

## A Point Mutation in the Coding Region of Uroporphyrinogen Decarboxylase Associated With Familial Porphyria Cutanea Tarda

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Familial porphyria cutanea tarda (PCT) is inherited as an autosomal dominant trait caused by decreased activity of uroporphyrinogen decarboxylase (URO-D). In most families with PCT, URO-D mRNA levels are normal but both catalytic activity and immunologic reactivity of URO-D are half normal. We have cloned and sequenced 8 URO-D cDNA transcripts derived from a pedigree member with familial PCT. Three of the cDNAs had sequences encoding normal URO-D but five cDNA's contained a point mutation resulting in a gly → val substitution at amino acid position 281. An oligonucleotide probe complementary to the mutant sequence hybridized to DNA from affected individuals within the pedigree, but not to DNA from normal individu-

als. Measurements of pulse labeled URO-D in Epstein-Barr virus transformed lymphocytes indicated that the mutant protein has a half-life in vivo of less than four hours. In vitro measurements utilizing labeled URO-Ds generated in a reticulocyte lysate system revealed a 12-hour half-life for the mutant protein compared with a 102-hour half-life for normal URO-D. This is the first URO-D mutation to be characterized in a pedigree with familial PCT. This mutation was not detected in affected individuals from seven other PCT pedigrees, suggesting that PCT can result from different mutations.

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**P**ORPHYRIA CUTANEA TARDA (PCT) is the most common form of porphyria in humans and is characterized clinically by dermal photosensitivity.<sup>1</sup> Biochemical characteristics include excess hepatic accumulation and urinary excretion of uroporphyrin and 7-carboxyl porphyrin. PCT can be inherited as an autosomal dominant trait (familial PCT).<sup>1</sup> The mutation responsible for familial PCT results in half-normal catalytic activity and immunoreactivity of uroporphyrinogen decarboxylase (URO-D).<sup>2</sup> URO-D is a cytosolic heme biosynthetic enzyme 367 amino acids in length (molecular weight [mol wt] 40,831). The human URO-D locus is on the p arm of chromosome 1<sup>3,4</sup> and a complete human URO-D cDNA has been cloned and sequenced.<sup>5</sup>

URO-D deficiency is also responsible for the much rarer human disease, hepatoerythropoietic porphyria (HEP), which is inherited as an autosomal recessive trait.<sup>2</sup> Affected homozygotes usually have URO-D activity of only 5% to 10% of normal. Characterization of a URO-D cDNA derived from an affected homozygote was sequenced and revealed a mutation changing the codon for amino acid 281 from gly to glu,<sup>6</sup> a substitution that decreases the stability of URO-D.<sup>7</sup> This mutation could not be detected by hybridization studies using an oligonucleotide probe complementary to the mutation in any of 13 pedigrees with familial PCT.<sup>8</sup>

Although the amount of URO-D protein is half-normal in patients with familial PCT, URO-D mRNA levels were normal in cultured lymphoblasts from PCT patients in two unrelated pedigrees.<sup>9</sup> No deletions, rearrangements, or

restriction fragment length polymorphisms have been detected at or near the URO-D locus in four pedigrees with familial PCT.<sup>10</sup> There is no evidence that a splicing error occurs affecting URO-D mRNA, making it likely that a point mutation in a coding region is responsible for the defect.

Because URO-D mRNA levels are normal in cells derived from individuals with PCT, we anticipated that mRNA transcripts of both the normal gene and the mutated gene would be present. We have cloned and sequenced eight URO-D cDNA clones derived from RNA extracted from Epstein-Barr virus transformed lymphocytes of the proband of a pedigree with familial PCT (Pedigree B<sup>9</sup>). The proband, a 46 year-old white woman, has the characteristic combination of photosensitivity and uroporphyrinuria (24-hour urinary uroporphyrin excretion of 2058 μg; normal value <50). The catalytic activity of URO-D was half normal in an erythrocyte lysate and in the cultured lymphoblastoid cells.

### MATERIALS AND METHODS

**cDNA cloning and sequence analysis.** Total RNA was extracted from lymphoblasts with guanidine HCl. Poly A + RNA was purified by oligo dT cellulose chromatography and used to construct a cDNA library that was cloned into lambda gt10.<sup>11</sup> The library was screened with a <sup>32</sup>P-labeled 226 base pair (bp) *EcoRI-PstI* fragment of normal URO-D cDNA that corresponds to the extreme 5' end of the cDNA. URO-D cDNA clones were subcloned into M13 mp8 following digestion by *EcoRI*. Sequencing was performed completely in both directions using the Sequenase system (US Biochemical Corp, Cleveland) in conjunction with synthetic oligonucleotide primers. URO-D cDNA-derived amino acid sequences were analyzed using the Genepro program (Riverside Scientific Enterprises, Seattle). Secondary structure predictions followed the method of Chou and Fasman.<sup>12</sup> Analysis of hydrophathy followed the algorithm of Kyte and Doolittle.<sup>13</sup> Uroporphyrinogen decarboxylase cDNA nucleotide and protein numeration follow those used in reference 5.

**Oligonucleotide hybridization analysis.** DNA of each clone in M13mp8 was digested with *EcoRI* and run on a 1% agarose gel that was blotted to nylon membrane, and hybridized<sup>8</sup> for 36 hours at 42°C with 8 × 10<sup>5</sup> counts/mL of a <sup>32</sup>P-labeled oligonucleotide (nucleotides 852-868, 5'-AGTCAAGCACACCACC-3') that corresponds to the 281 gly → val mutation (see Results). The blot was washed three times in 5X SSC (0.75 mol/L NaCl, 0.075 mol/L Na<sub>3</sub>C<sub>6</sub>H<sub>5</sub>O<sub>7</sub>), 0.1% sodium dodecyl sulfate (SDS) at room temperature for 15 minutes followed by a 15-minute wash in 2X SSC, 0.1%

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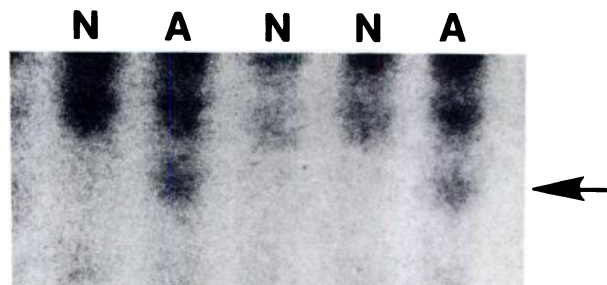
SDS at 60°C and again at 65°C for two minutes. The blots were exposed to XAR5 film (Kodak, Rochester, NY) overnight with a Cronex (Dupont, Wilmington, DE) intensifying screen. Ten micrograms of genomic DNA from each pedigree member was digested to completion with an excess of Taq I. Samples were treated as above except that hybridization was performed at 37°C and the final wash was performed in 5X SSC, 0.1% SDS at 60°C for two minutes, and exposed to film for nine days. A control experiment was performed with the same blot in which an oligonucleotide probe was used that corresponded to the normal sequence (nucleotides 852-868, 5'-AGTCAAGCCCCAACACC-3'). Polymerase chain reaction (PCR) amplification of a 180-bp region of the URO-D gene from normal and affected individuals was performed by the method of Saiki et al.<sup>14</sup> Twenty cycles were performed manually (denatured at 90°C for one minute, annealed at 42°C for two minutes, and extended at 65°C for three minutes). The amplified DNA resulting from 500, 100, 50 and 10 ng of template DNA was slot blotted to nitrocellulose and hybridized with both probes as above. The membranes were washed three times in 5X SSC at room temperature for 15 minutes, once in 5X SSC, 1% SDS at 60°C for two minutes, and once in 5X SSC on ice for 15 minutes, and exposed to film for 48 hours.

**Quantification of URO-D and labeling experiments.** The amount of URO-D present in lymphoblasts from both normal and affected pedigree members was determined by rocket gel immunoelectrophoresis<sup>9</sup> with a polyclonal rabbit anti-URO-D serum. Newly synthesized URO-D was measured by incubating  $2 \times 10^7$  cells derived from normal or affected individuals with 1 mCi of <sup>35</sup>S-methionine for four hours in methionine free culture media. The cells were immediately lysed in buffer containing the protease inhibitor aprotinin (Sigma Chemical Co, St Louis). Immunoprecipitation was performed on lysates from normal and affected cells, normalized to contain equal amounts of TCA precipitable counts.<sup>7</sup> Immunoprecipitation was performed with the polyclonal rabbit anti-URO-D serum. A commercial polyclonal rabbit anti-ferritin serum (Boehringer Mannheim, Indianapolis) was used as an internal control. The immunoprecipitated material was run on a 10% to 20% gradient SDS acrylamide gel, treated with Enhance (New England Nuclear, Boston), dried, and exposed to Kodak XAR-5 film for ten days. The band corresponding to URO-D or ferritin was quantified by densitometry.

**In vitro degradation.** <sup>35</sup>S-Methionine labeled URO-D was translated from RNA transcripts of cloned normal and mutant URO-D cDNA as described.<sup>6</sup> Six microliters of the translation mix was added to 160  $\mu$ L of lysate (0.46 mg/mL protein) from lymphoblasts derived from the proband and aliquots of 20  $\mu$ L were removed after 0, 6, 12, 18, and 24 hours of incubation at 37°. The samples were electrophoresed on SDS gels and the amount of labeled URO-D quantified for each time point by densitometry of the autoradiogram.<sup>6</sup>

## RESULTS

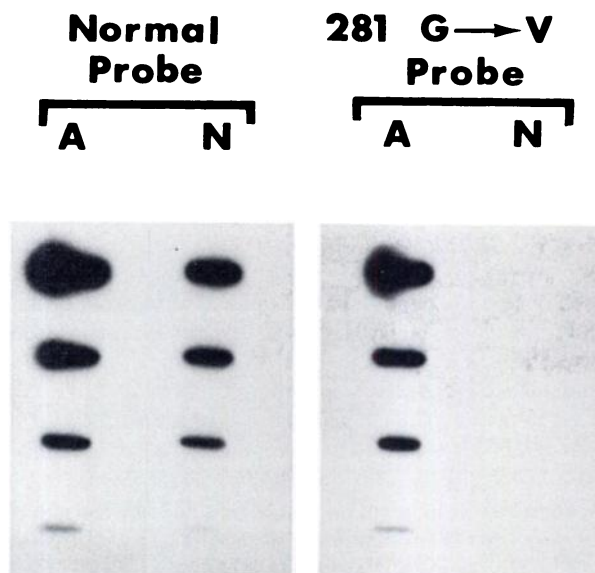
Eight cDNA clones that contained the entire coding region of URO-D were isolated from a cDNA library made from lymphoblasts derived from an affected pedigree member. Five of the clones contained a G  $\rightarrow$  T substitution at nucleotide position 860 that would change amino acid 281 from gly to val. This substitution was found on five independently derived clones. A synthetic oligonucleotide of 17 bases containing this substitution hybridized to the five mutant cDNAs but not the three normal cDNAs (data not shown), indicating that the mutation was genuine and not a sequencing error.



**Fig 1.** Hybridization of an oligonucleotide probe corresponding to the 281 gly  $\rightarrow$  val mutation to Taq I digested genomic DNA from pedigree members. Pedigree members were classified as normal (N) or affected (A), based on erythrocyte URO-D activity. The arrow indicates the 2.0 kb Taq I fragment containing the URO-D gene.<sup>8</sup> The other bands represent non-URO-D sequences that also hybridize to the oligonucleotide probe.

Two oligonucleotides were used as hybridization probes to confirm that the 281 gly  $\rightarrow$  val mutation was present in genomic DNA. The oligonucleotide corresponding to the 281 gly  $\rightarrow$  val mutation hybridized to DNA only from the proband and other pedigree members with half-normal URO-D activity (Fig 1). The oligonucleotide corresponding to the normal sequence hybridized to DNA from all members of the pedigree (data not shown).

Similar hybridizations were performed with DNA derived from seven other unrelated pedigrees with familial PCT. The normal oligonucleotide hybridized with DNA from all individuals. The 281 gly  $\rightarrow$  glu mutant oligonucleotide did not



**Fig 2.** PCR amplification products were slot blotted onto nitrocellulose (PCR products from 0.5, 0.1, 0.05, and 0.01  $\mu$ g template DNA from top to bottom) and probed with either an oligonucleotide probe complementary to the normal sequence or to the 281 gly  $\rightarrow$  val mutation. Left: PCR products derived from DNA of an affected individual (A) and a normal individual (N) hybridized to the normal oligonucleotide probe. Right: PCR products from an affected individual (A) and a normal individual (N) hybridized to a probe complementary to the 281 gly  $\rightarrow$  val mutation.

Table 1. Steady State and Newly Synthesized URO-D in Cultured Lymphoblasts

	Affected	Normal	Ratio Affected/Normal
Steady state URO-D* ( $\mu\text{g}$ URO-D/mg total soluble protein)	0.498	1.061	0.47
Relative amount labeled URO-D following 4 h labeling period†	1227	2505	0.49
	1172	2032	0.58
Relative amount of labeled ferritin control following labelling period†	605	444	1.36
	472	467	1.01

\*Average of ten measurements made on rocket gels (see Materials and Methods) in normal cells (SD = 0.37) and seven measurements in affected cells (SD = 0.16).

†Measurements were made by quantification of immunoprecipitated labeled URO-D or ferritin following the labeling period (see Materials and Methods). Duplicate points determined, all data shown.

hybridize to DNA from any of the 23 normal or 29 affected pedigree members, indicating that different mutations were present in these pedigrees.

The same results were obtained using PCR-amplified URO-D DNA (Fig 2). The normal oligonucleotide probe hybridized to DNA from both normal and affected individuals in the pedigree of the proband described above, while the mutant probe hybridized to amplified DNA only from affected individuals. The mutant probe did not hybridize to amplified DNA from affected individuals from four other pedigrees.

All eight cDNA clones contained two previously described silent mutations (T  $\rightarrow$  C) at nucleotide positions 87 and 931.<sup>6</sup> A third silent T  $\rightarrow$  C mutation at position 1027 was also noted. An A  $\rightarrow$  G substitution at nucleotide position 325, resulting in a ser  $\rightarrow$  gly substitution at codon 103 was also observed. The 103 ser  $\rightarrow$  gly substitution has been previously described and does not affect the stability or function of URO-D.<sup>6</sup>

Rocket gel immunoelectrophoresis indicated that steady state URO-D levels are half-normal in affected members of the pedigree (Table 1). Pulse labeling experiments, followed by quantification of labeled URO-D, indicated that cells derived from affected individuals already have half-normal levels of URO-D immediately following a four-hour pulse (Table 1). Shorter labeling periods of 0.5, one, and two hours were attempted but measurements of the relative amount of labeled URO-D were not reproducible. The *in vitro* degradation experiments (Fig 3) showed that the normal URO-D protein has a half-life of 102 hours, the 281 gly  $\rightarrow$  glu mutant has a half-life of 15 hours, and our 281 gly  $\rightarrow$  val mutant has a half-life of 12 hours.

#### DISCUSSION

The G  $\rightarrow$  T substitution (nucleotide 860) in the mutant cDNAs from the pedigree studied occurs at the exact position as the mutation in the URO-D cDNA of some patients with HEP<sup>6,8</sup> but substitutes a val rather than a glu. The gly normally present at amino acid position 281 is probably critical for proper folding of the protein as substitution of glu at that position results in a rapidly degraded protein.<sup>7</sup> Although analysis of the URO-D protein sequence indicates that the 281 gly  $\rightarrow$  val substitution does not alter the charge, hydrophobic profile, or Chou-Fasman prediction of secondary structure along the chain, this substitution also affects protein stability.

To determine if the mutant gene we characterized produces an unstable URO-D protein, we measured steady state URO-D and newly synthesized URO-D in lymphoblasts. Both were half-normal in cells from affected individuals within the pedigree even though steady state levels of total URO-D mRNA were normal. This suggests that either the mutant mRNA is not translated, or the half-life of the mutant URO-D is much less than the four-hour pulse period used in our *in vivo* labeling experiments. If the mutant mRNA is not translated, an additional mutation 5' to the coding region might be responsible. This is unlikely because our cDNA clones contained up to 16 nucleotides 5' of the start codon corresponding to the major transcriptional start signal of the URO-D gene<sup>15</sup> and there was no difference between the normal and mutant alleles. We favor the hypothesis that the 281 gly  $\rightarrow$  val mutation causes very rapid degradation of the mutant protein. Mutant proteins with

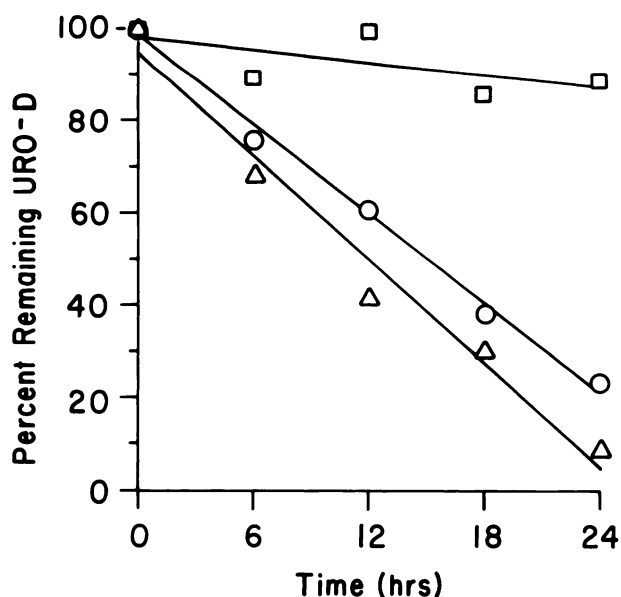


Fig 3. The results of a representative *in vitro* degradation experiment. Normal and mutant cDNAs were cloned into a transcription vector and URO-D RNA transcribed. Labeled URO-D was translated from the RNA in a rabbit reticulocyte lysate system and exposed to a lysate prepared from cultured lymphoblasts. The amount of URO-D remaining was determined by densitometry of autoradiograms made from SDS gels (see text).  $\square$  = Normal URO-D;  $\circ$  = 281 gly  $\rightarrow$  glu URO-D;  $\triangle$  = 281 gly  $\rightarrow$  val URO-D.

half-lives of less than ten minutes have been described.<sup>16</sup> We attempted to measure relative URO-D levels in cultured lymphoblasts following labeling periods of less than four hours but failed to obtain reproducible results. The *in vitro* degradation experiments (Fig 3) indicate a much longer half-life of 12 hours compared with 102 hours for the normal protein. We conclude that under the conditions of our *in vitro* half-life experiments our 281 gly → val mutant is more stable than *in vivo* where its half-life is less than four hours (Table 1). Differences between *in vitro* and *in vivo* degradation rates have been described for other proteins.<sup>17</sup> In contrast, the 281 gly → glu mutant was found to have the same half-life *in vitro* as *in vivo*.<sup>6,7</sup> Although the 281 gly → val mutation was not found in other pedigrees with familial PCT, other point mutations that affect protein stability are likely to be present. An oligonucleotide corresponding to the third possible substitution at nucleotide 860 was also prepared (nucleotide 860 G → C resulting in a potential 281 gly → ala mutation). This mutant oligonucleotide did not hybridize to DNA from any of four pedigrees tested (data not shown). It seems clear that substitutions at nucleotide 860 lead to URO-D proteins with shortened half-lives but substitutions elsewhere in the coding region must have the same effect.

The 281 gly → glu mutation was found in three of five pedigrees with HEP.<sup>8</sup> The clinical and biochemical symptoms of HEP occur early in life and are much more severe

than in familial PCT. HEP is inherited as an autosomal recessive trait and it has been suggested that HEP is the homozygous form of familial PCT.<sup>7</sup> The finding of point mutations that appear to affect URO-D stability in both diseases supports this contention. Familial PCT, however, is not a rare disease but only 16 cases of HEP have been described to date.<sup>18</sup> If most cases of familial PCT are due to URO-D instability caused by a collection of different point mutations, there may be variations in the degree of instability. An embryo homozygous for a mutation causing a very unstable URO-D protein might not be able to synthesize sufficient heme to survive. Thus, HEP patients may represent individuals homozygous for mutations that result in URO-D proteins stable enough to partially meet the requirements for heme biosynthesis. The 281 gly → glu mutation found in HEP results in a decrease in half-life from the normal 80 hours to eight hours (in our hands, 102 hours to 15 hours). The 281 gly → val mutation which we have found likely results in a protein with a much shorter half-life *in vivo*.

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