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Enhancers located in the vitamin D receptor gene mediate transcriptional autoregulation by 1,25-dihydroxyvitamin D_3

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Abstract

The regulatory actions of 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) on target genes are mediated by the vitamin D receptor (VDR). Interestingly, one of the genomic targets of $1,25(OH)_2D_3$ action is the VDR gene itself; however, the mechanism underlying this regulation is unknown. We investigated VDR autoregulation by screening the mouse VDR locus from 20 kb upstream of the transcriptional start site (TSS) to 10 kb downstream of the last exon using chromatin immunoprecipitation (ChIP)-DNA microarray analysis (ChIP/chip). Three potential VDR binding sites were located within introns lying downstream of the TSS and their activities confirmed through direct ChIP analysis. Further exploration revealed that one of these intronic regions was capable of conferring $1,25(OH)_2D_3$ response to both a downstream heterologous promoter and the minimal VDR promoter. Importantly, this regulatory region contained a classic vitamin D response element and was highly conserved within the human gene. We also demonstrated using ChIP analysis that the binding of VDR is associated with co-localization of RXR and the enhanced entry of RNA polymerase II. Thus, each of these sites appears likely to contribute to VDR autoregulation. Our studies using ChIP/chip analysis coupled to more traditional approaches define a direct mechanism whereby the VDR gene is upregulated by $1,25(OH)_2D_3$.

Keywords: ChIP; ChIP/chip analysis; Vitamin D receptor gene; Vitamin D autoregulation; VDRE; RNA polymerase II recruitment center; Histone acetylation; Transactivation

1. Introduction

1,25-Dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) regulates mineral homeostasis in vertebrates through its ability to control the expression of gene networks in the kidney, intestine, and bone. Its actions are mediated by the vitamin D receptor (VDR), which functions as an RXR heterodimer and aids in the recruitment of coregulatory proteins necessary for chromatin modification and transcriptional activation [1]. Numerous genes are responsive to the hormone including those involved in the transport of calcium and phosphate, bone formation, and bone resorption [2]. $1,25(OH)_2D_3$ is also able to regulate the expression of the VDR gene itself. VDR autoregulation has been observed in a variety of bone-related culture models and in certain vitamin D_3 target tissues in vivo [3]. While there is sufficient evidence to support the upregulation of VDR by $1,25(OH)_2D_3$, the mechanism is still unclear due to the complexity of the promoter region(s) in the human and mouse genes and the absence of any identifiable VDREs.

In the present study, we used high resolution ChIP-chip analysis to identify three conserved VDR binding regions located substantial distances downstream of the gene's transcriptional start site (TSS). One of these regions, S1, was able to confer $1,25(OH)_2D_3$ regulation to both a heterologous and a native promoter, which allowed us to identify the functional VDRE located within. Furthermore, this region exhibited high sequence homology to a region located within the human VDR gene, which also showed transcriptional activity when transfected into cultured bone cells. Finally, ChIP analysis indicated that VDR binding was accompanied by differential RXR and enhanced RNA polymerase II (RNA pol II) recruitment. Our results identify a direct autoregulatory mechanism whereby $1,25(OH)_2D_3$ is able to induce the synthesis of VDR mRNA.

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2. Methods

2.1. Tiled oligonucleotide microarray analysis

ChIP/chip analysis was carried out as described by others [4–6]. In brief, DNA was isolated by specific immunoprecipitation using the ChIP methodology described previously [7] and then subjected to ligation mediated PCR [8]. The resulting ~500 bp amplicons were labeled with Cy3 or Cy5 dyes using an indirect labeling protocol [8]. Cy3 and Cy5 labeled DNA samples were then mixed in the presence of CoT-1 DNA, denatured and co-hybridized to a custom oligonucleotide microarray (Nimblegen Systems Inc, Madison, WI) as described [4]. The microarray probes consisted of maskless array, in situ-synthesized 50-mer oligonucleotides at 2 bp intervals representing an 84 kb screen of the mouse VDR gene from 20 kb upstream of the gene's TSS to 10 kb downstream of the final 3′ non-coding exon. After sample co-hybridization, the microarrays were washed extensively and scanned using an Axon 4000B scanner at the appropriate wavelengths. The logarithmic enrichment ratio of Cy5 to Cy3 hybridization intensities (log 2) were plotted as a function of chromosome nucleotide position.

2.2. Transfection assays

MC3T3-E1 cells were cultured in α-MEM containing 10% FBS. Transfections were carried out in a 24-well plate (5×10^4 cells/well) using Lipofectamine PLUS (Invitrogen Corporation, Carlsbad, CA) as described by the manufacturer to introduce DNA and siRNA duplexes (Dharmacon RNA technologies, Lafayette, CO) into the cells. After transfection, the cells were cultured in medium supplemented with 20% FBS with or without $1,25(OH)_2D_3$. Cells were harvested 24 h after stimulation and the lysates assayed for luciferase and β-galactosidase activities as previously described [9]. Luciferase activity was normalized to β-galactosidase activity in all cases.

2.3. Statistical analyses

All values are expressed as mean ± S.E.M. We evaluated differences between experimental groups by using an unpaired *t*-test or one way analysis of variance (ANOVA) with Dunnett's multiple comparison post test. All statistical calculations were performed using the GraphPad PRISM Version 4 statistical software package.

3. Results and discussion

Previous attempts to define a vitamin D regulatory region in either the mouse or human VDR genes have been uniformly unsuccessful. Based upon this failure, we elected to utilize a chromatin immunoprecipitation method combined with DNA microarray analysis (ChIP-chip analysis) [4] to scan the entire mouse VDR gene locus as documented in Fig. 1A (the VDR gene is transcribed on the reverse strand). Given that VDR upregulation has been documented extensively in cultured osteoblastic cell lines, including our own observations in both ST2 and MC3T3-E1 cells [10], we isolated DNA from ST2 cells treated with vehicle or $1,25(OH)_2D_3$ for 6 h using standard ChIP methods. Following amplification, the DNA was co-hybridized to a high density oligonucleotide DNA microarray (see Section 2). A comparison of hybridization signals generated from the scan can be seen in Fig. 1B.

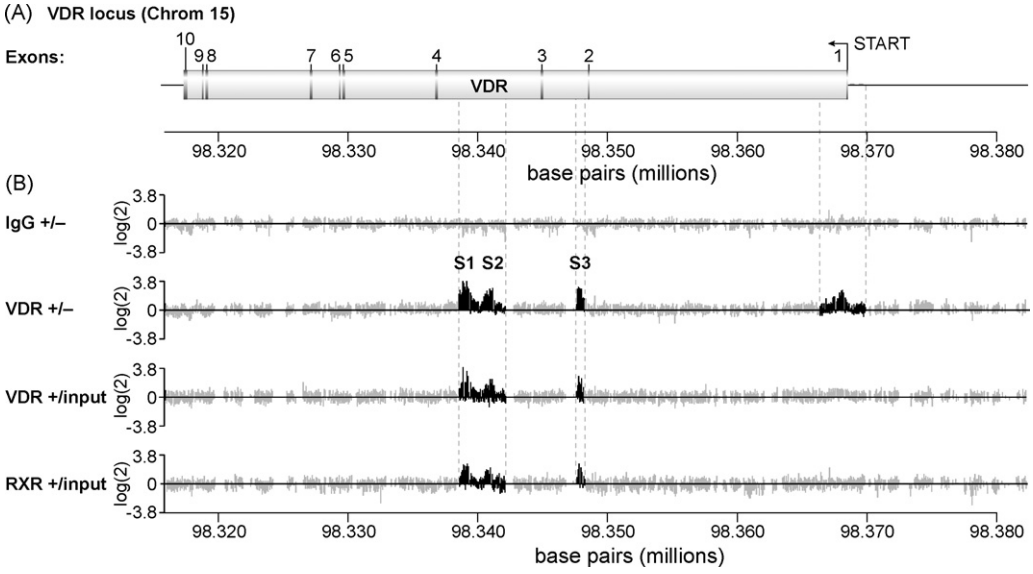

Fig. 1. ChIP/Chip analysis of the VDR locus identifies VDR-binding regions. (A) Schematic diagram of the mouse VDR gene and its 10 exons. The nucleotide base pairs indicate nucleotide location on chromosome 15 (May 2004 assembly). (B) Individual data tracks representing the enrichment ratios of Cy5 to Cy3 hybridization intensity ((log(2)) for each comparison as indicated.