JOURNAL OF BONE AND MINERAL RESEARCH
Volume 19, Number 1, 2004
Published online on December 15, 2003; doi: 10.1359/JBMR.0301204

The Effects of 1α,25-Dihydroxyvitamin D_3 on the Expression of DNA Replication Genes

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ABSTRACT

To identify key genes in the antiproliferative action of $1,25(OH)_2D_3$, MC3T3-E1 mouse osteoblasts were subjected to cDNA microarray analyses. Eleven E2F-driven DNA replication genes were downregulated by $1,25(OH)_2D_3$. These results were confirmed by quantitative RT-PCR in different cell types, showing the general nature of this action of $1,25(OH)_2D_3$.

Introduction: 1α,25-Dihydroxyvitamin D_3 [$1,25(OH)_2D_3$] has a potent antiproliferative action characterized by a blocked transition from the G1- to the S-phase of the cell cycle. This study aims to identify genes whose expression is markedly altered after $1,25(OH)_2D_3$ treatment in parallel with or preceding the observed G1-arrest.
Materials and Methods: The cDNA microarray technique was used, and the expression of approximately 4600 genes in MC3T3-E1 mouse osteoblasts was studied 6 and 12 h after treatment with 10^{-8} M $1,25(OH)_2D_3$. Quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analyses were performed on MC3T3-E1 cells and on wildtype and vitamin D receptor (VDR) knockout primary murine epidermal keratinocytes (VDR^{wt}MEKs, $VDR^{-/-}$MEKs) and murine mammary tumor cells (GR) to confirm the microarray data.
Results and Conclusions: After 12 h of treatment, in parallel with the $1,25(OH)_2D_3$-induced G1 arrest, a particular set of DNA replication genes including a cell division cycle 6 homolog, a DNA polymerase alpha subunit, proliferating cell nuclear antigen, two DNA polymerase delta subunits, and flap-structure specific endonuclease 1, was downregulated at least 2-fold. These genes are known targets of the E2F family of transcription factors, which are probably the central mediators of this action of $1,25(OH)_2D_3$. Indeed, as shown by transfection assays with an E2F reporter construct, 12- and 24-h treatment of MC3T3-E1 cells with $1,25(OH)_2D_3$ reduced E2F activity by 49% and 73%, respectively. Quantitative RT-PCR analyses confirmed the downregulation of these DNA replication genes by $1,25(OH)_2D_3$ in MC3T3-E1, GR, and VDR^{wt}MEKs cells, but not in $VDR^{-/-}$MEKs cells, showing that this $1,25(OH)_2D_3$-driven antiproliferative action is of a general nature and depends on a functional VDR.
J Bone Miner Res 2004;19:133–146. Published online on December 15, 2003; doi: 10.1359/JBMR.0301204

Key words: vitamin D, osteoblasts, arrays, DNA replication, E2F

INTRODUCTION

AFTER DIETARY INTAKE or photosynthesis in the skin, vitamin D is sequentially hydroxylated to yield the biologically active metabolite 1α,25-dihydroxyvitamin D_3 [$1,25(OH)_2D_3$]. High affinity binding of $1,25(OH)_2D_3$ to the nuclear vitamin D receptor (VDR), followed by dimerization of the liganded VDR with the retinoid X receptor (RXR) and binding of the VDR-RXR heterodimer to specific vitamin D responsive elements (VDREs) in the promoter region of vitamin D target genes, regulates the transcription of a large and diverse set of genes.[1–3] The number and variety of target genes reflect the pleiotropic effects of $1,25(OH)_2D_3$, ranging from the classic influence on bone metabolism and calcium and phosphate homeostasis to nonclassic antiproliferative and pro-

The authors have no conflict of interest.

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differentiating effects on a wide variety of normal and malignant cells.[4]

The role of $1,25(OH)_2D_3$ in bone resorption and bone formation has been described at length and involves key players such as parathyroid hormone (PTH), osteopontin, osteocalcin, osteoprotegerin, and osteoprotegerin ligand, whereas the $1,25(OH)_2D_3$-driven calcium absorption in the intestine and reabsorption in the kidney depends on the activity of calbindinD-9K, calbindinD-28K, the plasma membrane calcium pump (PMCA), and the recently described new calcium channels ECaC1 and CaT1.[5]

The potent antiproliferative and pro-differentiating effects of $1,25(OH)_2D_3$ are less well understood and are undoubtedly an intriguing issue. Treatment with $1,25(OH)_2D_3$ affects growth and differentiation of numerous cancer cell types including prostate, breast, and colon cancer cells. The induced growth reduction is accompanied by an impaired transition from the G1 to the S phase of the cell cycle, leading to an accumulation of cells in the G1 phase.[6,7] Cyclin-dependent kinase (cdk) inhibitors like $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ are assumed to be the main mediators of this cell cycle arrest through their interaction with different cdks. Ultimately, active cyclin D-cdk4/6 complexes are required for cells to pass the G1/S restriction point as hyperphosphorylation of the retinoblastoma protein (pRb) by these complexes results in the release of E2F transcription factors and the subsequent transcription of genes needed for cell cycle progression and DNA replication.

The DNA replication process starts in G1 phase by the assembly of pre-replication complexes and ultimately leads to accurate continuous or discontinuous DNA synthesis on the leading or the lagging strand during S phase.[8–11] At the origin of DNA replication, cell division cycle 6 (Cdc6) binds to the origin recognition complex (Orc) and recruits mini-chromosome maintenance (Mcm) proteins. The Mcm family of proteins contains six members (Mcm 2–7) that form hexameric complexes at the replication origin. Subcomplexes of Mcm 4, 6, and 7 are shown to have DNA helicase activity needed to unwind duplex DNA, therefore creating two separate DNA strands.[12] The polymerase α-primase complex starts DNA synthesis by producing an RNA-DNA primer. This primer is elongated by the highly processive DNA polymerases δ and ϵ. To switch between the polymerase α-primase complex and polymerases δ and ϵ, proliferating cell nuclear antigen (PCNA) is loaded onto the DNA. After binding polymerases δ and ϵ, this toroid structure slides along the DNA strands. Ultimately, the RNA primer synthesized by the polymerase α-primase complex is removed by RNase H1. However, one ribonucleotide is left at the RNA-DNA junction and is subsequently removed by flap-structure specific endonuclease 1 (FEN1).

Orc1, Cdc6, Mcm proteins, DNA polymerase α (Polα), and PCNA, as well as genes involved in nucleotide biosynthesis such as thymidine kinase (Tk), dihydrofolate reductase, thymidylate synthetase, and ribonucleotide reductase, are examples of well-known E2F-regulated genes.[13,14] Blocked transcription of E2F target genes because of sequestration of free E2F by dephosphorylated pRb leads to cell cycle arrest. This mechanism most likely underlies the antiproliferative effect of $1,25(OH)_2D_3$, because treatment of different cell types with $1,25(OH)_2D_3$ leads to dephosphorylation of pRb, and therefore, to lower levels of free activating E2F transcription factors.[6,15–18]

In an attempt to further elucidate the mechanism of action of $1,25(OH)_2D_3$, we used a microarray approach to study the expression of approximately 4600 genes in MC3T3-E1 mouse osteoblasts at 6 and 12 h after treatment with a single dose of $1,25(OH)_2D_3$. After a 12-h treatment, we detected the clear downregulation of at least 11 E2F regulated genes involved in DNA replication. We were able to confirm these microarray data concerning the $1,25(OH)_2D_3$-mediated downregulation of these genes in two different bone-unrelated murine cell types other than MC3T3-E1 and to link them to actual changes in E2F activity induced by $1,25(OH)_2D_3$ treatment. Furthermore, the observations made in VDR knockout primary murine epidermal keratinocytes ($VDR^{-/-}$MEKs) suggest a pivotal role for a functional VDR in the $1,25(OH)_2D_3$-induced downregulation of DNA replication genes.

MATERIALS AND METHODS

Cell culture

MC3T3-E1 cells (Riken Cell Bank, Tsukuba, Japan) were maintained in α-MEM with 2 mM glutaMAX-I containing 10% fetal bovine serum (Biochrom KG, Berlin, Germany), 100 U/ml penicillin, and 100 μg/ml streptomycin. Fibroblastic NIH-3T3 (ATCC, Manassas, VA, USA) were maintained in DMEM/F-12 supplemented with 4.5 mg/ml D-(+)-glucose (Sigma, Bornem, Belgium) and the same additives as mentioned for MC3T3-E1. Mouse mammary tumor cells (GR) were obtained from Gordon Ringold and cultured as previously described.[19] Wildtype and VDR knockout primary murine epidermal keratinocytes (VDR^{wt}MEKs and $VDR^{-/-}$MEKs) were isolated and cultured as previously described.[20] Cells were seeded in Mg^{2+}-free KBM medium (BioWhittaker, Walkersville, MD, USA) containing bovine pituitary extract, epidermal growth factor, transferrin, epinephrine, gentamicin, amphotericin-B, bovine insulin and hydrocortisone, and 0.05 mM Ca^{2+}. The experiments with MEKs were conducted after formal approval by the ethical committee of the Katholieke Universiteit Leuven. All reagents used for cell culture, except for those indicated otherwise, were purchased from Invitrogen (Merelbeke, Belgium). Cells were seeded at 1×10^4 cells/cm^2 or at 5×10^4 cells/cm^2 for MEKs for downstream applications. All cells were treated with $1,25(OH)_2D_3$ (10^{-8} M), a gift of JP van de Velde (Solvay, Weesp, The Netherlands) or vehicle (ethanol) 24 h after seeding.

Total RNA extraction

Total RNA from MC3T3-E1 cells used for microarray analysis was extracted using TRizol LS reagent (Invitrogen). Total RNA from all cell types used for quantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis was prepared using the RNeasy kit (Qiagen, Hilden, Germany). Both RNA extraction methods were performed as specified by the manufacturer.

Construction of microarrays

The mouse gene set contained in total 4608 cDNA fragments spotted in duplicate, distant from each other, on type VIIstar silane-coated slides (Amersham BioSciences, Buckinghamshire, UK). The clone set was composed from the 6K collection of Incyte (Mouse Gem I; Incyte). The complete set can be found on the Flanders Interuniversity Institute for Biotechnology (VIB) web site (www.microarrays.be). The cDNA inserts were PCR amplified using M13 primers, purified with MultiScreen-PCR plates (Millipore, Bedford, MA, USA), and arrayed on the slides using a Molecular Dynamics Generation III printer (Amersham BioSciences). Slides were blocked in 3.5% SSC, 0.2% SDS, and 1% bovine serum albumin for 10 minutes at 60°C.

RNA amplification and labeling

Antisense RNA amplification was performed using a modified protocol of in vitro transcription as described earlier.[21] For the first-strand cDNA synthesis, 5 μg of total RNA was mixed with 2 μg of a high-performance liquid chromatography (HPLC)-purified anchored oligo-dT + T7 promoter (5′-GGCCAGTGAATTGTAATACGACTCACTATAGGGAGGCGG-T_{24}(ACG)-3′) (Eurogentec, Seraing, Belgium), 40 U RNAseOUT (Invitrogen), and 0.9 M D(+)trehalose (Sigma) in a total volume of 11 μl and heated to 75°C for 5 minutes. To this mixture, 4 μl 5× first strand buffer (Invitrogen), 2 μl 0.1 M DTT, 1 μl 10 mM dNTP mix, 1 μl 1.7 M D(+)trehalose (Sigma), and 200 U SuperScript II (Invitrogen) were added in 20 μl final volume. The sample was incubated in a Biometra-UnoII thermocycler at 37°C for 5 minutes, 45°C for 10 minutes, 10 cycles at 60°C for 2 minutes, and at 55°C for 2 minutes. To the first-strand reaction mix, 103.8 μl water, 33.4 μl 5× second-strand synthesis buffer (Invitrogen), 3.4 μl 10 mM dNTP mix, 1 μl of 10U/μl *E. coli* DNA ligase (Invitrogen), 4 μl 10 U/μl *E. coli* DNA Polymerase I (Invitrogen), and 1 μl 2 U/μl *E. coli* RNAse H (Invitrogen) were added, and the reaction mix was incubated at 16°C for 2 h. The synthesized double-stranded cDNA was purified with Qiaquick (Qiagen). Antisense RNA synthesis was performed by AmpliScribe T7 high yield transcription kit (Epicenter Technologies, Madison, WI, USA) in a total volume of 20 μl according to the manufacturer's instructions. The RNA was purified with an RNeasy purification kit. From this RNA, 5 μg was labeled by RT using random nonamer primers (Genset, Paris, France), 0.1 mM d(G/T/A)TPs, 0.05 mM dCTP, 0.05 mM Cy3-dCTP or Cy5-dCTP (Amersham BioSciences), 1× first-strand buffer, 10 mM dithiothreitol (DTT), and 200 U SuperScript II (Invitrogen) in 20 μl total volume. The RNA and primers were denatured at 75°C for 5 minutes and cooled on ice before adding the remaining reaction components. After a 2-h incubation at 42°C, mRNA was hydrolyzed in 250 mM NaOH for 15 minutes at 37°C. The sample was neutralized with 10 μl of 2 M 3-(*N*-morpholino) propane sulfonic acid (MOPS) and purified with Qiaquick.

Array hybridization and post-hybridization processes

The probes were resuspended in 30 μl hybridization solution (50% formamide, 5× SSC, 0.1% SDS, 100 μg/ml salmon sperm DNA) and prehybridized with 1 μl poly-dT (1 mg/ml) at 42°C for 30 minutes to block hybridization on the polyA/T tails of the cDNA on the array. Mouse COT DNA (1 mg/ml; Invitrogen) was added to the mixture, which was placed on the array under a glass coverslip. Slides were incubated for 18 h at 42°C in a humid hybridization cabinet (Amersham BioSciences). Post-hybridization washings were performed for 10 minutes at 56°C in 1×SSC, 0.1% SDS, two times for 10 minutes at 56°C in 0.1×SSC, 0.1% SDS, and for 2 minutes at 37°C in 0.1×SSC.

Scanning and data analysis

Arrays were scanned at 532 and 635 nm using a Generation III scanner (Amersham BioSciences). Image analysis was performed with ArrayVision (Imaging Research Inc., Ontario, Canada). Spot intensities were measured as AR volume, which corresponds to the artifact-removed density value for each spot multiplied by the area of the spot. In addition, pixels with values exceeding a user-defined threshold value (4.0 × median of absolute deviations) were excluded and were replaced with estimated values, derived by interpolation from neighboring pixels. This measure removed the influence of image artifacts (e.g., dust particles) on density estimation.

Quantitative real-time PCR

RNA (1.5 μg) was reverse transcribed at 42°C for 80 minutes using 150 ng random primers and 200 U SuperScript II (Invitrogen). PCR reactions contained 1× TaqMan buffer A, 200 μM dNTPs, 2 or 5 mM $MgCl_2$, 0.65 U AmpliTaq Gold (Applied Biosystems, Foster City, CA, USA), 300 nM forward primer, 300 nM reverse primer, 200 nM of a dual-labeled detection probe (Eurogentec), and 0.5 μl cDNA or 0.5 μl of a corresponding RNA sample that was not reverse transcribed and served as a negative control. Amplification reactions were performed in triplicate in an ABI-prism 7700 sequence detector (Applied Biosystems) at previously described conditions.[22] For all samples, a detection probe for β-actin was used to normalize the obtained data. For each detection probe used, plasmid clones containing partial target cDNA sequences were made using standard molecular cloning techniques. These plasmid clones represent known amounts of target cDNA, and serial dilutions of the plasmid clones served as standard curves. Quantification of the amount of target cDNA in the samples was done using these standard curves.

Sequences of forward primers (FW), reverse primers (RV), and detection probes (P) were as follows:

Cdc6: TTCTGTGCCCGCAAAGTGT (FW), CTGGCTCCTGACATCCGACT (RV), CGCTTTACGGATGTCTCCTGAAACAGCA (P)

FEN1: GCAGAACGAGGAGGGTGAGA (FW), CGTACACAGGCTTGATGCCA (RV), TGATGGGCATGTTCTACCGTACCATCC (P)

Polδ1: GAGGACGTTCAGCACAGCATC (FW), AAGGCGTCCTTCAGGCAGTA (RV), TCACCGACCTGCAGAATGGGAACG (P)

Polδ2: GCAGTCAAAATGCTGGACGA (FW), AAACTCGCCTGGCATCACAT (RV), ATCCTTCTGCAACTGAGTGCCTCGGTAC (P)
Polα2: GACTGAGGACGGGATGGTCA (FW), GTCTTTGGAGGCACTGTGCC (RV), TGAGCTCATCGCCTTCTGCACCAG (P)
PCNA: ACAGCTTACTCTGCGCTCCG (FW), GGACATGCTGGTGAGGTTCAC (RV), AGGCTTCGACACATACCGCTGCGA (P)
P21: CGCTGTCTTGCACTCTGGTG (FW), AAATCTGTCAGGCTGGTCTGC (RV), AGCGGCCTGAAGATTCCCCGG (P)
P27: ACAATCAGGCTGGGTTAGCG (FW), GCCCTTTTGTTTTGCGAAGA (RV), CGCTTCCTCATCCCTGGACACTGCT (P)
Ccnd1: ACCGCACAACGCACTTTCTT (FW), AATCTGTTCCTGGCAGGCAC (RV), CCAGAGTCATCAAGTGTGACCCGGACT (P)

All sequences are in the 5′-3′ direction, and all detection probes are labeled with a fluorescent reporter at the 5′ end (FAM; 6-carboxyfluorescein) and a quencher dye at the 3′ end (TAMRA; 6-carboxytetramethylrhodamine).

Cell cycle analysis

At 6, 12, and 48 h after treatment with 10^{-8} M $1,25(OH)_2D_3$ or vehicle, approximately 1×10^6 cells were washed with PBS twice and fixed in ice-cold 70% ethanol for 30 minutes. After fixation, cells were washed twice with PBS containing 0.05% Tween-20 and resuspended in PBS containing 0.05% Tween-20, 0.5 mg/ml propidium iodide, and 1 mg/ml RNase A (Sigma). Analysis of samples was done using the CellQuest and Modfit program on a FACSort flow cytometer (Becton Dickinson, Erembodegem, Belgium).

Transient transfection assays using E2F reporter constructs

MC3T3-E1 cells were seeded at approximately 4×10^4 cells in 24-well plates. Triplicate wells were transfected 24 h after seeding with 40 ng of the β-galactosidase expression vector pcDNA3.1(-)/Myc-His/*lac*Z (Invitrogen) and 400 ng of a luciferase reporter vector containing 6E2F binding sites[23] or 400 ng of a minimal TK-TATA luciferase reporter vector using Fugene6 Transfection Reagent (Roche Diagnostics, Mannheim, Germany). A 6:1 ratio Fugene6:plasmid DNA was used to obtain maximum transfection efficiency. Twenty-four hours after transfection, cells were treated with $1,25(OH)_2D_3$ (10^{-8} M) or vehicle and assayed for luciferase activity at the indicated times. Luciferase activity was normalized to β-galactosidase activity, which was measured by means of the Galacto-Light Plus System (Applied Biosystems).

Statistical analysis

Statistical analysis was performed with the software program NCSS (NCSS, Kaysville, UT, USA). All results are expressed as means ± SEM of at least two independent experiments. ANOVA analyses followed by Fisher's least significant differences (LSD)-multiple comparison tests or Student's *t*-tests were carried out to detect significant differences; $p < 0.05$ was accepted as significant.

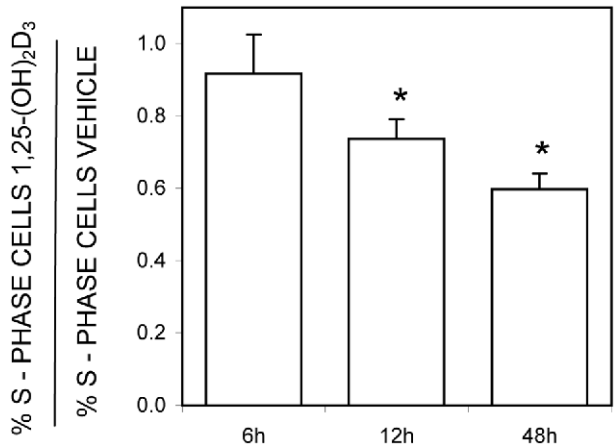

FIG. 1. Cell cycle analysis of MC3T3-E1 cells treated with $1,25(OH)_2D_3$. MC3T3-E1 cells were treated with 10^{-8} M $1,25(OH)_2D_3$ or vehicle and stained with propidium iodide at 6, 12, or 48 h thereafter. For each of the indicated times, the ratio between the number of S-phase cells in $1,25(OH)_2D_3$-treated cells and the number of S-phase cells in vehicle-treated cells is given. Bars represent the mean ratios ± SEM of at least three independent experiments. *$p < 0.05$, $1,25(OH)_2D_3$-treated vs. vehicle-treated (Student's *t*-test).

RESULTS

Cell cycle analysis of MC3T3-E1 cells after treatment with $1,25(OH)_2D_3$

Treatment with $1,25(OH)_2D_3$ blocks the cell cycle at the transition from the G1 to the S phase. To quantify this phenomenon in MC3T3-E1 mouse osteoblasts, we stained these cells with propidium iodide after treatment with 10^{-8} M $1,25(OH)_2D_3$ and counted the percentage of cells present in the S phase (Fig. 1). At 6 h after treatment, there was no significant difference between the number of S phase cells in a $1,25(OH)_2D_3$-treated sample and the number of S phase cells in a vehicle-treated sample. However, after 12 h of treatment, the number of S phase cells in a $1,25(OH)_2D_3$-treated sample was only 74% of the number of S phase cells in a vehicle-treated sample. This drop increased at 48 h after treatment, yielding only 60% of S phase cells in a $1,25(OH)_2D_3$-treated sample compared with a vehicle-treated sample. The complementary rise of G1 phase cells was also maximal at 48 h after treatment (data not shown).

Microarray analysis of gene expression in MC3T3-E1 after treatment with $1,25(OH)_2D_3$

The cDNA microarray technique was applied as a starting point to study changes in gene expression profile in MC3T3-E1 cells after treatment with 10^{-8} M $1,25(OH)_2D_3$ at two different time-points, namely 6 and 12 h. A control experiment was performed to identify the background fluctuations in gene expression resulting from experimental variables in the production, the hybridization, or the scanning of the cDNA microarray. Hybridization of a sample of MC3T3-E1 cells at 6 h after vehicle treatment versus itself

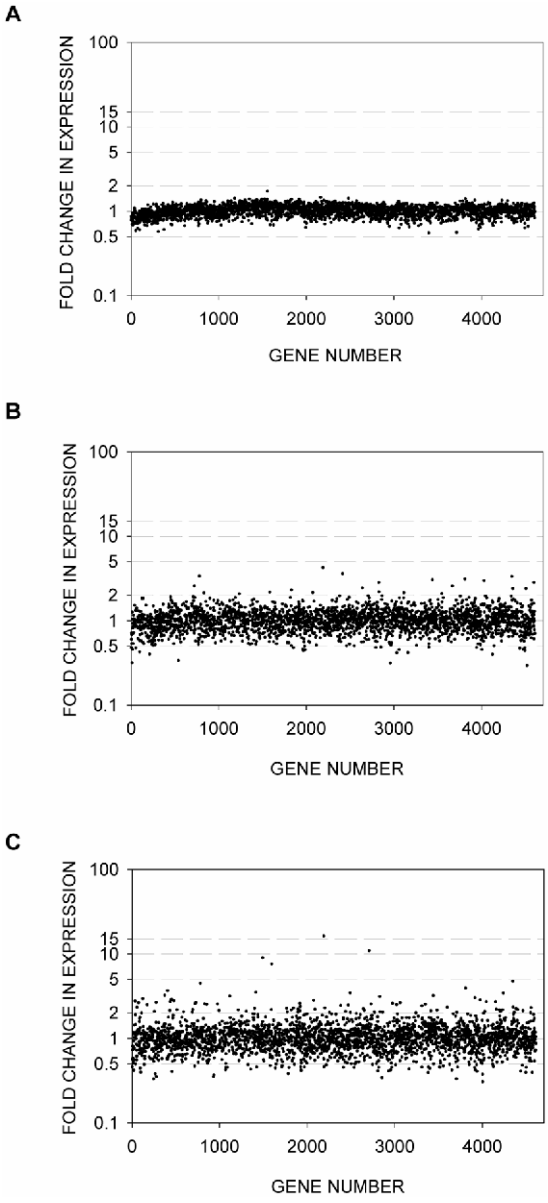

FIG. 2. cDNA microarray analysis on MC3T3-E1 cells. Scatterplots represent the fold change in expression on a logarithmic scale between MC3T3-E1 cells treated with $1,25(OH)_2D_3$ (10^{-8} M) and vehicle-treated cells for each of the 4608 cDNA fragments on the array. (A) Control experiment: a sample of MC3T3-E1 cells after a 6-h incubation with vehicle was hybridized vs. itself. (B) $1,25(OH)_2D_3$-treated MC3T3-E1 cells hybridized vs. vehicle-treated cells at 6 h after treatment. (C) $1,25(OH)_2D_3$-treated MC3T3-E1 cells hybridized vs. vehicle-treated cells at 12 h after treatment.

demonstrated that changes in expression level caused by these experimental variables were less than 2-fold for all genes, and except for one upregulated and three downregulated genes, background noise never exceeded 1.7-fold (Fig. 2A). However, we used a threshold of a 2-fold change in expression level to identify the most relevant upregulation or downregulation of genes at 6 h as well as at 12 h after treatment with 10^{-8} M $1,25(OH)_2D_3$ (Figs. 2B and 2C). From this selection, we only retained the genes with a signal that significantly differed from local background in both duplicate spots for further analysis. Hence, after a 6-h treatment with $1,25(OH)_2D_3$, we found 19 downregulated and 18 upregulated genes, whereas a 12-h treatment with $1,25(OH)_2D_3$ resulted in the downregulation of 61 genes and the upregulation of 73 genes (Table 1).

Downregulation of DNA replication genes in MC3T3-E1

The 6-h as well as the 12-h incubation with $1,25(OH)_2D_3$ resulted in the up- or downregulation of heterogeneous groups of genes encoding unknown or known functions, some of which might be players in the antiproliferative action of $1,25(OH)_2D_3$ (e.g., β-catenin, insulin-like growth factor 2 binding protein 3). However, almost 20% (11 of 61) of the genes downregulated after a 12-h incubation period with $1,25(OH)_2D_3$ were genes known to play key roles in DNA replication (Table 1). The microarray data showed a 3-fold downregulation for the gene encoding Cdc6 and a more than 2-fold downregulation for three of the Mcm family members, namely Mcm 2, 6, and 7. Moreover, the 68-kDa subunit (Polα2) of the polymerase α-primase complex as well as the 125-kDa catalytic subunit (Polδ1) and the 50-kDa regulatory subunit (Polδ2) of DNA polymerase δ and subunit 2 of DNA polymerase ϵ (Polϵ2) showed a more than 2-fold decrease. Similarly, FEN1 showed a 3-fold downregulation, whereas Tk1 was 2.4-fold downregulated. Although PCNA was only 1.9-fold downregulated after a 12-h incubation with $1,25(OH)_2D_3$ and therefore did not exceed the cut-off value of 2.0, we included PCNA in our further study because it is a known proliferation marker along with Tk1 and FEN1. In addition to the 11 DNA replication-related genes, the gene encoding cyclin D1 (Ccnd1) was also found to be more than 2-fold downregulated. Although present on the array, the genes encoding the cdk inhibitors $p21^{CIP1/WAF1}$ and $p27^{KIP1}$ did not show any upregulation at 6 or at 12 h after treatment with $1,25(OH)_2D_3$.

Time-course analysis of DNA replication genes in MC3T3-E1 using quantitative RT-PCR

Microarray analysis revealed clear changes in expression of several DNA replication-related genes at 12 h after $1,25(OH)_2D_3$ treatment. To expand and to confirm this basic information, we selected six of these genes, namely Cdc6, Polδ1, Polδ2, Polα2, PCNA, and FEN1, and studied their expression in MC3T3-E1 cells at times ranging from 1 to 72 h after treatment with $1,25(OH)_2D_3$ by means of quantitative RT-PCR (QRT-PCR; Fig. 3). To link the downregulation of these six genes to $1,25(OH)_2D_3$-induced changes in the expression of key cell cycle regulators, QRT-PCR analyses on Ccnd1, p21, and p27 were included as well. Neither a 1-h treatment (data not shown) nor a 3-h treatment conferred any change in expression of the abovementioned genes. A decrease in expression, although only minor for Polδ2 and Polα2, was seen at 6 h after treatment. Except for Polδ2 (1.8-fold), the decrease at 12 h was at least 2-fold, with a maximum downregulation for the *Cdc6* gene (3.0-fold; Fig. 3). These ratios are an almost perfect match of

TABLE 1. GENES REGULATED BY $1,25(OH)_2D_3$ IN MC3T3-E1 CELLS, BASED ON cDNA MICROARRAY ANALYSIS

Fold induction	*Accession No. or UniGene No.*	*Gene name*
Downregulated after a 6-h incubation period with $1,25(OH)_2D_3$		
−2,02	W18828	Dihydropyrimidinase-like 3
−2,06	AA008515	RIKEN cDNA 1110036H20 gene
−2,06	AA116515	Protein-kinase, interferon-inducible double stranded RNA dependent inhibitor
−2,08	W89883	Procollagen, type III, alpha 1
−2,08	W80177	Matrix metalloproteinase 2
−2,09	AA049551	RIKEN cDNA 6720485C15 gene
−2,17	AA050647	Similar to Rho GTPase activating protein 1
−2,18	Mm.1377	TβRII
−2,18	W13213	Protein kinase C, beta
−2,20	AA003252	Expressed sequence AL024221
−2,21	AA145458	Fibronectin 1
−2,21	W16254	Tubulin, beta 5
−2,25	AA049376	Angio-associated migratory protein, related sequence
−2,25	W10023	Catenin beta
−2,39	W48116	Inhibitor of DNA binding 4
−2,40	AA145784	Tissue inhibitor of metalloproteinase 3
−2,50	AA060238	U1 small nuclear ribonucleoprotein 70 kDa polypeptide A
−3,19	AA050726	Paired related homeobox 1
−3,41	AA061285	Expressed sequence AA960365
Downregulated after a 12-h incubation period with $1,25(OH)_2D_3$		
−1,92	**AA116947**	**Proliferating cell nuclear antigen (PCNA)**
−2,01	**AA008627**	**DNA polymerase epsilon, subunit 2**
−2,01	AA030521	Expressed sequence AI836659
−2,02	AA465936	RIKEN cDNA 2310061B02 gene
−2,02	AA170417	RIKEN cDNA E130115I21 gene
−2,03	**AA064230**	**Mini-chromosome maintenance deficient 7 (*S. cerevisiae*)**
−2,03	AA008549	Ubiquitin conjugating enzyme 7 interacting protein 4
−2,04	W15720	Synaptogyrin 2
−2,04	W83609	Retinol binding protein 1, cellular
−2,04	AA386769	RAD51 associated protein 1
−2,04	W99925	B-cell CLL/lymphoma 11A (zinc finger protein)
−2,05	W66710	ESTs
−2,10	AA008134	Dynein, cytoplasmic, light chain 1
−2,10	W14540	Histocompatibility 2, K region
−2,13	W82989	Ancient ubiquitous protein
−2,14	AA108822	RIKEN cDNA 1300019P08 gene
−2,15	AA060238	U1 small nuclear ribonucleoprotein 70 kDa polypeptide A
−2,17	AA044532	Bystin-like
−2,17	Mm.29350	Synd2(syndecan2)
−2,17	W99968	Potassium intermediate/small conductance calcium-activated channel, subfamily N, member 4
−2,17	Mm.34988	Exonuclease 1
−2,17	W36781	Similar to KIAA0595 protein
−2,18	AA036380	Snail homolog 1, (Drosophila)
−2,18	AA111722	**Cyclin D1**
−2,19	AA058225	RIKEN cDNA 3010002G01 gene
−2,19	W10023	Catenin beta
−2,20	AA119255	Clone IMAGE:574251 mRNA
−2,20	AA060802	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2, p49/p100
−2,20	W87102	RIKEN cDNA 4833427B12 gene
−2,20	W64238	Apelin
−2,20	AA060537	Peptidylprolyl isomerase B
−2,20	AA061468	Hypothetical protein MGC36662
−2,20	W16221	Procollagen, type VI, alpha 1
−2,21	AA030271	RIKEN cDNA 2010012C24 gene
−2,22	W33596	Interleukin 25
−2,22	AA038556	Hypothetical protein MGC36621
−2,25	W65559	Mitochondrial ribosomal protein S11
−2,26	W64706	RIKEN cDNA 2600017H24 gene
−2,27	AA122891	Glyceraldehyde-3-phosphate dehydrogenase
−2,29	W48116	Inhibitor of DNA binding 4

TABLE 1. (CONTINUED)

Fold induction	*Accession No. or UniGene No.*	*Gene name*
−2,31	**AA016759**	**Mini-chromosome maintenance deficient 6 (*S. cerevisiae*)**
−2,36	AA058182	Angio-associated migratory protein, related sequence
−2,36	W36002	RIKEN cDNA 2210010N10 gene
−2,38	**AA011839**	**Mini-chromosome maintenance deficient 2 (*S. cerevisiae*)**
−2,38	**Z21848**	**Polymerase (DNA directed), delta 1, catalytic subunit (125 kDa)**
−2,38	**AA041834**	**Thymidine kinase 1**
−2,39	AA541870	Arsenate resistance protein 2
−2,40	**D13546**	**Polymerase (DNA directed), alpha 2**
−2,42	W75898	Hypothetical protein MGC37115
−2,43	**AA035902**	**Polymerase (DNA directed), delta 2, regulatory subunit (50 kDa)**
−2,44	AA030294	Frizzled homolog 1, (Drosophila)
−2,48	W15971	FK506 binding protein 2 (13 kDa)
−2,53	AA049376	Angio-associated migratory protein, related sequence
−2,62	AA038052	Immediate early response 2
−2,63	**AA050378**	**DNA polymerase alpha 2, 68 kDa**
−2,66	AA003252	Expressed sequence AL024221
−2,71	W91387	Clone IMAGE:424021
−2,85	AA050169	Protein phosphatase 4, catalytic subunit
−2,85	AA002760	ESTs
−2,92	AA008515	RIKEN cDNA 1110036H20 gene
−3,02	**AA048426**	**Cell division cycle 6 homolog (*S. cerevisiae*)**
−3,02	**AA034561**	**Flap structure specific endonuclease 1**
Upregulated after a 6-h incubation period with $1,25(OH)_2D_3$		
3,58	W81912	Cellular retinoic acid binding protein II
3,35	W85374	ESTs
3,11	AA142685	Adducin 3 (gamma)
3,05	AA137467	Clone IMAGE:5039248, mRNA
2,97	AA011759	Glutamine synthetase
2,82	W98906	ESTs, Weakly similar to T12543 hypothetical protein DKFZp434M154.1
2,57	AA027510	RIKEN cDNA 1110036H21 gene
2,57	AA118841	RIKEN cDNA 9030412M04 gene
2,44	AA124340	RIKEN cDNA 1110036H20 gene
2,40	W97477	Similar to CG15168 gene product, clone MGC:36859 IMAGE:4459181, mRNA, complete cds
2,30	AA059521	ESTs
2,20	W98550	ESTs, Moderately similar to SNX8_HUMAN SORTING NEXIN 8
2,17	W11916	RIKEN cDNA 2010003I05 gene
2,15	W91158	p53-regulated PA26 nuclear protein
2,14	W13152	RIKEN cDNA 1110038L14 gene
2,13	W16053	Adenosine deaminase, RNA-specific, B1
2,10	W54436	ESTs
2,08	W54448	RIKEN cDNA 2010003O02 gene
Upregulated after a 12-h incubation period with $1,25(OH)_2D_3$		
16,22	AA038156	RIKEN cDNA 4631401E18 gene
4,72	W85374	Cell adhesion molecule-related/down-regulated by oncogenes
4,47	Mm.4913	Follistatin
3,94	AA142685	Adducin 3 (gamma)
3,65	AA474390	Expressed sequence AI987801
3,51	AA036347	Kruppel-like factor 9
3,43	AA003823	RIKEN cDNA 1110025J15 gene
3,43	AA388323	DNA segment, Chr 8, Brigham & Women's Genetics 1112 expressed
3,21	AA137467	Clone IMAGE:5039248, mRNA
3,12	W98906	Polyglutamine-containing protein
3,03	W34612	Transglutaminase 2, C polypeptide
2,93	W82737	Neural precursor cell expressed, developmentally down-regulated gene 9
2,89	W29916	Hypothetical protein MGC28180
2,86	W15931	Glioblastoma amplified sequence
2,86	W97059	Tweety homolog 2 (Drosophila)
2,81	W91158	p53 regulated PA26 nuclear protein
2,72	AA080236	MAP kinase-interacting serine/threonine kinase 1
2,71	AA008573	Clone IMAGE:5009979, mRNA, partial cds
2,65	AA058055	Clone IMAGE:480196

TABLE 1. (CONTINUED)

Fold induction	*Accession No. or UniGene No.*	*Gene name*
2,64	AA125030	RIKEN cDNA 3110001A13 gene
2,63	AA178305	Adducin 3 (gamma)
2,63	W36545	ESTs
2,59	AA059892	RIKEN cDNA 4930506D01 gene
2,57	AA118841	RIKEN cDNA 9030412M04 gene
2,55	W11395	Expressed sequence AW050020
2,55	AA021816	Adducin 3 (gamma)
2,52	AA061408	RIKEN cDNA 6330406I15 gene
2,52	W62513	ESTs
2,50	AA123360	Copper chaperone for superoxide dismutase
2,50	AA003120	EST AI425994
2,48	AA003739	RIKEN cDNA 4930431E10 gene
2,45	W34106	RIKEN cDNA 2310047A01 gene
2,42	AA031238	RIKEN cDNA 1110005L02 gene
2,38	X13664	N-ras
2,34	AA052699	Synaptosomal-associated protein, 25 kDa, binding protein
2,34	W97732	Clone IMAGE:422403
2,29	AA058302	Insulin-like growth factor 2, binding protein 3
2,28	AA097379	ESTs
2,25	AA032344	Expressed sequence AW494241
2,25	AA030833	RIKEN cDNA 5830406C15 gene
2,24	AA002322	Expressed sequence AI430822
2,24	AA124340	RIKEN cDNA 1110036H20 gene
2,21	AA108992	SH3-domain binding protein 5 (BTK-associated)
2,21	AA003633	Cullin 1
2,20	AA004013	RIKEN cDNA 2310008J16 gene
2,17	W82406	Laminin, gamma 1
2,17	AA139308	Expressed sequence AI197390
2,17	AA413119	Adenylyl cyclase-associated CAP protein homolog 1 (*S. cerevisiae, S. pombe*)
2,16	AA051309	Expressed sequence AW228844
2,15	AA467571	Clone IMAGE:3485144, mRNA
2,14	AA144080	Sloan-Kettering viral oncogene homolog
2,13	AA426897	Oocyte specific homeobox 1
2,13	AA030925	Expressed sequence AW228844
2,12	AA033193	Sirtuin 6 (silent mating type information regulation 2, homolog) 6 (*S. cerevisiae*)
2,12	AA174620	Histocompatibility 2, class II antigen E beta
2,11	AA122711	Solute carrier family 25 (mitochondrial carrier; peroxisomal membrane protein, 34 kDa), member 17
2,11	AA116946	Translocase of inner mitochondrial membrane 8 homolog a (yeast)
2,09	W90822	Hypothetical protein, clone 1-82
2,09	AA052723	ESTs
2,08	AA011759	Glutamine synthetase
2,07	AA137848	Isocitrate dehydrogenase 3 (NAD+) alpha
2,06	AA023461	Clone IMAGE:445127
2,06	W41175	Glycerol phosphate dehydrogenase 1, mitochondrial
2,06	W98550	ESTs, Moderately similar to SNX8_HUMAN SORTING NEXIN 8
2,04	AA108501	ESTs, Weakly similar to PGG2 RAT CHONDROITIN SULFATE PROTEOGLYCAN NG2 PRECURSOR [*R. norvegicus*]
2,04	AA051124	Glia maturation factor, beta
2,03	AA125367	Protein tyrosine phosphatase, non-receptor type 16
2,03	W47923	Expressed sequence AW046014
2,02	AA049697	ESTs, Moderately similar to PUR4_HUMAN PHOSPHORIBOSYLFORMYLGLYCINAMIDINE SYNTHASE
2,02	AA003402	RIKEN cDNA 4833439O17 gene
2,02	W98657	ESTs, Weakly similar to RIKEN cDNA 2810408B13
2,01	AA144003	Hypothetical protein MGC18754
2,00	AA002452	ESTs

Genes that are more than 2-fold regulated and have signals that significantly differ from local background in each of the duplicate spots are listed.

Bold represents genes encoding functions in DNA replication. Although the fold-induction of PCNA does not exceed the cut-off value, PCNA is included in the list.

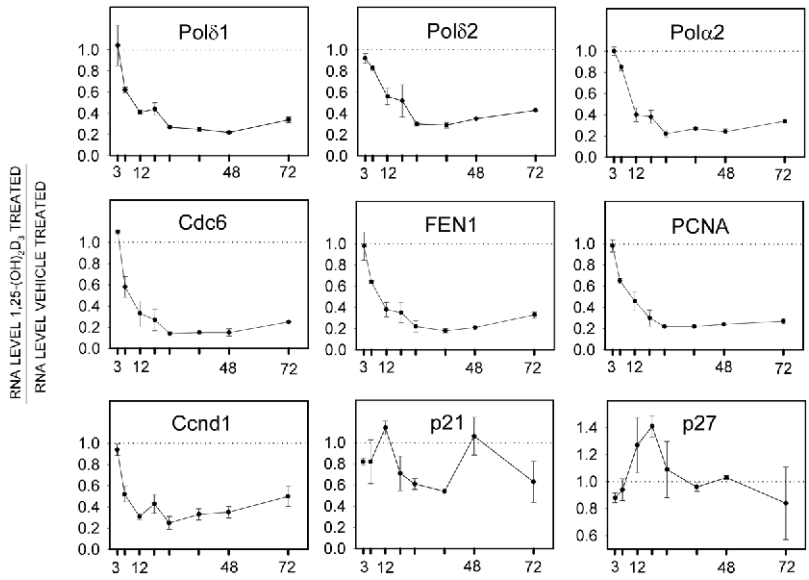

FIG. 3. QRT-PCR analyses on MC3T3-E1 cells. MC3T3-E1 cells were treated with $1,25(OH)_2D_3$ (10^{-8} M) or vehicle for 3, 6, 12, 18, 24, 36, 48, or 72 h. At each of these timepoints, gene expression was assessed by QRT-PCR analysis, normalized to β-actin RNA levels, and expressed as a ratio between $1,25(OH)_2D_3$-treated and corresponding vehicle-treated samples. Data are the means ± SEM of two independent experiments performed in triplicate. The 6- and 12-h samples used for QRT-PCR are independent of those used for cDNA microarray analysis. For all six DNA replication genes and for Ccnd1, the overall downregulation by $1,25(OH)_2D_3$ was found to be significant according to Fisher's LSD-multiple comparison test ($p < 0.05$). For p21 and p27, there was no significant upregulation according to the same test.

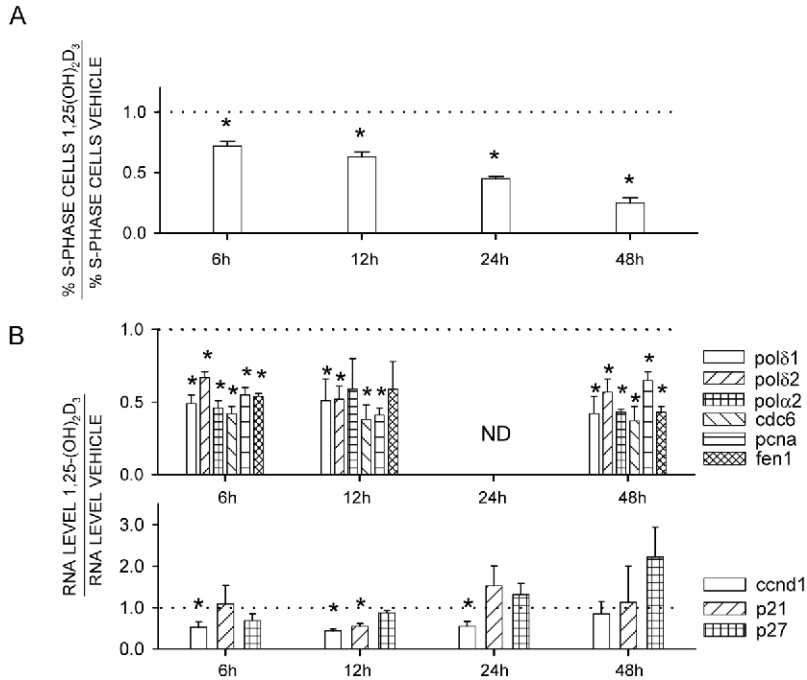

FIG. 4. Effects of $1,25(OH)_2D_3$ on GR cells. (A) Cell cycle analysis of GR cells treated with $1,25(OH)_2D_3$. Cells were treated with $1,25(OH)_2D_3$ (10^{-8} M) or vehicle and stained with propidium iodide at the indicated times. Bars represent mean ratios ± SEM between the number of S-phase cells in $1,25(OH)_2D_3$-treated samples and the number of S-phase cells in vehicle-treated samples from a representative experiment performed in duplicate. $*p < 0.05$, $1,25(OH)_2D_3$-treated vs. vehicle-treated (Fisher's LSD-multiple comparison test). Dotted line indicates a 1:1 ratio. (B) QRT-PCR analyses. Cells were treated with $1,25(OH)_2D_3$ (10^{-8} M) or vehicle for the indicated times. Target gene RNA levels were measured, normalized to β-actin RNA levels, and expressed as a ratio between $1,25(OH)_2D_3$-treated and corresponding vehicle-treated samples. Bars represent mean ratios ± SEM of at least two independent experiments performed in triplicate. $*p < 0.05$, $1,25(OH)_2D_3$-treated vs. vehicle-treated (Student's *t*-test). Dotted line indicates the 1:1 ratio.

those obtained by microarray analysis (from 1.9-fold [PCNA] to 3.0-fold [Cdc6 and FEN1] downregulation; Table 1). The 24- to 48-h interval showed the most pronounced downregulation for all six DNA replication genes. At these times, Cdc6 was affected the most by treatment with $1,25(OH)_2D_3$, resulting in a 7.1-fold decrease in expression, whereas expression of Polδ2 was repressed only 3.4-fold (Fig. 3). Downregulation of Polα2, PCNA, Polδ1, and FEN1 varied between these two values. Repression of all six DNA replication genes slightly decreased at 72 h after treatment, possibly because of the increasing confluence of the vehicle-treated cells, resulting in reduced growth.

Ccnd1 showed a 2-fold decrease in expression from 6 h after treatment with $1,25(OH)_2D_3$. p21, however, did not show any significant upregulation by $1,25(OH)_2D_3$ at the indicated times, and an apparent rise in p27 expression was only observed around 18 h after treatment.

Effect of $1,25(OH)_2D_3$ treatment on DNA replication genes in other cell types

To investigate the general nature of this effect, we studied the expression of these six DNA replication-related genes and Ccnd1, p21, and p27, as well as the G1/S-block after

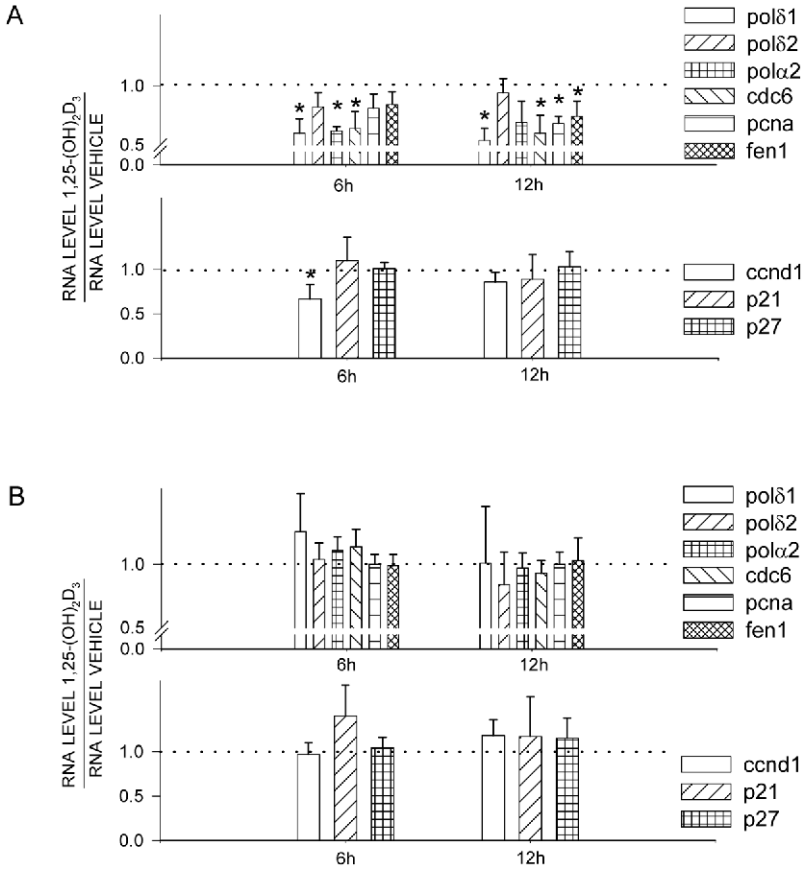

FIG. 5. QRT-PCR analyses on primary MEKs. Cells were treated with $1,25(OH)_2D_3$ (10^{-8} M) or vehicle for the indicated times. Target gene RNA levels were measured, normalized to β-actin RNA levels, and expressed as a ratio between $1,25(OH)_2D_3$-treated and corresponding vehicle-treated samples. Bars represent mean ratios ± SEM of at least two independent experiments performed in triplicate. $*p < 0.05$, $1,25(OH)_2D_3$-treated vs. vehicle-treated (Student's *t*-test). Dotted line indicates the 1:1 ratio. (A) VDR^{wt}MEKs. (B) $VDR^{-/-}$MEKs.

treatment with $1,25(OH)_2D_3$ in other cell types including murine mammary tumor cells (GR), VDR wildtype primary murine epidermal keratinocytes (VDR^{wt}MEKs), and fibroblastic NIH-3T3 cells by means of QRT-PCR. VDR knockout MEKs ($VDR^{-/-}$MEKs) were subjected to the same analysis to determine the dependence of $1,25(OH)_2D_3$-induced effects on a functional VDR.

Cell cycle analysis of $1,25(OH)_2D_3$-treated GR cells revealed a significant drop in the number of S-phase cells ranging from 28% at 6 h to 75% at 48 h after treatment compared with vehicle-treated cells (Fig. 4A). This decrease in S-phase cells was mirrored by a significant downregulation of all six DNA replication genes from 6 h after treatment with $1,25(OH)_2D_3$ (Fig. 4B). The expression of Ccnd1, however, was clearly, although statistically not significantly, reduced at 3 h after treatment with $1,25(OH)_2D_3$ (1.6-fold downregulation compared with vehicle-treated cells, $p = 0.056$, data not shown). At 6, 12, and 24 h, $1,25(OH)_2D_3$ caused a significant 2-fold decrease in Ccnd1 expression. Only the cdk inhibitor p27 showed an apparent rise in expression, albeit after 48 h of treatment (Fig. 4B).

In primary VDR^{wt}MEKs, a 6-h treatment resulted in the significant downregulation of Polδ1, Polα2, Cdc6, and Ccnd1, whereas a 12-h treatment repressed the expression of Polδ1, Cdc6, PCNA, and FEN1. At none of the indicated times was the expression of p21 or p27 altered (Fig. 5A). None of the six DNA replication genes, Ccnd1, p21, or p27 showed changes in expression in primary $VDR^{-/-}$MEKs treated with $1,25(OH)_2D_3$ for 6 or 12 h (Fig. 5B). A similar observation was made for fibroblastic NIH-3T3 cells; until 48 h after treatment, none of the DNA replication genes were downregulated, and no changes in expression were observed for Ccnd1, p21, or p27 (data not shown). Accordingly, there was no observable G1/S block until 48 h after treatment with $1,25(OH)_2D_3$ (data not shown).

$1,25(OH)_2D_3$-induced changes in E2F activity

Because genes involved in DNA replication are generally known to be E2F regulated, we investigated if these changes in expression profile induced by $1,25(OH)_2D_3$ treatment could be linked to changes in E2F activity. MC3T3-E1 cells were transiently transfected with an E2F reporter construct containing six E2F responsive sites. Cells were treated with a single dose of $1,25(OH)_2D_3$ (10^{-8} M) or vehicle and assayed for luciferase activity 6, 12, or 24 h later. A 12- or 24-h incubation period with $1,25(OH)_2D_3$ resulted in a clear reduction of E2F activity (2.0- and 3.7-fold, respectively), in contrast to a 6-h incubation period (Fig. 6). However, changes observed at 12 and 24 h probably reflect changes in E2F activity that occur earlier in time. The time span between actual lowering of E2F activity by $1,25(OH)_2D_3$ and the resulting decrease in the luciferase expression is unknown. Similar results were obtained using an E2F reporter construct with 4 E2F responsive sites (data not shown).

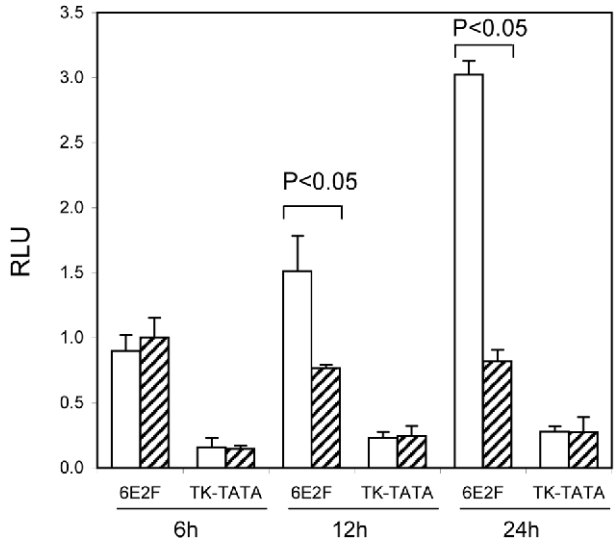

FIG. 6. Effect of $1,25(OH)_2D_3$ on E2F activity in MC3T3-E1 cells. MC3T3-E1 cells were transiently transfected with a luciferase reporter construct containing six E2F binding sites or with a minimal TK-TATA luciferase reporter construct. Cells were treated with $1,25(OH)_2D_3$ (10^{-8} M; hatched bars) or vehicle (open bars) and assayed for luciferase activity at the indicated times. Luciferase activities were corrected for β-galactosidase activity from a cotransfected β-galactosidase expression construct and are presented as relative luciferase units (RLU). A representative experiment of three independent experiments performed in triplicate is shown; bars are mean RLUs ± SEM. $p < 0.05$ indicates significant differences between vehicle-treated and $1,25(OH)_2D_3$-treated cells according to Student's *t*-test.

DISCUSSION

cDNA microarray studies on MC3T3-E1 cells investigating changes in gene expression at different moments during proliferation, differentiation, or mineralization were recently described.[24–26] This study concerns the effect of $1,25(OH)_2D_3$ on gene expression in MC3T3-E1 cells using the cDNA microarray technique. More in particular, we focused on differential expression of genes that might be involved in the antiproliferative effect of $1,25(OH)_2D_3$, an effect described in tumor cells[7,17,27] as well as in normal cells. [15,28] The blocked transition from the G1 phase to the S phase of the cell cycle is a known, although not fully understood, feature of this antiproliferative effect.[6,7] Quantification of this G1/S block in MC3T3-E1 cells revealed a reduction of S-phase cells by 25% after a 12-h incubation period with $1,25(OH)_2D_3$. The data from the cDNA microarray analysis show that $1,25(OH)_2D_3$, after a 12-h incubation period, decreased the expression of several DNA replication genes including those encoding Cdc6, Polδ1 (catalytic subunit, 125 kDa), Polδ2 (regulatory subunit, 50 kDa), Polα2 (68 kDa subunit), FEN1, and PCNA. The downregulation of these six genes in MC3T3-E1 cells is far more than a short transient phenomenon because quantitative RT-PCR analyses showed a decreased expression of these genes starting at 6 h and lasting at least up to 72 h after treatment.

In addition to MC3T3-E1 cells, other cell types were examined to determine if this mechanism of action of $1,25(OH)_2D_3$ is typical of MC3T3-E1 cells or common to different normal and malignant cell types. Therefore, we selected VDR wildtype primary murine epidermal keratinocytes and mouse mammary tumor cells, both characterized by a $1,25(OH)_2D_3$-induced growth inhibition.[19,29] The results showed a substantial decrease in expression of all six aforementioned genes in these cell types and hence demonstrated this effect of $1,25(OH)_2D_3$ to be of a general nature. In addition, failure of $1,25(OH)_2D_3$ to repress the DNA replication genes in $VDR^{-/-}$MEKs points toward a prominent role for a functional VDR in this $1,25(OH)_2D_3$-induced effect.

Chen et al. studied the expression of DNA replication genes during $1,25(OH)_2D_3$-induced differentiation of leukemic HL-60 cells and showed that thymidine kinase and Polα mRNA levels decreased no earlier than 3 days after $1,25(OH)_2D_3$ treatment. From their data, they infer the downregulation of these genes to be a consequence of rather than a cause for discontinued cell proliferation.[30] In contrast, the QRT-PCR analyses on MC3T3-E1 and on GR cells performed in this study showed a repression of DNA replication genes before (MC3T3-E1) or at least in parallel with (GR) an observable G1/S block in these cells. Moreover, in primary VDR^{wt}MEKs also, all six genes (except for *Polδ2*) were clearly downregulated at 6 and 12 h after treatment. Conversely, in fibroblastic NIH-3T3 cells, which were less sensitive to $1,25(OH)_2D_3$ treatment and did not show any repressed expression of the six DNA replication genes until 48 h after treatment, there was no observable G1/S block until 48 h. The data on these four divergent cell types suggest that downregulation of DNA replication genes precedes and therefore may contribute to the observed proliferation arrest. Notwithstanding these findings, the downregulation of these genes is unlikely to be the first event in $1,25(OH)_2D_3$-induced growth inhibition. More extensive microarray-based studies on earlier time-points could be used to identify the primary mediator(s).

Surprisingly, neither $p21^{CIP1/WAF1}$ nor $p27^{KIP1}$, previously assumed mediators of the $1,25(OH)_2D_3$-induced cell cycle arrest, showed a significant increase in expression at 6 or 12 h after treatment with $1,25(OH)_2D_3$, according to the microarray analysis. QRT-PCR analyses confirmed these findings in MC3T3-E1 cells as well as in GR cells and in primary VDR^{wt}MEKs. These data confirm our previous findings in human MCF-7 cells in which increased $p21^{CIP1/WAF1}$ transcript levels are only detected after 24 h of treatment together with a clear induction of $p21^{CIP1/WAF1}$ protein production.[7] Although Liu et al. showed a rapid increase in expression of $p21^{CIP1/WAF1}$ by $1,25(OH)_2D_3$ through a VDRE in its promoter region,[27] attempts to further elucidate the involvement of $p21^{CIP1/WAF1}$ in $1,25(OH)_2D_3$-mediated cell cycle arrest have generated conflicting results[31–33]; even the functionality of the VDRE has been questioned.[34] Moreover, recent studies in $p21^{-/-}$ and $p27^{-/-}$ mouse embryonic fibroblasts show a clear $1,25(OH)_2D_3$-induced growth inhibition in primary and immortalized $p21^{-/-}$ cells and in immortalized $p27^{-/-}$ cells.[35] In LNCaP prostate cancer cells, $1,25(OH)_2D_3$ causes growth inhibition not by a direct effect on $p21^{CIP1/WAF1}$ but by an upregulation of insulin-like growth factor binding protein-3 (IGFBP-3), which in turn, acts on $p21^{CIP1/WAF1}$[36]; a

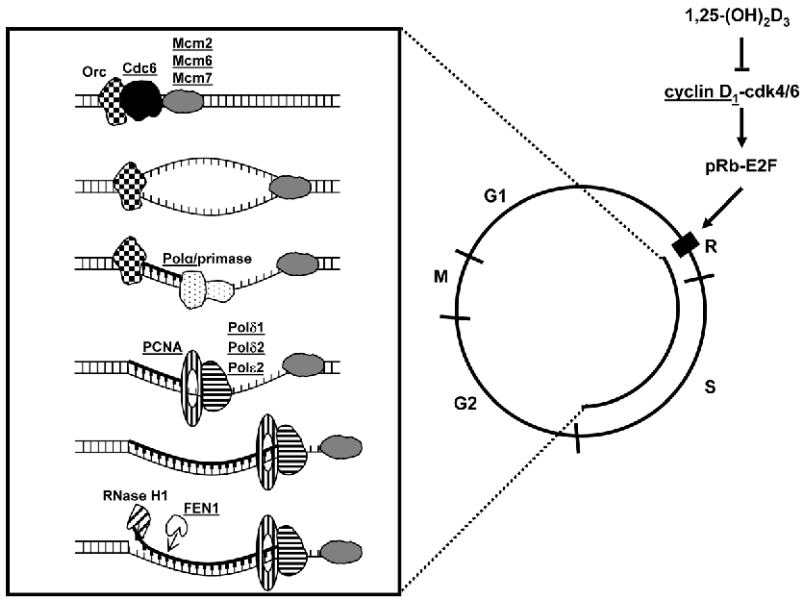

FIG. 7. Sequence of events leading to downregulation of DNA replication machinery by $1,25(OH)_2D_3$. Reduction of E2F activity by $1,25(OH)_2D_3$, possibly through inhibition of cyclin D_1-cdk4/6, leads to downregulation of DNA replication genes in parallel with the G1 arrest. Magnification on the left gives a schematic and simplified overview of the DNA replication process. Underlined genes were found to be more than 2-fold downregulated after 12 h of $1,25(OH)_2D_3$ treatment according to cDNA microarray analysis. (R = restriction point).

finding that has been confirmed recently by a microarray-based study in these cells.[37] Similarly, the microarray analysis on MC3T3-E1 revealed a more than 2-fold upregulation for IGF2BP-3 at 12 h after treatment with $1,25(OH)_2D_3$.

Because none of the six investigated DNA replication genes are known to contain functional VDREs in their promoter sequences, the observed effect of $1,25(OH)_2D_3$ probably constitutes an indirect effect. However, $1,25(OH)_2D_3$ provokes a similar effect on the expression of Cdc6, Polδ1, Polδ2, Polα2, FEN1, and PCNA most likely through a pathway shared by these six genes. The most obvious candidate to mediate the effect of $1,25(OH)_2D_3$ on these genes is the complex between the E2F family of transcription factors and the retinoblastoma susceptibility protein pRb. This E2F-pRb complex is the major gatekeeper of the G1 to S phase transition in cycling cells, and its role in cell cycle control and cancer has been extensively described.[38–40] Briefly, initial hyperphosphorylation of pRb by active cyclin D1-cdk4/6 followed by a second wave of phosphorylation by cyclin E/A-cdk2 releases E2F from the complex. In late G1 phase, these free E2F transcription factors promote the expression of genes required by cells to pass the G1/S gate and to complete the cell cycle. Inability to phosphorylate pRb keeps this G1/S gate closed and causes cells to accumulate in G1.

E2F target genes act on two different yet complementary levels. A first set of E2F responsive genes regulates cell cycle progress and contains, among others, E2F-1, E2F-2, cyclin E, cyclin A, and cdk2; genes through which E2F establishes a positive feedback mechanism that makes onset of S phase irreversible. In a recent study, Jensen et al. showed cyclin A and cyclin E mRNA levels as well as cdk2 protein levels to be lower in $1,25(OH)_2D_3$-treated MCF-7 cells than in control cells.[18] Similarly, treatment of human NCI-H929 myeloma cells with the $1,25(OH)_2D_3$ analog EB1089 results in lowering of cyclin A and cdk2 protein levels.[41] A second group of E2F responsive genes encodes proteins acting in the complex processus of DNA replication. Cdc6, Mcm, Orc1, DNA polymerase α, PCNA, dihydrofolate reductase, thymidine kinase, and thymidylate synthase are known E2F targets in mammalian cells.[13,14] Recent microarray-based studies have drastically increased the number of known E2F target genes and prove *FEN1*, *Polδ1*, and *Polδ2* to be E2F-driven genes.[42–44] Hence, all six genes we focused on are E2F target genes. Moreover, Tk1 and Mcm 2, 6, and 7 were also present on the cDNA microarray and were more than 2-fold repressed at 12 h after $1,25(OH)_2D_3$ treatment. Similarly, subunit 2 of DNA polymerase ϵ, whose human homolog contains two overlapping E2F binding sites,[45] showed a 2-fold reduction in expression. One must keep in mind, however, that the list of DNA replication genes we found to be downregulated by $1,25(OH)_2D_3$ is most probably not complete because an array containing only 4600 cDNA fragments was used.

Such a relatively rapid and simultaneous change in the expression of a large number of DNA replication genes has not been described in the field of steroid research, except for a comprehensive microarray analysis recently conducted by Lobenhofer et al.,[46] who focused on the mitogenic effects of estrogens on hormone-responsive breast cancer MCF-7 cells. They were able to link the estrogen-induced proliferation of MCF-7 cells to the upregulation of a large group of DNA replication related genes containing many of the genes we found to be downregulated in MC3T3-E1 after $1,25(OH)_2D_3$ treatment according to our microarray data. They also ascribe these findings to changes in E2F activity after estrogen stimulation. Interestingly, their data show a significant upregulation of cyclin D1 at 4 h after estrogen stimulation, preceding the rise in expression of the DNA replication genes. In agreement with this finding, our microarray analysis revealed a decrease in cyclin D1 expression in response to $1,25(OH)_2D_3$, albeit at 12 h after treatment. In MC3T3-E1 cells, a more extensive QRT-PCR–based time course analysis of *Ccnd1* expression after

$1,25(OH)_2D_3$ treatment revealed a 2-fold downregulation at 6 h, coinciding with the onset of repressed expression for the DNA replication genes. Moreover, in GR cells, an apparent drop in *Ccnd1* expression occurred from 3 h after treatment onward and thus preceded the downregulation of DNA replication genes. Taken together, these data show an obvious correlation between Ccnd1 and DNA replication genes as to changes in their expression after $1,25(OH)_2D_3$ treatment. Unchanged expression levels for Ccnd1 as well as for DNA replication genes in $VDR^{-/-}$MEKs and NIH-3T3 cells at different times after $1,25(OH)_2D_3$ treatment contribute to this idea.

Dephosphorylation of pRb by $1,25(OH)_2D_3$ has previously been described in different cell types.[6,15–18] We now quantified the effects of $1,25(OH)_2D_3$ on E2F activity in MC3T3-E1 cells and demonstrated a link between these changes in E2F activity and the repression of the aforementioned genes. Transfection studies with an E2F-luciferase reporter construct showed that at 12 h after treatment with $1,25(OH)_2D_3$, the drop in E2F activity was 2.0-fold, and at 24 h, it was at least 3.7-fold. These data support the idea that $1,25(OH)_2D_3$ represses the expression of the aforementioned genes through hypophosphorylation of pRb and subsequent lowering of free E2F (Fig. 7).

Beside $1,25(OH)_2D_3$, several other steroid hormones are known to either promote or inhibit cell cycle progression possibly by affecting the E2F-pRb pathway like all-*trans* retinoic acid and thyroid hormone.[47,48] Whether these hormones induce similar simultaneous changes in the expression of a large number of DNA replication genes as do estrogens (upregulation) or $1,25(OH)_2D_3$ (downregulation) needs further study, but these simultaneous changes might well be one of nature's logical solutions to answer growth inhibitory or growth promoting stimuli in response to ligand activated nuclear transcription factors using E2F-pRb as the central mediator to control the complete DNA replication process.

In conclusion, we show, by using cDNA microarrays, that the antiproliferative effect of $1,25(OH)_2D_3$ on MC3T3-E1 mouse osteoblasts was accompanied by a significant downregulation of several well-known E2F-driven DNA replication genes. The downregulation of these genes was confirmed in normal as well as malignant murine cell types, showing the general nature of this mode of action of $1,25(OH)_2D_3$.

ACKNOWLEDGMENTS

This work was supported by the Flemish Fund for Scientific Research (FWO G.0242.01). LV and KM are postdoctoral fellows from the Flemish Fund for Scientific Research. The authors thank Kurt Hofman for his gift of the E2F-luciferase reporter constructs and Suzanne Marcelis, Ingrid Stockmans, and Biauw Keng Tan for excellent technical assistance.

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Received in original form February 17, 2003; in revised form August 7, 2003; accepted August 11, 2003.