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TRANSCRIPTIONAL ACTIVATION THROUGH THE VITAMIN D RECEPTOR IN OSTEOBLASTS

Dennis M. Kraichely and Paul N. MacDonald

Department of Pharmacological and Physiological Science, Saint Louis University Health Sciences Center, St. Louis, Missouri 63104

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1. ABSTRACT

Osteoblasts are bone-forming cells that play an essential role in the development and maintenance of a mineralized bone extracellular matrix and they are target cells for vitamin D. Osteoblasts express vitamin D receptors (VDR) and 1,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] regulates the expression of osteoblastic-specific genes such as osteocalcin and osteopontin. VDR is a ligand-inducible transcription factor which heterodimerizes with retinoid X receptor (RXR) and binds as a heterodimer to vitamin D-responsive elements (VDREs) in the promoter region of vitamin-D responsive genes, ultimately leading to their increased transcription. Important structural aspects of the VDR and the role that each functional domain plays in mediating VDR action in the context of the osteoblast are discussed. A summary of the potential molecular mechanisms involved in VDR-activated transcription highlighting the importance of interactions between the VDR and general transcription factors (GTFs), TBP-associated factors (TAF_{II}s), and nuclear receptor coactivator and corepressor proteins are reviewed. These interactions have a role in linking the VDR-RXR heterodimer to the transcriptional pre-initiation complex (PIC) and in regulating the transcription of vitamin D-dependent genes. In addition, recent findings suggest that these interactions are important for regulating the accessibility to promoters by modifying the acetylation state of histones. The complex interplay that occurs between VDR and these various factors to determine the overall transcriptional activity of vitamin D-responsive genes will be summarized.

2. INTRODUCTION

Vitamin D is required for normal skeletal development and for maintaining bone integrity. This critical role in bone physiology is most obvious in the deficiency state where a lack of vitamin D produces rickets in children and osteomalacia in adults. Vitamin D is a steroid hormone and it is the vitamin D endocrine system that plays an integral role in skeletal homeostasis. The parathyroid glands respond to hypocalcemic challenges by synthesizing and secreting parathyroid hormone (PTH). One of the many important actions of parathyroid hormone is to stimulate renal synthesis and release of $1,25(OH)_2D_3$, the bioactive metabolite of vitamin D. $1,25(OH)_2D_3$ acts on mineral regulating target tissues such as intestine, kidney, parathyroid glands, and bone to participate in maintaining calcium and mineral homeostasis. Its predominant role is to enhance the intestinal absorption of dietary calcium and phosphorous. In the absence of adequate dietary calcium, reserves in the skeleton are mobilized to maintain appropriate serum calcium levels. Thus, one key physiological role of vitamin D is to preserve skeletal calcium by ensuring an adequate absorption of dietary calcium.

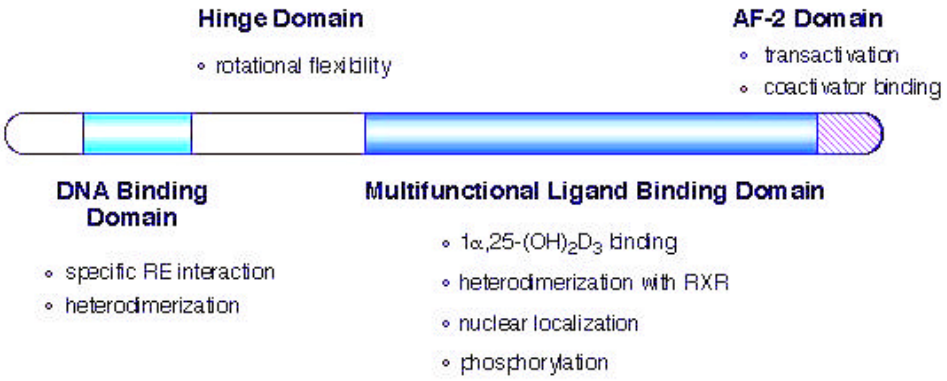

Figure 1. Schematic representation of the structural domains of the vitamin D receptor. This diagram highlights the various domains and functional role each plays in the VDR. VDR domains are defined as the N-terminal A/B domain, region C or the DNA-binding domain (DBD), the hinge region, and the multifunctional ligand-binding domain (LBD). A more detailed description of the functions ascribed to each domain is found in the text.

In addition to the calciotropic role of this steroid hormone, vitamin D also functions in a number of other complex systems, such as promoting cellular differentiation. In skeletal tissue, $1,25(OH)_2D_3$ increases osteoclast number by inducing the differentiation of preosteoclasts into mature bone-resorbing cells (1, 2). Vitamin D is also a known regulator of osteoblast differentiation and function. Osteoblasts express specific receptors for $1,25(OH)_2D_3$ and vitamin D acts directly on the osteoblast to alter the transcription of osteoblast-associated genes including osteocalcin, osteopontin, and alkaline phosphatase. Thus, it is through an integrated series of diverse effects that vitamin D is thought to preserve and maintain the integrity of mineralized tissues.

The biological effects of $1,25(OH)_2D_3$ are mediated through a nuclear receptor protein termed the vitamin D receptor (VDR). The VDR binds $1,25(OH)_2D_3$ with high affinity and high selectivity. In target cells such as the osteoblast, the interaction of the $1,25(OH)_2D_3$ hormone with VDR initiates a complex cascade of molecular events culminating in alterations in the rate of transcription of specific genes or gene networks. Binding of $1,25(OH)_2D_3$ to the VDR induces heterodimerization of VDR with retinoid X receptor (RXR) and it is this heterodimeric interaction that permits VDR to bind with high affinity binding to specific DNA sequence elements (VDREs) in vitamin D responsive genes and ultimately influences the rate of RNA polymerase II-mediated transcription. Thus, it is the VDR-RXR heterodimer that functions as the active transcriptional enhancer in vitamin D-activated transcription. However, much less is known of this process following VDR-RXR interaction with the VDRE. Recent data suggest that protein-protein interactions between the VDR-RXR heterodimer and the transcription machinery play a critical role in the mechanism of vitamin D-mediated gene expression.

This manuscript discusses structural, functional, and mechanistic aspects of VDR-mediated transactivation in osteoblasts. The emphasis is on the macromolecular interactions that are required for the regulatory activity of the VDR on osteoblast gene expression. These macromolecular interactions include ligand binding, DNA interaction, RXR heterodimerization, and protein-protein contacts that may comprise the communications links between VDR and the transcription pre-initiation complex. These contacts include direct or indirect interaction of VDR with components of the transcription complex including transcription factor IIB and putative coactivator/corepressor proteins. This final aspect has been a fertile area of recent research in the nuclear receptor field and its implications in the mechanism of transcriptional regulation by the VDR in osteoblasts are now being realized.

3. STRUCTURAL AND FUNCTIONAL ASPECTS OF THE VDR

The VDR is a member of the superfamily of nuclear receptors. This large family of proteins share an overall structural relatedness in which discrete functional domains have been ascribed based on structure/function data. The various sub-domains of the receptors were originally assigned based on the cloning of the human estrogen receptor and were given the following general designations: A/B, C, D, and E/F (3). A brief summary of the various domains for the VDR is presented in figure 1.

3.1. N-terminal A/B Domain

The A/B domain is a hyper-variable region at the amino terminus of the receptor. Although the precise functional role of the A/B domain is not well understood, its variability between receptors suggests that this domain may be important for hormone- or receptor-selective functions. The A/B domain of most receptors contains a ligand-independent transactivation function (AF-1) that is required for the full transcriptional activity of the receptor (4-9) In addition, the human progesterone receptor contains a unique third activation function (AF-3) which lies at the extreme N-terminus of the receptor (10). The VDR is unique among the nuclear receptors in that its A/B domain is small, consisting of only 20 amino acid residues. Deletion of these 20 residues does not affect VDR-mediated transcription, indicating that VDR function is independent of the A/B domain and its intrinsic activation domains (11). Whether other ligand-independent activation domains comparable to AF-1 and AF-3 reside elsewhere in the VDR is presently unknown.

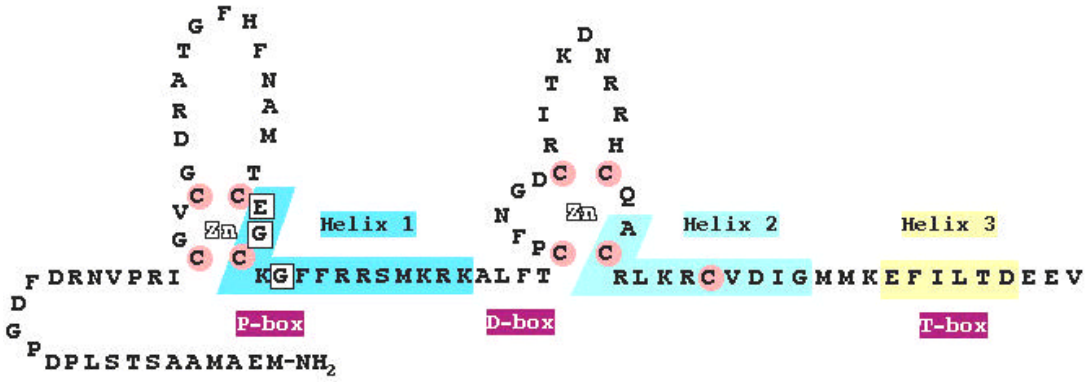

Figure 2. Amino acid composition and important subdomains within the DNA-binding domain of the vitamin D receptor. Residues that are critical for target sequence selectivity form the P-box. The D-box contains residues that are important for homodimerization of class I nuclear receptors. The T-box is essential for both DNA-binding and transactivation of the VDR; this region may also be important for dimerization with RXR for class II nuclear receptors.

3.2. The DNA Binding Domain

Region C or the DNA-binding domain (DBD) is the most highly conserved domain among the nuclear receptors. There are nine cysteine residues that are conserved throughout the members of the nuclear receptor superfamily. The first eight of these cysteines (counting from the N-terminus) tetrahedrally coordinate two zinc atoms to form two zinc binding modules that function as a DNA binding motif (figure 2). Mutagenesis studies of the VDR DBD has provided strong support for this zinc coordination scheme. Mutation of the first eight of the nine cysteine residues (Cys $\rightarrow$ Ser) eliminated VDR binding to both non-specific and specific DNA sequences and eliminated VDR-mediated transactivation (11). The mutation of the ninth cysteine residue (C84S) had little effect on VDR function suggesting that this residue is not functionally analogous to the first eight cysteines.

Much of what is known of the nature of VDR-DNA interactions is modeled after functional and structural data of other related nuclear receptors. Three important sub-domains within the DBDs of nuclear receptors are required to recognize and bind specific nucleotide sequences of DNA (see figure 2). The first region is an alpha-helical domain referred to as the proximal or P-box which confers target sequence selectivity for the glucocorticoid receptor (GR) and the estrogen receptor (ER) (12-14). A second region is known as the distal or D-box which is important for homodimerization of the GR subfamily of receptors (14). A third alpha-helical region, referred to as the T-box, resides just C-terminal to the second zinc finger and it mediates homodimer and monomer interactions with DNA (15, 16). Mutations in the T-box of the VDR show a dramatic reduction in both VDR binding to DNA and in transactivation indicating an important role for this alpha-helical domain in VDR function (17). However, altering the P-box or D-box residues of VDR to those of the GR did not confer GR target gene selectivity to the VDR (17). Moreover, these mutations did not significantly affect VDR interaction with DNA or VDR-activated transcription. Thus, it is apparent that the specificity determinants for VDR are more complex than previously thought; perhaps owing to the heterodimeric nature in which VDR binds DNA compared to homodimeric interactions of ER or GR with their response elements.

Three-dimensional structural analysis of the purified DBDs for the glucocorticoid receptor, estrogen receptor, retinoic acid receptor-beta , and retinoid X receptor-alpha has provided detailed insights into the mechanism of receptor-response element interaction (15, 18-21). A common structural feature of the DBDs for all these receptors is the folding of two alpha-helices in the carboxyl terminal portion of the each zinc finger into a single DNA-binding domain. The first alpha-helix in the amino terminal finger (denoted helix 1 in figure 2) lies across the major groove of DNA making specific contacts with the DNA binding site and, as mentioned above, it is this region that contains the residues that determine response element specificity. The second alpha-helix (denoted helix 2 in figure 2) folds across the first in a perpendicular arrangement. The DBD is rich in the positively charged amino acids, lysine and arginine, several of which form favorable electrostatic interactions with the negatively charged phosphate backbone of the DNA helix. The crystal structure of the TR-RXR heterodimer bound to DNA has been solved and structural aspects for VDR-RXR binding were predicted (22). One key feature is that the T-box residues of VDR (denoted helix 3 in figure 2) make direct contact with the D-box residues of RXR providing additional support for the importance of the VDR T-box in binding to DNA.

3.3. The Hinge Region

The hinge region, which lies between the DNA-binding domain and the ligand-binding domain, is designated as the D domain. The hinge region is proposed to serve as a highly flexible link to impart a high degree of rotational freedom allowing the nuclear receptors to bind a variety of response elements (23, 24) However, since the identification of transcriptional corepressor proteins for the nuclear receptors and the realization that the hinge domain is central for corepressor interaction (section 4.5), this region of nuclear receptors has received much greater scrutiny.

3.4. The Multifunctional LBD

The E/F domain of the nuclear receptors represents a multifunctional domain that is generally referred to as the ligand-binding domain or LBD. Comparing crystallographic data obtained from several nuclear receptors, it is clear that the LBDs are structurally similar. In general, the LBDs consist of three layers comprised of twelve alpha-helices and several beta-strands that are organized around a lipophilic ligand-binding pocket (25-28). This arrangement has been termed an anti-parallel alpha-helical sandwich (25). In addition to serving as a binding site for the $1,25(OH)_2D_3$ ligand, it also fulfills several other critical roles. A prominent role of this region of VDR is to mediate interaction with RXR, the heterodimeric partner that is required for high order binding of VDR to DNA. Key serine residues in this domain serve as sites of phosphorylation that may be important in regulating the transcriptional activity of the VDR. Finally, this region of VDR also plays a central role in forming part of a protein-protein interaction surface through which VDR contacts other proteins that are important for VDR-mediated transcription, such as TFIIB and transcriptional coactivators. These various functional aspects of the VDR LBD are discussed below.

3.4.1. Ligand binding

As its name implies, one of the important roles of this domain in the vitamin D receptor is to bind the small, lipophilic $1,25(OH)_2D_3$ ligand. This binding event is complicated by the inherent conformational flexibility of the $1,25(OH)_2D_3$ seco-steroid and the ability of the apo- and holo-VDR to assume different conformations. Thus, major goals of vitamin D research are to obtain a detailed understanding of the ligand-receptor interaction at the molecular level and to determine the three-dimensional structure of $1,25(OH)_2D_3$-VDR complex. Predictions of residues in the vitamin D receptor which are important in forming the ligand-binding pocket for $1,25(OH)_2D_3$ have been made based on the crystal structures of the liganded RAR-gamma LBD and the estrogen receptor LBD bound by either 17 beta-estradiol or a selective antagonist (raloxifene) (26, 28, 29). From these data the ligand-binding contacts in VDR are proposed to involve helices H3, H5, H11, and H12, plus portions of helices H6 and H7 along with their intervening loop.

3.4.2. RXR Heterodimerization

In addition to hormone binding, the LBD has a central role in mediating heterodimerization of VDR with receptor auxiliary factors (RAFs) such as retinoid X receptor (RXR). VDR-RXR heterodimer formation is required for high affinity interaction of the receptor with VDREs and at least three putative regions in the LBD of VDR mediate protein-protein contacts with RXRs and RAFs (30, 31). A predominant, C-terminal heterodimerization domain resides between residues 382 and 403 in the hVDR sequence (30). Mutagenesis of several specific residues in this domain (Lys382, Met383, and Glu385) disrupted VDR-RAF and VDR-RXR interaction *in vitro* and eliminated transcriptional activation by the VDR. A second putative interaction domain was identified between residues 318 and 339 (30). These regions correspond to helices H10 and H11 and helices H7 and H8, respectively. A third putative heterodimerization surface exists in the amino terminal segment of the LBD between amino acids 244 and 263 (31). Selected point mutations within this region do not interfere with ligand binding, but they affect the ability of the VDR to heterodimerize with RAFs or RXRs and disable transcription from vitamin D responsive constructs. One important outcome of these studies is that, in all the receptor mutants examined, heterodimerization of VDR with RXR was required for VDRE interaction and for $1,25(OH)_2D_3$/VDR-mediated transcriptional activation suggesting that heterodimerization between VDR and RXR is a requisite step in this mechanism.

The crystal structure of the RXR-RXR homodimeric complex provides additional insight into the putative heterodimerization surface of the VDR-RXR complex (25). The RXR dimer is symmetrically arranged with the interaction surface being formed mainly by helix H10 and, to a lesser extent, by helix H9. Helix H10 of RXR corresponds to the C-terminal region of VDR identified by Nakajima *et al*. as being crucial for heterodimer formation (aa 382-403)(30). Interestingly, a natural mutation in helix H10 of the VDR LBD (R391C) was described recently in patients with hereditary hypocalcemic vitamin D resistant rickets (32). This mutation abrogates VDR-RXR heterodimer formation in these patients and this further suggests that helix H10 may directly contact helix H10 of RXR to comprise the major interaction surface yielding a structurally symmetrical VDR-RXR heterodimeric complex.

3.4.3. VDR Phosphorylation

Phosphorylation is widely regarded as a key means of regulating cellular processes. Most of the steroid/thyroid hormone receptors, including the VDR, are phosphoproteins and key residues that serve as substrates for phosphorylation reside in the E/F domain. VDR present in mouse 3T6 cells is hyperphosphorylated in response to physiologic concentrations of $1,25(OH)_2D_3$ (33). Ligand-dependent phosphorylation has also been demonstrated in ROS 17/2.8 osteoblasts and in chick duodenal organ culture, two relevant target systems for vitamin D action (34, 35). In this last system, the effect is observed within 15 min following the addition of $1,25(OH)_2D_3$. Since this precedes most other cellular responses to $1,25(OH)_2D_3$, it is likely that phosphorylation of VDR may play an initiating event in the transcriptional processes mediated by the VDR.

The major phosphorylated residues of the VDR have been determined. Using domain-specific antibodies, Brown and Deluca mapped the major phosphorylation site(s) to the N-terminal region of the LBD in porcine VDR (36). Studies in ROS 17/2.8 cells revealed that the main phosphorylated domain of hVDR resided between Met197 and Val234 in hVDR (35). Within this domain is a cluster of serine residues, many of which resemble consensus sites for casein kinase II. Indeed, hVDR is an effective substrate for *in vitro* phosphorylation by purified casein kinase II and site-directed mutagenesis defined Ser208 as the site phosphorylated by casein kinase II *in vitro* and *in vivo* when VDR was transiently expressed in COS-7 cells (37). Furthermore, co-expression of casein kinase II in this system augmented VDR phosphorylation of Ser208 showing that this kinase could also phosphorylate Ser208 in the cell (38).

Hilliard et. al. systematically identified this same Ser208 residue as the main phosphorylated residue of VDR using phosphopeptide mapping studies (39). Interestingly, in this study, phosphorylation at Ser208 was augmented 8-fold when the cells were treated for 4 h with $1,25(OH)_2D_3$. Thus, Ser208 is the major phosphorylated residue of VDR and likely represents the hormone-dependent phosphorylation site observed in earlier studies. A second alternate site of phosphorylation is Ser51which resides between the two zinc finger motifs in the DBD of the hVDR (40). Ser51 is a consensus site for protein kinase C (PKC) and it is selectively phosphorylated by the PKC-beta isoform *in vitro* and *in vivo*.

Although the global phosphorylated state of the cell clearly affects VDR-mediated transcriptional activity (41, 42), demonstration that VDR phosphorylation per se is important for vitamin D-mediated transcription has remained elusive. For example, mutations that disrupt phosphorylation at Ser208 and Ser51 do not affect VDR-activated transcription (38, 43). One caveat here is that mutations in one serine residue may actually promote phosphorylation of an adjacent serine residue to compensate (39). Thus, more detailed studies are required to define the precise functional roles of Ser208 and Ser51 phosphorylation by casein kinase II and by protein kinase C in VDR function.

3.4.4. The AF-2 helix, a ligand dependent transactivation domain

The E/F region of nuclear receptors contains a highly conserved ligand-dependent transactivation domain (AF-2) which is essential for receptor-mediated transcriptional activation (8, 44-56). The AF-2 domains of the nuclear receptors are generally characterized by defining receptor mutations that selectively abolish ligand-activated transcription without disrupting other receptor functions such as ligand binding, response element interaction, dimerization, or nuclear localization. An important feature of the AF-2 domain is that its activity is transferable. That is, fusing the activation domain itself to a heterologous DNA-binding domain results in a fusion protein that activates transcription autonomously (9, 57, 58). The AF-2 transactivation domain resides at the extreme carboxyl-terminus of the VDR (residues 416-423). Based on structural determinations of related nuclear receptors (RXR, RAR, TR, and ER) (25-27, 29), the highly-conserved AF-2 domain of VDR is an amphipathic alpha-helix (helix H12) that may form a part of an important protein interaction interface for transcriptional mediators or intermediary factors that are required for nuclear receptor-dependent transcription (59, 60)(sections 4.3-4.4).

These findings create a dilemma; namely, how does the binding of ligand regulate transcriptional activation by an autonomously active domain in these receptors? The answer likely resides in the ligand-induced conformational changes that these receptors undergo (61). For some time it was speculated that in the absence of ligand, the AF-2 domain might be imbedded in an inactive state in the hydrophobic LBD core and that the binding of ligand unmasked the AF-2 domain thereby exposing it for the binding transcriptional mediators. However, since the elucidation of the crystal structures for apo-RXR alpha LBD, holo-RAR gamma LBD, and holo-TR alpha LBD, a new model has emerged. By comparing the crystal structures of the unliganded RXR-alpha with that of the liganded RAR-gamma, it is evident that in the unliganded state, the AF-2 domain (helix H12) projects out away from the globular core of the LBD and in the liganded state the AF-2 domain is repositioned, folding back on helix H11 and interfacing with the surface of the LBD core. (25-28). Consequently, helix H12, which contains the AF-2 domain, apparently moves from a relatively solvent exposed position to a position in which it is folded or packed onto the LBD in the ligand-activated receptor.

The repositioning of helix H12 seems to play at least two important roles in mediating ligand-activated transcription by the receptor. First, the folding down of helix H12 appears to act as a "hinged door," sealing off the channel through which the lipophilic ligand enters the ligand-binding pocket. Second, this ligand-induced repositioning of helix H12 may lock the AF-2 domain into a stable conformation, with the hydrophobic residues of the helix facing toward the ligand-binding cavity and the charged residues exposed to the solvent possibly serving as a surface for interaction with coactivator proteins (25-29) (sections 4.3-4.4).

4. MECHANISMS INVOLVED IN VDR-MEDIATED TRANSACTIVATION

The VDR is a ligand-inducible transcription factor which binds ligand, heterodimerizes with retinoid X receptors (RXRs), and binds as a VDR-RXR complex to vitamin D responsive elements (VDREs) in promoters of genes that are regulated by vitamin D. However, beyond these initial steps, little is known of the molecular mechanisms or signals that link the receptor heterodimer to the transcription PIC. Recent approaches have focused on defining the protein-protein interactions between VDR and components of the transcriptional machinery that establish a physical communication link between the heterodimer and the PIC. Mechanistically, these interactions may: 1) facilitate the recruitment of one or more limiting factors into the PIC, 2) enhance the stability of the PIC, 3) cause a conformational change which triggers initiation by RNA polymerase II (i.e., the promoter clearance phase), 4) enhance the re-assembly of the complex for subsequent rounds of transcription (re-initiation), or 5) a combination of these possibilities. Which, if any, of these mechanisms apply to vitamin D-mediated transcription in bone cells is presently unclear. In addition, several key transcriptional components that contact VDR and other nuclear receptors have been described recently and these interactions are believed to play central roles in VDR-mediated transcription. The transcriptional components that are known to interact with VDR and nuclear receptors may be classified into three general categories; 1) the general transcription factors (GTFs), 2) the TBP-associated factors (TAF_{II}s), and 3) the comodulator proteins which include coactivators and corepressors.

4.1. VDR interactions with GTFs

Central players in the activated transcriptional process are the general transcription factors and their ordered assembly into the transcription preinitiation complex [reviewed in (62)]. PIC assembly begins with TATA-binding protein (TBP, a subunit of TFIID) binding to the TATA

element of class II promoters in a process that is facilitated by TFIIA. Then, in what may be the rate-limiting step, TFIIB enters the complex by direct interaction with TBP. RNA polymerase II, in association with TFIIF, binds to this early complex by contacting TFIIB. Thus, TFIIB serves as a bridging protein between TBP and RNA polymerase II. Further association with TFIIE and other general factors results in a complex capable of accurately initiating RNA synthesis.

Transcription initiated by these minimal components represent basal level transcription which can be stimulated (or repressed) by sequence-specific, trans-acting factors such as the VDR. Transactivators interact with a variety of GTFs in the PIC and of these, TFIIB appears to be a central target. We and others have demonstrated a direct interaction between TFIIB and the VDR (63, 64). This interaction is functionally important since TFIIB expression augments vitamin D-activated transcription in transient gene expression studies and a dominant-negative inhibitor of TFIIB-VDR complexes selectively impairs VDR-activated transcription (63, 65). These studies indicate that the formation of the VDR-TFIIB complex is important for VDR-activated transcription and they further suggest that one line of communication between the VDR-RXR heterodimer and the PIC, may be a direct, specific protein-protein contact between VDR and TFIIB. A second GTF, TFIIA, was recently shown to contact VDR and $1,25(OH)_2D_3$ was found to promote the formation and recruitment of TBP:TFIIA into higher-mobility complexes on a VDRE-linked promoter (66). Although these studies establish that the interaction of VDR with GTFs is central for appropriate transcription mediated by $1,25(OH)_2D_3$, the molecular details of the mechanism are still under investigation.

4.2. VDR interactions with TAF_{II}s

A second target for VDR and other nuclear receptors in the transcription complex are the TBP-associated factors or TAF_{II}s (67, 68). At least ten distinct eukaryotic TAF_{II} proteins have been identified which associate tightly with TBP (69). This large complex of TBP and the TAF_{II}s collectively form the RNA polymerase II transcription factor TFIID. *In vitro* biochemical studies point to a role for TAF_{II}s in activated transcriptional processes since purified TBP, in the absence of TAF_{II}s, supports basal, but not activated transcription (70). In contrast, recent genetic studies in yeast have suggested that TAF_{II}s may be expendable for activated transcription of most genes (71-75), although it is possible that functional redundancies between TAF_{II}s or other unidentified components may occur in these *in vivo* experiments.

Importantly, TAF_{II} proteins interact directly with a variety of transcriptional activator proteins including members of the nuclear receptor superfamily (67, 68, 76, 77). An illustrative example is the interaction of the estrogen receptor with $TAF_{II}30$ and the demonstration of the essential nature of that interaction in ER-activated transcription (78). Moreover, the expression of human $TAF_{II}28$ in COS cells significantly augments hormone-dependent transcriptional activation by the estrogen, vitamin D, and retinoid X receptors (79). Receptor selectivity was observed with $hTAF_{II}135$ which was shown to potentiate RAR, TR, and VDR-activated transcription without affecting ligand-activated transcription by ER and RXR (80). These data indicate that TAF_{II}s possess the ability to selectively and directly interact with nuclear receptors including the VDR and that interaction enhances transcriptional activation. Again, the mechanism may involve the facilitated recruitment or stabilization of the PIC through a VDR-TAF_{II} bridging interaction, but the details remain to be elucidated.

4.3. VDR interactions with nuclear receptor coactivator proteins

In addition to the GTFs and TAF_{II}s, a third class of proteins has been proposed for efficient nuclear receptor-mediated transcription. This hypothesis was based on the observation that one nuclear receptor interferes with the transcriptional activation pathway of another nuclear receptor without affecting basal transcription or the transcription initiated on unrelated promoters (59, 60, 81, 82).

Nuclear receptor coactivators interact with the receptor and augment ligand-activated, RNA polymerase II-directed transcription (59, 60, 70, 83-87). The prototypical example of a transcriptional coactivator protein is the steroid receptor coactivator (SRC-1), which was identified in a yeast two-hybrid screen using the the LBD of PR as the bait (88). SRC-1 interacts with the PR in an agonist-dependent manner and augments PR-dependent transcription when transiently expressed in mammalian cell lines. SRC-1 also enhances ER, GR, TR, RXR, and RAR-mediated transcription, suggesting a general role of this coactivator in nuclear receptor-mediated transcription.

Since the initial identification of SRC-1, a number of proteins with nuclear receptor coactivator properties have been described for the VDR. Many of these proteins were identified by screening cDNA libraries in the yeast two-hybrid system or by *in vitro* biochemical approaches. Putative coactivators for VDR include: SRC-1 (89), RIP140 (89), TIF 1 (90), and GRIP 1/TIF 2 (91-93). Many of these coactivators interact with the AF-2 transactivation domain of the receptors (section 3.4.4). Indeed, our laboratory recently described a $1,25(OH)_2D_3$-dependent interaction between VDR and the SRC-1 and RIP140 coactivator proteins and that both interactions are mediated through the AF-2 domain (89). In the vitamin D receptor, two independent studies by our laboratory and Haussler's group demonstrated that the mutation of two critical residues in the AF-2 domain L417S/L417A and E420Q/E420A did not affect the binding of $1,25(OH)_2D_3$, heterodimerization with RXR, or binding to a VDRE, but did disrupt transcriptional activation (89, 94). Thus, there is a strong correlation between the transcriptional activity of VDR mutants and the ability of these mutants to interact with SRC-1 and RIP140. Another coactivator protein that contacts VDR is glucocorticoid receptor-interacting Protein 1 (GRIP1); an 86-kDa protein named for its interaction with the GR (91, 92). As with SRC-1, GRIP1, is rather promiscuous in that it interacts with a variety of nuclear receptors and augments their transcriptional pathways. The VDR contacts GRIP1 in a $1,25(OH)_2D_3$-dependent fashion and again this interaction is mediated, in part, through the AF-

2 domain. Interestingly, in addition to a strong intrinsic activation function, the ability of GRIP1 to inhibit basal transcription suggests that it may contact the basal transcriptional machinery, indicating that GRIP1 may be a pure bridging protein between the VDR and PIC (91).

While the AF-2 domain is centrally involved in mediating ligand-dependent interactions of nuclear receptors with coactivators, in all likelihood, the coactivator-binding surface of VDR is comprised of the AF-2 domain and surrounding residues. Experimental data from several laboratories show that the conserved AF-2 core domain is important, but not sufficient for full transactivation when assayed outside of the context of the full-length receptor (45, 89). From crystal structural determination of related nuclear receptors, candidate regions for these other areas include those immediately surrounding the AF-2 core, such as exposed residues on the surfaces of H3, H4, the loop between H11 and H12, and the region between H1 and H3 comprising the omega loop (25-29).

The presence of additional domains which may form part of this coactivator-binding surface is supported by recent findings which suggesting the existence of a second class of transcriptional coactivator proteins which act independent of the AF-2 domain. TRAM-1 is a novel 160-kDa thyroid hormone receptor activator molecule which exhibits properties that are distinct from representative members of the AF-2 interacting coactivators (i.e. SRC-1) (95). TRAM-1 retains a strong ligand-dependent interaction with an AF-2 mutant of TR (E457A), while SRC-1 failed to interact with this mutant. Interestingly, TRAM-1 interaction with the TR is disrupted by a helix H3 mutation (K288A) suggesting that regions of helix H3 may be crucial for mediating interactions with this second class of coactivator proteins.

Recently, our laboratory identified a novel coactivator protein termed NCoA-62 that contacts the VDR and that augments VDR-activated transcription (96). NCoA-62 is a 62,000 Da protein that forms protein-protein contacts with the VDR LBD both in yeast and *in vitro* in GST-VDR pulldown assays. Interestingly, NCoA-62 interaction with VDR does not require the AF-2 domain, as the deletion of the VDR AF-2 domain does not affect NCoA-62 binding indicating that this novel coactivator may contact other important transactivation domains in the VDR LBD. Although unrelated based on sequence, NCoA-62 may be functionally similar to TRAM-1 and clearly falls into the class of AF-2 independent coactivators. NCoA-62 also interacts with retinoid receptors and its expression enhanced retinoic acid, estrogen, and glucocorticoid-mediated gene expression. NCoA-62 is highly related to nuclear proteins *in Drosophila melanogaster*. In fact, the *Drosophila* homologue is involved in ecdysone-mediated transcription suggesting a high functional conservation of NCoA-62 between lower forms and mammals.

4.4. Mechanisms of VDR-coactivator interactions

Although the studies described above have begun to identify potentially important players in the mechanism of VDR-mediated transcription in osteoblasts, the next facet of our understanding must extend beyond the mere identification of factors and begin to characterize the mechanisms through which these proteins function. One hypothesis states that the coactivators function as macromolecular bridges between the transcriptional activators and the PIC. Their interaction with the PIC (either direct or indirect) may promote PIC assembly or enhance the stability of the PIC, thereby leading to activated transcription. Indeed, O'Malley and colleagues have shown that the progesterone, glucocorticoid, and estrogen receptors activate transcription *in vitro* by increasing the formation of a more stable preinitiation complex (97-99). However, the role of coactivator proteins such as SRC-1 in the stabilization of the PIC remains to be established.

An emerging property of several coactivator proteins including CREB binding protein (CBP) and SRC-1 is that these coactivators possess intrinsic histone acetyltransferase (HAT) activity (100, 101). For years, it has been appreciated that the acetylated state of histones is highly correlated with promoter activity. The hypothesis states that histone acetylation results in a disruption or loosening of the chromatin structure making promoters more accessible to the transcription machinery and ultimately leading to an increase in the rate of transcription (102-112). The ability of nuclear receptor coactivators to express HAT activity provides an attractive model for nuclear receptor-mediated transactivation. Specifically, nuclear receptors interact in a ligand-dependent manner to recruit enzymes that modify chromatin structure at a particular promoter. Acetylation of histones around a promoter results in a disordered structure, increasing the accessibility of the transcriptional machinery to the promoter ultimately leading to activated transcription (108, 113)(see figure 3).

4.5. VDR interactions with nuclear receptor corepressor proteins

In addition to transcriptional coactivator proteins, corepressor proteins such as SMRT (silencing mediator of retinoic acid and thyroid hormone receptor) and N-CoR (nuclear receptor corepressor) are known to interact with several members of the nuclear receptor superfamily including TR, RAR, and most recently VDR. Corepressors function in a ligand-independent manner to repress the transcription of target genes (114-124). Ligand binding disrupts the receptor-corepressor complex and relieves the inhibition of basal transcription. Recently, Yen and Chin demonstrated that the unliganded VDR represses basal transcription and that it exerts a dominant-negative effect on thyroid hormone-mediated transcription (125). Interestingly, the AF-2 domain of the nuclear receptor is required both for the interaction with the majority of coactivators and for the dissociation of corepressor proteins, suggesting a mechanistic link between transcriptional suppression and activation (23, 114). Moreover, the recent finding that corepressor proteins possess intrinsic histone deacetylase activity suggests that corepressors may play an even more important role in the regulation of the transcriptional activity of nuclear receptors than first proposed (126-131).

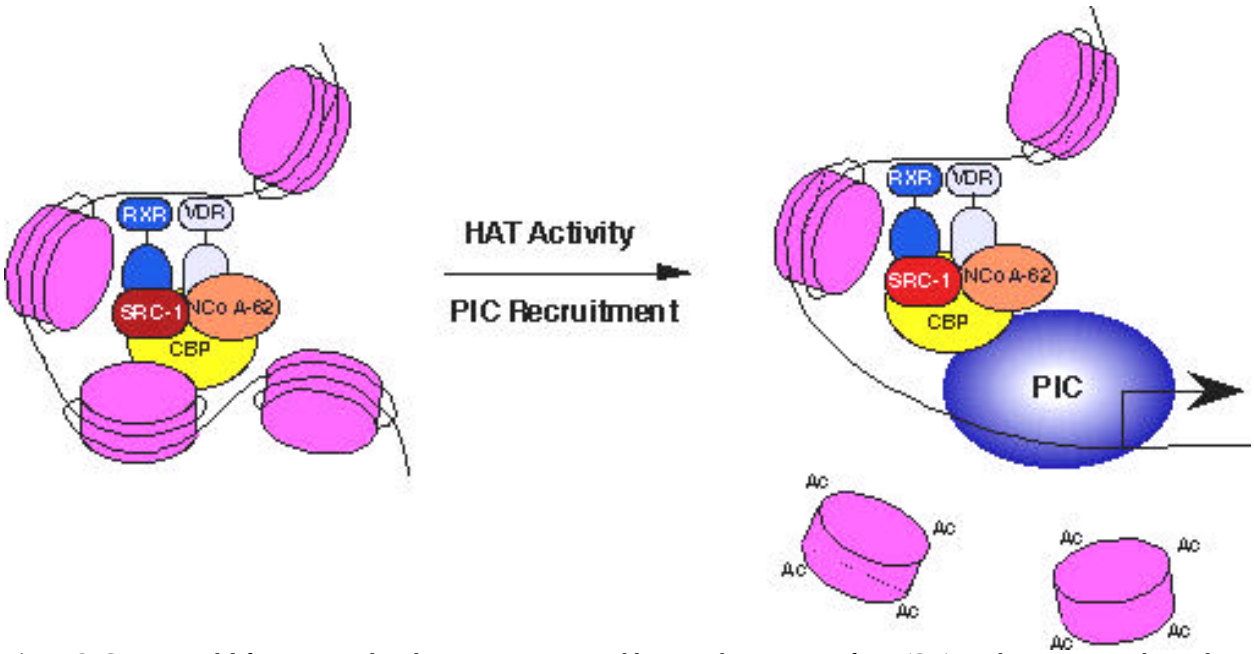

Figure 3. Current model for VDR-mediated transcription in osteoblasts. In the presence of $1,25(OH)_2D_3$, the VDR-RXR heterodimer contacts coactivator proteins which contain intrinsic HAT activity. Acetylation of histones leads to a loosening of the chromatin structure increasing the accessibility of the transcriptional machinery to the promoter, leading to activated transcription.

5. MODEL OF VDR-MEDIATED TRANSCRIPTION IN OSTEOBLASTS

Based on these studies, a current model for VDR-mediated transcription is proposed. This model incorporates several properties of VDR that were discussed in this section. The initial event in this model is high affinity binding of the $1,25(OH)_2D_3$ ligand to the VDR. Ligand binding induces VDR/RXR heterodimerization and the heterodimer specifically binds VDREs in the promoter regions of vitamin D responsive genes. The VDR portion of the heterodimer contacts TFIIB to form a ternary complex of proteins. GTFs and coactivator proteins form protein-protein contacts between VDR and the PIC and it is the interaction with and the communication between VDR, RXR, TFIIB, and other ligand-dependent coactivator proteins such as SRC-1 and NCoA-62 that may determine the overall transcriptional activity of a vitamin D-responsive gene. Furthermore, nuclear receptor-regulated gene transcription may consist of a combination of hormone-dependent derepression mediated by corepressor proteins such as N-CoR, and transactivation mediated through coactivators such as SRC-1. Understanding the complex interplay that occurs between these various factors is crucial to unraveling the complexities of activated or repressed transcription mediated by vitamin D and the VDR.

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Send correspondence to: Paul N. MacDonald, Ph.D., Saint Louis University School of Medicine, Department of Pharmacological & Physiological Science, 1402 South Grand Saint Louis, MO 63104. Tel: (314)-268-5318, Fax: (314)-577-8233, E-mail: macdonal@slu.edu