Am. J. Hum. Genet. 58:712–721, 1996

Uroporphyrinogen Decarboxylase: Complete Human Gene Sequence and Molecular Study of Three Families with Hepatoerythropoietic Porphyria

M. J. Moran-Jimenez,[1] C. Ged,[1] M. Romana,[3] R. Enriquez de Salamanca,[4] A. Taïeb,[2] G. Topi,[5] L. D'Alessandro,[5] and H. de Verneuil[1]

[1]Département de Biochimie Médicale et Biologie Moléculaire, UPR-ES Pathologie Moléculaire et Thérapie Génique, Université de Bordeaux II, and [2]Unité de Dermatologie Pédiatrique, Hôpital Pellegrin-Enfants, Bordeaux; [3]Inserm U-91, Hôpital H. Mondor, Créteil; [4]Hospital Universitario 12 de Octubre, Madrid; and [5]Istituto Ospitaliero Dermosifilopatico di S. Maria e S. Gallicano, Centro Studi per le Porfirie, Rome

Summary

A deficiency in uroporphyrinogen decarboxylase (UROD) enzyme activity, the fifth enzyme of the heme biosynthetic pathway, is found in patients with sporadic porphyria cutanea tarda (s-PCT), familial porphyria cutanea tarda (f-PCT), and hepatoerythropoietic porphyria (HEP). Subnormal UROD activity is due to mutations of the UROD gene in both f-PCT and HEP, but no mutations have been found in s-PCT. Genetic analysis has determined that f-PCT is transmitted as an autosomal dominant trait. In contrast, HEP, a severe form of cutaneous porphyria, is transmitted as an autosomal recessive trait. HEP is characterized by a profound deficiency of UROD activity, and the disease is usually manifest in childhood. In this study, a strategy was designed to identify alleles responsible for the HEP phenotype in three unrelated families. Mutations of UROD were identified by direct sequencing of four amplified fragments that contained the entire coding sequence of the UROD gene. Two new missense mutations were observed at the homoallelic state: P62L (proline-to-leucine substitution at codon 62) in a Portuguese family and Y311C (tyrosine-to-cysteine substitution at codon 311) in an Italian family. A third mutation, G281E, was observed in a Spanish family. This mutation has been previously described in three families from Spain and one from Tunisia. In the Spanish family described in this report, a paternal uncle of the proband developed clinically overt PCT as an adult and proved to be heterozygous for the G281E mutation. Mutant cDNAs corresponding to the P62L and Y311C changes detected in these families were created by site-directed mutagenesis. Recombinant proteins proved to have subnormal enzyme activity, and the Y311C mutant was thermolabile.

Received August 30, 1995; accepted for publication January 12, 1996.

Address for correspondence and reprints: Dr. C. Ged, Département de Biochimie Médicale et Biologie Molécularie, UPR-ES Pathologie Molécularie et Thérapie Génique, Université de Bordeaux, 146 rue Léo Saignat, 33076 Bordeaux Cedex, France.

0002-9297/96/5804-0007$02.00

Introduction

Uroporphyrinogen decarboxylase (UROD) is a cytosolic enzyme that catalyzes the sequential decarboxylation of the four acetic acid substituents of uroporphyrinogen III to form coproporphyrinogen III. Decreased activity of UROD is responsible for three diseases, sporadic porphyria cutanea tarda (s-PCT), familial porphyria cutanea tarda (f-PCT), and hepatoerythropoietic porphyria (HEP) (Verneuil et al. 1984; Kappas et al. 1995). Subnormal UROD activity in s-PCT is restricted to the liver. In f-PCT, UROD activity is approximately half that of normal levels in all tissues. HEP is a severe form of cutaneous porphyria that generally occurs early in infancy and is characterized clinically by photosensitivity, bullae, cutaneous erosions, hypertrichosis, and red-colored urine. A severe deficiency of UROD activity is observed in HEP patients. The catalytic activity of UROD in erythrocytes derived from patients with HEP is generally <10% of normal levels (Kappas et al. 1995). HEP has been considered as a homozygous form of f-PCT. Mutant UROD proteins present in patients with f-PCT and HEP have been divided into three groups based on reactivity of the UROD protein with anti-UROD antibodies and on enzymatic activity. These three groups include cross-reactive immunologic material (CRIM)–negative cases, CRIM-positive cases, and CRIM-super-negative cases (absence of detectable protein associated with a significant enzyme activity) (Verneuil et al. 1984; Fujita et al. 1987; Toback et al. 1987; Koszo et al. 1990).

Cloning of a human UROD cDNA and the gene encoding UROD has demonstrated that UROD protein is encoded by a single gene, with no evidence of organ-specific isozymes (Romeo et al. 1986; Romana et al. 1987). UROD mutations responsible for HEP and f-PCT have proved to be heterogeneous. The first mutation described in HEP, the substitution mutant G281E, was first detected in a Tunisian family, and the mutation was shown to result in an unstable UROD protein (Verneuil et al. 1986). Subsequently, the same mutation was identified in several Spanish families (Verneuil et al. 1988; Roberts et al. 1995). Four other point mutations—E167K, R292G, V134Q, and H220P—and a large dele-

tion have also been found in three additional families (Romana et al. 1991; Verneuil et al. 1992; Meguro et al. 1994). In pedigrees with f-PCT, two point mutations have been reported: a splice-site mutation causing a deletion of exon 6 (Garey et al. 1989) and the substitution mutant G281V (Garey et al. 1990). S-PCT is not due to mutations at the UROD locus but appears to result from the generation of an inhibitor of UROD activity that is restricted to the liver (Garey et al. 1993). In addition to UROD mutations described in humans, the molecular basis of UROD deficiency has been characterized in 13 yeast (uroporphyric) mutants (Garey et al. 1992; Chelstowska et al. 1992).

In this report, we describe the complete sequence of the human UROD gene and the molecular analysis of the UROD gene defect in three new HEP families. Mutations were detected by direct sequencing of amplified genomic fragments. Mutant cDNAs were then constructed by site-directed mutagenesis. Mutant UROD proteins were expressed in a bacterial expression system, and the properties of the mutant proteins were defined.

Patients and Methods

Patients

The probands of the three new pedigrees described here developed the typical photocutaneous dermatosis of HEP. All probands had striking elevations of urinary uroporphyrin and heptacarboxylic porphyrin excretion, and isocoproporphyrin was noted in the feces in all cases.

The three new HEP pedigrees are outlined in figure 1. In family A, from Portugal, the clinically affected members (II-1 and II-2) developed mutilating photocutaneous sensitivity with sclerodermoid changes in childhood. Both had normal hepatic function, as judged by biochemical testing. Patient II-2 was treated with chloroquine with benefit. Patient II-1 had only venesections with no obvious clinical effect. At age 5 years, the child of patient II-1 was clinically normal. Peripheral blood samples were obtained from the two clinically affected members of this pedigree and from eight other family members. Measurements were made of erythrocyte UROD activity, and molecular analysis of the UROD gene was performed.

Family B was an Italian family. The proband (IV-1) developed cutaneous symptoms in the 3d decade of life. Symptoms appeared after the administration of estrogens and iron for the treatment of iron deficiency anemia and dysmenorrhea. A clinical and biochemical improvement was noted after venesection; the hepatic function was normal. Blood samples were obtained from the proband, her clinically normal parents, and her clinically normal sister, and measurements were made of erythrocyte UROD activity and erythrocyte UROD immunoreactivity. DNA analysis was done as well.

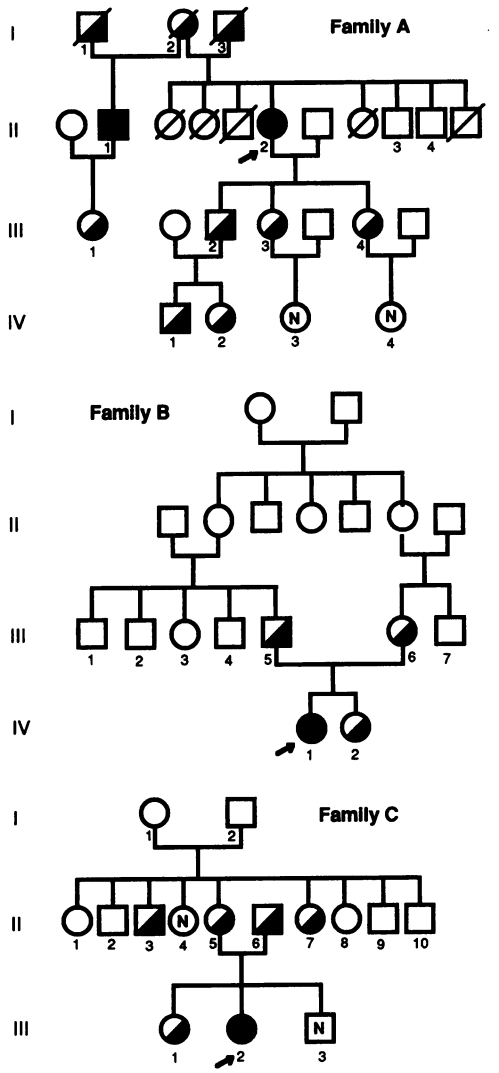

Figure 1 Pedigree of the three HEP families. In family A, deceased individuals I-1, I-2, and I-3 are obligate carriers. Individuals III-3 and III-4 were not available for DNA or enzymatic analysis. In family B, DNA and enzymatic analyses were performed in individuals III-5, III-6, IV-1, and IV-2. The parents of the proband are first-degree relatives. In family C, DNA and/or enzymatic analyses were performed in individuals II-3, II-4, II-5, II-6, II-7, III-1, III-2, and III-3. Open symbols containing the letter N denote normal individuals. Half-filled symbols denote carrier or f-PCT individuals. Blackened symbols denote HEP patients. Completely open symbols denote individuals who were not tested.

In family C, a Spanish family, HEP was detected in the proband (III-2) at the age of 2 years, following a varicella infection. Her paternal uncle (II-3) also contracted varicella at the same time and subsequently developed typical features of f-PCT. Subject II-3 also had serological evidence for an exposure to the hepatitis C virus. At the time HEP was detected in the proband, the proband's mother was pregnant, and DNA analysis was performed on cord blood of her newborn infant. Two maternal aunts, a maternal uncle, and the sister of the

Table 1

Primers Used in Study

Type of Analysis and Primer	Sequence
DNA Analysis	
1 UDP1	5′-GGAGTCCGATCATGTGATCTTCA-3′
2 UDI1	5′-GCCTTATGAACCCGCGCTTT-3′
3 UDE31	5′-GCCTGCTGTGAACTGACTC-3′
4 UDI3	5′-GAAAAGTTTTCGAGGGGCAG-3′
5 UDI4B	5′-B-GGCGTCCTTGGATTATATTC-3′
6 UDI4	5′-CTCCTGGAATGAGCTGAACA-3′
7 UDI51	5′-ccggaattCTGGCTTCTGTGACACCAT-3′
8 UDI6B	5′-B-TACAGACCAAATCCCAGCCT-3′
9 UDI6	5′-CATTTGGGGAAAATTGAGGT-3′
10 UDI70	5′-GCAAGGCCCTCTGTAGCCTGA-3′
11 UD292	5′-AGAAAGCCCGGTAAGCC-3′
12 UDI9B	5′-B-CAGTAACAGGACACACAGAG-3′
13 UDI9	5′-CTAGTATATATAGGGAGGAC-3′
14 UDI10B	5′-B-CTTTCACAAACAAAACTACAC-3′
Site-Directed Mutagenesis	
15 62 MutS	5′-GCTCTCtTGAGGCCTGCTG-3′
16 62 MutAS	5′-GCCTCAaGAGAGCGACACG-3′
17 311 MutS	5′-CCTTGTgTGCATCTGAGGA-3′
18 311 MutAS	5′-GATGCAcACAAGGCACAGG-3′
19 GEX1	5′-gaagatctGAAGCGAATGGGTTGGGAC-3′
20 UDE31	5′-GCCTGCTGTGAACTGACTC-3′
21 UDE62	5′-ccggaattcGTCCTGGAAACGATCAATC-3′

NOTE.—The location of primers 1–14 on UROD gene is shown by arrows in fig. 2, and the corresponding sequences are underlined in fig. 3. B stands for biotine; lowercase letters indicate either a mutated base, in "Mut S" and "MutAS" primers, or an added sequence containing a restriction site.

proband also underwent molecular analysis of the UROD gene.

UROD Gene Sequence

As described by Romana et al. (1987), a genomic clone was obtained from a human genomic DNA library in λEMBL4 bacteriophage by use of a full-length human UROD cDNA as a probe (Romeo et al. 1986). The structure of the gene was determined by restriction mapping and hybridization of genomic fragments with UROD cDNA. The complete gene sequence was determined by successive subcloning of the original clone containing UROD by use of a commercial T_7 sequencing kit (Pharmacia). Sequencing data were used to design specific primers (table 1) for mutation detection and expression studies.

UROD Assay

UROD enzyme activity was measured in hemolyzed whole blood by use of high-performance liquid chromatography with pentacarboxyl porphyrinogen I as substrate (Verneuil et al. 1984). Results are expressed as nanomoles of coproporphyrin I formed per hour and per milligram of hemoglobin. Hemoglobin was measured by the cyanmethemoglobin method (Boehringer). Immunoblot assays to quantify UROD protein were done in family B by Prof. S. Sassa (Rockefeller University, New York) as described elsewhere (Chang and Sassa 1985).

DNA Analysis

DNA was prepared from whole blood by phenol-chloroform extraction and used for in vitro amplification (PCR) of four genomic segments (fig. 2). In each primer pair, the antisense oligonucleotide was biotinylated to allow the purification of the PCR product as single-stranded DNA immobilized on Streptavidine M-280 (Dynal). PCR conditions were 200–500 ng of DNA, 10 pmol of each primer, 1.5 mM $MgCl_2$, 200 μM of each dNTP, 1 U *Taq* polymerase (Promega) in 1 × buffer supplied by Promega in a final volume of 50 μl. After an initial denaturation at 94°C for 5 min, 30 cycles were performed, including 15 s at 92°C, 30 s at 52°C–56°C, depending on the Tm of the primers, and 60 s at 72°C, before a final extension at 72°C for 5 min. For subsequent sequencing, 40 μl of the PCR product were incubated with the beads during 15 min at room temperature, denatured in 8 μl of 0.1 N NaOH, and resuspended in 10 μl of H_2O after neutralization. The sequencing reaction was performed directly on the template immobilized on the beads, by the dideoxy chain–termination method using a T_7 sequencing kit (Pharmacia). For restriction analysis, one fifth of the PCR product was digested with 10 U of the appropriate enzyme, as recom-

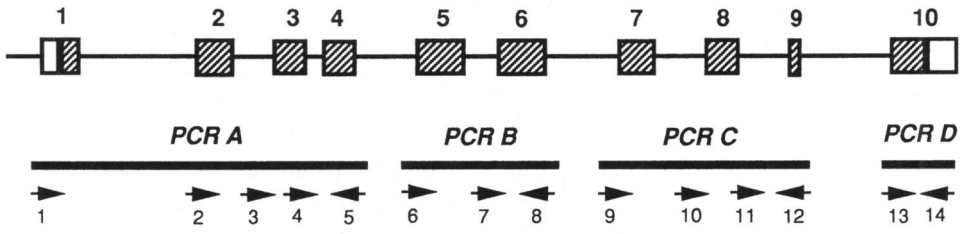

Figure 2 UROD gene structure and sequencing strategy. The 10 exons are amplified from genomic DNA by use of four primer sets—(1/5), (6/8), (9/12), and (13/14)—yielding PCR products A, B, C, and D, respectively. Antisense primers (5, 8, 12, and 14) are biotinylated at the 5′ end; sense primers (1–4, 6–7, 9–11, and 13) are used for sequencing.

Table 2

Summary of DNA and Enzymatic Analysis in Erythrocytes of Three Independent Families

Family and Individual	DNA Analysis	UROD Activity (% of normal value)	UROD Concentration (% of normal value)
Family A:			
II-1	P62L/P62L	12	nd
II-2	P62L/P62L	14	nd
III-1	P62L/+	27	nd
III-2	P62L/+	nd	nd
III-3	P62L/+	45	nd
III-4	P62L/+	nd	nd
IV-1	P62L/+	33	nd
IV-2	P62L/+	38	nd
IV-3	+/+	82	nd
IV-4	+/+	117	nd
Family B:			
III-5	Y311C/+	70 (50–89)	65
III-6	Y311C/+	71 (69–73)	36
IV-1	Y311C/Y311C	14 (13–15)	29
IV-2	Y311C/+	70 (62–79)	85
Family C:			
II-3	G281E/+	26	nd
II-4	+/+	nd	nd
II-5	G281E/+	34	nd
II-6	G281E/+	44	nd
II-7	G281E/+	nd	nd
III-1	G281E/+	44	nd
III-2	G281E/G281E	3	nd
III-3	+/+	nd	nd

NOTE.—For family B, erythrocytic UROD activity was measured twice, and results are expressed as the mean of the two values (in parentheses). nd = no determination. Normal values for UROD activity: 100–190 nmol coproporphyrin I/h/mg hemoglobin.

mended by the supplier and analyzed on 2.5% Nusieve agarose gels (Tebu).

Site-Directed Mutagenesis

The same method was used to construct P62L and Y311C mutant cDNAs, by sequential PCR steps (Ausubel et al. 1994). For each mutant, two overlapping fragments containing the desired mutation were obtained by PCR from the normal UROD cDNA in the pGEM7Zf vector. The PCR primer pairs 62MutS/UDE62 and GEX1/62MutAS were used to construct the P62L mutant. The primers 311MutS/UDE62 and UDE31/311MutAS were used for the Y311C mutant. The resulting PCR products were purified from agarose gels using Wizard PCR preps (Promega) and used as templates for a new PCR step using GEX1/UDE62 and UDE31/UDE62 amplimers for the mutants P62L and Y311C, respectively. The PCR product obtained with GEX1/UDE62 contained the entire cDNA sequence between a 5′ *Bgl*II and a 3′ *Eco*RI restriction site to facilitate cloning in the expression vector pGEX-2T (Pharmacia) by use of *Bam*HI and *Eco*RI sites. The PCR product obtained with UDE31/UDE62 was cleaved by *Bam*HI and *Eco*RI and cloned by exchange in a normal pGEX-UD plasmid, linearized by the same enzymes. The integrity of the constructs was checked by sequencing; the clones containing extra mutations caused by PCR artifacts were discarded. Finally, the normal and mutant pGEX constructs were grown overnight prior to a 3-h induction with 2 mM isopropyl thiogalactoside (IPTG). Bacterial cells were washed in PBS and lysed by sonication for enzyme assay.

Results

UROD Gene Sequence

The sequence of genomic UROD is depicted in figure 3: the 10 exons are located within a 3-kb region and the 9 introns have short sequences (70–740 bp). Two transcription start sites have been identified at positions designated as −12 and −18 from the initiator ATG, leading to a very short untranslated region in the mRNA (Romana et al. 1987). The 5′ flanking region contains a putative TATA box (5′-TTAAATT-3′) and a GC box (5′-GGGGCGGAGC-3′), two consensus sequences which are usually found in the promoters of housekeep-

Figure 3 Nucleotide sequence of the UROD gene. Coding regions and introns are shown by upper-and lowercase letters, respectively, 5′UTR and 3′UTR by italics. The regions corresponding to amplimers or sequencing primers are underlined and notified in the right margin, together with exon location. The initiation and termination codon, in exon 1 and 10, respectively, as well as the polyadenylation signal, are in boldface.

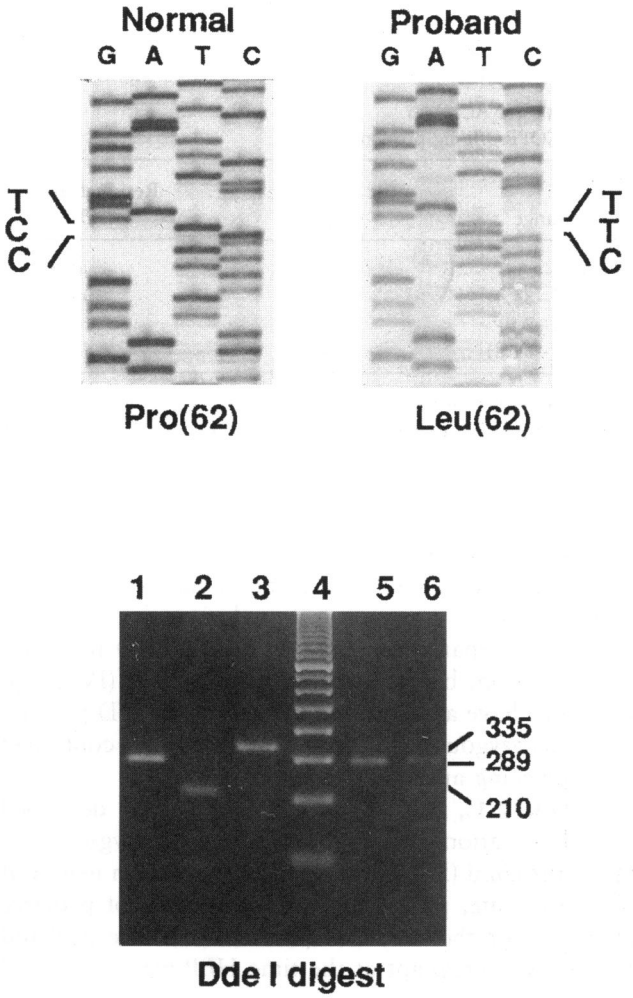

Figure 4 Sequence and restriction analysis of the mutation P62L. The proband is homozygous for the substitution TCC→TTC, at codon 62 (proline→leucine). A 335-bp PCR product was obtained with primers 3 and 5 (fig. 1) and digested with *Dde*I enzyme whose recognition site is abolished by the mutation. A 289-bp fragment is observed in the patient (lane 1), her affected brother (lane 5), and her daughter, who is an obligate carrier (lane 6). A 210-bp fragment is present in a normal grandson (lane 2). Lane 3 contains the undigested PCR product (335 bp) obtained in the proband. Lane 4 contains a 100-bp ladder (Pharmacia).

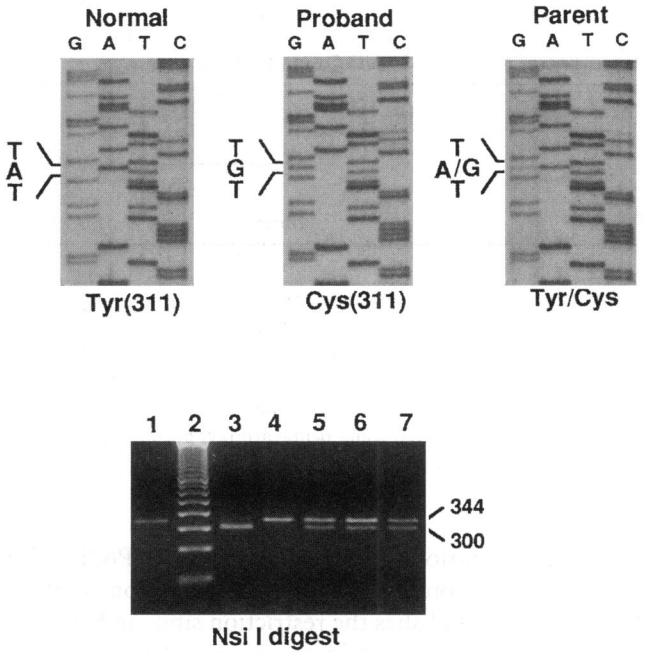

Figure 5 Sequence and restriction analysis of the mutation Y311C. The proband is homozygous for the substitution TAT→TGT at codon 311 (tyrosine→cysteine). A 344-bp PCR product (lane 1) was obtained from primers 10 and 12 (fig. 1) and digested with *Nsi*I enzyme whose recognition site is abolished by the mutation. The PCR product remains uncut in the proband (lane 4), while both cut and uncut bands (300 and 344 bp) are seen in heterozygous family members: mother, father, and sister in lanes 5, 6, and 7, respectively. The normal restriction profile is shown in lane 3 (300 bp). A 100-bp ladder is loaded in lane 2.

ing genes. The gene-sequencing data allowed us to select specific oligonucleotides for the amplification of the complete coding sequence from genomic DNA and direct sequencing of all exons and intron-exon boundaries of the UROD gene for the identification of mutations in HEP patients (fig. 2).

Mutation Analysis in Three HEP Families

In each proband, the entire coding sequence of UROD gene was analyzed by direct sequencing of the four genomic fragments shown in figure 2. The presence of the mutation in each proband was confirmed by restriction analysis performed not only on the proband but also in family members. Family trees and summarized data for enzymatic studies and DNA analysis are presented in figure 1 and table 2, respectively. A good correlation was observed between the genotypic analysis and the enzyme assays.

In family A, a new mutation was identified at the homozygous state in the proband (II-2) and her half-brother (II-1). A C→T substitution at nt 185 resulted in

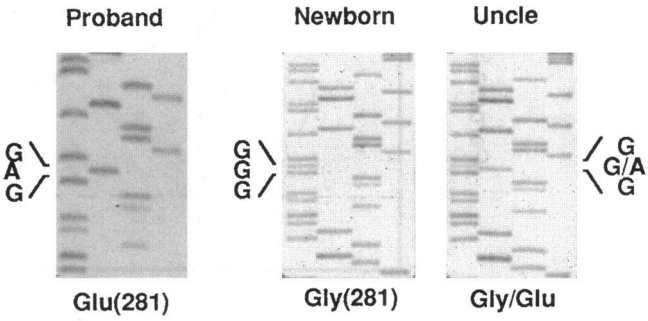

Figure 6 Sequence analysis of the mutation G281E. The proband is homozygous for the substitution GGG→GAG at codon 281 (glycine→glutamic acid). The brother (newborn) and the uncle have a normal and a heterozygous profile, respectively.

Table 3

Expression of Mutated GST-UD Fusion Proteins

Plasmid	Specific Activity (nmol COPRO/h/mg protein)				Residual Activity (%)
	Mean	SD	Range	Assays	
pGEX	.24	±.11	(.11–.38)	$n = 5$	0
pGEX-UROD	46.6	±23.9	(17.3–84.4)	$n = 6$	100
pGEX-UROD-P62L	2.18	±.66	(1.1–2.9)	$n = 6$	4.7
pGEX-UROD-Y311C	26.7	±21.2	(4.5–61.8)	$n = 5$	58.2

Note.—Bacterial lysates were obtained after an overnight culture followed by induction for 3 h with 2 mM IPTG and UROD specific activity (SA) was measured using pentacarboxyl porphyrin I permethyl ester as substrate. Residual activity was determined dividing 100 × [SA − SA (pGEX)] by [SA (pGEX-UROD) − SA (pGEX).

a missense mutation, Pro→Leu at codon 62 (P62L). The mutation was confirmed by *Dde*I restriction analysis (the mutation abolishes the restriction site), and the carrier status was assessed in family members in whom samples were available (table 2). Sequence and restriction analyses are shown in figure 4. The degree of consanguinity in this family is not known.

In family B, a novel mutation was observed at the homozygous state in a young girl (IV-1) born of consanguineous parents. An A→G substitution at nt 932 was detected, which results in a missense mutation Tyr→Cys at codon 311 (Y311C). The mutation abolishes a *Nsi*I restriction site. Parents of the proband (III-5 and III-6) and the proband's sister (IV-2) proved to be heterozygous for the mutation (fig. 5). Erythrocyte UROD activity was measured twice, and the values were in good agreement with the genotypic analysis (table 2). However, a discrepancy was observed in UROD immunoquantification, because the proband's sister (IV-2) appeared to have a subnormal amount of UROD protein. The heterozygous state in individual IV-2 was confirmed by sequencing and restriction analysis.

In family C, from Spain, the previously described G281E mutation was observed in the homozygous state in the proband (III-2). The mutation created a new *Alu*I restriction site, permitting rapid screening of pedigree members for the mutation (not shown). The proband mother was pregnant at the time HEP was recognized

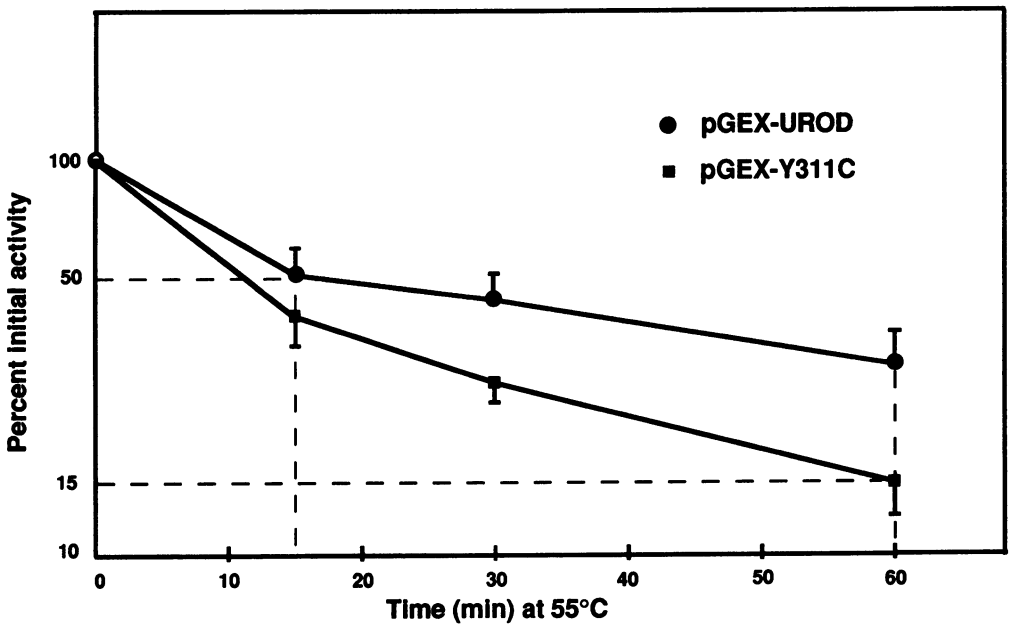

Figure 7 Thermostability of Y311C mutant expressed in *E. coli*. Supernatants of bacterial lysates were incubated at 55°C for 15 and 60 min before enzyme assay. Results are expressed as the percentage of initial activity at zero time. One hundred percent activity is 46.6 and 26.7 $nmol.h^{-1}.mg^{-1}$ in the normal and the Y311C mutant, respectively (table 2).

Table 4

Frequency of Mutations in 14 Independent HEP Families

Mutation	Ancestry	*n*	Allelic Status	Allele Frequency	References
P62L	Portuguese	1	Homoallelic	2/28	Present study
Y311C	Italian	1	Homoallelic	2/28	Present study
G281E	Spanish/Tunisian	9[a]	Homo/Hetero	17/28	Roberts et al. 1995
E167K	Italian	1	Homoallelic	2/28	Romana et al. 1992
R292G	Dutch	1	Heteroallelic	1/28	Verneuil et al. 1992
Deletion	Dutch	1	Heteroallelic	1/28	Verneuil et al. 1992
V134Q	British-German	1	Heteroallelic	1/28	Meguro et al. 1994
H220P	British-German	1	Heteroallelic	1/28	Meguro et al. 1994

[a] Seven Spanish and one Tunisian homoallelic patients and one Spanish heteroallelic patient.

in the proband, and cord-blood analysis revealed that the newborn (III-3) was normal at the UROD locus. A maternal uncle of the proband (II-3) developed clinical symptoms of PCT at the age of 32 years. Genomic sequencing of individual II-3 demonstrated this individual was heterozygous for the G281E mutation (fig. 6).

Prokaryotic Expression of the P62L and Y311C Mutants

The pGEX expression vector was used to express normal and mutated UROD proteins in *Escherichia coli.* In this vector, the inserted cDNA is in frame with the glutathione S-transferase gene and under the control of a strong tac promoter, which is inducible by IPTG. The promoter is responsible for the overexpression of the fusion protein, which can be easily purified by affinity chromatography on glutathione-sepharose columns. A recognition sequence for thrombin is located at the junction between glutathione and the UROD insert in the construct. Site-directed mutagenesis by sequential PCR was used to construct mutated plasmids containing either the P62L or the Y311C mutation. The enzymatic activity of normal and mutant UROD proteins was measured in bacterial lysates. As shown in table 3, the P62L mutant had very little residual activity, whereas the Y311C mutant had values that were approximately half those of normal levels. Similar results were obtained when the GST-UROD fusion protein was cleaved by thrombin before measuring UROD activity. The Y311C mutant, however, was thermolabile at 55°C as compared to the normal UROD protein (fig. 7).

Discussion

HEP is a rare disease. Approximately 30 patients with HEP have been described, and the clinical entity is often difficult to distinguish from congenital erythropoietic porphyria (Cruces-Prado et al. 1980; Verneuil et al. 1992). When the phenotype of HEP is mild, the disease may be mistaken for f-PCT. The frequency of UROD mutations observed in 14 independent HEP families is shown in table 4. In unrelated Spanish HEP, a striking genetic homogeneity has been observed. With the exception of one compound heterozygote, the probands of all Spanish subjects have carried the G281E mutation in the homozygous state. In families from other countries, the UROD mutations identified have been heterogeneous, and each family has carried one or two private mutations (Verneuil et al. 1993; Roberts et al. 1995). In most pedigrees reported, consanguinity among the parents of the proband has been found explaining the unusually high proportion of homozygosity for UROD mutations. Until recently, HEP and f-PCT were considered to be two different types of porphyria, since the parents of patients with HEP had not been noted to develop the clinical phenotype of f-PCT. In addition, the mutations observed in HEP patients had not been observed in pedigrees with f-PCT (Verneuil et al. 1993). Recently, however, the G281E mutation has been described in the heterozygous state in members of HEP pedigrees who subsequently developed clinically overt PCT (Roberts et al. 1995). A pedigree from Spain reported here also contained a member who was heterozygous for the G281E mutation and developed clinical symptoms of PCT. Two mutations recently described in HEP, V134Q and H220P (Meguro et al. 1994), and the new mutations described in the pedigrees reported here (P62L and Y311C) produce a relatively mild phenotype. These mutations have a relatively mild effect on UROD activity and were accompanied by clinical expression of the photosensitive skin disease only in homozygotes or in compound heterozygotes.

In order to confirm that the newly described mutations were responsible for the phenotype, we used the pGEX expression system, which provides a simple way to purify the recombinant mutant proteins. The mutant proteins derived from these constructs clearly demonstrated a decrease in catalytic activity for both the P62L and Y311C mutants and thermoinstability for the Y311C mutant. The P62L mutation corresponds to a change in a conserved amino acid

in human, rat, *Bacillus subtilis,* and *Synechococcus* species strain PCC 7942 but not in yeast or in *E. coli* (Nishimura et al. 1993). The P62L mutant (corresponding to position 55 in the yeast UROD gene) is located quite close to position 59 and 62 in the yeast gene. Mutations at position 59 and 62 have resulted in markedly impaired catalytic activity in yeast UROD (Chelstowska et al. 1992). The P62L substitution apparently modifies the spatial structure of the UROD protein because of the rigidity of the proline heterocycle. The mechanism by which the Y311C mutation influences thermostability of the UROD protein and clarification of the functional effects of all of the UROD mutations described in the human UROD gene await crystallographic studies of human UROD.

The identification of the molecular defects in UROD gene does contribute to the analysis of unclassified cases of porphyria and allows genetic counseling. Finally, in severe HEP forms, which may lead to gene therapy in the future (Verneuil et al. 1995), a precise knowledge of the molecular lesions is a prerequisite to any protocol of gene transfer. At the present time, more work is needed to evaluate the best delivery system of the therapeutic gene and the ideal target tissue (i.e., liver and/or bone-marrow). In the future, the availability of a mouse model of the disease would permit in vivo or ex vivo gene therapy experiments to clarify these points.

Acknowledgments

We are thankful to Prof. S. Sassa for UROD analysis by immunoblot in family B, S. Labatut and M.-H. Dealbert for technical assistance, and M.-T. Sanchez for typing the manuscript. The sequence reported in this work has been assigned the Genbank accession number U30787. This work was supported by grants from the Ministère de la Recherche et de la Technologie, the Institut National de la Santé et de la Recherche Médicale (INSERM-CRI95042), and the Conseil Régional d'Aquitaine.

References

Ausubel FM, Brent R, Kingston RE, Moore D, Seidman JG, Smith JA, Struhl K (eds) (1991) Current protocols in molecular biology. Vol 1, Suppl 15. J Wiley and Sons, New York

Chang CS, Sassa S (1985) δ-aminolevulinate dehydratase in human erythroleukemia cells: an immunologically distinct enzyme. Blood 65:939–944

Chelstowska A, Zoladek T, Garey J, Kushner J, Rytka J, Labbe-Bois R (1992) Identification of aminoacid changes affecting yeast uroporphyrinogen decarboxylase activity by sequence analysis of HEM12 mutant alleles. Biochem J 288:753–757

Cruces-Prado MJ, Enriquez de Salamanca R, Hernando MV, Payero MLP, Beltran TC, Aguilar AR (1980) Two cases of infantile and familial porphyria cutanea tarda. Dermatologica 161:205–210

Fujita H, Sassa S, Toback AC, Kappas A (1987) Immunochemical study of uroporphyrinogen decarboxylase in a patient with mild hepatoerythropoietic porphyria. J Clin Invest 79:1533–1537

Garey JR, Franklin KF, Brown DA, Harrison LM, Metcalf KT, Kushner JP (1993) Analysis of uroporphyrinogen decarboxylase complementary DNAs in sporadic porphyria cutanea tarda. Gastroenterology 105:165–169

Garey JR, Hansen JL, Harrison LM, Kennedy JB, Kushner JP (1989) A point mutation in the coding region of uroporphyrinogen decarboxylase gene associated with familial porphyria cutanea tarda. Blood 73:892–895

Garey JR, Harrison LM, Franklin KF, Metcalf KT, Radisky ES, Kushner JP (1990) Uroporphyrinogen decarboxylase: a splice-site mutation causes the deletion of exon 6 in multiple families with familial porphyria cutanea tarda. J Clin Invest 86:1416–1422

Garey JR, Labbe-Bois R, Chelstowska A, Rytka J, Harrison L, Kushner J, Labbe P (1992) Uroporphyrinogen decarboxylase in *Saccharomyces cerevisiae* HEM12 gene sequence and evidence for two conserved glycines essential for enzymatic activity. Eur J Biochem 205:1011–1016

Kappas A, Sassa S, Galbraith RA, Nordmann Y (1995) The porphyrias. In: Scriver CR, Beaudet AL, Sly WS (eds) The metabolic basis of inherited disease, 7th ed. McGraw Hill, New York, pp 2103–2159

Koszo F, Elder GH, Roberts A, Simon N (1990) Uroporphyrinogen decarboxylase deficiency in hepatoerythropoietic porphyria: further evidence for genetic heterogeneity. Br J Dermatol 122:365–370

Meguro K, Fujita H, Ishida N, Akagi R, Kurihara T, Galbraith RA, Kappas A, Zabriskie JB, et al (1994) Molecular defects of uroporphyrinogen decarboxylase in a patient with mild hepatoerythropoietic porphyria. J Invest Dermatol 102:681–685

Nishimura K, Nakayashiki T, Inokuchi H (1993) Cloning and sequencing of the *hemE* gene encoding uroporphyrinogen decarboxylase (UPD) from *Escherichia coli* K-12. Gene 133:109–113

Roberts AG, Elder GH, Enriquez de Salamanca R, Herrero C, Lecha M, Mascaro J (1995) A mutation (G281E) of the human uroporphyrinogen decarboxylase gene causes both hepatoerythropoietic porphyria and overt familial porphyria cutanea tarda: biochemical and genetic studies on Spanish patients. J Invest Dermatol 104:500–502

Romana M, Dubart A, Beaupain D, Chabret C, Goossens M, Romeo PH (1987) Structure of the gene for human uroporphyrinogen decarboxylase. Nucleic Acids Res 15:7343–7356

Romana M, Grandchamp B, Dubart A, Amselem S, Chabret C, Nordmann Y, Goossens M, et al (1991) Identification of a new mutation responsible for hepatoerythropoietic porphyria. Eur J Clin Invest 21:225–229

Romeo PH, Raich N, Dubart A, Beaupain D, Pryor M, Kusher J, Cohen-Solal M, et al (1986) Molecular cloning and nucleotide sequence of a complete human uroporphyrinogen decarboxylase cDNA. J Biol Chem 261:9825–9831

Toback AC, Sassa S, Poh Fitzpatrick MB, Schechter J, Zaider E,

Harber LC, Kappas A (1987) Hepatoerythropoietic porphyria: clinical, biochemical, and enzymatic studies in a three-generation family lineage. N Engl J Med 316:645–650

Verneuil H de, Beaumont C, Deybach JC, Nordmann Y, Safar Z, Kastally R (1984) Enzymatic and immunological studies of uroporphyrinogen decarboxylase in familial porphyria cutanea tarda and hepatoerythropoietic porphyria. Am J Hum Genet 36:613–622

Verneuil H de, Bourgeois F, de Rooij F, Siersema PD, Wilson JHP, Grandchamp B, Nordmann Y (1992) Characterisation of a new mutation (R292G) and a deletion at the human uroporphyrinogen decarboxylase locus in two patients with hepatoerythropoietic porphyria. Hum Genet 89:548–552

Verneuil H de, Ged C, Boulechfar S, Moreau-Gaudry F (1995) Porphyrias: animal models and prospects for cellular and gene therapy. J Bioenerg Biomembr 27:239–248

Verneuil H de, Grandchamp B, Beaumont C, Picat C, Nordmann Y (1986) Uroporphyrinogen decarboxylase structural mutant (Gly^{281}→Glu) in a case of porphyria. Science 324:232–234

Verneuil H de, Hansen J, Picat C, Grandchamp B, Kushner J, Roberts A, Elder G, et al (1988) Prevalence of the 281(Gly→Glu) mutation in hepatoerythropoietic porphyria and porphyria cutanea tarda. Hum Genet 78:101–102

Verneuil H de, Moreau-Gaudry F, Bensidhoum M, Hombrados I, Ged C (1993) Porphyria cutanea tarda and hepatoerythropoietic porphyria: recent advances in molecular pathology. In: Polli EE (ed) Molecular bases of human diseases. Elsevier Science, Amsterdam, pp 289–293