

# Regulation of the human *cyclin C* gene via multiple vitamin D<sub>3</sub>-responsive regions in its promoter

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## ABSTRACT

The candidate human tumor suppressor gene *cyclin C* is a primary target of the anti-proliferative hormone 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> [1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>], but binding sites for the 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> receptor (VDR), so-called 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> response 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 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> 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 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>, but four independent promoter regions showed a consistent, 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>-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 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>-responding genes.

## INTRODUCTION

The biologically most active vitamin D metabolite, 1 $\alpha$ ,25-dihydroxyvitamin D<sub>3</sub> [1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub>], 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). 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> levels are tightly controlled by the monooxygenase vitamin D 24-hydroxylase (*CYP24*), which metabolizes the active hormone. The *CYP24* gene is also the most responsive primary 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> target gene and shows at the mRNA level up to 1000-fold inducibility by the hormone (3). Most other known primary 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> target genes are much less responsive and often show an inducibility of 2-fold or less after short-term treatment with 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> (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 protein (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 G<sub>0</sub> to G<sub>1</sub> 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 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> receptor (VDR) is the only nuclear protein that binds 1 $\alpha$ ,25(OH)<sub>2</sub>D<sub>3</sub> with high affinity ( $K_d = 0.1$  nM). Corepressor proteins, such as NCoR, SMRT and Alien (12), link non-liganded, 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).

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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  $1\alpha,25(\text{OH})_2\text{D}_3$ -triggered protein-protein interactions is the location of at least one activated VDR molecule close to the basal transcriptional machinery of a  $1\alpha,25(\text{OH})_2\text{D}_3$ -responding gene. This is traditionally achieved through the specific binding of the VDR to a  $1\alpha,25(\text{OH})_2\text{D}_3$  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 RGTKSA (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 everted repeat with nine intervening nucleotides (ER9-type VDRE) have been described previously (21). Although individual VDREs have been shown to be able to induce transactivation 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  $1\alpha,25(\text{OH})_2\text{D}_3$  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,  $1\alpha,25(\text{OH})_2\text{D}_3$  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,  $1\alpha,25(\text{OH})_2\text{D}_3$ -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, NCoA3, 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% CO<sub>2</sub> 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  $1\alpha,25(\text{OH})_2\text{D}_3$  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 RXR $\alpha$  (30) were subcloned into the T<sub>7</sub>/SV40 promoter-driven pSG5 expression vector (Stratagene, La Jolla, CA). The same constructs were used for both T<sub>7</sub> 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 AGAGGT-CATGAAGGACA (31) and GAAGTTCATGAGAGTCA (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 oligodT<sub>18</sub> 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 MgCl<sub>2</sub> 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  $2^{-(\Delta\Delta Ct)}$ , where  $\Delta\Delta Ct$  is the  $\Delta Ct_{[1\alpha, 25(OH)_2D_3]} - \Delta Ct_{(Ethanol)}$ ,  $\Delta Ct$  is  $Ct_{(cyclin\ C\ or\ CYP24)} - Ct_{(ARPO)}$  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'-TGCCTACATGTAGCCTGTGT-3' and reverse 5'-GCTGTAGCTAGAGTTCTGAC-3' (242 bp), *CYP24* gene forward 5'-CAAACCGTGGAAAGGCC-TATC-3' and reverse 5'-AGTCTTCCCCTTCCAGGATCA-3' (70 bp) (33), *acidic riboprotein P0* (*ARPO*, also known as *36B4*) control gene forward 5'-AGATGCAGCAGATCCG-CAT-3' and reverse 5'-GTGGTGATACCTAAAGCCTG-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 KH<sub>2</sub>PO<sub>4</sub> and 8.1 mM Na<sub>2</sub>HPO<sub>4</sub>·2H<sub>2</sub>O). 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, 1.1% 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-1008), RXR $\alpha$  (sc-553), NCoA3/RAC3 (sc-7216), MED1/TRAP220 (sc-5334) and phosphorylated Pol II (sc-13583) were obtained from Santa Cruz Biotechnologies (Heidelberg, Germany). The immuno-complexes were collected with 60 µl of protein A agarose slurry (Upstate Biotechnology) for 2 h at 4°C with rotation. The beads were pelleted by centrifugation for 1 min at 4°C at 100 g and washed sequentially for 5 min by rotation with 1 ml of the following buffers: low-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 150 mM NaCl and 20 mM Tris-HCl, pH 8.1), high-salt wash buffer (0.1% SDS, 1% Triton X-100, 2 mM EDTA, 500 mM NaCl and 20 mM Tris-HCl, pH 8.1) 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 (1 mM EDTA and 10 mM

Tris-HCl, pH 8.0). For re-ChIP, the immuno-complexes were eluted by adding 200 µl re-ChIP elution buffer (10 mM DTT) for 30 min at room temperature with rotation, the supernatant was diluted 1:40 in ChIP dilution buffer and the antibody against the second protein of interest was added, the new immuno-complexes were allowed to form by incubating overnight at 4°C on a rocking platform, the immuno-complexes were collected by incubating with 60 µl protein A agarose slurry for 2 h at 4°C on a rocking platform and finally washed as indicated above. In both cases, the immuno-complexes were then eluted by adding 250 µl elution buffer (1% SDS and 100 mM NaHCO<sub>3</sub>) and incubated for 15 min at room temperature with rotation. After centrifugation, the supernatant was 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 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 (Fuji).

### 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 [<sup>35</sup>S]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 <sup>32</sup>P-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

Region nos	Annealing temperature (°C)	Location	Primer sequences
1	60	+97 to -220	5'-CTGCCATGGAACACAGCTTG-3' 5'-CGAGCTCTCGTGAACACTAG-3'
2	58	-117 to -617	5'-ATCGACAAAAGTTCGGGCC-3' 5'-GAGAAATCGCTTGAACCTCTG-3'
3	60	-329 to -1196	5'-CGAAGTGGCTCAGATACGGG-3' 5'-GCAATGAGCCTAGATAGCGC-3'
4	60	-1176 to -1842	5'-GCGCTATCTAGGCTCATTGC-3' 5'-ACACCCTAATTGGTCTCCAC-3'
5	60	-1385 to -1842	5'-CGGCCGCGATCTTAATGGAG-3' 5'-ACACCCTAATTGGTCTCCAC-3'
6	60	-1786 to -2176	5'-CAGTCAGCATCTGAGGCAAC-3' 5'-GCCACCACACTCCGCTAATT-3'
7	60	-2118 to -2453	5'-GGGACTTATGGGAGTGACG-3' 5'-GCCAACATGGTAAAACCTG-3'
8	60	-2433 to -2885	5'-CCTCCAAGTAGCTAGGATC-3' 5'-CGTCACTCCATAAAAGTCCC-3'
9	60	-2783 to -3456	5'-GGCCTGGAGTTTCTTAAGGTAG-3' 5'-CATGGCCAGCATGGTGAAC-3'
10	58	-3169 to -3456	5'-GGCCTGGAGTTTCTTAAGGTAG-3' 5'-GAGACCATCCTGGCTAACAC-3'
11	60	-3437 to -3867	5'-ACCTTAAGAACTCCAGCC-3' 5'-GGCTGAGGTGGTGGATTAA-3'
12	58	-3801 to -4197	5'-GTGGAAGCAGAAACCAGGTC-3' 5'-GGGGTTTCACCATGTTGGTC-3'
13	58	-4177 to -4758	5'-GACCTGGTTCTGCTTCCAC-3' 5'-GCTTGAGATGATGGATACCC-3'
14	60	-4509 to -4988	5'-TTTCAGACCTCACTACCCAC-3' 5'-ATTCCAGGTATTCTTGCCGC-3'
15	58	-4924 to -5374	5'-GGAGCTAGAAGGTTAGTGGC-3' 5'-GAGAGCCTCTCTGTATC-3'
16	58	-5217 to -5746	5'-GTAGTCATCCCCTGTACCTC-3' 5'-AGCCTCAAAGGGCCATGAAG-3'
17	60	-5628 to -6039	5'-TTCGTGCAAGGTTGCTGTGG-3' 5'-GGCCTCTGCCTTCATGAATG-3'
18	60	-5960 to -6354	5'-AAGACGGGGCCAACTC-3' 5'-CTATGTATCAGGCACTGCTC-3'
19	58	-6286 to -6788	5'-GGTAAGTCATAGGGTTGTGG-3' 5'-GGCCATTTCTTGATCCTGGG-3'
20	58	-6728 to -7202	5'-GAGCCAGATCTTCAATCC-3' 5'-CCAGAGTAGCTATCTTCAGC-3'
21	58	-7082 to -7522	5'-TGTGCCACAGCAAGATGGTG-3' 5'-CCCTCTGTCCTCAGAGTTTC-3'
22	60	-7464 to -7877	5'-GGTTCATCATGGAGTGTGGG-3' 5'-CATCCAGACTCCAGACACAG-3'
23	58	-7825 to -8383	5'-AGTCCCAAGCATAACAGG-3' 5'-CTAAGTCTGTGGCCAGAG-3'
1 <sub>CYP24</sub>	66	-64 to -480	5'-GTCCAGGCTGGGGTATCTG-3' 5'-CCTCCGCCCTCTCTGCG-3'

### 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  $\mu$ g) with 10  $\mu$ g *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  $\mu$ l. After dilution with 900  $\mu$ l phenol red-free DMEM, the liposomes were added to the cells. Phenol red-free DMEM supplemented with 500  $\mu$ l of 15% charcoal-stripped fetal bovine serum was added 4 h after transfection. At this time, 100 nM  $1\alpha,25(\text{OH})_2\text{D}_3$  or solvent was also added. The cells were lysed 16 h after the onset of stimulation using the reporter

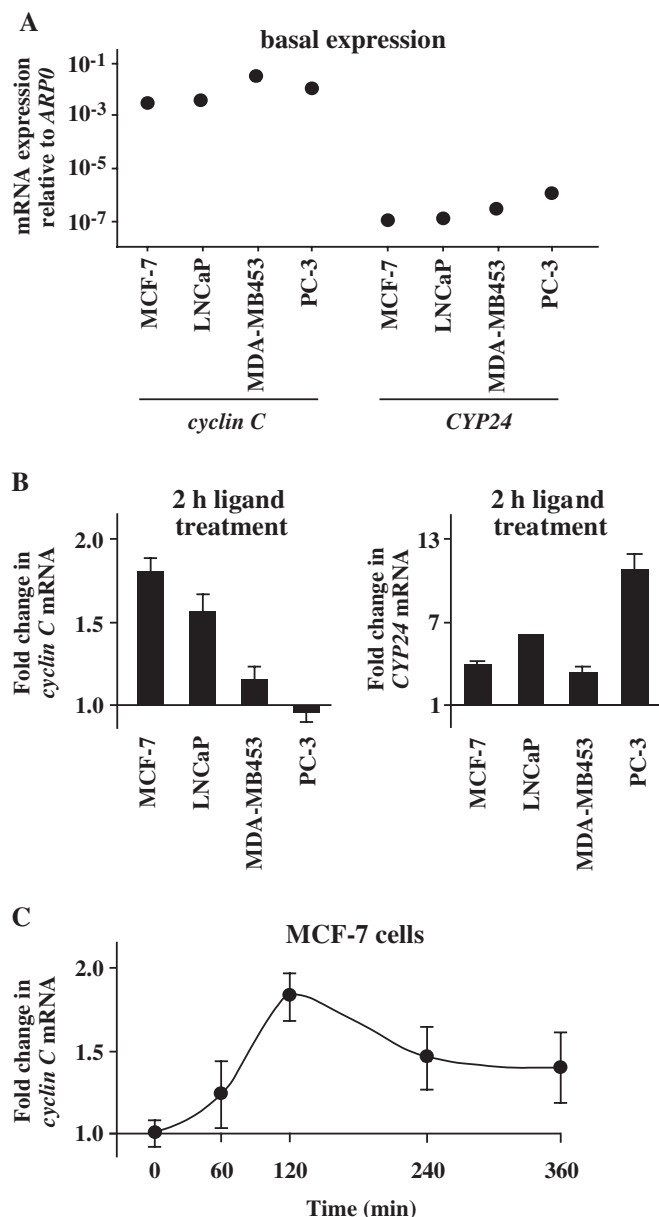
gene lysis buffer (Roche Diagnostics) and the constant light signal luciferase reporter gene assay was performed according to the supplier's recommendation (Canberra-Packard, Groningen, The Netherlands). The luciferase activities were normalized with respect to protein concentration and induction factors 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  $1\alpha,25(\text{OH})_2\text{D}_3$  target genes, *cyclin C* and *CYP24*, were monitored by real-time

quantitative PCR in relationship with the control gene *ARPO* 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



**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 *ARPO* 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  $1\alpha,25(\text{OH})_2\text{D}_3$  was measured. (C) The time course of *cyclin C* mRNA expression in response to 10 nM  $1\alpha,25(\text{OH})_2\text{D}_3$  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.

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.

#### $1\alpha,25(\text{OH})_2\text{D}_3$ inducibility of *cyclin C* and *CYP24* in different cancer cell lines

All four cell lines were treated for 2 h with 10 nM  $1\alpha,25(\text{OH})_2\text{D}_3$  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 *ARPO* 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  $1\alpha,25(\text{OH})_2\text{D}_3$ -induction of the human *cyclin C* promoter.

#### Whole *cyclin C* promoter screening for histone 4 acetylation levels

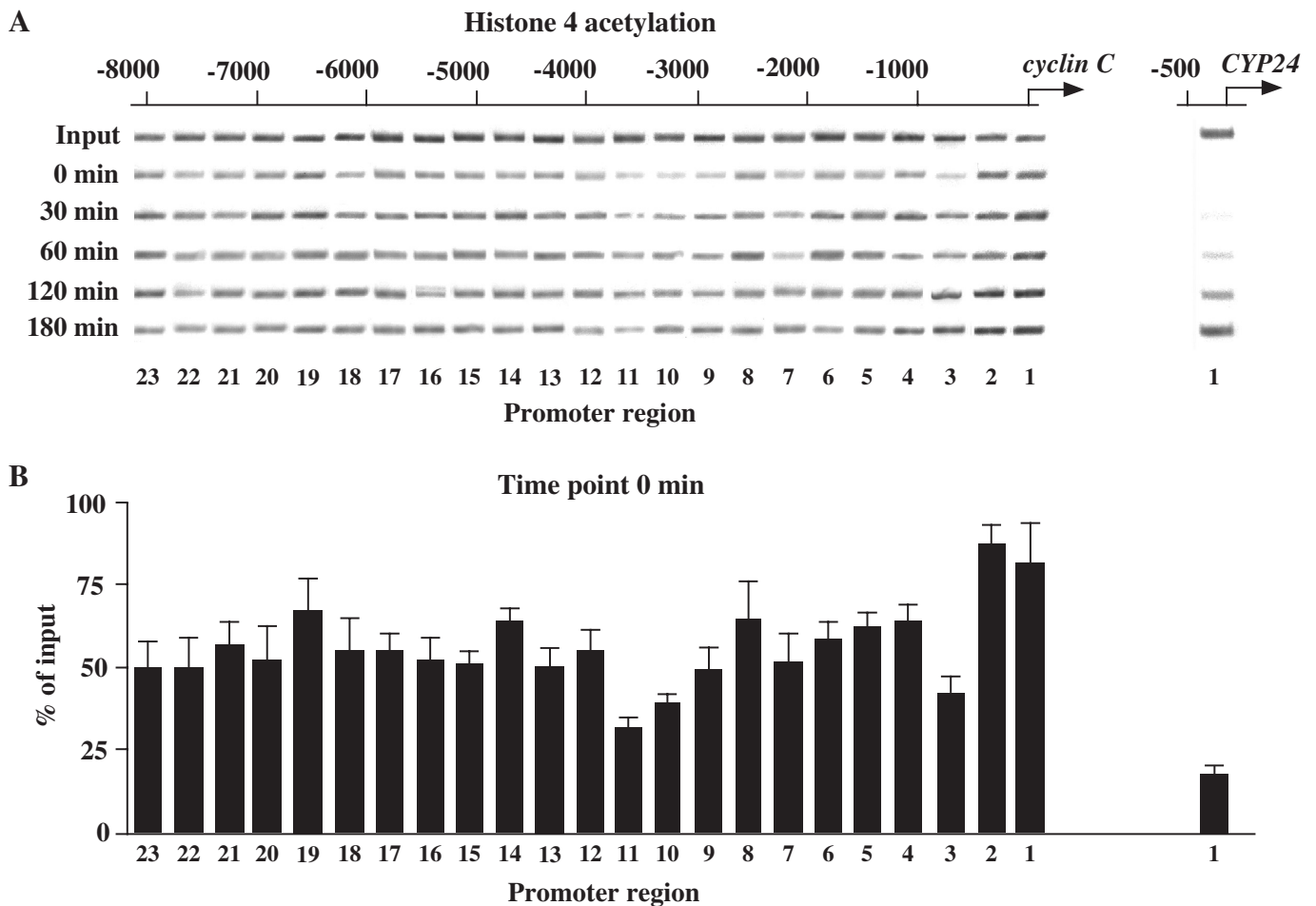
We hypothesized that a transient upregulation of the *cyclin C* gene should result in dynamic changes in the chromatin activation state on a wider region of the promoter of the gene in response to  $1\alpha,25(\text{OH})_2\text{D}_3$  stimulation. In order to test this hypothesis, we designed 23 overlapping 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

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  $1\alpha,25(\text{OH})_2\text{D}_3$  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

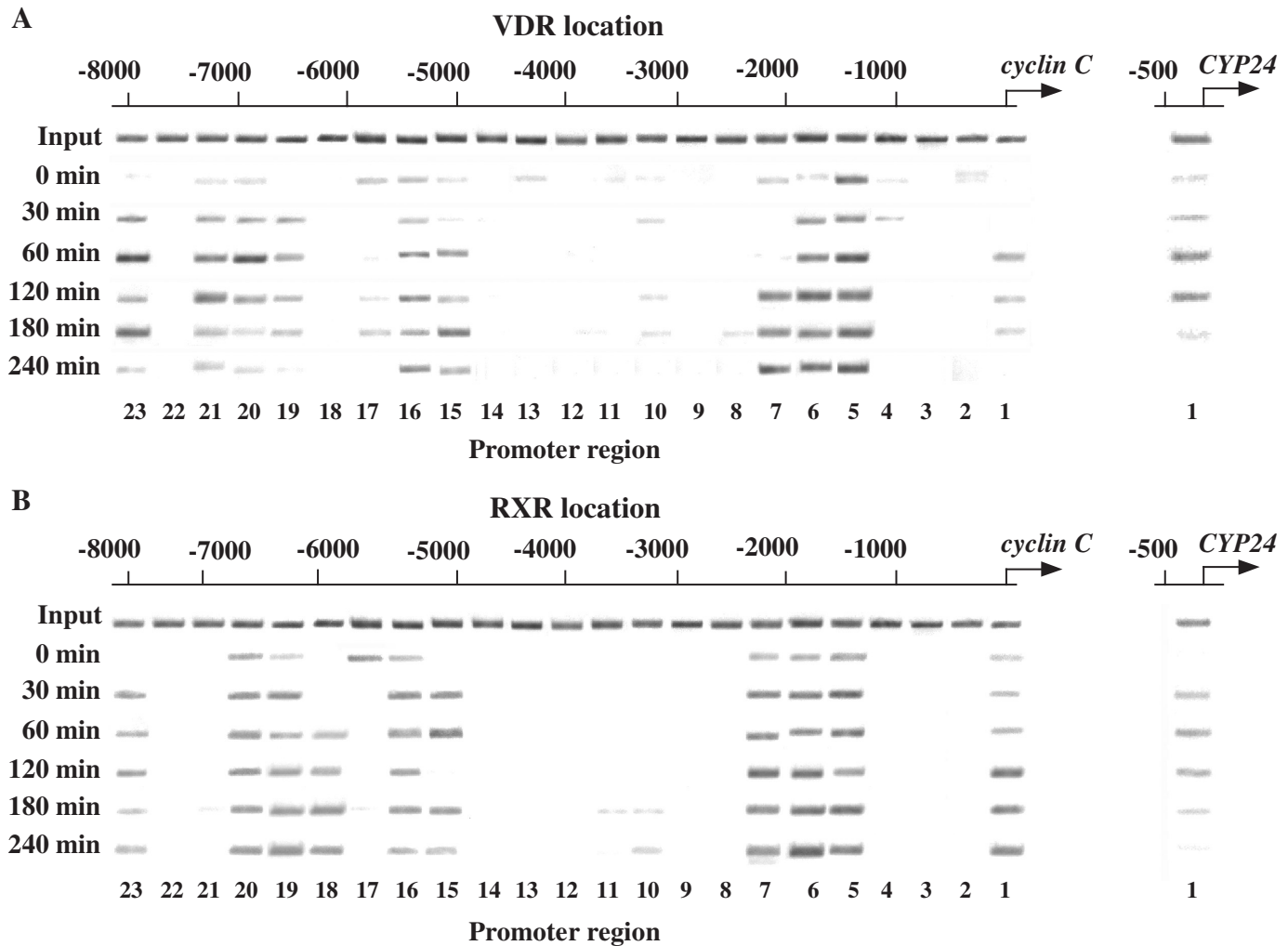
significantly more association with AcH4. The latter was highest at the TSS and within the first 617 bp of the promoter (regions 1 and 2) and lowest at promoter regions 3, 10 and 11. However, the histone 4 acetylation level of all regions of the *cyclin C* promoter did not vary more than by a factor 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  $1\alpha,25(\text{OH})_2\text{D}_3$  (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  $1\alpha,25(\text{OH})_2\text{D}_3$ . 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



**Figure 2.** Chromatin activity of the human *cyclin C* promoter. Chromatin was extracted from MCF-7 cells that had been treated for indicated time periods with 10 nM  $1\alpha,25(\text{OH})_2\text{D}_3$ . ChIP assays using an antibody against AcH4 were performed. The histone 4 acetylation states of 23 overlapping regions representing the first 8.4 kb of the human *cyclin C* promoter was monitored for the five treatment times in comparison with the activity of the proximal promoter of the *CYP24* gene (promoter region 1). (A) Representative agarose gels are shown. (B) The basal activity of the promoter regions (time point 0 min) was determined by quantifying the ethidium bromide-stained PCR products on a FLA-3000 reader in relationship with the chromatin input. Columns indicate the means of at least six independent cell treatments and the bars represent standard error of mean.



**Figure 3.** Ligand-modulated VDR and RXR binding to the human *cyclin C* promoter. Chromatin was extracted from MCF-7 cells that had been treated for indicated time periods with 10 nM  $1\alpha,25(\text{OH})_2\text{D}_3$ . ChIP assays using an antibody against VDR (A) and RXR (B) were performed. The VDR and RXR association of the 23 regions of the human *cyclin C* promoter was monitored for the six treatment times in comparison with that of the proximal promoter of the *CYP24* gene (promoter region 1). Representative agarose gels are shown.

a stimulation period of 240 min with 10 nM  $1\alpha,25(\text{OH})_2\text{D}_3$ , 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  $1\alpha,25(\text{OH})_2\text{D}_3$ . Taken together, the ChIP assay results indicate that, in contrast to histone 4 acetylation, VDR association is better suited to monitor  $1\alpha,25(\text{OH})_2\text{D}_3$ -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  $1\alpha,25(\text{OH})_2\text{D}_3$  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

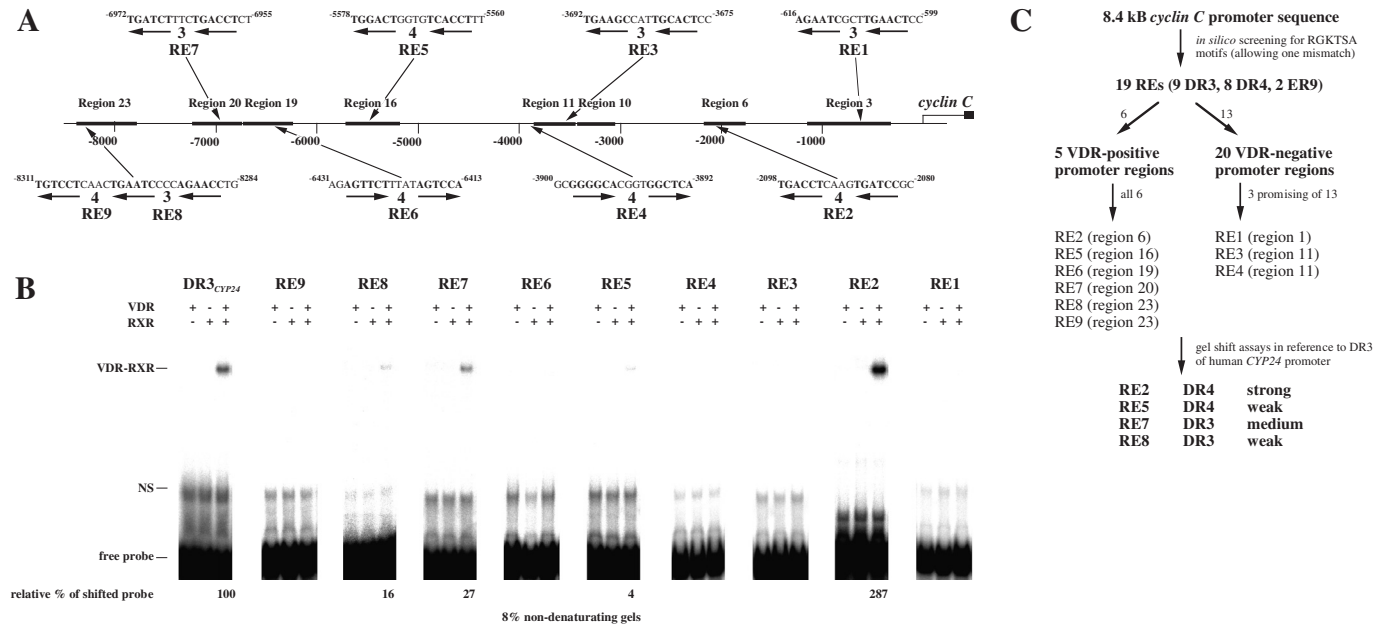
significant effects of  $1\alpha,25(\text{OH})_2\text{D}_3$  on the association level of RXR with these regions could be detected. In contrast, on the proximal *CYP24* promoter RXR binding displayed a clear maximum between 60 and 120 min. In summary, RXR location fits almost perfectly VDR location and 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<sub>A</sub> 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 <sup>32</sup>P-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  $1\alpha,25(\text{OH})_2\text{D}_3$  (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.

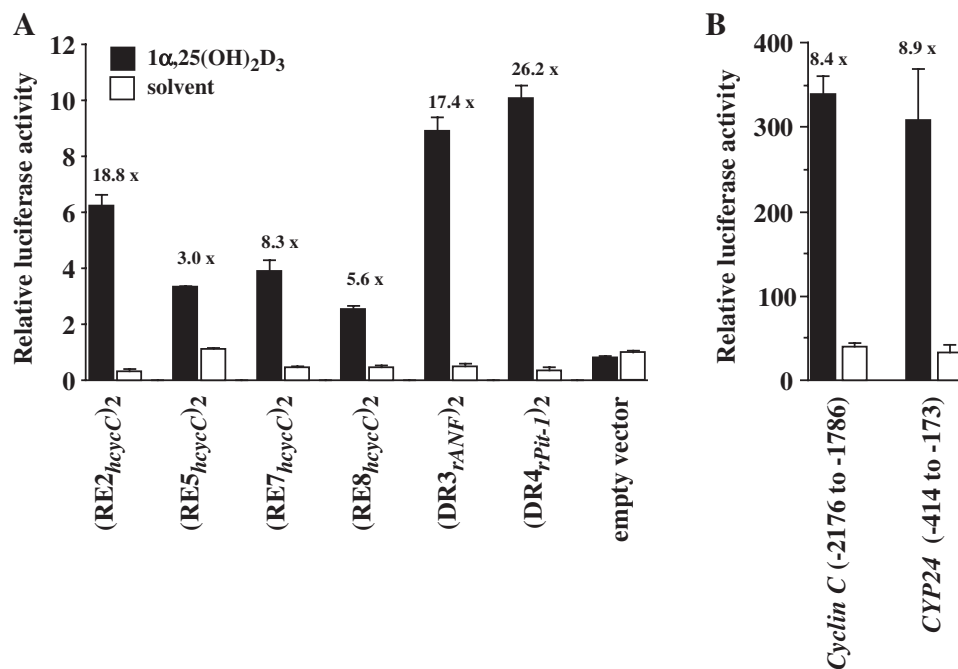
The functionality of RE2 in its natural promoter context was tested by analyzing the inducibility of a 390 bp fragment of the human *cyclin C* promoter (from -2176 to -1786) in MCF-7 cells measured by luciferase reporter gene assays. We obtained a 8.4-fold induction of luciferase activity after stimulation with 100 nM  $1\alpha,25(\text{OH})_2\text{D}_3$  (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

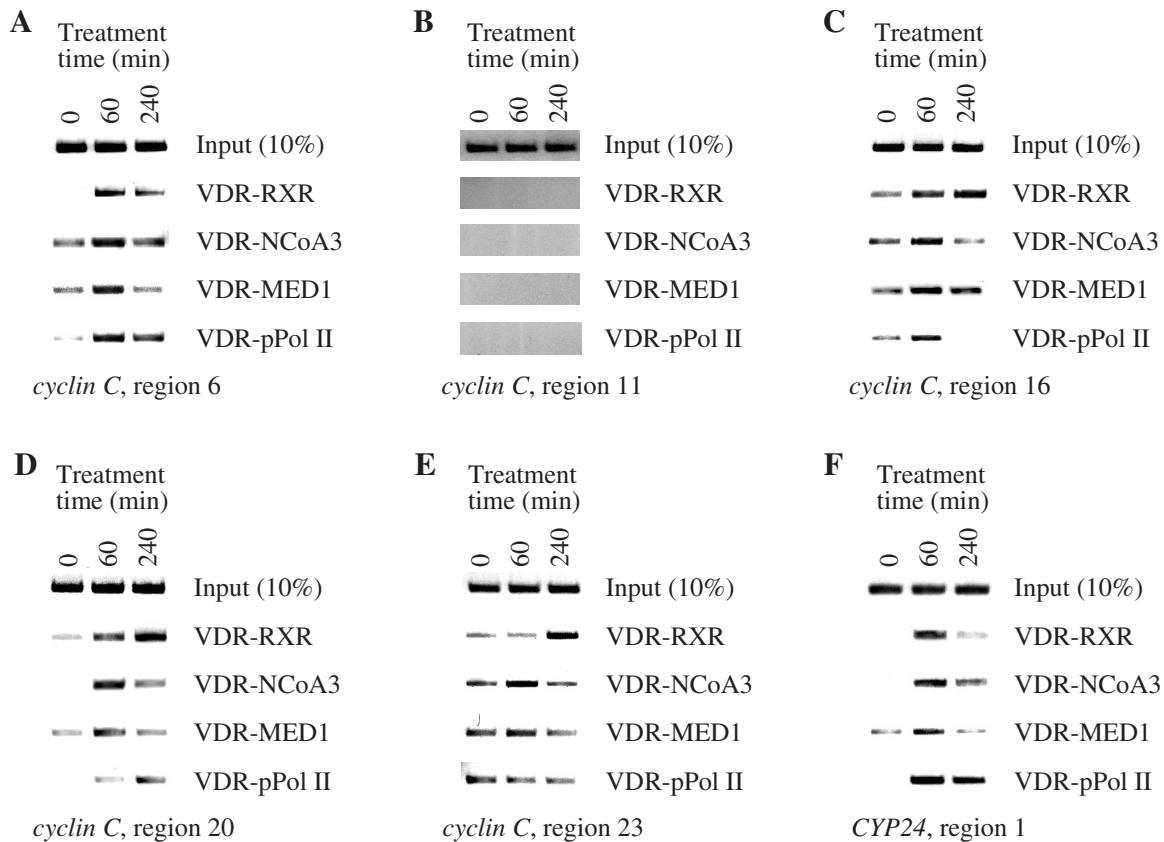
An additional test for the functionality of the VDREs in the chromatin context of MCF-7 cells was performed by re-ChIP assay (Figure 6). In this assay, first the anti-VDR antibody and then antibodies against RXR, NCoA3, MED1 or phosphorylated Pol II were used for immuno-precipitation, so that the enriched 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  $1\alpha,25(\text{OH})_2\text{D}_3$ -responsive *cyclin C* promoter regions.

### DISCUSSION

This study describes a deeper understanding of the regulation of the *cyclin C* gene by  $1\alpha,25(\text{OH})_2\text{D}_3$ . The *cyclin C* gene is an interesting  $1\alpha,25(\text{OH})_2\text{D}_3$ -responding gene, since its function is not linked to the classical endocrine functions of  $1\alpha,25(\text{OH})_2\text{D}_3$ , such as the regulation of calcium homeostasis and bone mineralization, but to the regulation of cellular



**Figure 5.** Functionality of VDREs. Reporter gene assays were performed with extracts from MCF-7 cells that were transiently transfected with a *luciferase* reporter construct containing two copies of RE2, RE5, RE7, RE8, the DR3-type VDRE of the rat *ANF* gene and the DR4-type RE of the rat *pit-1* gene (A) or 390 bp fragment of the human *cyclin C* promoter containing RE2 and a comparable fragment of the human *CYP24* promoter including the DR3-type VDRE (B) and an expression vector for human VDR. Cells were treated for 16 h with either solvent or 100 nM  $1\alpha,25(\text{OH})_2\text{D}_3$ . Relative luciferase activity is shown and fold inductions are indicated above the columns. Columns represent means of at least three experiments and bars indicate standard deviations.



**Figure 6.** VDR complexes in  $1\alpha,25(\text{OH})_2\text{D}_3$ -responsive promoter regions. Chromatin was extracted from MCF-7 cells that had been treated for indicated time periods with 10 nM  $1\alpha,25(\text{OH})_2\text{D}_3$ . 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  $1\alpha,25(\text{OH})_2\text{D}_3$  should provide a more detailed understanding of the mechanisms of how  $1\alpha,25(\text{OH})_2\text{D}_3$  regulates cellular functions, such as proliferation, differentiation and apoptosis. However, despite some function in the  $G_0$ – $G_1$  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  $G_1$  (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  $1\alpha,25(\text{OH})_2\text{D}_3$  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  $1\alpha,25(\text{OH})_2\text{D}_3$  signaling, because its protein product leads to the degradation of  $1\alpha,25(\text{OH})_2\text{D}_3$  and, therefore, the eventual extinction of the transcriptional signal. VDR–RXR heterodimers are the central transcriptional 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  $\kappa\text{B}$  (NF $\kappa\text{B}$ ) 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 NF $\kappa\text{B}$  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  $1\alpha,25(\text{OH})_2\text{D}_3$  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  $1\alpha,25(\text{OH})_2\text{D}_3$ -responding 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  $1\alpha,25(\text{OH})_2\text{D}_3$ . 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  $1\alpha,25(\text{OH})_2\text{D}_3$  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  $1\alpha,25(\text{OH})_2\text{D}_3$  is largely not understood, although microarrays and other gene expression profiling methods (4,5) suggest that approximately half of all  $1\alpha,25(\text{OH})_2\text{D}_3$ -responding genes are downregulated by the hormone. In this respect, the finding that the protein product of the primary  $1\alpha,25(\text{OH})_2\text{D}_3$ -responding gene *cyclin C* is a component of transcriptionally repressive MED complexes (16), may provide a mechanism to explain how the downregulation of secondary  $1\alpha,25(\text{OH})_2\text{D}_3$ -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  $1\alpha,25(\text{OH})_2\text{D}_3$ . 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  $1\alpha,25(\text{OH})_2\text{D}_3$ -responding genes with an impact on cellular growth, differentiation and apoptosis.

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