

INACTIVATING MUTATIONS IN THE 25-HYDROXYVITAMIN D₃ 1 α -HYDROXYLASE GENE IN PATIENTS WITH PSEUDOVITAMIN D-DEFICIENCY RICKETS

SACHIKO KITANAKA, M.D., KEN-ICHI TAKEYAMA, M.S., AKIKO MURAYAMA, M.D., TAKASHI SATO, M.S.,
KATSUZUMI OKUMURA, PH.D., MASAHIRO NOGAMI, M.S., YUKIHIRO HASEGAWA, M.D., HIROO NIIMI, M.D., PH.D.,
JUNN YANAGISAWA, PH.D., TOSHIKI TANAKA, M.D., PH.D., AND SHIGEAKI KATO, PH.D.

ABSTRACT

Background Pseudovitamin D-deficiency rickets is characterized by the early onset of rickets with hypocalcemia and is thought to be caused by a deficit in renal 25-hydroxyvitamin D₃ 1 α -hydroxylase, the key enzyme for the synthesis of 1 α ,25-dihydroxyvitamin D₃.

Methods We cloned human 25-hydroxyvitamin D₃ 1 α -hydroxylase complementary DNA (cDNA) using a mouse 1 α -hydroxylase cDNA fragment as a probe. Its genomic structure was determined, and its chromosomal location was mapped by fluorescence in situ hybridization. We then identified mutations in the 1 α -hydroxylase gene in four unrelated patients with pseudovitamin D-deficiency rickets by DNA-sequence analysis. Both the normal and the mutant 1 α -hydroxylase proteins were expressed in COS-1 cells and were assayed for 1 α -hydroxylase activity.

Results The gene for 25-hydroxyvitamin D₃ 1 α -hydroxylase was mapped to chromosome 12q13.3, which had previously been reported to be the locus for pseudovitamin D-deficiency rickets by linkage analysis. Four different homozygous missense mutations were detected in this gene in the four patients with pseudovitamin D-deficiency rickets. The unaffected parents and one sibling tested were heterozygous for the mutations. Functional analysis of the mutant 1 α -hydroxylase protein revealed that all four mutations abolished 1 α -hydroxylase activity.

Conclusions Inactivating mutations in the 25-hydroxyvitamin D₃ 1 α -hydroxylase gene are a cause of pseudovitamin D-deficiency rickets. (N Engl J Med 1998;338:653-61.)

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HEREDITARY pseudovitamin D-deficiency rickets, also known as vitamin D-dependent rickets type I, is characterized clinically by hypotonia, weakness, growth failure, and hypocalcemic seizures in early infancy.¹ The patients also have hypocalcemia, radiologic findings typical of rickets, elevated serum parathyroid hormone concentrations, and generalized aminoaciduria.^{2,3} It is inherited as an autosomal recessive trait.

Two other inherited types of rickets are known,⁴ and the mutated genes in patients with these diseases have been identified. The diseases are hypocalcemic vitamin D-resistant rickets, also known as type II vitamin D-dependent rickets, in which the gene for the vitamin D receptor is mutated,⁵⁻⁷ and X-linked

hypophosphatemic vitamin D-resistant rickets, in which the *PEX* gene (phosphate-regulating gene with homology to endopeptidases on the X chromosome) is mutated.⁸ In contrast, the molecular basis of pseudovitamin D-deficiency rickets has remained unclear, although the disease locus has been mapped to chromosome 12q14 by linkage analysis.⁹

Pseudovitamin D-deficiency rickets is distinguished from other types of hereditary rickets in that affected patients have low serum concentrations of 1 α ,25-dihydroxyvitamin D and normal or high concentrations of 25-hydroxyvitamin D.^{10,11} In these patients, physiologic doses of 1 α ,25-dihydroxyvitamin D₃ but massive doses of vitamin D or 25-hydroxyvitamin D₃ are required to cure the rickets.¹² These findings suggest that the activity of renal 25-hydroxyvitamin D₃ 1 α -hydroxylase,^{13,14} which converts 25-hydroxyvitamin D₃ to 1 α ,25-dihydroxyvitamin D₃, is defective in patients with pseudovitamin D-deficiency rickets. Whether this defect is due to abnormalities in the 25-hydroxyvitamin D₃ 1 α -hydroxylase gene or to genetic abnormalities in other factors required for the full activity of this enzyme is unknown.^{13,15} Since direct enzymatic measurement of 1 α -hydroxylase activity in these patients is difficult, the human 1 α -hydroxylase gene was cloned in the present study to investigate genetic mutations in patients with pseudovitamin D-deficiency rickets.

Using a novel vitamin D receptor-mediated expression cloning method,¹⁶ we recently cloned 1 α -hydroxylase complementary DNA (cDNA) from the kidneys of vitamin D receptor-deficient knockout mice in which the 1 α -hydroxylase gene was overexpressed.¹⁷ In the present study, we cloned human 1 α -hydroxylase cDNA, determined the structure of the gene and the chromosomal locus, and analyzed whether this gene is mutated in patients with pseudovitamin D-deficiency rickets.

From the Institute of Molecular and Cellular Biosciences, University of Tokyo, Tokyo (S. Kitanaka, K.T., A.M., T.S., J.Y., S. Kato); Core Research for Evolutional Science and Technology, Japan Science and Technology Corporation, Saitama (S. Kato); the Faculty of Bioresources, Mie University, Mie (K.O., M.N.); Tokyo Metropolitan Kiyose Children's Hospital, Tokyo (Y.H.); the Department of Pediatrics, Chiba University School of Medicine, Chiba (H.N.); and the Department of Endocrinology and Metabolism, National Children's Medical Research Center, Tokyo (T.T.) — all in Japan. Address reprint requests to Dr. Kato at the Institute of Molecular and Cellular Biosciences, University of Tokyo, Yayoi, Bunkyo-ku, Tokyo 113-0032, Japan.

METHODS

Patients

We studied four unrelated Japanese patients with pseudovitamin D–deficiency rickets and four of their unaffected parents, one sibling, and one nephew. The diagnosis of pseudovitamin D–deficiency rickets in these patients was based on the early onset of hypocalcemia, radiologic findings characteristic of rickets, and the requirement of high doses of vitamin D and physiologic doses of 1 α -hydroxyvitamin D₃.^{2,3} The clinical data on these patients are summarized in Table 1. The diagnosis was confirmed by low serum concentrations of 1 α ,25-dihydroxyvitamin D determined when the patients were 10 to 20 years old, after the cessation of therapy with vitamin D₂ or 1 α -hydroxyvitamin D₃. The parents of Patient 1 were second cousins; those of Patient 2 were first cousins; those of Patient 3 were unrelated and came from the same remote area; those of Patient 4 were unrelated and came from different areas.

The studies were approved by the appropriate institutional review committees, and all subjects gave informed consent.

Isolation of 1 α -Hydroxylase cDNA and Gene

A human kidney cDNA library was prepared from poly(A)⁺RNA purified from normal kidney tissue.¹⁹ A total of 1 \times 10⁶ plaques were screened by hybridization with a mouse 1 α -hydroxylase cDNA N-terminal fragment (0.7 kb).¹⁶ Two positive plaques were subcloned and sequenced automatically in a Prism 377 DNA sequencer (Applied Biosystems, Foster City, Calif.), with AmpliTaq DNA polymerase FS (Perkin–Elmer, Norwalk, Conn.) and dye terminator.

The genomic sequence of human 1 α -hydroxylase was determined by sequencing the polymerase-chain-reaction (PCR) products amplified with three sets of primers specific to the exon and the untranslated region, with normal human leukocyte DNA as a template.

Fluorescence in Situ Hybridization

Three human genomic fragments covering all areas of the 1 α -hydroxylase gene were used as probes. Fluorescence in situ hybridization was carried out on more than 20 chromosomes in prometaphase.²⁰

PCR Amplification and Sequence Analysis of the 1 α -Hydroxylase Gene

Genomic DNA of all patients and family members was extracted from peripheral white cells. Exons of the 1 α -hydroxylase gene were amplified by PCR with specific primers derived from intronic sequences. Information about the sequence of the primers is available elsewhere.* AmpliTaq Gold (Perkin–Elmer) and its standard buffer were used in all reactions. All exons were amplified in a PCR thermocycler (Perkin–Elmer) by initial denaturation at 95°C for nine minutes, followed by 30 cycles at 95°C for one minute, 60°C for one minute, and 72°C for one minute. The corresponding PCR products were purified and sequenced directly in both directions.

Plasmid Construction

The wild-type human 1 α -hydroxylase cDNA was introduced into the expression vector pCDNA3 (Invitrogen, San Diego, Calif.), and the patients' mutations were introduced with a site-directed mutagenesis kit (Quick Change, Stratagene, La Jolla, Calif.). The

expression plasmid for the ligand-binding domain of the vitamin D receptor (VDR) fused to the GAL4 DNA binding domain [GAL4-VDR(DEF)] was prepared as described elsewhere.²¹

Assay of 1 α -Hydroxylase Activity

1 α -Hydroxylase activity was assayed both by vitamin D receptor–mediated transactivation assay and by high-performance liquid chromatography.¹⁶ Briefly, for the vitamin D receptor–mediated transactivation assay, COS-1 cells were transiently transfected with 0.5 μ g of GAL4-VDR(DEF), 1 μ g of 17M2-G-CAT reporter plasmid,²² 0.2 μ g each of the adrenodoxin and adrenodoxin reductase expression plasmids, 1 to 2 μ g of either wild-type or mutant 1 α -hydroxylase expression plasmid, and 1 μ g of β -galactosidase expression plasmid pCHI10 (Pharmacia Biotech, Uppsala, Sweden). Twelve hours after transfection, the ligands were added to the medium. After incubation for an additional 36 hours, cell extracts were prepared and used for chloramphenicol acetyltransferase assays.²³

For analysis by high-performance liquid chromatography, COS-1 cells were transfected with 0.5 μ g each of adrenodoxin and adrenodoxin reductase expression plasmids and 3 μ g of either wild-type or mutant 1 α -hydroxylase expression plasmid and were then incubated with tritiated 25-hydroxyvitamin D₃ for six hours. The incubation medium and cells were extracted and analyzed by normal-phase and reverse-phase high-performance liquid chromatography. The eluent fractions were collected, and the radioactivity was estimated by liquid-scintillation counting.^{16,24,25} Authentic vitamin D derivatives were chromatographed, and the retention times were determined by ultraviolet absorption at 264 nm.

RESULTS

Isolation of 25-Hydroxyvitamin D₃ 1 α -Hydroxylase cDNA and Gene

A 2.4-kb cDNA clone was isolated from a normal human kidney cDNA library by using the mouse 1 α -hydroxylase cDNA fragment as a probe. This cDNA contains a 1527-bp open reading frame predicted to encode a protein of 508 amino acids. The sequence of this cDNA was verified by determination of the genomic sequence.

The sequence of the coding region was 82 percent identical to that of mouse 1 α -hydroxylase at both the nucleotide and the amino acid levels.¹⁶ The deduced amino acid sequence of this cDNA has substantial homology with members of the mitochondrial P450 family,²⁶ particularly human vitamin D₃ 25-hydroxylase (*CYP27*, 40 percent),²⁷ 25-hydroxyvitamin D₃ 24-hydroxylase (*CYP24*, 32 percent),²⁸ P450_{sc} (*CYP11A*, 33 percent),²⁹ and 11 β -hydroxylase (*CYP11B1*, 30 percent).³⁰

The structure of the human 1 α -hydroxylase gene was determined by PCR amplification of normal human leukocyte DNA, with use of exon-specific primers designed from the cDNA sequence, on the basis of the finding that the known members of the mitochondrial P450 family usually have short introns.³¹ The 1 α -hydroxylase gene consisted of nine exons spanning a region of approximately 4.8 kb.

Chromosomal Localization of the 1 α -Hydroxylase Gene

Fluorescence in situ hybridization revealed that the 1 α -hydroxylase gene was located on chromosome band 12q13.3 (Fig. 1). Since the gene respon-

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PSEUDOVITAMIN D-DEFICIENCY RICKETS DUE TO 1 α -HYDROXYLASE MUTATIONS

TABLE 1. CLINICAL AND BIOCHEMICAL FEATURES IN FOUR PATIENTS WITH PSEUDOVITAMIN D-DEFICIENCY RICKETS.*

PATIENT No.	YEAR OF BIRTH	CLINICAL PRESENTATION	AGE AT DIAGNOSIS	SERUM CALCIUM	SERUM PHOSPHORUS	SERUM 1 α ,25-DIHYDROXY-VITAMIN D†	SERUM 25-HYDROXY-VITAMIN D†	AMINO-ACIDURIA	THERAPY	
									VITAMIN D ₂	ALFACALCIDOL‡
				mg/dl		pg/ml	ng/ml		IU/day	μ g/day
1	1971	Bone deformity	1 yr	6.5	3.8	<5	28	Yes	50,000–150,000	1.5–2.0
2	1965	Unable to walk	2 yr	5.8	4.2	12	10	Yes	30,000–50,000	1.0–2.0
3§	1954	Unable to walk, seizures	1 yr 11 mo	5.1	4.2	13	48	Yes	300,000	2.0
4	1970	Unable to walk, poor growth, seizures	1 yr 2 mo	5.7	5.1	ND	ND	Yes	25,000–70,000	1.0–2.0
Normal range				8.7–10.1	2.4–4.3	20–76	10–55			

*All patients had normal renal function at the time of diagnosis. To convert values for serum calcium to millimoles per liter, multiply by 0.25; to convert values for serum phosphorus to millimoles per liter, multiply by 0.32; to convert values for serum 1 α ,25-dihydroxyvitamin D to picomoles per liter, multiply by 2.4; and to convert values for serum 25-hydroxyvitamin D to nanomoles per liter, multiply by 2.5. ND denotes not determined. Dose of therapy is that required for the remission of rickets and hypocalcemia.

†These values were obtained after the cessation of therapy (after three to six months for vitamin D₂ and one month for alfacalcidol).

‡Alfacalcidol is a preparation of 1 α -hydroxyvitamin D₃.

§Patient 3 was previously described by Sasaki et al.¹⁸

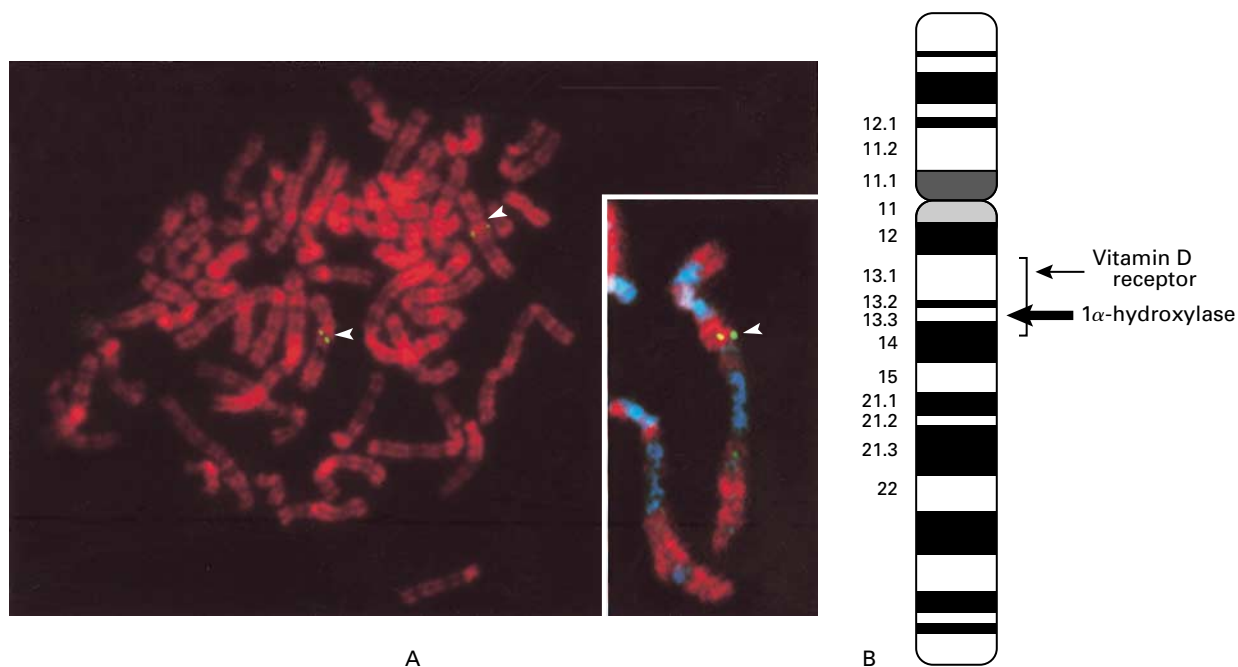


Figure 1. Chromosomal Localization of the 1 α -Hydroxylase Gene According to Fluorescence in Situ Hybridization.

Panel A shows fluorescence in situ hybridization with three genomic 1 α -hydroxylase fragments covering the entire coding region as probes on chromosome preparations obtained from phytohemagglutinin-stimulated blood lymphocytes. The probes were labeled by nick translation with biotin-16-deoxyuridine triphosphate and detected with avidin-fluorescein (Boehringer Mannheim, Mannheim, Germany).²⁰ Precise mapping of the gene was performed by using prophase-like chromosome spreads with higher-resolution banding. Hoechst (G-banding), propidium iodide (R-banding), and avidin-fluorescein images were merged and colored blue, red, and green, respectively. Fluorescence signals on the R-banded chromosomes are indicated by arrowheads. The inset is a larger image of chromosome 12 showing precise mapping of the gene. Panel B shows the idiogram of chromosome 12 indicating the localization of the 1 α -hydroxylase gene to 12q13.3. The vitamin D-receptor gene, the gene responsible for hypocalcemic vitamin D-resistant rickets, was previously mapped to 12q13–14, as indicated in the figure.³²

sible for pseudovitamin D–deficiency rickets has been mapped to this locus,⁹ this finding suggested that pseudovitamin D–deficiency rickets is caused by a defect in the structural gene encoding 1α -hydroxylase.

Tissue Expression of the 1α -Hydroxylase Gene

Using the human 1α -hydroxylase cDNA clone as a probe, we examined its expression in various human tissues by Northern blot analysis. The 1α -hydroxylase transcript (approximately 2.4 kb) was detected only in renal tissue (Fig. 2).

Homozygous Mutations in the 1α -Hydroxylase Gene in Patients with Pseudovitamin D–Deficiency Rickets

To determine whether the 1α -hydroxylase gene was mutated in patients with pseudovitamin D–deficiency rickets, we analyzed genomic DNA from the four patients. Four different missense mutations in four different positions were detected in these patients (Fig. 3A and 3B). Patient 1 had a mutation in exon 2, Arg107His (CGC to CAC), and Patient 2

had a mutation in exon 2, Gly125Glu (GGA to GAA). Patient 3 had a mutation in exon 6, Arg335Pro (CGG to CCG), that eliminates a restriction site for *Hpa*II. The presence of the mutation was further confirmed by digesting the PCR product of exon 6 with the enzyme (data not shown). Patient 4 had a mutation in exon 7, Pro382Ser (CCT to TCT). All these patients were homozygous for their mutations, and the parents and one sibling who were studied were all heterozygous carriers (Fig. 3B). A nephew of Patient 4 was a normal homozygote.

Functional Analysis of Wild-Type and Mutant 1α -Hydroxylase Proteins

The 1α -hydroxylase activities of the wild-type and the mutant enzymes in the four patients were assessed by examining the ligand-induced transactivation function of the vitamin D receptor. The vitamin D receptor was activated only when the 25-hydroxyvitamin D₃ added to the culture medium was converted to an active ligand, possibly $1\alpha,25$ -dihydroxyvitamin D₃ (Fig. 4A). The expression of wild-type

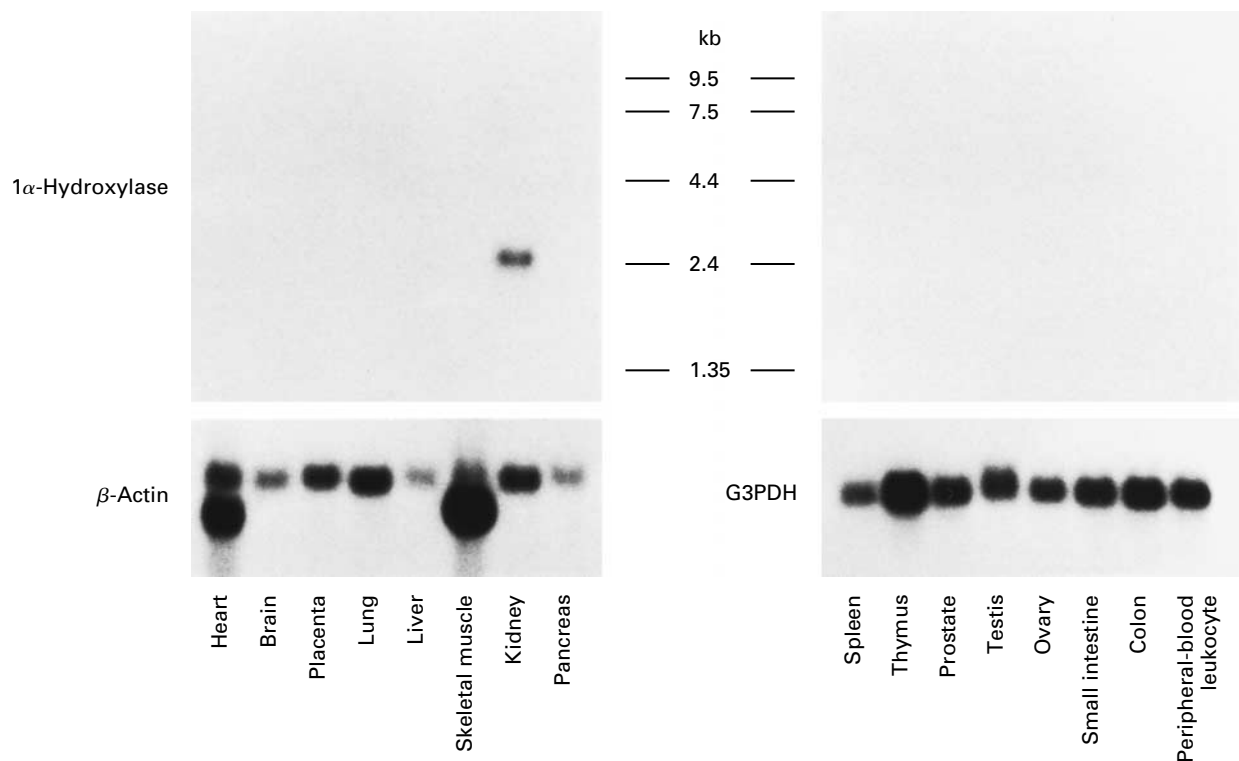


Figure 2. Northern Blot Analysis of 1α -Hydroxylase Gene Expression in Human Tissues.

The expression of the 1α -hydroxylase gene in various human tissues was analyzed by Northern blotting with 2 μ g of poly(A)⁺RNA from each tissue (Clontech, Palo Alto, Calif.).³³ The 0.7-kb cDNA fragment corresponding to the N-terminal region of human 1α -hydroxylase was used as a probe, and the levels of expression of β -actin and glutaraldehyde-3-phosphate dehydrogenase (G3PDH) served as internal controls.

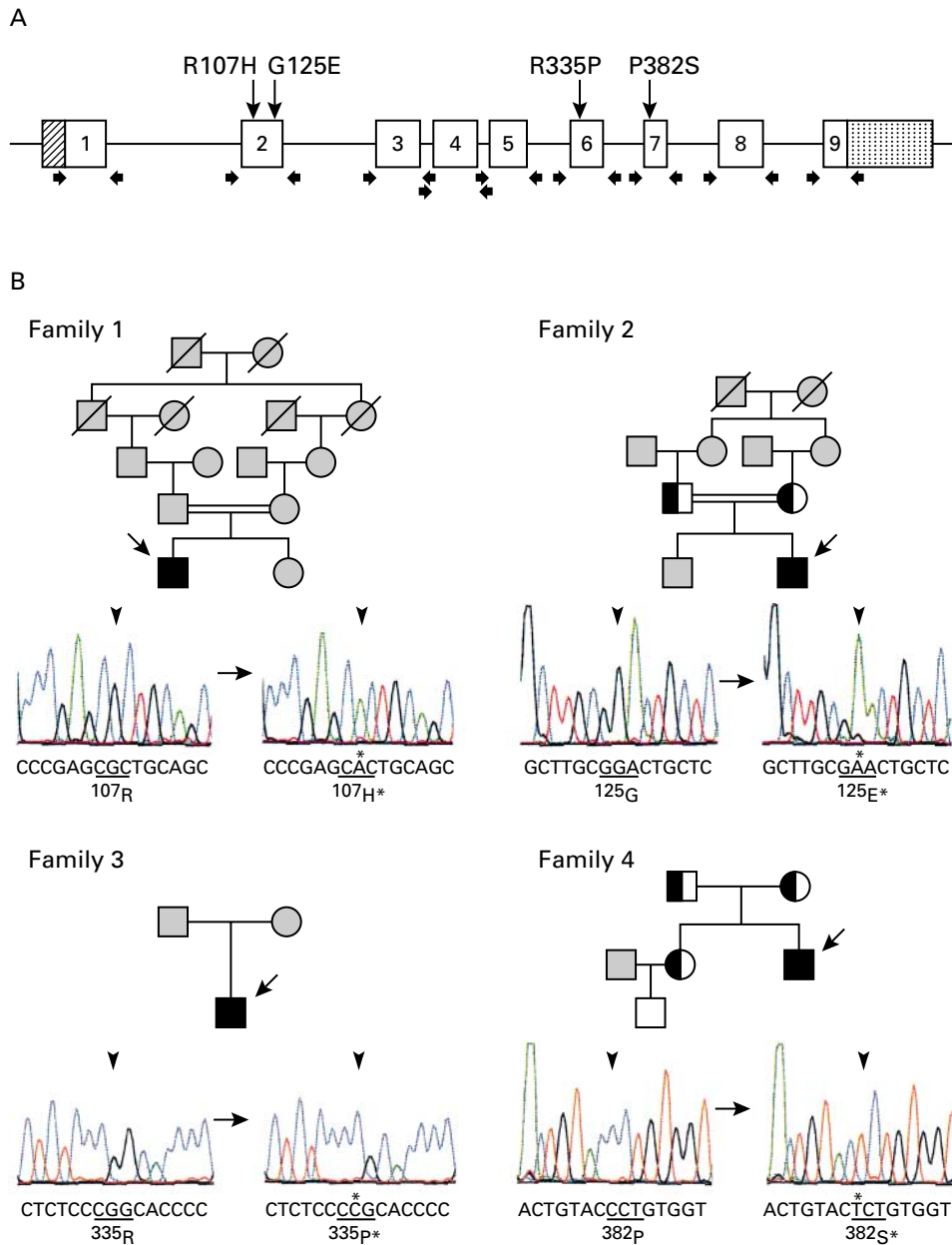


Figure 3. Mutation Analysis of the 1 α -Hydroxylase Gene in Four Families with Pseudovitamin D–Deficiency Rickets. Panel A shows the positions of the missense mutations detected in the four patients with pseudovitamin D–deficiency rickets. The exons are numbered, and the positions of the primers used for PCR amplification of each exon are indicated by the arrows under the diagram. Panel B shows the mutations detected in each family. The affected probands are indicated by arrows. Homozygotes are indicated by solid circles (females) or squares (males), heterozygotes by half-solid circles or squares, and the one tested family member with no mutation by an open square. Those not studied are indicated by gray symbols. Relevant portions of the sequencing chromatograms obtained from a normal subject (left) and the affected proband (right) are shown, with the position of each nucleotide mutation (arrowhead) and amino acid substitution (asterisk) indicated.

1α -hydroxylase activated the reporter gene (Fig. 4B, lanes 3 and 4), as in mouse 1α -hydroxylase¹⁶ (lane 5). In contrast, none of the mutants (Fig. 4B, lanes 8, 9, 10, and 11) increased the activity of the reporter gene. In vitro translation analysis indicated that the wild-type and the mutant plasmids were translated with similar efficiency at 55 kd in size (data not shown).

The 1α -hydroxylase activity of these mutants was assayed to determine the ability of the enzyme to catalyze the conversion of 25-hydroxyvitamin D₃ to $1\alpha,25$ -dihydroxyvitamin D₃. When tritiated 25-hydroxyvitamin D₃ was added to cells transfected with the wild-type 1α -hydroxylase expression plasmid, a metabolite was detected (Fig. 4C, lower panels) at a retention time identical to that of authentic $1\alpha,25$ -dihydroxyvitamin D₃ (Fig. 4C, upper panels). However, none of the mutants converted 25-hydroxyvitamin D₃ into $1\alpha,25$ -dihydroxyvitamin D₃ (Fig. 4D). These results indicate that the protein products of the four mutations found in the patients with pseudovitamin D-deficiency rickets had no 1α -hydroxylase activity.

DISCUSSION

The responsible genes and their mutations have been identified for two types of hereditary rickets besides pseudovitamin D-deficiency rickets.⁵⁻⁸ We isolated a human cDNA encoding 25-hydroxyvita-

min D₃ 1α -hydroxylase and identified different homozygous missense mutations in this gene in four patients with pseudovitamin D-deficiency rickets. Each of the four mutations abolished 1α -hydroxylase activity. The asymptomatic parents and sibling were heterozygous for the mutations, demonstrating the autosomal recessive inheritance of this disease. We conclude that pseudovitamin D-deficiency rickets is caused by inactivating mutations in the 1α -hydroxylase gene.

We found no correlation between phenotype and genotype; all four mutations abolished 1α -hydroxylase activity, and the clinical features in all four patients were similar. Clinical heterogeneity has been reported in pseudovitamin D-deficiency rickets,^{2,34} and there may be other mutations that only partially reduce enzyme activity, as is the case with other P450 enzymes.^{35,36} In a preliminary study of a patient who had pseudovitamin D-deficiency rickets with mild clinical features, we detected no mutations in the coding region (unpublished data), a fact that suggests that gene expression may be impaired by a mutation in the promoter region. Alternatively, there may be genetic heterogeneity in pseudovitamin D-deficiency rickets, and further analysis may reveal mutations in another gene. We found only homozygous mutations in the four patients, a result in agreement with the finding that in rare autosomal recessive diseases, homozygous mutations are fre-

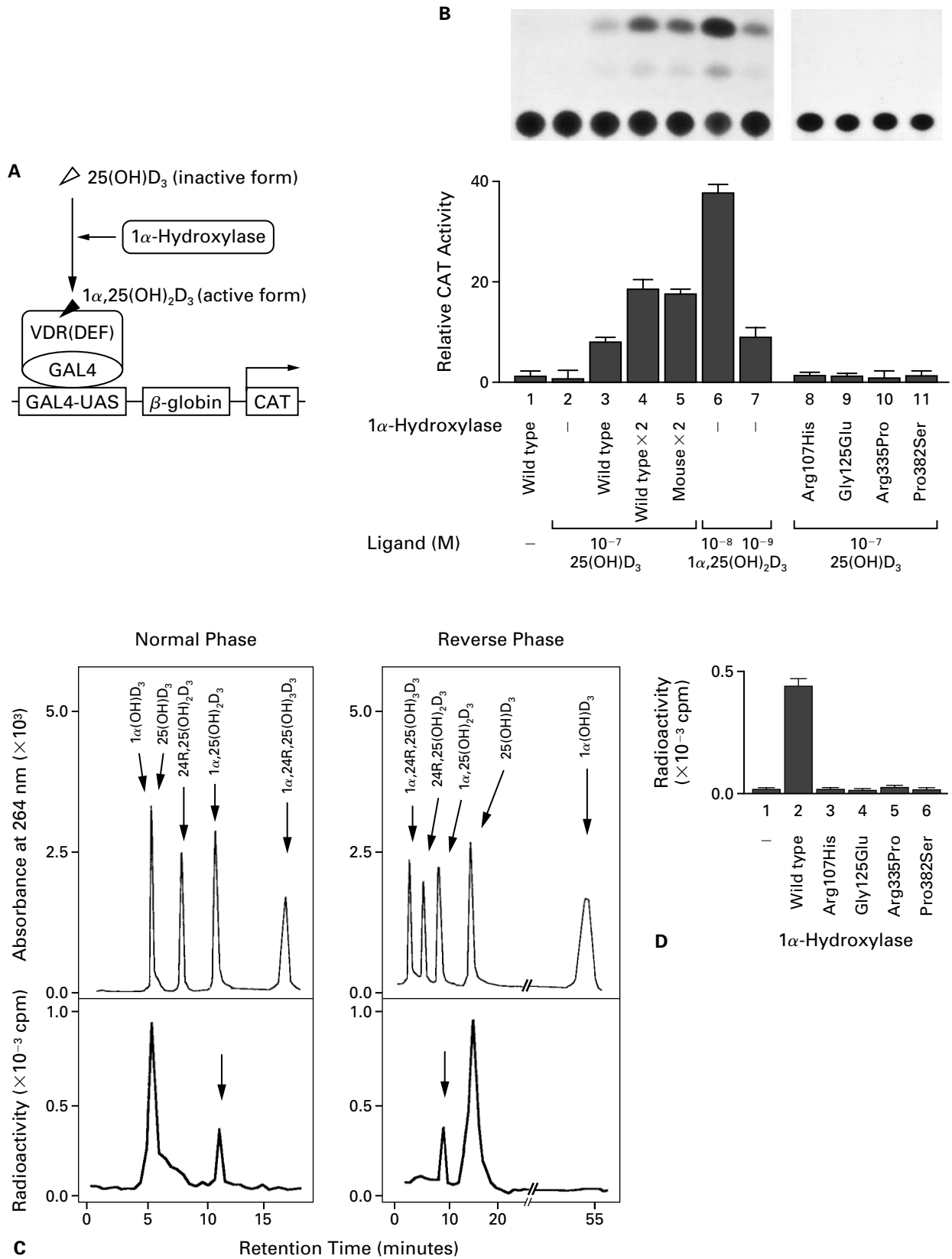
Figure 4. Functional Analysis of Wild-Type and Mutant 1α -Hydroxylase Proteins.

Panel A shows the schema of the assay system for 1α -hydroxylase activity. COS-1 cells were transfected with GAL4-VDR(DEF), 17M2-G-CAT, adrenodoxin, and adrenodoxin reductase expression plasmids together with wild-type or mutant 1α -hydroxylase expression plasmid in the presence of 25-hydroxyvitamin D₃ (25(OH)D₃). When the expressed 1α -hydroxylase converts 25-hydroxyvitamin D₃ into $1\alpha,25$ -dihydroxyvitamin D₃ ($1\alpha,25$ (OH)₂D₃), the activated GAL4-VDR(DEF) induces expression of chloramphenicol acetyltransferase (CAT) on the reporter gene (17M2-G-CAT) through the GAL4 upstream-activating sequence (GAL4-UAS), and 1α -hydroxylase activity can be analyzed by chloramphenicol acetyltransferase assay.

Panel B shows the 1α -hydroxylase activity of wild-type and mutant 1α -hydroxylase as determined by the assay shown in Panel A. Significant activation was not detected in the absence of 25-hydroxyvitamin D₃ (lane 1) or 1α -hydroxylase expression plasmid (lane 2); however, $1\alpha,25$ -dihydroxyvitamin D₃ effectively induced chloramphenicol acetyltransferase activity (lanes 6 and 7). The expression of wild-type 1α -hydroxylase induced chloramphenicol acetyltransferase activity in a dose-dependent way (lanes 3 and 4), as observed in mouse 1α -hydroxylase (lane 5).¹⁶ In contrast, none of the mutants increased the activity of the reporter gene (lanes 8, 9, 10, and 11). The results of one representative chloramphenicol acetyltransferase assay and a graph corresponding to the mean (\pm SE) values for three independent experiments are shown.

Panel C shows the conversion of 25-hydroxyvitamin D₃ to $1\alpha,25$ -dihydroxyvitamin D₃ by 1α -hydroxylase as determined by high-performance liquid chromatography. The results of normal-phase high-performance liquid chromatography are shown on the left and those of reverse-phase high-performance liquid chromatography on the right. The upper panels show the retention times of authentic vitamin D derivatives (1α -hydroxyvitamin D₃ [1α (OH)D₃], 25-hydroxyvitamin D₃, 24R,25-dihydroxyvitamin D₃ [24R,25(OH)₂D₃], $1\alpha,25$ -dihydroxyvitamin D₃, and $1\alpha,24R,25$ -trihydroxyvitamin D₃ [$1\alpha,24R,25$ (OH)₃D₃]) as determined by ultraviolet absorption at 264 nm. The lower panels show the production of $1\alpha,25$ -dihydroxyvitamin D₃ as determined by high-performance liquid chromatography when COS-1 cells were transfected with wild-type 1α -hydroxylase expression plasmid in the presence of tritiated 25-hydroxyvitamin D₃. Note that the retention times of a converted metabolite matched those of authentic $1\alpha,25$ -dihydroxyvitamin D₃ in both normal and reverse phases (arrows).

Panel D shows the conversion of 25-hydroxyvitamin D₃ to $1\alpha,25$ -dihydroxyvitamin D₃ by the wild-type (lane 2) and mutant (lanes 3, 4, 5, and 6) 1α -hydroxylase proteins. The activity of 1α -hydroxylase was determined by high-performance liquid chromatography, as in Panel C. Note that all the mutants failed to convert 25-hydroxyvitamin D₃ to $1\alpha,25$ -dihydroxyvitamin D₃ (lanes 3, 4, 5, and 6). Values corresponding to the mean (\pm SE) for three independent experiments are shown.



quent and compound heterozygotes are uncommon.³⁷

The mutated residue (Pro382Ser) in one of our patients was located in a conserved region among various P450 enzymes that is assumed to be required for substrate binding,^{38,39} and another mutated residue (Gly125Glu) was located in the region corresponding to the proposed substrate-recognition site in the P450 family (CYP2).⁴⁰ These mutations probably abolish the activity of 1 α -hydroxylase by reducing its affinity for 25-hydroxyvitamin D₃. We do not know how the other two mutations abolish 1 α -hydroxylase activity, but because these four residues are highly conserved among several mitochondrial P450 enzymes, they are likely to be important for the enzymatic function of this protein.

With the data obtained in this study, we now know the molecular basis of all three major types of hereditary rickets. The phenotypes of pseudovitamin D-deficiency rickets and hypocalcemic vitamin D-resistant rickets are similar, except for alopecia in the latter and differences in serum concentrations of vitamin D metabolites.⁴ There are many endogenous vitamin D derivatives with biologic activity,⁴¹ and therefore altered serum concentrations of vitamin D derivatives in these types of hereditary rickets may cause different changes.

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