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Receptor Mediated Genomic Action of the $1,25(OH)_2D_3$ Hormone: Expression of the Human Vitamin D Receptor in *E. coli*

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The nuclear vitamin D receptor (VDR) binds the 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) hormone with high affinity and elicits its actions to stimulate gene expression in target cells by binding to the vitamin D-responsive element (VDRE). VDREs in such positively controlled genes as osteocalcin, osteopontin, β_3 integrin and vitamin D-24-OHase are direct hexanucleotide repeats with a spacer of three nucleotides. The present studies of VDR/VDRE interaction utilized full-length human vitamin D receptor (hVDR) that was overexpressed in *E. coli*, purified to near homogeneity (>95%), and its authenticity confirmed by demonstrating high affinity hormone binding and reactivity to monoclonal antibody 9A7γ. The expressed hVDR displays strict dependence on the family of retinoid X receptors (RXRs) for binding to the vitamin D-responsive element (VDRE) in the rat osteocalcin gene. Similar overexpression in *E. coli* of the DNA binding domain (Δ134), containing only residues 4–133 of hVDR, generated a receptor species that possesses intrinsic DNA binding activity. Both full-length and Δ134 hVDRs retain similar DNA binding specificities when tested with several natural hormone responsive elements, indicating that the N-terminal zinc finger region determines hVDR–DNA sequence selectivity. The C-terminal region of the molecule is required for hormone binding and confers the receptor with the property of very high affinity DNA binding, via heterodimerization between hVDR and RXR. A natural ligand for the RXR co-receptor, 9-*cis* retinoic acid, suppresses both VDR–RXR binding to the VDRE and $1,25(OH)_2D_3$ stimulated transcription, indicating that 9-*cis* retinoic acid recruits RXR away from VDR to instead form RXR homodimers.

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INTRODUCTION

The vitamin D receptor (VDR) is a member of the steroid/retinoid/thyroid hormone receptor superfamily by virtue of amino acid homologies, particularly in the two conserved functional domains for DNA- and hormone-binding [1, 2]. VDR mediates the biological effects of its hormonal ligand, 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), in target cells by binding via N-terminal zinc fingers to a DNA enhancer sequence, termed the vitamin D responsive element (VDRE), and regulating gene transcription. As summarized in Fig. 1, VDRE sequences have been identified and characterized in the 5′-flanking region of various vitamin D-dependent genes, including the rat osteocalcin gene [3, 4], the human osteocalcin gene [5], the mouse *Spp*-1/osteopontin gene [6], the avian β_3 integrin gene [7] and the rat vitamin D 24-hydroxylase (24-OHase) gene [8–10]. In addition, it has been observed that high affinity interaction of yeast- or insect-expressed VDR with the VDRE *in vitro* requires a nuclear receptor auxiliary factor (RAF) [4, 11, 12]. Several groups have provided evidence that RAF is comprised of the family of retinoid X receptors (RXRs) [13–15].

As pictured in Fig. 2, the C-terminal domain of

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GGGTGA	ATG	AGGACA	rat osteocalcin	Terpening *et al.* (1991) MacDonald *et al.* (1991)
GGGTGA	ACG	GGGGCA	human osteocalcin	Ozono *et al.* (1990)
GGTTCA	CGA	GGTTCA	mouse osteopontin	Noda *et al.* (1990)
GAGGCA	GAA	GGGAGA	avian β_3 integrin	Cao *et al.* (1993)
GGTTCA	GCG	GGTGCG	rat 24-OHase-Distal	Zierold *et al.* (1994)
AGGTGA	GTG	AGGGCG	rat 24-OHase-Proximal	Ohyama *et al.* (1994) Jurutka *et al.* (1994)

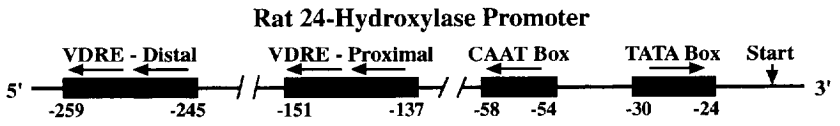

Fig. 1. Naturally occurring vitamin D responsive elements. The lower portion of the figure illustrates the positions of the proximal and distal VDREs in the rat 24-OHase promoter [8, 9, 10, 48].

human VDR (hVDR) not only binds the $1,25(OH)_2D_3$ ligand, but also contains regions responsible for heterodimerization with RXR, including residues 382–402 as well as 325–332 [16] and a highly conserved domain (E1) between amino acids 244 and 263 [17, 18]. The hormone binding domain and the DNA binding domain each contain a major phosphorylation site in hVDR, serines 208 and 51, respectively; these post-translational modifications may modulate the trans-activation function of hVDR [19–21]. Finally, several amino acids within hVDR (Fig. 2) have been identified that are required for transcriptional activation but do not necessarily co-map with the heterodimerization, hormone binding, or phosphorylation sites in hVDR [22]. Therefore, a current model of vitamin D action proposes that the hormone-bound VDR binds to the VDRE as a heterodimer with RXR, and the resulting VDR–RXR–VDRE complex then interacts with the transcriptional apparatus to regulate the expression of target genes [23].

With the goal of obtaining pure preparations of VDR in quantities adequate for structure–function studies and to confirm the above conclusions about VDR–DNA interaction, we have examined a number of *E. coli* expression vectors with various strategies of expression and purification. Freedman and coworkers [24, 25] reported *E. coli* systems for the expression and partial purification of both a 100 amino acid fragment containing the zinc fingers and of full-length hVDR. However, their studies differ from ours in that they neither examined $1,25(OH)_2D_3$ hormone binding kinetics nor characterized the expressed proteins immunologically. In the case of their 100 amino acid fragment VDR, Freedman and Towers [24] observed noncooperative binding to the natural mouse osteopontin VDRE, a perfect direct repeat, but only weak association with a human osteocalcin sequence which inexplicably contained only a single half element of the VDRE direct repeat. Utilizing 70–80% pure, full-length hVDR, Towers *et al.* [25] found putative homodimer binding to a direct repeat containing osteopontin-like half elements with a spacer of 3 bp, and identified several residues in the zinc finger region via site-directed mutagenesis that apparently mediate homodimerization of hVDR on this synthetic probe. We report in this paper the purification and characterization of the intact hVDR protein and a C-terminal truncation mutant containing primarily the two zinc fingers, each expressed in the BL21(DE3)plys S strain of *E. coli* utilizing a pT7-7 expression vector. In contrast to the work of Freedman and coworkers, we employed a natural VDRE from the rat osteocalcin gene, which is an imperfect direct repeat [3, 4], to probe the monomeric, homodimeric and heterodimeric DNA binding ability of *E. coli*-expressed hVDRs.

MATERIALS AND METHODS

Expression in E. coli and purification of hVDR and hRXRα

A 2 kb fragment of the hVDR cDNA was excised from our previously reported pSG5hVDR construct [26] and inserted into the unique EcoRI site of the expression vector pT7-7 which was a generous gift from Dr Stanley Tabor, Harvard Medical School, Boston, MA. The recombinant plasmid was then transformed into *E. coli* BL21(DE3)plys S (kindly supplied by Dr David Mangelsdorf). After growth to an A_{600} of 0.3–0.4 in LB medium with ampicillin (100 μg/ml) and chloramphenicol (25 μg/ml), 0.3 mM

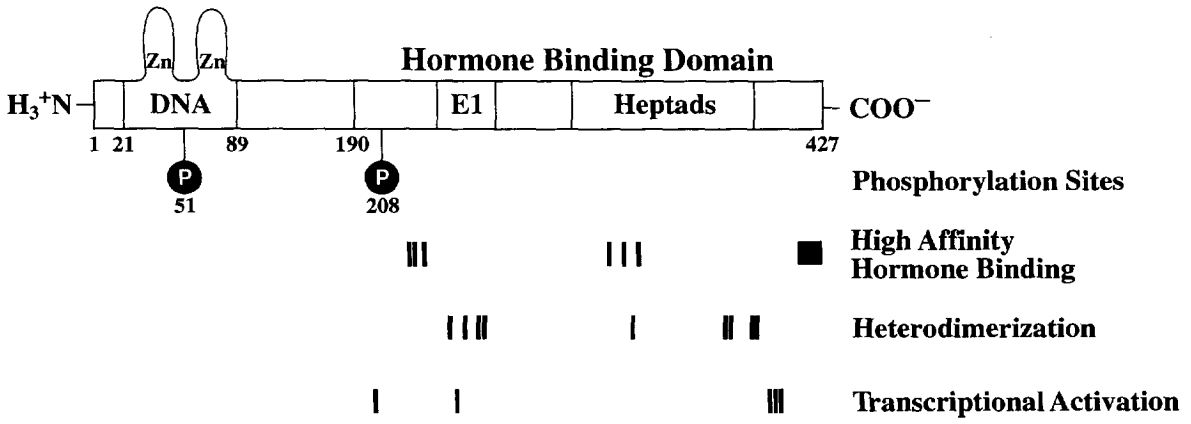

Fig. 2. Schematic depiction of human VDR, showing residues and domains which are involved in various functions. See text and Whitfield *et al.* [22] for details.

isopropyl-β-D-thiogalactopyranoside (IPTG) was added and incubation continued for an additional 3 h. Cells were then collected by centrifugation. Isolation of *E. coli*-expressed hVDR was carried out by the method of inclusion body-arrest essentially as described previously by Lin *et al.* [27]. The crude cell extract was applied to a hydroxylapatite column pre-equilibrated with 10 mM K_2HPO_4, pH 7.8, and eluted with a 100 ml linear gradient of 0.01–0.4 M K_2HPO_4. Binding activity for $1,25(OH)_2D_3$ eluted at an approximate salt concentration of 0.1 M K_2HPO_4. Human RXRα [28] was similarly expressed in *E. coli* using the pT7-7 vector and BL21(DE3)plys strain; it was expressed in a soluble form and enriched to approx. 70% purity by chromatography employing successive heparin–Sepharose and DNA–cellulose columns.

Gel mobility shift assay

Synthetic oligonucleotides [4] containing the VDRE sequence of the rat osteocalcin gene were as follows:

Wild-type CT5
5′-AGCTGCACTGGGTGAATGAGGACATTACA-3′

Mutant CT5
5′-AGCTGCACTGGtTGAATGAGcACATTACA-3′

The two six-base direct repeats are underlined. Shown in bold lower case in the mutant CT5 oligonucleotide are single bases in each motif which were altered to create a VDRE with reduced activity. Double-stranded CT5 and CT5M (mutant) oligonucleotides were labeled with [α-^{32}P]dCTP (3000 Ci/mmol) at the 5′-overhanging ends with the Klenow fragment of DNA polymerase I to a specific activity $>10^8$ cpm/μg DNA. Purified *E. coli*-expressed intact hVDR (100 ng) was incubated with or without rat liver nuclear extract, which contains RAF activity [16], in DNA binding buffer (10 mM Tris–HCl, pH 7.6, 100 mM KCl, 2 μg bovine serum albumin, 1 μg poly-(dIdC)) for 15 min at room temperature and then incubated with 0.5 ng of ^{32}P-labeled CT5 or CT5M for an additional 15 min. A 50- or 100-fold molar excess of unlabeled CT5 (30 min preincubation at room temperature) or 1 μg of monoclonal antibody against the VDR (9A7γ) was added for competition or binding inhibition experiments, respectively. The reaction mixtures were loaded onto a 4% non-denaturing polyacrylamide gel. Gels were run in 22.5 mM Tris–borate, 0.5 mM EDTA at 10 mA for 70 min, dried, and exposed for autoradiography.

RESULTS

Overexpression in E. coli and purification of hVDR

A 2 kb cDNA fragment containing the full-length hVDR sequence was inserted into several *E. coli* expression vectors, with the pT7-7 plasmid shown in Fig. 3(A) proving to be the most suitable for the expression of the hVDR gene (data not shown). The inserted hVDR cDNA was fused into the reading frame of the T7 gene 10 protein. The resulting construct, pT7-7hVDRI, contains six non-hVDR amino acids fused to the N-terminus. The pT7-7hVDRI plasmid was transformed into *E. coli* BL21(DE3)plys S, which contains a genomic copy of the viral T7 RNA polymerase under control of an IPTG-inducible UV5 promoter. After induction with 0.3 mM IPTG for 3 h, a high level of intact hVDR was expressed, estimated at 15% of the total protein [Fig. 3(B), lane 3]. To express a fragment of hVDR possessing the DNA binding fingers, a truncated Δ134 mutant hVDR (coding for residues 4–133) was also subcloned into pT7-7. The resulting plasmid was named pT7-7hVDRII. As shown in Fig. 3(C), the pT7-7 vector also mediated efficient expression of the Δ134 mutant hVDR with a high yield, estimated at 10% of the total protein.

Cell fractionation studies indicated that the full-length hVDR ($\sim$48 kDa) was predominantly confined to insoluble inclusion bodies (data not shown). For purification of this trapped protein, the inclusion

body-arrest method developed by Lin *et al.* [27] was employed to obtain soluble, bioactive protein. After denaturation/renaturation, the final dialysate contained highly enriched hVDR [Fig. 4(A), lane 4], which was subsequently purified to near homogeneity (>95%) by hydroxylapatite chromatography [Fig. 4(A), lanes 1 and 2]. The final yield of purified *E. coli*-expressed hVDR was about 5–10 mg/l of culture. The protein band of full-length hVDR obtained in this fashion reacted with anti-VDR monoclonal antibody 9A7γ [Fig. 4(B), lane 1] and comigrated exactly with the immunoreactive hVDR band generated from an extract

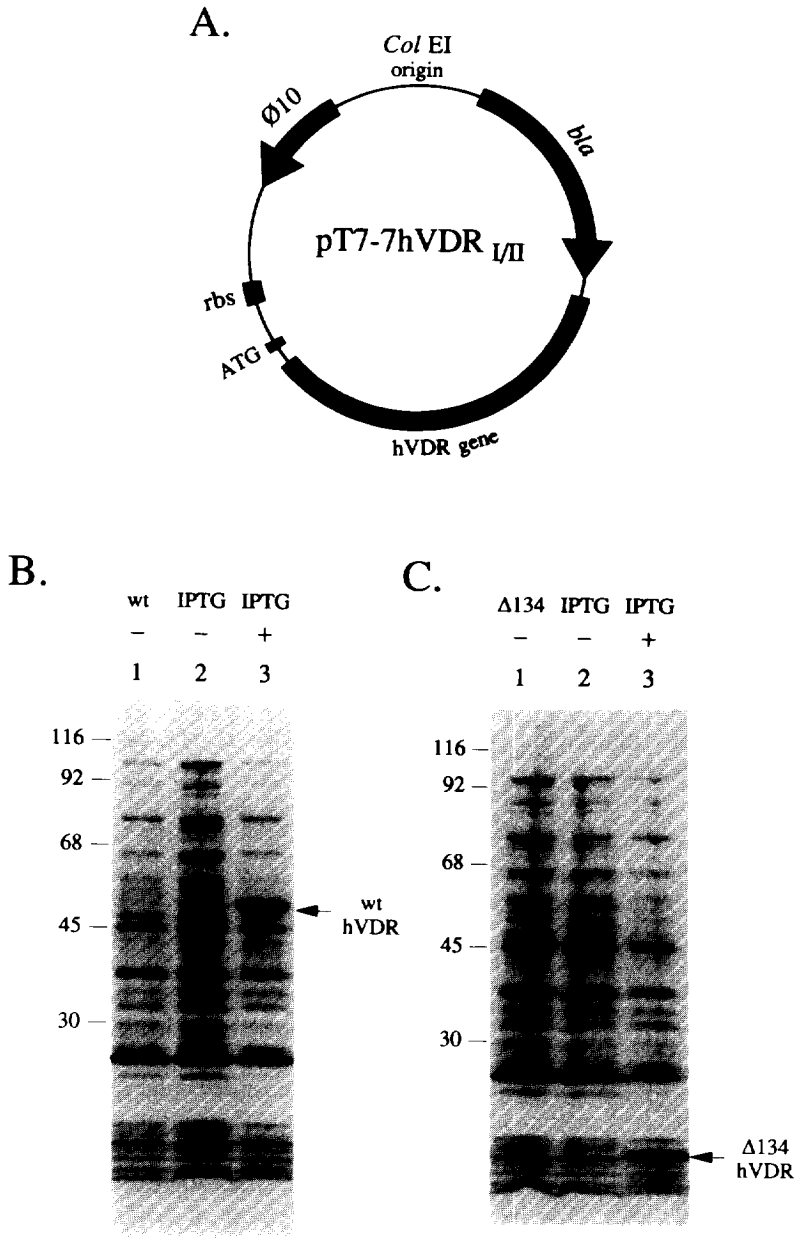

Fig. 3. Overexpression of the hVDR protein in *E. coli*. (A) Schematic diagram of the expression vector pT7-7hVDRI/II constructs. The ribosome binding site (rbs) is preceded by the T7 ϕ10 promoter and the ampicillin-resistance gene is denoted by *bla*. (B) Electrophoretic detection of overexpressed full-length hVDR. Extracts from BL21(DE3)plys S strain cells bearing pT7-7hVDRI grown without (lane 2) and with (lane 3) IPTG induction for 3 h were analyzed by 10% SDS–PAGE followed by Coomassie blue staining. Lane 1 represents an expression vector control that contains no hVDR cDNA insert. Molecular weight standards are designated in kDa on the left of the gel. (C) Overexpression of the C-terminally truncated Δ134 hVDR mutant. Whole cell extracts were analyzed on a 12.5% SDS-polyacrylamide gel. Lane 1 represents expression of a vector control containing no Δ134 insert; lane 2 is without IPTG induction and lane 3 is with IPTG induction.

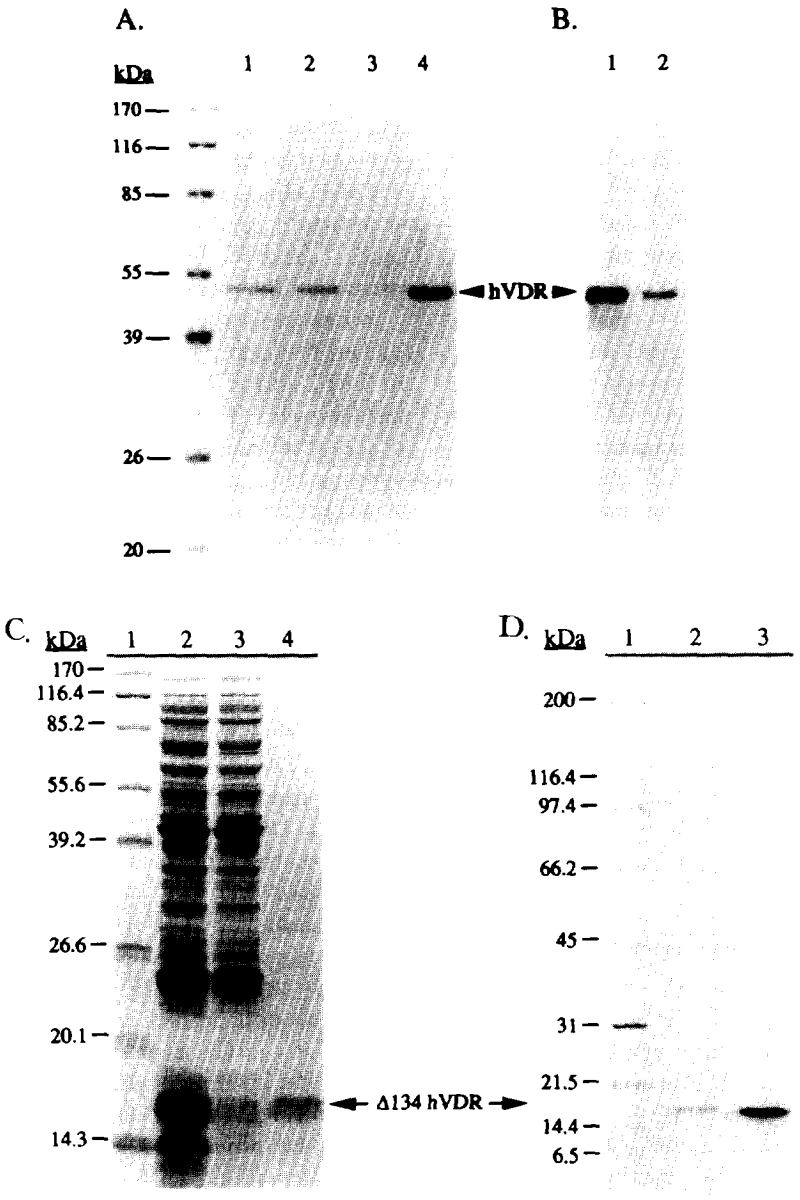

Fig. 4. Analysis of purified *E. coli*-expressed, full-length and Δ134 hVDRs by denaturing gel electrophoresis and Western blotting. (A) Coomassie-stained 10% SDS/polyacrylamide gel of purified *E. coli*-expressed wild type hVDR. Lanes 1 and 2, equal aliquots of pooled hVDR fractions eluted from a hydroxylapatite chromatographic column; lane 3, pooled flow-through fractions from the column; lane 4, extract from inclusion body-arrest before application to the hydroxylapatite column. (B) Western immunoblot analysis of the expressed hVDR. 1 μg of purified, *E. coli*-expressed hVDR (lane 1) and 40 μg of crude protein extract from COS-7 cells which were transfected with an hVDR expression plasmid, pSG5hVDR (lane 2), were analyzed via immunoblotting with monoclonal antibody 9A7γ as described elsewhere [21]. (C) Coomassie-stained 5–20% gradient SDS/polyacrylamide gel showing purification of *E. coli*-expressed Δ134 hVDR. Lane 2, crude extract before application to the DNA–cellulose column; lane 3, pooled flow-through fractions from the column; lane 4, pooled hVDR fractions eluted from the column. (D) Western immunoblot analysis of purified Δ134 hVDR. Lane 2, 40 μg of crude protein extract from COS-7 cells that were transfected with a pSG5Δ134 plasmid; lane 3, 1 μg of purified, *E. coli*-expressed Δ134 hVDR. Lane 1 in C and D consists of molecular weight standards.

of COS-7 cells [Fig. 4(B), lane 2] which had been transfected with the hVDR expression plasmid pSG5hVDR [26].

In contrast to full-length hVDR, Δ134 hVDR is largely soluble and was therefore extracted by the method of Alroy and Freedman [29] and then purified directly by DNA–cellulose chromatography [4]. The Δ134 hVDR was enriched to >80% purity [Fig. 4(C),

lane 4] and its authenticity was confirmed by immunoblot detection with VDR specific monoclonal antibody 9A7γ [Fig. 4(D)].

Hormone binding activity of E. coli-expressed hVDR

As expected, under no conditions tested did the Δ134 hVDR exhibit binding of the $1,25(OH)_2D_3$ ligand (data not shown). Initial tests of $1,25(OH)_2[^3H]D_3$ binding to *E. coli*-expressed, full-length hVDR yielded poor binding kinetics, characterized by relatively low affinity, non-saturable binding [30]. A dramatic improvement in the specific ligand binding activity occurred when rat liver nuclear extract was included in the assay [30]. Since rat liver nuclear extract does not itself contain detectable $1,25(OH)_2D_3$ binding activity [30], we have concluded that the hVDR requires some uncharacterized factor(s) for optimal $1,25(OH)_2D_3$ binding. Upon inclusion of rat liver nuclear extract, a classic $1,25(OH)_2D_3$ binding/saturation curve was obtained, and a Scatchard plot of those data revealed an apparent equilibrium dissociation constant (K_d) of 0.22 nM and a maximum concentration of receptor binding sites (N_{max}) of approx. 2.0 nmol/mg protein [30]. A second preparation of *E. coli*-expressed hVDR attained a specific activity of 3.43 nmol/mg protein (data not shown). This exceeds the concentrations achieved with other reported VDR expression systems, namely baculovirus [4, 31] and yeast [32], where the levels are 90–2000 pmol/mg protein and 50–200 pmol/mg protein, respectively.

DNA binding activity of E. coli-expressed hVDR

After incubating *E. coli*-expressed, full-length hVDR with ^{32}P-labeled wild-type VDRE (CT5) oligonucleotide and a RAF-containing fraction (rat liver nuclear extract), two major DNA–protein complexes were observed [Fig. 5(A), lanes 3 and 8]. Neither RAF nor *E. coli*-expressed hVDR alone showed specific DNA binding as evidenced by the lack of shifted complexes [Fig. 5(A), lanes 1 and 2]. Thus, unlike the results of Freedman and coworkers [25, 33] who employed vast excesses of hVDR, we find no evidence for the specific binding of homodimeric VDR complexes to a VDRE. The present data demonstrate that a RAF from mammalian nuclear extracts is absolutely required for a physiologic concentration of *E. coli*-expressed hVDR to bind efficiently to the natural VDRE of the rat osteocalcin gene. Both of the DNA–protein complexes shown in Fig. 5(A) can be inhibited by a specific monoclonal antibody (lane 7) which binds to an epitope near the DNA binding fingers of hVDR, confirming the specific involvement of the hVDR protein. DNA binding activity was reduced when *E. coli*-expressed hVDR was incubated with a mutated VDRE (CT5M) oligonucleotide [Fig. 5(A), lane 4] and by a 50- or 100-fold excess of unlabeled-CT5 oligonucleotide (lanes 5 and 6), indicating specific, high-affinity and saturable DNA binding.

Initial experiments indicated that the Δ134 hVDR formed two complexes with the VDRE in the absence of RAF, and several titration analyses demonstrated that complex I appears between 0.125 and 0.25 μl of receptor fragment solution while the formation of complex II requires between 0.5 and 1 μl [see Fig. 5(B) for a representative experiment]. Complex I is assumed to represent binding of the truncated receptor as a monomer, while complex II most likely represents the noncooperative association of two monomeric Δ134 hVDRs with the VDRE [24]. Using 0.75 μl (315 ng) of Δ134 hVDR preparation, we confirmed that unlike the full-length receptor, Δ134 hVDR does not require RAF for DNA binding [Fig. 5(C), compare lanes 1 and 2]. The Δ134 hVDR does, however, exhibit selectivity between the CT5 and CT5M probes [Fig. 5(C), compare lanes 1 and 4], characteristic of intact hVDR. The fact that both complexes can be inhibited and partially supershifted by 9A7γ specific antibody [Fig. 5(C), lane 3] confirms that the expressed Δ134 hVDR is the binding species involved in these interactions. Although Δ134 hVDR clearly possesses intrinsic VDRE binding ability, a careful analysis of lanes 1 and 2 of Fig. 5(C) reveals that RAF appears to slightly enhance the binding of the Δ134 DNA binding domain to the VDRE. However, control experiments with buffer alone indicated that this small effect was variable and not dependent on RAF or its components such as the RXRs. In contrast, in the case of full-length hVDR, addition of a highly enriched preparation of *E. coli*-expressed hRXRα elicited the striking appearance of a single VDR–RXR–VDRE complex (Fig. 6, lane 1). This result illustrates that hRXRα functions as an effective RAF or co-receptor with hVDR but does not bind to the VDRE as either a monomer or homodimer (Fig. 6, lane 3).

Fig. 5—*opposite*. Characterization of specific DNA binding by purified, full-length and Δ134 hVDRs using gel mobility shift analysis. (A) VDRE binding of full-length hVDR (100 ng). The constituents of each incubation are indicated in the lower portion of the figure; Ab is anti-VDR monoclonal antibody 9A7γ (1 μg). Lanes 3 and 8, full-length hVDR plus RAF yields two major retarded bands (denoted B and A). These bands may be comprised of VDR complexed with two different RXR isomers that are present in rat liver nuclear extract or with a single RXR which is partially proteolyzed. (B) Titration of DNA binding of Δ134 hVDR in a gel shift assay. Various amounts of purified Δ134 hVDR preparation (0.42 μg/μl) were examined to determine optimal conditions for binding to the CT5 VDRE. (C) VDRE binding of Δ134 hVDR. All reactions received 0.75 μl of Δ134 hVDR (0.42 μg/μl). Lane 2 shows Δ134 hVDR incubated with RAF (1 μg total protein). Lane 3 represents a reaction including 9A7γ monoclonal antibody (1 μg).

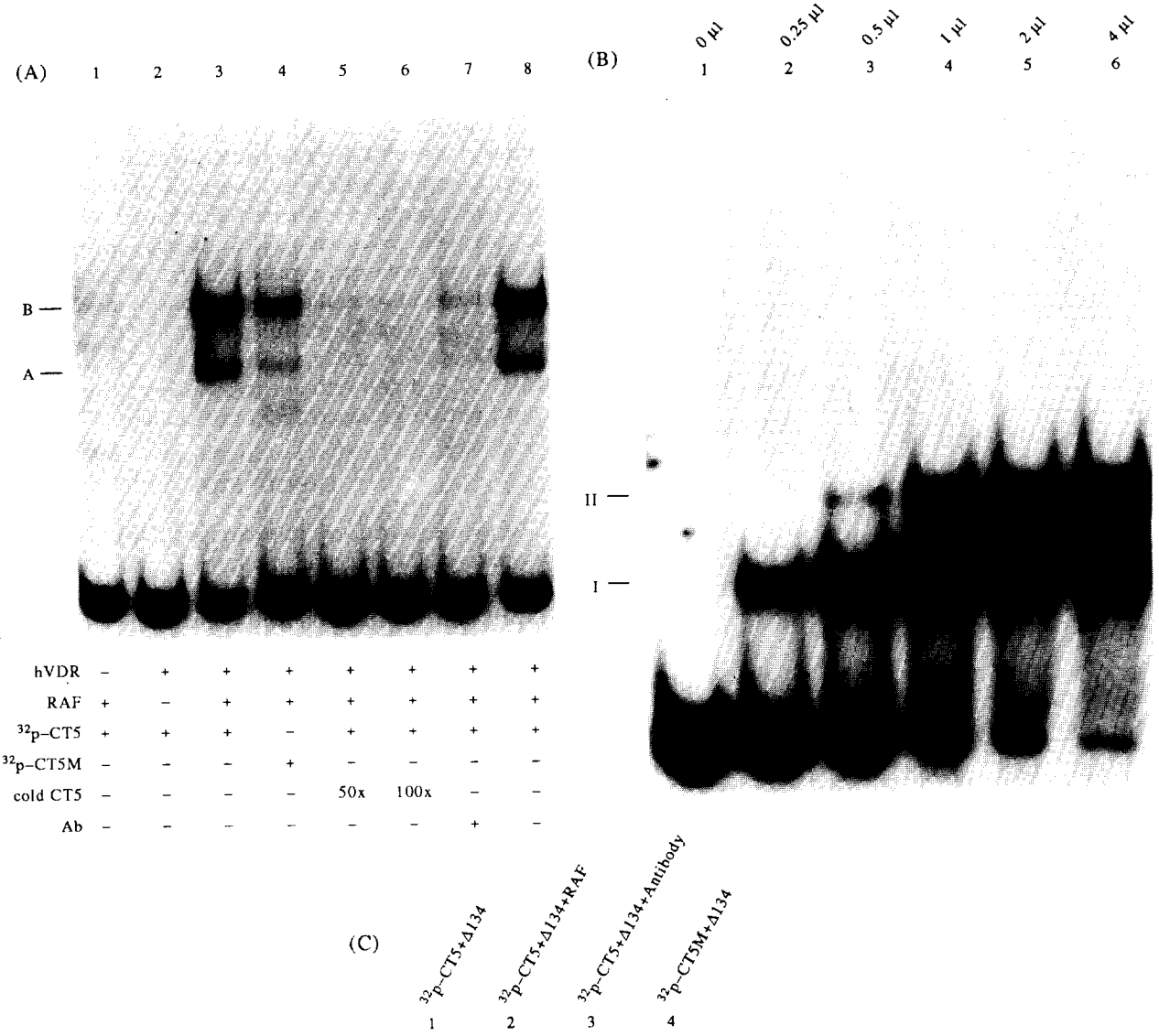

Fig. 5—*legend opposite.*

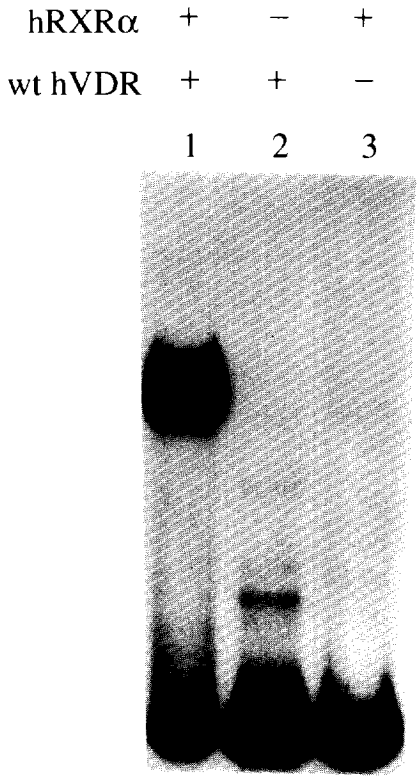

Fig. 6. Evaluation of the interaction of full-length hVDR with hRXRα via gel mobility shift analysis. The interaction of *E. coli*-expressed, full-length hVDR (100 ng) and hRXRα (100 ng) on the rat osteocalcin VDRE was characterized. Lanes 2 and 3 represent 100 ng of each receptor alone. The rapidly migrating retarded band in lane 2 may represent minor proteolysis of full length hVDR to yield a DNA-binding fragment analogous to Δ134 hVDR.

Finally, to evaluate the selectivity of *E. coli*-expressed hVDRs, several established hormone responsive elements (HREs), including those for glucocorticoids (GRE) [34] and thyroid hormone (TRE) [35], were examined for their interaction with full-length and Δ134 hVDRs using the gel mobility shift assay. The TRE contains sequence motifs similar to the VDRE half elements, GGGTGA and AGGACA, with a spacing of four nucleotides (see legend to Fig. 7) and, in fact, a VDRE in mouse calbindin-D_{28k} gene has been reported that possesses a spacer of four nucleotides [36]. The results from gel mobility shift experiments [Fig. 7(A and B)] show that *both* wild type and Δ134 hVDRs can form complexes with the TRE to some degree (lane 7), but exhibit little or no interaction with GRE (lane 5). Thus, no major variation in the specificity of DNA binding was detected between the full-length and Δ134 hVDRs. However, while competition of full-length hVDR is complete at a 100-fold excess of unlabeled probe [Fig. 7(A), lane 2], competition of Δ134 hVDR binding to the CT5 probe using 100-fold molar excess of unlabeled CT5 oligonucleotide revealed that Δ134 hVDR–VDRE interactions were not reduced and complex II actually increased (data not shown). As illustrated in Fig. 7(B, lane 2) a 1000-fold excess of radioinert probe is required to eliminate complex II and to significantly diminish complex I. This suggests that Δ134 hVDR possesses a lower affinity for the VDRE than does the intact hVDR–RAF heterocomplex, where as low as a 50-fold excess of unlabeled probe efficaciously eliminated VDRE interactions [Fig. 5(A), lane 5]. Confirmation of this conclusion was obtained by assessing hVDR–RAF–VDRE interactions at several concentrations of labeled nucleic acid probe. The K_d for full-length hVDR–RAF binding was approx. 2 nM while that for the Δ134 hVDR was $\geqslant$20 nM and difficult to assess because saturation could not be achieved (data not shown).

DISCUSSION

While our work was in progress [37], Freedman and coworkers [24, 25] and Kumar *et al.* [38] utilized alternative T7 promoter-containing vectors to express hVDR in *E. coli*. The data of Kumar *et al.* [38] are more in concert with our results in that a cell nuclear extract was reported to be necessary to facilitate hVDR binding to the human osteocalcin VDRE. Moreover, a human retinoic acid receptor (RAR) and a thyroid hormone receptor (TR) have been overexpressed in *E. coli* using combinations of T7 promoter and BL21(DE3)plys S host similar to that reported in the present communication. Like the expression of hVDR described here, TR [27] was formed within insoluble inclusion bodies, but RAR [39] was expressed almost completely in soluble form, raising interesting questions about what determines the solubility/insolubility of these highly homologous proteins. An unexpected observation reported elsewhere [30] was that *E. coli*-expressed, full-length hVDR does not appear capable of efficiently binding $1,25(OH)_2D_3$ with high affinity, and we discovered that expressed hVDR requires a nuclear extract for optimal $1,25(OH)_2D_3$ binding. One novel possibility is that RAF not only elicits efficient VDRE association via heterodimerization with the hVDR C-terminal domain [16, 18], but that it also confers this domain of hVDR with the property of high affinity ligand binding, either by stabilizing the hormone binding domain via protein–protein interaction or even by contributing a part of the ligand pocket in the heterodimer. The ecdysone receptor provides a precedent for such a phenomenon, since the insect RXR homologue, *ultraspiracle*, is a necessary co-receptor that endows the ecdysone receptor with high affinity sterol binding and responsiveness [40]. Based upon other experiments [30], relatively poor ligand binding also occurs with purified baculovirus-expressed hVDR, indicating that the requirement for a co-factor is not limited to hVDR expressed in bacteria; this argues against the possibility that improper hormone binding is the result of the denaturation/renaturation step for the *E. coli*-expressed hVDR.

However, other phenomena could potentially explain our observations, such as low solubility of the $1,25(OH)_2D_3$ ligand or purified hVDR instability at the very low protein concentrations of the ligand binding assay.

The DNA binding activities of *E. coli*-expressed, full-length hVDR and Δ134 hVDR were characterized in a series of experiments which included the presence/absence of RAF, a mutated VDRE (CT5M), antireceptor monoclonal antibody, and responsive elements such as the GRE and TRE. Two significant differences were observed between the binding of full-length vs Δ134 hVDR: (i) the full-length hVDR absolutely required RAF from mammalian nuclear extract or purified hRXRα to bind efficiently to the VDRE of the rat osteocalcin gene, while Δ134 hVDR possesses intrinsic DNA binding activity; (ii) intact hVDR, in the presence of RAF, bound to the VDRE with a higher apparent affinity than did the Δ134 hVDR mutant. The second noted difference was based upon competition experiments with excess radioinert probe and direct assessment of the kinetics of binding of each species. One explanation of these findings is that while deletion of the C-terminal region seemingly removes a repressor domain that masks the DNA binding zinc fingers in the intact hVDR, some important structural or conformational elements in the C-terminal region of hVDR are lost in the Δ134 hVDR truncation, resulting in reduced affinity for the VDRE. Therefore, true high affinity binding of hVDR to the rat osteocalcin VDRE requires heterodimerization of the C-terminal region of the receptor with RAF components like RXR, making the biological significance of putative homodimer associations of full length hVDR with synthetic, VDRE-like elements [25, 41] unclear at present.

The findings in the present communication therefore distinguish the VDR from the glucocorticoid receptor and other classic steroid hormone receptors which bind to DNA as homodimers and whose DNA binding determinants, as revealed by structural analysis, appear to reside primarily in the zinc finger domain [42]. The region of VDR immediately C-terminal of the zinc fingers (which is present in Δ134 hVDR), probably also contains a protein–DNA or protein–protein interaction surface because this segment includes residues that constitute the T- and A-boxes in orphan nuclear

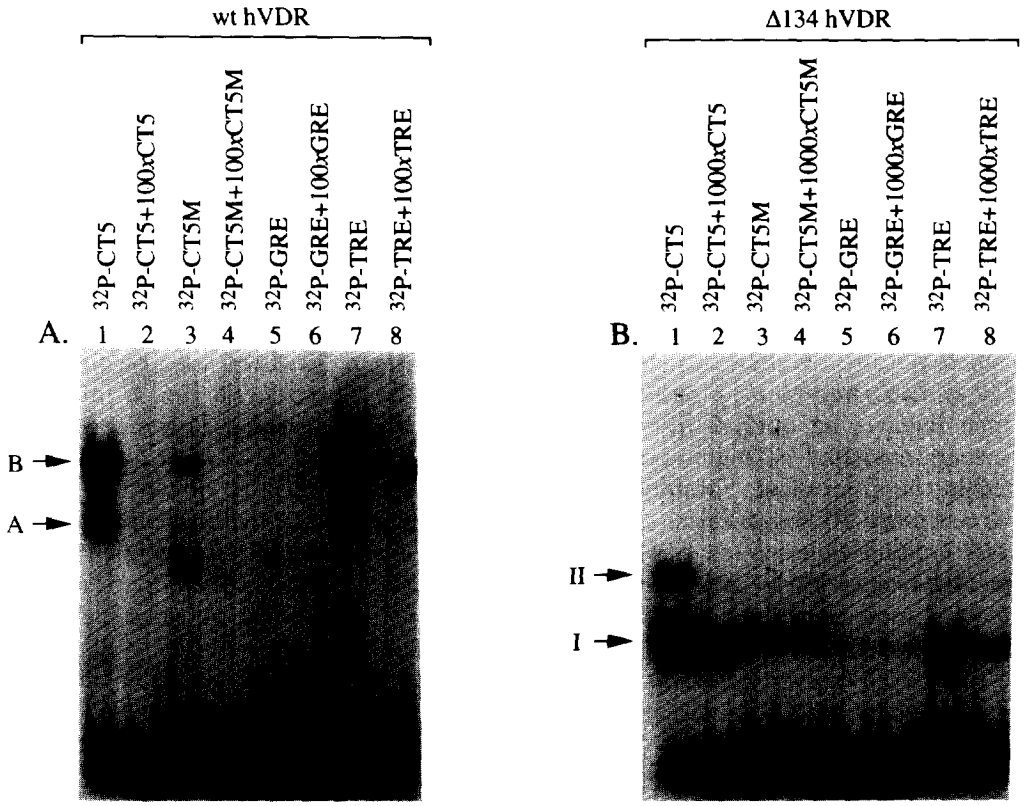

Fig. 7. Gel mobility shift analysis of hVDR binding to the VDRE and related HREs. The GRE sequence used was 5′-GATCCTGTACAGGATGTTCTAGCTA-3′ (lanes 5 and 6), and the TRE sequence used was 5′-TTTGGCTCTGGAGGTCACAGGAGGACAGC-3′ (lanes 7 and 8). Motifs similar to the VDRE half elements in the rat osteocalcin gene (CT5) are underlined. (A) Interactions between full length hVDR plus RAF and HREs. Lanes 2, 4, 6, and 8 represent incubations with a 100-fold molar excess of unlabeled HREs. (B) Complex formation between Δ134 hVDR and HREs. Lanes 2, 4, 6, and 8, represent incubation with a 1000-fold molar excess of unlabeled HREs.

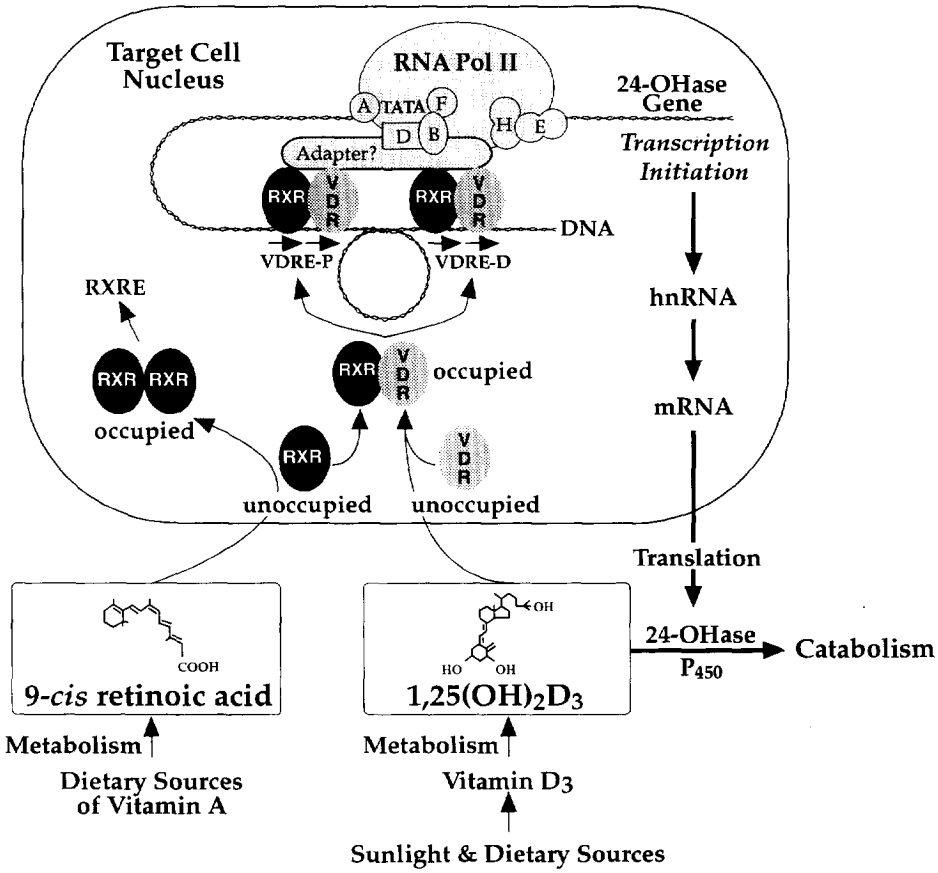

Fig. 8. Genomic action of the vitamin D hormone. VDRE-P refers to the proximal VDRE and VDRE-D refers to the distal VDRE in the rat 24-OHase gene as described in Fig. 1. Note that this model differs significantly in the early steps from that of Cheskis and Freedman [33] who maintain that unoccupied homodimeric hVDR binds initially to the VDRE and that $1,25(OH)_2D_3$ elicits dissociation of this inactive homodimer and favors formation of the active $1,25(OH)_2D_3$–VDR–RXR heterocomplex on the VDRE.

receptors such as NGF1-B [43]. Further, the binding of the VDR and other related receptors, namely the TR and RAR, to imperfect direct repeats as heterodimers with RAF/RXR probably represents a more complex association which requires an optimal interaction of both dimer partners mediated by heterodimerization domains located in the C-terminal, hormone-binding portion of these receptors. Although expression, truncation and DNA binding experiments such as those presented here can suggest these interactions, their ultimate elucidation must await a determination of the 3-dimensional structure of the heterodimeric receptor–DNA complex. The overexpression of hVDR and hRXRα reported here will eventually make such structural analyses feasible.

Utilizing osteocalcin VDREs and more recently the rat and human vitamin D 24-OHase VDREs, we [10, 15, 23] and others [44, 45] have been able to formulate a general hypothesis for the mechanism of VDR action in mediating transcriptional stimulation by the $1,25(OH)_2D_3$ hormone. The rat 24-OHase is unique in that its promoter region (see the lower portion of Fig. 1 for a schematic representation) contains two VDREs [8–10] and induction of 24-OHase P_{450}-initiates catabolic elimination of active vitamin D_3 metabolites [2]. This gene has been used by our group to develop the model illustrated in Fig. 8 for the action of the $1,25(OH)_2D_3$ hormone. This model is an extension of that provided by MacDonald *et al.* [23] for the rat osteocalcin VDRE. It shows that the $1,25(OH)_2D_3$ ligand may bind to the unoccupied VDR monomer or associate preferentially with a binary complex of VDR and RXR; a third possibility is that $1,25(OH)_2D_3$ functions analogously to ecdysone and allosterically facilitates heterodimerization [40]. What is certain is that the $1,25(OH)_2D_3$ ligand enhances the ability of the VDR/RXR heterodimer to bind to VDREs [9, 11, 15, 33]. In the case of the rat 24-OHase promoter, we propose that the VDR/RXR heterodimer associates with both the proximal and distal VDREs and that these occupied *cis* elements may cooperatively stimulate the transcription of the 24-OHase gene. In a process that is not understood at the molecular level, the hormone–receptor complexes enhance the initiation of transcription by RNA polymerase II and its associated transcription factors (Fig. 8).

Furthermore, we originally reported that a novel retinoid, 9-*cis* retinoic acid, which is a natural ligand for RXR [46], significantly attenuates $1,25(OH)_2D_3$-mediated transcriptional activation from the rat osteocalcin VDRE [15]. Recently, this conclusion has been verified for other vitamin D-regulated genes, including human osteocalcin [44], human 24-OHase [45] and rat 24-OHase (Jurutka, Thompson, Hsieh and Haussler, unpublished results). As shown in Fig. 8, 9-*cis* retinoic acid suppresses vitamin D action apparently by diverting unoccupied RXR away from heterocomplexes with VDR to instead bind as occupied homodimers to the RXRE. This concept also applies to thyroid hormone action via TR/RXR heterodimers, which is decreased by 9-*cis* retinoic acid via a mechanism involving RXR homodimer formation [47]. Therefore, in a mechanism that likely occurs in all vitamin D target cells, the action of the $1,25(OH)_2D_3$ hormone can be limited in either of two ways: (i) induction of the 24-OHase P_{450} which initiates the rapid catabolism of the ligand; or (ii) the intervention of 9-*cis* retinoic acid to divert the RXR silent partner from participation with VDR in the activation of gene transcription.

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