The vitamin D hormone and its nuclear receptor: molecular actions and disease states

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Abstract

Vitamin D plays a major role in bone mineral homeostasis by promoting the transport of calcium and phosphate to ensure that the blood levels of these ions are sufficient for the normal mineralization of type I collagen matrix in the skeleton. In contrast to classic vitamin D-deficiency rickets, a number of vitamin D-resistant rachitic syndromes are caused by acquired and hereditary defects in the metabolic activation of the vitamin to its hormonal form, 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), or in the subsequent functions of the hormone in target cells. The actions of 1,25(OH)₂D₃ are mediated by the nuclear vitamin D receptor (VDR), a phosphoprotein which binds the hormone with high affinity and regulates the expression of genes via zinc finger-mediated DNA binding and protein–protein interactions. In hereditary hypocalcemic vitamin D-resistant rickets (HVDDR), natural mutations in human VDR that confer patients with tissue insensitivity to 1,25(OH)₂D₃ are particularly instructive in revealing VDR structure/function relationships. These mutations fall into three categories: (i) DNA binding/nuclear localization, (ii) hormone binding and (iii) heterodimerization with retinoid X receptors (RXRs).

That all three classes of VDR mutations generate the HVDDR phenotype is consistent with a basic model of the active receptor as a DNA-bound, 1,25(OH)₂D₃-ligated heterodimer of VDR and RXR. Vitamin D responsive elements (VDREs) consisting of direct hexanucleotide repeats with a spacer of three nucleotides have been identified in the promoter regions of positively controlled genes expressed in bone, such as osteocalcin, osteopontin, β₃-integrin and vitamin D 24-0Hase. The 1,25(OH)₂D₃ ligand promotes VDR-RXR heterodimerization and specific, high affinity VDRE binding, whereas the ligand for RXR, 9-cis retinoic acid (9-cis RA), is capable of suppressing 1,25(OH)₂D₃-stimulated transcription by diverting RXR to form homodimers. However, initial 1,25(OH)₂D₃ liganding of a VDR monomer renders it competent not only to recruit RXR into a heterodimer but also to conformationally silence the ability of its RXR partner to bind 9-cis RA and dissociate the heterodimer. Additional probing of protein–protein interactions has revealed that VDR also binds to basal transcription factor IIB (TFIIB) and, in the presence of 1,25(OH)₂D₃, an RXR-VDR-TFIIB ternary complex can be created in solution. Moreover, for transcriptional activation by 1,25(OH)₂D₃, both VDR and RXR require an intact short amphipathic α-helix, known as AF-2, positioned at their extreme C-termini. Because the AF-2 domains participate neither in VDR-RXR heterodimerization nor in TFIIB association, it is hypothesized that they contact, in a ligand-dependent fashion, transcriptional coactivators such as those of the steroid receptor coactivator family, constituting yet a third protein–protein interaction for VDR. Therefore, in VDR-mediated transcriptional activation, 1,25(OH)₂D₃ binding to VDR alters the conformation of the ligand binding domain such that it: (i) engages in strong heterodimerization with RXR to facilitate VDRE binding, (ii) influences the RXR ligand binding domain such that it is resistant to the binding of 9-cis RA but active in recruiting coactivator to its AF-2 and (iii) presents the AF-2 region in VDR for coactivator association. The above events, including bridging by coactivators to the TATA binding protein and associated factors, may position VDR such that it is able to attract TFIIB and the balance of the RNA polymerase II transcription machinery, culminating in repeated transcriptional initiation of VDRE-containing, vitamin D target genes. Such a model would explain the action of 1,25(OH)₂D₃ to elicit bone remodeling by stimulating osteoblast and osteoclast precursor gene expression, while concomitantly triggering the termination of its hormonal signal by inducing the 24-0Hase catabolizing enzyme.

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Introduction

Classic nutritional rickets is caused by the simultaneous deprivation of sunlight exposure and dietary vitamin D. As depicted in Fig. 1, the pathways comprising the metabolic activation of the vitamin to its hormonal form and subsequent functions in target tissues present a number of additional steps where defects elicit vitamin D-resistant rachitic syndromes. Two of these disorders involve the inadequate bioactivation of 25-hydroxyvitamin D$_3$ (25(OH)D$_3$) to 1,25-dihydroxyvitamin D$_3$ (1,25(OH)$_2$D$_3$) by the kidney as catalyzed by the 1-OHase enzyme (Fig. 1). Acquired chronic renal failure results in renal rickets and secondary hyperparathyroidism (renal osteodystrophy) when the compromising of renal mass reduces 1-OHase activity (Haussler & McCain 1977). The etiology of pseudo-vitamin D-deficiency rickets (PDDR) apparently involves a hereditary defect in the gene coding for the 1-OHase enzyme (Labuda et al. 1992). Interestingly, the PDDR locus is resolvable from that of the vitamin D receptor (VDR) but maps very close to it on chromosome 12 in the 12q13-14 region (Labuda et al. 1992). Recently, a cDNA was cloned for the rat 1-OHase (St-Arnaud et al. 1996) and it is expected that the human renal 1-OHase gene will soon be cloned and its chromosomal location determined. The likelihood that both the gene encoding the enzyme that generates the 1,25(OH)$_2$D$_3$ hormone and the cognate hormone receptor gene lie in close proximity on chromosome 12 invites speculation about the evolution of the vitamin D ligand-receptor system.

The traditional actions of vitamin D, via its 1,25(OH)$_2$D$_3$ hormonal metabolite, are to effect calcium and phosphate homeostasis to ensure the deposition of bone mineral on type I collagen matrix (summarized in Fig. 1). 1,25(OH)$_2$D$_3$ stimulates intestinal calcium and phosphate absorption, bone calcium and phosphate resorption, and renal calcium and phosphate reabsorption, all resulting in a sufficient Ca$^{2+}$-PO$_4$ ion product to precipitate hydroxyapatite. Failure to achieve normal bone mineral accretion by these mechanisms leads to rachitic syndromes. Recently, a breakthrough has occurred in our understanding of what was originally known as hypophosphatemic vitamin D-resistant rickets, a familial disorder of renal phosphate wasting more appropriately referred to as dominant X-linked hypophosphatemic (HYP) rickets (Fig. 1). The gene defect responsible for HYP rickets has been fine mapped in the Xp22.1 region, harboring a gene identified as PEX, or phosphate regulating gene with homologies to endopeptidases located on the X-chromosome (Francis et al. 1995). One hypothesis is that PEX codes for an endopeptidase that apparently correctly processes a peptide precursor to yield a novel, as yet unidentified, phosphate retaining hormone. The normal function of this hormone may be to oppose the action of parathyroid hormone (PTH) and stimulate phosphate reabsorption by the renal tubule by inducing the Na$^+$-phosphate cotransporter. However, the existence of tumor-induced osteomalacia, an acquired disorder that closely resembles the phosphate wasting of HYP rickets and is characterized by low circulating 1,25(OH)$_2$D$_3$ (Parker et al. 1981), combined with renal cross-transplantation (Nesbitt et al. 1992) and
parabiosis (Meyer et al. 1989) studies in normal and hyp mice, indicates strongly that the HYP phenotype is caused by excessive amounts of a phosphaturic hormone in the circulation. This humoral peptide is distinct from PTH and has been named phosphatonin (Cai et al. 1994, Econs & Dreznner 1994). Thus, instead of PEX mutations resulting in insufficient generation of a novel phosphate-retaining peptide, they may instead elicit the appearance of abnormally high circulating levels of phosphatonin, with the normal role of the PEX gene product postulated to be the proteolytic inactivation of this phosphaturic principle. Most germane to the vitamin D endocrine system is the fact that serum 1,25(OH)₂D₃ levels are inappropriately low for the prevailing phosphate concentrations in HYP rickets and patients can be cured with a therapeutic combination of phosphate and 1,25(OH)₂D₃ (Harrel et al. 1985). Because it is well known that hypophosphatemia stimulates 1,25(OH)₂D₃ production (Hughes et al. 1975), the PEX/phosphatonin system might constitute yet another regulatory loop in maintaining normal phosphate homeostasis. One could hypothesize that under hypophosphatemic conditions, when 1,25(OH)₂D₃ levels are elevated, the sterol hormone not only increases intestinal phosphate absorption (Fig. 1) and suppresses PTH synthesis (DeMay et al. 1992) to conserve phosphate, but also induces the PEX gene product (Rowe et al. 1996) to cleave phosphatonin and further promote renal phosphate reclamation.

1,25(OH)₂D₃ is primarily recognized as a calcemic hormone, perhaps due to the abundance of dietary phosphate, or because calcium homeostasis is more vitamin D-dependent than the regulation of extracellular phosphate. Regardless of the mechanism, traditional vitamin D-deficiency and clinically significant defects in the vitamin D receptor lead invariably to hypocalcemia and secondary hyperparathyroidism, with phosphate being somewhat less affected. As illustrated in Fig. 1, target tissue insensitivity to 1,25(OH)₂D₃ is known as hereditary hypocalcemic vitamin D-resistant rickets (HVDRR) and is caused by defects in the gene on chromosome 12 coding for the VDR. A review of the etiology of HVDRR and the natural mutations in the VDR that confer tissue insensitivity and clinical resistance to 1,25(OH)₂D₃ is particularly instructive in illuminating the physiologic relevance of the 1,25(OH)₂D₃-VDR hormone-receptor complex as well as structure/function relationships in the receptor itself.

**Natural mutations in the nuclear vitamin D receptor**

Clinically significant hereditary hypocalcemic vitamin D-resistant rickets is an autosomal recessive disorder resulting in a phenotype characterized by severe bowing of the lower extremities, short stature and, often, alopecia (Rut et al. 1994). The serum chemistry in HVDRR includes frank hypocalcemia, secondary hyperparathyroidism, elevated alkaline phosphatase, variable hypophosphatemia and markedly increased 1,25(OH)₂D₃. The symptoms of HVDRR, with the exception of alopecia, mimic classic vitamin D-deficiency rickets, suggesting that VDR not only mediates the bone mineral homeostatic actions of vitamin D but may also participate in the differentiation of hair follicles in utero. Recently, VDR knockout mice have been created (Yoshizawa et al. 1996), revealing apparently normal heterozygotes but severely affected homozygotes (VDR−/−), 90% of which die within 8–10 weeks. Surviving mice lose their hair and possess low bone mass, hypocalcemia, hypophosphatemia and 10-fold elevated 1,25(OH)₂D₃ coincident with extremely low 24,25(OH)₂D₃. All of these parameters in the VDR knockout mouse mimic the phenotype of patients with HVDRR, confirming that VDR normally mediates all of the bone mineral regulating functions of vitamin D. Interestingly, although natural point mutations in other receptors related to VDR, such as thyroid hormone receptor β (TRβ) (Collingwood et al. 1994), are characterized by dominant negative receptors that generate the thyroid hormone resistant phenotype in the heterozygotic context, no natural, dominant negative mutations have yet been identified in HVDRR, patients (Whitfield et al. 1996). Thus, all HVDRR cases studied to date are homozygous for the particular VDR mutation.

Figure 2 illustrates a number of point mutations in VDR that have been detected in HVDRR patients (reviewed in Rut et al. 1994, Haussler et al. 1995). Three of these genetic alterations result in nonsense mutations that introduce stop codons in VDR (R73stop, Q152stop and Y295stop), creating truncated VDRs that lack both hormone- and DNA-binding (heterodimerization) capacities and are associated with unstable mRNAs. More revealing are the series of missense mutations (Fig. 2) that can be classified according to three of the basic molecular functions of VDR: (i) DNA binding/nuclear localization by the N-terminal zinc finger region, (ii) 1,25(OH)₂D₃ hormone binding by the C-terminal domain and (iii) heterodimerization with retinoid X receptors (RXRs) through subregions of the C-terminal domain. As depicted schematically in Fig. 2 and discussed in detail later, VDR is a ligand-dependent transcription factor that controls gene expression by heterodimerizing with RXR and associating specifically with vitamin D responsive elements (VDREs) in target genes. Since VDR is a member of the steroid, retinoid, thyroid hormone receptor superfamily, and belongs to the VDR/retinoic acid receptor (RAR)/TR subfamily of RXR heterodimerizing species (Haussler et al. 1991), it is reasonable to draw from data on RAR and TR for comparison with VDR.

The greatest number of VDR natural mutations characterized to date are localized to the DNA binding, zinc finger region (Fig. 2). The first two discovered, G33D and...
Figure 2 Natural mutations in the human vitamin D receptor leading to 1,25(OH)₂D₃ hormone resistance. See text for details and citations. N37, K91 and F92 are not sites of VDR natural mutations, but are so designated because they are heterodimerization contacts that lie within the DNA binding domain (Hsieh et al. 1995, Rastinejad et al. 1995). The eight cysteine residues (C) that tetrahedrally coordinate two zinc atoms in the finger structure are also denoted.

R73Q (Hughes et al. 1988), reside at the ‘tips’ of the fingers and affect charge–charge interactions between VDR and the phosphate backbone of DNA. When viewed in toto, the zinc finger region mutations in HVDRR (Fig. 2) have the following two general properties: (i) they occur in residues conserved across the entire nuclear receptor superfamily and (ii) most lie within α-helices on the C-terminal side of the first and second fingers which are intimately involved in DNA base recognition and phosphate backbone contacts respectively (Rastinejad et al. 1995). These observations suggest that many of the clinically significant mutations in VDR which are still compatible with life may not greatly perturb the fundamental structure of the DNA binding domain of the receptor, but instead compromise its ability to recognize DNA with specificity and high affinity. Whether HVDRR cases with mutations in zinc finger region residues unique to VDR will be uncovered depends upon the properties of such alterations, which could range from innocuous to lethal.

Mutations located within the hormone binding domain of VDR also elicit the HVDRR phenotype (Fig. 2), including R274L (Kristjansson et al. 1993) and H305Q (Malloy et al. 1995). Transcriptional activation by R274L and H305G VDR is attenuated as a result of inefficient 1,25(OH)₂D₃ binding, ranging from severe in the case of R274L to a modest increase in Kₐ for H305Q. In both instances, transcriptional activation is restored when the dose of 1,25(OH)₂D₃ is raised to pharmacologic levels (10⁻⁶ M) in transfection experiments (Kristjansson et al. 1993, Malloy et al. 1995). Our laboratory has recently characterized two novel VDR hormone binding domain mutations in HVDRR patients, I314S and R391C, that significantly affect the heterodimerization of VDR with RXR (Whitfield et al. 1996). Both of these C-terminal replacements (Fig. 2), however, do display some degree of what may be a hormone binding deficit, a phenomenon not observable in typical in vitro ligand binding kinetic assays at 4 °C. Thus, only at 37 °C in intact cells do R391C and I314S exhibit apparent slight and significant impairment of 1,25(OH)₂D₃ high affinity retention respectively (Whitfield et al. 1996). Further, the two mutations in question are situated in or adjacent to heptad repeats (Fig. 2), hypothetical coiled-coil-like structures that were originally proposed to participate in the heterodimerization of VDR, RAR, and TR with RXR (Forman & Samuels 1990, Nakajima et al. 1994). Consistent with this concept, both R391C and I314S VDRs do not bind RXR with normal affinity when assayed in vitro, with the greatest impairment of heterodimerization occurring with R391C (affinity reduced by one order of magnitude) (Whitfield et al. 1996). Additional evidence supporting blunted RXR heterodimerization by these two mutant VDRs is provided by transfection experiments in
which 1,25(OH)\textsubscript{2}D\textsubscript{3}-stimulated transcription can be restored to that of normal fibroblasts when fibroblasts from patients harboring either the R391C or the I314S mutation are cotransfected with exogenous RXR. Yet this apparent RXR rescue of the mutated VDRs requires approximately 10-fold elevated 1,25(OH)\textsubscript{2}D\textsubscript{3} doses compared with the response to hormone in normal fibroblasts (Whitfield et al. 1996). This latter observation reveals that the hormone binding and heterodimerization functions of VDR are not entirely separable, an aspect which is also apparent from fundamental biochemical analysis of the hormone dependency of VDR–RXR heterodimer binding to VDREs as discussed in detail below.

Understanding the molecular properties of natural VDR mutations in HVDRR allows us to comprehend why the patients respond differentially to therapy with massive doses of 1,25(OH)\textsubscript{2}D\textsubscript{3}, or suitable analogs. For example, cases with zinc finger region aberrations are unresponsive to the hormone because DNA binding is precluded by the absence of structural complementarity between VDR and the VDRE, regardless of the 1,25(OH)\textsubscript{2}D\textsubscript{3} liganding or heterodimerization of the receptor in solution. Conversely, patients harboring mutations in the hormone binding/heterodimerization domain can be responsive to pharmacologic doses of 1,25(OH)\textsubscript{2}D\textsubscript{3} or analogs, even though the hormone already is increased in the circulation because of the hypocalcemia caused by tissue insensitivity. For example, patient I314S was essentially cured by excess vitamin D metabolite, indicating that compensating for the hormone binding deficit was able to override the milder heterodimerization defect and allow sufficient VDRE binding by the VDR–RXR heterodimer. Conversely, patient R391C responded only modestly to treatment with excess 1,25(OH)\textsubscript{2}D\textsubscript{3} analog, presumably because the fundamental heterodimerization defect could not be overcome and therefore normal VDRE binding could not be achieved (Whitfield et al. 1996).

The final insights gained from the natural VDR mutations summarized in Fig. 2 are structural in nature. We have discussed previously that the zinc finger mutations are confined to absolutely conserved residues. In the crystal structure of the DNA binding domain heterodimers of RXR\textalpha{} and TR\textbeta{} (Rastinejad et al. 1995), the lysine and arginine residues corresponding to K45 and R50 in human VDR (hVDR) make direct base contacts with DNA, while the arginines corresponding to R73 and R80 in hVDR make direct DNA phosphate backbone contacts. That mutations in these four residues are clinically important in the etiology of HVDRR argues for structural congruity between the VDR finger region and that of TR. Rastinejad et al. (1995) have extended this assumption to include a modeling of RXR–TR vs RXR–VDR bound to DNA which accommodates the fact that TR binds as a heterodimer to a direct hexanucleotide repeat spaced by four nucleotides (DR+4), while VDR binds as a heterodimer to a similar set of half elements spaced by three nucleotides (DR+3). In addition to verifying the common protein–DNA interfaces, their modeling predicts that hVDR residues N37 in the first finger and K91/E92 C-terminal of the second finger (see Fig. 2) engage in heterodimeric contacts with residues in the second zinc finger of RXR, to form effectively a stable, DNA-supported heterodimer. Indeed, recent site-directed mutational studies (Hsieh et al. 1995) indicate that the alteration of K91 and E92 in hVDR in fact grossly reduces transactivation while moderately attenuating heterodimerization and DNA binding, thus confirming the importance of K91 and E92. An additional surprising finding was that the K91/E92 double mutant manifested dominant negative characteristics (Hsieh et al. 1995), distinguishing it from the natural HVDRR replacements discussed above. Apparently, the K91/E92 mutant VDR is able to bind DNA sufficiently through its native zinc finger and strong heterodimerization function in the ligand binding domain such that it can block binding by wild type receptor, but is rendered inactive in stimulating transcription because of a presumed conformational perturbation initiated by unstable or improper alignment of the heterodimer on the VDRE.

Based upon recently reported X-ray crystal structures of the ligand binding domains of ligand-occupied hRAR\textgamma{} (Renaud et al. 1995), agonist-occupied rat TR\textalpha{}, (Wagner et al. 1995) and unoccupied, but dimeric hRXR\textalpha{} (Bourguet et al. 1995), it is also possible to incorporate the HVDRR mutations in the hormone binding domain (Fig. 2) into a hypothetical structural context. Figure 3 constitutes a schematic compilation of the existing crystallographic data and compares them with natural and artificially generated mutations in hVDR. At the top of Fig. 3, the residue numbers for VDR in the ligand binding domain appear in relation to the older heptad repeat nomenclature (heptads 1–9, dotted boxes). At least some of these heptads, particularly heptads 4 and 9, are thought to facilitate heterodimerization (Nakajima et al. 1994). The E1 region is a highly conserved area that supports heterodimerization (Whitfield et al. 1995b). The helices depicted schematically in Fig. 3 (open boxes) are those determined for hRAR\textgamma{}; this general pattern of \textalpha{}-helices and \textbeta{}-strands (solid boxes) appears to be well conserved across the TR, RAR and RXR members of the subfamily crystallized thus far (Bourguet et al. 1995, Renaud et al. 1995, Wagner et al. 1995). Although the heterodimerization domains have yet to be elucidated by structural analysis, the homodimerization domain of RXR is comprised of helices 7, 9 and 10 (Fig. 3 and Bourguet et al. 1995). Flanking the dimerization region are clusters of ligand binding contacts, shown for RAR and TR in Fig. 3, which paint a picture of hormone binding involving helices 3, 5, 11 and 12 plus portions of helices 6 and 7 along with their intervening loop, as well as the loop between \textbeta{}-strands 1 and 2. As summarized in Fig. 3 and
discussed by Whitfield et al. (1995a, 1996), a number of artificially generated mutants in hVDR support the concept that the dimerization and hormone binding regions in VDR are well aligned with those in RXR, RAR and TR. Of even greater interest and relevance to the present monograph, the four clinically important hVDR mutants under consideration correspond to pertinent locations in the known structures of the retinoid and thyroid hormone receptor ligand binding domains. We postulate that this general structural organization represents that of the VDR ligand binding domain. As shown in Fig. 3, the pure hormone binding mutant hVDRs, namely R274L and H305Q, are located precisely within ligand clusters in helix 5 and in the loop between helix 6 and 7 respectively. I314S, which endows hVDR with combined defects in hormone retention and heterodimerization, lies within helix 7 at a presumed interface of ligand binding and dimerization activities of the receptor (Fig. 3). Finally, R391C is positioned well within the helix 10 dimerization surface, but not far removed from C-terminal ligand binding contacts that are likely influenced by replacement of this amino acid in hVDR. Thus, at least within the context of the assumed structural organization of VDR derived from that of other subfamily members, the I314S and R391C mutations are situated precisely where they would be predicted to lie, given the biological properties of the mutant receptors and the phenotype of the patients. These results not only have profound implications concerning the putative structure of VDR in relation to its closest relatives, but prove unequivocally that the calcemic actions of 1,25(OH)₂D₃ are mediated by the vitamin D receptor, existing as a 1,25(OH)₂D₃-ligated heterodimer with RXR that is bound to DNA.

Physiology and cellular actions of 1,25(OH)₂D₃

In order to delineate the physiologic roles for the vitamin D hormone, it is appropriate first to place the VDR mediator into the context of vitamin D metabolism and cellular actions. Figure 4 summarizes the integration of vitamin D metabolism and cellular actions introduced in Fig. 1, with physiologic regulatory events now superimposed on the metabolic pathway and the inclusion of an expanded list of physiologic actions for the 1,25(OH)₂D₃ hormone. The conversion of vitamin D₃ to 25(OH)D₃ by the liver is a constitutive metabolic step, followed by the 1-hydroxylation of 25(OH)D₃ to 1,25(OH)₂D₃, a reaction under exquisite control (Haussler & McCain 1977). When blood calcium is low, activation of this latter step occurs, either as a result of the hypocalcemic state per se, or in response to elevated PTH, each of which serves independently to enhance renal 1-OHase activity. Low phosphate is also capable of separately upregulating the 1-OHase enzyme. To limit activation, the hormonal product, 1,25(OH)₂D₃, effects an ultra-short feedback loop to suppress its own biosynthesis in the kidney and also represses PTH synthesis to remove the peptide hormone stimulus of the 1-OHase via a longer feedback loop (Fig. 4). However, the dominant negative feedback controls of 1-OHase activity appear to result from the concerted actions of 1,25(OH)₂D₃ to stimulate bone mineral resorption and to promote intestinal calcium and phosphate absorption, which together elicit an increase in blood calcium and phosphate levels, each of which down-regulates the 1-OHase.

The process by which 1,25(OH)₂D₃ causes bone remodeling is complex, involving stimulation of osteoclast
differentiation and osteoblastic production of osteopontin, both of which activate resorption in part through the recognition of bone matrix osteopontin by osteoclast surface α₃β₃-integrin. The resorption effect is supported by 1,25(OH)₂D₃-elicited suppression of bone formation via the induction of osteocalcin and the repression of type I collagen. This latter insight that the normal function of osteocalcin is to curtail bone matrix formation arises from the creation of osteocalcin knockout mice (Ducy et al. 1996). In addition to stimulating the transcription of bone-related genes such as osteopontin and osteocalcin, the 1,25(OH)₂D₃ hormone also induces its own catabolism in kidney as well as other target tissues like bone by enhancing the expression of the vitamin D-24-OHase enzyme. 24-Hydroxylation of 1,25(OH)₂D₃ is the first step in deactivating the hormone, which is eventually metabolized by side chain cleavage to calcitriol acid (Haussler 1986). Thus, the synthesis of 1,25(OH)₂D₃ is not only governed by feedback mechanisms that sense 1,25(OH)₂D₃, calcium, PTH and phosphate concentrations, but the hormone induces the termination of its own signal in target tissues, qualifying 1,25(OH)₂D₃ as a *bona fide* hormone by any definition.

As introduced in the section on HVDRR, mediation of the cellular functions of 1,25(OH)₂D₃ requires that VDR bind the hormonal ligand specifically and with high affinity (Fig. 4). Upon such binding, VDR becomes hyperphosphorylated (Jurutka et al. 1993, Haussler et al. 1994) and recruits RXR into a heterodimeric complex that binds strongly to DNA (Fig. 4). The 1,25(OH)₂D₃-ligated RXR-VDR heterocomplex selectively recognizes VDREs in the promoter regions of positively controlled genes such as osteocalcin (MacDonald et al. 1991), osteopontin (Noda et al. 1990), vitamin D-24-OHase (Ohyama et al. 1994) and β₃-integrin (Cao et al. 1993). Negative VDREs (Haussler et al. 1995) exist in the 5'-regions of the genes for type I collagen (Pavlín et al. 1994), bone sialoprotein (Li & Sodek 1993), PTH (DeMay et al. 1992) and PTH-related peptide (Falzon 1996, Kremer et al. 1996). The mechanisms whereby VDR accomplishes positive and negative control of DNA transcription after VDRE association are not well understood, although substantial progress has been made in comprehending the stimulation of transcription as detailed in later sections of this article. Moreover, as summarized in Fig. 5, a number of VDREs have been definitively characterized. The prototypical VDRE is found in the osteocalcin gene, consisting of an imperfect direct repeat of hexanucleotide estrogen responsive element (ERE)-like, half-sites with a spacer of three nucleotides (DR+3). Classic EREs possess a central GT core at positions 3 and 4 of the hexanucleotide, but this feature is only partially conserved in the six natural positive VDREs listed in Fig. 5. There is, however, absolute conservation of the A in position 6 of the 5' half-element and of the G at position 2 of the 3' half-element. A preliminary working consensus for the positive VDRE can be derived from these natural VDREs (see boxed sequence in Fig. 5). This generalization is supported, in part, by PCR experiments that were designed to select, from random oligonucleotides, the highest affinity DNA ligand for the RXR-VDR heterodimer (Nishikawa et al. 1994, Colnot et al. 1995).
The random selection process yields an identical VDRE 5' half-element of GGGTCA (Fig. 5, bottom), which is also a preferred RXR target when RXR homodimers bind to DNA (Yang et al. 1995). This observation is in concert with the conclusion (Jin & Pike 1996) that, with respect to association of RXR-VDR with VDREs, RXR lies on the 5' half-element whereas VDR is situated on the 3' half-element. Examination of both consensus sequences suggests that the G at position 3 of the spacer is important in VDR binding, a deduction consistent with the finding (MacDonald et al. 1991) that this base is partially protected by RXR-VDR in methylation interference assays. However, interesting differences arise when one compares the most frequently encountered 3' half-element bases in natural VDREs, namely the GGGGCA composite which actually occurs in human osteocalcin, with the GGGTCA random consensus selection for the 3' half-element (Fig. 5). Clearly, GGGTCA represents a potent VDR binding site, a supposition that is bolstered by the fact that osteopontin, which possesses a perfect DR+3 of GGGTCA, is the highest affinity VDRE we have tested (data not shown). Intriguingly, Ts at positions 3 and 4 in the 3' VDR half-site occur infrequently in the balance of natural VDREs (Fig. 5). The paucity of Ts in the 3' half-element could be related to a need for varying potency of VDREs in regulated genes, or may even provide for a repertoire of different VDR conformations that could be induced by contact with distinct 3' half-site core sequences. This postulated range of VDR conformations might endow the receptor with the ability to recruit a variety of different coactivators and corepressors, or even to favor the binding of one vitamin D metabolite ligand over another. Irrespective of the above considerations, it is evident that the primary VDRE is a DR+3 recognition site in DNA that directs the VDR to the promoter region of 1,25(OH)2D3 regulated genes, ultimately altering the functions of target cells as a result of transcriptional control of gene expression.

**Significance of lipophilic ligands in the association of RXR-VDR with DNA**

Dimeric complexes are a feature commonly employed in the regulation of eukaryotic transcriptional systems. This process of protein dimerization often will generate novel heterodimeric complexes which display highly cooperative binding to DNA as well as an altered target sequence specificity (Glass 1994). Among the classical steroid hormone receptors, dimerization results in the formation of symmetrical homodimeric protein complexes on palindromic DNA half-sites. Dimerization has been shown to be mediated in part by residues within the DNA binding domain of the receptor (Luisi et al. 1991) and is enhanced by residues within the ligand binding domain (Falwell et al. 1990). The other subfamily of nuclear hormone receptors, including VDR, TR and RAR, apparently binds with highest affinity to direct repeat elements either as homodimers or, more commonly, as heterodimers with RXR (Kliwer et al. 1992). In both subgroups of nuclear receptors, protein–protein interactions serve to align the DNA binding domains so that they are optimally positioned to bind to their specific DNA target sequences (Kurokawa et al. 1993, Perlmann et al. 1993, Rastinejad et al. 1995). The ligand binding region of these receptors is multifunctional, in that this domain not only binds the cognate ligand, but also it possesses a dimerization surface as well as the ligand-dependent transactivation function, AF-2 (Gronemeyer 1991, Chambon 1994). The
dimerization surface consists of packed helices which are stabilized by hydrophobic heptad repeats interspersed throughout the structure. Ligand apparently can influence different functional components, including the dimerization interface, and the activating AF-2 domain (Reinaud et al. 1995, Wagner et al. 1995). Therefore, a likely role for ligand is to regulate the association and dissociation of dimeric protein complexes and hence regulate specific binding to DNA target sequences.

In this regard the following three questions remain regarding 1,25(OH)2D3-mediated control of positively regulated genes: (i) does VDR bind as a homodimer (Freedman et al. 1994, Nishikawa et al. 1994) as well as a heterodimer to DR+3 VDREs? (ii) What is the effect of the 1,25(OH)2D3 ligand on VDR or VDR–RXR binding to VDREs? (iii) What role does 9-cis retinoic acid, the RXR ligand, play in RXR–VDR binding to VDREs and enhanced transcription of 1,25(OH)2D3-responsive genes?

It is generally accepted that TR forms homodimers as well as heterodimers with RXR on thyroid hormone responsive elements (TREs), although recent data suggest that the TR homodimer, when unoccupied by thyroid hormone, operates as a repressor of transcription (Chin & Yen 1996, Schulman et al. 1996). Thyroid hormone is proposed to associate TR homodimers to facilitate TR–RXR heterodimerization on the TRE and stimulate transcription. In contrast, RAR does not appear to be capable of forming homodimers on DR+5 retinoic acid responsive elements (RAREs) (Perlmann et al. 1996), instead cooperating exclusively with RXR in RARE association and vitamin A metabolite-responsive transcription. When present in excess in gel mobility shift DNA binding assays in vitro, both TR and RAR display RXR heterodimeric association with their respective hormone responsive elements (HREs) in the absence of added lipophilic ligand. These in vitro studies are consistent with immunocytochemical data indicating that, unlike classic steroid hormone receptors that reside in the cytoplasm complexed with Hsp-90 and other proteins in their unoccupied state, unliganded TR, RAR and VDR (Clemens et al. 1988) exist in the nucleus in general association with DNA. These findings have led to the dogma that ligand is not required for TR, RAR, and VDR to associate with target HREs. Indeed, we have observed that addition of 260 ng baculovirus–expressed hVDR to a gel shift reaction generates weak homodimeric VDR as well as strong VDR–RXR–heterodimeric binding to a rat osteocalcin VDRE probe, both of which are independent of the presence of 1,25(OH)2D3 (Nakajima et al. 1994). However, in vivo footprinting experiments (Blanco et al. 1996, Chen et al. 1996) have led to the conclusion that, at least in the case of RAR–RXR heterodimers, RAR ligands are required for RARE binding. We, therefore, sought to devise an in vitro gel shift assay that would more accurately reflect the in vivo situation, primarily consisting of the use of physiologic salt (0-15 mM KCl) concentrations and limited amounts of partially purified, baculovirus–expressed VDR and RXRs (Thompson et al. 1997). Utilizing this assay, we have addressed the three questions regarding VDR/RXR listed above, namely heterodimer versus homodimer, the potential role of 1,25(OH)2D3, and the effect of 9-cis retinoic acid (9-cis RA).

When 20 ng VDR (~10 nM) or 20 ng RXR plus 20 ng RXR are incubated with either the rat osteocalcin or mouse osteopontin VDREs (see Fig. 5), no DNA-bound homodimeric VDR species is apparent, but a VDRE complexed VDR–RXR heterodimer occurs that is strikingly dependent upon the presence of the 1,25(OH)2D3 ligand (Thompson et al. 1997). Thus, at receptor levels approaching that in a typical target cell, a VDR ligand–dependent heterodimer with RXR is the preferred VDRE binding species. Only when VDR or VDR plus RXR levels are raised to 100 ng of each receptor with the mouse osteopontin VDRE (Thompson et al. 1997), or 260 ng with the weaker rat osteocalcin VDRE (Nakajima et al. 1994), can faint homodimers of VDR bound to the probe be visualized. In addition, at these greater amounts of receptors, neither the VDR homodimer nor the VDR–RXR heterocomplexes are modulated significantly by inclusion of 1,25(OH)2D3 in the incubation (Thompson et al. 1997). We, therefore, conclude that higher receptor levels in vitro generate artifactual VDR homodimers as well as attenuate the normal physiologic ligand dependence of VDR–RXR binding to the VDRE. To explain seemingly ligand-independent VDR–RXR association with the VDRE, we postulate the existence of a subpopulation of VDR that is unstably activated in the absence of 1,25(OH)2D3 (Schulman et al. 1996) and therefore capable of heterodimerization to generate a positive gel mobility shift under conditions of vast receptor excess. In contrast, our physiologically relevant gel shift assay at ≤10 nM receptor levels and 0-15 mM KCl reflects the presumed in vivo events of ligand triggered heterodimerization (Blanco et al. 1996, Chen et al. 1996), and extends earlier in vitro data showing that 1,25(OH)2D3 enhances VDR–RXR complex formation (Sone et al. 1991, MacDonald et al. 1993, Ohyama et al. 1994).

Next, we tested the effect of 9-cis RA in this gel shift assay. A spectrum of data exists on the role of 9-cis RA in 1,25(OH)2D3-stimulated transcription, including demonstration of synergistic action with 1,25(OH)2D3 (Carlberg et al. 1993, Schrader et al. 1994, Kato et al. 1995, Sasaki et al. 1995), negligible action (Ferrara et al. 1994), or an inhibitory effect (MacDonald et al. 1993, Jin & Pike 1994, Lemon & Freedman 1996). These marked differences likely result from varying transfection and ligand addition protocols, as well as cell and species specificity. Employing the physiologic gel shift procedure with biochemically defined components, we obtained clear evidence that 9-cis RA is a potent inhibitor of 1,25(OH)2D3–enhanced, VDR–RXR binding to VDREs such as osteocalcin, with dramatic attenuation by the retinoid occurring at
concentrations as low as $10^{-7} \text{ m}$ (Thompson et al. 1997). Previous gel shift data had also hinted at 9-cis RA inhibition (MacDonald et al. 1993, Chesiks & Freedman 1994), even though higher concentrations of 9-cis RA were utilized in these earlier studies. One somewhat puzzling finding, however, was that the suppressive effect of 9-cis RA seemed more pronounced \textit{in vitro} than \textit{in vivo}. To probe for distinct routes of assembly, we varied the order of addition of VDR, RXR, 1,25(OH)$_2$D$_3$ and 9-cis RA in the gel shift assay for VDRE binding (Thompson et al. 1997). The results showed that 9-cis RA is a potent inhibitor of VDR-RXR heterodimerization on the VDRE in all situations except when VDR alone is preincubated with 1,25(OH)$_2$D$_3$ followed by addition of RXR (Thompson et al. 1997). To explain these data, we have developed the model depicted in Fig. 6, which hypothesizes two alternative allosteric pathways for the interaction of VDR–RXR with the VDRE. In pathway A (Fig. 6), 1,25(OH)$_2$D$_3$ occupies monomeric VDR, altering the conformation of the ligand binding domain such that it recruits RXR for heterodimeric binding to DNA and subsequent VDRE recognition. Importantly, we postulate that previously occupied VDR conformationally influences RXR in the resulting heterodimer such that it is incapable of being liganded by 9-cis RA (pathway A, Fig. 6). This action to abolish RXR ligand responsiveness both silences the ability of 9-cis RA spuriously to trigger vitamin D hormone signal transduction, and prevents 9-cis RA from dissociating the RXR-VDR complex in order to divert RXR for retinoid signal transduction. On the other hand, as illustrated in pathway B (Fig. 6), we propose that RXR exists in a different, 9-cis RA-receptive, allosteric state in most other circumstances, such as when present as a monomer, in an apoheterodimer with VDR, or even when the apoheterodimer of RXR and VDR is subsequently liganded with 1,25(OH)$_2$D$_3$. This latter species of RXR-VDR-1,25(OH)$_2$D$_3$ (pathway B) is hypothesized to be fully competent in VDRE recognition, but the 9-cis RA binding function of the RXR partner has not been conformationally repressed, rendering this form sensitive to dissociation by 9-cis RA, which would then favor the formation of retinoid-occupied RXR homo- dimers. Therefore, unless VDR monomers are first occupied by 1,25(OH)$_2$D$_3$ (pathway A), 9-cis RA can operate to divert or dissociate RXR and direct it to form RXR homodimers (pathway B). It is tempting to speculate that the 1,25(OH)$_2$D$_3$-liganded heterodimer in pathway A is more potent in transcriptional stimulation than the analogous species in pathway B, perhaps because the AF-2 function of the RXR partner is allosterically activated only in the former instance. The 1,25(OH)$_2$D$_3$-occupied VDR–RXR in pathway B has the advantage of flexible regulation because it is effectively a two-ligand switch. It likely occurs \textit{in vivo} because, as stated above, the fact that 9-cis RA blunting of 1,25(OH)$_2$D$_3$ responsiveness is
significant but incomplete suggests that at least two populations of RXR–VDR heterodimers exist. Finally, when our model (Fig. 6) is compared with those for RXR–RAR and RXR–TR (Forman et al. 1995), it is evident that VDR is closer in mechanism of action to the TR, where 9-cis RA inhibits TR signal transduction by diversion of RXR (Lehmann et al. 1993). Also analogous is the fact that thyroid hormone occupation of the TR partner abolishes 9-cis RA binding to the RXR counterpart (Forman et al. 1995). Finally, the action of RXR–RAR heterodimers seems to be fundamentally different from that of RXR–VDR in that RAR liganding by a retinoid facilitates RXR occupation by its retinoid ligand, resulting in cooperative stimulation of gene transcription by the repertoire of vitamin A metabolites.

**VDR protein–protein interactions that effect gene transcription**

Although we now have at least a rudimentary understanding of ligand-induced VDR binding to a VDRE, the next logical question is how does VDR regulate the machinery for gene transcription? In the basal state of DNA transcription, the TATA-box binding protein (TBP) and its associated factors (TAFs) are bound to the TATA box at approximately position −20 in the 5’ region of controlled genes, but the frequency of transcriptional initiations is very low because the RNA polymerase II–basal transcription factor IIB (TFIIB) enzyme complex is not stably associated with TBP–TAFs. The recruitment of the TFIIB–RNA polymerase II complex appears to be the rate limiting step in preinitiation complex formation, and is stimulated dramatically when a transactivating factor or factors bind to upstream enhancers. In a process involving DNA looping, transactivators are thought to attract TFIIB and also interact with TAFs, forming a stable preinitiation complex that executes repeated rounds of productive transcription. Recent data indicate that the activation function in the hormone binding domain of the estrogen receptor, AF-2, associates specifically with a TAF known as TAF130 (Jacq et al. 1994) and that the estrogen receptor (ER) binds to TFIIB in vitro (Ing et al. 1992). In collaboration with Ozato and associates and Tsai and O’Malley, we have observed that hVDR also specifically associates with hTFIIB (Blanco et al. 1995). In this work, Blanco et al. (1995) showed that VDR binds to a TFIIB–glutathione S transferase fusion protein linked to glutathione-laden beads. Additionally, it was observed that both TRα and RARα interact with hTFIIB (Blanco et al. 1995), but that RXR does so only very weakly (P W Jurutka, L S Remus and M R Haussler, unpublished results). This last result suggests that, while the ligand binding partners in the VDR/TR/RAR subfamily provide a hard-wired connection to the assembly and enhancement of the transcription machinery, the RXR partner is not primarily engaged in TFIIB contact.

Independent data obtained by MacDonald et al. (1995) using the powerful yeast two-hybrid system to detect protein–protein interactions also revealed that hVDR binds efficiently to TFIIB. Moreover, MacDonald et al. (1995) further exploited the yeast two-hybrid system to prove that, while hVDR and RXR interact, no homodimeric association occurs for hVDR alone, providing further evidence against the existence of physiologically significant VDR homodimers. Utilizing fusion protein technology, they also showed that VDR interacts directly with RXR to form a heterodimer in solution in the absence of DNA and, further, that this process was enhanced 8-fold by the presence of 1,25(OH)2D3 hormone (MacDonald et al. 1995). Because hVDR–TFIIB association is not dependent upon the 1,25(OH)2D3 ligand (Blanco et al. 1995, MacDonald et al. 1995), the role of 1,25(OH)2D3 can now be further resolved to an early participation in conforming VDR such that it attracts RXR followed by the targeting of the resulting RXR–VDR heterodimer to VDREs (see Fig. 6).

Interestingly, the presence of RXR further facilitates VDR–TFIIB association, especially in the presence of 1,25(OH)2D3 (P W Jurutka, L S Remus and M R Haussler, unpublished results). In fact, because of its capacity to enhance VDR–RXR heterodimerization, the 1,25(OH)2D3 ligand is capable of generating high levels of an RXR–VDR–TFIIB ternary complex in solution, significantly in excess of that occurring with either RXR and TFIIB or even with VDR and TFIIB (P W Jurutka, L S Remus and M R Haussler, unpublished results). These data not only reaffirm the interaction of VDR with TFIIB, but also they imply that the 1,25(OH)2D3-ligated VDR–RXR complex is the most efficient binder of TFIIB. This latter effect may be the result of positive conformational influences of RXR on liganded VDR, since VDR is the primary attachment moiety for TFIIB.

Because VDR–TFIIB interactions have been detected either in vitro or in the yeast system where certain mammalian cell restrictions may be relaxed, it was important to confirm the relevance of VDR–TFIIB association in mammalian cells. Blanco et al. (1995) have reported functional studies which, for the first time, show the interaction of TFIIB with a member of the steroid receptor superfamily in ligand-dependent activation of transcription in intact cells. In pluripotent P19 mouse embryonal carcinoma cells, transfection of hVDR or hTFIIB alone produced no better than a 2-fold induction of VDRE–luciferase reporter expression by 1,25(OH)2D3. However, when transfected together, hVDR and hTFIIB mediated a synergistic transcriptional response of approximately 30-fold when 1,25(OH)2D3 was added, an effect which was absolutely dependent on the presence of the VDRE in the luciferase construct. It should be noted that the VDR–TFIIB positive cooperation appears to be cell-specific because similar experiments in contact-inhibited NIH/3T3 Swiss mouse embryo cells resulted in
Indeed, as illustrated in Fig. 7, the region of VDR from residues 416 to 422 possesses a high degree of similarity to the analogous sequences in the entire nuclear receptor superfamily. One hallmark of this conserved sequence is the glutamic acid residue at position 420 of hVDR (Fig. 7) included in a consensus of \( \Phi\Phi\Theta\Phi\Theta\Phi \) (where \( \Phi = \alpha \)-hydrophobic amino acid) for this domain (Renaud et al. 1995, Wagner et al. 1995). Allowing for conservative replacements, it seems virtually certain that hVDR forms an amphipathic helix (corresponding to helix 12 in the other receptors) surrounding glutamic acid-420 that is analogous to the ligand-dependent activation function (AF-2) characterized for TR (Barettino et al. 1994), RAR (Renaud et al. 1995), RXR (Leng et al. 1995) and ER (Danielian et al. 1992). Although this AF-2 domain is capable of autonomously activating transcription (Leng et al. 1995), that such activity is modest may be because of the fact that the AF-2 region is proposed to operate in a ligand-dependent fashion, involving a structural rearrangement to reposition the AF-2 for both intramolecular and intermolecular protein–protein interactions. Specifically, based upon the crystal structure of unliganded RXR (Bourguet et al. 1995) and liganded RAR (Renaud et al. 1995) and TR (Wagner et al. 1995), helix 12/AF-2 appears to protrude outward from the more globular ensemble of helices 1–11 in the absence of ligand, such that it is unable to interact efficiently with coactivator/transcription factor. Upon liganding, a conformational signal is then transmitted to helix 12 that causes it to fold back on helix 11 and attach to the face of the globular ligand binding domain. The pivoting of helix 12 seemingly accomplishes two feats that mediate ligand-activated transcription by the receptor: (i) closing of a ‘door’ on the channel through which the lipophilic ligand enters the internal binding pocket of the receptor, and (ii) locking helix 12 into a stable conformation that facilitates its interaction with coactivator/transcription factor. Ligand binding contacts on or near helix 12 (see Fig. 3) probably are significant in maintaining this active positioning of helix 12, essentially trapping ligand in the binding pocket to effect more sustained transactivation events.

In order to evaluate the relevance of the above proposed mechanism for VDR action, we (Jurutka et al. 1997) have altered E-420 and L-417 (see Fig. 7) individually to alanine residues, which preserves the putative \( \alpha \)-helical character of this region. The altered VDRs bind ligand near-normally, with only a mild increase (about 3-fold) in the \( K_d \) for the E420A receptor. Both E420A and L417A hVDRs also heterodimerize efficiently with RXR and associate with VDREs similarly to wild-type hVDR, yet their capacity for 1,25(OH)\(_2\)D\(_3\)-stimulated transcription is abolished, even at high doses of ligand (Jurutka et al. 1997). These point mutations, therefore, identify a C-terminal AF-2 in VDR which corresponds to similar activation domains in other nuclear receptor superfamily members. Because VDR interacts with TFIIB, one of the first squelching of transcription by TFIIB. Therefore, in more differentiated cells, perhaps including osteoblasts or fibroblasts, accessory coactivators may be present to modulate TFIIB or bridge between VDR and TFIIB.

In summary, VDR and TFIIB are hypothesized to exist in a multi-subunit transcription complex which also contains TAFs and/or coactivators that may be promoter- or tissue-specific. Further characterization of this complex will require the discovery of cell type and promoter-specific components via transfection and biochemical interaction studies. Ultimately, an in vivo transcription system must be devised which utilizes defined components to replicate faithfully 1,25(OH)\(_2\)D\(_3\)-stimulated gene expression.

One subdomain of VDR that likely interacts with coactivators and/or basal transcription factors is the extreme C-terminus. We have previously shown that A403 hVDR, a truncated receptor that lacks the C-terminal 25 amino acids, binds 1,25(OH)\(_2\)D\(_3\) ligand with reasonable affinity and heterodimerizes normally with RXR, but is devoid of transcriptional activity (Nakajima et al. 1994). These data suggest that VDR contains a transcriptional activation domain near its C-terminus.
questions we asked was whether the VDR AF-2 constitutes a contact site for this basal transcription factor. Although some very preliminary evidence existed for an association between TFII B and the C-terminus of hVDR (MacDonald et al. 1995), we observed that neither the E420A nor the L417A mutant VDRs are impaired in their interaction with TFII B as probed with glutathione-S-transferase–TFII B fusion protein binding technology (Jurutka et al. 1997). Thus, the domain(s) of VDR that interfaces with TFII B apparently lies elsewhere in the receptor, possibly in the N-terminal portion of the ligand-binding region (Blanco et al. 1995), in the hinge (MacDonald et al. 1995), or in the vicinity of the DNA-binding zinc fingers.

The present experiments with VDR are in concert with recent insight into the function of AF-2 in other nuclear receptors, which is to recruit coactivators of the type of steroid receptor coactivator-1 (SRC-1) (Ôrâte et al. 1995). A number of candidate coactivators have been isolated in addition to SRC-1 (Halachmi et al. 1994, Banaihmad et al. 1995, Cavailles et al. 1995, Lee et al. 1995, Hong et al. 1996) and, in several cases, interaction with nuclear receptors requires intact AF-2 core regions (Banaihmad et al. 1995, Cavailles et al. 1995). Moreover, AF-2 mutations act as dominant negative receptors, for example in the case of hRARγ (Renaud et al. 1995). Indeed, we have observed that VDR AF-2 mutants E420A and L417A exhibit dominant negative properties with respect to transcriptional activation (Jurutka et al. 1997). Such AF-2 altered receptors are inactive transcriptionally, but can bind 1,25(OH)2D3 ligand and heterodimerize normally on VDREs, the consequence being competition with wild-type VDR–RXR heterodimers for VDRE binding. These data argue that the AF-2 of the primary VDR partner in an RXR–VDR heterodimer is absolutely required for the mediation of 1,25(OH)2D3-activated transcription, not only for its intrinsic activation potential, but also because of its presumed role in stabilizing the retention of 1,25(OH)2D3 ligand in the VDR binding pocket.

What part, if any, is played by the AF-2 domain (Fig. 7) of the RXR ’silent’ partner in the RXR–VDR-1,25(OH)2D3 signal transduction pathway? To investigate this phenomenon, AF-2 truncated mutants of RXRα or RXRβ were created and tested for their ability to function as dominant negative modulators of 1,25(OH)2D3-stimulated transcription (Blanco et al. 1996). Because previous data with RXR–RAR control of gene expression seemed to indicate that the RXR AF-2 was dispensable (Durand et al. 1994), we were surprised to find that AF-2 truncated RXRs were potent dominant negative effectors of 1,25(OH)2D3 action in transfected cells (Blanco et al. 1996). We, therefore, conclude that although the RXR ’silent’ partner in VDR signaling apparently is not occupied by retinoid ligand (see Fig. 6), its AF-2 does play an active role in transcriptional stimulation. A similar conclusion has also been reached recently by two other groups studying RXR–RAR action (Chen et al. 1996, Schulman et al. 1996), with the use of RAR-specific ligands precluding ligand binding by the RXR partner. However, Schulman et al. (1996) have introduced a caveat to the above theory as they point out that AF-2-truncated RXRs in heterodimers become strong, constitutive binders of corepressors like the silencing mediators of retinoid and thyroid hormone receptors (SMRTs). Thus, an alternative explanation to an active coactivator–binding role for RXR AF-2 in heterodimers is that it plays a more passive role in excluding corepressors. In this latter scenario, truncation or point mutation (Schulman et al. 1996) of RXR AF-2 generates spurious corepressor binding rather than compromising coactivator contact. Only additional research into coactivator and corepressor associations of VDR–RXR heterodimers will resolve this issue.

**General mechanism for vitamin D hormone action on transcription**

In order to provide a working hypothesis for 1,25(OH)2D3 action at the molecular level, we have developed the model illustrated in Fig. 8. It is based primarily on data from our laboratory and others studying 1,25(OH)2D3 and VDR, and it relies on the assumed similarities between VDR action and that of TR and RAR. VDR is proposed to exist in target cell nuclei, perhaps very weakly associated with DNA, in a monomeric, inactive conformation with the C-terminal AF-2 domain extended away from the hormone binding cavity. Upon liganding with 1,25(OH)2D3, VDR assumes an active conformation, with the AF-2 pivoted into correct position for both ligand retention and coactivator contact. In addition, the hormone facilitates interaction of VDR and RXR through a stabilized heterodimerization interface. In turn, 1,25(OH)2D3-occupied VDR may itself function as a kind of allosteric regulator of RXR, perhaps by conveying a conformational signal through the juxtaposed dimerization domains to induce the AF-2 of RXR into an active conformation for coactivator binding. As discussed above (see Fig. 6), the joining of preliganded VDR and unliganded RXR apparently renders the RXR partner unresponsive to binding and either activation or dissociation by 9-cis RA. Alternatively, if 9-cis RA encounters RXR monomer first (Fig. 8), or binds to RXR that is complexed with VDR in an apoheterodimer (Fig. 6), the retinoid is able to divert the RXR to generate homodimers and effectively blunt 1,25(OH)2D3-driven transcription.

In the primary activation pathway pictured in Fig. 8, the RXR–VDR–1,25(OH)2D3 complex recognizes and targets the genes to be controlled through high affinity association with the VDRE in a gene promoter region. Coactivators that are presumed to bind to VDR and RXR...
AF-2s are then postulated to link with TAFs/TBP, thereby looping out DNA 5' of the TATA box. This series of events positions VDR such that it can independently recruit TFIIIB to the promoter complex, a process that initiates the assembly of the RNA polymerase II holoenzyme into the preinitiation complex. Precedents exist for transcription factors independently attracting TFIIIB, such as hepatocyte nuclear factor-4 (Malik & Karathanasis 1996), as well as for a sequential, two-step pathway for activator-stimulated transcriptional initiation (Struhl 1996, Stargell & Struhl 1996). Using the latter model as an analogy, the VDR activator would contact both TBP/TAFs (via coactivator bridges) and TFIIB in order to initiate RNA polymerase II holoenzyme assembly. The order of attachment of these two 'arms' of activation has not been determined but, at least, in the case of acidic activators, recruitment to the TATA element precedes interaction with components of the initiation complex (Stargell & Struhl 1996). It is of interest that the mechanism of 1,25(OH)₂D₃ action depicted in Fig. 8 is not only essential for induction of bone remodeling and other vitamin D functions, but is also self-limiting via 24-OHase induction. In addition, these actions of 1,25(OH)₂D₃ would be blunted under conditions within a cell where 9-cis RA concentrations dominate over those of 1,25(OH)₂D₃.

The above described molecular mechanism whereby the vitamin D hormone controls gene expression requires further experimental evaluation. To advance our understanding of the structure/function relationships in VDR, a physical characterization of the structure of VDR via X-ray crystallography will be required. Furthermore, in order to comprehend the genomic action of vitamin D in calcium homeostatic and other target cells, it will be necessary to elucidate the detailed involvement of various RXR isoforms, specific TAFs and novel coactivators/corepressors that might influence the regulation of different vitamin D-controlled promoters. This information in its entirety should assist in determining the potential role for VDR and 1,25(OH)₂D₃ in the pathophysiology of osteoporosis and other endocrine-related bone diseases.

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