Proc. Natl. Acad. Sci. USA
Vol. 94, pp. 121–126, January 1997
Biochemistry

YY1 regulates vitamin D receptor/retinoid X receptor mediated transactivation of the vitamin D responsive osteocalcin gene

BO GUO*, FAUZIA ASLAM*, ANDRÉ J. VAN WIJNEN*, STEFAN G. E. ROBERTS†, BARUCH FRENKEL*, MICHAEL R. GREEN†, HECTOR DELUCA‡, JANE B. LIAN*, GARY S. STEIN*, AND JANET L. STEIN*§

*Department of Cell Biology and Cancer Center, and †Howard Hughes Medical Institute, Program in Molecular Medicine, University of Massachusetts Medical Center, 55 Lake Avenue North, Worcester, MA 01655; and ‡Department of Biochemistry, University of Wisconsin–Madison, 420 Henry Mall, Madison, WI 53706

Communicated by Arthur B. Pardee, Dana–Farber Cancer Institute, Boston, MA, November 4, 1996 (received for review September 13, 1996)

ABSTRACT The responsiveness of genes to steroid hormones is principally mediated by functional interactions between DNA-bound hormone receptors and components of the transcriptional initiation machinery, including TATA-binding protein, TFIIB, or other RNA polymerase II associated factors. This interaction can be physiologically modulated by promoter context-specific transcription factors to facilitate optimal responsiveness of gene expression to hormone stimulation. One postulated regulatory mechanism involves the functional antagonism between hormone receptors and nonreceptor transcription factors interacting at the same hormone response element. Here we demonstrate that the multifunctional regulator YY1 represses 1,25-dihydroxyvitamin D_3 (vitamin D)-induced transactivation of the bone tissue-specific osteocalcin gene. We identify YY1 recognition sequences within the vitamin D response element (VDRE) of the osteocalcin gene that are critical for YY1-dependent repression of vitamin D-enhanced promoter activity. We show that YY1 and vitamin D receptor (VDR)/retinoid X receptor heterodimers compete for binding at the osteocalcin VDRE. In addition, we find that YY1 interacts directly with TFIIB, and that one of the two tandemly repeated polypeptide regions of TFIIB spanning the basic domain is responsible for this interaction. TFIIB and VDR can also interact directly, and these factors synergize to mediate transactivation. Our results suggest that YY1 regulates vitamin D enhancement of osteocalcin gene transcription *in vivo* by interfering with the interactions of the VDR with both the VDRE and TFIIB.

Steroid hormones are key physiological mediators of development and homeostasis. Understanding cross talk between steroid hormone-dependent and -independent signaling pathways is critical for gaining further insight into integration of cellular regulatory cues that modulate development and tissue-specific gene expression. The biological effects of steroids and related hormones, including derivatives of vitamins A and D_3, are mediated through their cognate receptors. These receptors are members of a large group of ligand-activated proteins that act as transcriptional activators or repressors for their target genes (1, 2). 1,25-Dihydroxyvitamin D_3 (vitamin D), the active form of vitamin D, binds with high affinity to the vitamin D receptor (VDR). Liganded VDR forms nuclear VDR/retinoid X receptor (RXR) heterodimers and modulates expression of vitamin D responsive genes (1, 3, 4).

Vitamin D is a principal regulator of calcium homeostasis and has been reported to affect hormone secretion, differentiation, and cell proliferation (2–4). In skeletal tissue, a primary target for this steroid hormone, vitamin D regulates bone remodeling and modulates the levels of osteocalcin (OC), a bone-specific calcium-binding protein (5–9). In normal diploid osteoblasts, OC is synthesized only by mature postproliferative cells in which the OC gene is transcriptionally activated at the onset of extracellular matrix mineralization. The OC gene promoter is a paradigm for investigating endocrine mediated transcriptional regulation, as well as the mechanisms by which the OC gene is rendered vitamin D responsive upon induction of tissue-specific basal levels of transcription (5–7). However, to understand vitamin D-dependent modulation of OC gene transcription, it is necessary to define interrelationships between the VDR and nonsteroid hormone-related transcription factors.

MATERIALS AND METHODS

Gel Mobility-Shift Assays. Nuclear extracts were prepared (10) from rat osteosarcoma (ROS) 17/2.8 cells at confluency and cultured as described (11) in the absence or presence of 10^{-8} M $1,25(OH)_2D_3$ 24 hr before harvest. Protein–DNA binding reactions were accomplished as described (ref. 12 and references therein). Gel shift immunoassays were performed with 1.0 μg YY1 antibody, which blocks the formation of the YY1/DNA complex, or an E2F antibody as control (SC-281X, SC-251X, respectively; Santa Cruz Biotechnology).

Transient Transfection Assays. ROS 17/2.8 cells were plated at a density of 0.7×10^5 per 35-mm well in 6-well plates. Transfections were performed by the DEAE–Dextran method using 2.0 μg reporter plasmid, 0–2.0 μg pCMV-YY1 expression plasmid, and 0.3 μg RSV-luciferase plasmid as an internal control to correct the variations in transfection efficiency. The pCMV vector lacking YY1 insert was used as carrier DNA with the total amount of plasmid DNA equivalent to 6.0 μg. Vitamin D treatment was carried out immediately after removal of DEAE–Dextran and subsequent wash with PBS, by adding fresh medium supplemented with charcoal-stripped serum and 10^{-8} M $1,25(OH)_2D_3$ or ethanol. After 48 hr, cells were harvested and total cellular extracts assayed for luciferase and chloramphenicol acetyltransferase (CAT) activity. CAT activity was normalized by luciferase activity and/or total cellular protein content. Each set of experiments was repeated at least three times, and similar results were obtained. The results presented are the average of the three transfections.

Purification of Bacterially Expressed Proteins. His-YY1 was synthesized in the *Escherichia coli* K-12-derived

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Copyright © 1997 by THE NATIONAL ACADEMY OF SCIENCES OF THE USA
0027-8424/97/94121-6$2.00/0
PNAS is available online at **http://www.pnas.org**.

Abbreviations: GST, glutathione *S*-transferase; VDR, vitamin D receptor; RXR, retinoid X receptor; OC, osteocalcin; rOC, rat OC; ROS, rat osteosarcoma; CAT, chloramphenicol acetyltransferase; VDRE, vitamin D response element; EMSA, electrophoretic mobility-shift assay; OP, osteopontin.

§To whom reprint requests should be addressed at: Department of Cell Biology, University of Massachusetts Medical Center, 55 Lake Avenue North, Worcester, MA 01655-0106. e-mail: janet.stein@ummed.edu.

M15[pREP4] strain transformed with His-YY1 plasmid and purified as described (ref. 12 and references therein). The glutathione *S*-transferase (GST)-hTFIIB series, GST-YY1 (a gift from E. Seto, University of Texas, San Antonio), GST-hRXR alpha, and hVDR (both provided by Leonard P. Freedman, Sloan–Kettering Institute, New York) were expressed in the *E. coli* strain BL21 (DE3; Novagen) and induced with isopropyl β-D-thiogalactopyranoside (1 mM). After 4 hr, cells were harvested by centrifugation and resuspended in 1/20 of the original volume using buffer A containing 1.0 M KCl (20 mM Hepes, pH 7.5/5 mM $MgCl_2$/1 mM DTT/0.5 mM phenylmethylsulfonyl fluoride/0.5% Nonidet P-40) and stored at −70°C. The frozen cells were thawed and broken by a French press. For the GST-TFIIB series, cell debris was removed by centrifugation and the supernatant used directly for GST protein-binding assays. hVDR was partially purified by 30% $(NH_4)_2SO_4$ precipitation. For GST-RXR alpha and GST-YY1, GST fusion proteins were affinity-purified with glutathione–Sepharose 4B (Pharmacia) and then used for protein–DNA binding reactions.

Protein Binding Assays. GST fusion protein beads were prepared by incubating lysate with glutathione–Sepharose beads at 4°C for 1 hr and washed twice with buffer A containing 1.0 M KCl and then twice with buffer A containing 0.1 M KCl. Fusion protein beads (30 μl) were incubated with equal amounts of either His-YY1 or VDR or nuclear extract for 1 hr at 4°C in 0.2 ml buffer A containing 0.1 M KCl. The beads were subjected to a salt-step gradient involving consecutive washes with 0.15 M, 0.5 M, 0.8 M KCl, and bound proteins were eluted by 2–3 column volumes of SDS/PAGE sample buffer. The eluate was resolved by SDS/PAGE and proteins were visualized by Western blot analysis using the YY1, GST (SC-281X, Sc-138; Santa Cruz Biotechnology), or VDR (IVG8C11) (13) antibodies, respectively.

RESULTS AND DISCUSSION

YY1 Interacts with the Proximal Steroid Half Element of the OC Vitamin D Response Element (VDRE). The OC gene promoter contains multiple positive and negative regulatory elements, including a VDRE (14–19). The VDRE plays a key role in the transcriptional regulation of OC gene expression in osteoblasts both *in vivo* and *in vitro* (20–23). Vitamin D mediated up-regulation of OC gene transcription is functionally linked to the vitamin D inducible binding of VDR/RXR complexes to the OC-VDRE (20–23). Interestingly, examination of the OC-VDRE sequences reveals two binding motifs for the transcriptional regulator YY1, which overlap the VDR/RXR recognition sequences (Fig. 1*A*). YY1 is a multifunctional zinc finger protein that activates or represses gene transcription in a promoter–context dependent manner and is capable of acting as an initiator of transcription (24). YY1 consensus elements have a central conserved core motif (5′-CAT-3′) and occasionally overlap the recognition sequences for other transcription factors (12, 24).

To examine whether these YY1 motifs are functional for YY1 binding, we first analyzed protein–DNA interactions at the OC-VDRE using the electrophoretic mobility-shift assay (EMSA). Binding reactions were carried out using the OC-VDRE oligonucleotide as probe, to detect VDRE binding proteins present in vitamin D-treated ROS 17/2.8 osteosarcoma cells (Fig. 2*A*). The most prominent complex interacting with the VDRE represents the vitamin D-inducible VDR/RXR heterodimer (25). A secondary complex is observed that exhibits a higher electrophoretic mobility. This complex is mediated by YY1, as determined by DNA competition assays and immunoreactivity with a YY1-specific antibody (Fig. 2*A*), and is not modulated by vitamin D treatment (Fig. 2*B*).

We next designed a panel of mutant VDRE oligonucleotides to determine which of the two YY1 core motifs (5′-CAT-3′)

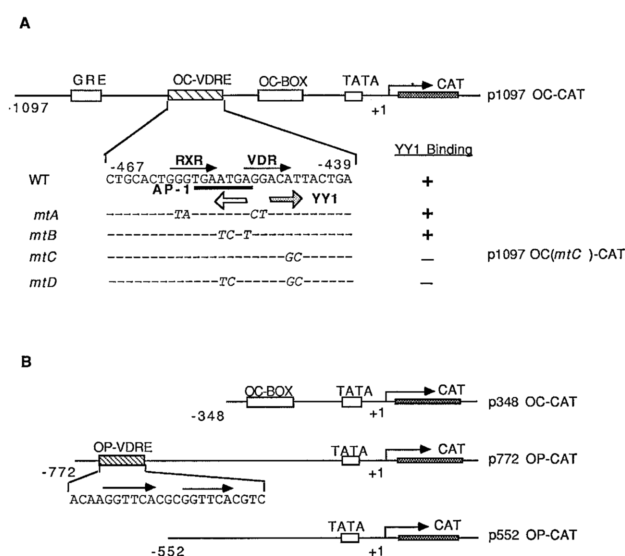

FIG. 1. Constructs and oligonucleotides used for analysis of YY1 effects on the steroid hormone responsive transactivation of bone-related genes. (*A*) Schematic diagram of the 1097-bp rat OC (rOC) promoter CAT construct. The rOC-VDRE is located between −467 and −439 bp of the OC promoter. Positions of other OC promoter regulatory elements (open boxes) are also shown relative to the transcriptional start point. The OC-VDRE that contains two steroid half elements is depicted by the hatched box. Recognition sequences for VDR/RXR and AP-1 are indicated by the long arrows and solid bar, respectively. The proximal steroid hormone half element that mediates YY1 binding is indicated by the short dotted arrow, whereas the putative YY1 core motif located on the antisense strand is indicated by the short open arrow. Oligonucleotides with designated mutations in each of the recognition motifs are displayed below the OC-VDRE sequence. Nucleotide substitutions are indicated by italics. The right column shows the binding ability of YY1 to the corresponding oligonucleotides. (*B*) Deletion mutants of the rOC promoter as well as the mouse osteopontin (OP) promoter. The p1097 OC-CAT construct (*A*) versus p348 OC-CAT, and p772 OP-CAT versus p552 OP-CAT, differ in the presence of VDRE containing promoter segments. Similar to the OC-VDRE, the OP-VDRE (hatched box) contains two steroid half elements (arrows) but no YY1 motifs.

interacts with YY1 (Fig. 1*A*). These VDRE oligonucleotides contain point mutations in the distal YY1 motif (mtB), the proximal YY1 motif (mtC), or both (mtD). For comparison, we designed an oligonucleotide (mtA) in which mutations were introduced that abolish binding of VDR/RXR to the OC-VDRE. EMSA competition assays using these oligonucleotides were performed with either nuclear proteins from vitamin D-treated ROS 17/2.8 cells (Fig. 3) or purified YY1 (data not shown). Both the mtA and mtB oligonucleotides compete for YY1 binding to the OC-VDRE. In contrast, the mtC and mtD oligonucleotides, in which the proximal CAT core is disrupted, do not compete for YY1 binding but still compete for VDR/RXR binding to the VDRE. Therefore, YY1 recognizes only the proximal YY1 core motif, which overlaps the proximal steroid half element, although binding is also influenced by mutations adjacent to the 5′-CAT-3′ motif. Interestingly, this proximal element of the VDRE is predominantly bound by VDR, based on the polarity of the VDR/RXR heterodimer interaction with the OC-VDRE (25–28).

YY1 Inhibits Vitamin D Enhancement of OC Transcription. To investigate the functional role of YY1 in the regulation of OC gene transcription, we cotransfected ROS 17/2.8 osteosarcoma cells with construct p1097 OC-CAT (which encompasses the VDRE) or p348 OC-CAT (in which VDRE containing sequences are deleted) (Fig. 1) in combination with a YY1 expression plasmid (pCMV-YY1). As shown in Fig. 4*A*, YY1 represses vitamin D-induced activity of p1097 OC-CAT in a dose-dependent manner, but has no significant effect on

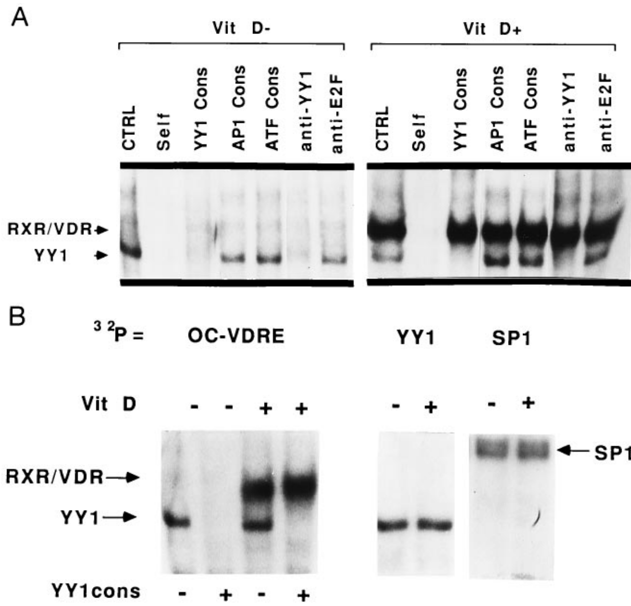

FIG. 2. Identification of VDR/RXR and YY1 complexes formed with the OC-VDRE. EMSAs were performed with nuclear proteins (1.5 μg) from control and vitamin D (10^{-8} M)-treated ROS 17/2.8 cells using rOC-VDRE as probe. (*A*) Characterization of the YY1/VDRE complex using oligonucleotide competition and immunoassays. Competition assays were performed with a 50-fold molar excess of unlabeled competitor oligonucleotides including the OC-VDRE, as well as consensus oligonucleotides for YY1 (5′-CGCTCCGCGGGCC-ATCTTGGCGG-3′), AP-1 (5′-CGCTTGATGACTCAGCCGGAA-3′), and ATF (5′-TGCCTGACGTCAGAGAGCTAG-3′). Gel shift immunoassays were performed with a YY1 specific antibody, which blocks the formation of the YY1/VDRE complex, and an E2F antibody as nonspecific control. (*B*) Treatment of ROS 17/2.8 cells with 10^{-8} M 1,25$(OH)_2D_3$ does not affect YY1 DNA binding activity. EMSAs were performed using the ^{32}P end-labeled rOC-VDRE, YY1 consensus, and SP1 consensus oligonucleotides, respectively, as probe. Nuclear extracts from control or 1,25$(OH)_2D_3$ (10^{-8} M, 24 hr)-treated ROS 17/2.8 cells at confluency were used in the binding reactions as indicated above the lanes. (*Left*) A 50-fold excess of unlabeled YY1 consensus oligonucleotides (self) was used as competitor DNA to identify the YY1 complex.

the basal activity in the absence of vitamin D (data not shown). Deletion of VDRE containing sequences (p348 OC-CAT) results in the loss of both vitamin D inducibility and YY1 repression of OC promoter activity. To determine whether YY1 repression is OC promoter selective, we also analyzed the promoter activity of the OP gene in cotransfection assays. The VDRE of the OP gene does not contain a YY1 binding motif (Fig. 1*B*), and we observe that the OP-VDRE does not compete for YY1 binding to the OC-VDRE (Fig. 3). We therefore used the construct p772 OP-CAT, which encompasses the OP-VDRE, and the p552 OP-CAT, which lacks the OP-VDRE, as reporter gene constructs (Fig. 1*B*). Neither p772 OP-CAT nor p552 OP-CAT promoter activity is influenced by coexpression of YY1 in the absence or presence of vitamin D (Fig. 4*A*). We conclude that YY1 does not repress vitamin D induction of OP gene transcription and that the inhibitory effect of YY1 is selective for the OC promoter.

To determine whether the inhibitory effect of YY1 acts through the YY1 binding site within the OC-VDRE, the YY1-specific point mutation mtC (Fig. 1*A*) was introduced into the OC-VDRE using PCR mediated mutagenesis (Fig. 1*A*). The results of cotransfecting p1097(mtC)-CAT and pCMV-YY1 clearly show that YY1 does not repress the vitamin D enhanced activity of the mutant OC promoter (Fig. 4*B*). However, the substituted nucleotides did reduce VDR/RXR binding activity to the OC-VDRE resulting in reduced vitamin D responsiveness of OC promoter (2.6-fold for mtC versus 5.0-fold for wild type). Thus, the proximal YY1 recognition motif in the OC-VDRE is required for YY1 repression. These results indicate that YY1 may interfere with VDRE mediated transcriptional activation by directly occupying the proximal steroid half element that contacts the VDR.

To ascertain that YY1 repression of VDR transactivation occurs in the absence of tissue-specific factors, which contribute to OC gene transcription, transfection assays were performed in VDR deficient COS-7 cells. The rat VDR expression plasmid pSV_2-VDR was cotransfected with the p1097 OC-CAT reporter. We observed that overexpression of VDR results in transient activation of OC promoter activity, which is further enhanced with vitamin D (data not shown). Coexpression of YY1 suppresses the vitamin D dependent enhancement of VDR transactivation. Thus, the OC promoter-selective repression of YY1 does not require the activities of osteoblast-specific factors.

To establish whether the OC-VDRE alone is capable of mediating YY1 repression of vitamin D-enhanced OC gene transcription, we performed cotransfection experiments with a construct in which multiple copies of OC-VDRE sequences were fused directly to a minimal basal promoter [(VDRE)4L^d-LUC]. This (VDRE)4L^d-LUC construct displays significant vitamin D induction (24-fold) when transfected into osteoblastic cells (Fig. 4*C*). The magnitude of the vitamin D response is related to four tandemly arranged copies of the OC-VDRE, which permits detection of synergistic, vitamin D-dependent transactivation. The results indicate that YY1 effectively antagonizes this high level of transcriptional activation, which is mediated by endogenous levels of VDR in osteoblastic cells. Thus, the OC-VDRE sequences alone are sufficient to support YY1 repression of VDR mediated transactivation. Furthermore, when YY1 and VDR coexpression experiments are performed in VDR-deficient COS-7 cells with a construct containing a single copy VDRE [(VDRE)1*tk*CAT], repression of vitamin D enhancement by YY1 is again observed (data not shown). Hence, a single VDRE is capable of mediating repression of vitamin D enhancement of OC gene transcription.

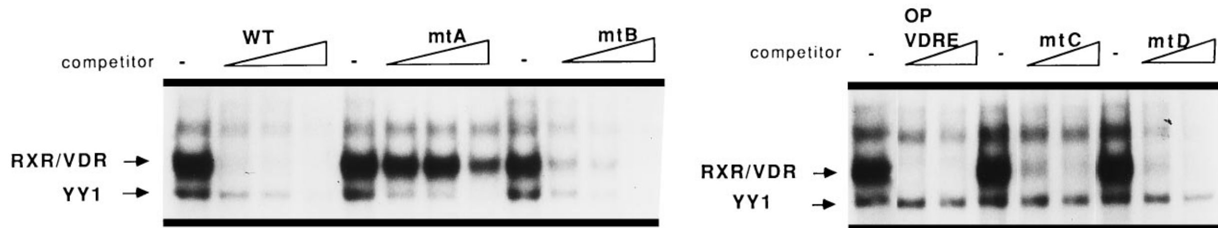

FIG. 3. YY1 recognizes the proximal 5′-CAT-3′ core motif. EMSAs were carried out to determine the location of the YY1 binding motif within the rOC-VDRE using a panel of nucleotide substitution mutations: mtA, mtB, mtC, and mtD (Fig. 1*A*). These mutants were designed to abrogate either the distal or proximal YY1 binding motif, or both. Binding reactions contain the rOC-VDRE as probe and nuclear proteins from vitamin D (10^{-8} M, 24 hr)-treated ROS 17/2.8 cells. All competition assays were performed using a 10-, 25-, or 50-fold molar excess of competitor oligonucleotides (as indicated above the lanes), with the exception of the right portion with 25- or 50-fold molar excess, respectively.

Comparison of established VDREs and other hormone response elements (data not shown) reveals that several VDREs contain potential YY1 recognition sites. The presence of YY1 core motifs within VDREs suggests that YY1 may play a general role in the transcriptional regulation of hormone responsive genes.

VDR and YY1 Compete for Interaction both with the VDRE and TFIIB. Our results indicate that two distinct transcription factors, YY1 and VDR, recognize one common element within the OC-VDRE and that YY1 interferes directly with VDR/RXR mediated transactivation. Because mutation of the YY1 site in the VDRE abolishes this interference event, it appears that YY1 functions in a DNA binding-dependent manner. To address this question directly, we performed competitive protein–DNA binding assays using purified GST-YY1, GST-RXR, and hVDR. Fig. 5 clearly establishes that increasing the VDR concentration results in a dose-dependent displacement of YY1 from the VDRE by VDR/RXR heterodimers. This event may resemble the physiological situation following induction of nuclear VDR/RXR heterodimers upon vitamin D stimulation. Therefore, YY1 and VDR/RXR heterodimers are in direct competition for occupancy of the VDRE.

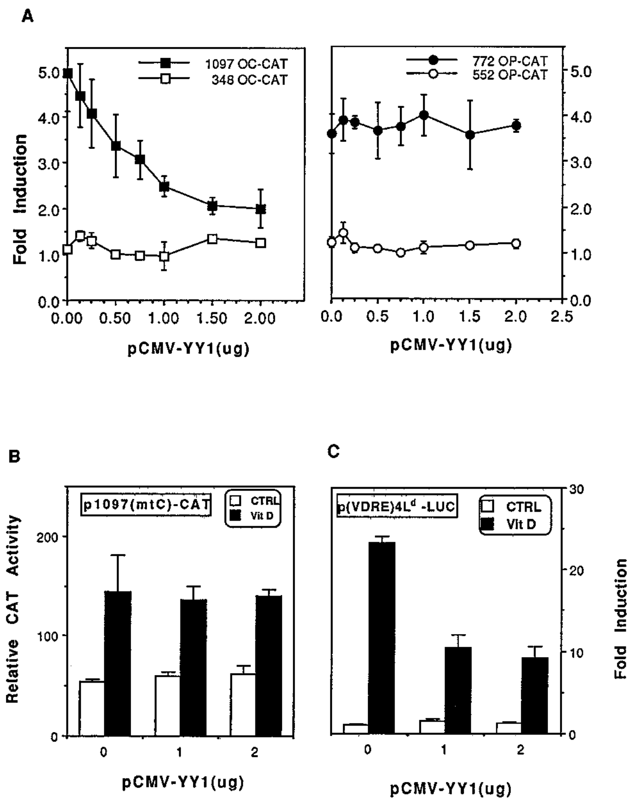

FIG. 4. Overexpression of YY1 selectively represses the vitamin D induction of OC promoter activity. Vitamin D induction of a panel of CAT reporter gene constructs (see Fig. 1) was determined in the absence or presence of different amounts of pCMV-YY1 expression plasmid. ROS 17/2.8 cells were transfected by p1097 OC-CAT or p348 OC-CAT reporter gene construct (*Left*). The OP promoter CAT constructs p772 OP-CAT and p552 OP-CAT (Fig. 1*A*) were used as reporter for similar transfection assays (*Right*). (*B*) Cotransfection experiments were performed as described in *A* using the p1097 OC(mtC)-CAT construct, which contains a YY1-specific mutation (mtC) in the OC-VDRE sequences. (*C*) Results of cotransfection experiments with ROS 17/2.8 using a reporter gene construct containing four copies of the OC-VDRE fused to a heterologous promoter [(VDRE)4L^d-LUC] and the YY1 expression plasmid (pCMV-YY1; amounts indicated below the graph). All values are relative to the control in the absence of vitamin D and pCMV-YY1. Fold inductions were calculated by dividing promoter activity (in relative luciferase units) in the presence of vitamin D (10^{-8} M) by promoter activity in the absence of vitamin D control.

Nuclear receptor mediated gene transcription can involve coactivation by the CBP/p300 class of transcriptional cofactors (29). The coactivator p300, which is a close homologue of CBP, has been shown to interact with YY1. Therefore, we addressed whether YY1 repression of vitamin D enhanced OC gene transcription is related to competition by YY1 and VDR/RXR for limiting amounts of p300. The results indicate that *in vivo* coexpression of p300 does not relieve YY1-dependent repression (data not shown).

Similar to other ligand-dependent receptors, VDR transactivates by physically interacting with both the VDRE and the general transcription factor TFIIB (1, 30, 31). Interestingly, YY1 can also functionally (32) and physically interact with TFIIB (Fig. 6). TFIIB is an essential component of the transcriptional initiation complex and acts as a bridge between the TATA-binding protein–RNA polymerase II complex and steroid hormone receptors (1). Therefore, we postulate that under physiological conditions YY1 may interfere with VDR mediated transactivation by targeting both the VDR/VDRE protein–DNA interaction and the VDR/TFIIB protein–protein interaction. The VDR has been shown to interact via its ligand binding domain with the C terminus of TFIIB, which encompasses the TFIIB basic domain between amino acids 178–201 (30, 31). Identification of the TFIIB domain, which interacts with YY1, is essential for understanding the mechanism by which YY1 modifies the TFIIB/VDR interaction.

To delineate the TFIIB domain responsible for the association with YY1, we analyzed the binding of YY1 to a panel of GST-TFIIB fusion proteins (Fig. 6). We find that deletion of either amino acids 118–174 or amino acids 178–201 abolishes the YY1/TFIIB interaction. These protein segments together encompass the first of two tandemly repeated polypeptide domains of TFIIB (33). Deletion of amino acids 202–269 or amino acids 273–297, which span the second repeat domain of TFIIB, modestly reduces binding of YY1 to TFIIB (Fig. 6*B*). Taken together, our results show that YY1 and TFIIB interact via a unique domain of TFIIB, which spans the TFIIB basic region. Interestingly, the TFIIB basic region is also required for VDR binding (30, 31), suggesting that YY1 and VDR may compete for binding to this region of TFIIB. Therefore, we performed competitive protein binding assays with VDR and YY1 isolated from ROS 17/2.8 cells using GST-TFIIB as the binding substrate. Fig. 6*C* shows that the YY1/TFIIB interaction is disrupted in the presence of VDR. We conclude that

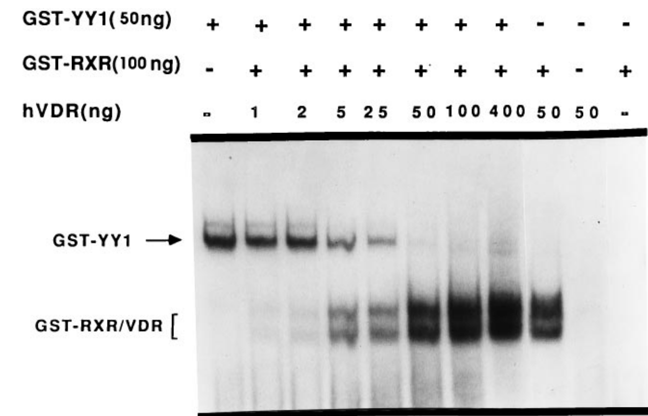

FIG. 5. Mutually exclusive binding of YY1 and VDR/RXR heterodimers to the OC-VDRE. GST-YY1 and GST-RXRα were affinity-purified on glutathione-Sepharose beads (14). DNA binding assays were carried out in the absence or presence of GST-YY1 (50 ng) and GST-RXRα (100 ng) in the presence of increasing amounts of hVDR. All DNA–protein binding reactions were performed in the presence of 10^{-8} M $1,25(OH)_2D_3$ for 30 min.

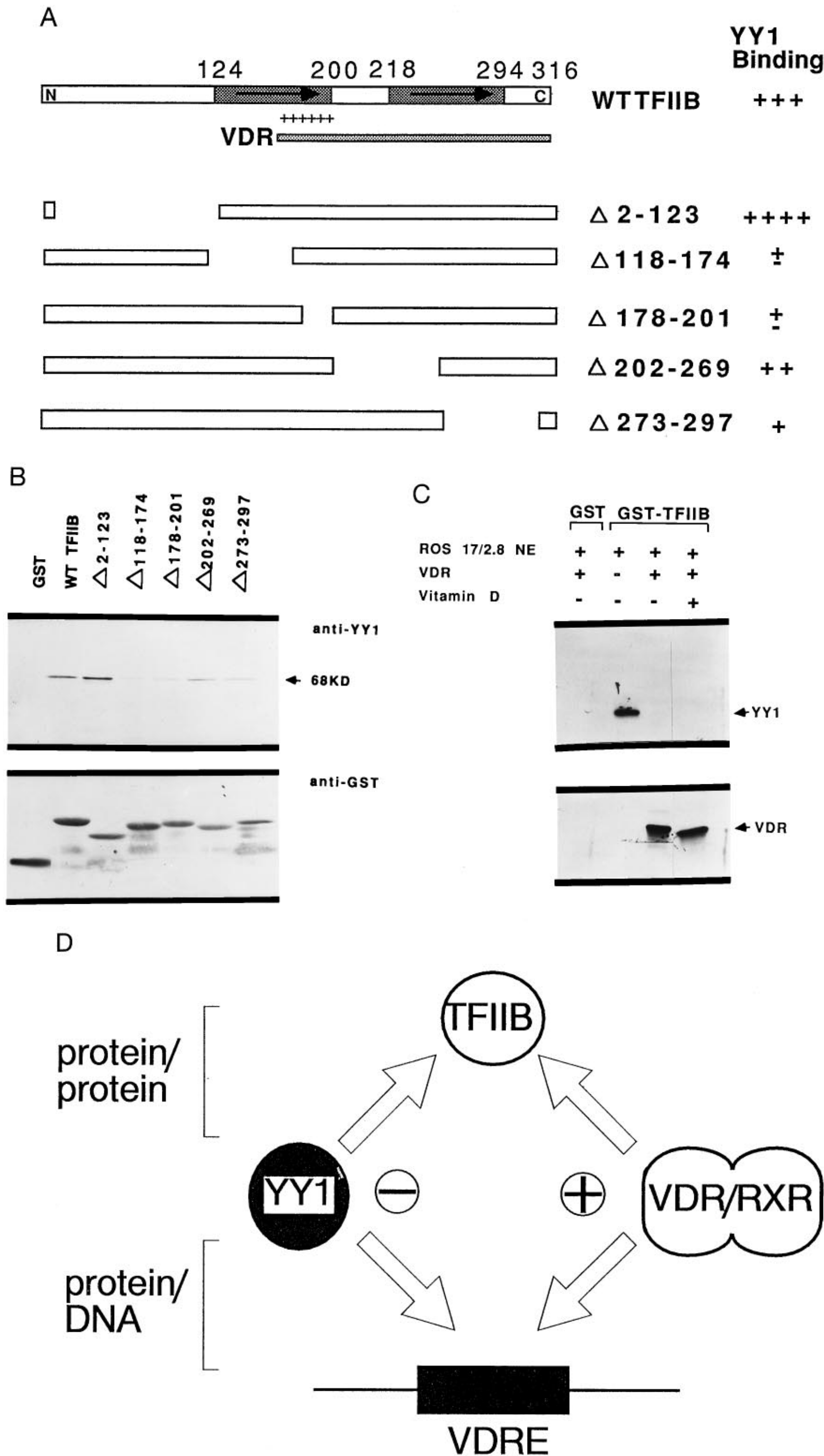

FIG. 6. YY1 binds to the basic region of TFIIB and competes for the VDR/RXR interaction with TFIIB. (*A*) Schematic representation of the TFIIB protein and a panel of wild-type and truncated GST-TFIIB fusion proteins. Indicated in the TFIIB diagram are the TFIIB repeat regions (arrows) and basic domain (+). The ability of truncated TFIIB proteins to interact with YY1 is shown in the right column. The TFIIB domains required for the interaction with VDR (cross-hatched bars) is indicated below the diagram of the wild-type protein. (*B*) Protein binding assay using a panel of GST-TFIIB fusion proteins linked to glutathione-Sepharose beads that were incubated with purified his-YY1. The YY1 signal, which is observed after Western blotting of protein bound to the beads, is indicated by the arrow. (*C*) YY1 and VDR compete for binding to TFIIB. ROS 17/2.8 nuclear extracts were incubated at 4°C with matrices containing GST or GST-TFIIB in the absence or presence of VDR (1 μg) with or without addition of 10^{-8} M vitamin D. (*D*) A model illustrating the mechanism by which YY1 may influence vitamin D dependent enhancement of OC transcription. This model suggests that YY1 exerts its inhibitory effect by interfering with the binding of VDR/RXR to VDRE, as well as by interfering with the interaction between DNA bound VDR/RXR and TFIIB.

YY1 repression of vitamin D enhanced transcription may involve competition at both protein–DNA and protein–protein interaction levels (Fig. 6*D*). Further mapping of the TFIIB binding domain within YY1 will provide additional insight into the mechanism associated with control of vitamin D enhancement of OC gene transcription.

CONCLUSION

In proliferating osteoblasts in which the OC gene is not transcribed nor vitamin D responsive, the ubiquitous regulator YY1 is present at constitutive levels and the representation of VDR/RXR heterodimers is very low (5–7). As a result, YY1 may associate with the OC-VDRE and prevent hormone receptor access to impede premature vitamin D dependent transactivation. This function of YY1 would contribute to maintaining fidelity of vitamin D responsiveness and prevent the precocious induction of OC gene transcription by VDR mediated mechanisms under physiological conditions in which the gene should not be expressed. There have been other examples of competition between steroid hormone receptor and nonreceptor transcription factors for DNA binding (34). Recently, Alroy *et al.* (35) reported that the VDR contributes to the regulation of interleukin 2 gene transcription by com-

peting for DNA binding of an NFATp-AP1 complex as well as by blocking formation of this complex. These interactions involve a different mechanism of regulation than for the OC gene. For interleukin 2 gene regulation, the VDR blocks formation of the specific transactivation complex at the regulatory element. For OC gene regulation, YY1 interferes with both DNA binding and critical protein–protein interactions between the VDR and basal transcriptional machinery necessary for vitamin D enhancement. Vitamin D mediates homologous upregulation of the VDR and induces VDR/RXR heterodimers (3, 5–7). The subsequent increased nuclear concentration of VDR/RXR heterodimers may result in displacement of YY1 from the OC-VDRE and modification of the protein–protein bridge between the distally located OC-VDRE and the proximal OC promoter. Thus, YY1 may modulate the interactions between the VDR and VDRE, as well as between VDR and TFIIB to attenuate vitamin D responsiveness.

In conclusion, our results show that YY1 is a key functional modulator of vitamin D responsiveness of the OC gene. YY1 appears to be a component of regulatory options that collectively influence positive and negative physiological control of this bone tissue-specific gene. These findings may contribute to understanding molecular mechanisms mediating steroid hormone regulation of skeletal development and provide a potential basis for elucidating regulatory perturbations associated with skeletal pathologies.

We thank Liz Buffone for technical support and Judy Rask for manuscript preparation. We gratefully acknowledge Thomas Last for many stimulating discussions and expert technical advice. This work was supported by National Institutes of Health Grants AR42262, AR39588, and DK46721.

1. Tsai, M.-J. & O'Malley, B. W. (1994) *Annu. Rev. Biochem.* **63,** 451–486.
2. Evans, R. M. (1988) *Science* **240,** 889–895.
3. Darwish, H. & DeLuca, H. F. (1993) *Crit. Rev. Eukaryotic Gene Expression* **3,** 89–116.
4. Luisi, B. F., Schwabe, J. W. & Freedman, L. P. (1994) *Vitam. Horm.* **49,** 1–47.
5. Stein, G. S. & Lian, J. B. (1993) *Endocr. Rev.* **14,** 424–442.
6. Stein, G. S. & Lian, J. B. (1995) *Endocr. Rev. Monograph* **4,** 290–297.
7. Lian, J. B. & Stein, G. S. (1993) in *Nutrition and Gene Expression,* eds. Berdanier, C. D. & Hargrove, J. L. (CRC, Boca Raton, FL), pp. 391–429.
8. Hauschka, P. V., Lian, J. B., Cole, D. E. C. & Gundberg, C. M. (1989) *Physiol. Rev.* **69,** 990-1047.
9. Lian, J. B. & Gundberg, C. M. (1988) *J. Clin. Orthop. Relat. Res.* **226,** 267–291.
10. Dignam, J. D., Lebovitz, R. & Roeder, R. (1983) *Nucleic Acids Res.* **11,** 1475–1489.
11. Majeska, R. J., Rodan, S. B. & Rodan, G. A. (1980) *Endocrinology* **107,** 1494–1503.
12. Guo, B., Odgren, P. R., van Wijnen, A. J., Last, T. J., Fey, E. G., Penman, S., Stein, J. L., Lian, J. B. & Stein, G. S. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 10526–10530.
13. Brown, T. A. & DeLuca, H. F. (1991) *Arch. Biochem. Biophys.* **286,** 466–472.
14. Hoffmann, H. M., Catron, K. M., van Wijnen, A. J., McCabe, L. R., Lian, J. B., Stein, G. S. & Stein, J. L. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 12887–12891.
15. Aslam, F., Shalhoub, V., van Wijnen, A. J., Banerjee, C., Bortell, R., Shakoori, A. R., Litwack, G., Stein, J. L., Stein, G. S. & Lian, J. B. (1995) *Mol. Endocrinol.* **9,** 679–690.
16. Banerjee, C., Hiebert, S. W., Stein, J. L., Lian, J. B. & Stein, G. S. (1996) *Proc. Natl. Acad. Sci. USA* **93,** 4968–4973.
17. Towler, D. A., Rutledge, S. J. & Rodan, G. A. (1994) *Mol. Endocrinol.* **9,** 1484–1493.
18. Towler, D. A. & Rodan, G. A. (1995) *Endocrinology* **136,** 1089–1096.
19. Ducy, P. & Karsenty, G. (1995) *Mol. Cell. Biol.* **15,** 1858–1869.
20. Kerner, S. A., Scott, R. A. & Pike, J. W. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 4455–4459.
21. Demay, M. B., Gerardi, J. M., DeLuca, H. F. & Kronenberg, H. M. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 369–373.
22. Morrison, N. A., Shine, J., Fragonas, J.-C., Verkest, V., McMenemy, M. L. & Eisman, J. A. (1989) *Science* **246,** 1158–1161.
23. Markose, E. R., Stein, J. L., Stein, G. S. & Lian, J. B. (1990) *Proc. Natl. Acad. Sci. USA* **87,** 1701–1705.
24. Shrivastava, A. & Calame, K. (1994) *Nucleic Acids Res.* **22,** 5151–5155.
25. Staal, A., van Wijnen, A. J., Birkenhager, J. C., Pols, H. A. P., DeLuca, H., Gaub, M.-P., Lian, J. B., Stein, G. S., van Leeuwen, J. P. T. M. & Stein, J. L. (1996) *Mol. Endocrinol.* **10,** 1444–1456.
26. Perlmann, T., Rangarajan, P. N., Umesono, K. & Evans, R. M. (1993) *Genes Dev.* **7,** 1411–1422.
27. Schrader, M., Muller, K. M., Nayeri, S., Kahlen, J. P. & Carlberg, C. (1994) *Nature (London)* **370,** 382–386.
28. Nishikawa, J., Kitaura, M., Matsumoto, M., Imagawa, M. & Nishihara, T. (1994) *Nucleic Acids Res.* **22,** 2902–2907.
29. Kamei, Y., Xu, L., Heinzel, T., Torchia, J., Kurokawa, R., Gloss, B., Lin, S.-C., Heyman, R. A., Rose, D. W., Glass, C. K. & Rosenfeld, M. G. (1996) *Cell* **85,** 403–414.
30. Blanco, J. C. G., Wang, I.-M., Tsai, S. Y., Tsai, M.-J., O'Malley, B. W., Jurutka, P. W., Haussler, M. R. & Ozato, K. (1995) *Proc. Natl. Acad. Sci. USA* **92,** 1535–1539.
31. MacDonald, P. N., Sherman, D. R., Dowd, D. R., Jefcoat, S. C., Jr., & DeLisle, K. (1995) *J. Biol. Chem.* **270,** 4748–4752.
32. Usheva, A. & Shenk, T. (1994) *Cell* **76,** 1115–1121.
33. Roberts, S. G. E. & Green, M. R. (1994) *Nature (London)* **371,** 717–720.
34. Miner, J. N. & Yamamoto, K. R. (1992) *Genes Dev.* **6,** 2491–2501.
35. Alroy, I., Towers, T. L. & Freedman, L. P. (1995) *Mol. Cell. Biol.* **15,** 5789–5799.