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Multiple enhancer regions located at significant distances upstream of the transcriptional start site mediate RANKL gene expression in response to 1,25-dihydroxyvitamin D_3

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Abstract

One of the primary regulators of receptor activator of NF-κB ligand (RANKL) is 1,25-dihydoxyvitamin D_3 ($1,25(OH)_2D_3$). To elucidate the mechanism whereby $1,25(OH)_2D_3$ activates RANKL expression we screened some 300 kb of the RANKL gene locus using a ChIP on chip analysis and identified five potential regulatory regions lying significant distances upstream of the transcription start site (TSS), the farthest over 70 kb from the TSS. A direct ChIP analysis confirmed the presence of the VDR/RXR heterodimer at these sites. The binding of the VDR was associated with histone modification and enhanced entry of RNA polymerase II, indicating an important functional consequence to the localization of these transcription factors in response to $1,25(OH)_2D_3$. The region −76 kb upstream from the TSS, termed D5, was capable of mediating VDR-dependent transcriptional output in response to $1,25(OH)_2D_3$ in luciferase assays. The identified VDRE in this region was able to confer dramatic $1,25(OH)_2D_3$ sensitivity to heterologous promoters. This region was highly evolutionarily conserved and functionally active in the human RANKL gene as well. We propose that the RANKL gene is regulated via multiple enhancers that while located at significant distances from the TSS, likely form a chromatin hub centered on the *RankL* promoter.

Keywords: RankL; ChIP; ChIP/chip; Vitamin D receptor; Enhancer; VDRE; Glucocorticoids; RNA polymerase II recruitment center; Osteoblasts; 1,25-Dihydroxyvitamin D_3

1. Introduction

RankL is the molecule that is now considered to be both necessary and sufficient for osteoclastogenesis *in vivo* and *in vitro*. RankL is a TNF-like factor that is produced by stromal cells and osteoblasts as well as a variety of other cell types [1]. This factor actively promotes not only the process of osteoclast differentiation, but is also required for the cell's bone resorbing activity and for its survival [2]. It is evident that RankL and its receptor Rank are essential in osteoclast formation, which is most strongly supported by the skeletal phenotypes of both *RankL*- and *Rank*-null mice, neither of which are capable of producing osteoclasts *in vivo* [3,4]. Abnormalities in the expression of RankL are implicated in a variety of bone diseases as extensively reported. RankL is synthesized and expressed on the surface of regulatory cells in response to 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) and other stimulators. Despite earlier studies [5,6], regulatory sites within the *RankL* gene that mediate the actions of $1,25(OH)_2D_3$ remain unclear [7,8].

The absence of *bona fide* target sites for VDR action within the first 8 kb of the *RankL* gene, as reported by us and others [7,8], prompted a more expansive approach to delineate *RankL* regulatory regions. We therefore used contemporary ChIP/chip analysis in this endeavor. We discovered five sites of action located at upstream regions from the mouse *RankL* gene TSS, the most distant at approximately 76 kb. This latter region, termed D5, was transcriptionally active in transfection studies and contained a unique VDRE sequence. This region as well as the VDRE sequence itself was highly evolutionarily conserved among species and functionally active in the human *RANKL* gene as well, suggesting a role of this region in the regulation of this gene by other transcriptional

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activators. We propose that the RankL gene is regulated via the D5 region through interaction with additional upstream VDR binding sites that create a chromatin loop for transferring pre-initiation complexes (PIC) to the downstream *RankL* promoter.

2. Materials and methods

2.1. Cell culture

Mouse MC3T3-E1 and ST2 osteoblastic cells were cultured in α-MEM and MEM-α medium, respectively. Primary calvarial osteoblasts (mOBs) were obtained as previously described [9] and cultured in α-MEM. Human osteosarcoma MG63 cells were grown in Dulbeccos's modified Eagle's medium (DMEM) supplemented with 1% non-essential amino acids. COS-7 fibroblasts were also cultured in DMEM. Each medium was supplemented with 10% fetal bovine serum obtained from Hyclone (Logan, UT), 100 U/ml penicillin and 100 μg/ml streptomycin.

2.2. Chromatin immunoprecipitation (ChIP) assay

Chromatin immunoprecipitation assays were performed as previously described [10].

2.3. Tiled oligonucleotide microarray analysis

ChIP/chip analysis was carried out as described by others [11–14]. In brief, DNA was isolated by ChIP methodology followed by ligation mediated PCR as described by Oberley et al. [14]. The resulting ∼500 bp amplicons were then labeled with Cy3 or Cy5 dyes using an indirect labeling protocol and mixed in the presence of CoT-1 DNA, denatured and co-hybridized to custom oligonucleotide microarrays (Nimblegen Systems Inc., Madison, WI) as described. The microarrays were washed extensively and scanned using an Axon 4000B scanner at the appropriate wavelengths.

Custom oligonucleotide arrays were synthesized by Nimblegen Systems, Inc (Madison, WI). The microarray probes consisted of maskless-array, *in situ*-synthesized 50-mer oligonucleotides at 2 bp intervals representing a screen of over 300 kb of the mouse RANKL gene locus from 200 kb upstream of the gene's TSS to 100 kb downstream of the final 3′ non-coding exon. The tiled arrays were synthesized in duplicate in both the forward as well as reverse directions, providing four independent measurements at each site within the gene. In addition, each analysis was carried out using two independently derived ChIP DNA samples. After sample co-hybridization, the logarithmic enrichment ratio of Cy5 to Cy3 hybridization intensities (log 2) were plotted as a function of chromosome nucleotide position. While all the peaks representative of enhanced VDR or RXR binding either in the presence of $1,25(OH)_2D_3$ or as compared to input DNA are presented as raw data, a peak finding algorithm was utilized to score the relative level of binding in the five regions identified [15].

2.4. Plasmids

The mouse RankL D5 (−76045/−74973) and human RankL D5 (−96903/−95805) regions were amplified from genomic DNA and cloned into the *Hin*dIII/*Bam*HI sites of the TK-luc vector. The mRL-VDRE (−75620/−75590) containing several overhanging 5′ and 3′ nucleotides were synthesized, annealed and similarly cloned into the *Hin*dIII/*Bam*HI sites of pTK-luc. All plasmid constructs were sequenced to verify successful cloning.

2.5. Transfection assays

MC3T3-E1 and/or ST2 cells were seeded into 24-well plates at appropriate densities and cultured in α-MEM or MEM-α medium containing 10% FBS. Cells were transfected 24 h later with Lipofectamine PLUS in serum and antibiotic-free medium. Individual wells were transfected with 250 ng of a luciferase reporter vector, 50 ng of pCH110-β-gal and 50 ng of pcDNA-hVDR (unless otherwise indicated). After transfection, the cells were cultured for 24 h in a medium supplemented with 20% FBS and treated with vehicle, $1,25(OH)_2D_3$, Dex or combination of both. Cells were then harvested and the lysates assayed for luciferase and β-galactosidase activities as previously described [16]. Luciferase activity was normalized to β-galactosidase activity in all cases.

3. Results and discussion

Earlier studies indicated that $1,25(OH)_2D_3$ induces *RankL* expression in a variety of osteoblast-like cells including the mouse ST2 cell line [6]. In order to find VDRE sequences responsible for induction of this gene, we used ChIP–chip analysis to scan the entire mouse *RankL* gene locus for VDR binding sites. ST2 cells were first treated for 6 h with either vehicle or $1,25(OH)_2D_3$ and then subjected to a standard chromatin immunoprecipitation (ChIP) using antibodies to VDR, RXR or a non-specific IgG. Fig. 1 documents the hybridization signals generated across this tiled array for the following comparisons: (1) IgG in the presence and absence of hormone and (2) VDR in the absence or presence of hormone. As highlighted in the figure, no preferential enrichment of *RankL* DNA was evident when a hormone/vehicle comparison was made using DNA derived from immunoprecipitations with control IgG. In contrast, however, five regions of DNA enrichment were observed when similar comparisons were made using ChIP DNA derived from anti-VDR immunoprecipitation. Interestingly, all of these potential sites of VDR binding were located at significant distances upstream of the *RankL* TSS: −16 kb (mRLD1), −22 kb (mRLD2), −60 kb (mRLD3), −69 kb (mRLD4) and