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# Vitamin D receptor interaction with specific DNA requires a nuclear protein and 1,25-dihydroxyvitamin $D_3$

(vitamin D response element/steroid receptor/gene activation/osteocalcin promoter/nuclear accessory factor)

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ABSTRACT **The regulation of osteocalcin gene expression by 1,25-dihydroxyvitamin $D_3$ is mediated by the vitamin D receptor and a cis-acting DNA response element that has been identified within the 5′ region of the osteocalcin promoter. In this report, we show that vitamin D receptors derived from nuclear extracts of mammalian cells bind directly to this cis-acting element *in vitro* and do so in a manner requiring hormone. Vitamin D receptors derived from reticulocyte lysate translations *in vitro* or from extracts of a *Saccharomyces cerevisiae* strain that expresses the recombinant protein also bind the osteocalcin responsive element, but only when nuclear extracts of mammalian cells are provided. The vitamin-D-receptor-DNA-binding accessory factor is isolated by salt extraction, labile to temperature, and sensitive to tryptic digestion. These studies suggest that the high-affinity interaction of the vitamin D receptor with the osteocalcin vitamin D response element *in vitro* requires both 1,25-dihydroxyvitamin $D_3$ and an accessory protein derived from the mammalian cell nucleus.**

The vitamin D receptor (VDR) is a member of the steroid, thyroid, and retinoic acid receptor gene family of proteins that mediates the transcriptional activities of their respective ligands through transactivation (1, 2). Interaction with specific DNA within this family is determined largely through a region comprised of two zinc-coordinated finger structures (3). The activity of this region *in vivo* appears to be regulated through a complex carboxyl-terminal domain that functions, in part, to bind activating ligand (1–4). Indeed, the expression of recombinant human (h) VDR and of mutant versions of hVDR as well as functional analysis have confirmed the structural organization predicted by the primary sequence of the receptor (5, 6).

Progress in understanding the molecular action of the vitamin D hormone 1,25-dihydroxyvitamin $D_3$ [1,25$(OH)_2D_3$] and the specific transcriptional role of the receptor in that mechanism has been enhanced through definition of a vitamin D response element (VDRE) located within the promoter region of the rat and human genes encoding osteocalcin (OC) (7–9). The cis-acting DNA sequence in the human gene is located 500 base pairs upstream of the transcriptional start site and exhibits many of the characteristics typical of steroid hormone enhancer elements, including an ability to transfer vitamin D response to heterologous promoters (7). The requirement for VDR in the activation of the OC promoter provides strong evidence that this protein mediates vitamin D action and that both are required for transcriptional stimulation (6). In the present studies, we show that VDR binds directly to the OC VDRE *in vitro*, an interaction that requires the presence of both 1,25$(OH)_2D_3$ and an activity derived from extracts of mammalian cells that facilitates VDR–DNA binding. The biochemical properties of this activity suggest it to be a protein factor of nuclear origin. These experiments suggest that the molecular action of the vitamin D hormone on transcription may require not only the VDR but an additional nuclear accessory factor (NAF, termed the VDR-NAF) as well.

## MATERIALS AND METHODS

**Reagents.** 1,25$(OH)_2D_3$ was a kind gift of Milan Uskokovic (Hoffmann-La Roche). CV-1 and COS-1 cell lines were obtained from American Type Culture Collection. Molecular cloning reagents and restriction enzymes were obtained from Boehringer Mannheim.

**Transcriptional Cotransfection Assay.** CV-1 cells were cultured in Dulbecco's modified Eagle's medium supplemented with 10% (vol/vol) fetal calf serum and antibiotics. Cells were plated in 60-mm dishes and transfected 24 hr later using Polybrene as described (10). Plated cells received 10 μg of pBL-TKCAT containing human OC sequences from position −568 to position −345 [pTK-OC(−568/335)] and 1.0 μg of either pAV-hVDR or parent vector. Parallel plates received ethanol or ethanol containing 1,25$(OH)_2D_3$ (10 nM). Cells were harvested 48 hr later and assayed for protein and chloramphenicol acetyltransferase (CAT) activity (11). CAT activity was normalized by protein and assessed in an overnight assay at 37°C.

**Nuclear Extracts of Transfected COS-1 Cells.** Nuclear extracts were obtained from COS-1 cells transfected with or without the hVDR expression vector pAV-hVDR using DEAE-dextran as described (12). Cells were harvested 72 hr after transfection and 2 hr after exposure to 1,25$(OH)_2D_3$ and nuclear extracts were prepared as described by Shapiro *et al.* (13). Western blot analysis revealed the VDR to be present only in extracts of cells transfected with pAV-hVDR.

**Translation of VDR *in Vitro*.** The plasmid p7Zf-hVDR, containing a full-length copy of the human VDR cDNA, was linearized with *Xba* I and utilized to produce uncapped RNA from the SP6 promoter. VDR RNA was translated in a reticulocyte lysate system as outlined by the manufacturer (Promega). Translation in the presence of [$^{35}$S]methionine revealed a protein of the expected 50-kDa size after denaturing polyacrylamide gel analysis. Translations of hVDR RNA with radioinert methionine produced lysate that bound [$^3$H]1,25$(OH)_2D_3$ with high affinity.

***Saccharomyces cerevisiae*-Derived VDR.** Plasmid YEpV1 containing the hVDR cDNA under the control of the copper-inducible *CUP1* promoter as described (14) was transformed into the protease-deficient yeast strain BJ3505. Cells were grown to an $OD_{600}$ of 1 and then induced for 6 hr with 0.1 mM $CuSO_4$ in the presence of 0.1 μM 1,25$(OH)_2D_3$. Extracts were prepared by disruption with glass-bead homogenization in 10

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Abbreviations: 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin $D_3$; VDR, vitamin D receptor; CAT, chloramphenicol acetyltransferase; VDRE, vitamin D response element; OC, osteocalcin; NAF, nuclear accessory factor; h, human.

mM Tris·HCl, pH 7.6/0.3 M KCl/5 mM dithiothreitol. VDR was purified to >95% homogeneity using calf thymus DNA-cellulose by techniques to be described elsewhere (38). All protein preparations were stored at −70°C prior to use.

**VDR Binding to Specific DNA *in Vitro*.** Specific DNA binding was assessed by band-shift assays, as described (15). The DNA fragment −568/−413 containing the VDRE was utilized as a probe and was prepared by digesting a vector containing this DNA insert with *Eco*RI, end-labeling with $^{32}P$ by a filling-in reaction, and then digesting with *Xba* I. The DNA fragment ($10^8$ cpm/$\mu$g) was isolated and recovered after resolution on a 4% polyacrylamide gel. DNA probe (5000–20,000 cpm per reaction) was incubated with COS-1 cell extract, reticulocyte lysate, yeast extract, or purified VDR, as indicated in the figure legends in the following buffer: 25 mM Tris·HCl/15 mM Hepes, pH 7.9/40 mM NaCl/5.5 mM KCl/3 mM $MgCl_2$/4.5 mM EDTA/6% (vol/vol) glycerol/0.08% Tween 20/1 mM $\beta$-mercaptoethanol/1 $\mu$g of poly(dI-dC). Samples were incubated for 20 min at room temperature and then electrophoresed on a 5% polyacrylamide gel prepared in 50 mM Tris/380 mM glycine, pH 8.2, at a constant 200 V. Gels were dried and autoradiographed overnight.

## RESULTS

A vitamin D responsive locus has been identified (7) within the 5′ flanking region of the human OC gene promoter. Fig. 1*A* demonstrates the functional response of a herpes simplex virus thymidine kinase promoter CAT reporter plasmid in which OC DNA fragment (−568/−345) containing this VDRE has been inserted. As can be seen, a 10-fold induction of CAT activity was obtained when CV-1 cells were cotransfected with pAV-hVDR and treated with $1,25(OH)_2D_3$. In contrast, no induction was observed in the absence of transfected hVDR or in the absence of $1,25(OH)_2D_3$.

As illustrated in Fig. 1*B*, several DNA–protein complexes were apparent when nuclear extracts of COS-1 cells were incubated with a shorter version of this DNA fragment (−568/−413) and then subjected to nondenaturing gel electrophoresis in band-shift analysis. A major protein–DNA complex designated band 3, however, was present only in extracts of cells transfected with pAV-hVDR and was absent from extracts derived from cells that were mock-transfected or remained untransfected. Untransfected COS-1 cells contain ≈1600 VDRs per cell whereas pAV-hVDR transfected cells contain over $2.5 \times 10^5$ copies per cell (5). As seen in Fig. 1*C*, although band 1 was unaffected by either antibody and band 2 was nonspecifically affected by both, possibly due to protein interaction, only band 3 was specifically lost when the protein–DNA complexes were formed in the presence of the anti-VDR monoclonal antibody 9A7. This antibody is known to interact immediately on the carboxyl side of the DNA binding domain of the VDR and to alter or block the receptor's capacity to interact with DNA (16). Fig. 1*D* reveals that the interaction of the VDR with the large OC DNA fragment could be competed efficiently with an excess of duplex oligonucleotide whose sequence matches that of the VDRE from position −512 to position −483. Thus these data suggest that band 3 represents an OC DNA complex that contains the VDR and that the site of binding is that of the previously defined VDRE at nucleotides −509 to −489. Other protein–DNA complexes seen here are likely due to interactions with other regions of this probe.

To further examine the VDR–DNA complex, we performed band-shift analysis with VDR synthesized from reticulocyte lysate translations *in vitro*. As observed in Fig. 2, VDR derived from this *in vitro* source and preincubated with $1,25(OH)_2D_3$ was unable to bind to the OC DNA probe. Surprisingly, however, VDR regained the ability to bind the DNA probe when nuclear extracts of untransfected COS-1 cells were included in the incubations. Importantly, this complex comigrated with the VDR–DNA complex derived from COS-1 cells transfected with pAV-hVDR. Reticulocyte lysates from control *in vitro* translations in the absence of exogenously added RNA did not exhibit complex formation (data not shown). These results suggest that an activity derived from the nuclear fraction of mammalian cells is

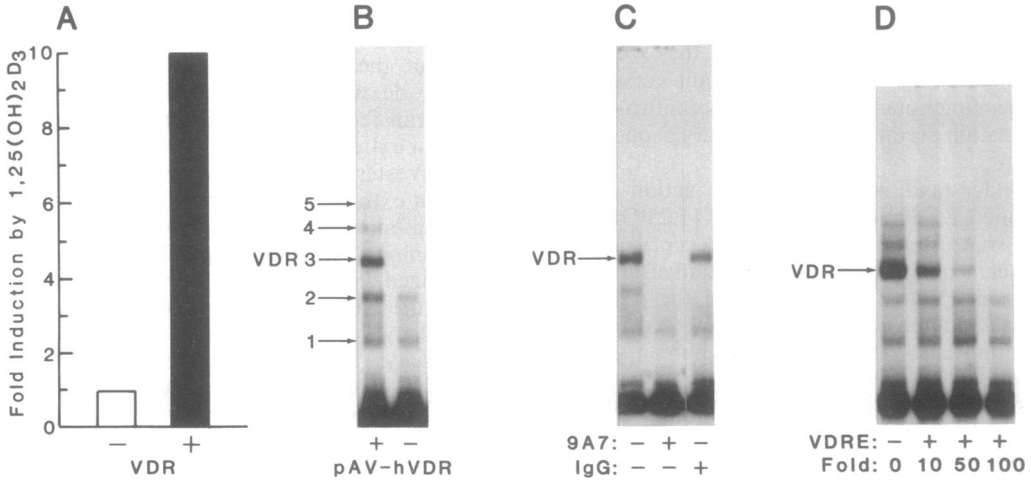


FIG. 1. Interaction of the VDR with the OC gene promoter. (*A*) Cotransfection of phOC(−568/−335)TKCAT with pAV-hVDR. CV-1 cells were transfected with 10 $\mu$g of phOC(−568/−335) containing the VDRE and 1 $\mu$g of either pAV-hVDR (solid bar) or parent vector (open bar) and treated with or without $1,25(OH)_2D_3$ (10 nM). Data are plotted as a ratio of induced to uninduced CAT activity (fold induction) averaged from several transfections. (*B*) Protein–DNA complexes unique to pAV-hVDR transfected COS-1 cells. An end-labeled OC DNA fragment from position −568 to position −413 was incubated with nuclear extracts derived from $1,25(OH)_2D_3$-treated COS-1 cells that had been transfected with pAV-hVDR (+) or mock-transfected (−). Free and protein-bound DNA probe were resolved. Protein–DNA complexes were designated bands 1–5, where band 3 was consistently unique to pAV-hVDR-transfected cells. (*C*) Inhibition of protein–DNA complex in band 3 by anti-VDR monoclonal antibody. The OC probe was incubated with nuclear extracts of pAV-hVDR transfected COS-1 cells as in *B* in the presence (+) or absence (−) of 12 ng of anti-VDR monoclonal antibody (9A7) or 180 ng of an irrelevant rat monoclonal antibody of identical class (IgG). (*D*) VDR binds to the VDRE locus. Protein–DNA complexes were formed as in *C* in the absence (−) or presence (+) of increasing molar excess (Fold) of an oligonucleotide of sequence 5′-TTGGTGACTCACCGGGTGAACGGGGGCATT-3′ that matched the VDRE locus from position −512 to position −483. Significant competition of band 3 was observed with a 10-fold molar excess of VDRE.

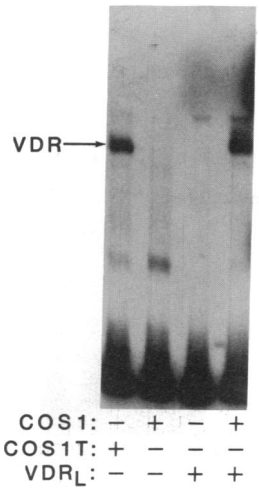


FIG. 2. VDR translated *in vitro* requires mammalian cell nuclear factor for specific DNA recognition. Reticulocyte lysate containing *in vitro*-translated VDR ($VDR_L$) was incubated with an OC DNA probe in the presence (+) or absence (−) of nuclear extracts derived from untransfected COS-1 cells (COS1). Comigration of the VDR–DNA complex is observed with VDR–DNA complex derived from pAV-hVDR-transfected cells (COS1T).

permissive for specific VDR–DNA binding and that band 3 contains not only VDR but an additional factor(s) as well.

High-salt cellular extracts of yeast strain BJ3505 transformed with plasmid YEpV1 overproducing hVDR (14) were next examined for interaction with the OC DNA probe. As observed in Fig. 3*A*, VDR–1,25$(OH)_2D_3$ complexes from this source were similarly unable to associate with the DNA probe during band-shift analysis in the absence of mammalian cell extract. Nevertheless, as with reticulocyte lysate translated VDR, the inclusion of increasing concentrations of untransfected COS-1 cell nuclear extract led to clear recovery of binding activity. Importantly, control extracts of a similar yeast strain transformed with YEpE2 expressing the human estrogen receptor (39) did not exhibit binding to the OC DNA fragment even in the presence of mammalian cell extract. The DNA complex formed with VDR-containing yeast extracts and untransfected COS-1 cell extracts comigrated with the complex observed with pAV-hVDR transfected COS-1 cell extracts were incubated alone. As observed in Fig. 3*B*, the VDR–DNA complex was enhanced when both VDR-containing yeast extracts and transfected cell extracts were mixed. Finally, formation of the complex was lost both when yeast VDR and COS-1 extracts were coincubated with labeled DNA probe and a 50-fold molar excess of VDRE oligonucleotide (Fig. 3*C*) and in an incubation that contained monoclonal antibody 9A7 (data not shown). These experiments support the hypothesis that binding of the VDR to a VDRE sequence within the OC promoter requires the presence of a nuclear factor limited in these experiments to high-salt nuclear extracts of mammalian cells.

As documented in Fig. 4*A*, the binding of highly purified VDR (>90%) to the OC DNA probe also required the presence of nuclear extracts. We utilized this purified receptor to further assess the possibility that the VDR–DNA binding enhancing activity was a protein, a hypothesis supported by the fact that the activity required solubilization from the nuclear pellet. As observed in Fig. 4*B*, preincubation of the nuclear extracts with trypsin led to a loss of VDR binding to the OC DNA fragment, a loss blocked by the inclusion of trypsin inhibitor. Furthermore, the extracts were sensitive to elevated temperatures. Preincubation of nuclear extracts with increasing temperature led to a progressive loss of DNA enhancing activity and complete loss at 50°C (Fig. 4*C*). These experiments, therefore, provide strong evidence that the nuclear activity we have identified in COS-1 cells that enhances the interaction of VDR with specific DNA is the result of the action of a nuclear protein. Finally, the fact that the reaction occurs to near completion within 2 min (data not shown) suggests that these observations are due to a VDR–protein interaction and not a posttranslational modification *in vitro*.

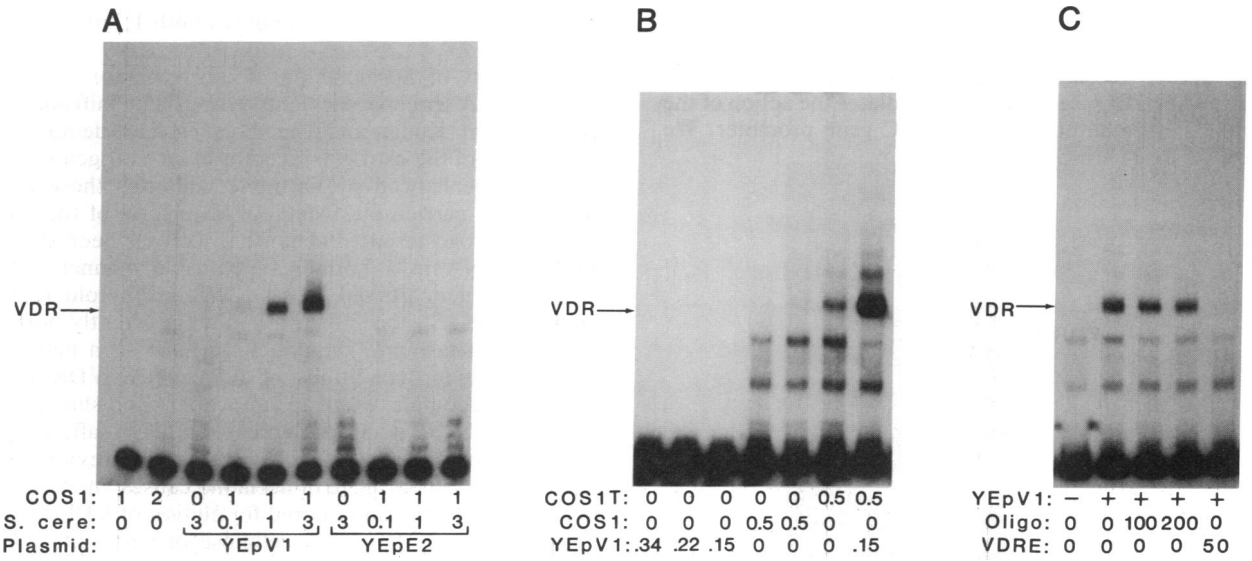


FIG. 3. VDR expressed in *S. cerevisiae* requires mammalian cell nuclear factor for specific DNA recognition of the VDRE. (*A*) VDR synthesized in yeast requires COS-1 nuclear extracts. Cytosol extracts from yeast strains producing VDR (YEpV1) (14) or estrogen receptor (YEpE2) (39) were prepared as described (14) and incubated with probe −568/−413 in the absence or presence of nuclear extracts of untransfected COS-1 cells and resolved. The VDR–DNA complex is designated with the arrow. Units are relative. (*B*) Comigration of the yeast VDR and mammalian VDR DNA complexes. Cytosol from the yeast strain transformed with YEpV1 was incubated with OC DNA probe in the presence of nuclear extracts from pAV-hVDR-transfected COS-1 cells (COS1T) or untransfected COS-1 cells (COS1). The mammalian- or yeast-derived VDR–DNA complex is indicated by the arrow. (*C*) Yeast VDR is incubated with nontransfected COS-1 cell nuclear extract and OC DNA probe in the presence of increasing molar excess of nonspecific oligonucleotide (Oligo) or VDRE oligonucleotide. Only the VDRE competes after resolution of free and bound probe.

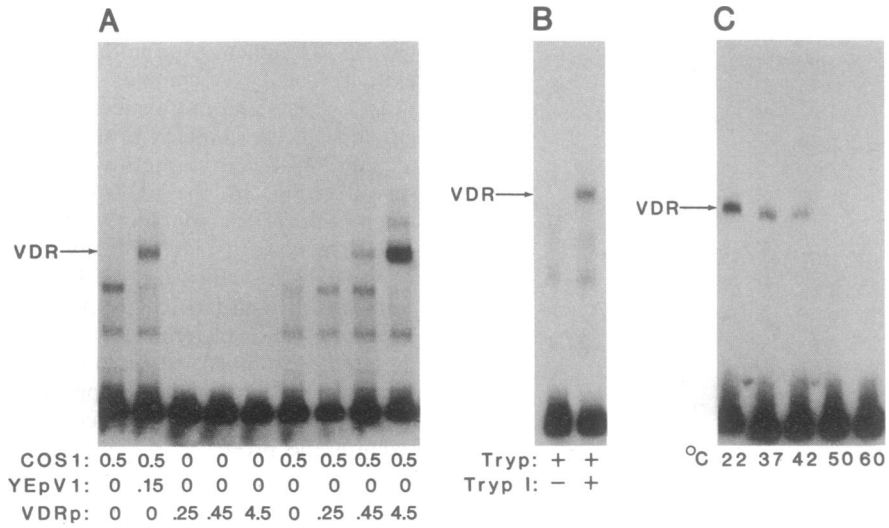


FIG. 4. Purified VDR requires a nuclear protein sensitive to trypsin and labile to heat for DNA association. (*A*) VDR purified from yeast cytosols by DNA-cellulose chromatography ($VDR_p$) and OC DNA probe were incubated in the absence and presence of nuclear extracts of nontransfected COS-1 cells (COS1). Crude cytosol from a VDR-producing yeast strain (YEpV1) is shown. (*B*) Purified VDR and DNA probe were incubated with COS-1 nuclear extracts pretreated with either trypsin at 40 μg/mg of nuclear protein for 30 min at room temperature and then a 4-fold molar excess of trypsin inhibitor (Tryp) or both trypsin and a 4-fold molar excess of trypsin inhibitor (Tryp I). VDR–DNA complex was observed only when trypsin inhibitor was included with trypsin at the beginning of the incubation. (*C*) Purified VDR and OC DNA probe were incubated with COS-1 nuclear extracts pretreated for 30 min at the temperatures indicated. Complex is lost at 50°C or above.

Reticulocyte lysates containing the VDR were unable to bind to the OC DNA probe in the absence of $1,25(OH)_2D_3$ (data not shown). High-salt extracts of pAV-hVDR transfected COS-1 cells that contained both nuclear factor and unoccupied VDR were, therefore, examined for DNA binding activity after a preincubation in the absence or presence of increasing concentrations of $1,25(OH)_2D_3$. As illustrated in Fig. 5, whereas unoccupied VDR exhibited minimal capacity to interact with the OC DNA probe, occupied VDR bound the probe in a concentration-dependent manner. Thus, at least one role of the $1,25(OH)_2D_3$ hormone is to enhance the affinity of the receptor for specific DNA.

## DISCUSSION

In this report, we characterize the interaction of the VDR with a specific DNA sequence that mediates the action of the vitamin D hormone on the human OC gene promoter. We show that nuclear extracts derived from COS-1 cells transfected with a vector that allows expression of the human VDR contain a protein that binds to the VDRE by band-shift analysis. The lack of this binding activity in untransfected cells and its inhibition by an anti-VDR monoclonal antibody that interferes with VDR–DNA binding suggests that the protein is the VDR. Further, VDRE DNA binding activity requires the presence of $1,25(OH)_2D_3$ for maximal interaction. In addition, we show that VDR derived by *in vitro* translation or from extracts of a VDR-expressing yeast strain is capable of binding the VDRE only in the presence of a mammalian cell protein factor apparently of nuclear origin. Thus, the interaction of the VDR with a response element from the OC gene promoter requires both $1,25(OH)_2D_3$ and VDR-NAF.

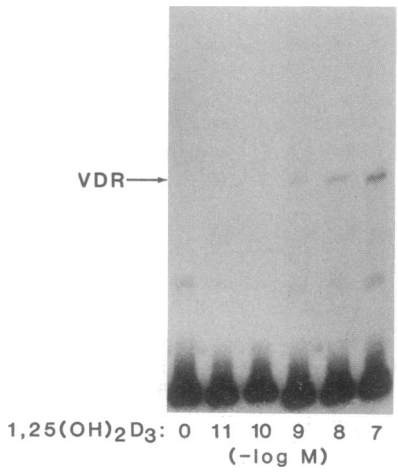


FIG. 5. Association of mammalian cell VDR to specific DNA is dependent upon $1,25(OH)_2D_3$. Cytosol prepared in high salt derived from pAV-hVDR-transfected COS-1 cells was incubated for 20 min at room temperature with an OC DNA probe and with the indicated molar concentrations of $1,25(OH)_2D_3$ and resolved. VDR–DNA complex is observed in this short incubation at 1 nM hormone.

The effect of hormone on steroid receptor binding to specific DNA sequence elements during band-shift analysis is controversial. Kumar and Chambon (17) have demonstrated the binding of the estrogen receptor to an estrogen response element is enhanced by hormone, although these experiments were carried out using a mutant form of the protein (18). The progesterone receptor has likewise been shown to bind to DNA in a hormone-responsive manner (19). In contrast, glucocorticoid (20) as well as thyroid (21) and retinoic acid (22) receptors bind in an apparently hormone-independent fashion. The effect we have seen here is not absolute—under conditions of unoccupied VDR excess, VDR–DNA complexes are apparent after band-shift analysis, suggestive of an acquired increase in DNA affinity. This apparent affinity change is consistent with previous observations that the presence of hormone causes an elevation in the salt concentration required for elution of VDR from cell nuclei (23) and from DNA cellulose *in vitro* (23, 24). The effect of hormone on steroid receptor activation is currently speculative, although the hormone may cause dissociation from heat shock protein (25), promote dimerization (17, 26), induce conformational change (27), allow posttranslational modification (28), or effect any combination thereof. Perhaps an effect of $1,25(OH)_2D_3$ on the VDR may be to enhance receptor affinity for the nuclear factor described here.

Although mammalian VDR associates with the VDRE, our studies using reticulocyte lysate and yeast-derived receptors

show conclusively that an additional component is required for specific high-affinity interaction. As all receptor-containing complexes comigrate in the band-shift assays presented here, VDR-NAF is likely present also in the VDR–DNA complex obtained from mammalian cell extracts. It is of interest that avian (29), mammalian (24), and yeast (14) VDR bind and elute from calf thymus DNA-cellulose in an identical fashion. Thus, heterologous DNA binding and specific VDRE binding are phenomena that are separable by the requirement for an additional protein in the latter reaction that produces a high-affinity complex stable under band-shift conditions. High-affinity complexes are not evident during heterologous DNA binding. Clearly, the isolation of NAF from the nucleus, its sensitivity to enzymatic digestion, and its instability to high temperature suggest that it is a protein, likely of nuclear origin.

The concept that the stability and activity of certain DNA binding proteins can be enhanced by and/or dependent upon additional protein–protein interactions is strikingly exemplified by the mechanism of action of the activator protein 1 (AP-1) gene family and the formation of Jun/Fos heterodimers (30). This concept has been strengthened with the observation that the DNA binding activity and/or transcriptional activity of additional proximal promoter basal transactivators such as CCAAT/enhancer binding protein (C/EBP) (31), Jun/Fos complex (32), and the chicken ovalbumin upstream promoter transcription factor (COUP-TF) (33) are all enhanced through interaction with additional protein factors. For COUP-TF, a member of the steroid receptor gene family (34), a clear association has been established between the presence of a nuclear factor and the transcriptional capacity of COUP-TF protein. Recent observations also suggest that nuclear factors may be required for high-affinity interactions between thyroid (35, 36) and retinoic acid (37) receptors and their respective specific DNA elements. Indeed, some evidence suggests that more than one activity may be present. As both the above receptors and the VDR lack a substantial N-terminal domain such as that found within glucocorticoid and progesterone receptors, perhaps heterodimeric function supplies this missing N-terminal function in the former class of proteins. We have not presented evidence to suggest that VDR-NAF described here is specific for the VDR alone or facilitates VDR transactivation. Clearly, further efforts will be required to demonstrate the relevance of this factor as well as the role of the $1,25(OH)_2D_3$ hormone in VDR transcriptional activity.

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