The Number of Vitamin D Receptor Binding Sites Defines the Different Vitamin D Responsiveness of the CYP24 Gene in Malignant and Normal Mammary Cells

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Primary 1α,25-dihydroxyvitamin D₃ (1α,25(OH)₂D₃)-responding genes are controlled by the vitamin D receptor (VDR) binding to specific sites (VDREs) that are located within the regulatory regions of these genes. According to previous studies, the gene encoding 25-dihydroxyvitamin D₃ 24-hydroxylase, CYP24, which is the strongest known 1α,25(OH)₂D₃-responsive gene, has multiple VDREs that locate within the proximal and the distal promoter. However, it has remained unclear, what is the biological role of these regions and how they participate in the regulation of transcription. In this study, we found a different CYP24 expression profile in normal (MCF-10A) and malignant (MCF-7) human mammary cells. Moreover, CYP24 mRNA showed to be three times more stable in MCF-7 cells than in MCF-10A cells. We studied the mechanism of this difference using expression profiling, quantitative chromatin immunoprecipitation and chromosome conformation capture assays. Interestingly, the number of functional VDREs was higher in MCF-7 cells than in MCF-10A cells. Three functional VDREs in MCF-7 cells are connected to linear mRNA accumulation, whereas only one VDRE seems to lead to stepwise CYP24 mRNA accumulation in MCF-10A cells. The distal VDREs were involved in transcriptional regulation via ligand-dependent, dynamic chromatin looping, which brings cyclically the distal elements together either individually or simultaneously next to the transcription start site. In conclusion, our data suggest that in comparison to normal cells, clearing of 1α,25(OH)₂D₃ is enhanced in malignant cells due to differences in transcriptional regulation of CYP24 and metabolism of CYP24 mRNA.

The biologically most active form of vitamin D, 1α,25-dihydroxyvitamin D₃ (1α,25(OH)₂D₃), has an essential role for mineral homeostasis and skeletal integrity as well as for controlling the cell growth and differentiation in various tissues (1). Because of its importance to normal functions of the body, the levels of 1α,25(OH)₂D₃ are tightly controlled by a feedback regulation that is designed to rapidly metabolize the excess hormone. The first step in the pathway of degradation of 1α,25(OH)₂D₃ involves activation of the CYP24 gene encoding the 25-dihydroxyvitamin D₃ 24-hydroxylase. CYP24 belongs to the cytochrome P450 (CYP) family, which encodes a wide variety of enzymes that are needed in the oxidative metabolism of many endogenous and exogenous compounds (2).

The physiological effects of 1α,25(OH)₂D₃ are mediated through the vitamin D receptor (VDR), which is a member of the nuclear receptor (NR) superfamily. VDR and other steroid receptors are ligand-activated NRs that control gene expression in response to binding of steroidal or other lipophilic compounds. The binding of 1α,25(OH)₂D₃ induces a conformational change within the ligand-binding domain of the VDR (3). This change enhances the interaction of VDR with its heterodimeric partner, the retinoid X receptor (RXR). The ligand-dependent conformational change also modulates interactions of NRs with a number of different nuclear proteins, such as co-activators (CoAs) and co-repressors (CoRs) (4). CoR proteins, such as NCoR1 (5) and SMRT/NCoR2 (6), link non-liganded, DNA-bound NRs to enzymes with histone deacetylase (HDAC) activity, which causes chromatin condensation. After a ligand-dependent conformational change, interactions with CoA proteins, such as SRC-1, are favored (7). These CoAs link the ligand-activated NRs to enzymes displaying histone acetyltransferase (HAT) activity, such as CBP, which leads to chromatin decondensation (8). In a second step, mediator proteins, such as Med1, build a bridge to the basal transcriptional machinery containing RNA polymerase II (pol II) and TATA-box binding protein (TBP).

The liganded VDR-RXR heterodimer binds with high affinity and selectivity to vitamin D response elements (VDREs) in the regulative regions of VDR target genes (9). Traditionally, response elements (REs) are thought to be located relatively close to the transcription start site (TSS) of target genes. However, several studies have recently suggested that both positively and negatively regulated genes may have multiple REs...
that locate not only within proximal promoters but also in more distal regions (10, 11) and within coding regions (12–14). These data suggest that transcriptional regulation via multiple REs may be a general concept more than an exception. However, the mechanism on how these REs work in concert to regulate gene activity is still unclear.

We reported previously that in MCF-7 human breast cancer cells the human CYP24 gene has multiple VDREs within its proximal and distal promoter, which all participate in the transcriptional regulation of the gene (15). However, we were not able to answer the question, how the distal elements are involved in transcriptional regulation. In this study, we have clarified the biological role of multiple REs as well as the mechanism of transcriptional regulation of the human CYP24 gene via multiple REs by using quantitative chromatin immunoprecipitation (ChIP) and chromosome conformation capture (3C) analysis (16). Our data suggest that the number of functional VDREs within the CYP24 gene is higher in MCF-7 cells than in MCF-10A cells. The number of functional VDREs mirrors to the CYP24 mRNA expression profile, because a higher number of VDREs is connected to linear mRNA accumulation, whereas a lower number of VDREs leads to a more controlled, stepwise mRNA accumulation. The distal VDREs are involved in transcriptional regulation by forming an enhancer via ligand-dependent, dynamic chromatin looping, which cyclically brings the distal elements in contact with the TSS either individually or simultaneously.

EXPERIMENTAL PROCEDURES

Cell Culture—MCF-7 human breast cancer cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) containing 5% fetal bovine serum (FBS), 2 mM L-glutamine, 0.1 mg/ml streptomycin, and 100 units/ml penicillin in a humidified 95% air/5% CO₂ incubator. MCF-10A human mammary epithelial cells were cultured in a mixture of DMEM and Ham’s F12 medium (1:1) with 20 ng/ml of epidermal growth factor, 100 ng/ml of cholera toxin, 10 µg/ml insulin, 500 ng/ml hydrocortisone, and 5% horse serum. Before use under “Experimental Procedures,” FBS was stripped of lipophilic compounds, such as endogenous nuclear receptor ligands, by stirring it with 5% activated charcoal for 3 h at room temperature. Charcoal was then removed by centrifugation, and the medium was sterilized by filtration (0.2 µm pore size). Prior to mRNA or chromatin extraction, the cells were grown overnight in phenol red-free DMEM, supplemented with 5% charcoal-stripped FBS, to reach 50–60% confluency. MCF-10A cells were maintained for experiments in phenol red-free DMEM/Ham’s F12 medium with above supplements instead horse serum, which was replaced by 5% charcoal-stripped FBS. For ChIP or 3C assays the cells were first treated with 2.5 µM α-amanitin (Sigma-Aldrich) for 2 h, followed by exposure to either solvent (ethanol, 0.1% final concentration) or 10 mM 1α,25(OH)₂D₃ (kindly provided by Milan Uskokovic, Bioxell Inc, Nutley), after the removal of the α-amanitin.

Total RNA Extraction, cDNA Synthesis, and Real-time PCR—Total RNA was extracted using the High Pure RNA Isolation kit (Roche, Mannheim, Germany) and cDNA synthesis was performed using Transcriptor High Fidelity cDNA Synthesis kit (Roche) according to the manufacturer’s instructions. Real-time quantitative PCR was performed with LightCycler480 apparatus (Roche) using the TaqMan Gene Expression Assays (Applied Biosystems, Carlsbad, CA). PCR cycling conditions were: 10 min at 95 °C, 50 cycles of 15 s at 95 °C, 7 min at 60 °C. Fold inductions were calculated using the formula 2⁻⁰·⁰₅ΔCₚ, where ΔCₚ is ΔCₚ(stimulus) − ΔCₚ(solvent), ΔCₚ is Cₚ(CYP24) − Cₚ(RPLP0), and the Cₚ is the cycle at which the threshold is crossed.

ChIP Assays—ChIP assays were performed as previously described (11). The antibodies against acetylated H4 (Ach4, 06-866), dimethylated histone H3K4 (H3K4me2, 07-030), trimethylated histone H3K27 (H3K27me3, 07-449), and histone H3 C terminus (H3CT, 07-690) were from Upstate Biotechnology (Lake Placid, NY). Nonspecific IgG (sc-2027) and the antibodies against Brg-1 (sc-10768), VDR (sc-553), CBP (sc-369), TPB (sc-204), MED1 (sc-5334), p40 (sc-13583), Sin3A (sc-994), SMRT (sc-1610), NCoR1 (sc-8994), HDAC1 (sc-6298), HDAC2 (sc-6296), and HDAC3 (sc-11417) were obtained from Santa Cruz Biotechnologies (Santa Cruz, CA). The ChIP templates were analyzed by quantitative real-time PCR.

Micrococcal Nuclease Digestion Assay—Chromatin was fragmented to mono-nucleosome level by M13nase digestion using the method described by Soutouglou and Talianidis (17) with small changes. Prior to the MNase digestion, MCF-7 cells (10⁶ cells) were washed twice with 1× phosphate-buffered saline and suspended to 1 ml of lysis buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 5 mM MgCl₂, 0.1% Nonidet P-40) and incubated on ice for 10 min. The nuclei were collected by centrifugation (1500 × g, 5 min) and washed once with lysis buffer. The nuclei were suspended to MNase reaction buffer (50 mM Tris, pH 7.5, 15 mM NaCl, 3 mM MgCl₂, 5 mM KCl, 1 mM CaCl₂, protease inhibitors) and 30 units of MNase (Merck, Germany) was added. After 30 min of incubation at 37 °C, 2 µl of proteinase K (10 mg/ml, Merck) was added, and the samples were further incubated overnight at 64 °C. Finally, the DNA was recovered by phenol-chloroform extraction followed by ethanol precipitation and analyzed by quantitative PCR using primers and hydrolysis probes, which are listed in supplemental Table S1.

Quantitative PCR Analysis of Immunoprecipitated and MNase-digested DNAs—Quantitative PCR analysis was performed by using BHQ1-FAM hydrolysis probes provided by Eurogentec (Liege, Belgium). The sequences of primers and hydrolysis probes are listed in supplemental Table S1. The qPCR reaction was performed with LightCycler480 apparatus (Roche) following the following PCR profile: 10 min at 95 °C, 50 cycles of 20 s at 95 °C, 1 min at 60 °C. Results were normalized with respect to input and nonspecific IgG results were subtracted by using formula 2⁻⁰·⁰₅ΔCₚ*100 (specific antibody) − 2⁻⁰·⁰₅ΔCₚ*100 (nonspecific IgG), where ΔCₚ is the Cₚ immunoprecipitated DNA − Cₚ (input) and Cₚ is the cycle where the threshold is crossed. Two-tailed Student’s t-tests were performed using Prism4.0c software and p values of the percent of input were calculated in reference to time point 0 min.

3C-linked ChIP—Prior to 3C analysis, ligand treatment, α-amanitin treatment, and formaldehyde cross-linking were done as described for the ChIP analysis. The nuclei from 2 × 10⁶ cells were collected as described for the MNase assay. The nuclei were suspended to Histone reaction buffer (10 mM Tris-
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HCl, pH 8.5, 10 mM MgCl₂, 100 mM KCl, 0.1 mg/ml BSA, protease inhibitors) and sonicated briefly to break down the nuclear envelope. In each sample, 20 units of HinfI was added, and the samples were incubated for overnight at 37 °C. The reaction was stopped by heating the samples to 65 °C for 20 min. The samples were diluted 1:10 with ligase buffer (40 mM Tris-HCl, pH 7.8, 10 mM MgCl₂, 10 mM dithiothreitol, 0.5 mM ATP), 25 units of T4 DNA ligase was added, and the samples were incubated for 1 h at room temperature. The reaction was stopped by heating the samples at 65 °C for 10 min and 2 µg of anti-VDR antibody (sc-1008, Santa Cruz Biotechnologies) was added to each sample. The samples were incubated for overnight at 4 °C and the immuno-complexes were collected as described in ChIP assay. The recovered DNA was analyzed by PCR using primers listed in supplemental Table S2 and FastStart SYBR Green Master mixes (Roche). Digestions performed with a large CYP24 promoter fragment, which was subcloned in the pGL4 plasmid (Promega, Madison, WI), served as a positive control.

RNAi and Immunoblotting—MCF-7 cells were transfected with either nonspecific siRNA oligomers or Stealth™ siRNAs targeting the VDR mRNA (Invitrogen, Carlsbad, CA) by using RNAiMAX reagent (Invitrogen) according to the instructions of the manufacturer. The cells were seeded into 6-well plates and grown in phenol red-free DMEM supplemented with 5% charcoal-stripped FBS until they reached a confluency of 40–50%. Liposomes containing control or VDR siRNA were formed by incubating 100 pmol of each siRNA duplex with 5 µl of RNAiMAX for 20 min at room temperature in a total volume of 500 µl of phenol red-free DMEM without antibiotics. The liposomes were added to the cells and siRNA treatment was continued for 48 h, after which the cells were treated with ethanol or 10 nM 1α,25(OH)₂D₃ for 50 min. Silencing of VDR at the protein level was verified by Western blotting using 25 µg (total protein) of whole cell extract from MCF-7 cells and anti-VDR antibody (sc-1008, Santa Cruz Biotechnologies). Cellular proteins were separated using 12% SDS-polyacrylamide gel electrophoresis. The blotted proteins were detected by using IR800 fluorescence labeled secondary antibodies (Thermo Scientific, Rockford, IL) and on an Odyssey reader (Li-Cor Biotechnology, Lincoln, NE).

RESULTS

CYP24 mRNA Expression Profile and Stability in Malignant and Normal Breast Cells—We measured ligand-dependent CYP24 mRNA expression in MCF-7 human breast cancer cells (Fig. 1A) and in MCF-10A normal human mammary epithelial cells (Fig. 1B) with 15-min intervals up to 4 h after the onset of 1α,25(OH)₂D₃ treatment. In MCF-7 cells the CYP24 mRNA expression started to increase continuously without signs of cycling 45 min after onset of ligand treatment. In this cell line ligand induction was nearly 700-fold after 4 h and over 6000-fold after 48 h. In MCF-10A cells CYP24 mRNA expression started to increase 45 min after the onset of 1α,25(OH)₂D₃ treatment and, but mRNA accumulation was significantly faster than in MCF-7 cells, because the maximum of 10,600-fold induction was reached already after 4 h. Then CYP24 mRNA levels started to decrease, being only 7000-fold after 24 h of ligand treatment. The accumulation of CYP24 mRNA in MCF-10A cells showed a stepwise profile suggesting that the CYP24 expression is under more careful control in normal cells than in cancer cells.

The stability of CYP24 mRNA was determined in both cell lines by treating the cells with actinomycin D 24 h after ligand induction (Fig. 1C). The half-life of the CYP24 mRNA was 3.3 h...
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A schematic presentation of the locations and sequences of proximal (black box) and distal (blue and red boxes) VDREs. For quantitative ChIP analysis chromatin was extracted from the cells that were treated for indicated times with 10 nM 1α,25(OH)2D3, and ChIP was performed using anti-VDR antibody. The recruitment of VDR to the proximal and distal VDREs of the human CYP24 promoter in MCF-7 cells (B) and MCF-10A cells (C) was detected for each treatment time with a LightCycler480 apparatus using BHQ1-FAM hydrolysis probes. Two-tailed Student's t-tests were performed using Prism4.0c software, and p values of the percent of input were calculated in reference to time point 0 min (*, p = 0.01 to 0.05; **, p = 0.001 to 0.01; ***, p < 0.001). In each panel, n is at least 3. Error bars indicate S.D.

FIGURE 2. Ligand-induced recruitment of VDR to proximal and distal VDREs on the CYP24 promoter in MCF-7 and MCF-10A cells. A, schematic presentation of the locations and sequences of proximal (black box) and distal (blue and red boxes) VDREs. For quantitative ChIP analysis chromatin was extracted from the cells that were treated for indicated times with 10 nM 1α,25(OH)2D3, and ChIP was performed using anti-VDR antibody. The recruitment of VDR to the proximal and distal VDREs of the human CYP24 promoter in MCF-7 cells (B) and MCF-10A cells (C) was detected for each treatment time with a LightCycler480 apparatus using BHQ1-FAM hydrolysis probes. Two-tailed Student's t-tests were performed using Prism4.0c software, and p values of the percent of input were calculated in reference to time point 0 min (*, p = 0.01 to 0.05; **, p = 0.001 to 0.01; ***, p < 0.001). In each panel, n is at least 3. Error bars indicate S.D.

in MCF-7 cells, which was more than 3-fold longer than that in MCF-10A cells. For comparison, the basal CYP24 mRNA expression in MCF-7 cells was 3-fold lower than in MCF-10A cells (supplemental Fig. S1). This suggests that clearing of 1α,25(OH)2D3 may be enhanced in malignant cells in comparison to normal cells because of more stable CYP24 mRNA.

Number of Functional CYP24 VDREs in Malignant and Normal Breast Cells—We reasoned that the different CYP24 mRNA expression profiles in malignant and normal mammary cells are based on differences in the transcriptional regulation of the gene. Therefore, we performed a detailed quantitative ChIP analysis to both MCF-7 and MCF-10A cells using anti-VDR antibody (Fig. 2). VDR showed to be associated with both proximal and distal VDREs in MCF-7 cells (Fig. 2B), but in MCF-10A cells only with the proximal VDRE1/2 (Fig. 2C).

Based on above results, additional ChIP assays were performed with antibodies targeted against acetylated H4 and the nuclear proteins VDR, RXR, CBP, MED1, and TBP using ligand treatment times 0, 40, and 80 min (Fig. 3). The proximal VDRE1/2 showed in both cell types a similar profile in the recruitment of all tested proteins. In contrast, in MCF-10A cells only minor recruitment to the distal VDRE3 and VDRE4 was observed, which did not differ significantly from association to control regions. This suggests that the CYP24 gene uses in breast cancer cells a higher number of functional VDREs for its regulation by 1α,25(OH)2D3 than in normal mammary epithelial cells. To verify that the different utilization of the VDREs was not due to unequal expression of VDR and RXR in MCF-7 and MCF-10A cells, we studied the respective mRNA expression levels in both cells (supplemental Fig. S1). RXR expression was similar in both cells, but VDR expression was 11-fold higher in MCF-10A cells than in MCF-7 cells. Still, the distal VDRE3 and VDRE4 were not functional in MCF-10A cells, even in the presence of significantly more VDR. This emphasizes the different utilization of these elements in different cells.

Rapid Ligand Response of Chromatin on the CYP24 Promoter—Because the number of functional VDREs was higher in malignant cells, we decided to study the mechanism of transcriptional regulation via multiple REs in detail in MCF-7 cells. The changes in chromatin activity related to the regulation of CYP24 transcription were determined by ChIP analysis with antibodies against acetylated or methylated histones or against Brg-1, a component of SWI/SNF family of ATP-dependent chromatin remodeling complexes, and by micrococcal nuclease (MNase) assay (Fig. 4).

In all cases, the changes in the chromatin status were assessed every 10 min up to 100 min after onset of ligand treatment. The level of general acetylation of histone H4 (H4ac) changed cyclically and ligand-dependently at all three VDREs (Fig. 4A). For all three VDREs the highest acetylation levels were reached at time points 40 and 80 min. In the first cycle, the highest acetylation level could be observed at the distal VDRE3 and in the second cycle at the proximal VDRE1/2.

The level of dimethylation of histone H3 at K4 (H3K4me2) showed ligand-dependent and cyclical behavior at all VDREs (Fig. 4B). At VDRE1/2 the H3K4me2 level was rather high already in the absence of the ligand, but increased significantly after 10 min of ligand treatment. The next peaks occurred at time points 60 and 100 min. The highest H3K4me2 levels at VDRE3 and VDRE4 were reached at time points 30 and 80 min, which is significantly different to that of VDRE1/2. Thus, at the distal VDREs but not at the proximal VDRE cycling of H3K4me2 followed that of H4ac.

At all VDREs the amount of trimethylation of histone H3 at K27 (H3K27me3), a marker for inactive chromatin, was high in the absence of the ligand and decreased after addition of the ligand (Fig. 4C). The overall methylation status was highest at VDRE1/2 and lowest at VDRE3. All VDREs showed lowest H3K27me3 levels at time points 40 and 80 min. Thus, the markers for active and inactive chromatin gave opposite periodicity at both distal VDREs, but at VDRE1/2 the H3K4me2 profile was similar to repressive marker H3K27me3.
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The MNase assay showed that the nucleosomes were present at all VDREs in the absence of 1α,25(OH)2D3 (Fig. 4D). Addition of ligand resulted in rapid disappearance of nucleosomes from all VDREs but they appeared again at time points 60 and 100 min. ChIP analysis with an antibody against the C terminus of histone H3 confirmed the results of the MNase assay, since the highest levels of histone H3 enrichment were achieved at all VDREs at time points 0, 20, 60, and 100 min (Fig. 4E). ChIP analysis with Brg-1 antibody indicated that the highest enrichment of SWI/SNF was achieved at all VDREs at time points 10, 60, and 100 min (Fig. 4F). These data are in good agreement with the observed changes in nucleosome density.

**Distal and Proximal VDREs Recruit Nuclear Proteins Cyclically**—Next we performed quantitative ChIP analysis with 10-min intervals over 100 min and measured the recruitment of VDR, CBP, MED1, TBP, and phosphorylated RNA polymerase II (pPolII) to the three VDREs (Fig. 5). VDR associated with all VDREs at time points 30–40 and 80 min (Fig. 5A). A remarkable difference between the three VDREs could be observed in the recruitment of the histone acetyltransferase CBP, which at time point 40 min was enriched at VDRE3 far more prominent than at VDRE1/2 and VDRE4 (Fig. 5B). This suggests that VDRE3 has a prominent role in the ligand-dependent shift of the chromatin on the CYP24 promoter to a permissive state. Otherwise the association of CBP to the proximal and distal VDREs was similar, showing highest enrichment at time points 10, 40, and 80 min. The highest associations of MED1, a component of the mediator complex, TBP and pPolII could be observed at all VDREs at time points 40 and 80 min (Fig. 5, C–E).

As a control the recruitment of repressing co-factors NCoR1, SMRT, Sin3A, HDAC1, HDAC2, and HDAC3 to the proximal and distal VDREs was investigated (Fig. 6). NCoR1 was ligand-dependently recruited to the distal VDRE3 and VDRE4, but not to the proximal VDRE1/2 (Fig. 6A). SMRT was enriched at VDRE3 in the absence of ligand and at VDRE4 in the presence of ligand, while no enrichment could be observed at VDRE1/2 (Fig. 6B). Sin3A was recruited ligand-independently to VDRE3 and ligand-dependently to VDRE1/2, but not at all to VDRE4 (Fig. 6C). HDAC1 was enriched ligand-dependently at VDRE1/2 and VDRE4, but not at VDRE3 (Fig. 6D). HDAC2 was recruited ligand-independently only to VDRE3 (Fig. 6E). HDAC3 was enriched ligand-independently at VDRE3 and ligand-dependently at VDRE1/2 and VDRE4 (Fig. 6E).

In summary, during the studied time period of 100 min the CYP24 gene showed two transcription cycles, which involved recruitment of transcription factors and activating as well as repressing cofactors to both proximal and distal VDREs. In addition, the remarkable similarities in the recruitment times suggest that all VDREs are connected to each other and to the TSS via chromatin looping. On the other hand, the individual repressive protein complexes at different VDREs suggest that they are not connected between active transcription cycles.

**Ligand-dependent, Dynamic Chromatin Looping**—To ensure that the VDREs of the CYP24 promoter are connected with each other and the TSS via chromatin looping, we performed a 3C analysis with 10-min intervals over 100 min (Fig. 7). According to our data, the VDRE3 was connected with the TSS at time points 10, 30, 60, and 100 min.
points 20, 50, and 80–100 min (Fig. 7, B and C). In contrast, VDRE4 was connected with the TSS at time periods 40–50 and 70–80 min. When VDR expression was silenced by specific siRNA, no chromatin looping could be observed suggesting that VDR was an anchoring point of at least one side of the loop (Fig. 7, D and E). This confirms our assumption that the looping of distal VDREs to the TSS is a ligand-dependent and cyclical process.

**DISCUSSION**

It is now getting more and more obvious that both positively and negatively regulated genes have multiple REs (10–14). However, it is not clear how these REs actually participate in coordinated gene regulation. In this study, we have clarified the biological role of the multiple VDREs of the CYP24 promoter in MCF-7 human breast cancer cells in comparison to MCF-10A normal human mammary epithelial cells. In addition, we investigated in detail how CYP24 gene transcription is regulated in MCF-7 cells via multiple VDREs.

It has been recently suggested that mRNA expression of inducible genes has a cyclical nature (18–20). In this study, no cycling could be observed, when the CYP24 mRNA accumulation was studied in MCF-7 cells. However, when the mRNA accumulation was studied in MCF-10A cells, a clearly different mRNA expression profile could be detected. The amount of CYP24 mRNA expression was still continuously increasing after ligand treatment, but not linearly as it was observed in malignant cells. Instead, a clearly stepwise accumulation could be detected, showing a periodicity of 40–50 min during the first 3 h. These data suggest that in normal mammary cells the transcription of CYP24 gene has evaluation points, where the need of gene product is considered. When the concentration of 1α,25(OH)2D3 is still high, the next cycle is initiated, but when the hormone is already metabolized to acceptable level, CYP24 transcription terminates. On the other hand, in malignant cells this control is apparently missing leading to continuously increasing mRNA levels.

Because of its anti-proliferative potential 1α,25(OH)2D3 has beneficial effects as a deterrent agent against various cancers (21). Thus, a defect in the normal metabolism of 1α,25(OH)2D3 may result in low hormone levels and via that higher risk of cancer. Epidemiological studies have revealed that the level of 1α,25(OH)2D3 in the circulation is not decreased with breast cancer patients in comparison to healthy persons. However, the level of 25(OH)D3, the precursor of 1α,25(OH)2D3 and substrate for the enzyme encoded by the CYP27B1 gene, was slightly decreased in breast cancer patients in comparison to healthy persons (22). Thus, if decreased 1α,25(OH)2D3 levels have any role in the carcinogenesis of mammary cells, there must be some local changes in the metabolism of 1α,25(OH)2D3, even on a single cell level. One possibility would be that at the beginning of carcinogenesis some changes would occur in the local metabolism of 1α,25(OH)2D3.
which would lead to low hormonal levels resulting in low anti-proliferative protection by the hormone. Our expression profiling data suggested that transcriptional regulation of the CYP24 gene must differ somehow in MCF-7 cells from that in MCF-10A cells. This finding directed us to compare, if the different VDREs were utilized differently in MCF-7 and MCF-10A cells at chromatin level. Our ChIP results suggest that the distal VDRE3 and VDRE4 were inactive in MCF-10A cells. This is an interesting result, because in MCF-7 cells the VDRE3 recruited highest level of the HAT CBP during transcriptionally active cycles and on the other hand, the repressive transcription factors were associated with it in the absence of ligand. Thus, the inactive VDRE3 could explain the higher basal activity of CYP24 as well as the quicker response to 1α,25(OH)2D3 in MCF-10A cells than in MCF-7 cells. Moreover, the data suggest that there is a connection between the mRNA expression profile and the number of functional VDREs. When the number of VDREs is high and they interact with the TSS individually, one after another via dynamic chromatin looping, they would con-

FIGURE 5. Ligand-induced recruitment of activating transcription factors to proximal and distal VDREs on the CYP24 promoter. For quantitative ChIP analysis, chromatin was extracted from MCF-7 cells that had been treated for 2 h with α-amanitin and then for indicated time periods with 10 nM 1α,25(OH)2D3. ChIP was done using antibodies against VDR (A), CBP (B), MED1 (C), TBP (D), or pPolII (E). The recruitment of above transcription factors to the proximal and distal VDREs of the human CYP24 promoter was detected for each treatment time with a LightCycler480 apparatus using BHQ1-FAM hydrolysis probes. Two-tailed Student’s t-tests were performed using Prism4.0c software and p values of the percent of input were calculated in reference to time point 0 min (*, p = 0.01 to 0.05; **, p = 0.001 to 0.01; ***, p < 0.001). In each panel, n is at least 3. Error bars indicate S.D.

FIGURE 6. Recruitment of repressive transcription factors to the proximal and distal VDREs of the CYP24 promoter. Chromatin was extracted from MCF-7 cells that had been treated first for 2 h with α-amanitin and then for 0 and 60 min with 10 nM 1α,25(OH)2D3. ChIP was performed using antibodies against the CoRs NCoR1 (A), SMRT (B), or Sin3A (C) as well as against HDAC1 (D), HDAC2 (E), or HDAC3 (F). The recruitment of these repressive proteins to the proximal and distal VDREs was detected for each treatment time with a LightCycler480 apparatus using BHQ1-FAM hydrolysis probes. Two-tailed Student’s t-tests were performed using Prism4.0c software, and p values of the percent of input were calculated in reference to time point 0 min (*, p = 0.01 to 0.05; **, p = 0.001 to 0.01; ***, p < 0.001). In each panel, n is at least 3. Error bars indicate S.D.
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FIGURE 7. Ligand-dependent, dynamic chromatin looping connects distal VDREs to the TSS. Prior to the 3C analysis, nuclei were extracted from MCF-7 cells, which had been treated for 2 h with α-amanitin and after that for indicated time periods with 10 μM 1α,25(OH)2D3. The nuclei were suspended to HindIII digestion buffer and the genomic DNA was digested with HindIII restriction enzyme (digestion sites are represented in panel A by vertical red lines) and ligated with T4 DNA ligase. After ligation, ChIP was carried out with anti-VDR antibody. PCR was performed on purified DNA template with primer a in combination with primers b, c, or d (location indicated in panel A by horizontal blue arrows). Digestions performed with a large CYP24 promoter fragment served as a positive control. B, representative gels of 3C analysis. C, semi-quantitative 3C data. The looping between distal VDREs and the TSS is presented as % of positive control. Two-tailed Student’s t-tests were served as a positive control. *p < 0.01 to 0.05; **p < 0.001 to 0.01; ***p < 0.001. In each measurement, n is at least 3. Error bars indicate S.D. D, silencing of the VDR at protein level by RNAi was ensured by Western blotting. Representative gels of three replicates are shown. E, effect of VDR silencing to the ligand-dependent chromatin looping.

continuously activate the basal transcriptional machinery, resulting in continuous transcription of the gene, i.e. a linear accumulation of mRNA. In conclusion, we suggest that a gene with multiple functional REs has a continuously increasing mRNA expression profile, whereas a gene with only one functional RE, shows a cyclical mRNA accumulation profile.

Another parameter defining the profile of accumulation of mRNA of a given gene would be the stability of mRNA. Our results indicate that the mRNA of CYP24 was three times more stable in MCF-7 cells than in MCF-10A cells, which suggests that in malignant cells the metabolism of CYP24 mRNA has been disturbed. Because of the higher stability of CYP24 mRNA the hydrolysis of 1α,25(OH)2D3 is more effective, leading to enhanced clearance of 1α,25(OH)2D3 in malignant cells in comparison to the normal cells.

The transcriptional activation of NR target genes involves complex enzymatic processes, where N-terminal histone tails are covalently modified to loosen the interactions between DNA and the nucleosomes. Previous studies have suggested that H4 acetylation and H3K4 dimethylation correlate with gene activation and H3K27 trimethylation with gene repression (23–26). Our ChIP results suggest that H4 was acetylated cyclically at all VDREs. H3K4me2 showed a similar profile as H4ac at both distal VDREs, but an opposite profile at the proximal VDRE. These data indicate that H3K4me2 may have a different role in transcriptional regulation close to the TSS than in more distal promoter regions of the CYP24 gene. Indeed, Heintzman et al. (23) have recently shown that methylation levels of H3K4 are different at distal enhancers, when compared with those close to the TSS. The repressive histone mark H3K27me3 showed an opposite profile to that of H4ac both at proximal and distal VDREs. Thus, our ChIP data suggest that the histone modifications at the proximal and the distal CYP24 VDREs are ligand-dependent and cyclical processes showing a periodicity of 40 min. However, there are some differences in the kinetics of histone modifications at the individual VDREs, which indicates that the different VDREs may recruit different protein complexes representing slightly distinct enzymatic activity.

In addition to the post-translational histone modifications, transcriptional activation often requires remodeling of nucleosomes within the regulatory regions and the TSS of a gene to be activated. This is achieved via action of ATP-dependent chromatin remodeling complexes, such as SWI/SNF (27–29). Our ChIP and MNoase data suggest that during transcription the nucleosomes were remodeled at all three VDREs ligand-dependently and cyclically. Each peak of activating histone tail modifications was preceded by a remodeling of nucleosomes by the ATP-dependent SWI/SNF complex, which dissociated from the promoter after remodeling. Just like the histone tail modifications, ligand-dependent nucleosome remodeling at the CYP24 promoter started only minutes after onset of ligand treatment and occurred cyclically with a periodicity of 40–50 min both at the proximal and distal VDREs. After each activating cycle the nucleosomes spontaneously returned back to their original positions and had to be remodeled again before the next activating cycle.

Métriev et al. (30) reported that during transcriptional activation of the pS2 gene the binding of transcription factors to the promoter occurs cyclically. Our data suggest that this applies also to transcription of the CYP24 gene. Although the core of the protein complex formed by key transcription factors was similar at all VDREs, the individual VDREs seem to recruit slightly different enzymatic activity during the transcriptionally active and inactive cycles, as could be observed from the
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In conclusion, our data suggest that in MCF-7 cells the transcriptional activation of CYP24 starts rapidly after onset of 1α,25(OH)₂D₃ treatment and that both proximal and distal VDREs are involved in the cyclic up-regulation of gene expression by forming an enhancer module next to TSS. The number of functional VDREs is lower in normal mammary epithelial cells than in breast cancer cells, which reflects to the mRNA expression profile. In addition, clearing of 1α,25(OH)₂D₃ is enhanced in malignant cells due to more stable CYP24 mRNA. The higher 1α,25(OH)₂D₃ clearance rate likely increases the proliferation rate of breast cancer cells, because the anti-proliferative effect of 1α,25(OH)₂D₃ remains lower than in normal mammary epithelial cells.

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