The Molecular Basis of Hereditary 1,25-Dihydroxyvitamin D_3 Resistant Rickets in Seven Related Families

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Abstract

Hereditary 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] resistant rickets (HVDRR) is an autosomal recessive disease caused by target organ resistance to the action of 1,25$(OH)_2D_3$, the active form of the hormone. The defect in target cells is heterogenous and commonly appears to be a mutation in the gene encoding the vitamin D receptor (VDR). We have studied cultured skin fibroblasts and Epstein-Barr virus transformed lymphoblasts of seven family branches of an extended kindred having eight children affected with HVDRR. We have previously shown that cells from three affected children in this group contain an "ochre" nonsense mutation coding for a premature stop codon in exon 7 within the steroid-binding domain of the VDR gene.

In the current studies, we found that cells from affected children failed to bind [^{3}H]1,25$(OH)_2D_3$ and had undetectable levels of VDR as determined by immunoblots using an anti-VDR monoclonal antibody. Measurement of VDR mRNA by hybridization to a human VDR cDNA probe showed undetectable or decreased abundance of steady-state VDR mRNA. Parents, expected to be obligate heterozygotes, showed approximately half the normal levels of [^{3}H]1,25$(OH)_2D_3$ binding, VDR protein, and mRNA. The mutation at nucleotide 970 (counting from the mRNA CAP site) results in the conversion of GTAC to GTAA, which eliminates an Rsa I restriction enzyme site and facilitates identification of the mutation. We found that polymerase chain reaction (PCR) amplification of exons 7 and 8 from family members and subsequent Rsa I digestion allows detection of the specific genotype of the individuals. When Rsa I digests of PCR-amplified DNA are subjected to polyacrylamide gel electrophoresis, children with HVDRR exhibit a homozygous banding pattern with loss of an Rsa I site. Parents exhibit a heterozygotic DNA pattern with detection of both normal and mutant alleles.

In summary, our data show that the genetic abnormality is a point mutation within the steroid-binding domain of the VDR in all seven related families with HVDRR. Analysis of restriction fragment length polymorphism at the 970 locus of PCR-amplified DNA fragments can be used to diagnose this mutation in both affected children and parents carrying the disease. (*J. Clin. Invest.* 1990. 86:2071–2079.) Key words: vitamin D resistance • rickets • vitamin D receptor • genetic diseases • mutation • restriction enzyme • polymerase chain reaction

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Introduction

Hereditary 1,25-dihydroxyvitamin D resistant rickets (HVDRR)[1] is a rare autosomal recessive disease characterized by target organ resistance to the action of the hormonal form of vitamin D, 1,25$(OH)_2D_3$ (1, 2). The disease typically has its onset in early infancy with the appearance of severe rickets, hypocalcemia, secondary hyperparathyroidism, and elevated circulating levels of 1,25$(OH)_2D_3$. A frequently associated and unexplained finding in HVDRR is total scalp and body alopecia (3), a feature said to be present in the more severely affected patients (4). Studies from a number of laboratories have revealed that the cause of the target organ resistance in HVDRR is a defect in the intracellular vitamin D receptor (VDR) (5–16).

The human VDR is a 50-kD protein and the cDNA has recently been cloned and sequenced (17) and found to belong to the steroid-thyroid-retinoic acid receptor superfamily of genes (18). Like the other members of this receptor family, VDR is comprised of at least two functional domains, a steroid hormone-binding domain and a DNA-binding domain (17–23). The steroid-binding domain stretches from amino acid 182 to the COOH-terminus at amino acid 427 and provides the molecule with high affinity, steroid-specific recognition of the 1,25$(OH)_2D_3$ metabolite. The DNA-binding domain, comprising amino acids 24–89, is a cysteine-rich region that contains two putative "zinc-fingers" (17). 1,25$(OH)_2D_3$ binding to the steroid-binding domain of the receptor "activates" the VDR to a form with high affinity for DNA. The DNA-binding domain of the VDR then binds to a vitamin D response element in the regulatory region of target genes to modulate transcription of responsive genes and thereby mediates hormone action (17–23).

Progress to elucidate the molecular basis of HVDRR began with the demonstration that rodent skin possessed VDR (24, 25) and that cultured human dermal fibroblasts derived from skin biopsies could be used as a model system for the study of the VDR from patients (26). It was soon demonstrated that defects in the VDR were the likely cause of the HVDRR syndrome (5) and that different families exhibited different defects (5–16). An additional useful finding was the demonstration that 1,25$(OH)_2D_3$ could induce the enzyme 25-hydroxyvitamin D-24-hydroxylase (24-hydroxylase) in multiple target tissues by a receptor-mediated process (27). It was subsequently found that 24-hydroxylase activity could be induced in cultured skin fibroblasts so that induction of 24-hydroxylase activity could be employed as a bioassay of the ability of cultured fibroblasts to respond to 1,25$(OH)_2D_3$ (5). Using this bioresponse marker, cells from a variety of patients with HVDRR

1. *Abbreviations used in this paper:* HVDRR, hereditary 1,25-dihydroxyvitamin D_3 [1,25$(OH)_2D_3$] resistant rickets; PCR, polymerase chain reaction; VDR, vitamin D receptor.

were shown, in vitro, to be resistant to the action of $1,25(OH)_2D_3$ (5, 8, 9, 11, 12, 15).

Multiple cases of HVDRR have now been investigated and the heterogeneous nature of the defect has become apparent as several types of abnormality in the VDR have been described (5–16). The most common defect has been undetectable [^{3}H]-$1,25(OH)_2D_3$ binding (5, 6, 9, 12). This phenotype could be due either to absent VDR or to defects affecting the steroid-binding domain of the VDR rendering it inactive in a ligand binding assay. A second type of defect, which is currently the best documented, is an abnormality in the DNA-binding domain of the VDR (11, 15, 16). The phenotype in these cases is normal [^{3}H]$1,25(OH)_2D_3$ binding but abnormal affinity of the $1,25(OH)_2D_3$-VDR complex for DNA (11, 15). Other types of VDR defects have been suggested (12–14) and more will no doubt be demonstrated as additional families are investigated.

In this paper, we describe an extended kindred with seven related family branches having eight children affected with HVDRR. Complex consanguinity is present among the families, there being multiple documented marriages between related individuals with other intermarriages likely. This inbreeding has led to an increased prevalence of an otherwise rare recessive disease. The mutation causing HVDRR in this extended kindred appears to be the same in all affected children and results in undetectable VDR by [^{3}H]$1,25(OH)_2D_3$ binding analysis. In a companion paper published elsewhere (28), we described the results of DNA sequence studies accomplished on polymerase chain reaction (PCR)-amplified DNA from affected children and parents in three of these families. The defect was found to be a point mutation causing a single C to A base substitution at nucleotide 970 (counting from the CAP site) in exon 7, which results in the conversion of the codon for tyrosine (TAC) into a premature stop codon (TAA) at amino acid 292. This "ochre" nonsense mutation causes a truncation of the VDR protein eliminating the translation of a large portion of the steroid-binding domain. In this paper we describe the pedigree of this extended kindred, the clinical findings and the course of the disease, and analyses of [^{3}H]$1,25(OH)_2D_3$ binding, VDR immunoblots (Western blots) and VDR mRNA hybridizations (Northern blots) in most of the families. Finally, identification of the same mutation by restriction enzyme mapping is demonstrated in all of the families except the one from which cells are not available.

Methods

Culture conditions. Fibroblasts derived from skin biopsies were grown on minimal essential medium containing 10% calf serum-iron supplemented (Hyclone Laboratories, Logan, UT). EBV transformed lymphoblasts were maintained on RPMI medium containing 10% serum.

Preparation of cytosol, ligand binding, and Western blotting. Fibroblasts and/or EBV-lymphoblasts were harvested and washed twice with PBS by centrifugation at 2,000 *g* for 5 min. Cell pellets were resuspended in KTEDM buffer (0.3 M KCl, 0.01 M Tris pH 7.4, 0.005 M EDTA, 0.005 M dithiothreitol, and 0.01 M sodium molybdate) and 200 U aprotinin added. Cells were disrupted by sonication on ice and cell extracts prepared by ultracentrifugation at 200,000 *g* for 30 min at 4°C.

For ligand binding, cytosols were diluted to a protein concentration of $\approx$ 1 mg/ml with KTEDM and incubated with 1 nM [^{3}H]-$1,25(OH)_2D_3$ in the presence or absence of a 500-fold excess of radioinert hormone. After 3 h on ice, bound and free steroid were separated by hydroxylapatite (9, 11, 15).

For Western blot analysis, cell extracts were electrophoresed on 10% polyacrylamide gel (acrylamide:bisacrylamide, 30.8:1) containing 0.1% SDS in the buffer system described by Laemmli (29). After electrophoresis, the proteins were transferred to nitrocellulose in the carbonate buffer system described by Dunn (30). The blots were then probed sequentially with the anti-VDR rat monoclonal antibody 9A7, rabbit anti-rat IgG and ^{125}I-labeled protein A (100,000 cpm/ml) as previously described (15).

Preparation of RNA and Northern blot analysis. Total cellular RNA was prepared by the LiCl, urea method of Auffray and Rougeon (31). Briefly, cells were collected and washed twice with PBS resuspended in 6 M urea, 3 M LiCl, and then homogenized for 15 s using a Polytron (Brinkmann Instrument Co., Westbury, NY). Homogenates were placed on ice for > 24 h and then the RNA was collected by centrifugation at 15,000 *g* for 30 min at 4°C. RNA pellets were resuspended in 0.01 M Tris pH 7.2, 0.001 M EDTA, and 0.1% SDS and extracted twice with an equal volume of phenol: chloroform:isoamylalcohol (25:24:1). The RNA was precipitated with one-half volume 7.5 M ammonium acetate and 2.5 vol ethanol and then resuspended in sterile water. Northern blotting was performed as described by Fourney et al. (32) with modifications. RNA samples were mixed with 3 vol of a solution composed of 500 μl 100% formamide, 175 μl 37.5% formaldehyde, and 100 μl 10× MOPS/EDTA buffer (1× MOPS/EDTA buffer: 0.04 M MOPS [3-(*N*-morpholino) propane sulfonic acid], 0.01 M sodium acetate, 0.001 M EDTA adjusted to pH 7.0) and denatured at 65°C for 15 min. After cooling, samples were supplemented with 1 μl of 1 mg/ml ethidium bromide plus loading dye and then applied to a 1% agarose gel prepared in 1× MOPS/EDTA buffer, pH 7.0, 0.66 M formaldehyde. The running buffer was 1× MOPS/EDTA buffer. Gels were electrophoresed at 80 V constant for 5 h at ambient temperature. After electrophoresis, gels were photographed, rinsed twice in 10× SSC for 20 min each, and the RNA transferred to nylon (Hybond; Amersham Corp., Arlington Heights, IL) in 10× SSC. After transfer, the nylon was baked at 80°C in vacuo for 2 h and then prehybridized in 7% SDS, 10% dextran sulfate, 0.5 M sodium phosphate, pH 7.2 at 60°C overnight.

To probe the blots, a 2.1-kb Eco RI DNA fragment containing the entire coding sequence for the human VDR (17) was labeled with [^{32}P]dATP by the random-prime method according to the manufacturer's instruction (Boehringer Mannheim, Inc., Indianapolis, IN) and $1–2 \times 10^6$ cpm/ml were added directly to the prehybridization solution. The blots were hybridized at 60°C for 16 h. After hybridization, the blots were washed as follows: once for 10 min in 2× SSC at 24°C; twice for 30 min in 2× SSC, 1% SDS at 60°C, and once for 30 min in 0.1× SSC, 1% SDS at 24°C. Blots were exposed to XAR film at −70°C and developed.

PCR and Rsa I restriction digest. The structural portion of the VDR is encoded by eight exons spanning 39 kb of genomic DNA (Kesterson, R. A., and J. W. Pike, unpublished results). As previously reported (28), the mutation in this kindred is found in exon 7. Exons 7 and 8 were amplified together by the PCR according to the manufacturer's instructions (Perkin-Elmer/Cetus Corp., Norwalk, CT). Genomic DNA (500 ng) obtained from cultured lymphoblasts or fibroblasts of available family members was used as a template. A pair of primers was designed to anneal to the intron-flanking regions of exon 7 and 8 at a site located 10–20 bp from the intron-exon boundaries (16). Oligonucleotides 7a (5′-GCGAATTCCGTTACTGGTAACCTGACCTCT-TC-3′) and 8b (5′-TGGAATTCATACACCCCGCTCCCCAGTCCC-TGAG-3′) were employed (see Fig. 4 *A*). The DNA was amplified with a 1-min denaturation at 94°C, a 1.5-min primer annealing at 55°C, and a 3-min polymerase extension at 72°C for 30 cycles. The PCR product was either purified by NENsorb (New England Nuclear, Boston, MA) affinity chromatography and directly sequenced (33), or it was subcloned into vector pGEM 7Zf- (Promega Biotec, Madison, WI) and sequenced by dideoxynucleotide chain termination (34).

Rapid diagnostic identification of the single-point mutation in VDR exon 7 was accomplished by Rsa I restriction enzyme digestion of the PCR-amplification product. The DNA sample was ethanol precipitated, resuspended in 25 μl H_2O, and an aliquot (5 μl) was digested

with Rsa I. Digestion products were analyzed on a standard polyacrylamide (6%) gel stained with silver stain (Bio-Rad Laboratories, Richmond, CA). Hae III digested $\phi \times 174$ DNA was used as a molecular size marker.

Results

Patients. The subjects of our study are a group of seven related Arab families living in a village near Haifa, Israel. The families are designated *C, E, F, H, J, K,* and *L* in the sequence in which their clinical disease was recognized and/or their cells were obtained for analysis. The pedigree showing the interrelationships of the seven families is illustrated in Fig. 1. Several additional consanguineous marriages are suspected but not certain. The male individuals in generation *I* and *III* (Fig. 1, asterisks) are probably brothers. There is no known relationship between either K_2 or J_4 lineages and the other families. Some description of the *C* family (5, 9) and the *H* family (35) has been reported previously. The affected children in all families had a similar phenotypic appearance and clinical presentation. Rickets and complete alopecia were noted before or by 3 mo of age. The clinical pattern in all cases included: hypocalcemia (serum calcium < 2.0 mmol/liter [< 8.0 mg/dl]; normal 2.18–2.62 mmol/liter [8.7–10.5 mg/dl]), hypophosphatemia (serum phosphorous < 1.13 mmol/liter [< 3.5 mg/dl] with a few cases between 1.13–1.29 mmol/liter [3.5–4.0 mg/dl]; normal for children > 1.29 mmol/liter [> 4 mg/dl]), elevated alkaline phosphatase (> 1,000 IU; normal 125–375), secondary hyperparathyroidism (serum PTH 136–174 μU/ml, normal 20–100 μU/ml) and elevated $1,25(OH)_2D_3$ levels before treatment (> 480 pmol/liter [> 200 pg/ml]; normal 48–96 pmol/liter [20–40 pg/ml]). All affected children developed severe dental caries by 2 yr of age (36).

The parents of the HVDRR children are all obligate heterozygotes (i.e., carriers of the defective VDR gene), but appeared to be phenotypically normal exhibiting no evidence of bone disease or alopecia and having normal blood chemistries. Serum $1,25(OH)_2D_3$ levels were within the normal range in those parents in whom it has been measured ($n = 5$).

All of the HVDRR children were originally treated with oral calcium supplements and supratherapeutic amounts of $1\alpha(OH)D_3$ (up to 60 $\mu g/m^2/d$). Serum $1,25(OH)_2D_3$ levels were substantially elevated by the hormone treatment in all cases, in some exceeding 1,500 pg/ml. In none of these children did the clinical picture appear to respond to the treatment regime. However partial improvement, apparently spontaneous, occurred at 7 yr of age in the older child of the *C* family (10). Over the past 3 yr, six of the children have been treated with intravenous calcium infusions on a daily basis (37), as reported by Balsan et al. (38) and Bliziotes et al. (39). The children treated for longer than 1 yr (C_1, C_2, and E_1), have shown remarkable clinical improvement with acceleration of growth, healing of rickets on x ray, normalization of hypocalcemia and hypophosphatemia, and disappearance of secondary hyperparathyroidism (37). The affected children in the *F* and *H* families are still improving after ~ 1 yr of IV calcium infusions while the *J* family child has recently been started on treatment. The *K* and *L* children will receive similar treatment as they reach the age of 2 yr. Although the rickets dramatically improved with calcium infusion, the alopecia has thus far not changed.

VDR binding studies. Available tissue for evaluation of these families has included either cultured skin fibroblasts or E-B virus transformed lymphoblasts. Fibroblasts classically have been used for this purpose (5–12, 14, 15). However, immortalized lymphoblasts have the advantage of being more

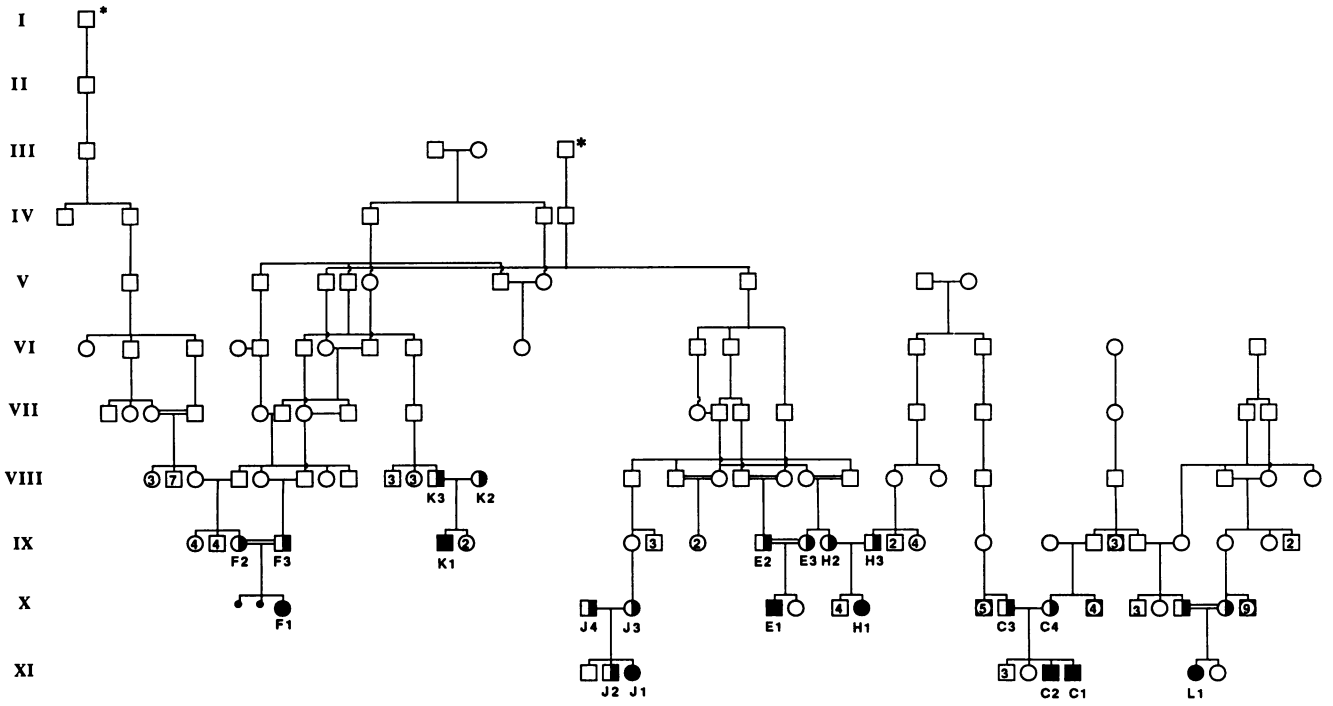

Figure 1. Pedigree of *C, E, F, H, J, K,* and *L* families. Affected children with HVDRR and alopecia are indicated by solid symbols (■), presumed heterozygotic parents by half-solid symbols (◨), and consanguineous marriages by double lines (=). (*)Indicate probable brothers in generation I and III.

convenient to obtain from patients, of growing faster, and of not being limited by a fixed number of cell divisions. We have introduced the lymphoblast system more recently and it appears to provide information similar to the fibroblasts on VDR status of HVDRR patients and family numbers (16).

As shown in Table I, the HVDRR patients thus far analyzed for [^{3}H]1,25(OH)$_2$D$_3$ binding showed low abundance of VDR near the level of detectability, i.e., not appreciably different from background levels. This was the case whether fibroblasts or lymphoblasts were examined. There was no change in VDR levels in cultured cells either after spontaneous clinical improvement or after treatment with prolonged calcium infusion. The parents, who are all expected to be heterozygous in this recessive disease, showed VDR abundance ranging from the lower limits of normal to approximately half-normal or less. It is of interest that heterozygotes showed approximately half normal values in lymphoblasts and values within the normal range in fibroblasts. In all cells of the obligatory heterozygotes in which it has been tested, the affinity of the VDR for [^{3}H]1,25(OH)$_2$D$_3$, measured by Scatchard analysis, has been within the normal range.

Western blot analysis. As shown in Figs. 2 and 3, the normal VDR can be detected in extracts of either fibroblasts (Fig. 2 *A*) or lymphoblasts (Fig. 3 *A*) by the monoclonal antibody 9A7 as a 50-kD band on immunoblots. However, other proteins are also detected by this antibody, presumably because cross reactive epitopes are revealed under denaturing conditions of PAGE. As can be seen in the figures, no 50-kD VDR band was detected in any of the HVDRR children. The mutation in this kindred has been shown to be a premature termination codon at amino acid 292 (28). This defect would be expected to result in a truncated VDR that would migrate at 32 kD (28). Specifically, no band representing this shortened VDR is detectable. It is of interest that the heterozygotic parents show approximately the same abundance of VDR protein on immunoblot as by ligand binding. This varies from less than 50% to close to the normal range.

The recreated mutant protein was over expressed in transfected CV-1 cells and the truncated protein could be demonstrated by immunoblot as a 32-kD band as predicted from the site of the mutation (28). The absence of the truncated protein in cell extracts from homozygotes and heterozygotes is of interest and the possible reasons will be reviewed in the Discussion section below.

Northern blots. Northern blots to measure steady-state levels of VDR mRNA demonstrated low abundance or no detectable hybridizing bands in children with HVDRR (Figs. 2 *B* and 3 *B*). Heterozygotic parents showed the normal sized 4.4-kb VDR mRNA (17), but at decreased abundance. The amount of VDR mRNA which was detected was roughly comparable to the magnitude of VDR demonstrated by both ligand binding (Table I) and Western blot (Figs. 2 *A* and 3 *A*). In the case of one family (the *F* Family), mRNA was detectable on Northern blot from the affected child (F_1) and was of nor-

Table I. Summary of Data Obtained from Cells of Patients with HVDRR and Family Members

Family designation*	Phenotype‡	[^{3}H]1,25(OH)$_2$D$_3$ Binding‖		Western¶	Northern¶	Sequence**	Restriction analysis‡‡
		Fibroblasts	Lymphoblasts				
	Normal	20–45	20–30	++++	++++	TAC	n
C_1 male '75	HVDRR	2	2.6	ND	ND	TAA	m
C_2 male '80	HVDRR	—	2.4	ND	ND	TAA	m
C_3 father	nl	13	6.6	+	+	TAC/TAA	n/m
C_4 mother	nl	—	13.5	++	++	TAC/TAA	n/m
E_1 male '82	HVDRR	—	5.0	ND	ND	TAA	m
E_2 father	nl	—	9.0	++	++	—	—
E_3 mother	nl	—	—	—	—	TAC/TAA	n/m
F_1 female '86	HVDRR	—	4.5	ND	+	TAA	m
F_2 mother	nl	—	10.5	+++	+++	TAC/TAA	n/m
H_1 female '73	HVDRR	—	4.8	ND	ND	TAA	m
H_3 father	nl	—	9.5	++	+	TAC/TAA	n/m
J_1 female '87	HVDRR	3	—	ND	ND	TAA	m
J_2 male	nl	26	—	++++	++++	TAC/TAA	n/m
J_3 mother	nl	22	—	++++	++	—	n/m
J_4 father	nl	24	—	++++	+++	—	n/m
K_1 male '88	HVDRR	—	—	—	—	—	—
K_2 mother	nl	22	—	—	—	—	n/m
K_3 father	nl	28	—	—	—	—	n/m
L_1 female '87	HVDRR	—	—	—	—	—	—

* Designation refers to pedigree shown in Fig. 1 and includes year of birth of affected children. ‡ Phenotype based on clinical impression. nl, normal. ‖ [^{3}H]1,25(OH)$_2$D$_3$ binding, expressed in fmol/mg protein from cultured dermal fibroblasts or EBV transformed lymphoblasts. ¶ Western blot data evaluating VDR protein and Northern blot data evaluating VDR mRNA expressed semiquantitatively on a + to ++++ scale. ND indicates not detectable. ** Sequence refers to DNA nucleotide sequence performed on PCR amplified DNA from lymphoblasts at codon 292 of exon 7. TAC codes for Tyr while TAA is a termination codon (ochre) signaling a premature stop in translation. TAC/TAA indicates a heterozygotic pattern with both normal and mutant alleles. ‡‡ Restriction analysis refers to the banding pattern found when exons 7 and 8 were subjected to digestion with Rsa I. The normal (n) bands are 201 and 101 bp and the mutant (m) band is 302 bp. n/m is the heterozygotic pattern indicating both n and m alleles. — Indicates cells not available or not tested.

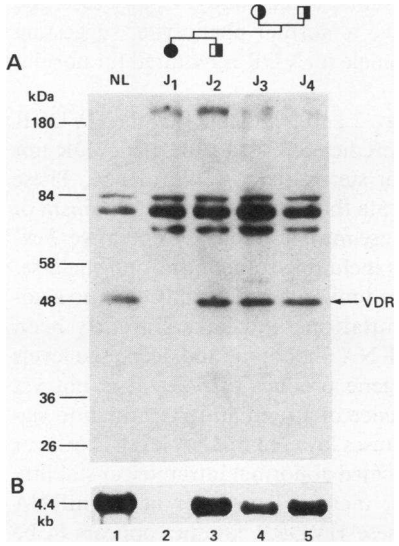

Figure 2. Western blot and Northern blot analyses of cell extracts from fibroblasts of HVDRR children and parents. (*A*) KTEDM extracts (100 μg protein) from fibroblasts were resolved by SDS-PAGE and electrophoretically transferred to nitrocellulose filters. The filters were sequentially incubated with 9A7 anti-VDR rat monoclonal antibody, rabbit anti-rat immunoglobin, and [^{125}I] protein A. Immunoreactive bands were visualized by autoradiography. Normal size VDR (50 kD) was detected in the normal control (lane *1*), in the unaffected sibling J_2 (lane *3*), and in the parents J_3 and J_4 (lanes *4* and *5*). No protein was detected in the HVDRR affected child J_1 (lane *2*). (*B*) Total RNA (20 μg RNA) was size fractionated on 1% agarose gels containing 0.66 M formaldehyde and transferred to nylon filters in 10× SSC. Filters were hybridized to a ^{32}P-labeled human VDR cDNA probe and washed as described in Methods. Lane *1* shows a 4.4-kb hybridizing band in the normal control sample and serves as a standard. VDR mRNA was undetectable in J_1 HVDRR sample (lane *2*), while the unaffected sibling J_2 and the parents' cells all had normal levels of VDR mRNA (lanes *3, 4,* and *5*). The integrity and equal loading of the RNA was determined by ultraviolet illumination of stained gels (data not shown).

mal size but decreased abundance. To determine whether the VDR mRNA in the other families was absent or merely reduced below the level of detectability, PCR amplification was used on the RNA of the HVDRR child (E_1) in the *E* Family. The amplified RNA, probed with VDR cDNA, showed the expected 4.4-kb band on RNA blots indicating that the mRNA was present but in decreased amounts (data not shown). The decreased level of VDR mRNA, as the decreased or absent truncated protein, would not be expected from the demonstrated nonsense mutation (28). As mentioned above for the absent truncated protein, the possible causes of this finding will be examined in the Discussion section.

Restriction enzyme mapping. The genetic defect in these families is the conversion of the nucleotide sequence GTAC to GTAA at 970 bp (exon 7) of VDR (28). This change introduces an ochre nonsense mutation into the VDR gene and eliminates a restriction enzyme recognition site. The enzyme Rsa I cuts at the GTAC sequence, which is altered by the mutation. As shown in Fig. 4, this change in DNA sequence provides another tool to detect the presence of the mutation using restriction enzyme mapping techniques. Genomic DNA from available family members was subjected to PCR amplification using oligonucleotide primers which flank the area of interest (Fig. 4 *A*). The amplified DNA product was restricted with Rsa I and the enzyme digest subjected to polyacrylamide gel electrophoresis. Wild-type DNA (Fig. 4 *B*, lane *2*) shows a pattern of four bands at 201, 195, 101, and 76 bp. This indicates there are three Rsa I sites in the normal DNA fragment which is confirmed by examining the known DNA sequence of this region (Fig. 4 *A*) (17). The mutation at nucleotide 970 deletes one of the three Rsa I sites in the DNA fragment so that patients homozygous for the mutant allele exhibit only three

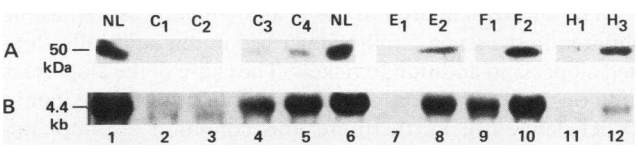

Figure 3. Composite of Western blot and Northern blot analyses of cells extracts from EBV lymphoblasts of HVDRR children and parents. (*A*) Western blot. (*B*) Northern blot. Methods were the same as described in Fig. 2. VDR protein and mRNA were undetectable in all affected children (C_1, C_2, E_1, and H_1) except for mRNA in F_1. The parents (C_3, C_4, E_2, F_2, and H_3) all exhibited detectable VDR protein and mRNA. A normal control (*NL*) is shown in lanes *1* and *6*.

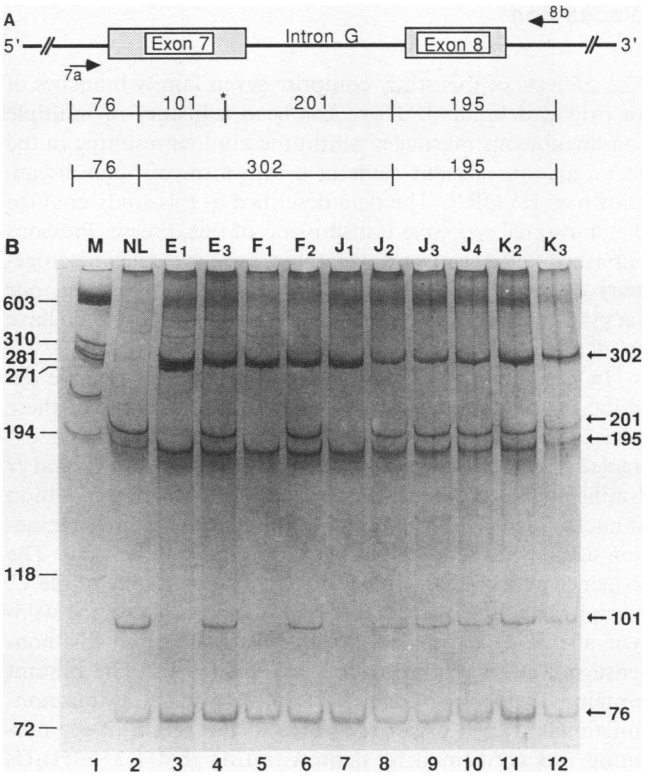

Figure 4. Restriction enzyme mapping of PCR amplified DNA from HVDRR family members. (*A*) Schematic representation of Rsa I digestion showing the genomic DNA and the Rsa I sites in the mutant (*lower*) and wild-type (*upper*) genes. The DNA was amplified by PCR using oligonucleotide primers 7a and 8b (*arrows*) to generate a 573-bp fragment. *Site of the mutation (GTAC to GTAA) in exon 7 obliterating a Rsa I site. (B) Polyacrylamide gel electrophoresis of PCR-generated DNA after Rsa I digestion. Digestion of normal (*NL*) PCR product with Rsa I yields four DNA fragments at 201, 195, 101, and 76 bp in lane *2*. DNA from patients with HVDRR show three diagnostic bands at 302, 195, and 76 bp in lanes *3* [E_1], *5* [F_1], and *7* [J_1]. Heterozygous parents in lanes *4* [E_3], *6* [F_2], *9* [J_3], *10* [J_4], *11* [K_2], and *12* [K_3] yield a banding pattern consistent with the presence of both mutant and wild-type alleles. Patient J_2, an unaffected sibling, was found to be a heterozygote by Rsa I digestion (lane *8*). Lane *1* shows Hae III digest of φX174 DNA which serves as size marker.

bands, at 302, 195, and 76 (Fig. 4 *B*, lanes *3, 5,* and *7*). On the other hand, the heterozygotes have both normal and mutant alleles present so that they exhibit five bands: one representing the mutant allele band at 302 and the four wild-type allele bands at 201, 195, 101, and 76 bp (Fig. 4 *B*, lanes *4, 6,* and *8–12*).

In the case of the *K* family, only cells from the parents (K_2 and K_3) were available for study. Restriction enzyme digests of PCR-amplified DNA identified these parents as obligate heterozygotes exhibiting the TAC/TAA genotype (Fig. 4 *B*, lanes *11* and *12*). The presence of both the normal and mutant genes allows us to predict that the child affected with HVDRR (K_1) in this family has the identical mutation as the other members of this extended kindred. The data from the *C* and *H* families is not shown. No cells have yet been obtained from members of the *L* family, but because of the relationships to the other families (see Fig. 1), we assume that the affected child with HVDRR (L_1) has the same genetic abnormality as the other members of the kindred.

Discussion

The subjects of this study comprise seven family branches of an extended kindred. There has been a history of multiple consanguineous marriages within the kindred resulting in the accumulation of eight cases of a rare form of bone disease known as HVDRR. The data described in this study confirm the autosomal recessive transmission of this disease. Previous published findings in various families have indicated heterogeneity of the defects causing HVDRR (5–16). However, our data indicate that all of the cases in this extended kindred have an identical defect.

In a companion paper published elsewhere (28), we describe the elucidation of the genetic defect in three of these cases. DNA sequence analysis of PCR-amplified DNA extracted from EBV transformed B-lymphocytes in *C, E,* and *H* families revealed the presence of a nonsense (ochre) mutation at nucleotide 970. This mutation causes a premature termination signal within the steroid-binding domain of the VDR. The sequence analysis confirmed the heterozygotic state of the *C, E,* and *H* parents by detecting both the mutant and the wild-type alleles. The truncated VDR that results from this nonsense mutation was expressed in COS-1 cells. The mutant protein was unable to bind [^{3}H]1,25(OH)$_2$D$_3$ and is thus nonfunctional. Target organ resistance to the action of the hormone was confirmed by demonstrating that 1,25(OH)$_2$D$_3$ treatment was unable to activate cells cotransfected with the recreated mutant VDR and a plasmid containing a VDR responsive element linked to a reporter gene. In contrast, the cells transfected with wild-type VDR, when treated with 10 nM 1,25(OH)$_2$D$_3$, successfully activated gene transcription in this experiment (28).

The [^{3}H]1,25(OH)$_2$D$_3$ ligand-binding data have been performed in either lymphoblasts or fibroblasts (Table I). In all cases, the cells from HVDRR cases show a negligible number of VDR sites. The heterozygotic parental cells differ somewhat, with lymphoblasts showing the expected level of approximately one-half the number of receptors, whereas the fibroblasts have higher values approaching or within the normal range. We have no explanation for this phenomenon in heterozygotic cells, but speculate that the VDR in fibroblasts is upregulated (40), while in the lymphoblasts they are not. This interesting difference warrants further study. In any case, the heterozygotic parents have a normal phenotype, suggesting that only a single normal allele for VDR is required for normal 1,25(OH)$_2$D$_3$ function.

The data shown in Figs. 2 and 3 indicate that the HVDRR cells contain neither the predicted 32-kD truncated VDR nor the normal abundance of steady-state VDR mRNA. These unexpected findings indicate that an additional mechanism of control beyond the nonsense mutation must be operative. Several other genetic diseases including some forms of β-thalassemia, Tay-Sachs disease, and muscular dystrophy are also associated with nonsense mutations and have similarly been shown to exhibit a low mRNA phenotype and decreased levels of the encoded protein gene product (41–43). It is not yet understood why the presence of a premature termination signal in the VDR mRNA causes lowered mRNA levels, however several studies have implicated abnormal intranuclear stability of the mutant mRNA or increased turnover of the mRNA (41–43). The defect in these HVDRR families appears to be similar in this regard. The nonsense mutation causes a truncated and nonfunctional VDR protein and leads to decreased accumulation of the defective mRNA. Any abnormal VDR protein that might be translated probably would be subjected to an accelerated turnover rate as well.

In previous analyses of two other unrelated kindreds with HVDRR from our laboratories (11, 15), a different genetic defect was demonstrated (16). In those families, designated *D* and *G*, the subject's clinical appearance was similar to the patients described in this paper, however analysis of VDR in cultured cells revealed a different type of defect. In contrast to the *C, E, F, H, J, K, L* families, the affected children in the *D* and *G* families had normal abundance of VDR determined by normal ligand binding. Moreover, the VDR was normal sized based on Western blots and sucrose gradients (11, 15). However, we were able to detect an abnormally low affinity of the 1,25(OH)$_2$D$_3$-VDR complex for DNA as demonstrated by VDR elution profile from DNA-cellulose (11, 15). This led to the prediction that the defect would be located within the DNA binding domain of the VDR. Indeed, sequence analysis of PCR-amplified DNA from cells of *D* and *G* family members revealed missense mutations within exons 2 and 3 which encode the putative zinc-finger structures in the DNA binding domain of the VDR (16). The mutation in the *G* family resulted in a Gly (GGC) changed to Asp (GAC) at amino acid 30 at the tip of the first zinc-finger, while the *D* family mutation resulted in Arg (CGA) changed to a Gln (CAA) at amino acid 70 at the tip of the second zinc-finger. In both cases the changes occurred in amino acids that are highly conserved across the steroid receptor gene superfamily (18), suggesting that these amino acids play an essential role in the function of the DNA-binding domain of the VDR.

The affected children in the *D* and *G* family as well as the children in the seven families described in this study all exhibited alopecia in addition to rickets. The cause of the alopecia is unknown but it is not associated with other forms of vitamin D deficiency (3). Furthermore, this condition does not improve when the defect in calcium metabolism and the rickets respond to treatment or improve spontaneously. Immunocytochemistry of the hair follicle has demonstrated the extensive presence of VDR indicating that this structure is likely a 1,25(OH)$_2$D$_3$ target tissue (44). It is also clear that among its bioresponses, 1,25(OH)$_2$D$_3$ induces the differentiation of various tissues including the skin (45, 46). One possible explana-

tion for the alopecia is an ectodermal defect due to absent $1,25(OH)_2D_3$ action during a critical stage of hair follicle development that precludes subsequent hair growth from the follicle.

In recent years the VDR has been identified in a number of new target tissues and the scope of hormone action has been extended beyond calcium metabolism (47). Target organ systems include various endocrine organs and the immune system. It is therefore of interest to note that children with HVDRR appear to have normal physiology except for their abnormal calcium homeostasis (48). Careful analysis has detected defective leukocyte fungicidal activity (49) and hematopoietic cell function (50), but there is no evidence of a clinically relevant abnormality. It is not clear whether this means that $1,25(OH)_2D_3$ is relatively unimportant in endocrine gland function or immunomodulation, or that other mechanisms take over and compensate for the lack of $1,25(OH)_2D_3$ action.

It is important to note that all of the metabolic and bone abnormalities appear to be reversed when the HVDRR patients are treated with IV calcium infusions (37–39). This suggests that the major mechanism by which $1,25(OH)_2D_3$ regulates bone formation is via its action on the intestine to absorb calcium. The implication is that, if mineral supply to the bone is normal, $1,25(OH)_2D_3$ action on bone cells may not be required for normal bone formation. This speculation is consistent with studies showing normal bone formation in vitamin D-deficient rats infused with calcium and phosphorous (51) and in the mostly catabolic bioresponses of $1,25(OH)_2D_3$ in isolated bone cells (52). However, detailed examination of the bone from HVDRR patients over a prolonged time course of therapy will be necessary before this hypothesis is substantiated.

It is of interest that PTH levels and other evidence of secondary hyperparathyroidism are corrected by normalization of mineral balance subsequent to intravenous calcium infusion. This supports the view that $1,25(OH)_2D_3$ action on the parathyroid gland is not essential to suppress PTH levels and that normalization of the serum calcium is sufficient to accomplish inhibition of parathyroid gland function.

Once the genetic abnormality in three of these families was elucidated by DNA sequence analysis (28), it became apparent that rapid detection of the mutation could be accomplished by restriction enzyme mapping of PCR-generated DNA. Similar approaches have been used in other genetic diseases to identify carriers (53, 54). In 9 of 14 parents of affected children that were tested by restriction enzyme mapping in this HVDRR extended kindred, all were shown to be heterozygotes as expected. The genotype of one asymptomatic sibling, J_2, could not be identified unambiguously as either normal or heterozygotic by analysis of the pedigree, phenotype or VDR receptor studies. Enzyme restriction mapping indicates that he is a heterozygote having both the normal and mutant VDR alleles.

As molecular techniques are applied to genetic diseases such as the steroid hormone-resistant states, substantial new information about the mechanism of hormone action will be elucidated. Our studies on HVDRR have demonstrated both nonsense and missense mutations as the basis of hormone resistance. These approaches applied to glucocorticoid (55), androgen (56–58), mineralocorticoid (59), and thyroid (60, 61) resistant patients should similarly provide further insight into additional mechanisms by which a mutation can inactivate the hormone-receptor system and cause clinical disease.

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