Congenital Erythropoietic Porphyria: Identification and Expression of Exonic Mutations in the Uroporphyrinogen III Synthase Gene

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Abstract

Congenital erythropoietic porphyria (CEP), an inborn error of heme biosynthesis, results from the deficient activity of uroporphyrinogen III synthase (URO-synthase). This autosomal recessive disorder is heterogeneous; patients with severe disease are often transfusion dependent, while milder patients primarily have cutaneous involvement. To investigate this phenotypic heterogeneity, exonic point mutations in the URO-synthase gene were identified in unrelated CEP patients. Four missense mutations were identified: (*a*) an A to G transition of nucleotide (nt) 184 that predicted a Thr to Ala substitution at residue 62 (designated T62A); (*b*) a C to T transition of nt 197 that encoded an Ala to Val replacement at residue 66 (A66V); (*c*) a T to C transition of nt 217 that predicted a Cys to Arg substitution at residue 73 (C73R); and (*d*) a C to T transition of nt 683 that resulted in a Thr to Met replacement at residue 228 (T228M). In addition, a G to A transition of nt 27 that did not change the encoded amino acid (A9A) was detected in an African patient. The T62A, C73R, and T228M alleles did not express detectable enzymatic activity, while the A66V allele expressed residual, but unstable activity. The C73R allele was present in eight of 21 unrelated CEP patients (21% of CEP alleles). In three patients, identification of both alleles permitted genotype-phenotype correlations; the A66V/C73R, T228M/C73R, and C73R/C73R genotypes had mild, moderately severe, and severe disease, respectively. These findings provide the first genotype-phenotype correlations and permit molecular heterozygote detection in this inherited porphyria. (*J. Clin. Invest.* 1992. 89:693–700.) Key words: porphyria • uroporphyrinogen I • mutation detection • missense mutations

Introduction

Congenital erythropoietic porphyria (CEP),[1] also known as Günther's disease, is an inborn error of heme biosynthesis that results from the markedly deficient activity of the fourth enzyme of the heme biosynthetic pathway, uroporphyrinogen III synthase [URO-synthase; also designated hydroxymethylbilane hydro-lyase (cyclizing); EC 4.2.1.75] (1, 2). This enzyme normally catalyzes the rapid cyclization of the linear tetrapyrrole, hydroxymethylbilane (HMB), to form the uroporphyrinogen III isomer, the physiologic precursor of heme (3, 4). In patients with this autosomal recessive disorder, the enzymatic defect leads to the accumulation of HMB which is nonenzymatically cyclized to the uroporphyrinogen I isomer and then oxidized to uroporphyrin I (URO I), a nonphysiologic and pathogenic compound (1). The accumulated URO I in erythrocytes leads to hemolysis, and the released porphyrin isomer is deposited in tissues and bones and excreted in the urine and feces. URO I is a photocatalytic compound, and exposure of the skin to sunlight, other forms of ultraviolet light, or trauma results in blistering and vesicle formation (5). Ruptured vesicles are prone to secondary infection leading to cutaneous scarring and deformities, particularly of the hands and face. Severely affected patients are transfusion-dependent, and have secondary hypersplenism and disfiguring cutaneous involvement, whereas mildly affected patients may have only cutaneous manifestations (6). Later-onset adult patients with mild disease symptoms also have been described (e.g., 7, 8).

Diagnosis of CEP patients can be reliably made by the demonstration of deficient URO-synthase activity in erythrocytes or cultured cells (9–11). Affected individuals are not totally deficient in URO-synthase activity since they must have sufficient residual activity to synthesize uroporphyrinogen III for the production of heme. Presumably, the amount of residual URO-synthase activity primarily determines the severity of the disease; however, at present it is not possible to biochemically predict disease severity (9). In addition, it is difficult to enzymatically identify heterozygous carriers of the disease gene due to the overlap of low-normal and high heterozygous values (9).

Human URO-synthase has been purified to homogeneity from erythrocytes, and shown to be a monomeric protein with an apparent molecular weight of 29.5 kD (12). Recently, the full-length cDNA encoding the human URO-synthase polypeptide of 265 amino acids was isolated, sequenced, and expressed in *Escherichia coli* (13). Using the cDNA as a probe, a single URO-synthase gene has been regionally assigned to a narrow region of chromosome 10q25.3 → q26.3 (14). Genomic clones have been obtained, and exon-intron junctions in the ~ 60 kb URO-synthase gene have been determined (Warner, C. A., S-F Tsai, and R. J. Desnick, unpublished results).

The availability of the URO-synthase cDNA provided the opportunity to investigate the molecular nature of the phenotypic variability in unrelated patients with CEP. While these studies were in progress, Deybach et al. reported two putative mutations (P53L and C73R) in two severely affected patients (15). In this communication, five exonic mutations (designated

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1. *Abbreviations used in this paper:* ASO, allele-specific oligonucleotide; CEP, congenital erythropoietic porphyria; HMB, hydroxymethylbilane; PCR, polymerase chain reaction; SSCP, single strand conformation polymorphism; URO, uroporphyrinogen.

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T62A, A66V, C73R, T228M, and a silent base substitution, A9A) were identified in unrelated CEP patients using reverse transcription and amplification of the URO-synthase message, and by analysis of the first four coding exons by the single strand conformation polymorphism (SSCP) technique. Each missense mutation was confirmed by expression studies. Of interest, the A66V allele expressed residual enzymatic activity, consistent with the proband's mild phenotype. In contrast, little, if any, URO-synthase activity was expressed by the other mutant alleles. In several patients, the identification and expression of both mutant alleles permitted the first genotype-phenotype correlations in this porphyria.

Methods

Patient specimens. Peripheral blood samples were collected from 21 unrelated CEP patients and their family members with informed consent. Lymphoid cell lines were established using cyclosporin A and Epstein-Barr virus as previously described (16). Cells were maintained by standard procedures in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin, and 1 mg/ml of streptomycin (Gibco Laboratories, Grand Island, NY).

cDNA amplification and sequencing of mutant URO-synthase alleles. Total RNA was isolated from cultured lymphoblasts using standard techniques (17). First-strand cDNA was reverse transcribed from ~ 5 μg of total RNA, using a cDNA synthesis kit according to the manufacturer's instructions (Bethesda Research Laboratories, Gaithersburg, MD). The cDNA (~ 10% of the total reaction) was amplified by the polymerase chain reaction (PCR) with *Taq* polymerase (Perkin-Elmer Cetus, Norwalk, CT) essentially as described by Saiki et al. (18) with the following modifications. After an initial denaturation of the RNA-DNA hybrid at 94°C for 5 min, amplification was performed for 30 cycles and consisted of denaturation at 94°C for 1 min, annealing at 58°C for 1.5 min, and extension at 72°C for 1.5 min. The sense primer (5'-ACTACTGAATTCTCCCGCGAGTGCCCTATAAG-3'), corresponding to URO-synthase cDNA nt − 35 to − 16 with an additional 12 nt containing an EcoRI site, and the antisense primer (5'-ACTACTGTCGACTTGAGGCAGGAGTCTGAC-3'), corresponding to cDNA nt 894 to 911 with an additional 12 nt including a SalI site, were synthesized on a synthesizer (model 380B; Applied Biosystems, Foster City, CA). After amplification, the 970-nt PCR product, which included the entire coding sequence, was extracted with phenol/chloroform and precipitated with isopropanol. The amplified DNA then was directionally subcloned into the pGEM-4Z vector (Promega Biotec, Madison, WI) at the EcoRI and SalI sites. Positive clones were identified by colony hybridization to an internal oligonucleotide probe (5'-TCTGGGAAAGGTCTCTG-3'), which corresponded to URO-synthase cDNA nt 245 to 261. The inserts, from six to eight independent subclones, were sequenced in both orientations by the dideoxy-chain termination method (19) using Sequenase (United States Biochemical Corp., Cleveland, OH). Computer-assisted sequence analyses were performed using the Microgenie software (Beckman Instruments, Inc., Palo Alto, CA).

Detection of URO-synthase mutations by SSCP. Mutations in the first four coding exons of the URO-synthase gene of CEP patients were detected by SSCP as described (20), with modifications (21). Each exon was amplified using the appropriate sense and antisense primers indicated in Table I. For each reaction, the 50-μl amplification mixture contained 100 ng of genomic DNA, 10 pmol of each primer, 12.5 μM dNTPs, 25 μCi [α-^{35}S]dATP, 25 μCi [α-^{35}S]dCTP, 50 mM Tris-HCl pH 9.0, 50 mM NaCl, 10 mM MgCl, 170 μg/ml bovine serum albumin (BSA), and 2 U of *Taq* polymerase (Promega Biotec). 30 cycles of amplification were performed with denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 30 s. The amplification products were diluted 1:25 in 0.1% SDS, 10 mM EDTA, and then 4 μl was mixed with 4 μl of formamide loading dye. Samples were denatured at 100°C for 5 min, and electrophoresed in 6% polyacrylamide gels containing 10% glycerol at 30 to 70 W and 4°C. The gels were dried and exposed to Kodak XAR-5 film for 1 to 4 d. The amplified products for SSCP analysis were engineered with EcoRI or KpnI restriction sites (Table I) for subcloning into pGEM-4Z and subsequent sequencing as described above.

Allele-specific hybridization of genomic DNA. Any nucleotide change that occurred in more than two subcloned PCR products was considered a candidate mutation and analyzed by dot-blot hybridization of allele-specific oligonucleotides (ASOs) with genomic DNA isolated from peripheral blood leukocytes or lymphoid cells (22, 23) from the patient and appropriate family members. Since the P53L, T62A, A66V, and C73R mutations all occurred within the same URO-synthase exon, genomic DNA was amplified with sense and antisense primer set 3 (Table I) as described above. Amplification for 30 cycles included denaturation at 94°C for 1 min, annealing at 50°C for 30 s, and extension at 72°C for 30 s. For detection of the T228M mutation in genomic DNA, primer set 5 was used (Table I). Optimal amplification was performed for 30 cycles by denaturation (94°C, 1 min), annealing (55°C, 1.5 min), and extension (72°C, 1.5 min). The standard amplification reaction mixture for all mutations was 3 μg genomic DNA, 1 μM of each primer, 200 μM dNTP, 50 mM Tris-HCl pH 9.0, 50 mM NaCl, 10 mM $MgCl_2$, 170 μg/ml BSA, and 2 U *Taq* polymerase (Promega Biotec). The PCR product (40 μl) was denatured in 360 μl of 0.4 M NaOH containing 25 mM EDTA for 5 min, and then 180-μl aliquots of each were transferred to duplicate Zeta Probe membranes (Bio-Rad Laboratories, Richmond, CA) using a Minifold I dot-blot apparatus (Schleicher and Schuell, Keene, NH). For allele-specific hybridization, the ASOs corresponding to the appropriate normal and mutant sequences (Table II) were end labeled with [γ-^{32}P]ATP (24), and the radiolabeled ASOs were added to the hybridization solution at a concentration of 10^6 cpm/ml. Note that the mutation-specific ASO for the T62A lesion required a second base change to reduce non-specific hybridization to the normal sequence. Prehybridizations for 3 h in 6X SSPE (1X SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4), 10X Denhardt's (1X Denhardt's is 0.1% Ficoll/0.1% polyvinylpyrrolidone/0.1% BSA), and overnight hybridizations for the A9A,

Table I. Primer Sets for PCR Amplification of URO-Synthase Coding Exons

Coding exon primer set	URO-synthase cDNA sequence amplified	Oligonucleotide primers*	
		Sense	Antisense
1	−26–63	5'-ACTACT(GAATTC)ttgcttaggaagagtct-3'	5'-ACTACT(GAATTC)ctgtgggataaggagtc-3'
2	64–147	5'-ACTACT(GAATTC)ggttttgcaaaacctcaga-3'	5'-ACTACT(GAATTC)gtccctctctggcttca-3'
3	148–248	5'-AGTAGT(GAATTC)gtctttattgctttttgg-3'	5'-AGTAGT[GGTACC]gacccaccctcacCTTC-3'
4	245–319	5'-ACTACT(GAATTC)atagtttgctttgctcaca-3'	5'-ACTACT(GAATTC)ctgcattcttatcagtagt-3'
5	661–1084	5'-ACTACT(GAATTC)cgtcacatgagcagtaacg-3'	5'-ACTACT(GAATTC)atacctgtctcctccctg-3'

* URO-synthase intronic and exonic sequences in lower and upper case letters, respectively. *Eco*RI and *Kpn*I recognition sites in parentheses and brackets, respectively. Underlined nucleotides represent additional non-URO-synthase sequence to facilitate restriction enzyme cleavage.

Table II. Allele-Specific Oligonucleotides for Dot-Blot Analyses

Mutation	Normal oligonucleotide	Mutation-specific oligonucleotide
A9A	5'-GAAGGATGCGAAGGAAG-3'	5'-GAAGGATGCAAAGGAAG-3'
P53L	5'-TTTCTCATCCTGAAGATTA-3'	5'-TTTCTCATCTTGAAGATTA-3'
T62A	5'-CTCATTTTTACCAGCCC-3'	5'-CTCATTATTGCCAGCCC-3'
A66V	5'-CCCCAGAGCAGTGGGAAGCA-3'	5'-CCCCAGAGTAGTGGAAGCA-3'
C73R	5'-GCAGAGTTATGTTTGGAGC-3'	5'-GCAGAGTTACGTTTGGAGC-3'
T228M	5'-GCCCCACTACGGCTCGC-3'	5'-GCGAGCCATAGTGGGGC-3'

* Sense strand sequences are indicated for all oligonucleotides except the mutation-specific T228M ASO which is an antisense sequence.

P53L, T62A, A66V, C73R, and T228M mutations were performed at 47, 40, 50, 55, 50, and 51°C, respectively. After hybridization, the blots were washed at room temperature for 15 min in 6X SSC (1X SSC is 0.15 M sodium chloride/0.015 M sodium citrate, pH 7.0), 0.1% SDS, and then twice for 30 min each in the same solution at the indicated temperatures for the respective normal or mutation-specific oligonucleotide probe (Table II). Membranes were exposed to Kodak XAR-5 film with an intensifying screen for ~ 3 h.

Prokaryotic expression and characterization of URO-synthase mutations. The normal and mutant URO-synthase alleles were expressed in *E. coli* using the pKK223-3 vector (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) as previously described (13). The AvaII fragment of the full-length URO-synthase cDNA was blunt-end ligated into the HindIII site of pKK223-3. This construct was designated pKK-UROS. The pKK-UROS-A66V and pKK-UROS-C73R constructs were synthesized by digesting the respective mutant cDNAs with HindIII, and exchanging the 225-nt fragment as a cassette into the corresponding position in pKK-UROS. Similarly, the pKK-UROS-T62A and pKK-UROS-T228M expression constructs were synthesized using the 586-nt BstXI fragment as the cassette. To construct pKK-UROS-P53L, primers corresponding to nt 139 to 164 (5'-TCTGAGAAGCTTTCT-CATCTTGAAGA-3') and nt 381-357 (5'-TTCTGCAAGCTTTT-CTGCATTTCCA-3'), respectively, were used to amplify a portion of the URO-synthase cDNA. These primers contained the HindIII sites present in the URO-synthase cDNA; the 5' primer had a C to T transition of nt 158 which altered codon 53 to encode a leucine residue. Amplification was performed for 30 cycles with denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 72°C for 30 s. After phenol/chloroform extraction, ethanol precipitation, and digestion with HindIII, the amplified product was ligated as a cassette into HindIII digested pKK-UROS. After transformation of *E. coli,* single colonies were isolated and the inserts were sequenced to confirm the presence of the desired mutation. Bacterial growth, IPTG induction, and URO-synthase assays were performed as previously described (13). For enzyme stability studies, samples from the bacterial lysates, equalized for enzymatic activity, were incubated at 37°C in the presence of 0.5 mg/ml of BSA for 60 min. Aliquots were removed at timed intervals, placed on ice, and the URO-synthase activity was determined by the coupled assay (9).

Results

Identification of the A66V, C73R, and T228M mutations by cDNA amplification and sequencing. Initial efforts to identify exonic lesions causing CEP focused on two unrelated patients who had about 10% of mean normal erythrocytic URO-synthase activity (9), and mild to moderate disease manifestations. Total RNA was isolated from cultured lymphocytes from each proband, reverse-transcribed into cDNA, and then the entire URO-synthase coding region was PCR-amplified. Nucleotide sequencing of eight subcloned PCR products from proband 1, a mildly affected 12-yr-old male, revealed two allelic missense mutations (Fig. 1). Five of the subcloned products had a C to T transition of nt 197 that predicted an alanine to valine substitution at residue 66 (designated A66V). The other three subclones had amplified products with a T to C transition of nt 217 which predicted a cysteine to arginine replacement at position 73 (designated C73R). Thus, proband 1 was heteroallelic for the A66V and C73R mutations.

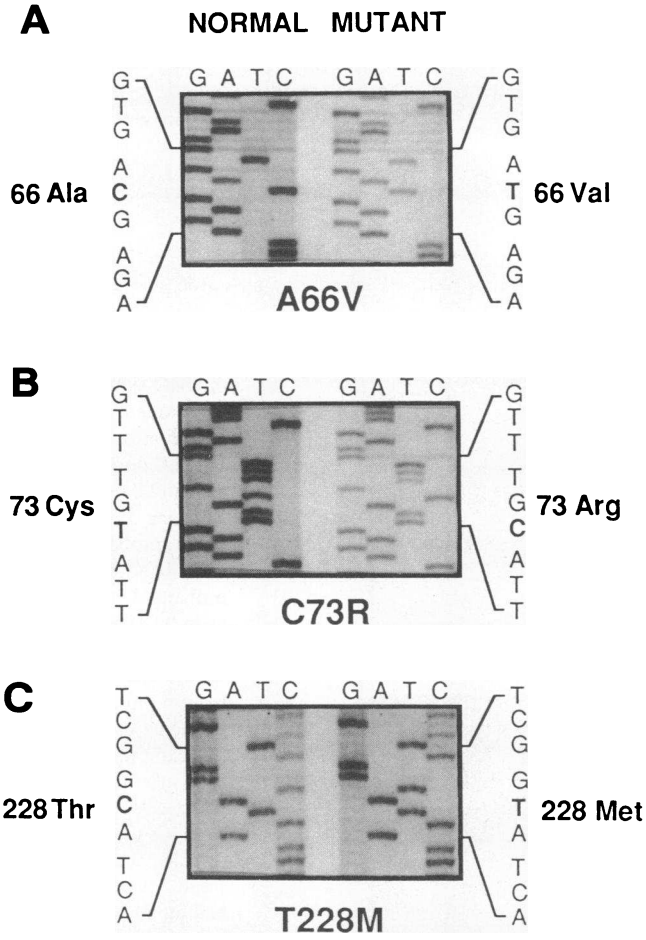

Figure 1. Identification of exonic mutations in the URO-synthase gene causing CEP. Partial DNA sequences of the reverse transcribed and amplified URO-synthase cDNAs showing (*A*) the C to T transition of nt 197 predicting an alanine to valine substitution in residue 66 (A66V); (*B*), a T to C transition of nt 217 encoding a cysteine to arginine replacement of residue 73 (C73R); and (*C*), a C to T transition of nt 683 resulting in a threonine to methionine substitution of residue 228 (T228M).

Nucleotide sequencing of six subcloned PCR products from proband 2, a moderately severe 15-yr-old male, also revealed two different allelic mutations (Fig. 1). In three subclones, the amplified products had the C73R mutation, while the other three subclones had a single mutation in a CpG dinucleotide, a C to T transition of nt 683 that predicted a threonine to methionine replacement in position 228 (designated T228M). In the PCR products from both probands, all other base substitutions occurred in only one or two subclones, and none of the amplified products had both mutations, consistent with both probands being heteroallelic for the identified lesions.

Identification of A9A and T62A mutations by SSCP. Based on the location of the intron/exon boundaries (Warner et al., unpublished results), each of the first four coding exons from 16 unrelated CEP patients was amplified for SSCP analysis. As shown in Fig. 2, the SSCP profiles for proband 1 (A66V/C73R) and proband 2 (C73R/T228M) revealed mobility shifts in the fourth coding exon (Fig. 2, *B*, lanes *3* and *4*, respectively). A mobility shift, located between the two major bands, was observed in the profiles of both probands, consistent with detection of the C73R lesion. Proband 1 also had a second mobility shift in the top band, consistent with detection of the A66V lesion. In addition, a third mobility shift was detected in the exon 4 profile from proband 3, a mildly affected 16-yr-old Japanese male (Fig. 2, *B*, lane *5*). When this exon was amplified from the proband's genomic DNA and sequenced, an A to G transition of nt 184 was detected (Fig. 3) that predicted a threonine to alanine substitution (designated T62A).

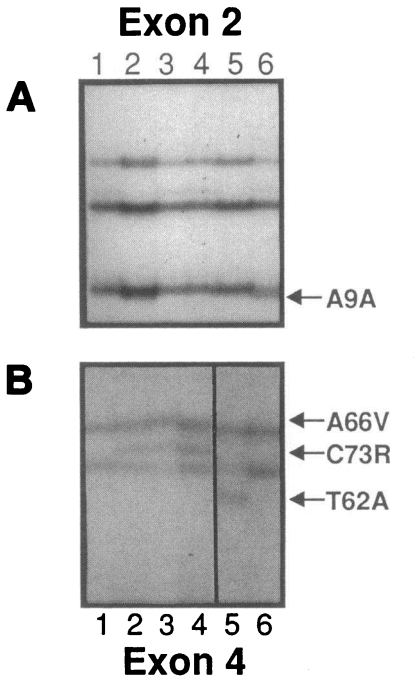

Figure 2. SSCP analysis of URO-synthase coding exons 2 and 4 from CEP homozygotes. Lane *1*, normal; lane *2*, proband 7 (see Table III); lane *3*, proband 1; lane *4*, proband 2; lane *5*, proband 3; lane *6*, proband 4. Analyses of exon 2 (*A*) revealed a mobility shift of the lower band from proband 4 (lane *6*). Sequencing of the amplified exon identified a base substitution designated A9A (*Fig. 3*). Analysis of exon 4 (*B*) revealed that probands 1 and 2 (lanes *4 and 3*) had an additional band between the two major bands found in controls. This shifted band correlated with the presence of the C73R mutation. The top band from proband 1 also was shifted, reflecting the presence of the A66V mutation. Proband 3 (lane *5*) had a more rapidly migrating band subsequently identified as a missense mutation, T62A (*Fig. 3*).

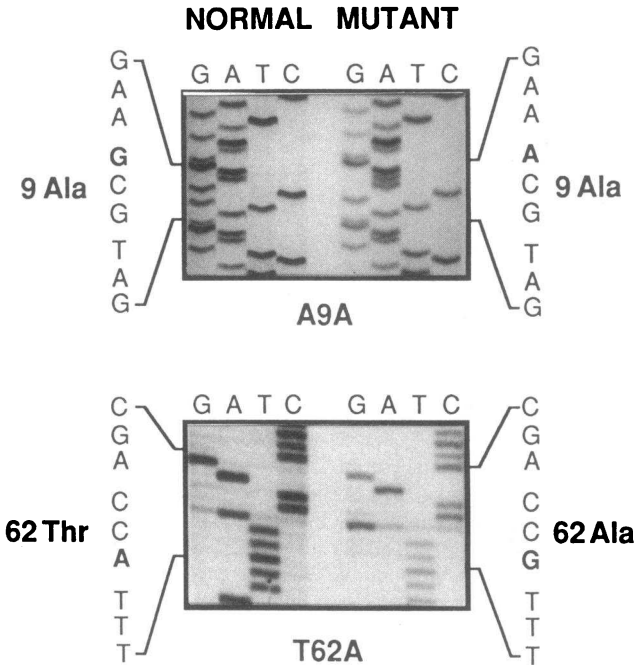

Figure 3. Partial DNA sequences of PCR-amplified genomic DNAs for URO-synthase exons 2 and 4. When compared with the normal sequence (*left*), proband 4 had a G to A transition of nt 27 that did not change the amino acid sequence (A9A), while proband 3 had an A to G transition of nt 184 that predicted a threonine to alanine substitution at residue 62 (T62A).

Examination of the exon 4 SSCP profiles of 14 other unrelated CEP patients revealed the occurrence of the C73R mobility shift in six (Table III). Amplification and sequencing of this exon from genomic DNA confirmed the presence of the C73R allele in each of these CEP patients (data not shown). Similarly, SSCP analyses of the first three coding exons from 16 unrelated CEP patients revealed only one other mobility shift. As shown in Fig. 2 (*A*, lane *6*), a shift was detected in the lower band of the exon 2 profile for proband 4, a moderately affected 35-yr-old South African Black female from the Baralong tribe, a subgroup of the Tswana chiefdom. Amplification and sequencing of this exon from genomic DNA of proband 4 identified a mutation in a CpG dinucleotide, a G to A transition of nt 27 (Fig. 3), which did not predict an amino acid change (designated A9A).

Confirmation of the identified mutations by dot-blot analyses. Initial confirmation of the above mutations was made by demonstrating these lesions in genomic DNA from probands 1, 2, 3 and 4, and their respective family members by dot-blot hybridization with ASOs. As shown in Fig. 4, amplified genomic DNA from proband 1 hybridized to both the normal and mutation-specific ASOs for A66V and C73R, confirming that this patient was heteroallelic for these point mutations. Proband 1 inherited the A66V and C73R alleles from his father and mother, respectively. Similarly, amplified genomic DNA from proband 2 hybridized with the mutation-specific ASOs

Table III. Genotype-Phenotype Correlations in Unrelated Patients with CEP

Proband	Age	Sex	Ancestry	Disease severity	Genotype
1	14 yr	M	German, Scottish, Irish	Mild	C73R/A66V
2	14 yr	M	Romanian	Moderately severe	C73R/T228M
3	16 yr	M	Japanese	Mild	T62A/
4	55 yr	F	African Black	Moderate	(A9A)*
5	5 mo	F	Cree Indian	Severe**	C73R/C73R
6	6 yr	F	English	Severe	C73R/
7	20 yr	M	English	Moderate	C73R/
8	10 yr	F	Alaskan Indian	Moderate	C73R/
9	6 yr	M	English	Moderate	C73R/
10	11 yr	F	German/Hungarian	Moderate	C73R/
11	37 yr	F	English, Irish, Polish	Mild	T228M/

* Mutation not identified; silent base subsitution in parenthesis. ** Died at age 5 mo due to sepsis.

for C73R (data not shown) and T228M (Fig. 4), also confirming heteroallelism for these two mutations. Proband 2 inherited the C73R and T228M mutations from his mother and father, respectively. Hybridization of amplified exon 4 from proband 3 and his parents with the normal and mutation-specific ASOs for T62A, revealed that the proband was heteroallelic for the mutation, having inherited this base substitution from his mother. Finally, hybridization analysis of genomic DNA from proband 4 revealed that she was heterozygous for the A9A allele. Since her mother did not have this substitution (Fig. 4), presumably this base change was inherited from her deceased father.

Prokaryotic expression of the URO-synthase mutations. To further characterize the URO-synthase missense mutations, pKK-UROS expression vectors for each of the mutant alleles were constructed and expressed in *E. coli,* and the enzymatic activity and stability of the recombinant proteins were determined. Table IV shows the URO-synthase activities of the expressed normal allele and the P53L, T62A, A66V, C73R, and T228M alleles following transformation and IPTG induction. Note that only the A66V mutation had detectable residual activity, while the activities of the other missense mutations were less than 1% of the mean level expressed by the normal allele. Fig. 5 compares the stabilities of the expressed A66V and normal URO-synthase proteins when incubated at 37°C. The half-life of the normal enzyme was about 37 min, whereas that for the A66V enzyme was only 14 min, indicating that the mutant protein had about half the stability of the normal enzyme at physiologic temperature.

Occurrence of the URO-synthase mutations in unrelated CEP families. To determine the frequencies of the A9A, T62A, A66V, C73R and T228M mutations in unrelated CEP families, genomic DNAs from 19 unrelated CEP patients were analyzed by dot-blot hybridization with mutation-specific ASOs (data not shown). Notably, a severely affected CEP patient (proband 5) who died at five months of age was found to be homoallelic for the C73R mutation; both parents of proband 5 were heterozygous for the C73R mutation. Five other unrelated patients were heterozygous for the C73R mutation, confirming the results of the exon 4 SSCP analyses described above. Thus, 9 of the 42 CEP alleles examined (21%) had the C73R mutation. In addition, a mildly affected 37-yr-old female was heteroallelic for the T228M mutation; thus, the allele frequency for the T228M mutation was about 5% (2 of 42 alleles studied). Analysis of 70 normal Caucasian, 40 Caucasian CEP, 28 American Black, and 70 South African Black (Tswana chiefdom) alleles for the A9A base substitution detected 1 Cau-

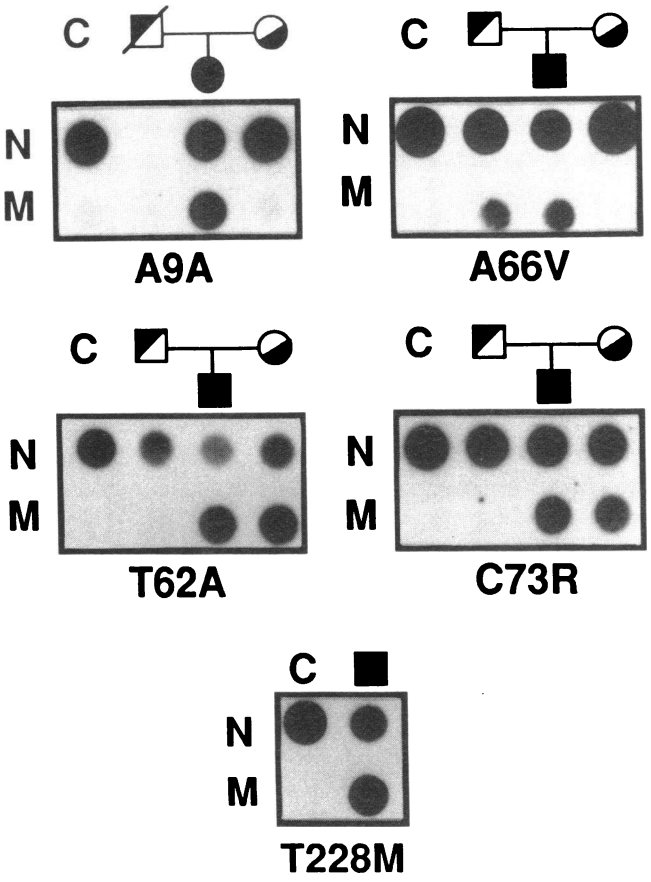

Figure 4. Confirmation of mutations in genomic DNA by hybridization with ASOs. Genomic DNAs isolated from lymphoblasts of the probands and their parents were amplified and tested for the presence of the A9A, T62A, A66V, C73R, or T228M mutations by hybridization with normal (*N*) and mutation-specific (*M*) oligonucleotides.

Table IV. Expression of URO-Synthase in E. coli

Construct	URO-synthase activity (U/mg) Mean	Range	N*	% of Mean normal level
pKK223-3	3.20	1.80–5.09	7	
pKK-UROS	86.61	44.0–136.4	7	100
pKK-UROS-P53L	3.42	1.68–5.47	3	<1.0
PKK-UROS-T62A	3.32	1.28–6.31	4	<1.0
PKK-UROS-A66V	15.6	11.4–19.8	4	14.5
pKK-UROS-C73R	2.40	1.71–4.82	4	<1.0
pKK-UROS-T228M	3.22	0.00–4.28	4	<1.0

* Mean and range of activities determined in *N* independent experiments. Induced for 3 h with 5 mM IPTG.

casian, 1 American Black, and no South African Black alleles, indicating that this base change was infrequent in these populations.

Discussion

In this communication, four missense mutations (T62A, A66V, C73R and T228M) in the URO-synthase gene causing CEP were identified and expressed. In addition, a silent base substitution (A9A) was detected in an African Black patient. Each of the URO-synthase base changes was confirmed in genomic DNA from the respective CEP proband and first degree relatives documenting the inheritance of these altered alleles (Fig. 4). Two of the five base substitutions (A9A and T228M) occurred at CpG dinucleotides, known hot spots for mutations (25, 26), which occur 13 times in the URO-synthase coding sequence.

These URO-synthase exonic base substitutions were detected by reverse transcription of mRNA to cDNA, followed by amplification and sequencing of the URO-synthase coding region, or by sequencing amplified regions of genomic DNA in which mobility shifts were observed by SSCP analyses. The latter detection technique was facilitated by the characterization of the intron/exon boundaries of the first four URO-synthase coding exons amplified by the PCR primer sets in Table I. Both mutation detection techniques offer different advantages for the identification of new mutations causing CEP. If cultured lymphoblasts are available from the patient, total RNA can be readily isolated for reverse transcription and amplification of the entire URO-synthase coding region as a single 970-bp product for direct sequencing or sequencing of independent subclones. In this way, all exonic base changes can be detected, as shown here for the A66V, C73R, and T228M lesions. Alternatively, knowledge of the intron/exon junctions permits the detection of all exonic base changes as well as splice junction defects by direct sequencing of amplified genomic DNA. When more 3′ intron/exon junctions in the URO-synthase gene are defined, SSCP analyses may identify additional mobility shifts for sequence analyses. It should be noted that certain substitutions will alter the SSCP profile by generating a new band (e.g., T62A and C73R), while other base changes will result in very subtle mobility shifts (e.g., A9A and A66V).

While these studies were in progress, Deybach et al. (15) identified two putative mutations in two severely affected CEP patients; one was homoallelic for the C73R mutation and the other was heteroallelic for C73R and P53L. Neither of these missense mutations was confirmed by expression studies. Thus, four of the five known mutations causing CEP (P53L, T62A, A66V, and C73R) are located within a 20-residue sequence of the URO-synthase polypeptide that is homologous to a region in the human α-globin chain involved in the binding of the D ring of heme (13, 27), the pyrrole group in HMB which undergoes rearrangement and ring closure catalyzed by URO-synthase. Since all four of these missense mutations are located in coding exon 4, they can be detected readily by amplification of this exon from genomic DNA (using primer set 3, Table I) and hybridization with ASOs, and/or restriction analyses with FokI (for P53L), and MaeII (C73R), which selectively recognize the mutant or normal sequences.

Prokaryotic expression of the five missense mutations, including P53L, resulted in levels of URO-synthase activity that could not be discriminated from the endogenous *E. coli* activity, with the notable exception of the A66V allele (Table IV). The A66V allele expressed a mutant protein with about 15% of normal activity and about half-normal stability when incubated at 37°C (Fig. 5). Since anti-human URO-synthase antibodies were unavailable, the presence of cross-reactive immunologically detectable enzyme protein was not determined. Therefore, it was not possible to assess whether the mutant alleles which had undetectable residual activity produced enzyme protein.

Of the 21 unrelated CEP patients studied, seven were heteroallelic and one was homoallelic for the C73R mutation. In this sample, the frequency of the C73R allele was 21%, indicating that this mutation is a common cause of CEP. When the

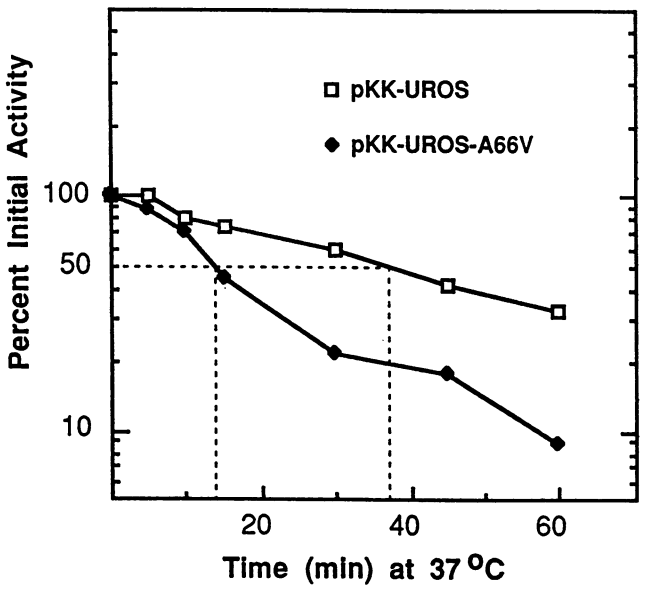

Figure 5. Thermostability of the enzymes expressed by pKK-UROS and pKK-UROS-A66V in *E. coli.* Cell extracts were incubated at 37°C for the indicated times, and the URO-synthase enzyme activities were determined. The results are expressed as the percentage of initial activity.

results of Deybach et al. (15) are included, the C73R allele occurred in 12 of 46 CEP alleles (an allele frequency of 26%). The other mutant alleles were detected only in the index probands with the exception of T228M, which was identified in a second patient. The P53L mutation was not detected in any of the 21 CEP homozygotes in this study.

The identification of these lesions permitted the first genotype-phenotype correlations in this disease. The common C73R allele had little, if any enzymatic activity (Table IV), and individuals homoallelic for this mutation had severe disease. In addition to the homoallelic C73R patient in this study who expired at five months of age, Deybach et al. also reported a severely affected, transfusion-dependent child who was homoallelic for the C73R mutation (15). It is likely that the cysteine residue in position 73 may be involved in disulfide bond formation, thereby being essential to the secondary structure of the normal enzyme. Subsequent studies of C73R/C73R patients may determine the relative level of cross-reactive immunologic material, thereby determining the relative stability of the mutant enzyme protein.

The phenotypes of individuals who are heteroallelic for the C73R mutation depend on the specific lesion in the other allele (Table III). For example, proband 1 is heteroallelic for C73R and A66V and has mild disease, as might be expected since the latter mutation expressed about 15% of normal activity in *E. coli* (Table IV). Thus, the presence of the A66V allele provided sufficient URO-synthase activity to attenuate the severe transfusion-dependent phenotype of patients homozygous for the C73R allele. Heteroallelism for C73R and P53L also resulted in a severe phenotype (15), indicating that the P53L allele encodes an altered protein with little, if any, enzymatic activity, consistent with the results of the prokaryotic expression studies (Table IV). Of interest, proband 2, who was heteroallelic for the C73R and T228M mutations, had moderately severe disease. Although prokaryotic expression of the T228M allele resulted in no detectable enzymatic activity, the fact that the proband was not transfusion-dependent, suggested that the T228M allele may produce active enzyme, albeit at very low levels. The threonine replacement may result in improper folding and/or instability of the mutant monomer. Since all CEP patients must synthesize heme, most non-consanguineous CEP patients should have at least one leaky mutation that expresses sufficient URO-synthase activity to maintain the minimal level of heme biosynthesis required for life. The amount of residual activity would determine the severity of the phenotype. This is consistent with the fact that disease severity in many inherited metabolic diseases is markedly altered by the presence of very low amounts of residual enzymatic activity (e.g., 28, 29). Thus, the mutations causing CEP are heterogeneous, and those which express residual enzymatic activity lead to milder phenotypes even when the allelic mutation produces no enzymatic activity.

In summary, these studies identified four missense mutations causing CEP, designated T62A, A66V, C73R and T228M. Expression of these mutant alleles permitted an in vitro assessment of their ability to produce active enzyme, and thereby modify disease severity. The identification and expression of these mutations should permit molecular heterozygote detection, accurate genetic counseling, and improved prenatal diagnosis for CEP families with these lesions, as well as facilitate genotype-phenotype correlations for this inherited porphyria.

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