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Using chromatin immunoprecipitation to monitor 1α,25-dihydroxyvitamin D_3-dependent chromatin activity on the human CYP24 promoter☆

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Abstract

We applied the chromatin immunoprecipitation (ChIP) method for the analysis of 1α,25-dihydroxyvitamin D_3 (1,25-D_3)-dependent chromatin activity on the human 24-hydroxylase (CYP24) promoter in MCF-7 human breast cancer cells. In this pilot study we concentrated on the proximal promoter (+22 to −424) of the CYP24 gene, which includes the known 1,25-D_3 response element (VDRE) cluster. A constitutively active region of the human histone 4a gene (−40 to +285) served for normalization. Chromatin activity snapshots were taken 0, 30, 60, 120, 180, 240 and 300 min after the onset of stimulation with 1,25-D_3 and anti-acetylated histone 4 antibodies were used for ChIP. Our results suggest that ChIP is suitable for monitoring 1,25-D_3-dependent changes of chromatin organization and can be used to reveal information about chromatin activity in living cells.

Keywords: Chromatin immunoprecipitation; CYP24; VDR; VDRE

1. Introduction

The 24-hydroxylase (CYP24) gene is in a variety of tissues a primary target of the nuclear hormone 1,25-D_3 [1]. The complex of the vitamin D receptor (VDR), the retinoid X receptor (RXR) and a VDRE is considered as the molecular switch of nuclear 1,25-D_3 signaling [2]. Simple VDREs are direct repeats (DRs) of two hexameric core binding motifs with 3 or 4 intervening nucleotides [3]. A cluster of two DR3-type VDREs is located in close (approximate position −300 to −140) proximity to the transcription start site (TSS) of both the human and the rat CYP24 gene [4,5]. This cluster is believed to mediate the strong transcriptional response of the CYP24 gene to 1,25-D_3.

According to the histone code, gene transcription is preceded by acetylation of N-terminal tails of histones 3 and 4 at specific lysine residues [6]. In active genes the N-terminal tails of both type of histones have higher degree of acetylation than in repressed genes. Histone acetylation neutralizes the positive charge of the lysines and thus the attraction for the negatively charged DNA, so that the chromatin is less densely packed. In the ChIP method [7] antibodies raised against acetylated histone 3 and 4 tails provide an efficient tool for monitoring the local activation status of the chromatin on promoter regions. In addition, by using antibodies targeted against all kinds of nuclear proteins, such as, e.g., the VDR, coactivator and corepressor proteins, one can take snapshots of the localization of these proteins within chromatin and thus get valuable information on gene activation mechanisms. In this pilot study we have used the ChIP method with anti-acetylated histone 4 antibodies to study how the activity of the promoter of the human CYP24 gene changes in response to 1,25-D_3 treatment. We found that the proximal CYP24 promoter was activated within 30 min after 1,25-D_3 stimulation before returning to an inactive, deacetylated state by 300 min.

2. Materials and methods

2.1. Cell culture

MCF-7 human breast cancer cells were grown overnight in phenol red-free DMEM supplemented with 5% charcoal-treated fetal bovine serum, 2 mM L-glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin. Prior to ChIP the cells

Abbreviations: 1,25-D_3, 1α,25-dihydroxyvitamin D_3; CYP24, 24-hydroxylase; ChIP, chromatin immunoprecipitation; DR, direct repeat; RXR, retinoid X receptor; TSS, transcription start site; VDR, vitamin D receptor; VDRE, 1,25-D_3 response element

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were treated for up to 300 min with 10 nM 1,25-D_3 dissolved in ethanol.

2.2. ChIP

Nuclear proteins were cross-linked to DNA by adding formaldehyde directly to the medium to a final concentration of 1% for 15 min. Cross-linking was stopped by adding glycine to a final concentration of 0.125 M and incubating at room temperature for 5 min on a rocking platform. The medium was removed and the cells were washed twice with ice cold PBS (140 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 8.1 mM $Na_2HPO_4{\cdot}2H_2O$). The cells were collected by scraping into ice cold PBS supplemented with a protease inhibitor cocktail (Roche, Mannheim, Germany). After centrifugation the cell pellets were resuspended in lysis buffer (1% SDS, 10 mM EDTA, 50 mM Tris–HCl, pH 8.1, protease inhibitors) and the lysates were sonicated to result in DNA fragments of 300–1500 bp in length. Cellular debris was removed by centrifugation and the lysates were diluted 1:7 in ChIP dilution buffer (0.01% SDS, 1.1% Triton X-100, 1.2 mM EDTA, 16.7 mM Tris–HCl, pH 8.1, 16.7 mM NaCl, protease inhibitors). Nonspecific background was removed by incubating the chromatin resuspension with a salmon sperm DNA/protein A agarose slurry (Upstate Biotechnology, Lake Placid, USA) at 4 °C for 30 min with agitation. The samples were centrifuged and the recovered chromatin solutions were incubated with 5 μl of anti-acetylated histone 4 antibody (Upstate Biotechnology) at 4 °C overnight with rotation. The immune complexes were collected with 120 μl of protein A agarose slurry at 4 °C for 2 h with rotation. The beads were pelleted by centrifugation at 4 °C for 1 min at 100 × *g* and washed sequentially for 5 min in rotating platform with 1 ml of the following buffers: low salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 150 mM NaCl), high salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris–HCl, pH 8.1, 500 mM NaCl) and LiCl wash buffer (0.25 mM LiCl, 1% Nonidet P-40, 1% sodium deoxycholate, 1 mM EDTA, and 10 mM Tris–HCl, pH 8.1). Finally, the beads were washed twice with 1 ml TE buffer (10 mM Tris–HCl, 1 mM EDTA, pH 8.0). The immunocomplexes were eluted by adding 250 μl elution buffer (1% SDS, 0.1 M $NaHCO_3$) and incubation at room temperature for 15 min with rotation. After centrifugation, the supernatant was removed to clean tubes and the elution was repeated. The supernatants were combined and the cross-linking was reversed by adding NaCl to final concentration of 200 mM and incubating at 65 °C overnight. The remaining proteins were digested by adding proteinase K (final concentration 40 μg/ml) and incubating at 42 °C for 1 h. The DNA was recovered by phenol/chloroform/isoamyl alcohol (25/24/1) extractions and precipitated with 0.1 volumes of 2.5 M NaAc, pH 5.2 and 2 volumes of EtOH using glycogen as carrier. Each DNA pellet was dissolved in sterile water and the concentrations were adjusted to 2 ng/μl. Finally, the chromatin DNA templates from differently treated cells were normalized to each other by PCR using H4a primers (Fig. 1A).

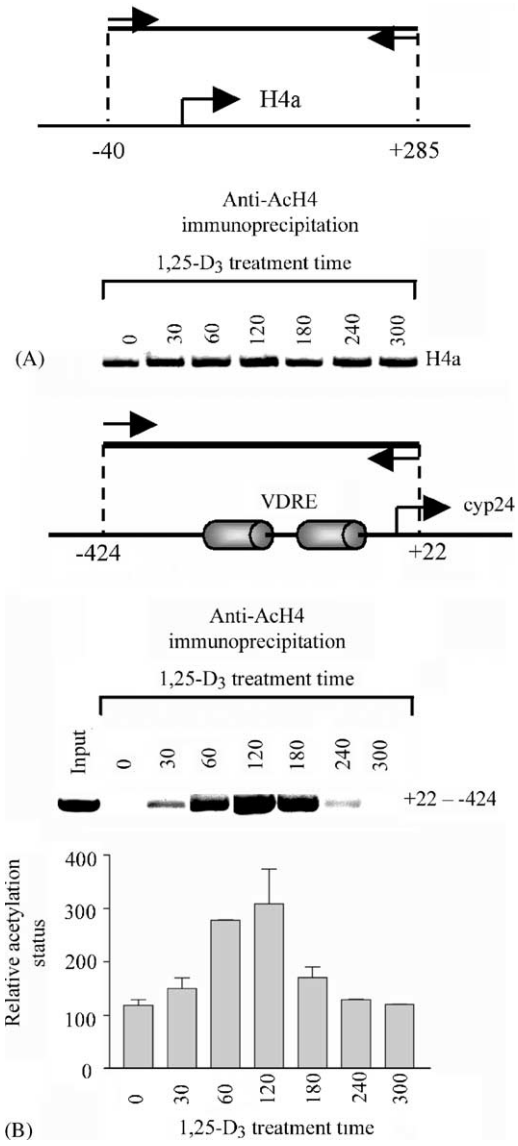

Fig. 1. ChIP analysis of the 1,25-D_3-dependent activity of the proximal CYP gene promoter in reference to the constitutively active histone 4a promoter. ChIP assays were performed with chromatin extracted from MCF-7 cells that had been treated for different times with 10 nM 1,25-D_3. A PCR primer pair that detects a constitutively active region of the human histone 4a gene was used to normalize the amount of chromatin DNA templates (A). Another PCR primer pair was specific for the proximal promoter of the human CYP24 gene (B). PCR results with both primer pairs are displayed by representative agarose gels. Quantitative data of the change of the acetylation state of the chromatin on the proximal CYP24 promoter are shown as normalized means of three independent cell stimulations. Bars indicate standard deviations.

2.3. Polymerase chain reaction

For normalization of the different immunoprecipitated genomic DNA templates, a primer pair (5′-CTATTCTCTC-

ACTTGCTCTTG-3′ and 5′-GTCCCTGGCGCTTAAGCG-CG-3′) for the promoter of the human histone H4a gene was designed that detected the region −40 to +285 relative to the TSS (Fig. 1A). For studying the chromatin activity of the CYP24 promoter, a primer pair (5′-CAGACGCGGCAGCTTTTCTG-3′ and 5′-CGTTTCCT-CCTGTCCCTCTC-3′) was used that detected the promoter region from +22 to −424 relative to the TSS (Fig. 1B). In PCR analysis, 10 ng of immunoprecipitated genomic DNAs were used as template and the cycling conditions were as follows: preincubation at 94 °C for 5 min, 50 cycles of denaturation at 95 °C for 30 s, annealing at 60 °C for 30 s and elongation at 72 °C for 30 s and one final incubation at 72 °C for 10 min. The PCR products were separated by electrophoresis through with 2.0% agarose supplemented with 0.5 μg/ml ethidium bromide and quantified using a Fuji FLA3000 reader (Tokyo, Japan) and Image Gauge software (Fuji).

3. Results

MCF-7 human breast cancer cells were stimulated for 0–300 min with 10 nM 1,25-D_3 and ChIP assay was performed using anti-acetylated histone 4. PCR was performed with chromatin DNA templates of the different time points using primers for the proximal CYP24 promoter. At time point 0 this promoter region was shown to be deacetylated and silent but the chromatin architecture changed rapidly due to 1,25-D_3 treatment (Fig. 1B). Thirty minutes after stimulation with 1,25-D_3 the proximal promoter fragment of CYP24 becomes acetylated revealing the VDREs and thus making transcription possible. The hormone-dependent acetylation status of the CYP24 promoter increased for the next 90 min, reaching a maximum at 120 min after starting of the 1,25-D_3 treatment. The proximal CYP24 promoter remained in a highly activated state for another 60 min and then rapidly deacetylated. Finally, at time point 300 min the chromatin activity on the investigated promoter region had returned to its basal level.

4. Discussion

The ChIP data demonstrated that the acetylation status of the chromatin on the proximal VDRE cluster of the human CYP24 gene promoter responds nicely to 1,25-D_3 stimulation. Before hormone treatment this chromatin region shows a low acetylation status, i.e. the proximal CYP24 promoter is inactive and the VDREs are masked by nucleosomes being tightly bound to DNA. ChIP assays with anti-VDR antibodies have indicated that in the absence of 1,25-D_3, VDR is not binding to this chromatin region (Väisänen and Carlberg, in preparation), i.e. the VDRE cluster seems not to be involved in active repression of the CYP24 before hormone treatment. Stimulation with 1,25-D_3 rapidly changes the chromatin structure on the proximal CYP24 promoter by induction of the acetylation of the N-terminal tails of histone 4. In addition, our data suggest that histone acetylation is not a short living process, while high acetylation status lasts for more than 3 h. This suggests that 1,25-D_3 treatment activates the proximal CYP24 promoter for at least 3 h. However, this does not solve the question, how the VDRE cluster originally gets acetylated. Our most recent data (Väisänen and Carlberg, in preparation) suggest that the known VDRE cluster is not the only 1,25-D_3 responsive region within the CYP24 promoter. The whole CYP24 promoter has several regions that are constitutively acetylated and also contains putative positive and negative VDREs (Väisänen and Carlberg, in preparation). Thus, the known VDRE cluster may be initially activated via these additional positive VDREs and finally inactivated via the negative VDREs.

In conclusion, this pilot study suggests that ChIP is a suitable method for the analysis of hormone-dependent changes of chromatin organization and can be used to retrieve valuable in vivo information about chromatin activity during transcription.

Acknowledgements

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