The primary function of activated T lymphocytes is to produce various cytokines necessary to elicit an immune response; these cytokines include interleukin-2 (IL-2), interleukin-4, and granulocyte-macrophage colony-stimulating factor (GMCSF). Steroid hormones and vitamin A and D3 metabolites act to repress the expression of cytokines. 1,25-Dihydroxyvitamin D3 (1,25-(OH)2D3) down-regulates activated IL-2 expression at the level of transcription, through direct antagonism of the transactivating complex NFAT-1/AP-1 by the vitamin D3 receptor (VDR). We report here that GMCSF transcription in Jurkat T cells is also directly repressed by 1,25-(OH)2D3 and VDR. Among four NFAT/AP-1 elements in the GMCSF enhancer, we have focused on one such element that when multimerized, is sufficient in mediating both activation by NFAT-1 and AP-1 and repression in response to 1,25-(OH)2D3. Although this element does not contain any recognizable vitamin D response elements (VDREs), high affinity DNA binding by recombinant VDR is observed. In contrast to VDR interactions with positive VDREs, this binding is independent of VDR’s heterodimeric partner, the retinoid X receptor. Moreover, VDR appears to bind the GMCSF element as an apparent monomer in vitro. Protease digestion patterns of bound VDR, and receptor mutations affecting DNA binding and dimerization, demonstrate that the receptor binds to the negative site in a distinct conformation relative to a positive VDRE, suggesting that the DNA element itself acts as an allosteric effector of VDR function. This altered conformation may account for VDR’s action as a repressing rather than activating factor at this locus.

The classical effects of the secosteroid 1,25-dihydroxyvitamin D3 (1,25-(OH)2D3) include the regulation of calcium absorption in the intestine, maintenance of mineral homeostasis in the kidney, and regulation of bone formation and remodeling (1–3). More recently, the scope of vitamin D action has broadened with the detection of the nuclear receptor for this ligand, vitamin D3 receptor (VDR), in tissues such as skin, testis, pancreas, colon, muscle, breast, prostate, thymus, and bone marrow. Generally, 1,25-(OH)2D3 acts as a growth inhibitor in many of these cell types. For example, the ligand is a potent inducer of differentiation of promyeloctic leukemia cells (4–6). The identification of the cyclin-dependent kinase inhibitor gene product, p21Waf1/Cip1, as a direct target of 1,25-(OH)2D3 action in myeloid cells (7) provides a direct link between the general actions of this ligand and growth control/differentiation in these cells. Transcription of the p21 gene is directly enhanced in response to 1,25-(OH)2D3 through the binding of VDR to specific regulatory sites in the p21 promoter. VDR, as typical of many members of the nuclear hormone receptor superfamily, binds to DNA and activates transcription as a heterodimeric complex with the retinoid X receptor (RXR) (8–11). VDR homodimers (12, 13) as well as monomers (9) are also capable of binding some vitamin D response elements (VDREs), such as that found in the mouse osteopontin gene promoter (14), but are probably not capable of transactivating (15). The architecture of this consensus positive VDRE is two directly repeating hexameric half-sites consisting of the sequence PuGG/TTCA; such VDREs are probably not capable of transactivating (15). The architecture of this consensus positive VDRE is two directly repeating hexameric half-sites consisting of the sequence PuGG/TTCA spaced by three nucleotides (DR3) (13, 16). Transactivation by VDR from a DR3 is strictly ligand-dependent, where the ligand stabilizes VDR-RXR formation (9, 10), as well as enhances interactions with components of the transcriptional preinitiation complex, such as TFIID (15) and perhaps as yet unidentified coactivators, as has been demonstrated for several other steroid and nuclear receptors (reviewed in Ref. 17).

Whereas considerable information exists describing transactivation by steroid/nuclear receptors, the mechanisms through which these receptors elicit repression of activated transcription, which we call here transrepression, are poorly understood. Several genes have been identified as targets of transrepression by various steroid and nuclear receptors (18–22). Genes that are down-regulated in response to 1,25-(OH)2D3 include those encoding human and chick parathyroid hormone (PTH) (23, 24), rat α1(I) collagen (25), human atrial natriuretic (26), interleukin-2 (IL-2) (27), and the rat bone sialoprotein (28). For the chick PTH and rat bone sialoprotein genes, imperfect DR3 elements have been identified at promoter proximal sites through which 1,25-(OH)2D3-mediated repression occurs. However, the promoters for the human PTH, human atrial natriuretic, and human IL-2 genes do not appear to contain canonsor; EMSA, electrophoretic mobility shift assay; DBD, DNA-binding domain; CMV, cytomegalovirus.

* This work was supported in part by National Institutes of Health Grants DK454460 (to L. P. F.) and CA08748 (to Sloan-Kettering). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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Repression of GMCSF Transcription by VDR

MATERIALS AND METHODS

Antibodies and Overexpressed Proteins—Rat monoclonal anti-VDR antibody was purchased from Affinity BioReagents (Golden, CO). Polyclonal anti-VDR antibody was generously provided by Affinity BioReagents. VDR was overexpressed in Escherichia coli using the pET system as described previously (9). To purify VDR, induced cell pellets were lysed in a buffer containing 50 mM Tris-Cl, pH 7.5, 1 mM EDTA, 50 mM NaCl, 5 mM phenylmethylsulfonyl fluoride (TED-500), plus 0.5 mg/ml lysozyme by Dounce homogenization and micro-tip sonication. Sodium deoxycholate was added to a final concentration of 0.05%, and the sample was centrifuged for 1 h at 30,000 rpm in a Ti45 fixed angle rotor. The supernatant was transferred to a fresh tube, and polymer P (Aldrich) was added to a final concentration of 0.2% over 10 min and incubated an additional 10 min at 4 °C. Samples were then centrifuged for 1 h at 30,000 rpm, and the supernatant was precipitated by addition of ammonium sulfate to 30%. The sample was incubated at 4 °C for 1 h, and centrifuged for 20 min at 20,000 rpm in a Ti45 rotor. The protein pellet was resuspended in TED-50 and purified by gel filtration analysis as described previously (9). The VDR DNA-binding domain derivative (DBD) was purified as described previously (38). The mutant receptors VDR-L262G and VDR-K45A were purified exactly as described for wild-type VDR. FLAG-RXRα was overexpressed in insect cells and purified as described previously (39).

Plasmids—The GMCSF reporter constructs used in transient transfection experiments were created as follows: 716GMCSF-LUC–pGL-2 was digested with Smal and NheI, alkaline phosphatase-treated, and gel-purified. The Pou1IbaxI, 1.4-kilobase pair fragment insert was derived from pHGM716CAT (provided by P. Cockerill, Hanson Center for Research, Australia) corresponding to the GMCSF promoter fragment from 2.6 kilobase pairs to 3.3 kilobase pairs as well as 600 bp of the GMCSF promoter. Positive clones were verified by dioxygenucleotide DNA sequencing. 3N3GMCSFLUC–pGL2 basic plasmid was digested with Sall and XhoI, filled in with Klenow enzyme, alkaline phosphatase-treated, and gel-purified. The Pou1IIStnl 600-bp GMCSF promoter fragment insert was derived from pHMG3N3CAT (provided by P. Cockerill). This fragment contained the NFAT1 site at position 550 within the 716 bp of the GMCSF minimal enhancer sequence, multimerized three times, yielding a 158-bp fragment fused to the 600-bp GMCSF promoter fragment. GMCSFLUC–pGL2 basic plasmid was digested with SacI and XhoI, filled in by Klenow enzyme, alkaline phosphatase-treated, and gel-purified. The Pou1IIStnl 600-bp GMCSF promoter fragment insert was derived from HMG3N3CAT. Expression plasmids were generated using the cytomegalovirus-driven pEc-CMV vector (Invitrogen); CMV-VDR (40) was constructed as described previously. VDR mutant constructs were subcloned as described for wild-type VDR.

Oligonucleotide-directed in Vitro Mutagenesis—Site-directed mutagenesis was carried out using pCMV-VDR as template, generating single-stranded DNA, and synthesizing the mutant strand with the mutagenesis primer. For VDR-L262G mutant VDR, the mutagenic oligonucleotide 5’-TACATCTGTTCTGCATGCTGCTGTCGATGACGTCCTG-3’ was used to change leucine 262 to a glycine residue. VDR-K45A was generated using the mutant oligonucleotide 5’-CTGAAAGGCTGCCGCTTCTC-3’ to change lysine 45 to an alanine residue. Mutant pools were screened for the appropriate codon changes by dioxygenucleotide DNA sequencing. The mutated VDR was transferred to a T7 overexpression vector as an Avidin-BiotinII fragment, and protein was produced and purified as described for wild-type VDR.

Electrophoretic Mobility Shift Analysis—VDR DNA binding was assessed by gel mobility shift electrophoresis using conditions described previously (11). The VDR from the mouse osteopontin gene (DR3) was generated as complimentary oligonucleotides of the sequence 5’-GATC CACAAGGTTCAAGGTTTACTAGCCTGCC-3’ (top strand). The NFAT/ AP-1 binding site position 550 within the 716 bp of the GMCSF enhancer (GM550) was synthesized as complimentary oligonucleotides of the sequence 5’-GATCCTTTATATGACTCTTCTGTTTTCCTCCTCCCTTA-3’ (top strand). The sequence of the NFAT-IL2 oligonucleotide top strand is 5’-CTCAGCAGAAAAGGAGGAAAAACGTCTTACATCAGAAAGGCTT-3’. Mutant oligonucleotides are listed in Fig. 3A. Equimolar amounts of complimentary strands were annealed and 32P-end-labeled as described previously (38). Overexpressed purified VDR was incubated with 12 fmol of the indicated oligonucleotide duplex for 20 min at room temperature together with 50 µg of poly(dI-dC)-mnl in binding buffer (20 mM Tris-Cl, pH 7.9, 1 mM EDTA, 50 mM KCl, 10% glycerol, 0.05% Nonidet P-40, and 1 mM dithiothreitol). Protein-DNA complexes were resolved by electrophoresis on 10% nondenaturing acrylamide gels run in 0.5× Tris borate-EDTA, at 250 V, constant voltage at 4 °C. Gels were dried and subjected to autoradiography. For supershift analyses, receptors...
were preincubated with preimmune sera or specific antibody for 10 min at room temperature. DNA was then added, and incubation proceeded for an additional 20 min at room temperature. Electrophoretic mobility shift assay (EMSA) was performed as described above.

**Proteolytic Clipping Assay—**EMSA was carried out as described above with the following modifications. 100 ng of recombinant VDR was incubated with 20 fmol of the indicated 32P-end-labeled oligonucleotide duplex for 20 min at room temperature. Trypsin protease (Life Technologies, Inc.) was made up in 137 mM NaCl at a stock concentration of 25 μg/μl. Trypsin protease was then added directly to the binding reactions to the indicated final concentration for 10 min at room temperature. For the time-course experiment, 10 ng/μl trypsin was added directly to the binding reactions for the indicated times. Complexes were resolved by electrophoresis on 10% nondenaturing acrylamide gels as described above, dried, and subjected to autoradiography.

**Cell Transfection and Reporter Assays—**The T cell line Jurkat was transfected by electroporation method using BTX (San Diego) 0.2 cuvettes. Cells were grown in RPMI medium containing sodium pyruvate, glutamine, and penicillin-streptomycin to a final concentration of 100 μg/ml. Fetal calf serum was added to 10%, and cells were maintained at a density of approximately 8 × 10^4 cells/ml. For transfection, cells were washed in RPMI medium and resuspended in this medium to a density of 3 × 10^6 cells/200 μl. Each transfection reaction contained 5 μg of reporter plasmid, 1.25 μg of internal control plasmid, 500 ng of producer plasmid, and 5 × 10^6 cells. All reactions are done in triplicate with two transfections. Therefore, six reactions were transfected per BTX cuvette in a final volume of 200 μl. BTX settings were as follows; T = 500 V, C = 1700 microfarads, R = 72 ohms, S = 126 V. After electroporation, cells were incubated for 30 min and the contents of each cuvette was then added to 6 ml of RPMI containing sodium pyruvate, glutamine, penicillin-streptomycin, and charcoal-stripped fetal calf serum to 10%. Cells were plated as 1 ml of transfection mix added to 14 ml of the same stripped serum containing medium and allowed to incubate 24 h (5% CO₂, 37 °C). At 24 h after transfection, cells were treated in one of the following ways for 9 h: (a) no treatment, (b) addition of activating agents phorbol myristate acetate (PMA, 50 ng/ml; Sigma) and phytohemagglutinin (PHA, 2 μg/ml; Sigma), (c) addition of 5 × 10^{-8} M 1,25-(OH)₂D₃ (Biomol), or (d) addition of activating agents and 1,25-(OH)₂D₃. Experiments were harvested and normalized to protein concentration as well as to β-galactosidase activity produced off the internal control plasmid CMV-β-gal included in each transfection. Equal amounts of total cell extract were added to luciferase assays, and results were quantitated as relative light units using a luminometer.

**RNase Protection Assay—**Jurkat cells were transiently transfected as described previously with the reporter constructs N3GMCSF/LUC, as well as the producer plasmids pRC-CMV or pCMV-VDR. Transfected cells were treated with the activating agents PMA (50 ng/ml) and PHA (2 μg/ml) alone or in the presence of 1 × 10^{-8} M 1,25-(OH)₂D₃. All cells were treated with 10 mM cycloheximide for the duration of the experiment. Cells were harvested after 6 h of treatment, and total RNA was isolated using Trizol reagent (Life Technologies, Inc.). The antisense luciferase probe was generated as described previously (27). 3 μg of RNA was ethanol-precipitated with 1 × 10^{-6} cpm of either luciferase antisense probe or an antisense β-actin probe. The reaction was then resuspended in hybridization buffer (Ambion RPA II kit), heated to 90 °C, and vortexed thoroughly. Hybridization reaction was incubated overnight at 45 °C. Digestion of unprotected RNA was performed by the addition of a 1:100 dilution of an RNase A/RNase T1 mixture, and allowed to incubate at 37 °C for 30 min. Protected fragments were ethanol precipitated, resuspended in a glycerol loading buffer and heated to 90 °C for 4 min. Reactions were analyzed on a 6% denaturing polyacrylamide gel run at 400 V at room temperature.

**Immunoblotting—**Transfected Jurkat cells were harvested and resuspended in 250 μl Tris buffer, pH 8.0. Whole cell extracts were prepared by repeated freeze/thaw lysis, and centrifuged at 14,000 rpm at 4 °C for 15 min in a microcentrifuge. Supernatants were retained, and protein concentration was determined by the Bradford method (Bio-Rad). 30 μg of whole cell extract was analyzed by SDS-polyacrylamide gel electrophoresis. Protein was transferred to polyvinylidene difluoride membrane (NEP Life Science Products) at 140 mA for 20 min in 25 mM Tris, 192 mM glycine, and 20% methanol. The membrane was blocked with 15% nonfat dry milk (Carnation) in phosphate-buffered saline overnight at room temperature. Monoclonal rat anti-VDR (Affinity Bioreagents) was dilute 1:6000 in phosphate-buffered saline, 1% nonfat dry milk, 0.1% Tween 20 and incubated for 3 h. The membrane was washed extensively in phosphate-buffered saline, 0.1% Tween 20. The membrane was then incubated in a secondary antibody solution consisting of a 1:2000 dilution of horseradish peroxidase-conjugated sheep anti-rat IgG (Amersham Pharmacia Biotech) in phosphate-buffered saline, 1% nonfat dry milk, 0.1% Tween 20 for 45 min. Membrane was washed as previously described and developed by using enhanced chemiluminescence (Amersham Pharmacia Biotech).

**RESULTS**

VDR Directly Mediates Repression of the GMCSF Locus by Acting through a NFAT/AP-1 Site in the GMCSF Enhancer—A
group of genes encoding the cytokines IL-2, GMCSF, and interferon-γ that are activated in response to T cell stimulation are also repressed by the secosteroid 1,25-(OH)₂D₃. We previously demonstrated that 1,25-(OH)₂D₃-mediated repression of IL-2 transcription is a direct, VDR-dependent effect (40). To determine if the down-regulation of GMCSF mRNA levels observed in response to 1,25-(OH)₂D₃ is also occurring at the level of transcription, a transient transfection experiment was performed with the promoter constructs depicted in Fig. 1A. 716 bp of the GMCSF enhancer located from position −2600 bp to −3316 bp from the start site of transcription were fused to the GMCSF promoter and subcloned into a luciferase reporter backbone construct yielding the construct −716GMCSF-LUC. This construct was used to transiently transfected, with or without a VDR producer plasmid, Jurkat cells, a transformed T cell leukemia cell line. In each transfection series, cells were (a) left untreated, (b) treated for 9 h with the activating agents PMA and PHA, (c) treated with 1,25-(OH)₂D₃ alone, or (d) treated with activating agents and 1,25-(OH)₂D₃. Activation levels were reduced by only 14% upon the addition of 1,25-(OH)₂D₃ in the absence of overexpressed VDR (Fig. 1B). It has been reported that VDR is not expressed in resting peripheral blood lymphocytes, and is only detectable following activation (33). We have been able to detect VDR expression in non-activated Jurkat cells but at very low levels (for example, see Fig. 7A). However, upon transient overexpression of VDR, activation levels were reduced by nearly 60% following addition of 1,25-(OH)₂D₃ (Fig. 1B). This repression was also ligand dose-dependent, with maximal repression of 98% occurring at 1 × 10⁻⁶ M 1,25-(OH)₂D₃ (data not shown). The increase in repression in response to VDR levels and ligand concentration indicates that the effects are both receptor- and ligand-dependent. The 1,25-(OH)₂D₃ repression is independent of de novo protein synthesis since the observed down-regulation of GMCSF mRNA as detected in ribonuclease protection assays was resistant to the presence of 10 μM cycloheximide (Fig. 1C).

The 716-bp region of the GMCSF enhancer used in the reporter assays contains several binding sites for the transcription factor NFAT-1 as well as for AP-1 family members (41, 42). Interestingly, Alroy et al. (27) showed that 1,25-(OH)₂D₃-mediated repression of IL-2 gene transcription was occurring through a NFAT/AP-1 composite site centered at −270 in the IL-2 enhancer. Since cis elements mediating activation could not be separated from putative cis-acting repressing sites, the IL-2 element alone wasomerized to assess its ability to repress. In this context, this site was sufficient to confer both activation and VDR-dependent repression to a minimal promoter. Since a similar situation exists in the GMCSF enhancer, we made an analogous construct consisting of the NFAT/AP-1 site located at position 550 within the 716-bp GMCSF enhancer, since it had been previously shown to be capable of functioning on its own as an enhancer element in activated T cells (42). The NFAT/AP-1 site wasomerized three times, fused to the GMCSF promoter, and subcloned into a luciferase reporter plasmid. This construct, called N3GMCSF-LUC (Fig. 1A), was used in transient transfection experiments to determine if this particular NFAT/AP-1 site in the GMCSF enhancer is sufficient to mediate the 1,25-(OH)₂D₃-dependent repression. As shown in Fig. 1B, the N3GMCSF-LUC reporter was repressed by 60% upon addition of ligand and overexpressed VDR. This level of repression was identical to that observed

![Image](image.png)

**Fig. 2.** RXR does not participate with VDR in binding to the GM550 element. A, binding analysis on a positive VDRE (DR3) (lanes 1–6) versus the negative GM550 element (lanes 7–12). Lanes 1 and 7, probe alone; lanes 2 and 8, 25 ng of purified FLAG-RXRα; lanes 3 and 9, 40 ng of purified VDR; lanes 4 and 10, 80 ng of VDR; lanes 5 and 11, 12.5 ng each of VDR and RXRα; lanes 6 and 12, 25 ng each of VDR and RXRα. The positions of the VDR-DNA and VDR-RXα-DNA complexes are indicated. B, comparison of the VDR and VDR-RXα binding profiles to two different negative elements. NFAT-IL2 is a composite site from the human IL-2 promoter necessary and sufficient to mediate both activation and 1,25-(OH)₂D₃-induced repression (27). Lanes 1 and 8 are probe alone; lanes 2 and 9, 30 ng of VDR; lanes 3 and 10, 14 ng of RXR; lanes 4–7 and 11–14, 30 ng of VDR and a titration of RXR from 14 ng to 35 ng. C, the DNA binding species bound to the negative element is VDR. In lanes 1–4, 40 ng of VDR were incubated with GM550 (lanes 1 and 2) or DR3 (lanes 3 and 4) probe in the absence or presence of 3 μg of a VDR-specific monoclonal antibody (VDR-MAb). In lanes 6–10, 40 ng of VDR were incubated with either DR3 (lanes 6 and 7) or GM550 (lanes 9 and 10) probe in the absence or presence of 3 μg of a FYN-specific monoclonal antibody. In lanes 12 and 13, 10 ng each of Jun and Fos proteins were incubated with GM550 probe in the presence or absence of VDR monoclonal antibody. 10 fmol of DR3 and 12 fmol of GM550 were used as indicated.
with the full 716 bp of the GMCSF enhancer element (Fig. 1B). This result confirms that this NFAT/AP-1 site is also sufficient to confer activation by PMA and PHA (42), and demonstrates that it is sufficient to mediate repression by 1,25-(OH)2D3. It is important to note that the GMCSF enhancer contains three additional NFAT/AP-1 sites within the 716-bp enhancer which may be able to confer activation, and therefore possibly 1,25-(OH)2D3-mediated repression, although we have yet to test these sites. We also observed a modest repressive contribution (25%) acting through the GMCSF promoter alone (Fig. 1B). The promoter contains several transcription factor binding sites, among them an element known as CLEO (43). Interestingly, the promoter also contains a YY1 element, which has been shown to display an antagonizing effect on VDR function (71). This element consists of a weak AP-1 site as well as an Ets element. The possibility exists that such sites could also contribute to the observed repression.

**VDR Is Capable of Binding to a Noncanonical Negative Element in the GMCSF Enhancer with High Affinity, Independent of RXR—**As mentioned, our previous study on the IL-2 enhancer demonstrated that the target for 1,25-(OH)2D3-transrepression was a composite NFAT/AP-1 element (27). Although this region lacks a readily identifiable vitamin D response element, at least as defined by the consensus positive VDRE, we found that VDR and VDR-RXR were in fact able to bind this NFAT/AP-1 site specifically (see Ref. 27 and Fig. 2B). Moreover, VDR mutants that disrupted specific DNA binding to the element in vitro were incapable of conferring transrepression in vivo. We therefore asked if the NFAT/AP-1-containing region at position 550 within the GMCSF enhancer, which confers 1,25-(OH)2D3-transrepression, also binds VDR selectively. Like the IL-2 element, the GMCSF site does not contain any recognizable VDREs. A 35-bp oligonucleotide duplex containing the NFAT/AP-1 site, termed GM550, was synthesized, and DNA binding was analyzed by EMSA. An additional oligonucleotide containing a positive DR3 VDRE served as a control for in vitro DNA binding by recombinant VDR and RXR. Fig. 2A demonstrates that VDR alone, but not RXR alone, bound both the DR3 and GM550 probes (lanes 2–4 and 8–10). Surprisingly, the high affinity VDR-RXR heterodimeric complex, which is the predominant species on the DR3, was not detected on GM550 (compare lanes 5 and 6 with lanes 11 and 12). Moreover, the VDR-GM550 complex migrated with a faster mobility than that of the VDR-DR3 complex (Fig. 2A, lanes 9 and 10 versus lanes 3 and 4). The complex bound to the GM550 element is indeed VDR, since an anti-VDR monoclonal antibody directed against the C-terminal region of the VDR DNA-binding domain blocked receptor binding to both the DR3 and GM550 sites (Fig. 2C, lanes 2 and 4), but a monoclonal antibody raised against an unrelated protein, the Src family member Fyn, was unable to disrupt both complexes (lanes 7 and 10). Moreover, the VDR monoclonal antibody was unable to perturb the Jun-Fos binding complex on the negative element, indicating that the loss of a binding complex by the anti-VDR antibody was specific to VDR. In addition, the VDR-GM550 complex is competed specifically by an excess of DR3 competitor oligonucleotide but not the analogous amount of an oligonucleotide containing a glucocorticoid response element (data not shown).

The absence of RXR in VDR-GM550 binding suggested that RXR is not involved in VDR-mediated transrepression. However, this generalization, however, does not seem to hold, since VDR bound predominately as a heterodimer with RXR to the NFAT-1/IL-2 element (Fig. 2B, lanes 1–7 versus 8–14). Heterodimers will form on the GM550 element at high concentrations; however, this binding is noncooperative and therefore unlikely to be functional. These results indicate that the VDR species at the negative site in GMCSF is distinct from that observed on both the positive DR3 VDRE and the negative NFAT/AP-1.
element in the IL-2 enhancer. These data also infer that VDR may possibly be a monomer and/or in an altered conformation when bound to the GM550 repressive element, based on its faster mobility relative to VDR on the DR3 in the EMSA.

The VDR Binding Site within GM550 Is a Core of Seven Base Pairs That Overlaps the NFAT-1 Binding Site—To further delineate the binding site for VDR in the GM550 element, a series of mutant oligonucleotide duplexes, shown in Fig. 3A, were synthesized and tested in gel mobility shift assays. As seen in Fig. 2B, mutation of the AP-1 site (lanes 16–20) had no effect on VDR binding. Similarly, alterations generated in the GM550 mutB oligonucleotide (lanes 21–25), which include the last two bases of the AP-1 site and three bases that separate the AP-1 and NFAT sites (Fig. 3A), also had little effect on VDR’s ability to bind. However, mutations that completely change the NFAT binding site as well as six additional bases that extend into the AP-1 binding site (GM550 mutA; Fig. 3A) completely abolished VDR binding (Fig. 3B, lanes 6–10). Taking into account that the GM550(–AP1) mutant oligonucleotide confers normal VDR affinity and mutA abolishes binding, we reasoned that the NFAT binding site may be critical for VDR recognition. We therefore generated GM550(–NFAT), where only the NFAT-1 binding site was mutated (Fig. 3A). As seen in Fig. 3B (lanes 11–15), VDR binding to this oligonucleotide is severely reduced. Thus, the lack of VDR binding to GM550 mutA and –NFAT mutant probes defines the sequence GCTTTCC, which superficially resembles an extended VDRE half-site, as the minimal requirement for VDR binding to the GM550 negative element.

The GM550 Negative Element Acts as an Allosteric Effector of VDR—The faster mobility profile exhibited by VDR when bound to the negative GM550 versus the positive DR3 observed in Fig. 2 suggested that VDR binding to the negative element was unique. To address this, several approaches were taken to compare VDR binding to each element. First, a gel mobility supershift assay was carried out. Fig. 4A illustrates that when VDR was prebound to the DR3, a polyclonal antibody raised against the receptor (distinct from that shown in Fig. 2B) was able to specifically supershift the receptor-DNA complex (lane 6). However, when VDR was preincubated with GM550, neither the preimmune serum nor this VDR-specific antibody supershifted the complex (lanes 11 and 12). As was observed in Fig. 2, the VDR-GM550 complex migrated with a faster mobility when compared with VDR bound to the DR3. Additional evidence that VDR utilizes an alternative strategy in binding the negative element was derived from a VDR mutant generated by site-directed mutagenesis termed VDR-K45A. This lysine, at residue 45, is absolutely conserved among all the known members of the steroid and nuclear receptor superfamily and lies within the specificity a-helix of the receptor DBD, immediately proceeding the first zinc finger. This lysine residue has been shown in several crystal structures to be making direct side-chain contacts on positive hormone response elements (44). As expected, DNA binding of the K45A mutant to the DR3 VDRE was completely abolished in the presence or absence of RXR (Fig. 4B, lanes 11–15). However, VDR-K45A was capable of binding to the negative GM550 element, albeit with a slightly lower affinity than the wild-type receptor (lanes 16–20). As with wild-type VDR, addition of RXR had no effect on the binding. This result suggests that a specific contact within the receptor DNA-binding domain essential for interaction with a positive response element is not making an equivalent contact on the negative GM550 site. Transient transfection of cells with the VDR-K45A mutant correlates with the in vitro DNA binding data. The K45A mutant receptor was unable to activate transcription from a DR3-regulated reporter, but repressed from the GM550 element (although repression is reduced to a similar extent as VDRE-DNA binding is decreased; data not shown). Taken together, the data reinforce the importance of DNA binding by VDR on the GM550 element but infer that VDR is associating with this element in a conformation that is distinct from how it binds a positive VDRE. This difference in VDR conformation would presumably be imposed by the DNA element itself.

To directly investigate whether the GM550 element can act as an allosteric effector of VDR, a proteolytic clipping assay was performed. As is evident from the results presented in Fig. 5, GM550 induced a pronounced conformational change in VDR relative to the receptor bound to the DR3 as assayed by protease sensitivity. VDR is highly resistant to the proteolytic effects of trypsin at amounts as high as 100 ng/μl, but only when it is prebound to the negative element (Fig. 5A, lanes 1–8). In contrast, preincubation of VDR with the DR3 yielded a progressive cleavage pattern with increasing trypsin concentrations (lanes 9–16). A nearly identical pattern was observed when a time
course of induction was carried out using a constant amount of protease (Fig. 5B). The resistant profile observed when VDR was bound to GM550 is indicative of a more compacted receptor conformation on the DNA. These allosteric effects induced by the negative element might create new surfaces on the protein, which may be necessary to elicit the repressive function. DNA-induced structural changes have been demonstrated with Jun and Fos (45).

VDR Binds to the GM550 Element as a Monomer—We have demonstrated that the high affinity DNA binding species on GM550 does not include RXR (Figs. 2 and 4B), indicating that the repressing VDR species is not a heterodimer. Cheskis and Freedman (9) demonstrated that, in addition to the usual heterodimeric binding, VDR is also capable of binding to the mouse osteopontin positive VDRE as both homodimers and monomers, whereby preformed homodimers on DNA are dissociated to monomers upon addition of 1,25-(OH)2D3. To address whether VDR is binding to GM550 as a homodimer or a monomer, we took advantage of a truncated version of VDR constituting only the DBD. In the absence of the strong dimerization interface that co-localizes to the C-terminal ligand-binding domain, the VDR DBD cannot form dimers in solution, and as such resolves as both monomers and cooperative dimers on a DR3 in gel shift assays (13). When gel mobility shifts were carried out comparing VDR DBD binding to the positive versus negative element, as shown in Fig. 6A, the VDR DBD yielded two bound species with the DR3 (lanes 1–6), representing monomeric and cooperative dimeric species (dbd1 and dbd2, respectively), but resolved only one bound band with the GM550 probe, running with the same mobility as the faster of the two bound species seen with the DR3 probe (lanes 7–12). This is consistent with a VDR monomer associated with the GM550 element. As a control for monomeric binding, we used an oligonucleotide duplex containing only one half-site from the DR3 element (DR1/2); this probe restricted binding to predominantly a single bound species that is presumably a VDR monomer (lanes 13–18). To further demonstrate this, a mixing experiment was carried out in which full-length VDR and VDR DBD were co-incubated, bound to each probe, and resolved by EMSA (Fig. 6B). Although an intermediate species consisting of a VDR DBD heterodimer bound to the positive DR3 element was readily apparent (lanes 6 and 7), no such species was detected on the negative GM550 element, even at high concentrations of both receptors (lanes 11 and 12). These data strongly suggest that the DNA binding species on the GM550 element is a VDR monomer.

A second approach we took to demonstrate that the VDR-GM550 complex is indeed monomeric utilized a dimerization mutant of VDR, VDR-L262G. This mutation, located within helix 4 of the LBD, renders VDR incapable of heterodimerizing with RXR and therefore unable to activate transcription from a reporter gene regulated by the osteocalcin
Repression of GMCSF Transcription by VDR

We therefore tested the ability of the VDR-L262G dimerization mutant to bind to both the positive and negative elements in vitro (Fig. 7B). As reported previously (46), VDR-L262G is unable to heterodimerize with RXR on the positive DR3 (lanes 9 and 10). Moreover, this mutant cannot homodimerize since no detectable binding was observed on the DR3 in the absence of RXR (lanes 7 and 8 versus 3 and 4). In contrast, the VDR-L262G mutant bound to the negative element GM550 with an apparent higher affinity than wild-type VDR and was unaffected by the addition of RXR (lanes 13–16 versus 17–20). Overall, this DNA binding profile correlates with the transfection data in that the dimerization mutant was able to transrepress from the GM550 element, but could not activate from the DR3 (Fig. 7A). Taken together, the DNA-binding domain and dimerization mutant experiments strongly suggest that the VDR-GM550 complex consists of a VDR monomer that is acting as the functional repressive complex at the GM550 element.

**DISCUSSION**

The paradigm for vitamin D$_3$ receptor binding to DNA elements that confer transcriptional activation in response to 1,25-(OH)$_2$D$_3$ is a dimeric receptor species comprising one molecule of VDR and one molecule of RXR bound to two directly repeating hexameric half-sites (consensus: PuGG/TTCAt), which are spaced by three nucleotides, and termed DR3 (reviewed in Ref. 47). Each monomeric subunit makes a series of asymmetric interactions at the two DNA-binding domains, and symmetrical contacts within each ligand-binding domain. The heterodimeric complex can then presumably interact directly with the preinitiation complex or indirectly through a number of coactivators (15, 48–53), whose identities and functions are just now being defined, yielding the net result of transcriptional activation. This of course is all contingent on ligand binding, and the role of ligand in all of this appears to be manifold. Ligand binding increases the affinity of VDR for RXR (9, 10, 54) and therefore for the cognate DNA binding element, it induces conformational changes in the tertiary structure of the receptor (55–57), and it enhances interactions with components of the transcriptional machinery, presumably resulting in the stabilization of the preinitiation complex so that productive initiation can occur.

Since transcriptional repression is essentially the antagonsism of all of these events, it is not surprising that the mechanism of transrepression by a nuclear receptor as we currently understand it is fundamentally different from activation. A number of genes have been described that are targets of repression by steroid and nuclear receptors, and a variety of mechanisms ranging from DNA-independent inhibition of positive factor (22, 58) to competition for common binding sites on a promoter have been proposed (18, 19, 21, 24, 28, 40, 59–61). However, what has not been adequately accounted for is how the same trans-factor, i.e. a steroid/nuclear receptor, can activate some target genes and repress others. An attractive hypothesis proposed by Yamamoto and others (62) is that in scenarios involving direct DNA binding, it is the DNA element itself that acts as an allosteric effector of receptor structure/function. This is feasible since in those cases where negative elements have been delineated, they rarely resemble positive elements (24, 26, 28, 61). Thus, the interaction of the receptor with a noncanonical recognition sequence might induce an alternative structure that would then be incapable of activating transcription, but has also gained a repressive function, possibly by precluding the binding of another, unrelated positive factor, such as Fos and Jun.

**Fig. 6. VDR binds the GM550 element as a monomer.** A, binding analysis of the VDR DBD (VDRdbd) resolves a single complex on the GM550 element. In each series, a concentration range of VDR DBD (5, 10, 20, 50, and 75 ng) was incubated with DR3, GM550 probes, or an oligonucleotide probe comprising a single hexameric AGGTCA half-site (lanes 1–7) and resolved by EMSA. B, mixing VDR and VDR DBD yields a VDR-DBD heterodimeric species (dbd/VDR) only when it is bound to the DR3 element. Lanes 1–7, binding to the DR3 probe, using 25 or 50 ng of VDR DBD (lanes 2 and 3), 30 ng or 60 ng of VDR (lanes 4 and 5), 25 ng of VDR DBD co-incubated with 30 ng of VDR (lane 6), and 50 ng of VDR DBD co-incubated with 30 ng of VDR (lane 7). Lanes 8–12, binding to the GM550 probe, using 50 ng of VDR DBD or VDR (lanes 9 and 10), 40 ng of VDR DBD co-incubated with 40 ng of VDR (lane 11), or 80 ng of VDR DBD co-incubated with 40 ng of VDR (lane 12). In all lanes, 10 fmol of probe was used. In both A and B, dbd refers to monomeric and dbd$_2$ to dimeric species, respectively.

VDRE (46). We generated the VDR-L262G mutant in the context of the full-length receptor and first tested its activity to transactivate from a positive VDRE in COS cells (Fig. 7A, left panel). COS cells were used to initially test VDR-L262G activity because these cells do not express endogenous VDR that might obscure the effect of the mutant receptor. VDR-L262G was unable to transactivate a reporter construct driven by the DR3 VDRE in response to 1,25-(OH)$_2$D$_3$, whereas wild-type VDR was able to transactivate this reporter more than 100-fold (an identical result was also observed with this reporter and VDR-L262G in Jurkat cells; data not shown). Since this mutant can still bind ligand with near wild-type affinity (46), and its expression in the transfected COS cells is identical to wild-type VDR (Fig. 7A), its inability to transactivate **in vivo** is due to the loss of its dimerization function. We then tested VDR-L262G's ability to repress transcription from the GM550 element by transiently transfecting Jurkat cells with the mutant receptor together with the N3GMCSF-LUC reporter construct described in Fig. 1. In contrast to the lack of transactivation from the positive VDRE, the L262G mutant is still able to transrepress activated transcription, albeit not quite to wild-type levels, from the GM550 element in Jurkat cells in response to 1,25-(OH)$_2$D$_3$ (Fig. 7A, right panel), suggesting that dimerization of VDR is not required for its repressing function.

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Four key pieces of data presented in this work are consistent with DNA acting as an allosteric effector as an explanation for how VDR represses the activated transcription of the GMCSF gene. First, we clearly observe specific receptor binding to an element we have defined to 7 bp at \(22758\) to \(22765\) consisting of the sequence GCTTTCC in the GMCSF enhancer. Second, VDR binding to this element induces a conformation in the receptor that is distinct from its apparent conformation when it binds a positive DR3 VDRE, as deduced from both limited protease digestion and from DNA binding and dimerization mutants that are unable to bind and activate from the DR3 but can bind and repress from the GMCSF negative element with near or equal affinity and potency as wild-type receptor. Third, whereas VDR binds to and activates transcription from the DR3 as a VDR-RXR heterodimer, RXR is completely excluded from the VDR binding species at the negative element. Fourth, VDR appears to bind the negative element as a monomer. The last two observations are consistent with what we have previously demonstrated concerning how \(1,25-(OH)_{2}D_{3}\) affects the dimerization state of VDR (9). In the presence of RXR, \(1,25-(OH)_{2}D_{3}\) affects the dimerization state of VDR (9).
Previous work from our laboratory identified the IL-2 gene as a direct target of 1,25-(OH)_{2}D_{3}-mediated repression. We believe this begins to explain the molecular basis of the immunosuppressive effects of 1,25-(OH)_{2}D_{3} at the level of the activated T cell, but not completely. The present study extends this work by identifying the GMCSF locus as an additional direct target of 1,25-(OH)_{2}D_{3}, and suggests a more diverse role of vitamin D action in the immune system. The primary role of GMCSF is to activate mature granulocytes and macrophages, thereby eliciting the body's response to infection and initiating inflammatory responses (67, 68). This response clearly must be tightly regulated such that activation occurs only at appropriate times, and that a gradual down-modulation of the response is initiated. We believe that the data presented here is consistent with the role of 1,25-(OH)_{2}D_{3} contributing directly to this gradual decline of the inflammatory response at the level of repressing the transcription of GMCSF in activated T cells. This would lead to a decrease in the levels of GMCSF protein in the tissue periphery. It is also likely that a similar regulatory process is occurring directly in macrophages, although we have yet to test this. In fact, the role of 1,25-(OH)_{2}D_{3} at the level of GMCSF and in macrophages is further linked by the inference of a 1,25-(OH)_{2}D_{3} feedback loop in this cell type. Activated macrophages have been shown to produce the enzyme 1-α-hydroxylase (69, 70). This enzyme is the critical regulatory enzyme required to convert the inactive vitamin D metabolite 25-(OH)D_{3} to the biologically active ligand 1,25-(OH)_{2}D_{3}. The ability of activated macrophages to promote an increase in 1,25-(OH)_{2}D_{3} levels would in essence lead to the deactivation of macrophages via repression of GMCSF expression. There are potentially important clinical implications to this autoregulatory loop. Dysregulation of GMCSF leads to pathological conditions in diseases such as juvenile chronic myeloid leukemia (37), acute myeloid leukemia (36), and rheumatoid arthritis. In these scenarios, it is conceivable that the regulatory actions of 1,25-(OH)_{2}D_{3} may not be operating normally, or the feedback loop may be inactive. The close association of 1,25-(OH)_{2}D_{3} and GMCSF as demonstrated here suggests that the role of this ligand be evaluated carefully in these and other diseases.

**Acknowledgments**—We thank Peter Cockerill for original GMCSF plasmids, Amy Wolven for providing the FYN monoclonal antibody, and Ben Luisi and Mercy Devasahayam for critically reading this manuscript. We are also grateful to Christophe Rachez, Bryan D. Lemon, and Robert Benezra for helpful discussions and insights.

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Repression of GMCSF Transcription by VDR

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