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REVIEW

Regulation of gene expression by 1,25-dihydroxy-vitamin D_3 in bone cells: exploiting new approaches and defining new mechanisms

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The biological actions of 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$) serve both to orchestrate calcium and phosphorus homeostasis in higher vertebrates and to regulate a diverse set of cellular functions unrelated to control of mineral metabolism. With regard to bone, mesenchymal lineage cells, including both early and late osteoblasts as well as osteocytes represent classic targets of the vitamin D hormone. Accordingly, much of the early information regarding our current understanding of the mechanism of action of 1,25$(OH)_2D_3$, of which gene regulation is central, derives from a broad array of studies in these cell types. Indeed, a gene that provided both the earliest and perhaps the most extensive information regarding this and additional mechanisms was that of osteoblast-specific osteocalcin. Subsequent work has provided much additional detail as to how 1,25$(OH)_2D_3$, through the vitamin D receptor (VDR), mediates the modulation of many bone cell genes. In recent years, however, a series of technical advances involving the coupling of chromatin immunoprecipitation (ChIP) to unbiased methodologies that involve next-generation DNA sequencing techniques (ChIP-seq) have opened new avenues in the study of gene regulation. In this review, we summarize early work and then focus on more recent studies that have used ChIP-seq analysis and other approaches to provide insight into not only the regulation of specific genes such as the *VDR, TNFSF11 (RANKL), LRP5, CBS* and *CYP24a1*, but overarching genome-wide principles of gene regulation as well. The results of these studies highlight the value of these new approaches and the increased insight that can be gained.

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Introduction

Early studies of the biological activities of 1,25-dihydroxy-vitamin D_3 (1,25$(OH)_2D_3$) in the intestine, kidney and bone revealed that this hormone likely functioned following nuclear localization by regulating the expression of genes. This hypothesis was strengthened when it was discovered that 1,25$(OH)_2D_3$ interacted selectively with an intestinal binding protein, later termed the vitamin D receptor (VDR), and that this interaction prompted translocation of the protein to the nucleus.[1–3] Subsequent studies demonstrated that the VDR could be found not only in the intestine, but also in the parathyroid gland,[3] kidney[4] and bone,[5] and later in specific cell types in many if not most bodily tissues.[6–8] Further characterization of the VDR as a DNA-binding protein supported its role as the primary mediator of vitamin D action in all tissues.[9] This idea was eventually confirmed when the VDR was cloned and shown to belong to the nuclear receptor family of transcription factors[10–12] and through the discovery that mutations in the VDR led to the human syndrome of hereditary 1,25$(OH)_2D_3$-resistant rickets,[13,14] a phenotype replicated through genetic deletion of the VDR in mice.[15,16] Interestingly, although the initial actions of vitamin D were found in the intestine, it was in bone cells and on bone cell-specific genes that the most striking molecular advances have been made.

Mechanistically, 1,25$(OH)_2D_3$ prompts VDR interaction directly with DNA sequences located within regulatory regions of target genes. This interaction requires heterodimer formation with either related steroid receptor family members RXRα or β[17,18] and occurs with high affinity on selected sequences comprising two directly repeated AGGTCA hexameric half-sites separated generally by 3 bp.[19–21] The formation of this protein–DNA complex, and in particular the exposure of unique binding sites on the surface of both the VDR and RXR, provides

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a platform for the recruitment of additional multi-protein co-regulatory complexes, a structure that has now been deduced from three-dimensional analysis.[22] These complexes function directly through various epigenetic mechanisms to modulate regions of the chromatin environment surrounding the promoter of a target gene, to regulate the essential recruitment of RNA polymerase II and likely through additional mechanisms as well.[23] Osteocalcin was the first gene for which a vitamin D response element or VDRE was identified,[19,24] a finding followed thereafter by the discovery of a VDRE located in the *SPP1* (osteopontin) gene[20] and subsequently in the *CYP24A1* gene.[25–27] Although VDR/RXR DNA-binding sites that diverge from this overall organizational motif have emerged from time to time,[28] the discovery of an osteocalcin/osteopontin type VDRE sequence in many subsequent genes firmly established this motif as a classic VDR/RXR-binding site for genes that are induced by $1,25(OH)_2D_3$. Many additional features and details of this overall mechanism have been identified. Together, they have firmly established the molecular fundamentals of $1,25(OH)_2D_3$ action at target genes, an overall process that is not unlike that of most steroid hormones and indeed most other transcription factors as well.

The development of chromatin immunoprecipitation (ChIP) methods coupled first to site-specific PCR analysis and shortly thereafter to methods capable of detecting in an unbiased manner the abundance of immunoprecipitated DNA segments on a genome-wide basis (tiled microarrays (ChIP-chip) and then massively parallel DNA sequencing (ChIP-seq)) are changing traditional molecular biological approaches to the study of transcriptional regulation (**Figure 1**). The primary advantage of the current ChIP-seq approach is the facile ability to detect proteins at endogenous sites on the genome in a largely unbiased and genome-wide manner. This approach has been exploited extensively over the past decade by many investigators, including those belonging to the ENCODE Consortium to not only explore specific pathways of gene regulation, but to also provide new annotation to the genome.[29] A specific area of progress has also been a significant advance in our understanding of the genetics and epigenetics of cellular differentiation and reprogramming.[30,31] In this review, we document advances that have been made using ChIP-chip and ChIP-seq approaches in understanding the regulation of bone cell gene expression by $1,25(OH)_2D_3$.

Observations

Although early mechanistic studies of the regulation of gene expression by $1,25(OH)_2D_3$ defined sites of VDR/RXR interaction on genes such as osteocalcin and osteopontin, regulatory sites could not be detected on many genes whose RNAs were known to be regulated by the vitamin D hormone. They include the VDR gene itself as well as genes for receptor activator of NF-κB ligand (RANKL), low-density lipoprotein receptor-related protein 5 (LRP5), cystathionine β-synthase (CBS) and a number of others as well. Indeed, this feature still categorizes the majority of targets for $1,25(OH)_2D_3$. The absence of sites suggests either that alternative mechanisms are at play or that the regions that control the expression of these genes are located outside those being explored. Although both are possible, the latter seemed the most likely as traditional methods are highly biased and limit investigative focus almost exclusively to regions near the transcriptional start sites (TSSs) of candidate genes. To begin to explore this question, we applied the unbiased technique of ChIP-chip analysis (confirmed via ChIP-seq analysis) to the study of $1,25(OH)_2D_3$'s regulation of the genes mentioned above. We also re-examined the CYP24A1 gene to determine if the regulatory mechanism previously defined for this gene was complete. In addition to these specific genomic loci, ChIP-seq analysis is inherently genome-wide, therefore we will also discuss the principles of $1,25(OH)_2D_3$ regulation of the entire transcriptome in osteoblasts and other cell types.

The regulation of specific genes. *Specific targets*. The *VDR* gene. The VDR gene is highly expressed in both early and late osteoblasts and terminally differentiated osteocytes[32] and is believed to be synthesized in osteoclasts[33] as well. Through the VDR, $1,25(OH)_2D_3$ regulates a wide variety of genes which underlie both catabolic and anabolic cellular characteristics. Studies two decades earlier suggested that the VDR gene was transcriptionally regulated by several hormones including the glucocorticoids, estrogens and retinoic acid, and might be autoregulated by $1,25(OH)_2D_3$ as well.[34] More recent studies *in vivo* suggest that $1,25(OH)_2D_3$ upregulates the VDR almost exclusively in bone cells (Meyer and Pike, unpublished). Despite this compelling evidence, studies using traditional approaches failed to identify binding sites for the VDR at regions surrounding the gene's TSS either *in vitro* or *in vivo*. As the mouse *Vdr* gene, like that of the human gene[13,35] is complex, spanning some 54 kilobases (kb) on chromosome 15 (**Figure 2a**), we conducted in osteoblasts an unbiased ChIP-chip analysis across the *Vdr* gene locus that spanned several hundred kilobases upstream as well as downstream of the *Vdr* transcriptional unit.[36,37] These initial studies as well as an additional unpublished investigation using ChIP-seq analysis (see **Figure 2a**) revealed the $1,25(OH)_2D_3$-induced presence of both the VDR and RXR at two separate intronic sites approximately +19 and +29-kb downstream and at one intergenic site −6-kb upstream of the TSS. This group of regulatory sites was generally conserved in both the mouse and the human genes and their properties extensively characterized including identification of the VDREs contained within each region.[36] Interestingly, retinoic acid receptor binding was induced by retinoic acid at the intronic +19-kb site while cAMP response element binding protein (CREB) binding was induced by protein kinase A activators (including PTH) at the upstream −6-kb site.[37] A number of additional transcription factors including both runt-related transcription factor 2 (RUNX2) and CCAAT/enhancer-binding protein β (C/EBPβ) were also found at subsets of these sites as well. These results provided the first evidence that VDR-binding sites in genes could be located at remote sites distal to gene promoters and that multiple regions with unique regulatory properties could participate.

The *TNFSF11* (RANKL) gene. Early studies by Suda *et al.*[38] suggested that a factor derived from osteoblast lineage cells regulated osteoclast differentiation. Importantly, the expression of this factor was controlled by $1,25(OH)_2D_3$ as well as other calciotropic agents such as PTH and the inflammatory cytokines. This factor was identified and cloned in 1999 and designated RANKL.[39,40] RANKL is a membrane bound and sometimes soluble tumor necrosis factor (TNF)-like factor

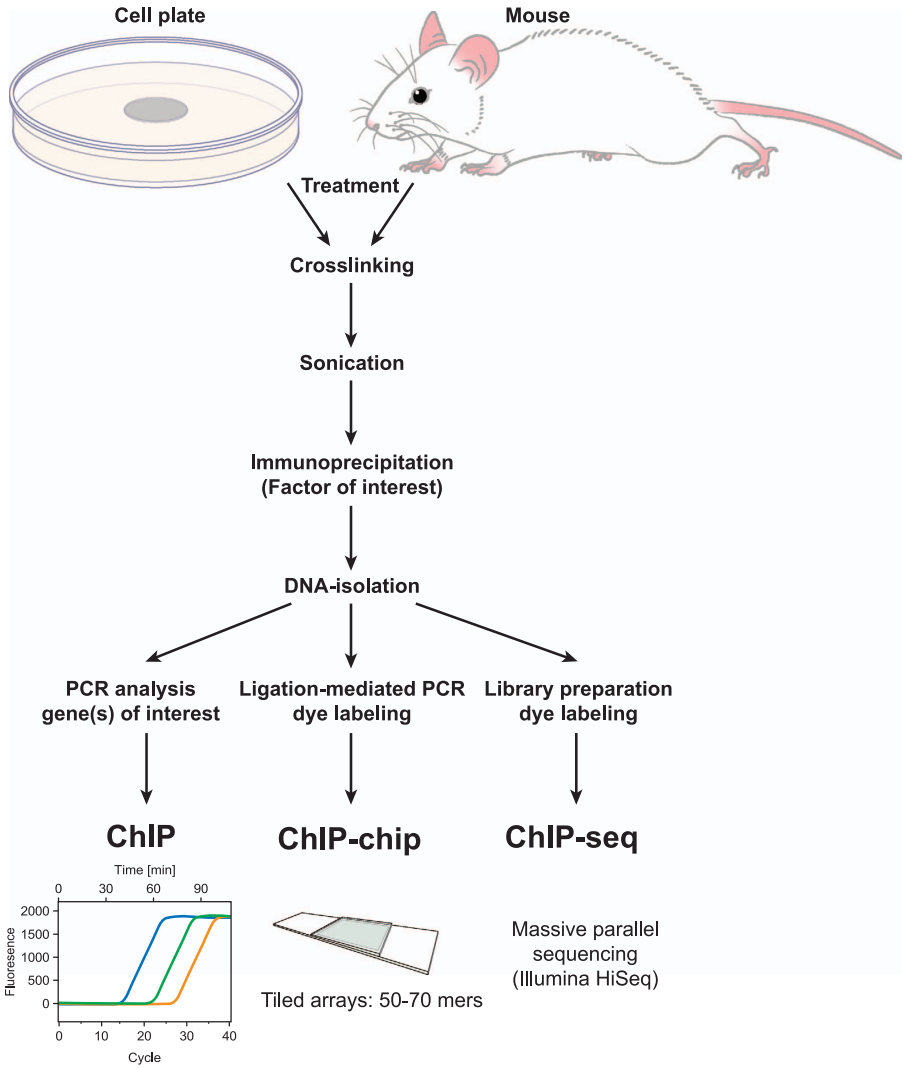

Figure 1 Site-specific and genome-wide methodologies associated with chromatin immunoprecipitation (ChIP) methods. ChIP-chip, ChIP linked to tiled microarray analysis; ChIP-seq, ChIP linked to DNA deep sequencing methods.

derived from the *TNFSF11* gene that strongly induces osteoclast differentiation from hematopoietic precursors. Indeed, the signaling pathway that mediates activation of this complex differentiation pathway is now well described.[41] Despite considerable effort, however, attempts using traditional methods to identify regions mediating the regulation of RANKL by $1,25(OH)_2D_3$ as well as PTH and cytokines such as interleukin-6 and oncostatin M were largely unsuccessful. As the RANKL gene is located in a gene desert and bounded by intergenic segments of nearly 200 kb of DNA (**Figure 2b**), we explored this gene in osteoblasts for regulation by the vitamin D hormone by conducting a ChIP-chip analysis for both VDR and RXR spanning over 500 kb of DNA surrounding the mouse *Tnfsf11* transcription unit.[42] This initial study, confirmed through subsequent studies using ChIP-seq analysis (**Figure 2b**), was highly revealing. Although neither VDR nor RXR were present near the promoter region following administration of $1,25(OH)_2D_3$, both were strikingly present at -16 (D1), -22 (D2), -60 (D3), -69 (D4) and -75 (D5) kb upstream of the *Tnfsf11* gene promoter. Each of these regions, as well as a newly defined site at -40 kb (D7), was extensively characterized and the VDR-binding sites that mediated vitamin D hormone action were identified in a subset. Similar ChIP-chip studies of PTH action on RANKL expression through CREB were also carried out.[43] As with the VDR, these studies revealed that PTH induced CREB binding at several but not all of these regulatory regions. Interestingly, O'Brien and Co-workers[44] also identified one these upstream regions using an entirely independent approach. Importantly, this region was deleted in the mouse genome and shown to affect both the basal expression of RANKL at specific skeletal sites and to ameliorate but not eliminate overall response to both $1,25(OH)_2D_3$ and PTH.[45] We also showed that cytokine induction, on the other hand, was mediated through signal transducer and activator of transcription 3 induction at a novel region located at -88-kb upstream.[46] Residual binding of both RUNX2 and C/EBPβ and other transcription factors were also observed.[47] These studies provide a conclusion similar to that observed in the VDR gene; $1,25(OH)_2D_3$ regulation of gene expression occurs through multiple regulatory regions that can be located many kilobases distal to the transcriptional start sites of genes and are frequently modular in their actions.

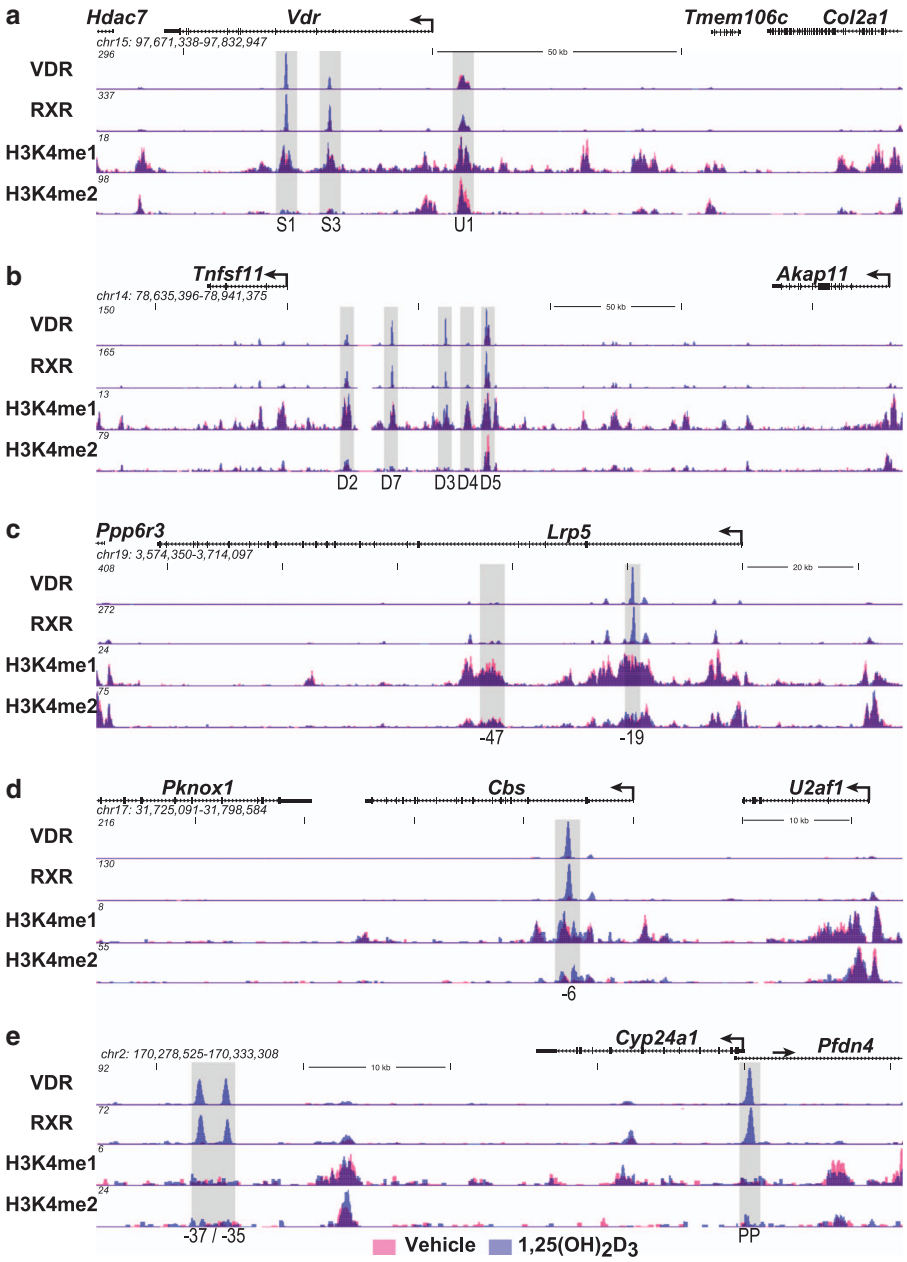

Figure 2 ChIP-seq profiles at specific gene loci. Mouse MC3T3-E1 cells were treated for 3 h with either vehicle or $1,25(OH)_2D_3$ (10^{-7} M) and then subjected to ChIP-seq analysis using antibodies to VDR, RXR, histone H3K4me1 or H3K4me2. ChIP-seq tag densities (normalized to 10^7 reads) were quantified and mapped to the mouse MM9 genome using MACS, HOMER and cistrome data analyses. The genomic loci (chromosome number and nucleotide interval are indicated) contain the *Vdr* (**a**), *Tnfsf11* (**b**), *Lrp5* (**c**), *Cbs* (**d**) and *Cyp24a1* (**e**) genes and their respective neighbors. Each data track (scales are indicated on the Y axis) contains two mapped data sets derived from vehicle- and $1,25(OH)_2D_3$-treated cells (red and blue, respectively). The TSS and direction of transcription for each gene is indicated by an arrow; exons are indicated by boxes. Shaded vertical columns highlight the locations and number/letter designation for each of the regulatory regions of the target genes. $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; CBS, cystathionine β-synthase; ChIP, chromatin immunoprecipitation; HOMER, hypergeometric optimization of motif enrichment; LPR5, lipoprotein receptor-related protein 5; MACS, model-based analysis for ChIP-seq; TSS, transcriptional start site; VDR, vitamin D receptor; PP, promoter proximal.

The *LRP5* gene. The canonical Wnt signaling pathway has a crucial role in osteoblast proliferation and differentiation, and thus appears to exert a significant anabolic role in bone formation.[48] It also has a central role in transducing mechanosensory inputs via the osteocyte.[49,50] Wnt activity is mediated through an interaction between specific Wnt family members and the cell surface receptor Frizzled. This interaction triggers downstream events that lead ultimately to stabilization and nuclear translocation of β-catenin, a dominant coactivator of the transcription factor 7-like 2/lymphoid enhancer-binding factor 1 family of transcription factors often prebound at target genes. LRP5 and LRP6 serve as coreceptors of Frizzled, facilitating positively the process of β-catenin activation. That LRP5 and LRP6 represent anabolic potentiators of Wnt activation was strengthened initially by the observation that hypermorphic alleles of LRP5 were associated with high bone

mass in humans.[51,52] As preliminary studies in osteoblasts revealed that 1,25(OH)$_2$D$_3$ prompted an upregulation of LRP5 mRNA, we explored the mechanism through which this hormone might operate using an initial ChIP-chip analysis of VDR/RXR spanning the mouse *Lrp5* locus similar to that conduced for the *Vdr* and *Tnfsf11* genes.[53] These studies revealed the presence of three potential regulatory regions, one located immediately downstream of the *Lrp5* TSS and two additional regions also located within separate introns at +19 and +47 kb (see **Figure 2c** for updated ChIP-seq data). Further characterization of these regions confirmed the ability of the regions at +19 kb to mediate 1,25(OH)$_2$D$_3$ activity; additional unpublished studies revealed similar binding sites for the VDR near the *Lrp5* promoter. These studies similarly demonstrate the enhanced frequency with which regulatory regions are located distal to gene promoters, although in this case two of these regions were accompanied by a regulatory region that was indeed located near the TSS. Perhaps more importantly, these studies provide support for the hypothesis that the actions of 1,25(OH)$_2$D$_3$ in bone cells may not be exclusively catabolic.

The *CBS* gene. Osteoporotic fracture risk is elevated during the aging process because of a multiplicity of factors that may include lifestyle, nutrition, and hormonal and genetic factors.[54] One additional factor may be a raise in circulating levels of the sulfur-containing amino-acid homocysteine.[55] Indeed, this increase in homocysteine can give rise to hyperhomocysteinemia, which negatively impacts collagen cross-linking and maturation thus affecting bone quality, bone strength and promotes bone resorption.[56,57] A key step in the elimination of homocysteine is the condensation of this amino acid with serine, a reaction that is catalyzed by the enzyme cystathionine β-synthase and which culminates via several additional steps in the synthesis of cysteine.[58] The importance of this enzyme in bone is highlighted by the observation that mice genetically deficient in CBS exhibit severe hyperhomocysteinemia, which is accompanied by elevated reactive oxygen species levels and a progressively aberrant skeletal phenotype.[59,60] Initial studies in osteoblasts revealed that 1,25(OH)$_2$D$_3$ induced the synthesis of CBS mRNA, prompting further examination of the mechanism through which this induction might occur. We therefore assessed VDR and RXR binding across the CBS gene locus using ChIP-chip analysis.[61] These and additional ChIP-seq analyses (**Figure 2d**) revealed the presence of a single potential regulatory region in the CBS gene located in the second intron approximately +6-kb downstream of the TSS. Examination of the DNA sequence within this downstream region led to the identification of a classic VDRE comprised of two hexameric half-sites separated by 3 bp. Further study revealed that this element was indeed capable of mediating 1,25(OH)$_2$D$_3$ response. These results provide additional support for the utility of this unbiased analysis in rapidly identifying regulatory sites of vitamin D hormone action. They also suggest that in addition to both anabolic and catabolic actions, 1,25(OH)$_2$D$_3$ is also capable of modulating internal pathways of amino-acid synthesis that are positive for bone structure and function.

The *CYP24A1* gene. *CYP24A1* encodes the 1,25-dihydroxyvitamin D$_3$-24 hydroxylase, a p450 enzyme that catalyzes the initial steps that results in the inactivation of 1,25(OH)$_2$D$_3$.[62] Accordingly, this enzyme represents a primary set point determinant of both the level and duration of response to 1,25(OH)$_2$D$_3$ that is achievable within all vitamin D target cells both *in vitro* and *in vivo*. Thus, global genetic deletion of CYP24A1 expression in mice or the production of mutant hydroxylases as observed in young children with idiopathic familial hypercalcemia results in exaggerated levels of 1,25(OH)$_2$D$_3$ that potentiate vitamin D hormone activity and can cause hypercalcemia.[63] As might be anticipated, *CYP24A1* expression is strongly regulated by 1,25(OH)$_2$D$_3$. Thus, traditional studies of this gene in 1993 quickly revealed the present of two highly conserved VDREs located within the first 300 bp of both the mouse and human homologs that were capable of binding both VDR and RXR and mediating strong induction by 1,25(OH)$_2$D$_3$.[25–27] The presence of these two sites of 1,25(OH)$_2$D$_3$ action were confirmed following extensive ChIP-chip and ChIP-seq analyses of VDR and RXR binding in mouse bone cells and in many other cell types as well[64] (**Figure 2e**). Interestingly, inspection of both VDR and RXR-binding profiles across the mouse *Cyp24a1* locus following these analyses also revealed the presence of a cassette containing a cluster of intergenic VDR/RXR-binding sites spanning +35 to +37 kb in the mouse (**Figure 2e**) and +50 to +66 kb in the human genomes.[64–66] Subsequently, detailed studies not only identified several of the multiple VDREs that were contained within these regulatory regions but linked their activity directly to enhancement of the *Cyp24a1* expression by 1,25(OH)$_2$D$_3$. Thus, these unbiased approaches are capable of not only confirming the presence of previously characterized regulatory regions, but identifying new sites as well. It is reasonable to conclude that the traditional methods used to study the regulation of many genes has underestimated both in number and complexity the components of regulation regions actually present in many genes, and that the application of unbiased methods described above will be required to more fully understand the regulation of these genes by 1,25(OH)$_2$D$_3$ and other factors.

Derived principles of vitamin D hormone action. The targeted ChIP-chip and subsequent ChIP-seq analyses of the above candidate genes provide strong validation for many of the principles of gene regulation established for 1,25(OH)$_2$D$_3$ over the past several decades using more traditional methods of experimentation.[65,66] Indeed, they reinforce, in part, the concepts that (1) VDR DNA binding involves an RXR partner, (2) classic binding sites are comprised of two directly repeated hexanucleotide half-sites separated by 3 bp, (3) VDRE-containing regulatory regions are modular, that is, contain adjacent binding sites for additional transcription factors and (4) diverse transcription factor occupancy at these regulatory regions correlates directly with the upregulation of the genes to which the corresponding *cis* elements are linked. Importantly, however, these data and additional studies[67–69] also point to several new principles of gene regulation by 1,25(OH)$_2$D$_3$ as well including the idea that regulatory regions of genes may be less frequently located near promoters than within introns or within intergenic regions often 10's if not 100's of kilobases distal to transcriptional start sites and furthermore that many such regions are generally involved. These approaches have also illuminated an additional concept, that is, that regulatory regions of gene (enhancers) are strongly highlighted by a

number of specific epigenetic histone marks, most predominant being histone H3K4 mono- and dimethylation (H3K4me1 and H3K4me2, respectively) and H3K27 acetylation (H3K27ac) (see **Figures 2a–e** for H3K4me1 and H3K4me2 marks at *Vdr*, *Tnfsf11*, *Lrp5*, *Cbs* and *Cyp24a1*).[70–72] Unique histone modifications also mark other features of genomic loci as well. These dynamic regulatory marks are imposed upon the genome by a diverse set of chromatin regulatory factors.[73] This epigenetic histone code for repeating features of the genome was identified and is supported by studies conducted through the ENCODE Consortium as well as other investigative groups.[70] Importantly, features of this code make it possible to localize regulatory regions of many genes without knowing precisely which transcription factors might participate in their expression.

Genome-wide principles of gene regulation by $1,25(OH)_2D_3$. As discussed above, ChIP-chip and now predominantly ChIP-seq analyses provide regulatory data for a diverse set of transcription factors on a genome-wide scale. This capability is particularly powerful because it can define thousands of sites of action for a specific factor, be used to understand the regulation of gene networks and enable the development of overarching principles of gene regulation.[70] It has also facilitated deep exploration of individual components involved in genome structure and function as well as those that predispose to disease. Of particular importance, this approach can be applied equally to studies conducted both *in vitro* and *in vivo*, permitting detailed molecular studies in animals that have not heretofore been possible. Along with others, we have conducted genome-wide studies of VDR/RXR binding in a variety of cell types. The following summary of these studies reveal a series of overarching principles that both confirm and extend those that were highlighted by the candidate genes described above. *Identification and quantitation of the VDR cistrome in osteoblasts and colorectal cancer cells*. Genome-wide analysis facilitates the identification and quantitation of all binding sites for a particular factor or feature on the genome of a specific target cell (termed a cistrome); it also facilitates detection of changes in that cistrome, which might occur as a function of an alteration in the cellular environment.[74,75] Accordingly, we conducted a genome-wide ChIP-chip analysis as well as subsequent ChIP-seq analyses of the VDR in osteoblasts and the colorectal cancer cell line LS180 in the absence and presence of $1,25(OH)_2D_3$, quantitating the number of binding sites identified on the genome and noting their relative locations.[65,66] In both cell types, VDR binding was low in the absence and strongly elevated in the presence of $1,25(OH)_2D_3$, reaching a limit in the presence of the hormone ranging from 2000 to 8000 sites per genome depending upon cell type. We also explored the locations of these individual binding sites, and, as seen for the five candidate genes described earlier, only a small percent were associated with transcriptional start sites; the vast majority (95%) was found either in introns or within intergenic regions adjacent to neighboring genes, often at significant linear distances. Both number and location were cell-type specific, a discovery not surprising in view of the fact that the regulation of genes by the vitamin D hormone is cell specific in nature.[65–67] Finally, *de novo* motif analysis revealed the frequent presence of motifs comprised of two directly repeated half-sites separated by 3 bp. Thus, on virtually thousands of regions across numerous cell types, VDR binding to DNA likely involves classic VDREs. The cell-specific nature, locations and presence of VDREs confirm properties that were observed in the candidate genes described above. *Features of the VDR/RXR cistrome*. Genome-wide analysis revealed numerous features of VDR-binding sites in additional to those above.[65,66] ChIP-chip/ChIP-seq analysis of RXR revealed that although the RXR cistromes tend to be less sensitive to $1,25(OH)_2D_3$ and larger than those of the VDR, likely reflecting the fact that RXR serves a complex role as heterodimer partner to a number of other nuclear receptors, the vast majority of the thousand or more VDR-binding sites per genome identified contain RXR. Genome-wide data reveal that VDR DNA binding is strongly influenced by the presence of $1,25(OH)_2D_3$, despite many examples where VDR is prebound to DNA in the absence of ligand and either unaffected or displaced in its presence. These and other experiments suggest that there may be several modes of VDR/RXR DNA binding. Whether these modes contrast mechanisms of gene activation vs suppression, dual roles that the vitamin D hormone performs equally well, are unknown. Indeed, where the mechanism of gene suppression by $1,25(OH)_2D_3$ has been investigated, both DNA-binding-independent (that is, CYP27B1 and PTH)[76,77] and DNA-binding-dependent[78–81] mechanisms have been identified. It should be noted as indicated above, however, that ChIP-seq analysis will be necessary to confirm these latter DNA-binding-dependent series of findings. Finally, an extensive analysis of the presence of epigenetic histone marks suggests that VDR/RXR binding occurs in regions where histone 3 is mono- and di-methylated at K4 and where histones 3 and 4 are extensively acetylated on several lysine residues including H3K27 (Meyer and Pike, unpublished). Moreover, the presence of the VDR at many of these sites upregulates the level of histone

Table 1 Overarching principles of vitamin D-mediated gene regulation

VDR-binding sites: 2000–8000 $1,25(OH)_2D_3$-sensitive binding sites/genome; cell-type specific.
Binding site locations: dispersed in CRMs (CRMs or enhancers) across the genome; located in a cell-type-specific manner near promoters, but predominantly within introns and distal intergenic regions; frequently located in clusters of elements.
Active transcription unit: the VDR/RXR heterodimer.
VDR/RXR-binding site sequence (VDRE): induction mediated by classic hexameric half-sites (AGGTCA) separated by 3 bp; repression mediated by divergent sites.
Mode of DNA binding: predominantly, but not exclusively, ligand dependent.
Modular features: CRMs contain binding sites for multiple transcription factors that facilitate both independent or synergistic interaction.
CRM signatures: defined by the dynamic presence of distinct epigenetic histone H3 and H4 modification.

Abbreviations: CRM, *cis*-regulatory module; VDR, vitamin D receptor; VDRE, vitamin D response element; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D3.

acetylation.[82] These findings support the overarching idea that on a genome-wide scale, like that seen for candidate genes, VDR/RXR-binding sites are located in regions of the genome that contain significant epigenetic enhancer features that provide strong regulatory potential. Overarching principles of $1,25(OH)_2D_3$ regulation, as determined on a genome-wide scale, are summarized in **Table 1**.

Linking regulatory regions to the genes they modulate. One consequence of the discovery that regulatory regions are frequently located large distances from the genes they control is that although their identification on the genome has become easier, identification of the genes they regulate has become much more difficult. The finding that chromatin structure is also exceptionally important also contributes to this difficulty. Although several ChIP-based approaches, including that of chromatin interaction analysis by paired-end tag sequencing, may ultimately provide detailed linkage at the genome-wide level, this highly bioinformatic-based linkage remains largely correlative.[83,84] We have used several approaches to link the actions of regulatory regions directly to the genes they regulate. These include the use of bacterial artificial chromosomes, which contain a specific target gene surrounded by as much as 250 kb of endogenous linear DNA.[37] The size of this segment of DNA frequently contains many candidate regulatory regions, which can be examined for their contribution to the internal gene of interest. A bacterial artificial chromosome clone can be recombineered (bacterial recombinant engineering) to contain a reporter function and then introduced in a stably integrated manner into the genomes of cells in culture and as transgenes into mice (**Figure 3**). Following characterization of the wild-type minigene in these backgrounds, enhancers can be removed and the consequence of this removal on activity assessed following reintroducing of the clone into the same cells or into the mouse genome. An additional approach has been to delete regulatory regions directly from the genome.[45] Although this approach is similarly useful, it is both expensive and difficult to thoroughly investigate genes at the genomic level if they contain multiple regulatory regions. Recent advances in genome-editing methods likely will expedite this approach and facilitate simultaneous examination of more than one region.[85] Along with others, we have used these methods to explore properties of both the *VDR* and *TNFSF11* genes, studies that have been described in depth previously. Importantly, these studies confirm the functional role of many of the regulatory regions that were identified initially by ChIP-chip and ChIP-seq methodologies.

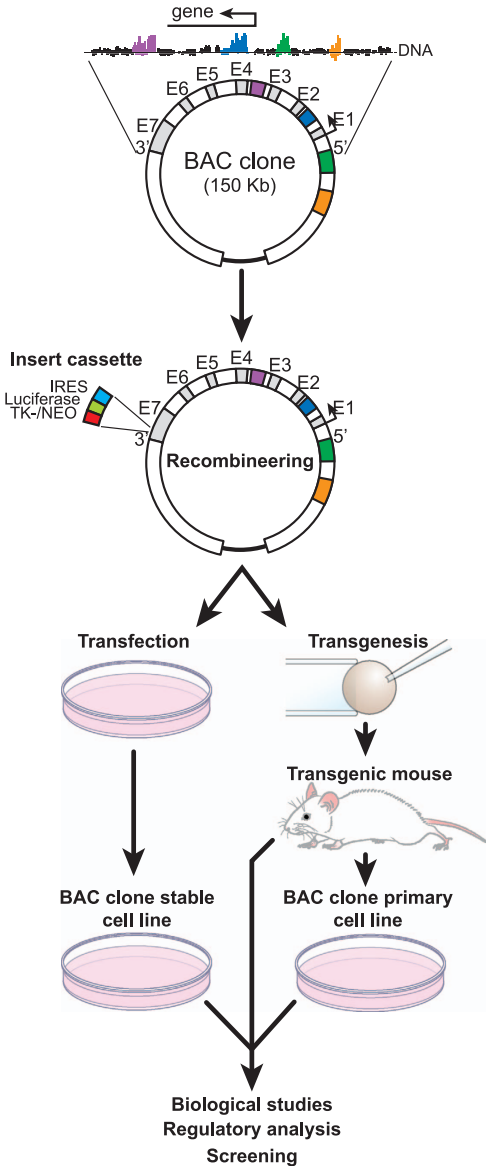

Figure 3 Methodology associated with the preparation and use of bacterial artificial chromosomes (BACs). Chromatin immunoprecipitation (ChIP)-seq analysis is used to define the regulatory locus of a specific gene. A BAC clone containing this segment of DNA is modified through recombineering methods and then introduced via stable transfection into culture cells or through transgenic methods into mice. Orientation of the target transcription unit (5′ and 3′) together with representative exons (E) is indicated. The recombineering cassette contains an internal ribosome entry site (IRES)/luciferase and a thymidine kinase promoter-neomycin gene (TK-NEO) component. Direction of transcription is indicated by the arrow.

Conclusions

Early studies of a few selected target genes provided significant insight into how $1,25(OH)_2D_3$ functions to regulate gene expression. The advent of ChIP, linked ultimately to unbiased methodologies that included the use of tiled microarrays and deep sequencing analysis, has revolutionized our approach to the study of gene regulation, and provided new insight into how $1,25(OH)_2D_3$ as well as other systemic and local factors operate to control the expression of genes. These unbiased methods have revealed not only novel features of VDR and RXR binding, but novel features of the regions to which they bind and how these features, both genetic and epigenetic, contribute to the regulation of gene expression. As a side note, many of these more recent observations suggest that the more traditional molecular biological approaches used in the study of transcription over the past several decades can no longer be relied upon to provide a complete and representative picture of how $1,25(OH)_2D_3$ functions. These findings do suggest, however, that much remains to be discovered in our ongoing efforts to understand how the vitamin D hormone functions to modulated the expression of genes and thus to direct the biological activities that are under its directive *in vivo*.

Conflict of Interest

The authors declare no conflict of interest.

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