Journal of Steroid Biochemistry & Molecular Biology 121 (2010) 130–135

Contents lists available at ScienceDirect

Journal of Steroid Biochemistry and Molecular Biology

journal homepage: www.elsevier.com/locate/jsbmb

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

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ARTICLE INFO

Article history:
Received 2 November 2009
Received in revised form 12 February 2010
Accepted 25 February 2010

Keywords:
Genome-wide analysis
ChIP-chip analysis
ChIP-seq analysis
VDR/RXR binding
Distal transcriptional regulation
Histone acetylation
RNA polymerase II recruitment
BAC clone evaluation

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.

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

☆ Special issue selected article from the 14th Vitamin D Workshop held at Brugge, Belgium on October 4–8, 2009.

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doi:10.1016/j.jsbmb.2010.02.036

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