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# Retinoid X Receptor:Vitamin $D_3$ Receptor Heterodimers Promote Stable Preinitiation Complex Formation and Direct 1,25-Dihydroxyvitamin $D_3$-Dependent Cell-Free Transcription

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**The numerous members of the steroid/nuclear hormone receptor superfamily act as direct transducers of circulating signals, such as steroids, thyroid hormone, and vitamin or lipid metabolites, and modulate the transcription of specific target genes, primarily as dimeric complexes. The receptors for 9-*cis* retinoic acid and 1,25-dihydroxyvitamin $D_3$ [1,25$(OH)_2D_3$], RXR and VDR, respectively, as members of this superfamily, form a heterodimeric complex and bind cooperatively to vitamin D responsive elements (VDREs) to activate or repress the transcription of a multitude of genes which regulate a variety of physiological functions. To directly investigate RXR- and VDR-mediated transactivation, we developed a cell-free transcription system for 1,25$(OH)_2D_3$ signaling by utilizing crude nuclear extracts and a G-free cassette-based assay. Transcriptional enhancement in vitro was dependent on purified, exogenous RXR and VDR and was responsive to physiological concentrations of 1,25$(OH)_2D_3$. We found that RXR and VDR transactivated selectively from VDRE-linked templates exclusively as a heterodimeric complex, since neither receptor alone enhanced transcription in vitro. By the addition of low concentrations of the anionic detergent Sarkosyl to limit cell-free transcription to a single round and the use of agarose gel mobility shift experiments to assay factor complex assembly, we observed that 1,25$(OH)_2D_3$ enhanced RXR:VDR-mediated stabilization or assembly of preinitiation complexes to effect transcriptional enhancement from VDRE-linked promoter-containing DNA.**

The coordinate expression of particular genes within a cell determines its developmental fate, and specific responses to external stimuli enable it to adapt to rapid changes in its environment. Part of this orchestrated response is the regulated transcription of genes which induce or maintain a differentiated state in a cell-type- or stage-specific manner. Many transactivating and transrepressing factors are employed by a cell to modulate the transcription of these genes by the basal transcriptional machinery. Regulation by such factors occurs through DNA binding, protein-protein interactions, and subsequent stimulation or inhibition of transcriptionally competent preinitiation complexes (PICs). In the traditional view, PIC formation involves an ordered sequential assembly of general transcription factors (GTFs) (TFIID [the TATA-binding protein {TBP} and its associated factors], TFIIA, TFIIB, RNA polymerase II [Pol II], TFIIF, TFIIE, and TFIIH) at core promoter sequences (reviewed in references 19, 34, 84, 110). In regulated transcription, the extent of assembly of PICs into productive complexes able to initiate and elongate mRNA synthesis is stimulated by multiple activators through specific interactions at rate-limiting steps with various components or associated cofactors of this machinery (18, 23, 31, 55, 56, 64, 80, 94, 104, 105). Recently, a simpler view of transactivation has been proposed; it involves activator recruitment or stimulation of TFIID, TFIIE, and large multisubunit complexes, termed holoenzymes, which have been found to contain RNA Pol II, TFIIB, TFIIF, TFIIH, the mediator/SRB complex (including suppressors of yeast RNA Pol B carboxy-terminal domain truncations), and other polypeptides, such as substoichiometric amounts of components of the SWI/SNF family of chromatin remodelers (10, 44, 47, 65, 74, 106). Holoenzyme complexes have been implicated as potential initiators of transcription in vivo which might alter chromatin structure, expedite the assembly of subsets of the transcriptional machinery at promoters of regulated genes, and thereby rapidly induce multiple rounds of transcription (4, 91, 92).

Among the various families of transcriptional regulators, the largest appears to be the steroid/nuclear hormone receptor superfamily. These intracellular receptors act as direct transducers of a wide variety of circulating signals, including steroids, thyroid hormone, retinoids, vitamin $D_3$, and lipid metabolites, by localizing to the nucleus and enhancing or repressing the transcription of specific target genes (68). Nuclear receptor proteins are organized into discrete functional domains, including a highly conserved DNA-binding domain consisting of two $Zn^{2+}$ ions, each tetrahedrally coordinated by four cysteines (28), and a less well-conserved ligand-binding domain that includes a short ligand-modulated activation domain at the extreme C terminus, AF-2 (20). The receptors for 9-*cis* retinoic acid (RA) and 1,25-dihydroxyvitamin $D_3$ [1,25$(OH)_2D_3$], RXR and VDR, respectively, are members of this superfamily. A heterodimeric complex of RXR and VDR binds cooperatively to specific DNA target sites, vitamin D responsive elements (VDREs), composed of directly repeating hexameric half-sites spaced by three nucleotides (DR3), to activate or repress the transcription of many diverse genes encoding proteins involved in regulating bone remodeling, cal-

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cium mobilization, suppression of T-cell proliferation, and growth inhibition and differentiation (2, 30, 59, 72, 75). $1,25(OH)_2D_3$ decreases the affinity of VDR for itself and DNA targets, enhances the formation of RXR:VDR heterodimers, and potentiates their affinity for and activation from VDREs (14, 15, 52). In contrast, 9-*cis* RA induces the formation of RXR:RXR homodimers, enhances transcription from RXREs, and simultaneously decreases both the affinity of RXR:VDR heterodimers for and their transactivation potential from VDRE-linked reporter genes (14, 15, 52, 62, 111). Taken together, this suggests there is cross talk at the level of receptor dimerization between retinoids and vitamin $D_3$. However, the effects on quaternary associations upon ligand binding may be a result of substantial tertiary structural changes that occur within the ligand-binding domain (6, 81, 103). Moreover, a number of studies have demonstrated ligand-dependent associations with putative transcriptional coactivators (9, 32, 40, 49, 51, 73, 101, 102) (reviewed in reference 37), suggesting that ligand effects on transcriptional activation are mediated in part by the recruitment or stabilization of key initiation factors at the promoters of regulated genes.

These observations infer that ligand-mediated conformational changes in the structure of receptor dimers result in multiple functional effects. This study utilized crude nuclear extracts and a G-free cassette-based assay to directly investigate transcriptional regulation by the nuclear receptors VDR and RXR in response to ligands. We established a cell-free transcription system for $1,25(OH)_2D_3$ signaling in which the enhancement of transcription was dependent on purified, exogenous RXR and VDR and was responsive to physiological concentrations of ligand. In addition, we observed that RXR and VDR transactivated selectively from VDRE-linked templates exclusively as a heterodimeric complex, since neither receptor alone enhanced transcription in vitro. Finally, using Sarkosyl to limit cell-free transcription to a single round and utilizing agarose gel mobility shift experiments to assay factor complex assembly, we found that $1,25(OH)_2D_3$ enhanced RXR:VDR-mediated stabilization or assembly of PICs to increase, at least in part, the transcription of VDRE-linked promoter-containing DNA.

## MATERIALS AND METHODS

**Reporter and G-free cassette plasmid construction.** The VDRE from the osteopontin/*Spp-1* gene was synthesized as complementary oligonucleotides 5′-gatccacaaGGTTCAcgaGGTTCAcgtccg-3′ and 5′-gatccggacgTGAACCtcgTGA ACCttgtg-3′ with *Bam*HI ends. Each 5′ end was phosphorylated, and equimolar amounts were annealed as previously described (52) and ligated into *Bam*HI-linearized pSP73 (Promega). Clones containing zero, one, two, and four tandem copies of the VDRE oriented in the same direction, as determined by *Sst*I restriction analysis and dideoxynucleotide sequencing, were selected and digested with *Asp*700 and *Xho*I, and the fragments containing VDREs were agarose gel purified. pE1b-CAT contains the adenovirus E1b gene minimal TATA promoter sequence 5′-gggTATAtaat-3′ immediately flanked by *Xba*I and *Bam*HI sites upstream of a cDNA for chloramphenicol acetyltransferase (CAT) in the context of a pSP72 (Promega) derivative and was kindly provided by R. Roeder (Rockefeller University, New York, N.Y.). pE1b-CAT was linearized with *Asp*700 and *Xho*I and used as the recipient for the VDRE-containing fragments described above to create a VDRExn(-131):E1b-CAT reporter series (whereby the nomenclature for VDRE distance is the distance from the center of the 3′-most VDRE to the start site of transcription). A VDRExn(-83):E1b-CAT reporter series was made by digestion of the VDRExn(-131):E1b-CAT reporters with *Pst*I and religation. Construction of the VDREx2:tk-CAT reporter construct (see Fig. 4A) was described previously (16).

A G-free 105-bp cassette was excised from $pG_5$E1b-Gless(105)-CAT (kindly provided by J. Licht, Mt. Sinai Medical Center, New York, N.Y.) by digestion with *Nco*I, made blunt ended, digested with *Eco*RI, and ligated into *Eco*RI- and *Eco*RV-linearized pSP73 to create pSP73-G(-)105. The E1b minimal TATA promoter was excised from pE1b-CAT by digestion with *Hin*dIII and *Eco*RI and ligated into similarly linearized pSP73-G(-)105 to create pE1b-G(-)105. pE1b-G(-)105 was linearized with *Eco*RI and *Bam*HI, made blunt ended, and religated to create pBS-G(-)105. The VDRExn(-83):E1b-CAT reporter series was digested with *Eco*RV and *Pst*I, and the VDRE-containing fragments were agarose gel purified and ligated into *Pvu*II- and *Pst*I-linearized pBS-G(-)105 to create one-, two-, and four-VDRE-containing G-free cassettes, called VDRExn(-84): BS-G(-)105, for cell-free transcription, called pML200 in this study, and pMLwt2 (78), kindly provided by R. Roeder, contain adenovirus major late promoter sequences from approximately −400 to +10 upstream of a G-free 220-bp cassette, and either was used as an internal reference for cell-free transcription. All templates and reporter plasmids were purified by cesium chloride gradient ultracentrifugation.

**Cell culture, nuclear extract preparation, and transient transfection.** Namalwa B cells (American Type Culture Collection [ATCC]) were cultured in 4-liter spinner flasks and maintained in log phase in RPMI supplemented with 10% fetal bovine serum (FBS) (Sigma), 300 μg of glutamate per ml, and 100 μg (each) of penicillin and streptomycin (Gibco-BRL) per ml. HeLa S-3 (ATCC) cells were maintained in spinner flasks in Joklik's minimal essential medium supplemented with 5% FBS. Namalwa and HeLa cells were harvested at densities between $1.0 \times 10^6$ and $1.2 \times 10^6$ cells/ml, and nuclear extracts were prepared by the method of Dignam et al. (22). HeLa (ATCC) cell culture and transient transfection by calcium phosphate coprecipitation were performed exactly as described in detail elsewhere (52). Sf9 cells were cultured in T-175 flasks (Nunc) and maintained in Grace's insect medium (Gibco-BRL), supplemented with 10% defined FBS (Hyclone), 1× lactalbumin hydrolysate (Gibco-BRL), 1× yeastolate ultrafiltrate (Gibco-BRL), and 100 μg (each) of penicillin and streptomycin (Gibco-BRL) per ml. The infection of Sf9 cells is described below.

**Overexpression and purification of receptors and GTFs.** A recombinant baculovirus expressing the human VDR (hVDR) from the polyhedrin promoter was a generous gift of N. Koszewski (University of Kentucky Medical Center, Lexington) and has been described elsewhere (48). A recombinant baculovirus expressing an epitope-tagged human retinoid X receptor-α from the polyhedrin promoter (FLAG-hRXRα [f-RXR in this work]) was previously described (26). Single-plaque isolates expressing high levels of hVDR or f-RXR, as assayed by gel mobility shift electrophoresis as described below, were amplified to create high-titer stocks used for overexpression and purification. Overexpression of f-RXR and hVDR by baculovirus infection was performed by plating $3.0 \times 10^7$ Sf9 cells in 15-cm-diameter tissue culture dishes, removing media, and adding 5 ml of serum-free medium-diluted high-titer viral stocks to a multiplicity of infection of 10. Cells were rocked intermittently at room temperature for 30 min to promote virus adsorption, the plating medium was replaced, and cells were grown at 27.5°C until they were harvested at 48 h postinfection.

Whole-cell lysates were prepared by resuspension and homogenization of cells on ice in 4 packed cell volumes of high-salt lysis buffer (500 mM NaCl, 50 mM Tris-HCl [pH 7.9], 1 mM EDTA, 5 mM dithiothreitol [DTT], 0.5 mM phenylmethylsulfonyl fluoride). Further purification of hVDR was performed similarly to *Escherichia coli* overexpression and purification (14), except that 0.05% Nonidet P-40 (NP-40; Sigma) was substituted for deoxycholate and Superdex-200 fractions containing hVDR were pooled, dialyzed against 100 volumes of TED-150 (150 mM KCl, 50 mM Tris-HCl [pH 7.9], 1 mM EDTA, 1 mM DTT)–20% glycerol twice for 2 h, aliquoted, snap frozen, and stored at −80°C. For purification of f-RXR, the supernatant after polyethylenimine precipitation of DNA in the hVDR purification protocol described above was dialyzed against 50 volumes of TED-150–20% glycerol twice for 2 h. Insoluble material was separated by centrifugation, and the supernatant was pooled and adjusted slowly to 300 mM KCl–50 mM Tris-HCl (pH 7.9)–20% glycerol–1 mM DTT–0.1% NP-40 in a final volume of 13.5 ml. The adjusted supernatant was rocked slowly at 4°C for 4 to 8 h in a 15-ml conical tube with 1 ml of preequilibrated anti-FLAG M2 affinity resin (3 mg of M2 monoclonal antibody per ml; IBI/Kodak). The affinity resin was pelleted at 700 × *g*, resuspended, and extensively washed and pelleted three times with 14 ml of buffer. Washed resin was transferred to two Eppendorf tubes and eluted in 0.5 volumes of TED-150–20% glycerol supplemented with 300 μg of FLAG peptide (D-Y-K-D-D-D-D-K; IBI/Kodak) per ml three times consecutively by rocking for 30 min to 1 h at 4°C. Fractions were pooled, aliquoted, snap frozen, and stored at −80°C.

Purified recombinant histidine-tagged GTFs hTBP, hTFIIAα/β, and hTFIIAγ and recombinant purified untagged hTFIIB were overexpressed in *E. coli* (a generous gift of M. Carey, UCLA School of Medicine, Los Angeles, Calif.) as previously described (17). Native TFIIA purified from HeLa cells as previously described (69) was provided by R. Roeder.

**Ligand.** Crystalline $1,25(OH)_2D_3$ was generously provided by M. Uskokovic (Hoffmann-La Roche, Nutley, N.J.) and was also purchased from BioMol. $1,25(OH)_2D_3$ was stored and delivered in ethanol to cell culture and in vitro assays.

**Oligonucleotide probes and in vitro DNA binding.** DNA binding to response elements (capitalized in sequences below) by receptor derivatives was assayed by gel mobility shift electrophoresis as described previously (1, 52). A self-annealing DNA oligonucleotide containing the osteopontin VDRE used for in vitro DNA binding and as a specific competitor for cell-free transcription has previously been described (52). A probe containing two consecutive VDREs separated by 10 bp, formed by annealing (52) DNA oligonucleotides 5′-gacaGGTTCAcgaG GTTCAcgacgtctgcGGTTCAcgaGGTTCAagt-3′ and 5′-ctagactTGAACCtcgTG AACCgcagacgtcgTGAACCtcgTGAACCgtgtctgca-3′, was used as a specific competitor of cell-free transcription (see Fig. 3A). A probe containing a glucocorticoid responsive element (GRE) used as a nonspecific, related competitor

oligonucleotide in cell-free transcription (see Fig. 3A) has been described elsewhere (1). Purified f-RXR and hVDR were incubated with 10 fmol of $^{32}$P-end-labeled probes for 30 min in cell-free transcription buffer (described below) together with 25 μg (each) of poly(dI-dC) and bovine serum albumin (BSA) per ml in a final reaction volume of 20 μl. One-half of each reaction mixture was loaded per lane and resolved at 375 V/12 mA on 10% nondenaturing gels (acrylamide-bisacrylamide, 75:1) in 0.5× Tris-borate-EDTA (pH 8.0) for 45 min.

For assays of GTF recruitment of f-RXR and VDR, in vitro DNA binding by agarose gel electrophoresis was performed as follows. A 295-bp VDREx4:E1b-G(-) probe was prepared by *Xho*I and *Bgl*II digestion of the VDREx4(-84):BS-G(-)105 plasmid described above. Approximately 100 ng of the probe was end labeled with Klenow enzyme (Promega) and 50 μCi of [α-$^{32}$P]dCTP (3,000 Ci/mmol; NEN-DuPont) and purified by Chromaspin TE-10 (Clontech) gel filtration chromatography. GTF binding assays were performed in 65 mM KCl–12 mM HEPES (pH 7.9)–0.5 mM Tris-HCl (pH 7.9)–12.5% glycerol–0.1% Tween 20–10 mM $MgCl_2$–1 mM DTT–0.5 mM phenylmethylsulfonyl fluoride–15 μg of poly(dG-dC) per ml–0.5 mg of BSA per ml in a final reaction volume of 13.5 μl. Zero or thirty nanograms of f-RXR:VDR (in equimolar amounts) in the absence or presence of $10^{-7}$ M $1,25(OH)_2D_3$ was incubated with 5 fmol of probe (ca. 40,000 cpm) for 15 min at 30°C, and purified GTFs (described above) were added in the indicated amounts (see Fig. 7) and incubated for an additional 25 min at 30°C prior to being loaded. One-half of each reaction mixture was loaded in each lane and electrophoresed in 1.5% low electroendosmosis agarose (Fisher Biotech) in 0.25× Tris-borate (pH 7.9) at 4 V/cm for 10 h.

All gels were dried on Whatman paper and exposed to Biomax-MR autoradiography film (Kodak) at −80°C with an intensifying screen for 2 to 4 h, and quantitation was performed by exposure to phosphorimager screens, gathering data on a Fujix BAS-1000 phosphorimager, and analysis by MacBAS version 2.0 bioimaging software. The results presented in Fig. 7 are from two separate assays resolved in the gel system described above and are representative of greater than 15 experiments performed with various amounts of recombinant, purified GTFs under various agarose and acrylamide electrophoresis conditions.

**Cell-free transcription.** Transcription assays were performed in transcription buffer (60 mM KCl, 25 mM Tris-HCl [pH 7.9], 12% glycerol, 0.2 mM EDTA, 0.01% NP-40) supplemented with 1.2 to 1.5 mg of crude nuclear extract from Hela or Namalwa cells per ml, 4 mM $MgCl_2$, 10 U of RNase block (Stratagene), 100 U of RNase $T_1$ (Gibco-BRL), 100 μM 3′-*O*-methyl-GTP (Pharmacia), 20 μM UTP, 0.5 mM ATP, 0.5 mM CTP, 10 mM phosphocreatine (Sigma), and 10 μCi of [α-$^{32}$P]UTP (800 Ci/mmol; NEN-Dupont) in a final reaction volume of 25 μl. All preincubation steps remained on ice until 5 min prior to addition to final reaction mixtures, at which time they were preincubated at 30°C. Equimolar amounts of receptor derivatives were preincubated with ligands (typically for 2 h) (unless otherwise indicated) from 0.5 to 15 μg/ml in transcription buffer–50 μg of BSA per ml. Unless otherwise indicated, 10-μl receptor-ligand binding reaction mixtures were added to 2 μl of template (0.5 to 1.7 pmol of VDRE-containing plasmid per ml, 0.1 to 0.25 pmol of internal reference plasmid per ml, and pSP73 carrier DNA to a final DNA concentration of 2 pmol/ml as indicated) in transcription buffer and incubated at 30°C for 15 min. Then nuclear extract, RNase block, and RNase $T_1$ were added in a volume of 8 μl in transcription buffer and incubated for an additional 25 min at 30°C. Finally, nucleoside triphosphates (NTPs) were added in a volume of 5 μl in transcription buffer to initiate transcription and reactions were allowed to proceed for 45 min. To limit transcription to a single round of initiations as previously described (33), the anionic detergent *N*-lauroyl sarcosine (Sarkosyl; Sigma) was added to a final concentration of 0.025% at various stages in some experiments (see Fig. 6).

Reactions were stopped by the addition of EDTA to 20 mM, and 25 μl of a mixture of 4,000 U of RNase $T_1$ per ml and 0.1 mg of BSA per ml in 0.1 M Tris-HCl (pH 7.5) was added to each reaction mixture and incubated at 37°C for 30 min to 2 h. After cleavage, 200 μl of TXN STOP buffer (7 M urea, 10 mM Tris-HCl [pH 7.8], 0.3 M NaAc, 0.5% sodium dodecyl sulfate [SDS], 0.1 M LiCl, 10 mM EDTA, 40 μg of yeast tRNA, and 200 cpm of a 502-bp DNA fragment recovery control [*Nde*I- and *Eco*RI-digested pRC-CMV {Invitrogen} $^{32}$P end labeled with Klenow enzyme]) was added, and samples were extracted with 250 μl of phenol-chloroform-isoamyl alcohol (25:24:1) by vortexing. The aqueous phase was extracted with an equal volume of chloroform-isoamyl alcohol (24:1), precipitated by inversion with an equal volume of isopropanol, incubated at room temperature for 30 min, and pelleted by microcentrifugation. The pellet was washed with 70% ethanol, dried, and resuspended on ice in 80% formamide–0.1% SDS–10 mM EDTA. Recovered products and 1,500 cpm of *Hae*III-digested pBR322 DNA ladder (Sigma) (dephosphorylated with calf intestinal alkaline phosphatase [Boehringer Mannheim] and $^{32}$P end labeled with T4 polynucleotide kinase [Boehringer Mannheim]) were incubated for 5 min at 90°C and immediately placed on ice. Products were separated by electrophoresis in 6% denaturing polyacrylamide (acrylamide-bisacrylamide, 20:1; 7 M urea) gels for approximately 45 min at 400 V in 1.0× Tris-borate-EDTA.

All gels were dried on Whatman paper and exposed to Biomax-MR autoradiography film (Kodak) at −80°C with an intensifying screen for 12 to 72 h. Quantitation was performed by exposure to phosphorimager screens, gathering data on a Fujix BAS-1000 phosphorimager, and analysis by MacBAS version 2.0 bioimaging software. The results presented in Fig. 2 through 6 are representative of greater than 60 individual experiments performed on different days with three separate HeLa nuclear extract preparations, four separate Namalwa nuclear extract preparations, two different DNA template preparations, four (each) RXR and VDR preparations, and two ligand stocks. Quantitation data are the means and standard deviations of repeated experiments expressed as fold transcription (test values/reference values) in response to receptors and ligand versus extract alone controls.

## RESULTS

**RXR and VDR mediate strong transcriptional enhancement from a natural VDRE in a cell-free system.** In order to examine the transcriptional activities of RXR and VDR in vitro, we overexpressed and purified each receptor by recombinant baculovirus infection of Sf9 cells (Fig. 1A). *E. coli*-expressed VDR was used as a marker. As shown in Fig. 1B, epitope-tagged hRXRα, expressed as a FLAG fusion (f-RXR) in Sf9 cells, and VDR, also expressed in Sf9 cells, bound as a heterodimer with high affinity to the VDRE from the osteopontin gene (*Spp-1*; lanes 6 and 7); 50 fmol of f-RXR and VDR shifted >50% of 10 fmol of the VDRE probe. In addition, VDR expressed in Sf9 cells bound to this VDRE as a homodimer (Fig. 1B, lanes 4 and 5), as we had previously demonstrated (14, 96). f-RXR also bound, albeit more weakly, to the osteopontin VDRE (Fig. 1B, lanes 2 and 3), as we predicted from transient-transfection studies with the same VDRE (52). The relative affinities by receptor dimeric complexes are presented in Fig. 1C and are more apparent at lower receptor concentrations.

To assay $1,25(OH)_2D_3$-mediated transcription in vitro, we created a VDRE-linked G-free cassette driven by the E1b minimal TATA promoter, called VDREx4:BS-G(-)105 (see Materials and Methods for description) (Fig. 2A). Creation of the BS-G(-)105 template series effectively placed the E1b minimal TATA promoter in close proximity to the G-free cassette, thereby specifying two transcriptional start sites within the G-free cassette for 84- and 82-base specifically initiated RNA products, which can be distinguished from a 105-base RNA product (Fig. 2B) which cryptically initiates elsewhere on the plasmid. Since RXR and VDR bind with highest affinity to the osteopontin VDRE in equimolar amounts (data not shown), we added increasing amounts of an equimolar mixture of purified f-RXR and VDR in the presence of $10^{-7}$ M $1,25(OH)_2D_3$ to the VDRE-linked G-free cassette and incubated it with crude nuclear extract from either Namalwa B cells (Fig. 2B, lanes 1 through 5) or HeLa cells (lanes 6 through 8) as a source of GTFs. A G-free cassette driven by the adenovirus major late promoter, pML200 (see Materials and Methods), was added as an internal reference for transcription. f-RXR:VDR was very active, as only a 2-fold receptor-to-response element ratio gave nearly a 5-fold transcriptional enhancement over nuclear extract alone (Fig. 2, lanes 1 and 2) and an approximately 16-fold receptor-to-response element ratio (equivalent to lane 7 in Fig. 1B) gave up to 20-fold enhancement (lane 5) when normalized to the reference template. In other experiments, we have observed strong f-RXR:VDR-dependent transcriptional enhancement in response to $1,25(OH)_2D_3$ from other VDRE-linked templates with natural start sites and minimal promoter sequences from −53 to +10 of the adenovirus major late gene (data not shown). Notably, Namalwa B-cell nuclear extracts were more responsive to exogenously provided receptors than were HeLa cell nuclear extracts (Fig. 2B and C).

**RXR:VDR-dependent transcription in vitro requires specific DNA binding and is enhanced by multiple VDREs.** To establish the specificity of RXR:VDR-mediated transcriptional enhancement in vitro, we used templates containing zero or four VDREs in the absence or presence of $1,25(OH)_2D_3$ as well as competed binding with molar excesses of DNA oligonucleotides in cell-free transcription assays. As shown in Fig.

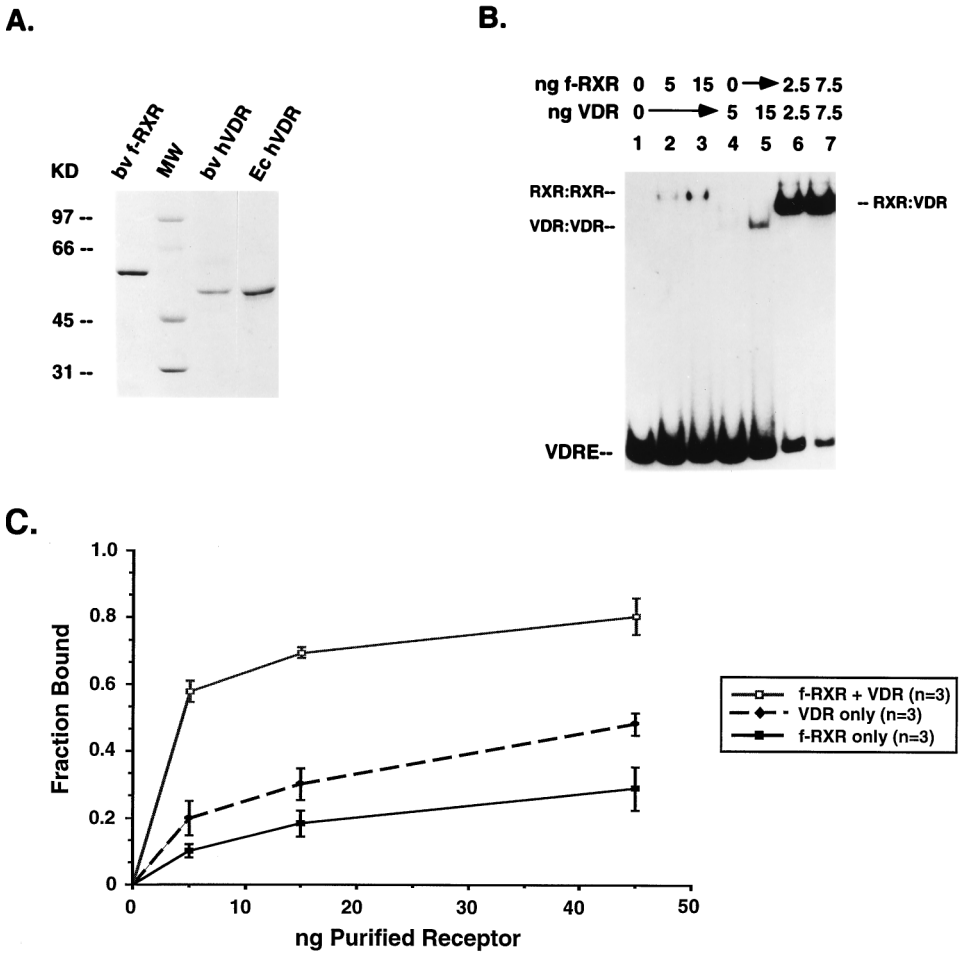


FIG. 1. Recombinant RXR and VDR proteins bind strongly to a natural VDRE. (A) FLAG-hRXRα (f-RXR) and hVDR proteins after baculovirus (bv) infection of Sf9 cells and partial purification. f-RXR was affinity purified to apparent homogeneity with anti-FLAG M2 monoclonal antibody resin as described in Materials and Methods. VDR was partially purified by gel filtration chromatography as described in Materials and Methods and elsewhere (14). Three micrograms of each purified preparation was resolved by electrophoresis on an SDS-10% polyacrylamide gel and stained with Coomassie brilliant blue. The apparent molecular mass (MW) of f-RXR is 53 kDa, and that of VDR is 48 kDa. Purified *E. coli*-expressed VDR (Ec hVDR) (14) is shown as a marker. Low-molecular-weight markers (MW; Bio-Rad) are indicated. (B) Purified f-RXR and VDR bind with high affinity as a heterodimer to the osteopontin/*Spp-1* VDRE, while VDR homodimers and f-RXR homodimers bind to the same element with decreased affinity. DNA binding by baculovirus-expressed f-RXR and VDR was assayed by gel mobility shift electrophoresis on a native 10% polyacrylamide gel as described in Materials and Methods. The indicated amounts (in nanograms; as estimated by Bradford assay and protein purity) of f-RXR alone (lanes 2 and 3), VDR alone (lanes 4 and 5), or both (lanes 6 and 7) were incubated with 10 fmol of $^{32}$P-end-labeled VDRE probe as previously described (52). The positions of the slowest-mobility, lowest-affinity f-RXR:f-RXR homodimers and the fastest-mobility, intermediate-affinity VDR:VDR homodimers are designated on the left, while that of the highest-affinity, intermediate-mobility f-RXR:VDR heterodimers is designated on the right. (C) Relative binding of dimeric complexes to the osteopontin/*Spp-1* VDRE. Quantitation data of three separate gel mobility shift assays are expressed as means ± standard deviations of the fraction of probe retarded by specific receptor binding.

3A, equimolar amounts of f-RXR and VDR in the absence or presence of $1,25(OH)_2D_3$ cannot enhance transcription from the E1b promoter in the absence of a VDRE (lanes 1 through 4). $1,25(OH)_2D_3$ also cannot enhance transcription from a VDRE-containing template without RXR:VDR exogenously provided to Namalwa nuclear extracts (Fig. 3A, lane 8). To further demonstrate the specificity of our cell-free system, we incubated DNA oligonucleotides containing two tandem VDREs (Fig. 3A, lanes 11 through 14) as a specific competitor or a DNA oligonucleotide containing a related binding site for a GRE as a nonspecific competitor (lanes 15 through 18). We observed that only a 10-fold molar excess of competitor binding sites (relative to template binding sites) with an oligonucleotide containing two VDREs was required to compete transcriptional enhancement by RXR:VDR from a template containing four VDREs in vitro by approximately 50%, and $1,25(OH)_2D_3$ responsiveness was nearly completely competed by a 100-fold molar excess (Fig. 3A; compare lanes 11 through 14 with lanes 9 and 10). The same molar excesses of a GRE-containing oligonucleotide had no effect on RXR:VDR-mediated transcriptional enhancement in vitro (Fig. 3A, lanes 15 through 18). The molar excess of DNA binding sites (including template) to 50 to 150 ng of exogenously added receptors was approximately 1.5- to 0.5-fold, respectively, for 10× competitor oligonucleotides and 15- to 5-fold, respectively, for 100× competitor.

In other studies, we observed ligand-dependent synergy by RXR:VDR with other nonreceptor transcription factors when a single VDRE was linked to binding sites for Sp1, NF-1, AP-1, and Oct-1 and also with each other when multiple VDREs were linked to promoters (52, 58). The same molar excess of binding sites from a single-VDRE-containing oligonucleotide cannot compete transcriptional activity as effectively as one containing two VDREs (data not shown), therefore implying a

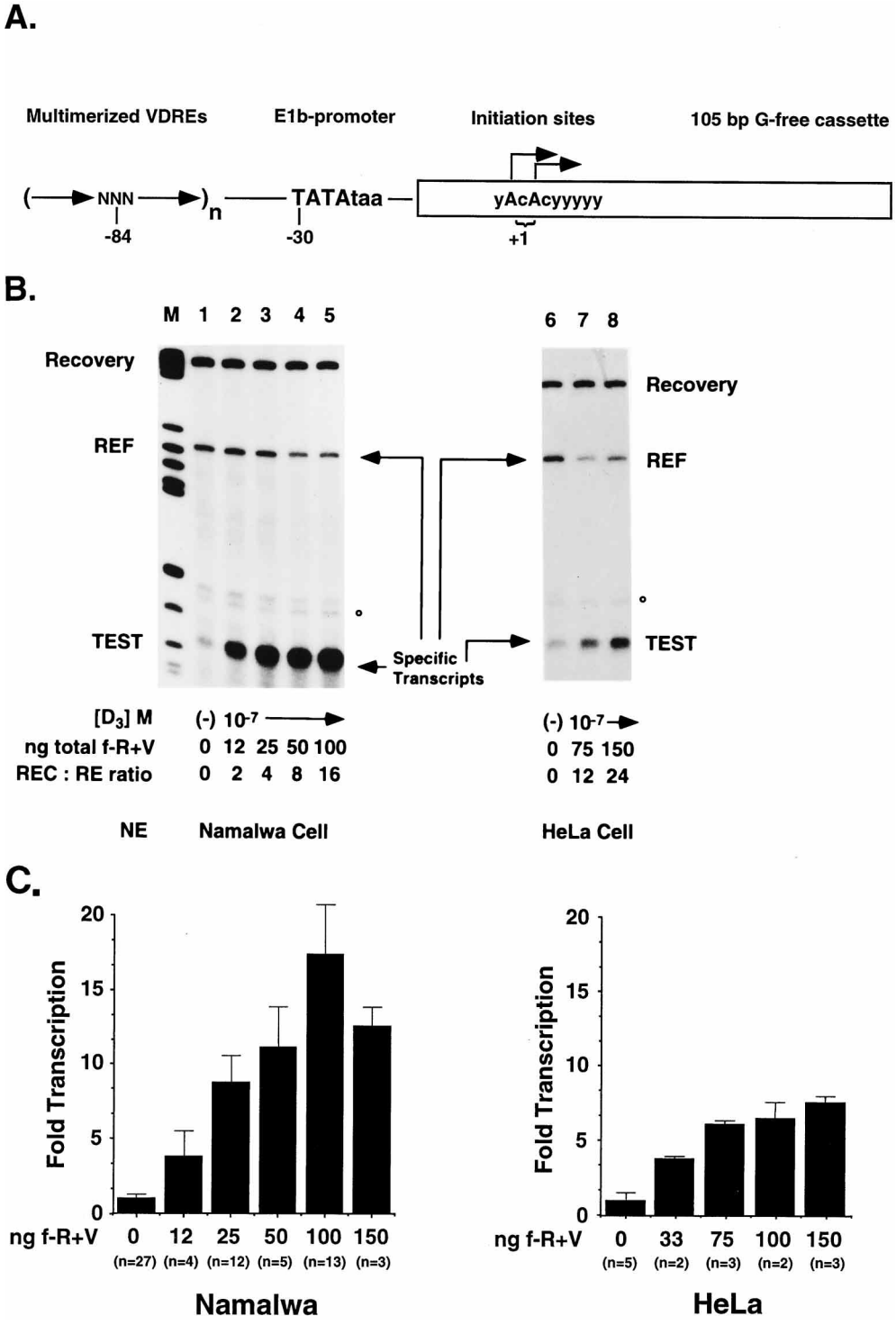


FIG. 2. Purified f-RXR and VDR proteins mediate strong transcriptional enhancement in the presence of $1,25(OH)_2D_3$ in a cell-free system. (A) Schematic of VDRE-linked test templates, VDRExn:BS-G(-)105. Osteopontin VDREs were multimerized upstream of E1b minimal TATA promoter sequences and a G-free cassette of 105 bp as described in Materials and Methods. Specific initiation directed from the E1b TATA occurs at two sites separated by 1 bp within the G-free cassette and generate 82- and 84-base RNA products. (B) f-RXR:VDR directs strong transcriptional enhancement from the osteopontin VDRE in the presence of $1,25(OH)_2D_3$ in crude nuclear extracts. The indicated total amounts (in nanograms) of an equimolar mixture of purified f-RXR:VDR (f-R+V) (0, 0.12, 0.25, 0.5, and 1 pmol for lanes 1 through 5, respectively, and 0, 0.75, and 1.5 pmol for lanes 6 through 8, respectively) were incubated with 30 ng (17 fmol) of VDREx4:BS-G(-)105 template (TEST), 6 ng (2.6 fmol) of pML200 reference (REF), and 35 μg of either Namalwa B-cell nuclear extract (NE) (lanes 1 through 5) or HeLa cell NE (lanes 6 through 8) in the presence of $10^{-7}$ M $1,25(OH)_2D_3$ [$D_3$], and transcription was allowed to proceed for 45 min as described in Materials and Methods. The positions of specifically initiated 82- and 84-base RNA products from the TEST template and of 220-base RNA products from the REF template are indicated by arrows, and those of cryptically initiated 105-bp products are denoted by circles. The molar ratios of RXR:VDR to VDRE binding sites (REC:RE ratio) are shown. A $^{32}P$-end-labeled DNA recovery control and molecular weight markers (see Materials and Methods) are also shown. (-), absence. (C) Namalwa nuclear extracts are more responsive than are HeLa nuclear extracts to exogenously added RXR:VDR in the presence of $10^{-7}$ M $1,25(OH)_2D_3$. Quantitation data from multiple experiments are expressed as means and standard deviations in response to the indicated amounts of added receptors and $10^{-7}$ M $1,25(OH)_2D_3$ for this amount of extract and template.

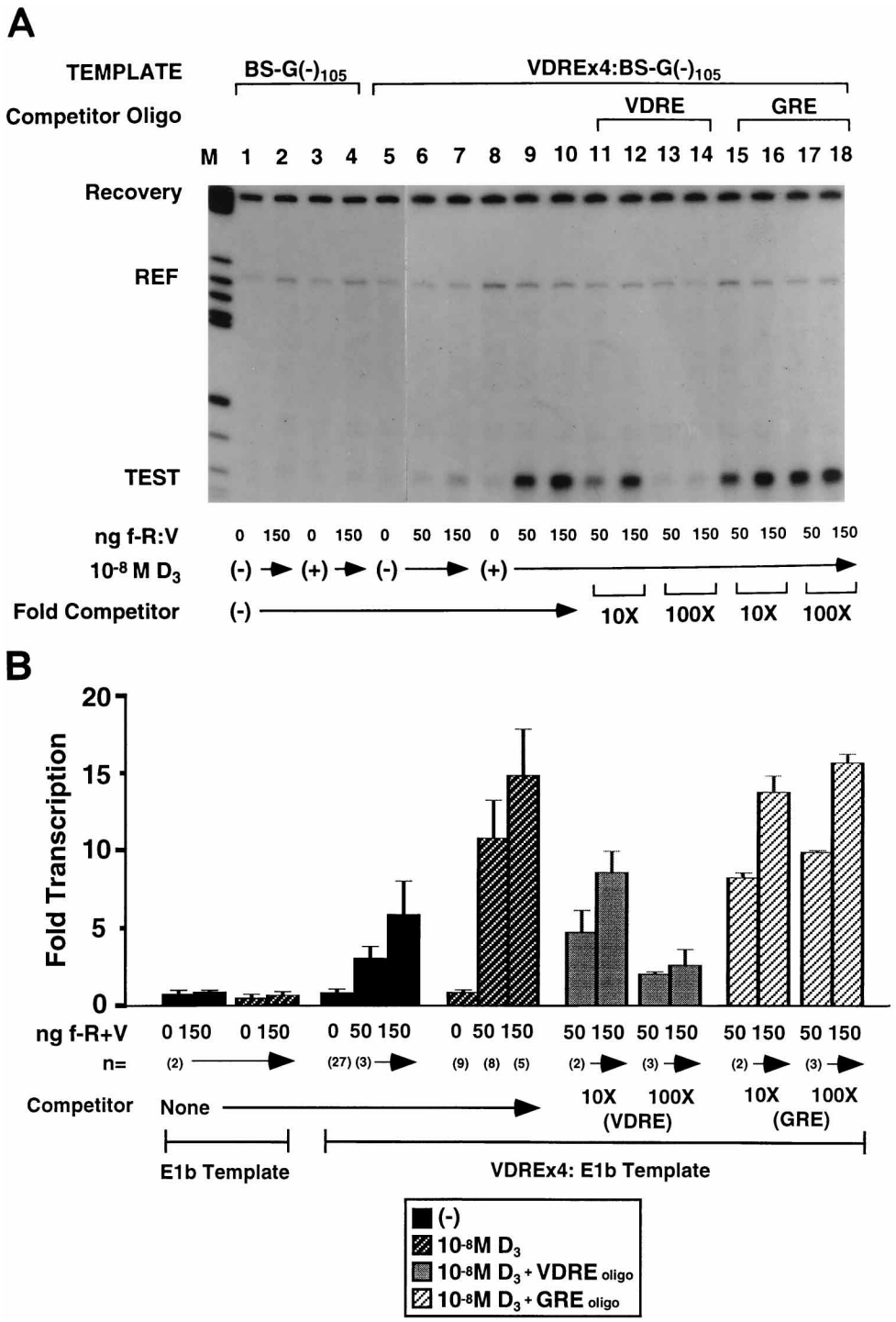


FIG. 3. RXR:VDR-mediated transcriptional enhancement requires specific DNA binding and increases with tandemly linked VDREs. (A) Receptor-dependent transcription in a cell-free system requires a VDRE and can be specifically competed by a VDRE-containing oligonucleotide but not by a nonspecific but related GRE-containing oligonucleotide. An equimolar mixture (0, 50, or 150 ng) of purified f-RXR and VDR (f-R:V) (0, 0.5, or 1.5 pmol, respectively) was incubated in the absence (−) or presence (+) of $10^{-8}$ M $1,25(OH)_2D_3$ ($D_3$) with 30 ng (17 fmol) of either a BS-G(−)105 test template containing only a minimal promoter with no VDREs (lanes 1 through 4) or a VDREx4:BS-G(-)105 test template (lanes 5 through 18) containing four VDREs and 6 ng (2.6 fmol) of pML200 reference (REF) template in the absence (lanes 1 through 10) or presence (lanes 11 through 18) of DNA competitor oligonucleotides (Competitor Oligo) (described below) with 35 μg of Namalwa B-cell nuclear extract, and transcription was allowed to proceed for 45 min. A 10- or 100-fold response element molar excess (relative to template binding sites) of a specific competitor DNA oligonucleotide containing either two consecutive VDREs (lanes 11 through 14) or a nonspecific competitor DNA oligonucleotide containing a GRE (lanes 15 through 18) was added during receptor and template preincubation prior to nuclear extract addition. Lane M, markers. (B) Quantitation data from multiple experiments are expressed as means and standard deviations in response to indicated receptor amounts and $10^{-8}$ M $1,25(OH)_2D_3$ for this amount of extract and template. (C) Receptor-dependent cell-free transcription increases with number of VDRE binding sites. The indicated total amounts (in nanograms) of an equimolar mixture of purified f-RXR and VDR (f-R+V) (0, 0.25, or 0.75 pmol in the absence or presence of $10^{-7}$ M $1,25(OH)_2D_3$) were incubated with 22 ng (12.5 fmol) of VDRExn:BS-G(-)105 test template containing a single VDRE (lanes 1 through 5), two VDREs (lanes 6 through 10), or four VDREs (lanes 11 through 15), 8 ng (3.4 fmol) of pML200 (REF) template, and 30 μg of Namalwa B-cell nuclear extract, and transcription was allowed to proceed for 45 min. Lane M, markers. (D) Quantitation data from multiple experiments are expressed as means and standard deviations in response to indicated receptor amounts and $10^{-7}$ M $1,25(OH)_2D_3$ for this amount of extract and template.

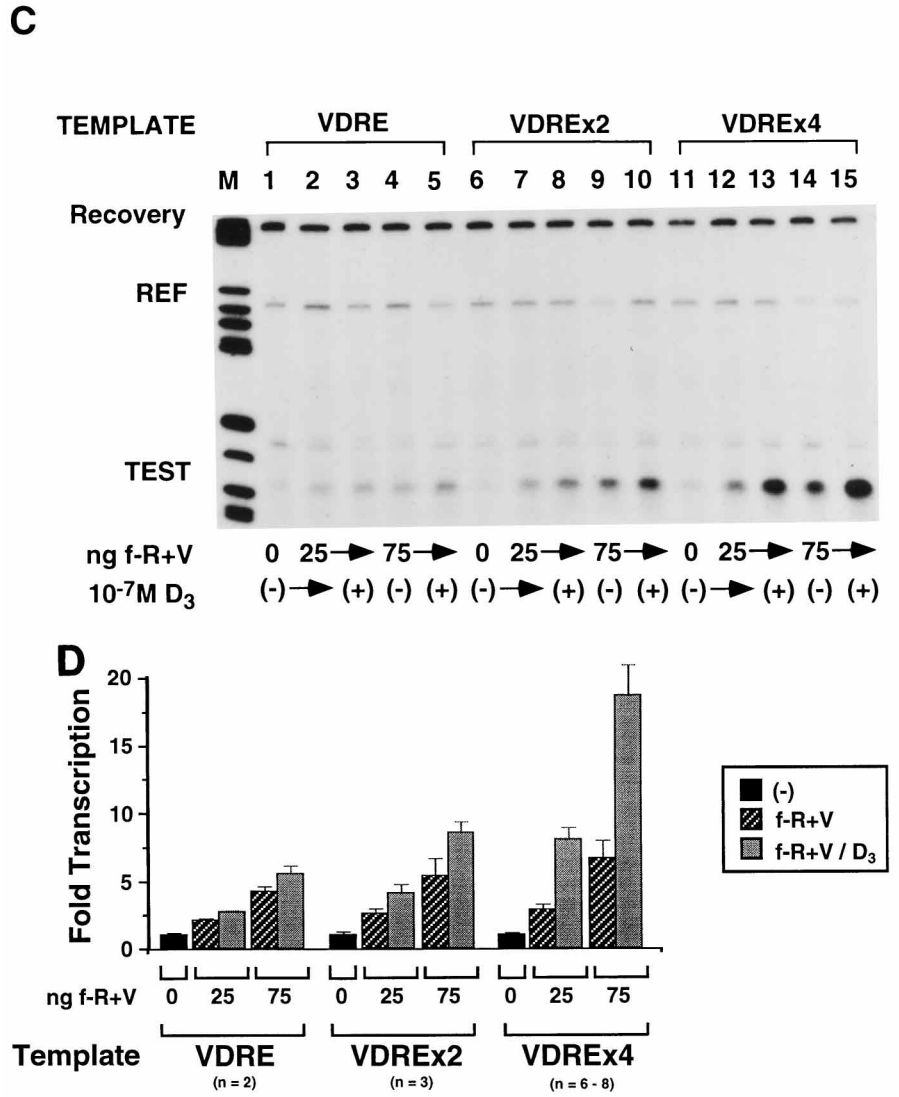


FIG. 3—*Continued.*

potential role for synergy in RXR:VDR-mediated transactivation in vitro. We tested this by comparing the transcriptional activities of RXR:VDR from templates containing one, two, or four VDREs (Fig. 3C). We observed, however, that total enhancement of transcription in vitro by f-RXR:VDR increased approximately linearly with response element copy number; however, ligand responsiveness was slightly greater with multiple VDREs (Fig. 3D). Equimolar amounts of f-RXR and VDR in the presence of $10^{-7}$ M $1,25(OH)_2D_3$ can increase transcription up to 5-fold from templates containing a single VDRE (Fig. 3C; compare lane 5 with lane 1), up to 10-fold from templates containing two VDREs (compare lane 10 with lane 6), and up to 20-fold from templates containing four VDREs (compare lane 15 with lane 11) linked in tandem to the E1b promoter when incubated with crude nuclear extracts.

We have also observed that "basal" activity from our templates reproducibly increases slightly from zero to four VDREs with extract alone (up to twofold in Namalwa extracts and nearly threefold in HeLa extracts) (data not shown). It is important to note that this increase of basal responsiveness is ligand independent and that all of our quantitation data are expressed as fold induction relative to extract alone on each template. By Western blotting, both Namalwa and particularly HeLa nuclear extracts contain detectable amounts of VDR (data not shown). We interpret the increase of basal activity in response to VDRE copy number to be due to endogenous RXR:VDR in crude extracts, which might account, in part, for both our inability to see strong synergy in vitro and the decreased fold responsiveness to exogenous receptors of HeLa extracts relative to that of Namalwa extracts (Fig. 2B and C).

We consistently observed modest ligand-independent enhancement of transcription by f-RXR:VDR in vitro (for example, Fig. 3A, lanes 6 and 7), as has been described for other nuclear receptors, including RXR:RAR heterodimers (100) and both RXR:thyroid hormone receptor (TR) heterodimers and RXR:RXR homodimers (50). Ligand-independent transcription in our cell-free system was typically two- to sixfold and generally increased with increasing amounts of RXR:VDR (see Fig. 2 through 6), whereas ligand-dependent enhancement of transcription was typically an additional two- to fourfold over that observed in the absence of $1,25(OH)_2D_3$ and generally decreased with increasing amounts of RXR:VDR.

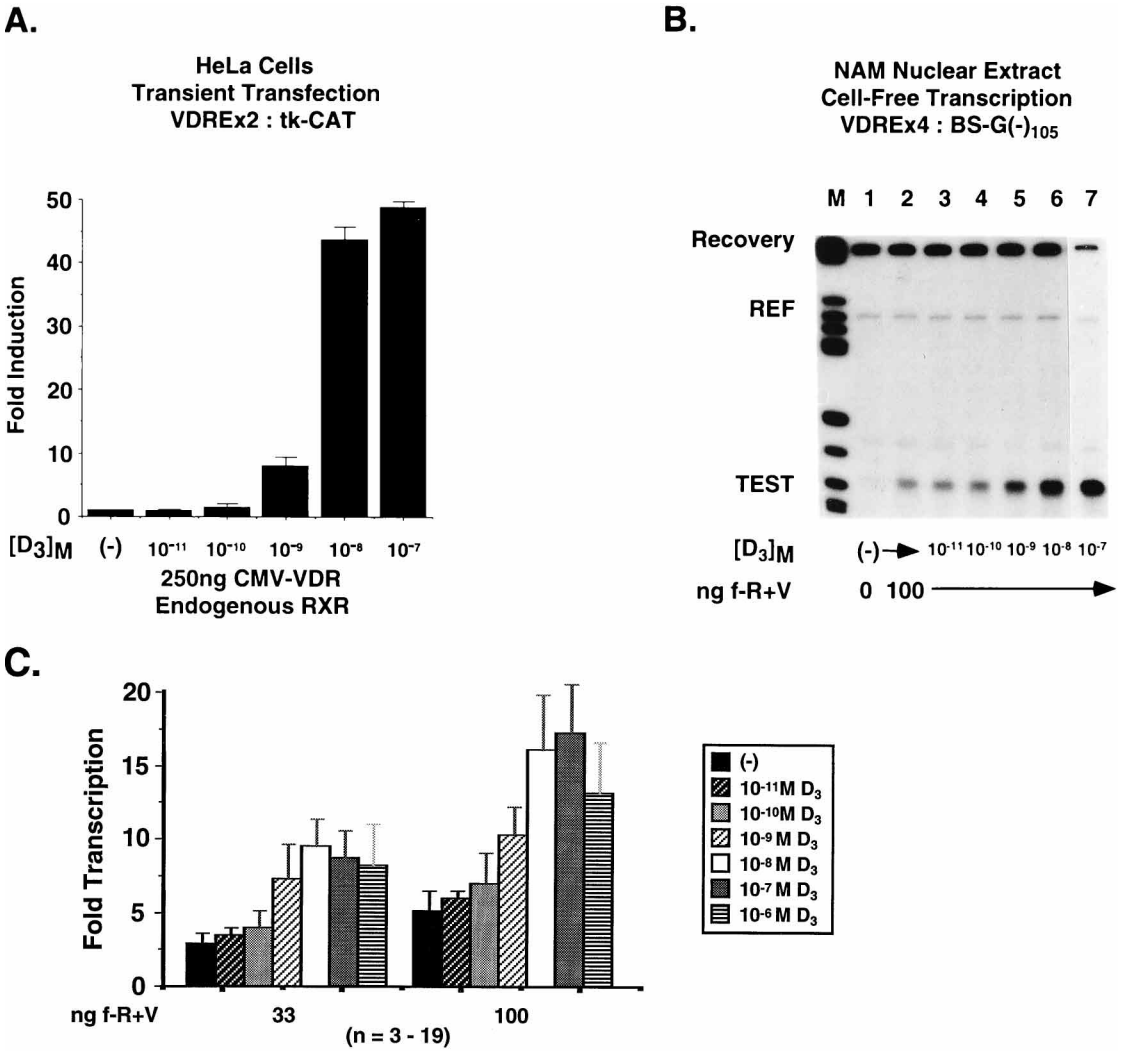


FIG. 4. Significant $1,25(OH)_2D_3$-mediated induction of transcription in vitro is identical to the dose-response curve observed in vivo. (A) In vivo dose-response curve to $1,25(OH)_2D_3$ in HeLa cells from a natural VDRE-containing reporter. HeLa cells were transiently transfected by calcium phosphate coprecipitation of 5 μg of VDRE:tk-CAT reporter plasmid, 2 μg of β-galactosidase expression plasmid, 250 ng of pCMV-VDR, and carrier DNA. $1,25(OH)_2D_3$ ($[D_3]$) was added in the indicated concentrations for 36 h, and cells were harvested at 48 h posttransfection and assayed for β-galactosidase and CAT activities as previously described (52). CAT activity was normalized to β-galactosidase activity, and data are the means and standard deviations of three experiments. (−), absence. (B) Receptor-dependent transcription in vitro is enhanced by ligand at physiologically responsive levels. The indicated total amounts (in nanograms) of an equimolar mixture of purified f-RXR and VDR (f-R+V) (0 for lane 1 and 1 pmol for lanes 2 through 7) in the absence (−; lanes 1 and 2) or presence (lanes 3 through 7) of increasing concentrations of $1,25(OH)_2D_3$ were incubated with 30 ng (17 fmol) of VDREx4:BS-G(-)105 test template, 6 ng (2.6 fmol) of pML200 reference (REF) template, and 35 μg of Namalwa B-cell nuclear extract, and transcription was allowed to proceed for 45 min. Lane M, markers. (C) Cell-free transcription responsiveness is squelched by both excess receptor (relative to binding site saturability) and excess ligand concentrations (relative to receptor saturability). Quantitation data from multiple experiments are expressed as means and standard deviations in response to 33 and 100 ng of receptors (equivalent to 8 and 24 nM, respectively) and the ligand concentrations indicated for this amount of extract and template.

DNA binding to the VDRE-linked template is not sufficient for transcriptional activation. The VDR DNA-binding domain (29) and the RXR DNA-binding domain (79), which bind with high affinity to the osteopontin VDRE, cannot substitute in any combination for exogenous f-RXR and VDR (data not shown), indicating that domains other than the DNA-binding domain are required for stimulatory interactions with either the basal transcription machinery or other coactivators in the extract.

**RXR:VDR-mediated transcription is enhanced by $1,25(OH)_2D_3$ at physiologically responsive concentrations.** In order to compare the ability of ligand to increase transcriptional enhancement by RXR:VDR in vitro to that observed in vivo, we performed ligand titrations in both cell-free transcription and transient-transfection assays. In vivo by transient expression of VDR with endogenous RXR in HeLa cells, we observed significant activation from a VDRE-linked reporter plasmid (see Materials and Methods) only at nanomolar $1,25(OH)_2D_3$ concentrations and this activation was maximal at $10^{-7}$ M (Fig. 4A). In vitro, an identical dose-response curve was seen; an observable enhancement of transcription in Namalwa B-cell nuclear extracts from a VDRE-linked template occurred at nanomolar $1,25(OH)_2D_3$ concentrations (the $K_d$ for VDR), and this activation was maximal at $10^{-7}$ M (Fig. 4B). In HeLa nuclear extracts, a similar dose response curve was seen (data not shown). Interestingly, as shown in Fig. 4C, at ligand concentrations above equimolar ligand/receptor ratios, the transcriptional response decreased. For example, with 33 ng of

receptors (8 nM), the response decreased at above 10 nM ligand. hundred nanograms of receptors (24 nM) required more ligand for a maximal response; a decrease in transcriptional response occurred at above 100 nM ligand. Moreover, at receptor concentrations approaching VDRE site saturation (>15:1 receptor-to-site ratio), a maximum transcriptional response was reached and then typically decreased at above that ratio (data not shown). A similar phenomenon was seen at $1,25(OH)_2D_3$ concentrations above $10^{-7}$ M in cell culture (data not shown). As ligand enhances RXR:VDR dimerization and by inference other ligand-dependent interactions, we attribute these receptor-dependent and ligand-dependent decreases in activity to squelching of limiting GTFs or other coactivators of DNA.

**$1,25(OH)_2D_3$-dependent transcription in vitro requires an RXR:VDR heterodimer.** In our previous studies both in vitro and in vivo in cell culture (14–16, 52), we found that VDR bound strongly to the osteopontin VDRE as a homodimer and that DNA binding was further increased by its association with RXR. However, arguing against a role for VDR:VDR homodimers and in favor of RXR:VDR heterodimers as the functional transactivating species from this binding element are our observations that (i) $1,25(OH)_2D_3$ decreased the affinity of VDR for itself and DNA targets, (ii) increased amounts of RXR increased $1,25(OH)_2D_3$-dependent transactivation from a VDRE-linked reporter in vivo, and (iii) $1,25(OH)_2D_3$ enhanced the association of RXR and VDR and increased their affinity for DNA targets in vitro. In these same studies, we demonstrated that 9-*cis* RA and RXR-specific ligands attenuated both $1,25(OH)_2D_3$-dependent transactivation from a VDRE-linked reporter in vivo and decreased the association of RXR and VDR in vitro. The decreased association of RXR and VDR we found was apparently a consequence of induction of the formation of RXR:RXR homodimers, which increased both their affinity for RXREs (directly repeating hexameric half-sites separated by 1 bp [DR1], as others have similarly found [53, 111]) and transactivation from RXRE-linked reporters in cell culture. Taken together, these results led to our hypothesis that RXR:VDR heterodimers are the active species for transactivation of VDRE-linked genes in vivo (52).

To directly address this question here, we assayed the abilities of exogenously added f-RXR alone, VDR alone, and f-RXR:VDR to mediate $1,25(OH)_2D_3$-dependent transactivation by in vitro transcription. We observed that $1,25(OH)_2D_3$-dependent transcriptional enhancement from a VDRE-linked template in vitro occurred only when both f-RXR and VDR were added exogenously to a crude nuclear extract (Fig. 5A; compare lanes 11 through 14 with lanes 1 through 10). Even a total amount of VDR equivalent to that of f-RXR and VDR added together cannot support $1,25(OH)_2D_3$-dependent transcriptional enhancement from the VDRE-linked template (Fig. 5A; compare lanes 15 through 18 with lanes 11 through 14). Weak ligand-independent enhancement was observed upon addition of either receptor alone (Fig. 5B) and again may be attributable to limiting amounts of endogenous partner receptor in the extract. These results are consistent with our previous observations in vitro and in cell culture and establish that it is the RXR:VDR heterodimer which is the transactivating species from the osteopontin VDRE, even though VDR can bind to this VDRE in the absence of RXR (Fig. 1B). Moreover, these findings are in agreement with recent cell-free studies demonstrating a functional requirement for RXR in the ligand-dependent transcriptional activation of the thyroid hormone receptor (26).

**$1,25(OH)_2D_3$ enhances RXR:VDR-mediated stabilization or assembly of PICs in a single-round transcription assay.** In order to further examine the relative effects of RXR:VDR stimulation of transcription in the absence and presence of $1,25(OH)_2D_3$, we employed the anionic detergent Sarkosyl at various stages in our cell-free system. In the absence of Sarkosyl, f-RXR:VDR heterodimers typically activated transcription and $10^{-8}$ M $1,25(OH)_2D_3$ enhanced this stimulation (Fig. 6A, lanes 1 through 3). When Sarkosyl was added to a final concentration of 0.025% after preincubation of f-RXR:VDR with the template but prior to extract addition, transcription was eliminated (Fig. 6A, lane 7), consistent with its ability to inhibit PIC formation (33). When Sarkosyl was added after both f-RXR:VDR and extract preincubation, effectively allowing PIC formation and, upon addition of NTPs, a single round of initiation but not reinitiation, we observed twofold stimulation of transcription by f-RXR:VDR and approximately fourfold stimulation of transcription in the presence of $10^{-8}$ M $1,25(OH)_2D_3$ (Fig. 6A, lanes 4 through 6). However, when Sarkosyl was added to templates preincubated with extract but prior to receptor addition, the ability of f-RXR:VDR to enhance transcription in vitro was abolished both in the absence and presence of $10^{-8}$ M $1,25(OH)_2D_3$ (Fig. 6A, lanes 8 through 10). Additionally, this loss of RXR:VDR-responsiveness was not merely a block to activation by inhibitors in the extract, as we observed nearly indistinguishable stimulation by the receptors in order-of-addition experiments comparing preincubation of templates with extract prior to or after receptor addition in the absence of Sarkosyl (data not shown). Notably, transcriptional enhancement by RXR:VDR requires a VDRE (Fig. 3A) and the concentrations of Sarkosyl necessary to inhibit PIC assembly completely block RXR:VDR DNA binding by gel mobility shift (data not shown). Taken together, these results are consistent with f-RXR:VDR heterodimers promoting the formation of PIC complex formation and with $1,25(OH)_2D_3$ enhancing this activity. Our results also suggest that the receptors may promote reinitiation of transcription because their effect was increased in the absence of Sarkosyl (Fig. 6A, lanes 1 through 3).

**$1,25(OH)_2D_3$ increases RXR:VDR-facilitated recruitment and assembly of GTFs.** Several classic activators, such as Gal4-VP16, and the Epstein-Barr virus-encoded activators Zta and Zebra have been shown to stabilize higher-mobility complexes containing TFIIA, TBP, or TFIID and the activator, as well as recruitment of TFIIB into these complexes in gel shift assays (17, 54). To investigate whether RXR:VDR can directly promote the formation of PICs by association with specific members of the general transcriptional machinery, we employed agarose gel mobility shift assays with purified recombinant GTFs overexpressed in *E. coli* or highly purified native TFIIA from HeLa cells and a 295-bp restriction fragment excised directly from our transcription template as described in Materials and Methods. $1,25(OH)_2D_3$ enhances RXR:VDR dimerization and their affinity for DNA targets (14, 15). At site-saturating amounts of receptors, however, ligand has little effect on total DNA binding, although mobility is altered (Fig. 7A, lanes 2 and 3). These conditions were used to examine relative effects of ligand on RXR:VDR-mediated recruitment of PIC complexes to VDRE-linked promoter DNA. The migrations of factor-specific complexes are depicted on the right in Fig. 7A (some complexes, particularly TBP, TBP-TFIIA, and f-RXR:VDR-TBP, can be seen only upon very long exposure or with greater amounts of GTFs; therefore, their relative mobilities from other exposures and/or experiments are noted for comparison).

As shown in Fig. 7A, RXR:VDR can facilitate the assembly

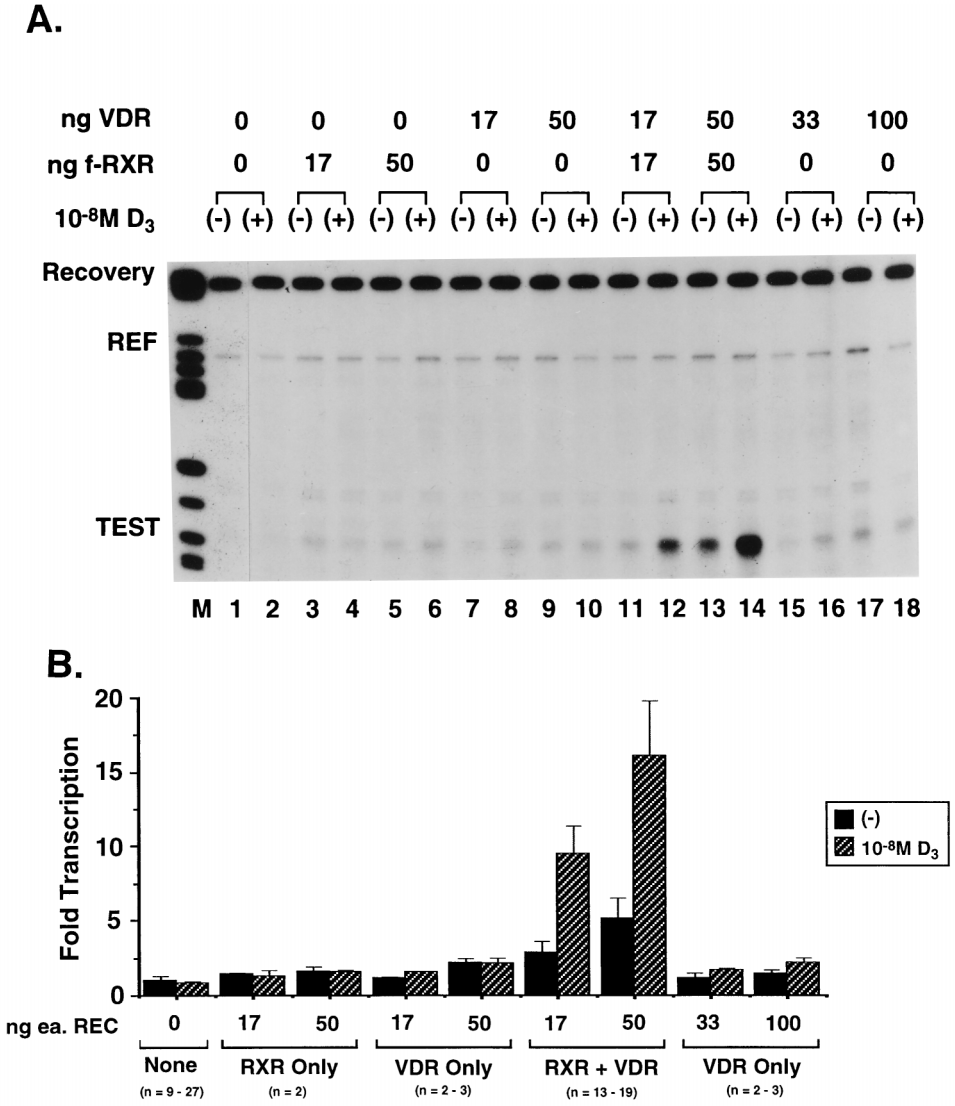


FIG. 5. Ligand-dependent transcription from a natural VDRE in a cell-free system requires an RXR:VDR heterodimeric complex. (A) Purified f-RXR alone (lanes 1 through 6), VDR alone (lanes 7 through 10), or equal amounts of both receptors added together (lanes 11 through 14) were incubated in the absence (−) or presence (+) of $10^{-8}$ M $1,25(OH)_2D_3$ ($D_3$) with 30 ng (17 fmol) of a VDREx4:BS-G(-)105 test template, 6 ng (2.6 fmol) of pML200 reference (REF) template, and 35 μg of Namalwa B-cell nuclear extract, and transcription was allowed to proceed for 45 min. In lanes 15 through 18, twice as much VDR alone was added (compared to lanes 7 through 10). Lane M, markers. (B) Quantitation data from multiple experiments are expressed as means and standard deviations in response to the indicated receptor (REC) amounts and $10^{-8}$ M, $1,25(OH)_2D_3$ for this amount of extract and template. ea., each.

of TBP, TFIIA, and TFIIB by recruiting them into higher-mobility complexes. The fraction of probe migrating in higher-mobility complexes in the presence of TBP and TFIIB was modestly enhanced by RXR:VDR (approximately 1.5-fold) and further increased with $1,25(OH)_2D_3$) over that migrating in TBP-TFIIA-TFIIB and TBP-TFIIB complexes bound in the absence of receptors (Fig. 7A, lanes 10 through 15, and B). However, particularly striking was the approximately 3-fold stimulation by RXR:VDR [and the subsequent increase to nearly 10-fold with $1,25(OH)_2D_3$] of higher-mobility complexes in the presence of TBP and native TFIIA relative to TBP-TFIIA in the absence of receptors (Fig. 7A, lanes 7 through 9). A requirement for TFIIA and perhaps TFIIB for RXR:VDR-stimulated binding of TBP was apparent, as TBP alone cannot be significantly recruited by the receptors (Fig. 7A, lanes 4 through 6). Similar results have been observed with purified recombinant TFIIA (52a). These results are consistent with those from the single-round transcription experiments (Fig. 6) and, in combination, suggest that RXR:VDR heterodimers promote the formation of PICs and that their direct interaction with members of the general transcription machinery is enhanced by ligand.

## DISCUSSION

Our previous studies have focused on ligand-mediated dimerization of the nuclear receptors RXR and VDR in vitro and their subsequent transcriptional activity in response to ligands in cell culture (14–16, 52). In those studies, we found that VDR bound to the osteopontin VDRE as a homodimer and that DNA binding was further increased by its association with RXR. The predominance of the RXR:VDR heterodimer's role in $1,25(OH)_2D_3$-mediated transactivation was suggested by our observations that $1,25(OH)_2D_3$ decreased the

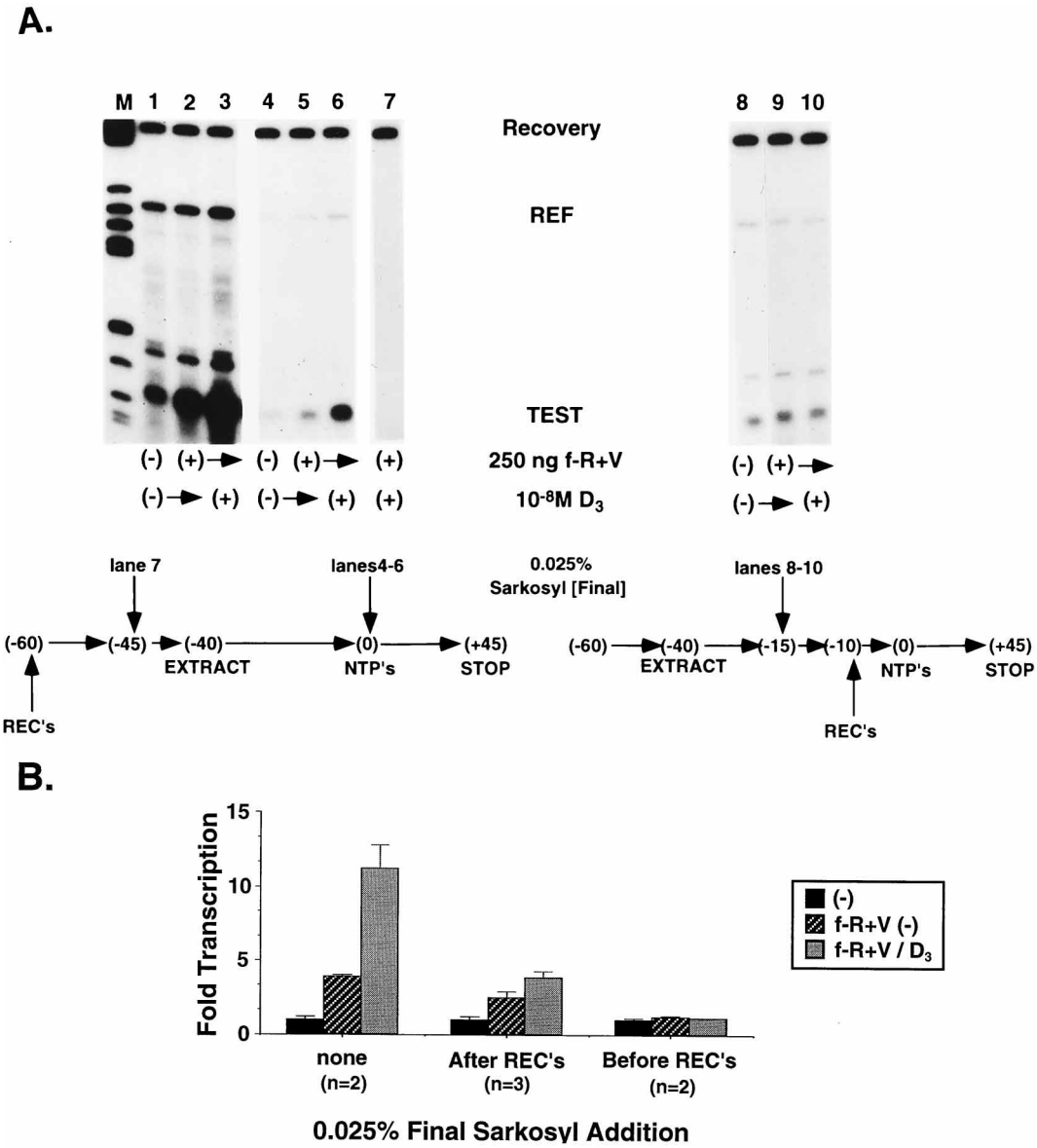


FIG. 6. $1,25(OH)_2D_3$ enhances RXR:VDR heterodimer-facilitated stabilization or assembly of PICs in a single-round transcription assay in vitro. (A) An equimolar mixture (0 or 250 ng) of purified f-RXR and VDR (f-R+V) (0 or 2.5 pmol, respectively) in the absence (−) or presence (+) or $10^{-8}$ M $1,25(OH)_2D_3$ ($D_3$) was incubated with 75 ng (43 fmol) of VDREx4:BS-G(-)105 test template, 15 ng (6.4 fmol) of pML200 reference (REF) template, 70 μg of Namalwa B-cell nuclear extract, and 20 μCi of [α-$^{32}$P]UTP in a final reaction volume of 50 μl, and transcription was allowed to proceed for 45 min in the absence (lanes 1 through 3) or presence (lanes 4 through 10) of 0.025% Sarkosyl (final concentration) added at the times indicated, as described in Materials and Methods. REC's, time of f-RXR:VDR addition; lane designations indicate times of Sarkosyl addition. Note that the signal was much stronger in the absence of Sarkosyl (lanes 1 through 3) since multiple rounds of transcription occurred during the 45-min incubation. NTP's, NTP addition; lane M, markers. (B) Quantitation data from two or three separate experiments for each determination are expressed as means and standard deviations in response to the indicated receptor amounts and $10^{-8}$ M $1,25(OH)_2D_3$ for this amount of extract and template.

affinity of VDR for itself and DNA targets, that increased amounts of RXR increased $1,25(OH)_2D_3$-dependent transcriptional activation from a VDRE-linked reporter in vivo, and that $1,25(OH)_2D_3$ enhanced both the association of VDR and RXR and their affinity for DNA targets in vitro. In addition, we showed that 9-*cis* RA and RXR-specific ligands both attenuated $1,25(OH)_2D_3$-dependent transactivation from a VDRE-linked reporter in vivo and decreased the association of RXR and VDR in vitro. In addition, we observed that 9-*cis* RA induced the formation of RXR:RXR homodimers, which both increased their affinity for RXREs, as others have reported (53, 111), and directed transactivation from RXRE-linked reporters in cell culture (52).

In this work, we sought to extend our previous observations by looking more directly at the transcriptional activity of RXR:VDR by a cell-free transcription assay dependent on exogenous receptors and responsive to ligand. We have detected up to 20-fold transcriptional enhancement from VDRE-linked templates with added recombinant, purified RXR and VDR and physiological concentrations of $1,25(OH)_2D_3$ to crude nuclear extracts. In addition, we have demonstrated that this enhancement is specific to VDRE-linked templates, increases

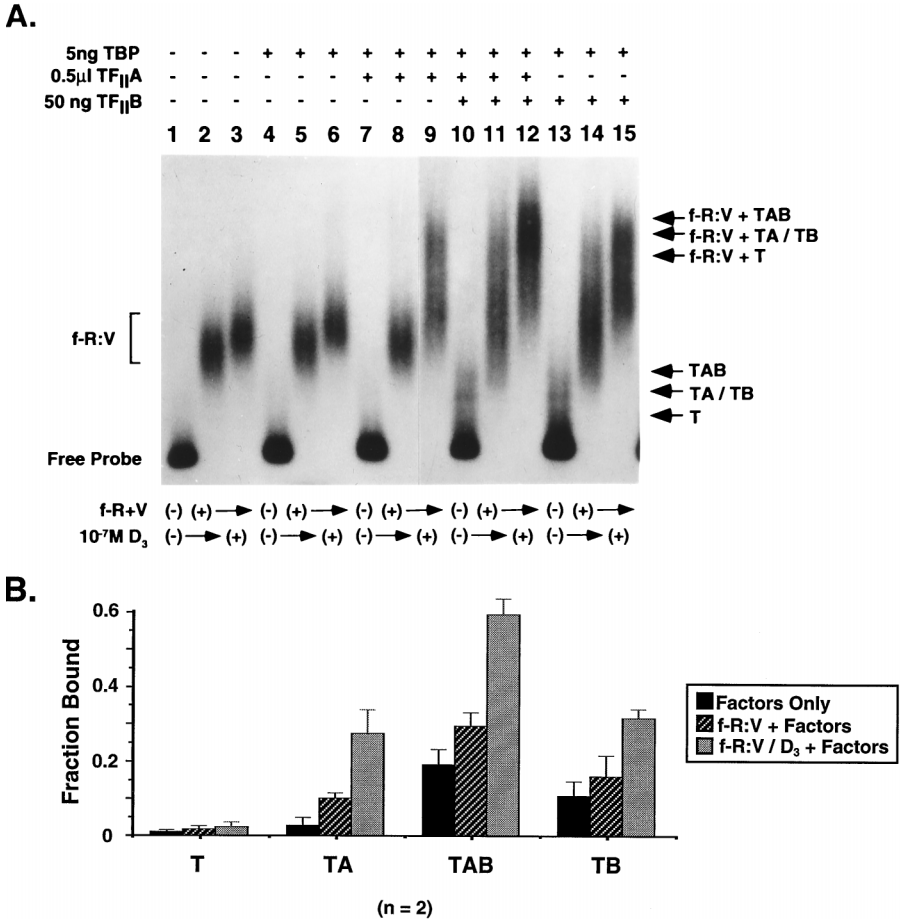


FIG. 7. RXR:VDR heterodimers increase and $1,25(OH)_2D_3$ enhances the formation of TBP-TFIIA (TA), TBP-TFIIA-TFIIB (TAB), and TBP-TFIIB (TB) complexes by recruitment into higher-mobility complexes. (A) Agarose gel mobility shift analysis with purified, recombinant GTFs hTBP, hTFIIA, hTFIIB, and 295-bp VDREx4:E1b probe in the presence (+) and absence (−) of RXR:VDR. Probe (5 fmol) was preincubated at 30°C with 0 (lanes 1, 4, 7, 10, and 13) or 30 ng of an equimolar mixture of purified f-RXR and VDR (f-R+V) in the absence (lanes 2, 5, 8, 11, and 14) or presence (lanes 3, 6, 9, 12, and 15) of $10^{-7}$ M $1,25(OH)_2D_3$ ($D_3$) for 15 min prior to incubation with 0 or 5 ng of purified recombinant TBP, 0 or 0.5 μl of purified native TFIIA, and 0 or 50 ng of purified recombinant TFIIB for 25 min at 30°C. Complexes were resolved on a 1.5% agarose gel as described in Materials and Methods. The migrations of specific complexes are indicated to the right of the gel (some complexes, particularly TBP (T), TA, and f-R:V+T can be seen only upon longer exposure or with greater amounts of GTFs, and their mobilities from other exposures and/or experiments are shown for reference). (B) Quantitation data of two separate experiments resolved under identical electrophoresis conditions are expressed as means and standard deviations of fraction of probe retarded in specific complexes.

with response element copy number, and is both receptor and ligand saturable.

**Exclusivity of the RXR:VDR heterodimer as the transactivator.** The fact that VDR can bind to elements such as the osteopontin VDRE as a homodimer and one report indicating that a VDR homodimer could activate transcription in transient transfections with insect cells (8) have led some investigators to suggest that VDR can transactivate in the absence of RXR. In our cell-free assays, transcriptional enhancement required an RXR:VDR heterodimer, as neither receptor added alone could support $1,25(OH)_2D_3$-dependent transactivation in vitro (Fig. 5). In other supporting experiments, we have observed that with limiting amounts of RXR, 9-*cis* RA and RXR-selective ligands can modestly attenuate transcription in vitro (data not shown). Taken together, these results are in agreement with our previous observations in vitro and in cell culture and, we believe, definitively establish that it is the RXR:VDR heterodimer which is the transactivating species from a DR3 VDRE (14, 52).

**A role for RXR:VDR during transcription factor complex assembly.** Many classes of activators have been shown to directly target GTFs in transcription assays, including TFIIB, TBP/TFIID, TFIIE, and TFIIF (18, 27, 35, 38, 41, 42, 54–57, 66, 71, 82, 83) or other general coactivators, including USA, TBP-associated factors, and other associated proteins (13, 69, 86, 90, 93). Moreover, activators such as Gal4-VP16 and the Epstein-Barr virus-encoded activators Zta and Zebra have been found to stabilize higher-mobility complexes containing TFIIA, TBP, TFIID, or TFIIB and the activator into these complexes (17, 54). By employing single-round transcription assays with the addition of Sarkosyl at various stages in our cell-free system, it appears that RXR:VDR heterodimers facilitate and $1,25(OH)_2D_3$ stimulates the assembly or stabilization of PICs, thereby increasing transcription (Fig. 6). In addition, by gel mobility shift assays with highly purified, recombinant GTFs (Fig. 7), we found that RXR:VDR facilitated the assembly of TBP:TFIIA, TBP:TFIIA:TFIIB, and TBP:TFIIB by recruitment into higher-mobility complexes on a VDRE-linked promoter and that $1,25(OH)_2D_3$ enhanced the formation of these complexes, particularly in the presence of TFIIA. Enhanced activator responsiveness mediated through TFIIA, especially in more defined transcription assays involv-

ing TFIID, has previously been demonstrated and may be involved in counteracting inhibitory components of TFIID (17, 61, 108). While we cannot rule out contributions of RXR:VDR to elongation of transcription, as has been described for other activators (107), our results suggest that direct stabilization of GTF complexes by RXR:VDR is one way in which the receptors mediate transcriptional enhancement. Our observations extend previous demonstrations of steroid and nuclear receptor interactions with TBP and TFIIB (3, 5, 27, 38, 63, 88) but also suggest there may be an important role for TFIIA as a coactivator.

**Auxilliary components of nuclear receptor control of transcription.** Recently, some nuclear receptors, including TR and RA receptor (RAR), have been found to associate with corepressors, N-CoR and SMRT, which are thought to enhance transcriptional repression by these receptors in the absence of ligand in vivo and in vitro (11, 12, 36, 95). However, other cell-free studies have suggested that TR can silence transcription as a homodimer in the absence of ligand by intrinsic repressor functions, independent of a corepressor protein, manifested through direct inhibitory interactions with components of the basal transcriptional machinery, notably TBP (25, 27). The known corepressor proteins do not associate with VDR (12, 36). Consistent with this, we were unable to detect basal repression by VDR in the absence of ligand in previous studies in cell culture (52) and here actually observed some degree of ligand-independent transcriptional enhancement by RXR:VDR in our cell-free system. Our apparent ligand-independent transcription is unlikely a function of residual concentrations of ligand in the nuclear extracts or from receptor preparations, since VDR mutants which cannot bind ligand enhanced transcription to similar levels (data not shown). Ligand-independent enhancement of transcription in vitro has also been reported for other members of the steroid/nuclear receptor superfamily, including the glucocorticoid receptor, progesterone receptor, RAR, TR, RXR, and the orphan receptors apolipoprotein AI liver-specific enhancer receptor protein (ARP-1) and hepatocyte nuclear factor 4 (46, 50, 66, 67, 87, 100).

Whereas transactivation in vivo is completely dependent on ligand, potentially some of the ligand-independent transcription observed in vitro could be from exogenous receptor titration of an inhibitory activity, such as the corepressors mentioned above, or basal repressors, such as Dr1/NC2 and DRAP1 (39, 43, 70). A more plausible explanation for the observed constitutive enhancement of transcription by RXR:VDR and other nuclear receptors in vitro is the lack of chromatin assembly in the crude nuclear extracts. By footprinting analysis, in vivo occupancy by RXR:VDR and RXR:RAR appears to require ligand (7, 21). In addition, members of the SWI/SNF family of chromatin remodelers have been shown to be essential for transactivation by steroid receptors in yeast cells (109). Taken together, these results suggest that chromatin antagonizes transactivation by nuclear receptors and that potential association with chromatin remodelers, such as NURF (98, 99) and SWI/SNF (45) relieves this repression in vivo. In support of this, the glucocorticoid receptor has been shown to stimulate transcription of histone H1-repressed templates in vitro (24) and to rearrange nucleosomes in vivo (97).

Recently, several members of the steroid/nuclear receptor superfamily have been shown to have ligand-dependent associations with putative cofactors which are not members of the general transcriptional machinery (9, 32, 40, 49, 51, 73, 101, 102). Many of these interactions appear to be mediated through a ligand-modulated activation domain at the extreme C terminus of the family, AF-2 (20). Few of these interacting proteins, with the notable exceptions of SRC-1, CBP, and TIF-2, have been demonstrated to have bona fide transcriptional coactivator activity with nuclear/steroid receptor family members (37, 40, 73, 101). Two of these putative coactivators, SUG-1/TRIP-1 and TIF-1, have been recently demonstrated to interact with VDR in a yeast two-hybrid assay (102); however, no functional activity has yet been ascribed to them in VDR-mediated transcription and SUG-1/TRIP-1's role as a potential transcription mediator may be more complex than originally thought, as it was found to be a component of the 26S proteosome (85).

$1,25(OH)_2D_3$ regulates a myriad of biological processes, from bone formation and calcium mobilization to growth inhibition and differentiation. Ultimately, many, if not all, of these physiological effects must be explained at the level of transcriptional control by VDR. This, in turn, raises the issue of determined tissue-specific and developmental-stage-specific actions by receptors, such as VDR, whose expression patterns appear to be rather ubiquitous. One possibility for more restricted regulatory effects by nuclear receptors is the relative availability of cofactors required for transcriptional activation and how these cofactors affect the structure and function of AF-2 and/or the entire ligand-binding domain (60). A cell-free transcription system such as we have described here will be necessary to address these questions in a direct manner. Ultimately, a highly purified, reconstituted system and the use of chromatin-assembled templates (76, 77, 89) will be invaluable to further characterize RXR:VDR-mediated transcription, to define $1,25(OH)_2D_3$ signaling and cross talk by other ligands in modulating interactions with potential coactivators, and perhaps to gain greater insights as to how other members of the nuclear receptor superfamily regulate the transcription of various target genes.

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