Identification and Characterization of 1,25-Dihydroxyvitamin D₃-responsive Repressor Sequences in the Rat Parathyroid Hormone-related Peptide Gene*

(Received for publication, December 29, 1995, and in revised form, April 3, 1996)

Richard Kremer‡, Michael Sebag, Céline Champigny, Karen Meerovitch, Geoffrey N. Hendy, John White, and David Goltzman

From the Departments of Medicine and Physiology, McGill University, Montreal, Quebec H3A 1A1, Canada and the Calcium Research Laboratory, Royal Victoria Hospital, Montreal, Quebec H3A 1A1, Canada

Parathyroid hormone-related peptide (PTHRP) gene transcription is suppressed by 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃), the active metabolite of vitamin D₃. In the present report, we examined 1,25(OH)₂D₃-mediated repression of PTHRP expression by transfection of PTHRP promoter/reporter constructs in normal human keratinocytes and by DNA binding. We localized an element conferring 1,25(OH)₂D₃-mediated repression in vivo to a 47-base pair (bp) region located –1121 to –1075 from the transcriptional start site. Mobility shift analysis revealed that this vitamin D response element (VDRE) forms DNA-protein complexes. The addition of a monoclonal antibody that recognizes the DNA binding region of the vitamin D receptor (VDR) attenuated binding of the receptor to the 47-bp sequence, whereas the addition of monoclonal antibody raised against the retinoid X receptor (RXR) further retarded the mobility of the protein-DNA complex. Consequently, the PTHPR promoter element binds a VDR-RXR heterodimer. Examination of this VDRE revealed complete sequence homology with a half-site of the human and rat osteocalcin VDRE (GGGTTA). Furthermore, mutation analysis suggests that a 16-bp domain consisting of an almost perfect repeat separated by a 3-base pair "spacer" GGGTGAGAGGGGTGA is responsible for the DNA-protein interaction within this 47-bp sequence. Our results therefore indicate the existence of an inhibitory VDRE within the PTHPR promoter that is similar in sequence composition and cellular factor requirement to classical up-regulatory VDREs.

1,25-Dihydroxyvitamin D₃ not only plays a major role in regulating calcium homeostasis, but it also has antiproliferative and differentiating effects in many types of cells (1, 2). The keratinocyte serves as a paradigm for autocrine/paracrine regulation inasmuch as it has the capacity to manufacture a number of factors that can then modulate its function through their respective receptors. Among these factors are 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) (3) and parathyroid hormone-related peptide (PTHRP) (4, 5). PTHRP is therefore produced by normal cells such as keratinocytes, in which it is thought to regulate growth and differentiation (6), as well as by cancer cells responsible for malignancy-associated hypercalcemia (7-9). We have previously shown that 1,25(OH)₂D₃ down-regulates PTHRP gene transcription (5, 10) and that this effect is likely to involve vitamin D-responsive elements (VDREs) in the 5′ promoter region of the gene. In addition, we demonstrated in the same system that mitogenic stimuli positively influence PTHRP gene transcription (5, 10). To address the questions of whether the regulation of PTHRP gene transcription in response to 1,25(OH)₂D₃ and mitogenic stimuli is mediated by upstream regulatory sequences in the promoter region of the PTHRP gene, we previously conducted transfection experiments in normal human keratinocytes (5) and demonstrated that these effects were mediated by a 1200-base pair (bp) sequence of the 5′-flanking region of the PTHRP gene. In the study presented here, we used functional promoter assays and gel retardation assays to map the regulatory components essential for down-regulation by 1,25(OH)₂D₃ and up-regulation by mitogenic stimuli. We found that transcriprional inhibition and activation require an 80-bp sequence that confers responsiveness to 1,25(OH)₂D₃ and mitogenic stimuli when fused to a heterologous promoter and that a distinct 47-bp sequence within the 80 bp confers responsiveness to 1,25(OH)₂D₃ and binds to a 1,25(OH)₂D₃ receptor (VDR)-retinoic acid X receptor (RXR) complex.

MATERIALS AND METHODS
DNA Constructs

Deletion Constructs—Restriction fragments HF and NF, encoding 1951 bp and 300 bp, respectively, of the rat PTHPR promoter region upstream of the transcription initiation site and +22 bp of the first exon were prepared and cloned into the plasmid pOgh (11) upstream of the reporter human growth hormone gene as described previously (5). Construct HF was used to create unidirectional (5'→3') deletions using exonuclease III. After digestion with HindIII, the 5' end was filled in with thiotriphosphate dNTPs to prevent 3'→5' exonuclease digestion at the 3' end of the enzymatic cut. Subsequently, digestion immediately downstream of the 5' end of the enzymatic cut was performed with XbaI, and the linearized plasmid was dissolved in exonuclease III buffer (15 mM Tris HCl, pH 8.5, 0.6 mM MgCl₂) and digested according to the manufacturer's specifications (Pharmacia Biotech Inc.) with 250–500 units of exonuclease III at room temperature. Aliquots were removed at 60-s intervals into tubes containing S1 nuclease in S1 buffer (16 mM sodium acetate, pH 4.6, 400 mM NaCl, 1.6 mM ZnSO₄, and 8% glycerol) and incubated at room temperature for 30 min. The reaction was then terminated by adding S1 stop buffer (0.8 mM Tris-HCl, pH 8.6, 10 mM NaCl, 0.1 mM EDTA, 8% glycerol) to a final concentration of 100 mM MgCl₂ and 200 units of S1 nuclease. Each restriction fragment was then digested with HindIII, XbaI, and the linearized plasmid was dissolved in exonuclease III buffer (15 mM Tris HCl, pH 8.5, 0.6 mM MgCl₂) and digested according to the manufacturer's specifications (Pharmacia Biotech Inc.) with 250–500 units of exonuclease III at room temperature. Aliquots were removed at 60-s intervals into tubes containing S1 nuclease in S1 buffer (16 mM sodium acetate, pH 4.6, 400 mM NaCl, 1.6 mM ZnSO₄, and 8% glycerol) and incubated at room temperature for 30 min. The reaction was then terminated by adding S1 stop buffer (0.8 mM Tris-HCl, pH 8.6, 10 mM NaCl, 0.1 mM EDTA, 8% glycerol) to a final concentration of 100 mM MgCl₂ and 200 units of S1 nuclease.

*This work was supported by Medical Research Council of Canada Grants MT-10839, MT-9315, and MT-5775 and by a grant from the National Cancer Institute of Canada. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

†To whom correspondence should be addressed: Calcium Research Laboratory, Royal Victoria Hospital, Room H4.67, 687 Pine Ave. West, Montreal, QC H3A 1A1 Canada.

The abbreviations used are: 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; PTH, parathyroid hormone; PTHRP, PTH-related peptide; VDRE, vitamin D response element; bp, base pair(s); VDR, vitamin D receptor; RXR, retinoid X receptor; GH, growth hormone; EGF, epidermal growth factor; FBS, fetal bovine serum; GH, growth hormone; OP, osteopontin; OP-mut, mutant of osteopontin; NHK, normal human keratinocyte.
EDTA, 80 mM MgCl₂). A fill-in reaction was then performed using 1 μl of Klenow enzyme mix and incubated for 15 min at 37 °C. The plasmids were then ligated as described previously using T₄ DNA ligase (12). Several deletion constructs were obtained, and those beginning at −1491, −1161, −840, −751, and −481 bp upstream of the transcription initiation site were chosen for further study (see Fig. 1A).

Polymerase Chain Reaction Constructs in TKGH Plasmids—A series of fragments spanning nucleotides −1161 to −690 were obtained by polymerase chain reaction: C1 (−1161 to −1079), C2 (−1047 to −944), and C3 (−1047 to −690). They were inserted into a blunt-ended HindIII site upstream of the thymidine kinase (TK) promoter of plasmid TKGH as described previously (13). In a similar manner, several smaller site upstream of the thymidine kinase (TK) promoter of plasmid TKGH were also made using complementary synthetic oligonucleotides.

All constructs were verified by restriction mapping and DNA sequencing.

Cell Culture and Transfection

Normal human keratinocytes (NHKs) were grown and maintained as described previously (14). Cells were grown to 60% confluency in T25 flasks in keratinocyte basal medium (KBM, Clonetics Corp., San Diego, CA) supplemented with 0.1 ng/ml of human recombinant epidermal growth factor (EGF) and 0.15 mM Ca²⁺. The cells were transfected with plasmid DNA using the cationic lipid, N-[1-(2,3-dioleyloxy)propyl]-N,N,N-trimethylammonium chloride (15) (Lipofectin reagent, Life Technologies, Inc.), according to the manufacturer’s specifications using minor modifications. Fifty μg of Lipofectin reagent and 25 μg of plasmid DNA (20 μg of PTHRP plasmid and 5 μg of β-galactosidase) were mixed in polystyrene tubes in a total volume of 180 μl, allowed to stand for 15 min at room temperature, and then added dropwise to fresh keratinocyte culture medium (3 ml of KBM containing 0.1 ng/ml of EGF, in a T25 flask). After 20–22 h of incubation at 37 °C, the medium was removed and replaced with medium containing various combinations of fetal bovine serum (FBS), EGF, and 1,25(OH)₂D₃.

Medium samples were collected 72 h after transfection, and growth hormone (GH) production was measured using a two-site immunoradiometric assay (Nichols Institute, San Juan Capistrano, CA). The limit of detection of the assay is 100 pg/ml, and background values with medium alone or with medium from cells transfected with control plasmid pOGH were less than 150 pg/ml. To control for transfection efficiency, results were normalized to β-galactosidase values and expressed as picograms of GH/unit of β-galactosidase. β-galactosidase measurements were performed on NHK cellular extracts using a colorimetric assay as described previously (12).

Fig. 1. Transient expression of deletion mutants of the rat PTHRP promoter in normal human keratinocytes and detection of promoter activity by growth hormone assay in conditioned media. A, boundaries of the deletion mutants created from the rat PTHRP promoter and was then transfected into normal human keratinocytes and incubated in basal medium in 0.15 mM Ca²⁺ with (open bar) or without (dark bar) EGF as described under “Materials and Methods.” The results shown are the mean ± S.E. of six determinations. The asterisk indicates a significant difference between EGF-treated and nontreated cells (*, p < 0.01). The results are representative of four separate experiments.

Cell Retardation Assays

Nuclear extracts were prepared from NHKs as described previously (17). Cellular extracts were also prepared from COS-1 cells at 30–50% confluence in 100-mm² culture plates. These cells had been transiently transfected with 10 μg of plasmid DNAs expressing the human RXRa and the human VDR (kind gifts of Drs. R. Evans and M. Haussler, respectively) using the lipofectamine technique. The cells were grown for 48 h and then washed with chilled phosphate-buffered saline, collected, and freeze-thawed in a buffer containing 20 mM Tris-HCl, 2 mM dithiothreitol, 20% (w/v) glycerol, 0.5 mM KCl. Following centrifugation at 105,000 × g for 1 h, supernatants containing whole cell extracts were retained and assayed for protein content. The following synthetic oligonucleotide sequences of the PTHRP promoter were used: R47, 5′-AGCAGCAGGCTCTTCGTTGTTGGAGGGAAGTTGAGGAGGAGAAGTC-3′; R28, 5′-GCTTCAGAGGAGGAGGGAAGGTAAGTC-3′; R28 Mut1, 5′-GCTTCAGAGGAGGAGGGAAGGTAAGTC-3′; R28 Mut2, 5′-GCTTCAGAGGAGGAGGGAAGGTAAGTC-3′; R28 Mut3, 5′-GCTTCAGAGGAGGAGGGAAGGTAAGTC-3′; R28 Mut4, 5′-GCTTCAGAGGAGGAGGGAAGGTAAGTC-3′. Underlined bases indicate those differing from the wild-type sequence. [32P]Labeled human osteocalcin or mouse osteopontin VDREs were used as controls: 5′-TTGTTGACTCACCGGGTGAGAAGGGGTGGTTGGAGGGAAGTTGAGGAGGAGAAGTC-3′ (18); 5′-GTACAAGTTCACAGGTTGACGCAGGGG-3′ (19).

Each binding reaction included 5 fmol of the [32P]-labeled oligonucleotide probe, 10 μg of nuclear or cellular proteins, 200 μg of 1,25(OH)₂D₃, and 5 μg of poly(dI-dC) as a nonspecific competitor of DNA. The binding reactions were performed at room temperature for 30 min in a binding buffer (10 mM Tris, 100 mM KCl, 5 mM MgCl₂, 0.5 mM glycerol, 1 mM EDTA, and 1 mM dithiothreitol). The complexes were resolved by electrophoresis through 4% polyacrylamide gels at 4 °C. For competition experiments, osteocalcin sequences or putative VDRE sequences were included in the binding reaction prior to the addition of the probe. Finally, a monoclonal antibody against the VDR (Affinity Bioreagents Inc, Newcastle Station, N.J.) or a polyclonal antibody against RXRa, β-, and γ (kindly provided by Dr. P. Chambon) were used to identify specific bands that contained receptor proteins. The VDR monoclonal antibody (clone 9A7) binds just C-terminal to the DNA binding domain of the receptor and was shown previously to inhibit binding of the VDR to an osteocalcin VDRE (20).

Statistical analysis was by one way analysis of variance by Student’s t test.

RESULTS

Effect of EGF and Serum on Activity of the PTHRP-GH Fusion Genes—We first assessed the potential of EGF and serum to regulate a series of PTHRP gene promoter deletion constructs that contained between 1951 and 300 bp of 5′-flanking sequence relative to the transcription start site, each fused to a GH reporter gene. These plasmids were transfected into NHKs, which normally express both the VDR and PTHRP.
Hormone-responsive Sequences in the PTHRP Gene

Constructs expressed both moderate basal promoter activity and a 2.5- to 4-fold increase in activity following treatment with EGF (Fig. 1) or FBS (data not shown). Furthermore, there was a maximum increase of activity in the presence of EGF, which peaked with the −1161 construct and then progressively decreased as the promoter was extended upstream to −1951 or downstream to −300 bp. This suggested the presence of repressor elements modulating both basal and stimulated promoter activity upstream toward −1951 bp and downstream toward −300 bp. No significant stimulation with EGF or serum was observed between −481 and −300 relative to the transcription start site.

Effect of 1,25(OH)2D3 on the Activity of the PTHRP-GH Fusion Genes—We previously reported the presence of a 1,25(OH)2D3-responsive region in the rat PTHRP promoter (5), localized to between −300 and −1161 bp upstream of the cap site. To pursue these findings, several deletion constructs spanning this region were transfected into NHKs. We first analyzed the −1161 construct under basal conditions (without EGF or FBS) and in the presence of either EGF or FBS. Both EGF and FBS produced marked increases in promoter activity relative to basal conditions (Fig. 2A). The effect of 1,25(OH)2D3 on promoter activity under these conditions was then determined. Significant inhibition of activity with 1,25(OH)2D3 was observed in both basal and stimulated conditions (Fig. 2A). We subsequently analyzed and compared the promoter activity of constructs truncated at positions −1161, −840, −751, −481, −300 in the presence of FBS, without or with 10 nM 1,25(OH)2D3 (Fig. 2B). The inhibitory effect of 1,25(OH)2D3 was only observed with the −1161 construct, suggesting that the vitamin D-responsive area was localized between −1161 and −840 bp.

Mapping of Promoter Sequences Involved in Repression by 1,25(OH)2D3—To determine in more detail which sequences within the PTHRP promoter were involved in 1,25(OH)2D3-inducible repression, fragments −1161 to −1079 (C2), −1047 to −944 (C3), −1047 to −690 (C4) were fused to the TK promoter located 5′ to the GH gene (TKGH) and analyzed for functional activity (Fig. 3). Only C1 was capable of conferring 1,25(OH)2D3 responsiveness. Furthermore, the C1 construct in the antisense as well as the sense orientation was also able to confer stimulation to EGF and inhibition in response to 1,25(OH)2D3.
1,25(OH)2D3 (Fig. 4B), suggesting that the responsive sequences can function in an orientation-independent manner.

We then tested the capacity of three synthetic oligonucleotides (R47, L47, and M40) spanning bp −1161 to −1079 to confer hormone responsiveness to the heterologous TK promoter. These were inserted into the TKGH vector and transfected into NHKs (Fig. 4A). Basal activity was much reduced with all three constructs in comparison with the −1161 to −1079 (C1) construct (Fig. 4B) but was not significantly different from the parent TKGH recombinant. Stimulation in the presence of EGF was observed with all three constructs. However, inhibition by 1,25(OH)2D3 was only significant with construct R47, suggesting that the inhibitory activity required critical sequences downstream of M40 (Fig. 4B). Furthermore, none of several shorter PTHRP gene sequences analyzed (C1a, C1b, C1c, and C1d; see “Materials and Methods”) were capable of conferring inhibition by 1,25(OH)2D3 in the TKGH assay (data not shown).

**Fig. 4. Mapping of the C1 region and identification of a vitamin D responsive element (VDRE).** Region −1161 to −1079 (C1) was cloned in both sense (C1-S) and antisense (C1-AS) orientations upstream of the TK promoter as described under “Materials and Methods.” Three overlapping fragments spanning the C1 area (L47, M40, and R47) were constructed using synthetic oligonucleotides and cloned into TKGH. NHKs were transfected with these constructs and were incubated in basal medium with either no FBS or EGF (dark bars), EGF (open bars), and EGF plus 10−8 M 1,25(OH)2D3 (gray bars) as described under “Materials and Methods.” At 72 h, aliquots of the medium were taken, and GH levels were measured by an immunoradiometric assay. Concentrations of GH in the medium of cells transfected with pOGH were equivalent to concentrations detected in the medium alone. The results shown are the mean ± S.E. of triplicate determinations. Asterisks indicate a significant difference between 1,25(OH)2D3−treated and nontreated cells (*, p < 0.01) that were incubated with EGF. Open circles indicate a significant difference from incubations performed in the presence and absence of EGF (○, p < 0.01). The results are representative of four separate experiments.

Gel Retardation Assays—To determine if the 1,25(OH)2D3 VDR interacted directly with DNA sequences active in transfection studies, we analyzed the interaction of R47 and a shorter fragment deleted at both the 5′ and 3′ ends (R28) in gel retardation assays.

First we examined the capacity of radiolabeled R47 to bind to an extract of COS-1 cells that had been enriched with transfected VDR-RXR. We compared this binding with that of a 32P-labeled osteopontin VDRE. R47 bound to the extract and was seen as a predominant band co-migrating with the bound osteopontin VDRE (Fig. 5, lanes 3 and 6). Similar results were obtained with the osteocalcin VDRE (Fig. 6, lanes 4 and 14). We then assessed the interaction of R47 with the 1,25(OH)2D3 receptor (VDR) in the nuclear extract using a monoclonal antibody to the VDR. This antibody has previously been shown to inhibit binding of an osteocalcin VDRE when preincubated with nuclear extracts containing VDR prior to adding the labeled osteocalcin VDRE (18). The inhibition is likely to be due to interaction of the antibody with the VDR close to its DNA binding domain. A similar inhibition of binding to labeled R47 was observed (Fig. 5, lane 7) when the VDR antibody was preincubated with COS-1 nuclear extracts enriched with VDR-RXR.

To assess the binding of R47 to RXR in the nuclear extract, we employed an antibody to RXR that binds but does not inhibit the RXR-DNA interaction. Preincubation with the RXR antibody produced a supershift when the osteopontin VDRE was used as a probe (Fig. 5, lane 5). A similar supershift was observed with the labeled R47 (Fig. 5, lane 8). Incubation in the presence of a nonspecific antiserum (lane 9) did not affect the binding of labeled R47 to the nuclear extract.

We then analyzed the binding to the nuclear extract of radiolabeled R28, a 3′ deletion fragment of R47. The analyses were performed with or without RXR and VDR antibodies or in the presence of cold competitor (Fig. 6). A predominant band was observed, which was inhibited by VDR antibody (Fig. 6, lanes 5 and 8) and supershifted by RXR antibody (Fig. 6, lanes 6 and 9). Increasing concentrations of cold R28 also inhibited the binding of labeled R28 to the nuclear extracts (Fig. 6, lanes 11–13), demonstrating the specificity of the binding. Similar results were observed with NHK nuclear extracts (data not shown).

The R28 sequence was compared with other known VDRE sequences (Fig. 7). A putative VDRE sequence containing a half-site homologous to the human and rat osteocalcin VDRE (GGGTTGA) was identified in the 3′ area (Fig. 7). Furthermore, an almost identical repeat of the downstream half-site (GGTGG) was observed 3 bp upstream. Mutants of these sites were
Hormone-responsive Sequences in the PTHRP Gene

We have identified a 47-bp sequence in the rat PTHRP gene promoter that is located at -1121 to -1075 bases upstream of the transcription start site, confers vitamin D responsiveness to a heterologous promoter, and binds to the vitamin D receptor. This sequence contains one motif that is entirely homologous to a half-site of the human and rat osteocalcin VDRE (GGGTGA) (18, 22). A nonconsensus direct repeat (GGGTGGA) is located three nucleotides upstream of this motif. This 47-bp sequence can repress gene transcription in response to 1,25(OH)₂D₃. Recently, another sequence that mediates repression in response to 1,25(OH)₂D₃ was identified in the human PTH gene but bears no homology with the sequence described here (23).

As expected from the sequence homology found with the rat PTHRP VDRE, the rat and human osteocalcin VDREs could compete with the rat PTHRP VDRE in binding to nuclear extracts as shown by gel retardation assays. This suggests strongly that osteocalcin and PTHRP VDREs bind to the same region of the vitamin D receptor. In addition, the DNA-PTHRP VDRE complex seen on gel retardation assays was strongly inhibited by the addition of a monoclonal antibody raised against the DNA binding domain of the VDR, indicating a specific and direct interaction of the VDR with this novel VDRE.

RXRs (24) have been shown to heterodimerize with the VDR, increasing both DNA binding and transcriptional function of the up-regulatory VDREs (osteocalcin and osteopontin). In the present study, RXR also interacted with the PTHRP VDRE-VDRE complex as evidenced by the supershift observed in gel retardation assays in the presence of RXR monoclonal anti-
tibodies. This observation tends to support the notion that the VDR\(\alpha\)RXR heterodimer is the major functional unit that mediates 1,25(OH)\(\beta\)D\(_3\) action in this model. This contrasts with the recently described inhibitory human PTH VDRE (16, 23), which was shown not to interact with RXR and has no homology with the PTHRP VDRE described here. However, another negative PTH VDRE was described in the avian (chicken) PTH gene, which interacts with a VDR\(\alpha\)RXR heterodimer (21). The described sequence, GGGTCAGGAGGGTGT, shows a striking homology with the negative PTHRP VDRE described here, and, taken together, our results are highly suggestive of a common 1,25(OH)\(\beta\)D\(_3\)-mediated down-regulatory mechanism for both PTH and PTHRP.

There was no consensus AP1 site identified within the 47-bp sequence reported here such as has been described previously within the stimulatory human osteocalcin VDRE (18). It is likely, however, that other sequences flanking the GGGTGA motifs are critical to its interaction with the 1,25(OH)\(\beta\)D\(_3\) receptor in order to provide specificity for this unique down-regulatory sequence.

Mutation of the half-site located 3 bp upstream of GGGTGA seems to indicate that this nonconsensus direct repeat, which differs only by a single base pair addition from the downstream half-site (GGGTGAGAGGGTGA), is required for transcriptional down-regulation. Our observation with functional promoter assays also supports the suggestion that sequences located upstream of the GGGTGA motif are critical for down-regulation, since this sequence alone (in C\(_{1d}\)) cannot confer down-regulation in such assays. Furthermore, short sequences upstream of GGGTGA (C\(_{1c}\) and M40) also cannot confer down-regulation by themselves. Only a combined sequence containing both regions (C\(_{1c}\) and C\(_{1d}\), i.e. R47) was able to permit down-regulation by 1,25(OH)\(\beta\)D\(_3\) in functional promoter assays.

In this study, we have also determined the presence of DNA sequences that confer augmented gene transcription in response to mitogenic stimuli. These are located within the same 80-bp sequence of DNA (C\(_1\)), and M40) also cannot confer down-regulation by themselves. Only a combined sequence containing both regions (C\(_{1c}\) and C\(_{1d}\), i.e. R47) was able to permit down-regulation by 1,25(OH)\(\beta\)D\(_3\) in functional promoter assays.

Although our study clearly shows that a direct interaction of the VDR-RXR heterodimer with the putative PTHRP VDRE occurs, the mechanism by which transcriptional repression takes place may involve interference with the binding of positive transcription factors stimulated in response to mitogenic stimuli. Our present data would favor this hypothesis, since transcriptional inhibition in response to 1,25(OH)\(\beta\)D\(_3\) was more pronounced in the presence of mitogenic stimuli. Therefore, the inhibition of transcription with 1,25(OH)\(\beta\)D\(_3\) may well depend on specific interaction of the VDR-RXR heterodimer complex
with other, as yet unidentified, transcription factors up-regulated in response to various mitogens. Such a mechanism has recently been proposed for the transcriptional repression of the interleukin-2 gene by 1,25(OH)₂D₃. In this model the VDR-RXR heterodimer blocks nuclear factor of activated T cells/AP1 complex formation, thus preventing transcriptional activation of the interleukin-2 gene (25).

In this context, it is important to note that the VDRE sequence reported here does not confer the same degree of inhibition by 1,25(OH)₂D₃ when fused to a heterologous promoter such as TKGH as it does in the native gene, suggesting that this VDRE functions best within its own gene context. Site-directed mutagenesis of this VDRE sequence, in the intact gene promoter, will be required to determine its exact role in the context of the native promoter. Other important elements outside the sequence reported here may also facilitate transcriptional repression by 1,25(OH)₂D₃. Additionally, other potential VDRE sequences remote from the VDRE sequence identified in the present study may also interact directly or indirectly with the VDR and act synergistically to confer maximal inhibition to the promoter in response to 1,25(OH)₂D₃. Further studies are required to examine these issues.

Acknowledgments—We thank I. Bolivar, Johnson Mak, and Harvey Miller for excellent technical assistance and C. Ferrara-Wilson and P. Kirk for preparation of the manuscript.

REFERENCES
Identification and Characterization of 1,25-Dihydroxyvitamin D₃-responsive Repressor Sequences in the Rat Parathyroid Hormone-related Peptide Gene
Richard Kremer, Michael Sebag, Céline Champigny, Karen Meerovitch, Geoffrey N. Hendy, John White and David Goltzman

doi: 10.1074/jbc.271.27.16310

Access the most updated version of this article at http://www.jbc.org/content/271/27/16310

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 23 references, 10 of which can be accessed free at http://www.jbc.org/content/271/27/16310.full.html#ref-list-1