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Additional Protein Factors Play a Role in the Formation of VDR/RXR Complexes on Vitamin D Response Elements

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Abstract The vitamin D receptor (VDR) elicits a transcriptional response to 1,25-dihydroxyvitamin D_3 by binding to specific response elements (VDRE) in the promoter of target genes. Retinoic X receptor (RXR) is required for formation of the VDR-VDRE complex when VDR is supplied at physiologic concentrations. When porcine intestinal nuclear extract is used as a source of VDR, two distinct complexes are always observed with native gel electrophoresis. Both complexes contain VDR and RXR. We now show that the faster-migrating complex requires another heretofore unknown nuclear factor for its formation. In addition, we provide evidence that the formation of the slower-migrating complex is enhanced by transcription factor IIB (TFIIB). Using ligand binding assays, we determined that both complexes contain the same ratio of VDR to VDRE. Using RXR subtype-specific antibodies in gel shift assays, we show that the complexes contain more than one RXR subtype. Therefore, the present results demonstrate VDR-RXR-VDRE complexes formed with pig intestinal nuclear extracts contain other proteins and that the complexes formed between VDR and VDRE are not simply heterodimers of VDR and RXR. J. Cell. Biochem. 71:515–523, 1998.

Key words: binding; complex formation; retinoic X receptor; TFIIB; vitamin D receptor; VDRE; steroid receptor; nuclear extract

1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), the physiologically active form of vitamin D, regulates gene transcription by binding to its nuclear receptor, which is a member of the steroid receptor superfamily. The vitamin D receptor (VDR) binds to vitamin D response elements in the promoter region of 1,25-$(OH)_2D_3$–regulated genes [DeLuca, 1991]. Binding of the VDR to vitamin D response elements (VDREs) requires retinoid X receptor (RXR), which is also involved in the binding of thyroid receptor and retinoic acid receptors to their respective response elements [Munder et al., 1995; Yu et al., 1991]. Several VDREs have been identified and characterized [Darwish and DeLuca, 1992; Demay et al., 1990; Morrison et al., 1989; Noda et al., 1990; Ozono et al., 1990; Zierold et al., 1994], and they generally consist of two 6 base pair (bp) half-sites separated by 3 bp. The sequence of the half-sites is not well conserved; however, the spacing between the half-sites is characteristic of VDREs and seems to contribute to receptor binding specificity [Umesono et al., 1991].

In recent years, much of the work has focused on understanding the molecular mechanisms of transcriptional regulation in the VDR-thyroid–retinoic acid receptor subfamily. Specifically, the concept of homodimerization and heterodimerization of the receptors when bound to DNA, their orientation, spacing between the HRE half-sites, and ligand effects have been extensively analyzed [Glass, 1994]. When nuclear extracts are used as a source of VDR, the formation of two complexes has been consistently observed. These two complexes have often been arbitrarily identified as homodimers and heterodimers. Both complexes formed with porcine intestinal nuclear extract (PNE) and

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VDRE contain VDR and RXR; thus, homodimers are not observed under near physiological conditions found with whole nuclear receptor extract [Munder et al., 1995]. Recently, YY1 has been shown to be in the complexes formed by nuclear extracts on the osteocalcin VDRE [Guo et al., 1997].

In an attempt to identify the distinguishing features between these complexes, we undertook the present study to further analyze the two VDR-RXR-VDRE complexes. Our results indicate that factors other than VDR and RXR are present in VDR-RXR-VDRE complexes. One of these factors may be transcription factor IIB (TFIIB). TFIIB has previously been shown to interact with VDR [Blanco et al., 1995; MacDonald et al., 1995], but it has not been shown to be a component of the receptor–response element complexes. In addition, an unidentified factor is involved in the formation of one of the VDR-RXR-VDRE complexes.

MATERIALS AND METHODS

Bacterially expressed TFIIB was purified using Whatman (Hillsboro, OR) phosphocellulose followed by MonoQ columns [Ha et al., 1991]. IIB8 and IIB12 anti TFIIB antibody [Thompson et al., 1995] was a generous gift from Dr. Burgess's laboratory (McArdle Laboratories, University of Wisconsin, Madison). IIB20 anti TFIIB antibody was purchased from Promega (Madison, WI). Purified recombinant human RXRβ (2 mg/ml) was purchased from Affinity Bioreagents (Neshanic Station, NJ). VDR antibodies (VD2-F12 and IVG8-C11) were generated in the DeLuca laboratory [Dame et al., 1986]. RXRα, β, γ-specific antibodies were a generous gift from Beth Allegretto at Ligand Pharmaceuticals (San Diego, CA) [Titcomb et al., 1994]. 4RX-ID12 was supplied by Dr. Pierre Chambon (Strasbourg, France) [Rochette-Egly et al., 1994]; 1,25-$(OH)_2$[26,27-^{3}H]D_3 (160 Ci/mmol) was obtained from DuPont-NEN (Boston, MA). Soluene-350 and Hionic Fluor were purchased from Packard (Meriden, CT). Baculovirus-expressed purified VDR used in Figure 6 was a generous gift from Panvera (Madison, WI).

Synthetic Oligonucleotides

All nucleotides were synthesized on an Applied Biosystems instrument (La Jolla, CA). Complementary strands containing the VDREs were synthesized and annealed. XbaI overhangs were included for labeling. The sequence of the rat distal 24-hydroxylase VDRE was 5′ ctagAGAG**CGCACC**CGC**TGAACC**CTGGGCt 3′ [Zierold et al., 1994].

Purification of VDR

Baculovirus-expressed receptor was purified as described [Li et al., 1994]. Bovine serum albumin (80 µg/ml) (Sigma, St. Louis, MO) and 50% glycerol was added to the purified receptor, and aliquots were stored at -80°C.

Gel Retardation Assay

The VDREs described in synthetic oligonucleotides were labeled by filling in recessed ends using [α–^{32}P]dCTP (DuPont-NEN) and the Klenow fragment of DNA polymerase Promega. Probe purification was done on a 6% acrylamide gel as described in Darwish and DeLuca [1992] with minor modifications. Binding reactions and electrophoretic separation were run as explained Darwish and DeLuca [1992] with either PNE or purified components. Antibodies were included in the incubation mixtures as indicated, and PNE stripped of VDR (SK PNE) was obtained by incubating PNE with anti-VDR-linked Sepharose (IVG8-C11) twice [Ross et al., 1992].

Quantitation of 1,25-$(OH)_2$[^{3}H]D_3 in VDR-VDRE Complexes

Twenty micrograms of PNE extract (2 fmol VDR/µg protein) were incubated with 1 µg poly-dIdC, glycerol loading buffer, 70,000 dpm of 1,25-$(OH)_2$[^{3}H]D_3, and 0.5% CHAPS to prevent the 1,25-$(OH)_2$[^{3}H]D_3 from sticking to the side of the tubes. After addition of saturating levels of 24-hydroxylase VDRE probe, the reactions were allowed to incubate on ice for at least 30 min. Reaction mixtures were separated on a 4% nondenaturing acrylamide gel; a background lane, which contained all components but no VDRE, was always included.

Following electrophoresis, each lane was cut into six 0.5 cm slices, 12 0.25 cm slices. These acrylamide slices were then incubated overnight in 2 ml of Soluene-350 to allow the 1,25-$(OH)_2$[^{3}H]D_3 to be released from the acrylamide. Hionic Fluor (10 ml) was then added and

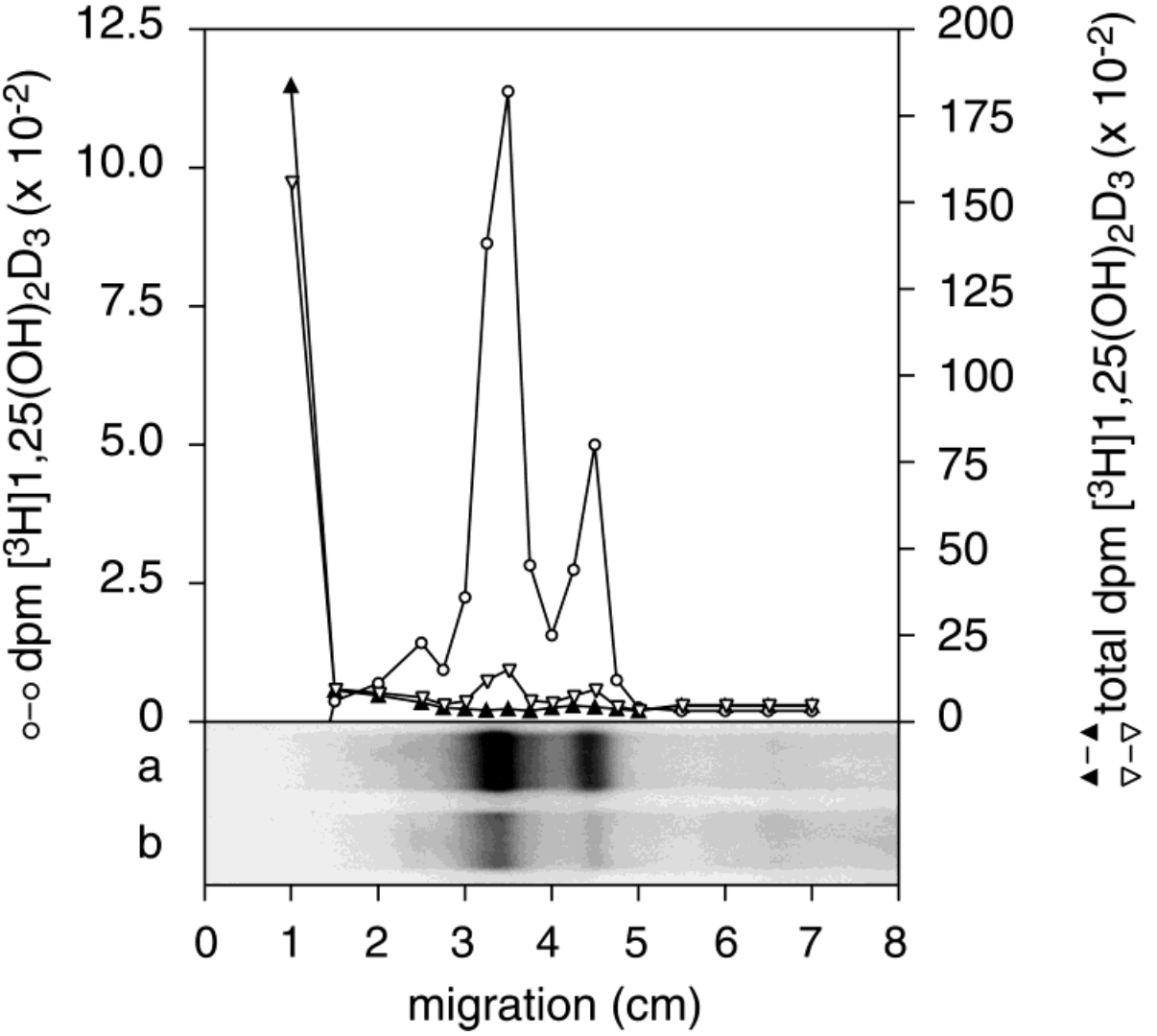

Fig. 1. Pig intestinal nuclear extract was incubated with 1,25-$(OH)_2[^3H]D_3$ and unlabeled 24-hydroxylase distal VDRE. VDRE bound complexes were separated on a nondenaturing gel. After electrophoresis, each lane was cut into 0.25 cm acrylamide pieces, dissolved in Soluene-350 overnight, and counted in a scintillation counter. Total radioactivity (dpm) in the presence (open triangles) or absence (background, closed triangles) of VDRE are shown. The counts in the peaks corresponding to the two complexes after background subtraction are also shown (open circles, different scale). In parallel experiments, $[^{32}P]$-labeled VDRE in the complexes was quantified by the PhosphorImager. A representative image is shown at the bottom of the figure: **lane a** contains 1 ng of labeled VDRE, while **lane b** contains 1 ng of labeled VDRE diluted with 3 ng of unlabeled VDRE.

the samples counted for 5 min in a Packard scintillation counter (model A1900 Tri-Carb). The radioactivity in the background lane was subtracted from all corresponding slices in the +VDRE lanes. The femtomoles of 1,25-$(OH)_2[^3H]D_3$ in each peak were then calculated based on the specific activity of the radiolabeled 1,25-$(OH)_2[^3H]D_3$. In parallel, the VDRE in the complexes was determined using ^{32}P quantitation with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

RESULTS

Complexes Formed on the Distal 24-Hydroxylase VDRE Have the Same Amounts of VDR per VDRE

The two complexes formed on the VDREs by PNE separated by native gel electrophoresis both bind 1,25-$(OH)_2[^3H]D_3$. The ligand bound appeared in direct proportion to the bound VDRE (Fig. 1). An attempt was made to determine exactly how many VDRs bind per VDRE, but accurate quantitations could not be made

TABLE I. Ratio of the Two Complexes Determined on the Basis of Amount of VDRE or Amount of Ligand

	Ratio[a]
VDRE (^{32}P-labeled)	2.42 ± 0.44[b]
1,25-$(OH)_2$[^{3}H]D_3	2.62 ± 0.10[b]

[a]Ratio fentomoles in slower-migrating complex over fentomoles in faster-migrating complex expressed as mean ± SD.
[b]These ratios are not significantly different at $P < 0.1$ as calculated by Student's *t*-test.

due to possible ligand dissociation from the VDR in the complex during electrophoresis. However, the ratio of ligand bound/VDRE was the same for both complexes (Table 1). The fact that these ratios are similar is a strong indication that there are equal amounts of VDR per VDRE in both complexes.

Of considerable interest is that receptor that is not bound to VDRE barely enters the gel (Fig. 1, high counts in the top centimeter of the gel). The accumulation of VDR at the top of the gel was also seen when the proteins separated on nondenaturing gels were transferred to a membrane and Western-blotted with anti-VDR antibody (data not shown). Using purified VDR in the same buffer showed identical localization of the VDR, suggesting that it is not other proteins that prevent VDR from migrating (data not shown). Thus, in the absence of VDRE, VDR either complexes with itself or has an overall conformation that does not allow migration under nondenaturing conditions. In a previous study, a similar behavior was observed: three forms of VDR were detected, one of which did not enter an electrofocusing gel under either acidic or basic conditions [Pierce et al., 1987].

RXR Subtypes in the Complexes

Quantitation of RXR in the complexes using [^{3}H]-9-cis-retinoic acid was attempted but was not successful. The major problem encountered was that [^{3}H]-9-cis-retinoic acid bound to many proteins in the nuclear extract, and thus specific peaks as seen with the VDR ligand were not detected. In addition, RXR, as a silent partner of VDR, might not bind [^{3}H]-9-cis-retinoic acid [Forman et al., 1995]. We used subtype-specific anti-RXR antibodies [Titcomb et al., 1994] to determine that more than 1 RXR is

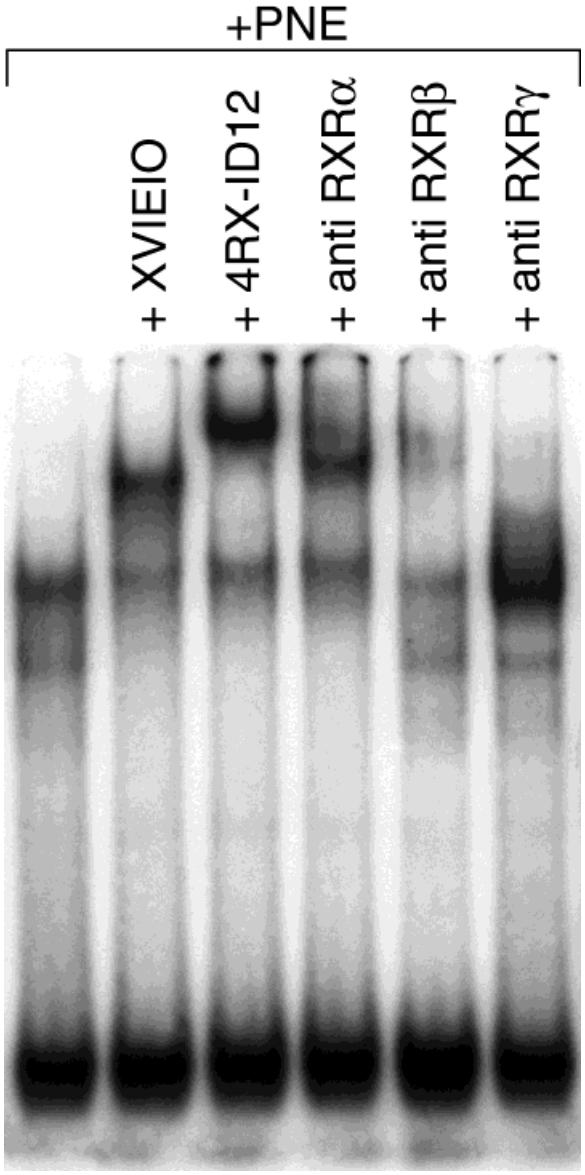

Fig. 2. Electrophoretic mobility shift assay was performed as described in Materials and Methods using the 24-hydroxylase distal VDRE as a probe and pig intestinal nuclear extract. Reactions in each lane were incubated with antibodies as indicated. Anti-VDR (XVIE10) was from Dr. DeLuca and anti-RXR (4RX-ID12) was from Dr. Pierre Chambon; all others were a generous gift from Beth Allegretto at Ligand Pharmaceuticals.

present in each complex. As seen in Figure 2, RXRα-specific antibody supershifts both complexes, and RXRβ-specific antibody prevents the formation of both complexes, indicating that both RXRβ and RXRα are present in both complexes. RXRγ antibodies do not affect the complexes due to the absence of RXRγ subtype in intestinal tissue [Mangelsdorf et al., 1992]. Pro-

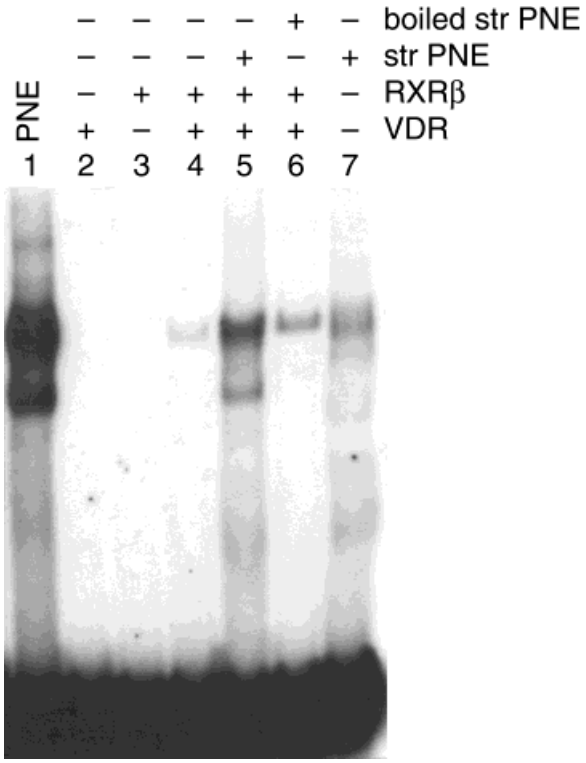

Fig. 3. Electrophoretic mobility shift assay was performed using the 24-hydroxylase distal VDRE as a probe in the presence of purified VDR (50 fmol) and purified RXRβ (1,000 fmol). PNE stripped of VDR (str PNE) was added where shown. In **lane 6,** str PNE was boiled for 5 min and chilled prior to addition to the reaction mixture.

vided that the subtype specificity is conserved in porcine extracts, these data show that more than one RXR is present in each complex.

Complexes Formed on the Distal 24-Hydroxylase VDREs Contain Other Proteins in Addition to VDR and RXR

Purified VDR and RXRβ were used to form the complexes on VDREs. Figure 3 shows that very little complex is formed on the VDRE with physiologic concentrations of purified VDR and purified RXRβ alone. Addition of PNE stripped of VDR markedly enhanced the formation of complexes. Boiling of the stripped PNE prior to addition to the purified components resulted in little complex formation, indicating that stripped PNE is supplying heat labile factors and not minerals, salt, or small organic molecules possessing no tertiary structure. Measurement of VDR in stripped PNE by ELISA showed trace amounts of VDR (data not shown).

Though pig intestinal nuclear extract is required for the formation of the faster-migrating complex, formation of the slower-migrating complex has been observed routinely in previous studies when baculovirus-expressed VDR or RXR was used. This suggests that the complex-enhancing factor can be supplied by nuclear extract of cells from other species like SF21 insect cells. TFIIB is ubiquitous and conserved [Hisatake et al., 1991], and in addition protein-protein interaction of VDR with TFIIB was shown to occur [Blanco et al., 1995; MacDonald et al., 1995]. This led us to test TFIIB in our in vitro binding studies. As can be seen in Figure 4, addition of TFIIB enhances the formation of the slower-migrating complex. In Figure 5, the presence of TFIIB in the complex formed by unpurified VDR with RXRβ is shown using anti-TFIIB antibodies. The IIB8 antibody supershifted the complex, while IIB20 impeded complex formation. Preincubation of TFIIB with TFIID and radioinert TATA oligonucleotide before addition of VDR and RXRβ also showed decreased complex formation (data not shown). It is not known why the IIB12 antibody does not react, but it is possible that its epitope is masked by the complex.

While the formation of the faster-migrating complex requires a heat labile factor in PNE, regardless of the source of VDR, we have observed that the effect of TFIIB is seen only when VDR is purified and used at physiological concentrations. In Figure 6, we used purified VDR from baculovirus supplied by Panvera Corp. (Madison, WI) to show that, when using 93 fmol of VDR per 15 µl reaction, TFIIB enhances complex formation, while at tenfold higher VDR concentrations no increase is observed. Using TFIIB with bacteria-expressed VDR, no increase in complex formation was found (data not shown).

DISCUSSION

The present study demonstrates that an unknown factor plays a significant role in the formation of the faster-migrating complex of VDR/RXR and VDRE. In addition, we show that TFIIB is present in the slower-migrating VDRE complex and that it enhances binding of highly purified VDR and RXRβ when tested at physiological concentrations found in nuclear extracts. The degree of binding enhancement is somewhat variable but always observed. We do

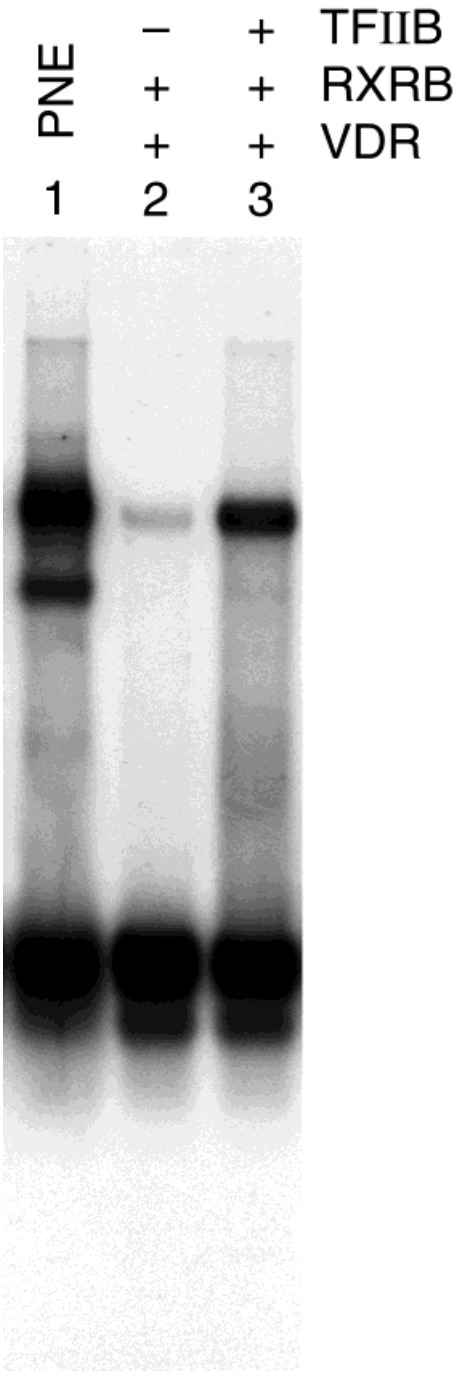

Fig. 4. Electrophoretic mobility shift assay was performed as described in Materials and Methods using the 24-hydroxylase distal VDRE as a probe. Purified source of VDR (50 fmol), RXRβ (1,000 fmol), and TFIIB (1,000 fmol) were used as shown. PNE was used in **lane 1** as a positive control.

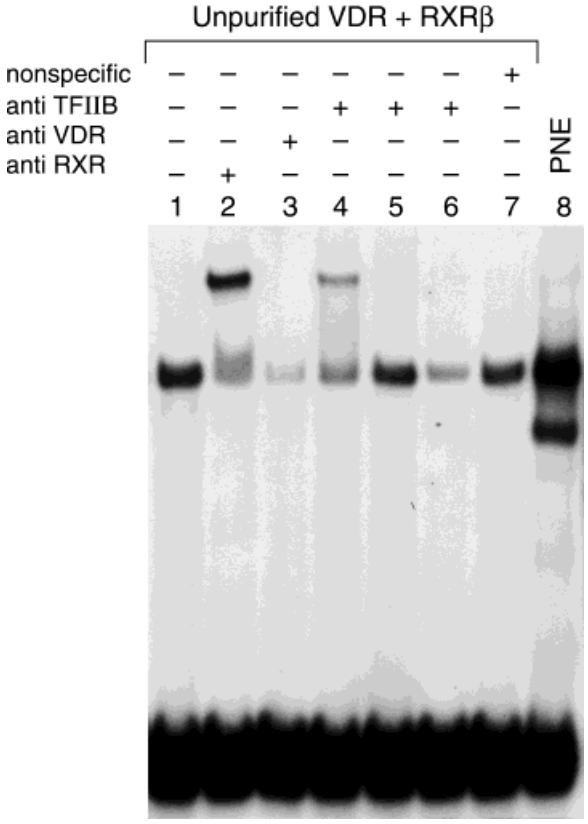

Fig. 5. Electrophoretic mobility shift assay was performed using unpurified VDR with RXRβ and various antibodies. Unpurified VDR (baculovirus expressed) and RXRβ were incubated with 4RX-D12 (**lane 2**), VD2-F12 (**lane 3**), IIB8 (**lane 4**), IIB12 (**lane 5**), IIB20 (**lane 6**), MOPC21 (**lane 7**). PNE only (**lane 8**) was included as a positive control.

not know why this variability is seen. The presence of TFIIB in the complex is consistent with previous reports that have demonstrated TFIIB binding sites on VDR [Blanco et al., 1995; MacDonald et al., 1995] as well as effects of TFIIB on 1,25-$(OH)_2D_3$–dependent transcription [Blanco et al., 1995]. In addition, thyroid receptor has also been shown to interact with TFIIB [Baniahmad et al., 1993]. So far, the presence of other factors in the formation of complexes may have been overlooked due to the use of nonpurified sources of either VDR or RXR or because the amounts of purified components used were manyfold higher than physiological [Freedman et al., 1994; Nakajima et al., 1994; Nishikawa et al., 1995; Towers et al., 1993].

The formation of two VDRE complexes differing in electrophoretic mobility has consistently been observed with crude PNE. Both of these complexes contain VDR and RXR, as shown by antibody-supershift experiments [Munder et al., 1995], and thus both are at least heterodimers, while neither complex can be a homodimer.

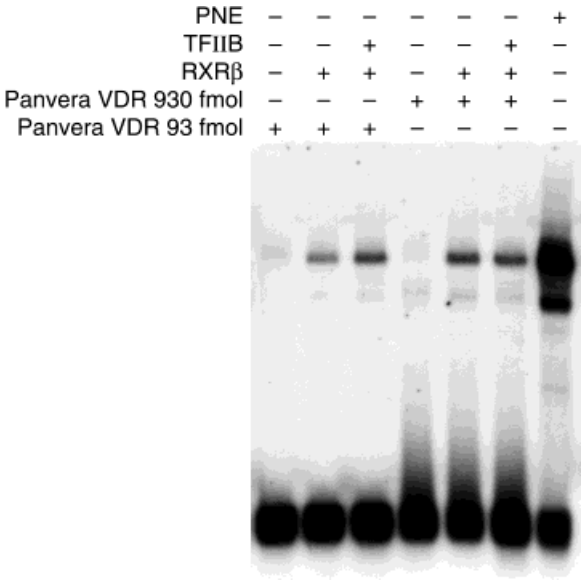

Fig. 6. Electrophoretic mobility shift assay was performed using purified baculovirus-expressed VDR from Panvera at near physiologic concentrations (93 fmol per reaction) and at tenfold higher concentrations with RXRβ and TFIIB as shown. PNE was included as a positive control.

Why then do the two complexes differ in electrophoretic mobility?

These studies show that the complexes differ in at least one unknown component. Shakoori et al. [1994] have shown that nuclear extracts from an osteosarcoma cell line (ROS17/2.8) allow more abundant formation of the slower-migrating complex, while nuclear extracts from osteoblasts form more of the faster-migrating complex with the rat osteocalcin VDRE. This evidence supports the idea that both complexes may form in vivo, and they may have different roles. Unfortunately, Bortell et al. [1993] were not able to show a correlation between osteocalcin mRNA production in ROS17/2.8 or osteoblasts with the differential formation of the two complexes even though they produce mRNA in slightly different patterns.

RXRα and RXRβ are found in both complexes. RXR has been shown to tetramerize in solution [Kersten et al., 1995], indicating that there are available sites for multiple protein interactions on RXR. The observation of multiple RXRs in VDRE complexes is based on the specificity of the antibodies provided by Ligand Pharmaceuticals. Whether the specificity demonstrated in human [Titcomb et al., 1994] extends to porcine RXRs has not been shown. Sedimentation velocity analysis of the complexes performed by Shakoori et al. [1994] has suggested tetrameric and trimeric complexes and the possibility of the presence of other factors, which agrees well with the present study. Physiologically, the reason for multiple RXRs in a complex may be to provide an additional site for regulation, though this study shows that in vitro binding can occur with only RXRβ in the presence of TFIIB. The relevance of these complexes to the final transcriptional event is still not clear, nor is it clear what the relationship is between the two complexes. It is our view that the formation of these complexes is an early event in the vitamin D activation or suppression of gene expression. Thus, the demonstration that TFIIB contributes to complex formation between VDR, RXR, and VDRE provides a closer link to the basal transcription machinery. Recently, it was shown that RXR interacts with the TATA-binding protein (TBP) and mediates transcription [Schulman et al., 1995], suggesting that another protein in the VDRE complex, RXR, may interact with the basal transcription machinery; however, so far interaction of TBP with RXR in VDR complexes has not been shown.

The sites of interaction between VDR and TFIIB have already been investigated [Blanco et al., 1995; MacDonald et al., 1995], but more work needs to be done to determine what other factors are needed and the exact sequence of events that ultimately leads to 1,25-$(OH)_2D_3$-dependent transcription. One factor may be phosphorylation, and others may be enhancers, as recently described by Masuyama et al. [1997]. Certainly, identification of the factor in PNE necessary for the formation of the faster-migrating complex may contribute to the determination of why two complexes form and what function they might have in transcription.

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