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Regulation of Estrogen Receptor-α Gene Expression by 1,25-Dihydroxyvitamin D in MCF-7 Cells

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Abstract This report describes an investigation of the role of 1,25-dihydroxyvitamin D (VD_3) in the regulation of estrogen receptor-α (ER) in the ER-positive breast cancer cell line, MCF-7. Treatment of cells with 10 nM VD_3 resulted in a 50% decline in the concentration of ER protein at 24 h. Scatchard analysis showed a corresponding decrease in the number of estradiol binding sites and no alteration in the binding affinity of estradiol for the ER (K_d = 0.08 nM in VD_3-treated cells compared with K_d = 0.07 nM in control cells). Vitamin D treatment also caused a 50% decrease in the steady state amount of ER mRNA, which was maximal by 18 h. In vitro transcription run-on experiments demonstrated a decrease of approximately 60% in transcription of the estrogen receptor gene. Transient transfections using an ER promoter-CAT construct also demonstrated a 40% decrease in CAT activity after VD_3 treatment. Sequence analysis identified a potential vitamin D response element (nVDRE) within the ER promoter. When this element was mutated, the ability of VD_3 to block transcription from the ER promoter was lost. When the nVDRE was placed upstream of a heterologous promoter, nVDRE-SV40-CAT, treatment with VD_3 resulted in a 50% decrease in CAT activity. Interestingly, co-transfection of either the ER promoter-CAT or the nVDRE-SV40-CAT construct and a vitamin D receptor expression vector into COS-1 or CV-1 cells showed an approximately 4-fold increase in CAT activity after VD_3 treatment. Taken together these data suggest that VD_3 inhibition of ER gene transcription is mediated through a nVDRE in the ER promoter. Inhibition appears to be cell specific. J. Cell. Biochem. 75:640–651, 1999.

Key words: ER; 1,25-dihydroxyvitamin 3; MCF-7 cells

1,25-Dihydroxyvitamin D (VD_3), the active metabolite of vitamin D_1, is a secosteroid hormone that exerts its biological activities through a specific intracellular receptor, the vitamin D receptor (VDR), which regulates gene transcription [Norman, 1995]. The vitamin D receptor is a member of the steroid/thyroid receptor gene superfamily [Haussler et al., 1981; Pike, 1991], which has been shown to activate or repress expression of hormone-responsive target genes [Evans, 1988; Green et al., 1988; Moore, 1990; Rodriguez et al., 1990; Moore et al., 1991].

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The classical target organs of VD_3 are intestine, bone, and kidney. In addition, other organs were demonstrated to be targets, such as pituitary [Perez-Fernandez et al., 1997; Horii et al., 1992], skin [Casado et al., 1998; Jones et al., 1998], pancreas [Jones et al., 1998; Faure-Dussert et al., 1997], brain [Veenstra et al., 1998], reproductive organs [Jones et al., 1998; Horii et al., 1992], parathyroid and thyroid [Horii et al., 1992], lymphocytes [Jones et al., 1998], keratinocytes [Jones et al., 1998], colon [Evans et al., 1998], and prostate [Schleicher et al., 1993], as well as many cancer cells, including melanoma [Norman, 1995], breast carcinoma [Demirpeuce et al., 1994; James et al. 1994; Narvaez et al. 1997; Simboli-Campbel et al., 1997], leukemia [Mangelsdorf et al., 1984; Reitsma et al., 1983], osteosarcoma [Christakos et al., 1979], fibrosarcoma [Minghetti et al., 1988], and colon carcinoma cells [Brehier et al., 1988]. Some of these tissues exhibit specific, saturable, high-affinity (K_d = 0.01–0.1 nM), but low binding capacity receptors for VD_3 [Christa-

kos et al., 1979; Minghetti et al., 1988; Walters, 1992]. Although many of the biological responses of vitamin D are mediated through genomic pathways [Christakos et al., 1979; Pike, 1991], it appears that some responses to VD_3 are mediated through nongenomic pathways that may involve the protein kinase A and C signal transduction pathways [Caffrey et al., 1989; Norman et al., 1992]. Genes regulated by VD_3 are associated with mineral homeostasis, the biosynthesis and catabolism of vitamin D secosteroids, differentiation events in skin and the immune system, and replication and cellular proliferation [Minghetti et al., 1988].

1,25-Dihydroxyvitamin D has been shown to induce differentiation and consequently inhibit the proliferation of several cell types [Walters, 1992; Reichel et al., 1989; DeLuca, 1991; Gudas, 1992; Bikle, 1992]. It has been shown to inhibit the growth of leukemia (HL-60) cells [Mangelsdorf et al., 1984; Reitsma et al., 1983], malignant melanoma cells [Colston et al., 1981], macrophages [Reichel et al., 1987], keratinocytes [Pillai et al., 1988], bone [Majeska et al., 1982], colon cancer cells [Brehier et al., 1988], and breast cancer cells [Morrison et al., 1989; Demirpeuce et al., 1994]. In breast cancer cells, vitamin D has been shown to induce differentiation [Demirpeuce et al., 1994; Koga et al., 1991; Davoodi et al., 1995]. VD_3 also inhibits tumor promotion by 12-O-tetra-decanoyl-phorbol-13-acetate and mezerein in mouse skin [Chida et al., 1995] and inhibits induction of ornithine decarboxylase by tumor promoters in the skin, stomach, colon, and liver [Chida et al., 1995; Hashiba et al., 1987; Kuroki et al., 1983]. However, in cell culture systems, VD_3 enhances chemical transformation of BALB 3T3 [Kuroki et al., 1983; Saski et al., 1986] and Syrian hamster cells [Jones et al., 1984] and induces anchorage-independent growth of JB6 [Hosoi et al., 1986], BALB 3T3, and NIH 3T3 [Huh et al., 1987] cells.

Several studies have demonstrated that treatment of breast cancer cells with vitamin D results in a decrease in estrogen receptor (ER) [James et al., 1994; Narvaez et al. 1997; Simboli-Campbel et al., 1997], however, the mechanism of regulation was not investigated. The purpose of this study was to define the mechanism by which VD_3 regulates the expression of the ER.

MATERIALS AND METHODS

Tissue Culture

The ER-positive breast cancer cell line MCF-7 was originally derived from a pleural effusion of a human breast carcinoma [Berthois et al., 1986]. Monolayer cultures of MCF-7 cells were grown in improved minimum essential medium (IMEM) supplemented with 5% fetal calf serum (FCS). When the cells were approximately 80% confluent, the medium was replaced with phenol red free IMEM, containing 5% (vol/vol) charcoal-treated calf serum (CCS). The calf serum was pretreated with sulfatase and dextran-coated charcoal to remove endogenous steroids. After 2 days in these conditions, cells were treated with 10 nM VD_3 or 1 nM estradiol, or both. Cells were harvested at the various times indicated.

Plasmids

The clones for the ER, pOR300, and Q7 [Saceda et al., 1988], and for 36B4 [Masiakowski et al., 1982], p36B4, have been previously described. POR 300 was constructed by subcloning a 300-base pair (bp) restriction fragment of the ER (pOR 3) into the pGem 4 polylinker region using the restriction enzymes *Pst*I and *Eco*RI. The clone p36B4 was constructed by subcloning a 220-bp fragment of 36B4 into the *Pst*I restriction site of the pGem 4 polylinker [Masiakowski et al., 1982]. The ER promoter-CAT vector, ER-128-CAT, was constructed by subcloning 128 bp of the proximal promoter of the ER gene (−128 to +1 nucleotides) into the *Hin*dIII and *Xba*I restriction sites of the pCAT enhancer vector (Promega, Madison, WI). Mutation of the nVDRE in the ER-128-CAT to a nonsense sequence (ER-128NS-CAT) was performed using in vitro mutagenesis. A synthetic oligonucleotide containing the negative VD_3 response element (AGGGCAAGGCAACAGTCCCTGGCCG) with *Bgl*II restriction sites on the 5′ and 3′ ends was cloned into the *Bgl*II restriction site upstream of the SV40 promoter of a CAT reporter vector (nVDRE-SV40-CAT). All vectors were sequenced using the Sequenase kit (USB, Cleveland, OH).

Estrogen Receptor Protein Assays

For ER protein analysis, MCF-7 cells were cultured and treated as described above. The concentration of receptor protein was assayed using an enzyme immunoassay kit from Abbott Laboratories (North Chicago, IL). To obtain to-

tal receptor protein, the cells were homogenized by sonication in a high salt buffer (10 mM Tris, 1.5 mM EDTA, 5 mM Na_2MoO_4, 0.4 M KCl, 1 mM monothioglycerol with 2 mM leupeptin). The homogenate was incubated on ice for 30 min and centrifuged at 100,000*g* for 1 h at 4°C. Aliquots of the total extracts were then analyzed according to the manufacturer's instructions.

In order to measure the number of estrogen binding sites and the dissociation constant (K_d) of the estradiol-ER complex, the whole cell multiple-dose ligand binding assay was used. Cells were plated in 6-well plates (50,000 cells/well) in IMEM containing 5% FCS. When the cells were 70% confluent, the medium was replaced with phenol red-free IMEM containing 5% CCS. After 2 days in these conditions, the cells were treated for 18 h with 10 nM VD_3. Cells were washed with HBSS medium and incubated for 1 h with various concentrations of [^{3}H]estradiol (0.2, 0.4, 0.8, 1.5, 2.5, and 4 nM) in the presence and absence of a 200-fold excess of diethylstilbestrol (DES) (nonspecific binding, B_N, and total binding, B_T, respectively). Unbound ligand was removed by washing and the cells were disrupted either by sonication or by freezing and thawing for three cycles. The radioactivity in each well, as well as the total radioactivity for each [^{3}H]estradiol concentration, was measured in a β-counter. Specific binding was determined as the difference between total and nonspecific binding ($B_S = B_T - B_N$), and the data were plotted according to the Scatchard equation [Scatchard, 1949]. The binding affinity (K_d) and capacity (B_{max}) were determined and the saturation curves obtained by plotting B_S versus the concentration of the radio labeled ligand. The protein concentration in each well was determined using the Bio-Rad method and IgG as standard. All points were done in triplicate.

Measurement of ER mRNA

Total cellular RNA was extracted from MCF-7 cells by homogenization in a 6 M guanidine isothiocyanate lysing buffer containing 5 mM sodium citrate, 0.1 M β-mercaptoethanol, and 0.5% sarkosyl. After centrifugation through a 5.7 M cesium chloride pad at 100,000*g* for 16 h at 20°C, the amount of ER and 36B4 mRNA were determined by an RNase protection assay. For this analysis, homogeneously ^{32}P-labeled antisense molecules (cRNA) were synthesized in vitro from pOR300 and p36B4 using T7 polymerase. A total of 60 µg of total RNA were hybridized for 12–16 h to the radiolabeled cRNA. After a 30-min digestion at 25°C with RNase A, ^{32}P-labeled cRNA probes protected by total RNA were separated by electrophoresis on 6% polyacrylamide gels. The bands were visualized by autoradiography and quantified by optical densitometry. The amount of ER mRNA was normalized to the amount of 36B4 mRNA that is constitutively expressed in the presence of estradiol [Masiakowski et al., 1982; Saceda et al., 1988], growth factors [Saceda et al., 1996; Stocia et al., 1997], activators of protein kinase C [Saceda et al., 1991], and VD_3 (data not shown).

Nuclei Isolation

MCF-7 cells were treated, harvested, and resuspended in 5 ml of 1.5 M sucrose buffer as previously described [Saceda et al., 1988]. The cells were then homogenized with 10 strokes in a Dounce homogenizer using pestle A. The homogenate was diluted to 15 ml with 1.5 M sucrose and centrifuged at 10,000*g* for 20 min at 4°C. The nuclear pellet was resuspended in 0.5 ml of nuclei storage buffer (20 mM Hepes, 75 mM NaCl, 0.5 mM EDTA, 0.85 mM DTT, 0.125 mM PMSF, 50% glycerol). The nuclei were stored at −70°C until the transcription run-on assay was performed.

Transcription Run-on Assay

The isolated nuclei were in vitro transcribed in the presence of nucleotide triphosphates, ATP, CTP, GTP, and ^{32}P-UTP as previously described [Saceda et al., 1988]. The radiolabeled RNA transcripts were isolated and hybridized to an excess of denatured plasmid DNA immobilized on a nitrocellulose filter. The denatured plasmids used for the detection of specific transcripts were exon I of ER (Q7) and 36B4. After the hybridization, the nitrocellulose blots were washed and exposed to X-ray film. Autoradiographs were analyzed by densitometry and the background was subtracted. Results were normalized to the transcriptional level of 36B4.

Transfection and CAT Assays

In the transfection assays, 10^6 MCF-7 cells were plated in 100-mm dishes and grown in IMEM supplemented with 10% CCS for 24 h before transfection. Calcium phosphate-DNA precipitates containing 5 µg of either the

ER-128-CAT vector, the ER-128NS-CAT vector, or the nVDRE-SV40-CAT vector; 2 μg of the β-galactosidase vector; and 23 μg of carrier DNA were prepared and the cells were transfected by the method of Chen and Okayama [Chen, 1987]. At 18 h after transfection, cells were washed and the medium was replaced with phenol red-free IMEM supplemented with 10% CCS. After 24 h in estrogen-depleted medium, 10 nM VD_3 was added. Cell lysates were prepared 6 and 24 h after VD_3 treatment and analyzed for CAT activity and β-galactosidase activity. β-galactosidase activity was determined as a measure of the transfection efficiency. The conversion of [^{14}C]chloramphenicol to its acetylated forms was determined by thin-layer chromatography (TLC). The plates were scanned by a phosphorimager. The amount of CAT activity was normalized to the amount of β-galactosidase activity. Results are expressed as percentage of control CAT activity. Comparisons between groups were conducted using a paired t-test with a value of $P < 0.05$ considered significant.

In co-transfection assays, COS-1 or CV-1 cells were plated in 100-mm dishes and grown in IMEM supplemented with 10% CCS for 24 h. Calcium phosphate- DNA precipitates containing 5 μg of either the ER-128-CAT vector, the ER-128NS-CAT vector, or the nVDRE-SV40-CAT vector, 2 μg of the β-galactosidase vector, 5 μg of VDR expression vector, and 18 μg carrier DNA were prepared and the cells were co-transfected as described above.

RESULTS

Effect of Vitamin D Treatment on the Estrogen Receptor

To define the role of vitamin D in the expression of the ER in breast cancer, the effects of VD_3 on the concentration of the ER protein in MCF-7 cells were determined. MCF-7 cells were treated with 10 nM VD_3 and the concentration of ER was measured using an enzyme immunoassay. The data presented in Figure 1A show that 10 nM VD_3 treatment resulted in a decline in total receptor protein by about 50%. Receptor protein declined from a concentration of approximately 360 fmol/mg protein in control cells to 170 fmol/mg protein in cells treated with VD_3. The amount of receptor decreased maximally by 24 h. To determine whether the effects of vitamin D were concentration dependent, cells were treated with 1, 10, and 100 nM VD_3 for

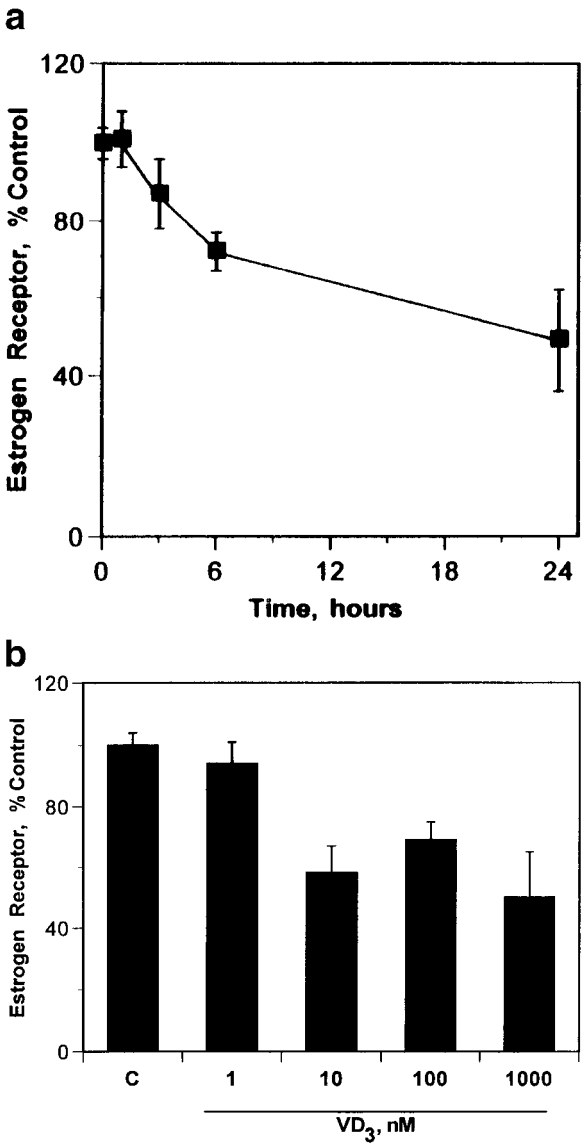

Fig. 1. Effect of VD_3 on the concentration of estrogen receptor (ER) protein. MCF-7 cells were grown in improved minimum esssential medium (IMEM) supplemented with 5% fetal calf serum. At 80% confluence, medium was changed to phenol red-free IMEM with 5% charcoal-treated calf serum for 2 days. Cells were treated with vitamin D_3. The concentration of ER was determined by the enzyme immunoassay as described under Materials and Methods. The results are presented as percentage control. Each point represents the mean value of at least six experiments (±SD). **A:** Time course of the effect of 10 nM VD_3. **B:** Effect of vitamin D_3 concentration.

24 h and the amount of ER was determined. After treatment with 1 nM VD_3 there was no significant change (less than 10%) in the concentration of the ER. When cells were treated with either 10 nM, 100 nM, or 1,000 nM of VD_3 there was a 50% decline in the ER concentration (Fig. 1B), suggesting that the decrease in the ER

protein was dependent on the VD_3 concentration. In the MCF-7 cells employed in this study, ER-α is the predominant isoform of the receptor, comprising approximately 98% of the total receptor messenger RNA (mRNA)(data not shown).

To determine whether the decrease in ER protein corresponded to a similar decrease in estradiol binding sites, the binding capacity and affinity of the ER were determined using a multiple-dose ligand binding assay. The saturation curves and the corresponding Scatchard plots for control and vitamin D-treated cells are presented in Figure 2A and B, respectively. After treatment with 10 nM VD_3, a significant decrease in the estradiol binding capacity was observed. The number of estrogen binding sites decreased from 417 fmol/mg protein in control cells to 150 fmol/mg of protein in cells treated with VD_3, indicating a 64% decrease in ER protein. These results are consistent with the results obtained with the enzyme immunoassay. Scatchard analysis of the data indicates that treatment with VD_3 did not alter the binding affinity of estradiol to the receptor (K_d = 0.07 ± 0.004 nM, n = 3, r = −0.894 in control cells compared with K_d = 0.08 ± 0.001 nM, n = 3, r = −0.784 in VD_3-treated cells). These results suggest that although VD_3 decreased the number of receptor sites, it did not affect the affinity of the receptor for its ligand.

Effect of Vitamin D Treatment on the Steady-State Level of the Estrogen Receptor mRNA

To determine whether the decline in ER to a new steady-state level was accompanied by a parallel decrease in ER mRNA, MCF-7 cells were treated with 10 nM VD_3 and the amount of ER mRNA was measured using an RNase protection assay. In these experiments, the amount of ER mRNA was normalized to the amount of 36B4 mRNA which is expressed constitutively in the presence of estradiol [Masiakowski et al., 1982; Saceda et al., 1988] and is not affected by VD_3 (data not shown). Changes in ER mRNA and 36B4 mRNA were quantified by scanning densitometry and the data are presented graphically in Figure 3 as the ratio of the integrated ER to the integrated 36B4 signal. The results are presented as percentage of control. In this study, VD_3 treatment resulted in a maximum suppression of the steady-state amount of ER mRNA to approximately 50% of control values by 18 h. These data demonstrate a close temporal relationship between the change in steady-state levels of ER protein and mRNA.

To investigate whether regulation of ER by VD_3 was common to other ER-positive breast cancer cells, Zr-75B cells were treated with various concentrations of VD_3 and the effect on ER mRNA was measured by the RNase protec-

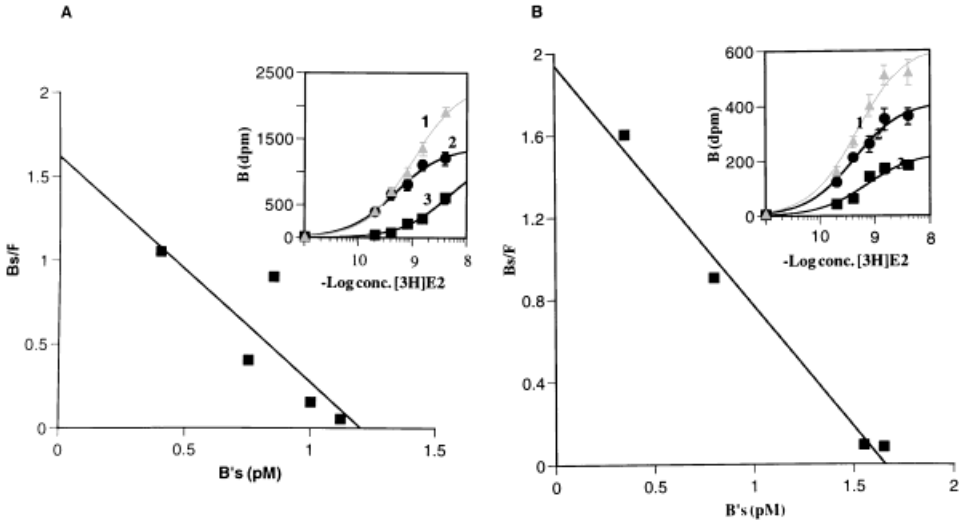

Fig. 2. Scatchard plot of [^{3}H]estradiol binding to the estrogen receptor (ER) in MCF-7 cells. MCF-7 cells were grown and treated as described in Fig. 1. The whole cell multiple-dose ligand binding assay was employed to measure the number of estrogen binding sites and the dissociation constant as described under Materials and Methods. The results are graphically represented according to the Scatchard equation: $B_S = B_T - B_N$, where B'_S = binding capacity in pM. B_T = total binding, B_N = nonspecific binding, and B_S = specific binding. A representative assay is shown. **A:** Control cells. **B:** VD_3-treated cells. **Inset:** Saturation curve of [^{3}H]E_2 binding to the ER; 1 = B_T, 2 = B_N, 3 = B_S. These experiments were repeated three times.

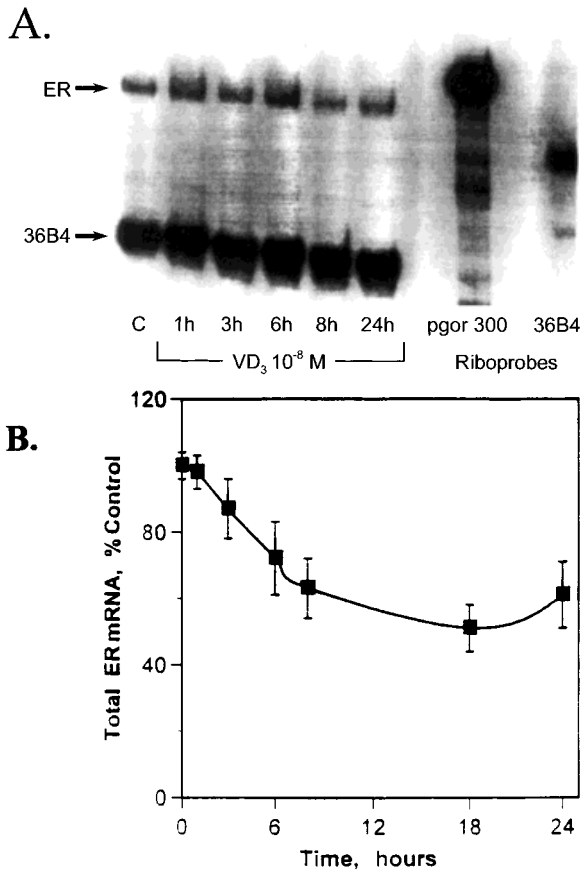

Fig. 3. Effect of VD_3 on the steady-state level of estrogen receptor (ER) mRNA. MCF-7 cells were treated as described in the legend to Fig. 1. Total RNA was isolated as described under Materials and Methods. A total of 60 μg of RNA was analyzed using an RNase protection assay as described under Materials and Methods. **A:** The autoradiographs were quantified by scanning densitometry and the values are presented as the ratio of integrated signals of ER and 36B4. The results are expressed as percentage control. **B:** The values are the mean of at least five experiments (±SD).

tion assay. There was an approximately 40% decrease in ER mRNA after treatment with either 1 or 10 nM VD_3. When cells were treated with 100 nM VD_3, ER mRNA decreased by approximately 60% (data not shown).

Effect of Vitamin D on the Transcription of the Estrogen Receptor Gene

To determine whether the effect of VD_3 on ER mRNA was due to regulation of ER gene transcription, an in vitro nuclear run-on assay was employed. Nuclei were isolated from cells treated with 10 nM VD_3, and the transcription elongation assay was performed. The level of transcription was measured by autoradiography and quantified by scanning densitometry.

The transcription of 36B4 was used as an internal control, and the relative change in ER transcription was normalized to the transcription of 36B4. The data presented in Figure 4 demonstrate that treatment with VD_3 results in a decrease in ER gene transcription to 40% of control values by 3 h. Transcription remained suppressed for up to 24 h. These data suggest that VD_3 decreased ER mRNA by inhibition of ER gene transcription.

To determine whether VD_3 inhibition of ER gene expression was mediated by the ER promoter, 128 bp of the proximal ER promoter were linked to the reporter gene chloramphenicol acetyltransferase (CAT) (ER-128-CAT, Fig. 5) and transfected into MCF-7 cells. The transfected cells were treated with 10 nM VD_3 for 6 and 24 h. Cells were harvested and assayed for CAT activity. To control for transfection efficiency, β-galactosidase activity was measured. The results are represented in Figure 6 as percentage of control. After VD_3 treatment, CAT activity decreased by approximately 40% ($P < 0.02$). Sequence analysis of this region of the ER promoter revealed a potential negative vitamin D hormone response element (nVDRE, −94 to −70). This element is an imperfect palindrome separated by 13 nucleotides and maps in

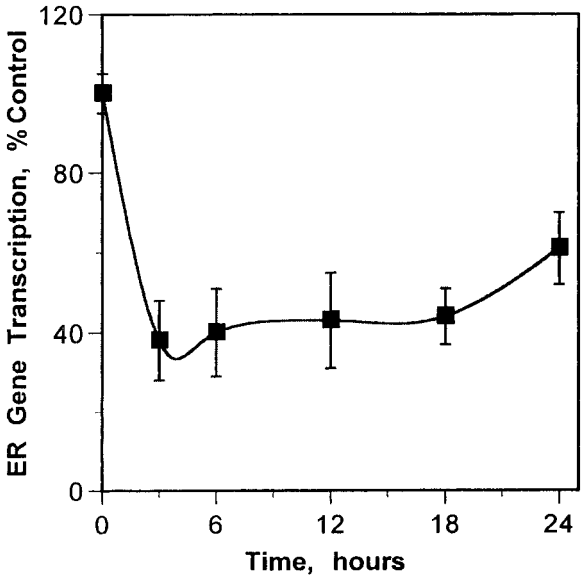

Fig. 4. Effect of VD_3 on the estrogen receptor gene transcription. MCF-7 cells were grown and treated as described in the legend to Fig. 1. The nuclei were isolated and the nuclear transcription run-on assay was performed as described under Materials and Methods. The autoradiographs were quantified by scanning densitometry and the level of transcription was expressed as the ratio of the integrated signals of ER and 36B4. The results are presented as percentage control. The values are the mean of three experiments (±SD).

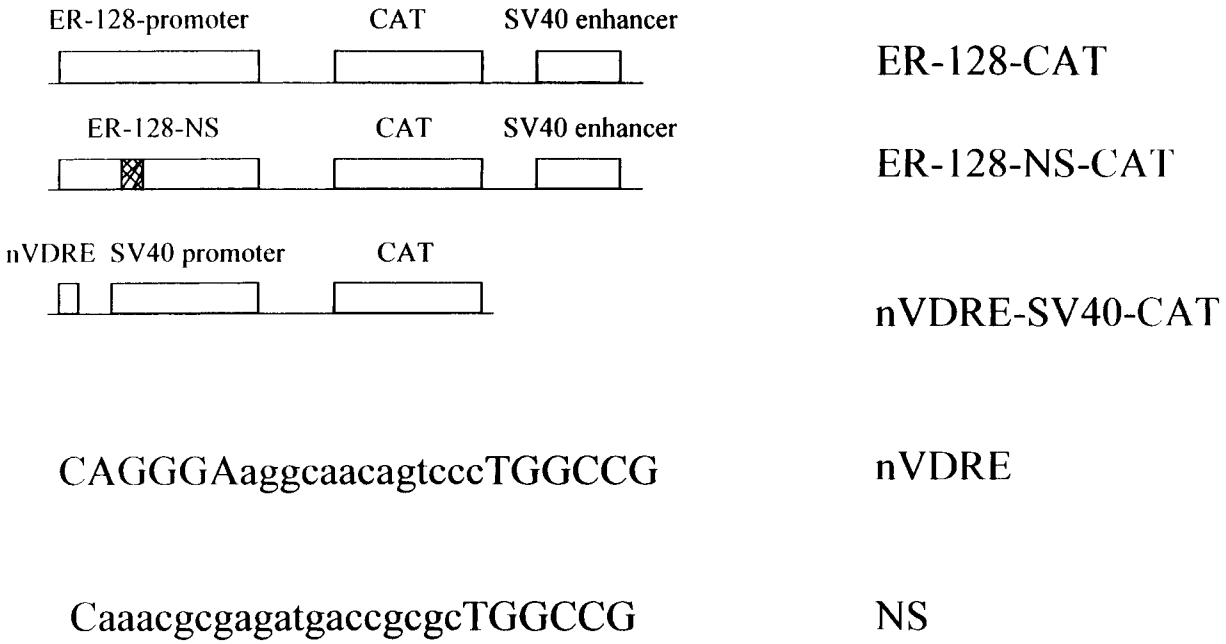

Fig. 5. Schematic diagram of chloramphenicol acetyltransferase (CAT) constructs.

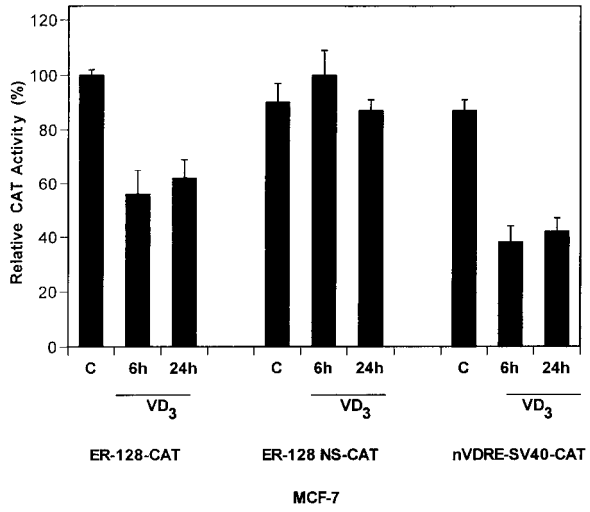

Fig. 6. Effect of VD_3 on the estrogen receptor (ER) promoter in MCF-7 cells. MCF-7 cells were transiently transfected with either the ER promoter-chloramphenicol acetyltransferase (CAT) vector, the nonsense ER promoter-CAT construct, or the nVDRE-SV40-CAT construct. After transfection, cells were treated with 10 nM VD_3 for 6 and 24 h. Cells were harvested and assayed for CAT activity as described under Materials and Methods. The results are expressed as percentage of ER-128-CAT in the absence of VD_3. The experiment was done in duplicate and repeated three times (±SD). C, control.

the region of CTF/NF1 site in the promoter region. It is located proximal to the CCAAT box and overlaps a potential NF1 site. To determine the role of this element in VD_3 regulation of ER gene transcription, two additional expression vectors were constructed. In the first vector, the putative response element was mutated to give a nonsense sequence (ER-128NS-CAT; Fig. 5). In this construct, the nucleotide composition of the response element was not altered, and the NF1 site was left intact. When the ER promoter containing the nonsense sequence was transfected into MCF-7 cells and the cells were treated with VD_3 there was no change in the amount of CAT activity (Fig. 6). In the second expression vector, the nVDRE was subcloned upstream of the SV40 promoter (nVDRE-SV40-CAT; Fig. 5). When the nVDRE-SV40-CAT vector was transfected into cells and the cells were treated with VD_3 there was an approximately 40% decrease in CAT activity ($P < 0.001$) (Fig. 6), suggesting that the negative VDRE functions as a repressor of a heterologous promoter.

To determine whether VD_3 inhibition of the ER promoter is dependent on the cell type, the promoter construct, ER-128-CAT, and a VDR expression vector were co-transfected into COS-1 and CV-1 cells. Cells were harvested 6 and 24 h after treatment with 10 nM VD_3 and assayed for CAT activity. The results are presented in Figure 7 as percentage of control. After VD_3 treatment, CAT activity increased about 4-fold in both the COS-1 and CV-1 cells. When the ER promoter containing the nonsense sequence was co-transfected with the VDR expression vector into COS-1 cells and treated

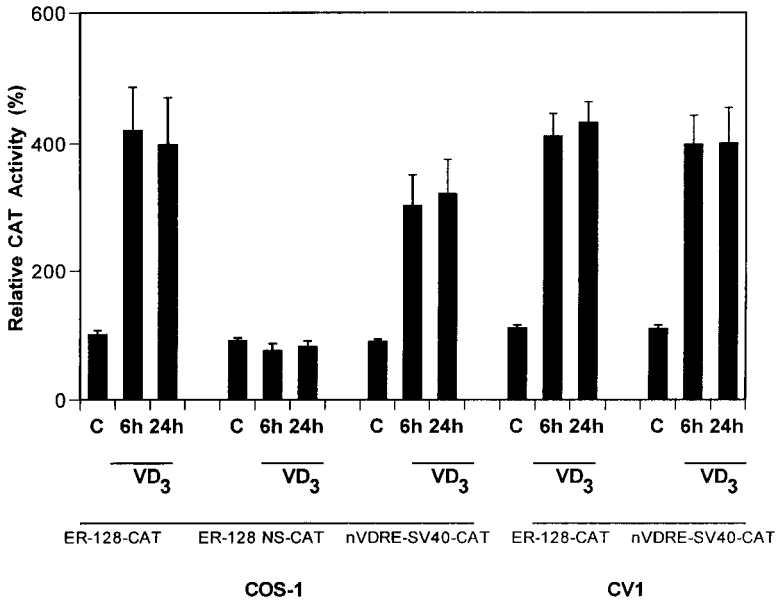

Fig. 7. Effect of VD_3 on the estrogen receptor (ER) promoter in COS-1 and CV-1 cells. The ER promoter-chloramphenicol acetyltransferase (CAT) vector, the nonsense ER promoter-CAT vector, and the nVDRE-SV40-CAT construct, together with a vitamin D expression vector, were transiently co-transfected into either COS-1 or CV-1 cells. The transfected cells were treated with 10 nM VD3 for 6 and 24 h, harvested, and assayed for CAT activity. The results are expressed as percentage of ER-128-CAT in the absence of VD_3. The experiment was done in duplicate and repeated three times (±SD). C, control.

with VD_3 there was no change in the amount of CAT activity (Fig. 7). When COS-1 and CV-1 cells were co-transfected with the nVDRE-SV40-CAT vector and the VDR expression vector and treated with VD_3, there was an approximately 4-fold increase in CAT activity. These data suggest that vitamin D inhibition of the ER promoter may be cell specific.

DISCUSSION

The purpose of this study was to define the mechanism by which VD_3 regulates the amount of ER. Therefore, the effects of VD_3 on the steady-state level of ER protein, mRNA, and gene transcription were examined. The results presented herein demonstrate that treatment with vitamin D resulted in a decrease in the steady-state level of ER protein and mRNA. The decline in protein and mRNA was due to a decrease in ER gene transcription as demonstrated by the nuclear run on assay. Analysis of the ER promoter suggested that the effects of vitamin D were mediated by a negative response element located at position −94 to −70 in the P1 promoter of the gene. The nVDRE is proximal to the CCAAT box and overlaps a potential NF-1 binding site. This negative element appears to function independent of the promoter and may function in a cell-specific manner.

When MCF-7 cells were treated with VD_3, there was a comparable decrease in the amount of ER protein and the number of estradiol binding sites. The concentration of ER, determined by the enzyme immunoassay, was approximately the same as the concentration of estradiol binding sites, measured by the ligand binding assay. In addition, treatment with VD_3 did not alter the binding affinity of estradiol for the ER or affect the activity of the receptor. Treatment of MCF-7 cells with VD_3 concentrations of 10–1,000 nM did not alter the amount of progesterone receptor in the cell and did not block the induction of progesterone receptor by estradiol (data not shown). The lack of effect of VD_3 on the affinity of estradiol for the ER is similar to the effect seen with transforming growth factor-β (TGF-β), another growth inhibitor of MCF-7 cells [Stoica et al., 1997], but is in sharp contrast to the effects seen with 12-O-tetradecanoyl-phorbol-13-acetate, which also inhibits the growth of breast cancer cells [Saceda et al., 1991]. TGF-β has no effect on the affinity of estradiol for its receptor, whereas, the phorbol ester blocks estradiol binding to the ER suggesting that growth inhibitors have different effects on the activity of the ER.

This report, showing a decline in ER protein after treatment of MCF-7 and Zr-75B cells with vitamin D, is in agreement with several earlier reports [James et al., 1994; Narvaez et al. 1997; Simboli-Campbel et al., 1997]. These studies showed a decrease in ER protein in MCF-7 cells after treatment with either 5 or 100 nM VD_3, using a ligand binding assay [James et al., 1994; Narvaez et al., 1997] or Western blot analysis [Simboli-Campbel et al., 1997], respectively. However, the mechanism of regulation was not investigated. Contrary to these find-

ings, vitamin D has been shown to have no effect on the amount of ER in MCF-7 [Demirpeuce et al., 1994], T47D [Davoodi et al., 1995] cells, in a yeast expression system [Santiso-Mere et al., 1993] and to increase the concentration of ER in a transfected ER negative cell line MDA-MB-321 [Davoodi et al., 1995] and in bone marrow-derived stromal cells [Bellido et al., 1993]. The reasons for the different results are unclear but may be due to different tissue culture conditions of the breast cancer cells and to differences between cell types similar to the differences between MCF-7 cells and the COS-1 and CV-1 cells seen here.

Transcriptional regulation of gene expression by vitamin D is mediated through sequence-specific interactions of the VD_3-VDR complex with DNA sites, termed vitamin D response elements (VDRE). Several VDREs that act as positive regulators have been identified and characterized in the promoters of the osteocalcin [Ozono et al., 1991; Demay et al., 1990], osteopontin [Noda et al., 1990], calbidin D-9K [Darwish et al., 1990], 24-hydroxylase [Hahn et al., 1994], and β3 integrin [Cao et al., 1993] genes. Less well characterized are the VDREs involved in negative regulation of gene expression. To date, negative VDREs have been described for the rat bone sialoprotein gene [Li et al., 1993] and the human and chicken parathyroid hormone (PTH) genes [Demay et al., 1992; Liu et al., 1996]. Alignment of the sequences of the VDREs provides a hexameric consensus core binding motif RRKNSA (R = A or G, K = G or T, S = C or G). The positive vitamin D response elements consist of direct repeats, palindromes, or inverted palindromes of the core motif on which VDR can function either as a homodimer or as a heterodimer with retinoid X receptor, retinoic acid receptor, thyroid receptor, or peroxisome proliferator activated receptor [Kahlen et al., 1994; Candeliere et al., 1996; Hsieh et al., 1995]. The negative VDREs also show a high degree of homology to the positive vitamin D response elements [Wurta et al., 1996]; for example, the human parathyroid hormone nVDRE contains a single copy of the hexameric consensus motif, and the chicken PTH nVDRE contains two imperfect direct repeats of the core sequence separated by 3 bp. The nVDRE in the ER promoter also contains two consensus core sequences, however, the consensus sequences are arranged as inverted repeats separated by 13 bp.

Several mechanisms have been proposed for negative regulation of transcription by steroid hormone receptors including competition for DNA binding of positive transcription factors, quenching, squelching, and direct repression. In the latter case, it has been suggested that binding to the negative response element causes a conformational change in the receptor which results in transcriptional repression instead of transcriptional activation. When the nVDRE from the ER promoter was tested in a heterologous cell type, it acted as a positive response element suggesting that binding of the VDR to the nVDRE did not induce a conformation change that converted the receptor from an activator to a repressor. A similar result was obtained with the nVDRE from the human parathyroid hormone receptor gene [Mackey et al., 1996]. The human PTH nVDRE mediates transcriptional repression in response to VD_3 in bovine pituitary (GH4C1) cells but not in ROS 17/2.8 cells [Mackey et al., 1996]. Transcriptional repression by VD_3 also does not appear to be due to squelching. When the nVDRE from the ER gene was mutated to a nonsense sequence, transcriptional repression was not observed after hormone treatment, suggesting that the VDR did not squelch the activity of other transcription factors. Binding of the VDR to the nVDRE also appears to be required for repression.

The requirement of other cellular factors for transcriptional repression has also been described for genes regulated by steroid hormone receptors. In some cases, transcriptional repression is caused by interference with the binding or activity of positive transcription factors. In other cases, repression is attributable to the presence of a co-repressor. The nVDRE in the ER promoter overlaps a potential NF-1 binding site raising the possibility that binding of the VDR to the nVDRE represses transcription of the ER gene by an interference mechanism. However, several lines of evidence suggest that this is not the mechanism. When the nVDRE was mutated to a nonsense sequence, there was no decrease in the basal activity of the promoter suggesting that the nVDRE does not contain an enhancer binding site. More importantly, when placed upstream of a heterologous promoter, the nVDRE acted as a negative response element, suggesting that it functioned independent of its position in the promoter. Although these data provide evidence that tran-

scriptional repression is not due to interference, it is still possible that binding of the VDR to the nVDRE also blocks the binding or activity, or both, of NF-1. However, it is unlikely that repression is due solely to interference with NF-1, since the nVDRE acts as a repressor of the SV40 promoter. Alternatively, repression may be attributable to the presence of a co-repressor. The vitamin D receptor is believed to repress transcription of the human parathyroid hormone gene by interacting with a cell specific factor and the nVDRE [Deway et al., 1992; Mackey et al., 1996]. It is thought that the vitamin D receptor binds to the negative response element as a heterodimer with a cell specific factor. The cell-specific factor is present in cells that repress parathyroid hormone transcription and is absent in cells that increase PTH in response to vitamin D. Data from this study suggest that VDR may also interact with a cell specific factor in breast cancer cells to inhibit transcription of the ER gene. In MCF-7 cells, the nVDRE functioned as a negative element in response to VD_3 treatment, whereas, in co-transfection assays in either CV-1 or COS-1 cells, the nVRE acted as a positive response element suggesting that the breast cancer cells contain an additional cellular factor necessary for transcriptional repression.

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