The Polymorphic N Terminus in Human Vitamin D Receptor Isoforms Influences Transcriptional Activity by Modulating Interaction with Transcription Factor IIB

Peter W. Jurutka, Lenore S. Remus, G. Kerr Whitfield, Paul D. Thompson, J.-C. Hsieh, Heike Zitzer, Poupak Tavakkoli, Michael A. Galligan, Hope T. L. Dang, Carol A. Haussler, and Mark R. Haussler

Department of Biochemistry
College of Medicine
University of Arizona
Tucson, Arizona 85724

The human vitamin D receptor (hVDR) is a ligand-regulated transcription factor that mediates the actions of the 1,25-dihydroxyvitamin D_3 hormone to effect bone mineral homeostasis. Employing mutational analysis, we characterized Arg-18/Arg-22, hVDR residues immediately N-terminal of the first DNA binding zinc finger, as vital for contact with human basal transcription factor IIB (TFIIB). Alteration of either of these basic amino acids to alanine also compromised hVDR transcriptional activity. In contrast, an artificial hVDR truncation devoid of the first 12 residues displayed both enhanced interaction with TFIIB and transactivation. Similarly, a natural polymorphic variant of hVDR, termed F/M4 (missing a *Fok*I restriction site), which lacks only the first three amino acids (including Glu-2), interacted more efficiently with TFIIB and also possessed elevated transcriptional activity compared with the full-length (f/M1) receptor. It is concluded that the functioning of positively charged Arg-18/Arg-22 as part of an hVDR docking site for TFIIB is influenced by the composition of the adjacent polymorphic N terminus. Increased transactivation by the F/M4 neomorphic hVDR is hypothesized to result from its demonstrated enhanced association with TFIIB. This proposal is supported by the observed conversion of f/M1 hVDR activity to that of F/M4 hVDR, either by overexpression of TFIIB or neutralization of the acidic Glu-2 by replacement with alanine in f/M1 hVDR. Because the f VDR genotype has been associated with lower bone mineral density in diverse populations, one factor contributing to a genetic predisposition to osteoporosis may be the F/f polymorphism that dictates VDR isoforms with differential TFIIB interaction. (Molecular Endocrinology 14: 401–420, 2000)

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INTRODUCTION

The traditional role of vitamin D, via its active hormonal metabolite 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), is to regulate calcium and phosphate metabolism to elicit normal bone mineralization and remodeling. 1,25-$(OH)_2D_3$ also appears to exert a number of nonclassical bioeffects in the immune, central nervous, and endocrine systems, as well as in epithelial cell differentiation (1). The generation of 1,25-$(OH)_2D_3$ from vitamin D_3, obtained initially from diet or derived from sunlight-initiated photobiogenesis in skin, involves sequential hydroxylations in liver and kidney (1). 1,25-$(OH)_2D_3$ ensures that the proper ion product of calcium and phosphate exists in the blood for optimal deposition of bone mineral by stimulating intestinal absorption, bone resorption, and renal reabsorption of these ions. A failure to achieve normal bone mineral accretion results in clinical rachitic syndromes, such as nutritional rickets, which arises from the simultaneous deprivation of sunlight exposure and dietary vitamin D_3, or hypocalcemic vitamin D-resistant rickets (HVDRR), which can be a consequence of inadequate enzymatic bioactivation of the vitamin. The molecular basis for a rare familial form of HVDRR, in which there is tissue insensitivity to 1,25-$(OH)_2D_3$, has been shown to reside in defects within the gene coding for the nuclear vitamin D receptor (VDR) (2, 3). The phenotype of these latter HVDRR patients, including hypocalcemia, secondary hyperparathyroidism, and severe osteopenia, mimics classic nutritional rickets, thus implicating VDR as the mediator of the bone

mineral homeostatic actions of 1,25-$(OH)_2D_3$. Further unequivocal evidence for the obligatory role of VDR in skeletal maintenance is detailed in two recent reports describing the VDR null mouse homozygote, which displays a phenotype similar to that of HVDRR patients (4, 5).

The VDR is a member of the nuclear receptor superfamily of proteins that contain amino acid homologies within two separate functional domains (6–8). The N-terminal region of VDR is configured into two zinc-coordinated fingers responsible for DNA recognition and binding, whereas the C-terminal segment binds the 1,25-$(OH)_2D_3$ hormone (9). This common modular structure reflects the similar molecular actions employed by the members of the nuclear receptor superfamily in translating a hormonal signal into a transcriptional response. Upon binding 1,25-$(OH)_2D_3$, VDR regulates specific gene transcription by binding as a heterodimer with the retinoid X receptor (RXR) (10–13) to a DNA enhancer sequence, termed the vitamin D-responsive element (VDRE), that is present within the promoter region of vitamin D-controlled genes (14–16). Thus, VDR belongs to the same subgroup of nuclear receptors that includes the thyroid hormone receptor (TR) and retinoic acid receptor (RAR), which also heterodimerize with RXR on their respective DNA-responsive elements (17).

In addition to its interaction with RXR, the VDR has been shown to associate with several additional proteins to form the active transcriptional complex required for gene regulation (1). These molecules, termed coactivators, include proteins of the p160 class that possess histone acetyl transferase (HAT) activity such as SRC-1 (18), GRIP1 (19), and ACTR (20). Other coactivators postulated to stimulate VDR-mediated transactivation are TIF1 (21), NCoA-62 (22), p65 (23), DRIP205/TRAP220 (24, 25), and components of the transforming growth factor-β (TGF-β) signaling pathway, including Smad3 (26, 27). Moreover, VDR has been reported to interact directly with components of the basal transcription machinery, such as TATA-binding protein associated factors $TAF_{II}135$ (28) and $TAF_{II}55$ (29), with concomitant enhancement in ligand-stimulated transcription. Finally, the basal transcription factor IIB (TFIIB) has been shown by several laboratories to interact both physically and functionally with this receptor (30–33), and one of the regions required for TFIIB association is localized within the C-terminal hormone-binding domain of the VDR (30, 31, 34).

The VDR gene harbors several polymorphisms, both in the coding and noncoding portions of the gene (1, 35). However, only one of these polymorphisms results in an actual change in the VDR primary sequence. This polymorphism occurs within the first ATG start codon of human VDR (hVDR) and contains a *Fok*I restriction endonuclease site (designated f). Absence of the *Fok*I site (denoted F) indicates that the first codon is ACG, resulting in translational initiation at an in-frame ATG three codons downstream (36, 37). Therefore, the *Fok*I polymorphism produces either a 424 (F) or a 427 (f) amino acid hVDR protein. These two isoforms are thus structurally distinct, unlike those hVDRs that contain polymorphisms present in the 3′-portion of the gene that are either silent codon changes or are found in introns or in the 3′-untranslated region (1).

Because of the central role of VDR in calcium and phosphate homeostasis to ensure the deposition of bone mineral, the *Fok*I polymorphism has been studied in the context of its potential influence on bone mineral density (BMD). In several different populations, including American and Japanese premenopausal women, as well as Mexican-American and Italian postmenopausal women, an association between enhanced BMD and the F allele has been reported (36–41), but no mechanism for this relationship has been proposed.

In the present study, we identify a novel region in the VDR N-terminal segment required for functional interaction with TFIIB and define specific residues that participate in transcriptional stimulation mediated by 1,25-$(OH)_2D_3$ via contact with TFIIB. Thus, similar to the estrogen and glucocorticoid receptors (42, 43), VDR appears to possess an activation function 1-like domain (AF-1) N-terminal of the zinc fingers. We also provide unique evidence that, as a direct result of differential interaction of the two receptor isoforms with TFIIB at this N-terminal region, the F hVDR possesses more potent transcriptional activity. This observation may provide the mechanistic basis for the enhanced BMD associated with the FF *vs.* ff genotype, and intimates that, unlike the inactivating mutations which generate the severe HVDRR phenotype, smaller differences in VDR activity over a lifetime could significantly impact the risk of bone fractures and osteoporosis.

RESULTS

hVDR Mutants Used to Probe Interactions with TFIIB

Previous reports by us and our collaborators (30) and others (31, 32) have demonstrated that VDR interacts both physically and functionally with TFIIB *in vitro*, in transfected mammalian cells, and in the yeast two-hybrid system. Furthermore, one region of hVDR that appears to be important for contacting TFIIB occurs within the C-terminal hormone binding domain (30, 34). In the present study, we endeavored to determine whether additional regions in hVDR are important for mediating the interaction between the receptor and TFIIB. A series of hVDR mutants was constructed as depicted in Fig. 1. These mutants included truncations of the hormone-binding (Δ161–427, Δ134–427, Δ115–427, Δ84–427)

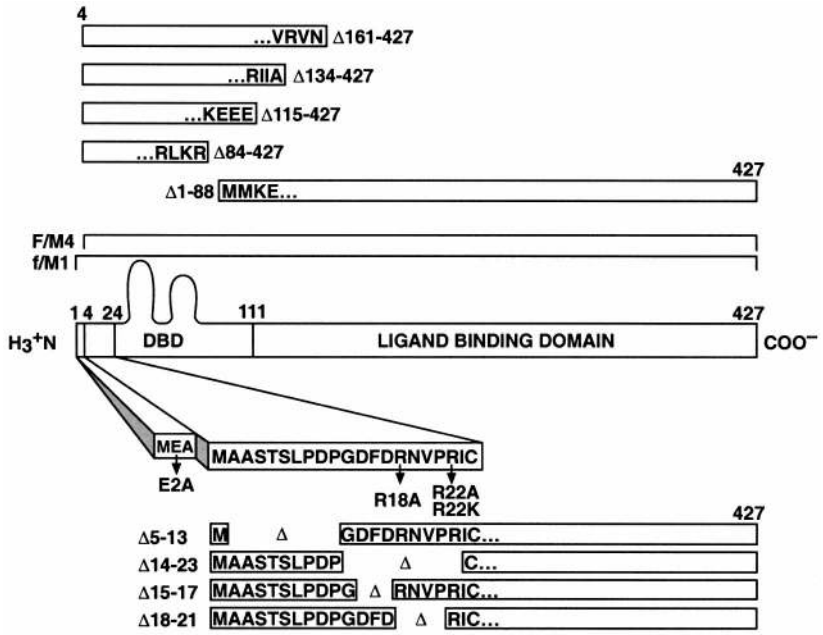

Fig. 1. Construction of hVDR Mutants Used to Probe Interactions with TFIIB

The *central* portion of the figure depicts a schematic representation of full-length hVDR (427 amino acids) containing an N-terminal DBD and C-terminal ligand binding region. Illustrated directly *above* the central hVDR molecule are the two polymorphic designations for the receptor: f/M1 encoding the full-length hVDR and F/M4, which codes for a shorter 424-amino acid protein isoform. Shown *above* the F/M4 hVDR are C- and N-terminal truncations that were generated in this receptor isoform by site-directed mutagenesis. The *bottom* portion of the panel depicts smaller internal deletions and point mutations (*arrows*) that were generated in the F/M4 hVDR isoform in this study. The *numbers after each delta symbol* represent the amino acids deleted in each case [using the Baker *et al.* (44) numbering system]. For point mutations (E2A, R18A, R22A, and R22K), the number of the mutated residue is designated along with the corresponding amino acid substitution.

or DNA-binding (Δ1–88) domains, as well as smaller internal deletions in the N-terminal region of VDR (Δ5–13, Δ14–23, Δ15–17, and Δ18–21). Specific point mutants were also synthesized that resulted in the loss of a positively charged arginine residue by replacement with alanine (R18A and R22A) or that contained a conservative change from arginine to lysine (R22K), thus maintaining a positive charge at this position. All of these mutants were constructed from the cDNA encoding a polymorphic form of hVDR known as F/M4, so denoted to indicate that this hVDR lacks the first three amino acids and thus translation begins from the fourth residue, a Kozak consensus methionine, in the amino acid sequence numbering convention described previously (44). Another polymorphic variant, termed f/M1, represents the full-length hVDR (amino acids 1–427) with translation commencing from the first residue, also a Kozak consensus methionine. The f/M1 receptor was created by mutagenesis using the F/M4 cDNA as a template and inserting the naturally occurring codons present in the f/M1 receptor [coding for the first three amino acids (MEA-) Fig. 1]. Finally, a point mutant within the context of the f/M1 polymorphic VDR was generated by converting glutamate to alanine at position 2.

Mapping Regions of hVDR Required for Interaction with hTFIIB

To define regions of hVDR that associate with TFIIB, we employed an *in vitro* coprecipitation or pull-down assay using glutathione-*S*-transferase (GST) fusion protein methodology and *in vitro* transcribed and translated (IVTT) receptor proteins. Figure 2A depicts the basic interaction between wild-type (WT) hVDR and a GST-TFIIB fusion protein bound to Sepharose beads (lane 4), with no interaction occurring when only GST-Sepharose (GST-S) is used (lane 3) or when the IVTT reaction contains only the pSG5 parent expression vector (lanes 1 and 2). The association between RXRα and TFIIB (lane 6) is much weaker than between VDR and TFIIB (lane 4), and the amount of RXRα that is coprecipitated does not increase in the presence of unliganded VDR (compare lanes 6 and 7). Importantly, inclusion of 1,25-$(OH)_2D_3$ markedly stimulates association of RXRα-VDR and TFIIB (lane 8). These observations indicate that the RXR-VDR heterodimer, which forms in solution in the presence of 1,25-$(OH)_2D_3$ and represents the functionally relevant molecular species in mediating the activation of VDRE-controlled genes, can readily interact with TFIIB in a presumed ternary complex.

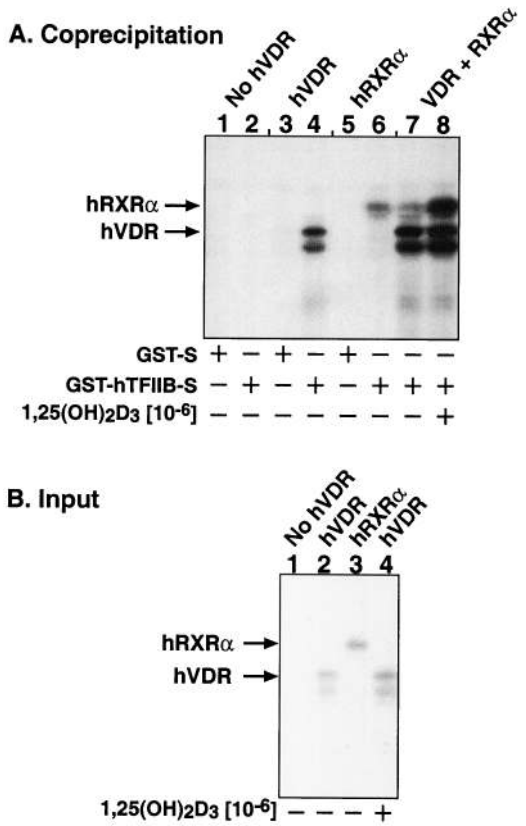

Fig. 2. Human TFIIB Interacts with hVDR and RXR in a 1,25-$(OH)_2D_3$-Dependent Ternary Complex

A, Plasmids (1.0 μg) expressing either hVDR or hRXRα were used as templates in IVTT reactions (see *Materials and Methods*) to generate [^{35}S]methionine-labeled hVDR and hRXRα proteins. Negative control IVTT reactions (lanes 1 and 2) employed the pSG5 template without a receptor cDNA insert. All reactions were then incubated with 10^{-6} M 1,25-$(OH)_2D_3$ (lane 8) or ethanol vehicle (lanes 1–7) for 1 h at 22 C, followed by incubation with either 20 μl of GST-hTFIIB fusion protein bound to Sepharose beads (GST-hTFIIB-S; lanes 2, 4, and 6–8) or 20 μl GST alone bound to Sepharose (GST-S; lanes 1, 3, and 5) for 1 h at 4 C. In lanes 7 and 8, equal portions of hVDR and hRXRα protein were mixed before incubation with 1,25-$(OH)_2D_3$. The beads were then washed extensively, and the amount of coprecipitated hVDR and hRXRα was detected by electrophoresis of denatured bead samples followed by autoradiography. B, The amount of extract analyzed as input (panel B) was 5% of the amount used in the coprecipitation reactions (panel A). The *arrows* indicate the migration position of hVDR and hRXRα.

We next evaluated the association of the WT hVDR and several C-terminal truncation mutants in the GST pull-down assay. Figure 3A reveals that removal of the hormone-binding domain results in a significantly attenuated interaction of hVDR with TFIIB (compare lanes 2 and 10), consistent with earlier evidence for a TFIIB contact site residing in the hormone-binding domain of hVDR, approximately between amino acids 257 and 355 (30). However, further truncation of the receptor enhances this level of association (lanes 6 and 8), with the most pronounced interaction occurring with the smallest truncation, namely Δ84, that possesses only the first 83 amino acids in hVDR (lane 4). These results suggest that an additional segment within the N-terminal portion of hVDR may be required for full TFIIB association. Indeed, removal of the first 88 amino acids (Δ1–88) in the receptor results in a dramatic decrease in the level of interaction with TFIIB (Fig. 3B, lane 4, and Fig. 4A, lane 6) as compared with that observed with the Δ84 truncation (Fig. 3A, lane 4), while the association of the C-terminal truncations (Fig. 3B, lanes 6, 8, and 10) is similar to that observed in Fig. 3A. Nonspecific binding to GST-S alone (odd-numbered lanes) is minimal. In addition, all of the truncations were expressed, based on analysis of 5% of the sample size employed in the coprecipitation reaction (input panels), although the level of expression for the truncations was generally less than that of the WT control. Taken together, these results strongly implicate the VDR DNA-binding domain (DBD), and/or its N-terminal extension, as important for TFIIB interaction, *in vitro*.

To define a smaller region in hVDR that participates in contacting TFIIB, a series of short internal deletants was constructed within the N-terminal extension of the DBD. Surprisingly, the Δ5–13 mutant displayed a striking enhancement in association with TFIIB (Fig. 4A, lane 8, and Fig. 4B, lane 4) compared with the WT receptor (Fig. 4A, lane 4, and Fig. 4B, lane 2), while the Δ14–23 deletant was attenuated in its interaction with TFIIB (Fig. 4A, lane 10, and Fig. 4B, lane 6). An additional pair of even shorter deletants revealed a similar pattern, with Δ15–17 displaying enhanced (Fig. 4B, lane 8) and Δ18–21 (lane 10) showing a reduced association with TFIIB. These results suggest that residues between amino acids 18 and 23 within the N-terminal domain of hVDR are mediators of TFIIB association, and that the apparent affinity of this interaction can be influenced by the composition of amino acids N-terminal of Arg-18.

Charged amino acids within the residue 18–23 segment of hVDR were selected for conversion to alanine by site-directed mutagenesis. Two such mutants, designated R18A and R22A, were evaluated in the GST pull-down assay. The results from these experiments, shown in Fig. 4C, indicate a significant loss in the ability of these two mutant hVDRs to interact with TFIIB (compare lanes 3 and 4 to lanes 5–8). No interactions occur when only GST-Sepharose is used (lane 2) or when the cDNA template in the IVTT reaction does not contain the hVDR insert (lane 1). In addition, the presence of the 1,25-$(OH)_2D_3$ hormone does not significantly influence the association with TFIIB (compare lanes 3 and 4, 5 and 6, and 7 and 8), suggesting that contact between TFIIB and the residues in the N-terminal domain of hVDR is independent of hormonal ligand.

Because the results described above were obtained with the pure protein-protein interaction system of IVTT GST pull-down, we also evaluated WT and mu-

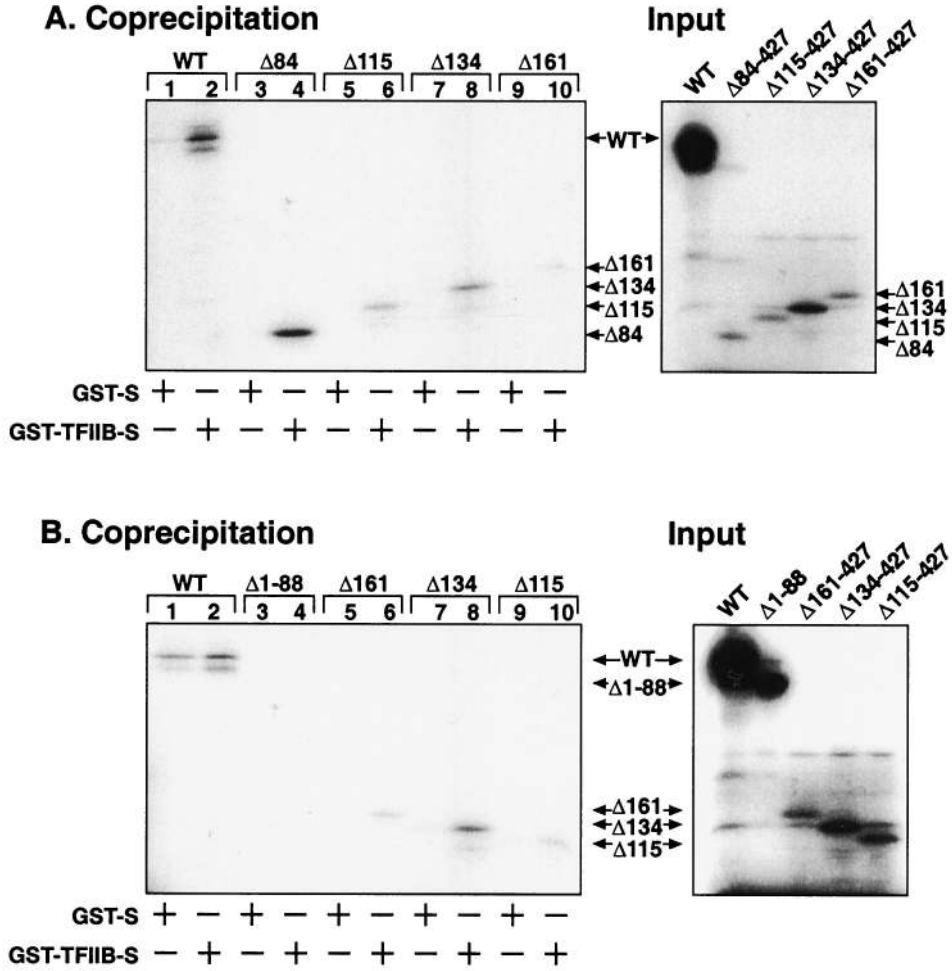

Fig. 3. Truncation Mutants Define a Region of hVDR Required for Contact with hTFIIB

A, WT and C-terminally truncated hVDR proteins were generated in the IVTT system and incubated with either 20 μl of GST-TFIIB-S (*even numbered lanes*) or 20 μl GST-S (*odd numbered lanes*) for 1 h at 4 C. The beads were then washed and analyzed as described in Fig. 2. B, Independent analysis of C- and N-terminal hVDR truncation mutants. A pattern of interaction comparable to that observed in panel A is detected between TFIIB and hVDR mutants lacking the hormone-binding domain (Δ115, Δ134, Δ161). In sharp contrast, an N-terminal truncation mutant (Δ1–88) that does not contain the zinc finger DNA-binding region and N terminus of hVDR does not interact appreciably with hTFIIB (lane 4), at least under the wash conditions employed. The amount of extract analyzed as input in each panel was 5% of the amount used in the coprecipitation reactions. The *arrows* indicate the migration position of each hVDR protein.

tant hVDR association with TFIIB in the context of cellular extracts employing a complementary coimmunoprecipitation protocol utilizing TFIIB antibody. In the experiment depicted in Fig. 4D, both overexpressed WT hVDR (lane 3) and the Δ5–13 mutant (lane 4) coimmunoprecipitate with TFIIB under these conditions, whereas R22A hVDR does not exhibit detectable binding to TFIIB (lane 5). Interestingly, when the level of TFIIB association with Δ5–13 is normalized to differences in expression of this mutant (input panel, *right*), Δ5–13 interacts more efficiently with TFIIB than WT hVDR, approximating the enhanced level of association observed when employing the IVTT system (Fig. 4, A and B). Thus, similar results for VDR-TFIIB interaction to those observed with the IVTT GST pull-down assay are obtained with an independent, and perhaps more physiologically relevant, methodology.

Transcriptional Activity of N-Terminal hVDR Mutants Is Related to the Magnitude of Receptor-TFIIB Interaction

Having demonstrated a requirement for the presence of the N terminus of hVDR to bind TFIIB optimally, *in vitro*, we next probed the functional significance of this domain in mediating 1,25-$(OH)_2D_3$-stimulated transcription of a reporter gene under the control of the rat osteocalcin VDRE (four tandem copies of the VDRE linked to GH gene, $[CT4]_4$-TKGH) in a variety of transfected mammalian cells. Figure 5A illustrates that, in

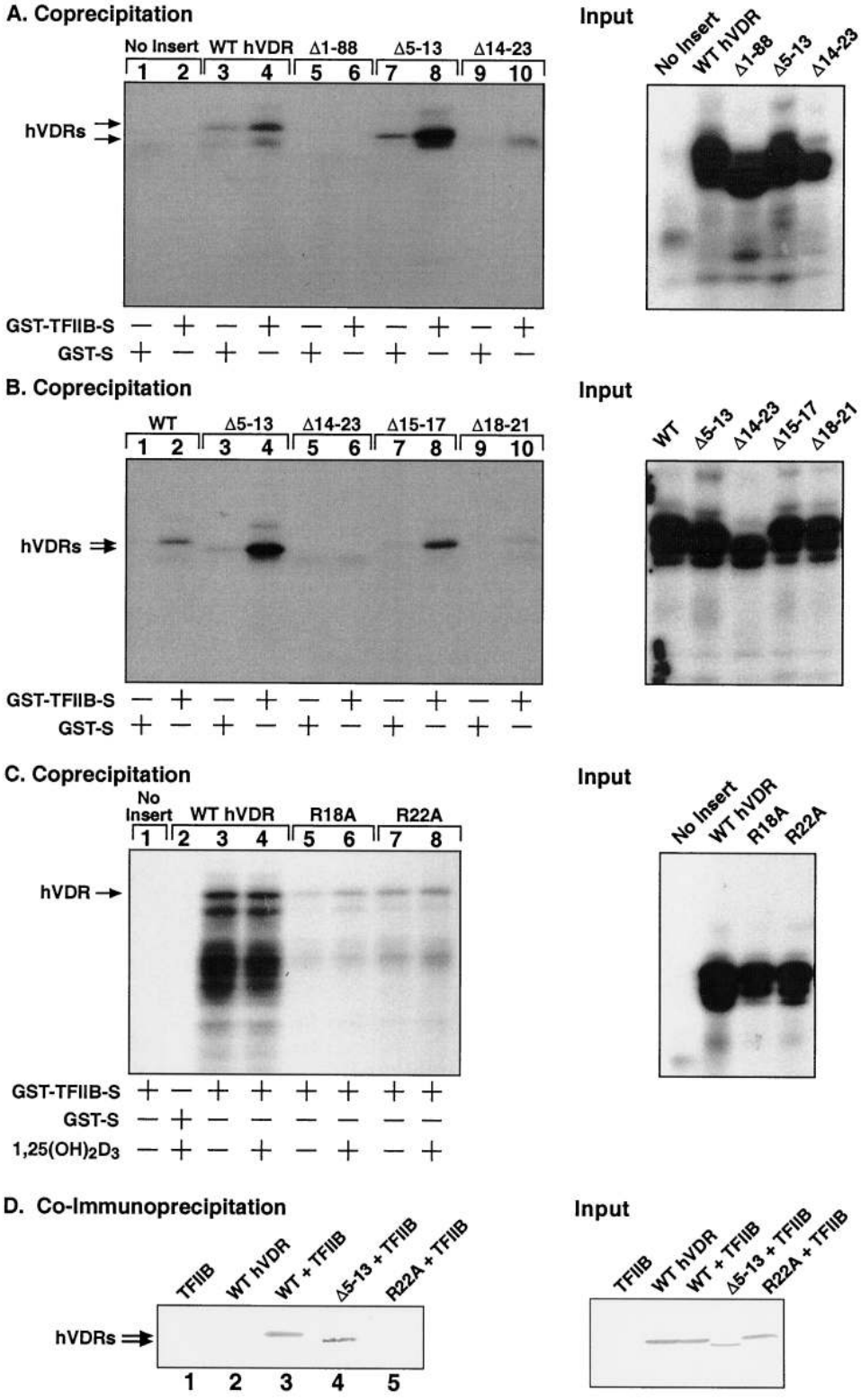

Fig. 4. Analysis of Internal Deletants and Point Mutants in the N-Terminal Region of hVDR and Identification of Residues Needed for Association with TFIIB

A, WT, N-terminally truncated (Δ1–88), and internally deleted (Δ5–13 and Δ14–23) hVDR proteins were synthesized in the IVTT system and incubated with either 20 μl of GST-TFIIB-S (*even numbered lanes*) or 20 μl GST-S (*odd numbered lanes*) for 1 h at 4 C. Lanes 1 and 2 contain an IVTT reaction that employed pSG5 template without the hVDR cDNA insert as a negative control. The beads were then washed and analyzed as described in Fig. 2. B, An additional set of internally deleted hVDRs (Δ15–17 and Δ18–21; lanes 7–10) was generated and analyzed in parallel with WT, Δ5–13, and Δ14–23 hVDRs (lanes 1–6) for interaction with

this cotransfection system, 1,25-$(OH)_2D_3$ (10^{-8} M) treatment of COS-7 cells overexpressing WT hVDR results in an approximate 56-fold increase in receptor-mediated transcription. In sharp contrast, the Δ14–23 and Δ18–21 deletants that displayed little interaction with TFIIB (Fig. 4, A and B) are moderately (Δ18–21) or severely (Δ14–23) impaired in their ability to activate transcription compared with the WT control. Interestingly, the Δ5–13 and, to a lesser extent, the Δ15–17 mutant, both of which displayed an enhanced association with TFIIB (Fig. 4, A, B, and D), showed a corresponding modest increase in transactivation in response to 1,25-$(OH)_2D_3$. In another set of similar experiments (Fig. 5B), also employing transfected COS-7 cells, R18A, R22A, and R22K point mutant hVDRs were evaluated. WT hVDR mediates a 23-fold increase in transcription of the GH reporter gene in the presence of ligand, while the R18A or R22A mutants exhibit only a 17-fold or 3-fold enhancement, respectively. A conservative replacement (arginine to lysine) at residue 22 preserves the activity at WT levels, suggesting that a basic charge is required at this position in hVDR for full transcriptional activity. Because interaction of R18A and R22A with TFIIB is attenuated (Fig. 4C), we overexpressed TFIIB together with these mutant hVDRs in a rescue experiment. The additional TFIIB was able to boost the level of 1,25-$(OH)_2D_3$-elicited transcription of WT receptor from 23- to 34-fold (Fig. 5B). Moreover, the activity of the R18A mutant, whose transactivation capacity is only mildly affected (17-fold *vs.* 23-fold for WT hVDR), can be almost completely restored by overexpression of TFIIB. However, the severely affected R22A mutant, with a 3-fold response to ligand, is boosted only slightly (up to 4-fold ligand stimulation) by TFIIB overexpression. A similar analysis in HeLa cells (Fig. 5C) and in a rat osteoblast-like osteosarcoma cell line, ROS 2/3 (data not shown), also employing the artificial VDRE, $[CT4]_4$-TKGH reporter, reveals a comparable pattern of transactivation by the mutant hVDRs in that R22A-mediated transcriptional stimulation is diminished compared with R18A and is less successfully rescued by excess TFIIB. ROS 2/3 cells were further analyzed with a reporter construct containing 1100 bp of upstream promoter sequence from the rat osteocalcin natural promoter, which contains a single VDRE, linked to the GH reporter gene (Fig. 5D). Responsiveness to 1,25-$(OH)_2D_3$ of this natural promoter construct was blunted compared to that occurring with multiple copies of the VDRE (Fig. 5, A–C), but the relative pattern of transcriptional activity displayed by the WT and mutant hVDRs was similar. Most importantly, excess TFIIB nearly restored the 1,25-$(OH)_2D_3$ transcriptional responsiveness of R18A and R22A hVDRs (Fig. 5D) without potentiating activation by the WT receptor in this setting of a natural promoter in a bone-derived cell type. These data support the contention that Arg-18 and Arg-22, two hVDR residues situated just N-terminal of the first DNA-binding zinc finger, play an important role in 1,25-$(OH)_2D_3$-elicited transactivation of VDRE-regulated genes (Fig. 5) via a mechanism that includes recruitment and contact with TFIIB (Fig. 4).

Neither Expression nor DNA and Hormone Binding Are Affected in hVDR Mutants Possessing Compromised Transcriptional Activity

One possible explanation for the reduced transcriptional activation observed with some of the mutant hVDRs is that the introduction of internal deletions or even point alterations within the VDR molecule could lead to changes in protein stability. We therefore examined the level of VDR protein expression (Fig. 6) in transfected cells employed for the transcriptional assays (Fig. 5). Since assessment of transcriptional activity involves assay of the culture medium, the same cells can be lysed and analyzed by Western blotting with an anti-VDR monoclonal antibody (9A7γ). These immunoblots revealed that the internal deletants were all expressed at levels comparable to the WT receptor (Fig. 6A). In fact, a mutant that displayed enhanced transcriptional activity (Δ5–13) is slightly less expressed than the WT hVDR (Fig. 6A, compare lanes 1 and 2), while a transcriptionally inactive mutant (Δ14–23) is somewhat enhanced in its expression (lane 3). Similarly, the functionally defective point mutant VDRs (R18A and R22A) are well expressed (Fig. 6B, compare lanes 1–3), as are the R22K and E2A mutants (data not shown). Since rescue experiments (Fig. 5) involved the overexpression of TFIIB, we assessed the expression of endogenous and transfected TFIIB utilizing a polyclonal TFIIB antibody (Fig. 6C). Overexpression of TFIIB leads to a dramatic enhancement in the level of

GST-TFIIB-S or GST-S. C, WT and two point mutant hVDRs (R18A and R22A) were generated in the IVTT system. A control IVTT reaction (lane 1) employed the pSG5 template without the hVDR cDNA insert. Reactions were then incubated with 10^{-6} M 1,25-$(OH)_2D_3$ (*even numbered lanes*) or ethanol vehicle (*odd numbered lanes*) for 1 h at 22 C followed by incubation with either 20 μl GST-TFIIB-S (lanes 1 and 3–8) or 20 μl GST-S (lane 2) for 1 h at 4 C. The beads were then washed and analyzed as described in Fig. 2. D, Extracts from cells transfected with either TFIIB alone (lane 1), WT hVDR alone (lane 2), or TFIIB and WT or mutant hVDRs (lanes 3–5) were immunoprecipitated with a TFIIB antibody-protein A/G-Sepharose complex. The immunoprecipitates were subjected to 5–20% SDS/PAGE followed by immunoblotting with an anti-VDR monoclonal antibody (9A7γ) to detect the level of hVDR interaction with TFIIB in a cellular context. The amount of extract analyzed as input in each panel was 5% of the amount used in the coprecipitation reactions. The *arrows* indicate the migration position of the hVDR proteins. The bands *below* the migration position of full-length hVDR represent proteolytic products in each reaction. The results are representative of three independent trials for panel C and two independent trials each for panels A, B, and D.

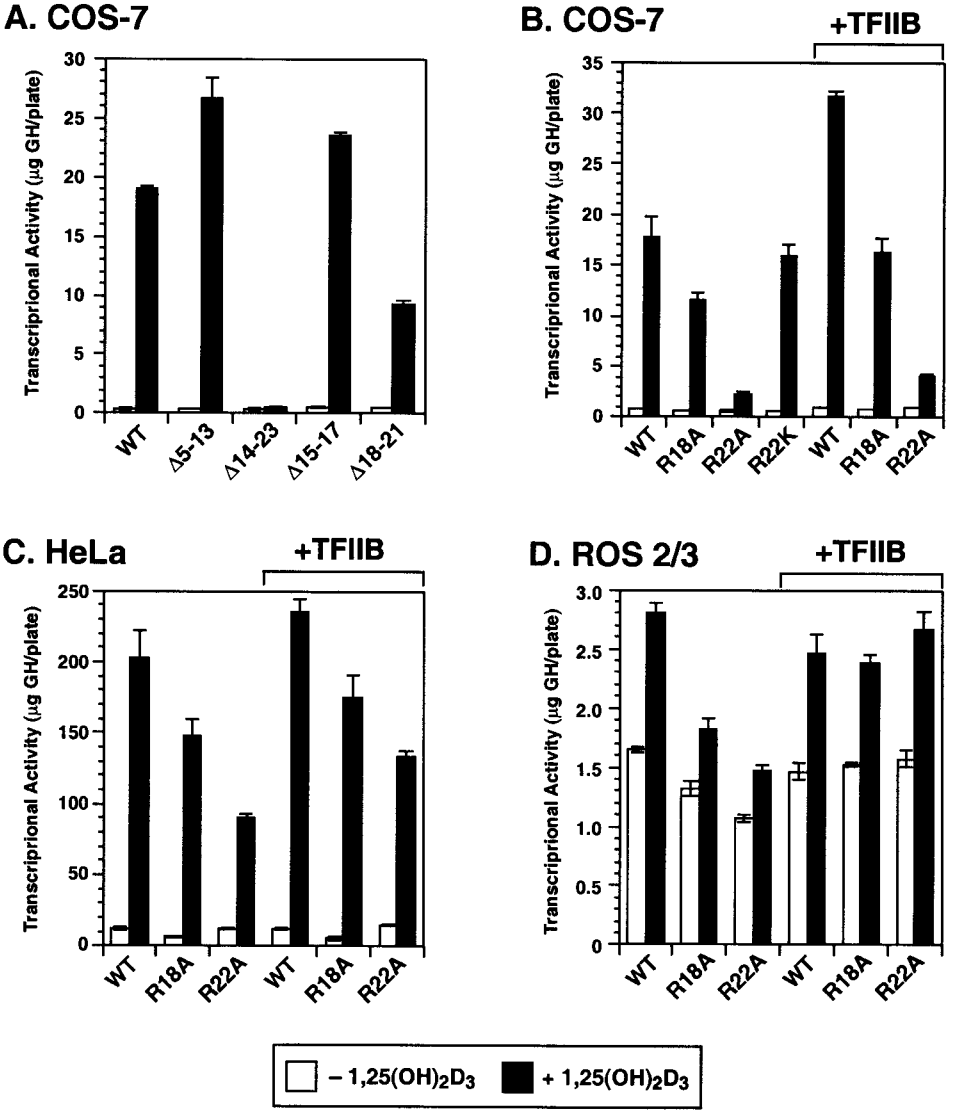

Fig. 5. Transcriptional Activity of N-Terminal hVDR Mutants Is Related to the Magnitude of Receptor-TFIIB Interaction

A, COS-7 cells were cotransfected by calcium phosphate-DNA coprecipitation with expression vectors for either the WT or internally deleted hVDRs and a reporter plasmid containing four osteocalcin VDREs linked to the human GH gene ($[CT4]_4$-TKGH). Cells were treated for 24 h posttransfection with 10^{-8} M 1,25-$(OH)_2D_3$ or ethanol vehicle. The level of GH secreted into the culture medium, which serves as an index of transcriptional activity, was assessed by RIA of each plate. B, Expression vectors for either the WT or indicated point mutant hVDRs and $[CT4]_4$-TKGH were cotransfected into COS-7 cells as in panel A followed by treatment with 10^{-8} M 1,25-$(OH)_2D_3$ for 24 h. An additional group of cells was further cotransfected with an expression plasmid for TFIIB in "rescue" experiments. The transcriptional activity of WT, R18A, and R22A was similarly monitored in HeLa cells (panel C) and in a rat osteoblast-like osteosarcoma cell line, ROS 2/3 (panel D), except employing a reporter vector that contains approximately 1100 bp of upstream sequence from the rat osteocalcin promoter in ROS 2/3. Each treatment group consists of triplicate samples, and each panel is representative of at least three independent experiments. *Error bars* represent the SEM.

detected protein above endogenous amounts in transfected cells (Fig. 6C, lanes 4–6 *vs.* 1–3). Importantly, overexpression of TFIIB does not affect the expression of WT or mutant hVDRs (Fig. 6B, lanes 4–6).

The residues implicated in TFIIB contact and in mediating a transcriptional response to 1,25-$(OH)_2D_3$ are located in the N-terminal domain of hVDR, in close proximity to the DNA binding zinc fingers of the receptor. We therefore performed a hormone-dependent electrophoretic mobility shift assay with extracts from COS-7 cells expressing limiting amounts of VDR proteins that approximate levels found in 1,25-$(OH)_2D_3$ target tissues (3, 45). Under these near-physiological conditions, both the heterodimeric DNA binding and the hormone association of the WT and mutant receptors can be assessed. As shown in Fig. 7A, both the WT and hVDR mutants formed a VDR-containing shifted complex

(lanes 3, 6, and 8) that was enhanced by the addition of the 1,25-$(OH)_2D_3$ ligand (lanes 4, 7, and 9). Significantly, the level of augmentation by 1,25-$(OH)_2D_3$ (~4-fold) based on densitometric scanning of the autoradiographs in Fig. 7A was similar for the WT receptor and each mutant hVDR (data not shown). Also, the level of expression of each receptor was essentially equivalent and unmodified by ligand as monitored by immunoblotting (data not shown). The nonspecific (NS) nature of the two lower protein-DNA complexes was deduced by their appearance in the lanes that used extracts from non-VDR transfected cells (lanes 1 and 2), as well as by their lack of elimination by the VDR antibody (lane 5). Similar data were obtained when employing the R18A or R22A hVDR (Fig. 7B). Taken together, these results strongly suggest that deletion of hVDR residues between positions 15 and 21 or modification of amino acids 18 or 22 to alanine does not decrease heterodimeric DNA binding by the VDR protein. Therefore, altered VDRE association cannot account for the attenuation of transactivation by these mutants. Additionally, the nearly equivalent enhancement in DNA binding elicited by the 1,25-$(OH)_2D_3$ hormone suggests that ligand binding properties of the mutant hVDRs remain unchanged.

The Polymorphic N Terminus of hVDR Affects Both the Interaction with TFIIB and the Level of Transactivation

The N-terminal hVDR *Fok*I polymorphism results in the formation of either a full-length 427-amino acid hVDR (denoted either "f" to indicate the presence of the *Fok*I restriction site or designated "M1" for translation from the first methionine in the primary sequence), or a shorter 424-amino acid protein (termed either "F" for the absence of the *Fok*I site or named "M4" to indicate translational initiation from the methionine at the fourth position in the primary sequence). These isoforms have previously been associated with differences in BMD in diverse populations (36–41). Having identified specific residues located near the N terminus of hVDR as important for TFIIB interaction and transactivation, we next investigated the potential impact, if any, of this polymorphic N terminus on the TFIIB contact domain nearby in the primary sequence. First, extracts from human fibroblasts were evaluated by Western blotting to determine the extent of expression of the two endogenous hVDRs in these cells. As shown in Fig. 8A, both polymorphic forms of the receptor are well expressed (lanes 1 and 2), and in the case of the heterozygote, both proteins are apparently synthesized (lane 3). The electrophoretic mobility of the M1 and M4 hVDRs can be distinguished in these denaturing gels, with the shorter M4 receptor displaying a slightly faster migration (compare lane 2 to 1). We then constructed expression vectors containing the coding sequence for both the M1 and M4 hVDRs. Western blot analysis

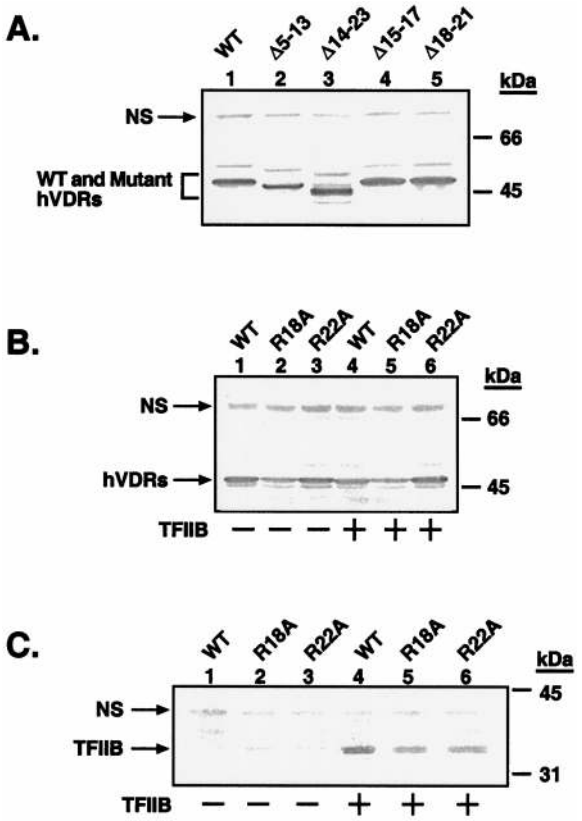

Fig. 6. N-Terminal hVDR Mutants and hTFIIB Are Well Expressed in Transfected Cells

A, Whole-cell extracts (40 μg protein equivalents) of COS-7 cells transfected with the indicated hVDR expression plasmids were analyzed by immunoblotting employing the anti-VDR monoclonal antibody, 9A7γ (64) as described in *Materials and Methods*. Note the faster migration of the Δ5–13 and Δ14–23 mutants that contain larger deletions. NS denotes nonspecific bands. B, Immunoblotting was performed as in panel A utilizing COS-7 cells transfected with expression vectors for WT or point mutant hVDRs in the absence or presence of an additional expression plasmid containing TFIIB. C, A parallel set of extracts was probed with a polyclonal antibody directed against TFIIB.

of extracts from COS-7 cells transfected with either of these plasmids demonstrates the expression of the appropriate hVDR isoform (Fig. 8B), with little difference in the expression levels of either species. As in the case of the endogenous hVDR, the overexpressed polymorphic receptors display distinct electrophoretic mobilities (compare lanes 1 and 2 or 4 and 5). Similar to the experiments shown in Fig. 7, we evaluated the heterodimeric DNA binding activity and hormone binding capacity of the M1 and M4 hVDRs in the hormone-dependent gel mobility shift assay and found no detectable differences in either DNA or 1,25-$(OH)_2D_3$ association between these two receptor isoforms (data not shown).

Since both polymorphic forms of hVDR are well expressed, either endogenously in cultured human fibroblasts, or in transfected cells, we tested the tran-

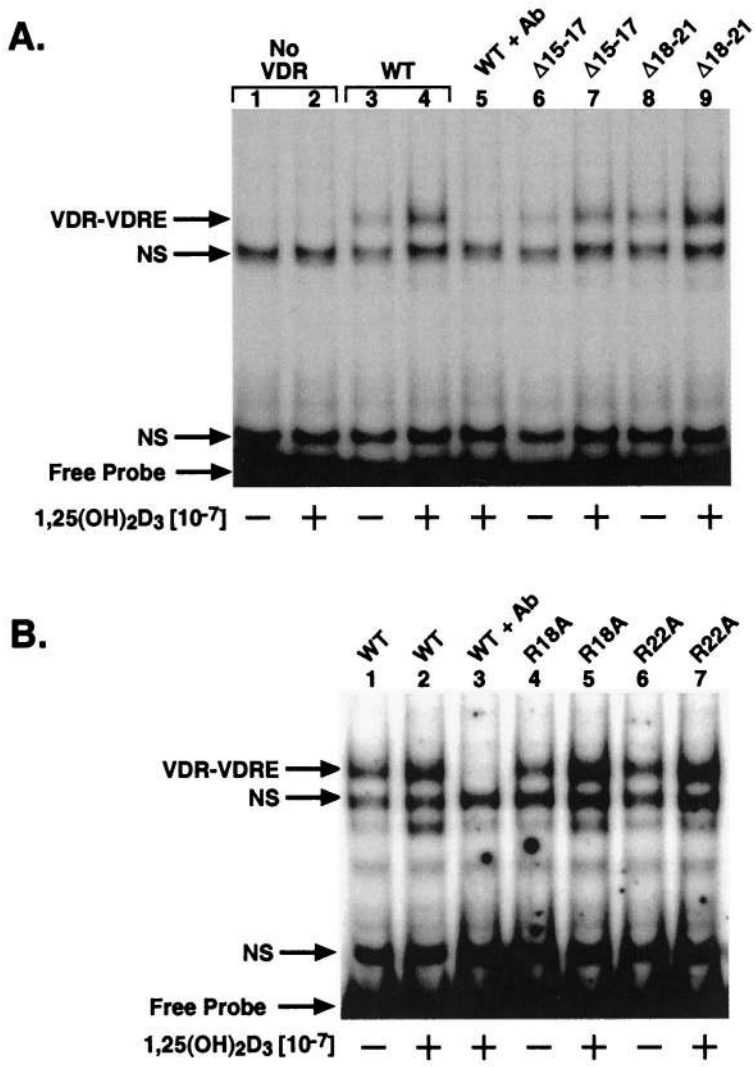

Fig. 7. DNA and Hormone Binding Are Not Affected in hVDR Mutants Possessing Compromised Transcriptional Activity

A, A gel mobility shift assay was performed using extracts (5 μg total protein) from COS-7 cells transfected with WT or internally deleted hVDR expression plasmids (0.1 μg) along with a labeled oligonucleotide containing the VDRE from the rat osteocalcin gene as described in *Materials and Methods*. Extracts were incubated with 10^{-7} M 1,25-$(OH)_2D_3$ (lanes 2, 4, 5, 7, and 9) or ethanol vehicle (lanes 1, 3, 6, and 8) for 0.5 h at 22 C before incubation with the VDRE oligonucleotide. Lane 5 also contained an anti-VDR monoclonal antibody (9A7γ) that is directed against an epitope in the DBD and disrupts the interaction of VDR with the VDRE (12). Lanes 1 and 2 contain extracts from COS-7 cells transfected with the expression vector lacking the hVDR insert. *Arrows* indicate the migration of the specific VDR-VDRE complex (as a heterodimer with RXR), nonspecific complexes (NS), or free probe. B, The gel mobility shift assay was performed as in panel A but employing point mutant hVDRs.

scriptional activity in response to varying doses of 1,25-$(OH)_2D_3$ of these two receptors in different transfected cell lines. Figure 9A illustrates that in transfected COS-7 cells, 1,25-$(OH)_2D_3$ (10^{-9} and 10^{-8} M) treatment results in an approximate 27- and 78-fold increase, respectively, in receptor-mediated transcription with the M4 receptor. In contrast, the M1 protein only displayed an 8.5- and 38-fold increase in activity at these two ligand concentrations. Interestingly, overexpression of TFIIB did not lead to a statistically significant potentiation of transcription with the more active M4, but did increase the activity of M1 (up to 13.3- and 66-fold). Similar results were obtained with the $[CT4]_4$-TKGH reporter in HeLa cells (Fig. 9B) and in murine P19 embryonal carcinoma (EC) cells or a rat osteoblast-like osteosarcoma cell line, ROS 2/3 (data not shown). ROS 2/3 cells were further analyzed with a reporter construct containing 1100 bp of the rat osteocalcin natural promoter linked to the GH reporter gene (Fig. 9C). In this experiment, 1,25-$(OH)_2D_3$ (10^{-9} and 10^{-8} M) treatment results in an approximate 3.0- and 3.5-fold increase, respectively, in receptor-mediated transcription with the M4 receptor, but only a 1.8- and 2.6-fold effect was noted with M1. Again, the

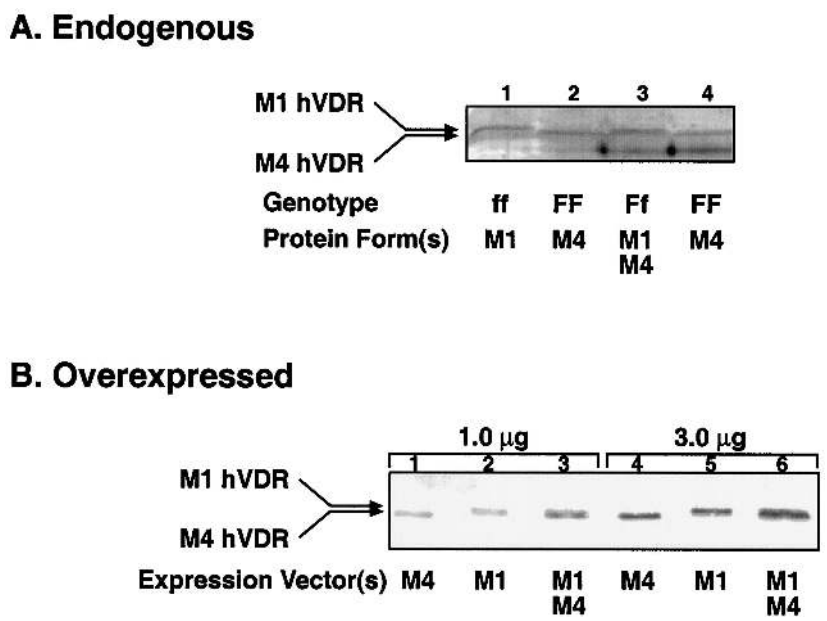

Fig. 8. Both N-Terminal Polymorphic Variants of hVDR Are Expressed in Human Fibroblasts and in Transfected Cells

A, Whole-cell extracts (40 μg protein equivalents) of human fibroblasts were analyzed by immunoblotting employing the anti-VDR monoclonal antibody, 9A7γ. The genotype (see *Materials and Methods* for details) of each fibroblast line is indicated along with the corresponding protein form. The *arrows* reveal the positions of the faster migrating M4 hVDR (424 amino acids) and the slower migrating M1 receptor variant (427 amino acids). B, Whole-cell extracts (40 μg protein equivalents) of COS-7 cells transfected with the M1 (lanes 2 and 5), M4 (lanes 1 and 4), or both M1 and M4 (lanes 3 and 6) hVDR expression plasmids were analyzed as in panel A. Two different amounts of expression plasmid (1.0 and 3.0 μg) were used in the transfections.

overexpression of TFIIB does not enhance transactivation by M4 (2.4- and 3.6-fold hormone effect) but does lead to a small but significant elevation in M1 activity (3.0- and 3.4-fold stimulation). Examination of the sequence differences between the M1 and M4 hVDR reveals that the three amino acid N-terminal extension of M1 (MEA-) contains a glutamate at position 2. Replacement of this residue with an uncharged alanine residue results in the complete restoration of 1,25-$(OH)_2D_3$-elicited transcription (at both 10^{-9} and 10^{-8} M hormone) in transfected ROS 2/3 cells (Fig. 9D).

Finally, we employed the *in vitro* coprecipitation assay to determine directly if the two hVDR isoforms display a differential level of interaction with TFIIB. Figure 9E depicts the association between M4 (lanes 1 and 3) and M1 (lanes 2 and 4) hVDR and a GST-TFIIB fusion protein bound to Sepharose beads. The M4 hVDR displays a 2-fold greater interaction with TFIIB compared with the M1 protein, based on quantitative densitometric scanning of the images shown in Fig. 9E and taking into account the minor difference in hVDR protein expression as depicted in the input panel. These results imply that the observed differences in transactivation capacity between M1 and M4 hVDR are likely the result of a polymorphic N terminus that selectively modulates the interaction of hVDR with TFIIB.

DISCUSSION

The VDR is a ligand-dependent transcription factor that regulates a number of genes, many of which are involved in calcium and phosphate mineral homeostasis and bone remodeling. Like many other members of the nuclear receptor superfamily, the VDR binds to DNA as a heterodimer with RXR and controls the transcription of its target genes by interacting with other coregulatory proteins. One of these proteins, basal transcription factor IIB, has previously been shown to interact both physically and functionally with VDR (30, 31). In the present study, we have localized within the N-terminal segment of VDR a critical site of interaction with TFIIB and demonstrated that key, evolutionarily conserved residues in this region are required for both TFIIB association and 1,25-$(OH)_2D_3$-dependent transactivation. We also explored the relevance of these findings to a naturally occurring polymorphism at the N terminus of hVDR that results in two structurally distinct isoforms of the receptor. Not only do these two forms (f/M1 and F/M4) display a differential pattern of response to 1,25-$(OH)_2D_3$ in eliciting VDRE-mediated transactivation in a number of cell lines including osteoblasts, but they also exhibit a positive correlation between their transcriptional activity and their ability to interact with TFIIB. Because

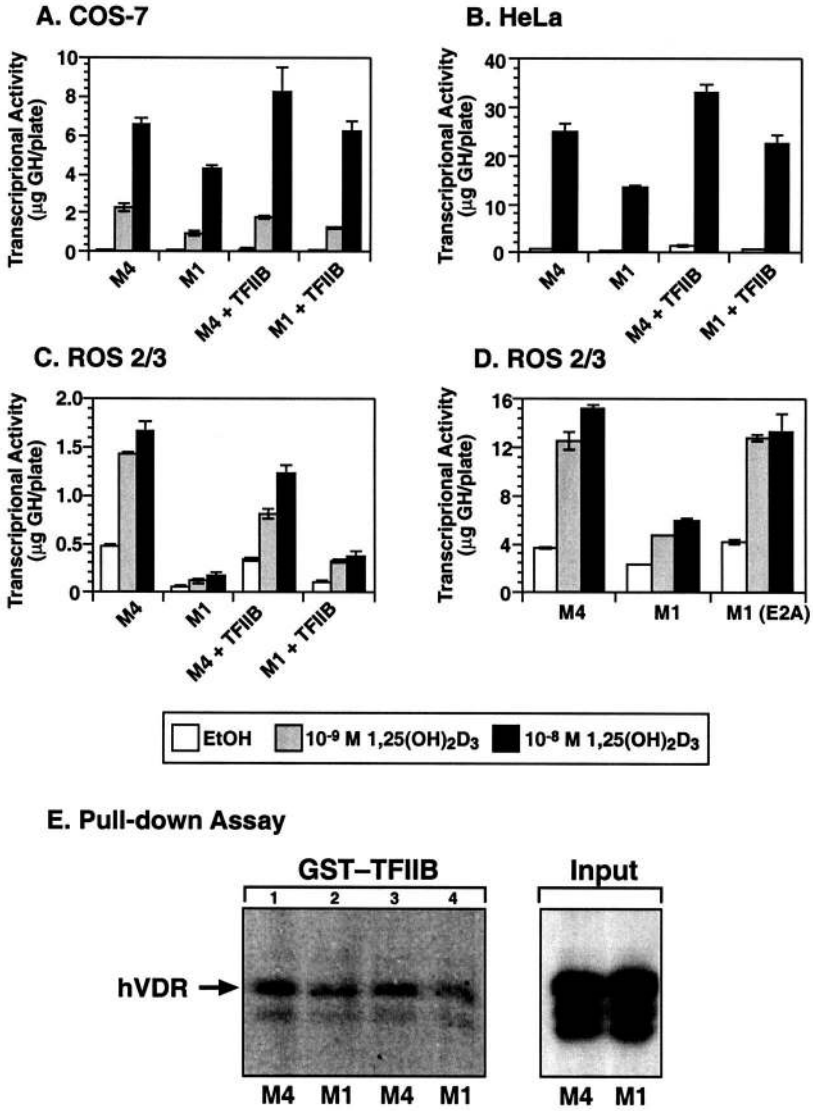

Fig. 9. Transcriptional Activity of the f/M1 Polymorphic hVDR Is Reduced as Is Association of this Variant Receptor with hTFIIB

Expression vectors for either the M1 (427 amino acids) or M4 (424 amino acids) polymorphic hVDR and $[CT4]_4$-TKGH were cotransfected into COS-7 (panel A) or HeLa (panel B) cells as in Fig. 5 followed by treatment with the indicated concentration of 1,25-$(OH)_2D_3$ for 24 h. A parallel group of cells was further cotransfected with an expression plasmid for human TFIIB in "rescue" experiments (+TFIIB). The transcriptional activity of M1 and M4 hVDRs was also monitored in a rat osteoblast-like osteosarcoma cell line, ROS 2/3 (panel C), employing a reporter vector that contains approximately 1100 bp of upstream sequence from the rat osteocalcin promoter linked to the GH reporter gene. D, Expression vectors for M1 and M4 polymorphic hVDR, or a mutant M1 (containing an alanine at position 2 instead of a glutamic acid; E2A) along with a natural osteocalcin promoter-reporter construct were cotransfected into ROS 2/3 cells as in panel C, followed by treatment with the indicated concentration 1,25-$(OH)_2D_3$ for 24 h. Each treatment group consists of triplicate samples, and each panel is representative of at least three independent experiments. *Error bars* represent the SEM. E, M1 (lanes 2 and 4) and M4 (lanes 1 and 3) hVDR proteins were generated in the IVTT system and incubated with 20 μl of GST-hTFIIB-S for 1 h at 4 C. The beads were then washed and analyzed as described in the legend to Fig. 2. The amount of extract analyzed as input was 5% of the amount used in the pull-down reactions.

these hVDR isoforms have previously been associated with differences in BMD in certain populations, one factor contributing to a genetic predisposition to osteoporosis therefore may involve the varying potency in interaction between polymorphic hVDRs and the basal transcriptional machinery.

Initial Mapping of a TFIIB Contact Site in VDR

Previous studies have identified TFIIB as a VDR-interacting protein, employing both pull-down assays (30–34) and the yeast two-hybrid system (31, 34). Yeast two-hybrid data from our laboratory confirm this inter-

action (C. Encinas and P. W. Jurutka, unpublished data). Furthermore, mapping studies have been performed using truncation and deletion methodology with both the GST pull-down (30) and yeast assays (31, 34). The results of these initial mapping experiments pointed to the hormone-binding domain in hVDR as possessing one site of interaction for TFIIB, likely encompassing primarily amino acids 257–355 (30). More recently, utilizing an alternative strategy, the hormone-binding domain (hVDR residues 93–427) was employed to generate a random point mutant library that was used to screen for TFIIB interaction-deficient mutants in the yeast two-hybrid system. The results implicated 11 individual residues between amino acids 228 and 345 in VDR, the majority of which are hydrophobic, as being critical for TFIIB association (46).

Multiple TFIIB Interaction Sites and Functional Significance

Several factors argue, however, that the C-terminal domain of hVDR may not be the only site of TFIIB interaction. The limitation of using gross deletions and truncations (30, 31) is that, once any critical interacting region has been removed, other, perhaps equally important, domains may go undetected. Also, in the above cited point mutant screen with the hVDR hormone-binding region (46), the entire N-terminal region of hVDR (residues 1–92) was not present during the analysis. These caveats, plus the current results using small N-terminal deletions and point mutations, lead us to propose that, in addition to hydrophobic amino acids within the hormone-binding region of hVDR, charged residues in the N-terminal domain of the molecule are required for optimal TFIIB association and functionally competent VDR-mediated transactivation (see also Fig. 10).

The functional significance of TFIIB interaction with VDR is supported by transactivation studies that demonstrate a cooperative effect of these two molecules. In experiments employing P19 EC cells, 1,25-$(OH)_2D_3$ treatment results in only a minor stimulation of reporter gene expression when either hVDR or TFIIB alone is cotransfected into these cells. Concomitant overexpression of VDR and TFIIB causes a dramatic ($>$30-fold) enhancement in reporter gene transcription (30). Another report (34) revealed that expression of a TFIIB mutant that was nonfunctional but still retained the ability to interact with hVDR resulted in a selective dominant negative effect on 1,25-$(OH)_2D_3$-elicited transcription in an osteoblast-like cell line. This inhibition could be reversed by simultaneous expression of WT TFIIB. Neither an inhibition nor a stimulation was observed with glucocorticoid-induced transcription of a glucocorticoid responsive element-reporter construct, indicating that excess TFIIB did not exert nonspecific effects on transcription. Thus, one requirement for 1,25-$(OH)_2D_3$-mediated transcription appears to be specific physical interaction of hVDR with TFIIB, which we propose involves both C- and N-terminal domains in the receptor.

Previous investigations of the putative functional role for the N terminus of hVDR have suggested that, unlike some of the other nuclear receptors that possess a larger N-terminal domain, perhaps this region of the hVDR may be functionally dispensable. Removal of N-terminal residues up to and including amino acid 21 in hVDR did not have a significant impact on transcriptional activity in transfected COS-1 cells using a human osteocalcin gene promoter-reporter construct (47). However, this hVDR construct still possesses Arg-22, a residue that is vital for transcriptional activity in all of the cell lines we tested. One might therefore deduce from our results that exposure of Arg-22 at the extreme N terminus of the (Δ1–21) truncation mutant (47) might accentuate its role, even obviating the need for Arg-18, the other basic residue we found to play a significant role in hVDR-TFIIB binding.

Other Nuclear Receptors Bind to TFIIB

The present proposal that hVDR has a second, N-terminal interaction domain with TFIIB, and that this domain contains crucial basic residues, has a precedent from the results concerning interaction between TR and TFIIB. Chicken TRα contains 50 residues N-terminal of the first zinc finger, including a cluster of basic amino acids (KRKRK) at positions 23–27 that were shown to be crucial for both TFIIB interaction and transactivation by this receptor (48). Thus, like hVDR, chicken TRα requires N-terminal basic residues for contacting TFIIB and for transactivation. The fact that this cluster of positively charged amino acids is well conserved among all known TRα homologs from vertebrate species [including fish (GenBank accession no. BAA08201)] is indeed consistent with such an important functional role for these residues. Significantly, positively charged residues are conserved at positions corresponding to 18 and 22 in all published VDR sequences, ranging from human to the recently cloned *Xenopus* VDR (GenBank accession no. AAB58585), strongly implying that these residues must subserve some crucial function.

Moreover, human TRβ also interacts specifically with TFIIB and, similar to our proposal for VDR (Fig. 10), deletion analysis revealed two contact sites in the TRβ molecule, one located in the N-terminal region and the other positioned in the ligand-binding domain (49). Each of these two distinct regions in TRβ was demonstrated to interact with different sites in TFIIB (49). It therefore appears that VDR and TR, in addition to sharing other features, such as heterodimerization with RXR on direct-repeat responsive elements as well as some sequence homology (27%), also possess dual interaction interfaces with TFIIB. Of course, it is possible that, in the tertiary structures of full-length TR and VDR, these two interaction regions combine to form a single docking scaffold.

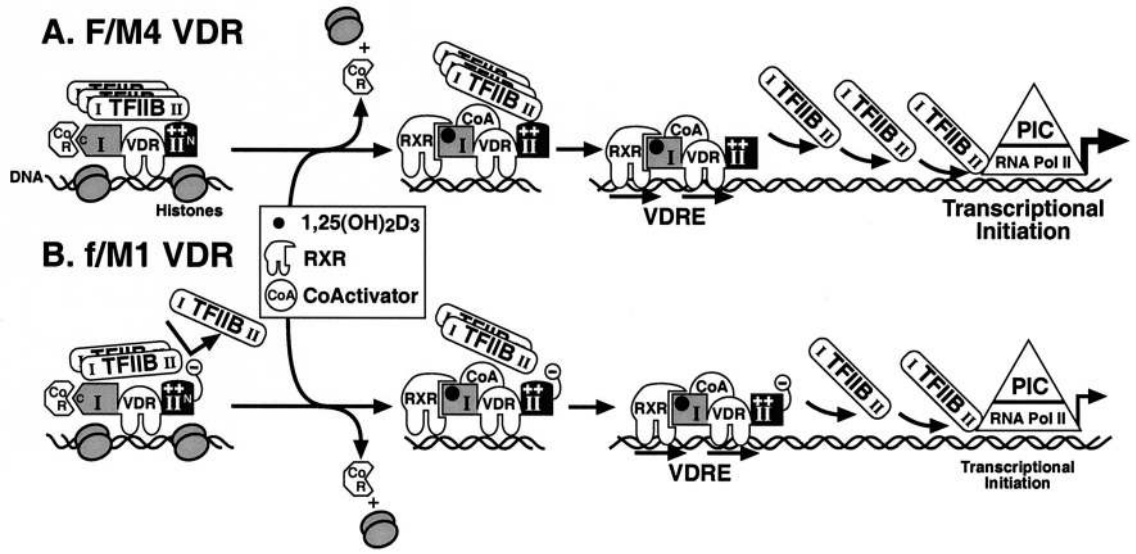

Fig. 10. Model of Interaction between hVDR and TFIIB and Its Consequences for Transactivation by the hVDR-RXR Complex on the VDRE

M1 and M4 hVDR refer to genetic variants of the hVDR gene, also distinguished by the presence (f) or absence (F) of a *Fok*I site in the genomic DNA. A, The M4 variant of hVDR, lacking a Glu residue at position 2, can interact optimally with hTFIIB via two conserved basic residues (Arg-18 and Arg-22) in the Site II (denoted "II") N-terminal interaction domain. This interaction with hTFIIB is augmented by a previously described Site I interacting region, denoted "I", located within the C-terminal ligand-binding domain of hVDR (30, 34). TFIIB is thereby efficiently delivered to the transcriptional initiation complex, leading to robust transcriptional activation. Also part of the activation process is the dissociation of a corepressor ("CoR"), as well as the binding of a coactivator (CoA), like SRC-1, which aids in derepression of the gene by acetylating histones and reorganizing chromatin in the promoter region (as suggested by the "dissociation" of histones; see text for references). B, The M1 variant of hVDR contains three additional amino acids at the extreme N terminus, including a Glu residue (Glu-2), which is proposed to weaken the interaction of Arg-18 and Arg-22 with hTFIIB. Subsequent steps in transactivation by the hVDR-RXR complex on the VDRE can still occur but are attenuated by the weaker nature of the f/M1 hVDR contact with hTFIIB in the Site II region.

A further example of a nuclear receptor that interacts with TFIIB via an N-terminal domain is the homodimerizing ER (50). This study of ER, as well as a similar evaluation of the orphan nuclear receptor hepatocyte nuclear factor-4 (HNF-4) (51), concludes that association of TFIIB with the respective receptor facilitates the assembly of transcriptional preinitiation components, particularly the TATA-box binding protein (TBP). Thus, a picture of nuclear receptor transactivation is beginning to emerge in which TFIIB plays a crucial role.

Significance of the TFIIB Interaction in Transactivation by VDR

There appear to be at least two distinct sets of protein-protein interactions involved in transcriptional activation by hVDR. One is represented by a group of coactivators that bind to a cleft comprised of the Tyr-236 to Lys-246 (helix 3) region combined with the helix 12 AF-2 platform in VDR (52) and effect derepression of chromatin nucleosome organization via HAT-catalyzed displacement of histones at the active promoter site. In our current model, contact by these coactivators with components of TBP-TAF and RNA polymerase II, with the possible participation of a cointegrator analogous to CBP/p300 (53), facilitates transcriptional activation. A second crucial interaction appears to be the recruitment of TFIIB to the promoter via the VDR-RXR heterocomplex (see Figs. 2 and 10). The delivery of TFIIB would then stabilize the RNA polymerase II preinitiation complex and allow for repeated rounds of transcription of the regulated target gene.

Clinical Impact and Functional Significance of hVDR Polymorphisms

The *Fok*I site polymorphism in exon 2 of the hVDR gene is distinct from all other reported VDR polymorphisms in that the two biallelic variants actually differ in protein sequence (f/M1 being 3 amino acids longer). The shorter F/M4 receptor apparently arose after the divergence of hominids from apes and has been dubbed a "neomorph" (1), yet it presently constitutes approximately 65% of VDR alleles in human subjects (36–41, 54, 55). This predominance of the relatively recent F/M4 allele suggests an evolutionary advantage in humans. In support of this notion, the African-American population, which has a significantly lower incidence of osteoporosis than Caucasians, also has a greater prevalence (>80%) of the F/M4 variant (38).

A direct association between the VDR *Fok*I polymorphism and BMD has been reported in several studies. In a group of Mexican-American/Caucasian women,

subjects with the ff genotype had a 12.8% lower BMD at the lumbar spine than FF subjects, with heterozygotes possessing an intermediate BMD (36). This study also showed an increased 2-yr rate of bone loss from the femoral neck in the ff *vs.* FF women. Similarly, in a population of premenopausal Japanese women, BMD in the lumbar spine was 12% less for the ff genotype *vs.* FF (37). Recently, in a large cohort that consisted of a group of 400 postmenopausal women of Italian descent, the FF genotype was associated with enhanced lumbar BMD (41). Interestingly, the effect of the FF genotype on enhanced lumbar BMD was greatest in women within 5 yr of menopause, progressively declining afterward, and the low BMD ff genotype was significantly overrepresented in patients with osteoporotic vertebral fractures (relative risk 2.58). In yet a fourth population of American premenopausal women (both Caucasian and African-American), ff women had femoral neck BMD that was 7.4% lower than that of FF women (38). The association of BMD and the *Fok*I polymorphism also has been extended to include a population of healthy growing children. Subjects that were FF homozygotes had a total body BMD that was 8.2% higher and a mean calcium absorption that was 41.5% greater than ff individuals (40). Thus, the *Fok*I polymorphism may be related to several VDR-dependent parameters of bone metabolism, including intestinal calcium absorption and BMD. However, one study found no significant relationship between the F/f genotype and BMD in a population of French premenopausal women (55), although as the authors point out, the association of BMD and this polymorphism may be masked by various regional factors, such as a high calcium diet. Indeed, an influence of dietary calcium on the impact of the *Fok*I polymorphism on BMD has been suggested by another group (39).

Comparative activities of the M1 *vs.* M4 hVDRs have been investigated directly via cotransfection of expression plasmids for these isoforms together with 1,25-$(OH)_2D_3$-responsive reporter vectors. One such report (37) suggested a 1.7-fold greater activity of F/M4 over f/M1 hVDR in transfected HeLa cells. We observe (Fig. 9) that the F/M4 hVDR isoform is 1.5- to 2.5-fold more transcriptionally active than the f/M1 protein, with the most marked difference resulting when the isoforms are assayed in osteoblast-like cells employing a VDRE in the setting of its natural, bone-specific promoter. However, in another study (56), the activities of hVDRs were comparable, both in transfected COS-7 cells employing reporter gene constructs and utilizing Northern blot analysis of vitamin D 24-hydroxylase mRNA induction by 1,25-$(OH)_2D_3$ in human fibroblasts. The disparate results observed between Gross *et al.* (56) and our present report or that of Arai *et al.* (37) may be because of differences between the reporter constructs used in each study. While we employed both a synthetic rat osteocalcin VDRE and a construct containing the natural promoter region from the osteocalcin gene, Gross *et al.* (56) used a synthetic human osteocalcin reporter and a construct that contained a large portion of the vitamin D 24-hydroxylase promoter. Importantly, the negative study of Gross *et al.* (56), and the earlier work by Arai *et al.* (37) demonstrating a functional difference between M1 and M4 hVDR, were both conducted using transient transfection experiments with a single cell line. In the present study, the functional variance between M1 and M4 hVDR was derived from experiments in four different cell lines, including an osteoblast-like osteosarcoma line (ROS 2/3). In addition, we have provided a functional linkage between the variance in M1/M4 hVDR activity and the differential interaction of these two isoforms with TFIIB, an observation that is consistent with the proximity of the hVDR polymorphic N terminus and the novel TFIIB interaction domain localized within the N-terminal segment of hVDR described in the present study. Finally, recent investigations in our laboratory have found that the F/M4 VDR is more transcriptionally active than the f/M1 receptor by analyzing endogenous VDR function in 17 different human fibroblast lines with varying genotypes at the *Fok*I locus but a constant genotype in terms of relevant VDR polymorphisms in the 3′-UTR (G. K. Whitfield, L. S. Remus, P. W. Jurutka, H. Zitzer, A. K. Oza, H. T. L. Dang, C. A. Haussler, M. A. Galligan, M. L. Thatcher, and M. R. Haussler, unpublished data). Therefore, we conclude that the F/M4 neomorph represents a more transcriptionally potent VDR isoform.

Integrative Model for Transactivation by Polymorphic hVDRs

Figure 10 represents a schematic working model for transcriptional control by 1,25-$(OH)_2D_3$ that incorporates the new findings presented in this manuscript. The model highlights a novel role for conserved basic residues in the N-terminal region of VDR for contacting TFIIB and provides an explanation for the differential effect of the polymorphic N terminus on both the interaction of the receptor with this basal transcription factor and on the activity of VDR. Panel A (*far left*) depicts the shorter 424 amino acid F/M4 hVDR in association with TFIIB at two sites within the receptor (the three molecules of TFIIB do not imply that this number of TFIIB molecules interact with one hVDR but rather are meant to indicate the difference in the level of interaction between TFIIB and M4 *vs.* M1 hVDR). Site I is localized in the C-terminal hormone-binding region of hVDR (30, 34) while site II represents the N-terminal domain identified in the present results, specifically conserved basic residues Arg-18 and Arg-22 (denoted by ++). Also associated with VDR is a corepressor (CoR), likely SMRT (signal mediator and repressor of transcription), which is thought to contact the receptor in the C-terminal ligand-binding domain (57). The VDR-TFIIB complex is loosely bound to DNA via the VDR DNA binding zinc fingers, but the regulated gene is illustrated in the repressed state because

of chromatin nucleosome structure (shown schematically as an association of DNA and histones).

Upon binding to the 1,25-$(OH)_2D_3$ ligand (panel A, *middle*), the M4 VDR is postulated to undergo a conformational change with the following consequences: 1) release of TFIIB from site I (34), 2) release of the corepressor and concomitant association of VDR with the RXR heteropartner (11, 45, 57) and a coactivator/HAT protein (CoA), the latter interaction being facilitated by the AF-2 of VDR (18–20, 58), and 3) derepression of the target gene by coactivator/HAT-mediated chromatin nucleosome reorganization (schematically depicted as a release of the histones). As a result of this derepressive effect, the active RXR-VDR-TFIIB complex can now associate with high-affinity VDRE binding sites located in the promoter region of the regulated gene (panel A, *right*) and "deliver" TFIIB to the preinitiation complex (PIC), resulting in stabilization of the PIC followed by subsequent rounds of RNA polymerase II-directed transcription of the downstream target gene. It is conceivable that the TFIIB delivery process is also facilitated by a VDRE (DNA)-induced change in the conformation of the N-terminal domain of VDR, thereby releasing TFIIB as the rate-limiting factor in the formation of the PIC.

Panel B depicts a similar mechanism of action for the longer 427-amino acid M1 hVDR. However, this isoform of VDR does not interact with TFIIB as well as M4 hVDR (schematically denoted as only two molecules of TFIIB), presumably because of the presence of a negatively charged glutamate (*minus sign enclosed by circle*) localized within the three amino acid N-terminal extension of M1 hVDR. We speculate that the molecular mechanism whereby the negatively charged Glu-2 residue attenuates TFIIB binding could involve either an intermolecular repulsion between f/M1 hVDR and presumed negative residues in TFIIB that bridge to VDR Arg-22/18, or a nonproductive intramolecular interaction of Glu-2 in f/M1 hVDR with the Arg-22/18 TFIIB site II, thus precluding TFIIB contact. Regardless, the hypothesized net result is that the M1 isoform delivers less TFIIB to the PIC, with a subsequently reduced amount of transcriptional initiation and mRNA synthesis from the target gene. Experimentally, the activity of M1 hVDR can be raised to the level of the more potent M4 receptor either by 1) overexpression of TFIIB to boost the endogenous levels of this protein and thus overcome the lower affinity for hVDR and the resulting diminished local concentrations that are delivered by M1 hVDR (Fig. 9), or 2) by "neutralization" of the glutamic acid residue at position 2 in M1 hVDR via mutagenesis to an alanine (Fig. 9D).

In summary, we have elucidated a novel domain in hVDR, located near the N terminus and adjacent to the DNA-binding zinc finger motif, that is required for 1,25-$(OH)_2D_3$-elicited transcriptional activity. Within this region, two basic residues, Arg-18 and Arg-22, were identified as critical for transactivation and contact with the basal transcription factor IIB. A polymorphic variant of hVDR that encodes a shorter, 424-amino acid protein (F/M4), which has been associated with enhanced BMD in diverse populations, is more transcriptionally active and is shown herein to associate more avidly with TFIIB compared with the 427-amino acid f/M1 isoform. Given the central role of 1,25-$(OH)_2D_3$ in calcium and mineral homeostasis, the varying potency of interaction between polymorphic hVDRs and components of the basal transcriptional machinery is likely one of several factors contributing to a genetic predisposition to osteoporosis.

MATERIALS AND METHODS

Construction of Mutant hVDR Plasmids

The hVDR expression vector, pSG5-hVDR (59), was employed in synthesizing point mutants by *in vitro* site-directed mutagenesis. Alteration of specific residues, deletions, as well as truncations of hVDR, were created via the method outlined in the Chameleon Double-Stranded, Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA) employing double-stranded hVDR cDNA [which contains amino acids 4–427, designated M4; see Baker *et al.* (44)]. The mutations generated in M4 hVDR for this study include the following point mutations: R18A, R22A, and R22K and internal deletions Δ5–13, Δ4–23, Δ15–17, and Δ18–21. Truncations produced were Δ161–427 (which contains amino acids 4–160), Δ134–427 (amino acids 4–133), Δ115–427 (amino acids 4–114), Δ84–427 (amino acids 4–83), and Δ1–88 (amino acids 89–427). Finally, one insertional mutation was created using M4 hVDR (amino acids 4–427) cDNA to include all 427 residues of the f hVDR (designated M1 or full-length hVDR). M1 hVDR cDNA was then used to construct E2A, a point mutant with alanine in place of glutamic acid at position 2.

Transfection of Cultured Cells and Transcriptional Activation Assay

COS-7 monkey kidney epithelial cells (800,000 cells per 60-mm plate) were transfected with 0.1 μg of WT or mutant pSG5-hVDR expression plasmid (59) and 10 μg of a reporter plasmid ($[CT4]_4$-TKGH) containing four copies of the rat osteocalcin VDRE (16) inserted upstream of the viral thymidine kinase promoter-GH reporter gene (Nichols Institute Diagnostics, San Juan Capistrano, CA) by the calcium phosphate-DNA coprecipitation method as described previously (60). The pTZ18U plasmid was used as carrier DNA, and each transfection contained a constant amount of total DNA (20 μg). In TFIIB "rescue" experiments, an additional 0.2 μg of pSG5-human (h)TFIIB vector was cotransfected into the cells. Sixteen hours later, the transfected cells were washed, then refed in DMEM (Life Technologies, Gaithersburg, MD) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and various concentrations of 1,25-$(OH)_2D_3$ in ethanol vehicle. After 24 h of incubation at 37 C, the level of GH secreted into the culture medium was assessed by RIA using a commercial kit (Nichols Institute Diagnostics) according to the manufacturer's protocol. Treatment of HeLa cells was carried out in a similar manner except that they were transfected with 1.5 μg of pSG5-hVDR (or mutant) expression plasmid and, when indicated, 1.5 μg of pSG5-hTFIIB. These cells were cultured in MEM supplemented with 10% FBS and antibiotics. In some experiments a rat osteoblast-like osteosarcoma cell line, ROS 2/3 (61), which contains only 100 VDR molecules per cell (62, 63), was employed. The ROS 2/3 cells were transfected with 1.0 μg of WT or mutant pSG5-hVDR in the absence or presence of 1.0 μg pSG5-

hTFIIB. The cells were later washed and then refed in DMEM and Ham's F-12 (DMEM/F12, 1:1) supplemented with 10% FBS, 100 U/ml penicillin, 100 μg/ml streptomycin, and various concentrations of 1,25-$(OH)_2D_3$ in ethanol vehicle.

Preparation of Cellular Extracts and Immunoblotting

Transfected COS-7 cells (as described above) were lysed directly in loading buffer (2% SDS, 5% β-mercaptoethanol, 125 mM Tris-HCl, pH 6.8, and 20% glycerol), and 40 μg of cellular protein were run on 5–15% gradient SDS/polyacrylamide gels. After electrophoretic fractionation, proteins were electrotransferred to Immobilon-P membranes (Millipore Corp., Bedford, MA) using a Transblot apparatus (Bio-Rad Laboratories, Inc. Richmond, CA) in 25 mM Tris-HCl, pH 7.4, 192 mM glycine, 0.01% SDS, and 20% methanol. The membrane was then blocked by incubation for 3 h with 3% blotto (3% dry milk, 10 mM Tris-HCl, pH 7.5, 150 mM NaCl). Immunodetection of bound hVDR or hTFIIB proteins was then performed using the monoclonal anti-VDR antibody, 9A7γ (64) or an anti-TFIIB polyclonal antibody (SI-1; Santa Cruz Biotechnology, Inc., Santa Cruz, CA). After the first antibody treatment, the Immobilon-P membrane was washed and treated at room temperature for 1 h with goat antirat IgG conjugated to biotin followed by four 15-min washes. A 5-ml mixture of biotinylated alkaline phosphatase and neutravidin (Pierce Chemical Co., Rockford, IL; in a ratio of 1:4) was preincubated for 45 min at 22 C in 1% blotto. The mixture was diluted to 30 ml with 1% blotto and added to the membrane for a 2-h incubation with rocking at room temperature and then was washed four more times, followed by a fifth wash with biotin blot buffer (0.1 M Tris-HCl, pH 9.5, 0.1 M NaCl, 2 mM $MgCl_2$, 0.05% Triton X-100). Finally, the blot was exposed to color reagent containing 50 μg/ml of 5-bromo-4-chloro-3-indolyl-phosphate and 100 μg/ml of 4-nitroblue tetrazolium chloride. The color reaction was stopped by washing with distilled water.

Preparation of Cellular Extracts and Gel Mobility Shift Assays

The hVDR proteins used for gel mobility shift assays were obtained from whole-cell extracts of COS-7 cells transfected with WT or mutant pSG5-hVDR plasmids. The transfected cells were washed and then refed in DMEM supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin but in the absence of 1,25-$(OH)_2D_3$. After 24 h of incubation at 37 C, the cells were washed twice with PBS (136 mM NaCl, 26 mM KCl, 8 mM Na_2HPO_4, 1.5 mM KH_2PO_4, pH 7.2), and scraped into 200 μl of KETZD-0.3 buffer (10 mM Tris-HCl, pH 7.6, 1 mM EDTA, 0.3 mM $ZnCl_2$, 0.3 M KCl, 10% glycerol, 1 mM dithiothreitol, 0.1 mM phenylmethylsulfonyl fluoride, 15 μg/ml aprotinin, 1 μg/ml leupeptin, 1 μg/ml pepstatin A). After sonication, samples were centrifuged at 16,000 × *g* for 15 min at 4 C and the hVDR-containing supernatant was used in electrophoretic mobility shift assays as described previously (45, 65). Briefly, 5 μl of transfected COS-7 cell lysate were incubated with 10^{-7} M 1,25-$(OH)_2D_3$ in DNA-binding buffer [10 mM Tris-HCl, pH 7.6, 150 mM KCl, 1 mg/ml acetylated BSA, 50 μg/ml poly(deoxyinosinic-deoxycytidylic acid)] for 30 min at 22 C followed by the addition of 0.5 ng of ^{32}P-labeled rat osteocalcin VDRE (5′-AGCTGCACTGGGTGAATGAGGACATTACA-3′; half-sites comprising an imperfect direct repeat are *underlined*) and incubation for another 20 min. Electrophoresis and autoradiography conditions were as described elsewhere (66).

GST Coprecipitation and Coimmunoprecipitation Assays

Human transcription factor IIB (hTFIIB)-GST fusion protein was expressed from pGEX-2T-hTFIIB (49), and GST alone was expressed from pGEX-4T, both in *Escherichia coli* strain DH5α. The overexpressed proteins were coupled to glutathione Sepharose (1.0 μg protein/μl resin) according to the protocol of the manufacturer (Pharmacia Biotech, Uppsala, Sweden) and stored as a 50% slurry in KETZD-0.3 buffer (0.3 M KCl) containing 30% glycerol at −20 C. For the GST pull-down assays, the desired cDNAs for WT or mutant hVDRs or human RXRα were used to generate [^{35}S]methionine-labeled proteins, utilizing the TNT Coupled Reticulocyte Lysate kit, an *in vitro* transcription/translation system (Promega Corp., Madison, WI). The GST or GST-TFIIB Sepharose beads (20 μl each) were incubated in KETZD-0.15 buffer containing 0.1% Tween-20, 150 mM KCl, 1 mg/ml BSA, and the following protease inhibitors: aprotinin, leupeptin, pefabloc SC, and pepstatin (wash buffer) at 4 C for 1 h on a rocking platform. The desired ^{35}S-labeled protein(s) was then incubated with the beads in the absence or presence of 1,25-$(OH)_2D_3$ (10^{-6} M). Next, the unbound proteins were washed from the beads four times with 1 ml each of wash (KETZD-0.15) buffer. The bound proteins were extracted from the beads into 40 μl loading buffer, boiled for 3 min and separated by gradient (5–20%) SDS-PAGE, and visualized via autoradiography. The amount of extract analyzed as input was 5% of the amount used in the coprecipitation reactions. For coimmunoprecipitation assays, TFIIB and WT or mutant hVDRs were overexpressed in COS-7 cells (as described above), followed by preparation of cellular extracts in KETZD-0.3 buffer employing sonication. The lysates were incubated with 2 μg of anti-TFIIB polyclonal antibody and 25 μl of Protein A/G-Plus Agarose (Santa Cruz Biotechnology, Inc.) for 2 h at 4 C. The immunoprecipitates were then washed extensively in wash buffer and resuspended in 50 μl loading buffer, followed by immunodetection of TFIIB-bound VDRs (as described above).

Genotyping of Human Fibroblasts

DNA was prepared from cultured human fibroblasts (10^7 cells) using the QIAmp tissue kit (QIAGEN, Valencia, CA) according to the manufacturer's instructions. The isolated genomic DNA (500 ng) was dissolved in a total volume of 50 μl that also included 100 ng each of primer 2a and 2b (36), 5 μl of 10× buffer (Perkin-Elmer Corp., Norwalk, CT) with 1.5 mM $MgCl_2$ and 2.5 mM each of dATP, dCTP, dTTP, and dGTP and 0.25 ml *Taq* DNA polymerase (Roche Molecular Biochemicals, Indianapolis, IN). PCR conditions were 10 cycles at 94 C for 30 sec, 60 C for 60 sec (with −0.1 C/cycle) and 72 C for 60 sec. This was followed by 25 cycles at 94 C for 30 sec, 59 C for 60 sec, and 72 C for 60 sec. Approximately 200 ng of unpurified PCR product were then incubated with 1 μl *Fok*I enzyme (New England Biolabs, Inc., Beverly, MA) and 1 μl 10× buffer in a total volume of 10 μl for 1.5 h at 37 C. The digestion mixture was then electrophoresed on a 4% NuSieve (3:1) agarose gel in Tris-borate-EDTA buffer and analyzed as described previously (36).

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Address requests for reprints to: Mark R. Haussler, Ph.D., Department of Biochemistry, College of Medicine, University of Arizona, Tucson, Arizona 85724. E-mail: haussler@u.arizona.edu.

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