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THE T-BOX NEAR THE ZINC FINGERS OF THE HUMAN VITAMIN D RECEPTOR IS REQUIRED FOR HETERODIMERIC DNA BINDING AND TRANSACTIVATION

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The T-box mediates binding of retinoid X receptor (RXR) homodimers to DNA while the P- and D-box in the zinc fingers of steroid hormone receptors play roles in DNA-binding specificity and homodimerization, respectively. We investigated the function of these elements in the human vitamin D receptor (hVDR) by mutating a Lys-Glu pair of amino acids in the T-box, and by altering the P- and D-boxes to the corresponding residues of the glucocorticoid receptor (GR). The T-box mutant hVDR displayed attenuated vitamin D responsive element (VDRE) binding in the presence of RXR and was severely compromised in transcriptional activation. In contrast, GR P/D-box mutant hVDRs bound to the rat osteocalcin VDRE and elicited near normal transcriptional activation. The T-box mutant uniquely exhibited dominant negative properties, highlighting the significance of this region of hVDR for heterodimeric transcriptional activation.

The superfamily of nuclear hormone receptors controls target gene transcription by binding as dimers to specific hormone responsive elements (1). Based on the interactions between nuclear hormone receptors and their natural responsive elements, this superfamily of receptors can be divided into GR and VDR subfamilies (2). Receptors in the GR subfamily (including glucocorticoid, mineralocorticoid, androgen, progesterone and estrogen receptors) bind to palindromic responsive elements as homodimers, while receptors in the VDR subfamily (including retinoic acid receptors (RARs), thyroid hormone receptors (TRs) and VDR) recognize direct repeats (DRs) via heterodimerization with RXRs (3). There are two regions in the zinc finger DNA binding domain that play important roles in responsive element binding for receptors in the GR subfamily. The first region is known as the proximal or P-box, which has been shown to confer target gene specificity in GR and estrogen receptor (ER) (4). The second region, known as the distal or D-box, partially mediates homodimerization of the GR subfamily of receptors (4). A third important region has been identified in mouse RXRβ, called the T-box,

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Abbreviations used: RXR, retinoid X receptor; hVDR, human vitamin D receptor; GR, glucocorticoid receptor; VDRE, vitamin D responsive element; RARs, retinoic acid receptors; TRs, thyroid hormone receptors; DRs, direct repeats; ER, estrogen receptor; ERE, estrogen responsive element; GRE, glucocorticoid responsive element; TAT, tyrosine amino transferase; $1,25(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; hGH, human growth hormone; TRE, thyroid hormone responsive element; RARE, retinoic acid responsive element.

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which coincides with an α-helical structure just C-terminal of the zinc finger region (5). Further, it has been demonstrated that the T-box is the region of an RXR-related orphan receptor, NGF1-B, that mediates homodimer binding to tandem repeats of estrogen responsive element (ERE) half-sites (6). In the present study, we evaluate the potential functions of the T-, P- and D-boxes in the full-length human VDR.

MATERIALS AND METHODS

In vitro site-directed mutagenesis. Three mutagenic oligonucleotides were used to generate the P-, D- and T-box hVDR mutants, respectively, by site-directed mutagenesis as described previously (7).

Construction of a glucocorticoid responsive element-linked TKGH reporter plasmid. A synthetic oligonucleotide, 5'-TATCCTGTACAGGATGTTCTAGCT-3' containing the glucocorticoid responsive element (GRE) sequence from the rat tyrosine amino transferase (TAT) gene (8) was cloned into the unique HindIII site of the TKGH plasmid (9), which contains a herpes simplex virus thymidine kinase promoter directing the transcription of a human growth hormone (hGH) reporter gene. An isolate (TAT GRE-TKGH), which contains three copies of the GRE, was chosen to evaluate transcriptional activity.

Cotransfection of COS-7 cells and transcription assays. Transcriptional activity was measured in COS-7 cells. Cotransfections were performed by the calcium phosphate DNA coprecipitation method with 7.5 μg of pSG5hVDR expression plasmid (7) and the rat osteocalcin VDRE-containing reporter plasmid $(CT4)_4$TKGH (5 μg) (10) or with 7.5 μg of pSG5mGR expression plasmid (3) and the TAT GRE-TKGH (5 μg) reporter vector. Following transfection, cells were treated for 16 h with either 10 nM 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$), 1 μM dexamethasone, or ethanol (vehicle) as a control. Medium was analyzed by radioimmunoassay for the expression of hGH using a kit from Nichols Institute Diagnostics (San Juan Capistrano, CA), and cells were harvested for immunoblot analysis. Immunoblotting was performed as previously described (11).

Gel mobility shift assay. The hVDR utilized for the gel mobility shift assay was obtained from whole cell extracts of COS-7 cells transfected with wild-type or mutant pSG5hVDR plasmids. Rat liver nuclear extract containing 1 μg of protein, which alone does not form complexes with the VDRE (12), was preincubated with the hVDR-containing COS-7 cell extract (2 μg of total protein) and assayed for its ability to retard a radiolabeled rat osteocalcin VDRE (5'-GCACTGGGTGAATGAGGACATTAC-3') as described previously (12).

RESULTS

The zinc finger region of hVDR is shown in Fig. 1 along with a comparison of the amino acid sequences of the P-, D- and T-boxes of hVDR, hTRβ, hRXRα, hER and hGR. To probe the significance of the P-, D- and T-boxes in the full-length hVDR, we altered the P- and D-box amino acids to the corresponding residues in GR (Fig. 1). In addition, two amino acid residues within the T-box of hVDR, namely Lys-91 and Glu-92, were mutated to Asn-Gln (Fig. 1).

Cotransfection results showed (Fig. 2A) that in the presence of a GR ligand, dexamethasone, the wild-type mouse GR readily activates transcription from the TAT GRE. The T-, D- and P-box mutant hVDRs were unresponsive to $1,25(OH)_2D_3$ in the GRE-reporter transcription system, even though the P-box mutant contains the residues that specify GR binding to a GRE. These data show that the target gene specificity of hVDR cannot be converted to that of GR by the simple exchange of amino acid residues in the P-box region of the first zinc finger. The same result occurred with a mutant hVDR in which residues in both the P-box and the D-box were mutated to those in GR (data not shown). To examine whether these mutants retain VDRE-mediated transcriptional activity, *in vivo*, we cotransfected COS-7 cells with a rat osteocalcin VDRE-linked reporter plasmid, $(CT4)_4$TKGH, and the hVDR expression vectors. This VDRE-

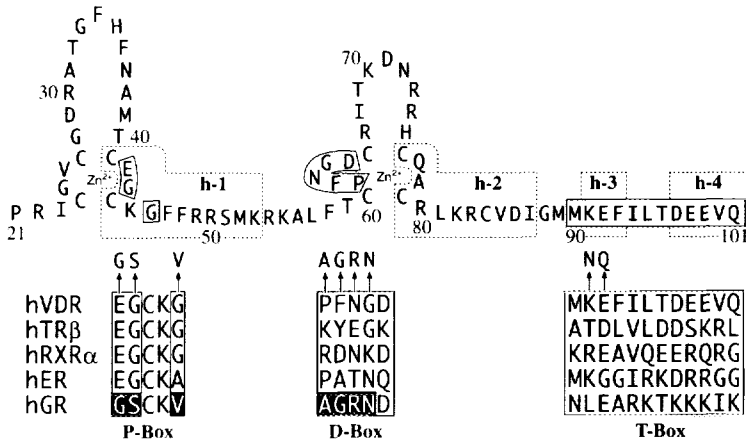

FIG. 1. Schematic representation of the DNA binding domain of hVDR (Pro^{21}-Gln^{101}). Four putative α-helices (h-1 to h-4) are boxed in with dashed lines, inferred from the recently elucidated crystal structure of the TRβ DNA binding domain (13). The P-, D- and T-boxes are delineated in the lower portion of the figure and compared between several nuclear receptors. The central portion of the figure depicts the mutations generated in the present study.

mediated transcription assay (Fig. 2B) revealed that the P-box and D-box mutants display an ability to activate transcription close to that of the wild-type receptor, with the P-box mutant being slightly more effective and the D-box mutant slightly less effective. In contrast, the T-box mutation abolishes about 90% of the transcriptional activity of hVDR.

To probe the DNA binding capacity of the P-, D-, and T-box mutant hVDRs, the VDRE from the rat osteocalcin gene and a rat liver nuclear extract containing RXR factors (12) were utilized in a gel mobility shift assay. The results indicated that the P-box mutant exhibits a 10% increase and the D-box mutant displayed a 25% reduction in binding to the VDRE as compared to wild-type hVDR (Fig. 3A, lanes 2, 3 and 4), while the T-box mutant was inhibited by 60% in its VDRE binding ability (Fig. 3A, lane 5). These differences in VDRE binding activity were not

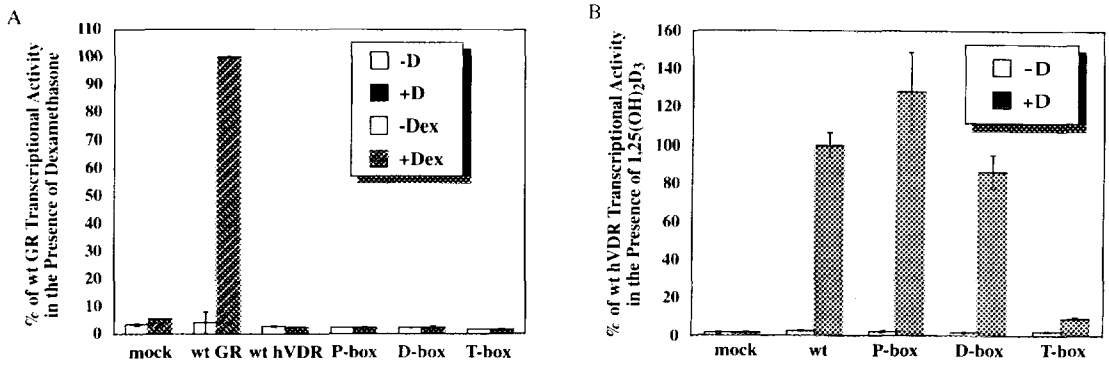

FIG. 2. Transcriptional activity of P-, D- and T-box mutant hVDRs using a GRE reporter (A) or a VDRE reporter (B). Following an 8 h transfection, COS-7 cells were treated for 16 h with 1 μM dexamethasone (+ Dex), 10 nM $1,25(OH)_2D_3$ (+D) or ethanol (-D or -Dex) as a control. The level of transcription with wt GR in the presence of Dex was arbitrarily set at 100% (A) as was transcription with wt VDR in the presence of $1,25(OH)_2D_3$ (B). In mock transfections, no receptor expression plasmid was included.

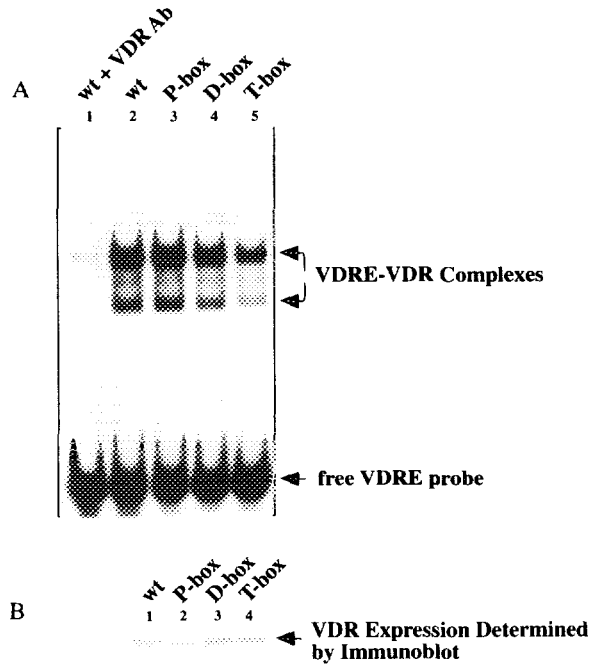

FIG. 3. Interaction of wild-type, P-, D- and T-box hVDRs with the rat osteocalcin VDRE using the gel mobility shift assay. (A) An assay was performed in the presence of a mammalian cell nuclear extract to probe hVDR-VDRE complexes formed when whole cell extracts of transfected COS-7 cells were incubated with an authentic VDRE probe. Two major retarded complexes were observed, both of which can be inhibited by VDR-specific monoclonal antibody, 9A7γ (lane 1). Quantitation of the results in Fig. 3A was accomplished by liquid scintillation spectrometry of the excised retarded bands. (B) The expression of wild-type and mutant hVDRs as examined by immunoblot analysis.

the result of variable expression of the mutant hVDRs (Fig. 3B). VDRE-mediated transcription data, *in vivo* (Fig. 2B), correlated well with the *in vitro* VDRE binding results from gel mobility shift analysis (Fig. 3A). We therefore conclude that the T-box contributes significantly to heterodimeric receptor DNA binding and transactivation functions of hVDR.

To extend our understanding of its mode of action, we next examined whether the T-box mutant could act in a dominant negative fashion. Cotransfection experiments were designed utilizing a VDRE-reporter vector and varying levels of the competing mutant hVDR plasmids up to a 15-fold excess relative to a constant level of the wild-type hVDR plasmid. The results (Fig. 4) revealed, as expected, that the P-box mutant hVDR does not inhibit the transcriptional activity of the wild-type receptor, but rather yields a slight enhancement. Consistent with the previous transactivation results, the D-box mutant elicited a slight inhibitory effect at high concentrations. In contrast, increasing the expression of the T-box mutant relative to wild-type hVDR caused a dramatic suppression of hVDR-mediated transactivation (Fig. 4). This effect occurred even at low ratios of T-box mutant to wild type hVDR (Fig. 4 and data not shown), revealing that the T-box hVDR mutant is a potent dominant negative receptor.

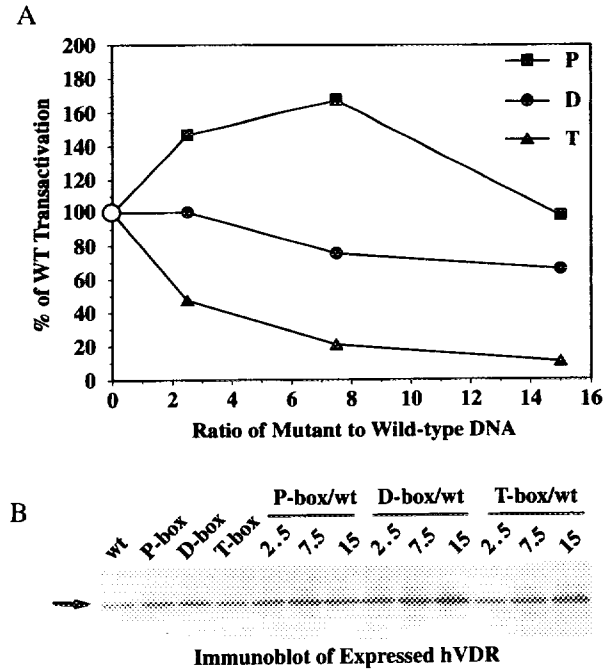

FIG. 4. Dominant negative mutation analysis of P-, D- and T-box variant hVDRs. (A) COS-7 cells were cotransfected with the reporter plasmid $(CT4)_4TKGH$ (5 μg) and the amount of transfected mutant hVDR expression vectors was increased (2.5, 7.5 and 15 μg) relative to the wild-type hVDR expression vector (1 μg). Eight hours after transfection, cells were incubated either in the presence or absence of 10 nM $1,25(OH)_2D_3$ hormone for an additional 16 h prior to harvesting. The transcriptional activity assay was performed as described in Methods. (B) Immunoblot analysis to assess hVDR expression (indicated by the arrow) in this representative experiment was performed as described previously (11). P-box/wt refers to the ratio of P-box mutant to wild-type receptor expression plasmids used in the transfection, etc.

DISCUSSION

A recent report presenting the crystal structure of the complex formed by the DNA binding domains of RXRα and TRβ associated with a DR4 thyroid hormone responsive element (TRE) (13) included a hypothetical model for the interaction of RXR-VDR with a DR3 element. A crucial prediction in this model was that VDR T-box residues Lys-91 and Glu-92 mediate heterodimerization via the formation of salt bridges with residues in the D-box of RXR (13). The present data strongly support this model and provide the first direct evidence for the fundamental importance of these two T-box amino acids in the function of VDR. Although other heterodimerization domains exist in the hormone binding region of hVDR (12, 14), it is now evident that optimal VDR-RXR interaction and selection of natural DR3 VDREs requires a DNA-supported, asymmetric dimerization interface created in part by Lys-91 and Glu-92. It should be noted that a VDR nuclear localization domain has recently been reported (15) that also spans the T-box. Although a decrease in nuclear localization by the T-box mutant might explain the discrepancy between the observed reductions in VDRE binding (60% in Fig. 3) and transactivation (90% in Fig. 2B), the dominant negative study (Fig. 4) suggests that the T-box mutant does in fact translocate to the nucleus, where it competes with wild-type hVDR, probably

by sequestering endogenous RXRs via other dimerization domains in the hormone binding region (12).

The current data on hVDR substituted with the GR D-box also support another feature of the model of Rastinejad *et al.* (13). In heterodimerizing receptors like TR and VDR (16), with the RXR partner positioned on the 5' half-element of the direct repeat, the D-box of the 3' receptor is neither involved in protein-protein nor DNA contacts (13). In concert with this prediction, our results reveal only very slight inhibition of hVDR functions by substituting GR D-box residues (Figs. 2B, 3 and 4).

Structural analysis of cocrystals of the RXR-TR-DR4 (13), ER-ERE (17) and GR-GRE (18) all predict some involvement of P-box residues in DNA binding by the respective receptors. In support of this notion, Predki *et al.* (19), altered the P-box of hRARα to that of GR and observed that the RAR no longer bound its natural retinoic acid responsive element (RARE) 3' half site in a DR2 element, but rather bound a half site matching that of a GRE. However, when the same approach was tested with a DR5 element, the chimera still bound, albeit with reduced affinity, to the native RARE, acquiring only a very weak affinity for a GRE half site in the 3' position. The present results are more comparable to this latter finding, in that alteration of all three P-box residues in hVDR to those corresponding to GR had a somewhat positive effect on both complex formation with RXR on the VDRE as well as on VDRE-mediated transactivation. A similar conclusion has been reached for hTRβ (20), where alteration of the TR P-box to that of the GR preserved the ability of the mutant TR to bind to and transactivate from a natural TRE. One common feature of the TRE used by Hartong *et al.* (20) and the VDRE used in the present study is a fortuitous resemblance of the 3' half site (AGGACA, occupied by TR or VDR, respectively) to a GRE half site (AGAACA) in classic GREs. Thus it is possible that the interaction (18) between the fourth base pair (AGGACA) and the valine substituted in from the GR P-box (GSxxV) may be strong enough to compensate for other P-box changes in VDR as well as TR. In another study, however, Nelson *et al.* (21) systematically altered the second and third residues in the TR P-box (EGxxG) to residues found in other members of the superfamily, and concluded that these alterations did not compromise binding of TR to a consensus AGGTCA half site, but rather affected binding to other non-consensus half sites, such as the AGGACA variant used in our study. Thus, it is possible that while an hVDR with a GR P-box retains its ability to bind to the natural rat osteocalcin VDRE, its capacity for recognizing the full range of natural VDREs might still be impaired. Finally, our findings with hVDR point mutants suggest that perhaps the presence of the RXR heterodimeric partner, as well as the participation of other subregions within the DNA binding domain, may override the P-box in providing DNA binding specificity under certain conditions. In place of the P-box residues, it appears that, for hVDR, adjacent conserved basic residues, which also contribute important base contacts in RXR-TR-DR4 (13) and ER-ERE (17) cocrystals, play a more prominent role in hVDR than is the case for GR. The existence of two natural mutations in hVDR, at Lys-45 (22) and Arg-50 (23), respectively (see Fig. 1 for sequence), both of which cause DNA binding defects that render the patients resistant to the $1,25(OH)_2D_3$ hormone, supports this concept. However, in order to confirm and extend the present conclusions it will be necessary to obtain RXR-VDR-VDRE cocrystals for analysis.

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