The Vitamin D Response Element in the Distal Osteocalcin Promoter Contributes to Chromatin Organization of the Proximal Regulatory Domain

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SUMMARY

The vitamin D receptor (VDR) and Runx2 are key regulators of tissue-specific gene transcription. Using the bone-related osteocalcin (OC) gene, we have previously shown that Runx2 is required for the extensive chromatin remodeling that accompanies gene activation. Here, we have addressed the direct contribution of the VDR to chromatin remodeling events necessary for regulation of OC transcription using mutational analysis. Our studies demonstrate that both the distal and proximal DNaseI hypersensitive sites characteristic of the transcriptionally active OC promoter are not enhanced in the absence of a functional vitamin D response element (VDRE). Furthermore, restriction enzyme accessibility studies reveal that nucleosomal reorganization of the proximal promoter occurs in response to vitamin D and this reorganization is abrogated by mutation of the VDRE. These findings indicate that binding of liganded VDR in the distal promoter directly impacts the chromatin structure of the proximal promoter. We find that in the absence of functional Runx sites, the VDR cannot be recruited to the OC promoter and therefore, the VDRE is not competent to mediate vitamin D responsiveness. On the other hand, chromatin immunoprecipitation assays show that Runx2 association with the OC promoter is not significantly impaired when the VDRE is mutated. ChIP assays also demonstrate that basal levels of histone acetylation occur in the absence of Runx2 binding but that the VDRE and vitamin D are required for enhanced acetylation of histones H3 and H4 downstream of the VDRE. Together our results support a step-wise model for chromatin remodeling of the OC promoter and show that binding of the liganded VDR/RXR directly impacts both the distal and proximal regulatory domains.
INTRODUCTION

The *in vivo* effects of vitamin D are largely mediated by a specific vitamin D receptor (VDR), which belongs to the steroid and thyroid hormone receptor superfamily (1-3). The VDR is a ligand-dependent transcription factor that recognizes and binds to cis-acting vitamin D response elements (VDREs) in the promoter regions of target genes to induce or repress their transcription (4;5). Bone is one of the major physiologic sites for the biological action of vitamin D.

Osteocalcin (OC) is a bone-specific gene whose transcription is directly regulated by vitamin D (6-8). Transcriptional analysis has established the presence of a VDRE between nucleotides -461 and -445 of the rat osteocalcin promoter (9-12). Enhancement of OC expression in response to vitamin D occurs only after basal tissue-specific transcription is initiated by bone-specific factors, and the extent of responsiveness is inversely related to the level of basal expression.

Initiation of basal transcription of the rat osteocalcin gene, as well as enhancement by vitamin D, is accompanied by changes in the structural properties of chromatin (13;14). In proliferating osteoblasts and cell lines that do not express OC, the promoter is uniformly packed in nucleosomes, which suppress transcription. However, in differentiated osteoblasts in which the OC gene is transcriptionally active, rearrangement of chromatin in the promoter region results in the appearance of two DNaseI hypersensitive (DHS) sites. The DHS sites are localized to regions of the promoter that contain important transcriptional elements: the distal site (-600 to -400 bp) spans the vitamin D response element (VDRE), two Runx binding sites and a YY1 binding motif, while the proximal site (-170 to -70 bp) includes a Runx element, a C/EBP binding site and a Dlx5/Msx2 homeodomain motif (15-18). These changes in DNaseI
accessibility are accompanied by changes in nucleosome placement; in fact when OC is being actively transcribed there is a single positioned nucleosome between the distal and the proximal DHS domains (13). Treatment of ROS 17/2.8 cells with vitamin D results in significant intensification of the DHS sites, concomitant with increased OC transcription (13). Recent studies have shown that the transcriptionally active rat OC gene promoter is associated with acetylated histones H3 and H4 and that acetylation of these histones is increased in response to vitamin D (19).

Binding of transcription factors to their cognate elements on a promoter allows the recruitment of coactivators, chromatin remodeling complexes and/or histone modifying enzymes, which result in the reorganization of the chromatin necessary for transcriptional activation of a gene (20-26). Indeed, studies have shown that the bone tissue-specific factor Runx2 interacts with liganded VDR to and the histone acetyltransferase p300 to activate the OC promoter (27,43). Moreover, mutation of all Runx binding sites results in loss of both vitamin D responsiveness and DNaseI hypersensitivity (28). It has also been reported that liganded VDR transactivates by physically interacting with both the VDRE and the general transcription factor TFIIB, which is an essential component of the transcriptional initiation complex (4,29,30).

Given these examples of crosstalk between the VDR and key transcriptional regulators, it is important to determine the role that the VDRE, and binding of liganded VDR to the OC promoter, may have in the chromatin remodeling process that accompanies OC transcriptional activation.

In these studies we demonstrate that the vitamin D response element in the OC promoter influences the nucleosomal organization and the DNase hypersensitivity of not only the distal VDRE region, but also the proximal promoter domain. While it has been shown that Runx2 and
the VDR functionally interact to regulate OC gene transcription, our chromatin
immunoprecipitation studies demonstrate that the VDRE is not essential for recruiting Runx2 to
the OC promoter. However, Runx2 is required for vitamin D-dependent VDR association in the
OC VDRE. Because we find that mutation of the VDRE abrogates essential chromatin
alterations in both the proximal and distal OC promoter, our results suggest that binding of the
VDR/RXR contributes to establishment of a remodeled chromatin state that assures
physiological, vitamin D-mediated transcription.

EXPERIMENTAL PROCEDURES

Plasmids---Construction of the reporter plasmids pOCZCAT, VDRE mutant (mVDRE originally
named mSHE) and mRunx (all Runx sites mutated, originally named mABC) has been
previously described (28;31).

Construction of Stable Cell Lines---ROS 17/2.8 cells with genomically integrated wild type,
Runx and VDRE mutant constructs were generated by the calcium phosphate method (32). For
each construct, four 100 mm plates were transfected with 15 µg of the -1.1 OC-CAT plasmid and
5 µg of pCEP-4 (Invitrogen, San Diego, CA) encoding the Hygromycin B phosphotransferase
gene. Cells were harvested at 95% confluency and replated for selection in media (F12
supplemented with 5% fetal calf serum) containing 55 U/ml Hygromycin B (Calbiochem, La
Jolla, CA) based upon preliminary killing curves. Resistant colonies (60-75) from each plate
were pooled and propagated as polyclonal cell lines. Each pool was expanded until 2x10^8 cells
were available for preparation of frozen stocks. Monoclonal cell lines were generated by limited
dilution of different polyclonal parental cell lines. Cells were routinely maintained in media
containing Hygromycin B for measuring CAT activity and responsiveness to steroid hormones and growth factors.

Stable cells were plated at 2x10^5 cells/well in a six well plate and treated with 10^-8 M vitamin D for 24 hours. Cells were washed twice with phosphate buffered saline and lysed by adding 300 µl of Reporter Lysis Buffer (Promega Corp., Madison, WI) at room temperature for 30 minutes. CAT activity assays were performed as described previously (33).

**Transient Transfection and CAT Reporter Assays**---ROS 17/2.8 monoclonal stable cell lines were plated at a density of 8x10^4 cells/well in six well plates and transfected 24 hours later with 0.7 µg of Runx expression construct. Cells were transfected with DNA in the presence of 7 µl/well of SuperFect (Qiagen Inc., Valencia, CA) and incubated at 37°C for 2.5 hours with occasional swirling. The transfection mix was aspirated and cells were washed twice with phosphate buffered saline (PBS) and then incubated at 37°C in F12 medium supplemented with 5%(v/v) fetal calf serum for 24-48 hours. Cells were washed twice with ice cold PBS and lysed with 300 µl of Reporter Lysis Buffer (Promega Corp., Madison, WI) at room temperature for 30 minutes. Cell lysates were collected and stored at -70°C or used immediately for CAT assay as described previously (33).

**Studies of DNaseI Hypersensitive Sites and Restriction Enzyme Accessibility**---DNaseI digestion and restriction enzyme analysis were performed according to the indirect end labeling method (34). ROS 17/2.8 cells were plated at a density of 1x10^6 cells per 100 mm plate and nuclei were isolated on day 9 by Dounce homogenization (loose pestle) in eight volumes of RSB buffer (10 mM Tris HC1 pH 7.4; 10 mM NaCl; 3 mM MgCl2) with 0.5%(v/v) NP-40. To evaluate cell lysis, an aliquot of nuclei was stained with 0.4%(w/v) Trypan blue 1:1(v/v).
The nuclear suspension was diluted by adding an equal volume of RSB buffer and nuclei were collected by centrifugation. The pelleted nuclei were resuspended in RSB buffer and the DNA concentration was estimated by absorption at 260 nm. Aliquots of twenty A260 units were digested with increasing concentrations of DNase I (0 to 5 units) (Worthington Biochemicals, Freehold, NJ) in 1 ml final volume for 10 minutes at room temperature, or with 500 U/ml of restriction enzyme, in their corresponding buffer, for 30 min at 37°C. The reaction was stopped by adding EDTA, SDS, RNase One (Promega, Madison, WI) and Proteinase K (Fisher Biotech, Fairlawn, NJ) to a final concentration of 25 mM, 0.5%(v/v), 1 U/ml and 200 µg/ml, respectively, and incubated at 37°C overnight. The samples were extracted once with phenol:chloroform:isoamyl alcohol (25:24:1) and twice with chloroform:isoamyl alcohol (24:1). Nucleic acids were precipitated with 2.5 volumes of ethanol at -70°C for at least 4 hours and then resuspended in 25 mM Tris pH 7.8. DNA was digested with BamHI or XbaI (4 units/µg of DNA) to release a 4.3 kb fragment from the endogenous OC gene (14) or a 2.7 kb fragment from the chromosomally integrated pOCZCAT wild-type or mutated fusion genes, respectively (35). The digested DNA was extracted with phenol:chloroform, precipitated with ethanol and resuspended in 25 mM Tris pH 7.8. DNA samples (10 µg) were electrophoresed in a 1.2% agarose gel (Biorad, Hercules, CA) and then transferred to a nylon membrane (Hybond N+, Amersham Pharmacia Biotech, Arlington Heights, IL) according to the manufacturer’s instructions. Hybridization probes were prepared by restriction digestion of pOC 3.4 (8), containing the rat OC gene and flanking sequences, with XbaI-BamHI and pOCZCAT with XbaI-NcoI. The probes were labeled by the random primer method using α32P-dCTP and the Stratagene Prime-It II kit (Stratagene, La Jolla, CA). Hybridization was carried out at 65°C with
1 ng of probe (10⁹ cpm/µg specific activity) per 10 cm² membrane. The blots were analyzed by autoradiography or by using a STORM Phosphorimager (Molecular Dynamics, Sunnyvale, CA).

**Chromatin Immunoprecipitation (ChIP) Assays**—Chromatin Immunoprecipitation (ChIP) assay was performed as described elsewhere (36) with some modifications. Cells were treated with 1%(v/v) formaldehyde at 37°C for 10 min. Crosslinking was stopped by adding glycine to a final concentration of 0.125 M. Cells were washed with ice cold PBS, collected by centrifugation at 165 xg for 5 min at 4°C. The cell pellet was resuspended in lysis buffer (25 mM HEPES pH7.8, 1.5 mM MgCl2, 10 mM KCl, 0.1%(v/v) NP-40, 1X C®mplete, 25 µM MG-132) and incubated on ice for 10 min. Following Dounce homogenization (20 strokes, pestle A), the nuclei were collected by centrifugation at 750 xg for 5 min, resuspended in sonication buffer (50 mM HEPES pH7.9, 140 mM NaCl, 1 mM EDTA, 1%(v/v) Triton X-100, 0.1%(v/v) Na-deoxycholate, 0.1%(v/v) SDS, 1X C®mplete, 25 µM MG-132) and sonicated on ice to a DNA size of 200-800 bp. For ChIPs using the αVDR antibody, RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS, 1X PBS) was used to resuspend and sonicate nuclei. The samples were centrifuged at 16,000 xg for 15 min and precleared with A/G plus- agarose beads precoated with 2 µg/ml sonicated salmon sperm DNA and 1 mg/ml BSA. Thirty A260 units of the precleared chromatin were immunoprecipitated with 5 µg of antibody and the immuno complexes were collected by binding to A/G plus- agarose beads. The beads were washed twice with each of the following buffers: sonication buffer, sonication buffer containing 500 mM NaCl, LiCl buffer (20 mM Tris pH 8, 1 mM EDTA, 250 mM LiCl, 0.5%(v/v) NP-40, 0.5%(v/v) Na-deoxycholate) and 10 mM Tris pH 8. The immuno complexes were eluted in 50 mM Tris pH 8, 1 mM EDTA and 1%(v/v) SDS at 65°C for 15 min, adjusted to 200 mM NaCl.
and incubated overnight at 65°C to reverse the crosslinking. Following treatment with RNase One (1 U/ml) and Proteinase K (20 µg/ml), the samples were purified using PCR purification kit from Qiagen Inc. One tenth of the immunoprecipitated DNA and input DNA were analyzed by PCR using oligos described in Table 1. PCR amplifications (26 cycles) were performed in the presence of 0.1 µCi α32P-dCTP per reaction and the products were resolved in 4% polyacrylamide gels.

The antibodies used were: αAcetylated H3 and αAcetylated H4 from Upstate Biotechnology Inc. (Lake Placid, NY), αVDR (GR37) from Oncogene (Calbiochem Novabiochem-Oncogene, (Cambridge, MA), αRNA polymerase 2(8WG16), αPhosphorylated RNA polymerase II CTD-Ser-2P (H5) from Covance (Berkeley, CA), and αRunx2 (M-70) from Santa Cruz Biotechnology Inc. (Santa Cruz, CA).

RESULTS

In order to assess the contribution of the VDRE in regulating native OC promoter activity and its potential role in the chromatin remodeling that accompanies OC gene expression, we established a new series of monoclonal stable cell lines of rat osteoblastic ROS 17/2.8 cells. To permit quantitation of chromatin alterations, these cells harbor fewer than ten copies each of a 1.1 kb OC promoter fused to the CAT reporter with point mutations in each of the two steroid half elements of the VDRE (mVDRE) (Fig. 1) or in the three Runx binding sites (mRunx, (28)). Although both YY1 and AP-1 binding sites partially overlap the VDRE, the introduced mutations do not affect binding of AP-1 or YY1 to their respective binding motifs (31).

We initially tested the ability of vitamin D to induce promoter activity of either the wild type (pOCZCAT) or the mutated transgenes in the chromatin context of each cell line (Fig. 2).
The mVDRE-CAT transgene completely lacks responsiveness to vitamin D, confirming the loss of VDRE function and VDR-DNA interaction. In contrast, the wild-type transgene shows a 4-fold increase in promoter activity upon treatment with vitamin D (Fig. 2). For comparison, we also examined the stable cell line carrying the mutant mRunx transgene (where all Runx binding sites are mutated). The mRunx transgene has an intact VDRE, yet exhibits a complete loss of vitamin D responsiveness and DNaseI hypersensitivity (Fig. 2 and (28)). The effectiveness of vitamin D treatment in these cell lines was monitored by measuring secreted osteocalcin levels as a representation of endogenous OC expression. All cell lines presented a 3-6-fold induction of the endogenous OC gene in response to vitamin D (data not shown). Thus, these results demonstrate that all cell lines maintain their osteoblastic phenotype and that the loss of vitamin D responsiveness of the mVDRE and mRunx OC transgenes is due to the absence of these functional binding sites.

**Mutation of VDRE or Runx Elements Differentially Alters the Chromatin Structure of the OC Promoter**---Previous studies from our laboratory have shown the presence of DNaseI hypersensitive sites in the endogenous OC gene and in transgenes of stable cell lines with an integrated OC promoter (28;37). Because the distal hypersensitive site (DHS II) encompasses the VDRE and vitamin D induction of OC transcriptional activity results in increased DNaseI accessibility of the OC promoter, we examined the effect of the VDRE mutation on nuclease hypersensitivity under basal conditions and in response to vitamin D-induction (Fig. 3). Figure 3A (middle panel) shows the results of DNaseI digestion of nuclei isolated from the ROS 17/2.8 cell line carrying the mVDRE transgene. Although the mutated VDRE element no longer binds the VDR/RXR complex (31), both the distal and the proximal DHS sites are present. However,
we note that in contrast to the endogenous wild type OC gene (Fig. 3B), the distal DHS encompassing the mVDRE is relatively weaker than the proximal DHS site (compare controls Fig. 3A middle panel and Fig. 3B). Furthermore, while vitamin D treatment results in enhanced DNaseI accessibility of the WT OC gene (Fig. 3B and (14)), the mVDRE OC transgene becomes less hypersensitive in response to vitamin D, especially at the distal DHS (Fig. 3A middle panel). The presence of the DNaseI hypersensitive sites in the mVDRE transgene is in striking contrast to the complete absence of nuclease hypersensitivities of the OC promoter upon mutation of the Runx sites flanking the VDRE and in the proximal promoter (mRunx, Fig. 3A lower panel).

Taken together, these results demonstrate that appearance of the two DHS associated with initial chromatin remodeling during OC gene activation does not require VDR binding to the VDRE. Nonetheless, the altered relative DNase hypersensitivity of the distal and the proximal promoters in the absence of VDR/RXR interaction shows that the VDRE contributes to the normal vitamin D-induced chromatin organization of the OC gene.

To further characterize the role of the VDRE in supporting chromatin structure of the OC gene, we examined the accessibility of the mVDRE transgene to restriction endonucleases. This approach identifies modifications in nucleosome positioning because the presence of a nucleosome at a restriction enzyme site interferes with cleavage (38). We selected restriction sites in the proximal and distal promoter regions that undergo extensive remodeling during both OC gene activation and vitamin D mediated transcriptional enhancement (14). The digestions were performed on nuclei isolated from ROS 17/2.8 stable cell lines carrying either the mVDRE transgene or, for comparison, the mRunx transgene (Fig. 4). As expected, the endogenous OC gene is highly accessible to all the restriction enzymes tested (Fig. 4, top panel, 4.3 kb parental band), and vitamin D treatment results in an enhanced cleavage by PstI, PvuII and HincII, but
not Bgl II (compare the relative intensities of the bands in the (-) versus (+) lanes; see also ref. (14)). Quantitation of the results (percent digestion by each enzyme) under basal conditions and in response to vitamin D treatment is presented in Table 2. Thus, treatment with vitamin D results in altered nucleosome organization of both the distal and proximal regions of the endogenous OC promoter.

In striking contrast to the wild-type OC gene the mRunx transgene, which neither presents DHS sites nor responds transcriptionally to vitamin D (Figs. 2, 3A bottom panel and (28)), exhibits a significant decrease in accessibility to all of the restriction enzymes assayed (see Table 2). Especially noteworthy is the nearly complete loss of cleavage by HincII, which indicates that a nucleosome resides over the distal OC promoter in the absence of Runx binding. As expected, no changes in restriction accessibility are observed upon treatment with vitamin D. Therefore, our results demonstrate that the presence of an intact VDRE (as in the mRunx promoter) is not sufficient to ensure vitamin D mediated chromatin remodeling.

Mutation of the VDRE (mVDRE), on the other hand, does not alter the overall pattern of restriction enzyme accessibility seen for the endogenous OC gene under basal conditions (Fig. 4 and Table 2, compare control values for the WT versus mVDRE). However, we observe that the extent of digestion by PstI, PvuII (within the proximal promoter), and BglII (between the VDRE and the positioned nucleosome) is lower when the VDRE is mutated (Table 2, compare mVDRE and WT control rows), while the level of HincII digestion (in the distal promoter) is essentially the same in the wild type and the mVDRE promoters (Table 2). We observe that the mVDRE transgene shows no change in restriction enzyme cleavage in either the distal or proximal promoter upon vitamin D treatment (Fig. 4 and Table 2), reflecting the loss of a transcriptional
response to vitamin D (Fig. 2). Importantly, our results reveal that the distal VDRE is necessary for vitamin D-dependent chromatin reorganization of the proximal OC regulatory domain.

Taken together, the decreased DNase hypersensitivity in the distal OC promoter (Fig. 3) and the altered restriction enzyme accessibility downstream of the mVDRE (proximal promoter, Fig. 4) demonstrate a significant contribution of the WT VDRE to the chromatin organization required for accessibility of regulatory factors to both the proximal and distal promoter domains. These findings of chromatin alterations influenced by mutation of the VDRE are consistent with known interactions of the distal VDR with the TFIIB and TFIIA basal transcription factors to support physiological levels of OC transcription.

Chromatin Dynamics at the OC Promoter are Altered by Mutation of the VDRE—Chromatin remodeling at the WT OC promoter in response to vitamin D, which results in enhanced transcription, is accompanied by increased acetylation of both histones H3 and H4 (19). Thus, to understand the contribution of the VDRE to vitamin D-induced histone modifications at the OC promoter, we performed chromatin immunoprecipitation (ChIP) assays. To distinguish the endogenous OC promoter from the transgene, we designed primers with 3’ nucleotides carrying either the wild type or the mutated sequences (Table 1). These primers were first tested for their ability to selectively amplify the mutant versus the wild type gene. As shown in figure 5A (right panel), under the PCR conditions used, each set of primers selectively amplifies the respective promoter.

We then proceeded to evaluate the acetylation status of the histones associated with the WT and mutated (mVDRE and mRunx) OC promoters in response to vitamin D. Results from ChIP studies are shown in figure 5A with quantitative analysis from three experiments shown in
We find that the endogenous OC gene and each of the mutant transgenes are associated with acetylated histones under basal conditions. Thus, histone acetylation at the OC promoter in osteoblastic cells occurs in the absence of VDR or Runx2 binding. For comparison, we examined histone modifications at the myogenin locus, a muscle specific gene; acetylated H3 and H4 histones were not detected, consistent with the silent state of this gene in osteoblasts (Fig. 5A). Vitamin D treatment results in 2 fold increased acetylation of both H3 and H4 histones at the endogenous OC locus (19). However, no significant changes in acetylation were observed at either the mVDRE or the mRunx transgene promoters in response to vitamin D (Figs. 5A and 5B). Our results also show that RNA pol II is present on both the wild type and the mutant promoters; however, in response to vitamin D, a significant increase in association of RNA polymerase II was observed only for the wild-type OC promoter (Figs. 5A and 5B). Similar results were obtained using an antibody that detects the hyperphosphorylated (elongating) form of RNA pol II (data not shown). Thus, the absence of increased histone acetylation and pol II recruitment are consistent with the loss of vitamin D response of the mVDRE and mRunx promoters for transcription, DNaseI, and restriction enzyme accessibility.

We next addressed whether loss of vitamin D responsiveness of the mVDRE and mRunx promoters is directly attributable to absence of binding of the VDR or is a consequence of failed recruitment of Runx2 to the promoters. We performed ChIP assays with the WT, mVDRE and mRunx promoters using antibodies against VDR and Runx2 in control and vitamin D treated cells (10^{-8} M for 4 hours, Fig. 6). Our results demonstrate that VDR association with the OC promoter is dependent on the presence of vitamin D and a wild type VDRE; no binding of the VDR to the mVDRE promoter was detected (Fig. 6A). We also find that Runx2 is recruited equally to the WT and mVDRE promoters and its presence is reproducibly increased (1.5-2 fold)
at the WT promoter in response to vitamin D. Interestingly, when the Runx sites are mutated, there is a complete loss of VDR binding as well as the expected absence of Runx2 association with this promoter (Fig. 6B). This result provides a mechanism for the lack of a vitamin D transcriptional response of the mRunx promoter. Our findings support the conclusion that the increase in histone acetylation in response to vitamin D in the WT promoter is related to VDR interaction at the distal VDRE, and not to altered Runx2 binding.

Taken together, all the parameters of chromatin structure that we have examined show that association of the liganded VDR with the distal VDRE alters chromatin dynamics of the proximal, as well as the distal, OC promoter.

DISCUSSION

We have previously shown that extensive chromatin remodeling accompanies both transcriptional activation and vitamin D-mediated up regulation of the OC gene (13;14). This remodeling results in a positioned nucleosome separating two DHS domains one in the distal and the other in the proximal promoter. Here we have addressed the role that liganded VDR plays in the chromatin reorganization that occurs in the OC promoter upon induction with vitamin D. Our studies have established that in the absence of a functional VDRE the two DHS sites remain in the promoter. However, their intensities are not enhanced in response to vitamin D, which is a signature of the WT OC promoter. Interestingly, this key observation suggests that chromatin remodeling is impaired both in the distal domain where the VDRE is located, and in the proximal promoter region. Further analysis using restriction enzyme accessibility of the mVDRE OC transgene promoter (see figure 4 and Table 2) demonstrates that binding of the liganded VDR to
the VDRE contributes to maintenance of the positioned nucleosome that resides between the distal and the proximal regulatory domains in the transcriptionally active OC gene. The lack of responsiveness of the mVDRE promoter to vitamin D at the proximal DHS I and at the restriction sites assayed, both in the distal and proximal regions, suggests that binding of the liganded VDR may serve as a bridge between the proximal and distal domains. Several in vitro and in vivo studies with different vitamin D responsive genes have shown that the VDR/RXR heterodimer can simultaneously interact with the VDRE and the basal transcription factor TFIIB (29;30;39). Our findings are in agreement with and provide evidence for a working model proposed for OC transcriptional activation, according to which the distal and proximal promoter crosstalk through protein-protein interactions and the presence of a positioned nucleosome between these promoter regions gives the structural flexibility to allow their interaction (40).

It has been reported that upon treatment with vitamin D, a general increase in H3 and H4 histone acetylation is observed both at the whole cell level and at the OC locus (19). However, the lack of increased acetylation after vitamin D induction at the mRunx and mVDRE transgenes reported here demonstrates that the vitamin D mediated enhancement of H3 and H4 histone acetylation observed in the WT OC promoter requires the binding of liganded VDR (Fig. 5 and (19)). Interestingly, our ChIP studies demonstrate that binding of Runx2 to the OC promoter is not prevented by mutation of the VDRE (Fig. 6). As previously reported (28), an OC promoter carrying mutations in all Runx sites (mRunx) does not respond to vitamin D treatment and does not exhibit DNaseI hypersensitivity. In the present work, we show that the accessibility of the mRunx promoter to all the restriction enzymes tested is dramatically reduced, resembling the digestion pattern obtained when the gene is inactive (14). In contrast, histone acetylation levels are similar to those in the WT promoter (Fig. 5) reflecting the basal level of transcription
observed for this transgene. A key finding of our studies is that the VDR cannot associate with the VDRE when the Runx sites are mutated, indicating that the chromatin of the mRunx OC promoter, although acetylated, is incompetent for VDR recruitment.

The sequence of events associated with gene activation has been studied in several genes and it appears that there is no obligate order for function of ATP-dependent remodelers and covalent modifiers that is general for all promoters (41). The precise order seems to depend upon the nature of the promoter, the complement of recruited transcription factors, and the chromatin structure in which the promoter resides. Moreover, it seems that there is no set order of action for chromatin-modifying complexes and complexes of the general transcription machinery (36;42). Taken together, our results support the idea that chromatin remodeling at the OC locus is a step-wise process (Fig. 7). Because acetylated histones are present even when the Runx sites are mutated, we propose that acetylation is an early event in remodeling of the OC promoter. However, in the mutant Runx transgene the DHS sites are not formed and the nucleosome is not positioned, indicating that recruitment of Runx2 is a requirement for these subsequent events. In response to vitamin D the WT OC gene promoter exhibits increased DNaseI hypersensitivity, increased acetylation, and enhanced restriction enzyme accessibility. None of these events occurs in the distal or the proximal promoter when the VDRE is mutated. Taken together these findings provide evidence that chromatin modifications associated with liganded VDR binding stabilize the promoter to facilitate functional interactions between the proximal and distal regulatory domains for physiological control of osteocalcin gene expression.

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FIGURE LEGENDS

Table 1 Primers Used for PCR Reactions: Top panel: The PCR primer pairs used for the detection of the endogenous and mutant OC promoter in chromatin immunoprecipitations assays are illustrated schematically. Bottom panel: Sequence, position and annealing temperature for each one of the primers used.

Table 2 Percent of Restriction Enzyme Digestion: Restriction enzyme accessibility of the endogenous OC and mutant transgenes (mVDRE and mRunx) was assayed in three different monoclonal cell lines for each promoter. Digestion products were analyzed by Southern blots (Fig 5), quantitated by PhosphoImager analysis and expressed as Percent Digestion \[100\times\text{product}/(\text{product} + \text{parental band})\].

Figure 1. Rat Osteocalcin promoter: The position of the VDRE relative to Runx, C/EBP and the two primary transcriptional elements required for basal transcription, the OC-box and the TATA box, are indicated. Lower panel shows the wild type and mutated VDRE sequences used to generate the mVDRE plasmid. The introduced mutations do not affect the binding to the overlapping YY1 and AP-1 motifs in the VDRE (31).

Figure 2. Vitamin D responsiveness of stable cell lines: Loss of vitamin D responsiveness of the rat OC promoter with mutation either in VDRE (mVDRE) or in the three Runx binding sites (mRunx). Five independent monoclonal stable cell lines for each transgene were treated 3 days after plating with $10^{-8}$M 1,25(OH)$_2$D$_3$ and assayed 24 h later for CAT activity. The CAT values
were normalized to total protein in the cell lysate. Each bar represents the mean value of at least three independent determinations for each cell line. The values are plotted as fold induction (Vitamin D/Control).

**Figure 3. DNaseI-hypersensitivity profile of the OC-CAT transgenes and endogenous OC gene:**  
(A) Top panel: Diagrammatic illustration of the OC promoter-CAT transgene showing the region used as probe. Middle and bottom panels: Nuclei isolated from untreated (control) or vitamin D treated (10⁻⁸ vitamin 1,25(OH)₂D₃ for 24 h) ROS 17/2.8 monoclonal stable cell lines were incubated with increasing amounts of DNaseI for 10 min at room temperature. The DNaseI concentrations (U/ml) are designated above each lane of the Southern blot. The DNA was then purified and digested with XbaI to detect the transgene. All samples (10µg) were resolved on a 1.2% agarose gel, transferred to a Hybond N+ membrane and hybridized with the NcoI-XbaI probe. The two DNaseI hypersensitive sites (DHS I and DHS II) are indicated by the solid filled arrows.  
(B) Top panel: Schematic representation of the endogenous OC gene showing the Bam HI-Xba I fragment used as probe. Bottom panel: DNaseI digestion profile for the endogenous OC gene. ROS 17/2.8 cells were processed as described in (A). The DNA was then purified and digested with BamHI to detect the endogenous OC gene. All samples (10µg) were resolved on a 1.2% agarose gel, transferred to a Hybond N+ membrane and hybridized with the XbaI-BamHI probe. The two DNaseI hypersensitive sites (DHS I and DHS II) are indicated by the solid arrows.

**Figure 4. Restriction enzyme digestion of endogenous and mutated OC promoters:** ROS 17/2.8 monoclonal stable cell lines were treated with vitamin D for 24 h. Nuclei were isolated
and digested with different restriction enzymes for 30 min at 37°C. Purified DNA (10 µg) was then digested either with BamHI (endogenous gene) or XbaI (transgenes) to release the indicated gene fragment (4.3 and 2.8 kb respectively), fractionated electrophoretically in a 1.2% agarose gel, transferred to Hybond N+ membrane and hybridized with the corresponding probe (shown in schematics, right panel). Top panel: Restriction digestion profile of endogenous OC promoter from mVDRE cell line. Middle panel: Restriction digestion profile of the mVDRE transgene. Bottom Panel: Restriction digestion profile of mRunx transgene. Right side of the figure has a schematic representation of each promoter showing the mutant binding sites and enzyme restriction digestion sites. The ratio of the digestion products to the parental band (4.3 or 2.8 kb) indicates the extent of nuclease accessibility (see Table 2). Arrows indicate the restriction sites for the enzymes used; dots indicate the position of the three fragments released from the transgenes by digestion with Pvu II.

**Figure 5. Association of acetylated Histone H3, H4 and RNA polymerase II with endogenous and mutated OC promoters:** Chromatin immunoprecipitation assays were performed with formaldehyde-crosslinked chromatin isolated from control or vitamin D treated monoclonal ROS17/2.8 stable cell lines. The antibodies used were against either acetylated H3, acetylated H4 or RNA polymerase II. (A) Representative autoradiographs of PCR products obtained from the chromatin immunoprecipitated with the indicated antibodies. The right panel (plasmids) shows the specificity of primers in selective amplification of wild type versus mutant plasmid (- no template). (B) Data in A were quantitated by PhosphoImager analysis, normalized vs. input levels, and plotted as fold induction (Vit. D/Control). Values represent the average of at least three independent experiments.
Figure 6. **Binding of transcription factors to the OC promoter:** ChIPs were carried out with antibodies against VDR, Runx2, or normal Ig (as negative control) on crosslinked chromatin isolated from control or vitamin D treated monoclonal mVDRE (A) and mRunx (B) stable cell lines. The immunoprecipitated DNA was amplified using oligos specific either for the endogenous OC gene or the transgenes (Table 1).

Figure 7. **Step-wise chromatin remodeling of the OC gene:** Comparison of WT, mRunx and mVDRE promoters provides evidence for the involvement of Runx2 and VDR/RXR transcription factors in the specific sequence of events that occurs during chromatin reorganization at the OC locus.
**TABLE 1**

**PRIMERS USED FOR PCR REACTIONS**

| Oligonucleotide | Sequence 5’-3’                                      | T Ann. (°C) |
|-----------------|-----------------------------------------------------|-------------|
| AwtF            | (-619) GCTCTCCCCCATCAAAACC (-620)                   | 60          |
| AmF             | (-619) GCTCTCCCCCATCAAAAAG (-602)                   | 55          |
| BwtR            | (-421) AAACCCCAAGAAGGAGCGG (-439)                   | 60          |
| BmR             | (-421) AAACCCCAAGAAGGAGCAA (-439)                   | 55          |
| VDRE.2F         | (-475) AGCTGCCCTGCACTGGG (-459)                     | 60          |
| SHE.2F          | (-476) GAGCTGCCCTGCACTGTA (-459)                    | 60          |
| OC.7R           | (-319) CATAGCCTAGAGAGGGTACA (-338)                  | 60          |
| CwtF            | (-141) GTCACCAACCACAGCATCTTTTGTG (-119)             | 60          |
| LMOC.3          | (+20) AGCAGAGAGGGGTCCTCATG (-1)                     | 60          |
| LMCAT.3         | (+45) TAGCTCCTGAAAATCTCGCCAAG (+23)                 | 60          |
| rMYOG F1        | (28) CGACCTGATGGAGCTGTATG (47)                      | 60          |
| rMYOG R         | (201) GGACAAATGCTAGGGGTCC (220)                     | 60          |
### TABLE 2

**PERCENT OF RESTRICTION ENZYME DIGESTION**

| Cell Line     | Hinc II (-536) | Bgl II (-346) | Pvu II a (-282) | Pvu II b (-151) | Pvu II c (+168) | Pst I (-148) |
|---------------|----------------|---------------|-----------------|-----------------|-----------------|---------------|
| **WT** (Endogenous) | C 28±1.4       | 55±3          | 66±3.7          | 67±2            | na              |               |
| D 50±0.9      | 56±2.5         | 79±4          | 76±1            | na              |                 |               |
| **mRUNX**    | C 9±0.3        | 31±1.5        | 26±1.2          | 22±1            | 12±.5           |               |
| D 11±1        | 31±0.6         | 25±1.7        | 21±2            | 12±1            |                 |               |
| **mVDRE**    | C 33±2.3       | 40±2.3        | 42±1.6          | 44±1            | 18±0.8          |               |
| D 28±0.7      | 37±0.8         | 43±1.7        | 45±2            | 18±1.2          |                 |               |

**nd**: no determined  
**na**: no applicable

Restriction enzyme accessibility of wild type and endogenous promoters was assayed in three different monoclonal cell lines for each promoter. Digestion products were analyzed by Southern blots (Fig 5), quantitated by PhosphoImager analysis and expressed as Percent Digestion [100*product/(product + substrate)].
**OC VDRE:**

\[
\begin{array}{c}
\text{GGGTGA ATG AGGACATT} \\
-461 \quad -445
\end{array}
\]

**OC mVDRE:**

\[
\begin{array}{c}
\text{GtaTGA ATG ActACATT} \\
-461 \quad -445
\end{array}
\]

Figure 1
Figure 2

Fold Induction of Vitamin D/Control

Cell Lines

pOCZCAT  mVDRE  mRunx
Figure 3A
Figure 3B
Figure 4

Vit. D (24 Hrs)

Endogenous OC

Transgene mRunx

Transgene mVDRE

4.3 kb

2.8 kb

Probe

A

VDRE

B

C

C/EBP

OC

Probe

A

VDRE

B

C

C/EBP

CAT

Probe

A

VDRE

B

C

C/EBP

CAT

Probe

A

VDRE

B

C

C/EBP

CAT

Probe

A

VDRE

B

C

C/EBP

CAT

Probe

A

VDRE

B

C

C/EBP

CAT

Figure 4
A

| Plasmids   | Control | Vit. D |
|------------|---------|--------|
| WT         | [Image] | [Image] |
| mt         | [Image] | [Image] |
| −          | [Image] | [Image] |

| Endogenous | [Image] | [Image] |
| mVDRE      | [Image] | [Image] |
| mRunx      | [Image] | [Image] |
| Myogenin   | [Image] | [Image] |

Input IgG αH3 Ac aH4 Ac aRNA pol

B

| Fold Vit. D/Control |
|---------------------|
| OC Endogenous       |
| mVDRE               |
| mRunx               |

Legend: □ H3 Ac, □ H4 Ac, □ RNAPol

Figure 5
Figure 6
Figure 7
The vitamin D response element in the distal osteocalcin promoter contributes to chromatin organization of the proximal regulatory domain
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