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The Vitamin D Receptor: New Paradigms for the Regulation of Gene Expression by 1,25-Dihydroxyvitamin D_3

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SYNOPSIS

The actions of the vitamin D hormone 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) are mediated by the vitamin D receptor (VDR), a ligand-activated transcription factor that functions to control gene expression. Following ligand activation, the VDR binds directly to specific sequences located near promoters and recruits a variety of coregulatory complexes that perform the additional functions required to modify transcriptional output. Recent advances in transcriptional regulation, which permit the unbiased identification of the regulatory regions of genes, are providing new insight into how genes are regulated. Surprisingly, gene regulation requires the orchestrated efforts of multiple modular enhancers often located many kilobases upstream, downstream or within the transcription units themselves. These studies are transforming our understanding of how $1,25(OH)_2D_3$ regulates gene transcription.

Keywords

Transcription; ChIP-chip analysis; distal enhancers; RNA polymerase II; histone H4 acetylation; VDR gene

INTRODUCTION

Research during the past two decades has established that the diverse biological actions of 1,25-dihydroyxyvitamin D_3 ($1,25(OH)_2D_3$) are initiated through precise changes in gene expression which are mediated by an intracellular vitamin D receptor (VDR) (1). Activation of the VDR through direct interaction with $1,25(OH)_2D_3$ prompts the receptor's rapid binding to regulatory regions of target genes, where it acts to nucleate the formation of large protein complexes whose functional activities are essential for directed changes in transcription (2). In most target cells, these actions trigger the expression of networks of target genes whose functional activities combine to orchestrate specific biological responses. These responses are tissue-specific and range from highly complex actions essential for homeostatic control of mineral metabolism to focal actions that control the growth, differentiation and functional activity of numerous cell types including those of the immune system, skin, the pancreas and bone as well as many other targets that are described in this issue devoted to vitamin D (3). In these tissues, gene targets are numerous. New studies combined with new techniques are now revealing a surprising increase in mechanistic complexity wherein multiple regulatory regions,

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frequently located many kilobases upstream, within or downstream of a target gene's transcription unit, appear to participate in transcriptional modulation (4–7).

VDR STRUCTURE AND FUNCTION

The VDR is structurally organized to mediate changes in transcription in response to 1,25 $(OH)_2D_3$

Despite nearly two decades of extensive biochemical characterization of the VDR following its discovery in 1974 (8,9), it was the cloning of this receptor's gene and the subsequent analysis of recombinant protein that led to key insights into both its structure and its function (10,11). As depicted in Figure 1A, the VDR protein is comprised of three distinct regions, an N-terminal dual zinc finger DNA binding domain, a C-terminal ligand-binding activity domain and an extensive and unstructured region that links the two functional domains of this protein together. The C-terminal region of the molecule, whose three dimensional structure has been solved by X-ray crystallography (12,13), is the most complex and is comprised of 12 α-helices as illustrated in Figure 1B. Amino acid contacts within a subset of these α-helices, as shown in Figure 1C, form a dynamic ligand binding pocket, as shown in Figure 1C. Importantly, selective occupancy by 1,25$(OH)_2D_3$ leads to the formation of two independent protein interaction surfaces on the VDR protein, one that facilitates interaction with a heterodimer partner required for specific DNA binding and one that is essential for the recruitment of large coregulatory complexes required for gene modulation (14). Additional studies suggest that the VDR can also be post-translationally modified through phosphorylation, an alteration in the protein that may be capable of modulating and fine-tuning its transcriptional activity (15–17). Collectively, these domains within the VDR create a macromolecule receptive to physiologically relevant levels of circulating 1,25$(OH)_2D_3$ and capable of directing cellular regulatory machinery to specific subsets of genes whose protein products are key to 1,25$(OH)_2D_3$ response.

The VDR specifies target genes through its DNA binding properties

The zinc-finger containing DNA binding domain of the VDR is typical of that found in all members of the steroid receptor gene family including those for the estrogens, androgens, and the glucocorticoids as well as for thyroid hormone, retinoid acid, and other lipophilic regulators (18,19). The VDR is now known to recognize a specific DNA sequence or vitamin D response element (VDRE) comprised of two hexameric nucleotide half-sites separated by three base pairs (1,20). Other response element structures also occur, although these appear much less frequently (21). The two DNA half-sites accommodate the binding of a heterodimer comprised of a VDR molecule and a retinoid X receptor (RXR) molecule (19). The latter forms a heterodimer with other members of the steroid receptor family as well, including receptors for retinoic acid (RA) and thyroid hormone (T3), thus linking the activities of several different endocrine systems. Recent studies, to be described below, suggest that RXR is independently bound to many sites on the genome in the absence of an activating ligand, thereby "marking" potential regulatory sites for subsequent activation by 1,25$(OH)_2D_3$. 1,25$(OH)_2D_3$ via its receptor also suppresses the transcriptional expression of numerous genes (1,22). The requirements for direct VDR DNA binding and for heterodimer formation with RXR in the suppression of gene activity are currently unclear.

The VDR regulates transcription through its ability to recruit coregulatory complexes

Selective VDR DNA binding in a cell serves to highlight that subset of genes within a genome whose transcriptional activities are targeted under a specific set of conditions for modification by 1,25$OH)_2D_3$. Changes in gene expression are not mediated directly via the VDR, however, but rather indirectly through the protein's ability to facilitate through its transactivation domain the recruitment of large and diverse coregulatory machines that directly mediate such changes (2,23). This recruitment is often gene-specific, suggesting a role for additional and as yet

unidentified components. Coregulatory complexes generally contain one VDR-interacting component as well as many additional subunits, several of which can contain inherent enzymatic activity. These complexes include machines with ATPase-containing nucleosomal remodeling ability, enzymes such as acetyl- and deacetyltransferases and methyl- and demethyltransferases containing selective chromatin histone modifying capabilities, and complexes that play a role in RNA polymerase II (RNA pol II) recruitment and initiation such as Mediator, as documented in Figure 2. Each of these groups of proteins identifies a key step in the process of transcription regulation and many more are likely to be identified in the future. The details of how these machines operate to enhance or suppress the expression of these gene targets are only now beginning to emerge.

VITAMIN D TARGET GENES

1,25(OH)$_2$D$_3$ regulates networks of genes in a tissue/cell-specific fashion

As described above, the role of ligand-activated VDR is to direct cellular transcription machinery to specific sites on the genome where these complexes can influence the production of RNA which encodes proteins that are integral to specific biological activities. It is in this manner that 1,25(OH)$_2$D$_3$ plays a central role in regulating mineral metabolism via its actions in intestinal and kidney epithelial cells and in specific bone cells. Surprisingly, while many target genes which play important roles in calcium and phosphorus homeostatic have been identified, additional targets important to these processes continue to be discovered. These include the calcium and phosphate transporters and their associated basolaterally-located, energy-driven ion pumps in the intestine and kidney (24–26), and the osteoblast-synthesized osteoclastogenic differentiation factor receptor activator of NF-κB ligand (RANKL) (27), which stimulates the activity of existing bone-resorbing osteoclasts, prolongs their lifespan and induces the formation of new replacements (28). Vitamin D also regulates gene networks involved in bile acid metabolism in the colon (29), the degradation of xenobiotic compounds in several tissues (24), the differentiation of keratinocytes in skin (30), the development and cycling of dermal hair follicles (31), and the functions of key cell types involved in both innate and adaptive immunity (32). The genes and gene networks that have been identified as responsible for these biological actions of 1,25(OH)$_2$D$_3$ are extensive. Indeed, many have emerged as a consequence of contemporary genome-wide analyses that are almost routinely conducted by investigators currently and which are capable of measuring the effects of the hormone on entire cellular or tissue transcriptomes. Importantly, many of these gene networks are regulated by the hormone in a tissue-specific fashion. Perhaps most interesting is the intricate regulatory controls exerted directly by 1,25(OH)$_2$D$_3$ and its receptor at genes involved in both the vitamin D ligand's production and degradation, actions that contribute to the maintenance of biologically active levels of intracellular 1,25(OH)$_2$D$_3$. Thus, as outlined in Figure 3, 1,25(OH)$_2$D$_3$ both suppresses the renal expression of *Cyp27b1* (33), whose protein product is responsible for its synthesis, and induces *Cyp24a1* (33,34) whose product is responsible for its degradation to calcitroic acid. In addition to these activities, 1,25(OH)$_2$D$_3$ also autoregulates the expression of its own receptor gene (Figure 3), thus modulating not only levels of the ligand, but of the VDR as well (5,35). Some of the mechanistic details of this regulation will be discussed below. Thus, 1,25(OH)$_2$D$_3$ also contributes directly to the maintenance of the key signaling components essential for generating and mediating hormonal response.

Traditional studies were initiated by identifying target genes and defining regions that mediate regulation by the vitamin D hormone

Identifying the site(s) of action of 1,25(OH)$_2$D$_3$ at a target gene locus represents the first step in defining the molecular processes that are essential for altering a gene's transcriptional output. This step is also important because it often leads to the identification of a region that is likely

to provide important regulatory control following activation through other signaling pathways as well. Early studies of the osteocalcin gene and its regulation by $1,25(OH)_2D_3$ in bone cells provide an excellent example of this principle. Based upon the ability of $1,25(OH)_2D_3$ to induce osteocalcin in bone cells, our early molecular studies, using a traditional human osteocalcin promoter-reporter plasmid approach coupled to classic protein-DNA interaction analyses, revealed the first DNA binding site for the VDR (20,36). This site was located approximately 485 base pairs upstream of the human gene's transcriptional start site (TSS) and was comprised of two directly repeated 6 bp sequences separated by three base pairs. Follow on studies confirmed the general location and highly conserved nature of this vitamin D responsive region in the rat (37) and mouse genes (38) as well. Interestingly, the latter was functionally suppressed by $1,25(OH)_2D_3$ as a result of a strategic change in the regulatory element's base structure thereby highlighting an important species-specific difference in vitamin D response. An extensive series of studies conducted over a decade or more following these initial discoveries firmly established that this general region was a direct target for many different transcription factors some of which were activated by either separate or overlapping signal transduction pathways (39). The ability of these proteins to influence response to $1,25(OH)_2D_3$ and for the vitamin D hormone and its receptor to influence their actions was characterized. Perhaps the most important transcription factor to be discovered at the osteocalcin promoter was RUNX2, a regulatory protein now known to be essential to the formation and bone-forming activity of osteoblasts (40,41). During the ensuing years, many genes have been explored for the location of regulatory sites that are capable of mediating $1,25(OH)_2D_3$ action, binding the VDR and its heterodimer partner, and recruiting coregulatory complexes necessary for changes in transcriptional output. These include the genes for osteocalcin, osteopontin, bone sialoprotein, *TRPV6*, *PTH*, *PTHrp*, *Cyp24a1* and *Cyp27b1* as well as many others. In the case of *Cyp24a1*, two sites located within 300 bp of the TSS were identified as significant mediators of the actions of $1,25(OH)_2D_3$ (34).

NEW APPROACHES REVEAL NEW INSIGHTS INTO VITAMIN D_3-MEDIATED GENE REGULATION

Development of new approaches to the study of transcription research

The study of gene regulation over the past several decades has relied heavily upon the analysis of transcriptional activity generated from gene promoter/reporter plasmids transfected into host cells to identify key components of regulatory processes. These analyses, together with biochemical assays that assess direct protein-protein and protein-DNA interactions have provided considerable insight into how genes are regulated. These approaches are inherently biased, however, since they rely on cellular transfection, involve the analysis of short segments of target genes that are not in context with the gene's normal chromatin environment, are often dependent upon co-expression and/or over-expression of DNA binding proteins and/or specific coregulators for measureable activity and, in many cases, utilize concentrations of reactants that are many fold higher than that normally found in cells. These deficiencies as well as many others prompted the development and application of new techniques to assess the molecular details of transcriptional regulation. Perhaps the most important has been the development of chromatin immunoprecipitation (ChIP) analysis, a technique summarized in Figure 4 that permits the detection of regulatory proteins and/or the appearance of covalent activity at specific DNA targets in unmodified cells or tissues (42,43). It has been the analysis of amplified ChIP products using tiled microarrays (ChIP-chip) (44) or massively parallel sequencing techniques (ChIP-seq) (45–47), as also seen in the figure, which has provided the most important new insights yet into how genes are regulated, however. These latter extensions to ChIP analysis impose no restrictions on the regions within the genome that can be evaluated. Thus, they can be used to examine at an equally high level of resolution both a single gene locus of several hundreds of kilobases or an entire genome comprised of several billion bases.

These techniques are currently being used to re-examine the mechanisms whereby 1,25 $(OH)_2D_3$ regulates known targets of vitamin D action, to explore regulated genes for which the underlying mechanisms have yet to be discovered, to identify and assess new gene targets, and to establish overarching principles of VDR gene regulation at the genome-wide level. One principle that appears to be emerging from these studies is that the regulation of most genes, including those that are targets of vitamin D action, is mediated by multiple regulatory regions often located many kilobases from their respective gene's start site. In the sections below, we provide specific examples of how ChIP-chip and ChIP-seq analyses are being used to illuminate the transcriptional actions of $1,25(OH)_2D_3$. As was hinted at above, many of our preconceived notions of how this hormone regulates transcription were only partially correct.

Regulation of *Cyp24a1* expression by $1,25(OH)_2D_3$ - before and after ChIP-chip analysis

As indicated above, $1,25(OH)_2D_3$ regulates the expression of both *Cyp27b1* and *Cyp24a1*. Since the expression of these genes is central to the maintenance of an effective vitamin D endocrine system, the diverging mechanisms whereby $1,25(OH)_2D_3$ suppresses the expression of *Cyp27b1* while inducing the expression of *Cyp24a1* have receive considerable attention. While many details remain to be worked out, it appears that $1,25(OH)_2D_3$ prompts the displacement of a key transcription factor at the *Cyp27b1* proximal promoter that is responsible for basal expression (48). This displacement suppresses the expression of *Cyp27b1*. In the case of *Cyp24a1*, numerous studies have shown the presence of two regulatory elements (VDREs) located approximately 150 and 250 bp upstream of the TSS that mediate the inducing capability of $1,25(OH)_2D_3$ via the VDR and its partner RXR (34,49). Several additional regulatory sites are also present in this proximal region that contribute to the upregulation of *Cyp24a1*, including sites for the transcription factor C/EBPβ and for Ets-1 (50). Interestingly, PTH also regulates *Cyp24a1*, although this action is indirect and mediated via either a modification of $1,25(OH)_2D_3$ response and/or through post translational events (51,52). Recent ChIP studies of $1,25(OH)_2D_3$-induced activation of *Cyp24a1* reveal that the hormone induces rapid binding of both VDR and RXR to the proximal promoter elements and that this binding leads to the recruitment of coregulators such as the p160 family members, the integrators CBP and p300, the Med1 cofactor TRAP220 and RNA polymerase II (RNA pol II) (53). This region undergoes rapid histone H4 acetylation as well, likely the result of the appearance of the p160 family members. Interestingly, the appearance of these factors at the *Cyp24a1* proximal promoter is cyclic within the first 3 hrs, with a periodicity of approximately 45 minutes (54). This periodicity has been observed for other nuclear receptors and its mechanism recently modeled for PPARγ in HEK293 cells (55). These and other studies provide excellent overviews of *Cyp27b1* and *Cyp24a1* regulation by $1,25(OH)_2D_3$.

In recent studies, we used ChIP-chip and ChIP-seq analyses to examine the ability of 1,25 $(OH)_2D_3$ to induce not only VDR and RXR binding to the human *CYP24A1* promoter, but also to stimulate the recruitment of RNA pol II to the gene's TSS and to promote changes in histone H4 acetylation (Meyer, Goetsch, and Pike, submitted). These studies confirmed the above findings of a region located immediately proximal to the *CYP24A1* promoter to which the VDR/RXR heterodimer bound upon induction by $1,25(OH)_2D_3$. The hormone also induced both an increase in H4 acetylation and the recruitment of RNA pol II at this region and, interestingly, at sites within the transcription unit as well. Surprisingly, ChIP-chip analysis also revealed that $1,25(OH)_2D_3$ induced VDR/RXR heterodimer binding to a robust cluster of intergenic sites located 50 to 70 kb downstream of the human *CYP24A1* gene. H4 acetylation and RNA pol II recruitment were increased across these sites in a fashion similar to that identified at the proximal promoter. Importantly, this cluster of $1,25(OH)_2D_3$-regulated enhancers was also conserved, both in position and function, in the mouse *Cyp24a1* gene locus as well. Functional analysis of these regions using large recombineered BAC clones containing the entire mouse and human *CYP24A1* gene loci confirmed the contribution of these

downstream clusters of enhancers. Thus, ChIP-chip analysis has revealed unexpectedly that *CYP24A1*, a quintessential target of $1,25(OH)_2D_3$ action, is regulated by multiple enhancers located not only proximal but also downstream of and distal to the promoter as well. This characteristic of the *CYP24A1* gene is emerging as typical of most highly regulated genes, and highlights as revealed by ChIP-chip analysis an important new feature of gene regulation.

$1,25(OH)_2D_3$ autoregulates the expression of the VDR gene through intronic and upstream enhancers

The VDR is an absolute determinant of the biological activity of $1,25(OH)_2D_3$ (1). Thus, the receptor's expression in cells is a requirement for response, and the receptor's concentration itself a key component of sensitivity to the hormone as well. While little is known of the molecular determinants of basal expression of the VDR in cells, the *VDR* gene is known to be regulated by a variety of hormones including PTH, retinoic acid, and the glucocorticoids (56). Perhaps most interesting is the ability of $1,25(OH)_2D_3$ to increase the level of *VDR* gene expression itself. Despite the discovery of this autoregulatory feature of the *VDR* gene several decades ago (10,35,57), a general lack of a regulatory response to $1,25(OH)_2D_3$ at the promoter for the *VDR* gene left the mechanism unresolved. To elucidate this mechanism, however, we turned to ChIP-chip analysis and explored the entire mouse *Vdr* gene locus for the presence of regions that might mediate the inducing actions of $1,25(OH)_2D_3$. This analysis revealed the presence of several enhancers that bound the VDR and its heterodimer partner RXR that were located in two separate introns approximately 20 and 30 kb downstream of the gene's TSS (5). No activity was observed at the *Vdr* gene's proximal promoter thus confirming the lack of activity observed in earlier studies. At least one of these regions contained a functional VDRE capable of mediating vitamin D hormone action when analyzed independently in host cells. More recent studies have now identified additional sites of regulation as well, at least one of which is located many kilobases upstream of the *Vdr* gene's TSS (Zella, Meyer, Lee and Pike, submitted). Importantly, subsets of these enhancers also mediate the actions of PTH, retinoic acid and the glucocorticoids, through the binding of the transcription factors CREB, RAR and GR, respectively, thus underscoring a previously known characteristic of enhancers, that of modularity. Further examination resulted in the identification of additional transcription factors such a C/EBPβ which likely participate in the basal expression of the VDR in selected cell types. Subsequent BAC clone analysis, as described above, has confirmed the roles of these enhancers in the regulation of *Vdr* gene expression. Current studies are focused upon the use of these large DNA constructs to recapitulate *Vdr* gene expression *in vivo* in transgenic mice.

$1,25(OH)_2D_3$ and PTH regulates the expression of the mouse *Rankl* gene through multiple upstream distal enhancers

Rankl is a TNFα-like factor that is produced by stromal cells and osteoblasts and which regulates the differentiation, activation and survival of osteoclasts, cells responsible for bone resorption (28,58–60). The expression of this factor in osteoblast lineage cells is regulated by the two primary calciotropic hormones $1,25(OH)_2D_3$ and PTH as well as several of the inflammatory cytokines including IL-1, TNFα and IL-6. These actions on *Rankl* expression facilitate the normal bone remodeling function of $1,25(OH)_2D_3$ and PTH, in particular, but also highlight the bone loss that is associated with increased levels of these hormones as well. As with the genes discussed above, early studies aimed at understanding the regulation of *Rankl* gene expression focused upon the proximal promoter and regions immediately upstream. While 1, $25(OH)_2D_3$ was shown to manifest activity at the proximal promoter, this activity was modest and difficult to interpret (61–63). Activity as a consequence of PTH treatment was not be detected. These features of both the mouse and human *RANKL* proximal promoters suggested the possibility that the genes might be regulated through additional unidentified control regions. To explore this possibility, we conducted a ChIP-chip analysis and explored the ability of $1,25(OH)_2D_3$ to induce VDR binding across the mouse *Rankl* gene locus. This

analysis revealed the presence of five regions capable of mediating the regulatory activity of the vitamin D hormone (4). Surprisingly, these regions were located 16, 22, 60, 69 and 75 kilobases upstream of the *Rankl* TSS. The region at 75 kb was shown to contain several VDREs and was particularly active. Studies in parallel by O'Brien and colleagues revealed that a region immediately upstream of the enhancer at −75 kb mediated the actions of PTH through CREB as well (64). This combined enhancer was thus termed the distal control region or DCR. Subsequent studies suggested that the actions of PTH were not limited to the DCR, but were also observed at several of the more proximal enhancers identified for the VDR as well (65). Interestingly, while basal levels of H4 acetylation were noted at many of these enhancers, both $1,25(OH)_2D_3$ and PTH induced a striking increase in this epigenetic activity. The vitamin D hormone also induced an increase in RNA pol II at these sites as well (4,65). These studies suggested that the binding of VDR and CREB to these sites initiated changes in chromatin structure and function, thus supporting the hypothesis that they represent true regulatory enhancers. The central role of the enhancer located at −75/76 prompted O'Brien and colleagues to delete this region in the mouse genome (66). Surprisingly, this deletion resulted in both a significant suppression of the basal expression of *Rankl* in osteoblasts as well as limited responsiveness to exogenous $1,25(OH)_2D_3$ and PTH. In addition, these mice displayed a modest decrease in bone mineral density in adults that was similar to that observed in PTH-null mice. These studies support a distinct biological role for a unique *Rankl* enhancer in both basal and inducible Rankl gene expression and highlight the utility of ChIP-chip analysis in identifying this as well as additional regulatory regions. These results reinforce the emerging concept that many if not most genes are regulated through the actions of multiple enhancers that can be located in often remote regions surrounding a gene's transcription unit. More recent studies have now identified an even more distal region, located 88 kb upstream of the mouse *Rankl* TSS that mediates the actions of the gp130-activating cytokines such as IL-6 through the STAT3 transcription factor (Bishop, Meyer, and Pike, submitted).

GENOME-WIDE STUDIES REVEAL OVERARCHING PRINCIPLES OF GENE REGULATION BY STEROID HORMONES AND BY $1,25(OH)_2D_3$

ChIP-chip analyses on a genome-wide scale have been conducted recently for several steroid hormones and their respective receptors (43,44,46,47,67,68). These studies include an examination of binding sites for the estrogen, androgen, and peroxisome proliferator-activated receptors. These studies have revealed new insight into the sites of action of these transcription factors and are currently establishing not only new gene targets but new principles through which hormones activate genomic targets. In several cases, investigators have identified the impact of transcription factor binding on RNA pol II recruitment and changes in epigenetic marks as well. Genome-wide studies of VDR binding sites in tissues and cells are currently in progress and have yet to be published. However, an extensive analysis of subsets of known $1,25(OH)_2D_3$ target genes has been examined, and these studies together with the above observations on *CYP24a1*, *Vdr*, *Rankl* as well as *Lrp5* (6) indicate several common features. Importantly, these features confirm those reported through the genome-wide studies conducted for other endocrine systems. First, it is now clear that the expression of target genes is commonly regulated by multiple control regions. While many of these regulatory regions are located proximal to promoters, the vast majority are situated many kilobases from their respective promoters both upstream and downstream as well as at intronic and exonic sites within the transcription unit itself. Second, while the binding of the VDR to these regulatory regions is largely, although not exclusively, dependent upon activation by $1,25(OH)_2D_3$, RXR, the VDR's heterodimer partner, can be found frequently at these regulatory sites prior to activation. Thus, as indicated earlier in this chapter, RXR may "mark" certain regulatory sites for subsequent activation by $1,25(OH)_2D_3$. RXR also forms homodimers with itself as well as heterodimers with other members of the steroid receptor family. Accordingly, the presence of

RXR at a specific site could alternatively represent the means for gene activation by other endocrine factors as well. Third, bioinformatic analysis of these regulatory sites of VDR/RXR activity has revealed that they are almost always associated with a recognizable regulatory element (VDREs) to which the heterodimer complex can directly bind. Functional studies of these elements have generally confirmed the validity of these projected binding sites. Fourth, the binding of the VDR/RXR heterodimer to regulatory sites within genes can be demonstrated by ChIP-chip analysis to be associated with subsequent genetic activity and frequently with a change in gene expression. Thus, VDR/RXR binding at enhancers correlates with the recruitment of many of the coregulators described earlier, including acetyltransferases, co-integrators such as CBP, corepressor such as SMRT or NCoR and members of the Mediator complex. The appearance of regulatory complexes at these sites of VDR action are likely responsible for striking increases in histone H4 acetylation or methylation that are observed at these sites and for the increase in RNA pol II that is recruited to these sites and to transcriptional start sites as well. Thus, the binding of the VDR facilitates downstream molecular activities that are integral to changes in the transcriptional output of target genes. Finally, an investigation of the regulation of these same genes by other hormones and signaling pathways demonstrates that these regulatory regions bind other transcription factors as well, thereby supporting the idea that regulatory regions are modular in nature and mediate the activity of multiple signaling inputs at target genes. These and additional features of gene regulation which have emerged as a result of ChIP-chip analyses provide new perspective into the underlying mechanisms through which the expression of target genes is controlled.

SUMMARY

This chapter represents a summary of what is known of the VDR protein and its molecular mechanism of action at target genes. New methodologies now employed, such as ChIP-chip and ChIP-seq as well as novel reporter studies using large BAC clones stably transfected into culture cells or introduced as transgenes in mice are providing new insight into how 1,25 $(OH)_2D_3$-activated VDR modulates the expression of genes at both single gene loci and also at the level of gene networks. Many of these insights are unexpected and suggest that gene regulation is even more complex than previously appreciated. These studies also highlight new technologies and their central role in establishing fundamental biological principles.

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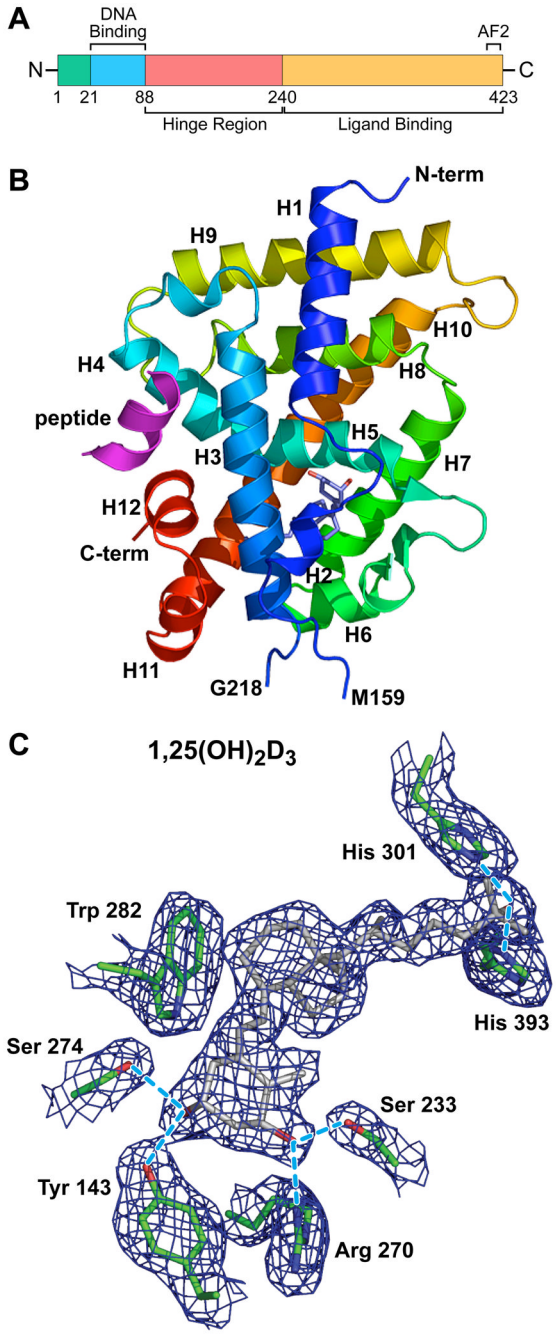

Figure 1.

Structure and key features of the VDR. A. Schematic of the VDR protein comprised of a DNA binding domain, a large ligand binding domain and a hinge region that links the two functional domain of the protein together. N, amino terminal end; C, carboxy terminal end; AF2, activation function 2. Amino acid numbers are shown. B. Crystal structure of the VDR ligand binding domain comprised of 12 α-helices (H1–H12). The N-terminal and C-terminal portions of the molecule are show. A deletion in the molecular from G218 to M159 was required to achieve the formation of crystals. The position of $1,25(OH)_2D_3$ is shown in the ligand binding pocket as a stick figure. The ligand binding domain was crystallized in the presence of a short peptide (indicated) representing a key LxxLL motif located in all coregulatory proteins that

interact directly with the VDR. The repositioning of H12 as a consequence of $1,25(OH)_2D_3$ binding provides the structural change necessary for interaction of the VDR with the LxxLL motif. C. An electron density map of $1,25(OH)_2D_3$ and adjacent amino acids within the VDR protein that make direct contact with the ligand. See Vanhooke et al. (13) for details.

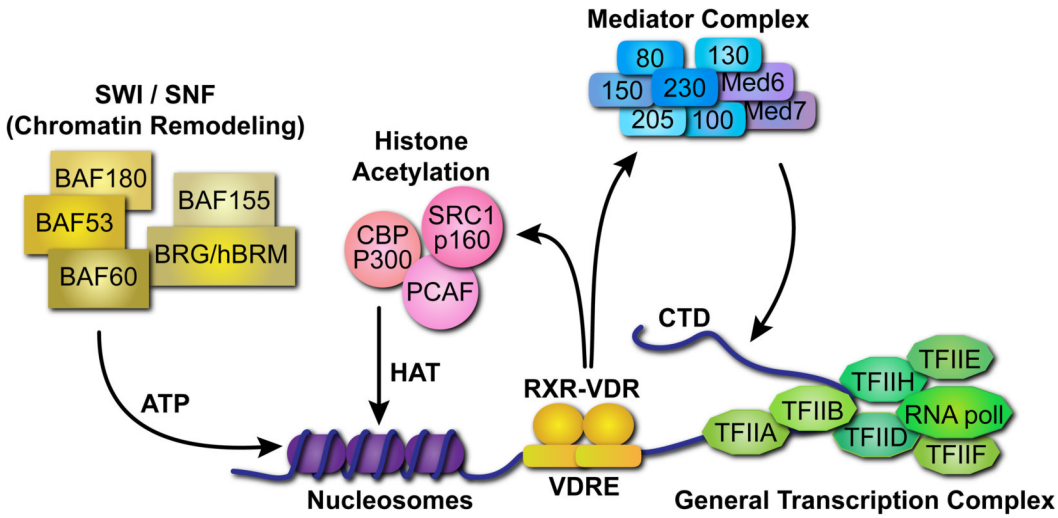

Figure 2.

Schematic model of coregulatory complexes that are involved in mediating the actions of 1,25 $(OH)_2D_3$ and the VDR. The general transcriptional apparatus is shown at the TSS and the VDR/RXR heterodimer is shown bound to its regulatory vitamin D response element or VDRE. Three regulatory complexes are shown that interact with the VDR: an ATPase-containing, chromatin remodeling complex termed SWI/SNF, a histone acetylation complex containing histone acetyltransferases (HAT) and Mediator complex. The latter facilitates the activation of RNA pol II through its C-terminal domain (CTD). Nucleosomes as well as individual proteins that comprise the individual coregulatory complexes are indicated.

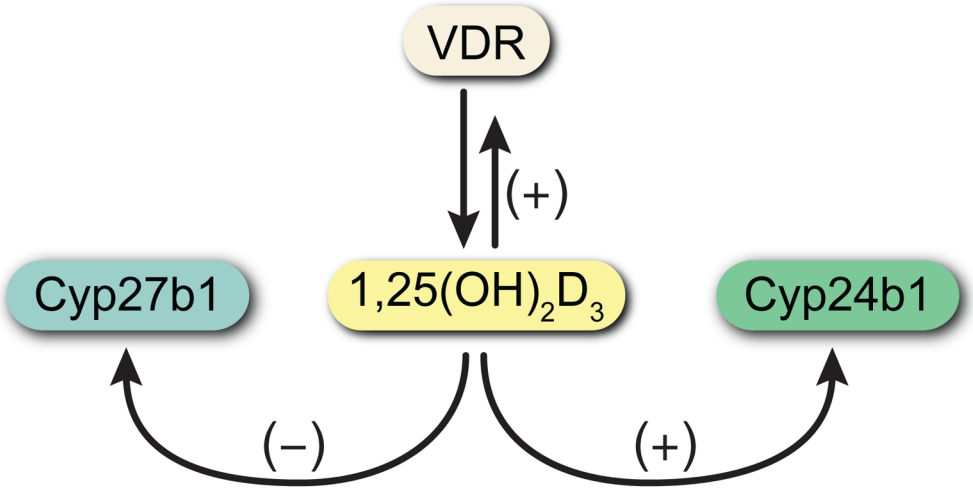

Figure 3.
Regulatory control of the synthesis (*Cyp27b1*), degradation (*Cyp24a1*) and mediation of activity (*Vdr*) of $1,25(OH)_2D_3$ The concentration of $1,25(OH)_2D_3$ in cells is determined through its synthesis and its degradation. Its functional activity is determined by the presence and intracellular concentration of the VDR.

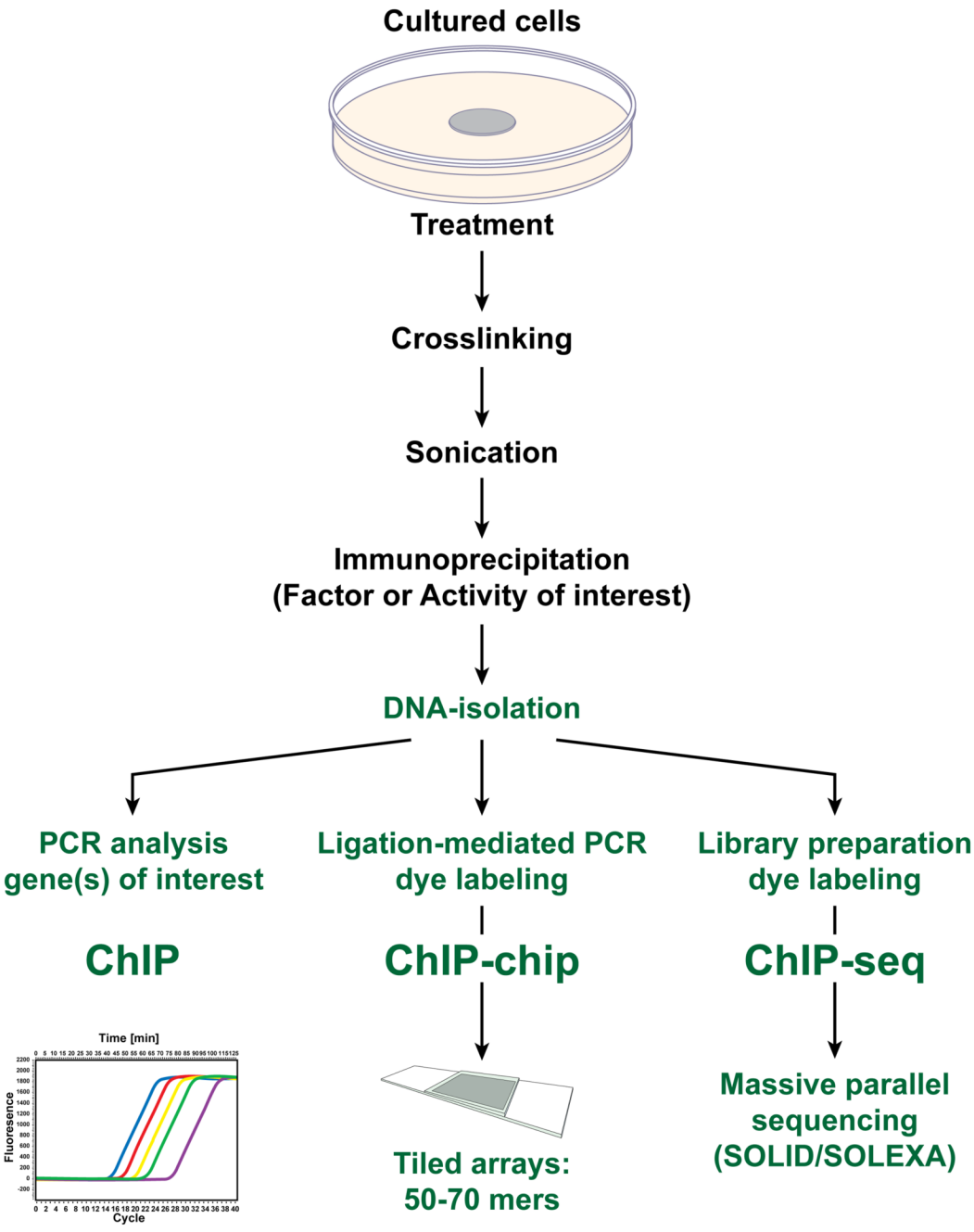

Figure 4.

Methodology associated with chromatin immunoprecipitation (ChIP) analysis and subsequent ChIP-DNA microarray (ChIP-chip) or massive parallel sequencing (ChIP-seq) analyses. Biological samples are cross-linked, sonicated to prepare discrete size chromatin fragments, and then subjected to immunoprecipitation using selected antibodies. The precipitated DNA is then isolated and evaluated by PCR analysis or amplified and then subjected to either ChIP-chip or ChIP-seq analyses.