Novel Molecular Defects of the δ-Aminolevulinate Dehydratase Gene in a Patient With Inherited Acute Hepatic Porphyria

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Cloning and expression of the defective gene for δ-aminolevulinate dehydratase (ALAD) from the second of 2 German patients with ALAD deficiency porphyria (ADP), who had been originally reported by Doss et al. in 1979, were performed. Cloning of cDNAs for the defective ALAD were performed using Epstein-Barr virus (EBV)-transformed lymphoblastoid cells of the proband, and nucleotide sequences of cloned cDNA were determined. Two separate mutations of ALAD cDNA were identified in each ALAD allele. One was G457A, termed "H1," resulting in V153M substitution, while the other was a deletion of 2 sequential bases at T^{818} and C^{819}, termed "H2," resulting in a frame shift with a premature stop codon at the amino acid position of 294. Using allele-specific oligonucleotide hybridization, the mother of the proband was shown to have an H1 defect, while using genomic DNA analysis, the father was shown to have an H2 defect. Expression of H1 cDNA in Chinese hamster ovary cells produced an ALAD protein with only a partial activity (10.65% ± 1.80% of the normal), while H2 cDNA encoded no significant protein. These data thus demonstrate that the proband was associated with 2 novel molecular defects of the ALAD gene, 1 in each allele, and account for the extremely low ALAD activity in his erythrocytes (~1% of normal). (HEPATOLOGY 2000;31:704-708.)

δ-Aminolevulinate dehydratase (E.C.4.2.1.24 [ALAD]) is a cytosolic enzyme in the heme biosynthetic pathway that catalyzes the condensation of 2 molecules of δ-aminolevulinic acid (ALA) to form a monopyrrole, porphobilinogen.[1] The enzyme activity is present in great excess in normal cells; thus, a partial deficiency of this enzyme activity is not usually accompanied by any clinical consequences.[2,3] However, a markedly decreased enzyme activity caused by certain chemicals, *e.g.*, lead, succinylacetone, or by an inherited enzyme deficiency may result in a clinical condition resembling those of severe acute hepatic porphyria.[4] Four unrelated cases of inherited ALAD deficiency porphyria (ADP) have been reported to date, *i.e.*, 2 German male patients who developed the disease at adolescence,[5] 1 Swedish boy who suffered from severe ADP from his birth,[6] and 1 Belgian male patient who developed the disease at the age of 63.[7] Molecular analysis of the gene defects have been reported for 2 of the 4 patients, *i.e.*, a German patient B[8] and a Swedish boy,[9] while others have yet to be defined. The molecular defects of the first 2 patients were both heteroallelic mutations of the ALAD gene, which are all distinct, indicating that the molecular defect in this disorder is highly heterogeneous. In the present study, we describe the molecular defects in the second ADP German patient, "H," who was also shown to have 2 novel and separate mutations of the ALAD gene, 1 inherited from each parent. In addition, examination of ALAD activity of the mutant proteins expressed in Chinese hamster ovary (CHO) cells conclusively established that both mutations identified in this study are in fact responsible for decreased ALAD activity, and that 1 of the 2 mutations is a *null* mutant, such that no ALAD protein was recognizable by an antibody against normal ALAD.

PATIENTS AND METHODS

Proband and Family Members Studied. Clinical and biochemical features of this proband "H" and his family member were described previously.[5] The proband developed signs and symptoms of ADP at the age of 15,[5] and is still alive and well, nearly 30 years after the first acute attack of ADP.[10]

Cell Cultures and Northern Blot Analysis of ALAD mRNA. Isolation of lymphocytes, transformation of cells with Epstein-Barr virus (EBV), and cultivation of lymphoblastoid cells were performed, as described previously.[11] Total RNA was isolated from lymphoblastoid cells or CHO cells transfected by human ALAD cDNAs according to the method of Cathala et al.,[12] and 10 μg of total RNA was used in Northern blot analysis to measure ALAD mRNA. Human ALAD cDNA was inserted into pGEM-T Easy Vector, and a radiolabeled probe was prepared according to the method previously described.[13] Hybridized Zeta probe filters were treated with RNase A (1 μg/mL) for 7 minutes at 37°C and then washed under stringent conditions. ALAD mRNA was detected by exposing the membrane to a sheet of x-ray film, and quantified by densitometry using an LKB Ultroscan XL Enhanced Laser Densitometer. An equivalent amount of RNA loading was confirmed by ethidium bromide staining of 28S and 18S rRNA in the gel.

Synthesis and Amplification of ALAD cDNA. The first-strand DNA was synthesized using avian myeloblastosis virus (AMV) reverse-transcriptase XL primed with Oligo dT-3 sites Adaptor Primer (3′-Full Race Core Set, TaKaRa, Tokyo, Japan). The first polymerase chain reaction (PCR) amplification of ALAD cDNA was performed by using a set of two oligomers, *i.e.*, ALAD21, which correspond to the beginning of exon 1A (Table 1) of ALAD cDNA, and 3 sites Adaptor (3′-Full Race Core Set; TaKaRa), as primers. The PCR product was collected by ethanol precipitation and then used as a

Abbreviations: ALAD, δ-aminolevulinate dehydratase; ALA, δ-aminolevulinic acid; ADP, ALAD deficiency porphyria; CHO, Chinese hamster ovary; EBV, Epstein-Barr virus; PCR, polymerase chain reaction; ASO, allele-specific aligonucleotide.

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TABLE 1. Oligonucleotides Used for Amplification of ALAD cDNA or Genomic DNA

Primer	Sequence	Location
ALAD 3	5′-GCAATCCATCTGTTGAGGA-	exon 5
ALAD 5	5′-AAGTCAAGCCCAGCTTTTGG-	exon 9
ALAD 6	5′-GCCAGGCCTCGTGCTCCAGG-	exon 9
ALAD 21	5′-GGGAGACCGGAGCGGGAGACA-	exon 1A
ALAD 22	5′-CAGGAGCAGCGGCCGGGAG-	exon 1A
ALAD 26	5′-GCCCTACCTGGACATCGTGCG-	exon 10
ALAD 28	5′-TCATTCCTCCTTCAGCCACTGCA-	exon 12
ALAD 29	5′-CTGTAGCTCATCACCGATAC-	exon 8

template for the second PCR amplification, which was performed using 2 sets of primers (Table 1), *i.e.*, ALAD22 and ALAD29, or ALAD3 and 3 sites Adaptor. Using the former sets of primers, ALAD cDNA containing the position at −109 ∼ 590 of the coding sequence was amplified, while using the latter sets, cDNA containing the position at 310 ∼ poly A tail was amplified.[14,15] Amplifications were performed 3 times in 3 separate experiments, and further steps were processed independently. PCR was performed using a DNA Thermal Cycler (Perkin Elmer-Cetus Corp., Norwalk, CT), employing a thermal cycle program, as described previously.[16]

cDNA Cloning. PCR products were purified using a GeneClean kit (BIO 101 Inc., La Jolla, CA), followed by cloning into pGEM-T Easy Vector (Promega, Madison, WI).

Amplification of ALAD Genomic DNA. Genomic DNA was isolated from EBV-transformed lymphoblastoid cells. Regions containing deletion detected in cDNA were amplified by PCR. The first PCR was performed using a set of primers, ALAD 5 and ALAD 28, which correspond to regions in exon 9 and exon 12 (Table 1). PCR products were purified and used as a template of the second PCR, which was performed using ALAD 26 (corresponding to a region in exon 10) and ALAD 28 as primers (Table 1). Cloning was performed as described above.

Nucleotide Sequencing Analysis. DNA sequencing was performed by the dideoxy chain-termination method using a genetically engineered T7 DNA polymerase (SequiTherm Long-Read Cycle Sequencing Kits LC., Epicentre-Technologies, Madison, WI), according to the manufacturer's instructions.

ASO Hybridization. Message amplification study was performed according to the method described previously.[17,18] Total RNA was reverse-transcribed with SuperScript RNase H^- reverse transcriptase (Gibco-BRL, Gaithersburg, MD), using random hexamers as primers. ALAD cDNAs were then amplified by PCR, using a set of primers, ALAD3 and ALAD6 (Table 1), corresponding to regions that contained the point mutation at the position 457. After 30 cycles of amplification, products were subjected to hybridization with allele-specific oligonucleotides (ASO) which were radiolabeled with [γ-^{32}P] adenosine triphosphate using T4 polynucleotide kinase (Promega).

Expression of Mutant ALAD cDNAs. The expression vector, pdKCR-Neo,[19] which contains the SV40 early gene promoter, donor, and acceptor sites, and polyadenylation sites derived from the rabbit β-globin gene and the SV40 early gene, was used for expression of ALAD proteins.[8] Cloned ALAD cDNA was cut off with *Eco* RI, followed by purification with a GeneClean kit, and inserted into the *Eco* RI site of the pdKCR-Neo vector. The recombinant plasmids were introduced into CHO cells by coprecipitation with calcium phosphate (CellPhect Transfection kit; Pharmacia Fine Chemicals, Uppsala, Sweden), followed by selection with G418 (Geneticin; Gibco-BRL) (800 μg/mL) starting 24 hours after transfection.

Western Blot Analysis and ALAD Assay. ALAD protein in erythrocytes and those that had been expressed in CHO cells by transfection were analyzed by Western blot analysis, as described previously.[8] ALAD in erythrocytes was partially purified using diethylaminoethyl cellulose chromatography, and concentrated approximately 100-fold by a Minicon concentrator B (Amicon, Inc., Beverly, MA) at 4°C, before Western blot analysis.[20] Fifty micrograms of protein was loaded onto each lane, in a 10% polyacrylamide gel–0.01% sodium dodecyl sulfate electrophoresis. CHO cells (n = 10^6) were used in sodium dodecyl sulfate–polyacrylamide gel electrophoresis. Following electrophoresis, proteins were blotted onto a sheet of nitrocellulose membrane. Immunological detection of human ALAD was made by using a monospecific IgG against human ALAD,[21] and chemiluminescent detection was made by using the ECL detection system (Amersham Life Science Ltd., Buckinghamshire, England), according to the manufacturer's instructions. Protein concentration was determined by the method of Lowry et al.[22] ALAD activity was determined using 10^6 cells per assay, as described previously.[23]

RESULTS

ALAD mRNA Level. Northern blot analysis of EBV-transformed lymphoblastoid cells demonstrated that ALAD mRNA in the proband's cells was similar, both in size and in content, to that of both parents and a normal sister-in-law (data not shown). This finding suggests that the proband's ALAD deficiency is likely the result of point mutations in the ALAD gene, rather than of its gene deletion or an unstable mRNA.

Cloning of the Mutant ALAD cDNA. First, by nucleotide sequence analysis, a substitution of A for G at nucleotide 457 in the ALAD gene was identified (Fig. 1A). This mutation, termed H1, accompanied an amino acid change from Val to

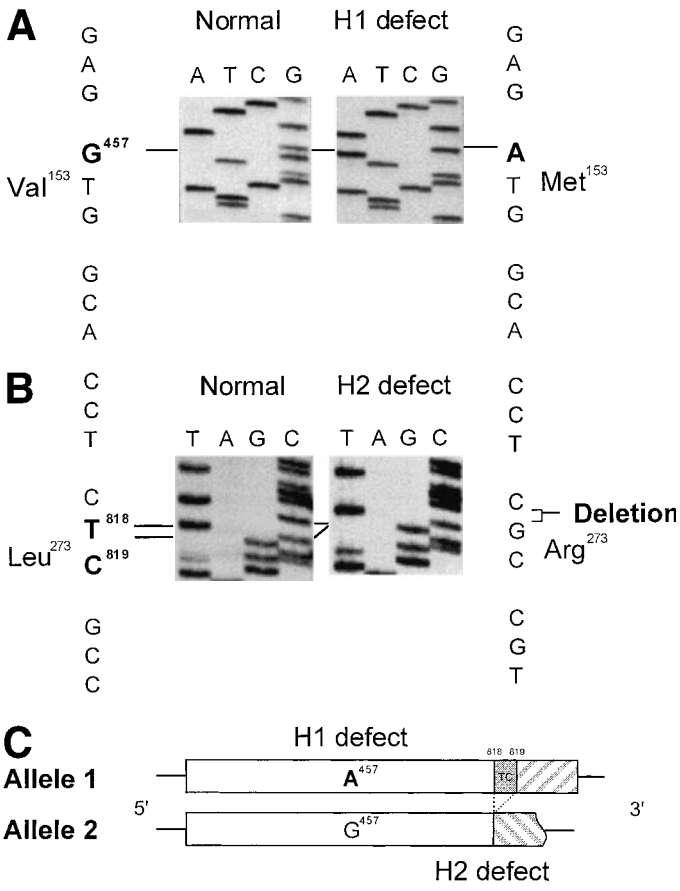

FIG. 1. Identification of exonic mutations in the ALAD gene. Nucleotide sequence analysis of reverse-transcription PCR products is shown. (A) G-to-A transition at nucleotide position 457 (H1 defect). (B) T and C deletions at nucleotide position 818 and 819 (H2 defect). (C) Schematic representation of the proband's ALAD cDNA. The proband has heteroallelic mutation of ALAD, consisting of H1 and H2 defects. H1 defect accompanies a valine-to-methionine substitution at amino acid residue 153, while H2 defect results in a frame shift, with a premature stop codon at amino acid residue 294.

Met at residue 153. Next, by nucleotide sequence analysis of H1 negative clones, another mutation, H2, was identified that was a deletion of 2 sequential bases at nucleotide T^{818} and C^{819} (Fig. 1B). Accordingly, the H2 defect resulted in a frame shift after Leu^{273} and a premature stop codon at amino acid position 294. Thus, the protein encoded by the H2 defect would be ~4 kd shorter than the normal protein, if it is expressed. Frequencies of 2 mutant ALAD cDNA clones were similar (52% and 48%, for alleles 1 and 2, respectively, for over 30 clones from 3 independently prepared libraries), suggesting that the abundance of each abnormal ALAD mRNA may be equivalent.

ALAD Genotype Studies. Using H1 and normal ALAD-specific oligonucleotides as probes in ASO hybridization (Fig. 2), we performed genotype studies of the ALAD gene in the proband's parents. Partial sequence of cDNA from the mother's cells, as well as the proband's cells, hybridized with the oligomer specific to the H1 defect, while cDNA from his father and a normal sister-in-law did not hybridize with H1 oligomer. In contrast to H1 defect, H2 defect was identified in the genomic DNA amplified from the proband's and the father's, but not in the genomic DNA from the mother or his sister-in-law (data not shown). These findings indicate that H1 defect is inherited from the mother, while H2 defect was inherited from the father of the proband.

Western Blot Analysis. Levels of ALAD protein were determined in erythrocytes of the proband and his father by Western blot analysis. ALAD protein expressed by H2 cDNA, which should have a molecular mass of 32 kd, was entirely undetectable both in the proband and in his father (Fig. 3). To establish quantitative relationship of ALAD protein between the proband, his father, and the normal control, immunoblot analysis of ALAD protein was performed using its serial dilution. By serial dilution of samples, the levels of ALAD protein, which showed a single band at 36 kd, in erythrocytes of the proband and the father were estimated to be 10.9% and 43.5% of the normal subject, respectively (Fig. 3).

ALAD Expressed in CHO Cells by Mutant ALAD cDNA. Human ALAD was expressed by transfection of cDNAs into CHO cells. Human ALAD protein was specifically detected and quantified by a rabbit IgG for human ALAD that reacted with the human, but not with CHO ALAD protein. The activity/mass ratio of the mutant was expressed as the percent of the normal control. The activity/mass ratio of H1 cDNA was 10.65% ± 1.80% of the normal control (mean ± SD, n = 3), while H2 cDNA did not express any protein or enzyme activity (Fig. 4). These findings show that H1 cDNA encoded ALAD with a partial enzyme activity, while H2 defect encoded no significant protein. To examine whether H2 defect encoded an unstable mRNA or an unstable protein, we performed Northern blot analysis. The results demonstrated that H2 mRNA is expressed as a normal-sized ALAD mRNA at a concentration comparable with the normal ALAD mRNA (Fig. 4). These findings thus indicate that the absence of a detectable H2 protein is not the result of the lack of its mRNA, but results from the abnormally unstable protein leading to a total loss of an immunologically reactive material, or lack of cross-reactivity with the antibody against normal ALAD protein.

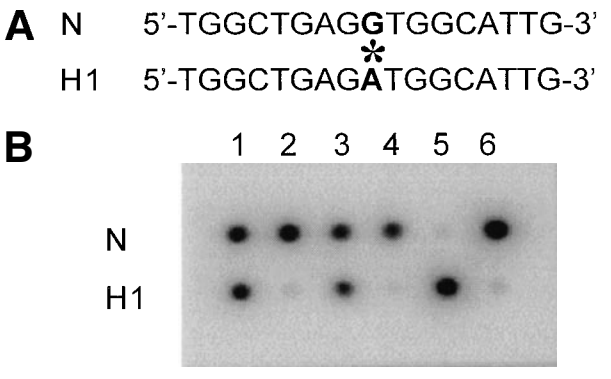

FIG. 2. ALAD genotype studies by ASO hybridization. (A) Sequence of the different oligonucleotides complementary to the normal and H1 sequences. (B) Dot-blot analysis of the amplified cDNA fragments hybridized with the different ASO. *Lane 1*, proband; *lane 2*, father; *lane 3*, mother; *lane 4*, sister-in-law (normal); *lane 5*, clone encoding H1 defect; *lane 6*, normal clone.

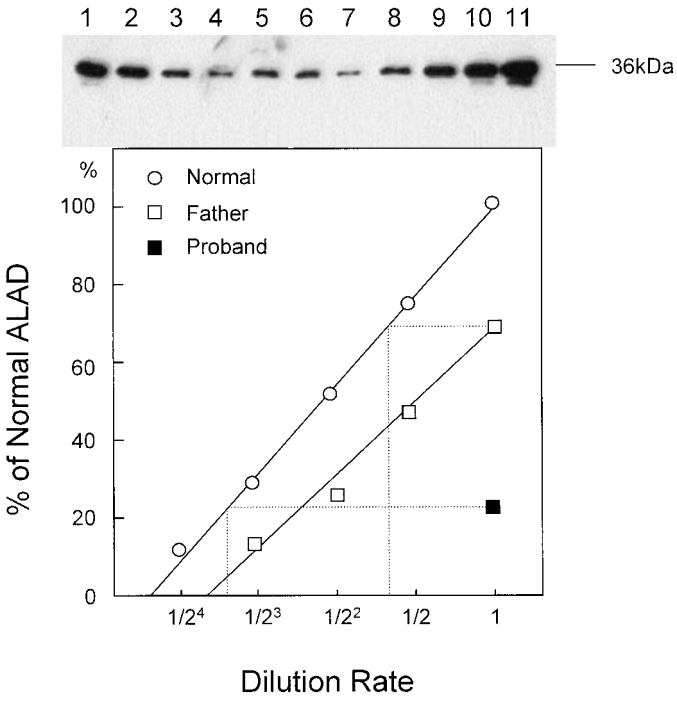

FIG. 3. Immunochemical study of ALAD protein in erythrocytes from the proband and his father. The proband's and his father's ALAD protein prepared from erythroid cells were quantitated by Western blot analysis using rabbit anti-human ALAD antibody. Results from the father's samples diluted to 1-fold (no dilution), 2-, 4-, or 8-fold are shown in *lanes 1-4*. The proband's sample is shown in *lane 5* (no dilution). Normal samples diluted to 16-, 8-, 4-, 2-, or 1-fold are shown in *lanes 7-11*. Purified ALAD is shown as the standard in *lane 6*.

DISCUSSION

Our findings in this study defined 2 novel mutations of the ALAD gene in the second German patient, with ADP, "H," who was 1 of the 2 German patients originally described by Doss et al.[5] Namely, the H1 defect was an G-to-A nucleotide transition at 457, resulting in a Val-to-Met substitution at amino acid residue 153, while H2 defect encoded 2 base deletions at 818 and 819, resulting in a frame shift with a premature stop codon at amino acid residue 294 (Fig. 1).

The proband "H" had markedly deficient erythrocyte ALAD activity that was <1% of the normal control, and a markedly elevated urinary excretion of ALA (1,163 μmol/d; normal: <49).[24] Immunoblot analysis of his erythrocytes

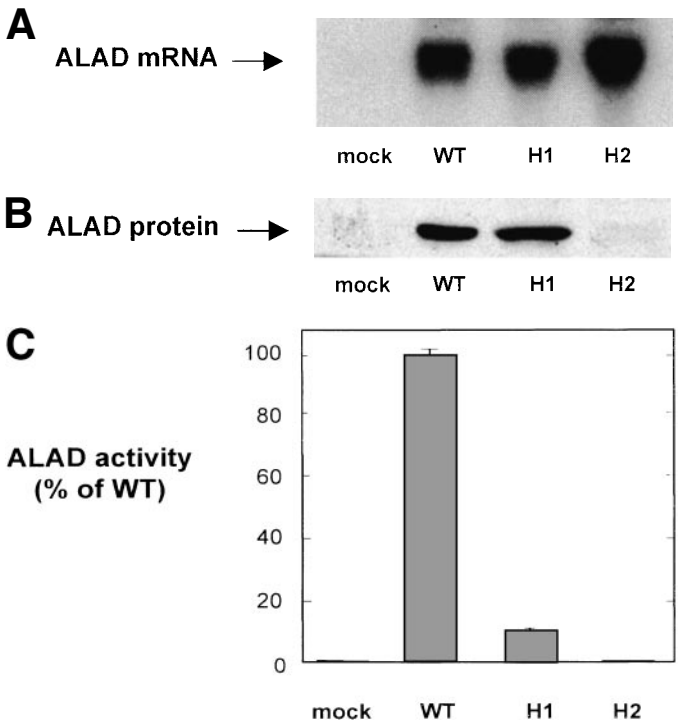

FIG. 4. Expression of human ALAD in CHO cells. Transfection of CHO cells with human ALAD cDNAs, quantitation of human ALAD mRNA, protein, and determination of ALAD activity were performed as described in Patients and Methods. (A) ALAD mRNA by Northern blot analysis. (B) ALAD protein by Western blot analysis. (C) ALAD activity. Data are the mean ± SD of triplicate assays. Human ALAD expressed by transfection with anti-sense ALAD cDNA (mock), ALAD cDNA (WT), H1 cDNA (H1), and H2 cDNA (H2).

demonstrated a significant amount of a cross-reactive immunological material (CRIM) (~11% of the normal control) (Fig. 3), suggesting the existence of a structurally abnormal noncatalytic enzyme. Clinically, the proband was compatible with homozygous ALAD deficiency, because ALAD activity in his erythrocytes was extremely low, while both parents and grandmother had enzyme activity ~50% of normal,[24] consistent with an autosomal recessive inheritance. Northern blot analysis of ALAD mRNA from the proband's lymphoblastoid cells showed a normal size and a normal amount of ALAD mRNA, indicating that the H2 ALAD mRNA itself is not unstable. Consistent with this finding, the frequency of cloned ALAD cDNA having H1 or H2 defect was approximately equal, suggesting that both mutant ALAD mRNAs had comparable stability. On the other hand, H2 cDNA expressed in CHO cells showed no detectable ALAD protein (Fig. 4), suggesting that the aberrant H2 protein is extremely unstable. Because the first German patient with ADP was also compound heterozygous for 2 different ALAD mutations,[8,16] 1 encoded a nonfunctional enzyme, and the other encoded an unstable protein, it is clear that both German patients with ADP resulted from heteroallelic mutations of the ALAD gene. Compound heterozygosity was also reported in a Swedish infant with ADP.[9] These findings indicate that most ALAD mutations are unique, suggesting a highly heterogeneous nature of the defect in ADP.[23]

Genotype studies of ALAD indicated that the proband's mother had H1 defect (Fig. 2), while his father had H2 defect. Erythrocyte ALAD activities of the mother and the father were ~50% of normal, consistent with the heterozygosity for the ALAD gene defect. Both mutations lie outside the substrate-binding site as well as the zinc-binding domain[14]; thus, their contribution to the loss of enzymatic activity is probably not a direct effect. On the other hand, because the activity/mass ratio of H1 ALAD expressed in CHO cells was ~11% (Fig. 4), the mutant protein may have a significant conformational alteration, resulting in a decreased catalytic activity.

In contrast to H1 defect, H2 defect should encode a structurally far more deleterious protein, because it has a large substitution and deletion in the C-terminal 58 amino acids. While ALAD expressed in CHO cells by H2 cDNA showed a normal size and a normal amount of ALAD mRNA, no immunologically detectable protein or enzyme activity was detected (Fig. 4), indicating that the mutant protein may have lost its cross-reactivity with a polyclonal ALAD antibody, or the mutant protein is extremely unstable. Consistent with this conclusion, the amount of ALAD protein in erythrocytes of the father was ~50% of the normal level, and the protein translated from the H2 allele could not be detected in the erythrocytes of either the proband or the father (Fig. 3).

The unique feature of this study is the identification and characterization of 2 novel mutations in the ALAD cDNA, and the description of an ALAD mutant with no significant protein. These findings also underscore the marked genetic heterogeneity in the nature of ALAD mutations.

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