the G/C element is the only functional sequence in the

deleted 63 bp region. Although it does not rule out the presence of other functional sequence elements, the magnitude of the increase in NIH3T3 cells is consistent with the G/C element being the only functional element in those cells. However, in the ROS17/2.8 cells, the increase in promoter activity after deletion of the 63 bp (Fig. 5) suggests that there may be DNA-protein interactions involving positive regulatory factor(s) in this osteoblastic cell line. The reverse CCAAT motif upstream of the ORE-1 which is conserved in the rat promoter and contained within the 63 bp sequence in question may contribute to transcriptional activity in

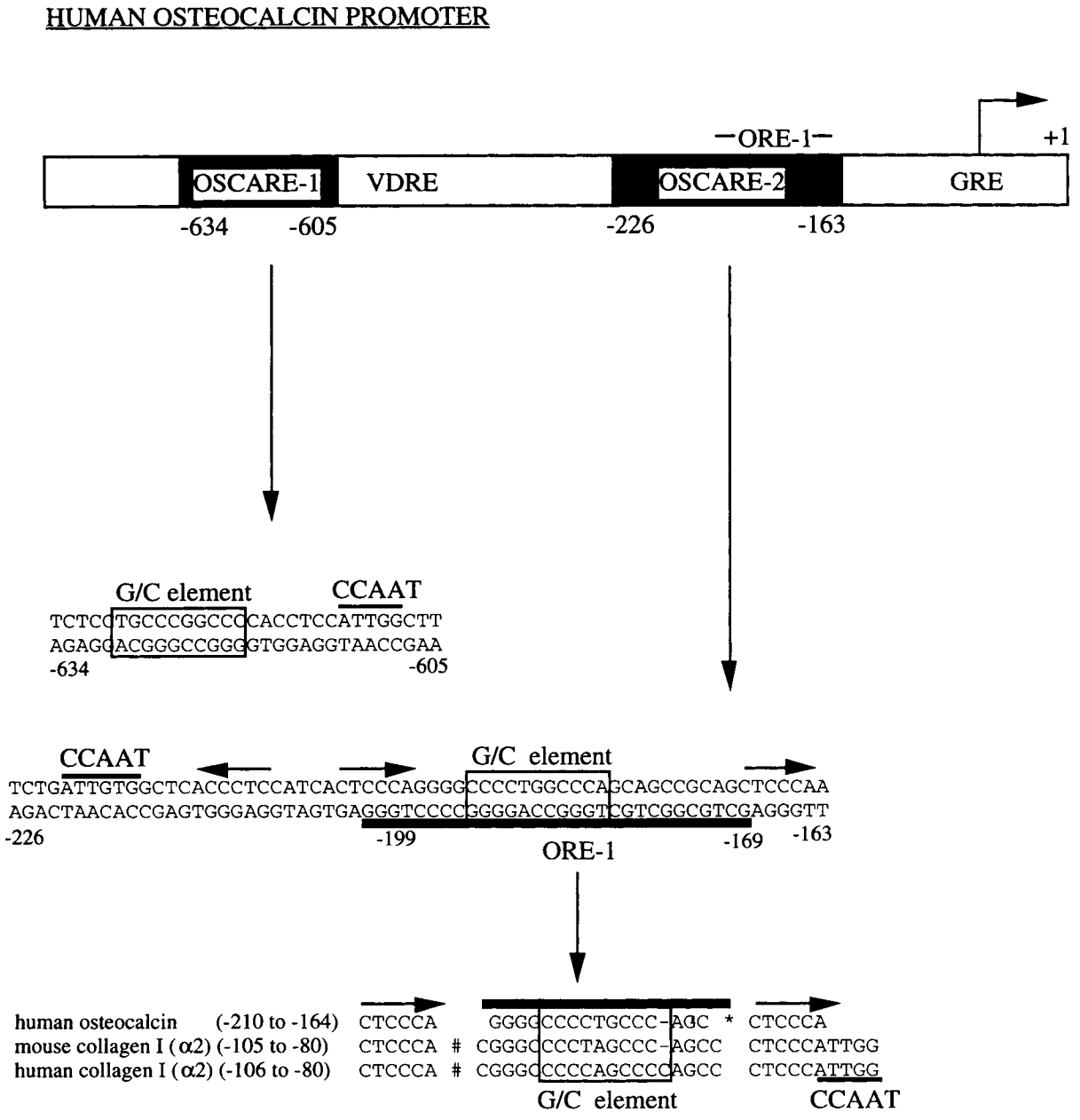

Fig. 6. The G/C-CCAAT regulatory unit is found in osteocalcin and collagen promoters. The G/C-CCAAT unit has a reverse CCAAT motif adjacent to a G/C-rich element. In the osteocalcin promoter there are two functional G/C-CCAAT units, and in the collagen type I (α2) promoter is a potential unit. CTCCCA motifs (*arrowed*) flanking the ORE-1 sequence (*underlined*) are conserved in the homologous region of the human and mouse collagen type I (α2) promoters (*overlined*). Dashes and spaces are inserted to best align the CTCCCA motifs. # represents 12 bp not shown from the mouse and human collagen type I (α2) promoters and * represents the sequence AGCCGCAG not shown from the human osteocalcin promoter.

ROS17/2.8 cells. Other studies have shown that the conserved sequence (TCTGATTGTGT) binds a multisubunit CAAT factor complex and contributes to high basal expression of the rat osteocalcin promoter in these cells [Towler et al., 1994]. Furthermore, the demonstration that the reverse CCAAT motif can function as a control element (see next paragraph) support this suggestion.

In the collagen I (α1) promoter, the G/C-rich sequence overlaps a reverse CCAAT motif. It has been suggested that these two elements function together to modulate basal promoter activity in NIH3T3 cells, with repressor proteins binding the G/C-rich sequence and enhancer factors binding the reverse CCAAT motif [Nehls et al., 1992]. A similar G/C-CCAAT regulatory unit in the distal region of the osteocalcin promoter (OSCARE-1) (Fig. 6) also showed repression through the G/C-rich sequence in NIH3T3 cells, with the positive activity of this unit in ROS17/2.8 cells being attributed both to the absence of repressor protein and to the ubiquitous presence of the CCAAT binding factor [Goldberg et al., submitted]. The results of the present study suggest that the G/C-CCAAT unit in the proximal osteocalcin promoter, designated here as OSCARE-2, may also fit this model (Fig. 6).

The G/C-CCAAT unit is conserved between species in the osteoblast-expressed collagen type I (α1) gene promoter [Brenner et al., 1989]. In collagen type I (α2) there is also a region conserved between species that is highly homologous to the ORE-1 sequence, possessing a G/C-rich region close to a functional reverse CCAAT motif (−84 to −80) [Dickson et al., 1985; Schmidt et al., 1984] (Fig. 6). Another highly conserved sequence motif, CTCCCA, present in multiple copies in both osteocalcin and collagen type I (α2) promoters, may play an as yet undefined role in osteocalcin regulation (Fig. 6). A similar motif, ACCTCTCT, found in the first exon of the rat osteocalcin gene promoter has been recently assigned an antisilencer function [Frenkel et al., 1994]. Perhaps the CTCCCA motifs in OSCARE-2 have similar roles.

In summary, the repressed basal activity of the proximal osteocalcin promoter in nonosteoblastic NIH3T3 cells compared to osteoblastic ROS17/2.8 cells may be the sum of the activities of several different regulatory elements. We show that a 30 bp region has strong repressive activity in NIH3T3 cells and suggest that a G/C-rich sequence within this region binds a repressor factor in activity similar to that of the G/C-rich inhibitory element in the collagen type I (α1) promoter. In agreement with studies of the collagen promoter, we propose that the G/C element that binds repressor factors acts in combination with a nearby reverse CCAAT motif which binds ubiquitous positive factors [Nehls et al., 1992]. The identification of this G/C-CCAAT regulatory unit in three osteoblast-specific genes—collagen type I (α1) and (α2) and osteocalcin—may indicate that this unit plays a regulatory role in osteoblast-specific gene expression.

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