IBMS BoneKEy. 2009 May;6(5):169-180
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doi: 10.1138/20090376

PERSPECTIVES

New Techniques in Transcription Research Extend Our Understanding of the Molecular Actions of the Vitamin D Hormone

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Abstract

Interest in vitamin D and its hormonal derivative 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) has increased in the past few years as a result of the hormone's general actions at the skeleton, but perhaps more so because of its potential ability to regulate cell growth and differentiation. These actions suggest a possible therapeutic role for the vitamin D hormone in a wide variety of diseases of growth control, immune function, and in cancer. The biological actions of 1,25$(OH)_2D_3$ are mediated by the vitamin D receptor (VDR), which functions as a transcription factor at multiple target genes. Chromatin immunoprecipitation (ChIP) coupled to either tiled microarray analysis (ChIP-chip) or massive parallel sequencing (ChIP-seq) promises to revolutionize the field of transcriptional regulation. We discuss these techniques and their utility in the context of several vitamin D target genes including the *Vdr*, *Cyp24a1* and the osteoclastogenic cytokine receptor activator of NF-κB ligand (*Rankl*). We find that 1,25$(OH)_2D_3$ and its receptor modulate the expression of these and other genes through multiple enhancers that are frequently located at remote sites many kilobases from their genes' transcriptional start sites. *IBMS BoneKEy*. 2009 May;6(5):169-180.
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Introduction

General interest in vitamin D has surged during the past few years due primarily to the protection the vitamin's hormonal derivative provides to the skeleton (1) but also because of its potential ability to reduce infection (2;3) and to prevent autoimmune diseases (3) and cancer (4). Although a role is highly speculative at present, it has been suggested that vitamin D might even prevent Alzheimer's and Parkinson's diseases (5), autism and influenza (6). Much of the interest in vitamin D is rooted in solid discoveries that have emerged over the last several decades that provide strong evidence that vitamin D regulates fundamental cellular processes such as proliferation, differentiation and survival. The overall impact of these activities is particularly important since it was found recently that much of the U.S. population, as well as populations in other parts of the world, are deficient in circulating levels of vitamin D_3 and its downstream metabolite 25-hydroxyvitamin D_3 (7;8). Indeed, it appears as though FDA recommendations for daily supplementation may be woefully inadequate to maintain effective levels of the vitamin in both children and adults (8). A recent review by Bouillon and colleagues provides an excellent summary of the scientific support for the diverse roles of vitamin D in mammals (9); thus, we will not considered these activities in detail here. As noted in the review, however, the biologic actions of vitamin D are well highlighted through the phenotypes that have emerged both in humans with defective vitamin D receptors (VDRs) (as observed in vitamin D-resistant rickets) (10) and in mice with engineered genetic deletions in this gene as well (11). These phenotypes are particularly compelling since they emerge as a result of the absence of vitamin D's fundamental transcriptional mediator. General features of the mechanisms through which vitamin D regulates mineral homeostasis, impacts the musculoskeletal, cardiovascular, immune, reproductive and central and peripheral nervous systems, and modulates skin function and energy metabolism are known (12). Critical details remain to be elucidated, however, and as they emerge are likely by

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all indications to reveal much greater complexity than originally envisioned. Despite this current absence of molecular detail, the biological activities of vitamin D provide numerous opportunities to develop useful therapeutics for a wide variety of human disease indications. These opportunities currently drive the synthesis and exploration of a range of novel vitamin D compounds and other structural mimetics potentially capable of modulating the vitamin signaling pathway (13). Most therapeutic opportunities have yet to be realized, however, largely because vitamin D's central role in regulating systemic calcium levels can also lead to hypercalciuria and hypercalcemia.

Mechanisms of Action of $1,25(OH)_2D_3$ and Its Receptor

Biological Processes and Networks

The common mechanism whereby 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), the hormonal derivative of vitamin D_3, regulates biological systems outlined above involves the coordinated regulation of both genes and gene networks (Fig. 1). In the case of mineral homeostasis, for example, $1,25(OH)_2D_3$ coordinates the expression of a number of genes whose products are integral to the uptake of calcium and phosphorus from the diet, the recovery and conservation of calcium and phosphorus though the kidney, and bone remodeling and mineralization at the skeleton (9;14;15). These target genes include *RANKL*, *TRPV5*, *NCX-1*, *TRPV6*, *S100g*, *PMCA1b*, and certainly many others as well. $1,25(OH)_2D_3$ also regulates the activity of various immune cells such as macrophages, dendritic cells, B cells, and a series of T cell subsets that comprise both helper cells and other regulatory subtypes. At these cell types, $1,25(OH)_2D_3$ is known to regulate the expression of a wide variety of growth factors, cytokines, and chemokines that play direct roles in immune cell regulation including IL-2, IL-4, IFNγ, GM-CSF and many others (16). In the skin, $1,25(OH)_2D_3$ controls gene networks involved in the differentiation of keratinocytes and genes that are involved in the control of the hair cycle (17). In the latter case, an interesting interaction is highlighted wherein the VDR regulates the activity of *Hr* (Hairless), a key transcription factor involved in hair follicle development and cycling (18). $1,25(OH)_2D_3$ also regulates the general proliferation and differentiation of numerous different cell types; indeed, both p21 and p27 have been shown to represent key targets of vitamin D hormone action to promote cell cycle arrest (19). These represent but a few examples of the biological processes and the gene networks that are under $1,25(OH)_2D_3$'s control. It is also important to note that a detailed molecular examination of many of the genes described above has been conducted and a fundamental understanding of where and how the VDR operates to regulate these genes has emerged. As will be seen, however, we believe that these findings are likely to represent only the basic rudiments of a molecular mechanism through which $1,25(OH)_2D_3$ controls transcription.

New Approaches to the Study of Gene Regulation

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. The analyses are tedious, generally restricted to the examination of single genes and often dependent upon co-expression and/or over-expression of DNA binding proteins and/or specific coregulators for interpretation. These approaches are also inherently biased as well, since analyses are limited to short segments of DNA, usually promoters and several kilobases of proximal upstream sequence, and generally out of context relative to the gene's normal chromosomal environment. Thus, it is of importance that recent approaches to the study of gene regulation have emerged that promise to solve many of these problems. These techniques involve the application of chromatin immunoprecipitation (ChIP) coupled to DNA

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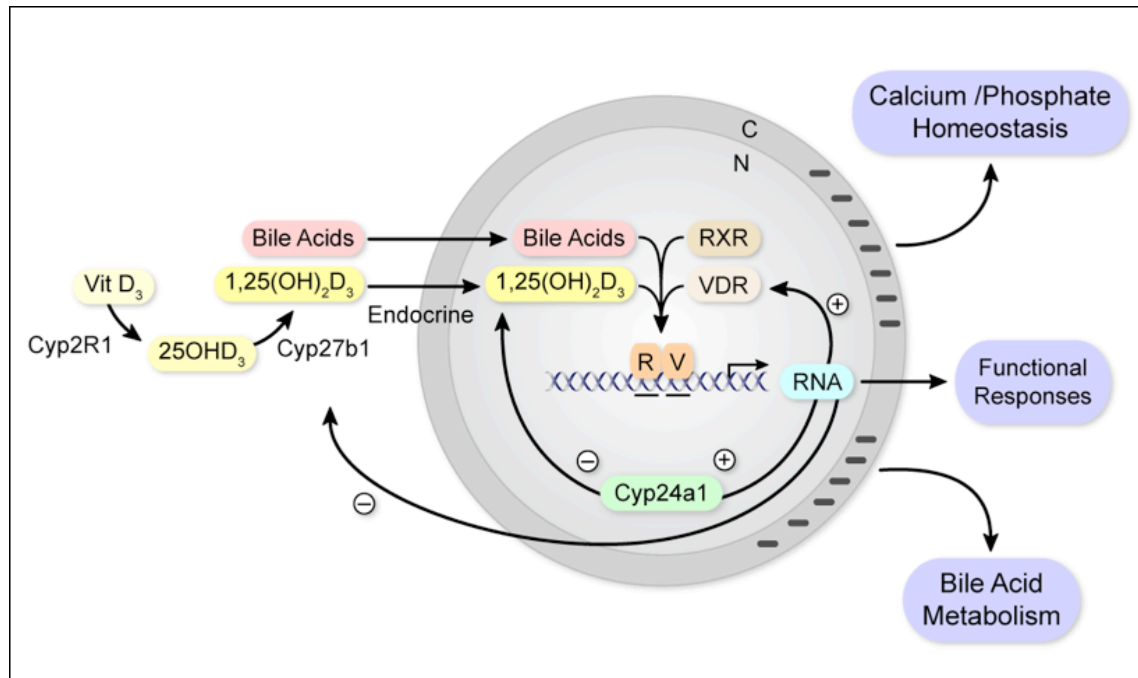

Fig. 1. Mechanism of action of hormonal $1,25(OH)_2D_3$. Ligand-activated VDR forms a heterodimer with RXR and binds directly to regulatory regions in the target gene locus. *Vdr, Cyp24a1* and *Cyp27b1* are also direct targets of $1,25(OH)_2D_3$. Changes in the expression of other target genes result in biological modification. C, cytoplasm; N, nucleus; R, RXR: V, VDR.

microarray analysis (ChIP-chip) or parallel DNA sequencing (ChIP-seq) (20). For each of these techniques (Fig. 2), regulatory proteins are first chemically fixed *in situ* at their endogenous sites of action on the chromosome, the chromatin then sonicated into fragments, and the protein-chromatin complexes precipitated using antibodies directed towards the factor of interest. Samples are then examined directly by PCR analysis or further developed for ChIP-chip analysis. For this technique, the DNA fragments are isolated, the segments amplified by ligation-mediated PCR, fluorescently labeled and the samples then hybridized directly to DNA microarrays containing synthetic oligonucleotides tiled with high resolution across a genomic region(s) of interest. This approach leads to the direct identification of sites of transcription factor interaction (20). Importantly, ChIP-chip analysis can also be used to evaluate the consequences of regulatory action on cofactor recruitment, histone modification, and covalent modifications made directly to the DNA. The abundance of the ChIP-derived DNA fragments can also be determined directly using massive parallel sequencing techniques (ChIP-seq) that generate 25-35 bp nucleotide reads whose identities can be mapped directly to the genome under investigation (21). While both ChIP-chip and ChIP-seq techniques are being applied to the study of specific gene loci, perhaps most exciting is their application to the study of complex gene networks, complete chromosomes and the entire genomes of organisms. Thus, these approaches reveal both transcription factor binding regions and expression signatures for both single genes as well as entire annotated genomes. They are also capable of identifying novel features of genomes, including epigenetic marks that define gene boundaries, identify regulatory regions of genes and highlight locations of functional promoters. Many unexpected insights into the fundamental nature of the genome are emerging as well. Thus, these approaches provide detailed snapshots of specific gene regulation but also identify overarching principles that govern the regulation of genes on a genome-wide scale. We have used these approaches at each level, as documented below, to explore the actions of $1,25(OH)_2D_3$ in osteoblasts and other cell types. We highlight here only a few of these initial observations.

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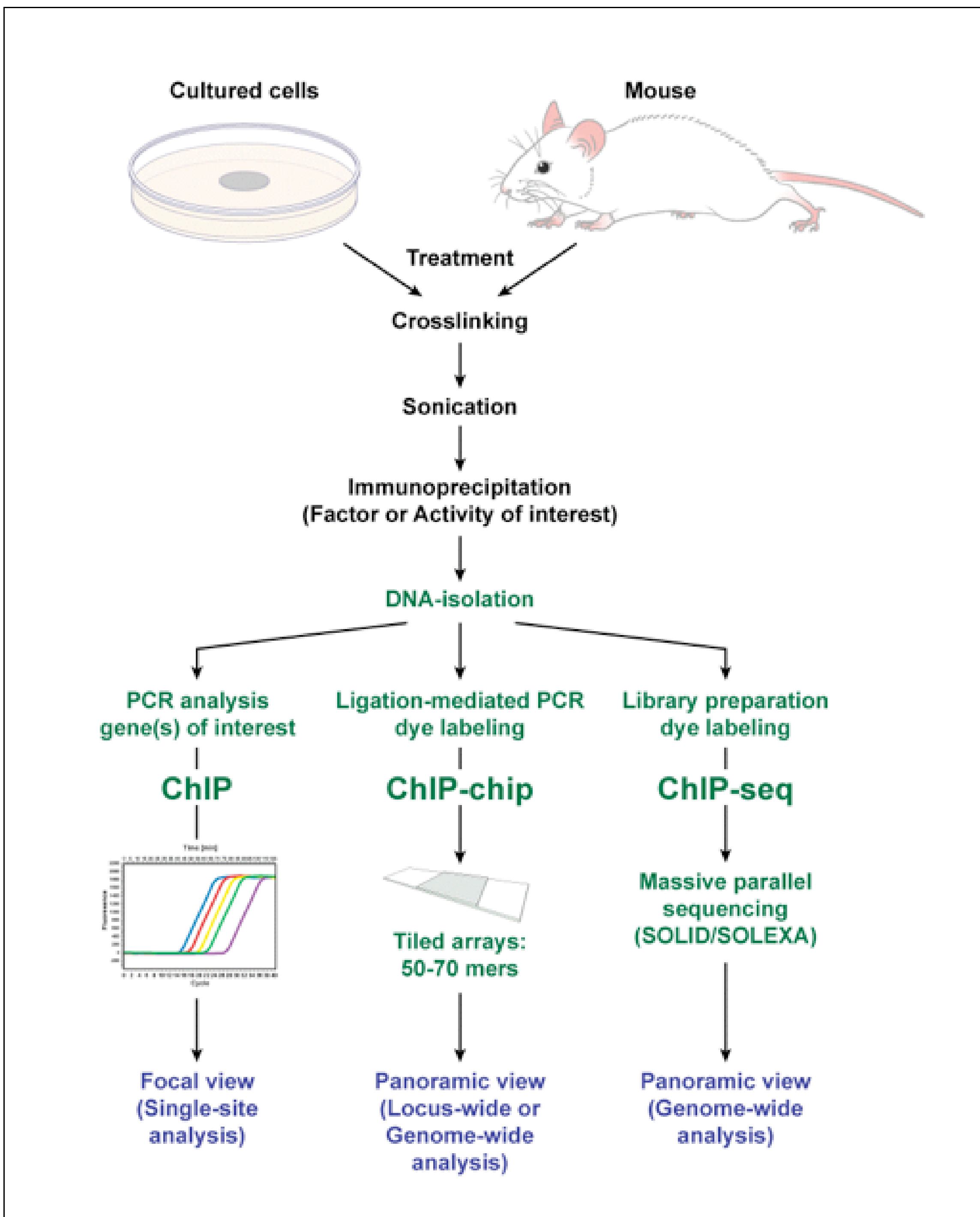

Fig. 2. Methodology associated with the techniques of ChIP, ChIP-chip and ChIP-seq.

Nevertheless, our preliminary studies have provided significant insight and we believe additional studies are likely to reveal even more detail into the molecular processes whereby the vitamin D hormone regulates gene expression.

Mediators of Vitamin D Action

The biological actions of 1,25$(OH)_2D_3$ at the transcription networks described above are influenced mechanistically by three primary gene products. These include Cyp27b1, the mitochondrial cytochrome P450 enzyme that

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is expressed in the kidney and other cell types and synthesizes the hormonal form of vitamin D (22), VDR, the regulatory factor that mediates the actions of the hormone (23), and Cyp24a1, the mitochondrial cytochrome P450 enzyme that is expressed in virtually all target tissues where it functions to degrade $1,25(OH)_2D_3$ and is itself induced by vitamin D (22). Under normal conditions, we surmise that the overall activities of the two P450 enzymes likely lead to the maintenance of unique, although potentially dynamic, steady state levels of $1,25(OH)_2D_3$ in specific target tissues. The VDR, of course, directly controls the expression of target genes. Since the relative expression of each of these genes is fundamental to the control of expression of all downstream gene and network targets, we summarize what is known of the regulation of these genes first, beginning with the VDR.

As indicated earlier, the VDR is expressed in virtually all vitamin D target tissues. Indeed, its expression represents an absolute determinant for biological response to vitamin D and its concentration is a key component of tissue sensitivity to the hormone. The VDR gene is known to be controlled by a number of regulatory factors including PTH, glucocorticoids, various retinoids and $1,25(OH)_2D_3$ itself (24). Despite this, little is known of the determinants for basal and/or tissue-specific expression of the VDR and almost nothing of the mechanisms that facilitate its upregulation by the above steroid and peptide agents. Recently, however, we employed ChIP-chip analysis to search for regulatory regions at the VDR gene locus that were involved in VDR-mediated autoregulation (25). We hypothesized that if enhancers that mediated $1,25(OH)_2D_3$ action could be found, they would likely play a role in VDR gene activation by other factors as well. Accordingly, we identified several key enhancers that mediated the unique actions of $1,25(OH)_2D_3$ and characterized their properties. Surprisingly, these enhancers were located in several large introns within the VDR gene between 8 and 21 kb downstream of its transcriptional start site (TSS). Both the VDR and retinoid X receptor (RXR) localized to these regions, supporting the idea that a VDR/RXR heterodimer, known to activate many VDR target genes, was also involved. Vitamin D response elements (VDREs) were subsequently identified and characterized as well. More recently, we have also identified enhancers in the VDR gene that mediate the actions of PTH, retinoic acid and the glucocorticoids via CREB, RAR and GR, respectively (Zella and Pike, unpublished data). Several of these enhancers overlapped those involved in VDR action; others were unique. Thus, our original hypothesis regarding the likelihood of enhancer modularity proved to be correct. Additional studies show that the binding of these transcription factors facilitates coregulator recruitment, alters the gene's chromatin epigenetic environment, and changes the distribution of RNA polymerase II across the gene. We were initially surprised at the locations and functional activities of these enhancers. During the past few years, however, it has become increasingly apparent that many if not most genes are regulated by complex modular enhancer regions located at distal sites within both introns and exons of the transcription unit and/or upstream or downstream of the gene transcription unit as well (26). Indeed, some of these regulatory regions are located tens if not hundreds of kilobases from transcription start sites. These types of studies and those by many other investigators are likely to increase our understanding of the mechanisms that control basal, tissue-specific and inducible expression of a wide number of genes.

Cyp27b1 is expressed in the kidney, positively regulated by PTH and negatively regulated by $1,25(OH)_2D_3$, the product of its actions. Studies by Kato and colleagues provide a regulatory mechanism whereby PTH induces CREB, which promotes activation of the gene, and $1,25(OH)_2D_3$ induces VDR binding, which functions to displace a key positive regulator (27;28). *Cyp27b1* is also expressed in other cell types as well, including those of the immune system, the skeleton, and skin (29). Little is known of the regulation of *Cyp27b1* at these sites, although the negative feedback regulation exerted by $1,25(OH)_2D_3$ does not appear to be a characteristic of the gene in these cell types. Studies across the

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Cyp27b1 locus using the techniques to be described below have not yet been conducted. Thus, we believe it is likely that our current understanding of how *Cyp27b1* is regulated in either the kidney or in extra-renal cells will undergo considerable modification in the near future.

The third gene that regulates vitamin D activity is *Cyp24a1*, a gene known for several decades to be expressed in cells that contain the VDR and to be regulated directly by $1,25(OH)_2D_3$ itself. Indeed, early studies of *Cyp24a1* regulation indicated that this gene was induced almost exclusively by $1,25(OH)_2D_3$, providing a specific mechanism whereby cellular levels of the hormone could be modulated negatively in relationship to its concentrations in specific tissues. While a VDRE capable of mediating transcriptional upregulation was identified first in the human osteocalcin gene (30), those mediating upregulation of *Cyp24a1* were found shortly thereafter and shown to be located within several hundred base pairs of the *Cyp24a1* promoter (31;32). Interestingly, our recent studies using both ChIP-chip and ChIP-seq have revealed that the *Cyp24a1* gene is regulated not only via these proximal promoter sites, but also through a cluster of elements located many kilobases downstream of the gene's final exon (Meyer, Goetsch, and Pike, unpublished data). As a result, mutations in both of the two proximal regulatory elements at the *Cyp24a1* gene promoter are not sufficient to prevent *Cyp24a1* induction by $1,25(OH)_2D_3$. Thus, the ChIP-chip technique has revealed new insight into the regulation of not only the *Vdr* but also of *Cyp24a1*, a gene long regarded as a prototypic target for vitamin D action.

The above studies indicate that while the *Cyp27b1*, *Vdr* and *Cyp24a1* genes are central to the activity of $1,25(OH)_2D_3$ in cells, their regulation by the vitamin D hormone itself is also a key component of tissue sensitivity. As will be described below, the mechanisms underlying the regulation of these genes typifies vitamin D's mechanism of action at other target genes as well. Moreover, the location of the regulatory sites identified using ChIP-chip techniques not only reveals additional insight but provides glimpses into a complexity of vitamin D action that was unanticipated.

Additional Targets of Vitamin D Action – RANKL

The studies outlined above have revealed important new insight into how the VDR regulates gene expression at two direct gene targets both by identifying sites of action of the VDR and its heterodimer partner and by defining several consequences of VDR/RXR binding in these regions. While the *VDR* and *Cyp24a1* provide several examples, others have been explored including the vitamin D targets *Lrp5* (33) and *Rankl* (34). We utilize the ability of vitamin D and other regulators to induce the expression of *Rankl* in bone cells described below as an illustrative example of the overall applicability of ChIP-chip and ChIP-seq in providing new insight into the mechanisms of action of $1,25(OH)_2D_3$ and its receptor at an exceedingly complex gene locus.

RANKL is a TNF-like factor that controls the differentiation, fusion, activation and survival of osteoclasts (35). While other factors play co-stimulatory roles in events associated with processes in osteoclastogenesis, RANKL is the key player (36). This central role is highlighted not only by the absence of osteoclasts in *Rankl*(-/-) mice (37), but also through its relevance as a therapeutic target to humans with osteoporosis as well (38). RANKL is expressed in stromal cells, osteoblasts, and T lymphocytes and in the case of the former two cell types, acts in a paracrine fashion to regulate both osteoclast formation from hematopoietic precursors and their eventual activation and survival as well. The expression of RANKL is regulated by the systemic hormones $1,25(OH)_2D_3$ and PTH as well as by the inflammatory cytokines including TNFα, IL-1β, and IL-6. Indeed, the ability of $1,25(OH)_2D_3$ to induce RANKL in the skeleton is fundamental to its calcium homeostatic actions. Early studies that attempted to delineate the molecular mechanism whereby *Rankl* gene expression was regulated focused upon the proximal promoter (39;40). These studies suggested the possibility that $1,25(OH)_2D_3$ might induce the *Rankl* gene, but the results were modest

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and difficult to reproduce. As a result of these observations, we explored the possibility that $1,25(OH)_2D_3$ action might be mediated by regulatory regions outside the proximal promoter. Accordingly, we conducted a ChIP-chip analysis of the mouse *Rankl* locus extending from the gene's upstream to its downstream neighbor, a distance of almost 500 kb (34). This scan revealed five distinct regions to which the VDR bound (D1-D5), all of them positioned exclusively upstream of the *Rankl* TSS. The most proximal region (D1) was located at 16 kb, the most distal (D5) at 75 kb. Further studies demonstrated the presence of specific functional binding sites for the VDR in three of the five enhancers. Deletion of the most distal of these in the mouse genome resulted in a striking reduction in inducibility of $1,25(OH)_2D_3$ and PTH *in vivo* (41). PTH response was also mapped to this distal region by Fu and colleagues (42). Subsequent studies indicated that these regions, as well as an additional upstream enhancer (D6), also mediated the actions of PTH and a subset mediated the actions of the inflammatory cytokines IL-6 and OSM (43). The transcription factor binding activity that we have identified to date at the *Rankl* gene using ChIP-chip analysis is summarized in the accompanying figure (Fig. 3). All of these distal regulatory regions were conserved in the human homolog and function in a similar, although not identical, manner (44). For example, in the human gene, although the most distal region (located at 99 kb) was active, a more proximal enhancer at 25 kb appeared to be the predominant mediator of $1,25(OH)_2D_3$ action in osteoblastic cells. Given the locations of these regulatory regions, confirmed both *in vitro* and *in vivo*, it seems highly unlikely that they would have been identified through the use of traditional analytical approaches.

The identification of novel regulatory regions in the *Rankl* gene provided a unique opportunity to examine the mechanistic roles of these regions in mediating *Rankl* gene expression as well (Martowicz, Meyer and Pike, unpublished data). Interestingly, the most striking response to VDR/RXR binding was a change in the level of histone H4 acetylation (H4ac). Accordingly, $1,25(OH)_2D_3$ induced a significant upregulation of H4ac at each of the five regulatory regions, a modification that spreads broadly across the *Rankl* locus. We conclude from this finding that at least one function of these distal regulatory regions is to facilitate significant structural changes in the chromatin domain across the *Rankl* locus. Surprisingly, VDR/RXR binding also resulted in the recruitment of RNA pol II at these enhancers rather than at the gene's TSS. This finding suggests that these *Rankl* enhancers might function as recruitment centers for RNA pol II, delivering the enzyme complex via a looping mechanism to the TSS where it becomes immediately engaged in transcript elongation. Perhaps the changes in acetylation following $1,25(OH)_2D_3$ treatment reflect this chromatin rearrangement. Regardless of the interpretation, the ability of the VDR to induce the recruitment of transcription factors such as RNA pol II and to provoke epigenetic changes at these sites provides strong support for a significant and perhaps complex role for these enhancers in *Rankl* gene regulation.

Genome-wide Analyses of $1,25(OH)_2D_3$ Gene Targets

Perhaps the most exciting opportunity provided by ChIP-chip and ChIP-seq technologies is the ability to map transcription factor activity in a particular cell or under a particular treatment across the entire genome. The term "cistrome" has been coined recently by Dr. Myles Brown at the Dana Farber Cancer Institute for this collection of sites assessed for a specific transcription factor in a specific cell type under a unique set of conditions. While the cistromes for a number of transcription factors have been identified recently, most notably for factors such as those capable of reprogramming pluripotency from somatic-derived cells (45), perhaps the most relevant here are the cistromes identified by Brown and colleagues for the estrogen receptor (ER) (46;47) and Stunnenberg and colleagues (48) for PPARγ and its RXR partner. Because these types of studies yield data at a much higher level, they have both confirmed previous discoveries and

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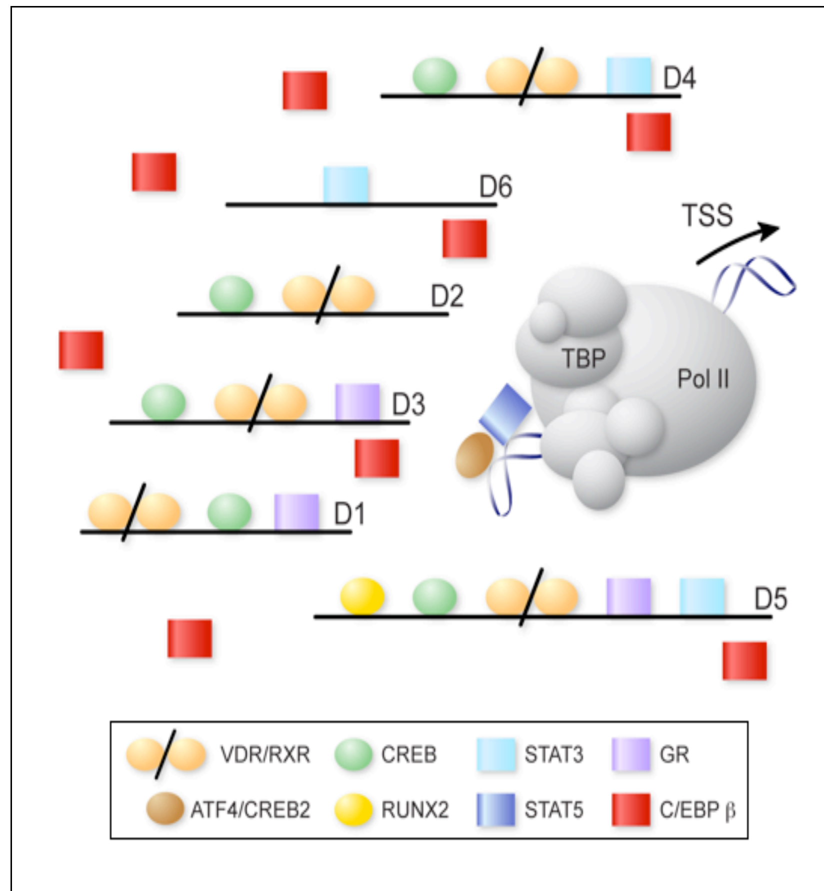

Fig. 3. Schematic summary of the mouse *Rankl* promoter and its multiple upstream regulatory regions. ChIP-chip studies have defined six highly conserved regulatory regions (D1-D6) located from 16 to 88 kb upstream of the *Rankl* gene promoter that mediate the actions of $1,25(OH)_2D_3$, PTH, inflammatory cytokines such as IL-6 and other agents in stromal cells/osteoblasts. Further studies have identified the transcription factors that bind to these regions and the regulatory elements that are located within. Depicted is the *Rankl* proximal promoter to which RNA polymerase II (pol II), TBP, and other proteins are bound. Also depicted are the six upstream regulatory regions that control *Rankl* expression and the transcription factors that become associated with these regions following activation. Individual transcription factors are color-coded. ATF4 and STAT5 have been shown by others to bind to the proximal *Rankl* promoter using traditional analyses. Note the ubiquitous presence of C/EBPβ, which is bound both to the indicated regulatory regions as well as to additional undefined sites. The three dimensional organization of the upstream regulatory regions is unknown and thus depicted randomly in the cartoon. Studies are ongoing to define the individual functions of these distal *Rankl* enhancers.

revealed a plethora of new concepts. The first is a general confirmation that like *Rankl*, *Cyp24a1* and *Vdr*, most genes are regulated through multiple enhancers located at sites both proximal as well as distal to the TSS. Indeed, proximal regulation often makes up less than 5-10% of the regulatory regions that control most genes. A second is that many transcription factors operate in synergy with additional transcription factors whose cistromes extensively overlap that of the primary regulatory factor. Specific examples include FoxA1 with the ER (46) and C/EBPα with PPARγ (48). Genome-wide ChIP-chip and ChIP-seq techniques can also be performed to assess the recruitment of coregulators and RNA polymerases and to track a wide variety of static and dynamic epigenetic marks across the entire genome; the latter are often signatures of gene status with regard to activation or suppression.

The application of genome-wide ChIP-chip and ChIP-seq techniques to the study of vitamin D action in target cells has not yet been reported. It seems likely, however, that these approaches will be used to identify

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VDR/RXR cistromes in target cells in the near future and to uncover new insights into 1,25(OH)$_2$D$_3$ action at the genome-wide level. Indeed, studies in this laboratory are well along in defining such principles of 1,25(OH)$_2$D$_3$ action in cells and tissues. We anticipate that these studies will provide significant insight into how the vitamin D hormone regulates gene expression at both single as well as multiple gene targets.

Summary

We have attempted in this short *Perspective* to describe several new revolutionary approaches to the study of gene regulation. These approaches promise to change the way we investigate the molecular actions of not only 1,25(OH)$_2$D$_3$ but other systemic hormones and local factors as well. These approaches can be utilized in a focused manner to understand the regulation of a single gene or on a larger scale to gain insight into how factors regulate gene expression at a genome-wide level. The capacity to conduct ChIP analysis using tissues derived from studies conducted *in vivo* suggests that detailed mechanistic studies such as those described above will no longer be limited to cell models in culture but can now be extended to animal models *in vivo* as well.

Conflict of Interest: None reported.

Peer Review: This article has been peer-reviewed.

References

1. Bischoff-Ferrari HA, Kiel DP, Dawson-Hughes B, Orav JE, Li R, Spiegelman D, Dietrich T, Willett WC. Dietary calcium and serum 25-hydroxyvitamin D status in relation to bone mineral density among U.S. adults. *J Bone Miner Res*. 2008 Dec 29. [Epub ahead of print]

2. Grant WB, Garland CF. The role of vitamin D3 in preventing infections. *Age Ageing*. 2008 Jan;37(1):121-2.

3. Walker VP, Modlin RL. The vitamin D connection to pediatric infections and immune function. *Pediatr Res*. 2009 Jan 28. [Epub ahead of print]

4. Holick MF. Vitamin D: a D-Lightful health perspective. *Nutr Rev*. 2008 Oct;66(10 Suppl 2):S182-94.

5. Evatt ML, Delong MR, Khazai N, Rosen A, Triche S, Tangpricha V. Prevalence of vitamin d insufficiency in patients with Parkinson disease and Alzheimer disease. *Arch Neurol*. 2008 Oct;65(10):1348-52.

6. Cannell JJ, Vieth R, Umhau JC, Holick MF, Grant WB, Madronich S, Garland CF, Giovannucci E. Epidemic influenza and vitamin D. *Epidemiol Infect*. 2006 Dec;134(6):1129-40.

7. Calvo MS, Whiting SJ, Barton CN. Vitamin D intake: a global perspective of current status. *J Nutr*. 2005 Feb;135(2):310-6.

8. Whiting SJ, Calvo MS. Dietary recommendations for vitamin D: a critical need for functional end points to establish an estimated average requirement. *J Nutr*. 2005 Feb;135(2):304-9.

9. Bouillon R, Carmeliet G, Verlinden L, van Etten E, Verstuyf A, Luderer HF, Lieben L, Mathieu C, Demay M. Vitamin D and human health: lessons from vitamin D receptor null mice. *Endocr Rev*. 2008 Oct;29(6):726-76.

10. Malloy PJ, Pike JW, Feldman D. The vitamin D receptor and the syndrome of hereditary 1,25-dihydroxyvitamin D-resistant rickets. *Endocr Rev*. 1999 Apr;20(2):156-88.

11. Li YC, Pirro AE, Amling M, Delling G, Baron R, Bronson R, Demay MB. Targeted ablation of the vitamin D receptor: an animal model of vitamin D-dependent rickets type II with alopecia. *Proc Natl Acad Sci U S A*. 1997 Sep 2;94(18):9831-5.

12. Pike JW, Meyer MB, Watanuki M, Kim S, Zella LA, Fretz JA, Yamazaki M, Shevde NK. Perspectives on mechanisms of gene regulation by 1,25-dihydroxyvitamin D3 and its receptor. *J*

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Steroid Biochem Mol Biol. 2007 Mar;103(3-5):389-95.

13. Pike JW, Yamamoto H, Shevde NK. Vitamin D receptor-mediated gene regulation mechanisms and current concepts of vitamin D analog selectivity. *Adv Ren Replace Ther*. 2002 Jul;9(3):168-74.

14. Bouillon R, Van Cromphaut S, Carmeliet G. Intestinal calcium absorption: Molecular vitamin D mediated mechanisms. *J Cell Biochem*. 2003 Feb 1;88(2):332-9.

15. Bouillon R, Bischoff-Ferrari H, Willett W. Vitamin D and health: perspectives from mice and man. *J Bone Miner Res*. 2008 Jul;23(7):974-9.

16. Adorini L. Regulation of immune responses by vitamin D receptor ligands. In: Feldman D, Pike JW, Glorieux FH, eds. *Vitamin D*. 2nd ed. New York: Elsevier/Academic Press; 2005:631-48.

17. Demay MB, MacDonald PN, Skorija K, Dowd DR, Cianferotti L, Cox M. Role of the vitamin D receptor in hair follicle biology. *J Steroid Biochem Mol Biol*. 2007 Mar;103(3-5):344-6.

18. Hsieh JC, Sisk JM, Jurutka PW, Haussler CA, Slater SA, Haussler MR, Thompson CC. Physical and functional interaction between the vitamin D receptor and hairless corepressor, two proteins required for hair cycling. *J Biol Chem*. 2003 Oct 3;278(40):38665-74.

19. Liu M, Lee MH, Cohen M, Bommakanti M, Freedman LP. Transcriptional activation of the Cdk inhibitor p21 by vitamin D3 leads to the induced differentiation of the myelomonocytic cell line U937. *Genes Dev*. 1996 Jan 15;10(2):142-53.

20. Gilchrist DA, Fargo DC, Adelman K. Using ChIP-chip and ChIP-seq to study the regulation of gene expression: Genome-wide localization studies reveal widespread regulation of transcription elongation. *Methods*. 2009 Mar 9. [Epub ahead of print]

21. Valouev A, Johnson DS, Sundquist A, Medina C, Anton E, Batzoglou S, Myers RM, Sidow A. Genome-wide analysis of transcription factor binding sites based on ChIP-Seq data. *Nat Methods*. 2008 Sep;5(9):829-34.

22. Prosser DE, Jones G. Enzymes involved in the activation and inactivation of vitamin D. *Trends Biochem Sci*. 2004 Dec;29(12):664-73.

23. Baker AR, McDonnell DP, Hughes M, Crisp TM, Mangelsdorf DJ, Haussler MR, Pike JW, Shine J, O'Malley BW. Cloning and expression of full-length cDNA encoding human vitamin D receptor. *Proc Natl Acad Sci U S A*. 1988 May;85(10):3294-8.

24. Krishnan AV, Feldman, D. Regulation of vitamin D receptor abundance. In: Feldman D, Glorieux FH, Pike JW, eds. *Vitamin D*. San Diego: Academic Press; 1997:179-200.

25. Zella LA, Kim S, Shevde NK, Pike JW. Enhancers located within two introns of the vitamin D receptor gene mediate transcriptional autoregulation by 1,25-dihydroxyvitamin D3. *Mol Endocrinol*. 2006 Jun;20(6):1231-47.

26. Chan CS, Song JS. CCCTC-binding factor confines the distal action of estrogen receptor. *Cancer Res*. 2008 Nov 1;68(21):9041-9.

27. Murayama A, Takeyama K, Kitanaka S, Kodera Y, Kawaguchi Y, Hosoya T, Kato S. Positive and negative regulations of the renal 25-hydroxyvitamin D3 1alpha-hydroxylase gene by parathyroid hormone, calcitonin, and 1alpha,25(OH)2D3 in intact animals. *Endocrinology*. 1999 May;140(5):2224-31.

28. Murayama A, Kim MS, Yanagisawa J, Takeyama K, Kato S. Transrepression by a liganded nuclear receptor via a bHLH activator through co-regulator

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switching. *EMBO J*. 2004 Apr 7;23(7):1598-608.

29. Zehnder D, Bland R, Williams MC, McNinch RW, Howie AJ, Stewart PM, Hewison M. Extrarenal expression of 25-hydroxyvitamin d(3)-1 alpha-hydroxylase. *J Clin Endocrinol Metab*. 2001 Feb;86(2):888-94.

30. Kerner SA, Scott RA, Pike JW. Sequence elements in the human osteocalcin gene confer basal activation and inducible response to hormonal vitamin D3. *Proc Natl Acad Sci U S A*. 1989 Jun;86(12):4455-9.

31. Ohyama Y, Ozono K, Uchida M, Yoshimura M, Shinki T, Suda T, Yamamoto O. Functional assessment of two vitamin D-responsive elements in the rat 25-hydroxyvitamin D3 24-hydroxylase gene. *J Biol Chem*. 1996 Nov 29;271(48):30381-5.

32. Zierold C, Darwish HM, DeLuca HF. Two vitamin D response elements function in the rat 1,25-dihydroxyvitamin D 24-hydroxylase promoter. *J Biol Chem*. 1995 Jan 27;270(4):1675-8.

33. Fretz JA, Zella LA, Kim S, Shevde NK, Pike JW. 1,25-Dihydroxyvitamin D3 regulates the expression of low-density lipoprotein receptor-related protein 5 via deoxyribonucleic acid sequence elements located downstream of the start site of transcription. *Mol Endocrinol*. 2006 Sep;20(9):2215-30.

34. Kim S, Yamazaki M, Zella LA, Shevde NK, Pike JW. Activation of receptor activator of NF-kappaB ligand gene expression by 1,25-dihydroxyvitamin D3 is mediated through multiple long-range enhancers. *Mol Cell Biol*. 2006 Sep;26(17):6469-86.

35. Lacey DL, Timms E, Tan HL, Kelley MJ, Dunstan CR, Burgess T, Elliott R, Colombero A, Elliott G, Scully S, Hsu H, Sullivan J, Hawkins N, Davy E, Capparelli C, Eli A, Qian YX, Kaufman S, Sarosi I, Shalhoub V, Senaldi G, Guo J, Delaney J, Boyle WJ. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell*. 1998 Apr 17;93(2):165-76.

36. Kobayashi Y, Udagawa N, Takahashi N. Action of RANKL and OPG for osteoclastogenesis. *Crit Rev Eukaryot Gene Expr*. 2009;19(1):61-72.

37. Kong YY, Yoshida H, Sarosi I, Tan HL, Timms E, Capparelli C, Morony S, Oliveira-dos-Santos AJ, Van G, Itie A, Khoo W, Wakeham A, Dunstan CR, Lacey DL, Mak TW, Boyle WJ, Penninger JM. OPGL is a key regulator of osteoclastogenesis, lymphocyte development and lymph-node organogenesis. *Nature*. 1999 Jan 28;397(6717):315-23.

38. Lewiecki EM. Emerging drugs for postmenopausal osteoporosis. *Expert Opin Emerg Drugs*. 2009 Mar;14(1):129-44.

39. Kitazawa S, Kajimoto K, Kondo T, Kitazawa R. Vitamin D3 supports osteoclastogenesis via functional vitamin D response element of human RANKL gene promoter. *J Cell Biochem*. 2003 Jul 1;89(4):771-7.

40. Mori K, Kitazawa R, Kondo T, Maeda S, Yamaguchi A, Kitazawa S. Modulation of mouse RANKL gene expression by Runx2 and PKA pathway. *J Cell Biochem*. 2006 Aug 15;98(6):1629-44.

41. Galli C, Zella LA, Fretz JA, Fu Q, Pike JW, Weinstein RS, Manolagas SC, O'Brien CA. Targeted deletion of a distant transcriptional enhancer of the receptor activator of nuclear factor-kappaB ligand gene reduces bone remodeling and increases bone mass. *Endocrinology*. 2008 Jan;149(1):146-53.

42. Fu Q, Manolagas SC, O'Brien CA. Parathyroid hormone controls receptor activator of NF-kappaB ligand gene expression via a distant transcriptional enhancer. *Mol Cell Biol*. 2006 Sep;26(17):6453-68.

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43. Kim S, Yamazaki M, Shevde NK, Pike JW. Transcriptional control of receptor activator of nuclear factor-kappaB ligand by the protein kinase A activator forskolin and the transmembrane glycoprotein 130-activating cytokine, oncostatin M, is exerted through multiple distal enhancers. *Mol Endocrinol*. 2007 Jan;21(1):197-214.

44. Nerenz RD, Martowicz ML, Pike JW. An enhancer 20 kilobases upstream of the human receptor activator of nuclear factor-kappaB ligand gene mediates dominant activation by 1,25-dihydroxyvitamin D3. *Mol Endocrinol*. 2008 May;22(5):1044-56.

45. Mathur D, Danford TW, Boyer LA, Young RA, Gifford DK, Jaenisch R. Analysis of the mouse embryonic stem cell regulatory networks obtained by ChIP-chip and ChIP-PET. *Genome Biol*. 2008;9(8):R126.

46. Carroll JS, Liu XS, Brodsky AS, Li W, Meyer CA, Szary AJ, Eeckhoute J, Shao W, Hestermann EV, Geistlinger TR, Fox EA, Silver PA, Brown M. Chromosome-wide mapping of estrogen receptor binding reveals long-range regulation requiring the forkhead protein FoxA1. *Cell*. 2005 Jul 15;122(1):33-43.

47. Carroll JS, Meyer CA, Song J, Li W, Geistlinger TR, Eeckhoute J, Brodsky AS, Keeton EK, Fertuck KC, Hall GF, Wang Q, Bekiranov S, Sementchenko V, Fox EA, Silver PA, Gingeras TR, Liu XS, Brown M. Genome-wide analysis of estrogen receptor binding sites. *Nat Genet*. 2006 Nov;38(11):1289-97.

48. Nielsen R, Pedersen TA, Hagenbeek D, Moulos P, Siersbaek R, Megens E, Denissov S, Børgesen M, Francoijs KJ, Mandrup S, Stunnenberg HG. Genome-wide profiling of PPARgamma:RXR and RNA polymerase II occupancy reveals temporal activation of distinct metabolic pathways and changes in RXR dimer composition during adipogenesis. *Genes Dev*. 2008 Nov 1;22(21):2953-67.

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