MOLECULAR AND CELLULAR BIOLOGY, Oct. 1995, p. 5789–5799 Vol. 15, No. 10
0270-7306/95/$04.00+0

Transcriptional Repression of the Interleukin-2 Gene by Vitamin D_3: Direct Inhibition of NFATp/AP-1 Complex Formation by a Nuclear Hormone Receptor

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Received 30 March 1995/Returned for modification 17 May 1995/Accepted 13 July 1995

T-lymphocyte proliferation is suppressed by 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], the active metabolite of vitamin D_3, and is associated with a decrease in interleukin 2 (IL-2), gamma interferon, and granulocyte-macrophage colony-stimulating factor mRNA levels. We report here that 1,25$(OH)_2D_3$-mediated repression in Jurkat cells is cycloheximide resistant, suggesting that it is a direct transcriptional repressive effect on IL-2 expression by the vitamin D_3 receptor (VDR). We therefore examined vitamin D_3-mediated repression of activated IL-2 expression by cotransfecting Jurkat cells with IL-2 promoter/reporter constructs and a VDR overexpression vector and by DNA binding. We delineated an element conferring both DNA binding by the receptor in vitro and 1,25$(OH)_2D_3$-mediated repression in vivo to a short 40-bp region encompassing an important positive regulatory element, NF-AT-1, which is bound by a T-cell-specific transcription factor, NFATp, as well as by AP-1. VDR DNA-binding mutants were unable to either bind to this element in vitro or repress in vivo; the VDR DNA-binding domain alone, however, bound the element but also could not repress IL-2 expression. These results indicate that DNA binding by VDR is necessary but not sufficient to mediate IL-2 repression. By combining partially purified proteins in vitro, we observed the loss of the bound NFATp/AP-1-DNA complex upon inclusion of VDR or VDR-retinoid X receptor. Order of addition and off-rate experiments indicate that the VDR-retinoid X receptor heterodimer blocks NFATp/AP-1 complex formation and then stably associates with the NF-AT-1 element. This direct inhibition by a nuclear hormone receptor of transcriptional activators of the IL-2 gene may provide a mechanistic explanation of how vitamin derivatives can act as potent immunosuppressive agents.

1,25-Dihydroxyvitamin D_3 [1,25$(OH)_2D_3$], the active metabolite of vitamin D_3, is a seco-steroid hormone that binds with high affinity to a nuclear receptor, the vitamin D_3 receptor (VDR). This receptor selectively associates with recognition sequences in the promoter region of target genes, thereby regulating the transcription of those genes. The principal functions of 1,25$(OH)_2D_3$ are the stimulation of intestinal calcium and phosphorus absorption, mediation of bone remodeling, and conservation of minerals in the kidney (for reviews, see references 46 and 63). In addition to its action in these tissues, however, 1,25$(OH)_2D_3$ has been found in skin, testes, breast, muscle, pancreas, endocrine glands, thymus, and bone marrow, suggesting additional regulatory functions for the hormone. Notably, 1,25$(OH)_2D_3$ appears to play an important role in modulating the growth of cells of the immune system: the hormone can induce the differentiation of myeloid leukemia cells along a monocyte/macrophage lineage (1, 4, 6, 49) and can inhibit T-lymphocyte proliferation and activation both in vivo and in vitro (8, 10, 37, 43, 44). Interleukin-2 (IL-2), gamma interferon, and granulocyte-macrophage colony-stimulating factor (GM-CSF) mRNA levels all decrease after T cells are exposed to 1,25$(OH)_2D_3$, but the molecular mechanisms mediating these changes have not been fully described (9, 58, 66–69, 79).

T-cell activation is a key step in the initiation of an immunological response. Upon receipt of the appropriate stimulus, a complex signaling cascade is initiated, resulting in cell proliferation and secretion of cytokines that enhance the immune response. One of the first genes to be expressed postactivation is the IL-2 gene. The lymphokine IL-2 exerts its influence by interacting with the IL-2 receptor on the surface of activated T cells, and this interaction is required for progression through the cell cycle (transition from G_1 to S phase). The kinetics of IL-2 induction are quite rapid, with transcripts detectable within 30 to 45 min after activation. A complex enhancer, which includes 275 bp just downstream from the transcription start site (−52 to −326), has been exhaustively studied (16, 24, 75). Binding sites for several ubiquitous and T-cell-specific transcription factors were defined in this region, and the proteins that bind to these sites, including Oct-1, AP1, NF-κB, and NFATp/c, have been identified (65).

Several agents, including the drugs cyclosporin A and FK506 (18, 51), as well as glucocorticoids (25, 80, 81) and retinoids (13, 19) appear to act as immunosuppressors by targeting IL-2 expression. 1,25$(OH)_2D_3$ inhibits the entry of activated T cells into S phase (68); similar blocks at this point in the cell cycle have been demonstrated for other inhibitors of IL-2 synthesis, such as the synthetic glucocorticoid dexamethasone (7), further implicating IL-2 as a target for vitamin D_3's immunosuppressive effect.

These observations demonstrate an important role for 1,25$(OH)_2D_3$ in the immunomodulation of T lymphocytes but not the actual mechanism by which this regulation is carried out. Since the signal transduction pathway of vitamin D_3 works directly through a hormone-inducible transcription factor, the VDR, we have focused our attention on how this nuclear receptor might mediate T-cell suppression. Interestingly, only

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phytohemagglutinin (PHA)-activated, normal human T lymphocytes express VDR (8). Two potential ways the suppression might occur are (i) VDR enhances the transcription of a negative regulator of a key gene involved in T-cell activation, such as IL-2, or (ii) VDR directly represses the transcription of such a gene; several members of the steroid/nuclear receptor superfamily have been shown to act as both activators and repressors of transcription, depending on the particular gene and/or cell type (for reviews, see references 56, 62, 64, 73, and 82).

We demonstrate here that repression of IL-2 transcription by $1,25(OH)_2D_3$ is a direct, VDR-dependent effect. Further, we delineate a region within the IL-2 promoter that mediates the repression; this region coincides with the binding site for a complex critical for IL-2 transcriptional activation, NFAT (nuclear factor of activated T cells). NFAT induction requires two activation-dependent events; the cyclosporin A-sensitive translocation of a lymphoid-specific, preexisting component, NFATp, and the protein kinase C-mediated stimulation of Jun and Fos (13, 26, 65). VDR, together with its heterodimeric partner RXR (retinoid X receptor), targets this complex by blocking NFATp/AP-1 complex formation. This process appears to require DNA binding by VDR to the composite NFATp/AP-1 element. The net result is a loss of activated transcription of the IL-2 gene.

MATERIALS AND METHODS

Antibodies. Rabbit anti-Jun polyclonal antibody was purchased from Santa Cruz Biotechnology Inc. Rat anti-VDR monoclonal antibody 9A7 (5) was the kind gift of J. W. Pike.

Proteins. *Escherichia coli*-expressed purified NFATp DNA-binding domain (DBD; residues 1 to 297 of NFATpXS [32]) was generously provided by A. Rao. His-tagged, *E. coli*-expressed purified full-length c-Jun and c-Fos were supplied both by G. Parsons and K. R. Yamamoto and by T. Kerppola and T. Curran. RXRβ was the kind gift of K. Ozato. Purification of *E. coli*-expressed VDR and a protein containing only the DBD of human VDR (VDRF) has been described previously (12, 23). VDR-R50G purification was exactly as described for VDR.

Plasmids. A minimal promoter/luciferase reporter, pE1b-LUC, was constructed by cloning the E1b TATA box from pE1b-CAT (obtained from R. Roeder) as a *Bam*HI-*Xho*I fragment into the *Hin*dIII-*Xho*I sites in pGL2-basic (Promega). Both *Bam*HI in pE1b-CAT and *Hin*dIII in pGL2-basic were blunt ended by Klenow enzyme. All upstream IL-2 reporter constructs were generated from pUPSTIL2-CAT. The latter construct was derived from p41 (30), containing 3.7 kb of the human IL-2 promoter and coding region. p41 (provided by U. Siebenlist) was digested with *Cla*I, and the fragment was cloned into pSP73, generating p41-SP73. An *Nde*I-*Pst*I fragment of p41-SP73 was then cloned into pOTCO, a chloramphenicol acetyltransferase (CAT) reporter plasmid, generating p41-TCO. The resulting construct contained 200 bp of the coding sequence of the IL-2 gene which were deleted by exonuclease III digestion. The resulting construct, pUPSTIL2-CAT, contained 2 kb of IL-2 enhancer sequences, the IL-2 RNA start site, and 45 bp of the IL-2 leader. To construct p(−1000)IL2-CAT, pUPSTIL2-CAT was digested with *Bgl*II and *Hin*dIII (which was filled in with Klenow enzyme), deleting 1,000 bp of the upstream region. The fragment was ligated into pOTCO which was cut with *Sal*I (the ends blunted by Klenow enzyme) and *Bgl*II. The new construct encompassed 1,000 bp of the IL-2 enhancer/promoter. Both p(−800)IL2-CAT and p(−428)IL2-CAT were generated by exonuclease III digestion of p(−1000)IL2-CAT. p(−321)IL2-CAT was generated by digesting pUPSTIL2-CAT with *Xmn*I, and the ends were filled in by Klenow enzyme. Subsequently, the vector was cut with *Bgl*II, and a 350-bp fragment was ligated into pOTCO which was cut with *Sal*I and *Bgl*II. p(−321)IL2-LUC was generated from p(−321)IL2-CAT by digestion with *Hin*dIII, and the ends were filled in by Klenow enzyme. The DNA was then cleaved with a second restriction enzyme, *Bgl*II. The fragment was cloned into pGL2-basic (Promega) which was digested with *Sma*I and *Bgl*II. To construct $p(100)_3$E1b-LUC, 100 bp from the IL-2 enhancer (−319 to −215, relative to the start site) were cloned 5′ to the E1b TATA box in the pE1b-LUC reporter vector (45). To construct $p(NFAT\text{-}1)_5$E1b-LUC, an NFAT-1 oligonucleotide duplex (top strand, 5′-CTAGCAGAAAGGAGGAAAAACTGTTTCATACAGAAGGCGTT-3′) was phosphorylated with T4 kinase (Bethesda Research Laboratories) and then cloned into the *Nhe*I site of pE1b-LUC (the annealed oligonucleotide was designed to have an *Nhe*I site at the 5′ end and an *Xba*I site at the 3′ end). After dideoxy sequencing, it was established that the phagemid contained five copies of the NFAT-1 oligonucleotide, all in the same orientation.

The pSP65-β-gal construct was generated by cloning the *Kpn*I-*Pvu*I fragment from the β-galactosidase (β-Gal) gene (168 bp) (pCH110; Pharmacia) into the polylinker of pSP65.

Expression plasmids were generated using the cytomegalovirus-driven pRC vector (Invitrogen). To create pCMV-hVDR, the VDR coding sequence was isolated from the *E. coli* overexpression vector pT7-hVDR (78) as an *Nhe*I (filled in)-*Apa*I fragment into pRc-CMV, which had been digested with *Xba*I, filled in by Klenow enzyme, and then cut with *Apa*I. To construct pCMV-VDRF, VDRF was cloned as an *Xba*I-*Eco*RI fragment from the *E. coli* overexpression vector pT7-VDRF (22) into pSELECT-1 (Promega), (creating pSELECT-VDRF). pRC-CMV was first cut with *Xba*I, and the 5′ overhang was blunt ended with Klenow enzyme and then digested with *Hin*dIII. VDRF was then cloned into this plasmid as an *Eco*RV-*Hin*dIII fragment from pSELECT-VDRF. pCMV-RXRα was obtained from R. Evans (Salk Institute).

Oligonucleotide-directed in vitro mutagenesis. Single-stranded DNA preparation and double-primer mutagenesis were carried out as described previously (3). To generate mutations in the DBD, base changes were introduced into pCMV-hVDR by using the mutagenic oligonucleotides that changed E-42, G-43, and G-46 simultaneously to G, S, and V, or R-50 to G, or G-46 and R-49 to A and K, respectively. A detailed description of the construction of these mutants will appear elsewhere (22). The mutated VDR cDNAs were also transferred to a T7 overexpression vector, pAR3040, and overexpressed in *E. coli* and purified as previously described (12).

Cell transfections and reporter assays. The T-cell line Jurkat was transfected by the DEAE-dextran/chloroquine method. Cells were grown in RPMI medium containing sodium pyruvate, glutamine, and penicillin-streptomycin. Fetal calf serum was added to 10% and cells, were maintained at a density of $\sim 5 \times 10^5$/ml. Cells were gently suspended (10^7/ml) in transfection cocktail (0.6 mM chloroquine diphosphate [Sigma]), 0.25 mg of DEAE-dextran [Pharmacia] per ml in minimal essential medium containing 10 μg of reporter plasmid per ml and 2 μg of producer plasmid per ml). Cells were incubated in transfection cocktail for 2.5 h in an incubator (5% CO_2, 37°C), and then the cells were washed. Subsequently, the cells were resuspended (10^7/15 ml) in RPMI-sodium pyruvate-glutamine-penicillin-streptomycin medium containing 10% charcoal-stripped fetal calf serum. At 40 h posttransfection, cells were treated in one of the following ways for 8 h: no treatment; addition of 2×10^{-8} M $1,25(OH)_2D_3$ (Biomol); addition of PHA (2 μg/ml; Sigma) and tetradecanoyl phorbol acetate (TPA; 50 ng/ml; Sigma) (activating agents); or addition of horm one and activating agents together. Experiments were normalized to protein concentration when CAT reporters were used; when luciferase reporters were used, Rous sarcoma virus-CAT supercoiled DNA (10 μg/ml) was added to the transfection cocktail and relative light units were normalized to CAT activity. To determine CAT activity, cell extracts were obtained and assayed by ethyl acetate phase extraction of ^{14}C-acetylated chloramphenicol and quantitated by scintillation counting. When luciferase constructs were transfected, the cell pellet was divided into two; half was used for CAT assays as described above, and the second half was used to determine luciferase activity as described previously (72).

RNA analysis. Jurkat cells (10^7 per treatment) were transfected with p(−321)IL2-LUC (10 μg), pCMV-VDR (2 μg), and pRSV-β-gal (10 μg) vectors as described for the transfection protocol. At 40 h posttransfection, the cells were treated with different combinations of 10 mM cycloheximide (10 mM), PHA (2 μg/ml), and TPA (50 ng/ml), and $1,25(OH)_2D_3$ (2×10^{-8} M), as specified. At the end of each treatment (total of 4 h), the cells were washed twice in ice-cold 1× phosphate-buffered saline and cytoplasmic RNA was prepared by Nonidet P-40 lysis. Luciferase riboprobe was synthesized with T7 RNA polymerase from plasmid pGEM-LUC (Promega) linearized with *Eco*RV; the probe extended 400 bp, which were all protected by luciferase RNA. The β-gal riboprobe was transcribed by T7 RNA polymerase from pSP65-β-gal linearized by *Eco*RI, resulting in 200 bp, 180 of which hybridized to β-gal RNA. RNA from the indicated treated transfected cells was analyzed by RNase protection assay of luciferase and β-gal antisense probes (50 and 20 μg cytoplasmic RNA, respectively) except that 40 μg of RNase A (Sigma) per ml and 2 μg of RNase T_1 (Bethesda Research Laboratories) per ml were used.

Gel mobility shift electrophoresis. VDR DNA binding was assessed by gel mobility shift electrophoresis as described previously (3, 21). For gel shifts from Jurkat cell extracts, nuclear extracts were prepared from Jurkat cells (2×10^6/ml, 400 ml) as described previously (16). Purified VDR, mutant protein derivatives, and nuclear extract were incubated with 0.1 to 0.5 ng (30,000 to 50,000 cpm) of labeled NFAT-1 oligonucleotide probe at room temperature for 20 min, together with 1 ng of poly(dI-dC) and a binding buffer to final concentrations of 20 mM Tris-HCl (pH 7.9), 1 mM EDTA, 0.05% Nonidet P-40, 10% glycerol, 50 mM KCl, and 1 mM dithiothreitol. Protein-DNA complexes were then resolved by electrophoresis on 6% nondenaturing acrylamide gels (75:1 acrylamide/bisacrylamide) run in 0.5× Tris-borate-EDTA, at 26 V/cm at 4°C. Gels were dried and subjected to autoradiography overnight with a screen. When purified Jun, Fos, and NFATp proteins were used, the binding buffer was supplemented with bovine serum albumin to 0.3%. VDR and its derivatives and/or RXR were preincubated with Jun and Fos for 10 min at 30°C unless otherwise indicated. Tubes were transferred to ice and cooled for 5 min, and the protein mixture was added to DNA. NFATp was then added, and the proteins were incubated with DNA for 20 min on ice. Protein-DNA complexes were separated from free DNA on a 4% nondenaturing polyacrylamide gel in 0.25× Tris-borate-EDTA.

DNase I footprinting. A 600-bp fragment encompassing the IL-2 enhancer/promoter (−321 to +45) was Klenow labeled at the 5′ end. Approximately 50,000 cpm of labeled DNA was incubated for 30 min at room temperature with

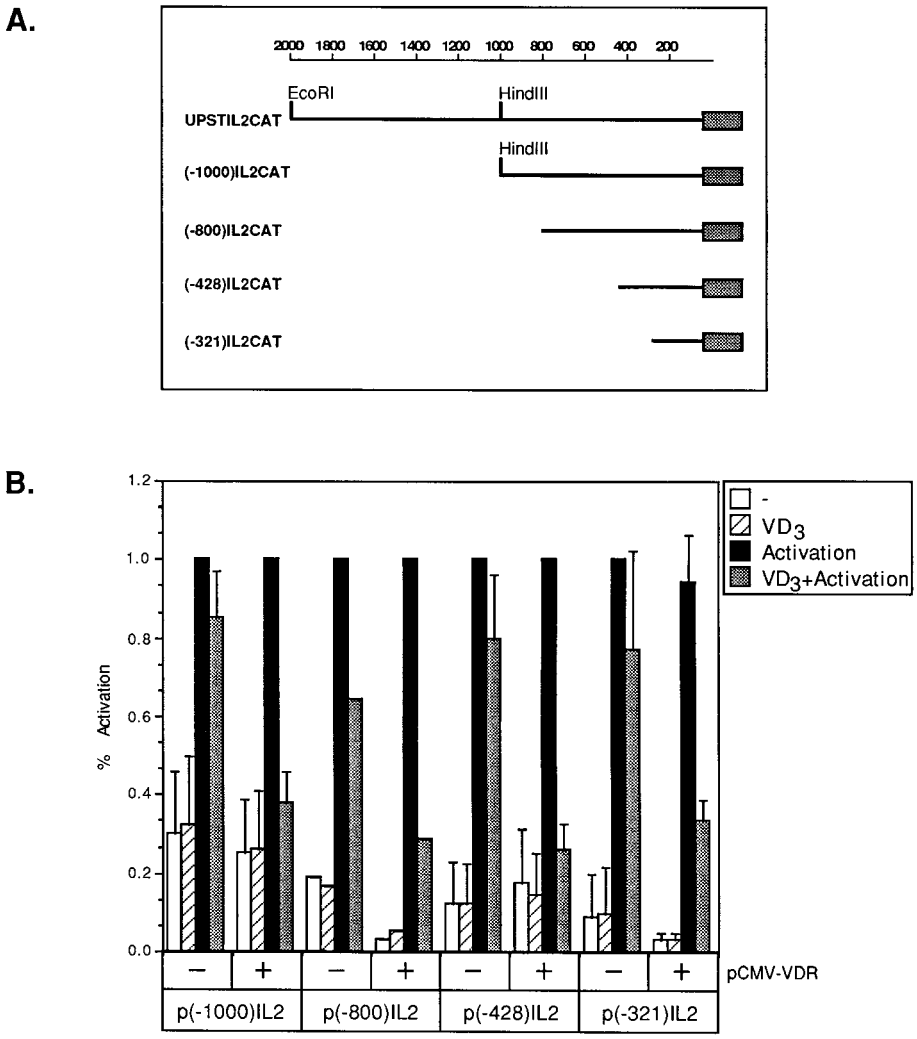


FIG. 1. (A) Exonuclease-generated IL-2 enhancer constructs. The IL-2–CAT reporters were generated beginning with 2 kb of the promoter/enhancer as described in Materials and Methods. (B) VDR represses transcriptional activation of the IL-2 enhancer/promoter. A VDR producer plasmid (pCMV-VDR) was used to cotransfect the human T-cell line Jurkat with the indicated CAT reporter constructs, p(−1000)IL2-CAT, p(−800)IL2-CAT, p(−428)IL2-CAT, and p(−321)IL2-CAT. The cells were transfected by the DEAE-dextran method, using 10 μg of reporter and 5 μg of producer DNA. At 40 h posttransfection, the cells were untreated (−) or treated with $1,25(OH)_2D_3$ (VD_3; 2×10^{-8}M), with PHA (2 μg/ml) and TPA (50 ng/ml) (Activation), or with both PHA-TPA and hormone. Cells were incubated for an additional 8 h and then harvested. CAT enzyme activity was quantitated, and values were normalized to protein concentration. Percent activation was calculated by dividing normalized values for each treatment, i.e., −, VD_3, and Activation + VD_3 by the value for Activation. The data represent the averages of three different experiments.

the indicated amounts of VDR protein in a reaction mixture containing 20 mM Tris-HCl (pH 7.9), 1 mM EDTA, 0.1% Nonidet P-40, 1 mM dithiothreitol, and 50 mM KCl (100 μl, final volume). An equal volume of a mixture of 10 mM $MgCl_2$ and 5 mM $CaCl_2$ was added at the end of the incubation, and samples were digested for 1 min at room temperature with DNase I (5 μl of a 1:20,000 dilution) (Bethesda Research Laboratories). Immediately at the end of this incubation, 200 μl of a solution containing 200 mM NaCl, 40 mM EDTA, 1% sodium dodecyl sulfate (SDS), 100 μg of proteinase K per ml, and 125 μg of glycogen (Boehringer Mannheim) per ml was added, and the reaction mixture was incubated for 15 min at 37°C. The samples were then extracted with phenol-chloroform-isoamyl mixture and then ethanol precipitated. Subsequently, equal values of precipitated counts from all samples were separated on an 8 M urea–8% polyacrylamide gel, and the gel was dried and exposed to film with a screen at −70°C.

RESULTS

Repression by VDR is mediated directly on the IL-2 enhancer. To localize the region conferring inhibition by $1,25(OH)_2D_3$, four deletion derivatives of the IL-2 enhancer fused to the CAT gene, p(−1000)IL2-CAT, p(−800)IL2-CAT, p(−428)IL2-CAT, and p(−321)IL2-CAT (Fig. 1A), were generated by exonuclease digestion of the upstream 2,000 nucleotides; these constructs were used to cotransfect Jurkat cells with or without a VDR producer plasmid. The cells were untreated, treated for 8 h with the activating agents phorbol myristate acetate (PMA) or TPA and with phytochemagglutinin (PHA), with $1,25(OH)_2D_3$ alone, or with activating agents and $1,25(OH)_2D_3$. Activation levels were reduced 70 to 80% in the presence of $1,25(OH)_2D_3$ and VDR in all of the IL-2 enhancer/deletion constructs (Fig. 1B), indicating that the proximal 321 bp of the enhancer contained a region which is sufficient to mediate the repression by the receptor. The $1,25(OH)_2D_3$-mediated repression was always stronger when the VDR producer plasmid was cotransfected, since T cells express VDR only 6 to 8 h postactivation (8). This finding also indicates that VDR is limiting in these cells for the repression.

The 321-bp region corresponds to the minimal enhancer region which was previously characterized as mediating IL-2-specific activation in response to signals originating from the cell surface (16, 24, 75).

Repression of IL-2 transcription by VDR could occur by one of at least three mechanisms: (i) VDR binds directly to an element within the IL-2 enhancer and/or interacts with positive factors that bind to this element, inhibiting the action of that factor (direct effect); (ii) VDR represses the expression of a key positive regulator of IL-2 expression (indirect); or (iii) VDR induces the expression of a repressor that in turn inhibits IL-2 transcription (indirect). If the effect is direct, then VDR-mediated repression should not be expected to be sensitive to protein synthesis inhibitors. Repression of IL-2 transcription by $1,25(OH)_2D_3$ was therefore analyzed in Jurkat cells in the presence of cycloheximide and anisomycin. Cells were cotransfected with an IL-2 reporter carrying the IL-2 promoter/enhancer driving luciferase expression [p(−321)IL2-LUC], a VDR producer (pCMV-VDR), and RSV-β-gal as a normalizing reporter. In all cases, cells were allowed to express VDR for 40 h following transfection prior to treatment with the protein synthesis inhibitors and/or hormone (Fig. 2A). No effect of VDR was expected during this time period, since the receptor was expressed in the absence of any added ligand in cells growing in charcoal-stripped serum. Activation of IL-2 and luciferase message synthesis by PHA and phorbol myristate acetate in the presence and absence of $1,25(OH)_2D_3$ was then monitored by RNase protection. IL-2 activation itself is dependent on de novo protein synthesis and thus served as a positive control for inhibition by cycloheximide and anisomycin. Cells were treated with protein synthesis inhibitors 30 min before activation, and as expected, no IL-2 mRNA was detected in response to activation (Fig. 2B, lane 3). When cells were activated in the presence of $1,25(OH)_2D_3$, the luciferase-specific mRNA was inhibited (compare lanes 4 and 5). Importantly, this inhibition was apparent even when $1,25(OH)_2D_3$ treatment was preceded by the addition of protein synthesis inhibitors (Fig. 2B, lanes 6 and 7). Thus, repression of IL-2 mRNA synthesis by $1,25(OH)_2D_3$ is independent of de novo protein synthesis and represents a direct effect on the IL-2 enhancer by VDR.

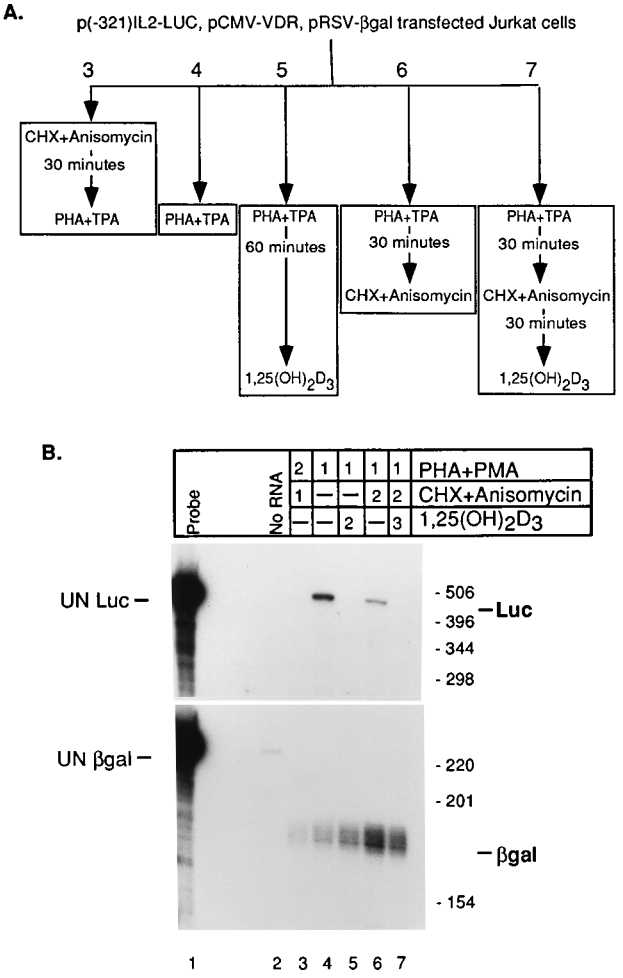


FIG. 2. VDR-mediated repression on the IL-2 promoter constitutes a direct effect. (A) Schematic outline of the cell treatments. Numbers correspond to lanes indicated in the gel shown in panel B. CHX, cycloheximide. (B) RNase protection assay of cells transfected with p(−321)IL2-LUC, pCMV-VDR, and pRSV-β-gal as an internal control. Cell treatments are indicated above each lane; numbers correspond to the order of addition (see panel A). The positions of the undigested riboprobes (UN) are indicated at the left; on the right, the positions of the digested probe are shown. Lane 2 contains a negative control (no RNA was included in the hybridization). Numbers on the right correspond to sizes (in bases) of DNA molecular weight markers. Luc, luciferase.

A short subregion in the IL-2 enhancer mediates repression by VDR. The promoter-proximal 321-bp segment of the IL-2 promoter contains several binding sites for a variety of transcription factors that mediate the induction of IL-2 expression (13, 26, 28). We were concerned that any attempt to delete further downstream in order to map the subregion mediating vitamin D_3 repression might compromise activation, resulting in a concomitant lower sensitivity to repression. We therefore chose to delineate the repression site by assaying for in vitro DNA binding by the receptor, initially using the 321-bp IL-2 enhancer element as a probe in a DNase I footprinting assay (Fig. 3A). Two closely linked protected regions were identified; the first footprint is from −283 to −278, and the second spans −266 to −252. The footprinted region overlaps an important positive IL-2 recognition element, called NF-AT-1 (52), that contains a weak AP1 site and a binding site for an inducible factor in T cells, NFATp (65). A 41-bp oligonucleotide duplex corresponding to the sequence shown in Fig. 3A and containing both the NF-AT-1 element and the putative VDR-binding site was synthesized and used as a probe in a gel shift assay; as little as 20 ng of purified VDR shifted this element (Fig. 3B). To test if the NF-AT-1 site alone could mediate $1,25(OH)_2D_3$-specific repression in vivo, five copies of this oligonucleotide were cloned into an E1b TATA-driving luciferase reporter and used in transfection assays. At least three copies of the NF-AT-1 element have been previously shown to confer strong transcriptional activation in response to cell surface activation signals (17). When Jurkat cells were cotransfected with this construct and with a VDR producer, $1,25(OH)_2D_3$-specific repression of luciferase activity was observed (Fig. 3C). A much lower, but detectable, repression due to endogenous VDR was also seen. These results demonstrate that VDR can bind to and mediate its repressive effect through the same minimal element is required for IL-2 transcriptional activation, namely, the NFAT-1 site.

RXR enhances the inhibition conferred by VDR. RXR is considered to be the heterodimeric partner of VDR. It strongly enhances VDR's binding to a positive vitamin D response element in vitro and has been shown to be important in mediating $1,25(OH)_2D_3$ transcription activation in vivo (12, 39, 48, 76). Therefore, RXR's contribution to transcriptional repression was tested (Fig. 4). An RXR overexpression plasmid

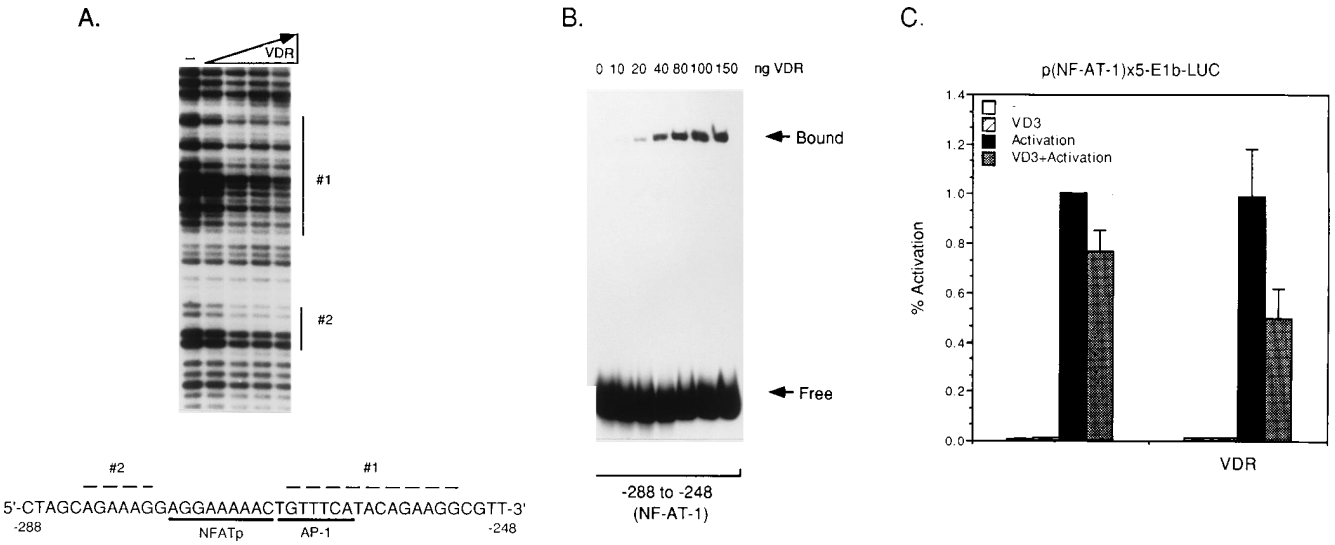


FIG. 3. DNA binding and transcriptional repression by VDR is localized to the NFAT-1 region of the IL-2 enhancer. (A) DNase I protection of the IL-2 enhancer by VDR. *E. coli*-expressed purified VDR protein (50 to 1,000 ng) was used in the DNase I footprint analysis on the proximal 321 bp of the IL-2 enhancer, which conferred inhibition in the transactivation assays. Indicated below is the NF-AT-1 element and surrounding sequences. The NFATp- and Jun-Fos-binding sites are underlined, and the two VDR protected regions are overlined. (B) VDR binding to a 41-bp element containing the distal NF-AT-1 site. An oligonucleotide duplex (top strand, 5′-CTAGCAGAAAGGAGGAAAAACTGTTTCATAGAGAAGGCGTT-3′) was used as a probe and incubated with 0 to 150 ng of purified VDR, and binding was assayed by gel shift. (C) VDR mediates repression of IL-2 activation in vivo through the NF-AT-1 site. Cells were transfected with a luciferase reporter plasmid containing five copies of the NF-AT-1 oligonucleotide [p(NF-AT-1)$_5$-E1b-LUC] and with pCMV-VDR or pRC-CMV. Cell treatment and normalization were as described for Fig. 1B. The data represent the averages of three independent experiments.

was used to cotransfect Jurkat cells with wild-type VDR together with the p(−321)IL2-LUC reporter, and cells were treated as before. 1,25(OH)$_2$D$_3$ repression was nearly twofold higher when both VDR and RXR were cotransfected than when VDR was transfected alone. Levels of repression with VDR alone are lower in this assay than in previous experiments (i.e., 40% in this assay versus 60 to 70% in the assay shown in Fig. 2B) since half as much VDR producer plasmid was used in this experiment. Transfection of the RXR producer alone augmented the repression seen by the endogenous VDR receptor somewhat but not to the level observed when VDR is overexpressed, indicating that VDR and not RXR is the limiting factor for repression (Fig. 4). RXR therefore plays a role in the vitamin D$_3$ repression of IL-2 transcription, presumably through heterodimerization with VDR. Consistent with this inference, in vitro DNA binding of VDR to the NFAT-1 element is enhanced by RXR (see Fig. 5B).

VDR DNA binding alone is necessary but not sufficient for repression. Transcriptional repression by steroid and nuclear receptors appears to require an intact DBD and in some cases DNA binding; however, in several examples, protein-protein interactions independent of DNA binding are sufficient for repression (27, 38, 40, 73, 83). To determine the contributions of both the DBD and direct DNA binding by VDR to the NFAT-1 site in mediating the IL-2 repression, several mutant VDR derivatives were generated (Fig. 5A). Changes were introduced at various positions within the first zinc module of the VDR DBD that forms a specificity α-helix; in a variety of nuclear receptor DBD crystal structures, side chain contacts with bases occur exclusively within this subregion of the DBD (47). Thus, the expectation was that amino acid changes at G-46 and R-49 to A and K, respectively (residues found in several other nuclear receptors at these positions), changing R-50 to G, or changing the P-box triplet EGG at positions 42, 43, and 46 to GSV (the corresponding residues in the glucocorticoid receptor) would drastically alter or abolish VDR-DNA binding. Three such mutant derivatives, EGG→GSV, G46A/R49K, and R50G, were all overexpressed in *E. coli*, purified to homogeneity, and tested for their relative abilities to bind to the NFAT-1 element in vitro. As shown in Fig. 5B, all three mutant proteins were unable to bind the NFAT-1 probe, in the presence or absence of RXR, under conditions in which the wild-type VDR bound the element with high affinity. Importantly, the EGG→GSV mutant bound a chimeric direct repeat element with high affinity in the presence of RXR, indicating that these amino acid changes did not disrupt the overall fold of the DBD (42) but rather only altered its specificity (22). A fourth derivative, the VDR DBD alone (VDRF; residues 14 to 114), which binds selectively and with high affinity to a positive vitamin D response element (23), was also assayed for binding to the NF-AT-1 element. In contrast to the other mutants, this derivative was able to bind strongly to the NF-AT-1 probe (Fig. 5B, lanes 14 to 18).

To test the activities of these mutant VDRs in vivo, Jurkat cells were cotransfected with wild-type VDR, the three mutant VDRs, or VDRF, together with p(−321)IL2-LUC, and repression of activation was assessed. As seen in Fig. 5C, the ability of each of the mutants and VDRF to repress IL-2 expression was abolished or significantly less than that of wild-type VDR. The residual repression detected from the mutant receptors most likely derives from the 10% inhibition attributed to the endogenous VDR in Jurkat cells. One mutant, G46A/R49K, partially repressed beyond the endogenous level (i.e., 33%) and was in fact able to bind very weakly but reproducibly to the NFAT-1 element in vitro (Fig. 5B, lane 10, and data not shown). Note that VDRF, which lacks the ligand-binding domain, should have repressed independently of hormone if it were functional. Immunoblotting revealed that these proteins were expressed at the same levels as wild-type VDR following transfection (data not shown). Taken together, these results indicate that DNA binding by VDR is necessary for the repression of IL-2 expression, since the VDR DBD mutants were

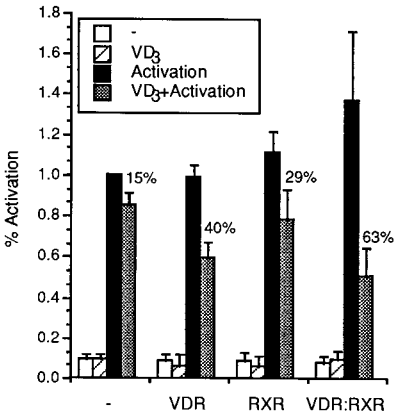
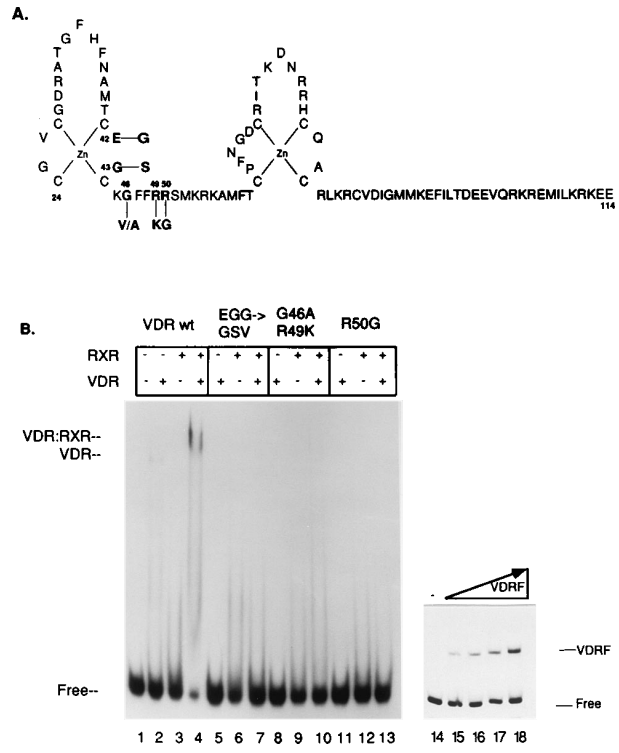


FIG. 4. 1,25(OH)$_2$D$_3$-mediated repression is enhanced by RXR. Jurkat cells were cotransfected with an RXRα or VDR producer plasmid (1 μg) alone or together, or with the parent vector pRc-CMV (−), and with p-321(IL2-LUC). Cells were also transfected with RSV-CAT (10 μg), and luciferase activities (light units) were normalized to CAT levels, as described for Fig. 1B.

unable to either bind in vitro to the NF-AT-1 element or repress in vivo. DNA binding alone, however, is apparently not sufficient, because the VDR DBD bound the element but nevertheless could not repress activated IL-2 transcription.

VDR inhibits NFAT complex formation on DNA in vitro. The results shown in Fig. 5 suggest that other interactions in addition to simple DNA binding by VDR are implicated in 1,25(OH)$_2$D$_3$-mediated repression of IL-2-activated transcription. This would most likely involve protein-protein contacts between VDR and the positive components that bind to the NF-AT-1 site, namely, NFATp and Jun-Fos (11, 33–35, 54, 55, 60, 61). To more carefully examine VDR's role in NFAT complex formation, nuclear extracts were made from resting and activated Jurkat cells, and their levels of binding to the NF-AT-1 oligonucleotide probe were assessed by gel mobility shift assay. A PMA- and PHA-inducible complex was detected with nuclear extracts from activated cells and to a much lower extent with extracts from resting cells (Fig. 6A; compare lanes 1 and 3). The weak shift detected with extracts from resting cells was probably due to some leaking of NF-ATp protein from the cytoplasm during extract preparation. Interestingly, both activated and resting cells extract shifts were inhibited when *E. coli*-expressed purified VDR was added to the reaction (lanes 2 and 4).

When the *E. coli*-expressed and purified components of the NFAT-1 binding complex, i.e., the DBD fragment of murine NFATp and full-length human Jun and Fos, were incubated together with the NF-AT-1 oligonucleotide probe, two specific shifts were detected (Fig. 6B, lane 9). The lower complex corresponds to the NFATp protein bound to DNA, and the more slowly migrating complex corresponds to a Jun-Fos-NFATp-DNA complex (54). Jun and Fos were unable to bind on their own to this element (Fig. 6B, lane 2), since the AP-1 site here constitutes a very weak recognition sequence (55). When VDR alone or the VDR-RXR heterodimer was combined with Jun, Fos, and NFATp, complex formation was significantly inhibited, with the heterodimer inhibiting to a greater extent (compare lanes 3 and 7 with lane 9). Neither the DNA-binding-defective VDR-R50G, the DBD (VDRF), nor RXR alone inhibited the formation of the NFATp-Jun-Fos complex (lanes 4 to 6). In addition, the inhibition by VDR-RXR was specific for the functional heterodimer, since VDR-R50G together with RXR was unable to affect the complex



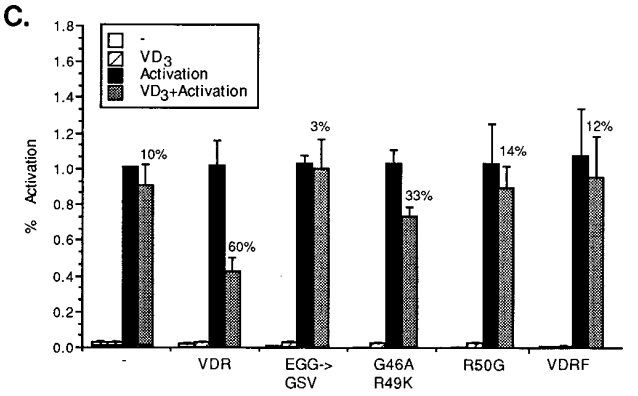


FIG. 5. Mutations in the VDR DBD abolish in vitro DNA binding and in vivo IL-2 repression. (A) Mutations generated in the VDR DBD. Numbering corresponds to that of the translated full-length human VDR cDNA. (B) Binding profiles of wild-type (wt) VDR (lanes 2 to 4) or mutants EGG→GSV (lanes 5 to 7), G46A/R49 (lanes 8 to 10), and R50G (lanes 11 to 13) to the NF-AT-1 element. In each series, 40 ng of purified VDR or mutant VDR was incubated alone or together with 80 ng of glutathione *S*-transferase–RXR and then mixed with the NF-AT-1 oligonucleotide probe. Lane 1 contains the probe alone. The positions of VDR and VDR-RXR complexes are indicated. In lanes 14 to 18, binding of the VDR DBD (VDRF) to the NF-AT-1 probe is shown; 0, 10, 20, 40, and 80 ng of purified VDRF protein was used. (C) In vivo regulation of VDR derivatives on IL-2 expression in Jurkat cells. Cells were cotransfected with 10 μg of p(−321)IL2-LUC reporter and the indicated VDR derivatives or the parent vector, pRc-CMV (−; 2 μg). Cells were also transfected with pRSV-CAT (10 μg), and luciferase activities (light units) were normalized to CAT levels. Cell treatment and normalization were as described for Fig. 1B. The data represent the averages of five independent experiments.

(lane 8). These results recapitulate the transfection data in that the most potent species for repression appears to be VDR-RXR. VDR-R50G and VDRF, both of which were unable to inhibit IL-2 transcriptional activation in vivo, could not block formation of an NFAT complex on DNA in vitro.

NFATp binds to DNA on its own and then recruits Jun and

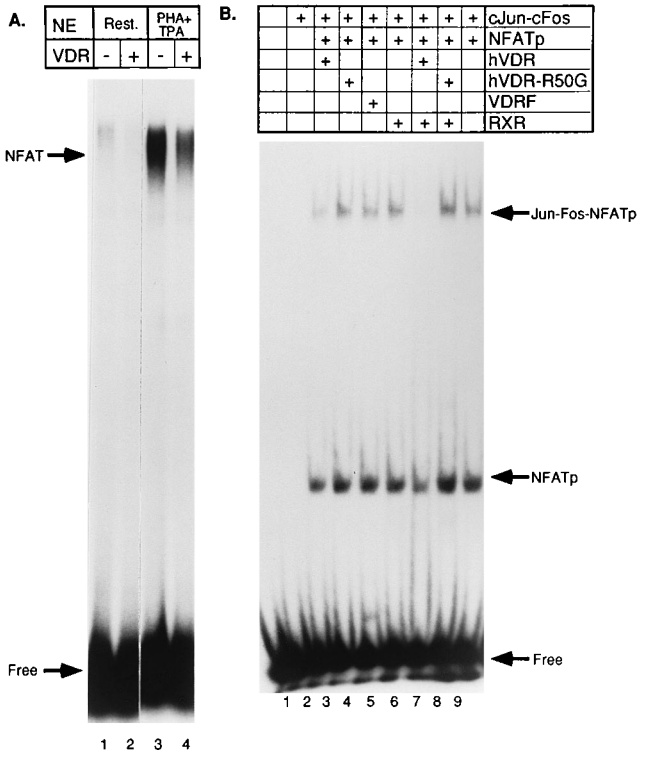


FIG. 6. *E. coli*-expressed purified VDR is able to inhibit NFAT complex formation on DNA in vitro. (A) Nuclear extracts (NE; 4 μg) from either resting (lanes 1 and 2) or PHA (2 μg/ml)- and TPA (50 ng/ml)-activated (lanes 3 and 4) Jurkat cells were incubated with the NFAT-1 oligonucleotide probe in the presence or absence of purified VDR (50 ng) (lanes 2 and 4). Free and bound DNA were resolved by gel shift analysis. (B) Inhibition of Jun-Fos-NFATp complex formation by VDR. Gel mobility shift assay with purified c-Jun (10 ng) proteins (lanes 2 to 9), 2 ng of NFATp DBD (amino acids 1 to 297) (lanes 3 to 9), human VDR (hVDR; 50 ng) (lanes 3 and 7), VDR-R50G (50 ng) (lanes 4 and 8), or VDRF (20 ng; equal molar amount to VDR) (lane 5) and baculovirus-expressed RXRβ (1.5 mg/ml; 1:20 dilution) (lanes 6 to 8). Indicated above each lane are the proteins used in the binding reaction. VDR, VDR-RXR, or one of the VDR derivatives was preincubated for 10 min with Jun and Fos in binding buffer. NFATp was then added for a 5-min incubation, after which the NFAT-1 oligonucleotide probe was added for a further 5-min incubation. Arrows indicate positions of protein-DNA complexes and free DNA.

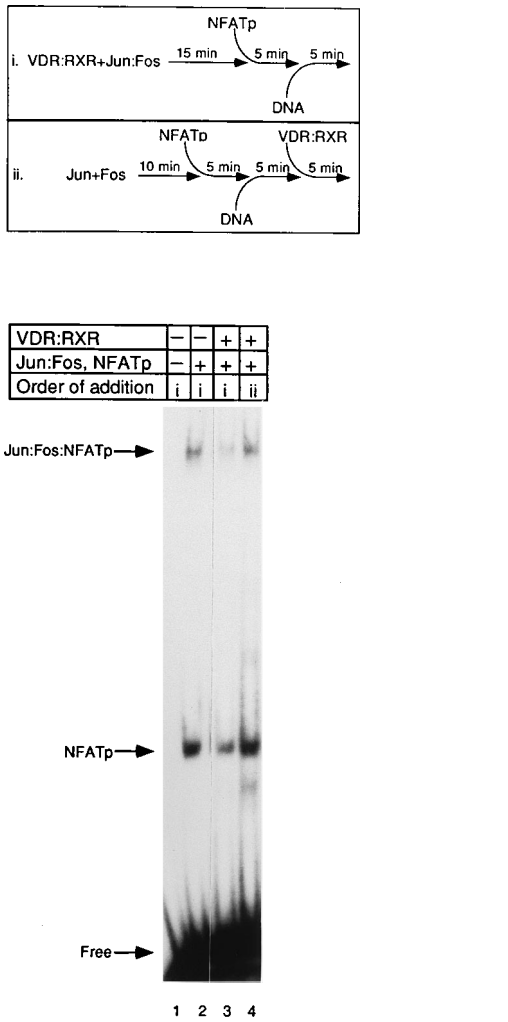


FIG. 7. VDR and RXR act to destabilize the interaction between Jun-Fos and NFATp prior to DNA binding. A schematic representation of the order of addition of proteins and DNA in the gel mobility shift assay is depicted above the gel. The gel mobility shift assay conditions and the amounts of proteins used are exactly as described in the legend to Fig. 6. Above each lane, the order of addition is indicated.

Fos to DNA, thus stabilizing the Jun-Fos-NFATp complex on the NF-AT-1 element (34, 55). The VDR-RXR heterodimer may act to inhibit the interaction between NFATp and Jun-Fos prior to their DNA binding, or it may destabilize the complex itself once bound to DNA. To distinguish between these two possibilities, the VDR-RXR heterodimer was added to the gel shift reaction either before or after the NFATp-Jun-Fos complex was combined with the DNA probe. As shown in Fig. 7, the VDR-RXR heterodimer was able to repress the NFAT-specific complex only when added before, not after, the addition of DNA (compare lanes 3 and 4). This result suggests that the receptor interferes with the NFATp-mediated recruitment of Jun-Fos to DNA but cannot displace the complex once it is bound.

To examine this observation in more detail, an off-rate experiment was carried out by simultaneously mixing Jun, Fos, NFATp, and DNA in the presence or absence of VDR-RXR and allowing them to incubate together for 1 h. At the end of this incubation, equal aliquots were removed and added to a 20-fold molar excess of unlabeled NF-AT-1 competitor DNA for times ranging from 0 to 60 min. As shown in Fig. 8, in the absence of VDR-RXR, a 30-min half-life for the NFATp-Jun-Fos complex was observed; NFATp alone was considerably more unstable (half-life of <2 min). When VDR-RXR was included in the reaction, however, very little NFATp-Jun-Fos complex was detected, even at the earliest time point. Instead, VDR and VDR-RXR complexes were apparent and were stable through 60 min of competition. The NFATp complex alone displayed the same kinetics as it does in the absence of VDR-RXR, implying that on its own, this protein is not the target of the receptors. Taken together, these results indicate that VDR-RXR can block NFAT complex formation on DNA both by interfering with the assembly of the complex on DNA and by slow-kinetic binding to an overlapping DNA sequence. Since some residual NFATp is always bound to DNA (Fig. 7 and reference 55), an effective way to block the recruitment of Jun and Fos to the NFAT-1 element is by the formation of a stable VDR-RXR complex on DNA, which may subsequently displace NFATp from DNA and preclude additional binding.

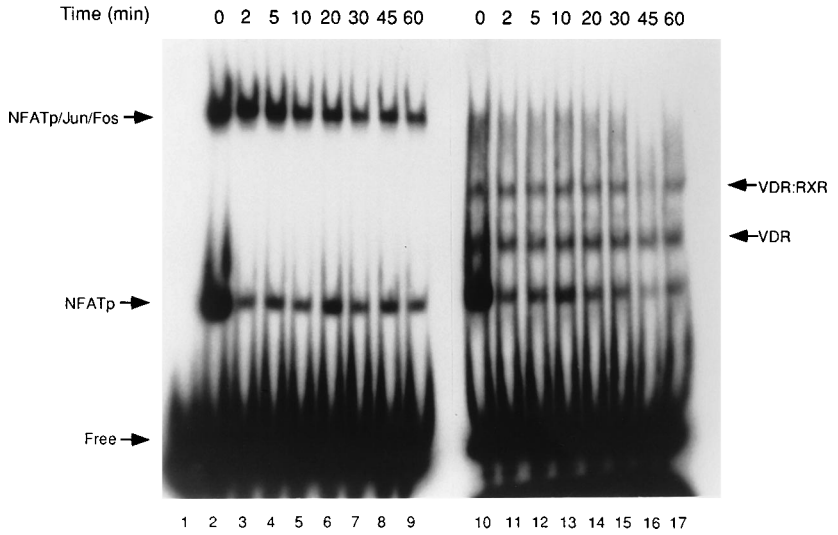


FIG. 8. The VDR-RXR heterodimer inhibits NFATp-Jun-Fos complex formation and stably binds to the NF-AT-1 element over a 60-min period. The NF-AT-1 oligonucleotide probe was incubated with Jun (10 ng), Fos (10 ng), and NFATp (2 ng) in the absence (lanes 2 to 9) and presence of VDR (50 ng) and RXRβ (50 μg of total protein) (lanes 10 to 17) for 1 h; equal aliquots were removed, added to a 20-fold molar excess unlabeled NF-AT-1 oligonucleotide, and incubated for 0 to 60 min as indicated. At the end of the incubation, reaction mixtures were loaded on a 4% native polyacrylamide gel. The positions of protein-DNA complexes and free DNA are indicated by arrows.

DISCUSSION

Repression of T-cell proliferation and IL-2 gene expression by the active metabolite of vitamin D, $1,25(OH)_2D_3$, has been documented in the literature. However, the molecular mechanism underlying this repression has not been characterized. In this work, we have attempted to elucidate the mechanism of IL-2 transcriptional repression by this hormone.

We have found that the repression of IL-2 transcriptional activation by $1,25(OH)_2D_3$ is mediated directly on the IL-2 promoter by VDR. Both transfection and in vitro DNA-binding assays indicate that the receptor confers this repression most effectively in the form of a VDR-RXR heterodimer. The specific target for VDR is a protein complex composed of a lymphoid cell-specific transcription factor, NFATp, and the ubiquitous transcription factors Jun and Fos. This complex binds to a site centered at −270 in the IL-2 enhancer and alone can mediate IL-2 activation. This short element is therefore able to confer both the activation and repression of IL-2 transcription.

Different repression mechanisms have been proposed for steroid/nuclear receptor family members. In some cases, DNA binding is required (2, 14, 15, 31, 70, 71, 84), while in other examples, protein-protein interactions appear to be sufficient in conferring repression (36, 38, 40, 41, 70a, 74, 83, 85). We have demonstrated here that DNA binding by VDR occurs within or just proximal to the NF-AT-1 element and that DNA binding is necessary but does not appear to be sufficient for the repression. On the one hand, we introduced into the VDR DBD several point mutations that abolish both in vitro DNA binding to the NF-AT-1 element and transrepression of IL-2-activated transcription (Fig. 5). On the other hand, regions outside the VDR DBD must also be required, since a derivative encompassing just the DBD, VDRF, was capable of binding the NF-AT-1 element with high affinity but was unable to inhibit IL-2 gene transcription (Fig. 5). These results suggest that VDR may interact with NFATp, Fos, or Jun, in addition to binding directly to DNA. Coimmunoprecipitation experiments in the presence of a reversible cross-linker have been carried out that indeed demonstrate an in vitro interaction of Jun or Fos with VDR in the absence of DNA (2a).

Of the transcription factors that bind to the NF-AT-1 element, it is NFATp that primarily determines the DNA-binding specificity of the NFAT complex in vivo and in vitro, whereas at least a portion of the transcriptional activity is provided by Jun and Fos (54, 64a). NFAT complex assembly occurs via high-affinity binding of NFATp to DNA, which in turn recruits Jun and Fos and in the process stabilizes the whole complex on DNA (59). The interactions that take place between these factors and VDR were tested in a gel mobility shift blocking assay. Purified, full-length VDR was able to inhibit NFAT complex formation on DNA when the source of NFAT was an activated T-cell nuclear extract. Similarly, VDR inhibited the assembly of purified Jun, Fos, and NFATp on the NF-AT-1 element (Fig. 6). The addition of RXR enhanced the ability of VDR to inhibit NFATp-Jun-Fos complex formation on DNA. This result is in agreement with our transfection results, which indicated that RXR enhanced the observed repression of IL-2 transcription by VDR. Moreover, the VDR-RXR heterodimer was able to inhibit complex formation only when added before the addition of DNA; a prebound NFATp-Jun-Fos complex was not destabilized by the receptor (Fig. 7). Importantly, both VDRF and VDR-R50G, neither of which could repress IL-2 expression in vivo, were unable to inhibit NFATp-Jun-Fos-DNA complex formation in vitro (Fig. 6B). That one of these VDR mutants binds DNA (VDRF) and the other does not (VDR-R50G) again points to a repression model that requires both protein-protein interactions and DNA binding.

How then might VDR mediate the $1,25(OH)_2D_3$-dependent repression of IL-2 transcription in T cells? The data presented here indicate that VDR-RXR heterodimers, which would form in response to the intracellular presence of $1,25(OH)_2D_3$, could interfere with the assembly of the transcriptionally active NFATp-Jun-Fos-DNA complex by precluding the interaction between Jun-Fos and NFATp, since prebound NFATp-Jun-Fos cannot be destabilized by VDR-RXR (Fig. 7). Even if, as suggested from the off-rate experiment shown in Fig. 8, VDR

inhibits only Jun-Fos and permits NFATp protein to bind the DNA element, NFATp's half-life on the NFAT-1 element is extremely short (33), and so it would in turn dissociate from the DNA. The stable association of VDR-RXR on the NF-AT-1 element (Fig. 8) would presumably block any subsequent assembly of the NFAT complex.

The NF-AT-1 site in the IL-2 promoter could be an example of a complex composite DNA recognition element for NFATp, Jun-Fos, and VDR, since it appears to selectively bind all of these factors. Similar composite elements have been described for other examples of steroid receptor-mediated repression (14, 56, 57, 73). Given that glucocorticoids are known to be potent inhibitors of IL-2 transcription and that the glucocorticoid receptor interacts with Jun-Fos, it is possible that the mechanism that we propose here for VDR might be a paradigm for how steroids and vitamin derivatives (i.e., vitamin D_3 and retinoids) down-regulate the transcription of cytokines such as IL-2, resulting in a suppression of the immune response.

Several cytokine genes other than the IL-2 gene may be regulated by NFATp or a growing list of NFAT family members, such as NFATc (20), NFATx (50), NFAT-3, and NFAT-4 (29). GM-CSF, IL-3, IL-4, and tumor necrosis factor alpha all have NFAT elements in their promoters (65). The NFAT-binding sites identified in the GM-CSF promoter differ from the IL-2 element in that the AP-1 sites are of high affinity and the NFAT sites are weak. The IL-4 enhancer harbors five independent NFAT sites, and at least four of these sites, including one strong NFATp-binding site, are required for promoter induction and also may form complexes with Jun and Fos (64a). The NFAT element in the tumor necrosis factor alpha gene appears to have diverged considerably from other NFAT-binding sites; it binds two molecules of NFATp as well as Jun but does not contain Fos (52). It is not known whether VDR represses the transcription of these genes, but GM-CSF mRNA levels are negatively regulated by $1,25(OH)_2D_3$, as assessed by Northern (RNA) blot analysis (77). The work presented here suggests that the presence of an AP-1 site as part of a composite NFAT element in these cytokine promoters may mediate $1,25(OH)_2D_3$ repression or repression by other steroid/nuclear hormone receptors in a manner similar or identical to what we have described here for IL-2.

ACKNOWLEDGMENTS

We thank M. Jasin, U. Siebenlist, R. Roeder, R. Evans, A. Rao, G. Parsons, K. Yamamoto, T. Kerppola, T. Curran, K. Ozato, and J. W. Pike for cell lines, plasmids, proteins, and antibodies. We are especially grateful to A. Rao, R. Benezra, and M. Jasin, as well as members of the Freedman laboratory, for ongoing suggestions and criticisms during the course of this work, and we thank B. Lemon and M. Bommakanti for help with experiments.

This work was supported by Public Health Service grants CA-08748 and CA-29502 from the National Cancer Institute. L.P.F. is a Scholar of the Leukemia Society of America.

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