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PERSPECTIVES ON MECHANISMS OF GENE REGULATION BY 1,25-DIHYDROXYVITAMIN D₃ AND ITS RECEPTOR

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Abstract

1,25-Dihydroxyvitamin D₃ (1,25(OH)₂D₃) functions as a systemic signal in vertebrate organisms to control the expression of genes whose products are vital to the maintenance of calcium and phosphorus homeostasis. This regulatory capability is mediated by the vitamin D receptor (VDR) which localizes at DNA sites adjacent to the promoter regions of target genes and initiates the complex events necessary for transcriptional modulation. Recent investigations using chromatin immunoprecipitation techniques combined with various gene scanning methodologies have revealed new insights into the location, structure and function of these regulatory regions. In the studies reported here, we utilized the above techniques to identify key enhancer regions that mediate the actions of vitamin D on the calcium ion channel gene TRPV6, the catabolic calcium-mobilizing factor gene RankL and the anabolic Wnt signaling pathway co-receptor gene LRP5. We also resolve the mechanism whereby 1,25(OH)₂D₃ autoregulates the expression of its own receptor. The results identify new features of vitamin D-regulated enhancers, including their locations at gene loci, the structure of the VDR binding sites located within, their modular nature and their functional activity. Our studies suggest that vitamin D enhancers regulate the expression of key target genes by facilitating the recruitment of both the basal transcriptional machinery as well as the protein complexes necessary for altered gene expression.

Keywords

ChIP scanning; ChIP-chip scanning; enhancer modules; VDR/RXR DNA binding; distal transcriptional regulation; chromatin looping; histone acetylation; RNA polymerase II recruitment

1. Introductory background

Two sequential hydroxylations of vitamin D₃ in the liver and kidney lead to the formation of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), a hormone whose primary function is to control mineral homeostasis in higher organisms [1]. Like other hormones in this class, the biological effects of 1,25(OH)₂D₃ are achieved through the regulation of gene expression [2]. These activities are mediated by the vitamin D receptor (VDR), a member of the steroid receptor family of genes that control the diverse biological actions of numerous small molecule hormones [3]. The DNA targets of the VDR can be found in a number of induced genes including those for osteocalcin, osteopontin, several p450-containing genes and p21 as well as

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in genes that undergo suppression such as PTH and PTHrp [4,5]. Although considerable diversity exists, the general consensus sequence for VDR DNA binding sites is AGGTCA xxx AGGTCA [4]. Surprisingly, the VDR does not interact with DNA as a homodimer, but rather as a heterodimer with nuclear retinoid X receptor (RXR) [4,6]. The localization of VDR and RXR to target gene binding sites in intact cells in response to 1,25(OH)₂D₃ is both rapid and highly dynamic [7]. Thus, the mechanism of action of 1,25(OH)₂D₃ is typical of that of other steroid hormones that function to target hormone-sensitive genes for changes in transcriptional output.

The modulation of gene expression is not mediated directly by VDR/RXR DNA binding. Rather, it is implemented through the capacity of this heterodimer to recruit a multiplicity of coregulatory protein complexes whose various enzymatic activities are essential to the molecular changes necessary to increase (or decrease) gene expression [8,9]. At least six major protein coregulator complexes are known to participate in the actions of the VDR, each with distinct molecular functions [9]. Association of these complexes with the VDR/RXR heterodimer is highly dynamic [7], and mediated via the activation domains (AF-2) of the two nuclear receptors [10,11] and LXXLL or FXXLF motifs located in key components within the coregulatory complex [12]. Although the consequences of these interactions on gene expression are now emerging, the mechanisms that govern the selectivity and dynamics of multiple coregulator recruitment still remain obscure.

Cell transfection of plasmids containing promoter segments fused to reporter genes has been a cornerstone of transcriptional analysis over the past several decades, and has contributed significantly to our understanding of both basal as well as inducible gene activation. Perhaps most important has been the use of this method to delineate the location of *cis* regulatory elements within a promoter and to explore the inherent properties of those elements in the context of transcription. More recently, however, chromatin immunoprecipitation analysis (ChIP) has emerged as a highly favorable technique for studying key features of gene regulation [7,13–15]. The technique's popularity derives from its capacity to examine protein-DNA interactions under a variety of conditions at the level of a natural chromatin setting in intact cells. Accordingly, it has been used to demonstrate the dynamic properties of transcription factor interaction on DNA and the consequence of that interaction on cofactor recruitment, chromatin structure and entry of RNA polymerase II [7,13–15]. Perhaps as important, the coupling of ChIP analysis to advanced DNA interrogation methods such as PCR scanning [16,17] and DNA microarray scanning [18–24] has enabled a search for and the discovery of novel regulatory control regions. This approach is yielding new information about the structure and function of gene enhancers. In this report, we describe the use of these and other techniques to delineate important control regions of genes that are both known targets as well as new targets of 1,25(OH)₂D₃ action. We explore the properties of these regulatory regions further to delineate potentially new functions of these enhancers.

2. The methodologic approach

As outlined above, we have used ChIP analysis [7] as well as both PCR scanning (ChIP-PCR) [17] and DNA microarray (ChIP-chip) [22–24] analyses to identify control regions of several novel vitamin D target genes. We then characterized the properties and features of these control regions more extensively using both ChIP analysis and more traditional molecular and biochemical techniques.

2.1. Chromatin immunoprecipitation analysis

ChIP analysis was carried out as previously described [7,22]. The overall method is illustrated in Fig. 1. In brief, cultured cells are treated with either vehicle or inducer for specific periods of time, subjected briefly to a crosslinking reagent such as formaldehyde, lysed, and then

sonicated to solubilize defined chromatin fragments ranging from 500 to 2000 base pairs. Samples are taken at this stage to ensure that the amount of input DNA is equivalent. Protein bound chromatin is then immunoprecipitated using specific antibodies capable of recognizing various DNA binding proteins, tethered transcription factors, cofactors, or uniquely modified histones that serve a regulatory role in transcriptional output. Various IgG's are used as negative precipitation controls. Following immunoprecipitation, the co-precipitated chromatin-DNA fragments are isolated and the presence of unique segments of DNA assessed using PCR analysis. PCR analysis can be carried out in either a semi-quantitative fashion or by using qPCR approaches. The abundance of co-precipitated DNA fragments is indicative of the amount of target protein present at the site during the crosslinking treatment [13]. A highlight is the ability of this method to detect the accumulation of a unique factor at a specific region of DNA under *in vivo* conditions. The resolution of this method in mammalian cells, however, is limited to 500 to 1000 bp.

2.2. ChIP-PCR scanning

ChIP-PCR scanning was conducted as previously described [17]. Briefly, however, immunoprecipitated DNA is interrogated directly by using a series of PCR primers capable of assessing the presence of DNA fragments across a defined region of the chromosome, as documented in Fig. 1. The extent of this analysis is determined by intent of the experiment, but if it involves a search for regulatory elements it is often restricted to a region located upstream of the start site of transcription (TSS). In this method, PCR primers are designed to detect the presence and abundance of contiguous 500 to 2000 base pairs DNA fragments which extend over a relatively short expanse of DNA (1–10 kb) [17].

2.3. ChIP-chip scanning

Perhaps most striking is the use of immunoprecipitated DNA to interrogate large expanses of the chromosome using DNA microarrays and ChIP-chip analysis [18,19,21]. Current studies have extended this approach to an evaluation of not only single gene loci (22–24), but to complete chromosomes as well [19,21]. Indeed, full genome wide scans are now being successfully conducted with the aim to locate all start sites of transcription and to identify full sets of binding sites for individual transcription factors as well as for sets of regulatory factors that orchestrate response to specific extracellular signals [25,26]. The resolution of this type of chromosomal interrogation is in the range of 20 to 50 nucleotides supporting the incredible power of this approach. ChIP-chip scanning was conducted as previously described [22]. As seen in Fig. 1, however, ChIP-chip analysis involves linear amplification of selectively immunoprecipitated DNA using ligation-mediated PCR, conjugation to fluorescent dyes and hybridization to custom DNA microarrays. The microarrays are then scanned and fluorescent signal intensity plotted as a function of chromosomal location.

3. Experimental results and discussion

3.1. Scanning target genes using ChIP-chip analysis

The molecular mechanism whereby $1,25(\text{OH})_2\text{D}_3$ modulates the expression of many target genes is unknown, largely because the regions that control transcriptional output within these target genes have not been identified. We therefore applied ChIP-chip technology as an initial approach to identify such regions in several genes, most notably the VDR gene itself [22], the intestinal calcium ion channel gene TRPV6 [17] and the TNF-like factor RankL that promotes the formation of calcium resorbing osteoclasts [23]. The well known target genes Cyp24a1 and Opn were used as controls [7]. Fig. 2 depicts the results we obtained when DNA's derived from cells treated with either vehicle or $1,25(\text{OH})_2\text{D}_3$ and precipitated with VDR antibody were used to screen the mouse RankL gene locus from over 200 kilobases upstream of the TSS to over 100 kilobases downstream of the final exon at 50 bp resolution. Further details of

additional precipitations that were used as controls for these and other studies can be obtained in recently published work [17,22–24]. As can be seen, the VDR binds to five regions of the RankL gene, all located at significant distances upstream of the RankL TSS at -16 kb (D1), -22 kb (D2), -60 kb (D3), -69 kb (D4) and -76 kb (D5). When evaluated further by direct ChIP analysis, we confirmed these findings and demonstrated that the VDR and its RXR partner could be shown to localize to each of these regions in a time- and dose-dependent manner but not at intervening sites located between the demonstrated control regions. Additional studies using cloning and transfection analysis revealed the presence of a unique and highly active VDRE in the D5 region and regulatory sites that mediate the actions of the parathyroid hormone and osteoclastogenic cytokines as well [23,27]. Based upon these findings, we have termed this region the RankL distal control region or RL-DCR.

Similar studies were also used to identify and confirm vitamin D regulatory regions located within the VDR gene [22] and the TRPV6 gene [17], and to discover that the Wnt signaling co-receptor LRP5 [28] was also a gene target for vitamin D [24]. RXR was a binding participant at all the identified sites within these genes. Interestingly, while the TRPV6 gene contained multiple upstream regulatory elements much like that of the RankL gene, all of these sites were located proximal to the TSS and within the first 5 kilobases [17]. Regulatory regions in the VDR and LRP5 genes, in contrast, were located at significant distances downstream of the TSS and positioned within large introns [22,24]. Fig. 3 depicts the overall location of regulatory regions within each of these as well as previously known vitamin D target genes. It is clear from these studies that the VDR/RXR heterodimer can modulate the expression of vitamin D target genes through regulatory regions or enhancers positioned at diverse locations within gene loci, some at apparent distances of up to 76 kb.

3.2. VDRE structure and sequence

The sequences of functional VDREs located within the regulatory regions identified in the RankL, TRPV6, VDR and LRP5 genes, together with a subset of VDREs identified previously in genes such as osteocalcin, Cyp24a1 and Opn, are documented in Table 1. The newly discovered regulatory elements confirm the importance of the overall VDRE motif comprised of two directly repeated hexanucleotide half-sites separated by three base pairs [3,4], which was documented originally in the human osteocalcin gene in 1990 [29]. They also provide new insight into the overall core motif by revealing that although certain bases within the motif are highly conserved, others demonstrate a rather high degree of degeneracy. Perhaps most interesting is the overall nature of the VDREs that have been identified. While previously identified VDREs are seen to occur in combination, the RL-DCR region of the RankL gene contains a composite VDRE that is comprised of two VDREs separated by a single base pair [23]. Not surprisingly, this VDRE is highly active. Multiple elements (at least 5) are located within the proximal promoter region (-5 kb) of the TRPV6 gene [17]. These elements conform in general to the typical VDRE motif. Therefore, the question arises here as to why the TRPV6 gene requires this number of vitamin D regulatory elements? The VDREs located in the VDR [22] and LRP5 [24] genes conform similarly to that of a typical VDRE. Perhaps most intriguing are the VDRE sequences found in these genes that do not represent functional binding sites for the VDR/RXR heterodimer. It seems likely that the chromatin structure surrounding these sites prevents their utilization, although this speculation will require experimental evidence. Finally, the most striking feature of many of these VDREs and the regulatory regions in which they are found is their distance upstream or downstream of start sites of transcription. It is almost certain that these linear distances are illusionary and that chromatin looping and chromatin structure play a critical role in juxtaposing these regulatory enhancers next to the promoters of the gene's they regulate [21].

3.3. Regional and VDRE conservation

The regulatory regions identified within the TRPV6, RankL, VDR and LRP5 gene loci are all highly conserved in the genomes of other mammalian species [17,22–24]. The bulk of the identified VDREs in these genes are also highly conserved, particularly those located in the TRPV6, RankL and VDR genes. In the human RankL gene, for example, the overall structure of the two VDREs is identical, and only three base pairs differ from that in the mouse [23]. Importantly, this sequence conservation extends to that of function; comparable regions of the human RankL and VDR genes were cloned and evaluated, and demonstrate a similar capacity to mediate induction by $1,25(\text{OH})_2\text{D}_3$. Interestingly, the sole exception to this sequence conservation lies in the LRP5 gene, wherein the VDRE we have identified in the mouse LRP5 gene is not conserved in the human genome due to a three base pair substitution [24]. Whether the LRP5 gene is regulated by vitamin D through other enhancer regions has not been explored. Regardless, both sequence and functional conservation across species enhances our general hypothesis that the regulatory regions we have identified in the above genes by ChIP PCR scanning and ChIP-chip scanning represent authentic regulatory enhancers for their respective genes.

3.4. The modular nature of regulatory enhancers

Enhancer regions facilitate changes in gene expression through their capacity to integrate the signal-modified activities of multiple regulatory factors. Thus, we hypothesized that if vitamin D regulatory regions could be identified in the genes described above, these regions might mediate the actions of not only $1,25(\text{OH})_2\text{D}_3$ but other hormones and control factors as well. Indeed, this hypothesis has proven to be correct using the RankL gene as an example. Thus, extensive studies using ChIP analysis as well as a cloned DNA fragment approach have revealed that in addition to $1,25(\text{OH})_2\text{D}_3$, both the peptide hormone PTH ([27] and data not shown) and the glucocorticoids (GC) [23] are able to upregulate the expression of RankL via the RL-DCR (the D5 region). These actions are mediated in *trans* through the transcription factor CREB which localizes to the RL-DCR in response to protein kinase A activation by PTH and through the GC receptor (GR) that can be detected at the RL-DCR in response to the synthetic glucocorticoid dexamethasone ([27] and data not shown). Similar studies which focused upon the regulatory regions of the TRPV6 and VDR genes revealed that the vitamin D enhancers were similarly modular in nature. These findings support the concept that enhancers are capable of integrating the actions of multiple transcription modifying inputs at the level of a target gene.

3.5. VDR/RXR binding to regulatory enhancers stimulates covalent changes in histone structure

DNA binding proteins do not act independently to modulate transcriptional output. Their primary function, rather, is to facilitate the recruitment of multi-protein complexes that are in turn capable of altering chromatin architecture through their capacity to promote unique histone modifications [8,9]. These epigenetic changes in chromatin structure include both acetylation and methylation, each of which is able to change the level of chromatin condensation [30]. We therefore examined whether the binding of the VDR and its RXR partner to each of the above described genes produced a change in the level of acetylation at histone 4. The results suggest an interestingly impact of $1,25(\text{OH})_2\text{D}_3$ and its receptor on histone modification at the Cyp24a1 promoter [7] and in the TRPV6 [17], RankL [23], LRP5 [24] and VDR [22] genes. Thus, an increase in this epigenetic modification in response to $1,25(\text{OH})_2\text{D}_3$ was observed at many of the regulatory regions within these genes. In several cases, however, histone modifications were observed but did not correlate directly with the sites of VDR/RXR binding [23]. These data suggest that while dependent upon transactivator binding, changes in chromatin

modification may be influenced to a large degree by the three dimensional nature of the chromatin within a specific gene locus and through spreading.

3.6. Enhancers function as recruitment centers for RNA polymerase II and the basal transcriptional apparatus

Upregulation of gene expression is accompanied by an increase in the level of basal transcription factors at the start site of transcription and an increase in the recruitment of RNA polymerase II (RNA pol II) to this site as well. We therefore tested that hypothesis by assessing the appearance of RNA pol II at the TSS of the RankL [23], LRP5 [24] and VDR [22] genes following $1,25(\text{OH})_2\text{D}_3$ treatment using ChIP analysis. As can be seen in Fig. 4 using the RankL gene as an example, RNA pol II is indeed recruited to the TSS in response to $1,25(\text{OH})_2\text{D}_3$ [23]. RNA recruitment to the other genes we investigated was observed as well. Interestingly, RNA pol II is also recruited to the distal enhancer regions of the RankL gene [23] and to the regulatory regions of the VDR [22], TRPV6 [17] and LRP5 [24] genes as well. This finding supports the emerging idea that in addition to their role in promoting chromatin modifications, enhancers may also function as recruitment centers for general transcriptional machinery [31]. If correct, these results suggest the need for an additional mechanism whereby the newly recruited raw materials for enhanced transcription can be transferred to the authentic gene promoter. They also highlight the novel role of chromatin structure and restructuring in the control of gene expression.

4. Concluding remarks and future perspectives

The use of ChIP scanning methods, direct ChIP analysis and transcriptional evaluations has revealed new insights into how $1,25(\text{OH})_2\text{D}_3$ acts to regulate the transcriptional output of genes such as Cyp24, OPN, TRPV6, RankL, VDR and LRP5. Accordingly, these studies have revealed much about the nature of vitamin D regulatory elements, their proximity to binding sites for other transcription factors, their conservation within genomes from other species and their unusual and often distant locations either upstream or downstream of transcriptional start sites. It is our belief that despite the complexity, the results described here strongly support a role for the enhancers we have identified in the regulation of their respective genes by $1,25(\text{OH})_2\text{D}_3$. An unexpected feature of the regulatory enhancers we have discovered is their unusual and often distant locations relative to the gene's TSS. Our studies coupled to those of other investigators suggest that distant properties of these enhancers may be more the norm than the exception. As documented in Fig. 5, the number and locations of these regulatory regions strongly supports a chromatin looping mechanism whereby these regulatory regions can be brought into close proximity with the gene's natural promoter [32]. Finally, we have also discovered several new properties of vitamin D-regulated enhancers. Perhaps most important is the observation that enhancers may function not only to modify chromatin structure, but to act as recruitment centers for the machinery necessary to alter a gene's transcriptional output. Future studies will be essential in determining whether this working hypothesis is correct.

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Fig. 1. ChIP analysis and the techniques of ChIP-PCR scanning and ChIP-DNA microarray (ChIP-chip) scanning.

Fig. 2.

ChIP analysis of the mouse RankL gene locus. A, Arrangement of the mouse RankL gene locus and position of flanking genes. The RankL gene is transcribed on the reverse strand (right to left). B, ChIP-chip scan of the RankL locus. ST2 cells were treated for 6 hr with either vehicle or 1,25(OH)₂D₃ (10⁻⁷ M) and subjected to ChIP. The precipitated DNA was subjected to chip using tiled oligonucleotide arrays containing 50-mers spanning the RankL gene locus (see text). The array data includes only a comparison of IgG (+/- 1,25(OH)₂D₃) and VDR (+/- 1,25(OH)₂D₃) over the RANKL upstream regions. See reference 23 for further detail.

Fig. 3. Organization of enhancer modules in vitamin D regulated genes. The locations of regulatory enhancers are identified within vitamin D target genes. Two independent distance scales are noted.

Fig. 4.

Recruitment of RNA pol II to mouse RankL enhancers. ST2 cells were treated with vehicle or the indicated hormones for 6 hrs and then subjected to ChIP analysis using antibodies to VDR, RNA pol II or control IgG. Precipitated DNA was evaluated for RankL gene enrichment. The primer code is identified at the top of the Figure.

Fig. 5. Model for potential chromatin looping at the mouse RankL gene. Chromatin looping juxtaposes the distant regulatory enhancers for the RankL gene at the proximal promoter.

TABLE 1

VDRE sequences in vitamin D target genes

Name	Species	Position	Sequence
osteocalcin	Human	–500	GGGTGA acg GGGGCA
osteopontin	Mouse	–750	GGTTCA cga GGTTCa
		–2000	GGGTCA tat GGTTCa
Cyp24a1	Mouse	–165	AGGTGA gtc AGGGCG
		–265	GGTTCA gcg GGTGCG
VDR	Mouse	(S1)	GGGTTA gag AGGACA
		(S2)	GGGTCT tcc AGTGCA
		(S3)	TCTTCA atg AGATCA
TRPV6	Human	(–1.2)	AGGTCA ttt AGTTCA
		(–2.1A)	AGGTCT tgg GGTTCa
		(–2.1B)	GGGTCA gtg GGTTCG
		(–3.5)	GGGGCA gag AGGTCA
		(–4.3A)	GGGGTA gtg AGGTCA
		(–4.3B)	CAGTCA ctc GGTTCa
		(–5.5)	AGGTCA aca GGTCTA
RankL	Mouse	(D5)	GAGTCA ccg AGTTGA
			GGTTGC ctg AGTTCA
LRP5	Mouse	(P2-B)	GGGTCA tgc AGGTTC