Regulation of the human *cyclin C* gene via multiple vitamin D$_3$-responsive regions in its promoter

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Received January 13, 2005; Revised and Accepted March 22, 2005

**ABSTRACT**

The candidate human tumor suppressor gene *cyclin C* is a primary target of the anti-proliferative hormone 1α,25-dihydroxyvitamin D$_3$[1α,25(OH)$_2$D$_3$], but binding sites for the 1α,25(OH)$_2$D$_3$ receptor (VDR), so-called 1α,25(OH)$_2$D$_3$ responsive elements (VDRES), have not yet been identified in the promoter of this gene. We screened various cancer cell lines by quantitative PCR and found that the 1α,25(OH)$_2$D$_3$ inducibility of *cyclin C* mRNA expression, in relationship with the 24-hydroxylase (CYP24) gene, was best in MCF-7 human breast cancer cells. To characterize the molecular mechanisms, we analyzed 8.4 kb of the *cyclin C* promoter by using chromatin immunoprecipitation assays (ChIP) with antibodies against acetylated histone 4, VDR and its partner receptor, retinoid X receptor (RXR). The histone 4 acetylation status of all 23 investigated regions of the *cyclin C* promoter did not change significantly in response to 1α,25(OH)$_2$D$_3$, but four independent promoter regions showed a consistent, 1α,25(OH)$_2$D$_3$-dependent association with VDR and RXR over a time period of 240 min. Combined *in silico/in vitro* screening identified in each of these promoter regions a VDRE and reporter gene assays confirmed their functionality. Moreover, re-ChIP assays monitored simultaneous association of VDR with RXR, coactivator, mediator and RNA polymerase II proteins on these regions. Since cyclin C protein is associated with those mediator complexes that display transcriptional repressive properties, this study contributes to the understanding of the downregulation of a number of secondary 1α,25(OH)$_2$D$_3$-responding genes.

**INTRODUCTION**

The biologically most active vitamin D metabolite, 1α,25-dihydroxyvitamin D$_3$ [1α,25(OH)$_2$D$_3$], is essential for mineral homeostasis and skeletal integrity (1), but also has important roles in the control of cell growth and differentiation in normal and malignant tissues (2). 1α,25(OH)$_2$D$_3$ levels are tightly controlled by the monoxygenase vitamin D 24-hydroxylase (CYP24), which metabolizes the active hormone. The CYP24 gene is also the most responsive primary 1α,25(OH)$_2$D$_3$ target gene and shows at the mRNA level up to 1000-fold inducibility by the hormone (3). Most other known primary 1α,25(OH)$_2$D$_3$ target genes are much less responsive and often show an inducibility of 2-fold or less after short-term treatment with 1α,25(OH)$_2$D$_3$ (4,5). One of these genes is *cyclin C* (6). Cyclin C belongs to the cyclin protein superfamily, whose members control cell cycle transitions through activation of cyclin-dependent kinases (CDKs). Human and *Drosophila* cyclin C proteins share a high degree of homology (72% identity), which suggests an important role for this gene product that is reflected in its conservation in diverse animal species (7). Interestingly, the cyclin C–CDK8 complex was found to be associated with the RNA polymerase II (Pol II) basal transcriptional machinery (8), and is considered as a functional part of those mediator proteins (MED) complexes that are involved in gene repression (9). This observation suggests a general role for cyclin C in reducing the transcriptional activity of a cell. Another role of cyclin C, in complex with CDK3, seems to be the regulation of the G0 to G1 transition of the cell cycle through specific phosphorylation of the retinoblastoma protein pRb (10). Moreover, the fact that the *cyclin C* gene, being located in chromosome 6q21, is deleted in a subset of acute lymphoblastic leukemias, suggests its involvement in tumorigenesis (11).

The 1α,25(OH)$_2$D$_3$ receptor (VDR) is the only nuclear protein that binds 1α,25(OH)$_2$D$_3$ with high affinity (8 $K_d = 0.1$ nM). Corepressor proteins, such as NCoR, SMRT and Alien (12), link non-ligated DNA-bound VDR to enzymes with histone deacetylase activity that results in chromatin condensation (13). This provides VDR with intrinsic repressive properties comparable with both retinoic acid and thyroid hormone receptors. Ligand binding to the VDR causes a conformational change within its ligand-binding domain, which results in the replacement of corepressors by coactivator proteins of the p160 family, such as nuclear coactivators (NCoAs) 1, 2 and 3 (14).
These coactivators link the ligand-activated VDR to enzymes displaying histone acetyltransferase activity, which results in chromatin relaxation and thereby reversing the action of unliganded VDR (15). In a subsequent step, ligand-activated VDR changes rapidly from interacting with the coactivators of the p160-family to those of the MED complexes, of which cyclin C-CDK8 (16) is a part. The MED complex acts as a bridge from activated VDR to the basal transcriptional machinery (17). In this way ligand-activated VDR executes two tasks, the modification of chromatin and the regulation of transcription.

An essential prerequisite for the direct modulation of transcription via 1α,25(OH)2D3-triggered protein–protein interactions is the location of at least one activated VDR molecule close to the basal transcriptional machinery of a 1α,25(OH)2D3-responding gene. This is traditionally achieved through the specific binding of the VDR to a 1α,25(OH)2D3 response element (VDRE) (18). The DNA-binding domain of the VDR contacts the major groove of a double-stranded hexameric DNA sequence with the consensus sequence RGKTSA (R = A or G, K = G or T, and S = C or G). In most cases the heterodimeric partner of VDR is the retinoid X receptor (RXR), another nuclear receptor superfamily member, which also contacts DNA. Therefore, simple VDREs are often formed by a direct repeat of two hexameric core binding motifs spaced by 3 nt (DR3-type VDRE) (19). In addition, strong DNA binding of VDR–RXR heterodimers to two hexameric motifs arranged as a direct repeat spaced by 4 nt (DR4-type VDRE) (20) or as an inverted repeat with nine intervening nucleotides (ER9-type VDRE) known to date at its core (22), has been reproducibly.

An essential prerequisite for the direct modulation of transcription via 1α,25(OH)2D3-triggered protein–protein interactions is the location of at least one activated VDR molecule close to the basal transcriptional machinery of a 1α,25(OH)2D3-responding gene. This is traditionally achieved through the specific binding of the VDR to a 1α,25(OH)2D3 response element (VDRE) (18). The DNA-binding domain of the VDR contacts the major groove of a double-stranded hexameric DNA sequence with the consensus sequence RGKTSA (R = A or G, K = G or T, and S = C or G). In most cases the heterodimeric partner of VDR is the retinoid X receptor (RXR), another nuclear receptor superfamily member, which also contacts DNA. Therefore, simple VDREs are often formed by a direct repeat of two hexameric core binding motifs spaced by 3 nt (DR3-type VDRE) (19). In addition, strong DNA binding of VDR–RXR heterodimers to two hexameric motifs arranged as a direct repeat spaced by 4 nt (DR4-type VDRE) (20) or as an inverted repeat with nine intervening nucleotides (ER9-type VDRE) have been described previously (21). Although individual VDREs have been shown to be able to induce transcription on their own, the presence of multiple VDREs in any given gene promoter suggests that they may act synergistically. A VDRE cluster, containing the most potent human DR3-type VDRE known to date at its core (22), has been reported in the proximal promoter of the human CYP24 gene (23). However, the DR3-type VDRE of the rat osteocalcin gene (24) is the only VDR binding site that is presently understood in its promoter context, where chromatin organization and flanking binding sites for other transcription factors, such as Runx2 and YY1, are taken into consideration (25).

The major protein constituents of chromatin are histones, and the covalent modifications of lysines at their N-terminal tails neutralize their positive charge and thus their attraction for the negatively charged DNA is diminished (26). This influences the packaging grade of the chromatin and regulates the access of transcription factors to their potential binding sites. More than 10 specific modifications of histones are known, but the acetylation of the lysine at position 8 of histone 4 correlates strongly with the activation of chromatin on a promoter preceding the initiation of transcription (27). Therefore, in most cases, the histones associated with active regions of promoters have a higher degree of acetylation at certain positions than in repressed or silent regions. To date, most studies on transcriptional regulation have been concentrated on isolated promoter regions or proximal promoters, where binding sites of nuclear receptors and other transcription factors have been localized (28).

We have previously identified the human cyclin C gene as a primary 1α,25(OH)2D3 target (6). Since neither VDREs nor chromatin packaging of the promoter of this gene was known, we analyzed, in MCF-7 human breast cancer cells, 8.4 kb of the human cyclin C promoter by using chromatin immunoprecipitation assay (ChIP) with antibodies against acetylated histone 4 (AcH4), VDR and RXR. Interestingly, 1α,25(OH)2D3 treatment did not change the acetylation status of histone 4 on any region of the cyclin C promoter. In contrast to this finding, up to five promoter regions showed a consistent, 1α,25(OH)2D3-dependent association with VDR and RXR over time and in four of these regions re-ChIP assays confirmed the simultaneous association of VDR with RXR, NCaA3, MED1 and Pol II. Furthermore, in silico screening, gel-shift and reporter gene assays identified in each of these four regions a DR3- or DR4-type VDRE.

**MATERIALS AND METHODS**

**Cell culture**

MCF-7 and MDA-MB453 human breast cancer cells and LNCAp and PC-3 human prostate cancer cell were grown in phenol red-free DMEM and RPMI, respectively, supplemented with 5% charcoal-treated fetal bovine serum, 2 mM l-glutamine, 0.1 mg/ml streptomycin and 100 U/ml penicillin, in a humidified 95% air/5% CO2 incubator. Before mRNA extraction or ChIP assay, the cells were treated at a density of 50–60% confluency for 0–360 min with 10 nM 1α,25(OH)2D3 dissolved in ethanol (kindly provided by Dr Lise Binderup, LEO Pharma, Ballerup, Denmark) or vehicle (ethanol, final concentration 0.01%).

**DNA constructs**

Full-length cDNAs for human VDR (29) and human RXRα (30) were subcloned into the T7/SV40 promoter-driven pSG5 expression vector (Stratagene, La Jolla, CA). The same constructs were used for both T7 RNA polymerase-driven *in vitro* transcription/translation of the respective cDNAs and for viral promoter-driven overexpression in mammalian cells. Two copies of the VDREs derived from the human cyclin C, rat atrial natriuretic factor (ANF) and rat pit-1 gene promoters were fused with the thymidine kinase promoter driving the firefly luciferase reporter gene. Each fragment of the human cyclin C promoter [from −2176 to −1786 relative to transcription start site (TSS)] and the human CYP24 promoter (from −414 to −173) were cloned by PCR from human genomic DNA and also fused with the luciferase reporter gene. All constructs were verified by sequencing. The core sequences of the cyclin C VDREs are indicated in Figure 4A and the core sequences of the rat ANF and rat pit-1 REs were AGAGG-CATGAAAGGACA (31) and GAGGTTCCATGAGAGTTCA (32), respectively.

**RNA extraction and real-time quantitative PCR**

Total RNA and mRNA were extracted using Tri-reagent (Sigma-Aldrich, St Louis, MO) and Oligotex mini mRNA kit (Qiagen, Hilden, Germany), respectively. An aliquot of 100 ng of mRNA was used as a template in cDNA synthesis reaction using 100 pmol of oligo(dT)18 primer in the presence of reverse transcriptase (Fermentas, Vilnius, Lithuania). The reaction was performed for 1 h at 37°C. Real-time quantitative PCR was performed in an IQ-cycler (Bio-Rad, Hercules, CA).
by using the dye SybrGreen (Molecular Probes, Leiden, The Netherlands). In PCRs, 3 mM MgCl₂ was used for all primers. The PCR cycling conditions used were: 40 cycles of 30 s at 95°C, 30 s at 58°C (62°C for CYP24) and 40 s at 72°C. Fold inductions were calculated using the formula 2^ΔΔCT, where ΔΔCT is the ΔCT ([Ct(+) − Ct(OH)] − ΔCT(ethanol)). ΔCT is Ct(cyclin C or CYP24) − Ct(ARP0) and Ct is the cycle at which the threshold is crossed. The gene-specific primer pairs (and product sizes) for the gene analyzed here were as follows: cyclin C gene forward 5′-TGGCTCATGGACGCTGTG-3′ and reverse 5′-GCTGTAGCTAGAGTTGAC-3′ (242 bp), CYP24 gene forward 5′-CAAACCGTGGAAGGCTTAC-3′ and reverse 5′-AGTCTCCCCTCCAGGGATCA-3′ (70 bp) (33), acidic riboprotein P0 (ARP0, also known as 36B4) control gene forward 5′-AGATGCAGCACATGCT-3′ and reverse 5′-GTGGTGAACATCCGTCCTG-3′ (318 bp). PCR product quality was monitored using post-PCR melt curve analysis.

**ChIP assays**

Nuclear proteins were cross-linked to DNA by adding formaldehyde for 15 min directly to the medium to a final concentration of 1%. 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 KH₂PO₄ and 8.1 mM Na₂HPO₄·2H₂O). The cells were collected by scraping in ice-cold PBS supplemented with a protease inhibitor cocktail (Roche Diagnostics, Mannheim, Germany). After centrifugation, the cell pellets were resuspended in lysis buffer (1% SDS, 10 mM EDTA, protease inhibitors and 50 mM Tris–HCl, pH 8.1) and the lysates were sonicated to obtain DNA fragments of 300–1000 bp in length. Cellular debris was removed by centrifugation and the lysates were diluted 1:10 in ChIP dilution buffer (0.01% SDS, 1% Triton X-100, 1.2 mM EDTA, 16.7 mM NaCl, protease inhibitors and 16.7 mM Tris–HCl, pH 8.1). Non-specific background was removed by incubating the chromatin resuspension with a salmon sperm DNA/protein A agarose slurry (Upstate Biotechnology, Lake Placid, NY) for 30 min at 4°C with agitation. The samples were centrifuged and the recovered chromatin solutions were incubated with 5 μl of indicated antibodies overnight at 4°C with rotation. The antibody against AcH4 was from Upstate Biotechnology (06-866), whereas antibodies against VDR (sc-13583) and phosphorylated Pol II (sc-13583) were obtained from Santa Cruz Biotechnology (Heidelberg, Germany). The immuno-complexes were collected and the elution was repeated. The supernatants were combined and the cross-linking was reversed by adding NaCl to a final concentration of 200 mM and incubated overnight at 65°C. The remaining proteins were digested by adding proteinase K (final concentration 40 μg/ml) and incubated for 1 h at 45°C. The DNA was recovered by phenol/chloroform/isoamyl alcohol (25:24:1) extractions and precipitated with 0.1 vol of 3 M sodium acetate, pH 5.2 and 2 vol of ethanol using glycogen as a carrier.

**PCR of chromatin templates**

For a complete coverage of the first 8.4 kb of the human cyclin C promoter, 23 primer pairs were designed (Table 1), optimized and controlled by running PCRs with 25 ng genomic DNA (input) as a template. When running immuno-precipitated DNA (output) as a template, 10 ng was used in the optimized conditions for each PCR with the following profile: preincubation for 5 min at 94°C, 40 cycles of 30 s denaturation at 95°C, 30 s annealing at primer-specific temperature (see Table 1) and 40 cycles of 30 s elongation at 72°C, with one final incubation for 10 min at 72°C. The PCR products were separated by electrophoresis through 2.0% agarose gels supplemented with 0.5 μg/ml ethidium bromide and quantified using a FLA-3000 reader (Fuji, Tokyo, Japan) with Image Gauge software (Fujifilm).

**Gel-shift analysis**

In vitro translated VDR and RXR proteins were generated by coupled in vitro transcription/translation using their respective pSG5-based full-length cDNA expression constructs (29) and rabbit reticulocyte lysate as recommended by the supplier (Promega, Madison, WI). Protein batches were quantified by test translation in the presence of ['³⁵S]methionine. The specific concentration of the receptor proteins was adjusted to ~4 ng/μl (10 ng corresponds to ~0.2 pmol) after taking the individual number of methionine residues per protein into account. Gel-shift assays were performed with 10 ng of the appropriate in vitro translated proteins. The proteins were incubated for 15 min in a total volume of 20 μl binding buffer (150 mM KCl, 1 mM DTT, 0.2 μg/μl poly(dI–dC), 5% glycerol and 10 mM HEPES, pH 7.9). Constant amounts (1 ng) of ['³²P]-labeled double-stranded oligonucleotides (50 000 c.p.m.) corresponding to one copy of a dimeric RE were then added and incubation was continued for 20 min at room temperature. Protein–DNA complexes were resolved by electrophoresis through 8% non-denaturing polyacrylamide gels in 0.5× TBE (45 mM Tris, 45 mM boric acid and 1 mM EDTA, pH 8.3) and quantified on a FLA-3000 reader using Image Gauge software.
Table 1. Genomic PCR primer sequences and their location within the human cyclin C and CYP24 promoters

<table>
<thead>
<tr>
<th>Region nos</th>
<th>Annealing temperature (°C)</th>
<th>Location</th>
<th>Primer sequences</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>60</td>
<td>+97 to −220</td>
<td>5'-CTGCCATGGAAACACAGTTCG-3'</td>
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<tr>
<td>2</td>
<td>58</td>
<td>−117 to −617</td>
<td>5'-ATCGCAAAAAGTTCGGCCC-3'</td>
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<tr>
<td>3</td>
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<td>60</td>
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<td>5'-CGGGCGCGGTATGAGACG-3'</td>
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<tr>
<td>6</td>
<td>60</td>
<td>−1786 to −2176</td>
<td>5'-CATGACATCTGAGGCAAC-3'</td>
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<tr>
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Transfection and luciferase reporter gene assays

MCF-7 cells were seeded into six-well plates (10^5 cells/ml) and grown overnight in phenol red-free DMEM supplemented with 5% charcoal-stripped fetal bovine serum. Plasmid DNA containing liposomes were formed by incubating a reporter plasmid and expression vector for human VDR (each 1 μg) with 10 μg N-[1-(2,3-dioleoyloxy)-propyl]-N,N,N-trimethylammonium methylsulfate (DOTAP) (Roth, Karlsruhe, Germany) for 15 min at room temperature in a total volume of 100 μl. After dilution with 900 μl phenol red-free DMEM, the liposomes were added to the cells. Phenol red-free DMEM supplemented with 500 μl of 15% charcoal-stripped fetal bovine serum was added 4 h after transfection. At this time, 100 nM 1α,25(OH)_2D_3 or solvent was also added. The cells were lysed 16 h after the onset of stimulation using the reporter gene lysis buffer (Roche Diagnostics) and the constant light signal luciferase reporter gene assay was performed according to the supplier’s recommendation (Canberra-Packard, Groningen, The Netherlands). The luciferase activities were normalized with respect to protein concentration and induction factors were calculated as the ratio of luciferase activity of ligand-stimulated cells to that of solvent controls.

RESULTS

Basal expression of cyclin C and CYP24 in different cancer cell lines

The basal expression levels of the two primary 1α,25(OH)_2D_3 target genes, cyclin C and CYP24, were monitored by real-time
quantitative PCR in relationship with the control gene ARP0 in MCF-7 and MDA-MB453 human breast cancer cells and in LNCaP and PC-3 human prostate cancer cells (Figure 1A). MCF-7 and LNCaP are less aggressive estrogen- and testosterone-dependent cell lines, respectively, while MDA-MB453 and PC-3 are sex-hormone-insensitive cells. The basal expression of the cyclin C gene was found to be comparable in MCF-7 and LNCaP cells and was ~30,000-fold higher than that of the basal mRNA expression of the CYP24 gene. The expression of the cyclin C gene was nearly 10-fold higher in MDA-MB453 cells than in MCF-7 and LNCaP cells, while the CYP24 mRNA level was ~3-fold elevated in MDA-MB453 cells compared with the two other cell lines. This results in a nearly 100,000-fold difference in the basal mRNA expression of cyclin C and CYP24 in these cells. Finally, in PC-3 cells the cyclin C mRNA level was found to be 3-fold higher than that in both MCF-7 and LNCaP cells, but the CYP24 gene expression was 10-fold elevated. This means that the two genes differ in their expression only by a factor of 10,000-fold in this cell line.

Figure 1. Comparison of cyclin C and CYP24 mRNA expression. Real-time quantitative PCR was used to determine the ratio of the basal levels of cyclin C and CYP24 mRNA relative to the control gene ARP0 in MCF-7 and MDA-MB453 human breast cancer and in LNCaP and PC-3 human prostate cancer cells (A). A logarithmic scale is employed on the y-axis to better present the data. (B) In the same four cell lines, the induction of cyclin C and CYP24 mRNA after 2 h treatment with 10 nM 1α,25(OH)2D3 was measured. (C) The time course of cyclin C mRNA expression in response to 10 nM 1α,25(OH)2D3 was determined in MCF-7 cells. Data points (A and C) and columns (B) indicate the means of at least three independent cell treatments and the bars represent the standard error of mean. The standard deviations in (A) are too small to be visible in relationship with the data points.

1α,25(OH)2D3 inducibility of cyclin C and CYP24 in different cancer cell lines

All four cell lines were treated for 2 h with 10 nM 1α,25(OH)2D3 and the fold change of normalized cyclin C and CYP24 mRNA was measured in relationship with the solvent control (Figure 1B). After this short-term stimulation the cyclin C mRNA level was found to be 1.8-fold upregulated in MCF-7 cells, 1.6-fold in LNCaP cells, 1.2-fold in MDA-MB453 cells and slightly reduced in PC-3 cells. In comparison, CYP24 gene expression was increased to 3.9-fold in MCF-7 cells, 6.1-fold in LNCaP cells, 3.3-fold in MDA-MB453 cells and 10.8-fold in PC-3 cells within the same time period. This means that MCF-7 cells showed not only the highest absolute induction of the cyclin C gene, but also monitor, in comparison with the CYP24 gene, the highest relative induction. Since cyclin C mRNA molecules are more abundant (reflected in the high basal level of the mRNA relative to the ARP0 control gene), we estimate that MCF-7 cells synthesized within the 2 h stimulation period ~7600-fold more cyclin C mRNA molecules than CYP24 mRNA molecules. A 6 h time course of cyclin C expression in MCF-7 cells (Figure 1C) suggests that the upregulation of the gene was very transient and showed a maximum after 2 h stimulation. This is in contrast to the CYP24 gene, which showed a steady increase in mRNA amount up to >400-fold induction after 6 h stimulation (data not shown). Taken together, the real-time quantitative PCR results suggest that MCF-7 cells are most suited for investigations of the 1α,25(OH)2D3-induction of the human cyclin C promoter.

Whole cyclin C promoter screening for histone 4 acetylation levels

We hypothesized that a transient upregulation of the cyclin C gene should result in dynamic changes in the chromatin activation state on a wider region of the promoter of the gene in response to 1α,25(OH)2D3 stimulation. In order to test this hypothesis, we designed 23 overlapping primer pairs that cover evenly the first 8.4 kb of chromosomal DNA upstream of the TSS of the cyclin C gene (Table 1). As a reference, a primer pair covering the proximal promoter of the CYP24 gene, containing the known VDRE cluster (34), was used. To minimize the number of primers in repetitive sequences, we employed the web-based CENSOR server screening service (35) to identify repetitive sequences in the promoter sequence. We determined that the repetitive sequence content of this segment of human chromosome 6 is ~46%. The remaining 54% unique sequence was used to design the overlapping sequence.
PCR primer pairs. Chromatin was extracted from MCF-7 cells that were grown overnight in the presence of 5% charcoal-treated fetal bovine serum, stimulated for 0, 30, 60, 120 and 180 min with 10 nM 1α,25(OH)2D3 and then cross-linked for 15 min in the presence of formaldehyde. ChIP assays were performed using an antibody against AcH4 and representative agarose gels of the PCR products from all treatment times are shown in Figure 2A. Comparable detection sensitivity for the 23 different cyclin C promoter regions and the proximal CYP24 promoter was demonstrated by the representative PCR products that were obtained using DNA liberated from the recovered chromatin by reverse cross-linking (input lane). PCR products obtained with AcH4-enriched chromatin visualized the acetylation status of each of the 23 regions of the cyclin C promoter in comparison with that of the proximal CYP24 promoter. Cell treatment, chromatin extraction and PCR were performed at least six times and the basal histone 4 acetylation level was quantified in relationship with their respective chromatin input (Figure 2B). Interestingly, while the proximal promoter of the CYP24 gene showed a low acetylation level, all 23 regions of the cyclin C promoter displayed significantly more association with AcH4. The latter was highest at the TSS and within the first 617 bp of the promoter (regions 1 and 2) and lowest at promoter regions 3, 10 and 11. However, the histone 4 acetylation level of all regions of the cyclin C promoter did not vary more than by a factor of 2. Moreover, for each of the 23 regions of the cyclin C promoter the histone 4 acetylation level did not change significantly in response to 1α,25(OH)2D3 (Figure 2A). In contrast, AcH4 association with the proximal CYP24 promoter showed a steady increase after 60 min ligand stimulation. In summary, the ChIP assay results demonstrate that the histone 4 acetylation level of the cyclin C promoter is, in contrast to that of the CYP24 promoter, not suited to monitor regulatory effects of 1α,25(OH)2D3. However, the high-basal levels reflect the fact that the cyclin C gene is highly transcribed in the absence of the hormone (Figure 1A).

VDR location on the cyclin C promoter

We next screened the whole cyclin C promoter for regions that precipitated with anti-VDR antibodies (Figure 3A). Over
a stimulation period of 240 min with 10 nM 1α,25(OH)₂D₃. VDR association centered at promoter regions 1, 6, 16, 20 and 23. These regions were separated by regions 2–4, 8–14, 18 and 22, which showed no or only very faint association with VDR. Since a portion of the chromatin template fragments were significantly larger than the amplified PCR products, the signals obtained at regions 5 and 7, 15 and 17, and 19 and 21 are interpreted as flanking effects. Promoter regions 1, 6, 20 and 23 as well as the proximal CYP24 promoter showed a significant increase in VDR association over time with a maximum between 60 and 180 min. In contrast, promoter region 16 displayed constitutive association with VDR, which was not significantly increased in response to 1α,25(OH)₂D₃. Taken together, the ChIP assay results indicate that, in contrast to histone 4 acetylation, VDR association is better suited to monitor 1α,25(OH)₂D₃-dependent changes of the cyclin C and CYP24 promoters. These results also indicate that up to five regions within the cyclin C promoter are nucleation points for activated VDR.

**RXR location on the cyclin C promoter**

We next screened the whole cyclin C promoter with ChIP assays using an antibody against the VDR partner receptor RXR over a stimulation period of 240 min with 10 nM 1α,25(OH)₂D₃ to identify potential VDR–RXR heterodimer binding regions (Figure 3B). From the results, it was found that the pattern of RXR location on the cyclin C promoter was nearly identical to the pattern obtained with the anti-VDR antibody (Figure 3A). RXR association centered at promoter regions 1, 6, 16, 19 and 23 that were isolated from each other by the non-associating regions 2–4, 8–14, 17, 21 and 22. Signals observed at regions 5, 7, 15 and 18 might be explained by being regions that flank positive regions. The only difference between the RXR and VDR association patterns was observed at regions 18–21, where the RXR patterns suggest region 19 as the center while the VDR pattern suggested region 20. In general, RXR binding to the different regions of the cyclin C promoter showed to be rather constitutive and no
significant effects of 1α,25(OH)2D3 on the association level of RXR with these regions could be detected. In contrast, on the proximal CYP24 promoter RXR binding displayed a clear maximum between 60 and 120 min. In summary, RXR location fits almost perfectly VDR location and suggests that promoter regions 1, 6, 16, 19/20 and 23 are able to bind VDR–RXR heterodimers.

Identification of VDREs on cyclin C promoter

The ChIP assays with anti-VDR and anti-RXR antibodies (Figure 3) suggest that the human cyclin C promoter may contain up to five VDREs. In order to challenge this prediction, we performed in silico screening of the first 8.4 kb of the cyclin C promoter for DR3-, DR4- and ER9-type response elements (REs) with the consensus sequence RGKTS and allowing for one mismatch per hexameric sequence. Applying this rule we found nine DR3-type REs, eight DR4-type REs and two ER9-type REs. From these 19 REs, 6 were found in the area of the VDR–RXR binding promoter regions 6, 16, 19/20 and 23. These were two DR3-type REs (RE7 and RE8 in regions 20 and 23, respectively) and four DR4-type REs (RE2, RE5, RE6 and RE9 in regions 6, 16, 19 and 23, respectively) (Figure 4A). From the remaining 13 putative VDREs outside of these 5 promoter regions, only the sequences of the 2 DR3-type REs 1 and 3 (in regions 3 and 11, respectively) and 1 DR4-type RE4 (close to region 11) were found interesting enough for further investigations. Surprisingly, no high-quality putative RE was found in region 1 and no promising ER9-type RE in the whole promoter area. Although RE8 and RE9 in region 23 form a cluster, the constituting DR3- and DR4-type REs were tested individually to examine their relative contribution to potential VDR–RXR binding.

On all of the nine putative VDREs, gel-shift assays were performed with in vitro translated VDR and RXR protein, either alone or in combination (Figure 4B), under conditions identical to our earlier DR3-type VDRE comparative study (22). RE2 bound 2.87-fold more effective VDR–RXR heterodimers than the reference DR3-type VDRE of the proximal human CYP24 promoter (23), while the relative binding of VDR–RXR to RE5, RE7 and RE8 was only 4, 27 and 16%, respectively, of the reference element. Neither VDR nor RXR homodimers was observed on any of the 10 tested REs. The binding strength of the DR4-type RE2 in region 6 belongs to the strongest known VDRE class (i.e. type I), while the DR4-type RE5 in region 16, the DR3-type RE7 in region 20 and the DR3-type RE8 in region 23 are class II-type VDREs (22). In contrast, the isolated REs 1, 3, 4, 6 and 9 did not show any binding of VDR–RXR heterodimers. This suggests that the additional 10, more degenerate, putative REs outside of regions 3, 6, 11, 16, 19/20 and 23 may not allow any in vitro binding of VDR–RXR heterodimers. Taken together, our in silico in vitro scanning for functional VDREs in the cyclin C promoter (summarized in Figure 4C) indicated that from 19 putative DNA-binding sites of the VDR only REs 2, 5, 7 and 8 in regions 6, 16, 20 and 23, respectively, showed significant VDR–RXR heterodimer binding. Since these four regions also show ligand-dependent association with VDR in ChIP assays, we conclude that the VDREs are responsible for the VDR and RXR association.

Figure 4. In silico and in vitro screening for VDREs in the human cyclin C promoter. (A) In silico promoter analysis indicated nine putative VDREs (RE1–RE9) found within seven regions of the human cyclin C promoter. The two hexameric half sites of each VDRE core sequence are shown in boldface and their relative orientation and number of spacing nucleotides are indicated. (B) Gel-shift experiments were performed with in vitro translated human VDR and human RXR alone or in combination and in the presence of 32P-labeled REs. Protein–DNA complexes were resolved from free probe through non-denaturing 8% polyacrylamide gels. Representative gels are shown. The relative amount of VDR–RXR heterodimer complex formation was quantified on a FLA-3000 reader in relationship with the DR3-type VDRE of the proximal promoter of the CYP24 gene. NS indicates non-specific complexes. (C) The order and the results of the screening procedure are schematically depicted in a flow chart.
Functionality of cyclin C VDREs in MCF-7 cells

In order to test the functionality of the REs 2, 5, 7 and 8, two copies of each of these REs were fused with the thymidine kinase promoter driving the firefly luciferase reporter gene, transfected into MCF-7 cells and stimulated for 16 h with 100 nM 1α,25(OH)2D3 (Figure 5A). Two copies of the rat ANF DR3-type VDRE and the rat pit-1 DR4-type RE, which are the best REs in their categories (22), mediated a 17.4- and 26.2-fold induction of reporter gene activity, respectively. The VDREs of the cyclin C promoter did not show that high a response to ligand treatment, but the 18.8-, 3.0-, 8.3- and 5.6-fold inductions after ligand stimulation for REs 2, 5, 7 and 8, respectively, are still representing 72, 17, 48 and 15% of the inducibility of their respective DR4- and DR3-type reference VDREs.

The functionality of RE2 in its natural promoter context was tested by analyzing the inducibility of a 390 bp fragment of the human cyclin C promoter (from −2176 to −1786) in MCF-7 cells measured by luciferase reporter gene assays. We obtained a 8.4-fold induction of luciferase activity after stimulation with 100 nM 1α,25(OH)2D3 (Figure 5B). This is very much comparable with the 8.9-fold induction of a reference promoter fragment of the human CYP24 promoter containing the DR3-type VDRE used in Figure 4B. In summary, reporter gene assays confirmed the functionality of the DR3- and DR4-type VDREs 2, 5, 7 and 8 in promoter regions 6, 16, 20 and 23, respectively, and the most potent VDRE, RE2, of the cyclin C promoter showed in its natural promoter context the same ligand inducibility as the DR3-type VDRE of the human CYP24 promoter.

Co-localization of VDR and RXR on cyclin C promoter regions

An additional test for the functionality of the VDREs in the chromatin context of MCF-7 cells was performed by re-ChIP assay (Figure 6). In this assay, first the anti-VDR antibody and then antibodies against RXR, NCoA3, MED1 or phosphorylated Pol II were used for immuno-precipitation, so that the enriched chromatin templates should have been associated with both VDR and its partner proteins at the same time. From this double-fractionated chromatin template, the cyclin C promoter regions 6, 16, 20 and 23 were amplified, but not the negative control region 11. The association of VDR with its partner proteins showed an individual profile on the cyclin C promoter regions 6, 16, 20 and 23 as well as on the proximal CYP24 promoter in their relative strength, but most of them showed their maximum at the time point 60 min after ligand treatment. In summary, re-ChIP assays confirmed the simultaneous association of VDR with RXR, NCoA3, MED1 or phosphorylated Pol II on all four 1α,25(OH)2D3-responsive cyclin C promoter regions.

DISCUSSION

This study describes a deeper understanding of the regulation of the cyclin C gene by 1α,25(OH)2D3. The cyclin C gene is an interesting 1α,25(OH)2D3-responding gene, since its function is not linked to the classical endocrine functions of 1α,25(OH)2D3, such as the regulation of calcium homeostasis and bone mineralization, but to the regulation of cellular
Figure 6. VDR complexes in 1α,25(OH)₂D₃-responsive promoter regions. Chromatin was extracted from MCF-7 cells that had been treated for indicated time periods with 10 nM 1α,25(OH)₂D₃. Re-ChIP experiments were performed with a first immuno-precipitation with anti-VDR antibody and a second precipitation with anti-RXR, anti-NCoA3, anti-MED1 or anti-phosphorylated Pol II antibodies. The association of VDR and its partner proteins was monitored on the human cyclin C promoter regions 6 (A), 11 (B) (negative control), 16 (C), 20 (D), 23 (E) and region 1 of the CYP24 promoter (F) (positive control). Representative agarose gels are shown.

growth. Being identified as a member of the cyclin family, the regulation of cyclin C by 1α,25(OH)₂D₃ should provide a more detailed understanding of the mechanisms of how 1α,25(OH)₂D₃ regulates cellular functions, such as proliferation, differentiation and apoptosis. However, despite some function in the G₀–G₁ transition (10), the main function of cyclin C protein seems to be acting as a component of MED complexes. This suggests that cyclin C regulates the general transcription rate rather than the cell cycle. Therefore, it is possible that the effects that cyclin C has on the cell cycle are secondary in nature and derive from an effect on the build up of other products involved more intimately in cell cycle regulation. Interestingly, cyclin C has been reported to be contained only in those MED complexes that have a transcriptionally repressive function (16). The cyclin C–CDK8 complex phosphorylates the C-terminal domain of Pol II (8) and the basal transcription factor TFIIH (36) and both of the phosphorylations terminate transcription. Nevertheless, the impact of a gene that represses mRNA transcription in general is at least as high as that of a gene that influences the cell cycle.

In the context of both functions the transient expression of the cyclin C is important and makes sense. Cyclin C mRNA accumulates periodically during the cell cycle, peaking in G₁ (37). Moreover, cyclin C protein was shown to have a half-life of 4 h (38) and in this study we have shown that the increase in cyclin C mRNA expression in response to 1α,25(OH)₂D₃ is very transient (Figure 1C). In contrast, for an enzyme, such as the monooxygenase CYP24, it is important to stay active for a longer time period and its expression should be independent of the cell cycle.

The reference gene of this study, CYP24, is the most responsive primary VDR target gene and is expressed in numerous tissues. It has a special role in 1α,25(OH)₂D₃ signaling, because its protein product leads to the degradation of 1α,25(OH)₂D₃ and, therefore, the eventual extinction of the transcriptional signal. VDR–RXR heterodimers are the central transcription factors on the CYP24 promoter, so that it is not surprising that the basal expression of the gene in the absence of an activating signal for VDR–RXR heterodimers is very low (Figure 1A). In contrast, the basal expression of the cyclin C gene is 10 000–100 000-fold higher than that of the CYP24 gene (Figure 1A), suggesting that transcription factors other than VDR–RXR heterodimers contribute to the activity of the gene. An in silico screening for putative transcription factor binding sites highlighted each two nuclear factor κB (NFκB) and AP-1 binding sites located between positions −5800 and −6800 of the human cyclin C promoter. Interestingly, cyclin C was listed recently as a NFκB responding gene (39). The high-basal activity of the cyclin C gene is also demonstrated by the overall high level of histone 4 acetylation
throughout the whole 8.4 kb of its promoter (Figure 2). Moreover, the fact that a treatment with 1α,25(OH)₂D₃ is visible on the level of histone 4 acetylation of the proximal CYP24 promoter but not on the whole cyclin C promoter (Figure 2A), confirms the dominating role of VDR–RXR heterodimers for the CYP24 gene regulation and relativizes their impact for the cyclin C gene. However, since the behavior of most primary 1α,25(OH)₂D₃-responsive genes resembles more than that of the cyclin C gene, with a modulation of the mRNA amount by a factor of only 1.5–2.0, the strong response of the CYP24 gene has to be considered as an exception.

A number of nuclear receptor target gene promoters, such as the CYP24 or the estrogen-induced pS2 gene (28), appear to have one dominating RE and the analysis of the chromatin status has mostly concentrated on these core promoter regions. In this study, we also found one strong VDRE (RE2) within the cyclin C promoter, which in addition is the RE closest to the TSS (position –2100). This VDRE may be considered as sufficient for understanding the full response of the cyclin C gene to 1α,25(OH)₂D₃. However, in addition to promoter region 6, in which RE2 is located, ChIP screening with anti-VDR and anti-RXR antibodies consistently identified four additional VDR–RXR associating regions, three of which contain classical VDREs. The ChIP signal obtained for region 1, which contains the TSS may be due to the interaction of the VDR–RXR bound to the VDREs at promoter regions 6, 16, 20 and 23 via traditional ‘DNA looping model’ (40), i.e. a false positive result.

Accepting that the cyclin C promoter has up to four functional VDREs, raises the question of their purpose. Although the in vitro DNA-binding affinity of VDR–RXR heterodimers to REs 5, 7 and 8 and their inducibility in MCF-7 cells was clearly lower than that of RE2, on chromatin level all four VDRE-containing promoter regions show equal association strength with VDR or RXR (Figure 3). Other transcription factors that bind in the vicinity stabilize a VDR–RXR heterodimer to a VDRE, which is physically weak under the stringent in vitro binding conditions. Such a phenomenon has already been described to explain the binding of VDR–RXR heterodimers to the proximal VDRE of the rat CYP24 gene promoter (41). Alternatively, the four distinct REs could come together simultaneously and work co-operatively as has been already observed with the RE clusters of other nuclear receptor target genes, such as CYP2B6 and CYP3A4 (42). Thus, REs can act synergistically in the activation of the respective genes, which could also be the case for the cyclin C promoter.

The strong DR4-type VDRE at position –2100 relative to the TSS, RE2, belongs to the most potent known VDR–RXR heterodimer binding sites. Under the same stringent evaluation criteria as applied in a comparative study of all known VDREs (22), the VDRE shows 25% of the VDR–RXR heterodimer binding strength of the DR4-type RE of the rat pit-1 promoter (32) and even 72% of its inducibility in MCF-7 cells. The latter RE is formed by two perfect hexameric half-site with optimized 5′-flanking sequences (43) and has served as a reference in a number of comparative nuclear receptor studies (22,43,44), but its general physiological impact can be questioned, since it is located in a chromatin region that is only active during a short time in embryonic development. The DR4-type VDRE of the human cyclin C promoter is nearly 3-fold more potent compared with the DR3-type VDRE of the human CYP24 proximal promoter (Figure 4B), which is presently the best human VDRE (22). RE2 of the human cyclin C promoter is also comparable in its strength with the DR3-type VDREs of the rat ANF gene (31) or the mouse osteopontin gene (45). This makes the cyclin C VDRE the most potent known human VDRE. Therefore, it is likely that in future it will serve as a reference to a number of studies on novel VDREs. Moreover, the examples of the VDREs of the cyclin C gene, the CYP24 gene and other VDR target genes demonstrate that equally potent VDR–RXR heterodimer binding ability can result in completely different 1α,25(OH)₂D₃ inducibility of the respective gene. Therefore, as an additional parameter for a prediction of ligand responsiveness the basal activity of the respective gene’s promoters has to be taken into account.

The mechanisms of downregulation of genes by 1α,25(OH)₂D₃ is largely not understood, although microarrays and other gene expression profiling methods (4,5) suggest that approximately half of all 1α,25(OH)₂D₃-responding genes are downregulated by the hormone. In this respect, the finding that the protein product of the primary 1α,25(OH)₂D₃-responding gene cyclin C is a component of transcriptionally repressive MED complexes (16), may provide a mechanism to explain how the downregulation of secondary 1α,25(OH)₂D₃-responding genes is mediated. Experiments investigating this hypothesis are currently underway in our laboratory.

In conclusion, our study provided insight into the regulation of the cyclin C gene by 1α,25(OH)₂D₃. We demonstrated that whole promoter ChIP screening with anti-VDR and anti-RXR antibodies is suitable for RE identification, found within 8.4 kb of the cyclin C promoter one strong and three weaker VDREs and monitored in MCF-7 cells the association of VDR–RXR with the promoter regions containing these VDREs. This may help to understand the downregulation of a number of secondary 1α,25(OH)₂D₃-responding genes with an impact on cellular growth, differentiation and apoptosis.

**ACKNOWLEDGEMENTS**

We would like to thank Dr Lise Binderup for 1α,25(OH)₂D₃ and Dr Thomas W. Dunlop for critical reading of the manuscript. Grants from the Academy of Finland, the Finnish Cancer Organisation and the Finnish Technology Agency TEKES (all to C.C.) supported this research. Funding to pay the Open Access publication charges for this article was provided by the Academy of Finland.

Conflict of interest statement. None declared.

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