Highly Potent Transcriptional Activation by 16-ene Derivatives of 1,25-Dihydroxyvitamin D₃

LACK OF MODULATION BY 9-CIS-RETINOIC ACID OF RESPONSE TO 1,25-DIHYDROXYVITAMIN D₃ OR ITS DERIVATIVES*

Received for publication, June 24, 1993, and in revised form, September 15, 1993

From the Department of Physiology, McGill University, Montreal, Québec H3G 1Y6, the Calcium Research Laboratory, Royal Victoria Hospital, McGill University, Montreal, Québec H3A 1A1, Canada, and Hoffmann-LaRoche Inc., Nutley, New Jersey 07110-1199

John Ferrara, Kimberly McCuaig, Geoffrey N. Hendy,§, Milan Uskokovic, and John H. White

Although several studies have been performed on the biological activities of analogs of 1,25-dihydroxyvitamin D₃ (1,25-(OH)₂ D₃) at the whole animal and cellular levels, little work has been done to analyze their transcriptional activation properties. A highly inducible 1,25-(OH)₂ D₃-responsive promoter composed of three copies of the mouse osteopontin vitamin D₃ response element (VDRE3) inserted upstream of a herpes simplex virus thymidine kinase promoter has been constructed, and its transcriptional properties have been analyzed by transient transfection into the monkey kidney cell line COS-7 and the rat osteoblast-like osteosarcoma line ROS 17/2.8. We have studied systematically transcriptional activation by a number of 1,25-(OH)₂ D₃ analogs, particularly those substituted at positions 16, 23, 26, and 27, sites that are targets for metabolism. Strikingly, except for derivatives that bind the 1,25-(OH)₂ D₃ receptor (VDR) very weakly, we find no parallel between the potency of action of a derivative as a transcriptional inducer and its affinity for the VDR. Derivatives substituted by multiple bonds at positions 16 and/or 23, although having varying affinities for the VDR, all stimulate transcription more potently than D₃ in some cases at 100-fold lower concentrations. The peak transcriptional activity observed varies by only approximately 20% among different active analogs, indicating little difference in the activity of the VDR once bound to ligand. Gel retardation assays with ROS 17/2.8 nuclear extracts suggest that the VDR binds to the mouse osteopontin VDRE predominantly as a heterodimer with retinoid X receptor(s) (RXR(s)). We find that 9-cis-retinoic acid, the cognate ligand for RXRs, does not have a significant effect on the response of the VDRE3 promoter to 1,25-(OH)₂ D₃ or a number of its derivatives in ROS 17/2.8 or in COS-7 cells, under conditions in which promoters containing retinoid X response elements are activated. This suggests that 9-cis-retinoic acid may not act on the response to 1,25-(OH)₂ D₃ or its derivatives by directly influencing the transcriptional activity of VDR/RXR heterodimers. This promoter/reporter system should be useful for analyzing the tissue-specific transcriptional activity of 1,25-(OH)₂ D₃ and its derivatives in any cell type amenable to transient transfection.

Vitamin D₃ is a secosteroid whose precursor is synthesized in the skin through the cleavage of the B ring of 7-dehydrocholesterol by nonenzymatic photolysis and isomerization (Holick, 1981). The resulting product is hydroxylated in the liver to 25-hydroxyvitamin D₃ (Ponchon et al., 1969) and further 1α-hydroxylated in the renal proximal tubule to its most biologically active form 1α,25-dihydroxyvitamin D₃ (1,25-(OH)₂ D₃)³ (Holick et al., 1971; Lawson et al., 1971; Norman et al., 1971). Because of its lipophilicity 1α,25-(OH)₂ D₃ is capable of passing through cellular membranes and binding to the vitamin D₃ receptor (VDR) present in target cells (Hausser and McCain, 1977; Liao et al., 1990). The VDR is a member of the nuclear receptor family of transcriptional regulators and is part of a subgroup that includes receptors for thyroid hormone and retinoids. These receptors recognize specific DNA sequences, known as response elements, that are composed of direct repeats with the consensus sequence PurG/T/C/A. DNA binding and transcriptional activation studies have shown that the VDR recognizes direct repeats separated by 3 bp, 1,25-(OH)₂ D₃ response elements (VDREs) comprising this structure that have been identified upstream of osteocalcin genes and the mouse osteopontin (MOP) gene (Kerner et al., 1989; Morrison et al., 1989; Noda et al., 1990; Demay et al., 1992). The MOP VDRE is composed of direct repeats containing the consensus sequence GGGTC. Several studies have shown that the VDR requires an auxiliary factor for binding to its response element (Sone et al., 1991; Ross et al., 1992). This factor has been demonstrated to be the nuclear retinoid X receptor, RXR (Yu et al., 1991; Leid et al., 1991). The abbreviations used are: 1,25-(OH)₂ D₃, 1α,25-dihydroxyvitamin D₃; Pur, purine; bp, base pair(s); CAT, chloramphenicol acetyltransferase; MOP, mouse osteopontin; RA, retinoic acid; RAR, retinoic acid receptor; RARE, RA response element; RXR, retinoid X receptor; RXRE, retinoid X response element; tk, thymidine kinase; VDR, vitamin D₃ receptor; VDRE, vitamin D₃ response element; 24,25-(OH)₂ D₃, 24,25-dihydroxyvitamin D₃; 1,25-(OH)₂-16-ene D₃, 1,25-dihydroxy-16-enevitamin D₃; 1,25-(OH)₂-23-ene D₃, 1,25-dihydroxy-23-enevitamin D₃; 1,25-(OH)₂-16,23E-diene D₃, 1,25-dihydroxy-16,23E-dienovitamin D₃; 1,25-(OH)₂-16,23Z-diene D₃, 1,25-dihydroxy-16,23Z-dienovitamin D₃; 1,25-(OH)₂-25,26-diene D₃, 1,25-dihydroxy-25,26-dienovitamin D₃; 1,25-(OH)₂-16-23-ene D₃, 1,25-dihydroxy-16-23-enevitamin D₃; 1,25-

* This work was supported by Grants MT-11704 and MA-8315 from the Medical Research Council of Canada and the Kidney Foundation 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 1754 solely to indicate this fact.

§ Recipient of a scientist award from the Medical Research Council of Canada.

† Chercheur-Boursier of the Fonds de la Recherche en Santé du Québec. To whom correspondence should be addressed: Dept. of Physiology, McIntyre Medical Sciences Bldg., McGill University, 3655 Drummond St., Montreal, Que., H3G 1Y6, Canada.
Transcriptional Activation by Vitamin D<sub>3</sub> Derivatives

et al., 1992), which specifically binds the ligand 9-cis-retinoic acid (9-cis-RA) (Heyman et al., 1992; Levin et al., 1992). The VDR binds to response elements as a heterodimer with RXRs. RXRs have also been shown to homodimerize in vitro in the presence of 9-cis-RA and bind selectively to response elements composed of direct repeats separated by 1 bp (Zhang et al., 1992). Transcriptional activation studies have shown that these sequences can act as 9-cis-RA response elements (Mangelsdorf et al., 1991; Zhang et al., 1992). Recent results have suggested that 9-cis-RA can also augment the response to 1,25-(OH)<sub>2</sub>D<sub>3</sub> of a synthetic promoter in MOP VDR-expressing and upstream of a truncated herpes simplex virus thymidine kinase (tk) promoter (Carlberg et al., 1993). This suggests that 9-cis-RA may modulate transcription of a number of target genes containing different response elements and control a wide range of biological processes.

Ligand-bound VDR plays an essential role in regulating the expression of a number of genes controlling calcium homeostasis (Lawsom, 1979). In the intestinal epithelium, for example, 1,25-(OH)<sub>2</sub>D<sub>3</sub> induces the synthesis of several proteins that may control the transport of calcium (Wasserman et al., 1974; Mundy, 1989). 1,25-(OH)<sub>2</sub>D<sub>3</sub> also stimulates magnesium and phosphate transport in the intestine through different mechanisms (Clemens and O’Riordan, 1990). In addition, 1,25-(OH)<sub>2</sub>D<sub>3</sub> inhibits growth of certain malignant cell types and promotes differentiation (Abe et al., 1981; Colston et al., 1981; Roodman et al., 1985). Progression of breast carcinomas can be slowed by treatment with 1,25-(OH)<sub>2</sub>D<sub>3</sub> (Preake et al., 1981). 1,25-(OH)<sub>2</sub>D<sub>3</sub> can stimulate mouse myeloid leukemia cells to differentiate into macrophages (Abe et al., 1981). Human leukemia HL60 cells will also differentiate in the presence of 1,25-(OH)<sub>2</sub>D<sub>3</sub> (Bar-Shavit et al., 1988; Miyaura et al., 1981). Similarly, 1,25-(OH)<sub>2</sub>D<sub>3</sub> can induce immature basal layer skin cells to differentiate into mature keratinocytes (Hosomi et al., 1983).

Given its antiproliferative effects, 1,25-(OH)<sub>2</sub>D<sub>3</sub> is potentially useful clinically in the control of various cancers and in the treatment of psoriasis. However, because of its role in calcium mobilization, hypercalcemia results from 1,25-(OH)<sub>2</sub>D<sub>3</sub> treatment. Recent efforts have resulted in the development of a number of synthetic 1,25-(OH)<sub>2</sub>D<sub>3</sub> analogs displaying low calcemic activity, many of which carry various side chain substitutions (Zhou et al., 1990, 1991; Abe et al., 1991; Bouillon et al., 1992; Haq et al., 1993). Although these derivatives differ from 1,25-(OH)<sub>2</sub>D<sub>3</sub> in their calcemic properties, many have been shown to be highly effective in controlling cellular differentiation and maintain a high affinity for the VDR (Zhou et al., 1991). Certain nonhypercalcemic derivatives activate transcription of a chimeric osteocalcin promoter/bacterial chloromphenicol acetyltransferase (CAT) reporter recombinant in stably transfected ROS 17/2.8 osteosarcoma cells (Morrison and Elsman, 1991).

Here, we have constructed a highly inducible promoter/reporter recombinant for analyzing the transcriptional activity of 1,25-(OH)<sub>2</sub>D<sub>3</sub> and its analogs. This construct contains three MOP VDREs inserted upstream of the herpes simplex virus tk promoter. We have tested the transcriptional activity of a number of 1,25-(OH)<sub>2</sub>D<sub>3</sub> analogs with different side chain substitutions in the monkey kidney cell line COS-7 and in ROS 17/2.8 cells and have found that some of these derivatives activate transcription more potently than 1,25-(OH)<sub>2</sub>D<sub>3</sub> in some cases at 100-fold lower concentrations. Peak transcriptional activity varies little among derivatives, suggesting that the various substitutions do not affect the activity of the ligand-bound VDR. Although the MOP VDRE binds VDR/RXR heterodimers in vitro, we do not observe any effect of 9-cis-RA on transcriptional induction by 1,25-(OH)<sub>2</sub>D<sub>3</sub> or its derivatives in COS-7 or ROS 17/2.8 cells, suggesting that 9-cis-RA may not directly modulate the transcriptional activity of VDR/RXR heterodimers. This promoter/reporter system should be useful for analyzing the tissue-specific activity of 1,25-(OH)<sub>2</sub>D<sub>3</sub> and its derivatives in a wide variety of cell types.

EXPERIMENTAL PROCEDURES

Plasmids

The VDREtkCAT and VDRE3tkCAT recombinants were constructed byinserting a monocloner or directly repeated trimer, respectively, of the sequence 5’-GATCCGTACAAGGTTTACAGGTTTACAGGTCA-3’ containing the MOP VDRE, which is flanked by BamHI and BglII ends, into the BamHI site of pRSV-CAT (Klein and Ralston, 1981). As discussed above, the thymidine kinase promoter. VDREMUTtkCAT and VDRE3MTtkCAT recombinants were constructed in an identical manner using the sequence 5’-GATCCGTACAAGGGCCACGAGGCCCACGTTCTTA-3’, which contains two base changes per repeat of the VDRE. The VDR expression vector (VDR/pSFGS) was constructed by inserting a 2.1-kilobase EcoRI fragment containing the entire coding region of the human VDR into the EcoRI site of pSGS (Green et al., 1988). For expression in Escherichia coli, the VDR cDNA was amplified by polymerase chain reaction using oligonucleotides that introduced a Kpn1 site into the 5’ end of the cDNA replacing the initial methionine codon, and the Kpn1 site immediately after the 3’ end of the cDNA region. The resulting amplified fragment was cloned into the bacterial expression vector pRT32 (Mader et al., 1993) digested with Kpn1 and XhoI.

Methods

Cell Culture and Transfections—Both COS-7 and ROS 17/2.8 cells were propagated in 9-cm dishes in Dulbecco’s modified Eagle’s medium containing 5% fetal bovine serum (Life Technologies, Inc.). Fetal bovine serum was charcoal stripped for use in experiments with 9-cis-RA. For CAT assays, 5 μg of the MOP VDRE3tkCAT or related construct, 5 μg of β-galactosidase expression vector pCH110, and 10 μg of BlueScribe (Stratagene) as an internal control were transfected into plates of COS-7 or ROS 17/2.8 at approximately 30% confluence using the calcium phosphate coprecipitation technique (Baker et al., 1991). 1,25-(OH)<sub>2</sub>D<sub>3</sub> or one of its derivatives was added at final concentrations indicated in the figures. The medium was changed, and fresh hormone was added 24 h after transfection. Cells were harvested 44–48 h after transfection. For gel retardation assays, activity was defined as above with 10 μg each of the expression vectors VDR/pSFGS and RXRa/pSFGS and harvested 44–48 h after transfection.

Extracts of Mammalian Cells—Transiently transfected cells were harvested for gel retardation assays by washing cells with 5 ml of ice-cold phosphate-buffered saline followed by scraping the cells in 1 ml of phosphate-buffered saline. Cells were centrifuged at 2,000 rpm for 10 min at 4 °C, and the pellets were resuspended in 100 μl of high salt extraction buffer (25 mM Tris (pH 7.9), 0.3 mM dithiothreitol, 0.1 mM EDTA, 420 mM NaCl, 10% (v/v) glycerol). Cells were lysed by three cycles of freezing at −70 °C and thawing at room temperature, then centrifuged at 10,000 rpm for 10 min at 4 °C. Supernatants were stored at −70 °C. Extracts were prepared for CAT assays as described (Webster et al., 1988). ROS 17/2.8 cell nuclear extracts were made by the protocol of Digman et al. (1983).

Expression of the VDR in E. coli—The VDR was expressed in E. coli using the bacteriophage T7 expression system (Studier and Moffatt, 1986). Cultures (500 ml) of BL21pLyS8 harboring the pRT32 control plasmid VDRpRT32 were grown at 37 °C to A<sub>600</sub> of 0.4 and induced with 0.4 mM isopropyl-1-thio-β-D-galactopropyranoside for an additional 2 h. Cells were centrifuged and the pellets resuspended in 5 ml of 25 mM Tris (pH 7.9), 0.3 mM dithiothreitol, 0.1 mM EDTA, 200 mM NaCl, 10% (v/v) glycerol, and 2.5 μg/ml of the protease inhibitors leupeptin, antipain, and pepstatin. Cells were lysed by sonication, and cleared lysates were recovered after centrifugation at 30,000 rpm for 45 min in a Beckman Ti-70 rotor. Cells expressing the VDR produced a polypeptide of 50 kDa visible on SDS-polyacrylamide gels (data not shown), then for a further 20 min at 30 °C after the addition of 50,000–100,000 cpm (5–10 fmol) of 32P-end-labeled double-stranded oligonucleotide. Samples were loaded on 5% polyacrylamide gels equilibrated in 25
Transcriptional Activation by Vitamin D₃ Derivatives

![Diagram of VDRE3tkCAT](image)

**Panel A**

**A**

**VDRE**

5' - GATCGTAGATTGCCAGCTGCTATGACATGTCCAAGTGCTCAAGTGCTCCAAGTGCAGAATCTAG-5'

**VDREM**

5' - GATCGTAGATTGCCAGCTGCTATGACATGTCCAAGTGCTCAAGTGCTCCAAGTGCAGAATCTAG-5'

**Panel B**

**RESULTS**

**A Highly Inducible 1,25-Dihydroxyvitamin D₃-responsive Reporter System**—A highly inducible promoter responsive to 1,25-(OH)₂ D₃ was constructed to study the transcriptional activity of a variety of clinically significant 1,25-(OH)₂ D₃ derivatives. Previous studies with natural and synthetic promoters responsive to nuclear receptors have shown that hormone response elements often act synergistically to mediate transcriptional activation (Jantzen et al., 1987; Schmid et al., 1989; Martinez and Wahli, 1989; Ponglikitmongkol et al., 1990; Mader and White, 1993). Therefore, a synthetic promoter containing multiple copies of a strong VDRE was constructed. The MOP VDRE is composed of a direct repeat of PurGGCTCA motifs separated by 3 base pairs (Fig. 1A, top; Noda et al., 1990), which conform exactly to the PurGGCTCA consensus motifs identified in elements responsive to 1,25-(OH)₂ D₃, thyroid hormone or retinoic acid (de The et al., 1990; Umesono et al., 1991). To create the recombinant VDRE3tkCAT (Fig. 1A), three copies of the MOP VDRE oligonucleotide were inserted immediately upstream of the herpes simplex virus tk promoter in the vector pBLCAT8+ (Klein-Hitpass et al., 1986). We also constructed derivatives containing either one copy of the MOP VDRE (VDREtkCAT), or one or three copies of mutated MOP VDRE (VDREmuttkCAT and VDRE3muttkCAT, respectively) containing two base changes per direct repeat (Fig. 1A, top) which disrupt VDR binding in vitro (Noda et al., 1990; see Fig. 7A).

Gel retardation assays demonstrate that the MOP VDRE provides a high-affinity binding site for heterodimers of the 1,25-(OH)₂ D₃ receptor (VDR) and RXRα. A retarded complex is formed which is dependent on the presence of both a bacterial extract containing the VDR and an extract of the monkey kidney cell line COS-7 transfected with a human RXRa expression vector (lanes 2–5) and whole cell extracts of COS-7 cells transfected with a human RXRa expression vector (lanes 7–10). This complex is formed by extracts of COS-7 cells cotransfected with VDR and RXRa-containing expression vectors (data not shown, but see Fig. 7A). A similar complex is not observed with VDR- or RXRα-containing extracts alone (Fig. 1B, lanes 1 and 5, respectively). This complex is not formed on a MOP oligonucleotide containing point mutations (Fig. 1A, top; Noda et al., 1990) that disrupt VDR binding (data not shown, but see Fig. 7A). A much weaker complex of similar mobility, which is not seen with COS-7 cell extracts alone, is observed if the VDR is incubated with an extract of untransfected cells, indicating the presence of endogenous RXR(s) in COS-7 cells (Fig. 1B, lanes 6–10). This result
2974

Transcriptional Activation by Vitamin D₃ Derivatives

Fig. 2. Induction of MOP VDRE-containing promoters by 1,25-(OH)₂ D₃ in COS-7 cells. Panel A, CAT assays of extracts of COS-7 cells transfected with a VDR expression vector (1 μg) and CAT reporter plasmids (5 μg) along with the β-galactosidase expression vector pCH110 (3 μg). Quantities of extracts used in CAT assays were normalized to β-galactosidase activity. 1,25-(OH)₂ D₃ (50 nM) was added to cultures as indicated. 1,25-(OH)₂ D₃ inducibility was tested with plasmids containing one or three mutant MOP VDREs (lanes 1 and 2, and lanes 3 and 4, respectively), three or one wild-type MOP VDREs (lanes 5 and 6, and lanes 7 and 8, respectively), or the parent plasmid pBLCAT8+. Panel B, graphic representation of the CAT assay shown in panel A. Spots corresponding to nonacetylated and acetylated chloramphenicol were excised from the thin layer chromatograph, and radioactivity was determined in a liquid scintillation counter.

is consistent with evidence provided by Northern blots that RXRα are present in COS cells (Mader et al., 1993).

Induction of the VDRE3tk CAT Promoter in COS-7 and ROS 17/2.8 Cells—The VDRE3tk CAT promoter is responsive to 1,25-(OH)₂ D₃ in transiently transfected COS-7 cells (Fig. 2). In cells transfected with a VDR expression vector along with reporter plasmids, VDRE3tkCAT and VDRE3tkCAT were both stimulated by 50 nM 1,25-(OH)₂ D₃, whereas the parent vector pBLCAT8+ and derivatives VDRE3-MUTtkCAT and VDRE3-MUTtkCAT were not responsive (Fig. 2). CAT activity in cells transfected with VDREtkCAT and VDRE3tkCAT and a VDR expression vector was inducible 10- and 30-fold, respectively, over background by 50 nM 1,25-(OH)₂ D₃ (Fig. 2). At least 4-fold more CAT activity was induced by 1,25-(OH)₂ D₃ in VDRE3tkCAT-containing cells than in cells transfected with the VDREtkCAT derivative. We have also found that the VDRE3tk CAT promoter was readily inducible in COS-7 cells by 1,25-(OH)₂ D₃ in the absence of cotransfected VDR expression vector (see Figs. 5, 6, and 8). Taken together, these results indicate that the observed transcriptional stimulation is dependent on ligand-activated VDRs present in COS-7 cells and, moreover, that synthetic promoters containing MOP VDRE(s) are highly inducible in these cells. The activity of VDRE3tkCAT was also tested in the rat osteoblast-like osteosarcoma cell line ROS 17/2.8 (Fig. 3). The promoter was induced 5-fold by 50 nM 1,25-(OH)₂ D₃ in these cells. The nonhypercalcemic 1,25-(OH)₂ D₃ analog MC903 induced CAT activity to a similar extent (Fig. 3, A and B). This observation is consistent with the results of Morrison and Eisman (1991) who observed similar levels of induction by 1,25-(OH)₂ D₃ and MC903 of a CAT gene under control of the human osteocalcin promoter in stably transfected ROS 17/2.8 cells.

Highly Potent Stimulation of Transcription by 1,25-(OH)₂ D₃ Derivatives Substituted at Positions 16 and 23—There has been considerable clinical interest in the action of various analogs of 1,25-(OH)₂ D₃ which do not elicit hypercalcemia in vivo. Many of these derivatives contain, among other substitutions, a double bond between positions 16 and 17 (16-ene derivatives; see Fig. 4). For example, 1,25-(OH)₂-16-ene-23-yne-vitamin D₃ has been shown to prolong the survival time of leukemic mice without inducing hypercalcemia (Zhou et al., 1990). We have used the VDRE3tk CAT promoter/reporter system to test transcriptional induction by a number of 1,25-(OH)₂-16-ene derivatives. COS-7 cells were chosen for these analyses because of the relatively low background and high inducibility of VDRE3tkCAT observed in this line (see Fig. 2). These experiments were performed in the absence of a VDR expression vector. Transcriptional induction was determined over a range from 10⁻¹² to 10⁻⁶ M ligand (10⁻³ M for 1,25-(OH)₂ D₃). A concentration curve was performed for 1,25-(OH)₂ D₃ for each independent transfection experiment so that transcriptional induction by a given ligand could be normalized to that observed with 10⁻⁶ M 1,25-(OH)₂ D₃.

All of the 1,25-(OH)₂-16-ene derivatives tested stimulated CAT activity at concentrations at least 10-fold lower than 1,25-(OH)₂ D₃ (Fig. 5 and Table 1). Half-maximal stimulation of CAT activity was observed with 10–20 nM 1,25-(OH)₂ D₃ (Fig. 5a), whereas similar levels of induction occurred with 10–20-fold lower concentrations of 1,25-(OH)₂-16-ene D₃ (Fig. 5b). Substitution at both positions 16 and 23 shifted further to the left the concentrations of derivative required for half-maximal induction. Introduction of a cis or trans double bond into 16-ene derivatives (1,25-(OH)₂-16,23Z-diene D₃ and 1,25-(OH)₂-16,23E-diene D₃, respectively) lowered the concentration re-
Panel A, CAT assay of extracts of ROS 17/2.8 cells transfected with 5 μg of VDRE3tkCAT (lanes 4-6) or 5 μg of parent plasmid pBLCAT8+ and 3 μg of β-galactosidase expression vector pCH110. Quantities of extracts used in CAT assays were normalized to β-galactosidase activity. 1,25-(OH)2 D3 (50 nM) or MC903 (50 nM) was added to cultures as indicated. Panel B, graphic representation of the results of duplicate CAT assays similar to that shown in panel A. Radioactivity was determined as in Fig. 2.

Effects of Removal of the 1α-Hydroxyl Group or Its Substitution with a Fluoro Group on Transcriptional Induction by Derivatives—The 1α-hydroxyl group of 1,25-(OH)2 D3 is critical for high affinity binding to the VDR. Analogs lacking a 1α-hydroxyl group are weak effectors of VDR-mediated transcription. The addition of 24,25-(OH)2 vitamin D3 leads to induction of CAT activity only at 1 μM (Fig. 6a). 25-Hydroxy-16,23E-diene D3 (Fig. 6b) is 1,000 times less effective than the corresponding 1,25-(OH)2 derivative (Fig. 5d). These results are consistent with the very low affinity of these derivatives for the VDR (Table I). Replacement of the 1α-OH group of 1,25-(OH)2-16-ene-23-yne D3 with a β-hydroxy group greatly reduces the induction of CAT activity, consistent with a sharp reduction in affinity for the VDR (compare Figs. 5d and 6c; Table I). However, replacement of the 1α-OH group with a fluoro group does not render the resulting derivative inactive. Although 1α-fluoro-25-hydroxy-26,27-hexafluoro-16-ene-23-yne D3 is a less effective activator of CAT activity than 1,25-(OH)2-26,27-hexafluoro-16-ene-23-yne D3 (Fig. 6, e and f), it is at least 10-fold more potent than 1,25-(OH)2 D3 (Fig. 5c). In agreement with these results, 1α-fluoro-25-(OH)-26,27-hexafluoro-16-ene-23-yne D3 competes 10-fold more efficiently for binding to 1,25-(OH)2 D3 to the VDR than 19,25-dihydroxy-16-ene-23-yne D3 (Table I).

Finally, note that derivatives tested in Figs. 5 and 6 which have low affinity for the VDR are generally weak inducers of CAT activity. However, for those analogs that have moderate to high affinity for the VDR, there is no strict correlation between the affinity for the receptor and the potency of induction of CAT activity (see Table I for a summary of the results of Figs. 5 and 6).

9-cis-Retinoic Acid, the Cognate Ligand for RXRs, Does Not Stimulate the Response of the VDRE3tk Promoter to 1,25-Dihydroxyvitamin D3 or 16-ene Derivatives—Previous results have provided evidence that the MOP VDRE binds heterodimers of the VDR expressed in bacteria and RXRs expressed in COS-7 cells in vitro (see Fig. 1). A similar protein-DNA complex is detected in nuclear extracts of ROS 17/2.8 cells (Fig. 7A, lane 1). This complex is not formed on a mutant of the MOP VDRE (Fig. 7A, compare lanes 1 and 3), its formation is stimulated by the addition of the VDR expressed in bacteria (Fig. 7A, lane 2), and it comigrates with the VDR/RXR-DNA complex observed in Fig. 1 (Fig. 7A, lane 5). These data strongly suggest that the retarded complex corresponds to a heterodimer of the VDR and RXR(s) expressed in ROS 17/2.8 cells. Northern analysis of poly(A)+ RNA from ROS 17/2.8 cells specifically detects the presence of bands corresponding to RXRα and RXRβ, but not RXRγ, mRNAs (Fig. 2B), as well as that of the VDR (data not shown). The sizes of the RXR bands observed here correspond closely to those detected by Northern analysis of rat tissues (Yu et al., 1991; Mangelsdorf et al., 1992). These results support the evidence that RXR(s) are present in the retarded complexes formed by the VDR on the MOP VDRE (Fig. 7A).

Given the above DNA binding data and Northern blots, we analyzed the potential modulatory effects of the cognate ligand for the RXR receptors, 9-cis-RA, on the response of the VDRE3tk promoter to 1,25-(OH)2 D3 and some of its analogs. Recent studies have suggested that 1,25-(OH)2 D3 and 9-cis-RA can stimulate transcription synergistically from a synthetic promoter containing a MOP VDRE (Carlborg et al., 1993). It was therefore of interest to determine if the VDR bound to different 1,25-(OH)2 D3 analogs responds differently to 9-cis-RA. COS-7 and ROS 17/2.8 cells were treated with 50 nM 9-cis-RA, a concentration that is sufficient for a specific response by RXRs (Heyman et al., 1992; Levin et al., 1992; Mangelsdorf et al., 1992). 50 nM 9-cis-RA alone had no effect on VDRE3tkCAT promoter activity in COS-7 or ROS 17/2.8 cells (Fig. 8, A and B, first and second lanes; and C and D, first and third lanes). In other experiments, the addition of 1 μM 9-cis-RA had no effect on the VDRE3tkCAT promoter activity (data not shown). Strikingly, in both COS-7 and ROS 17/2.8 cells the addition of 50 nM 9-cis-RA had no significant effect on the response to 1,25-(OH)2 D3 of the VDRE3tkCAT promoter (Fig. 8, A and B, first, third, and fourth lanes; and C and D, first, second, and fourth lanes; see also Fig. 9). Similar results were obtained in ROS 17/2.8 cells using the VDRE3tkCAT promoter which contains only one VDR binding site (data not shown). The addition of 9-cis-RA had no effect on the response of VDRE3tkCAT to MC903 in ROS 17/2.8 cells (Fig. 8C, lanes 5 and 6; Fig. 8D, fifth and sixth lanes). The action of 50 nM 9-cis-RA was tested further on the response to 1,25-(OH)2 D3 derivatives 1,25-(OH)2-16-ene-23-yne D3, 1,25-(OH)2-16-ene-23-yne-26,27-hexafluoro D3, and 1α-fluoro-25-(OH)-16-ene-23-yne-26,27-hexafluoro D3 in ROS 17/2.8 cells (Fig. 9). Consistent with the results of Fig. 8, 9-cis-RA had no significant effect on the activation of CAT activity observed in the presence of 10-7 M derivative or 10-6 M 1,25-(OH)2 D3. We have also repeated all of the experiments of Figs. 8 and 9 performed in ROS 17/2.8
cells with 1 μM instead of 50 nM 9-cis-RA and have obtained essentially identical results (data not shown).

The activities of 9-cis-RA and of RXRs in COS-7 and ROS 17/2.8 cells were tested using a promoter containing a single RXR response element composed of a direct repeat with 1-bp interrepeat spacing. This element has been shown to bind RXR homodimers in the presence of 9-cis-RA (Zhang et al., 1992). The promoter is stimulated 3-fold by 50 nM 9-cis-RA in transiently transfected COS-7 cells (Fig. 8E, lanes 1 and 3; Fig. 8F) and 2-fold in ROS 17/2.8 cells (Fig. 8E, lanes 4 and 5; Fig. 8F). All-trans-RA is inactive on the RXRE-containing promoter in COS-7 cells at 50 nM (Fig. 8E, lanes 1 and 2). Strong responses were observed in both cell lines to 1 μM 9-cis-RA using RXRE-containing promoters as well as an analogous recombinant containing the response element from the RARβ gene, RAREβ (data not shown). The results of Figs. 7 and 8 provide strong evidence for the presence of active RXR(s) in both COS-7 and ROS 17/2.8 cells and show that the 9-cis-RA is active and will stimulate a promoter containing a single RXR response element while not affecting an analogous promoter containing three VDREs.

Taken together the results of Figs. 1 and 7–9 suggest that although RXR(s) are active in both COS-7 and ROS 17/2.8 cells and apparently participate in binding of the VDR to the MOP VDRE, their cognate ligand 9-cis-RA does not contribute to the transcriptional response of promoters containing VDREs to 1,25-(OH)₂ D₃ or any of its derivatives.

**DISCUSSION**

A synthetic 1,25-(OH)₂ D₃-responsive expression vector has been constructed to analyze the transcriptional response to 1,25-(OH)₂ D₃ and a number of its derivatives. Expression is controlled by consensus VDREs and a truncated herpes simplex virus tk promoter, rendering the vector selectively responsive to 1,25-(OH)₂ D₃ and its derivatives. Vectors of this type have been used to study the transcriptional activity of a number of other nuclear receptors (Kumar et al., 1987; Yu et al., 1991). Transcription was analyzed in transiently transfected...
The VDRE3tk promoter has been used to test the transcriptional response to 1,25-(OH)₂ D₃ and 10 of its derivatives, many of them potentially clinically useful. A number of observations can be made based on the results of Figs. 5 and 6 and Table I. First, the peak transcriptional activation observed did not vary significantly among 1,25-(OH)₂ D₃ and any of its active derivatives, indicating that the transcriptional activity of the ligand-bound VDR was similar in each case. In other words, none of the compounds tested gave rise to a superactivated receptor. Second, the introduction of multiple bonds to 1,25-(OH)₂ compounds at positions 16 and/or 23 invariably gave rise to derivatives that stimulated CAT activity at 10- to more than 100-fold lower concentrations than 1,25-(OH)₂ D₃. These results are consistent with numerous observations that many of the derivatives tested here as well as related compounds are more potent inhibitors of cellular replication than 1,25-(OH)₂ D₃ (Tanaka et al., 1984; Eisman et al., 1986; Zhou et al., 1990, 1991). Derivatives with low affinities for the VDR were weak activators. However, for those derivatives with a moderate to high affinity for the receptor there was no strict correlation between the affinity of a given derivative for the VDR and the concentration at which half-maximal CAT activity was observed (Figs. 5 and 6). This indicates that parameters other than affinity for the receptor also control the transcriptional activity of a given derivative.

There are several possible explanations for the observations that 1,25-(OH)₂ D₃ derivatives activate transcription highly potently. The high affinity of 1,25-(OH)₂-16-ene D₃ for the VDR (Table I) suggests that introduction of a double bond at position 16 apparently fixes the side chain in a conformation that is similar to that bound by the receptor. In addition, recent studies have shown that both 1,25-(OH)₂ D₃ and 16-ene derivatives are metabolized to -24-oxo compounds. The 16-ene-24-oxo metabolite is stable in cells, whereas 1,25-(OH)₂-24-oxo D₃ is rapidly degraded to calcitriol. Interestingly, although calcitriol is inactive, 1,25-(OH)₂-16-ene-24-oxo D₃ is more potent than its parent compound in stimulating differentiation of the HL60 promyelocytic leukemia cells. Some derivatives may be metabolized to as yet unidentified compounds that have an unusually high affinity for the VDR or high stability. This could render some compounds selectively active depending on the metabolic products formed in a given cell type. Further substitution of 16-ene derivatives at positions 23 and 26 or 27 generates compounds that are more resistant to inactivation by metabolism. Introduction of multiple bonds at position 23 or fluorination at positions 26 and 27 provides resistance to inactivation by hydroxilation (Ikekawa, 1983). A 1,25-(OH)₂ D₃ derivative hexafluorinated at positions 26 and 27 is more potent and has longer lasting biological effects than its parent compound (Tanaka et al., 1984). It is also possible that derivatives are present in cells at higher concentrations because they are bound with less affinity than 1,25-(OH)₂ D₃ by component(s) of serum. We note in this regard that the curves of activation of CAT activity obtained with 1,25-(OH)₂ D₃ are not significantly affected if experiments are performed in 2% or 10% serum (data not shown). Finally the potency of a given derivative could be dependent on the specific components of vitamin D₃-dependent transcription complexes which may vary among different normal cell types and between normal and transformed cells.

Derivatives lacking a α-hydroxy group are generally inefficient activators (Fig. 6). The synthetic derivative 25-(OH)-16,23E-diene and 25-(OH)₂ D₃ both stimulated CAT activity equivalent to half-maximal activity observed with 1,25-(OH)₂ D₃ at 200 nm or higher. Although 25-(OH)-16,23E-diene was a
Summary of receptor binding and transcriptional activation characteristics of 1,25-(OH)_2 D_3 and derivatives

| Derivative | Efficiency of competition for [3H]1,25-(OH)_2 D_3 binding | EC_{50} transcriptional activation | Peak transcriptional activation |
|------------|----------------------------------------------------------|----------------------------------|---------------------------------|
| 1,25-(OH)_2 D_3  | 100% | 15 | 100 |
| 1,25-(OH)_2-16-ene D_3 | 240 | 0.9 | 118 |
| 1,25-(OH)_2-16,23E-diene D_3 | 145 | 0.15 | 93 |
| 1,25-(OH)_2-16,23-yne D_3 | 80 | 0.15 | 88 |
| 1,25-(OH)_2-23-yne D_3 | 51 | 0.01 | 96 |
| 24,25-(OH)_2 D_3 | 39 | 1.5 | 98 |
| 25-(OH)-16,23E-diene D_3 | 0.017 | >1,000 | 75* |
| 1β,25-(OH)_2-16-ene-23-yne D_3 | 0.12 | 150 | 118* |
| 1,25-(OH)_2-16,23-yne-26,27-F_6 D_3 | 0.4 | >1,000 | 50* |
| 1α-F-25-OH-16-ene-23-yne-26,27-F_6 D_3 | 54 | 0.01 | 86 |
| 24,25-(OH)_2 D_3 | 3.8 | 0.7 | 118 |

Fig. 7. Evidence for the presence of RXR(s) in specific complexes formed by the VDR on the MOP VDRE. Panel A, specific binding to the MOP VDRE by components of ROS 17/2.8 nuclear extracts. Gel retardation assays were performed with the MOP VDRE (lanes 1, 2, and 5) or a mutant MOP VDRE (lane 3). See Fig. 1 for sequences of oligonucleotides. Assays were performed using 1 µl of ROS 17/2.8 nuclear extract (ROS N/E; lanes 1–3), 0.25 µl of bacterial extract expressing the VDR (VDR/E. coli; lanes 2 and 4), and 1 µl of a whole cell extract of COS-7 cells transiently transfected with expression vectors for the VDR and human RXRα (RXR/VDR/COS; lane 5). Panel B, Northern blot of 5 µg of poly(A)+ RNA from ROS 17/2.8 cells performed as detailed under “Experimental Procedures.” The blots were exposed for 3 days. The estimated sizes of mRNAs (indicated by short arrowheads) are 5.5 kilobases for RXRα and 3.0 and 2.5 kilobases for RXRβ.

levels of the 1α-hydroxylase enzyme which would convert a 25-(OH) compound into the more active 1,25-(OH)_2-derivative. Substitution of the 1α-hydroxyl group of a derivative with a fluor group gave rise to a potent activator in the case studied. The derivative 1β-fluoro-25-hydroxy-26,27-hexafluoro-16-ene-23-yne stimulated CAT activity 10-fold more potently than 1,25-(OH)_2 D_3 although it was a less potent activator than its 1,25-dihydroxy counterpart (Fig. 6). In contrast, 1β-fluoro-25-(OH)_2-16-ene-23-yne was a very weak activator, consistent with its low affinity for the VDR (Fig. 6 and Table I). In summary, the above results demonstrate that 1,25-(OH)_2 derivatives substituted at positions 16 and 23 can be highly potent activators of the vitamin D_3 receptor and indicate that weak activator, its 1,25-dihydroxy counterpart was almost 100-fold more potent than 1,25-(OH)_2 D_3 (Fig. 5). This raises the possibility that derivatives lacking a 1α-hydroxyl group may be selectively active in tissues expressing both the VDR and high.
many parameters other than affinity for the VDR control the activity of a given compound. The assay developed here should be applicable to test rapidly the transcriptional activity of a large number of clinically significant derivatives of 1,25-(OH)$_2$D$_3$ in a wide variety of cell lines.

Several studies have shown that the VDR binds to response elements like the MOP VDRE as a heterodimer with a cofactor that has been identified as RXR (Sone et al., 1991; Yu et al., 1991; Ross et al., 1992). Here, RXRα and RXRβ, but not RXRγ, were detected in ROS 17/2.8 cells by Northern blotting, and evidence was provided that RXRs present in both COS-7 and ROS 17/2.8 cells heterodimerize with the VDR (Figs. 1 and 7). Transcriptional responses were observed in both cell lines with a promoter containing a single RXRE to both 50 nm and 1 μM 9-cis-RA, the cognate ligand for RXRs (Fig. 8 and data not shown). Taken together these results demonstrate the presence of functional RXR(s) in these cells which can form heterodimers with the VDR on the MOP VDRE. Several experiments were performed in both cell lines to test the effect of 9-cis-RA alone and in combination with 1,25-(OH)$_2$D$_3$ on transcription from the VDRE3tkCAT recombinant. We failed to observe a contribution of 9-cis-RA to the hormone response of the VDRE3 promoter, although a slight inhibitory effect of 9-cis-RA on the response to 1,25-(OH)$_2$D$_3$ was observed in some experiments in both cell lines (Fig. 8 and data not shown). Similar experiments in ROS 17/2.8 cells provided no evidence for modulation by 9-cis-RA of the response to several active 1,25-(OH)$_2$D$_3$ derivatives (Fig. 9). In addition, increas-
ing the concentration of 9-cis-RA from 50 nm to 1 μM had no significant effect on the response to 9-cis-RA (data not shown). These data indicate that under the conditions used here 9-cis-RA does not contribute significantly to the transcriptional response to 1,25(OH)2D3.

Recent studies have shown that 9-cis-RA activated a synthetic promoter containing the MOP VDRE both in the absence and presence of 1,25(OH)2D3 in transiently transfected Droso-
sophila S-3 cells, which lack endogenous RXRs (Carlb erg et al., 1993). The activation of a promoter containing a VDRE by 9-cis-RA alone, apparently through a heterodimer, suggests that under certain conditions this ligand may activate trans-
scripti on from promoters containing a wide variety of elements that bind RXR-containing heterodimers, including those responsive to all-trans-RA and thyroid hormone.

These observations are in apparent contrast to the results presented here. There are, however, several differences between the two studies. Our experiments were performed using endogenous levels of both VDRs and RXRs present in COS-7 and ROS 17/2.8 cells, which are very likely to be lower than those obtained in transiently transfected cells. It cannot be argued that the levels of RXRs are too low to observe a response to 9-cis-RA in the cells tested here. Our evidence and that of others suggests that the VDR binds to the MOP VDRE and activates as a heterodimer with RXRs. This indicates that RXR levels are sufficient to support a strong transcriptional response to 1,25(OH)2D3. We have also observed activation of the VDRE3tkCAT promoter by 9-cis-RA alone in the presence of a transiently transfected VDR expression vector in both COS-7 and ROS 17/2.8 cells (data not shown), suggesting that high concentrations of RXR are required for 9-cis-RA-dependent activation of promoters containing VDREs. RXR homodimers bound in vitro, albeit with low affinity, to a response element composed of a direct repeat of PurGGTCA motifs separated by 3 bp (Mader et al., 1993). It is possible, then, that the response of a promoter containing a VDRE to 9-cis-RA could be caused, at least in part, by the action of ligand-activated RXR ho-
modimers. Carlb erg et al. (1993) used a promoter containing a single VDRE, whereas VDRE3tkCAT contains three elements. However, we have performed experiments with 9-cis-RA in ROS 17/2.8 cells with VDREtkCAT which contains only one element and has obtained results similar to those presented in Fig. 8 (data not shown). Finally, there may exist intracellular factors known as coactivators or adapters (Lewin, 1990) which specifically link VDR/RXR heterodimers activated by 9-cis-RA to the transcription apparatus which are present in SL-3 cells but absent in the cell lines used here, raising the possibility that the response of VDRE-containing heterodimers to 9-cis-RA may be cell-specific.

Our results suggest that 1,25(OH)2D3 and 9-cis-RA activate transcription by separate pathways in ROS 17/2.8 and COS-7 cells. According to this hypothesis, promoters containing response elements like the MOP VDRE are bound selectively by heterodimers of the VDR and RXRs(s) which are activated by 1,25(OH)2D3, but not significantly affected by 9-cis-RA. Pro-
moters containing RXREs would be bound by RXR homodimers and activated by 9-cis-RA. It remains to be seen how generally this scheme can be applied to the wide variety of both normal and transformed cell types expressing the VDR.

Acknowledgments—We are grateful to Dr. Pierre Chambron for the VDR (cDNA), to Dr. J. Wesley Pike for the VDR cDNA, to Dr. Peter Sorter (Hoffmann LaRoche) for 9-cis-RA, to Dr. L. J. Fraber and Leo Laboratories (Ballup, Denmark) for MC903, and to Dr. J. McLane (Hoffmann LaRoche) for communicating unpublished results. We thank Drs. Sylvie Mader and Bernard Turcotte for critically reading the manuscript.
Transcriptional Activation by Vitamin D₃ Derivatives

Stein, G., and Lian, J. B. (1993) Endocr. Rev. 14, 424–442
Studier, F. W., and Moffatt, B. (1986) J. Mol. Biol. 189, 113–130
Tanaka, Y., DeLuca, H., Kobayashi, Y., and Ikekawa, N. (1984) Arch. Biochem. Biophys. 229, 349–354
Terpening, C. M., Haussler, C. A., Jurutka, P. W., Galligan, M. A., Kommm, B. S., and Haussler, M. R. (1991) Mol. Endocrinol. 5, 373–385
Tora, L., White, J. H., Brou, C., Tasset, D., Webster, N., Scheer, E., and Chambon, P. (1989) Cell 56, 477–487
Umesono, K., Murakami, K. K., Thompson, C. C., and Evans, R. M. (1991) Cell 65, 1255–1265
Uskokovic, M. R., Baggiozini, E., Shuey, S.-J., Iacobelli, J., Hennessey, B., Kiegel, J., Daniewski, A. R., Pizzolato, G., Courtney, L. F., and Horst, R. L. (1991) in Vitamin D: Gene Regulation, Structure-Function Analysis and Clinical Application (Norman, A. W., Bouillon, R., and Thomasset, M., eds) pp. 139–145.

Walter de Gruyter, New York
Wasserman, R. H., Corradino, R. A., Fulmer, C. S., and Taylor, R. (1974) Vit. Horm. 32, 299–324
Webster, N., Jin, J. R., Green, S., Hollis, M., and Chambon, P. (1986) Cell 54, 199–207
Yu, V. C., Delsert, C., Andersen, B., Holloway, J., Devary, O. V., Naar, A., Kim, S. Y., Beutin, J. M., Glass, C. K., and Rosenfeld, M. G. (1991) Cell 67, 1251–1266
Zhang, X.-K., Hoffman, B., Tran, P. B.-V., Graupner, G., and Pfah1, M. (1992) Nature 355, 441–444
Zhou, J.-Y., Norman, A. W., Chen, D.-L., Sun, G.-W., Uskokovic, M., and Koeffler, H. P. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 3929–3932
Zhou, J.-Y., Norman, A. W., Akashi, M., Chen, D.-L., Uskokovic, M., Arrucoechea, J. M., Dauben, W. G., Okamura, W. H., and Koeffler, H. P. (1991) Blood 1, 75–82