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Point Mutations in the Human Vitamin D Receptor Gene Associated with Hypocalcemic Rickets

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Hypocalcemic vitamin D–resistant rickets is a human genetic disease resulting from target organ resistance to the action of 1,25-dihydroxyvitamin D_3. Two families with affected children homozygous for this autosomal recessive disorder were studied for abnormalities in the intracellular vitamin D receptor (VDR) and its gene. Although the receptor displays normal binding of 1,25-dihydroxyvitamin D_3 hormone, VDR from affected family members has a decreased affinity for DNA. Genomic DNA isolated from these families was subjected to oligonucleotide-primed DNA amplification, and each of the nine exons encoding the receptor protein was sequenced for a genetic mutation. In each family, a different single nucleotide mutation was found in the DNA binding domain of the protein; one family near the tip of the first zinc finger (Gly→Asp) and one at the tip of the second zinc finger (Arg→Gly). The mutant residues were created in vitro by oligonucleotide directed point mutagenesis of wild-type VDR complementary DNA and this cDNA was transfected into COS-1 cells. The produced protein is biochemically indistinguishable from the receptor isolated from patients.

A GENETIC LESION IN THE PATHWAY of vitamin D action has been suspected in the human disorder of hypocalcemic vitamin D–resistant rickets (HVDRR) (*1*). This rare, autosomal recessive syndrome is characterized by hypocalcemia, secondary hyperparathyroidism, and early onset rickets, all of which develop despite an increase of the calcium regulating hormone 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$) in the plasma. In many cases total body alopecia is also present but its relation to vitamin D physiology is unknown. This constellation of features results from peripheral target tissue resistance to the hormone in a manner analogous to the clinical disorders of glucocorticoid (*2*), mineralocorticoid (*3*), and androgen resistance (*4*). While numerous patients have been reported with the HVDRR phenotype (*5–10*), progress in unraveling the molecular pathogenesis of this disorder has been hampered by the difficulty in obtaining receptor containing "target" tissue (usually intestine and bone) for which analysis could be performed. However, fibroblasts cultured from human skin have been shown to contain the 1,25-$(OH)_2D_3$ receptor (VDR) (*11*) and to exhibit biological response to this hormone (*9*). Skin fibroblasts from patients and family members with HVDRR have been evaluated, and a spectrum of molecular defects has emerged including decreased or absent 1,25-$(OH)_2D_3$ binding or decreased DNA binding of the VDR (*6–10*). All cases tested show an inability to induce the enzyme 25-hydroxyvitamin D-24-hydroxylase, a useful marker of receptor-mediated 1,25-$(OH)_2D_3$ action (*7–10*).

We have studied two families for clues to their VDR defects (*7, 8*). The first family (the D kindred) is the result of a consanguinous marriage in a black Haitian family and includes nine members, five of whom have been evaluated [(*7*) and our data]. The parents (D4 and D5) are phenotypically normal, as is one unaffected daughter (D3). Two affected daughters (D1 and D2) display severe rickets and the classic childhood phenotype of HVDRR. The second (the G kindred) is a family of Arabs living in the Middle East (*8*). The parents (G3 and G4) are first cousins and are phenotypically normal with no calcium or bone abnormalities. Of the six children, two males (G1 and G2) display the HVDRR phenotype.

Fibroblasts or lymphocytes (or both)

Table 1. Summary of physical and functional properties of vitamin D receptor in two kindreds with HVDRR.

Property	Wild type	D kindred			G kindred	
		Parents*	Unaffected siblings	Affected siblings	Parents	Affected siblings
M (kD)†	48–50	48–50	48–50	48–50	48–50	48–50
N_{max}‡	32 ± 12		41	32; 52	32; 37	20; 32
K_d ($\times 10^{-11} M$)§	5± 2		5	5.5; 6.3	2; 2	2; 3
DNA binding [peak salt elution (*M*)]	0.2	0.1; 0.2	0.2	0.1	0.1; 0.2	0.1
25-hydroxyvitamin D_3-24-hydroxylase activity	+		+	−	+	−

*Cells were Epstein-Barr–transformed lymphoblasts. †Molecular size. ‡N_{max}, in femtomoles per milligram of protein. §Dissociation constant (K_d) for 1,25-$(OH)_2D$; DNA-cellulose binding peak elution, with a monovalent salt; gradient values are ± S.E.

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were obtained from family members. The cells were transformed with Epstein-Barr virus and grown in culture for subsequent karyotype analysis, 1,25-$(OH)_2[^3H]D$ binding (*7, 8*), induction of 24-hydroxylase activity (*7–10, 12*), DNA-cellulose affinity (*7, 8*), and immunoblot analysis with a monoclonal antibody to VDR (*8*) (Table 1). The receptors from the affected children and obligate heterozygote parents display normal 1,25-$(OH)_2D_3$ binding (*7, 8*) and migrate at the expected molecular size of 48 to 50 kD when analyzed by immunoblot (*8*). However, receptors from the affected children elute from DNA-cellulose prematurely at 0.1*M* KCl instead of at the expected 0.2*M* KCl where the wild-type receptor elutes, suggesting that they have a lower affinity for DNA. These cells are also resistant in vitro to 1,25-$(OH)_2D_3$, as demonstrated by failure to induce 24-hydroxylase activity, while cells from the parents and unaffected sibling induce enzyme normally (*7, 8*). Thus, the phenotypic abnormality in these kindreds is likely to result from a defective VDR-DNA interaction in the affected children. In addition, the receptor is expressed from both normal and affected alleles in the obligate heterozygote parents. Routine cytogenetic analysis (G banding) revealed a normal karyotype in the affected children, excluding a major chromosomal rearrangement in these patients. Because hormone binding is normal and the receptor size is appropriate, we concluded that a minimal mutation in the receptor gene could represent the underlying genetic defect.

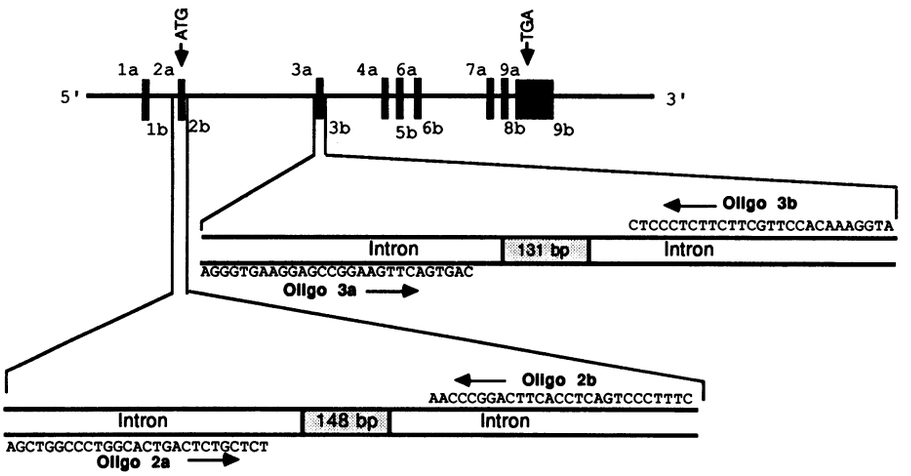

Fig. 1. Human VDR gene organization and exon amplification strategy. The nine coding exon sequences (black vertical bars) are separated along 45 kb of genomic DNA. Oligonucleotide primers (27 nucleotides) designed to anneal to 5′ flanking intron sequence are numbered 1a to 9b. In two instances (exons 4 to 5 and 7 to 8) the intron DNA is sufficiently short (<500 bp) to allow PCR to proceed through both exons plus the spanning intron from a single pair of primers. Hence, the nine exons from the patient were generated with seven paired sets of oligonucleotides that were allowed to anneal at 55°C for 2 minutes to 500 ng of heat-denatured genomic DNA. Primer elongation was accomplished at 72°C for 3 minutes in the presence of Taq I DNA polymerase and subsequent strand denaturation at 95°C for 1 minute. Twenty-two similar cycles (6 minutes each) were carried out (*15*) to produce amplified fragment DNA that was digested with Eco RI to produce cohesive ends and cloned into bacteriophage M13mp8 for sequencing.

We used the previously cloned human VDR cDNA (*13*), the sequence of the human chromosomal gene (*14*), and the DNA amplification technique of polymerase chain reaction (PCR) (*15*) to amplify specific regions of the chromosomal gene, isolate and sequence the amplified products, and interpret these data with regard to possible mutations. This approach avoids the tedium of generating a genomic or mRNA library from each family member, and subsequent screening, mapping, and sequencing the isolated clones.

The organization of the human VDR chromosomal gene and the two pairs of oligonucleotide primers used to amplify exons 2 and 3 of the nine exons comprising the full-length coding region of the gene are shown in Fig. 1. To identify potential splice site mutations, we designed primers to anneal to the intron flanking region of each

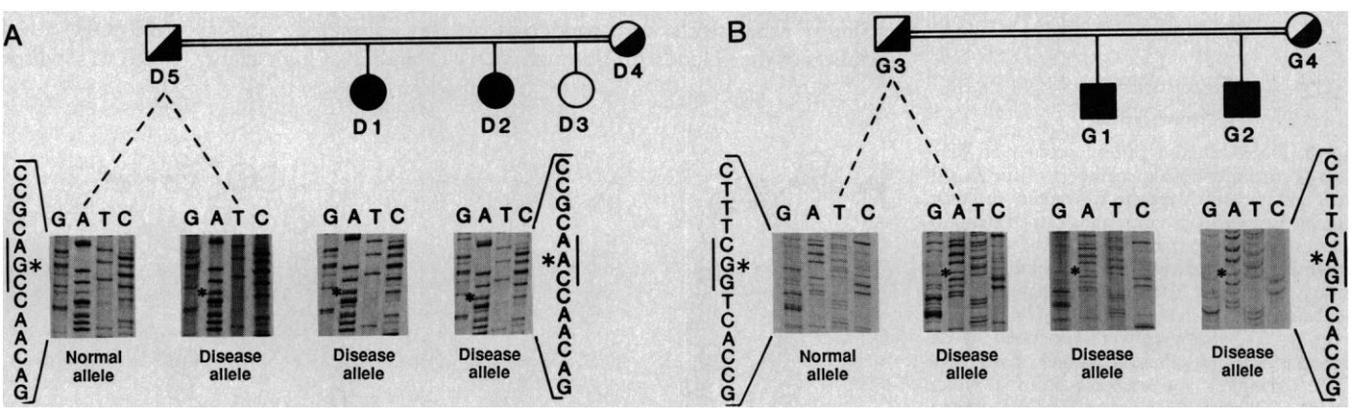

Fig. 2. Partial pedigree and corresponding VDR nucleotide sequence analysis of two families with autosomal recessive HVDRR. Squares represent males, circles indicate females, filled symbols signify obvious clinical disease phenotype, and partially filled symbols indicate heterozygote carrier state for the disease allele. The double bar between the parents indicates consanguinous matings. Genomic DNA was subjected to 22 cycles of PCR (*15*) and specific exon fragment yield was determined on a measured portion (10%) by electrophoresis in 5% acrylamide; the gels were stained with ethidium bromide and the intensity of the bands was compared. The reaction was then digested with Eco RI to generate cohesive ends and cloned into the Eco RI site of M13mp18 for sequencing by the dideoxynucleotide chain termination method (*28*). Mutations were verified by sequencing multiple clones in both strand orientations from independent amplification reactions. (**A**) Normal exon 3 sequence (left) and mutant sequence (right) are depicted for the D family. A single base substitution (asterisk) of G to A is the only identified sequence change in this kindred, and it is found in all clones from amplified exon 3 in patients D1 and D2, and approximately half of the parental clones. The normal and disease allele sequences from the father (D5) are shown and are identical to the maternal sequence. The phenotypically normal sibling (D3) carries only the normal wild-type allele. (**B**) A nucleotide transition (G to A) is seen in exon 2 from two affected members of the G kindred. Similarly the heterozygote parents displayed both the wild-type and mutant alleles.

exon so that the Taq I DNA polymerase would replicate 10 to 20 bp of genomic intron sequence prior to crossing the intron-exon boundary and generating the exon of interest. The 3′ ends of the oligonucleotide primers contained bases that produce an artificial Eco RI restriction site to facilitate cloning into M13 bacteriophage vector. Exons 4 and 5 as well as exons 7 and 8 are each separated by less than 250 nucleotides of intron and were amplified together with single pairs of oligonucleotides. In this manner, each of the nine exons and flanking intervening sequences from the VDR chromosomal gene were amplified and sequenced to identify a potential genetic mutation.

Having identified the receptor defect as an anomaly in DNA binding, we focused our attention on exons 2 and 3 (Fig. 1) of the VDR gene encoding this functional domain (*13, 14*). Together these exons encode for a 70 amino acid, cysteine rich area of VDR which is evolutionarily highly conserved (39 to 49% homology) throughout all members of the steroid receptor family. Moreover, in vitro mutations in this domain greatly reduce or abolish subsequent receptor-DNA interaction without disruption of hormone binding functions (*16*).

Genomic DNA from cells of each family member was subjected to PCR amplification, the products were cloned into a bacteriophage M13mp18 sequencing vector, and the clones were screened with either an end-labeled [^{32}P]oligonucleotide complementary to the middle of the amplified exon, or internally labeled (nick-translated) authentic VDR cDNA. Positive hybridizing clones were sequenced (Fig. 2). A reproducible single base mutation (CGA to CAA) was identified in the triplet codon for arginine in exon 3 from both affected children of the D kindred leading to the aberrant substitution of glutamine. The arginine at this position is positionally conserved throughout all of the steroid receptors sequenced to date (*16*). Although only the mutant codon was found in patients D1 and D2, parental genomic DNA was heterozygotic and generated both the mutant and the normal gene sequence. Random selection of PCR clones revealed 58% normal and 42% abnormal codons, a distribution consistent with the expected equal presence of both alleles in the heterozygote parents. The unaffected sibling (D3) showed only wild-type sequence and therefore is not a carrier of the disease.

Similar experiments were conducted on members of the G kindred. Although VDR exon 3 was entirely normal in this family, a point mutation was identified in exon 2 from the affected children; the triplet codon change of GGC to GAC results in the amino acid replacement of glycine with aspartic acid. Glycine also is a positionally conserved amino acid in all the steroid and thyroid receptors, suggesting that it plays a crucial role in protein-DNA interaction. As before, the G-kindred parents displayed both the normal and disease alleles.

The point mutations of exon 2 (G kindred) and exon 3 (D kindred) are the only VDR sequence variations uncovered in these families. We concluded that these single amino acid substitutions represent the molecular defects responsible for the HVDRR disease phenotype because, as would be expected, (i) with an autosomal recessive pattern of inheritance, the mutation predictably segregated in the family, and the parents displayed both the wild-type and mutant alleles; (ii) these were the only nucleotide changes found in the coding region for the receptor protein, and (iii) the observed mutations were located within the DNA binding domain of the receptor, consistent with the demonstrated biochemical defect.

We obtained final confirmation by recreating these nucleotide base changes in wild-type VDR cDNA. We used oligonucleotide directed point mutagenesis (*17*), which was followed by transfer of the in vitro generated mutant cDNA into the eukaryotic expression vector p91023b (*18*) and transfection into COS-1 monkey kidney cells (*13*). The expressed mutant VDR derived from sequence in the D kindred contained a glutamine substituted for arginine; it displayed normal 1,25-(OH)$_2$[^{3}H]D binding of high affinity, which was indistin-

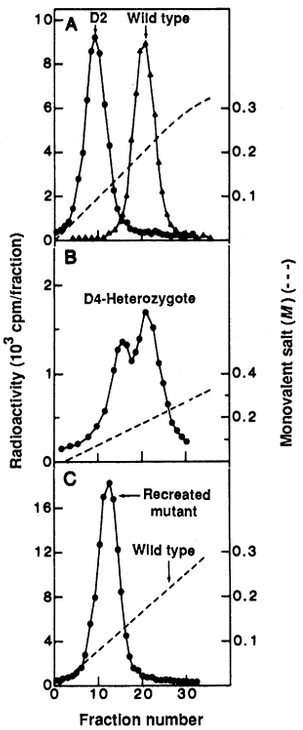

Fig. 3. DNA-cellulose elution profiles of VDR from normal individuals, members of the D kindred, and transiently transfected mutant receptor. Human Epstein-Barr virus transformed lymphocytes or transiently transfected COS-1 cells were grown in cell culture (*8, 13*), and soluble cellular extracts containing VDR were prepared (*8*) and incubated with 2 n*M* 1,25-(OH)$_2$[^{3}H]D for 3 hours at 0°C. Samples were processed as described in (*29*). (**A**) Elution profiles of wild-type VDR (triangles) and receptor from a typical patient (D2) with the classical HVDRR phenotype (circles). Analyses of the other affected individuals from the D and G kindreds similarly show early elution of the mutant VDR at 0.1*M* salt. (**B**) Profile of VDR isolated from an obligate heterozygote (HVDRR carrier) parent (D4) showing two receptor forms, one eluting at 0.2*M* salt coincident with wild-type receptor, and the other at 0.1*M* salt coincident with the elution position of VDR from the affected offspring. (**C**) Profile of deliberately mutated VDR after recreation of the site-specific mutation found in the D kindred (*17, 30*).

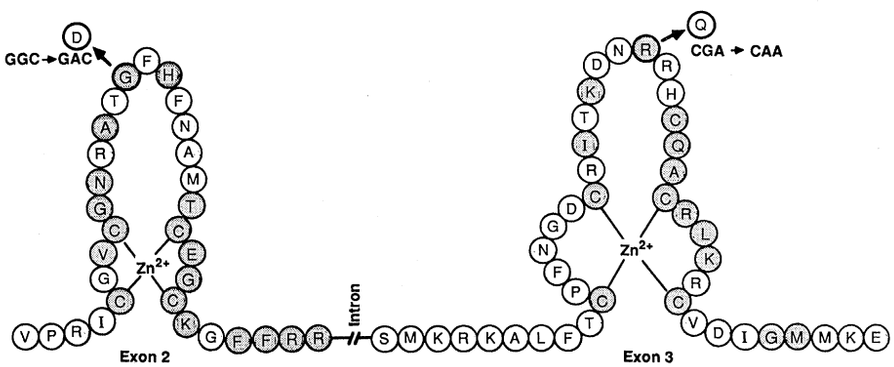

Fig. 4. Amino acid sequence and hypothetical structure of the VDR DNA-binding domain. The deduced amino acids from the VDR cDNA are shown as two potential zinc-finger arrays each encoded by separate gene exons. The conserved residues found in the other steroid and thyroid receptors (*16*) are shaded. The triplet codon change of GGC to GAC in VDR exon 2 results in a Gly^{30} conversion to Asp in the genomic DNA of G kindred members. A nucleotide transition (G to A) in VDR exon 3 replaces Arg^{70} for Gln in members of the D kindred. The residues are indicated in the diagram by the single letter abbreviations: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.

guishable from wild type. As predicted, the affinity of the mutant receptor was markedly decreased for DNA cellulose (Fig. 3C), consistent with that observed in both the affected patient (Fig. 3A) as well as the heterozygote parent (Fig. 3B). The mutant receptor eluted from DNA cellulose at approximately 0.1*M* KCl, in contrast to the normal receptor, which elutes at 0.2*M* KCl. The expressed mutant VDR from the G kindred, which contained an aspartic acid substituted for glycine, produced similar biochemical profiles. These results suggest that single nucleotide substitutions identified in the VDR gene from each of these two kindreds are responsible for the tissue resistance to $1,25\text{-}(OH)_2D_3$ and the HVDRR disease phenotype.

Steroid hormone molecules exert their effects on cellular functions via receptor proteins that contain discrete functional domains. A current model for the structure of the DNA binding domain is based on the conservation of certain hydrophobic and basic residues in an area of primary amino acid sequence rich in positionally conserved cysteine residues. A potential structural motif for the DNA binding domain in steroid receptor proteins is based on the discovery of tandemly repeated units in the transcription factor TFIIIA from *Xenopus* oocytes (*19*). The repeat domains (approximately 30 amino acids each) include paired cysteine and histidine residues which are thought to form fingerlike structures around a central zinc ion (*20, 21*). The zinc fingerlike structures are encoded by two separate exons in the chromosomal genes (Fig. 4) for both VDR (*14*) and progesterone receptor (*22*). The mutations associated with HVDRR in the patients considered here reside near the tip of each of the fingers, and emphasize the importance of these regions for functional interactions of receptor with DNA. The D-kindred mutation replaces a very basic arginine residue ($pK = 12$) for an uncharged glutamine at a prominent position in the second finger. In the G kindred, the mutation changes a neutral glycine to a negatively charged aspartic acid in the first zinc finger structure. Both arginine and glycine residues are present at these two positions in all of the presently identified members of the receptor supergene family (*16*), and this evolutionary conservation implies that these amino acids are critical to the interaction of receptor with DNA. It is unclear whether the abnormal function of these mutants is due to charge differences alone or whether the domain conformation in this critical region of the protein is altered. Point mutations of this nature may also affect protein-protein interactions such as the formation of receptor homodimers (*23*), or the interaction of receptor with other protein components of the transcriptional regulating complex (*24*), or the structural integrity of alternate motifs for DNA binding proteins (*25*).

Together with information determined for other members of the steroid-thyroid family of gene products by means of cell culture (*26*) and in vitro (*16, 27*) systems, the details of this complex process of protein-DNA interaction and receptor-mediated transcriptional regulation are beginning to emerge.

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1. This condition has been variably termed pseudovitamin D deficiency rickets, vitamin D–dependency rickets, vitamin D–resistant rickets, and vitamin D–dependent rickets type II. We prefer the name hypocalcemic vitamin D–resistant rickets (HVDRR), which emphasizes the target tissue resistance to $1,25\text{-}(OH)_2D_3$ as the fundamental biochemical defect. The reference to hypocalcemia is included to avoid confusion with X-linked hypophosphatemic rickets, a disorder of phosphate transport in the nephron and unrelated to mutations of the vitamin D receptor.
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29. Cellular extracts incubated with 2 n*M* $1,25\text{-}(OH)_2[^3H]D$ for 3 hours at 0°C were diluted with TEDZ buffer (10 m*M* tris, pH 7.4, 1 m*M* EDTA, 5 m*M* dithiotretiol, 0.3*M* $ZnCl_2$) to reduce the KCl concentration to 50 m*M*, and then added to 6 ml of 50 percent DNA-cellulose in TEDZ and incubated for 1 hour at 0°C. The DNA-cellulose, with VDR bound to it, was collected by centrifugation at 2000*g* and washed three times with TEDZ containing 1% Tween-80. The resuspended resin was used to make a column from which VDR was eluted with a 100-ml gradient of 0 to 400 m*M* monovalent salt (NaCl or KCl) in TEDZ. Fractions (2-ml) were collected, and proteins were assayed for $1,25\text{-}(OH)_2[^3H]D$.
30. Point mutagenesis of wild-type VDR cDNA was achieved with an oligonucleotide (25 nt) containing the desired base change (A for G) at nucleotide position 13. The flanking 12 bases of annealing sequence was to exon 2 (G kindred) or exon 3 (D kindred). The phosphorylated oligonucleotide primer was hybridized to full-length single (+) strand cDNA in M13mp2 vector. Second strand synthesis with T4 DNA polymerase, circular ligation, and transfection plating were performed (*18*). Colonies were screened by sequencing (*17*) a single nucleotide (A tracking) with subsequent mutant sequence confirmation of all four bases. The VDR cDNA altered by a single base was inserted into the Eco RI site of p91023(B) vector downstream of the adenovirus major late promoter. COS-1 cells were transfected with 12 μg of expression plasmid per 100-mm plate with DEAE-dextran (13). After 48 hours, cytosols from 2×10^7 cells were prepared and processed as described (*13*), and VDR was assayed for DNA-cellulose affinity by salt gradient elution.

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