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J Clin Invest. 1993;92(1):12-16. <https://doi.org/10.1172/JCI116539>.

Research Article

We have identified and characterized two mutations in the hormone binding domain of the vitamin D receptor (VDR) in patients with hereditary vitamin D-resistant rickets. One patient was found to have a premature stop mutation (CAG to TAG) in the hinge region affecting amino acid 149 (Q149X) and the other demonstrated a missense mutation (CGC to CTC) resulting in the substitution of arginine 271 by leucine (R271L) in the steroid binding domain. Eukaryotic expression analyses in CV-1 cells showed the inability of both patients' VDR to induce transcription from the osteocalcin hormone gene response element at 10^{-7} M 1,25-dihydroxyvitamin D3 (1,25(OH)₂D₃). Normal transcription levels could, however, be elicited by the missense mutated VDR (R271L) in the presence of 1,000-fold higher 1,25-(OH)₂D₃ concentrations than needed for the wild-type receptor. This shows that Arg 271 directly affects the affinity of the VDR for its ligand and its conversion to leucine decreases its affinity for 1,25(OH)₂D₃ by a factor of 1,000. Arg 271 is located immediately 3-prime to a 30 amino acid segment (VDR amino acids 241-270) that is conserved among members of the steroid/thyroid/retinoid hormone receptor superfamily. These results represent the first missense mutation identified in the hormone binding domain of VDR and further define the structure-function relationship of 1,25(OH)₂D₃ ligand binding to its nuclear receptor.

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Two Mutations in the Hormone Binding Domain of the Vitamin D Receptor Cause Tissue Resistance to 1,25 Dihydroxyvitamin D₃

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Abstract

We have identified and characterized two mutations in the hormone binding domain of the vitamin D receptor (VDR) in patients with hereditary vitamin D-resistant rickets. One patient was found to have a premature stop mutation (CAG to TAG) in the hinge region affecting amino acid 149 (Q149X) and the other demonstrated a missense mutation (CGC to CTC) resulting in the substitution of arginine 271 by leucine (R271L) in the steroid binding domain. Eukaryotic expression analyses in CV-1 cells showed the inability of both patients' VDR to induce transcription from the osteocalcin hormone gene response element at 10^{-7} M 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃). Normal transcription levels could, however, be elicited by the missense mutated VDR (R271L) in the presence of 1,000-fold higher 1,25-(OH)₂D₃ concentrations than needed for the wild-type receptor. This shows that Arg 271 directly affects the affinity of the VDR for its ligand and its conversion to leucine decreases its affinity for 1,25(OH)₂D₃ by a factor of 1,000. Arg 271 is located immediately 3-prime to a 30 amino acid segment (VDR amino acids 241-270) that is conserved among members of the steroid/thyroid/retinoid hormone receptor superfamily. These results represent the first missense mutation identified in the hormone binding domain of VDR and further define the structure-function relationship of 1,25(OH)₂D₃ ligand binding to its nuclear receptor. (*J. Clin. Invest.* 1993. 92:12-16.) Key words: rickets • calcitriol • steroid • genetic • transcription-genetic

Introduction

The biological effects of 1,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃),¹ the hormonal form of vitamin D, are mediated by its binding to a specific intracellular receptor present in target cells (1-3). The 48-kD receptor belongs to the family of steroid/thyroid/retinoid nuclear hormone receptors (4). These

This work has been reported in abstract form (1992. *Am. J. Hum. Genet.* 51:A131).

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Received for publication 14 December 1992 and in revised form 18 February 1993.

1. Abbreviations used in this paper: CAT, chloramphenicol acetyltransferase; HVDRR, hereditary vitamin D-resistant rickets; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; VDR, vitamin D receptor.

J. Clin. Invest.

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0021-9738/93/07/0012/05 \$2.00

Volume 92, July 1993, 12-16

receptors act in *trans* by binding to specific *cis*-acting DNA sequences in the promoter regions of hormone-responsive genes, thereby modulating their transcription (5). Receptors belonging to this superfamily comprise two functional domains: a ligand binding and a DNA binding domain (4). The DNA binding domains show high evolutionary conservation in a region of 60-70 residues that form two zinc fingers essential for interaction with DNA (4, 6-10). The high amino acid homology within this region predicts a three-dimensional organization common to the individual members of the gene family. The interaction of the glucocorticoid receptor DNA binding domain with its DNA hormone response element has recently been resolved by crystallographic analysis (11), and on this basis we have analyzed the stereochemical consequences of vitamin D receptor (VDR) mutations identified in patients with tissue resistance to 1,25(OH)₂D₃ (12).

Structure-function relationships of the hormone binding domain are much less well understood. The potential importance of individual amino acids in this domain is inferred by their evolutionary conservation throughout the receptor superfamily (4). For example, subdomains within the carboxy-terminal half of these receptors that affect ligand binding, receptor dimerization, and transcriptional activation have been identified (9, 13-17). Further insight has been obtained from mutations intentionally created in this domain, together with reports of receptor mutations in patients with tissue resistance to the various steroid hormones (9, 13, 15, 18-26). Only one of the published mutations in the human VDR has been in the hormone binding domain (25). Here we report the identification and characterization of two mutations located in the hormone binding domain of this receptor from patients with classical features of hereditary vitamin D-resistant rickets (HVDRR).

Methods

Patients. The patients presented in early childhood with classical features of tissue resistance to 1,25(OH)₂D₃, including severe bowing of weight-bearing bones (rickets), and one patient had alopecia. They both had elevated parathyroid hormone and very high levels of serum 1,25(OH)₂D₃. The patients were unrelated but both were of Middle Eastern origin and their parents were consanguineous. Neither showed any clinical improvement by high dose 1,25(OH)₂D₃ therapy (up to 50 µg/d). Biochemical evaluation was consistent in both patients with HVDRR. Detailed case histories have previously been published and receptor binding data showed no detectable cytoplasmic or nuclear 1,25(OH)₂[³H]D₃ association (27, 28). 25-Hydroxyvitamin-D-24-hydroxylase induction was measured in skin fibroblasts obtained by biopsies from patients and normal individuals. The conversion of 25-hydroxyvitamin-[³H]D to 24,25-dihydroxyvitamin-[³H]D by the 24-hydroxylase was determined as described (29) and plotted against the elution profile for a mixture of standard vitamin D metabolites. The production of radiolabeled 24,25-dihydroxyvitamin D₃ was expressed as femtomoles per hour per 10⁶ cells.

RNA isolation, cDNA synthesis, and PCR amplification. Total RNA was isolated from patient skin fibroblast cultures by standard protocols (RNAzol B; Cinna/Biotex Laboratories, Houston, TX). First strand cDNA synthesis was performed with Superscript RNase H-reverse transcriptase (Bethesda Research Laboratories, Gaithersburg, MD), 10 μ g total RNA, and a VDR primer specific to the early 3' untranslated sequence (primer 31b: 5'-GCCCTGGAGGAG-CAGCCC, 0.8 M final concentration; Fig. 1). The reaction was incubated at 45°C for 60 min in a total volume of 40 μ l. The full-length VDR coding region was amplified by PCR (30) using primers 31a (5'-GAGCACCCCTGGGCTCCA) and 31b (0.16 μ M final concentration) (Fig. 1) and 2 μ l of the reverse transcription reaction as template (standard buffer conditions of Perkin Elmer-Cetus, Norwalk, CT, and 2.0 mM MgCl₂ with 35 cycles of 90°C for 1 min, 50°C for 1 min and 20 s, and 72°C for 6 min). To facilitate complete sequencing, each half of the coding region was amplified by a nested PCR reaction, using primers 32a (5'-CCTGCCCTGCTCCTTC) and 32b (5'-AGGTCAG-CCAGGTGGGGC) for the 5' half and 33a (5'-CCCAGCTCTCCATG-CTGC) and 33b (5'-CCCAGGCACCGCACAGGC) for the 3' half (0.16 μ M primer concentration at 2.5 and 1.5 mM MgCl₂, respectively, for 35 cycles at 95°C for 1 min and 20 s and 72°C for 1 min and 40 s, with 4 s extension per cycle; Fig. 1).

Cloning and sequencing of amplified DNA. The PCR products were cloned into Bluescript II pSK- plasmid (Stratagene, La Jolla, CA). Positive clones were sequenced by the dideoxy chain termination method using fluorescence labeled M13 reverse primer on an automated sequencer (Applied Biosystems, Inc., Foster City, CA). The full-length VDR coding region was subcloned in a sense orientation into the EcoRI and XbaI sites of the expression vector pSVK3 (Pharmacia, LKB Biotechnology Inc., Piscataway, NJ) (31). For exclusion of Taq polymerase and sequencing errors, all steps were performed in duplicate, starting with the reverse transcriptase reaction, and several clones of each PCR product were sequenced in both orientations.

Reporter construct. A 881-bp 5'-untranslated fragment of the human osteocalcin gene including the VDR hormone response element (32) was amplified by PCR from human genomic DNA using the following primers: 5'-CTGCAGGGTCAGGAGGAGAATCG and 5'-GTGCGTCGGTGGGCTCT. Reaction conditions included standard Cetus buffer, 2.0 mM MgCl₂, and 0.2 μ M primer concentration for 35 cycles of 95°C for 1 min, 58°C for 1 min, and 72°C for 2 min. The PCR product was cloned into the reporter plasmid pCAT Basic (Promega Corp., Madison, WI), thereby fusing the osteocalcin gene promoter/enhancer region upstream of the chloramphenicol acetyltransferase (CAT) gene.

VDR cotransfection assay in CV-1 cells. VDR-deficient CV-1 cells were grown in DME supplemented with 10% FCS and L-glutamine (2 mM). 24 h before transfection the cells were plated to a density of 1×10^6 cells/10-cm plate. Transfection was carried out by the polybrene method as described (7) using 1 μ g of expression plasmid together with 5 μ g of human osteocalcin reporter plasmid. After a 30-s glycerol

shock, cells were cultured in DME-Nutridoma (Boehringer Mannheim Corp., Indianapolis, IN) supplemented with the desired concentrations of 1,25(OH)₂D₃ in ethanol (final concentration 0.1%). Cells were washed with PBS and harvested by scraping 72 h later, subjected to three freeze-thaw cycles (37°C/−135°C) in 0.25 M Tris-HCl, pH 7.8, and centrifuged, and the supernatant (20 μ g protein) was assayed for CAT activity (37°C overnight) (33). Products were resolved by thin layer chromatography and after autoradiography the relevant portions were excised and quantitated by liquid scintillation counting. The results are expressed as percentage CAT conversion and represent the average of three separate transfection experiments.

Results

Initial assessment of VDR bioactivity in fibroblasts of these patients was determined by 1,25(OH)₂D₃-mediated induction of the 25-hydroxyvitamin-D-24-hydroxylase (34). Cells from both patients failed to show any significant induction of 24-hydroxylase activity when cultures were exposed to 1,25(OH)₂D₃ at concentrations as high as 1 μ M (results not shown). This contrasts with normal control fibroblasts that produced 489 ± 119 fmol/h per 10^6 cells of 24,25-dihydroxyvitamin D₃ at 10 nM 1,25(OH)₂D₃ and is in agreement with the lack of response to vitamin D₃ therapy in these patients.

Mutational analysis. A different nucleotide point mutation was identified in each patient. One was found to have C → T transition at codon 149 (CAG to TAG; Fig. 2), replacing the normal glutamine codon with a termination codon and resulting in a truncated receptor missing 303 residues of the hormone binding domain. The other patient demonstrated a substitution of a G for a T in codon 271, resulting in a missense mutation (CGC to CTC), with exchange of a basic hydrophobic arginine for a nonpolar, hydrophobic leucine (Fig. 2). In both patients and in four others (unpublished results) we identified a single initiation codon (ATG) at nucleotide position 125, counting from the beginning of the published sequence (GenBank# J03258) (7). This is different from the originally reported sequence in which two putative transcription initiation sites (at 116 and 125 bp) were found (7). The former of these was absent in all of our patients, consistent with the findings of Saijo et al. (35). This could represent a polymorphism in the population resulting in two different receptor species that differ in length by 3 amino acids at the amino terminus. Our numbering of amino acids and their codons is from the single initiation site at position 125.

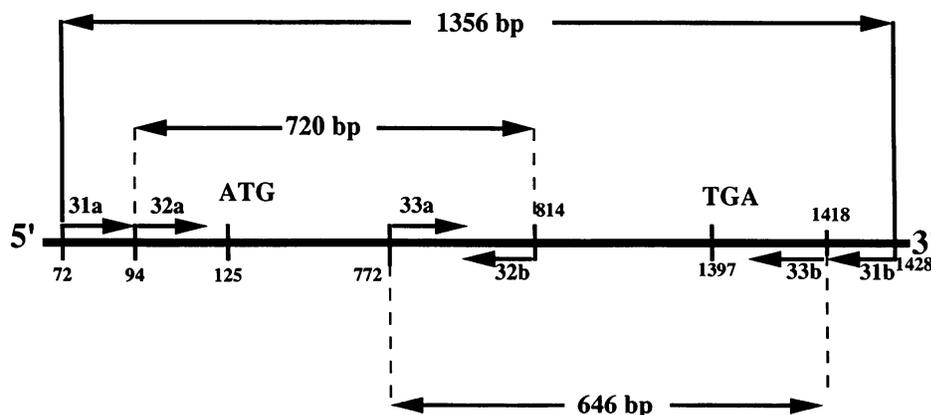


Figure 1. Reverse transcription and PCR amplification of the hVDR cDNA. cDNA was made from total RNA using primer 31b. The full-length coding region was amplified from the first strand cDNA by PCR using primers 31a and 31b. Each half of the gene was subsequently amplified by nested PCRs using primers 33a and 33b for the 3' half and 32a and 32b for the 5' half, giving rise to 646- and 720-bp fragments, respectively. The PCR products were then gel purified, cloned, and sequenced. Base-pair numbering refers to the location of the primer ends with respect to the cDNA sequence as originally published.

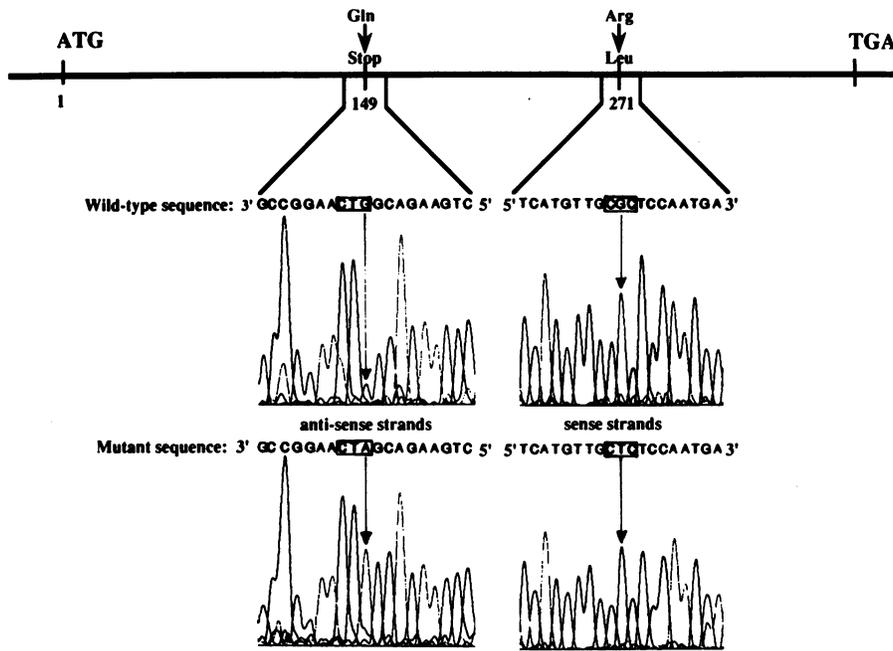


Figure 2. Sequencing data and location of mutations in two patients with HVDRR. One patient was found to have a C to T transition in codon 149 in the hinge region, changing it from CAG (glutamine) to TAG (termination codon). Shown is the actual sequence of the antisense strand from this patient with the involved codon boxed. The other patient had a substitution of a G for a T in codon 271 in the steroid binding domain, resulting in a missense mutation of CGC to CTC and an exchange of arginine for leucine. Shown is the actual sequence of the sense strand for this patient with the corresponding codons boxed.

Transcriptional activation by cotransfection in CV-1 cells. The ability of the mutant receptors to activate transcription was evaluated in receptor-deficient CV-1 cells cotransfected with VDR expression plasmid (patient or wild type) and human osteocalcin reporter plasmid. There was strong induction of CAT activity by 1 nM $1,25(\text{OH})_2\text{D}_3$ in CV-1 cells transfected with the wild-type VDR construct but no significant induction in those transfected with receptor cDNA from the patients (Fig. 3). A titration with $1,25(\text{OH})_2\text{D}_3$ concentration varying from 0.01 nM to $1 \mu\text{M}$ showed that CAT activity could be induced at ~ 50 nM hormone in the cells transfected with the missense mutated (R271L) VDR, reaching maximum induction at $1 \mu\text{M}$ $1,25(\text{OH})_2\text{D}_3$, i.e., the same as the maximum

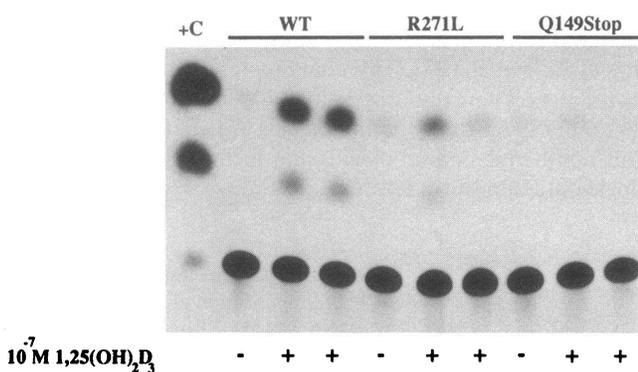


Figure 3. Comparing the transcriptional activation of the osteocalcin promoter by wild-type VDR and the two mutant VDR in the absence and presence of $1,25(\text{OH})_2\text{D}_3$ (10^{-7} M). Wild-type VDR and mutant VDR expression vector constructs were transfected into CV-1 cells along with a human osteocalcin plasmid. Cellular extracts prepared 72 h later were examined for CAT activity. Each lane represents a separate transfection. Cells in the first lane of each set received ethanol only, while cells in the other lanes received 100 nM $1,25(\text{OH})_2\text{D}_3$ in ethanol. Mutant VDRs show insignificant transcriptional activation of the osteocalcin promoter compared with the wild-type VDR when stimulated with $1,25(\text{OH})_2\text{D}_3$.

for the wild-type protein (Fig. 4). Consequently, mutation R271L decreases the affinity of the VDR for the ligand by $\sim 1,000$ -fold.

Discussion

It is now well established that HVDRR is caused by mutations in the VDR (25, 35, 36). With increasing knowledge about the structure-function relationship of the different members in the

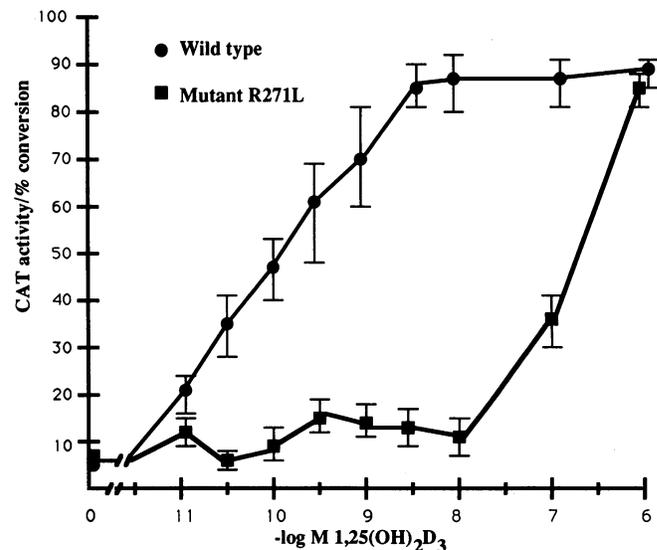


Figure 4. Effect of increasing concentrations of $1,25(\text{OH})_2\text{D}_3$ on transcriptional activation by wild-type and missense mutated (R271L) VDR. CV-1 cells were transfected with wild-type VDR expression plasmid or the R271L mutant VDR expression plasmid ($5 \mu\text{g}$ each) along with the osteocalcin reporter plasmid ($1 \mu\text{g}$). Cells were treated with ethanol only or ethanol containing the $1,25(\text{OH})_2\text{D}_3$ needed to give the concentrations indicated. Each point represents the average of three transfections.

steroid/thyroid/retinoid receptor superfamily, it is now possible to be more specific about the effects of various mutations. This is especially true for the DNA binding domain of the receptors but increasing evidence is also accumulating regarding the function of subdomains and individual amino acids in the hormone binding domain (9, 13, 17, 25). Deletion construct analysis has suggested that the critical point for NH₂-terminal deletions still giving high affinity wild-type binding of 1,25(OH)₂D₃ lies between residues 114 and 166 (9). Our mutation at codon 149 narrows this further by the fact that there is no detectable cytosolic binding of 1,25(OH)₂D₃ (27, 28). The COOH-terminal portion of the glucocorticoid receptor (37, 38), androgen receptor (18, 22), progesterone receptor (39), estrogen receptor (40, 41), and the VDR (9) have been shown to have a repressive effect on transcriptional activation in the absence of ligands. A VDR deletion mutant missing the carboxyl portion from residue 190 to the 3' terminus has been shown to exhibit considerable constitutive transcriptional activity (9). Similar data exist for the glucocorticoid receptor and the androgen receptor (18, 22, 37, 38). This has been interpreted as evidence for transcriptional domains being present in the amino-terminal portion of the hormone binding domains. This region of the VDR is rich in acidic residues (42) that have been implicated as being important in gene activation within yeast transcription factors (43) and steroid receptors (44). In the mammalian cotransfection analyses reported here, no transcriptional activation by the cDNA Q149X mutant was detected. This could be explained by deletion of these transcriptional activation domains. Additionally, a premature stop mutation at codon 149 not only deletes the entire portion of the molecule that is postulated to form the actual binding pocket for 1,25(OH)₂D₃, but it also deletes a dimerization domain that is essential for receptor binding to the VDR hormone response element. This domain (VDR residues 241–263; Fig. 5) is highly conserved between all the members of the superfamily and is involved in interaction with nuclear proteins (13, 15, 23), possibly RXRbeta (14, 16). Point mutations within this domain do not interfere with ligand binding but affect the ability of the receptors to form heterodimers, bind with their response elements, and initiate transcription (13, 15, 23). The

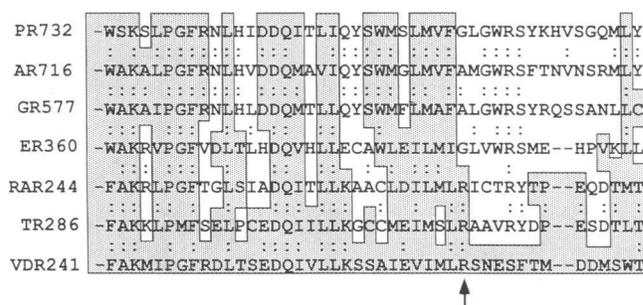


Figure 5. Conservation within a 49 amino acid sequence in the ligand binding domain of the steroid/thyroid/retinoid receptor superfamily. Shown is the sequence of the human VDR along with the homologous regions of the thyroid receptor (TR), retinoic acid receptor (RAR), estrogen receptor (ER), glucocorticoid receptor (GR), androgen receptor (AR), and progesterone receptor (PR). Numbers refer to the position of the first residue on the left for each receptor. Two dots indicate identical amino acid residues. Boxed and shaded areas show the residues conserved in VDR. Our mutation at position 271 (R271L) is indicated by the arrow.

one previously described stop mutation at amino acid codon 789 (25) is located 3-prime to all of the above-mentioned domains. It has the same effects, with total tissue insensitivity to 1,25(OH)₂D₃ and no specific 1,25-[³H](OH)₂D₃ binding. This suggests that the different domains form one functional unit and the ligand binding pocket is constructed by long stretches of three-dimensionally folded polypeptide. The R271L mutation is adjacent to the presumed dimerization domain which by homology with the other receptors can be extended up to Arg 271 (Fig. 5). This residue is conserved among the VDR, thyroid receptor, and retinoic acid receptor, and it is possible that it provides an important ligand contact or represents a polar residue critical for the three-dimensional structure of this domain. Our data do not clarify the role of Arg 271 in this regard, but the importance of this residue is emphasized by the recent observation that a mutation at this position (A746D) in the androgen receptor results in androgen insensitivity syndrome (26).

We have described two novel mutations in the hormone binding domain of the VDR that give further insight to the structure–function relationship of this part of the protein. There is increasing evidence that binding of 1,25(OH)₂D₃ to the VDR modifies protein–protein interaction and transcriptional activation through modification of the receptor's structure. The full understanding of the structure–function relationship of the hormone binding domains of the steroid/thyroid/retinoid receptors awaits the determination of their three-dimensional structure by x-ray crystallography.

Acknowledgments

DNA sequence analysis was carried out by the Nucleic Acids Core in the Institute for Molecular Genetics at Baylor College of Medicine.

This work was supported by grant NIDDK 41427 from the National Institutes of Health to Mark Hughes, and by the Medical Research Council (UK) to J. L. H. O'Riordan.

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