Familial Porphyria Cutanea Tarda: Characterization of Seven Novel Uroporphyrinogen Decarboxylase Mutations and Frequency of Common Hemochromatosis Alleles

Manuel Mendez,[1,2] Lonnie Sorkin,[1] Maria Victoria Rossetti,[1,2] Kenneth H. Astrin,[1] Alcira M. del C. Batlle,[2] Victoria E. Parera,[2] Gerardo Aizencang,[1] and Robert J. Desnick[1]

[1]Department of Human Genetics, Mount Sinai School of Medicine, New York, and [2]Centro de Investigaciones sobre Porfirinas y Porfirias, University of Buenos Aires, Buenos Aires

Summary

Familial porphyria cutanea tarda (f-PCT) results from the half-normal activity of uroporphyrinogen decarboxylase (URO-D). Heterozygotes for this autosomal dominant trait are predisposed to photosensitive cutaneous lesions by various ecogenic factors, including iron overload and alcohol abuse. The 3.6-kb URO-D gene was completely sequenced, and a long-range PCR method was developed to amplify the entire gene for mutation analysis. Four missense mutations (M165R, L195F, N304K, and R332H), a microinsertion (g10insA), a deletion (g645Δ1053), and a novel exonic splicing defect (E314E) were identified. Expression of the L195F, N304K, and R332H polypeptides revealed significant residual activity, whereas reverse transcription–PCR and sequencing demonstrated that the E314E lesion caused abnormal splicing and exon 9 skipping. Haplotyping indicated that three of the four families with the g10insA mutation were unrelated, indicating that these microinsertions resulted from independent mutational events. Screening of nine f-PCT probands revealed that 44% were heterozygous or homozygous for the common hemochromatosis mutations, which suggests that iron overload may predispose to clinical expression. However, there was no clear correlation between f-PCT disease severity and the URO-D and/or hemochromatosis genotypes. These studies doubled the number of known f-PCT mutations, demonstrated that marked genetic heterogeneity underlies f-PCT, and permitted presymptomatic molecular diagnosis and counseling in these families to enable family members to avoid disease-precipitating factors.

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Address for correspondence and reprints: Dr. Robert J. Desnick, Department of Human Genetics, Box 1498, Mount Sinai School of Medicine, Fifth Avenue at 100th Street, New York, NY 10029. E-mail: RJDesnick@vaxa.crc.mssm.edu

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Introduction

Familial porphyria cutanea tarda (f-PCT; MIM 176100) is an autosomal dominant disorder that results from the systemic half-normal activity of uroporphyrinogen decarboxylase (URO-D; E.C.4.1.1.37), the fifth enzyme in the heme biosynthetic pathway (Kushner et al. 1976; de Verneuil et al. 1978). The enzymatic defect leads to the accumulation of uroporphyrins and predisposes gene carriers to cutaneous manifestations including photosensitive dermatitis, mechanical fragility of the light-exposed skin, hyperpigmentation, and hypertrichosis (Grossman et al. 1979; Kappas et al. 1995; McGovern et al. 1996; Elder 1998). Homozygosity for URO-D mutations results in the rarer but more severe homozygous dominant disorder hepatoerythropoietic porphyria (HEP), which has clinical onset in childhood and a phenotype similar to that of congenital erythropoietic porphyria (Smith 1986; Koszo et al. 1990; Kappas et al. 1995). In addition, a more common acquired form, sporadic porphria cutanea tarda (s-PCT), is characterized by similar clinical features but is due to the isolated deficiency of hepatic URO-D activity (de Verneuil et al. 1978; Elder et al. 1978). The liver-specific enzyme deficiency in s-PCT does not result from mutations in the URO-D gene (Garey et al. 1993) but is induced by unknown mechanisms associated with iron overload, alcohol abuse, estrogens, and/or hepatitis C infection (Grossman et al. 1979; Fargion et al. 1992; DeCastro et al. 1993; Kappas et al. 1995).

URO-D catalyzes the conversion of uroporphyrinogen III to coproporphyrinogen III by the sequential removal of the four carboxylic groups of the acetic-acid side (Jackson et al. 1976; de Verneuil et al. 1980). The enzyme has been purified to homogeneity from human erythrocytes and has been shown to have a molecular weight of ~46 kD (de Verneuil et al. 1983). Recently, the crystal structure of human URO-D has been determined at 1.6-Å resolution (Whitby et al. 1998), which has revealed a homodimeric structure with a single active site. The enzyme is encoded by a single gene localized

to chromosomal region 1p34 (de Verneuil et al. 1984; Dubart et al. 1986). The URO-D cDNA was isolated in 1986 and contains an open reading frame of 1,104 nucleotides that encodes a 367-amino-acid polypeptide (Romeo et al. 1986). Portions of the genomic sequence have been reported (Romana et al. 1987; McManus et al. 1994), but only recently, while this research was being performed, was the entire 3-kb gene sequenced (Moran-Jimenez et al. 1996; Sorkin et al. 1996). The gene contains 10 exons and a TATA box and a GC box in the promoter region. There is no evidence for erythroid-specific and housekeeping forms of URO-D, as have been identified in porphobilinogen synthase and hydroxymethylbilane synthase (Grandchamp et al. 1987; Yoo et al. 1993; Kaya et al. 1994). The availability of the cDNA and genomic sequences encoding URO-D permits investigation of the mutations causing f-PCT. Such studies are particularly valuable for accurate presymptomatic diagnosis and counseling of gene carriers in f-PCT families. To date, only eight mutations, (L253Q, G281V, G281E, G318R, I334T, 828Δ31, 890ΔC, and IVS6^{+1}) have been identified in unrelated families with f-PCT (Garey et al. 1989, 1990; Roberts et al. 1995; McManus et al. 1996; also see Human Gene Mutation Database).

Although iron overload has been considered an important precipitating factor for s-PCT (Lundvall et al. 1970; Felsher and Kushner 1977), its role in f-PCT is not clear. The recent identification of the hemochromatosis (HFE) gene and its common C282Y and H63D mutations has permitted investigation of the frequency of these lesions in PCT patients (Feder et al. 1996). To date, the occurrence of these HFE mutations in f-PCT has not been investigated, but in the two European s-PCT populations studied to date, the frequency of either the C282Y or H63D mutation was increased in British and Italian s-PCT patients compared with that in the respective normal populations (Fargion et al. 1996; Roberts et al. 1997; Santos et al. 1997; Sampietro et al. 1998). The frequency of the C282Y allele in 41 British s-PCT patients was almost fivefold higher (30.5% vs. 5.9%) than that in normal British individuals, whereas there was no difference between the frequency of the H63D allele in the British s-PCT and that in normal British individuals (Roberts et al. 1997). Interestingly, the H63D allele was twofold more frequent (28.7% vs. 12.6%) in the 68 Italian s-PCT patients than in normal Italian individuals, whereas there was no difference between the frequency of the C282Y allele among Italian s-PCT patients and normal Italian individuals (Sampietro et al. 1998). In sum, the occurrence of heterozygosity or homozygosity for the HFE mutations among British and Italian s-PCT patients was 68.3% and 52.9%, respectively—frequencies higher than those in the respective normal populations.

In the present report, we describe an efficient long-range PCR method for amplifying the entire 3.6-kb genomic sequence as a single amplicon for detection of gene rearrangements and for sequencing. By this method, seven new URO-D mutations, including four missense mutations, a single base insertion, a deletion, and a novel splicing defect, were identified in 10 unrelated f-PCT patients from Argentina. Each missense mutation was characterized by expression studies, and the splice-site mutation was confirmed by reverse transcription–PCR (RT-PCR) analysis. Of interest, the microinsertion found in four presumably unrelated f-PCT families was shown by haplotype analysis to have arisen independently in at least three of the families. In addition, the occurrence of HFE C282Y and H63D mutations was determined in these probands with the inherited form of PCT, to assess their predisposition to iron overload and to correlate disease severity with their URO-D and HFE genotypes.

Material and Methods

Patient Specimens, Enzyme Assays, and DNA Isolation

Ten unrelated f-PCT patients from Argentina were studied. Fresh 24-h urine samples and peripheral blood samples were collected from each f-PCT patient and family member, for diagnostic and research purposes, after informed consent had been obtained, and the urinary porphyrin concentrations (Batlle 1997) and the erythrocyte URO-D activity of each patient were determined (Lim et al. 1983; Afonso et al. 1985). Cultured lymphoid cell lines were established and maintained as described elsewhere (Anderson and Gusella 1984). DNA was isolated from either peripheral blood or cultured lymphoblasts by the Puregene DNA Isolation Kit (Gentra Systems).

PCR Amplification of the Entire URO-D Gene

PCR primers for amplifying the introns of the URO-D gene were designed on the basis of the cDNA and intron-exon sequences (Romana et al. 1987; McManus et al. 1994; Moran-Jimenez et al. 1996), and the entire genomic sequence was determined. Here, the A of the initiation of translation ATG is numbered as 1; upstream nucleotides are indicated by negative numbers, beginning with C as −1. PCR and sequencing were performed as described elsewhere (Chen et al. 1994). On the basis of the information obtained from sequencing the entire gene, primers for long-range PCR and the sequencing of each exon and intron/exon boundaries were designed as shown in table 1.

Table 1

PCR Primers for Long-Range Amplification, Sequencing Reactions, and Site-Directed Mutagenesis of the URO-D Gene

Primer Type	Sequence
Long-range PCR:	
Upper	5′-TATGGACCTGGCTGGATAAGACTGTTGGT-3′
Lower	5′-GGGACAATCTTTCACAAACAAAACTACAC-3′
Sequencing, for each exon:	
1	5′-TACAGAAAGGGGCGGAGCCTGGACTGG-3′
2	5′-GGGAGCTGGCCTGGAGGAGGTAGATAG-3′
3	5′-GAGGAGAAAAGTTTTCGAGGGGCA-3′
4	5′-CAAAAGAGGGAAAGATTTATGCCTTCA-3′
5	5′-CTGAACAGAACCTTTCCTCCTGGATTC-3′
6	5′-GGGGTAGACAAAAGGAAGGGTCAGTC-3′
7	5′-GTGGATTTTGTATGTGGGGGAAACTTC-3′
8	5′-GGGATGGGTTGAGTGAAGGTGGTCCTG-3′
9	5′-GGAGCTGCCATGTATGCAGTTACCA-3′
10	5′-ATAGGGAGGACAAAGGCTTGCTGGT-3′
Site-directed mutagenesis:	
R332H-S	5′-CTGTGCCTTGTATGCATCTGAGGAGGAGATCGGGCAGTTGGTGAAGC-AGATGCTGGATGACTTTGGACCACATCACTAC-3′
R332H-AS	5′-ATCAGGCTGAAAATCTTCTCTCATCCGCCA-3′
N304K-S	5′-TGGCCAAGCAAGTGAAGGCCAGGTTGCGGGA-3′
N304K-AS	5′-TGCCCGATCTCCTCCTCAGATGCATACAAGGCACAGGGGTCCAGTTTGCC-3′
M165R-1	5′-TGTTGTACCCCAGGCACTGGGCATGGAGGT-3′
M165R-2	5′-CACCACCCTCAACCCTGTATGTCATCAGG-3′
M165R-3	5′-CCTGATGACATACAGGGTTGAGGGTGGTG-3′
M165R-4	5′-ATCAGGCTGAAAATCTTCTCTCATCCGCCA-3′
L195F-1	5′-TGTTGTACCCCAGGCACTGGGCATGGAGGT-3′
L195F-2	5′-CAGAGCATCAGTGAAGATGCGAAGCAGCT-3′
L195F-3	5′-AGCTGCTTCGCATCTTCACTGATGCTCTG-3′
L195F-4	5′-ATCAGGCTGAAAATCTTCTCTCATCCGCCA-3′
RT-PCR:	
RT-1	5′-GTTACAGACAGCTGACCATGGA-3′
RT-2	5′-CGATCAATCATCTGTGTTAGTGG-3′

Long-Range PCR and Cyclic Sequencing

The URO-D gene was amplified from genomic DNA (1 μg) in a single reaction using sense and antisense primers (table 1) and the GeneAmp XL PCR Kit (Perkin-Elmer), according to the manufacturer's instructions. For the first 16 cycles, annealing and extension were for 3 min each at 67°C, and for the last 12 cycles the times were increased 15 s each cycle, with a final extension cycle of 10 min at 72°C. A portion of the PCR product was analyzed by agarose-gel electrophoresis to determine that the long-range reaction was successful and to identify any gross gene rearrangements. For sequencing, each exon and the adjacent intron/exon boundary was PCR-cycle sequenced with the indicated primers (table 1), with 1 μl of the long-range PCR product used as the template and with the AmpliCycle Sequencing Kit (Perkin-Elmer) used according to manufacturer's instructions. The PCR sequencing program consisted of 30 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 1 min. The reactions were run on acrylamide sequencing gels. The gels were dried, placed against Kodak X-OAR film for 24 h, and then developed.

Prokaryotic Expression and Characterization of URO-D Point Mutations

The normal and mutant URO-D alleles were expressed in *Escherichia coli* strain λJM109, by the pKK223-3 expression system (Pharmacia Biotechology) as described elsewhere (Tsai et al. 1988; Warner et al. 1992; Chen et al. 1994; Xu et al. 1995). The *Eco*RI fragment of the full-length URO-D cDNA was blunt-end ligated into the *Hind*III site of pKK223-3. This construct was designated "pK-UROD." Each of the missense mutations was introduced into the pK-UROD plasmid by site-directed mutagenesis (Chen et al. 1994). For the R332H and N304K mutations, each expression construct was generated by amplification with the indicated sense and antisense primers (table 1). To introduce mutations M165R and L195F into the expression vector, two amplifications were necessary, since there were no

convenient restriction sites. First, each region containing a restriction site was amplified individually, and then the PCR products were ligated. For M165R, one set of primers was pM165R-1 and pM165R-2, whereas the other primer set was pM165R-3 and pM165R-4. After the two PCR products had been ligated together, the final construct was produced by primers 1 and 4. A similar procedure was used to construct an expression vector containing the L195F mutation. One primer set was pL195F-1 and pL195F-2, whereas the other set was pL195-3 and pL195-4. Primers 1 and 4 also were used to produce the final L195F construction. Each expression construct was sequenced, to ensure that only the desired mutation had been introduced and that the remainder of the sequence was correct.

Assay of URO-D Activity

Transfection, bacterial growth, and isopropylthiogalactoside (IPTG) induction were as described elsewhere (Tsai et al. 1988). The cells were washed twice with PBS, and the pellet was resuspended in 250 μl of lysis buffer (0.067 M sodium phosphate, pH 7.0, 0.1% Triton-X-100) and sonicated on ice. The extract was then centrifuged for 1 min at 13,000 *g,* and the supernatant was used as the enzyme source. The URO-D assay consisted of 0.67 M sodium phosphate, pH 7.0 (50 μl), 20 mM glutathione (50 μl), 1 mM EDTA (50 μl), uroporphyrinogen III (35 μg/ml; 50 μl), obtained by uroporphyrin reduction in 25 mM NaOH with 3% sodium amalgam, H_2O (250 μl), and bacterial extract (50 μl). The reaction was performed in tubes that had been filled with nitrogen gas and were incubated for 30 min at 37°C, with shaking, in the dark. The reaction was stopped by the addition of 55 μl of 50% trichloroacetic acid. The porphyrinogens were oxidized to porphyrins by exposure to white light for 20 min. The samples were then centrifuged for 1 min at 13,000 *g,* filtered through 0.2-μm Whatman filters, and analyzed by high-pressure liquid chromotagraphy (HPLC), according to the method of Lim et al. (1983). Specific activity was calculated as nmol of coproporphyrin III produced/h/mg protein. The nanomoles of coproporphyrin III were determined by extrapolation from a calibration curve of nanomoles of coproporphyrin III versus peak area.

Thermal Stability of pK-URO-D and pK-R332H

Bacterial lysates containing either pK-UROD or pK-UROD-R332H were induced with IPTG for 3 h. Extracts were diluted with 0.067 M sodium phosphate buffer, pH 7, to the same protein concentration (0.1–0.2 mg/ml). They were preincubated for various times, from 0 to 180 min, at 37°C, were placed on ice, and then were assayed for URO-D activity. Two enzyme preparations from independent inductions were assayed, and the results were averaged.

RT-PCR Studies of URO-D Mutation E314E

To confirm that mutation E314E was a splice-site mutation, RT-PCR was performed. Leukocytes were isolated from blood by Ficoll-Paque (Pharmacia Biotech), and RNA was isolated from the leukocytes by RNA Zol B (Tel-Test) and was reverse transcribed with M-MLV Reverse Transcriptase and Oligo$(dT)_{12–18}$ primers (Life Technologies), according to manufacturer's instructions. The total amount of cDNA was then amplified by primers RT-1 and RT-2 (table 1), and the PCR product was analyzed on 1.5% agarose gels. Each band was cut out of the gel separately, and the DNA was eluted by soaking the agarose in a small amount of Tris-EDTA buffer (10 mM Tris, 1 mM EDTA, pH 7.4). Each exon was then cycle sequenced by the *fmol* DNA cycle sequencing kit (Promega), according to manufacturer's instructions.

Analysis of the HFE C282Y and H63D Mutations

Genomic DNAs from the f-PCT probands were analyzed for the common HFE mutations, C282Y and H63D. DNA was PCR amplified by the primers and conditions described by Feder et al. (1996). The PCR products were cut by the restriction enzyme *Sau3A*I (New England Biolabs), in the case of H63D, and the restriction enzyme *Rsa*I, in the case of C282Y, and were analyzed by agarose-gel electrophoresis.

Haplotyping of f-PCT Probands with URO-D Mutation g10insA

To determine whether the four presumably unrelated probands from Argentina who had the g10insA mutation were related to each other, haplotyping was performed. Six CA-repeat markers from the chromosome 1p34 region (Dib et al. 1996) were obtained from Research Genetics; these six markers, from centromere to telomere, were D1S193 (AFM057xf4), D1S463 (AFM154xg11), D1S2713 (AFMa349yb5), D1S211 (AFM122xe1), D1S2724 (AFMb015wc9), and D1S451 (AFM248tf9). PCR amplification and gel analysis were performed as described by Gelb et al. (1995).

Results

Sequencing of the URO-D Gene

To facilitate identification of mutations in the URO-D gene, the entire genomic sequence was determined by primers based on the published cDNA and intron/exon boundary sequences (Romeo et al. 1986; Romana et al. 1987). Our sequence (Entrez Nucleotide Sequence

Table 2

Sequence Differences Identified in the URO-D Gene

Location and Genomic Number[a]	Difference[b]
5′ UTR:	
−225	insG
−222	delT
−207	insC
−213	T→G
−172	insC
−167	insG
−158	insC
Intron 1:	
120	delA
146	insG
236	T→C
237	C→T
317	delT
322	delG
474	insCACC
543	delG
593	delG
598	A→G
Intron 2:	
794	insG
Intron 6:	
1853	C→A
1855	G→C
1901	T→C
Intron 9:	
2768	delC
2790	delC
2848	C→A
2849	A→C
2858	C→A
2941	insG
2976	delT
2990	delC
3001	C→G
3003	insC
3′ UTR:	
3234	C→G
3235	C→T
3311	DelC

[a] Based on sequence in present study.

[b] Based on comparison of sequence in present study (Entrez Nucleotide Sequence Search, Genbank accession number AF047383) versus that reported by Moran-Jimenez et al. (1996).

Search, Genbank accession number AF047383) was 3,659 bp long, including both 330 bp 5′ upstream from the initiation of translation ATG and 96 bp of flanking sequence 3′ of exon 10. During our studies, the genomic structure of URO-D gene was published (Moran-Jimenez et al. 1996). Our exonic sequences were similar to the published genomic sequence, except for a 1-base difference in exon 4, a C at position g1043 in place of a T, changing codon 77 from CTT to CCT, which encoded a leucine-to-proline substitution. This was consistent with the previously published cDNA sequence (Romeo et al. 1986). Compared with the originally published cDNA sequence (Romeo et al. 1986), our genomic sequence had the five base changes previously noted by other investigators (McManus et al. 1994; Moran-Jimenez et al. 1996), in cDNA codons 23 (GCT→GCC), 305 (TTG→CTG), and 337 (TTG→CTG); these DNA changes did not alter the amino acid sequence. However, DNA changes at codons 103 (AGC→GGC) and 120 (GCG→CGC) did alter the amino acid sequence. In addition, 34 differences between the previously published (Moran-Jimenez et al. 1996) sequence and our sequence were identified, including insertions, deletions, and base changes (table 2). Our sequence was observed in the homozygous state in amplified genomic DNAs from normal individuals and in the normal alleles of the f-PCT patients studied. In addition, heterozygosity for an intron 6 polymorphism, g1835G→T, was identified in all 10 Argentinian f-PCT probands and in 1 of the 15 normal Argentinean individuals.

Identification of Mutations by Long-Range PCR and Cyclic Sequencing

On the basis of the URO-D genomic sequence, primers were designed to amplify the entire 3.6-kb gene as a single PCR product. Each exon was then cycle sequenced, with the long-range PCR product used as the template (fig. 1). By this method, the URO-D gene was amplified and sequenced from 10 Argentinean f-PCT probands whose biochemical diagnosis was based on

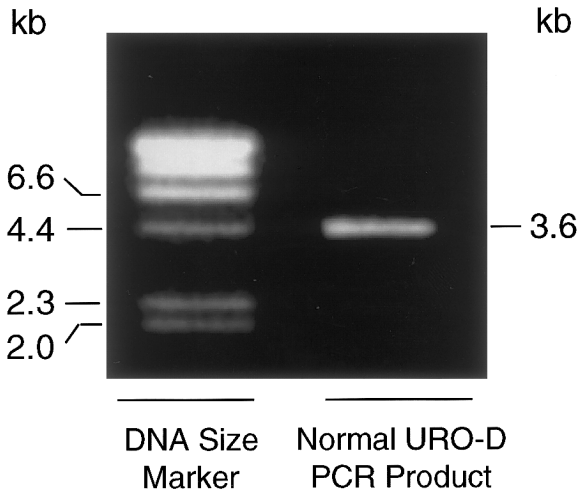

Figure 1 Agarose-gel electrophoresis of 3.6-kb URO-D long-range PCR product amplified from genomic DNA of a normal individual. The PCR product was electrophoresed in 1% agarose with Tris-Borate-EDTA buffer, pH 8.4. The DNA size marker is the lambda DNA-*Hind*III digest (New England Biolabs).

erythrocyte URO-D enzyme activity and on urinary and plasma porphyrin concentrations (table 3). Seven new mutations were identified, including four missense mutations: a T→G tranversion in exon 6, at genomic nt g1663 (cDNA nt 494), which predicted replacement of a methionine by an arginine at position 165 (a change designated "M165R"); a C→T transition in exon 6, at genomic nt g1752 (cDNA nt 583), which predicted substitution of a leucine by a phenylalanine at residue 195 (a change designated "L195F"); a C→A transversion in exon 9, at genomic nt g2711 (cDNA nt 912), which predicted an asparagine-to-lysine substitution of position 304 (a change designated "N304K"); and a G→A transition (at a CpG dinucleotide) of genomic nt g3124 (cDNA nt 995) in exon 10, which predicted an arginine-to-histidine substitution at residue 332 (a change designated "R332H") (table 3).

A novel splicing mutation (designated "E314E") was detected as a G→A transition (gDNA nt 2741; cDNA nt 942) of the last base of exon 9 (figs. 2*A*–2*C*). In addition, two gene rearrangements were identified. A single adenosine insertion 10 bp downstream from the initiation ATG in exon 1, at genomic nt g10 (cDNA nt 10; a change designated "g10insA" fig. 3*A*) caused a frameshift, which resulted in 13 different amino acids and in a termination signal in codon 17 of exon 2. The second gene rearrangement was a large deletion of 1,053 bases, designated "g645Δ1053"(fig. 3*B*). When the long-range PCR product amplified from genomic DNA of the proband was electrophoresed, two amplicons were observed, one the expected ~3.6-kb band and the other a smaller, ~2.6-kb band. Sequencing of the smaller product revealed a deletion of 1,053 bases, which included the region from the fifth base of exon 2 (g645) to g1698 in exon 6. A 10-bp direct repeat (GATCGCCAGA) from the intron 1/exon 2 junction was inserted at the 5′ breakpoint (see fig. 3*B*). Of note, the insertion mutation was found in four unrelated families from different areas of Argentina, whereas each of the other mutations was identified in only one Argentinean f-PCT family.

Prokaryotic Expression of the URO-D Missense Mutations

To further characterize the URO-D missense mutations, pK-UROD expression vectors for each of the mutant alleles were constructed and were expressed in *E. coli,* and the URO-D activities of the mutant proteins were determined, as shown in table 4. The activity of mutation M165R was <2% of the mean level expressed by the normal allele, whereas the N304K and L195F alleles expressed mutant polypeptides that had significant residual activity (19.5% and 32.1%, respectively, of the expressed normal mean activity). Interestingly, the R332H allele expressed more activity (127%) than the normal allele. However, stability studies indicated that the R332H mutant polypeptide was significantly less stable than the wild-type enzyme when incubated at 37°C, pH 7.0 (fig. 4). Compared with the ~105-min half-life of the normal enzyme, the half-life of R332H activity was ~40 min, which indicates that the mutant protein had a markedly reduced stability.

Characterization of Splice-Site Mutation E314E

Sequencing of the URO-D gene in the proband of family 1 identified a G→A transition in the last base of exon 9 (change E314E; fig. 2*A*), which did not alter the amino acid sequence. To determine whether this exonic

Table 3

Biochemical and Molecular Findings in Argentinean f-PCT Probands

Source (Ancestry/Sex) of Proband	Total Urinary Porphyrins (μg/24 h)	Plasma Porphyrin Index	URO-D Erythrocyte Activity (% of Normal[a])	URO-D Mutation
Normal controls	<250	<1.3	100	…
1 (Italian/F)	8,910	7.48	45	E314E
2 (Portuguese/M)	11,600	6.00	42	R332H
3 (Spanish/F)	3,040	3.10	50	L195F
4 (Italian/F)	5,010	6.75	46	M165R
5 (Spanish/F)	1,130	2.30	51	N304K
6 (Spanish/M)	2,030	2.10	42	g645Δ1053
7 (Arabic/F)	7,530	5.87	52	g10insA
8 (Spanish/M)	4,490	4.30	40	g10insA
9 (Spanish/M)	3,120	3.43	44	g10insA
10 (Spanish/M)	3,960	4.25	22	g10insA

[a] Normal mean activity of erythrocyte URO-D = 4.2 ± 0.6 nmol of coproporphyrinogen/h/ml packed erythrocytes. Data were obtained at the time of diagnosis; at present, all patients are on therapy and in remission.

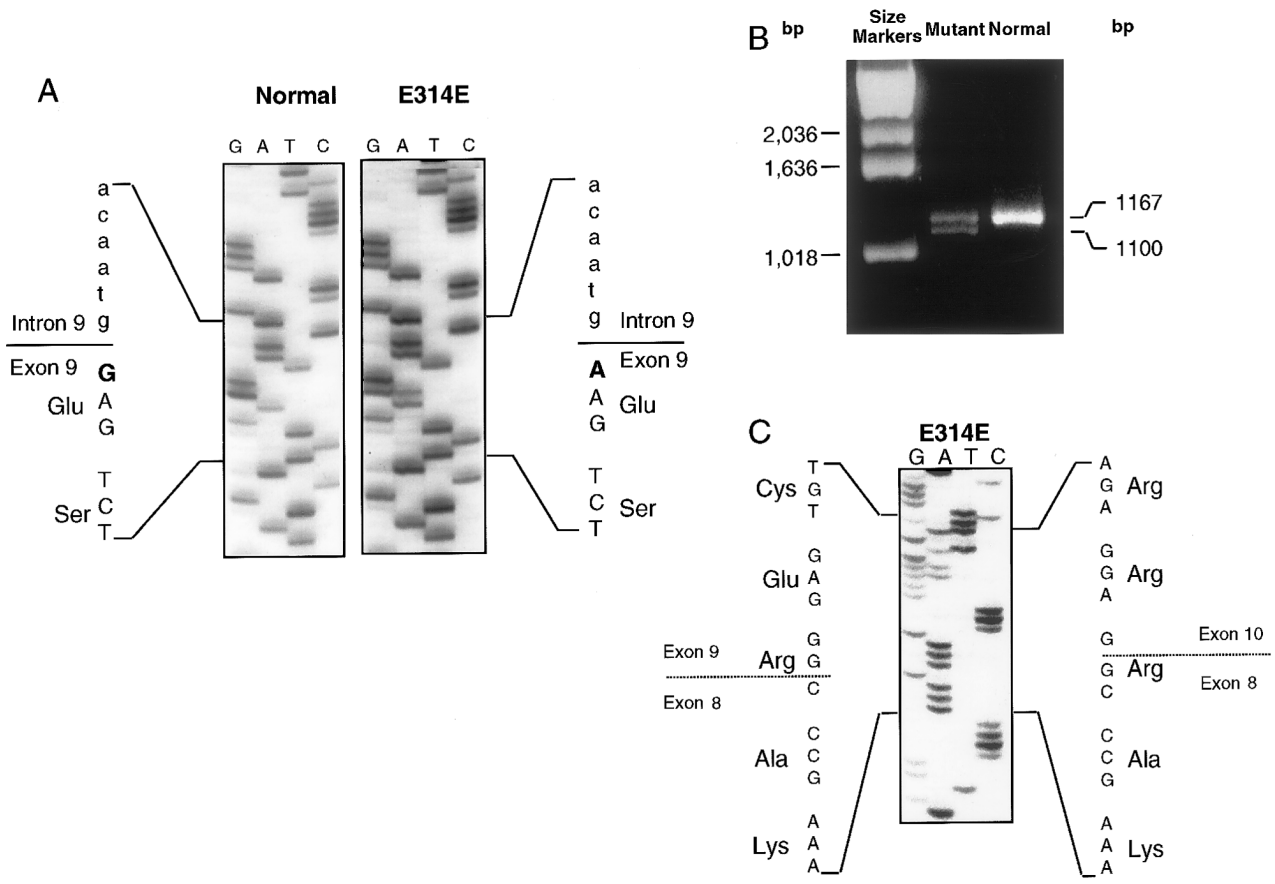

Figure 2 Characterization of splicing mutation E314E. *A*, Partial sequencing gel showing E314E due to a G→A transition at g2741, the last base of exon 9. Also shown are (*B*) agarose-gel electrophoresis and (*C*) partial sequencing of the URO-D RT-PCR product amplified from total lymphoblast RNA from a normal individual and from the f-PCT patient, which shows the deletion of exon 9.

mutation altered the splicing of intron 9, RT-PCR was performed on total mRNA from the leukocytes of the proband. Two PCR products were observed; one was of normal size, and the second was shorter by ~60–70 bp (fig. 2*B*). Sequencing of the RT-PCR products revealed that the normal-size product had the normal sequence, whereas the entire 67-bp exon 9 was deleted in the shorter amplicon and exon 8 was joined directly to exon 10 (fig. 2*C*)

Haplotype Analysis of the g10insA Alleles

The g10insA microinsertion was identified in four presumably unrelated PCT families. To determine whether these f-PCT families were related, haplotype analysis was performed with six polymorphic markers that mapped closely to the URO-D gene at 1p34 (Dib et al. 1996). Although families 7 and 10 were of Arab and Spanish descent, respectively, their probands had the same haplotype for all six markers (table 5), which indicates a common ancestry of their g10insA mutation.

Discussion

The identification and characterization of seven new mutations in the URO-D gene that cause f-PCT highlight the molecular heterogeneity underlying this porphyria. These mutations were detected by amplification of the entire genomic sequence and by cycle sequencing of the exons and adjacent intron/exon boundaries in the 3.6-kb amplicon. Four missense mutations, a coding-region splicing mutation, the first large deletion, and the first microinsertion causing f-PCT were identified. Figure 5 shows both the newly identified and all previously published URO-D mutations that cause f-PCT. The fact that the seven mutations reported in the present study were found in 10 f-PCT families from Argentina attests to the remarkable genetic heterogeneity underlying this inherited porphyria. That the g10insA mutation occurred in four Argentinean families suggests a possible common origin of the mutation. Indeed, haplotyping of polymorphic markers flanking the URO-D gene indicate that

the microinsertion in families 7 and 10, of Arab and Spanish descent, respectively, shared a common haplotype, whereas families 8 and 9, both of Spanish ancestry, had different haplotypes (table 5). These findings suggest either that the mutation is very old or, more likely, that the microinsertion arose independently at least three times.

Crystallographic studies have indicated that URO-D is active as a dimeric protein (Whitby et al. 1998), so that missense mutations can alter enzymatic function, subunit association, and/or stability of the dimer. Prokaryotic expression of the four newly discovered missense mutations (M165R, L195F, N304K, and R332H) revealed that each has a unique effect on enzymatic function. The M165R allele, in which a highly conserved hydrophobic and neutral methionine is replaced by a hydrophilic and basic arginine, expressed little, if any, URO-D activity in *E. coli,* which indicates that the mutant polypeptide is inactive, unstable, or both. In contrast, the other three missense mutations all expressed enzymatic activity in *E. coli,* which indicates that these polypeptides folded such that their active sites retained at least partial catalytic function. The L195F allele, in which a hydrophobic and neutral leucine is substituted by a hydrophobic, neutral, and aromatic phenylalanine residue, had ~30% of the mean activity expressed by the normal allele. Although this leucine has been highly conserved during evolution, it is notable that phenylalanine is in this position in tobacco and barley URO-D polypeptides (Swiss-Prot Annotated Protein Sequence Database trEMBL). Thus, it is not surprising that the human mutant protein retained significant activity when expressed in *E. coli* but that this lesion also rendered the mutant protein unstable (data not shown).

Table 4

Expression of URO-D Mutations in *E. coli*

Construct	Mean URO-D Activity[a] [Range] (U/mg)	% of Mean Normal Activity
pK223-3	.14 [.10–.18]	0
pK-UROD	16.4 [9.79–24.7]	100
pK-UROD-M165R	.21 [.18–.23]	1.36
pK-UROD-L195F	5.27 [2.60–10.3]	32.1
pK-UROD-N304K	3.21 [1.61–5.07]	19.5
pK-UROD-R332H	20.8 [13.7–27.5]	127.0

[a] Average of five to eight independent determinations.

The N304K mutant allele, in which a neutral asparagine is replaced by a hydrophilic and basic lysine, expressed an enzyme polypeptide that had ~20% of normal activity. This mutation occurred in the middle of a highly conserved block of seven amino acids that presumably is important for enzyme function and/or stability. It is notable that, in *E. coli,* the R332H allele, in which a hydrophilic and basic arginine is substituted by a hydrophilic, basic, and aromatic histidine, expressed activity that was 1.2-fold greater than normal. However, the R332H polypeptide was unstable at 37°C (fig. 4), which explains its causative role in f-PCT. It is interesting to note that only one other URO-D mutation, G318R, had normal activity and stability when expressed in *E. coli* (McManus et al. 1996). However, the frequency of the G318R lesion in the normal population was not determined. The fact that the substituted arginine was

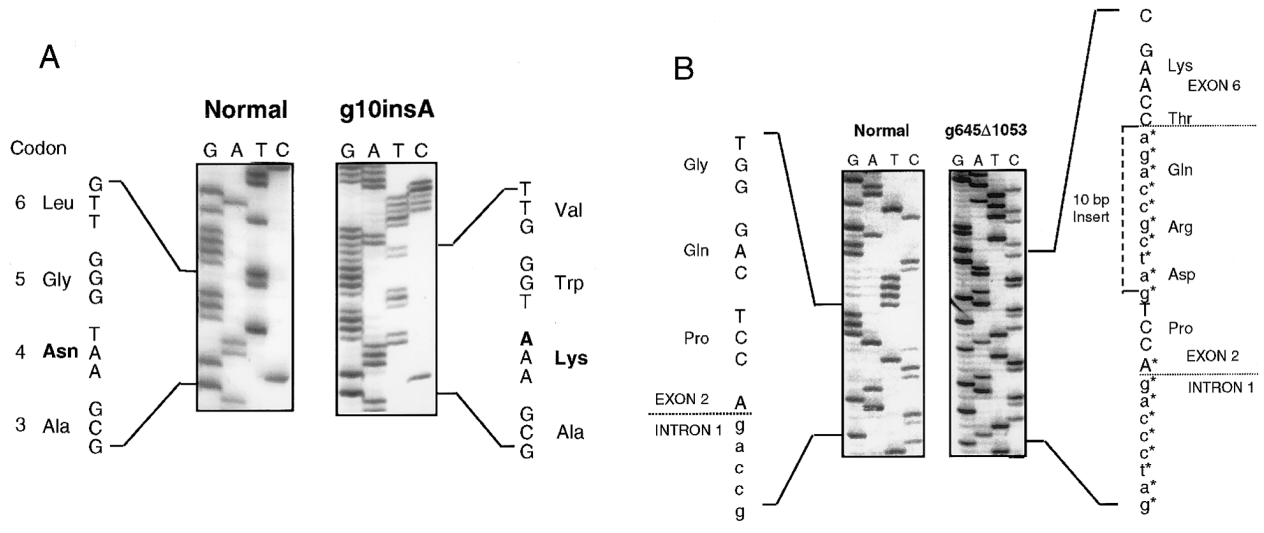

Figure 3 *A,* Partial sequencing gels showing the adenine insertion, g10insA, in exon 2. *B,* Partial sequencing gel showing the deletion of 1,053 bp, from g645, in exon 2, to g1698, in the middle of exon 6. Note that the deleted region was replaced by a 10-bp direct repeat of the starred intron 1/exon 2 sequence.

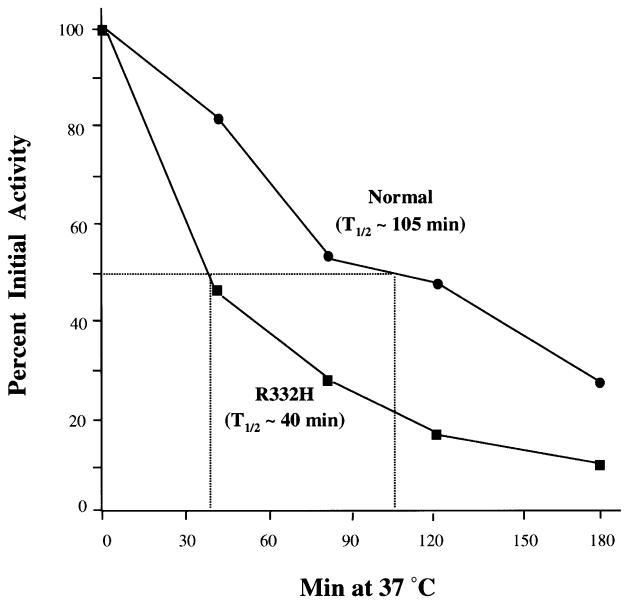

Figure 4 Thermostability of the URO-D activity expressed in *E. coli* by pK-UROD and pK-UROD-R332H. Cell extracts were incubated at 37°C, pH 7.0, for the times indicated, and the URO-D activities were determined. The results are expressed as the percentage of the initial activity, based on the mean of two independent assays each for the normal and mutant enzymes.

present in this position in a bacterial species suggests that this mutation may be a rare polymorphism. That both the G318R and the R332H alleles expressed high levels of activity in *E. coli* suggests that the mutations of these relatively unconserved amino acids are not critical for catalytic function, although the R332H substitution rendered the polypeptide markedly unstable. Other enzyme variants, although not expressed at greater than normal levels, have been shown to have high to normal activities when expressed in vitro but to be unstable under physiological conditions, thereby accounting for their pathogenicity (Chen et al. 1994; Xu et al. 1995; Lai et al 1998). Also, R332H is the only newly reported mutation that occurred at a CpG dinucleotide, a known hot spot for mutations (Cooper and Youssoufian 1988). Thus, missense mutations that retain some catalytic function would set a higher threshold for the ecogenic induction of clinical manifestations, whereas the "out-of-frame" insertion, deletion, and splicing mutations presumably would render the mutant mRNA or polypeptide unstable and/or totally inactive.

Another novel exonic point mutation (E314E) resulted from a G→A transition of the last base in exon 9 and caused the skipping of exon 9. Although the altered codon was degenerate and correctly encoded a glutamic acid residue, the base substitution changed the 5′ donor splice site from GAGgtaaca to GAAgtaaca. This nine-base sequence varied from the 5′ splice-junction consensus sequence, A/CAGgta/ga/gagt. Only 22% of a large number of recently analyzed mammalian junctions had an A in the last position of an exon (Krawczak et al. 1992). RT-PCR experiments demonstrated that the exonic G→A substitution prevented normal splicing of the URO-D mRNA and resulted in the joining of exons 8 and 10. The exon 9 deletion removed 22 amino acids from the translated polypeptide and altered the downstream reading frame. Since this is a major disruption of the enzyme polypeptide, it is presumed that the mutant protein was catalytically inactive and/or structurally unstable.

In addition to the point mutations in the URO-D gene, two gene rearrangements were detected. The g645Δ1053 mutation deleted ~30% of the 3.6-kb URO-D gene, including most of exon 2 to the middle of exon 6, a region that encoded 169 of the enzyme's 367 amino acids. The 10-bp direct repeat flanking the breakpoints suggests that slipped mispairing or intrachromosomal exchanges involving short direct repeats was responsible for the generation of this deletion (Kornreich et al. 1990). The second gene rearrangement, g10insA, caused a frameshift, which altered the amino acids predicted by codons 4–16 (except codons 7, 10, and 11) and created a stop signal (TGA) in codon 17. This insertion occurred before, between, or after two adenines, in positions g10 and g11. In the absence of either a longer string of adenines or a flanking direct repeat, it is likely that this insertion is due to polymerase stutter. Of note, this mutation was detected in four Argentinean families, with the mutant allele having possibly different ethnic origins. Haplotyping of the probands with the g10insA mutation indicated that two of the families were probably related whereas the other two were not, which suggests that the

Table 5

Haplotype Analysis of f-PCT Probands with URO-D Mutation g10insA

Marker (Map Position)[a]	Haplotype of Proband in[b]			
	Family 8 (Spanish)	Family 10 (Spanish)	Family 7 (Arab)	Family 9 (Spanish)
D1S193 (73.8 cM)	1-1	2-3	2-3	1-1
D1S463 (74.3 cM)	2-4	1-3	1-3	2-3
D1S2713 (74.8 cM)	1-2	3-4	3-4	3-4
D1S211 (75.3 cM)	2-2	2-3	2-2	1-1
D1S2724 (77.4 cM)	2-4	2-3	2-3	1-3
D1S451 (77.6 cM)	2-3	1-2	1-2	1-2

[a] Markers are those surrounding the chromosomal 1p34 locus of the URO-D gene. The genetic map position of each polymorphic CA-repeat marker is measured from the centromere on the short arm of chromosome 1.

[b] The order in which the families are listed is based on the relative identity of their haplotypes; shared haplotypes are underlined for families 7 and 10.

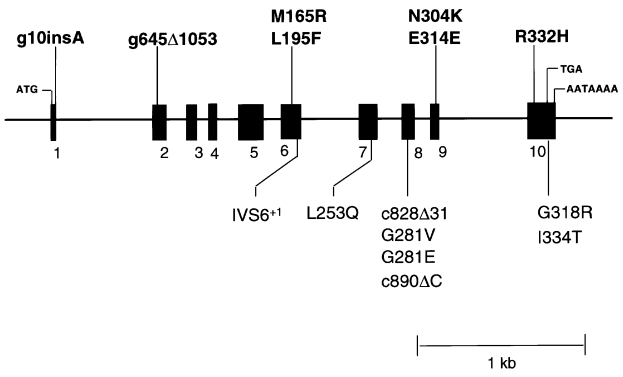

Figure 5 Diagram of the human URO-D gene, showing the relative location of the mutations causing f-PCT. New mutations described here are shown in boldface above the gene.

insertion mutation is quite old or occurred more than once. The identification of the g10insA mutation in three unrelated probands makes it the second most common mutation in f-PCT, since only a splice-site mutation in exon 6 has been identified in more (5 of 22) unrelated families (Garey et al. 1990). Additional studies will be necessary to determine both the true frequency of this mutation within the Argentinian population and whether this mutation is common within any other ethnic group.

If the 8 lesions that other investigators (Garey et al. 1989, 1990; Roberts et al. 1995; McManus et al. 1996) have identified in 11 f-PCT families are taken into account, a total of 15 URO-D mutations causing f-PCT now have been recognized. In families with homozygous dominant HEP, which is much rarer than f-PCT, nine missense mutations and a large deletion (de Verneuil et al. 1986*a*, 1986*b*, 1988; Romana et al. 1991; de Verneuil et al. 1992; Meguro et al. 1994; Roberts et al. 1995; McManus et al. 1996; Moran-Jimenez et al. 1996) have been identified. Of note, none of the HEP mutations have been identified in patients with f-PCT, and vice versa—except for G281E, which was identified in a man with f-PCT whose nephew had HEP (Roberts et al. 1995). These studies emphasize the underlying genetic heterogeneity in f-PCT, since most mutations were found in only one or two families, with the notable exceptions of the four families with the g10insA lesion and the five unrelated families with the exon 6 splice-site mutation (Garey et al. 1990). That some mutant alleles encode polypeptides with significant residual activity suggests that patients with these lesions may have either milder disease or a higher threshold for the induction of disease manifestations by various ecogenic factors such as iron overload or alcohol abuse.

To identify possible genotype-phenotype correlations, the erythrocyte URO-D activities, the plasma and urinary porphyrins, and the clinical records of the affected individuals from these 10 Argentinean families were reviewed. In addition, the presence of the recently identified common HFE mutations (C282Y and/or H63D), which cause iron overload, was determined in 9 of the 10 probands. Studies of patients with s-PCT have shown that, depending on the demographic region, one or the other of the HFE mutations, C282Y or H63D, is more frequent in individuals with s-PCT than it is in normal individuals (Roberts et al. 1997; Sampietro et al. 1998). In our study, only one patient (i.e., the individual with the g10insA mutation) was heterozygous for the C282Y mutation, for an allele frequency of 5.6%, whereas two patients (i.e., one individual with mutation E313E and one individual with mutation N304K) were homozygous and two patients (i.e., one individual with mutation g10insA and one individual with mutation L195F) were heterozygous for H63D, for an allele frequency of 33% (table 6). Thus, the frequency of f-PCT patients with one or two H63D alleles is four (44%) of nine, whereas only one of nine (11%) f-PCT patients had a C282Y allele. Although this is a small sample, the frequency of the H63D allele in the Argentinean f-PCT patients (33%) was similar to that in Italian s-PCT patients (28.7%), whereas the frequency of the C282Y allele in the f-PCT patients (5.5%) was slightly higher than that found in Italian s-PCT patients (1.1%). The similarity in the frequencies of these HFE mutations in the normal Argentinean and Italian populations may reflect the fact that most of the Argentinean f-PCT patients have Iberian or Italian ancestry. Further studies are needed to determine the frequency of the HFE mutations in normal individuals from Argentina; however, it is likely that the frequency of the HFE mutations is higher in f-PCT patients than in normal individuals and, thus, may be an important factor predisposing to f-PCT manifestations. Although the exact role of the H63D mutation in causing iron overload is less clear than that of the C282Y mu-

Table 6

HFE-Gene Mutations C282Y and H63D in Argentinean Patients with f-PCT

Family (Ancestry)	URO-D Mutation	Status of HFE-Gene Mutation[a]	
		C282Y	H63D
1 (Italian)	E314E	−/−	+/+
2 (Portuguese)	R332H	−/−	−/−
3 (Spanish)	L195F	−/−	−/+
4 (Italian)	M165R	−/−	−/−
5 (Spanish)	N304K	−/−	+/+
6 (Spanish)	g645Δ1053	ND	ND
7 (Arabic)	g10insA	−/−	−/−
8 (Spanish)	g10insA	−/−	−/−
9 (Spanish)	g10insA	+/−	−/+
10 (Spanish)	g10insA	−/−	−/−

[a] A plus sign (+) denotes presence, and a minus sign (−) sign denotes absence; ND = not determined.

tation, the Italian study suggests that the H63D mutation may cause a subtle iron-metabolism abnormality that results in hepatocellular accumulation of toxic iron species, thereby predisposing toward or precipitating the clinical manifestations of s-PCT (Sampietro et al. 1998).

It is interesting to note that the proband with the splice-site mutation, who is homozygous for the H63D mutation, had severe PCT clinical manifestations and that these began at the age of 5 years (A. M. de C. Batlle, unpublished data). The other patient homozygous for H63D had a point mutation in her URO-D gene and had milder PCT manifestations. However, overall, no clear genotype-phenotype correlations between the different URO-D mutations, clinical symptoms, and presence or absence of HFE mutations was observed in these Argentinean patients, which suggests that other important factors contribute to the onset, frequency, and severity of the clinical manifestations in PCT. Moreover, once the diagnosis is established, the institution of effective therapy by chronic phlebotomies and/or chloroquine obfuscates such correlations. In summary, the identification and characterization of these seven new mutations in the URO-D gene that cause f-PCT highlight the molecular heterogeneity underlying f-PCT, permit the precise diagnosis of asymptomatic heterozygotes in these Argentinean families, and provide the information for future structure-function studies of the human enzyme.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Entrez Nucleotide Sequence Search, http://www.ncbi.nlm.nih.gov/Entrez/nucleotide.html (for AF047383)

Human Gene Mutation Database, http://www.uwcm.ac.uk/uwcm/mg/hgmd0.html (for URO-D)

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nim.nih.gov/Omim (for porphyria cutanea tarda [MIM 176100])

Swiss-Prot Annotated Protein Sequence Database trEMBL, http://www.expasy.ch/sprot/sprot-top.html (for tobacco and barley URO-D polypeptides)

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