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Review

The Nuclear Vitamin D Receptor: Biological and Molecular Regulatory Properties Revealed

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INTRODUCTION

In the decade since the vitamin D receptor (VDR) was cloned[1] and recognized as a member of the superfamily of nuclear receptors that regulate gene expression in a ligand-dependent manner,[2,3] the central role of VDR in the biology of vitamin D action has been illuminated and is being defined at the molecular level. Following renal production as the hormonal metabolite of vitamin D, 1α,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) functions as the ligand for VDR, with the hormone–receptor complex inducing calcemic and phosphatemic effects that result in normal bone mineralization and remodeling. VDR not only mediates the action of $1,25(OH)_2D_3$ in calcium/phosphate translocating tissues, primarily intestine, but also elicits a myriad of apparent bioactivities in other major cell systems in the organism, including immune, neural, epithelial, and endocrine. The scope of this review will be limited to highlighting the actions of $1,25(OH)_2D_3$ mediated by nuclear VDR and discussing new developments in the structure/function analysis of the receptor, including the phenotype of VDR knockout mice and the biochemical classification of patients with point mutations in the receptor. These new advances, along with other recent research, will be interpreted to update our understanding of the molecular role of VDR, ranging from characterization of its natural gene and clinically significant polymorphisms, through its DNA contact sites and protein partners, to novel ligand analogs that hold the promise of influencing VDR conformation in a therapeutically beneficial fashion.

VDR BIOLOGY

Pathophysiology of the vitamin D endocrine system and bone mineral metabolism

The traditional action of vitamin D, via its $1,25(OH)_2D_3$ hormonal metabolite, is to effect calcium and phosphate homeostasis to ensure the deposition of bone mineral (summarized in Fig. 1A). $1,25(OH)_2D_3$ stimulates intestinal calcium and phosphate absorption, bone calcium and phosphate resorption, and renal calcium and phosphate reabsorption, thus increasing the blood $Ca \bullet PO_4$ ion product. Failure to achieve normal bone mineral accretion by these mechanisms leads to rachitic syndromes. Nutritional rickets, caused by the simultaneous deprivation of sunlight exposure[4] and dietary vitamin D, was ameliorated in classic experiments by administration of this fat soluble vitamin (reviewed in Ref. 5). Subsequently, it was recognized that the pathways comprising the metabolic activation of the vitamin to its hormonal form and consequent functions of the hormone in target tissues (Fig. 1) present additional steps where defects directly elicit vitamin D–resistant rachitic syndromes. Two such disorders involve the inadequate bioactivation of 25-hydroxyvitamin D_3 ($25(OH)D_3$), a constitutively produced intermediary metabolite, to $1,25-(OH)_2D_3$. This step is catalyzed by the 1α-OHase enzyme in kidney (Fig. 1A). Chronic renal failure results in renal rickets and secondary hyperparathyroidism when compromised renal mass reduces 1α-OHase activity,[6] whereas pseudo–vitamin D–deficiency rickets (PDDR) involves a specific hereditary defect in the gene coding for the 1α-OHase enzyme.[7] The latter conclusion has been verified by the recent cloning of cDNAs for the rat[8] and mouse[9]

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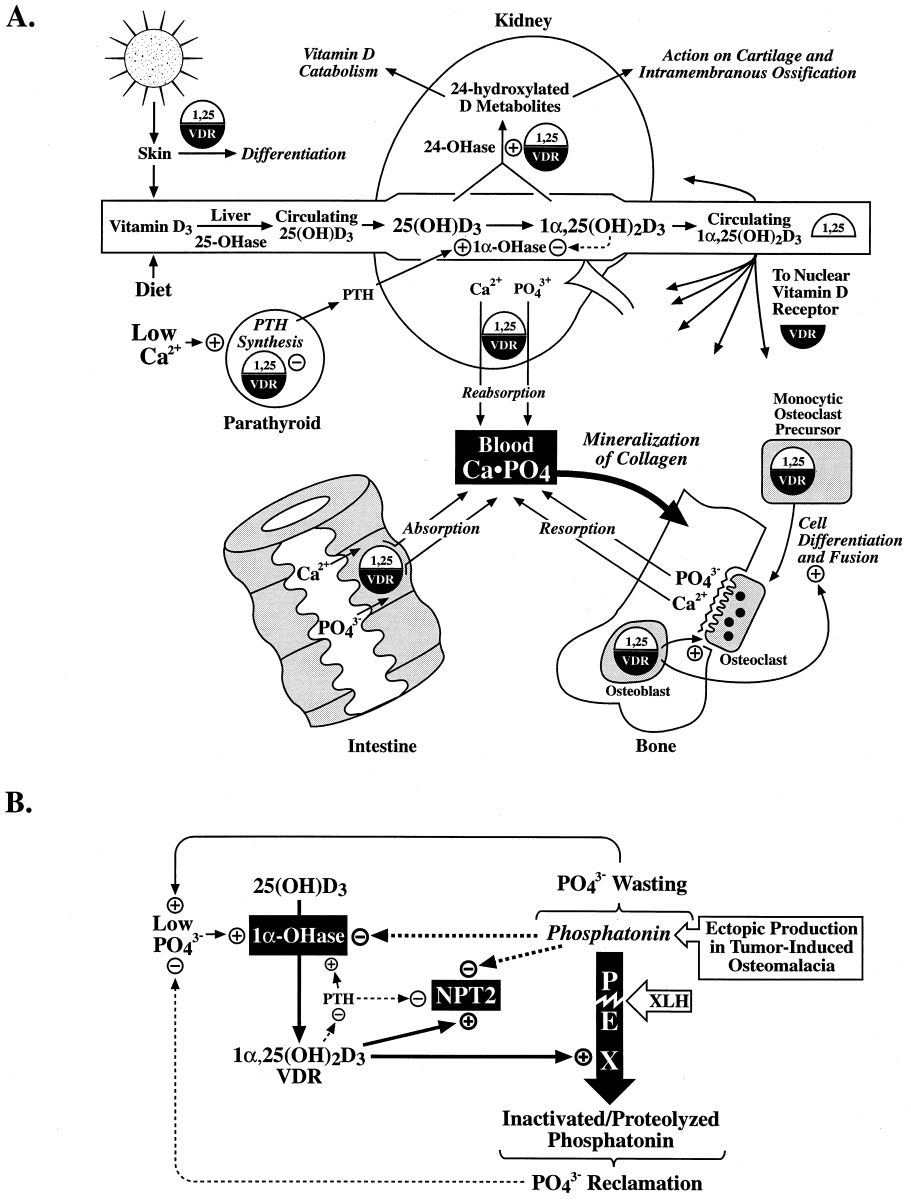

FIG. 1. Calcemic and phosphatemic biological actions of vitamin D in mammals. (A) Effects of vitamin D and its metabolites to ensure skeletal integrity, especially when calcium is limiting. (Central open box) Vitamin D_3, obtained from diet or derived from sunlight-initiated photobiogenesis in skin, is converted via two hydroxylation reactions to the $1,25(OH)_2D_3$ hormonal form that circulates in blood. The final step in bioactivation of vitamin to hormone is catalyzed by the renal 1α-OHase when stimulated by PTH under conditions of low calcium. (Lower portion) Integrated actions of the $1,25(OH)_2D_3$ metabolite, via binding to the intracellular VDR, to control calcium homeostasis in bone, intestine, kidney, and parathyroid as explained in the text. (Top left) Action of $1,25(OH)_2D_3$–VDR in skin cell differentiation. (Top center) Conversion of $1,25(OH)_2D_3$ or the preceding $25(OH)D_3$ metabolite to 24-hydroxylated forms in response to $1,25(OH)_2D_3$–VDR induction of the 24-OHase gene. This conversion serves to initiate catabolism of the vitamin D molecule, but may also produce 24-hydroxylated metabolites with novel hormonal activity with respect to chondrocyte differentiation and bone mineralization (see text). (B) The vitamin D bioactivation-phosphate homeostatic loop: proposed novel roles for phosphatonin, the PEX gene product, and NPT2. (Left and lower portion) Under normal physiologic conditions, low PO_4 enhances the synthesis of $1,25(OH)_2D_3$, which then acts through VDR to effect phosphate reclamation by suppressing PTH as well as inducing NPT2 and PEX gene expression. NPT2 acts directly to reabsorb PO_4, while the PEX enzyme eliminates phosphatonin. (Top right) Tumor-induced osteomalacia and XLH each elicit increased phosphatonin, an uncharacterized phosphaturic hormone that is postulated to inhibit both NPT2 and the 1α-OHase, to cause severe phosphate wasting.

1α-OHase P_{450} and the pinpointing of the human 1α-OHase gene to a chromosomal locus coincident with PDDR.[8] Interestingly, the 1α-OHase/PDDR locus maps rather closely to the VDR gene on chromosome 12 in the 12q13–14 region,[7] a proximity that may be relevant to the evolution and control of the vitamin D ligand–receptor system.

Hypocalcemic 1,25(OH)$_2$D$_3$ resistance

Clinical and molecular genetic data from the last decade have provided unequivocal evidence for the obligatory role of VDR in mediating the action of vitamin D. Familial target tissue insensitivity to 1,25(OH)$_2$D$_3$, known as hereditary hypocalcemic vitamin D–resistant rickets (HVDRR), is an autosomal recessive disorder resulting in a phenotype characterized by severe bowing of the lower extremities, short stature, and often alopecia.[10] In virtually all cases, the cause of this syndrome has been shown to be a defect in the gene encoding human VDR (hVDR) (reviewed in Refs. 10–13), although potential exceptions have been described.[14,15] The fact that the phenotype of HVDRR patients, excluding alopecia, mimics classic nutritional rickets indicates that 1,25(OH)$_2$D$_3$-liganded VDR not only executes all of the bone mineral homeostatic actions of 1,25(OH)$_2$D$_3$ but suggests that VDR itself also participates in the normal hair growth cycle in skin.

Generation of VDR null mice

Recently, VDR knockout mice have been created by two groups,[16,17] revealing apparently normal heterozygotes but homozygotes that display a phenotype very similar to HVDRR, including the progressive development of alopecia over 4–7 weeks of age. At various intervals after birth (differing somewhat between the two studies), VDR null mice acquired low bone mass, hypocalcemia, hypophosphatemia, hyperparathyroidism, and 10-fold elevated 1,25-(OH)$_2$D$_3$, coincident with extremely low 24,25(OH)$_2$D$_3$. Affected homozygotes died within 15 weeks[16] or exhibited near normal survival rates for up to 6 months.[17] The differing survival times may be related to diet or environmental variations, since ablation of exon II/first zinc finger[16] or exon III/second zinc finger[17] should produce equivalent functional consequences of VDR gene knockout. Despite a lack of VDR throughout early development, VDR null mice are born phenotypically normal, exhibiting symptoms of rickets/osteomalacia and secondary hyperparathyroidism primarily after weaning.[16] This observation suggests that the vitamin D endocrine system is principally required for maintaining bone mineral homeostasis when the organism is deprived of a consistent and plentiful supply of calcium, such as occurs after weaning in mammals, after hatching in birds, or after leaving the aqueous environment in the case of amphibians.[18]

In addition to the data reported in their abstract, Demay and coworkers[19] also presented preliminary results describing the prevention of many, but not all, of the phenotypic effects of VDR knockout by means of a "rescue diet," consisting of high levels of lactose, calcium, and phosphate. By manipulating blood calcium and phosphate levels in this manner, parathyroid hormone (PTH) was normalized and bone mineralization was greatly improved in the VDR knockout animals, to a degree that the histology of the growth plate was indistinguishable from that of normal littermates. However, alopecia and skin abnormalities, such as dermal cysts, persisted in the VDR null mice on the rescue diet, intimating that VDR plays an indispensable role in hair and skin development independent of bone mineral homeostasis. That normalizing circulating mineral concentrations via dietary intervention in VDR knockout mice prevents the rachitic phenotype is consistent with results in HVDRR patients whose bone abnormalities are resolved by frequent therapy with overnight intravenous calcium infusions.[20,21]

Thus, the generation of VDR null mice and the reversal of their bone abnormalities by diet dramatizes the concept that the major physiologic effect of 1,25(OH)$_2$D$_3$ is on intestinal absorption of calcium and phosphate, although certain calcium regulating end-points such as depressed renal calbindin-D_{9k} mRNA expression in kidney are not corrected when VDR null mice are maintained on the rescue diet (M. Demay et al., unpublished results). Moreover, in another preliminary study, Kato and colleagues[22] found that, when utilizing the coculture system of Suda and coworkers,[23] osteoblasts/stromal cells from VDR knockout mice will not support 1,25(OH)$_2$D$_3$-induced osteoclastogenesis of normal spleen cells, whereas the reverse experiment (normal osteoblasts/stromal cells and VDR null mouse spleen cells) results in the production of osteoclasts upon exposure of the coculture to 1,25(OH)$_2$D$_3$. Therefore, as depicted in Fig. 1(A), VDR appears to be essential for 1,25(OH)$_2$D$_3$ to elicit a paracrine signal from osteoblasts, which in turn facilitates osteoclast differentiation, at least in vitro. However, the fact that VDR null mice curiously possess normal, or even increased numbers of osteoclasts[16] suggests that other osteoclast-activating factors, such as PTH or interleukin-1 (IL-1), can still support osteoclastogenesis in the absence of functional VDR.

The participation of VDR in feedback control of the vitamin D endocrine system

Superimposed upon its pivotal role in controlling bone mineral transport and differentiation in hair follicles, another function of the 1,25(OH)$_2$D$_3$–VDR complex is to govern the level of the renal 1,25(OH)$_2$D$_3$ hormone by feedback regulation of its biosynthesis and by induction of a key catabolic enzyme. 1,25(OH)$_2$D$_3$ appears to effect a short feedback loop (Fig. 1A) to repress the 1α-OHase enzyme,[24] and is also a potent suppressor of the synthesis and secretion of PTH,[25] the primary tropic hormone stimulating the 1α-OHase (Fig. 1).[26] The mechanism whereby 1,25(OH)$_2$D$_3$ curtails PTH production involves a VDR-mediated silencing of PTH gene transcription.[27–29] Based upon preliminary data from dietarily rescued VDR null mice, excess calcium is able to adequately control PTH secretion and parathyroid cell growth, suggesting that the role of 1,25(OH)$_2$D$_3$ in these processes is cooperative with physiologic calcium levels but can be overridden in situations of calcium abundance.

Catabolism of 1,25(OH)$_2$D$_3$ and generation of 24-hydroxylated vitamin D metabolites

Turnover of 1,25(OH)$_2$D$_3$ is accomplished via several catabolic routes,[30,31] with 24-hydroxylation initiating the apparent primary pathway for elimination of vitamin D metabolites. The 24-OHase enzyme is markedly enhanced by 1,25(OH)$_2$D$_3$ through a VDR-dependent mechanism (Fig. 1A), a phenomenon predominating in kidney but also occurring in all 1,25(OH)$_2$D$_3$ target cells.[32,33] The dependency of 24-OHase activity on VDR action is emphasized by the extremely low levels of 24-hydroxylated metabolites found in VDR null mice, as discussed above.[16] Both the 25-(OH)D$_3$ precursor and the 1,25(OH)$_2$D$_3$ hormone serve as effective substrates for the 24-OHase enzyme, rendering the latter capable of catalyzing a potent attenuation of active vitamin D metabolite concentrations. In fact, homozygous 24-OHase null mice[34] display reduced 1,25-(OH)$_2$D$_3$ clearance, with the F1 progeny exhibiting signs of vitamin D intoxication, such as calcified kidneys. Interestingly, the F1 progeny also have defective intramembranous ossification. The failure of bones such as the calvaria and mandible to calcify in this situation could be a developmental consequence of excess 1,25(OH)$_2$D$_3$. Another possibility is that a 24-hydroxylated D-metabolite(s) is required for some aspect of cartilage or bone formation (Fig. 1A),[35–38] perhaps via pharmacokinetic interactions with 1,25-(OH)$_2$D$_3$ or through an uncharacterized novel receptor for the 24-hydroxylated metabolite.

The role of 1,25(OH)$_2$D$_3$–VDR in phosphate homeostasis

Other than negative feedback on PTH synthesis and secretion[27,39] to suppress this phosphaturic hormone, how does the 1,25(OH)$_2$D$_3$–VDR complex influence phosphate metabolism to maintain the Ca•PO$_4$ ion product? One mechanism seems to be the primary induction of phosphate translocating proteins in kidney and perhaps intestine (Fig. 1). An example is the renal sodium–phosphate cotransporter-2 (NPT2) (Fig. 1B),[40,41] a likely 1,25(OH)$_2$D$_3$-induced protein because its gene contains a vitamin D responsive element (VDRE) in the promoter region (see below). It is also well established that hypophosphatemia stimulates the 1α-OHase (Fig. 1B) to elevate circulating 1,25(OH)$_2$D$_3$ levels.[26] However, circulating 1,25(OH)$_2$D$_3$ is inappropriately low for the prevailing phosphate concentrations in patients with X-linked hypophosphatemic rickets (XLH),[42] a dominant familial disorder of renal phosphate wasting. Importantly, such patients can be cured with a therapeutic combination of oral phosphate and 1,25-(OH)$_2$D$_3$.[43] The defective gene responsible for XLH has been identified as PEX, or phosphate-regulating gene with homologies to endopeptidases located on the X-chromosome.[44] Further, there exists a significant number of cases of tumor-induced osteomalacia, an acquired disorder that closely resembles the phosphate wasting of XLH and is characterized by low circulating 1,25(OH)$_2$D$_3$.[45] Finally, renal cross-transplantation[46] and parabiosis[47] studies in normal and genetically hypophosphatemic mice demonstrate that a novel uncharacterized phosphaturic hormone is present in the circulation. Taken together, these observations argue strongly that the XLH and tumor-induced osteomalacia syndromes are both caused by excess amounts of this novel humoral factor, which is distinct from PTH and has been named phosphatonin (Fig. 1B).[48,49] Like PTH, phosphatonin is presumably a potent inactivator of NPT2 (Fig. 1B), but in contrast to PTH, this newly recognized factor apparently also inhibits the 1α-OHase, thus suppressing 1,25(OH)$_2$D$_3$. As depicted in Fig. 1(B), the normal role of the PEX gene product is postulated to be the proteolytic inactivation of this phosphaturic principle, such that inactivating PEX mutations in XLH elicit the appearance of abnormally high circulating levels of phosphatonin.[44] In the case of tumor-induced osteomalacia, the tumor appears to produce phosphatonin ectopically. Thus, as illustrated in Fig. 1(B), the PEX/phosphatonin system could participate in a novel regulatory loop for maintaining normal phosphate homeostasis, which becomes deranged in XLH, or when tumors directly secrete phosphatonin.

Another role of 1,25(OH)$_2$D$_3$/VDR in phosphate control is postulated to be the induction of PEX gene expression (Fig. 1B), thus creating an additional strategy for protection by 1,25(OH)$_2$D$_3$ against hypophosphatemia. Indeed, the PEX gene appears to possess a VDRE based upon Southwestern analysis of the relevant yeast artificial chromosome.[50] The present hypothesis is that a low phosphate level triggers an increase in circulating 1,25(OH)$_2$D$_3$, which in turn augments the PEX gene product to destroy phosphatonin, constituting a novel phosphate homeostatic loop that is overwhelmed when high levels of phosphatonin diminish 1,25(OH)$_2$D$_3$ synthesis and inhibit NPT2. This concept is consistent with the relatively depressed 1,25-(OH)$_2$D$_3$ levels observed either in patients with tumor-induced osteomalacia or in kindreds with XLH caused by inactivating PEX mutations. Given the present indications that phosphatonin represents a pathophysiologically relevant phosphaturic hormone that regulates vitamin D bioactivation, its characterization at the chemical level is eagerly anticipated.

Neoclassical 1,25(OH)$_2$D$_3$/VDR actions

As summarized in Table 1, the potential actions of 1,25(OH)$_2$D$_3$ via its nuclear VDR extend far beyond the bone mineral homeostasis realm pictured in Fig. 1. The following three independent methodologies have been employed to provide evidence that 1,25(OH)$_2$D$_3$ functions in a diverse array of cells: autoradiographic localization of the ligand following administration to vitamin D–deficient animals, immunohistochemical detection of VDR in the nucleus of target cells, and responsiveness of specific cell types in culture to 1,25(OH)$_2$D$_3$ and its active analogs. In many cases, these data are coupled to biological responses or to loss of function in VDR null mice, in vivo, for instance in the maintenance of insulin secretion by 1,25(OH)$_2$D$_3$,[51] the uterine hypoplasia reported in VDR knockout female mice apparently caused by suboptimal ovarian estrogen production,[16] and the exploitation of the prodifferentia-

TABLE 1. NEOCLASSICAL $1,25(OH)_2D_3$ TARGET SITES

Classification	*Target tissue or cell**	*Specific effects†*
Immune system	monocytes/macrophages[53] and T-lymphocytes (helper type 1)[53,57]	suppression of γ-interferon[202] and IL-1 through IL-6[53,159,203]
Central nervous system	dorsal root ganglia,[204] glial cells, and hippocampus[70,107]	production of NGF,[65,66,205] neurotrophin-3,[64] and leukemia inhibitory factor[63]
Epithelium	epidermal skin/keratinocyte[206]	differentiation[17,211]
	hair follicle[207]	differentiation[16,17,212]
	female reproductive tract[208]	uterine development[16]
	mammary[108]	↓ cell growth[213]
	prostate[209]	↓ cell growth[173]
	colon[210]	↓ cell growth[170,214]
	lung[108]	surfactant[215]
Endocrine system	thyrotrope[216]	TRH receptor[218]
	thyroid[108]	↓ TSH action[219]
	pancreatic β-cell[217]	insulin secretion[51]
	ovary[208]	folliculogenesis[16] and ↓ cell growth[220]
Muscle	heart[221]	↓ ANP[222,223]
Adipose	adipocyte[224]	lipoprotein lipase[225]
Many systems	diverse cells and cancer cell lines[173,226,227]	↓ cell growth (c-*fos*[179]; ↓ c-*myc*[58,157]), differentiation (p21[228]; p27[229]; *Mad*-1[230]) and apoptosis[213](↓ *Bcl*-2[231,232])

*Detected by autoradiographic ligand localization in the nucleus, VDR immunocytochemistry, or responsiveness of cultured cells.
†Effects of $1,25(OH)_2D_3$ are positive unless otherwise noted and are selected examples rather than a comprehensive list.

tion/antiproliferative actions of $1,25(OH)_2D_3$ in the treatment of psoriasis.[52]

One major neoclassical target for $1,25(OH)_2D_3$ is the immune system (reviewed in Ref. 53), with the suppression of IL-1 to IL-6 and interferon-γ constituting prominent in vitro $1,25(OH)_2D_3$ effects mediated by VDR (Table 1). Moreover, in vivo immunomodulatory actions of the hormone also have been documented (reviewed in Ref. 54), such as reduced macrophage and lymphocyte function in vitamin D–deficient rats.[55] $1,25(OH)_2D_3$ functions as a general suppressor of the immune system, especially of T-helper cells (subset type 1), suggesting that analogs of vitamin D might be useful therapeutic agents in procedures such as organ transplants[56] or in the treatment of autoimmune disorders.[57] In addition, $1,25(OH)_2D_3$ is thought to play an important role in the differentiation of cells in the hematopoietic lineage. Several illustrations of this action have been reported, including differentiation of a human promyelocytic leukemia cell line (HL-60) into macrophage-like cells,[58,59] and the development of osteoclasts in bone from colony forming unit–granulocyte/macrophage precursors (see Ref. 53 and references therein). Many of these effects of $1,25(OH)_2D_3$–VDR, although of potential therapeutic significance, may be biologically redundant with other immune modulators, perhaps offering survival advantages. The tentative conclusion of redundant immunoregulation is based upon the normal immune profile of VDR null mice at 7 weeks of age,[16] but it is reasonable to hypothesize that the potential immunomodulatory power of $1,25(OH)_2D_3$–VDR could become more significant during pathophysiological stress situations or under conditions of senescence.

Other apparent sites of action for $1,25(OH)_2D_3$ and VDR include the central nervous system (CNS) (Table 1), where one of the outcomes is immunosuppression. For example, $1,25(OH)_2D_3$ treatment elicits a partial improvement of symptoms in rodents with developing experimental allergic encephalomyelitis.[60,61] Moreover, $1,25(OH)_2D_3$ also has been shown to induce expression of the following neurotrophic hormones or their mRNAs: glial cell-derived neurotrophic factor (a protein that may be important in protecting certain types of neural tissue from degenerative processes),[62] leukemia inhibitory factor (a widely distributed protein in the brain with neurotrophic activity),[63] neurotrophin-3 mRNA (in primary astrocytes),[64] and nerve growth factor (both in primary newborn astrocytes[65] and in the hippocampus and cortex of the adult rat[66]). Furthermore, the activity of cholinergic acetyltransferase is elevated in specific brain regions in response to $1,25(OH)_2D_3$ administration.[67] In some neurons, analogous to the antiapoptotic effect of calbindin-D_{28k} in lymphocytes,[68] the induction of calbindins by $1,25(OH)_2D_3$ may protect against cell death in the face of repetitive calcium transients,[69] and in fact the expression of calbindin-D_{28k} mRNA is decreased in the hippocampus of Alzheimer's patients as assessed by in situ hybridization.[70,71] Taken together, these observations not only imply a modulatory role for VDR in neural cell growth and differentiation but also intimate a possible role for $1,25(OH)_2D_3$ in therapeutic intervention for neurodegenerative disorders.

Similarly, $1,25(OH)_2D_3$ appears to affect dramatically the maturation and functions of certain normal and neoplastic epithelial cells (Table 1). As discussed above, VDR plays a

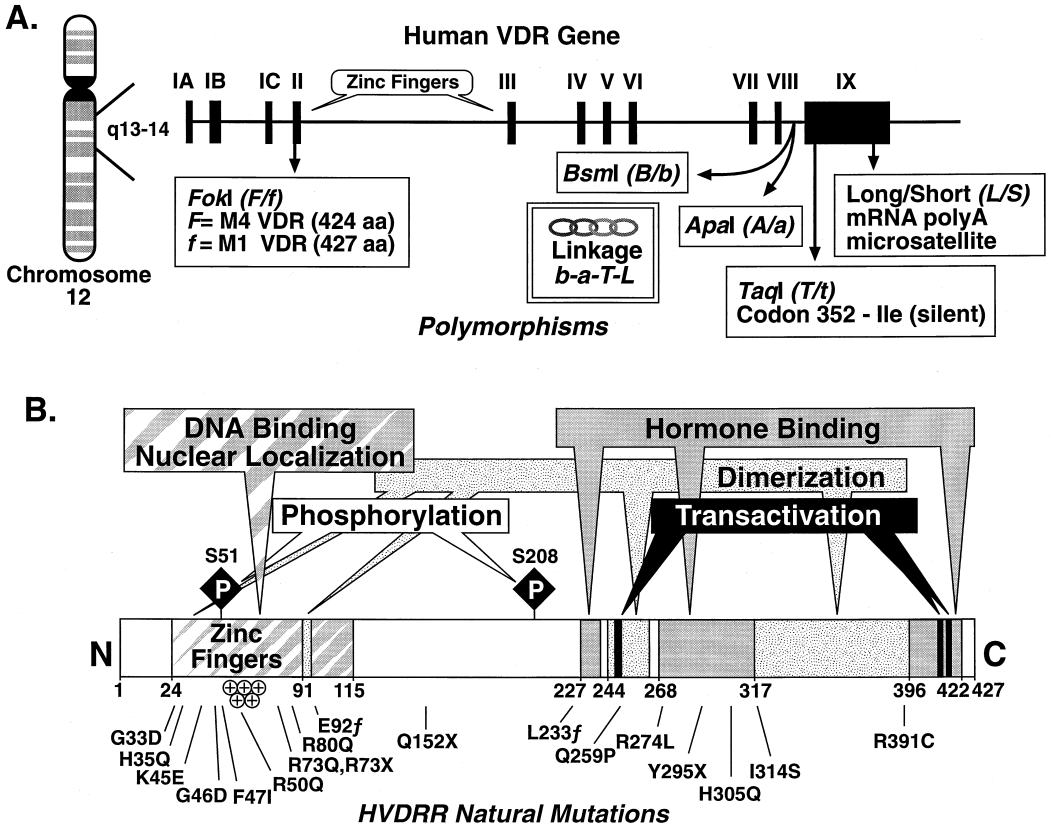

FIG. 2. Schematic view of genomic and deduced amino acid sequences for hVDR, displaying known natural variations. (A) The hVDR chromosomal gene, containing a total of 11 exons, three of which (IA, IB, and IC) encode 5′ UTR region and are variably present in VDR transcripts.[72] Several polymorphic variants, including a *Fok*I site in exon II, and a cluster of linked sites near or in exon IX, are discussed in the text. (B) Schematized linear amino acid sequence of hVDR, highlighting functional domains as currently understood from mutagenesis analysis. ⊕ signifies a cluster of five basic amino acids in the intervening sequence between the two zinc fingers. Two reported sites of modulatory serine phosphorylation at serine-51 and serine-208 are indicated by S51 and S208, respectively. Known natural point mutations in human patients with the HVDRR syndrome are indicated by single-letter abbreviations (e.g., G33D is a glycine to aspartate substitution at position 33). X refers to a premature stop codon, while *f* indicates a frame-shift mutation, leading to premature termination. Point mutants from positions 33–80 are defective in DNA binding, and all X and *f* mutants can neither bind hormone nor heterodimerize with RXR. Other point mutations in the C-terminal half of the receptor (from positions 259–391) display defects in either hormone-binding or heterodimer formation with RXR (or both) as discussed in the text.

key role in the hair growth cycle, an action related to the ability of $1,25(OH)_2D_3$ to stimulate the differentiation of keratinocytes (Table 1). Also, the proliferation of a number of epithelially derived cancer cells (e.g., mammary, prostate, and colon) is inhibited in culture by $1,25(OH)_2D_3$, with some cells being directed toward a more differentiated phenotype. This effect in neoplastic cells may be related to the reported ability of liganded VDR to arrest cells at the G_1 stage by influencing cell cycle regulatory proteins, such as p21 and p27, to control cell growth transcription factors such as c-*myc* and c-*fos,* or to elicit apoptosis by down-regulating *Bcl*-2 (Table 1 and references therein). Therefore, the $1,25(OH)_2D_3$ hormone seems to resemble its nutritionally derived, lipophilic ligand cousins, vitamin A and thyroid hormone, in possessing the ability to influence the program of cell development as well as to evoke classic metabolic and growth regulatory effects. $1,25(OH)_2D_3$ also reportedly affects several major endocrine processes, such as TRH/TSH action and pancreatic insulin secretion (Table 1). However, only further investigations of VDR null mice and other transgenic strategies, such as tissue-specific expression of a dominant negative VDR, will allow us to sort out which of the putative neoclassical effects of $1,25(OH)_2D_3$ mediated by VDR are biologically relevant. At this early juncture, of those sites enumerated in Table 1, only the skin/hair growth cycle and ovarian/uterine systems appear to be markedly affected in the VDR null mouse. However, the limited phenotype of VDR knockout mice does not preclude $1,25(OH)_2D_3$ and its analogs from being valuable as biological response modifiers useful in the treatment of hyperproliferative disorders, autoimmunity, and CNS deterioration, as well as traditional maladies of calcium and phosphorus metabolism such as renal osteodystrophy, hypoparathyroidism, and osteoporosis.

STRUCTURE/FUNCTION OF THE VDR GENE AND PROTEIN

hVDR chromosomal gene

The recently characterized gene encoding hVDR (Fig. 2A),[72] previously localized to chromosome 12,[73] is similar to other nuclear receptor genes[74] in that each of the two zinc fingers is encoded by separate exons (II and III), and the 5′ end of the gene exhibits some complexity in the form of alternate splice and/or translation start sites. For hVDR, alternate splicing of three exons (IA–IC) encoding portions of the 5′ untranslated region generates at least three mRNA variants,[72] while the presence of a polymorphic sequence in exon II determines the presence or absence of an alternative translation start site (see discussion of *Fok*I polymorphism below).[72] A unique feature of the hVDR gene is the presence of an additional exon (V) that is not found in other nuclear receptor genes (Fig. 2A)[72]; it resides near the center of the gene and encodes residues 155–194 in hVDR. This region of the VDR protein is more expansive than the corresponding segment in other nuclear receptors, suggesting that the VDR may have acquired a novel exon of unknown function as it diverged evolutionarily from other nuclear receptor genes.[72]

hVDR polymorphisms

One of the most intriguing, yet controversial, areas of bone-related genetic research in the past few years has been the discovery of common polymorphisms in the hVDR gene and their potential relationship to bone mineral density (BMD) and the pathophysiology of osteoporosis, hyperparathyroidism, and cancers of the breast and prostate. Morrison, Eisman, and colleagues[75] first reported that VDR alleles could predict BMD, contending that the occurrence of a *Bsm*I restriction site (Fig. 2A, denoted *b*) in the intron separating exons VIII and IX of the gene (Fig. 2A) is associated with enhanced lumbar spine BMD. Conversely, the absence of the *Bsm*I site (denoted *B*) in VDR was correlated with low BMD. In a population of Australian twins of Irish ancestry, Morrison et al.[75] concluded that the VDR genotype (*b* vs. *B*) accounted for up to 75% of the genetic component of BMD, although a correction/partial retraction of this report appeared recently.[76] Numerous subsequent studies with other population samples have found more modest,[77,78] little if any,[79–82] and even conflicting associations[83] of the *B* versus *b* alleles with BMD. A meta-analytic approach[84] that incorporated the results of 16 VDR polymorphic studies revealed a 1.5–2.5% decrease in BMD associated with *BB* (versus *bb*) homozygotes, far less dramatic than the 12% effect originally proposed.[75] Additional studies have also suggested a trend toward lower bone mass with the *B* allele,[85–88] but it has become clear that other confounding factors, such as age, estrogen status, ethnicity, and calcium intake, must be accounted for to reveal the true impact, if any, of this VDR polymorphism on BMD.

Further evaluation of the VDR gene has revealed a cluster of linked polymorphisms in the 3′ portion of the VDR gene (Fig. 2A),[89] including the aforementioned *Bsm*I site, a nearby *Apa*I site (in the same intron), and a silent mutation within codon 352 of the ninth exon that alters a *Taq*I site. These VDR polymorphisms have been linked not only to variations in bone-specific parameters but also to a higher occurrence of sporadic primary hyperparathyroidism in patients with the *b* allele,[90] as well as to prostate cancer, which possesses a particularly strong association with the *T* allele (lack of the *Taq*I site).[91] However, given that none of these polymorphisms change the encoded VDR protein in any way, the explanation for these findings has been unclear. Recently, an additional genetic variation in the hVDR gene was found in the form of a microsatellite poly(A) repeat in the 3′ UTR, approximately 1 kb upstream of the poly(A) tail (Fig. 2A). Multiple (≥12) allelic variants of this microsatellite were detected and classified into two groupings, long *(L)* and short *(S),* based upon the length of the repeat.[92,93] The *L* grouping (linked to *T*; see Fig. 2A) exhibits a strong association with prostate cancer incidence[93] but displays a contrasting protective effect against breast cancer.[94] It is still not established whether this poly(A) microsatellite is the functionally relevant locus, although by analogy to short tandem repeats in other genes[95] the length of the repeat may affect a crucial parameter, such as mRNA stability. Alternatively, the poly(A) microsatellite may be tightly linked to yet another site which is the true functional locus. Regardless of which scenario is correct, a complicating factor in interpreting earlier findings using the *Bsm*I, *Taq*I, or *Apa*I polymorphic restriction sites is that the linkage is imperfect between these restriction sites and the *L* versus *S* groupings, such that some ethnic groups exhibit a very tight linkage (thus displaying a clear correlation between BMD and the *Bsm*I site), while in others (e.g., African-Americans) the presence of the *Bsm*I or *Taq*I site is not a good predictor of the *L* versus *S* genotype,[92] leading to a loss of functional correlation between *Bsm*I or *Taq*I and BMD. Nonetheless, the study of the poly(A) microsatellite sequence may have brought us closer to a functional understanding of VDR genetic diversity, while also providing a partial explanation for the variability in association of *Bsm*I or *Taq*I genotypes with BMD and hyperproliferative disorders.

Near the 5′ end of the hVDR gene, a *Fok*I restriction endonuclease site has been identified,[96,97] the presence of which (denoted *f*) dictates that the 427-residue, M1 isoform of VDR is expressed (so named because it contains an ATG methionine translational start site corresponding to codon #1). An evolutionarily more recent polymorphism (Fig. 3),[98] or neomorph, has been reported in which both the *Fok*I restriction site and the ATG codon #1 are changed, causing an alternative 424-residue isoform of the receptor to be translated. The *F* neomorph, encoding M4 hVDR (for initiation of translation at the fourth codon) already constitutes approximately 65% of VDR alleles in human subjects (L. Remus and M. Haussler, unpublished results), intimating an evolutionary advantage in humans. The *Fok*I polymorphism, which is not linked to the aforementioned cluster of variations in the 3′ region of the gene, may therefore have significance for human bone pathology. Indeed, the M4 isoform is more transcriptionally active

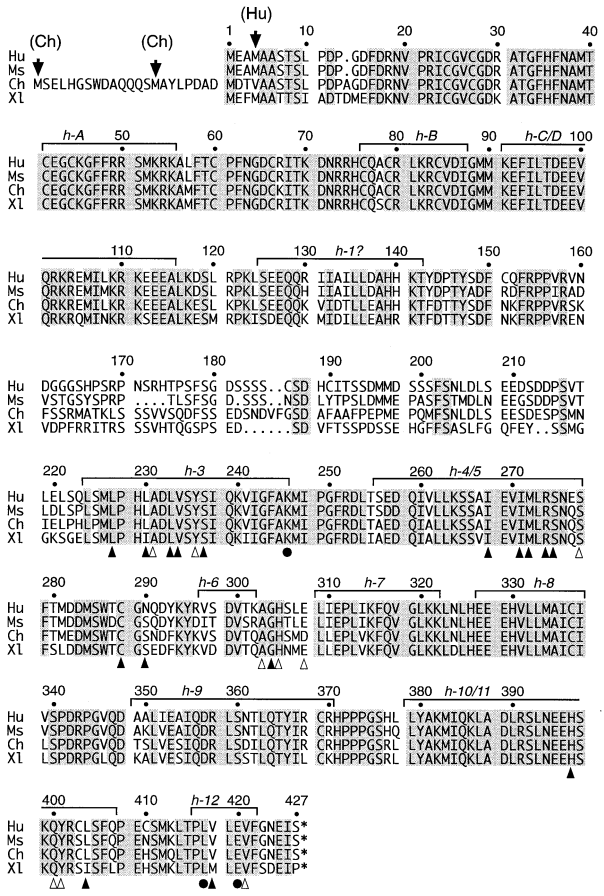

FIG. 3. Amino acid comparison among four of the six VDRs cloned to date. Shown are sequences from human (Hu),[147] mouse (*Mus musculus,* abbreviated Ms),[149] chicken (*Gallus gallus,* denoted Ch),[1,150,151] and *Xenopus laevis* (Xl).[18] Numbering is for the hVDR as published by Baker et al.[147] Not shown are sequences from rat[148] and Japanese quail,[151] which are similar to the mouse and chicken, respectively. Alternative translation start sites for chicken (Ch) or human (Hu) mRNAs are indicated by arrows. The approximate location of residues corresponding to α-helices (*h-A, h-1,* etc.) in the crystal structures of hTRβ,[102] rTRα[119] and hRARγ[118] are indicated by brackets above the sequences. By similar analogy, ligand contacts in rTRα[119] and hRARγ[118] are indicated by solid triangles below the sequences if found in both crystal structures, or by open triangles if found in only one structure. Artificial point mutations in hVDR that inhibit transcriptional activity without affecting either $1,25(OH)_2D_3$ binding or heterodimerization are denoted by solid circles under the sequences. An asterisk indicates the stop codon.

than the M1 VDR in a transient transfection assay employing a VDRE-linked reporter gene.[96] We have independently observed that the M4 hVDR is significantly more effective than the longer M1 receptor in activating the transcription of VDRE–reporter constructs in several transfected mammalian cell lines, including osteoblasts (L. Remus and P. Jurutka, unpublished results). The conclusion that the M4 *(F)* hVDR isoform is more transcriptionally potent is in concert with recent studies which suggest an association between the *F* hVDR allele and enhanced BMD in two different populations.[96,97] This raises the possibility that the *F* neomorphic VDR allele, and speculatively the *L* grouping of microsatellite variants (linked to varying degrees with *b*/*a*/*T* alleles), may provide partial protection against osteoporosis by ensuring enhanced BMD via the increased activity of hVDR throughout life.

Functional domains in the VDR protein

As depicted schematically in Fig. 2(B) and conceptualized later in this review, VDR is a $1,25(OH)_2D_3$-dependent transcription factor that controls gene expression by heterodimerizing with retinoid X receptors (RXRs) and associating specifically with VDREs in target genes.[99] The principal functional domains of hVDR (Fig. 2B) that effect this action include those for hormonal ligand binding, heterodimerization, DNA binding/nuclear localization, and transcriptional activation. The lower portion of Fig. 2(B) also illustrates the 18 point mutations in VDR detected to date in HVDRR patients (reviewed in Refs. 10–13 and 100). Five of these genetic alterations result in nonsense (X) or frameshift (*f*) mutations that introduce premature stop codons in the receptor (R73X, E92*f*,[101] Q152X, L233*f*, and Y295X), 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. 2B) that can be classified according to three of the major molecular functions of VDR: DNA binding/nuclear localization by the N-terminal zinc finger region (G33D through R80Q), $1,25(OH)_2D_3$ hormone binding by the C-terminal domain (R274L, H305Q, and I314S), and heterodimerization with RXRs through subregions of the C-terminal domain (I314S and R391C).

DNA binding

As with other nuclear receptors, all VDRs described to date possess a conserved DNA binding domain consisting of two zinc finger motifs (Fig. 2B and Fig. 3, residues 24–90 in hVDR). Although X-ray crystallographic data are not yet available for VDR, the solution of the cocrystal structure for the DNA binding domains of thyroid hormone receptor (TR)-RXR on a thyroid hormone responsive element (TRE)[102] provides an initial approximation of VDR structure/function in the DNA binding region. Accordingly, as detailed in Fig. 3, an α-helix resides on the C-terminal side of each zinc finger, with helix A and helix B constituting the DNA-recognition and phosphate backbone binding helices, respectively. Among the naturally occurring missense mutations in this segment of hVDR (Fig. 2B), his-35, lys-45, arg-50, arg-73, and arg-80 all correspond to DNA contacts in the TR-RXR–TRE cocrystal. That these alterations cause the HVDRR phenotype of $1,25(OH)_2D_3$ resistance in patients[10] argues strongly for the existence of a reasonable structural congruity between the VDR finger region and that of TR.

As a further analogy with the TR–RXR cocrystal,[102] the

region just C-terminal to the zinc fingers in VDR (Fig. 2B, residues 91–115 in hVDR), containing the T- and A-box helical region (denoted helices C/D in Fig. 3), likely also provides DNA contacts. The functional significance of putative helices A–D in DNA binding by VDR is emphasized by the near absolute conservation across species of the residues therein (Fig. 3). An interesting point concerning the VDR DNA binding domain is the presence, unique to VDRs, of a cluster of five basic amino acids in the intervening sequence between the two zinc fingers (Fig. 2B, ⊕; residues 49–55 in Fig. 3). Four of these positively charged residues (excluding lys-55) are hypothesized to make DNA contact based on the Rastinejad model,[102] but this region is also crucial for nuclear localization of the receptor.[103] In addition, this segment includes ser-51, a site of hVDR phosphorylation (Fig. 2B) by protein kinase C[104] that results in inhibition of VDRE binding by phospho-VDR, in vitro.[105] Thus, phosphorylation of ser-51 could conceivably modulate both DNA-binding and nuclear localization of VDR, in vivo.

Nuclear localization

Biochemical fractionation and in situ immunocytochemical localization studies have shown that VDR is predominantly a nuclear protein, even in the unoccupied state.[106–110] Two studies to date have examined potential nuclear localization domains in hVDR,[103,111] focusing mainly on stretches of basic amino acids, such as those that have been implicated in nuclear localization of other steroid hormone receptors.[112–114] The results of Luo et al.[111] suggest that a bipartite signal consisting of a cluster of basic residues at each end of the sequence between amino acids 79 and 105 of hVDR is required for nuclear accumulation. Hsieh et al.[103] have identified a separate basic sequence between the two zinc fingers of hVDR (residues 49–55) that represents a second nuclear localization signal. It remains unclear whether the identified three basic clusters of positively charged residues, which are absolutely conserved in helices A, B, and C (Fig. 3), function together or independently to effect nuclear translocation. The observations that point mutations causing HVDRR occur at arg-80[115] in the bipartite signal, and at arg-50[116] in the second signal, and that neither mutated receptor is competent in transferring ligand to the nucleus, strongly suggest a necessity for both nuclear localization signals. Finally, as denoted in Fig. 2(B), the entire DNA binding domain, as a functional unit, may be required for optimal retention of VDR in the nucleus as a result of the general interaction of VDR with DNA.[115,117]

Hormonal ligand binding

Based upon the partial X-ray crystal structures of human retinoic acid receptor γ (hRARγ),[118] rat TRα$_1$,[119] as well as unoccupied but homodimeric hRXRα,[120] the ligand binding domain of nuclear receptors consists of a "sandwich" of 12 α-helices and several short β-strands organized in three dimensions around a lipophilic hormone-binding pocket. Following this analogy, three regions of hVDR in closest proximity to the 1,25(OH)$_2$D$_3$ ligand would extend approximately from residues 227–240, 268–316, and 396–422 (Fig. 2B). As shown in Fig. 3, ligand contact sites at the individual amino acid level in the crystal structures of ligand-occupied hRARγ[118] and agonist-occupied rTRα$_1$[119] map to clusters in helix 3, helix 5, helix 11, and helix 12, as well as the β-strands (not delineated) that flank helix 6. Several of the mutations characterized in hVDR that compromise ligand binding coincide with hormone contact sites in RAR and/or TR. These include the natural mutations R274L[121] and H305Q,[13] as well as artificial hVDR mutations at leu-233,[122] cys-288,[123] and his-397.[124] The conclusion from these identified mutant hVDRs is that the structure of the VDR ligand binding domain, with the exception of relatively unconserved amino acids between residues 155 and 194 (exon V, see above and Fig. 3), closely resembles that of its nuclear receptor subfamily cousins, RAR and TR. A similar thesis has been presented independently by Wurtz et al.,[125] who modeled the hormone binding domain of liganded VDR utilizing coordinates from the X-ray crystal structure of holo-RARγ.

Heterodimerization with RXR

From large deletion studies with VDR, Nishikawa and coworkers[126] determined that VDR dimerization surfaces are found in the first zinc finger, in a region just C-terminal of finger-2, and in the large C-terminal ligand binding domain. Higher resolution mapping experiments have revealed that weak heterodimerization between VDR and RXR is facilitated by specific contact sites in the DNA binding domain of VDR (Fig. 2B). By site-directed mutagenesis, Hsieh et al.[127] have shown that lys-91 and glu-92, which are situated in the T-box region of hVDR (just C-terminal of finger-2 at the beginning of helix C in Fig. 3), mediate heterodimerization between the VDR and RXR DNA binding domains on the VDRE scaffold. This finding is independently supported by a modeling study,[102] which also identifies Asn-37, located in the first zinc finger of hVDR, as an amino acid that contacts residues in the second zinc finger of the RXR partner in determining selective association of VDR with a VDRE consisting of a direct repeat spaced by three nucleotides (DR3). Within the hormone binding domain, two regions of hVDR that are involved in strong, ligand-dependent heterodimerization with RXR have been deduced by mutagenesis experiments and by analogy to the RXR homodimer crystal (Fig. 2B).[120] These two subdomains consist of hVDR residues 244–263,[100,128–130] and amino acids 317–395,[124,130] corresponding to portions of helices 3–4 and 7–10, respectively (Fig. 3). The helix 7–10 region is positionally equivalent to contacts between hRXRα residues in a homodimer as determined via X-ray crystallography by Bourguet et al.[120] Thus, as illustrated in Figs. 2 and 3, the schematic picture of hVDR that is developing not only maintains the modular concept of receptor organization with general DNA and hormone binding domains, but also illuminates the complexity of regions within the ligand binding domain required for supporting both heterodimerization and hormone binding functions. The aforementioned I314S and

R391C natural mutations (Fig. 2B) are unique because they provide evidence for a potential structural interplay between heterodimerization and hormone binding of VDR. I314S, which endows hVDR with combined defects in both hormone retention and heterodimerization,[11] lies within helix-7, near a presumed juncture of ligand binding and heterodimerization subdomains of the receptor (Fig. 2B). Likewise, R391C is positioned within the helix-10 dimerization surface, but not far removed from C-terminal ligand binding contacts (Figs. 2B and 3), consistent with the observed phenotype of a primary heterodimerization defect and a milder, secondary ligand retention deficiency.[11] The conclusion is that the ligand binding and heterodimerization functions are interrelated within the context of the tertiary structure of VDR, likely via allosteric effects that ultimately generate an active receptor conformation.

Transactivation

The mechanism of transcriptional up-regulation employed by the agonist activated RXR–VDR heterodimer, once bound to DNA, is just now becoming understood. Functional evaluation of VDR points to at least two regions of the receptor required exclusively for transcriptional activation. One of these regions (residues 244–263) has already been noted for its role in heterodimerization (see above). However, embedded in this domain is lys-246 in hVDR (Fig. 3, denoted by a solid circle below the sequences), a residue not involved in heterodimerization, but whose alteration, even by conservative arginine replacement, severely compromises transactivation.[129] A recent report has extended this observation to the estrogen receptor,[131] and proposes, as did Whitfield et al.,[129] that this residue, which is highly conserved among nuclear receptors, forms part of the binding interface with transcriptional coactivators (see Fig. 4 and later discussion). The second region, by analogy with other nuclear receptors, is known as a ligand-dependent activation function or AF-2, and corresponds to helix 12 (Fig. 3) of the hormone binding domain.[132–135] Analysis of this domain in hVDR by site-directed mutagenesis has identified leu-417 and glu-420 (also denoted by solid circles in Fig. 3) as pure, hormone-dependent transcriptional activation residues, with their alteration having little effect on either hormone binding or heterodimeric DNA binding.[136,137] Like lys-246, leu-417 and especially glu-420 function to stimulate transcription by a mechanism involving coactivators.[136–138]

The transcriptional capacity of hVDR can be modulated in response to phosphorylation by kinases that participate in distinct signal transduction pathways.[139,140] For example, hVDR is phosphorylated by protein kinase A (PKA), presumably in the central portion of the molecule between the DNA and hormone binding regions, resulting in an attenuation of $1,25(OH)_2D_3$-activated transcription.[141,142] In addition, hVDR is phosphorylated by casein kinase II (CK-II), in vitro, at ser-208, the major hormone-regulated phosphorylation site, in vivo (Fig. 2B).[143,144] Overexpression of CK-II in mammalian cells effects a potentiation of $1,25(OH)_2D_3$-stimulated transcription,[145] indicating that the control of gene expression by $1,25(OH)_2D_3$–VDR can be fine-tuned via phosphorylation, probably allowing for cross-talk between the VDR and other signal transduction pathways, and perhaps even governing receptor activity at different stages of the cell cycle or during cell differentiation.[139,146]

Comparison of VDRs across species

To date, VDR cDNAs have been cloned from six species, namely human,[147] rat,[148] mouse,[149] chicken,[1,150,151] Japanese quail,[151] and frog *(Xenopus laevis)*.[18] As illustrated in Fig. 3, a direct comparison of the deduced amino acid sequences from four of these VDRs reveals significant sequence similarity. One variation that emerges from this comparison is that both avian VDRs (only the chicken sequence is shown) have a 22-residue N-terminal extension that possesses two alternative translational start sites which are positionally 5′ of those in other VDRs.[150,151] In contrast, a 3′ met-4 is an alternative translational start site in the *f* (M1) hVDR allele shown in Fig. 3. As detailed above in the human polymorphism section, in an apparently evolutionarily advantageous neomorph of hVDR (*F* or M4), the met-1 codon is mutated to render alternative met-4 the translational initiation site.

Major regions of hVDR display a striking degree of sequence similarity (Fig. 3), with the highest degree of conservation occurring in those domains of known functional significance. Prominent examples evident in Fig. 3 include the DNA-binding/nuclear localization domain (helices A–D), and the helix 3–12 ensemble that performs an intricate combination of $1,25(OH)_2D_3$ binding, RXR heterodimerization and transactivation as discussed above. Encompassing most of the amino acids encoded in exon V, there occurs a poorly conserved central region (approximately residues 159–201 in hVDR) between putative helices 1 and 3 of the hormone binding domain, which displays 47% identity between human and rat, 19% between human and *Xenopus,* and only 16% between human and chicken VDRs. Not surprisingly, this segment of VDR is also unique when compared with other nuclear receptors, a fact that prevents assignment of helix 2 in VDR based on structural data from TR, RAR, or RXR (Fig. 3). Nevertheless, the homology data in Fig. 3 provide compelling support for the current linear structure/function model of hVDR pictured in Fig. 2(B).

MECHANISM WHEREBY THE VDR–RXR HETERODIMER MEDIATES THE ACTION OF $1,25(OH)_2D_3$ TO CONTROL GENE TRANSCRIPTION

VDREs that mediate transactivation

Sequence and promoter analyses of several $1,25(OH)_2D_3$-regulated genes have led to the identification of VDREs, short DNA sequences to which the VDR binds as a heterodimer with RXR and subsequently exerts its influence on transcription. The top portion of Table 2 lists those VDREs identified in genes that are known to be transcriptionally activated by the $1,25(OH)_2D_3$ hormone, including

TABLE 2. VDRE SEQUENCES IN VDR-REGULATED GENES

Gene	Effect on transcription*	Vitamin D responsive element†			Reference(s)
		5′-RXR		3′-VDR	
Rat osteocalcin	+	**GGGT**GA	AT**G**	**AG**GA**CA**	(155, 233–235)
Human osteocalcin	+	**GGGT**GA	AC**G**	**GGGG**CA	(236–238)
Mouse osteopontin	+	**GG**T**TCA**	CGA	**GGTTCA**	(239)
Avian β_3 integrin	+	**G**AGG**CA**	GAA	**GG**GAG**A**	(240)
Rat 24-OHase-proximal	+	**AGGT**GA	GT**G**	**AGGGC**G	(33, 241)
Rat 24-OHase-distal	+	**GG**T**TCA**	GC**G**	**GGTGC**G	(242)
Mouse calbindin-D_{28k}	+	**GGGGGA**	TGT	**G**AGGAG	(243)
NPT2	+	**GGGGCA**	GCA	**AGGGCA**	(41)
p21 (Cdk inhibitor)	+	**AGG**GAG	ATT	**GGTTCA**	(228)
Random selection‡	+	**PuGGTCA**	NN**G**	**PuGTTCA**	(152, 153)
		VDR (RXR?)		RXR (VDR?)	
Avian PTH	−	**GGGTCA**	GGA	**GGG**T**GT**	(244)
Human PTH§	−	**GG**T**TCA**	AA**G**	CAGA**CA**	(29, 39)
Mouse osteocalcin	−	**GGG**CA**A**	AT**G**	**AG**GA**CA**	(162)
Rat bone sialoprotein‖	−	**AGG**GT<u>T</u>	<u>TAT</u>	**AGGTCA**	(245)
PKA inhibitor	−	**ATGT**TG	CT**G**	**AGGTCA**	(165)
Rat PTHrP-proximal	−	**AGGTTA**	CTC	**AGT**GA**A**	(246)
Rat PTHrP-distal	−	**GGGT**GG	AGA	**GG**GGTG	(247)

*VDREs activated by $1,25(OH)_2D_3$–VDR are shown in the upper portion and those repressed are listed in the lower portion.

†Bases depicted in darker type are those homologous to the randomly selected, highest affinity VDRE.

‡Consensus VDRE determined by binding of VDR–RXR heterodimers to randomly synthesized oligonucleotide sequences; Pu = purine.

§VDR heterodimerizes with a factor distinct from RXR on this VDRE.

‖Antisense TATA box is underlined.

osteocalcin and osteopontin (expressed in bone osteoblasts), β_3 integrin (found in bone osteoclasts and macrophages), 24-OHase and NPT2 (discussed above), calbindin-D_{28k} (from kidney) and p21 (an inhibitor of cyclin-dependent kinase [Cdk] in many tissues). In addition, two laboratories[152,153] have probed VDRE criteria by random selection of oligonucleotides that form complexes with VDR in combination with RXR (Table 2, center), providing results that are in general agreement with the natural VDREs. For positive transcriptional regulation, the VDRE is thus defined as a DR3, or direct repeat of two 6-base pair (bp) half elements that resemble estrogen-responsive element half-sites, separated by a spacer of three nucleotides. As depicted in Table 2 (top), VDR occupies the 3′ half-site of positive DR3 VDREs, while the 5′ half-site is bound by the RXR heteropartner.[154] Examination of both the natural and randomly selected VDRE sequences suggests that the G at position 3 of the spacer is important in VDR binding, a deduction consistent with the finding[155] that in the rat osteocalcin motif this base is partially protected by RXR–VDR in methylation interference assays. With the exceptions of osteopontin and p21, numerous sequence variations are evident in the 3′ half-element of natural VDREs when they are compared with the PuGTTCA random consensus selection for this half-element (Table 2). This variability could be related to a need for VDREs of graded potency 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.[156] This postulated range of VDR conformations might endow the receptor with the ability to recruit a variety of different coactivators. Finally, the presence of two VDREs in the 24-OHase gene promoter (Table 2) intimates that the regulation of this enzyme is even more complex and may manifest hypersensitivity in order to effect the rapid elimination of the $1,25(OH)_2D_3$ hormone. Irrespective of the above considerations, it is evident that the primary positive VDRE is a DR3 recognition site in DNA that attracts RXR–VDR to the promoter region of $1,25(OH)_2D_3$-regulated genes, ultimately adjusting the functions of target cells as a result of transcriptional control of gene expression.

VDREs that mediate transrepression

Certain genes are negatively controlled at the level of transcription by the $1,25(OH)_2D_3$ hormone, and this transrepression apparently occurs by several different mechanisms, including primary or secondary effects. For example, secondary effects involving the induction of intermediary proteins include $1,25(OH)_2D_3$ suppression of c-*myc* expression (Table 1),[157,158] and probably of atrial natriuretic peptide in cardiac myocytes (Table 1 and S. Chen et al., unpublished results). In what may be a primary effect, Alroy et al.[159] report that VDR binds, in vitro, to the human

Table 3. Effects of 9-*Cis* Retinoic Acid Ligand for RXR on Transactivation by the VDR–RXR Heterodimer

Antagonistic effects*

1. Blocking of $1,25(OH)_2D_3$ antiproliferative action on HT-29 colon cancer cells;[170] inhibition of $1,25(OH)_2D_3$ stimulated chondrogenesis of chick limb bud mesenchymal cells[248]
2. Inhibition of $1,25(OH)_2D_3$-induced osteocalcin mRNA accumulation in ROS 17/2.8 osteosarcoma cells[99]
3. Inhibition of $1,25(OH)_2D_3$-mediated VDR-RXR binding to osteocalcin and osteopontin VDREs[99,249,250]
4. Suppression of $1,25(OH)_2D_3$-elicited transactivation of a reporter gene from an osteocalcin or osteopontin-like VDRE in transfected cells[99,175]

Additive or synergistic effects*

1. Antiproliferative actions on the following cells in culture: human pancreatic carcinoma (Capan-1 and Capan-2),[169] colon cancer (Caco-2),[170] human promyelocytic leukemia (U937 and HL-60),[231,251–253] prostate cancer (LNCaP),[173] and breast cancer (MCF-7)[254]
2. Induction of 24-OHase mRNA in human skin[171] and colon cancer cells (HT-29)[170]
3. None reported
4. Transactivation in transient transfections employing the following VDREs: synthetic perfect DR3s consisting of GGGTGA[174] and AGGTCA[174,255] half elements, both rat 24-OHase VDREs,[256] mouse osteopontin VDRE,[174] human osteocalcin VDRE,[174] and a putative inverted repeat VDRE in the calbindin-D_{9k} gene[257]

*1. Cell growth and differentiation; 2. mRNA accumulation; 3. VDR-RXR heterodimerization; 4. transactivation.

IL-2 promoter to prevent NFATp/AP-1 complexing with the DNA, thereby effecting transrepression. However, unlike the rat osteocalcin gene, where liganded VDR has been shown to associate with the positive VDRE via in vivo footprinting,[160] no evidence has been provided that VDR directly binds to the IL-2 promoter in a ligand-dependent fashion, in vivo.

In other cases of transrepression by $1,25(OH)_2D_3$, there also exist data that VDR binds to the promoter in question, in vitro, and does so in a heterocomplex with RXR, or with another tissue-specific silencing coreceptor. For instance, VDR represses human PTH transcription in a $1,25-(OH)_2D_3$-dependent manner by complexing with a coreceptor distinct from RXR,[29] but on an element that resembles a degenerate DR3 VDRE with substitutions in the 3′ half-element (Table 2, lower portion). The putative avian PTH negative VDRE is also similar to the consensus positive VDRE, except that the G in the spacer and the last two bases (CA) in the 3′-half element are replaced by other nucleotides (Table 2). Interestingly, when Russell and coworkers[161] altered the two 3′ terminal bases (GT) back to the consensus CA, the avian PTH VDRE reverted from a negative to a positive VDRE. Moreover, the mouse osteocalcin VDRE, which unlike the rat and human osteocalcin VDREs confers negative control by $1,25(OH)_2D_3$,[162] also possesses only two base differences (positions 4 and 5 of the 5′ half-element) from that of the rat sequence (Table 2). A separate secondary mechanism for negative osteocalcin control by $1,25(OH)_2D_3$ in the mouse appears to involve suppression of an osteoblast-specific activator that binds to a *cis*-acting element in the promoter.[163] Nevertheless, based upon observations with VDREs in the avian PTH gene as well as mouse versus rat osteocalcin, simply changing 2 bp in either half-element of a VDRE reverses the direction of transcriptional regulation by $1,25(OH)_2D_3$ as mediated by the VDR. How is this on–off switch accomplished mechanistically? One possibility is that the polarity of the RXR–VDR heterocomplex on the VDRE is altered such that the VDR occupies the 5′ half-element when gene control is negative.[161] There is, in fact, precedent for binding orientation reversal conferring negative regulation in the case of the RAR–RXR heterocomplexes.[164] Another possibility is that base pair substitutions in either half-element conform the VDR complex such that it cannot recruit a coactivator, or perhaps retains a corepressor. The concept of VDRE association altering the tertiary structure of VDR is supported by the observation that a 2 bp change of TT to GA in positions 3 and 4 of the 3′ half-element, as in the mouse osteopontin versus rat osteocalcin VDREs, appears to alter the conformation of VDR as judged by antibody epitope accessibility and protease sensitivity.[156] Either or both of the potential mechanisms of reverse VDR/RXR orientation or negative VDR conformation could come into play in primary transrepression. Yet a third mechanism involving occlusion of an active site in the promoter apparently occurs with the bone sialoprotein gene upstream region, wherein the RXR–VDR or VDR–RXR complex binds to a VDRE overlapping an inverted TATA box (Table 2), presumably occluding TATA-binding protein association. A similar situation may occur in the case of the PKA inhibitor gene (Table 2), in which a putative negative VDRE is situated between two TATA sequences.[165] However, more research is clearly needed before we will have a comprehensive understanding of negative transcriptional regulation by VDR.

The effect of 9-cis retinoic acid, the ligand for RXR, on VDR signaling

An additional level of control of the VDR-RXR heterodimer could conceivably be exerted by RXR ligands, primarily the natural 9-*cis* retinoic acid (9-*cis* RA) hormone.[99] Precedents from other receptors that heterodimerize with RXR encompass a spectrum of effects. With respect to transactivation by all-*trans* RA as mediated by the RXR–RAR heterodimer, subsequent addition of RXR-specific ligands, referred to as rexinoids, further aug-

Table 4. Interaction of VDREs with Nearby or Overlapping *Cis* Elements That Bind Regulatory Proteins

Gene	*VDRE-type*	*Cis-trans modifier*	*Effect on transcription*	*Reference*
Rat osteocalcin	DR3	Inhibitory YY1 competes with VDR/RXR by overlapping the VDRE	−	(177)
Human osteocalcin	DR3	Upstream AP-1/*fos-jun*	+	(238)
Rat osteocalcin	DR3	Downstream ligand-dependent transcription sequence (GGTTTGG)	+	(258)
Mouse calbindin-D_{28k}	weak DR3	Adjacent butyrate responsive element	+	(243)
Rat 24-OHase	coordinate (two DR3s)	Ligand-dependent transcription sequence (AP-2-like) adjacent to proximal DR3	+	(259)
Mouse c-*fos*	composite	CAAT-binding transcription factor binds along with VDR and RXR	+	(179)

ments gene activation from a retinoic acid response element.[166–168] Conversely, the occupation of TR by thyroid hormone in an RXR–TR heterodimer silences the ability of that complex to bind and respond to 9-*cis* RA in terms of transactivation of a TRE-reporter construct.[166] Studies with VDR have variously indicated antagonistic, additive, or synergistic effects of 9-*cis* RA in combination with $1,25(OH)_2D_3$ (Table 3). In an attempt to reconcile these conflicting data, it is important to realize that these studies tend to assess four distinct endpoints: growth and differentiation of intact cells, accumulation of specific mRNAs, gel mobility shift assays of the heterocomplex binding to a VDRE, and transactivation from VDRE–reporter constructs in cotransfected cells. When the current body of data is considered, in toto, only the antagonistic effect of 9-*cis* RA on $1,25(OH)_2D_3$ action is demonstrable at all four of the above levels (Table 3). Rat osteocalcin control is a prominent example in which 9-*cis* RA attenuates the ability of $1,25(OH)_2D_3$ to elicit mRNA accumulation, heterodimeric VDRE binding, and transcription from a VDRE–reporter construct. Antagonism by 9-*cis* RA in the rat osteocalcin system may involve a simple RXR diversionary mechanism under conditions of high local levels of 9-*cis* RA and limiting RXR concentrations (see Fig. 4 below).

Positive effects of 9-*cis* RA on the action of $1,25(OH)_2D_3$ appear to be a more complex, though seemingly widespread phenomenon (Table 3), perhaps occurring at multiple steps in cell and gene regulation. For example, as listed in Table 3, the combination of 9-*cis* RA and $1,25(OH)_2D_3$ exerts additive growth inhibitory effects in select whole cell systems such as human pancreatic carcinoma (Capan) cells[169] and colon carcinoma (Caco-2) cells.[170] Synergistic actions of $1,25(OH)_2D_3$ and 9-*cis* RA have been noted mainly in cell growth and differentiation systems, in which a marked antiproliferative effect occurs in the presence of both ligands (Table 3). In addition, 24-OHase mRNA is synergistically enhanced in its accumulation when both ligands are present in two different cell systems.[170,171] However, in no case has 9-*cis* RA been shown to increase (directly) $1,25(OH)_2D_3$-mediated binding of VDR–RXR heterodimers to VDREs (Table 3). Furthermore, there is no definitive proof in many of these systems that the positive effect of 9-*cis* RA involves exclusively RXR–VDR heterodimeric action on VDREs. For instance, 9-*cis* RA binds to RAR as well as RXR, raising the possibility that actions of this pan-ligand for retinoid receptors could transpire via the RXR–RAR heterodimer, although two of the relevant investigations[172,173] demonstrating synergism utilized RXR-specific (rexinoid) ligands, obviating any binding to RAR.

Synergistic activation of transcription linked to selected natural and synthetic VDREs also has been reported when transfected cells are exposed to both 9-*cis* RA and $1,25-(OH)_2D_3$ (Table 3), including results with the osteopontin VDRE[174] that conflict with those of Lemon and Freedman[175] who reported antagonism (Table 3). Caveats to these VDRE experiments are that the transient transfection studies often involved synthetic VDREs lacking natural flanking regions, overexpression of both partner receptors well beyond physiologic concentrations, or the use of heterologous cell systems. In genes whose $1,25(OH)_2D_3$-induced expression is truly amplified by 9-*cis* RA and RXR-specific ligands, conceivable mechanisms could involve unique VDRE or flanking *cis*-element effects on the conformation of the RXR partner in the heterodimer such that it is receptive to the binding of rexinoids as agonists (see Fig. 4 and the next section).

The dual 24-OHase VDREs (Table 2) are again of particular interest in that, in contrast to the single rat osteocalcin VDRE, this VDRE pair confers synergistic activation by 9-*cis* RA and $1,25(OH)_2D_3$, both from a reporter construct and in the setting of the endogenous gene (Table 3). Other results[176] indicate that 9-*cis* RA alone can positively influence 24-OHase gene transcription, rendering the RXR ligand a regulator of vitamin D metabolite catabolism. Therefore, 9-*cis* RA could achieve an effective biological suppression of bone remodeling by blunting the positive action of $1,25(OH)_2D_3$ on osteocalcin transcription and by independently inducing the 24-OHase enzyme that in turn initiates the destruction of the $1,25(OH)_2D_3$ hormone (Table 3). Future research will be needed to clarify the mechanisms by which 9-*cis* RA influences $1,25(OH)_2D_3$–VDR signaling, and also to explain the largely unknown effects of 9-*cis* RA on VDR–RXR heterodimers that bind to negative VDREs.

Table 5. Candidate VDR-Interacting-Proteins (VIPs)

VIP	*Ligand dependency*	*Proposed function of protein or complex*	*Reference(s)*
Calreticulin	−	inhibitor of DNA binding	(260, 261)
ERC-55	−	calcium binding protein	(262)
SMRT	+	corepressor	(263)
RXR	+	active partner in DNA binding and transactivation	(99, 264)
SRC-1 (ACTR, TIF2, GRIP1, ERAP160)*	+	coactivator, histone acetylation	(137, 265, 266)
NCoA-62	−	coactivator	(267)
p65	+	coactivator	(268)
TIF1	+	chromatin remodeling	(269)
TAFII28	+	TBP-associated factor	(270)
TFIIB	±	basal transcription factor	(177, 264, 271, 272)
SUG1	+	receptor degradation	(186, 187)

*Synonyms are listed in parenthesis

Role of DNA sequences flanking or overlapping VDREs in positive VDR signaling

Beyond the various agonist/antagonist effects of the two receptor ligands and sequence variation within the DR3 VDRE, additional regulatory influences could be exerted by other *cis*-elements within the promoter region that bind generic or tissue-specific transmodulators. The simplest mechanism for such *cis*-elements to affect VDR-mediated transcription is to overlap the VDRE, analogous in theory to the bone sialoprotein negative VDRE (discussed above) that possesses an intrinsic TATA box sequence. In fact, such overlap occurs when the *trans*-repressor YY1[177] binds to the rat osteocalcin VDRE region during the early stages of osteoblast differentiation to prevent VDR action (Table 4). More commonly, adjacent *cis*-elements and their associated transfactors appear to positively modulate transcriptional activation by the VDR–RXR heterocomplex.[178] Specifically, $1,25(OH)_2D_3$-stimulated transcription from human and rat osteocalcin promoters is further augmented by the involvement of an upstream AP1, or a downstream GGTTTGG sequence, respectively (Table 4). Also, calbindin-D_{28k}, which is only modestly activated by $1,25(OH)_2D_3$ at the transcriptional level, possesses an adjacent butyrate responsive element (Table 4) that supplements the effect of the hormone. A more complex situation exists in the aforementioned case of 24-OHase transcription, where the two VDREs (Table 2), approximately 100 bp apart in the promoter, plus the cooperation of the proximal VDRE with an adjacent AP-2-like sequence, are all required for full transcriptional activation (Table 4). Another mechanism discovered for *cis-trans* modification of a VDR–RXR bioeffect is the transient expression of the c-*fos* gene.[179] This gene contains a composite VDRE distinct from a simple DR3, on which a bone cell-specific member of the CTF/NF-1 family of CAAT-box binding proteins forms an activating trimeric complex with VDR and RXR (Table 4). The examples discussed above indicate that there is potential for other signal transduction pathways to impinge on VDR function, and such cross-talk could be relevant in terms of the tissue and promoter selectivity of $1,25(OH)_2D_3$ action. As target gene parameters are more carefully dissected, additional examples of such regulatory complexity will likely emerge.

Integration of VDR interacting proteins into a model for transcriptional control by $1,25(OH)_2D_3$

To function as a ligand-dependent transcription factor, VDR must interact with a number of regulatory and signal transducing proteins (Table 5). The binding of VDR to DNA may be negatively controlled through association with calreticulin, a calcium-binding macromolecule capable of sequestering members of the nuclear receptor superfamily by contacting and masking the DNA recognition helix within the zinc finger region.[180] It has been proposed that calreticulin association with VDR might somehow coordinate $1,25(OH)_2D_3$ action with the calcium status of the cell (reviewed in Ref. 180). In addition to calreticulin, another putative calcium binding protein that interacts with VDR is ERC-55 (Table 5), although a functional role for this protein–protein contact has not yet been defined.

Unliganded VDR in the nucleus is likely bound to a corepressor related to the silencing mediator for retinoid and thyroid hormone receptors (SMRT) (Table 5 and Fig. 4).[181] It is proposed that, analogous to closely related receptors such as RAR,[182] the VDR–SMRT/corepressor complex is linked to histone deacetylase(s) that maintains chromatin in a repressed state with respect to transcription. As shown in Fig. 4, unliganded VDR is also envisioned to be weakly heterodimerized with RXR, and this multimer is postulated to be loosely and nonspecifically associated with DNA, perhaps sliding along the nucleic acid phosphate backbone. Upon VDR occupation by $1,25(OH)_2D_3$, the receptor undergoes a dramatic conformational change, resulting in the following events which are illustrated in Fig. 4: dissociation of the corepressor; repositioning of the VDR AF-2 to the stimulatory, closed configuration; stabilization of the active conformation of VDR by phosphorylation;

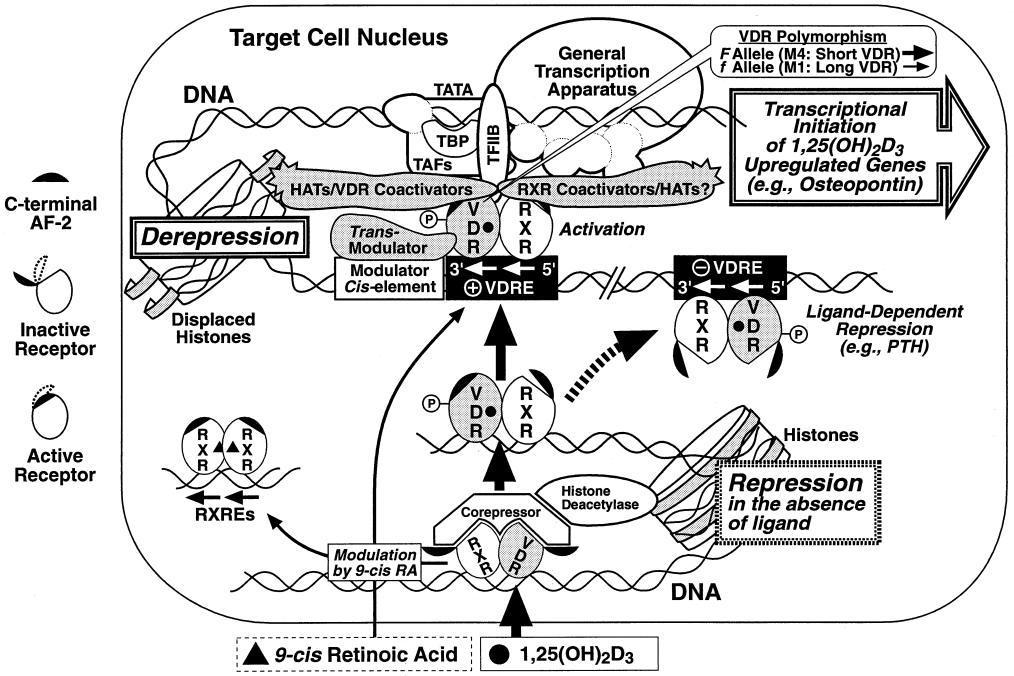

FIG. 4. Working model for transcriptional control by $1,25(OH)_2D_3$. (Bottom) Unliganded VDR binds weakly to RXR and DNA and may also bind to a putative corepressor that promotes silencing of gene expression via recruiting histone deacetylase activity. Histone deacetylase maintains histones such that they promote a repressive nucleosome configuration in chromatin. Heavy solid arrows denote the proposed major pathway in which $1,25(OH)_2D_3$ (●) enters the nucleus, binds VDR, and leads to high affinity association of the active $1,25(OH)_2D_3$–VDR–RXR complex with specific VDREs in the promoter regions of target genes. (Center and top) Liganded hVDR is transformed into an active conformation, with the AF-2 in the closed position (see legend at left), and also becomes phosphorylated on serine-208 in a $1,25(OH)_2D_3$-dependent reaction, which potentiates transactivation by VDR. There is a high affinity interaction of $1,25(OH)_2D_3$-occupied VDR with RXR, which is postulated to influence the structure of RXR by bringing its AF-2 into the active orientation. In this model, positive VDRE binding by the activated heterodimer, with VDR positioned on the 3′ half-element, leads to transcriptional derepression/stimulation of target genes via a combination of the following events: attraction of VDR coactivators that mediate derepression of chromatin nucleosome organization via histone acetyl transferase (HAT) catalyzed displacement of histones to unveil DNA, contact of VDR coactivators with TAF–TBP complex on the TATA box, and direct association of VDR with elements of the basal transcription machinery, such as TFIIB, to stabilize the preinitiation complex. Whether RXR also enlists coactivators that possess HAT activity and/or contact the general transcription apparatus has not been demonstrated, although the AF-2 of RXR in the heteropartner is absolutely required for transcriptional activation.[273] Conversely, as indicated with a heavy broken arrow, binding of the VDR–RXR heterodimer to negative VDREs may place VDR on the 5′ half-element, constituting one mechanism for ligand-dependent repression of target genes by interfering with transcription in an undefined way, perhaps by VDR–RXR existing in a conformation retaining corepressors or incapable of recruiting coactivators. (Top right) Polymorphic (*F* versus *f*) forms of VDR are shown to modify transcriptional activity, in vivo, as a result of differences in VDR potency (within the multimeric complex) between the short (M4) and long (M1) proteins encoded by these alleles. (Lower left) Modulation by 9-*cis* RA of $1,25(OH)_2D_3$-stimulated transactivation apparently occurs by either of two pathways. Antagonism ensues when RXR is diverted away as a liganded homodimer to its own target, RXRE-containing genes, in the presence of high local concentrations of 9-*cis* retinoic acid (▲). Alternatively, binding of 9-*cis* RA to the $1,25(OH)_2D_3$-occupied, DNA-bound heterodimer may be directed by distinct VDREs and/or modulatory *cis*-elements, resulting in an additive or synergistic effect of these two ligands on transcription.

high affinity heterodimerization with RXR (Table 5), with a concomitant allosteric effect to configure the AF-2 of RXR into an active conformation; and specific recognition and tight association with the VDRE. As discussed earlier in this review, DR3 VDREs can be either positive or negative in their influence on gene transcription (Table 2). In the simplest mechanism (Fig. 4), positive VDREs like that in the mouse osteopontin gene bind the heterodimer with VDR situated on the 3′ half-element. Conversely, negative VDREs such as in the avian PTH gene may be determined by a reverse orientation of binding, with VDR residing on the 5′ half-element. However, as discussed above, a more intricate mechanism for establishing positive or negative regulation by $1,25(OH)_2D_3$ could involve specific base pairs in either VDRE half-element dictating various receptor conformations that in turn are manifest as either transactivation or transrepression. As already outlined in Tables 3 and 4, and depicted in Fig. 4, the *cis*-environment of the

VDRE and associated transmodulators may be able to control both the 9-*cis* RA/rexinoid receptivity and magnitude of the $1,25(OH)_2D_3$ response, perhaps also governing promoter and cell selectivity, as well as allowing for cross-talk with other signal transduction pathways.

An exciting development in the action of nuclear receptors has been the recent isolation of coactivators,[182,183] most of which appear to interact with the AF-2 domain. Coactivator interactions with VDR are currently being investigated, and an association has been demonstrated with ligand-dependent coactivators such as SRC-1, as well as with ligand-independent coactivators like NCoA-62 (Table 5 and references therein; note the synonyms for SRC-1). Beyond performing a crucial bridging function to the general transcription apparatus, coactivators are now thought to enzymatically modify histones in the nucleosomes in order to liberate DNA for transcription. This step is catalyzed by histone acetyl transferases (HATs), which possess the newly recognized feature of facilitating transcriptional activation.[184] SRC-1 apparently possesses HAT activity, but supplemental HAT proteins like CBP and P/CAF,[182] as well as TIF1 (Table 5), also may be recruited to a supercomplex (Fig. 4) that both derepresses the promoter chromatin organization of the regulated target gene and links to the general transcription apparatus. In the case of the VDR–RXR heterocomplex, the primary receptor also appears to attract basal transcription factors like $TAF_{II}28$ and TFIIB (Table 5). Therefore, we hypothesize that by combining the bridging effects of coactivators and of VDR, activation is targeted to the appropriate site of transcriptional initiation, with subsequent recruiting of TFIIB to trigger preinitiation complex assembly. In humans, this action of VDR is seemingly more efficient with the M4 *(F)* neomorphic variant than with the longer M1 *(f)* receptor (Fig. 4, see VDR polymorphism section above), implying that VDR alleles could be involved in the determination of BMD.

Although little is known about the termination of the $1,25(OH)_2D_3$–VDR signal beyond 24-OHase catalyzed catabolism of the ligand, interaction of activated VDR with SUG1 (Table 5; not shown in Fig. 4 for simplicity) likely targets the receptor for ubiquitination and subsequent proteolysis by the 26S proteasome complex.[185–187]

The availability of a cell-free transcription system in which VDR–RXR heterodimers activate transcription in a $1,25(OH)_2D_3$-dependent fashion[188] should facilitate studies of transcriptional control by VDR, such as demonstrating direct effects by coactivators, promoter-specific factors, or corepressors. Ultimately, with such cell-free transcription systems it may also be feasible to incorporate elements of chromatin structure such as the aforementioned histones, in order to develop a system that truly mimics VDR action in vivo while at the same time permitting manipulations, in vitro.

Recent mechanistically relevant observations on $1\alpha,25(OH)_2D_3$ analogs

Because of the magnitude of vitamin D analogs that have been synthesized, comprehensive coverage in this review is not feasible, and the reader is therefore referred to excellent treatises on this topic[189,190] for a more detailed discussion. Nevertheless, the model in Fig. 4 suggests numerous facets that may differ between particular target genes and could therefore present opportunities for differential manipulation of bioeffects via $1,25(OH)_2D_3$ and/or 9-*cis* RA analogs. Indeed, some success has already been reported with this type of approach. Certain $1,25(OH)_2D_3$ side-chain analogs, such as 20-epimerized derivatives, raise calcium levels in the intact organism only slightly more than the $1,25(OH)_2D_3$ parent compound while at the same time stimulating transcription of VDRE-reporter constructs 1000-fold more effectively,[191] whereas other analogs, such as 22-oxa[192,193] or 16-ene[194] derivatives, exhibit sharply reduced calcemic activity but retain the ability to inhibit proliferation and induce cell differentiation. One analog in the latter general class, calcipotriol, is already employed in the treatment of psoriasis.[195] The advent of noncalcemic derivatives allows for the desired antiproliferative or other tissue-specific actions of $1,25(OH)_2D_3$ without creating unwanted hypercalcemia as a toxic side-effect. A recent report can serve as an example of what the future may hold. A 24-ethyl derivative of $1\alpha(OH)D_3$, namely $1\alpha(OH)D_5$, was shown to be a potent, nontoxic inhibitor of the proliferation of precancerous mammary lesions, in vivo,[196] suggesting that $1,25(OH)_2D_3$ derivatives may be used in cancer prevention as well as treatment.

The exact mechanistic basis for the unique effects of synthetic VDR ligands has not been elucidated, but there are indications that specific analogs can differentially affect ligand occupancy rates[191,197] or VDR conformational states that may elicit distinct interactions with comodulators.[198] For example, 20-epi analogs stabilize the receptor,[197] presumably by binding deeper than $1,25(OH)_2D_3$ in the ligand pocket, thereby slowing ligand dissociation and creating unique VDR (AF-2) conformational states that are superactive transcriptionally.[198] Heterodimerization between VDR and RXR and resultant DNA binding is also differentially influenced by 20-epi[191] and 16-ene[194] analogs. Finally, utilizing the yeast two-hybrid assay, it has been shown that ligand-enhanced VDR–RXR heterodimerization correlates with transcriptional activation by a series of $1,25(OH)_2D_3$ analogs.[199] The current challenge is to elucidate further the VDR-related events initiated by superactive and bioselective $1,25(OH)_2D_3$ analogs and to correlate these phenomena with pharmacologic profiles such that their molecular mode of action is understood.

SUMMARY AND PROSPECTIVE DEVELOPMENTS

The actions of $1,25(OH)_2D_3$–VDR that have emerged from recent studies include, but clearly transcend, the bone and calcium/phosphate homeostasis effects originally attributed to vitamin D. This new understanding now encompasses many of the tissues formerly reported to contain vitamin D receptors, but for which no function of the vitamin-derived hormone could be ascribed. The insights gained from VDR knockout and allelic variation research

confirm and extend these concepts, raising the prospect that, for the purpose of preventive measures, assessment of VDR polymorphisms may ultimately become part of a strategy to better identify and treat persons at risk for common disorders such as osteoporosis, breast cancer, or prostate cancer. We have learned much about VDR structure/function from natural mutations in 1,25-$(OH)_2D_3$-resistant patients and from site-directed mutagenesis experiments, as well as gained insight from the elucidation of analogous crystal structures for RAR, RXR, and TR. The next few years will no doubt witness a growing structural understanding of VDR, presumably culminating in the crystallographic resolution of physical details of liganded versus unliganded forms of VDR, perhaps in the absence and presence of various protein partners and/or VDRE binding sites. Such structural information should allow for the design of the next generation of synthetic analogs possessing the potential capability of selectively controlling individual $1,25(OH)_2D_3$ responses within the organism.

Fundamental investigations also have led to an enhanced appreciation of the sequence of events in nuclear VDR signal transduction, offering promise that we may be able to understand how diverse $1,25(OH)_2D_3$ agonists could differentially manipulate these steps in a promoter and/or tissue-specific fashion. Such analogs would therefore be endowed with therapeutic potential not only for classic VDR actions in calcium/phosphate homeostasis and bone mineralization, but also for hyperproliferative skin disorders and various types of cancer. A novel type of analog that will be useful in studies of VDR action is an authentic receptor antagonist, and preliminary reports[200,201] indicate that this reagent may be available soon. Another new frontier of VDR function at the molecular level is clearly that of characterizing VDR-interacting proteins, and identifying how they mediate $1,25(OH)_2D_3$ signaling within the context of specific gene promoters. These biochemical and genetic investigations may reveal mechanisms for potential interactions of $1,25(OH)_2D_3$ with signaling pathways for other hormones, growth factors, and cytokines that physiologically modulate the wide array of vitamin D bioeffects.

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