Identification of a vitamin D_3-response element that overlaps a unique inverted TATA box in the rat bone sialoprotein gene

Richard H. KIM*†§, Jack J. LI*, Yorimasa OGATA*, Masato YAMAUCHI*, Leonard P. FREEDMAN‡ and Jaro SODEK*†

*Medical Research Council Group in Periodontal Physiology, Faculty of Dentistry, 4384 Medical Sciences Building, and †Department of Biochemistry, University of Toronto, Toronto, Canada M5S 1A8, and ‡Cell Biology and Genetics Program, Memorial Sloan-Kettering Cancer Center, New York, NY 10021, U.S.A.

Bone sialoprotein (BSP), an early marker of osteoblast differentiation, has been implicated in the nucleation of hydroxyapatite during bone formation *de novo*. Our studies, using the osteoblastic cell line ROS 17/2.8, have revealed that rat BSP gene expression is suppressed by 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], which is a powerful regulator of bone formation and resorption. To determine the molecular basis of the transcriptional suppression of BSP gene transcription by 1,25$(OH)_2D_3$, we have conducted transient transfection analyses with chimaeric constructs of the rat BSP gene promoter linked to a luciferase reporter gene. 1,25$(OH)_2D_3$ suppressed expression in all constructs, including a short construct (pLUC 3; nt -116 to $+60$) that contained a putative vitamin D_3-response element (VDRE; **AGGGTT**TAT**AGGTCA**; nt -28 to -14) that overlaps a unique inverted TATA (TTTATA) box. Mobility shift assays demonstrated strong binding of recombinant human vitamin D_3 receptor protein (hVDR) to the VDRE. Point mutations introduced into each half-site and analysed for 1,25$(OH)_2D_3$-mediated suppression of transcription and for hVDR binding either decreased or increased both transcriptional suppression and binding. In comparison with activating VDREs, the rat BSP VDRE bound VDR–VDR homodimers more avidly than VDR–RXRα heterodimers (where RXR is retinoid X receptor). These studies have therefore identified a novel 1,25$(OH)_2D_3$ suppressor element that overlaps the inverted TATA box in the rat BSP gene and indicate that transcriptional suppression of the rat BSP gene by 1,25$(OH)_2D_3$ might involve competition between the VDR and the TATA binding protein (TBP).

INTRODUCTION

Bone sialoprotein (BSP) is a prominent component of the mineralized bone matrix that has been implicated in tissue mineralization [1]. Studies on the developmental expression [2], tissue localization [3–6], structural properties [7–11] and spatio-temporal deposition of BSP in newly forming bone [12,13] have shown that the expression of this glycoprotein is essentially restricted to differentiated cells in mineralizing tissues and that it might initiate hydroxyapatite formation during bone formation *de novo* [1,2,12,13]. Notably, BSP has been shown to nucleate hydroxyapatite crystal formation under steady-state conditions *in vitro* [14] and recent studies have shown BSP to be associated with the formation of ectopic hydroxyapatite microcrystals in malignant breast tumour tissues [15].

Consistent with a role in mineralized tissue formation, the expression of BSP has been shown to be induced by glucocorticoids [13,16,17] and bone morphogenetic proteins [18,19], which promote the differentiation of pre-osteoblastic cells into osteoblasts and induce bone formation [20–23]. In contrast, 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], which suppresses the differentiation of osteoblastic cells and subsequent bone formation [24], suppresses glucocorticoid-induced BSP expression [16] and type I collagen synthesis [24a]. However, 1,25$(OH)_2D_3$ is known to regulate a range of biological processes, including the maintenance of calcium homoeostasis, as well as regulating bone growth and remodelling and the modulation of cell growth and differentiation [25–27]. Thus the stimulated expression of some osteoblastic markers such as alkaline phosphatase [28–30], osteocalcin (OC) [31] and osteopontin [OPN] [32,33] by 1,25$(OH)_2D_3$ seems to reflect the complexity of the mechanisms involved in the homoeostasis of bone.

The direct effects of 1,25$(OH)_2D_3$ on gene transcription are mediated through a discrete *cis*-acting nucleotide sequence known as the vitamin D_3-response element (VDRE) [35]. The VDRE belongs to a subgroup of steroid-like hormone response elements, including the thyroid and retinoic acid response elements, that are characterized as direct repeats of two hexanucleotide half-sites separated by three (VDRE), four (thyroid response element) or five (retinoic acid response element) nucleotides (reviewed in [36]; [37]). A number of VDREs that enhance gene transcription have been identified in the promoters of the human [38] and rat OC [39] genes, and in mouse [33] and pig [10] OPN. Although the nature of VDREs that enhance transcriptional activity of genes has been well established, elements that mediate the suppression of gene transcription by 1,25$(OH)_2D_3$ [40] have yet to be fully characterized.

In previous studies we have isolated and sequenced the promoter regions of the rat [41] and human [42] BSP genes and have shown that a unique inverted TATA box exists within the highly conserved immediate promoter region [43]. Here we describe a novel VDRE that overlaps the inverted TATA box in the rat BSP gene promoter and show that the suppression of BSP gene transcription by 1,25$(OH)_2D_3$ is mediated through this VDRE.

MATERIALS AND METHODS

Cell culture

The rat osteosarcoma cell line ROS 17/2.8 was used as a source

Abbreviations used: BSP, bone sialoprotein; CAT, chloramphenicol acetyltransferase; CHX, cycloheximide; 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; GMS, gel mobility shift; h, human; m, mouse; OC, osteocalcin; OPN, osteopontin; p, pig; RXR, retinoid X receptor; TBP, TATA-binding protein; VDR, vitamin D receptor; VDRE, vitamin D_3-response element.

§ To whom correspondence should be addressed.

The nucleotide sequence reported will appear in DDBJ, EMBL and GenBank Nucleotide Sequence Databases under the accession number X86100 RNDNABSP.

of osteoblastic cells that synthesize BSP. Heat-inactivated fetal calf serum was purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and charcoal stripping was done as described [43a]. Cells were grown to confluence in 10 % (v/v) fetal calf serum and maintained for a further 24 h in 10 % (v/v) charcoal-stripped fetal calf serum. These cells were then incubated with or without 10 nM $1,25(OH)_2D_3$ in the presence or absence of 100 μM cycloheximide (CHX) for periods extending from 0 to 24 h to determine the direct and indirect effects of $1,25(OH)_2D_3$ on BSP mRNA expression.

Northern hybridization

Guanidinium thiocyanate was used to extract the total RNA, as described [44], from the ROS 17/2.8 cells. After purification, 20 μg aliquots of RNA were fractionated on 1.2 % (w/v) agarose gels and transferred to a Biotrans membrane (ICN), as previously described in detail [3]. Hybridizations were performed at 42 °C with randomly labelled rat BSP cDNA probe, prepared as described by Chen et al. [3]. After hybridization, membranes were washed four times, for 30 min each wash, at 55 °C in 2 × SSC (30 mM sodium citrate, 0.3 M NaCl, pH 7.0) containing 0.1 % SDS. This was followed by four 30 min washes at 55 °C in 0.1 × SSC and 0.1 % SDS. The hybridized bands, representing the two isoforms of rat BSP mRNA, were detected by autoradiography on Kodak X-Omat film at −70 °C and quantified by phosphorimaging (Molecular Dynamics). At the same time the agarose gels were quantified to normalize for the intensity of the hybridized bands in the Northern blots. Statistical differences were determined using Student's *t*-test.

Nuclear run-off transcription assay

Confluent, quiescent ROS 17/2.8 cells in 100 mm dishes were incubated with $1,25(OH)_2D_3$ and/or 10 nM dexamethasone. The dishes were washed twice in PBS, the cell layers scraped into 5 ml of PBS and the cells collected by centrifugation (500 ***g*** for 5 min at 4 °C). All subsequent steps were performed in ice as described [45]. Typically 5×10^6 d.p.m. were obtained from 5×10^7 cells. For hybridization to specific cDNA probes, equal amounts of radioactivity (5×10^6 d.p.m.) in 5 ml of hybridization buffer were incubated for 36 h at 42 °C with the cDNA species (200 ng) immobilized on nylon filters (Schleicher and Schuell, Keene, NH, U.S.A.). Hybridization conditions, together with phosphorimaging and radioautography, were as described for Northern hybridization analysis.

Construction of reporter plasmids

Luciferase constructs pLUC 1 to pLUC 5 were made by excising the rat BSP promoter inserts from pCAT 1 to pCAT 5, used in previous studies [41], with *Hin*dIII and *Sal*I and re-ligating the inserts into a pLUC B vector [46] at the same restriction sites. Luciferase constructs pLUC 6 to pLUC 13 were made by ligating the rat promoter segment −2992 (*Xba*I) to −801 (*Hin*dIII), including the 5′ phage arm, to the 5′-end of pLUC 5 [47]. This approx. 3 kb insert was then deleted unidirectionally with exonuclease III (Pharmacia), and the 5′ ends of each deletion were determined by nucleotide sequencing.

Site-directed mutagenesis

Site-directed point mutations were generated by PCR [48]. Briefly, two separate reactions were performed in the first round of amplification. One reaction contained 1.0 μg of plasmid pCAT 3 mixed with a 5′-flanking primer, an anti-sense mutant primer, plus the necessary PCR components. A second reaction contained identical components except for a 3′-flanking primer and the sense mutant primer. After 20 cycles of amplification, the mutant fragment with the mutation at the 3′ end and the second mutant fragment with the mutation at the identical 5′ end were generated. Those two PCR products were then separated on a low-melting-point agarose gel, sliced, combined and melted. A small volume (10 μl from approx. 200 μl) was taken to perform the second round of PCR with the 5′-flanking primer and the 3′-flanking primer used in the first round of PCR. The final PCR fragment containing the desired point mutation was subcloned into the pLUC-B vector that lacked promoters and enhancers. The designated mutations and fidelity of PCR were confirmed by nucleotide sequencing.

Transient transfection assays

All transfection assays were performed on exponentially growing ROS 17/2.8 cells. Cells at 30–50 % confluence were transfected with the DEAE-dextran method [45] 24 h after plating. Transfection included 1 μg of a luciferase construct, 2 μg of pCAT C (Pharmacia) as an internal control, and in some transfections 0.5 μg of a human vitamin D_3 receptor (hVDR) expression vector [49]. After the direct addition of the DNA–DEAE-dextran complex to the culture media, the cells were incubated for a further 3 h. The cells were then 'shocked' by exposure to 10 % DMSO in PBS for 2 min at room temperature (21 °C). The

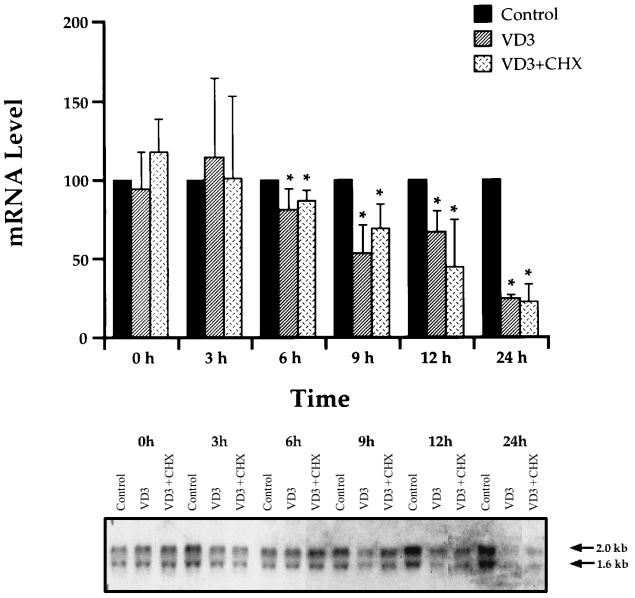

Figure 1 Northern hybridization analysis of vitamin D_3 effects on BSP mRNA expression

To demonstrate the effects of $1,25(OH)_2D_3$ on BSP expression, confluent cultures of ROS 17/2.8 cells were incubated without (Control) and with 10 nM $1,25(OH)_2D_3$ (VD3) in the absence or presence of 0.1 mM CHX (VD3 + CHX). Total RNA was isolated from triplicate cultures harvested after incubation times of 0, 3, 6, 9, 12 and 24 h and prepared for Northern hybridization analysis. Triplicate samples were analysed by Northern hybridization with a ^{32}P-labelled rat BSP cDNA probe and the BSP mRNA bands were quantified by phosphorimaging and expressed as a percentage of the control; the S.D. is shown. Significant differences ($P < 0.05$) compared with the controls at each time point are indicated with an asterisk. A representative autoradiograph of the Northern hybridization blot is shown below the graph.

Table 1 Nuclear run-off transcription analysis of the effects of 1,25(OH)$_2$D$_3$ on BSP gene transcription

Confluent cultures of ROS 17/2.8 cells were treated for 18 h with 0.1% ethanol (Control) or 10 nM dexamethasone (VD3). The cells were then incubated for 6 h in fresh medium containing 10 nM dexamethasone or 10 nM dexamethasone plus 10 nM 1,25(OH)$_2$D$_3$ (VD3), after which cells were harvested and the nuclei processed as described in the Materials and methods section. Triplicate 'run-off' assays were hybridized with radiolabelled RNA transcripts to cDNAs for rat BSP, OPN, α1 type I collagen, alkaline phosphatase and the plasmid vector pBluescript (pBST), immobilized on a Bio-Trans membrane. Hybridized membranes (triplicates) were quantified by phosphoimaging analysis. Values are expressed as means ± S.D. in arbitrary units above the pBST controls; significant differences ($P < 0.05$) are indicated with an asterisk.

	Control	VD3
BSP	6126 ± 2513	2555 ± 1056*
OPN	8246 ± 2449	20359 ± 1771*
Collagen type I	16308 ± 3228	12353 ± 2698
Alkaline phosphatase	6915 ± 1535	2919 ± 288*

dishes were washed, fresh media containing 10 nM 1,25(OH)$_2$D$_3$ were added and incubation was resumed for 48 h before harvesting. The luciferase assay was performed by the Promega protocol with a Berthold Lumat LB-9501 and the Luciferase Assay System (Fisher Scientific, Toronto, Ontario, Canada). The transfections were normalized by using the chloramphenicol acetyltransferase (CAT) assay with a CAT ELISA assay kit (Boehringer Mannheim).

Gel mobility shift (GMS) assays

The ability of the putative VDRE sequence to recognize the VDR protein was determined by GMS assays. Recombinant hVDR and RXRα–glutathione S-transferase fusion protein (where RXR is retinoid X receptor), prepared as described previously [50], were incubated with the complementary strands of 25-mer (or 21-mer) oligonucleotides, annealed by mixing the two strands in a 1:1 ratio, heating to 65 °C for 2 min and cooling at 21 °C for 30 min. The respective double-stranded oligonucleotides (3.5 pmol) were end-labelled with [γ-^{32}P]ATP (New England Nuclear) by using T$_4$ polynucleotide kinase (Pharmacia). Purified hVDR was incubated with 0.035 pmol of the labelled probe at 21 °C for 45 min under the binding conditions described by Cheskis and Freedman [50]. The components in the reaction mixture were resolved by electrophoresis on a 6% (w/v) non-denaturing acrylamide gel (38:2 acrylamide to bisacrylamide) run at 150 V at room temperature. After electrophoresis, the gel was dried and exposed to Kodak X-OMAT AR film for 3–24 h at −70 °C.

RESULTS

Previous studies have shown that 1,25(OH)$_2$D$_3$ suppresses BSP mRNA levels in embryonic rat calvariae [16], and in preliminary experiments we found that a similar suppression occurs in ROS 17/2.8 cells. To determine whether the observed down-regulation of the BSP mRNA results from altered transcription, we performed Northern hybridization analysis of total RNA extracted from osteoblastic ROS 17/2.8 cells treated with 10 nM 1,25(OH)$_2$D$_3$ for various periods. A decrease in BSP mRNA was first detected 6 h after 1,25(OH)$_2$D$_3$ treatment and progressively decreased to approx. 25% of control levels after 24 h (Figure 1). Because CHX did not block this decrease, a direct effect of the 1,25(OH)$_2$D$_3$ on BSP gene transcription was indicated and confirmed by nuclear run-off analyses (Table 1). The transcription of both BSP and alkaline phosphatase decreased to approx. 40% and that of type I collagen to approx. 75% after treatment of

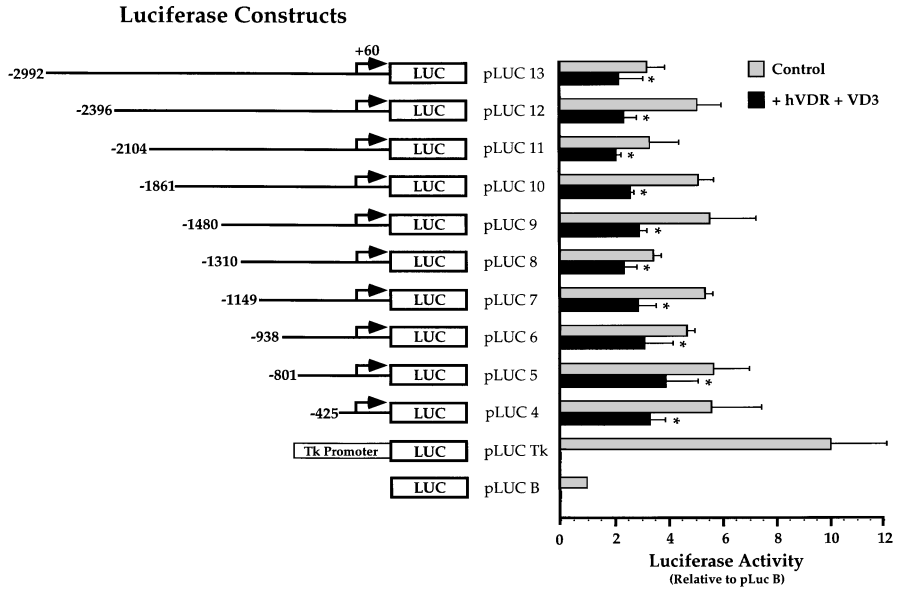

Figure 2 Characterization of the VDRE by transient transfection assays

Chimaeric constructs pLUC 13 to pLUC 4, encompassing various lengths of 5′-flanking sequences of the rat BSP gene, were produced by unidirectional deletions. These constructs (1 mg) and 2 mg of pCAT C (Pharmacia) were co-transfected with (+hVDR+VD3) or without (Control) 0.5 mg of hVDR expression vector into exponentially growing ROS 17/2.8 cells and treated with 10 nM 1,25(OH)$_2$D$_3$ (+hVDR+VD3) or not (Control) as described in the Materials and methods section. The histogram represents results from four or five independent experiments done in triplicate dishes. The relative light units obtained from the luciferase assays were normalized with values from the CAT assays and expressed relative to pLuc B; the S.D. is shown. Significant decreases ($P < 0.05$) in transcription are indicated with an asterisk.

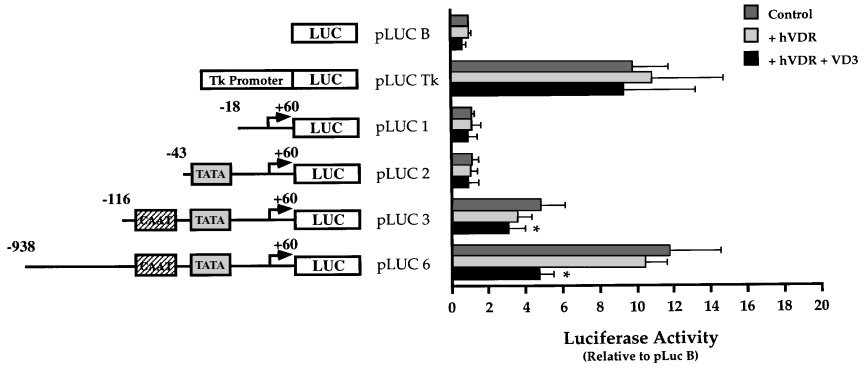

Figure 3 Localization of the VDRE to the TATA box region of the BSP gene promoter

Short chimaeric constructs pLUC B and pLUC 1, pLUC 2, pLUC 3 and pLUC 6, which include various lengths of the rat BSP promoter sequence as shown, were used in transient transfection assays to analyse for VDR effects on gene transcription as described in the legend of Figure 2. The relative light units obtained from the luciferase assays were normalized to values from the CAT assays and expressed relative to the pLUC B controls; the S.D. is shown. Results from three or four independent experiments, each done with triplicate dishes, are shown; significant decreases ($P < 0.05$) in transcription are indicated with an asterisk.

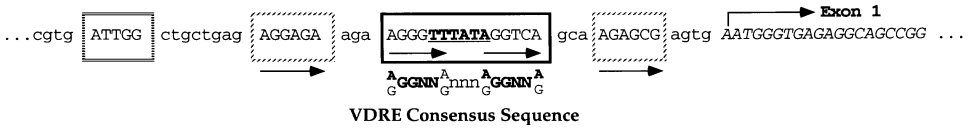

Figure 4 Potential sites of $1,25(OH)_2D_3$ regulation in the rat BSP promoter

A putative VDRE (solid outline) in the rat BSP promoter is shown overlapping the inverted TATA box (underlined and bold). Two hexanucleotide half-sites forming the VDRE (DR3) are indicated by arrows. Underneath, a consensus VDRE sequence is shown, with matching nucleotides shown in bold type. Immediately upstream of the putative VDRE is an inverted CCAAT box (ATTGG). The arrow indicates the transcription start site (+1) and the direction of transcription. Note the presence of two additional VDRE half-sites (enclosed in the hatched outlines) flanking the VDRE and separated from the VDRE by three nucleotides.

ROS 17/2.8 cells with $1,25(OH)_2D_3$, whereas transcripts of the OPN gene were increased 2.5-fold by $1,25(OH)_2D_3$ (Table 1).

Inspection of the promoter region (nt −2992 to +2282) of the rat BSP gene [41,47] revealed several VDRE half-sites but not a complete VDRE. Therefore to localize the site of $1,25(OH)_2D_3$ regulation a series of 5′ deletion mutants containing various lengths of the rat BSP promoter linked to a luciferase reporter gene vector were prepared and the effects of $1,25(OH)_2D_3$ on transcriptional activity analysed by transient transfection assays (Figure 2). Constructs pLUC 13 to pLUC 4, which included promoter sequences spanning nt +60 to −2992 deleted to nt +60 to −425, all showed suppression of transcription by $1,25(OH)_2D_3$ (Figure 2). Because suppression occurred in the shortest construct (pLUC 4; nt +60 to −425) a responsive site in the proximal promoter region was indicated. From an analysis of shorter constructs, encompassing the proximal promoter, transcription of pLUC 3 (nt −116 to +60), which includes the inverted CCAAT and TATA boxes, was found to be suppressed by $1,25(OH)_2D_3$ (Figure 3). Activities of shorter constructs lacking the inverted CCAAT box were too low to provide reliable results, so the specificity of the $1,25(OH)_2D_3$ response was determined with a Tk promoter linked to the same reporter vector (Figure 3). Because $1,25(OH)_2D_3$ did not affect the transcription of this construct it was deduced that the $1,25(OH)_2D_3$ was acting through the region encompassed by nt −116 to +60. Within this nucleotide sequence several hexanucleotides with similarities to the VDRE direct repeat sequence were present (Figure 4). Two of these direct repeats, separated by three nucleotides, overlapped the inverted TATA box. However, whereas the 3′ half-site conformed well to the consensus VDRE direct repeat sequence, the last nucleotide in the 5′ half-site was a T rather than an A or a G (Figure 4). Additionally, two additional half-sites were present on the 5′ and 3′ sides of the central VDRE and were separated by three nucleotides. Whereas

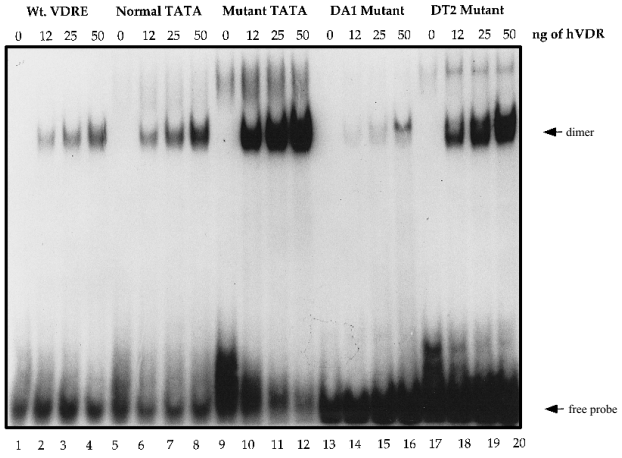

Figure 5 GMS assays of hVDR–VDRE binding

GMS assays, used to analyse hVDR binding, were performed with 5′ end-labelled 25 bp double-stranded oligonucleotides corresponding to the VDRE (wild-type; Wt.) and four mutated forms as shown in Table 2. The assays were performed with increasing amounts (0–50 ng) of hVDR protein as described in the Materials and methods section. Note that the hVDR binds as a dimer.

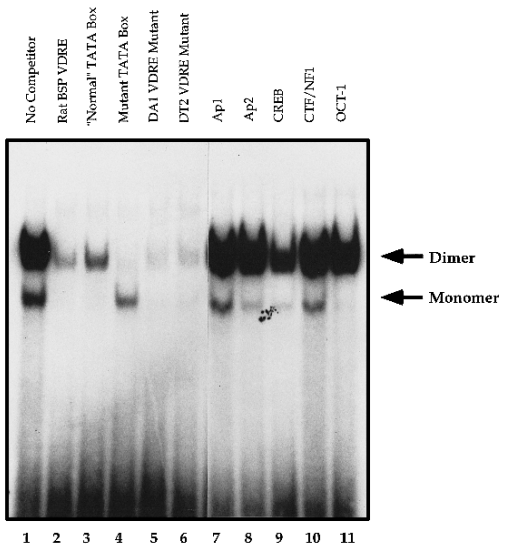

Figure 6 Competition GMS assays

Binding of 25 ng of hVDR to the wild-type VDRE (No Competitor) was competed with a 50-fold molar excess of unlabelled wild-type VDRE (lane 2) and various mutated oligonucleotides (lanes 3–6). Competitive binding was also performed with a number of consensus oligonucleotides (Promega) as indicated in lanes 7–11.

the 5′-side VDRE half-site sequence matched the consensus VDRE, the 3′-side half-site had a single mismatch in the third nucleotide.

To determine whether the putative VDRE spanning the TATA box was responsible for VDR regulation, a 25 bp oligonucleotide spanning this region was synthesized and used in GMS assays. Purified hVDR protein [50] was found to bind with high affinity, in a concentration-dependent manner, to the radiolabelled oligonucleotide (Figure 5, lanes 1–4). Notably the VDR was bound predominantly in the dimer form (Figure 5). To demonstrate that the binding of hVDR protein to the rat BSP VDRE was specific, the GMS analyses were repeated and the binding competed with the unlabelled rat BSP VDRE oligonucleotide and with consensus sequences of other *cis*-acting elements. Binding was effectively blocked by the unlabelled VDRE (Figure 6, lane 2) and by a consensus VDRE sequence (results not shown), but not by consensus oligonucleotides for Ap1, Ap2, CREB, CTF/NF1 and OCT-1 (Figure 6, lanes 7–11).

To determine whether the inverted TATA box sequence was required for regulation by $1,25(OH)_2D_3$, the inverted (wild-type) TATA box was converted into a 'normal' TATA box by two point mutations (TTTATA → TATAAA). This construct showed approx. 50% stronger luciferase activity; however, the degree of transcriptional suppression by $1,25(OH)_2D_3$ was similar to the wild-type sequence (Figure 7). Another mutated construct, in which the TATA box was 'knocked out' by a double mutation (TTTATA → TCTACA), had insufficient transcriptional activity to analyse the $1,25(OH)_2D_3$ effects. Double-stranded oligonucleotides with these mutated sequences were also prepared and tested for their ability to bind to the hVDR protein. The binding of the 'normal' TATA box mutant to the hVDR was similar to the wild-type VDRE (Figure 5, lanes 5 to 8), whereas the oligonucleotide with the mutated TATA box sequence had much stronger binding (Figure 5, lanes 9 to 12). Moreover, in competition GMS analyses both of these mutant oligonucleotides were able to compete with the binding of hVDR to the wild-type VDRE with equal intensity (Figure 6, lanes 3 and 4). These experiments indicated that the inverted TATA box sequence was not crucial for hVDR binding and hence for mediating the $1,25(OH)_2D_3$ effects.

To characterize further the VDRE sequence overlapping the TATA box, two point mutations, one in the 5′ half-site (AGGGTT → ATGGTT) and referred to as DA1 mutant, and a second in the 3′ half-site (AGGTCA → AGGTTA) and called DT2, were introduced into pLUC 3. These changes were chosen because G at the second position in the first half-site is conserved in the VDREs of OPN, OC and calbindin gene promoters, and C at the fifth position is conserved in the second half-site in all but the mouse calbindin VDRE. In transient transfection assays the suppression of transcriptional activity in the DA1 mutant by $1,25(OH)_2D_3$ was significantly lower than the wild-type, whereas significantly higher suppression was observed with the DT2 mutant (Figure 7). When double-stranded oligonucleotides corresponding to these two mutants were analysed for hVDR protein binding, the DA1 mutant showed much lower affinity for the hVDR than the wild-type VDRE, whereas the DT2 mutant had a higher affinity towards the hVDR (Figure 5 and Table 2).

Although the correlation between the results of transcription assays and GMS assays provided strong evidence that the VDRE overlapping the unique inverted TATA box is involved in the suppression of transcription of the rat BSP gene, we attempted further mutations aimed at completely abolishing the hVDR

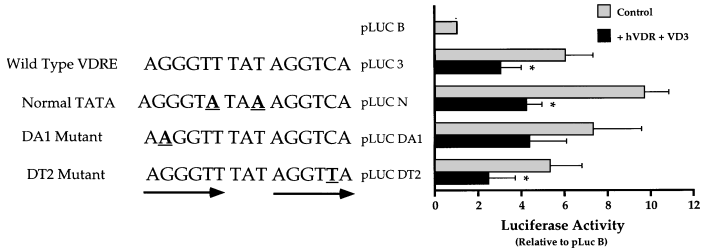

Figure 7 Luciferase assay of VDRE mutants

Point mutations, as shown on the left, were made in the pLUC 3 luciferase construct. These constructs (1 mg) and 2 mg of pCAT C (Pharmacia) were co-transfected, with (+hVDR+VD3) or without (Control) 0.5 mg of hVDR expression vector, into exponentially growing ROS 17/2.8 cells and treated with 10 nM $1,25(OH)_2D_3$ (+hVDR+VD3) or not (Control) as described in the Materials and methods section. The graph represents results from four independent experiments, each done in triplicate dishes. The relative light units obtained from the luciferase assays were normalized with values from the CAT assays and expressed as a value relative to pLuc B controls; the S.D. is shown. Significant differences ($P < 0.05$) compared with the controls are indicated with an asterisk.

Table 2 List of VDRE sequences

The following sequences were tested in GMS assays as described in the Materials and methods section. The values given are the amounts of each probe bound by the hVDR relative to the wild-type VDRE. Underlining indicates VDRE 1/2 sites; bold type indicates mutated nucleotides.

Name	Sequence	Binding affinity
Wild-type VDRE	5'-GAAGAAGGGTTTATAGGTCAGCAAG-3'	1.0
'Normal' TATA	5'-GAAGAAGGGT**A**TA**A**AGGTCAGCAAG-3'	0.9
'Knock-out' TATA	5'-GAAGAAGGGT**C**TA**C**AGGTCAGCAAG-3'	2.3
DA1 mutant	5'-GAAGAA**A**GGTTTATAGGTCAGCAAG-3'	0.4
DT2 mutant	5'-GAAGAAGGGTTTATAGGT**T**AGCAAG-3'	1.4
DblA mutant	5'-GAAGAA**A**GGTTTATA**A**GTCAGCAAG-3'	1.4
5′-DblT mutant	5'-GAAGA**TT**GTTTATAGGTCAGCAAG-3'	0.8
3′-DblT mutant	5'-GAAGAAGGGTTTATA**TT**TCAGCAAG-3'	1.0
5′/3′-DblT mutant	5'-GAAGA**TT**GTTTATA**TT**TCAGCAAG-3'	1.3

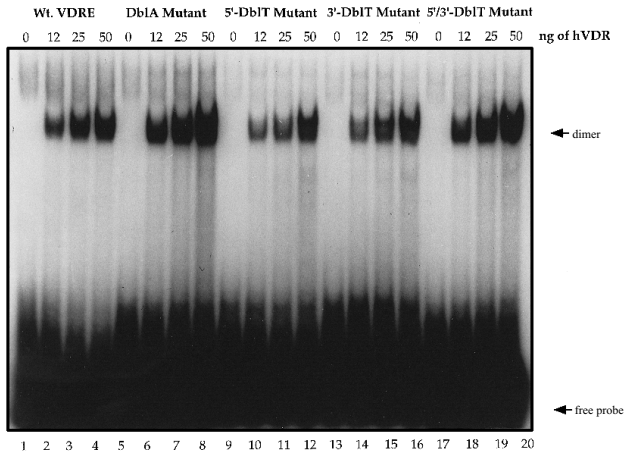

Figure 8 Binding of rat BSP VDRE mutants to hVDR

To determine the importance of specific oligonucleotides in the binding of the rat BSP VDRE to the hVDR, GMS analyses were performed with 25 bp double-stranded oligonucleotides in which nucleotide replacements had been incorporated into one or two positions as described in the Materials and methods section. The assays were performed with increasing amounts (0–50 ng) of hVDR. In each case hVDR binds as a dimer. Wt., wild type.

binding to the VDRE. Four additional mutant oligonucleotides were synthesized and tested for hVDR binding ability with the GMS assay (Figure 8). These mutations were again directed at conserved nucleotides in positions that are believed to be important for steroid receptor binding. Whereas two mutants, 5′-DblT and 3′-DblT, had slightly lower affinities for the hVDR than the wild-type sequence, the other two mutants, DblA and 5′/3′-DblT were found to have slightly higher affinities for the hVDR (Figure 8 and Table 2).

Because positive regulation through VDREs is mediated by VDR and RXRα heterodimer formation [50–53], GMS studies were performed to determine the relative affinities for homodimer (VDR–VDR) or heterodimer (VDR–RXRα) formation in activating [mouse osteopontin (mOPN) and pig osteopontin (pOPN)] and suppressing (BSP) VDREs. Notably in the presence of

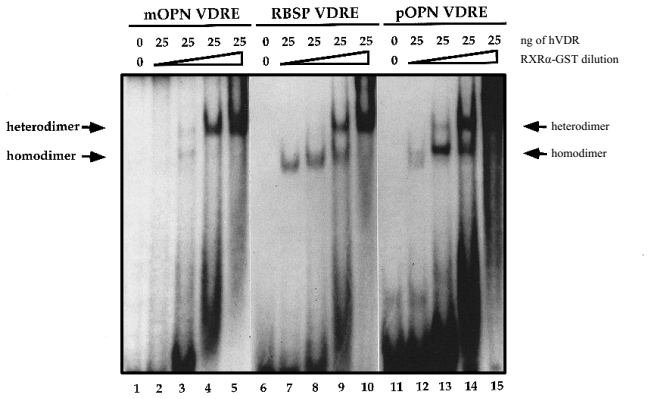

Figure 9 Comparison of VDR homodimer and VDR–RXRα heterodimer formation on positive and negative VDREs

To determine the effect of RXRα on hVDR–VDRE complex formation, GMS assays were performed with a constant amount of hVDR and various amounts of RXRα–GST fusion protein. The probes used were 25 bp double-stranded oligonucleotides with sequences corresponding to mOPN and pOPN VDREs, which are positive regulatory elements, and to the rat BSP VDRE, which is a negative regulatory element.

increasing amounts of RXRα the rat BSP VDRE bound homodimers more avidly than did the positive VDREs present in mOPN and pOPN (Figure 9).

DISCUSSION

We have identified a VDRE in the rat BSP promoter that seems to suppress gene transcription through a mechanism that involves competition with TATA-binding protein (TBP) in binding to the TATA box region of the promoter. Although previous studies have shown that physiological concentrations of $1,25(OH)_2D_3$ suppress BSP mRNA levels, previously up-regulated by glucocorticoid [16], we have shown, from the effects of CHX, nuclear run-off transcription assay and from the relative short time interval (less than 6 h) required to observe a decrease in BSP mRNA, that $1,25(OH)_2D_3$ exerts its effects on BSP gene transcription directly. This is further supported by the results of transient transfection assays in which $1,25(OH)_2D_3$ suppressed transcriptional activity of promoter constructs to an extent similar to that observed with the endogenous gene. However, in contrast with the effects of $1,25(OH)_2D_3$ on BSP expression in rat bone, this secosteroid has been reported to be without effect on BSP expression in human marrow stromal cells [54]. Although the sequence of the rat and human promoters are almost identical around the TATA box, a VDRE-like element might not be functional in the region of the human gene owing to an incomplete half-site in the VDRE-like sequence [42].

Although a VDRE consensus sequence was not immediately evident in the nucleotide sequence of the approx. 5 kb of promoter and first intron, transient transfections implicated the immediate promoter region as the site of $1,25(OH)_2D_3$ regulation. In this region, four possible half-sites were identified that were each separated by 3 nt (Figure 4). Whereas the first and third half-sites conformed to a VDRE consensus sequence, the second and fourth half-sites had single mismatches. The central pair of half-sites overlapped the inverted TATA box. Because of the uniqueness of the inverted TATA box and its possible significance in $1,25(OH)_2D_3$ regulation, we focused our studies on this region. Notably, complementary oligonucleotides corresponding to this putative VDRE were shown to bind strongly to a recombinant

form of the human VDR. However, despite the strong binding, attempts to map the VDRE binding site and to determine the involvement of adjacent sites by using DNAse protection analyses and methylation interference assays were unsuccessful. Although the reasons for this are not clear, similar problems with footprinting have been encountered with VDREs that down-regulate gene expression in the parathyroid hormone [39] and the interleukin 2 [55] gene promoters.

Analysis of mutations introduced into the TATA box to convert the inverted sequence into a consensus sequence with a normal orientation clearly demonstrate that the inverted sequence is not required for VDR binding, as indicated from GMS assays, or for mediating $1,25(OH)_2D_3$-directed suppression of transcription, as indicated by transient transfection assays. However, a slight increase in transcriptional activity was observed with the TATA in the normal orientation.

To ascertain whether the VDR binding and transcriptional suppression by $1,25(OH)_2D_3$ involved the same sequences, mutations were introduced into the two half-sites. Because the G in the second nucleotide position of the 5′ half-site seems to be conserved in OC, OPN and calbindin VDREs, a point mutation at this site was expected to abrogate the suppressive effect of $1,25(OH)_2D_3$. This G seems to be conserved in virtually all steroid hormone response element half-sites, presumably because an Arg residue, which is conserved in the DNA-binding domain of all members of the nuclear receptor superfamily, is making a critical side-chain contact with this nucleotide as deduced from structural analysis of both the glucocorticoid and oestrogen receptors [56,57]. Similarly, replacing the C in the fifth position, which is also highly conserved in the 3′ half-site, was also expected to block the $1,25(OH)_2D_3$ effect. However, whereas the 5′ mutation did indeed reduce VDR binding significantly, the construct with the 3′ mutation bound the VDR more strongly than did the wild-type sequence. Additional mutations also gave unpredictable results. In particular, the double mutants DblA, DblT and 5′/3′-DblT, all of which had the 5′ G replaced, gave stronger or weaker VDR binding, depending on the second point mutation (Table 2). Importantly, however, the affinity for the VDR correlated with results obtained for the transcription assays, providing strong evidence that this region is a functional VDRE.

In contrast with VDREs that enhance transcription, the rat BSP VDRE binds the hVDR strongly as a homodimer, which seems capable of binding to half-site sequences (J. J. Li, R. H. Kim and J. Sodek, unpublished work). This could explain our inability to block binding by point mutations completely. Moreover, the possibility that the adjacent VDRE half-sites could be involved in mediating the $1,25(OH)_2D_3$ effect is indicated by the relatively small effect observed on relieving transcriptional suppression in the DA1 mutant when VDR binding was decreased substantially. Thus multiple copies of the VDRE have been shown to be more efficient than a single VDRE in mediating the effects of $1,25(OH)_2D_3$ [31,49]. Moreover, the involvement of multiple contiguous VDRE half-sites in the $1,25(OH)_2D_3$-mediated suppression of the BSP gene might mirror the complex of glucocorticoid receptor binding sites involved in the suppression of the OC gene [58–60]; the mechanism of suppression in both genes apparently involves competition for the binding of hormone receptor and the TBP. Notably OC, like BSP, is considered a bone-specific protein that is expressed by differentiated osteoblasts. However, whereas BSP is expressed early in bone formation [2] and has been proposed to nucleate hydroxyapatite crystal formation [1,14], OC is expressed later than BSP and is believed to be important in bone resorption. In accord with these proposed functions, BSP expression is induced by glucocorticoids [13] and bone morphogenetic proteins [18], which promote bone formation, and is suppressed by $1,25(OH)_2D_3$ [16] and parathyroid hormone (J. J. Li, R. H. Kim and J. Sodek, unpublished work), which suppress bone formation and promote bone resorption. In contrast, OC expression is stimulated by $1,25(OH)_2D_3$ and suppressed by glucocorticoids [58]. That a competitive mechanism is operative in the $1,25(OH)_2D_3$-mediated down-regulation of the BSP gene is supported by the suppression, but never a complete block, in BSP gene transcription. In addition, interference with TBP binding is supported by an observation that the suppression is not overcome by upstream sequences containing enhancer sequences for transcription factor that promote BSP gene transcription.

That interference with the binding of basal transcription factors by steroid hormone receptors might be a more general mechanism of transcriptional regulation is also indicated from a number of earlier studies. Thus an oestrogen-response element that confers oestrogen inducibility in the ovalbumin gene promoter lies close to the TATA box [61] and different forms of the thyroid hormone receptor have been shown to regulate the growth hormone and the α subunit of the thyroid-stimulating hormone through a regulatory element that lies immediately 3′ to the TATA box [62,63]. In *Drosophila* the homoeobox protein from the gene *engrailed*, which is required for spatio-temporal gene expression during development, competes with transcriptional factor IID for binding to the TATA box in the hsp70 promoter and represses gene transcription [64,65].

The relatively small effect on suppression of transcription compared with the substantial reduction in binding affinity of mutated constructs of the BSP VDRE region could also be due to the stability of the TBP–TATA complex, which is believed to bind in a two-step mechanism [66]. After an initial low-affinity binding a slow isomerization step produces an extremely stable complex with a half-life of approx. 2 h under physiological conditions. Thus it is conceivable that the VDR is unable to bind until the TBP has detached, but once bound the VDR is able to compete with the low-affinity binding step required for subsequent TBP binding. Such a mechanism, requiring the initial displacement of the TBP, would also be consistent with the delayed effect of the $1,25(OH)_2D_3$ on BSP mRNA levels that were observed in the Northern hybridization analyses (Figure 1).

In conclusion, these studies have demonstrated that the expression of the rat BSP gene is regulated through a VDRE situated in the BSP promoter overlapping the TATA box. The identification and initial characterization of this VDRE should aid in the understanding of molecular mechanisms by which $1,25(OH)_2D_3$ regulates bone formation.

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