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Emerging regulatory paradigms for control of gene expression by 1,25-dihydroxyvitamin D_3 ☆

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ABSTRACT

1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) functions as a steroid hormone to modulate the expression of genes. Its actions are mediated by the vitamin D receptor (VDR) which binds to target genes and functions to recruit coregulatory complexes that are essential for transcriptional modulation. ChIP analysis coupled to tiled DNA microarray hybridization (ChIP-chip) or massively parallel DNA sequencing (ChIP-seq) is now providing critical new insight into how genes are regulated. In studies herein, we utilized these techniques as well as gene expression analysis to explore the actions of $1,25(OH)_2D_3$ at the genome-wide and individual target gene levels in cells. We identify a series of overarching principles that likely define the actions of $1,25(OH)_2D_3$ at most target genes. We discover that while VDR binding to target sites is ligand-dependent, RXR binding is ligand-independent. We also show that while VDR/RXR binding can localize to promoters, it occurs more frequently at multiple sites many kilobases from target gene promoters. We then describe a new method whereby the regulatory regions of complex genes can be evaluated using large recombineered bacterial artificial chromosomes. We conclude that these new approaches are likely to replace many of the traditional methods used to explore the regulation of transcription.

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1. Introductory background

1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) functions as a systemic endocrine signal in vertebrate organisms to control the expression of genes whose products are vital to the control of cellular growth, differentiation and function and to the maintenance of calcium and phosphorus homeostasis as well [1]. This capability is mediated by the vitamin D receptor (VDR), which binds as a retinoid X receptor (RXR) heterodimer to specific regulatory sequences near target genes where it functions to coordinate the diverse activities of a series of coregulatory complexes essential for altering transcriptional output [2]. The interaction of these complexes with the VDR/RXR heterodimer is mediated via the activation domains (AF-2) of both VDR and RXR [3,4] and LXXLL or FXXLF motifs located within key components of coregulatory complexes [5] that are highly dynamic [6]. Their functions include unique modifications of histones, the repositioning of nucleosomes and the recruitment of RNA polymerase II and likely other activities as well [7,8]. Despite a basic understanding of coregulator function, however, much of the detail related to how these molecular machines operate at regulatory sites to alter gene expression remains obscure.

The study of gene transcription as it relates to the above processes has relied heavily over the past several decades on the capacity to introduce synthetic gene-reporter chimeras into cells through transfection and to explore their transcriptional activity via cotransfection of cofactors and via mutagenesis. This approach has been aided by a number of biochemical protein–protein and protein–DNA interaction assays that have been used to provide additional mechanistic support. More recently, however, chromatin immunoprecipitation analysis (ChIP) has emerged as a highly favorable technique for studying key features of gene regulation [6,9–12]. Far more revealing, however, has been the coupling of the ChIP technique to tiled DNA microarray hybridization (ChIP-chip analysis) [13] or to direct massively parallel DNA sequencing (ChIP-seq analysis) [14–16] to interrogate large regions of the genome in an unbiased fashion. These techniques, together with the use of large bacterial artificial chromosomes (BAC clones), are now providing fundamental new insight into the overall structural organization of genes, the unique properties of gene promoters, the location and nature of regulatory components, and the mechanisms whereby coregulatory complexes function to alter the expression of the genes to which they are recruited. Surprisingly, many of the

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long held dogmas related to the regulation of gene transcription are proving to be fundamentally flawed. Applied at the genome-wide level and coupled to similarly scaled gene expression studies, these techniques promise to advance our understanding of mechanisms that are central to the cellular control of gene expression during development, growth and differentiation as well as to the mechanisms that lead to the loss of this control as a result of cancer.

In this report, we explore the genomic actions of $1,25(OH)_2D_3$ in bone and intestinal cells using both ChIP-chip, ChIP-seq and gene expression analyses at the level of the entire genome. We define a set of overarching principles that govern the actions of $1,25(OH)_2D_3$ at the genome-wide level, and then document several of our general principles at the level of the mouse *Vdr* gene, one key $1,25(OH)_2D_3$ target. We then utilize new analytical approaches to explore further hypotheses generated from our ChIP-chip and ChIP-seq studies.

2. The methodologic approach

As outlined above, we have used ChIP analysis [6] coupled to tiled DNA microarray hybridization (ChIP-chip) and/or massively parallel sequencing (ChIP-seq) analyses [13,16,17] to explore the actions of $1,25(OH)_2D_3$ and other hormonal regulators at both the genome-wide, targeted genome-wide, and individual gene levels in vitamin D target cells. The results of these studies are then further explored using a number of additional techniques that include enhancer fragment and BAC clone analyses.

2.1. ChIP analysis

ChIP analysis was carried out as previously described [7,22]. In brief, cultured cells were treated with either vehicle or inducer for specific periods of time, subjected briefly to formaldehyde, lysed, and then sonicated to solubilize defined chromatin fragments ranging from 500 to 2000 bp. Protein-bound chromatin was then immunoprecipitated using specific antibodies capable of recognizing various DNA binding proteins, tethered transcription factors, cofactors, or uniquely modified histones that serve a potential regulatory role in transcription. Following immunoprecipitation, the chromatin-DNA fragments were isolated and the presence of unique and identified segments of DNA examined using real time PCR analysis.

2.2. ChIP-chip analysis

ChIP-chip analysis is performed as previously described [18–20]. Briefly, DNA derived from the ChIP experiments conducted as above were amplified using ligation-mediated PCR, conjugated to Cy3 and Cy5 fluorescent dyes, and then hybridized to tiled mouse or human DNA microarrays. Genome-wide scans were conducted using Roche NimbleGen whole genome tiling arrays in the HD2 format (high density 2.1 million probe arrays) [23]. Targeted genome-wide custom arrays were designed to interrogate a collection of target genes determined previously by global gene expression analysis. These arrays were created in a cell-specific manner and spanned at least 200 kb of each genetic locus at approximately 100 bp resolution. Data were extracted using NimbleScan (version 2.5) software (Roche NimbleGen, Inc) and evaluated using lowess normalization in *R*. The $\log_2$ ratios of test vs experimental data were calculated for each point and peaks were called at an FDR of $p < 0.05$ using CMARRT algorithms [21]. Data shown are representative of two or more ChIP-chip analyses performed for each experimental design. All data were visualized using Gbrowse (www.gmod.org/wiki/Gbrowse) [22].

2.3. ChIP-seq analysis

ChIP-Seq analysis was performed as previously described [17] using a SOLiD Sequencer (ABI, Foster City, CA). Briefly, DNA derived from ChIP experiments were sonicated into 150 bp fragment size and then used to prepare libraries using the AB SOLiD System 2 Lower Input Fragment Library Preparation protocol. Samples were then sequenced. Corrected density files were merged and mapped at 30 bp resolution to the human hg18 reference genome (UCSC Genome Browser, http://www.genome.ucsc.edu) using MACS v1.3.5.

2.4. Enhancer and gene locus (BAC clone) analyses

The boundaries of specific gene regulatory regions identified by ChIP-chip and/or ChIP-seq were refined using sequence conservation analysis and the fragments (400–1500 bp) then cloned into either a heterologous reporter vector (pTK-luciferase) or a minimal promoter reporter vector wherein the promoter corresponded to the gene of interest as previously described [24,25]. Reporters were introduced into host cells using Lipofectamine. BAC clones were obtained commercially and modified through recombineering methods to contain an IRES-luciferase reporter/PGK-neomycin selection cassette in the final 3′ noncoding exon as described [26]. Further modifications were achieved using the GalK selection system as described [27]. Modified BAC clones were linearized, introduced into host cells using Lipofectamine and stable cell collections identified using G418 as previously documented [28].

3. Experimental results and discussion

3.1. Mechanisms of gene regulation by $1,25(OH)_2D_3$

We have used ChIP-chip and ChIP-seq analyses to identify under both basal and $1,25(OH)_2D_3$-treated conditions all VDR and RXR binding sites in the mouse MC3T3-E1 pre-osteoblastic cell genome (termed cistrome analysis) [23]. These techniques have also been used to examine similar activities in human colonic LS180 cells [29]. We have also explored the consequence of this genome-wide binding of VDR and RXR on site-specific coregulator and RNA polymerase II recruitment and changes in levels of histone H4 acetylation as well. Each of these activities was then correlated with the results of a classic gene expression analysis wherein the effects of $1,25(OH)_2D_3$ were assessed on the transcriptional output of individual genes within the genome. A set of overarching principles identified through these analyses is summarized in the following sections.

3.1.1. Overarching principles of VDR/RXR regulation

Genome-wide ChIP-chip analysis has revealed several overarching principles of regulation by $1,25(OH)_2D_3$ in MC3T3-E1 cells [23]. First, approximately 5000 overlapping VDR/RXR binding sites exist within this bone cell's genome at a false discovery rate (FDR) of $p < 0.05$. This number comprises most of the VDR binding sites identified but only a small portion of the RXR binding sites, thereby highlighting the diverse roles of RXR in activities independent of the vitamin D endocrine system. Second, while VDR binding to the genome is largely dependent upon activation by $1,25(OH)_2D_3$, RXR binding to these same sites is mostly, although not exclusively, ligand-independent. This finding is surprising in view of the existing dogma which posits that $1,25(OH)_2D_3$ either induces heterodimer formation followed by DNA binding or activates a receptor complex that is pre-bound to DNA. Third, the bulk of sites that bind both VDR and RXR can be shown through bioinformatic analyses to contain classic vitamin D response elements or VDREs comprised of a previously determined motif of two hexanucleotide

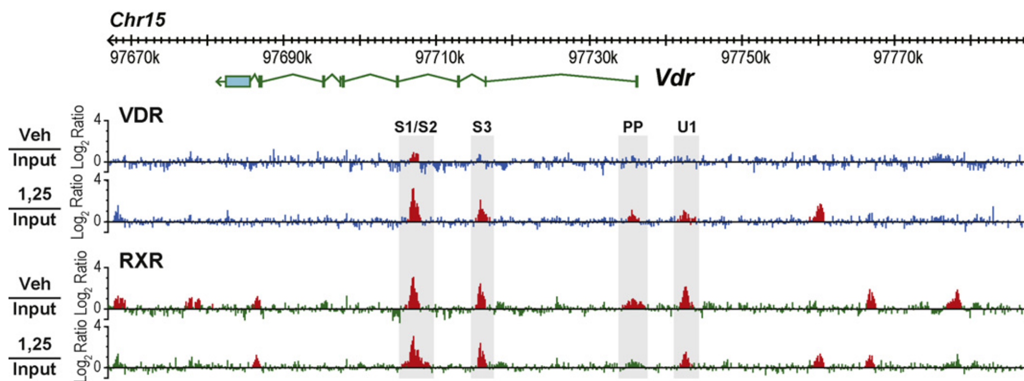

Fig. 1. ChIP-chip analysis of basal and 1,25$(OH)_2D_3$-induced VDR and RXR binding to the mouse *Vdr* gene. MC3T3-E1 cells were treated with either vehicle or 1,25$(OH)_2D_3$ for 3 h and then subjected to ChIP-chip analysis using antibodies to VDR or RXR. A schematic diagram of the mouse *Vdr* gene locus and its location on chromosome 15 is depicted at the top. Previously identified enhancers are designated S1/S2, S3, PP and U1 and marked through vertical shading. VDR data tracks represent the $\log_2$ ratios of fluorescence obtained from vehicle- or 1,25$(OH)_2D_3$-treated samples precipitated with antibody to the VDR vs corresponding sample input DNAs. RXR data tracks correspond to equivalent analyses. Red peaks represent statistically valid regions of VDR or RXR binding (FDR < 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

repeats separated by 3 bp [30,31]. Our analyses confirm that only one of these half-sites is likely to be highly conserved relative to the consensus sequence AGGTCA; the second is routinely degenerate. Fourth, binding sites can be located proximal to the start site of transcription (TSS), but are far more frequently located upstream, internal and/or downstream of the transcription unit. In most cases, they are located at sites that are 10s if not 100s of kilobases from the target gene's transcriptional start site. In addition, multiple distinct binding sites are generally present such that regulatory elements surround the target gene and often define the gene's extended locus. Fifth, the binding of both VDR/RXR is often associated with coregulator recruitment, changes in the levels of histone modifications and changes in RNA polymerase II density. Interestingly, the latter often occur at gene promoters in the absence of VDR/RXR binding sites, thus providing a link between upstream regulatory regions and their target genes. Changes in RNA polymerase II density are also routinely observed at sites associated with distal VDR/RXR binding as well. These observations indicate a potential role for enhancers as recruitment centers for the polymerase activity ultimately initiated at the gene's promoter. With regard to coregulator recruitment and changes in histone modification, both are generally, although not always, centered at VDR/RXR binding sites and modulated by the presence of 1,25$(OH)_2D_3$. Regardless of the details, these observations indicate collectively that VDR and RXR binding manifests downstream functional consequences on the transcriptional output of associated target genes. Thus, ChIP-chip analysis provides direct evidence for not only VDR/RXR binding activity, but for activities that arise as a consequence of this binding as well. Sixth, enhancers often contain binding sites for additional regulatory transcription factors. This feature is indicative of modularity, a classic characteristic of true enhancers. Seventh, bioinformatic analyses reveal that the regulatory regions defined by ChIP-chip analysis in MC3T3-E1 cells are located within 50 kb of approximately 3500 genes, 1000 of which are either induced or suppressed at least 2-fold by 1,25$(OH)_2D_3$. This observation suggests that the presence of one or more functional VDR/RXR enhancers reflects only the potential for this element to regulate the expression of a proximal gene. Whether it does so or not appears to be dependent upon a number of additional factors, most of them unknown. For example, perhaps VDR/RXR binding is permissive in some cases for the subsequent activity of an unrelated signaling pathway. Similar mechanistic principles have been identified for the actions of 1,25$(OH)_2D_3$ in human LS180 cells.

Our ChIP-chip and ChIP-seq analytical approaches in both MC3T3-E1 and LS180 cells have revealed in a fully unbiased manner a series of new principles through which 1,25$(OH)_2D_3$ functions to regulate gene expression. In view of the genome-wide scope of these studies across two cell lines, its seems likely that these principles will be generally applicable to the actions of 1,25$(OH)_2D_3$ in all cells and tissues. These results are strengthened by the very nature of the ChIP technique, which derives data from unmodified cells or tissues that express endogenous levels of both VDR and RXR as well as other components of the transcriptional regulating machinery and whose actions are measured on endogenous genes located in their normal position on their respective chromosomes. It is therefore not difficult to appreciate that this approach is leading to a sweeping change in the way investigators explore mechanisms of transcription or to imagine that this approach is likely to lead to many additional novel insights into how hormones such as 1,25$(OH)_2D_3$ and certainly other regulators selectively control the expression of genes.

3.1.2. Activities of 1,25$(OH)_2D_3$ on selected target genes

The above principles describe the activity of 1,25$(OH)_2D_3$ across all target genes within the genomes of MC3T3-E1 and LS180 cells. These principles are also generally reflected at a number of specific target genes whose regulation by 1,25$(OH)_2D_3$ is well known. These include such classic genes as osteocalcin (*Bglap*), *Cyp24a1*, and osteopontin (*Spp1*) as well as the genes for *LRP5*, *TRPV6*, and the *VDR* itself [31]. In the latter case, we speculate that autoregulation by 1,25$(OH)_2D_3$ serves to enhance and replenish VDR protein levels in cells where this phenomenon occurs. Fig. 1 depicts a ChIP-chip profile of VDR and RXR activity generated across the mouse *Vdr* gene in response to a 3-h treatment with either vehicle or 1,25$(OH)_2D_3$, and serves as a typical example of many of the principles identified in the above summary. The *Vdr* gene and its exons and introns are illustrated at the top of the figure relative to its position on chromosome 15, and the locations of previously described enhancers for the *Vdr* gene are indicated by S, PP or U [25,26]. Regions in red represent statistically valid peaks of VDR or RXR binding. As can be seen, VDR binding is generally absent under basal conditions, but strikingly increased at S1/S2, S3, PP and U1 following treatment with 1,25$(OH)_2D_3$. RXR, on the other hand, is almost fully bound to many sites on the *Vdr* gene, including those found at S1/S2, S3, PP and U1. Thus, treatment with 1,25$(OH)_2D_3$ leads to a general loss of RXR at ancillary sites but generally preserves RXR binding at enhancers where the VDR has been induced to bind. These results highlight the concept that while VDR binding is generally ligand-dependent, RXR binding is ligand-independent. They also demonstrate several additional

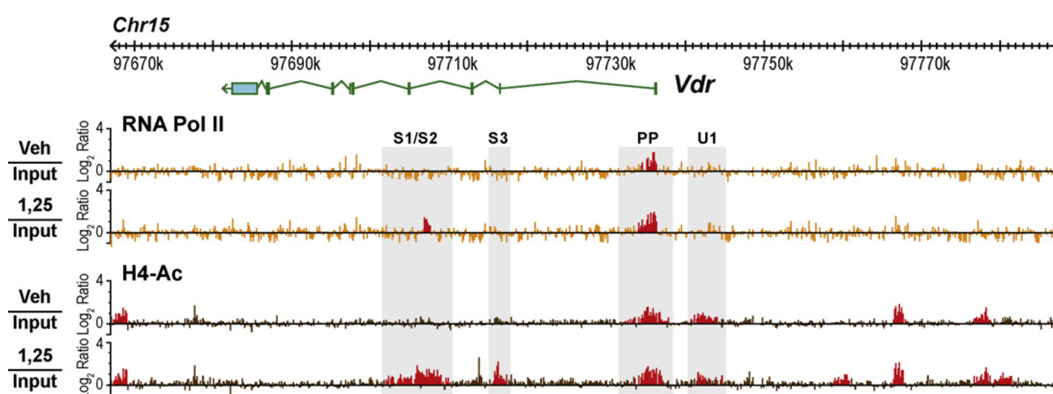

Fig. 2. ChIP-chip analysis of basal and $1,25(OH)_2D_3$-induced RNA polymerase II density and histone 4 acetylation analysis to the mouse *Vdr* gene. The schematic at the top of the figure is described in Fig. 1. RNA polymerase II data tracks (RNA pol II) represent the $\log_2$ ratios of fluorescence obtained from vehicle- or $1,25(OH)_2D_3$-treated samples precipitated with antibody to the RNA polymerase II vs corresponding sample input DNA's. Histone H4 acetylation data tracks (H4-Ac) correspond to equivalent analyses. Red peaks represent statistically valid regions of VDR or RXR binding (FDR < 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

features of gene regulation discovered as a result of our genome-wide analysis, that of number and distribution. Thus, at least four enhancers located intronically as well as significantly upstream of the gene's TSS are found to control *Vdr* gene expression. At the genome-wide level, intronic and upstream as well as downstream enhancers comprised over 80% of the regulatory sites observed [23].

Fig. 2 depicts ChIP-chip profiles of RNA polymerase II density and histone 4 acetylation levels in response to a 3-h treatment with either vehicle or $1,25(OH)_2D_3$. These measurements represent markers of gene activation and serve to highlight additional principles summarized in the above section. As can be seen, while RNA polymerase II is present at the *Vdr* gene promoter under basal conditions, likely due to the fact that the *Vdr* gene is expressed in MC3T3-E1 cells under these unstimulated conditions, its levels are increased significantly in response to $1,25(OH)_2D_3$, both at the promoter and at the S1/S2 enhancer. Likewise, H4 acetylation is also present under basal conditions and enhanced in response to $1,25(OH)_2D_3$. This upregulation of acetylation marks is apparent at all sites where both VDR and RXR were shown to bind (Fig. 1). These data therefore provide supportive evidence that the sites to which both VDR and RXR bind in response to $1,25(OH)_2D_3$ also display activities that are consistent with changes in gene expression. Thus, ChIP-chip analysis provides insight into not only the locations of VDR/RXR regulated enhancers, but measurements which are consistent with their transcriptional enhancing activities as well.

Fig. 3 summarizes the results of extensive ChIP-chip analyses of the activities of additional inducers of *Vdr* gene expression including retinoic acid, glucocorticoids and forskolin (a parathyroid hormone surrogate) [26]. As can be seen in this cartoon, retinoic acid-induced retinoic acid receptor (RAR), glucocorticoid-induced glucocorticoid receptor (GR) and forskolin-induced cyclic AMP-response element binding protein (CREB) can also be found at many of the enhancers identified originally for the VDR/RXR heterodimer. RAR and GR can be found at newly identified enhancer sites as well. A further examination of the presence of additional factors such as C/EBPβ and RUNX2, transcription factors involved in bone cell maturation and differentiation, revealed that they also can also be found at *Vdr* gene enhancers. Importantly, putative regulatory sequences known to bind each of these factors can also be found within these specific enhancer regions as well. These results support the concept of modularity, an additional characteristic of classic enhancer region.

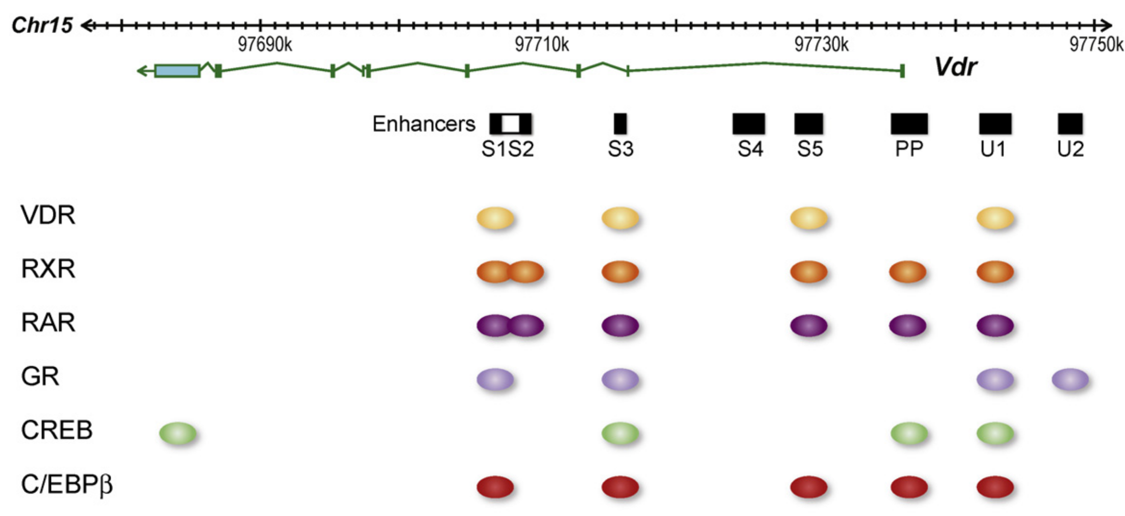

Fig. 3. Summary model documenting the interaction of transcription factors at the mouse VDR gene locus. MC3T3-E1 cells were treated with either vehicle, $1,25(OH)_2D_3$, retinoic acid, dexamethasone or forskolin for 6 h and then subjected to ChIP-chip analysis using antibodies to VDR, RXR, RAR, GR, CREB or C/EBPβ. Positive binding activity in the presence of the appropriate activating ligand is indicated by a colored circle at enhancers identified for the mouse *Vdr* gene. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

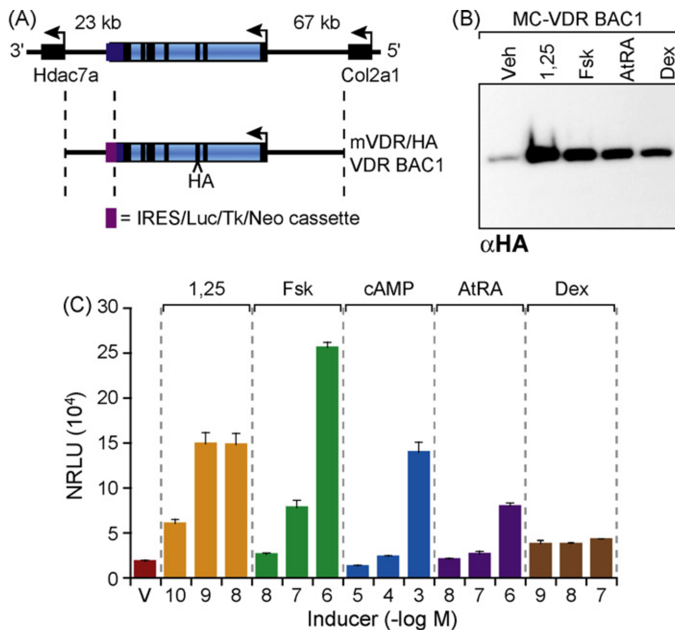

Fig. 4. BAC clone analysis of the mouse *Vdr* gene locus in stably transfected MC3T3-E1 cells. Cells were stably transfected with the recombinant *Vdr* BAC clone depicted in (A). (B) Western blot analysis of recombinant VDR expression in MC3T3-E1 cells stably transfected with the recombinant *Vdr* BAC clone depicted in (A) and its induction by $1,25(OH)_2D_3$, RA, Dex, forskolin. (C) Analysis of luciferase activity in MC3T3-E1 cells stably transfected with the recombinant *Vdr* BAC clone depicted in (A) and its dose dependent induction by $1,25(OH)_2D_3$, RA, Dex and forskolin. Data represent the average of a triplicate set of reactions ± SEM.

3.1.3. Exploring $1,25(OH)_2D_3$ regulatory activity in complex genes

The discovery by ChIP-chip analysis that many if not most genes are regulated by multiple enhancers often located far from their promoters adds significant complexity to subsequent studies aimed at assessing both the collective contribution of multiple enhancers as well as the unique contribution of individual enhancer to the expression of a linked gene. Indeed, this complexity at the genome-wide level also makes it difficult to determine which gene represents the specified target for a potential enhancer. The ability of a ligand to regulate the expression of one gene but not others in the vicinity can provide a clue as to the target of enhancer activity, although as indicated above, this is not absolute. To explore the contribution and roles of individual enhancers in the regulation of genes that are directly linked, we have turned to using BAC clones generally large enough (~200 kb) to contain not only the transcription unit itself but extensive sequence located both upstream and downstream as well [28]. These large DNA constructs can be modified using recombineering techniques to contain both an IRES-luciferase reporter and a selectable marker inserted into the final 3′ exon, transfected in a stable manner into host cells and evaluated for basal and inducible activity. Importantly, mutations can be introduced into these clones enabling an analysis of individual enhancers in the context of the entire gene locus. Perhaps most importantly, the genes are fully integrated into the genome, thus providing a normal chromatin environment in which to explore potential regulation. Their size together with the presence of insulators, which can be identified through detection of CTCF (CCCTC binding factor) binding sites, significantly reduces position effects. Fig. 4 summarizes the data we have obtained following construction of a recombineered mouse *Vdr* gene BAC clone containing a hemagglutinin (HA) tag at the translational start site and the preparation of stable MC3T3-E1 cells. The construct itself is depicted in Fig. 4A. As can be seen in Fig. 4B, the recombineered VDR protein is detected under basal conditions by western blot analysis and strongly induced by not only $1,25(OH)_2D_3$, but by retinoic acid (AtRA), dexamethasone (Dex), and forskolin (Fsk) as well. Analysis of the luciferase activity derived from the BAC clone stable cell line, as documented in Fig. 4C, confirms both basal expression of the BAC clone and its inducibility by each of the above regulators. We conclude that this large DNA construct recapitulates the regulatory capabilities observed from the endogenous mouse *Vdr* gene in MC3T3-E1 cells. Further studies reveal that deletion of the S1 enhancer attenuates but does not fully abrogate the inducible effects of $1,25(OH)_2D_3$, supporting a contributory role for the S1 enhancer in $1,25(OH)_2D_3$ mediated auto-induction of the *Vdr* gene [26]. Traditional analyses using isolated DNA fragments linked to a heterologous promoter/reporter plasmid provided additional data on several unique properties of each of the individual enhancers [20]. These and other approaches, such as deletion of enhancers directly in the mouse genome [32], are now being utilized to explore the roles of enhancers in gene activation *in vivo*.

4. Concluding remarks and future perspectives

The results described above outline new approaches to the exploration of mechanisms that underlie the regulation of gene expression by $1,25(OH)_2D_3$. An advantage of these approaches is that they can provide both a genome-wide as well as an individual gene perspective. With respect to the former, we have defined a new set of overarching principles whereby $1,25(OH)_2D_3$ likely acts to regulate the expression of virtually all genes. At a specific target gene level, we have highlighted several of these principles at the mouse *Vdr* gene itself while simultaneously providing specific details as to the location, properties and activity of individual regulatory regions that control this gene's expression. Finally, we provide evidence for a new approach to the downstream analysis of these complex genes using stably transfected BAC clones as well as isolated enhancer fragments linked to traditional reporters. It is clear from our own studies as well as those of others that these new approaches to transcription research will reveal not only fundamental new insight, but will likely also replace many of the traditional methods whereby mechanisms of gene regulation have been elucidated.

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