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Localization of a Negative Vitamin D Response Sequence in the Human Growth Hormone Gene

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1,25-Dihydroxyvitamin D_3 [1,25$(OH)_2$ D_3] exerts its biological effects by binding to the vitamin D receptor (VDR), which binds in turn to the vitamin D response elements located in the target gene's promoter. We have previously demonstrated that VDR binds *in vitro* with high affinity to the 5′-flanking sequence of the human growth hormone (hGH) gene. In this study, we analyzed the response to 1,25$(OH)_2$ D_3 of hGH-promoter constructs introduced by transfection into the MCF-7 human adenocarcinoma cell line. We found that the transcriptional activity of some of these constructs was markedly reduced by 1,25$(OH)_2$ D_3. Deletion analyses revealed that a 34-bp sequence located between positions −62 and −29 upstream of the transcription start site is sufficient for this repressive response. This conclusion was also confirmed by gel mobility shift assays. Our results indicate that vitamin D inhibits hGH gene transcription, directly or by interference with other transcription factors.

Key Words: **hGH; VDR; vitamin D; MCF-7; 1,25$(OH)_2$ D_3.**

The vitamin D receptor (VDR) belongs to the family of ligand-inducible nuclear receptors that mediate transcriptional regulation by binding to DNA sequences, termed vitamin D response elements (VDRE) (1, 2, 3). 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2$ D_3], the most active metabolite of vitamin D, can also induce biological responses via non-genomic pathways, involving protein kinase A and C signal transduction pathways and pathways based on Ca^{2+} channel regulation (4, 5).

The classical role of 1,25$(OH)_2$ D_3 is to stimulate transmembrane calcium transport in the intestine, bone and kidney. Moreover, specific nuclear receptors for 1,25$(OH)_2$ D_3 have been detected in several tissues, suggesting other functions beyond bone metabolism and mineral homeostasis (6, 7). Thus, VDR expression has been demonstrated in both the human pituitary and mammary glands (8, 9). Vitamin D enhances PRL synthesis and secretion in rat pituitary cells (10, 11) and reduces the transcription of growth hormone in these cells in response to T3 and retinoic acid (RA), by interfering with the binding of the T3 and RA receptors to the corresponding elements on the GH promoter (12). No data are available on the regulation of GH by vitamin D in the human mammary gland, although GH mRNA and GH receptors have been found in normal and neoplastic human breast (13, 14), and the MCF-7 human breast adenocarcinoma cell line expresses GH (15). Given the current lack of a human pituitary cell line for laboratory use, the presence of VDR, GH and Pit-1 in the MCF-7 cell line suggests that this cell line may be a useful model for studying the effects of 1,25$(OH)_2$ D_3 on human GH (hGH).

In a previous study, we found two high-affinity binding sites for the vitamin D receptor in the hGH gene promoter (16). In the present study, we evaluated the effect of 1,25$(OH)_2$ D_3 treatment on the transcriptional activity of hGH promoter constructs in the MCF-7 strain of the human breast adenocarcinoma cell line, and used gel mobility shift assays to localize a sequence that is required for this effect.

MATERIALS AND METHODS

Cell culture and transfection. MCF-7 human breast adenocarcinoma cells were obtained from the European Collection of Cell Cultures (Salisbury, U.K.). Stock culture was grown in 90-mm petri dishes in DMEM supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 2 mM L-glutamine in a 95:5 air-CO_2 atmosphere at 37°C. 12–24 h before transfection, the cells were cultured in DMEM containing 10% charcoal-stripped fetal calf serum. 75×10^3 cells per well were seeded in 6-well dishes and allowed to attach to these plates overnight. Transfections were carried out in wells containing 0.3 μl of Fugene (Roche Molecular Biochemicals, IN, USA) and 200 ng of total DNA, 100 ng of the different hGH promoter-luc constructs, and 100 ng Rous sarcoma virus β-galactosidase (pRSV-β-gal). Cells were incubated for 48 h with 100 nM of crystalline 1,25$(OH)_2$ D_3 diluted in ethanol in hormone-depleted medium. Each experiment was performed in triplicate cultures. The cells were

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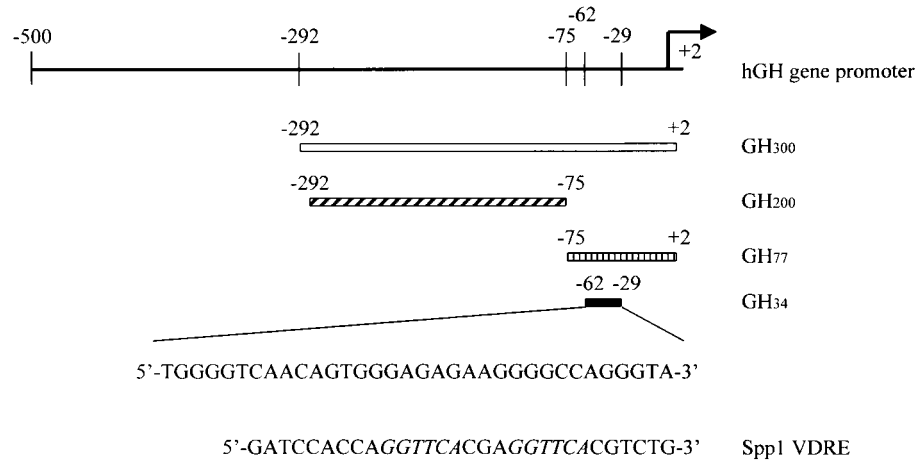

FIG. 1. Human GH fragments and oligonucleotides used in the gel mobility shift and transfection assays. For the gel mobility shift assay, the hGH fragments were obtained from the pUC8-hGH plasmid by digestion with *Bal*I/*Bam*HI (GH_{300}), *Bal*I/*Rsa*I (GH_{200}), or *Rsa*I/*Bam*HI (GH_{77}). The GH_{34} and Spp1 VDRE oligonucleotides were purchased from Amersham Pharmacia Biotech. For the transfection assay the GH_{200} fragment was obtained by PCR using pUC8-hGH as template, with *Kpn*I and *Bgl*II sites introduced at each end of the PCR product.

harvested in lysis buffer (25 mM Tris-PO_4 pH 7.8; 15% glycerol; 2% CHAPS; 1% lecithin; 4 mM EGTA; 8 mM $MgCl_2$, 1 mM DTT; 0.4 mM PMSF and 1% BSA), and luciferase activity was then measured using a luminometer. β-galactosidase activity was measured at λ_{420} nm using *o*-nitrophenyl-β-D-galactopyranoside as substrate.

Plasmid construction. The different fragments of the 5′ flanking region of the hGH gene were obtained from the pUC8-hGH plasmid and fused to the pGL3-Basic or pGL3-Promoter vector (Promega). Both vectors contain the luc + gene. Expression of luciferase in MCF-7 cells transfected with the pGL3-Basic vector depends on the insertion of a promoter sequence upstream of luc +. Expression of luciferase in cells transfected with the pGL3-Promoter vector does not depend on insertion of a promoter, and can be enhanced by insertion of an enhancer. Briefly, the pUC8-hGH plasmid was digested with *Bal*I and *Bam*HI, or with *Rsa*I and *Bam*HI, to obtain the GH_{300} fragment (−292 to +2) and the GH_{77} fragment (−75 to +2), respectively. Both the GH_{300} and GH_{77} fragments were inserted into the *Sma*I/*Bgl*II promoter-insertion site of the pGL3-Basic vector. The GH_{200} fragment (−292 to −75) was obtained by PCR using the following oligonucleotides: a) forward primer: 5′-GGGGTACCTGGCCACCATGGCCTGCGGC-3′ and b) reverse primer: 5′-GAAGATCTCCCACCTGTTTCTGTGCTAC-3′, with pUC8-hGH as template. *Kpn*I and *Bgl*II sites were introduced at each end of the PCR product, and the fragment was inserted into the *Kpn*I/*Bgl*II promoter-insertion site of the pGL3-Basic vector. The GH_{34} fragment (−62 to −29; 5′-TGGGGTCAACAGTGGGAGAGAAGGGGCCAGGGTA-3′) was obtained by synthesis, and *Sac*I and *Bgl*II sites were introduced at each end; the oligonucleotide was then inserted into the *Sac*I/*Bgl*II enhancer-insertion site of the pGL3-Promoter vector. The nucleotide sequences of all the cloned inserts were confirmed by sequencing. As positive transfection control we used the pGL2-$(Spp1)_2$ construct containing two copies of the mouse osteopontin/Spp1 VDRE (17).

Overexpression and purification of GST–hVDR fusion protein and gel mobility shift assays. The GST-hVDR fusion protein was overexpressed as described previously (18). Briefly, 500 ml of bacterial culture expressing the recombinant GST fusion protein was grown at 37°C to an OD_{600} of 0.3, at which time the temperature was reduced to 20°C. Cells were induced by the addition of 0.1 mM isopropyl-B-D-thiogalactopyranoside at OD_{600} 0.6. After 3.5 h, bacteria were collected by centrifugation and resuspended in 5 ml of lysis buffer (PBS containing 0.5 mM PMSF, 0.5 mg/ml leupeptin, and 1 mM DTT), sonicated, and centrifuged. Soluble extracts were incubated with glutathion-sepharose matrix (Amersham Pharmacia Biotech) for 30 min at 4°C before washing three times in lysis buffer. The amount of protein immobilized on beads was estimated by SDS–PAGE with Coomassie blue staining, by comparison with a titration of bovine serum albumin.

Gel mobility shift assays were performed as previously described (17, 18). The DNA probes (GH_{300}, GH_{200}, and GH_{77}) were isolated from pUC8-hGH by digesting the plasmid with a) *Bal*I and *Bam*HI, b) *Bal*I and *Rsa*I, or c) *Rsa*I and *Bam*HI, respectively, then gel-purifying the fragments and end-labeling with γ-[^{32}P]ATP using T4 polynucleotide kinase. The Spp-1/osteopontin vitamin D response element (5′-GATCCACAA*GGTTCA*CGA*GGTTCA*CGTCTG-3′) and the GH_{34} oligonucleotide (5′-TGGGGTCAACAGTGGGAGAGAAGGGGCCAGGGTA-3′) (purchased from Amersham Pharmacia Biotech) were also end-labelled with γ-[^{32}P]ATP using T4 polynucleotide kinase, and then purified through a MicroSpin G-25 column (see Fig. 1). For each gel mobility shift assay, 20,000 cpm of probe (10 fmol) was mixed with increasing amounts of bacterially expressed GST-VDR for 45 min at room temperature in a buffer consisting of 20 nM Tris (pH 7.9), 1 mM EDTA (pH 7.9), 10% glycerol, 0.05% NP-40, 50 mM KCl, 1 mM DTT, and 1 μg poly(dI:dC). In the supershift experiments, 1 μl of anti-VDR antibody (Chemicon International, CA, USA) was added to 200 ng of GST-VDR, incubated for 15 min and then incubated with 20,000 cpm of probe for 30 min. Half of the reaction mixture (10 μl) was electrophoresed at 15 V/cm at 4°C on 5% polyacrylamide gels. After electrophoresis, gels were dried and exposed to x-ray film.

Statistical analysis. Means were compared by one-way analysis of variance with the Tukey-Kramer multiple comparison test for post-hoc comparisons. Statistical significance is taken to be indicated by $P < 0.05$.

RESULTS

Effect of 1,25(OH)$_2$ D$_3$ Administration on the Transcriptional Activity of the hGH Promoter

To investigate the effects of vitamin D on the transcriptional activity of the hGH promoter, MCF-7 human breast adenocarcinoma cells were transfected with constructs linking 300, 77, or 200 base pairs of the hGH gene promoter to a luciferase reporter vector

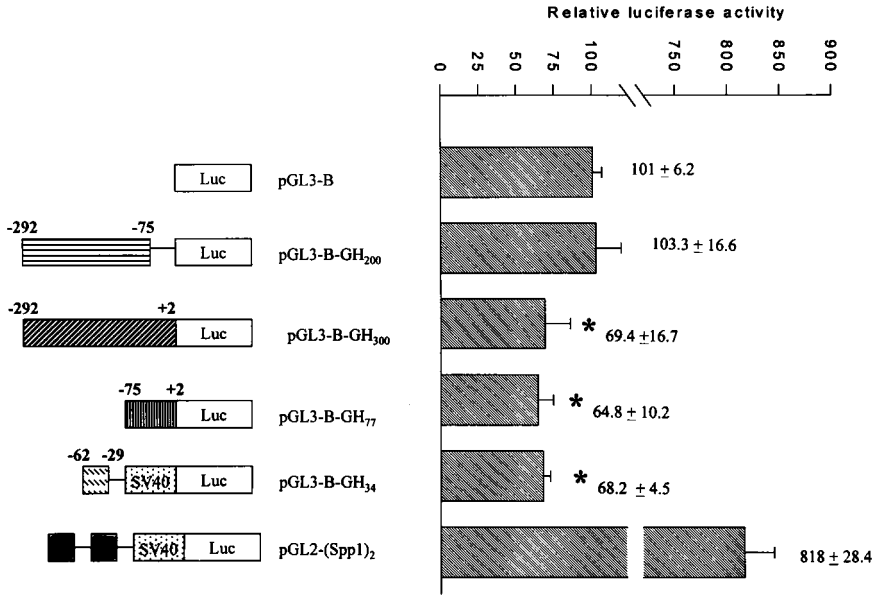

FIG. 2. Deletion analysis identified a 1,25(OH)$_2$ D$_3$-responsive region in the hGH gene promoter. The hGH fragments obtained from the pUC8-hGH plasmid were fused to the pGL3-Basic vector to obtain pGL3-B-GH$_{300}$ (−292/+2), pGL3-B-GH$_{200}$ (−292/−75), and pGL3-B-GH$_{77}$ (−75/+2). The GH$_{34}$ oligonucleotide was inserted into the pGL3-Promoter vector to obtain pGL3-P-GH$_{34}$. pGL2-(Spp1)$_2$, containing two copies of Spp1/osteopontin VDRE, was used as positive control. These constructs were transfected into MCF-7 cells using Fugene reagent, and the cells were then cultured in the presence of 100 nM 1,25(OH)$_2$D$_3$ or ethanol for 48 h in hormone-depleted medium. For each construct at least 3 experiments were performed, and in each of these experiments the control and vitamin D treatments were performed in triplicate. Relative luciferase activity was calculated as the ratio of luciferase activity in vitamin-D-treated cells to that in the corresponding control cells, and is indicated on the right side of each column (mean ± S.D.). Asterisks indicate significant differences ($P < 0.05$) with respect to relative luciferase activity calculated for cells transfected with pGL3-Basic.

(pGL3-Basic vector). After 24 h, 100 nM of 1,25(OH)$_2$ D$_3$ was added to the culture medium. The incubation was continued and cells were harvested 48 h later for measurement of reporter activity. As shown in Fig. 2, vitamin D treatment of cells transfected with the control plasmid (pGL3-Basic) had negligible effect on reporter activity, while vitamin D treatment of cells transfected with pGL3-B-GH$_{300}$ led to a statistically significant 30% reduction in reporter activity. Similarly, vitamin D treatment of cells transfected with pGL3-B-GH$_{77}$ led to a statistically significant 35% reduction in reporter activity (Fig. 2). However, vitamin D treatment of cells transfected with pGL3-B-GH$_{200}$ had no significant effect (Fig. 2). As positive control we used the pGL2-(Spp1)$_2$ construct, which contains two Spp1/mouse osteopontin VDRE sites. Vitamin D treatment of cells transfected with this construct led to an 8-fold increase in reporter activity (Fig. 2).

Gel Mobility Shift Assays Indicate Specific Binding of GST–VDR to GH$_{300}$ and GH$_{77}$ but Not to GH$_{200}$

In order to investigate VDR binding to the different hGH fragments, we used gel mobility shift assays to characterize the binding of GST-VDR to Spp-1 VDRE (used as positive control) and to GH$_{77}$, GH$_{300}$, and GH$_{200}$. It is well known that purified GST-VDR binds strongly to a VDRE derived from the Spp-1 gene (17). This target is composed of a direct repeat of the half-site 5′-GGTTCA-3′ with a 3-bp separation. As expected, purified recombinant GST-VDR bound to the Spp-1 VDRE.

Our results also indicate (Fig. 3) binding of GST-VDR to both the GH$_{77}$ and GH$_{300}$ fragments, with a similar binding pattern to that for Spp-1 VDRE. In contrast, GST-VDR did not bind to the GH$_{200}$ fragment (data not shown). In order to demonstrate that the protein binding to Spp-1 VDRE and the GH fragments was GST-VDR, we preincubated the GST-VDR with an anti-VDR antibody: as shown in Fig. 3, this treatment supershifted the Spp1 VDRE, GH$_{300}$, and GH$_{77}$ fragments in gel mobility shift assays.

Localization of the Suppressive Vitamin D Response Element in the −62 bp to −29 bp Region of the hGH Promoter

To locate the putative VDRE responsible for the observed suppression by vitamin D of the transcriptional activity of the hGH gene promoter, a 34-bp oligonucleotide (GH$_{34}$) corresponding to positions −62 to −29 bp of the hGH gene promoter was synthesized and used in both transfection and gel mobility shift assays (Fig. 4). Vitamin D treatment of cells transfected with pGL3-P-GH$_{34}$ led to a statistically significant 30% reduction in reporter activity (Fig. 2). Moreover, GST-hVDR was

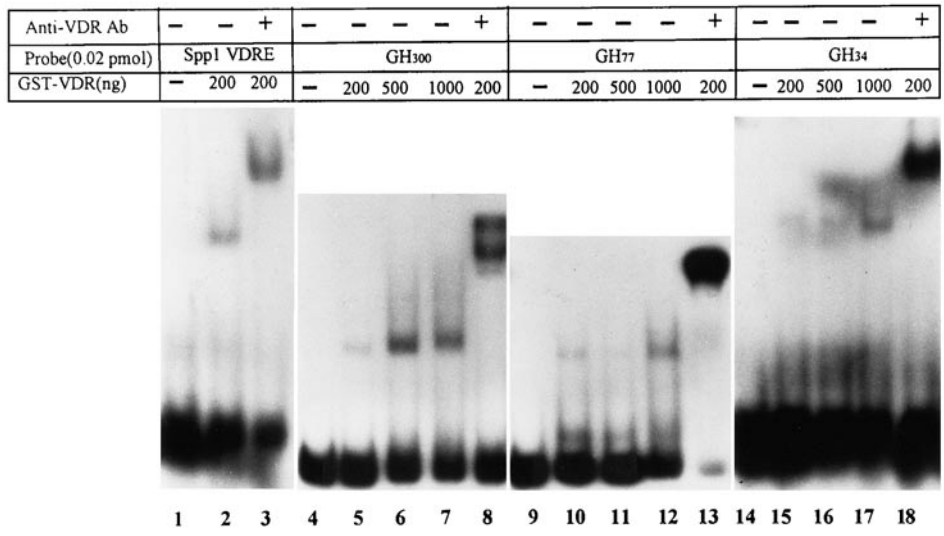

FIG. 3. GST–VDR binds to the hGH promoter within a region located 29 to 62 bp upstream the transcription start site. The figure shows the gel mobility shift profiles of GST–VDR bound to the different hGH fragments and the Spp1 VDRE control. Each line contains 20,000 cpm of labelled DNA fragment. 200 ng (lanes 2, 5, 10, and 15), 500 ng (lanes 6, 11, and 16), or 1 μg (lanes 7, 12, and 17) of GST–VDR were added to the different probes. For supershift assays, 200 ng of GST–VDR was preincubated with 1 μl of anti-VDR antiserum (lanes 3, 8, 13, and 18).

found to bind to radiolabeled GH_{34} (Fig. 3). After preincubation of the GST-VDR with anti-VDR antibody, the band observed in the gel mobility shift assay was supershifted, confirming that the ligand was VDR.

DISCUSSION

In this work, we found that $1,25(OH)_2$ D_3 exerted a suppressive effect on the human growth hormone gene, through binding of the vitamin D receptor to a sequence located between positions −62 and −29 bp with respect to the transcription start site.

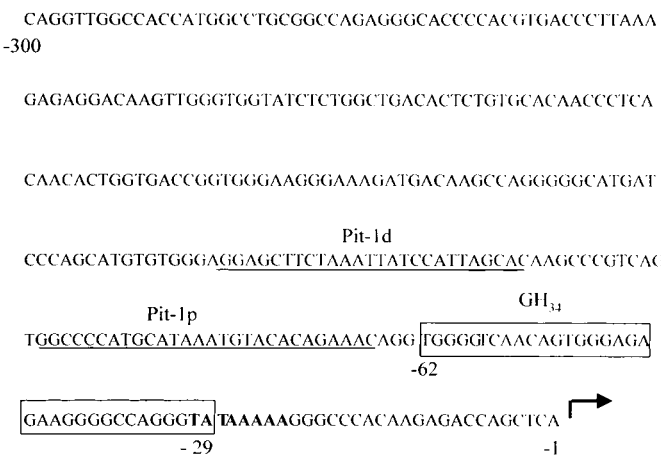

FIG. 4. Location of the suppressive vitamin D response sequence in the human GH promoter. The GH_{34} fragment (boxed region) is located between positions −62 and −29 bp from the hGH gene transcription start site. It is situated near the Pit-1 proximal response element (underlined) and overlapping the TATA box (in bold).

VDR mediates transcriptional activation in response to $1,25(OH)_2$ D_3 by binding to specific DNA sequences (VDREs), predominantly as a heterodimeric complex with a common partner, the retinoid X receptor (RXR). VDREs are typically composed of two hexameric half-sites organized as direct repeats, spaced by three nucleotides (the DR+3 rule) (19). Heterodimeric complexes with RXR can presumably interact with the preinitiation complex directly or indirectly through any of various coactivators (3, 20). However, some response elements for VDR and other receptors do not follow the DR+3 rule, and VDR can also regulate gene expression by binding in different configurations on palindromic or composite VDREs (21, 22).

By contrast, the mechanisms mediating transrepression by $1,25(OH)_2$ D_3 are poorly understood. Several genes have been identified as targets of negative vitamin D regulation, including hPTH (23), human atrial natriuretic peptide (24), rat bone sialoprotein gene (25) murine Id1 gene (26), and granulocyte-macrophage colony-stimulating factor gene (27). Although some sequences resembling the DR+3 element have been identified in these vitamin-D-responsive genes, other promoters do not contain such elements. Moreover, whereas VDR binds to and activates transcription from DR+3 as a VDR-RXR heterodimer, RXR is completely absent from the binding process, in at least some negative elements (27). The transcriptional repression can thus be explained through interaction of VDR with other regulatory transcriptional factors or with key components of the basal transcriptional machinery.

In this work, using different hGH fragments and oligonucleotides linking to a luciferase reporter vector,

we studied the transcriptional response to 1,25(OH)$_2$ D$_3$. We found that constructs containing a region of the hGH promoter located between positions −62/−29 in the proximal 5′-flanking region (GH$_{34}$) are sufficient for transcriptional repression in response to 1,25(OH)$_2$ D$_3$. Gel mobility shift assays confirmed that the full-length hVDR fused to GST binds to GH$_{34}$. These data are in line with previous results obtained by our group using the DNA-binding domain of the human vitamin D receptor (VDRF): specifically, we used gel mobility shift and foot-printing assays to demonstrate that VDRF binds with high affinity to GH$_{34}$ (16).

As mentioned above, one mechanism involved in the inhibition of hGH gene transcription could be the interaction of VDR with key components of the basal transcriptional machinery. Kim *et al.* (25) have identified a VDRE in the rat bone sialoprotein promoter that seems to suppress gene transcription through mechanisms that involve competition of VDR with TATA-binding protein (TBP) when binding to the TATA box region of the promoter. According to these data, the high levels of 1,25(OH)$_2$ D$_3$-dependent reporter activity in NIH 3T3 cells transfected with hVDR expression vector are significantly repressed by cotransfecting the hTFIIB transcription factor in a dose-dependent fashion, suggesting that an excess of one transcription factor sequesters another factor, reducing the formation of an active transcription complex (28). The interaction between human TATA-binding protein-associated factor 28 (hTAF$_{II}$28) and VDR has also been studied by Mengus *et al.* (29), who suggest that interactions between VDR and the basal transcription apparatus may contribute to transcriptional repression. The GH$_{34}$ sequence considered in the present study overlaps with the TATA box, and this could explain the repressive effects of VDR binding to the hGH gene promoter.

Vitamin D may also inhibit the transcriptional response of the hGH gene through interaction of VDR with other regulatory transcriptional factors. It is well known that the pituitary-specific transcription factor GHF1/Pit-1 plays a key role in pituitary GH gene expression. The synergistic regulation of the rat GH gene involves protein-DNA interactions, as well as physical association between nuclear receptors (RAR, RXR, and TR) and GHF-1/Pit-1. Thus, it has been shown that vitamin D interferes with growth hormone gene expression induced by T3 and retinoic acid in the rat GH$_4$C$_1$ pituitary cell line, probably through interference by VDR with the binding of the thyroid receptor or RA receptor to a common hormone response element (13). This repressive role of VDR is also observed in the retinoic acid-dependent activation of the retinoic acid receptor-β2 promoter (30). In our case, the VDRE located on the hGH promoter is only 3-bp downstream of the Pit-1 proximal site. For this reason, it is tempting to speculate that VDR binding to the hGH gene may also interfere with the binding of Pit-1 to its GH response element. This hypothesis is supported by the fact that MCF-7 cells express the Pit-1 transcription factor, as has recently been demonstrated by our group (15).

In conclusion, our results indicate that vitamin D inhibits human GH gene transcription in the MCF-7 strain of human breast adenocarcinoma cells. The 1,25(OH)$_2$ D$_3$ responsive region in the hGH gene is located between −62 and −29 bp upstream of the transcription start site. This region overlaps with the TATA box and is situated near the Pit-1 proximal response element site. Although our data do not demonstrate that the vitamin D receptor interferes with transcriptional factors to inhibit induction of transcription induction, this hypothesis should not be excluded.

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