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Vitamin D Represses Retinoic Acid-Dependent Transactivation of the Retinoic Acid Receptor-β2 Promoter: The AF-2 Domain of the Vitamin D Receptor Is Required for Transrepression*

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ABSTRACT

Retinoic acid (RA)-dependent activation of the RA receptor β2 (RARβ2) gene in embryonal carcinoma cells is mediated by binding of retinoid receptor heterodimers (RAR/RXR) to a RA response element (RARE) located closely to the TATA box. We have analyzed the effect of vitamin D on the response of the RARβ2 promoter to RA in pituitary GH4C1 cells that coexpress receptors for retinoids and vitamin D. Incubation with vitamin D markedly reduced the response to RA caused by transcriptional interference of the vitamin D receptor (VDR) on the RARE. This DNA element binds VDR/RXR heterodimers with high affinity, and these inactive heterodimers can displace active RAR/RXR from the RARE. Overexpression of RXR in GH4C1 cells, as well as incubation with BMS649 (a RXR-specific ligand), increased the inhibitory effect of vitamin D, suggesting that the VDR/RXR heterodimer is the repressive species and that titration of RXR is not responsible for this inhibition. Although DNA binding could be required for full potency of the inhibitory activity of VDR, it is not absolutely required because a truncated receptor (VDR Δ1–111), lacking the DNA binding domain, also displays repressor activity. Furthermore, the ability to mediate transrepression by vitamin D was strongly decreased when a mutant VDR in which the last 12 C-terminal aminoacids have been deleted (VDR ΔAF-2) was used. Because this region contains the domain responsible for ligand-dependent recruitment of coactivators, titration of common coactivators for VDR and RAR could be involved in the inhibitory effect of vitamin D. In agreement with this hypothesis, overexpression of E1A, which can act as a RARβ2 promoter-specific coactivator, significantly reversed repression by vitamin D. (*Endocrinology* **140:** 2898–2907, 1999)

THE NUCLEAR RECEPTORS for vitamin D (VDR) and retinoic acid (RAR) are ligand-inducible transcription factors that exert their actions by binding, preferentially as heterodimers with the retinoid receptor RXR, to hormone response elements (HREs) located in regulatory regions of target genes. These receptors are members of a larger group of factors that also include the thyroid and steroid hormone receptors, as well as orphan receptors (1–2). The nuclear receptors exhibit a modular structure consisting of regions A through E or F. The A/B region contains an autonomous ligand-independent transactivating function (AF-1). This is an atypical region in VDR because it contains only 20 aminoacids, and deletion of these residues does not seem to alter receptor function (3). Region C, the most conserved throughout the superfamily, contains the two zinc finger structures responsible for DNA binding; the flexible hinge D region connects the DNA binding domain (DBD) with region E, which contains the ligand binding domain (LBD), the dimerization domain, and a C-terminal ligand-dependent transactivation function (AF-2) (4–6).

Naturally occurring and synthetic HREs are normally composed of at least two copies of the consensus AGGTCA motif arranged as direct repeats (DRs), palindromes, or inverted repeats. The heterodimers bind with a specific polarity in the DRs (7, 8). Studies on the spacing of half-sites, arranged as DRs, have demonstrated that VDR preferentially mediates ligand-dependent transactivation via a DR separated by three nucleotides (DR3), whereas RAR transactivates via DRs with a two- or five-nucleotide gap (9). Although the orientation and spacing of the half-sites can determine selective transcriptional responses, specificity is not total, and some HREs can bind different heterodimers with high affinity. It has been shown that VDR/RXR can bind not only to DR3 but also to DR4, DR5, and palindromic elements (10, 11). In some cases, retinoic acid (RA) and vitamin D can cooperate to stimulate transcription through the same element. However, only a subset of receptor DNA binding elements function as response elements. Thus, VDR/RXR can bind to RA response elements (RAREs) in a transcriptionally inactive form; and under these circumstances, vitamin D can inhibit the response to RA (10). This suggests that transcriptional responses to RA could be selectively repressed in cells containing receptors for vitamin D.

The RARβ2 promoter has been extensively used as a model for transcriptional regulation by RA. This promoter contains a strong and well-characterized DR5-type RARE (βRARE) located at nucleotides −37 to −53, with respect to transcription initiation (12–14). More recent studies have

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shown the presence of a weaker RARE at positions −67 to −83 (15). The close proximity of the RAREs to the TATA box, rather than the arrangement of these elements, seems to be crucial to establish productive interactions of the receptor heterodimers with neighboring components of the basal transcriptional machinery (15). It has been shown that ligand-dependent transcriptional activation by nuclear receptors depends on the presence of an autonomous AF-2 in the LBD. Recent studies have solved the crystal structure of the LBDs of the apo RXR and holo RAR (16, 17). These studies have proven that RA binding induces a structural modification in helix 12 of the LBD that contains the AF-2. This change allows the recruitment of coactivator proteins and a ligand-dependent transcriptional activation. Different families of coactivators (including SRC-1/NCoA-1, TIF2/GRIP1/NCoA-2, pCIP/ACTR/AIB1/RAC3/TRAM1, and CBP/p300, a cointegrator of multiple signal transduction pathways) have been recently cloned (18, 19). Although these proteins can each serve as coactivators of several nuclear receptors, cell-type-specific or receptor-specific proteins may also play important roles in controlling transcriptional responses to different nuclear receptor ligands. The RARβ2 promoter is strongly activated by RA in undifferentiated embryonal carcinoma (EC) cells, and the TATA binding protein (TBP) functionally cooperates with RAR in the transactivation of this promoter. This cooperation seems to require the presence of a cell-specific E1A-like activity (E1A-LA), which is lost during differentiation (20, 21). Ectopic expression of the adenovirus E1A(13S) protein in differentiated cells is a prerequisite for TBP and RAR cooperation (20). It has been shown that E1A directly interacts with the AF-2 domain of RAR (22). Because E1A also associates with TBP (23), it has been proposed that E1A functions as a coactivator or bridging protein by interacting with both TBP and RAR, thereby stabilizing the preinitiation complex (22).

In this study, we have analyzed the influence of vitamin D on the activation of the RARβ2 promoter by RA in pituitary GH4C1 cells that coexpress RAR and VDR. Our results show that vitamin D exerts a strong dominant negative activity on RA-mediated transcriptional stimulation of this promoter. VDR/RXR heterodimers bind to the βRARE and can displace RAR/RXR binding in *in vitro* gel retardation assays. Although competition for DNA binding by transcriptionally inactive VDR/RXR heterodimers may contribute to this inhibitory response, our data also show that a mutant VDR, lacking the A/B domain and the DBD, also displays a dominant negative activity. Furthermore, an AF-2 defective C-terminally truncated VDR was unable to mediate transrepression, suggesting that titration of coactivators is involved in the inhibitory effect of vitamin D. In agreement with this hypothesis, overexpression of E1A significantly reverses the inhibition caused by vitamin D.

Materials and Methods

Plasmids

R-140 CAT construct, containing the fragment −124 to +14 of the human RARβ2 promoter, was obtained from the previously described R-140 Luc (14). The promoter fragment was obtained by PCR with the oligonucleotides 5′-GGGAAGCTTGGATCCTGGGAGTTGGT-3′ and 5′-GCTCTAGAGCTCACTTCCTACTAC-3′ and subcloned in *Hin*dIII and *Xba*I sites of pBLCAT8+, replacing the thymidine kinase (tk) promoter. The same strategy was used to obtain R-140 (M3) and R-140 (M7) from the parental constructs (14). In these plasmids, the 3′ (M3) and 5′ (M7) half-sites of the proximal RARE have been mutated. In the construct R-90 CAT, the sequences from −59 to +14 of the RARβ2 promoter were similarly cloned into the *Hin*dIII/*Xba*I sites of pBLCAT8+. In the plasmid Spp-1-tk-CAT, the oligonucleotide 5′-AGCTTGACCAA-CAAGGTTCACGAGGTTCACGTCTCT-3′, conforming the VDRE of the osteopontin promoter, was cloned into the *Hin*dIII and *Xba*I sites of pBLCAT8+ in front of the tk promoter. Expression vectors for RAR, RXR, and VDR contain the complementary DNA sequences of the α form of the human RAR (pRS-hRARα) (24), the human RXR α (25), and the human VDR (26). The expression vector for the ΔAF2 VDR mutant was constructed by PCR using the VDR expression plasmid as a template and the oligonucleotides 5′-GGAATTCCATGGAGGAGGAGGCCTTTG-3′ and 5′-CGGGATCCTCAGGAGATCTCATTGCC-3′ to generate a 872-bp fragment. This fragment was digested with *Bst*XI and *Bam*HI and cloned into the pSG5 expression vector. This receptor lacks the last 12 C-terminal aminoacids, which contain the AF-2 region. For the Δ1–111 VDR mutant, the oligonucleotides 5′-GGAATTCCATGGAGGAGGAG-GCCTTG-3′ and 5′-CGGGATCCTCAGGAGATCTCATTGCC-3′ were used to generate a 972-bp fragment, which was digested with *Eco*RI and *Bam*HI and subcloned into pSG5. This construct generates a truncated receptor lacking 111 N-terminal aminoacids which include the A/B region and the DBD (C region). The expression vectors for CBP, SRC-1, TFIIB, TBP, and E1A(13S) have been previously described (20, 27, 28)

Cell culture, transient transfections, and CAT assays

GH4C1 cells, cultured in DMEM containing 10% FCS, were transfected by electroporation, as previously described (10). Twenty-five micrograms of the reporter plasmids were mixed with 20–30 million cells and exposed to a high-voltage pulse (200–250 V, 960 μF). When indicated, the reporter plasmid was cotransfected with the same amount of expression vectors for the receptors VDR, RAR, or RXR. The cells from each electroporation were split into different culture plates in DMEM. COS-7 cells were cultured in DMEM containing 10% FCS and were plated 24 h before transfection into 60-mm dishes. The cells were then transfected with calcium phosphate with 5 μg of the reporter plasmid (plus 100 ng of a luciferase internal control plasmid). 2.5 μg of VDRs were used in cotransfection assays. In all cases, the total amount of DNA from each transfection was kept equal by addition of the corresponding empty expression vectors (RSV-0 and pSG5–0). Treatments with vitamin D and retinoids were administered in serum-free medium. After 48 h of treatment, CAT activity was determined by incubation of the cell extracts with [^{14}C]chloramphenicol. The unreacted and acetylated [^{14}C]chloramphenicol were separated by TLC and quantified with an Instantimager. Each treatment with the ligands was performed at least in duplicate cultures that normally exhibited less than 10% variation in CAT activity, and the experiments were repeated at least two or three times with similar relative differences in regulated expression. The results are normally expressed as the mean ± SD of the CAT values obtained.

Western blot analysis

Extracts from COS-7 cells, transfected with wild-type or mutant VDRs or with the noncoding vector pSG5–0, were run in 10% or 12% polyacrylamide gels. The proteins were transferred to a nitrocellulose membrane and incubated with either a 1:3000 dilution of the polyclonal antibody (sc-1008x; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) that recognizes the C-terminal region of VDR, or the same dilution of a polyclonal antibody (MAB1360, Chemicon International, Inc., Temecula, CA) raised against the DBD. The proteins were identified by chemoluminescence.

Gel retardation assays

The vectors for VDR, RAR, and RXR (cloned in pSG5) were used for *in vitro* transcription and translation with TNT Quick (Promega Corp., Madison, WI). The efficiency of expression, assessed in parallel reactions using ^{35}S[methionine], was similar for all of them. As a probe, we used the oligonucleotide 5′-GGGTAGGGTTCACCGAAAGTTCACTCG-3′ corresponding to the proximal RARE of the RARβ2 promoter. For the

binding reaction, the proteins were incubated on ice for 15 min in a buffer (10 mM HEPES-(KOH) (pH 7.9), 80 mM KCl, 1 mM dithiothreitol, 5% Ficoll) containing 3 μg poly (dI-dC) and then for 15–20 min at room temperature with approximately 50,000 cpm of labeled double-stranded oligonucleotide end-labeled with [^{32}P]cytidine 5′-triphosphate. For competition experiments, an excess of unlabeled doubled-stranded oligonucleotides were added to the binding reactions. As a DR-3 type, we used the Spp-1 VDRE 5′-ACAAGGTTCACGAGGTTCACGTCT-3′, and the sequence 5′-CCAGCCATGAATAAATGTTATAGGG-3′ was used as an unrelated oligonucleotide. For supershift experiments, 1 μl of specific antibodies against VDR (αVDR) and RXR (αRXR) were added to the binding reactions before the addition of the labeled fragment. Finally, DNA-protein complexes were resolved on 6% polyacrylamide gels in 0.5× Tris-Borate-EDTA buffer. The gels were then dried and autoradiographed at −70 C.

Results

Vitamin D inhibits the transcriptional response of the RARβ2 promoter to RA in GH4C1 cells

To analyze the effect of vitamin D on RA-mediated transactivation, the construct R-140 CAT containing the RARβ2 promoter was transfected into GH4C1 cells. Fig. 1A shows that treatment with 1 μM RA increased promoter activity by approximately 5-fold in these cells. Incubation with concentrations of vitamin D, ranging between 0.1 and 100 nM, did not affect basal activity of this plasmid, but it strongly reduced induction by RA. The inhibition of the transcriptional response to RA was dose dependent. A half-maximal inhibition was found at approximately 1 nM, and 10 nM vitamin D caused a maximal reduction. The influence of RA and vitamin D, in a construct that contains the DR-3 type VDRE present in the osteopontin promoter fused to the tk promoter (SPP-1-tk-CAT), was also analyzed in GH4C1 cells. Incubation with increasing concentrations of vitamin D transactivated this promoter with a dose dependence similar to that observed for transrepression of the RARβ2 promoter. A half-maximal effect was found at approximately 1 nM vitamin D, and a maximal stimulation of 6-fold was obtained at 10 nM. Interestingly, incubation with 1 μM RA increased basal SPP-1-tk-CAT activity, although less strongly than vitamin D, and potentiated the effect of vitamin D at the entire range of concentrations used. Therefore, vitamin D can either interfere with the response to RA on a DR-5 element, or it can have an additive effect with the retinoid on a DR-3 element.

The contribution of the two RAREs of the RARβ2 promoter in the regulation by RA and vitamin D was analyzed in GH4C1 cells transfected with the R-140 CAT plasmid, in which both RAREs are present, or else with a plasmid containing a shorter promoter fragment (R-90) in which the distal RARE has been deleted. In addition, two R-140 CAT constructs (M3 and M7) in which the 3′ or 5′ hemisites of the proximal RARE have been mutated were also used. As shown in Fig. 2B, deletion of the distal RARE did not decrease, but rather increased the response to RA in GH4C1 cells. In addition, vitamin D reduced the response of the shorter construct to the retinoid, showing that the proximal RARE is sufficient to mediate a potent transrepression by the vitamin. Mutation of the proximal RARE strongly reduced the response to RA, again demonstrating that this element is the main one responsible for transactivation by RA, and vitamin D blocked the residual stimulation of the mutant promoter. These data suggest that the proximal βRARE mediates the repressive effect of vitamin D. This was also demonstrated by the finding that vitamin D reduced similarly the effect of RA on the natural RARβ2 promoter, and in an heterologous construct (βRARE-tk-CAT), in which this RARE was cloned in front of the tk promoter (data not shown).

VDR/RXR heterodimers bind to the βRARE

To study whether the repressive action of VDR could involve competition for DNA binding, *in vitro*-translated VDR, RAR, and RXR were used for gel retardation assays with the βRARE element (Fig. 3). In the absence of RXR, neither RAR nor VDR bound to this element. However, in the presence of the heterodimeric partner, not only RAR (lane 5), but also VDR (lane 7), bound strongly to the βRARE. The identity of the VDR/RXR heterodimer was demonstrated by the finding that anti-VDR and anti-RXR antibodies (lanes 14 and 15, respectively) produced a supershift of the retarded band. In addition, the formation of the VDR/RXR heterodimer was efficiently competed by an excess of the βRARE and the strong SPP-1 VDRE element (DR3spp-1) but not by an unrelated oligonucleotide (NR).

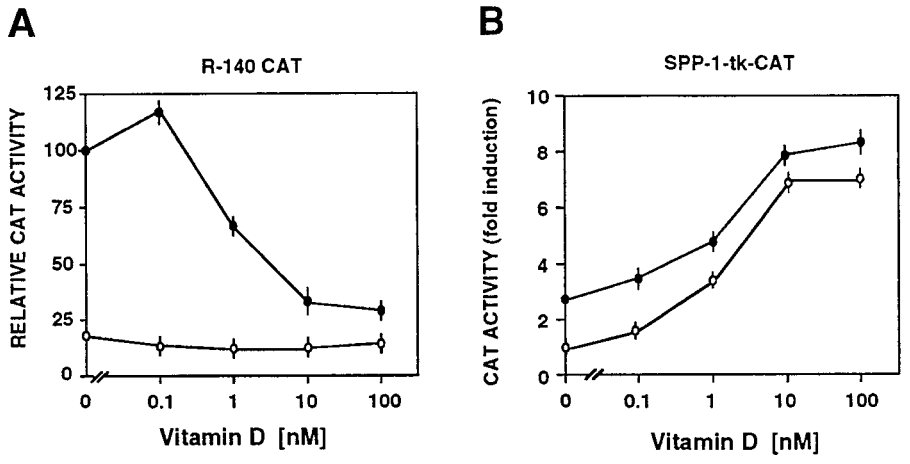

FIG. 1. Influence of vitamin D on transcriptional responses to RA in GH4C1 cells. A, Cells were transfected with the reporter plasmid R-140 CAT, which contains the fragment −124 to +14 of the RARβ2 promoter. After transfection, the cells were treated with increasing concentrations of vitamin D alone (*empty circles*) or in combination with 1 μM RA (*full circles*). CAT activity was determined after 48 h. The data are expressed as a percentage of the values obtained in the cells treated with RA in the absence of vitamin D. B, GH4C1 cells were transfected with Spp-1-tk-CAT, which contains the VDRE of the osteopontin promoter cloned upstream of the tk reporter. Treatments were the same as in A, and CAT activity is expressed as fold induction over the values obtained in the untreated cells.

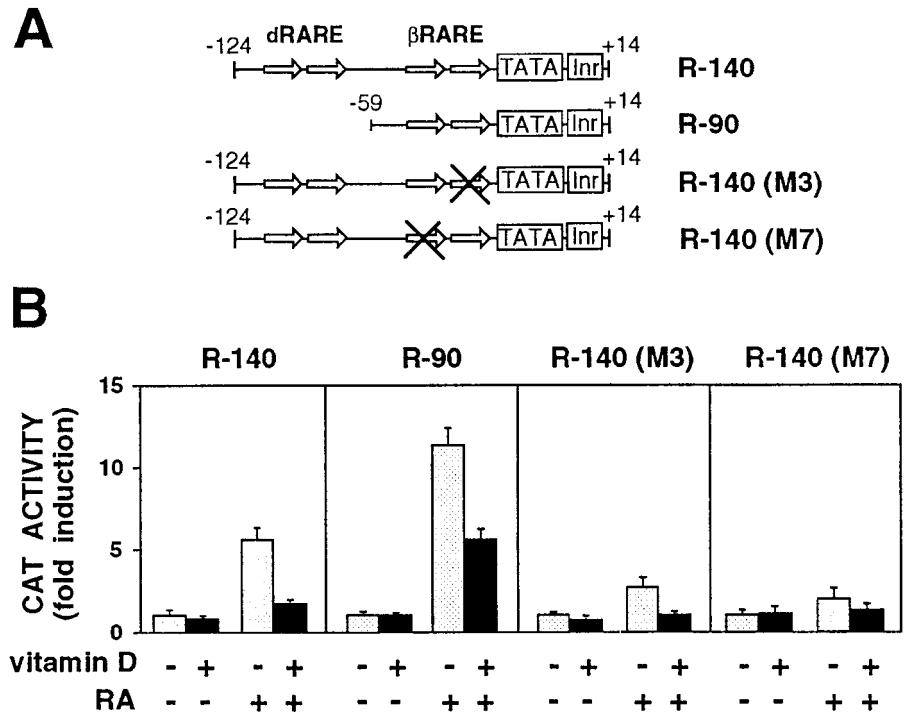

FIG. 2. Regulation of RARβ2 promoter constructs by RA and vitamin D. A, Schematic illustration of the constructs used for transient transfection in GH4C1 cells, showing the location of the TATA box, the initiator (Inr), and the proximal and distal RAREs (indicated by *arrows*). R-140 contains both RAREs, whereas R-90 contains only the proximal element. In R-140 (M3) and R-140 (M7), the 3′ and 5′ motifs of the proximal RARE have been mutated. B, CAT activity was determined in cells transfected with the constructs indicated above and incubated with 1 μM RA in the presence and absence of 100 nM vitamin D. CAT activity is expressed relative to the values obtained in the corresponding group of untreated cells.

Influence of overexpression of VDR and RAR on the repressive effect of vitamin D

The levels of VDR and RAR should determine the amount of each of these receptors bound to the response element, as well as the net transcriptional response of the RARβ2 promoter to vitamin D and RA. To analyze the effect of ratio variations of these receptors, the cells were cotransfected with the R-140 CAT plasmid and expression vectors encoding VDR or RAR. As shown in Fig. 4A, cotransfection with RAR significantly reverted the repression of the RA response caused by vitamin D. Under these conditions, 1 nM vitamin D did not reduce the response to RA, and 100 nM vitamin D had an effect similar to that elicited by 1 nM vitamin D in nontransfected cells. On the other hand, the dose-response curve shifted to the left in cells transfected with VDR, and the repressive effect of the vitamin was further enhanced. It should be noted that this repression was strictly ligand-dependent, because overexpression of VDR altered neither basal promoter activity nor the response to RA.

The effect of varying the concentrations of VDR and RAR in the presence of a constant amount of RXR on binding to the βRARE is illustrated in Fig. 4B. Lanes 4–8 show that VDR reduced RAR/RXR binding to DNA in a dose-dependent manner. This reduction was accompanied by an increase in VDR/RXR binding, although even at a VDR:RAR, 6:1 ratio (lane 8), binding of VDR/RXR was weaker than that found in the absence of RAR (lane 3). Similarly, increasing the amount of RAR in the assay reduced VDR/RXR binding. A complete inhibition was observed, even at a 1:0.75 ratio (compare lanes 10 and 11), but again the intensity of the RAR/RXR band in the presence of VDR (lane 14) was weaker than that found in the absence of VDR (lane 9). This suggests a formation of VDR/RAR heterodimers that would be unable to bind to the βRARE. On the other hand, these results imply that when the amounts of RXR are limiting, the binding of both active RAR/RXR heterodimers and inactive VDR/RXR heterodimers to the βRARE could be impaired.

Role of RXR on the response to VDR

In the experiment shown in Fig. 5A, it can be observed that transfection with RXR enhanced the repressive effect of vitamin D. This suggests that when RXR is not limiting the amount of VDR/RXR bound to the βRARE could increase. The influence of increasing concentrations of RXR on *in vitro* RAR and VDR binding to the βRARE was analyzed in gel retardation assays. As shown in Fig. 5B, when similar quantities of RXR, VDR, and RAR were used (lanes 6 and 7), RAR/RXR heterodimers were preferentially bound to the βRARE. However, the presence of an excess of RXR allows simultaneous binding of VDR/RXR and RAR/RXR heterodimers (lane 9). This binding was similar to that obtained when VDR (lane 3) or RAR (lane 4) was independently incubated in the presence of a high concentration of RXR. Lane 5 shows that binding of VDR/RAR to the βRARE was not observed.

The above observations suggest that the VDR/RXR heterodimer is responsible for the repressive effect of vitamin D on the response to RA. To analyze a possible role of the RXR ligand on this repression, the cells were treated with vitamin D in the presence and absence of 100 nM BMS649 (a RXR-specific ligand). Because RXR (although with less affinity than RAR) can also bind RA, in this experiment, the RARβ2 promoter was stimulated with the RAR-specific retinoid TTNPB. Incubation with BMS649 alone did not significantly alter basal promoter activity, and this activity was not affected by vitamin D (not illustrated). In contrast, as shown in Fig. 5C, treatment with 100 nM TTNPB increased promoter activity, and this response was repressed by vitamin D in a

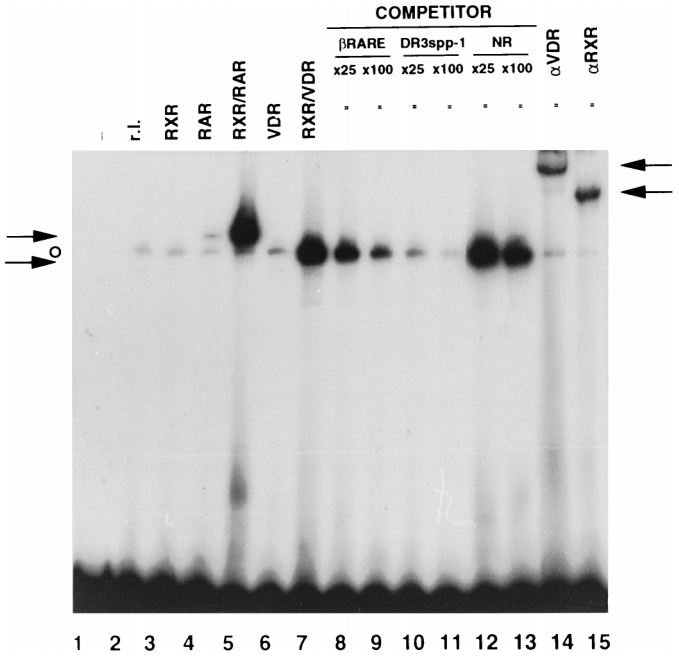

FIG. 3. VDR/RXR heterodimers bind to the βRARE. *In vitro* translated RAR, VDR, and/or RXR (1 μl) were used as indicated (lanes 3–7) in gel retardation assays with an oligonucleotide conforming the proximal RARE of the RARβ2 promoter (βRARE). Lane 1 shows the mobility of the unretarded oligonucleotide and lane 2 shows the nonspecific band formed by the unprogrammed reticulocyte lysate (r.l.). The position of this band is indicated by a *circle*, and the positions of RAR/RXR and VDR/RXR heterodimers are shown by *arrows* at the *left*. Increasing concentrations of the βRARE oligonucleotide, the DR3-type VDRE of the osteopontin promoter (DR3spp-1), and a nonrelated (NR) oligonucleotide (lanes 8–13) were used to compete the retardation caused by VDR/RXR (lane 7). In lanes 14 and 15, the VDR/RXR heterodimer was incubated with 1 μl of specific anti-VDR (αVDR) and anti-RXR (α-RXR) antibodies, and the mobilities of the corresponding supershifted bands are shown by *arrows* at the *right*.

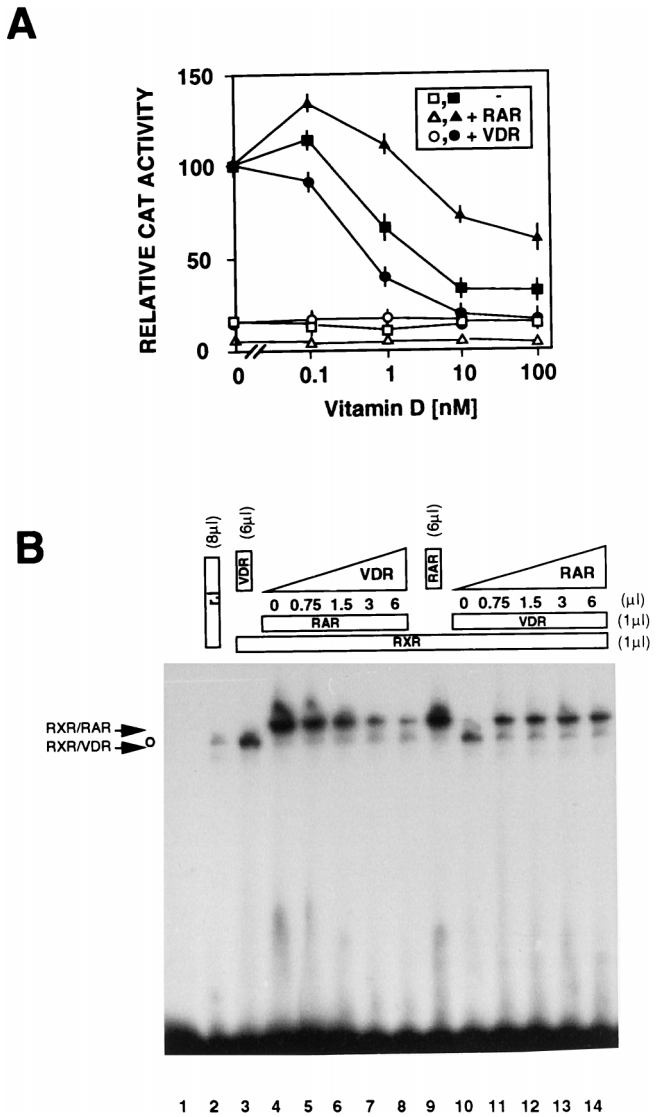

FIG. 4. The ratio between RAR and VDR determines the activation of the RARβ2 promoter and *in vitro* binding to the βRARE. A, GH4C1 cells were cotransfected with the R-140 CAT construct and an empty noncoding vector pSG5–0 (*squares*) or vectors expressing RAR (*triangles*) or VDR (*circles*). After transfection, the cells were treated for 48 h with the concentrations of vitamin D indicated, either alone (*empty symbols*) or in combination with 1 μM RA (*full symbols*). CAT activity obtained in cells treated with RA in the absence of vitamin D was considered in each case as a 100%. B, Gel retardation assays with the βRARE oligonucleotide and 1 μl of RXR in the presence of increasing concentrations (between 0 and 6 μl) of VDR and RAR. The amount of *in vitro* translated receptors or reticulocyte lysate used is indicated at the *top*. The *circle* shows the mobility of the nonspecific retarded band formed by the unprogrammed lysate.

dose-dependent manner. This repression was further enhanced when the cells were incubated with the combination of vitamin D and the RXR-specific ligand (Fig. 5C). The inhibitory effect of vitamin D on cells treated with 100 nM BMS649 plus TTNPB was similar to that obtained in cells treated with the same concentration of RA (illustrated by a *black triangle* in the figure).

The LBD of VDR mediates a significant repressive effect

To investigate whether the repressive effect of vitamin D *in vivo* depended on VDR/RXR binding to the RARβ2 promoter, we created a N-terminally truncated VDR (Δ1–111 VDR) that lacks regions A/B and C and that is, therefore, unable to bind DNA. This truncation renders a 35-kDa receptor that does not bind to the βRARE but is able to reduce binding of RAR/RXR because it contains the dimerization domain (data not shown). The influence of over-expression of this mutant receptor, as compared with the native VDR, on the response of the R-140 construct to RA in GH4C1 cells is shown in Fig. 6A. In agreement with results shown in Fig. 4A, the dominant inhibitory effect of vitamin D was further enhanced in cells transfected with VDR. In addition, the Δ1–111 VDR mimicked the effect of the wild-type receptor and also increased the repressive effect of vitamin D, although with a somewhat lower potency. This effect was again strictly ligand-dependent because transfection with native or truncated receptor affected neither basal promoter activity nor induction by RA in the absence of vitamin D (not illustrated).

To analyze whether the inhibitory influence of vitamin D on the response to RA was specific for pituitary cells, the effect of transfection with the native VDR and the Δ1–111

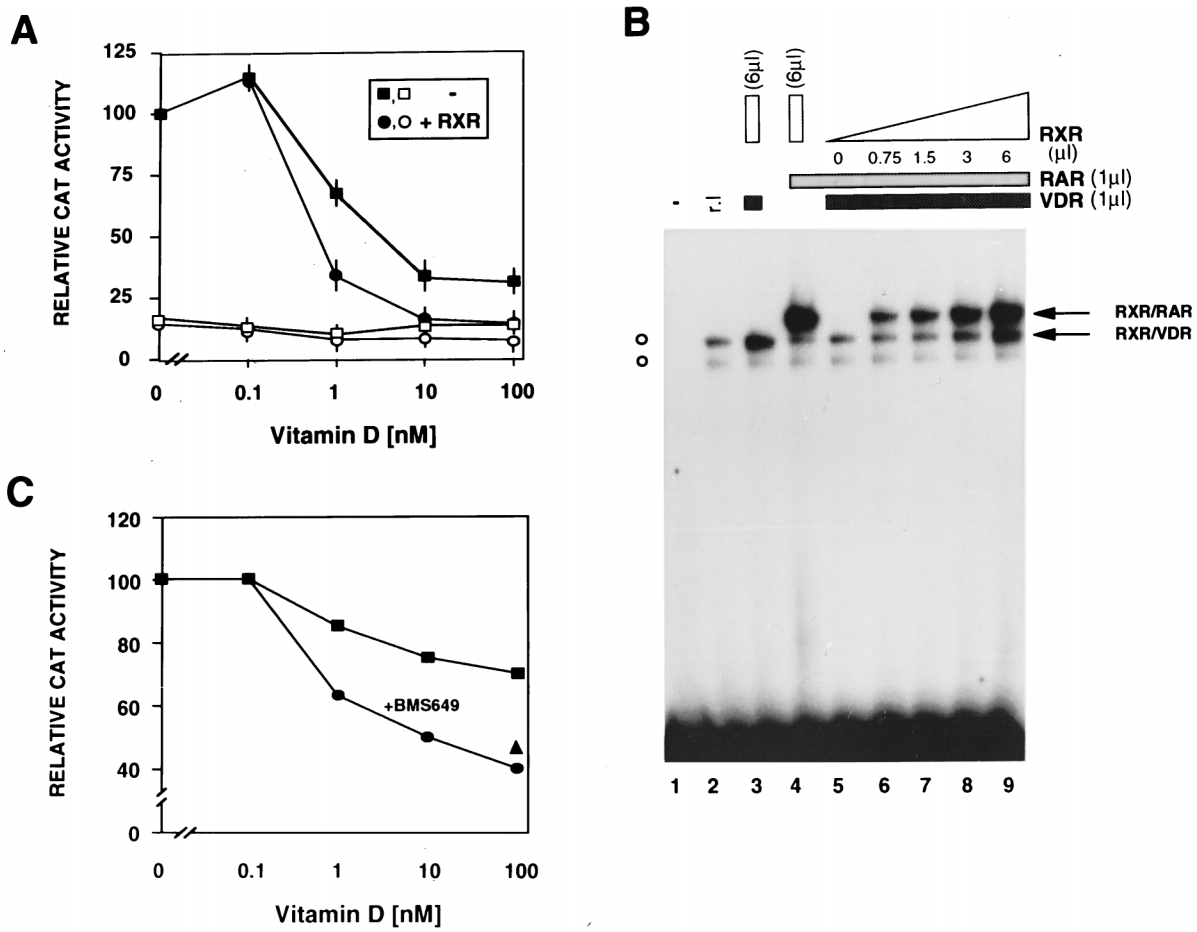

FIG. 5. Influence of RXR on RARβ2 promoter activation and binding of VDR and RAR to the βRARE. A, GH4C1 cells were cotransfected with the RARβ2 promoter in the presence (*circles*) and absence (*squares*) of an expression vector for RXR. The data are expressed as a percentage of the values obtained in the cells treated with RA alone in each condition. The cells were then treated for 48 h with vitamin D alone (*empty symbols*) or in combination with 1 μM RA (*full symbols*). CAT activity obtained in cells treated with RA in the absence of vitamin D was considered as a 100%. B, The βRARE oligonucleotide was used for retardation assays with 1 μl RAR or 1 μl VDR in the presence of increasing amounts of RXR (between 0 and 6 μl), as indicated. The mobility of two nonspecific bands produced by 8 μl reticulocyte lysate is shown by *circles* at the *left*. C, GH4C1 cells were transfected with R-140 CAT. After transfection, the cells were treated for 48 h with 100 nM TTNPB, a RAR-specific ligand, in the presence of the concentrations of vitamin D indicated, alone (*square symbols*) or in combination with 100 nM BMS649, a RXR-specific ligand (*circles*). CAT activity is expressed as the percentage of the values obtained in cells treated with TTNPB or TTNPB+BMS649 in the absence of vitamin D. The *black triangle* represents the data obtained in cells treated in parallel with 100 nM RA plus 100 nM vitamin D, as a percentage of the value obtained with RA alone. The data represent the mean of two independent transfections, with less than 10% variation.

mutant was also examined in COS-7 cells. The expression of endogenous VDR and the transfected receptors are shown in Fig. 6C. As analyzed by Western blot, VDR was undetectable in untransfected COS-7 cells, and both the native and truncated VDR were expressed at similar levels. Because these cells also express low levels of RARs, the RARβ2 promoter was always cotransfected with an expression vector encoding RAR. The *left* panel in Fig. 6B shows that, under these conditions, RA activated the promoter, and incubation with 100 nM vitamin D did not affect this response in the absence of exogenous VDR. The *middle* panel shows that, after expression of VDR, vitamin D caused a significant inhibition of the promoter response to RA, similar to that observed in GH4C1 cells that express high levels of endogenous VDR. As shown in the *right* panel, again the receptor lacking the A/B domain and the DBD was able to mediate a significant ligand-dependent transrepression.

Deletion of the AF-2 domain of VDR impairs transrepression by vitamin D

To analyze whether the AF-2 domain, which is required for ligand-dependent stimulation by VDR, is also involved in ligand-dependent transrepression, we constructed an AF-2-defective mutant receptor (ΔAF2 VDR). This receptor, which lacks only the last C-terminal 12 residues, heterodimerizes and binds normally to the βRARE (data not shown) and is expressed at the same levels as the native VDR after transfection in cells (Fig. 7B). However, as illustrated in Fig. 7A, the AF-2 mutant showed a reduced ability to mediate a ligand-dependent inhibition of the RA response. Whereas overexpression of VDR again significantly potentiated repression by vitamin D in GH4C1 cells, overexpression of ΔAF2 VDR had little effect on this inhibitory response. Similar results were obtained in COS-7 cells transfected with

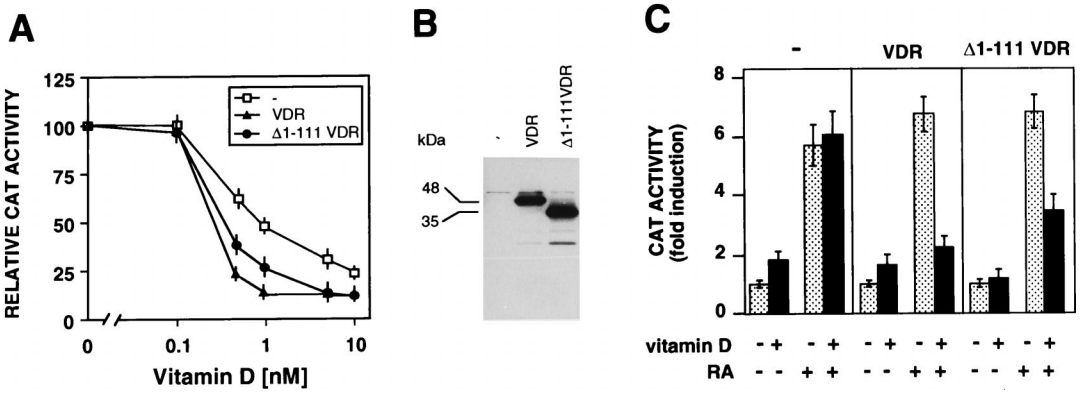

FIG. 6. The LBD of VDR mediates transrepression by vitamin D. A, GH4C1 cells were cotransfected with the RARβ2 promoter construct and expression vectors encoding wild-type VDR or a truncated receptor (Δ1–111 VDR), which lacks the A/B domain and the DBD. CAT activity was determined in cells treated with 1 μM RA in the presence of the indicated concentrations of vitamin D. B, Cos-7 cells were transfected with a noncoding vector or with expression vectors for VDR and Δ1–111 VDR. Expression of these proteins was assessed by Western blot, 48 h after transfection. The size of the receptors (in kDa) is shown at the *left*. C, R-140 CAT was cotransfected into COS-7, together with 0.2 μg of a RAR expression vector and the vectors expressing native VDR or Δ1–111 VDR. CAT activity was determined, after 48 h, in cells treated with 1 μM RA in the presence and absence of 100 nM vitamin D.

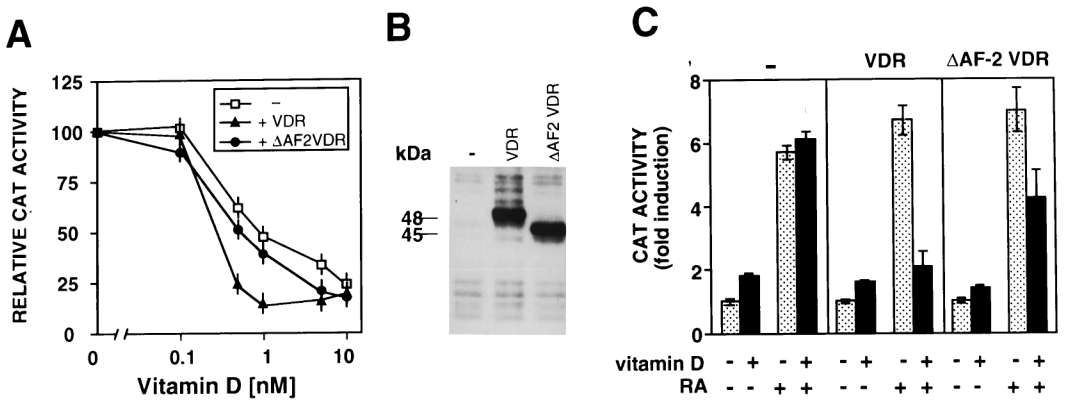

FIG. 7. The AF-2 domain of VDR participates in transrepression of the RARβ2 promoter by vitamin D. A, GH4C1 cells were cotransfected with R-140 CAT and a noncoding vector, an expression vector for wild-type VDR, or a vector expressing a receptor in which the last 12 C-terminal residues have been deleted (ΔAF-2 VDR). CAT activity was determined after 48 h of incubation with 1 μM RA in the presence of the indicated concentrations of vitamin D. B, Levels of VDR and ΔAF-2 VDR obtained in COS-7 cells transfected with vectors encoding these receptors. C, COS-7 cells were transfected with R-140 CAT and the VDR and ΔAF-2 VDR vectors. The cells also received 0.2 μg RAR. CAT activity was determined in cells treated with 1 μM RA and/or 100 nM vitamin D, and the data are expressed as the percentage of the values obtained in the corresponding untreated cells.

expression vectors for VDR and ΔAF2 VDR. Whereas incubation with 100 nM vitamin D almost totally inhibited RA-dependent activation of the R-140 CAT construct in cells transfected with native VDR, the inhibition mediated by the AF-2-defective receptor was significantly impaired (Fig. 7C).

E1A partially reverses the inhibitory response of vitamin D

The finding that the AF-2 domain seems to be required for the dominant negative activity of VDR suggests that titration of coactivators or common associated proteins that bind to this region may be involved in the inhibition of the RA response. If this hypothesis were true, overexpression of these factors should revert transrepression by vitamin D. A cellular counterpart of adenoviral E1A seems to play a coactivator role in the regulation of the RARβ2 promoter. E1A has been shown to cooperate with TBP in synergistically increasing the response of this promoter to RA. This is not observed in GH4C1 cells, in which transfection with E1A and/or TBP had little effect on the response to RA (Fig. 8). However, expression of E1A was able to significantly reduce the inhibitory effect of vitamin D on the RARβ2 promoter, both in the presence and absence of TBP, suggesting that titration of an E1A-like activity could contribute to the repression caused by vitamin D. In contrast with the effect of E1A on the RARβ2 promoter, expression of this protein did not alter the induction of the Spp1-tk-CAT construct by vitamin D, showing that E1A does not exert a nonspecific effect on vitamin D responses (data not shown). On the other hand, overexpression of the coactivators SRC-1 or CBP in GH4C1 cells altered neither the response of the RARβ2 promoter to RA nor the repressive effect of vitamin D. Similar results were obtained after transfection with TFIIB, which interacts with VDR and has been described to modulate transcriptional responses to vitamin D (data not shown).

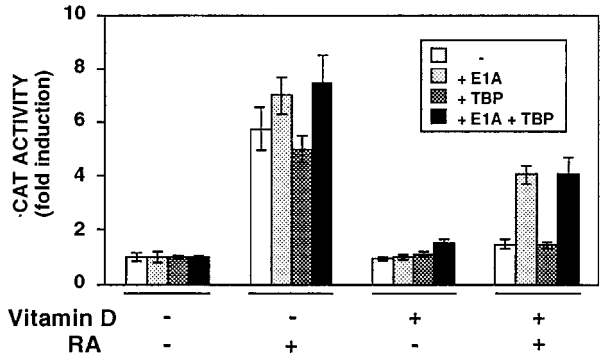

FIG. 8. Expression of E1A partially reverses the inhibitory effect of vitamin D on the RARβ2 promoter. GH4C1 cells were transfected with R-140 CAT and expression vectors for E1A (5 μg), the TBP (15 μg), or both. CAT activity was determined in cells treated with 1 μM RA and/or 100 nM vitamin D for 48 h.

Discussion

We show that vitamin D exerts an important repressive effect on RA-dependent transactivation of the RARβ2 promoter in pituitary GH4C1 cells that express functional receptors for both ligands (10). In these cells, nanomolar concentrations of vitamin D, which stimulate transcription of promoters containing a VDRE, effectively inhibited activation by RA. We found this inhibitory response to be specific, because vitamin D did not decrease basal RARβ2 promoter activity. Repression seems to be mediated by the RARE located between −37 to −53 on the promoter and can be transferred to a heterologous promoter. In EC cells, deletion of upstream sequences (from −124 to −59) which contain an imperfect RARE, diminish RA-dependent transactivation *in vitro* (29) and *in vivo* (15). However in GH4C1 cells, deletion of these sequences does not impair, but rather increases, transactivation. These results indicate that the distal RARE does not enhance the RA response in these cells, although this element could contribute to transrepression by vitamin D. A putative phorbol ester-responsive element at position −84 to −78 and a cAMP-responsive element at position −99 to −92 adjacent to the distal RARE have been described to be involved in RA-dependent RARβ2 promoter activation in EC cells (30, 31). These elements could be involved in the observed cell type-specific reduction of the RA response. In any case, vitamin D also reduced RA-dependent transactivation of a construct that does not contain these sequences, showing that the proximal βRARE is sufficient to confer the transrepressive effect. In the context of the longer promoter fragment, a mutation in the RXR 5′ half-site of the βRARE (reporter M7) essentially abolished the response to RA, whereas mutation of the 3′ half-site, which binds RAR (reporter M3), significantly decreased (but did not totally block) this response. A stronger effect of the mutation in the RXR-specific half-site has also been observed in *in vitro* transcription assays (29). Our results demonstrate that this residual response is also abolished by vitamin D in GH4C1 cells.

Our findings also show that VDR/RXR heterodimers bind to the βRARE with high affinity, although this binding is transcriptionally unproductive. The discrepancy between binding and activity is likely caused by the conformation acquired by the heterodimers in a DR-5 element, which may affect its interaction with other factors required for transcriptional activation. Other examples of receptor binding to HREs in a transcriptional inactive form have been previously described. In particular, we have observed unproductive binding of VDR/RXR to a palindromic HRE, which also binds RAR/RXR heterodimers (10). An attractive mechanism has been proposed to explain the lack of activation by RA of RAR/RXR heterodimers bound to a DR1 element. Ligand-transactivation by RAR on a DR5 element seems to require the dissociation of corepressors, as well as the recruitment of coactivators (32). In DR1 elements, because of an altered heterodimer polarity, corepressors remain associated, even in the presence of RA, resulting in constitutive repression (33). However, a mechanism of transrepression by vitamin D involving corepressor molecules is very unlikely, because VDR does not bind the corepressors SMRT or NCoR in solution (34, 35). Furthermore, we have shown that SMRT does not associate with RXR/VDR bound to the βRARE (A. M. Jimenez-Lara and A. Aranda, submitted for publication). The possibility that unidentified corepressors different from SMRT could be involved in transrepression cannot be dismissed; but the fact that, in the absence of ligand, VDR does not cause a repressive effect in the transfection assays makes this possibility highly unlikely. On the other hand, the promoter context in which a HRE is placed can be the determining factor for receptor selectivity. Thus, DR3 and DR4 elements, which normally mediate vitamin D and thyroid hormone responses, are converted to exclusive RAREs when placed in the RARβ2 promoter (15).

A competitive DNA binding mechanism between active RAR/RXR heterodimers and inactive VDR/RXR heterodimers could contribute to transrepression of the RA-dependent transactivation of the RARβ2 promoter by vitamin D. In fact, in *in vitro* assays, VDR displaced RAR/RXR binding to the βRARE. This competition occurred when RXR was limiting, because the presence of an excess of RXR allowed binding of both heterodimers. Transfection experiments suggested that RXR also plays a role in the promoter inhibition mediated by VDR and that the VDR/RXR heterodimer can be the *in vivo* transrepressing species. Furthermore, a RXR-specific ligand potentiated the negative inhibitory effect of vitamin D. It has been proposed that RXR ligands can inhibit transactivation by VDR/RXR heterodimers by promoting the formation of RXR homodimers (36, 37). The possibility that RXR homodimers could cause repression of the RA response can be dismissed, because incubation of GH4C1 cells with an RXR-specific ligand alone did not affect transactivation. Therefore, our results indicate that the ligands of both heterodimeric partners cooperate to transrepress the RARβ2 promoter.

Although DNA binding could be important for full potency-dominant inhibitory activity of VDR, other mechanism/s must contribute to this inhibition. This is based on the finding that a truncated receptor lacking the DBD also displays repressor activity in GH4C1 and COS-7 cells, although the repression is somewhat weaker than that mediated by the native receptor. The truncated receptor could act by sequestering RXR and, consequently, by causing a decrease in the amount of this receptor available for the formation of active RAR/RXR heterodimers. Titration of RXR has been de-

scribed to mediate repression of RA-dependent transactivation of the RARβ2 promoter by the thyroid hormone receptor (38). If competition for RXR were to be responsible for transrepression by vitamin D in GH4C1 cells, it would be expected that this inhibitory effect would disappear in the presence of excess amounts of RXR. However, vitamin D also blocked the response to RA after transfection of RXR.

On the other hand, the N-terminally truncated VDR also contains the AF-2 domain responsible for binding of coactivators and ligand-dependent transactivation. Our studies reveal that the C-terminal region of VDR, a putative amphipathic helix that contains the AF-2 domain (39, 40), is involved in transrepression, because a receptor lacking the last 12 aminoacids mediates little inhibition by vitamin D. A reduced ligand-binding affinity of the AF-2-defective VDR could explain the reduced transrepressive effect of this receptor. However, similar deletion mutants have been shown to bind ligand with high affinity (5, 40), and this mutant shows a clearly impaired ability to mediate repression in COS-7 cells treated with a saturating concentration of vitamin D. On the other hand, this VDR mutant binds to DNA and heterodimerizes normally with RXR. It has been recently demonstrated that deletion or mutation of the AF-2 domain abrogates ligand-dependent interaction of VDR with the coactivators SRC-1, SUG1, and RIP-140 (39, 40). E1A has been shown to play an important role in RA-dependent transactivation of the RARβ2 promoter (15, 20). E1A also contacts the AF-2 domain of RAR and may be considered to be a RARβ2 promoter-specific coactivator (22). Our results show that expression of E1A in GH4C1 cells significantly reduces transrepression by vitamin D without altering basal promoter activity. These findings suggest that VDR could titrate endogenous factors that can be substituted by the viral protein. The existence of a E1A-like activity in EC cells has been postulated (20), although this factor has not been purified or cloned yet. This E1A-like activity should exist in pituitary cells, because overexpression of E1A does not cause further stimulation of RA-dependent transactivation of the RARβ2 promoter, as it occurs in Cos cells that do not express this activity. E1A has a complex function in cells and can interact with a variety of effectors. Therefore, the nature of the factors that may be squelched by VDR in GH4C1 cells is unknown at the present. Another coactivator, which plays an important role in transcriptional activation mediated by different signaling pathways (including those modulated by nuclear receptors) is CBP/p300. This cointegrator contacts both the the AF-2 domain of the nuclear receptors and components of the basal transcriptional machinery, such as TBP or TFIIB (27, 41). Furthermore, CBP also binds E1A (42) and might act as a bridging factor between E1A-LA, RAR, and TBP. That led to the possibility that titration of this factor, which seems to be present in limiting amounts in several cell types (43), could be responsible for the inhibitory effect of vitamin D on the RA response. However, in GH4C1 cells, overexpression of CBP did not increase the response of the RARβ2 promoter to RA and did not revert repression by vitamin D. Thus, either CBP is not a good coactivator for this particular promoter or, most likely, this coactivator is expressed in sufficient amounts in these cells to elicit maximal responses. The same is true for other coactivators (such as SRC-1) or for some components of the basal transcriptional machinery (such as TBP or TFIIB), which regulate transcriptional responses to vitamin D (44, 45), because we did not observe modulation of the promoter response to RA or vitamin D after overexpression of these factors in GH4C1 cells. Therefore, other still unidentified coactivators that bind to both RAR and VDR could be involved in transrepression by vitamin D.

We had previously reported that RA and thyroid hormones induce an increase in GH transcripts in pituitary cells and that vitamin D interferes with this activation (10). This shows that transrepression also occurs with the endogenous genes. These findings, as well as the results obtained in the present work, demonstrate an important repressive role of VDR on some transcriptional responses to other nuclear receptors in this cell type. Although the role of vitamin D on pituitary ontogeny and function remains to be established, the interaction of vitamin D and retinoid signaling must be complex, because we have observed that (depending on the response element involved) these ligands can also cooperate to stimulate transcription. This seems to be the case with the pituitary-specific transcription factor GHF-1/Pit-1, which plays a key role in the expression of pituitary genes, as well as in pituitary development. The study of the GHF-1/Pit-1 gene promoter revealed the existence of a DR4 element that responds positively both to RA and vitamin D (46). A balance between stimulatory and inhibitory actions of vitamin D must create a complex and sensitive transcriptional network in the pituitary. It is clear that further studies on the physiological impact of the observed repression for the function or development of pituitary cells merits further investigation. The importance of transrepression by vitamin D is probably not restricted to these cells. We have observed here that RA-dependent activation of the RARβ2 promoter is also repressed by vitamin D in Cos-7 cells after expression of VDR. Therefore, it is expected that vitamin D could modulate RA responses in different cell types that coexpress VDRs and RARs.

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