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Two Basic Amino Acids C-Terminal of the Proximal Box Specify Functional Binding of the Vitamin D Receptor to Its Rat Osteocalcin Deoxyribonucleic Acid-Responsive Element

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Nuclear hormone receptor-responsive element binding specificity has been reported to reside predominantly in the proximal box (P-box), three amino acids located in a DNA-recognition α-helix situated on the C-terminal side of the first zinc finger. To further define the residues in the vitamin D receptor (VDR) DNA binding domain (DBD) that mediate its interaction as a retinoid X receptor (RXR) heterodimer with the rat osteocalcin vitamin D-responsive element (VDRE), chimeric receptors were created in which the core DBD of VDR was replaced with that of the homodimerizing glucocorticoid receptor (GR). Systematic alteration of GR DBD amino acids in these chimeras to VDR DBD residues identified arg-49 and lys-53, just C-terminal of the P-box within the base recognition α-helix of human VDR (hVDR), as the only two amino acids among 36 differences required to convert the GR core zinc finger domain to that of the VDR. Gel mobility shift and 1,25-dihydroxyvitamin D_3-stimulated transcription assays verified that an hVDR-GR DBD chimera is functional on the rat osteocalcin VDRE with only the conservative change of lys-49 to arg, and of the negatively charged glu-53 to a basic amino acid (lys or arg). Thus, for RXR heterodimerizing receptors like VDR, the P-box requires redefinition and expansion to include a DNA specificity element corresponding to arg-49 and lys-53 of hVDR. Examination of DNA specificity element amino acids in other nuclear receptors in terms of conservation and base contact in cocrystal structures supports the conclusion that these residues are crucial for selective DNA recognition. (*Endocrinology* 144: 5065–5080, 2003)

VITAMIN D IS KNOWN to be metabolized, primarily in the kidney, to its active hormonal form, 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2D_3$). The 1,25$(OH)_2D_3$ hormone functions as a nuclear receptor ligand, entering the nucleus of vitamin D target cells and binding to the vitamin D receptor (VDR) (1). Activated VDR then recruits retinoid X receptor (RXR) into a heterodimeric complex that establishes a high affinity interaction with vitamin D-responsive elements (VDREs), consisting of a tandem (direct) repeat of two hexad sequences separated by a spacer of three base pairs (DR3), in or near the promoters of vitamin D-regulated genes (2, 3). Analogous to the molecular signal transduction of other nuclear receptors, liganded VDR-RXR binds to coactivators with histone acetyl transferase (HAT) activity, such as steroid receptor coactivator-1 (SRC-1), that remodel and derepress chromatin in the vicinity of the VDRE (4). Subsequently, via contact with VDR-interacting proteins that are present in the multimeric mediator which anchors RNA polymerase II (5), and by delivering basal transcription factor IIB (TFIIB) to the preinitiation complex (6), liganded VDR-RXR stimulates the transcription of vitamin D target genes such as those encoding the bone remodeling proteins, osteocalcin and osteopontin, as well as a 24-hydroxylase (CYP24) that detoxifies the 1,25$(OH)_2D_3$ hormone (1). Based upon ablation of VDR in mice, besides regulating bone remodeling genes and vitamin D hormone catabolism, VDR also has major biological actions via gene expression/repression to control intestinal calcium absorption, epithelial cell differentiation (especially in skin), and the hair cycle (7, 8).

The purpose of the present study was to determine which residues in the human VDR (hVDR) mediate specific VDRE recognition, employing the rat osteocalcin (rOC) DR3 VDRE as a DNA platform, because the promoter for this gene unequivocally attracts the liganded VDR-RXR heterodimer in a biologically relevant fashion (2, 3, 9). With respect to DNA binding, most nuclear receptors sort into two major groups: 1) those that heterodimerize with RXR, including VDR, the thyroid hormone receptor (TR), the *all-trans*-retinoic acid receptor (RAR), as well as many others, and 2) the classic

Abbreviations: AR, Androgen receptor; CTE, C-terminal extension; CYP24, vitamin D 24-hydroxylase; DBD, DNA binding domain; D-box, distal zinc finger dimerization residues; DR3/4, direct repeat with a 3/4-bp spacer; ER, estrogen receptor; ERE, estrogen-responsive element; GR, glucocorticoid receptor; GRE, glucocorticoid-responsive element; GSV, gly-ser-val; HAT, histone acetyl transferase; hGH, human GH; HRE, hormone-responsive element; hTRβ, human TRβ; hVDR, human VDR; IR3, inverted repeat with a spacer of three base pairs; LBD, ligand binding domain; mGR, mouse GR; mOP, mouse osteopontin; MR, mineralocorticoid receptor; NR, nuclear receptor; 1,25$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; P-box, proximal box; PPAR, peroxisome proliferator-activated receptor; PR, progesterone receptor; PXR, pregnane X receptor; RAR, retinoic acid receptor; rOC, rat osteocalcin; RXR, retinoid X receptor; S-box, DNA specificity element; SRC-1, steroid receptor coactivator-1; TFIIB, transcription factor IIB; TR, thyroid hormone receptor; TRE, thyroid hormone responsive element; VDR, vitamin D receptor; VDRE, vitamin D-responsive element; VGV, VDR chimera with GR DBD.

steroid hormone receptors that homodimerize, such as the glucocorticoid receptor (GR), the estrogen receptor (ER), the androgen receptor (AR), *etc.* (10). A second generalization is that homodimerizing receptors bind to inverted repeat responsive elements, whereas heterodimerizing receptors bind to direct repeat-responsive elements with various spacers (10). Finally, the generic hexad DNA half-elements recognized by nuclear receptors fall into two primary categories, AGGTCA and AGAACA, in which the central two bases differ and facilitate selective responsive element binding by the receptors. The above outlined differences, plus numerous minor variations within each hexad sequence, create a large repertoire of responsive element platforms of variable specificity and strengths for the binding of nuclear hormone receptors to the genes they control.

With respect to the DNA binding specificity of the classic steroid hormone receptors, an illuminating set of experiments by Danielsen *et al.* (11), Mader *et al.* (12), and Umesono and Evans (13) revealed that, in the case of GR, altering just three residues on the C-terminal side of the proximal zinc finger, *i.e.* the proximal box (P-box), to those of ER not only eliminates glucocorticoid-responsive element (GRE) binding, but also confers GR with functional estrogen-responsive element (ERE) binding capability. Both GR and ER are homodimerizing receptors, and their generic responsive elements are inverted repeats with a spacer of three base pairs (IR3s) of AGAACA and AGGTCA, respectively. Because the P-box residues in GR are gly-ser-val (GSV), whereas those in ER are glu-gly-ala, it has become generally accepted that receptors with a GSV-like P-box interact specifically with an AGAACA half-element in DNA, and those with an glu-gly-ala-like P-box interact specifically with an AGGTCA half-element. This concept received further verification when DNA-binding domain (DBD) fragments of GR (14), ER (15), and TR-RXR (16) were cocrystallized with their canonical DNA-responsive elements. It was then revealed that P-box residues such as the val in GR, as well as the glu in ER and TR, indeed make direct base contacts with unique core residues in the respective AGAACA and AGGTCA half-elements.

As stated above, P-box exchange is capable of transforming a GR DBD into an ER DBD, and *vice versa*. In fact, entire DBD swaps are possible between the homodimerizing GR and ER, apparently because an important dimerization domain is also contained in the core DBD of these receptors, residing on the N-terminal side of the distal zinc finger (D-box) (11–13). However, DBD swaps between homodimerizing receptors such as GR or ER, and heterodimerizing receptors such as TR or VDR, are more difficult to interpret (17), likely because the heterodimerizing receptors do not use a D-box, and instead possess a more diffuse set of interaction sites in their DBD that contact the distal zinc finger in the tandemly oriented RXR heteropartner (16). As one example, Thompson and Evans (17) exchanged the core DBDs between GR and TR, showing that TR with a GR DBD was only about 10% as active transcriptionally as wild-type GR, yet GR with a TR DBD inserted was paradoxically almost 4-fold more active than wild-type TR. However, Thompson and Evans (17) used only inverted repeat elements as DNA platforms for their investigation, which likely continued to favor homodimerization of the chimeric receptor. TR has since been demonstrated to act primarily, if not exclusively, from a direct repeat with a spacer of 4 bp (DR4) element as a heterodimer with RXR (18).

Moreover, unlike in the GR- and ER-DNA cocrystals (14, 15), the C-terminal extension (CTE) beyond the core zinc fingers of TR makes extensive phosphate backbone contacts with DNA in the TR-RXR-thyroid hormone responsive element (TRE) cocrystal (16). This latter observation would seem to dictate that DBD swaps between heterodimerizing receptors such as TR and homodimerizing receptors include an appropriate number of CTE amino acids. Accordingly, Miyamoto *et al*. (19) recently showed that the VDR DBD core was inactive on DR3 hormone-responsive elements (HREs) when it was fused to the TR hinge (including the CTE) and ligand binding domain (LBD). Indeed, by mutational analysis we (20) found that three clusters of charged residues in the hVDR CTE were required for VDR-RXR binding to the rOC VDRE, and the pattern of these clusters in hVDR differs from the location of three clusters of positively charged residues in the TR CTE that are known to contact DNA. Therefore, precisely defining the P-box and other DBD residues crucial for HRE binding in the RXR heterodimerizing nuclear receptors has been difficult except where heterodimeric receptor-DNA cocrystals can be generated, as in the case of TR-RXR.

Adding to this uncertainty, we (21) have shown previously that VDR retains full activity in driving transcription from the rOC VDRE when its P-box (glu-gly-gly) is changed to that of GR (gly-ser-val). This suggests, as has been documented for TR using systematic point mutation of individual P-box residues (22), that RXR heterodimerization relieves some of the constraints on the compatibility of P-box sequences with DNA binding. Therefore, we sought to identify residues in the VDR DBD outside the P-box that are crucial specificity determinants for this receptor in binding as a heterodimer with RXR on the rOC VDRE. The strategy used in the current experiments was to construct VDR chimeras that contained the GR core zinc finger DBD but retained the VDR N terminus, CTE and LBD (denoted VGV chimeric receptors). We then deciphered which alterations were required in the core GR DBD of these chimeras to render them capable of driving transcription from a VDRE in response to $1,25(OH)_2D_3$.

Materials and Methods

Plasmid constructions and site-directed mutagenesis

Cloned cDNAs encoding hVDR (23), and mouse GR (mGR) (24) were subcloned into the expression plasmid pSG5 (25) as described previously (26). hVDR/mouse GR chimeric receptors were constructed by creating unique *Xho*I restriction enzyme recognition sites flanking the core regions of the DNA binding domains of each receptor [at amino acid residues 427 and 489 for mGR (2), and at residues 11 and 80 for hVDR (27)]. The DBDs were then exchanged by standard cloning techniques. Alteration of amino acid residues in hVDR/mGR chimeric receptors or full-length wild-type hVDR, mGR and human TRβ (hTRβ) was accomplished using a QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the protocol of the manufacturer. All receptor mutants were confirmed by DNA sequencing.

HRE-reporter plasmids were constructed using oligonucleotides containing the rOC DR3 VDRE [generating $(CT4)_4$TKGH, which contains four copies of the VDRE] (28), the rat tyrosine amino transferase IR3 GRE (5′-TATCCTGTACAGGATGTTCTAGCT-3′, with IR3 *underlined*) (29),

or the rat α-myosin heavy chain DR4 TRE (30), which were separately cloned into the unique *Hin*dIII site of the pTKGH reporter plasmid. Each of these constructs therefore includes a herpes simplex virus thymidine kinase promoter directing basal transcription of a human GH (hGH) reporter gene.

Cotransfection of COS-7 cells, transcription assays, and cell fractionation/immunoblotting

Transcriptional activity was monitored in transfected COS-7 cells. These cells were cotransfected by the calcium-phosphate DNA coprecipitation method with 2–5 μg/plate of the various pSG5 expression plasmids (26) including full-length, wild-type, and mutant hVDR, mGR, hTRβ (31), or mGR/hVDR chimeras, along with pTZ18U as carrier DNA, and the appropriate HRE TKGH reporter plasmid (see above). Cells were treated for 24 h after transfection with either 10 nM $1,25(OH)_2D_3$, 1 μM dexamethasone, or 10 nM T_3. Culture media were assayed by RIA for the expression of hGH using a kit from Nichols Institute Diagnostics (San Juan Capistrano, CA). To prepare whole cell extracts, cells were scraped, washed three times with PBS [136 mM NaCl, 26 mM KCl, 8 mM Na_2HPO_4, and 1.5 mM KH_2PO_4 (pH 7.2)], and suspended in KETD-0.3 buffer [10 mM Tris-HCl (pH 7.6), 1 mM EDTA, 300 mM KCl, 10% glycerol, 1 mM dithiothreitol], supplemented with 200 μg/ml pefabloc SC, 15 μl/ml aprotinin, 1 μg/ml leupeptin, and 1 μg/ml pepstatin-A (Roche Molecular Biochemicals, Indianapolis, IN). After sonication, samples were centrifuged at 16,000 × *g* for 15 min at 4 C. Protein concentrations were determined according to the method of Bradford (32). VDR immunoblotting of cell extracts was performed as described previously (20). When cells were fractionated to immunoassay VDR separately in cytosol and nuclear extracts, the subcellular isolation procedure was carried out as described elsewhere (33).

Gel mobility shift assay

The synthetic oligonucleotide CT5, 5′agctGCACTGGGTGAATGAGGACATTACA-3′, containing the VDRE sequence from the rOC gene (28), was used as the DNA probe; the direct hexanucleotide repeat (DR3) is *underlined* and the four-base overhang is shown in lower case. This oligonucleotide and its antisense complement (also including a four-base 5′ overhang) were annealed and labeled with [α-^{32}P]deoxy-CTP (3000 Ci/mmol) at the 5′-overhanging ends with Klenow fragment of DNA polymerase I to a specific activity more than 10^8 cpm/μg DNA. The hVDR mutants used in the gel mobility shift assay were obtained from whole cell extracts of COS-7 cells transfected with wild-type or mutant pSG5hVDR plasmids. The whole cell extracts were prepared as described above. *Escherichia coli*-expressed human RXRα (34) was preincubated with hVDR-containing COS-7 cell extracts in DNA binding buffer [10 mM Tris-HCl (pH 7.6), 150 mM KCl, 0.05 μg/μl BSA, and 0.025 μg/μl poly (deoxyinosine-deoxycytidine)] for 15 min at 22 C and then incubated with 0.5 ng of ^{32}P-labeled probe for an additional 15 min. The reaction mixtures were loaded onto 4% nondenaturing polyacrylamide gels containing 22.5 mM Tris-borate (pH 7.2), and 0.5 mM EDTA. Gels were run at 10 mA for 70 min, dried, and exposed for autoradiography. TR gel shift assays were performed analogously, employing extracts from COS-7 cells transfected with pSG5hTRβ along with a rat α-myosin heavy chain gene TRE DR4 as the labeled probe (5′-agctTGGCTCTGGAGGTGACAGGAGGACAGCA-3′, with the hexanucleotide repeats (DR4) *underlined* and the 5′ overhang in *lower case*).

Protein synthesis via in vitro transcription/translation

Synthesis of wild-type or mutant hVDR, hTRβ, or mGR was performed from the T7 promoter in each pSG5 receptor construct using a TNT coupled reticulocyte lysate system kit (Promega, Madison, WI) according to the protocol of the manufacturer. Briefly, master mix was first prepared with the following components: 12 μl of TNT buffer; 6 μl of amino acid mix; 6 μl of RNAsin; 3 μl of pefabloc SC; 3 μl of protease inhibitors (leupeptin/aprotinin); 13.8 μl of double-distilled water; 24 μl of ^{35}S-methionine (1200 Ci/mmol at 10 mCi/ml, NEN Life Science Products, Boston, MA); 10.2 μl of T7-polymerase; and 174 μl of rabbit reticulocyte lysate. One microgram of wild-type, mutant pSG5 receptor expression plasmid, or positive control luciferase vector was added directly to the TNT master mix and incubated in a 50-μl reaction for 1.5 h at 30 C. The final translation products were analyzed on a 10% sodium dodecyl sulfate/polyacrylamide gel. After electrophoresis, gels were fixed, dried, and exposed to Kodak XAR film at −70 C.

Results

Construction and transactivation properties of GR-VDR chimeric receptors

Data accumulated for the nuclear receptors indicate that their functional domains, such as the DBD and LBD, are modular in nature, and can be transferred to other proteins (35). To elucidate which amino acids in hVDR impart specific VDRE binding, chimeric receptors were generated in which the core DBDs of mGR and hVDR were exchanged, and then systematic mutagenesis was performed. Initially, two unique *Xho*I sites were introduced flanking the sequences encoding the core, zinc finger DBDs of hVDR and mGR, and the core DBDs were swapped to create G^XV^XG and V^XG^XV chimeric receptors. The chimeric receptors are named by letters referring to the origins of the N-terminal domain, DBD, and C-terminal LBD, respectively; for example, the V^XG^XV chimeric receptor has the N- and C-terminal portions of hVDR sandwiching the DBD of the mGR. The amino acid residue numbers shown in Fig. 1 indicate the location of *Xho*I sites (designated as x superscripts in the chimera nomenclature) that were engineered into mGR and hVDR to facilitate the exchange of core DBDs. As detailed in the legend to Fig. 1, when exchanged, the 70-amino acid VDR DBD and the 63-amino acid GR DBD create some degree of sequence variation flanking the core zinc fingers, which in addition to the inserted *Xho*I sites, must be considered when testing chimeric activity.

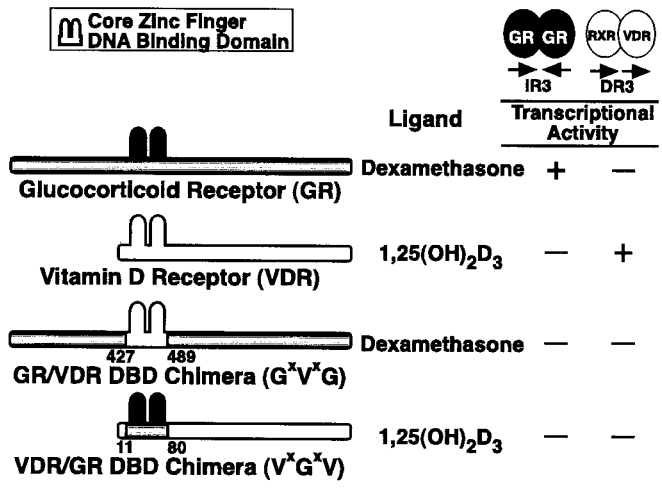

FIG. 1. Schematic structures and activities of vitamin D/glucocorticoid (V/G) receptor chimeras. Organization of receptor chimeras is shown on the *left*, and transactivation capacity in response to either VDR or GR ligands is summarized on the *right*. Note that the core DBDs varied in terms of the length of the retained N- and C-terminal extensions of the zinc fingers. The VDR DBD cassette contains an 11 amino acid N-terminal extension and only one amino acid C-terminal of the eighth cysteine. In contrast, the GR DBD cassette possesses no extension N-terminal of the first cysteine, but five amino acids C-terminal of the eighth cysteine. In data not shown, we also observed that the G^XV^XG chimera was nonfunctional on a VDRE arranged as an IR3 palindrome; likewise, the V^XG^XV chimera was inactive on a GRE arranged in a DR3 motif.

As summarized in Fig. 1, wild-type or chimeric receptors were transfected into COS-7 cells and were assayed for transcriptional activity using cotransfected VDRE-pTKGH and GRE-pTKGH reporter plasmids in the absence or presence of 10 nM $1,25(OH)_2D_3$ or 1 μM dexamethasone. As detailed in *Materials and Methods*, the VDRE used was a DR3 from the rOC gene, whereas the GRE (a typical IR3) was derived from the rat tyrosine aminotransferase gene (21). Whereas the native receptors were activated by cognate ligands to drive transcription from their respective responsive elements (Fig. 1, *right*), both chimeras were inactive in response to ligand on either HRE. Therefore, the core zinc finger regions are not exchangeable between VDR and GR, despite their generally similar structures, and the fact that the key P- and D-boxes from the GR can be swapped into VDR without loss of activity on the rOC VDRE (21). Apparently, other residues exist in the core DBD of VDR that differ from those in GR and discriminate DNA element binding to provide for VDR-VDRE specificity, assuming that minor alterations in sequences flanking the core zinc finger DBDs and the inclusion of engineered *Xho*I insertion sites are inconsequential.

Effects of progressive alteration of the GR core DBD in the VDR chimeric receptor, V^XG^XV, back to that of VDR

We next systematically repaired the DBD in the V^XG^XV chimeric receptor until essentially all the residues in the VDR DBD sequence were restored. Figure 2 illustrates in *red letters* the core DBD sequence of mGR, showing the location of the P-box and D-box. Those hVDR DBD residues that differ from those in mGR are depicted in black type adjacent to the mGR sequence; in total, there are 36 individual amino acid differences between mGR and hVDR in the region encompassing the nine conserved cysteines (Fig. 2, *top*). Beginning with the initial V^XG^XV chimera (Fig. 1), we first inserted 11 amino acids, PGDFDRNVPRI (Fig. 2, *lower left corner of upper panel*), which were lost from the hVDR immediately N-terminal of the first cysteine when the N-terminal *Xho*I cloning site was inserted to remove the VDR zinc finger cassette. This modified V^XG^XV chimera recreated the natural N terminus of hVDR, except for the presence of the *Xho*I site (generating codons for leu-glu) just N-terminal of this insertion in positions 11 and 12 of hVDR. Thus, for this and subsequent characterizations, hVDR residues 4–23 were normal in the V^XG^XV chimera except for positions 11 and 12, which constituted the residual leu-glu *Xho*I site instead of the native residues, which are pro-asp. Next, where the core mGR DBD differed in sequence from hVDR, it was progressively altered to the hVDR sequence in discrete units as depicted in the lower portion of Fig. 2, with the individual chimeras designated V^XG^XV1-VGV9. The units altered were: loop 1 (first Zn finger), P-box, M5 and M4 (between the Zn fingers), D-box, loop 2 (second Zn finger, altered in halves), and the C_8-C_9 region (conserved cysteines 8–9). In the latter five chimeras, one or both XhoI sites were repaired to the appropriate hVDR residues, as indicated by the absence of the x superscript (Fig. 2). Finally, in the last two chimeras (V^XGV8 and VGV9), the individual residue at position 49, which is a lysine in GR, was altered to an arginine (R49, circled in Fig. 2) to render it VDR-like.

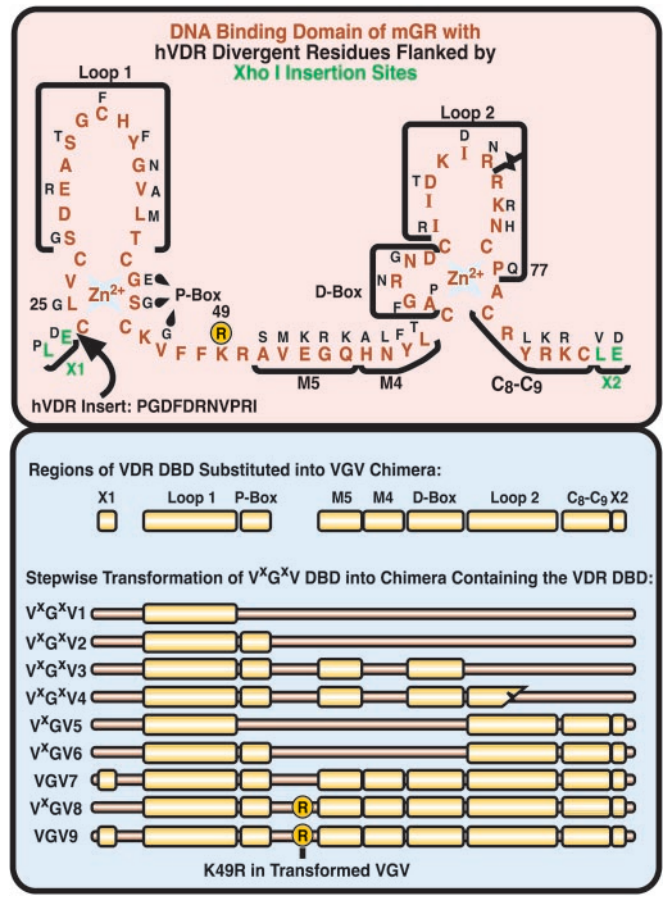

FIG. 2. Systematic alteration of the GR core DBD in the V^XG^XV chimeric receptor to the hVDR sequence. *Top panel*, Amino acid residues in the core zinc finger DBD are color coded as indicated at the *top* of the panel. Note that the hVDR sequence (in *black*, where it differs from mGR) contains an additional 11 residues at its N terminus, following the *Xho*I insertion site. These 11 hVDR amino acids were inserted first into the V^XG^XV1, and all subsequent chimeras. Brackets indicate receptor subdomain sequences that were substituted in various chimeras. *Lower panel*, A schematic diagram of subdomains that were altered in stages to transform the amino acid sequence of the mGR DBD to essentially that of the hVDR DBD. Thus, V^XG^XV1 is the V^XG^XV chimera, including both *Xho*I sites, in which the loop 1 subdomain has been altered to that of hVDR. In VGV9, at the other extreme, all subdomains, along with the K49 residue, have been changed to hVDR residues, regenerating the complete hVDR DBD sequence with the exception of a retained GR leucine at position 25.

Testing for transactivation capacity in response to $1,25(OH)_2D_3$ revealed that, of the first six modified V^XG^XV chimeras (Fig. 3A), only one (V^XGV5) displayed significant (approximately 25% of wild-type) transcriptional activity from the rOC VDRE. Surprisingly, this effect was lost in V^XGV6, which is V^XGV5 plus alteration of the P-box from that of GR to VDR. Nevertheless, there is a measurable gain in transactivation comparing V^XG^XV1 to V^XGV5, the latter of which contains additional hVDR sequences from the second half of loop 2, plus C_8–C_9. Thus, the second half of loop 2 plus the C-terminal α-helix traversing C_8 and C_9 apparently account for a portion of the specific DNA binding capacity of hVDR. The most notable difference between GR and VDR in this region is that GR possesses a proline, whereas VDR contains a glutamine, at position 77 (Fig. 2), which could

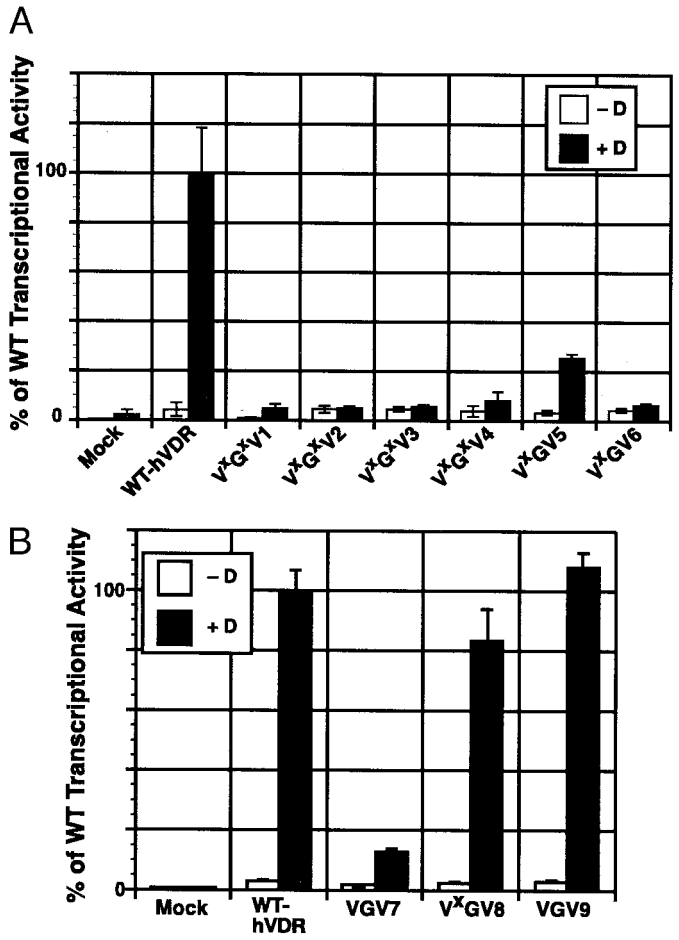

FIG. 3. Functional characterization of progressively transformed V^XG^XV mutants. A, Transcriptional activity of V^XG^XV1-V^XGV6 (see Fig. 2, *lower panel*, for a description of each chimera). COS-7 cells were cotransfected with a rOC VDRE-reporter plasmid $(CT4)_4TKGH$ (2 μg/plate), carrier pTZ18U DNA (23 μg/plate), and either the WT-pSG5hVDR or a mutant pSG5VGV expression vector (5 μg/plate). Transcriptional activation was quantitated using a growth hormone RIA, with the level of transcription by wild-type hVDR in the presence of 10 nM $1,25(OH)_2D_3$ (+D) arbitrarily set at 100%. All assays were performed in triplicate, and *error bars* represent ± SD; these results are representative of three independent experiments. Western blotting indicated that all mutant chimeras were expressed normally (data not shown), confirming that lack of activity was not caused by poor expression or protein instability. B, Transcriptional activity of VGV7-VGV9 (see Fig. 2, *lower panel*, for a description of these mutants). Transcription assays were performed and analyzed as described in (A).

generate alterations in the secondary structure. Inspection of hVDR *vs.* mGR revealed four remaining areas of sequence divergence that were not transformed in mutants V^XG^XV1-V^XGV6: the N-terminal *Xho*I cloning site, G25 (which is a leucine in GR), R49 (which is a conservatively replaced lysine in GR), and the M4 region. An independent experiment (data not shown) indicated that G25 in hVDR can be mutated to leucine without significant loss of hVDR activity, so the other three areas were targeted in transformed mutants VGV7-VGV9. The results (Fig. 3B) indicated that repair of the N-terminal *Xho*I site, plus substituting hVDR residues in units M5, M4 and the D-box, allowed for the recovery of 15% of wild-type transcriptional activity (mutant VGV7). Alteration of lysine to arginine at position 49 without *Xho*I site repair (V^XGV8) restored activity to 83% of wild type (Fig. 3B). Finally, the entire suite of replacements (VGV9), which regenerates the complete hVDR DBD sequence except for G25, which remains a leucine, yielded a receptor with approximately 105% of the activity of wild-type hVDR. The full restoration of hVDR activity in chimera VGV9 thus validates the integrity of the cloning steps.

The dramatic recovery of chimera V^XGV8 provided the first direct evidence for the fundamental importance of R49 in the specific DNA-binding/transactivation function of hVDR. Other regions, like the M5-M4-D-box cluster, appear also to be significant for VDR-VDRE binding (27). Within this cluster, the D-box has been demonstrated as unimportant to VDR DNA binding in independent studies (21) and can be eliminated from consideration; M4 constitutes an unconserved unit in nuclear receptors and is therefore also unlikely to be of significance in responsive element binding. The remaining M5 residues in hVDR have previously been shown to form the core of a nuclear localization signal for VDR that functions in conjunction with RXR heterodimerization to facilitate nuclear and subnuclear targeting (33, 36, 37), and M5 also contains a phosphorylatable serine that regulates VDR binding to the VDRE (27). Therefore, progressive mutation of the VDR-GR DBD chimera plus previous results establish that there are three regions unique to hVDR that facilitate rOC VDRE binding: 1) R49; 2) M5, a nuclear localization domain that contains a phosphorylatable serine and the basic sequence KRK; and 3) the C-terminal half of the distal zinc finger (loop 2), including the DNA phosphate backbone-binding α-helix traversing the eighth and ninth conserved cysteines.

Further analysis of the functional significance of R49 in full-length hVDR

Because, as is illustrated in Fig. 4, all of the homodimerizing receptors [GR, RXR, ER, progesterone receptor (PR),

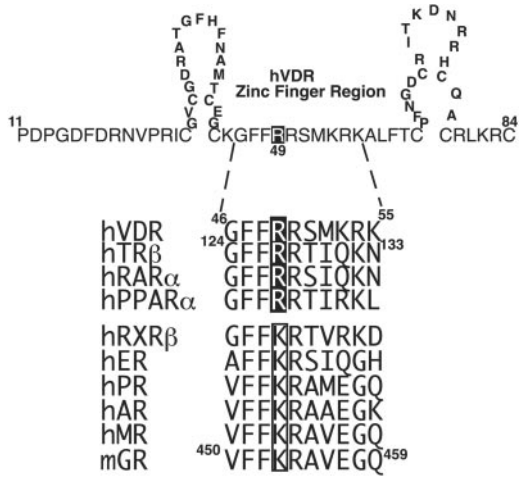

FIG. 4. Conservation pattern of hVDR R49. The residue corresponding to R49 in hVDR (*white letter on black background*) is positionally conserved in nuclear receptors that heterodimerize with RXR on direct repeat hormone-responsive elements (VDR, TR, RAR, PPAR) but is conservatively replaced with a lysine (*boxed*) in receptors that normally form homodimers on DNA, including the PR and MR.

AR, and mineralocorticoid receptor (MR)] possess a conservatively replaced lysine corresponding to R49 in hVDR and its subfamily members, it is conceivable that R49/K distinguishes, and perhaps helps to functionally segregate, the heterodimerizing from homodimerizing nuclear receptors. Considering its apparent pivotal role in VDRE recognition (Figs. 2 and 3B), and its striking conservation in RXR-heterodimerizing nuclear receptors (Fig. 4), R49 in hVDR was probed further. R49 in wild-type hVDR was mutated to various amino acids (D, P, L, S, and G) with uniform loss of hVDR transactivity (data not shown), and was conservatively replaced with a lysine, also with apparent total loss of transactivation (Fig. 5A) and VDRE binding (Fig. 5B) functions. This latter result is not surprising in light of the dramatic recovery of VDR activity in the V^XGV8 transformed chimera (Figs. 2 and 3B), in which position 49 in the DBD was changed from the lys of GR to the arg in VDR. Thus, the reverse experiment (Fig. 5), namely an R49K point mutation in hVDR, would be expected to produce an inactive receptor. However, unlike the D, P, L, S, and G substitutions for R49 in hVDR, which are stably expressed compared with wild-type receptor according to VDR immunoblot results (data not shown), R49K hVDR is completely proteolyzed to a 36-kDa fragment (Fig. 6A). A similar pattern of proteolysis of R49K hVDR, including 36- and 30-kDa fragments, occurs when the receptor is synthesized by *in vitro* transcription/translation (Fig. 6B). This degradation is not observed when either wild-type or R49G hVDR are synthesized under the same conditions (Fig. 6B). Western blotting results (Fig. 6A) also indicate that the 36-kDa immunoreactive R49K hVDR fragment partitions normally between cytosol and nucleus, suggesting that the DBD, CTE, and their nuclear localization signals (38)

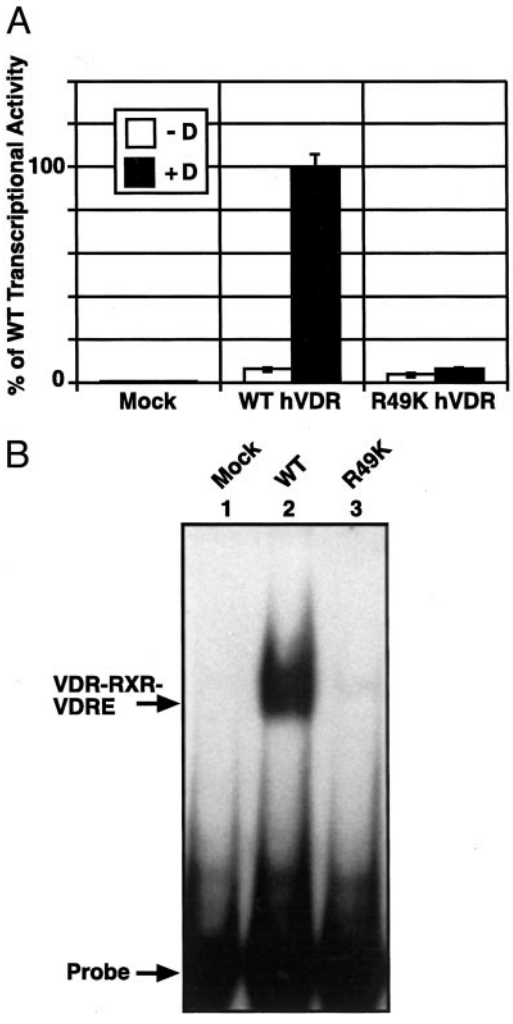

FIG. 5. Effect of alteration of hVDR R49 to lysine on receptor activity. A, Transactivation. Assays were performed as described in the legend to Fig. 3, ± 10 nM $1,25(OH)_2D_3$ (±D). B, Gel mobility shift analysis. Whole cell extracts (5 μg protein) from hVDR or mock transfected COS-7 cells were incubated with labeled VDRE probe in the presence of 50 ng of *Escherichia coli*-overexpressed human RXRα as described in *Materials and Methods*. Lane 1 represents an extract from mock transfected control cells, whereas lanes 2 and 3 contain extracts from cells transfected with wild-type and R49K hVDR, respectively.

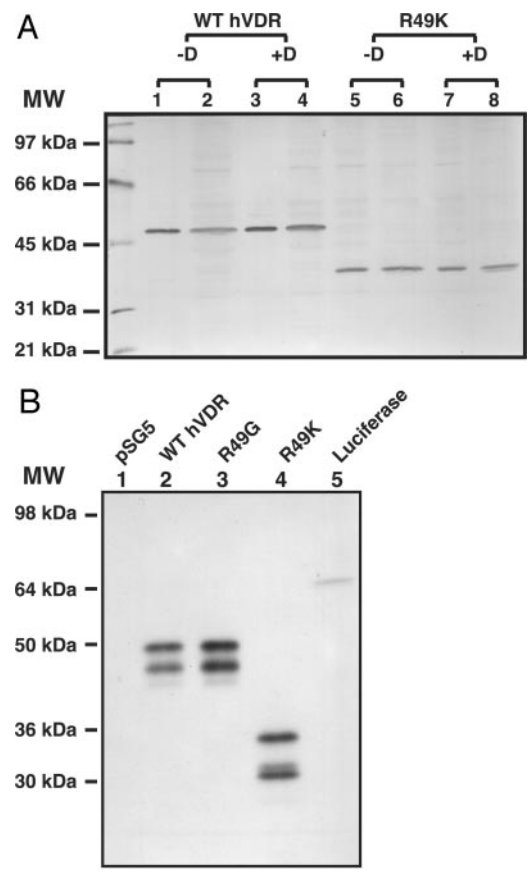

FIG. 6. Expression, subcellular distribution, and immunoreactivity of R49K mutant hVDR. A, Western blot of wild-type and R49K hVDR. Nuclear and cytoplasmic extracts from transfected COS-7 cells treated with ethanol vehicle (−D) or 10 nM $1,25(OH)_2D_3$ (+D) were prepared as described in a previous report (33). Sixty-microgram protein equivalents of cytosolic (lanes 1, 3, 5, and 7) or nuclear (lanes 2, 4, 6, and 8) extracts were loaded onto a 10% sodium dodecyl sulfate/polyacrylamide gel followed by electrophoresis and immunoblot analysis using a VDR-specific antibody, 9A7γ. B, Synthesis of R49G and R49K mutant, as well as wild-type, hVDRs employing an *in vitro* transcription/translation system. Coupled *in vitro* transcription/translation of hVDR from the T7 promoter in the pSG5hVDR vector (1 μg) was performed using a TNT rabbit reticulocyte lysate kit (Promega, Madison, WI) in the presence of [^{35}S]methionine as described in *Materials and Methods*.

remain intact, and that the mutated receptor protein is likely hydrolyzed in the C-terminal LBD between residues 200 and 300. Such proteolysis would eliminate hVDR ligand binding and RXR heterodimerization for VDRE binding (39), as well as remove the transactivation platform of helices-3 and -12 (40, 41). Thus, the artificial introduction of lysine at position 49 in hVDR generates a protease recognition site located distally in the LBD that precludes expression of active VDR. This influence of a specific lysine on stability is reminiscent of the human T cell lymphotrophic/leukemia virus type 1 $p12^{I}$ protein, in which a lysine variant at position 88 that is normally an arginine, causes ubiquitination and subsequent proteasomal degradation (42). Indeed, the proteasome inhibitor, MG-132, markedly retards R49K hVDR degradation (43). Although proteolysis could be used to explain the apparent lack of DNA-binding and transactivation with hVDR R49K (Fig. 5), the fact that other nonfunctional point mutations at hVDR position 49, such as R49G (Fig. 6B), are expressed normally and not proteolyzed, yet are still inactive, argues that the presence of a natural arginine residue at position 49 of hVDR is of fundamental importance to DNA binding.

To support the above conclusion, we generated the corresponding mutation in hTRβ, a nuclear receptor closely related to VDR both structurally and functionally. In hTRβ, R127 (which corresponds to hVDR R49) establishes direct contact via hydrogen bonds with the two GT core residues in the 3′ half-element of the TRE (16). Conservative replacement of R127 with a lysine in hTRβ eliminates its ability to transactivate (Fig. 7A) as well as to bind to the TRE as an RXR heterodimer (Fig. 7B), but in this case without rendering it susceptible to proteolysis (Fig. 7D). By analogy, this finding implies, but does not prove, that even if hVDR R49K were stable, it would still be unable to associate with the VDRE. Interestingly, carrying out the reverse experiment in mGR, namely mutating K453 to arg in the position corresponding to R49 in hVDR, produced a stable (Fig. 7D) and fully active receptor (Fig. 7C). This result is consistent with the fact that the rat GR residue equivalent to mGR amino acid K453 faces solvent rather than contacting a base in DNA in the GR-GR-GRE cocrystal (14).

Deduction of potential hVDR DNA contacts by comparison to GR- and TR-DNA cocrystal structures

Figure 8 depicts DNA contact residues in the TR-RXR-TRE and GR-GR-GRE cocrystals and compares the location of these sites within the primary sequences of the receptors. VDR is placed in the center of Fig. 8 to allow for inferences from the known x-ray structures. Except for the numerous DNA-interaction sites in the CTE of TR (note that the VDR CTE was retained in the VGV chimeras reported herein), there is a striking correspondence of positionally conserved (and presumably generic) DNA contact residues between TR and GR (indicated by *vertical blue boxes* in Fig. 8). Using hVDR as an index, the most prominent exceptions (illustrated as *orange vertical boxes* when unique to TR and a *green box* when unique to GR) include: 1) residues EG in the hVDR P-box, which are contacts in TR but not GR; 2) a DNA contact by the V in the GR P-box (the corresponding G in TR is not a contact); 3) R49 in hVDR that is a contact in TR but not GR as discussed above; 4) the $_{53}KR_{54}$ pair located in M5 of hVDR, which corresponds to contacts in TR but not GR; and 5) Q77 that is a contact in TR but not GR. We have already shown that alteration of hVDR P-box residues to those of GR can be tolerated without loss of activity on the rOC VDRE (21), but mutation of Q77 in hVDR chimera VGV9 (see Figs. 2 and 3B) to a proline (as in GR) causes a 90% loss in transactivation capacity (data not shown). Thus, Q77 is apparently of some significance in VDR DNA binding, although we cannot rule out a general disruptive effect of proline substitution on the DNA phosphate backbone binding α-helix in the distal zinc finger, which was determined to be important from the results in Fig. 3A.

What are the minimal residues that require alteration to convert the DNA binding specificity of a perfect GR DBD-VDR chimera to that of VDRE binding?

We next constructed a perfect (DBDs equivalently exchanged) GR DBD VDR chimera (VGV), containing the GR core DBD along with hVDR amino acids at the former two *Xho*I sites. With the knowledge gained from the results presented in Figs. 1–3 using the imperfect chimera, plus inferences from existing nuclear receptors (Figs. 4, 7, and 8), we proceeded to use the VGV chimera to discern the minimal number of amino acids required to restore VDR-like activity on the rOC VDRE. Despite the importance of R49 in hVDR documented above, the data in Fig. 9 reveal that altering only K49 to arginine in VGV is insufficient to restore either VGV binding to the rOC VDRE (Fig. 9B) or transactivation in response to $1,25(OH)_2D_3$ (Fig. 9C). However, when, in addition to K49R, E53, and G54 are mutated in VGV to the corresponding KR pair of amino acids in the M5 region of hVDR, significant VDRE binding is generated (Fig. 9B), and this VGV mutant attains a transactivity even greater than that of wild-type hVDR (Fig. 9C).

To further explore individual positions 53 and 54 in hVDR and to determine whether both basic residues are required for VDRE binding and transcriptional activation by $1,25(OH)_2D_3$, we next constructed a series of point mutants in the background of an R at position 49 in the perfect VGV chimera. As illustrated in Fig. 10A, the K49R/E53K/G54R triple mutant is markedly more active than wild-type hVDR when the rOC VDRE is used. Interestingly, only position 53 is crucial because, if in addition to the K49R change, glycine 54 is mutated to arginine (G54R) in VGV, the receptor is inactive, whereas altering only glutamic acid 53 to arginine (E53K) in the background of the K49R change allows the VGV mutant to acquire superactivity (Fig. 10A). Furthermore, residue 53, which is a negatively charged glutamic acid in GR, cannot simply be neutralized to yield VDR activity by replacing it with Q or G in the K49R altered VGV chimeric receptor (Fig. 10A), indicating that what is required for VDRE binding is not merely removing a negatively charged amino acid at position 53 in hVDR, but instead inserting a positively charged one such as lysine (Fig. 10A) or arginine (Fig. 10B). Finally, eliminating the negative charge at position

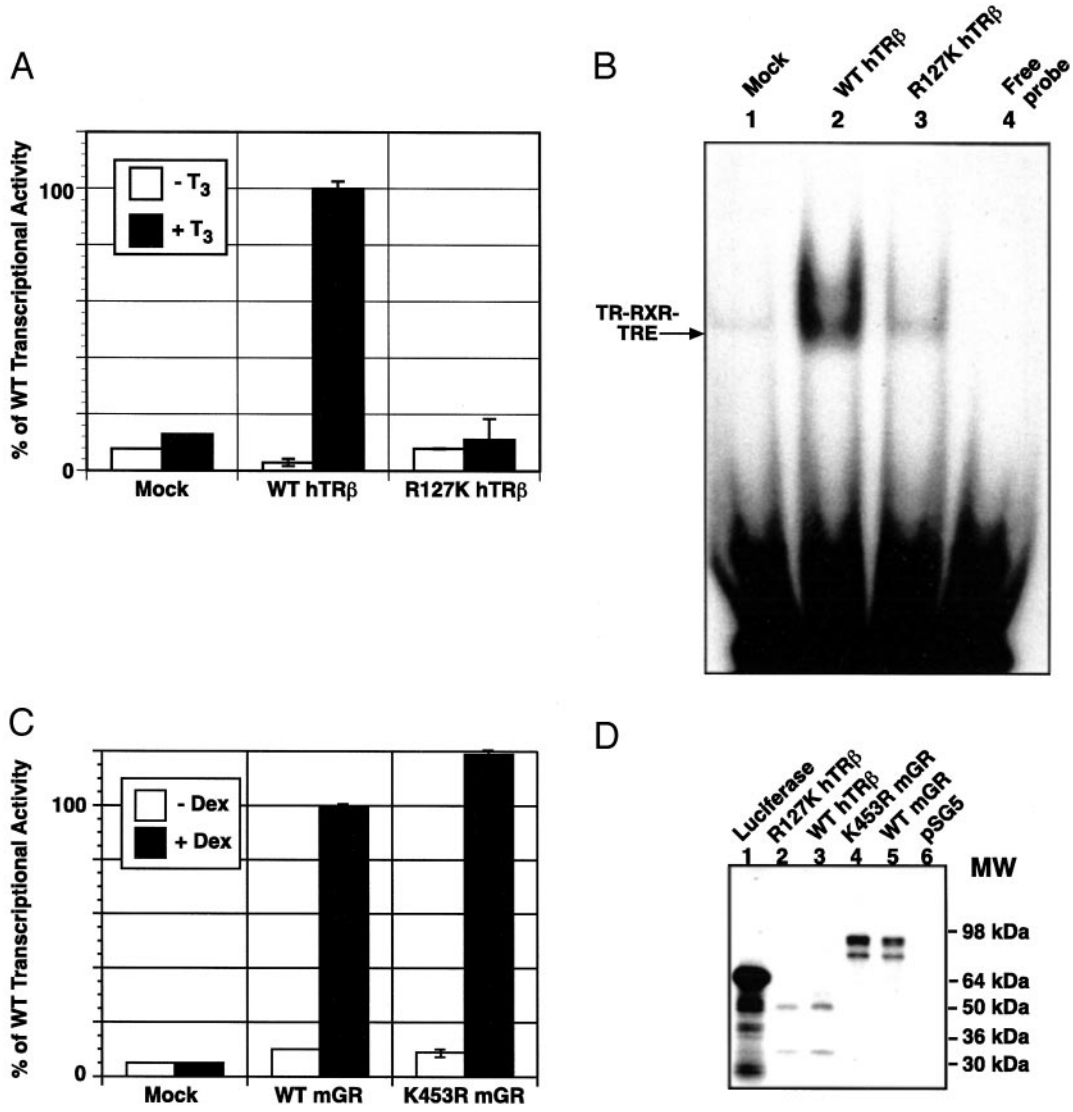

FIG. 7. Functional analysis of hTRβ and mGR mutated at the position corresponding to hVDR R49. A, Transcriptional activity of R127K hTR. COS-7 cells were cotransfected with a TRE-TKGH reporter (6 μg/plate) and expression plasmids for either wild-type hTRβ or the R127K mutant (2 μg/plate). Cultures were maintained in the absence or presence of 10 nM T_3 ($\pm T_3$), and media were assayed via RIA to determine the levels of the GH reporter. B, Assessment of the TRE binding ability of R127K hTRβ. Gel mobility shift analysis was performed as detailed in the legend to Fig. 5B, except that hTRβ wild-type and R127K mutant expression plasmids were employed, and the labeled TRE was the DR4 from the rat α-myosin heavy chain gene. C, Transcriptional activity of K453R mGR. COS-7 cells were cotransfected with a GRE-TKGH reporter (6 μg/plate) and expression plasmids for wild-type mGR or the K453R mutant (2 μg/plate). Cultures were maintained in the absence or presence of 1 μM dexamethasone ($\pm$Dex) and the media were monitored for the hGH reporter via RIA. D, Synthesis of R127K hTR and K453R mGR using an *in vitro* transcription/translation system. Coupled *in vitro* transcription/translation of hTRβ or mGR wild-type or mutants from the T7 promoter in the pSG5 vector (1 μg) was performed using a TNT rabbit reticulocyte lysate kit (Promega) in the presence of [^{35}S]methionine as described in *Materials and Methods*.

53, combined with introduction of a basic arginine at amino acid 54 (K49R/E53Q/G54R), does not compensate for the lack of a basic residue at amino acid 53 (Fig. 10B). The bottom line is that the core DBD of GR can be converted to that of VDR with respect to rOC VDRE activity by changing only two residues: 1) K49 to arginine (a conservative change) and 2) E53 to either lysine or arginine (a charge reversal). Therefore, we propose that residues 49 and 53 be referred to as the DNA specificity element (S-box) in VDR, a pair of residues that are required for, and complement the P-box in, VDRE recognition.

Examination of the P-box and S-box sequences in nuclear receptors

Figure 11 lists the sequences in the DNA base recognition α-helices of 13 nuclear receptors, categorized into RXR heterodimerizing, bifunctional, and homodimerizing members of the superfamily. There are two basic residues that are almost universally conserved in the nuclear receptor superfamily (*solid* and *open circles* in Fig. 11), K45 and R50 (numbered as in hVDR), that contact, respectively, the G in position 2 and the guanyl complement of the C in position 5 that

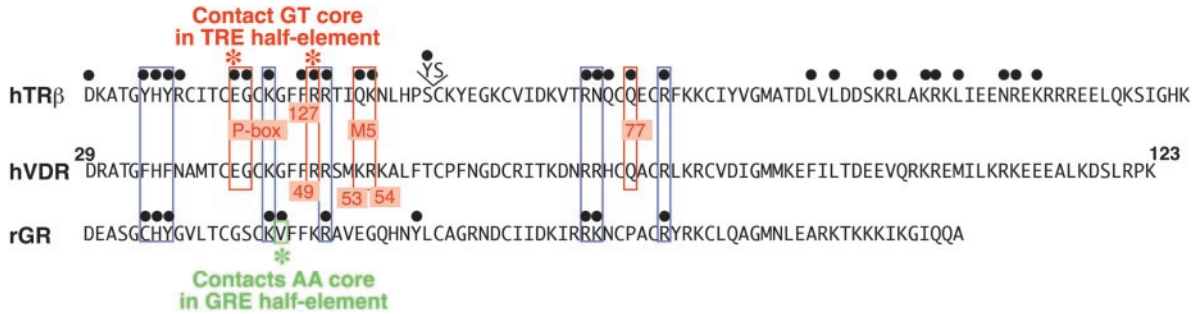

FIG. 8. Deduction of potential unique hVDR-VDRE DNA contacts by comparing the known amino acid-DNA contacts from the RXR-TR-TRE (*upper*) and GR-GR-GRE (*lower*) cocrystal x-ray structures. The region shown extends from the loop of the first zinc finger to the estimated C terminus of the CTE. DNA-interacting amino acids are designated with a solid circle above the residue. DNA contacts *boxed in blue* are general for the nuclear receptors (15, 16); they are expected to occur also in hVDR with respect to its interaction with the VDRE. The DNA contact *boxed in green* is unique to GR (15); note that mouse, rat, and human GRs have identical sequences in this region. DNA contacts *boxed in orange* include those unique to TR (16), and are likely candidates to occur also in VDR because of the structural and functional similarities between VDR and TR. The CTEs vary in length, and TR possesses numerous DNA interaction residues within its CTE. It is probable that analogous DNA contacts also exist in VDR (see text). *Asterisks* designate residues that contact core base pairs in the number 3 and 4 positions of the respective HREs.

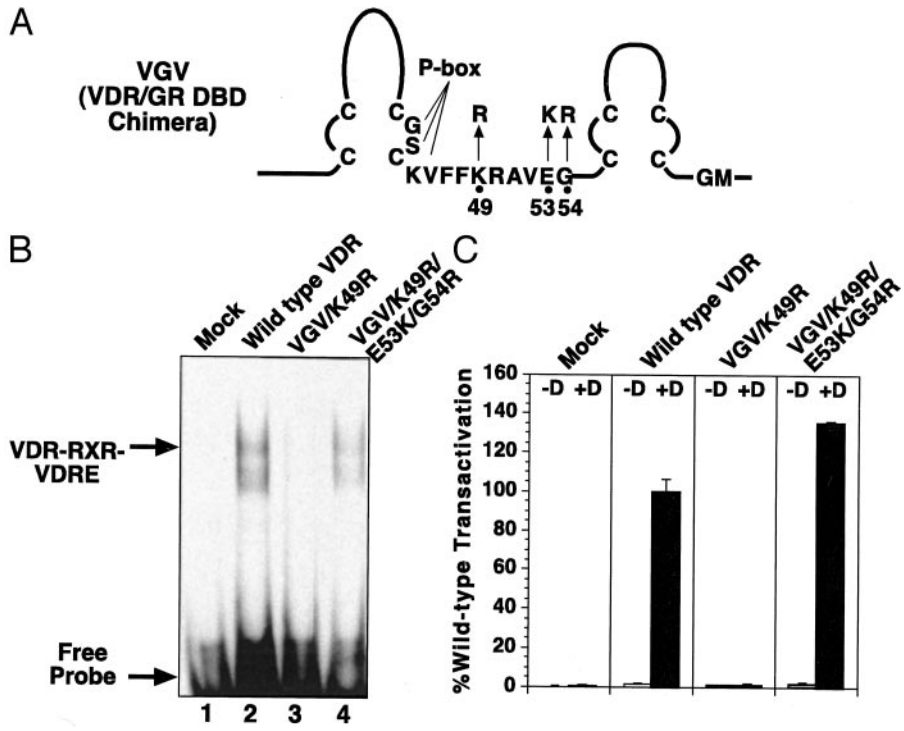

FIG. 9. Alteration of only three residues just C-terminal of the P-box restores transcriptional activity of the VGV chimera. A, Schematic representation of the central portion of the VGV chimera, showing the position of three residues between the two zinc fingers that were changed to hVDR amino acids. B, Gel mobility shift analysis, performed as described in the legend to Fig. 5B, using the rOC VDRE as a probe. Lanes 3 and 4 contain VGV chimeras with indicated single (K49R) and triple (K49R/E53K/G54R) alterations to the corresponding hVDR residues, respectively. C, Transcriptional activation assays using a rOC VDRE-reporter were carried out as detailed in the legend to Fig. 3. Receptors transfected are as in panel B, with the mock transfection receiving only the empty pSG5 vector. Absence or presence of 10 nM $1,25(OH)_2D_3$ is indicated across the *top* of each panel as −D and +D, respectively.

are generally present in all HRE half-elements. In contrast, the three P-box amino acids segregate according to the type of half-element recognized, specifically with the first residue in the glu-gly-gly/ala P-box interacting with the C complement of the G-C bp in position 3 of the TRE/ERE half-element (15, 16), and the third residue in the gly-ser-val P-box (14) contacting the T complement of the A-T bp in position 4 of the GRE half-element (see *triangles* in Fig. 11). Moreover, R49 in hVDR, the N-terminal residue in the S-box, is herein identified as being a pivotal amino acid dividing the RXR heterodimerizing nuclear receptors, for which this arginine is crucial to DNA binding, from the homodimerizing receptors that employ a lysine in this position (Fig. 11). Indeed, a survey of nuclear receptor sequences in GenBank revealed that the phe-phe-arg-arg cluster is conserved in all known group 1 nuclear receptors [NR1A throughout NR1K in the nuclear receptor nomenclature (44)], including evolutionarily ancient nematode proteins, as well as all known receptors that heterodimerize with RXR on direct repeat responsive elements. In contrast, nuclear receptor groups 2–6 (with the single exception of NR2A, the hepatocyte nuclear factor 4 orphan receptors), have the sequence phe-phe-lys-arg, with a lysine residue in the position corresponding to R49 in VDR, including receptors from organisms as ancient as coral polyps and jellyfish. None of these latter receptors form heterodimers with RXR, although several [*e.g.* TR2 (45) and *Drosophila* nuclear hormone receptor 78 (46)] have been reported to bind as homodimers to direct repeat elements. Note that RXR, which serves as a universal heteropartner, is also capable of homodimerizing on a DR1 element (and therefore

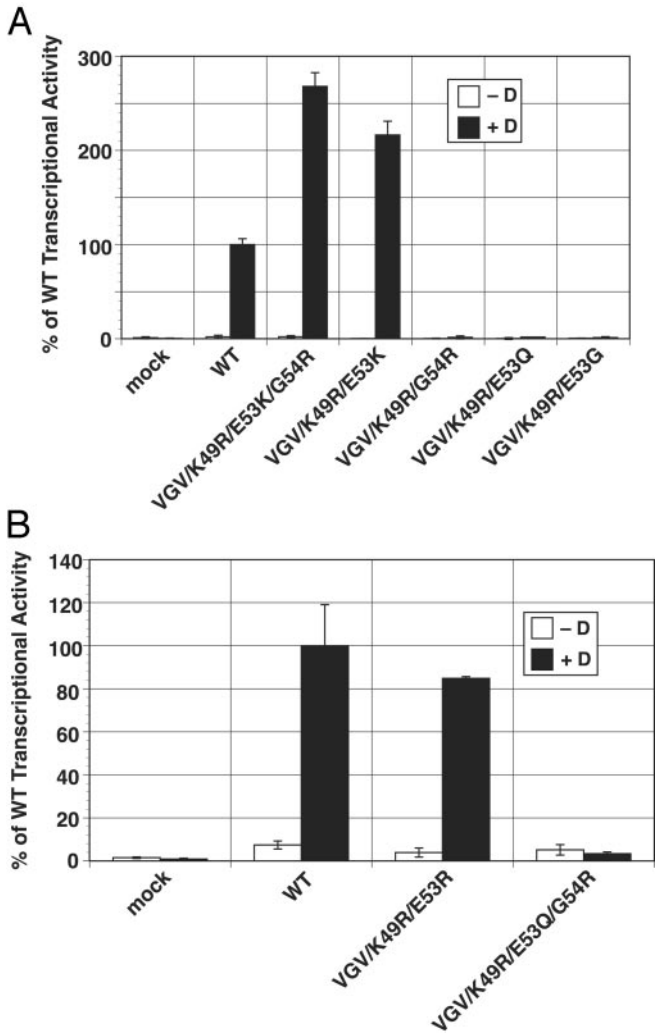

FIG. 10. Mutation of hVDR basic residues K53 and R54 in the background of an R at position 49 of the VGV chimera. A, Transactivation assay to determine whether K53 or R54 are individually required for hVDR activity, and if simple removal of a negative charge at position 53 (tested as E53Q and E53G) would activate the R49 VGV chimera. B, Transactivation assay to determine whether K53 in hVDR can be conservatively replaced with an arginine, and whether a basic residue (R) at position 54 can compensate for the absence of K53. All assays were carried out in triplicate, with *error bars* representing ± SD, and the results are representative of three independent experiments.

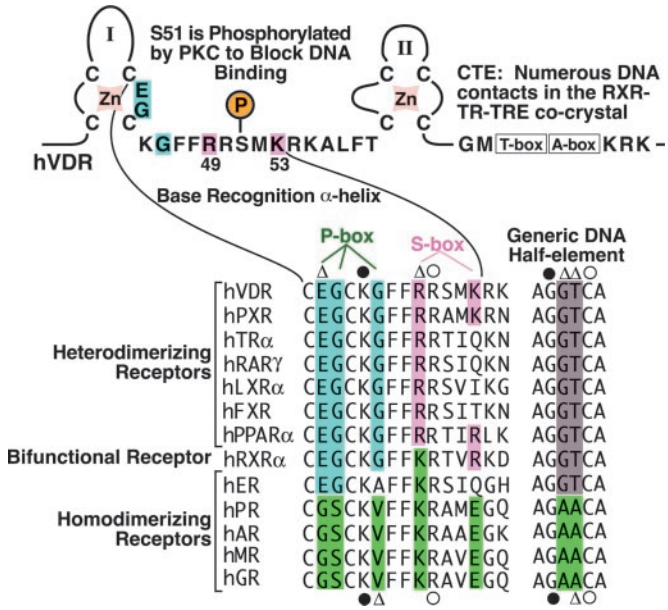

FIG. 11. Sequence comparison of the DNA base recognition α-helix in nuclear receptors: identification of a proposed S-box that contributes to DNA half-element specificity and distinguishes the heterodimerizing and homodimerizing receptors. The *top diagram* depicts the hVDR DBD, including the P-box (*blue*) and proposed S-box (*lavender*) residues within the DNA recognition α-helix. Also shown are the protein kinase C-phosphorylatable serine (residue 51) that blocks DNA binding of hVDR when phosphorylated (27), and the CTE that contains the T- and A-box sequences (20). Below the hVDR DBD schematic, residues in the P- and S-box regions are compared among the nuclear receptors, segregating the receptors into heterodimerizing and homodimerizing groups, with RXR being classified as bifunctional because it can both homodimerize and serve as a heterodimeric partner for other receptors. On the *lower right*, the corresponding generic half-elements are listed on which each receptor specifically docks when it binds to DNA. Only one DNA strand of the half-element is shown in each case, and amino acids in the receptors often contact bases on the complementary strand (not shown). Core bases (GT at positions 3 and 4) in the TRE/ERE-like half-element are highlighted with a *gray* background, and corresponding core residues (AA) in the GRE-like half-element are highlighted in *green*. Amino acids uniquely conserved in the homodimerizing receptors are also highlighted in *green*. ●, A universally conserved lysine and its base contact (position 2, a G) of the hexad half-element in DNA. ○, A universally conserved arginine and its base contact (position 5, the G complement of a C) in the hexad half-element in DNA. △, Distinguishing residues and their base contacts in the central core (positions 3 and 4) of the DNA half-element: *symbols above the sequence comparisons* designate E42 and R49 in the VDR subfamily and their contacts at positions 3 and 4 in the TRE/ERE-like AGGTCA half-element; *symbols below the sequence comparisons* designate a V residue in the GR subfamily P-box and its contact, which is the complementary base of the A at position 4 of the GRE half-element. Note that the hGR sequence in this region is identical to both the mGR sequence (employed experimentally herein) and the rGR (used in the GR x-ray crystallographic study cited in the text and in Fig. 12).

contains a lysine at the position equivalent to R49 in VDR), causing it to be classified as bifunctional (Fig. 11). Intriguingly, the C-terminal residue in the S-box, K53 in hVDR, is in stark contrast to the conserved glutamic acid present at that position in homodimerizing receptors that prefer the AGAACA half-element as a platform, but it is variable across the heterodimerizing and bifunctional receptors. Although this residue is never negatively charged (which would presumably cause repulsion of the DNA phosphate backbone), it ranges from a lysine in VDR and the pregnane X receptor (PXR), to an arginine in peroxisome proliferator-activated receptor (PPAR) and RXR, to a glutamine in TR, RAR, and ER, to a threonine in the farnesoid X receptor (FXR), and even to a nonpolar isoleucine in the liver X receptor (LXR) (Fig. 11).

K53 in hVDR lies at the C terminus of the base recognition α-helix, and this residue likely plays a significant part in VDRE association. The role of K53 in hVDR is probably one of phosphate backbone binding, which distinguishes it from the other S-box residue, corresponding to R49, which makes direct base contact with the GT core bases (16) in the TRE half-element (see *triangles* in Fig. 11).

Discussion

Site-directed mutagenesis of chimeric nuclear receptors was used in the current experiments to gain insight into the specific DNA recognition residues in the hVDR. Although there are 36 amino acid differences between the core zinc finger DBDs of GR and VDR, only two residues required alteration to convert the GR DBD into a VDR-like DBD capable of binding to, and mediating transactivation from, the rOC VDRE (Fig. 10). These data support the concept of structurally similar functional domains in nuclear receptors (47) and indicate that only a small number of evolutionarily changed amino acids can yield diverse bioactivity in this superfamily of transcription factors. Moreover, based upon x-ray crystallographic analysis of receptor fragment-responsive element cocrystals summarized in Fig. 12, there are only a limited number of residues in the DBDs of nuclear receptors that actually contact DNA. The average is approximately 14 per DBD, including only 10 contacts observed between the rat GR DBD and its half-site to as many as 29 contacts between the hTRβ DBD as part of a heterodimer with the RXR DBD on a DR4 element (16). The larger number of contacts by TR is mediated by a CTE of the DBD, which contains positively charged amino acids that interact with DNA just 5′ of the half-site hexamer (16, 48).

Several of these DNA contacts are found not only in most receptor-responsive element complexes studied to date, but also represent residues that are well conserved among all nuclear receptors that recognize DNA. These contacts, indicated in Fig. 12 with *white* or *black dots* above the sequences, include a histidine at position 35 (using hVDR numbering, see *bottom* of Fig. 12), an aromatic residue at position 36, a lysine at position 45, and arginines at positions 50, 73, and 80. Not surprisingly, these latter three basic residues mediate contacts to the negatively charged phosphate backbone of DNA. All of these residues exist within a conserved framework consisting of a portion of the loop of the first zinc finger along with two α-helices located on the C-terminal side of each finger. In addition to binding to a phosphate, the residue corresponding to R50 in hVDR also mediates direct contact with a base that is almost universally present in nuclear receptor-responsive elements, namely the complementary guanyl residue to the fifth cytidine base of the consensus half-sites AGGTCA or AGAACA. This contact is present in 18 of 19 receptor DBDs crystallized on DNA to date (Fig. 12). The base contact mediated by the lysine at position 45 (of hVDR) to the second guanidine is another feature of both consensus half-sites (AGGTCA or AGAACA); this contact is likewise found in 18 of the 19 crystallized DBD-DNA complexes. There are also two pervasive phosphate backbone contacts mediated by the arginines at positions 73 (18 of 19) and 80 (19 of 19). The residue at position 74, which can be either positively charged (arg or lys), or, in many other instances, is an asparagine (*e.g.* NGFI-B, RXR, RAR, or TR), also makes phosphate backbone contacts (18 of 19).

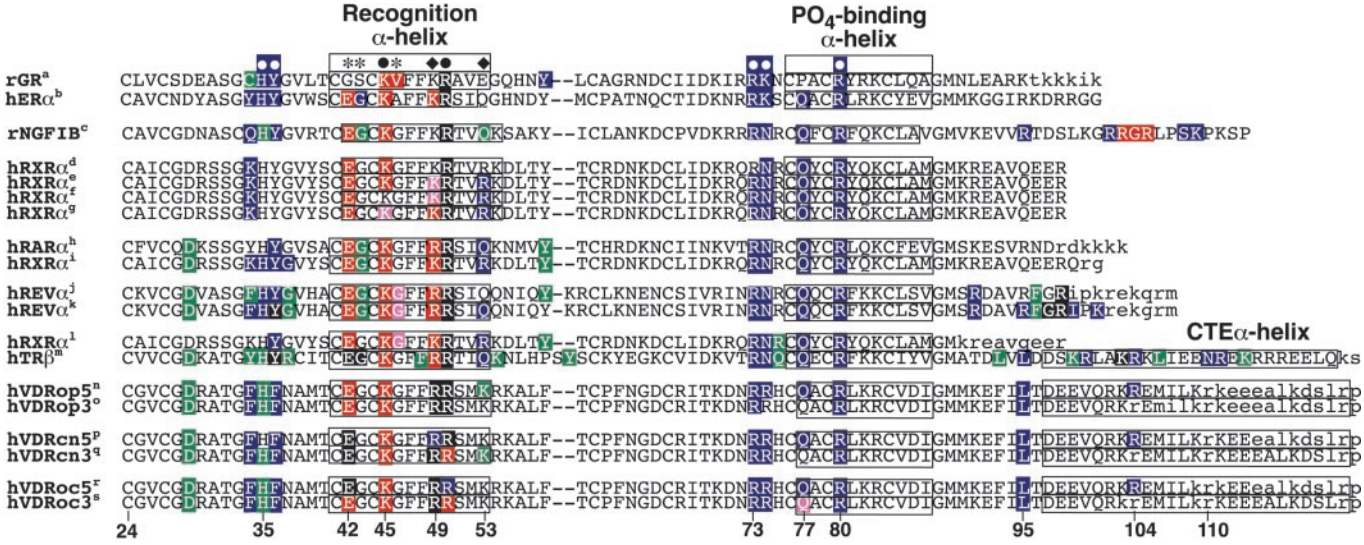

FIG. 12. Comparison of the DNA contact amino acids in the DBD fragments of nuclear receptors cocrystallized with HREs. Color highlighted residues constitute DNA contacts in the DBD, with all sequences aligned beginning with the N-terminal cysteine in the zinc finger region on the *left*. The color scheme for highlighted contacts is as follows: *black,* contacts both a base and the phosphate backbone; *red,* direct base contact; *purple,* indirect base contact via a water molecule; *blue,* direct phosphate contact; *green,* indirect phosphate contact via a water molecule. The three α-helices (recognition, phosphate binding, and CTE, where applicable) are *boxed*. The *symbols at the top* of the sequence are: *black dots,* universal base contacts; *white dots,* universal phosphate backbone contacts; *asterisks,* P-box residues; and *black diamonds,* S-box residues. The *numbering at the bottom* of the sequence corresponds to that of hVDR (23). The nuclear receptor DBDs listed were crystallized on DNA as follows: [a], rat GR on an artificial IR4 GRE as a homodimer (14); [b], human ERα on a consensus IR3 ERE as a homodimer (15); [c], rat NGFIB orphan receptor on an AAAAGGTCA element as a monomer (48); [d–g], four human RXRα DBDs lined up on a double consensus DR1 HRE separated by 2 bp (the DBD bound to the 5′-most half-element is listed first, and the DBD bound to the 3′-most half-element is listed last) (56); [h], human RARα on the 5′ half-element as a heterodimer with [i], human RXRα on the 3′ half-element of a DR1 HRE (65); [j and k], human RevErbAα as a homodimer on a consensus DR2 HRE (the DBD bound to the 5′ half-element is listed first) (57); [l], human RXRα on the 5′ half-element as a heterodimer with [m], hTRβ on the 3′ half-element of a consensus DR4 TRE (16); and [n–s], six hVDR DBDs on the mOP, a consensus DR3 (cn), or the rOC VDREs as homodimers (DBDs bound to the 5′ half-elements listed first in each case) (58). *Lower case letters* in the hVDR DBD CTE structures signify disordered amino acid side chains within the CTE α-helices (58).

A second category of contacts serves to differentiate the large group of receptors that usually binds AGGTCA-type elements from the more limited number that binds AGAACA half-sites [in addition to GR, only the PR, the MR and the AR, comprising group 3C of the unified nuclear receptor nomenclature (44)]. These contacts are mediated by residues in the P-box (13). As might be expected, these residues contact the central two base pairs that distinguish the half-sites of the two classes of receptors. Accordingly, the glutamate in the EGxxG motif contacts the complementary C of the third base in the AGGTCA half-element, whereas the valine in the GSxxV motif binds to the complementary T of the fourth base in the AGAACA half-element. Whereas the rat GR DBD is thus far the only DBD to be studied crystallographically on an AGAACA half-element, there are at present nine x-ray solutions of crystals containing AGGTCA or similar DNA half-sites, all of which are bound to receptors with an EGxxG/A P-box motif. In particular, a comparison of the rat GR DBD crystal (14) with that of the human ER DBD (15) lends strong support to the original conclusions of Umesono and Evans (13), that both P-boxes contribute important contacts to their respective cognate DNA half-elements.

Thus, it is somewhat of a surprise that VDR does not require the glutamate or either of the glycines in the EGxxG P-box motif for binding to the rOC VDRE (21). Indeed, a full-length hVDR in which the EGxxG residues have been mutated to the GSxxV GR P-box binds to, and transactivates from, the rOC VDRE even more efficiently than wild-type hVDR (21). This previously published result appears consistent with the present findings, in which the V^XGV5 chimera that still contains the GR P-box displays moderate transactivation ability, but the V^XGV6 chimera, in which the VDR P-box has been restored, does not (Fig. 3A). Part of the explanation for these results is evidently that the rOC VDRE 3′ half-site is AGGACA, with a complementary thymidine at the fourth position, exactly where the valine of the GR P-box makes its base contact (14). It is of note that, in addition to the rOC VDRE, three other natural VDREs (two of them negative elements) have a thymidine at this position of the 3′ half-element complementary strand (49–51).

The above outlined concept of the valine residue in the GR P-box favoring contact with a GRE-like T base complement to the fourth position of the rOC VDRE 3′ half-site also seems to explain why the VGV chimera with the K49R/E53K/G54R triple repair is superactive on the rOC VDRE relative to wild-type hVDR (Fig. 9C). However, the observation that the triply repaired VGV (which still has the GR P-box) is transcriptionally inactive on two other VDREs (data not shown), namely those from the mouse osteopontin (mOP) and the rat CYP3A23 genes, requires explanation. Unlike the 3′ half-element in the rOC VDRE, AGGACA, the 3′ half-element of the mOP VDRE is GGTTCA and the 3′ half-element of the CYP3A23 VDRE is very similar (AGTTCA). Thus, in both cases, the base complementary to the fourth position in the 3′ half-element to which the valine of the GR P-box would bind is no longer thymidine, but rather adenine. Previous mutagenesis experiments (52) have indicated that a valine in the third position in the P-box precludes binding of nuclear receptors to DNA half-sites with a T in the fourth position, apparently because they are unable to form a methyl group hydrophobic contact with the complementary adenine as normally occurs with thymine in the GR-GRE cocrystal (14). The basic conclusion is that the VDR P-box appears dispensable for binding to a subset of VDREs with an A, but not a T, in the fourth position of the 3′ half-site, at least when it is replaced with a GR-like P-box that possesses a compensatory valine in the third position. However, our observation (data not shown) that exchange of the VDR P-box into the VGV/R49K/E53K chimera destroys its activity on the rOC VDRE, but restores significant activity on the human CYP3A4 DR3 VDRE (53), which contains an AGTTCA 3′ half-element, suggests that both P- and S-boxes must be repaired in the VGV chimera for it to be functional on VDREs with a thymidine in the fourth position, as is the case with 5 of 11 established natural VDREs (1, 54, 55). Therefore, with the majority of VDREs characterized to date, a combination of the P- and S-boxes is required for specific DNA recognition.

The present results focus attention on proposed S-box hVDR residues R49 and K53 (indicated by *solid diamonds* at the top of Fig. 12) that are uniquely crucial for VDR to bind, and mediate transactivation from, the rOC VDRE. It is likely significant that R49 and K53 are part of the same hVDR DNA-recognition α-helix that also contains the P-box, but the fact that neither R49 nor K53 is completely conserved throughout the nuclear receptor superfamily is a preliminary indication that their roles may be receptor specific, or at least group specific. R49 is the better conserved of the two; as suggested in Figs. 4 and 12, this arginine is found in all group 1 receptors (VDR, TRs, RARs, and PPARs, but also RevErbs, RAR-related orphan receptors, and others), a large grouping that includes receptors which bind to DNA either as monomers or as heterodimers with RXR. RXR (in group 2) contains a lysine in this position, as do nearly all other nuclear receptors not in group 1. This list includes the classic steroid receptors, represented by GR and ER (group 3). Thus, the position corresponding to R49 in hVDR is occupied by either an arginine or its conservative replacement, lysine, in all nuclear receptors that bind DNA (unpublished data, G. Kerr Whitfield; see also http://receptors.ucsf.edu/NR/). Despite this high level of conservation, the role of this residue in DNA binding is quite variable among nuclear receptors, as evidenced by the crystallographic studies summarized in Fig. 12. At one extreme, the equivalent of R49 in hTRβ makes multiple base contacts on a DR4 element, including direct interaction with the GT core bases in the 3′ half-element (16). The equivalent to R49 in RevErbα also makes critical contributions to DNA binding as does the positionally conserved lysine in ER. However, at the other extreme, the residue corresponding to R49 does not contact DNA in either GR [homodimer on an IR4 (14)] or NGFI-B [monomer on an AAAAGGTCA element (48)] (Fig. 12). Regarding VDR, our conclusion, derived from the data presented herein, together with an analysis of the structural and functional roles of this positionally conserved arginine residue in other RXR heterodimerizing nuclear receptors (Fig. 12), is that R49 constitutes a critical determinant of VDRE recognition that operates in conjunction with the invariant base contact residues, K45 and R50.

The other residue in the proposed S-box for VDRE binding, corresponding to K53 in hVDR, is much more variable

than R49, both with respect to its conservation and its role in nuclear receptor DNA binding. Only receptors in the same subclass (1I) with VDR, *i.e.* PXR, contain a lysine at this position; other receptors possess either arginine (RXRs and PPARs) or glutamine (most other receptors—see Fig. 12 for examples). This residue commonly contacts the sense strand phosphate backbone at either the second or third position (AGGTCA). The most extensive contacts occur with RXR homodimers, where this residue (arginine) makes contact with either or both of the phosphate groups of these two guanyl residues (56). In those receptors where the residue corresponding to K53 is not basic, the situation is mixed. The glutamine in ER does not contact DNA, but in receptors other than VDR that bind to the 3′ half-site of a direct repeat type element [TR, RevErbα (16, 57)], or as monomers [rNGFI-B (48)], the glutamine usually makes a single contact, either with the second (RevErbα, NGFI-B) or third (TR) phosphate of the sense strand. Finally, in GR (as in the other group 3C receptors), the residue corresponding to K53 is a glutamate, a negatively charged residue that, of course, does not interact with the phosphate backbone of DNA. Indeed, the negatively charged glutamate in GR, MR, AR, and PR (group 3C receptors) at hVDR position 53 uniquely distinguishes this subclass of homodimerizing receptors that bind IR3-type elements consisting of AGAACA half-sites (Fig. 12). We hypothesize that the negative charge at this position prevents improper recognition of direct repeat HREs by the classic steroid hormone receptors. In summary, although not universal, there is sufficient precedent from other nuclear receptors to support the notion that K53 in hVDR is significant in VDRE binding, likely forming contacts with the phosphate backbone at the second and/or third position of the 3′ DNA half-element.

After the present research was completed, there appeared an x-ray crystallographic analysis of an hVDR DBD fragment on the rOC element, as well as on two other DR3 type elements (58). This report has clear implications regarding the present work, but the interpretation of the crystallographic data are complicated by the fact that all three cocrystals contain homodimers of hVDR DBDs rather than the physiologically significant VDR-RXR heterodimers. In fact, it was reported that heterodimeric RXR-VDR-VDRE cocrystals could not be formed using DBD fragments (58), likely because of the absence of the strong ligand-dependent heterodimerization interface present in the full-length receptors (2, 59). Importantly, considerable evidence points to the VDR-RXR heterodimer as the biologically relevant receptor complex (2, 3, 59–61), including recent studies showing that mutating (62), or pharmacologically blocking (63), the AF-2 transcriptional activation domain of RXRs simultaneously impedes VDR signaling upon $1,25(OH)_2D_3$ liganding. Moreover, RXR is absolutely required for VDR-mediated stimulation of transcription by $1,25(OH)_2D_3$ in an isolated chromatin system, *in vitro* (61), and based upon conditional RXR knockouts in skin (60), RXRα is required for VDR to facilitate normal hair cycling, *in vivo*. In addition, tests of binding affinity to DR3 elements have shown that homodimers of full-length VDR have a lower affinity (59), and a markedly faster off-rate (3), when compared with VDR-RXR heterodimers. Thus, there exist questions about the biological significance of homodimeric VDR-VDR-DNA cocrystals. Nevertheless, these data (58) represent the best information available to date concerning the details of VDR binding to VDREs of varying sequences.

On initial viewing, the VDR DBD homodimer seems to bind specifically to the two VDRE half-elements (58). However, close inspection reveals that the absence of the normal VDR-RXR dimer interface apparently causes considerable distortion in portions of the downstream VDR DBD. This aberration, which was recognized to some extent by the authors of this study (58), can be visualized clearly by comparing the cocrystal structure of the VDR DBD homodimer on a consensus DR3 element (58) with a model of the VDR-RXR DBD heterodimer, also on a consensus DR3, constructed by Rastinejad *et al.* (16) based upon the TR-RXR cocrystal on a TRE element. The heterodimer model (not shown) predicts an orderly dimer interface in which N37, K91, and E92 of the downstream hVDR DBD interact with residues in the upstream RXR DBD. N37 in hVDR is predicted to form H-bonds with three residues in RXR (R48, Q49, and R52), whereas K91 and E92 in VDR form salt bridges with D39 and R38, respectively, in RXR [human RXRα was used to generate the TR-RXR-TRE cocrystal on which this VDR-RXR model was based (16)]. Indeed, a K91 and E92 double mutant hVDR displays a dramatic loss of VDRE binding and transactivity (21), confirming that these two residues are essential for VDR DNA binding. However, as illustrated in Fig. 13A, when a second VDR DBD is present on the upstream half-site instead of RXR, as in the published VDR DBD x-ray crystallographic structures (58), the geometry of the dimer interface becomes drastically altered. N37 now interacts only with a His residue in the upstream VDR DBD, whereas both salt bridges (from K91 and E92) are lost, a structure inconsistent with the observation that K91 and E92 are required for VDRE binding (16, 21). Instead, K91 and E92 in the downstream VDR DBD of the homodimeric cocrystal face away from the dimer interface, creating a short α-helix and causing a rotation of the protein backbone that leads to a dramatic reorientation of the DBD C-terminal extension. As suggested by the illustration in Fig. 13B, the CTE of the VDR DBD bound to the 3′ half-site in the homodimer (shown in purple matching the balance of the VDR DBD) extends in a 5′ direction, crossing the antisense DNA strand at almost a right angle, and passing less than 10 nm below the VDR DBD bound to the 3′ half-site. It seems extremely unlikely that this configuration would exist if both bound VDRs were full-length receptors. In contrast, modeling (16) of a VDR-RXR-VDRE (DR3) structure based on the x-ray crystal of TR-RXR-TRE (DR4) depicts the VDR CTE passing nearly parallel to the DNA phosphate backbone (shown as a green helix in Fig. 13B), a conformation not only more compatible with the presence of the balance of the VDR protein, but also more amenable to multiple contacts between the CTE and the DNA phosphate backbone. Such interactions are a prominent feature of TR DNA binding (Fig. 12), in that four basic residues in the TR CTE contact DNA directly, and two others make contact via an intervening water molecule (16). Furthermore, the strict conservation of basic residues in a distinctive pattern of two clusters in VDR and PXR CTEs argues that at least some of these residues play an important functional role in responsive element binding

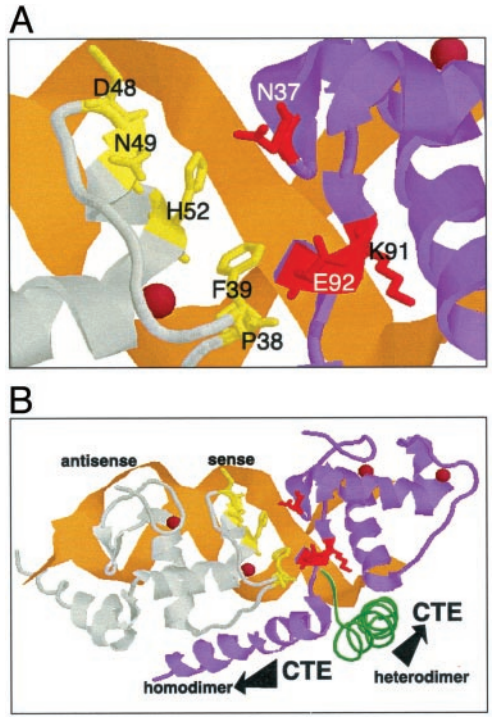

FIG. 13. Lack of H-bonds and salt bridges at the dimer interface, as well as apparent distortion and reorientation of the CTE of homodimerized hVDR DBDs crystallized on a DR3 VDRE. The DNA is depicted in *orange* as a ribbon in the sense orientation with the VDR DBD on the 5′ half-element shaded in gray (with *yellow* highlighted residues), and the VDR DBD on the 3′ element shaded in *purple* (with *red* highlighted residues). *Red spheres* represent the zinc atoms in the dual zinc finger structure. A, The proposed hydrogen bonding between N37 on VDR and R48/Q49/R52 on RXR (16) is diminished in the VDR homodimer, because the residues in VDR that correspond to RXR R48, Q49, and R52 are D48, N49 and H52, respectively (shaded *yellow*). Only one of these (H52) H-bonds with N37. Even more dramatic is the absence of two salt bridges, which were proposed between K91 (VDR) and D39 (RXR), and between E92 (VDR) and R38 (RXR) (16). The residues in VDR corresponding to RXR R38 and D39 are P38 and F39, respectively, neither of which can form salt bridges. B, In the hVDR DBD homodimer on a DR3, the CTE of the DBD on the 3′ half-site (*purple*) extends in the 5′ direction, crossing the antisense DNA strand perpendicularly (thus minimizing phosphate contacts) and passes below the VDR DBD on the 5′ half-site (58). This CTE orientation is in striking contrast to the alignment of the VDR CTE in the RXR-VDR heterodimer model on a VDRE (16), in which the CTE (depicted as a *green* α-helix), is turned such that it passes nearly parallel to the sense DNA strand, an orientation that is far more likely to support multiple phosphate backbone contacts in a fashion similar to TR in the RXR-TR-TRE cocrystal (16).

by these receptors as well as in TRs. Indeed, at least one basic residue in each of the two clusters of the hVDR CTE, namely $_{102}$RKR$_{104}$ and $_{109}$KRK$_{111}$, has been identified in mutational studies (20) as essential for DNA binding as well as transactivation. However, in the VDR homodimeric crystal on a consensus DR3 (58), only one basic residue (R104) in the upstream VDR DBD interacts with DNA, and there are neither contacts in the $_{109}$KRK$_{111}$ cluster nor any DNA CTE contacts in the downstream VDR DBD (Fig. 12). Therefore, we predict that, when solved, the RXR-VDR-VDRE cocrystal will differ from the VDR homodimer data of Shaffer and Gewirth (58) in that it will display more extensive DNA contacts by the VDR CTE.

Despite the limitations discussed above, the VDR DBD homodimer crystallographic data (58) support a crucial role for R49 in hVDR VDRE binding. As illustrated in Fig. 12, in five of the six VDR DBDs visualized in the Shaffer and Gewirth study (58), this residue makes a direct phosphate contact plus a water-mediated base contact with the third base of the half-site. The base contact occurs even when the third base, which is a guanine in the consensus half-site (AGGTCA), becomes a thymidine in the mOP VDRE (GGTTCA). Given this fact, the existence of this residue in the unique conformation of a VDR-RXR heterodimer on a DR3 element could conceivably be responsible for the ability to bind to, and display apparent preference for (64), VDREs containing a thymidine at the third position. In addition, R49 is one of only two residues that make both phosphate and base contacts (the other being the invariant R50 residue discussed above). All of these considerations, plus the current data demonstrating the necessity of this residue in the background of a GR DBD for binding to a VDRE, solidify a role for R49 in DNA binding by VDR and related receptors and appear to provide strong justification for its placement in the proposed S-box.

The second residue postulated for the S-box, namely K53 in hVDR, also participates in DNA binding, both based upon the results presented herein with VGV chimeras, and from the VDR DBD homodimer crystallographic data (58), in which K53 makes a contact in two of six potential VDRE half-sites studied (Fig. 12). The DNA interaction by K53 is a water-mediated contact to the phosphate of the second half-site residue in the GGTTCA or AGGTCA half-sites. Despite the observation that K53 does not contact DNA in four of the six possible half-sites studied with the hVDR DBD homodimer (see Fig. 12), the following evidence is marshaled to support a role for K53: 1) this residue, along with R49, was necessary and sufficient in the present study to restore the binding of a chimeric VGV receptor to the rOC VDRE; 2) this residue is conserved as a lysine in all known receptors that recognize a DR3 element, namely VDRs and PXRs; 3) a residue in this position (arginine in RXR, or glutamine in TR, RAR, and RevErbα) makes a direct phosphate contact in every DNA cocrystal of a receptor that normally binds on the 3′ half-site of a direct repeat responsive element; and 4) either a glutamine or an arginine is found at this position also in PPARs, RevErbs, RORs, as well as in the entire set of receptors from groups 2, 4, and 5. Taken together, these observations support an important role for the S-box, corresponding to R49 and K53 in hVDR, in those receptors that bind to direct repeat elements in general (arginine or glutamine at position 53), or to the DR3-binding receptors VDR and PXR in particular (lysine at position 53).

In conclusion, by employing site-directed mutagenesis of VDR-GR DBD chimeric receptors, coupled to functional DNA-binding and ligand-dependent transcription assays, we have identified R49 and K53 in hVDR as two previously unrecognized amino acids outside the P-box that comprise a proposed S-box that specifies binding of the VDR-RXR heterodimer to the natural rOC VDRE. Therefore, our current and previous (20) mutational analyses have revealed essential hVDR residues in the S-box and CTE, respectively, that in combination with VDR P-box amino acids, confer the

VDR-RXR with unique DNA binding properties. Recently published data on a VDR DBD fragment cocrystal with VDREs (58) support those conclusions in part, especially the significance of P-box residue E42 and S-box residue R49. However, we argue herein that because the VDR DBD fragment crystallized (58) homodimerizes only at high concentrations in the presence of a DR3 VDRE DNA platform, *in vitro*, it likely does not totally reflect the structural interface between a VDRE and the biologically relevant, full-length VDR-RXR heterodimer in a vitamin D target cell. In particular, as noted by the authors, only a very weak hydrophobic dimeric interface consisting of virtually all van der Waals contacts was observed for VDRE cocrystallized VDR-VDR DBD fragments (58), creating a dimer that possesses neither the numerous cross-unit stabilizing hydrogen bonds nor the DNA buttressing of polar side chains to stabilize the type of intersubunit interface that is observed for GR-GR (14), RXR-RXR (56), or RXR-TR (16). Therefore, whereas the VDR DBD homodimeric cocrystals with VDREs provide valuable insight into VDR-DNA contacts in the zinc finger region, they do not allow for analysis of key heterodimeric and DNA contacts in the CTE region. The resolution of the above questions concerning the biological implications and interpretation of the VDR DBD homodimer structure on DNA must await the successful crystallization of a physiologically relevant VDR-RXR heterodimer on a DR3 element.

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