

# Acute Intermittent Porphyria: Identification and Expression of Exonic Mutations in the Hydroxymethylbilane Synthase Gene

## An Initiation Codon Missense Mutation in the Housekeeping Transcript Causes "Variant Acute Intermittent Porphyria" with Normal Expression of the Erythroid-specific Enzyme

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### Abstract

Acute intermittent porphyria (AIP), an autosomal dominant inborn error, results from the half-normal activity of the heme biosynthetic enzyme, hydroxymethylbilane synthase (EC 4.3.1.8). Diagnosis of AIP heterozygotes is essential to prevent acute, life-threatening neurologic attacks by avoiding various precipitating factors. Since biochemical diagnosis is problematic, the identification of hydroxymethylbilane synthase mutations has facilitated the detection of AIP heterozygotes. Molecular analyses of unrelated AIP patients revealed six exonic mutations: an initiating methionine to isoleucine substitution (M1I) in a patient with variant AIP, which precluded translation of the housekeeping, but not the erythroid-specific isozyme; four missense mutations in classical AIP patients, V93F, R116W, R201W, C247F; and a nonsense mutation W283X in a classical AIP patient, which truncated the housekeeping and erythroid-specific isozymes. Each mutation was confirmed in genomic DNA from family members. The W283X lesion was found in another unrelated AIP family. Expression of each mutation in *Escherichia coli* revealed that R201W, C247F, and W283X had residual activity. In vitro transcription/translation studies indicated that the M1I allele produced only the erythroid-specific enzyme, while the other mutant alleles encoded both isozymes. These mutations provide insight into the molecular pathology of classic and variant AIP and facilitate molecular diagnosis in AIP families. (*J. Clin. Invest.* 1994. 94:1927–1937.) Key words: hydroxymethylbilane synthase • porphobilinogen deaminase • initiation of translation • mutation analysis • single strand conformation polymorphism

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Received for publication 5 May 1994 and in revised form 7 July 1994.

1. Abbreviations used in this paper: AIP, acute intermittent porphyria; ALA,  $\delta$ -aminolevulinic acid; ASO, allele-specific oligonucleotide; HMB-synthase, hydroxymethylbilane synthase; PBG, porphobilinogen; SSCP, single-strand conformational polymorphism.

*J. Clin. Invest.*

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0021-9738/94/11/1927/11 \$2.00

Volume 94, November 1994, 1927–1937

### Introduction

Acute intermittent porphyria (AIP),<sup>1</sup> an autosomal dominant inborn error of metabolism, results from the half-normal activity of hydroxymethylbilane synthase (EC 4.3.1.8; HMB-synthase), the third enzyme in the heme biosynthetic pathway (1–4). This enzyme, formerly known as porphobilinogen deaminase or uroporphobilinogen I synthase, catalyzes the head to tail condensation of four molecules of porphobilinogen (PBG) to form the linear tetrapyrrole, hydroxymethylbilane. Clinical onset of the disease typically occurs during or after puberty and is characterized by intermittent attacks of neurological dysfunction, including abdominal pain and other gastrointestinal complaints, hypertension, tachycardia, and various peripheral and central nervous system manifestations. Expression of the disease is highly variable, determined in part by environmental, metabolic, and hormonal factors that induce hepatic  $\delta$ -aminolevulinic acid synthase activity, the first and rate-limiting enzyme of heme biosynthesis, thereby increasing the production of the porphyrin precursors,  $\delta$ -aminolevulinic acid (ALA) and PBG (5–7). The half-normal hepatic HMB-synthase activity in AIP patients is insufficient to prevent precipitation of the acute, life-threatening symptoms of the disease. Thus, the diagnosis of AIP heterozygotes is crucial as the primary form of medical management is the avoidance of specific precipitating factors.

HMB-synthase is encoded by a single gene localized to the chromosomal region 11q24.1 → q24.2 (8, 9). Recently, the entire 11-kb gene was sequenced, including the 5' regulatory, 3' untranslated, and intronic regions (10). As shown in Fig. 1, the gene contains 15 exons and 2 distinct promoters that generate housekeeping and erythroid-specific transcripts by alternative splicing (11, 12). The housekeeping promoter is in the 5' flanking region and its transcript contains exons 1 and 3–15. The erythroid-specific promoter is in intron 1 and its transcript is encoded by exons 2–15. The housekeeping transcript is expressed in all tissues and its promoter has certain features characteristic of housekeeping promoters, while the intron 1 promoter is active in erythroid tissues and has regulatory elements similar to those of the  $\beta$ -globin gene promoter (11, 13, 14). cDNAs encoding the 42-kD housekeeping and 40-kD erythroid-specific isozymes, which differ only in their NH<sub>2</sub>-terminal amino acid sequences, have been isolated and characterized (12, 15). The enzyme from human erythrocytes has been purified to homogeneity and its physical and kinetic properties have been determined (16).

Previous biochemical and immunologic studies have revealed significant genetic heterogeneity in the mutations causing AIP (17–19). Two major subtypes of AIP have been delineated (7, 20). In classical AIP, the housekeeping and the erythroid-

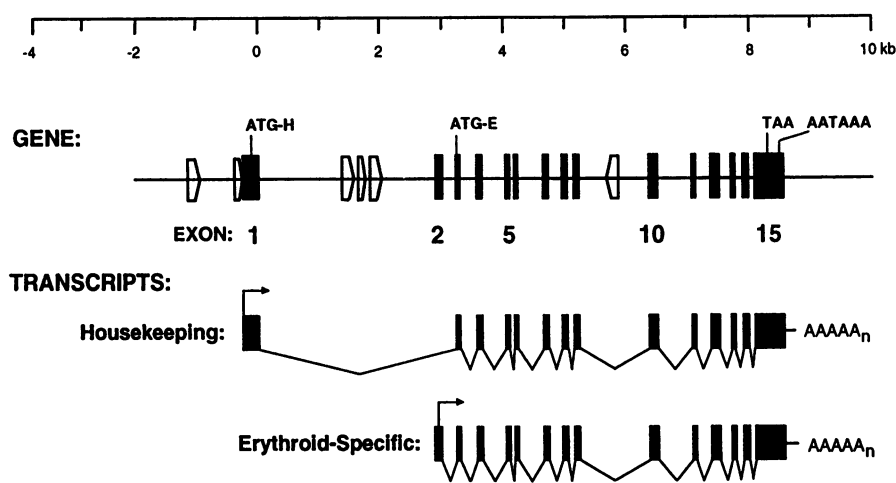


Figure 1. Genomic organization of the human HMB-synthase gene and alternative splicing of the housekeeping and erythroid-specific transcripts. Exons 1–15 are represented as solid rectangles, and the positions and orientations of the *Alu* repeat elements are indicated by the pentagonal figures. The gene has two promoter regions, 5' to exon 1 for the housekeeping transcript and 5' to exon 2 for erythroid-specific transcript. The initiation of translation sites for the housekeeping (ATG-H) and erythroid-specific (ATG-E) polypeptides are indicated.

specific enzymes both have half-normal activities, whereas in variant AIP (representing ~ 5% of AIP families) the housekeeping enzyme has half-normal activity, while the erythroid-specific enzyme is expressed at normal levels (21). To date, the molecular lesions in two unrelated families with variant AIP have been identified as different mutations in the intron 1 5' donor splice consensus sequence, which preclude normal splicing of the housekeeping transcript (22, 23). Symptomatic heterozygotes with classical or variant AIP, who excrete increased levels of the porphyrin precursors, ALA and PBG, can be identified easily, provided that the diagnosis is considered. However, the biochemical diagnosis of asymptomatic heterozygotes who usually have normal levels of urinary ALA and PBG has been problematic by enzyme assay, primarily due to the significant overlap between high heterozygote and low normal values (7, 24–27). Moreover, identification of asymptomatic heterozygotes for variant AIP is not possible as they have normal erythrocyte enzyme activities (21, 28). In view of the difficulties with biochemical diagnosis, investigators have turned to molecular techniques to identify diagnostically useful restriction fragment length polymorphisms (RFLPs) and specific mutations in the HMB-synthase gene.

To date, 24 different mutations in the HMB-synthase gene have been detected. Most of these mutations were either private or were found only in a few unrelated families, emphasizing the molecular heterogeneity of the mutations causing this disease (29–41). However, mutations W198X and R116W have been detected in a large number of Swedish and Dutch AIP families, respectively (33, 40). In addition, a variety of intragenic RFLPs have been identified which have proven useful for heterozygote diagnosis in informative families whose mutations have not been determined (42–48). However, there is marked linkage disequilibrium for the intron 1 and intron 3 RFLPs in northern European and American populations (49–51), with the exception of a recently identified common intron 10 *Hinf*I site (10).

The recent availability of the entire HMB-synthase genomic sequence (10) has facilitated mutation identification by the single-strand conformational polymorphism (SSCP) technique and by solid-phase direct sequencing. In this communication, six new mutations causing AIP are described. Of particular interest, a missense mutation in the initiation of translation codon of the

housekeeping transcript was identified as a novel cause of variant AIP.

## Methods

**Patient specimens, enzyme assays, and molecular screening.** Peripheral blood samples were collected from nine unrelated classical AIP patients, one patient putatively diagnosed as variant AIP, and their family members with informed consent. Lymphoid cell lines were established and maintained as described previously (52). From each proband, erythrocyte lysates were assayed for HMB-synthase activity (21) and genomic DNA was extracted from peripheral blood (53, 54) for detection of known HMB-synthase mutations (IVSI-1, IVSI+1, Q155X, R167Q, R167W, R173Q, W198X, and IVSI2-1) as described previously (29–35).

**Detection of HMB-synthase mutations by SSCP.** To detect mutations in HMB-synthase gene, SSCP was used as described (55) and modified (56). The 12 primer sets used for amplification of each exon and their flanking intronic sequences were synthesized on a synthesizer (model 380B; Applied Biosystems, Inc., Foster City, CA) and are indicated in Table I. Note that exons 2 and 3, 5 and 6, and 14 and 15 were amplified as single polymerase chain reaction (PCR) products which included the respective intervening introns. Amplifications were performed essentially as described by Saiki et al. (57) with the following modifications. Each 20- $\mu$ l reaction mixture contained 100 ng of genomic DNA; 10 pmol of each primer; 31.25 mM of each dNTP; 5  $\mu$ Ci [ $\alpha$ - $^{35}$ S]dATP; 10 mM Tris-HCl, pH 8.8; 50 mM KCl; 1.5 mM MgCl<sub>2</sub>; 0.1% Triton X-100, and 2.5 U of *Taq* polymerase (Promega Corp., Madison, WI). After an initial incubation at 94°C for 5 min, amplification (30 cycles) of each exon (except exon 1) was performed with denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 30 s. To amplify exon 1, the annealing temperature was raised to 66°C. Aliquots (1  $\mu$ l) of the amplified DNAs were diluted with 4.5  $\mu$ l of 0.1% SDS containing 10 mM EDTA and 4.5  $\mu$ l of 95% formamide loading dye. Samples (10  $\mu$ l) were denatured at 100°C for 5 min and then snap-frozen in ice water. After centrifuging for 30 s at 4°C, aliquots (3  $\mu$ l) were electrophoresed in 6% polyacrylamide gels containing 10% glycerol at 5–30 W and 4°C. The gels were dried and exposed to Kodak XAR-5 film for 1–2 d. Amplified fragments showing mobility shifts were subjected to solid-phase direct sequencing by the dideoxy chain termination method (58) using sequencing according to the manufacturer's instructions (United States Biochemical Corp., Cleveland, OH).

**Detection of HMB-synthase mutations by direct solid-phase sequencing.** To facilitate mutation detection directly from genomic DNA, primer sets (with one biotinylated) for the entire coding sequence and

Table I. Primer Sets for Amplification of HMB-Synthase Coding Exons for SSCP Analysis or Solid Phase Direct Sequencing

Coding exon primer set*	Genomic sequence amplified <sup>†</sup>	Oligonucleotide primers	
		Sense	Antisense
1A	-130-120	5'-gagaccaggagtcagactgt-3'	5'-agacgactgaggatggcaac-3'
1B	-130-120	5'-gagaccaggagtcagactgt-3'	5'-agacgactgaggatggcaac-3'
2-3A	2911-3309	5'- <u>GCCGCC</u> (GAATTC)tgacaactgccttggtc-3'	5'- <u>GCCGCC</u> (GAATTC)ctgttctgacaaacctct-3'
2-3B	2911-3360	5'- <u>cccaccctctctgtgccag</u> -3'	5'- <u>GCCGCC</u> (GAATTC)ctgttctgacaaacctct-3'
4A	3520-3710	5'- <u>GCCGCC</u> (GAATTC)gcctaacctgtgacagtct-3'	5'- <u>GCCGCC</u> (GAATTC)actgggcttcccattagct-3'
4B	3520-3770	5'- <u>GCCGCC</u> (GAATTC)gcctaacctgtgacagtct-3'	5'- <u>agcgggacggccttagta</u> -3'
5-6A	4012-4298	5'- <u>GCCGCC</u> (GAATTC)gagcactgattgattgac-3'	5'- <u>GCCGCC</u> (GAATTC)agacctagcactaggtg-3'
5-6B	3951-4298	5'-gcctctgtcccacatgaa-3'	5'- <u>GCCGCC</u> (GAATTC)agacctagcactaggtg-3'
7A	4546-4796	5'- <u>GCCGCC</u> (GAATTC)ggctccaccactgaagtag-3'	5'- <u>GCCGCC</u> (GAATTC)tcaggcccaaaaggaaag-3'
7B	4546-4796	5'-ggctccaccactgaag-3'	5'- <u>GCCGCC</u> (GAATTC)tcaggcccaaaaggaaag-3'
8A	4881-5119	5'- <u>GCCGCC</u> (GAATTC)cgagagagaatagaggtga-3'	5'- <u>GCCGCC</u> (GAATTC)acactagcagctcactgtt-3'
8B	4881-5119	5'- <u>GCCGCC</u> (GAATTC)cgagagagaatagaggtga-3'	5'- <u>GCCGCC</u> (GAATTC)acactagcagctcactgtt-3'
9A	5099-5293	5'- <u>GCCGCC</u> (GAATTC)aacagtgcactgcttaggt-3'	5'- <u>GCCGCC</u> (GAATTC)tgctttttcttgctgc-3'
9B	5061-5293	5'- <u>tggtccccaagaatgcagg</u> -3'	5'- <u>GCCGCC</u> (GAATTC)tgctttttcttgctgc-3'
10A	6295-6539	5'- <u>GCCGCC</u> (GAATTC)aaagacagactcaggcaga	5'- <u>GCCGCC</u> (GAATTC)taaggcagaaggagatgc-3'
10B	6295-6590	5'- <u>GCCGCC</u> (GAATTC)aaagacagactcaggcaga	5'- <u>atcgtttcacacaata</u> -3'
11A	7014-7258	5'- <u>GCCGCC</u> (GAATTC)catctcactgccaggtgct-3'	5'- <u>GCCGCC</u> (GAATTC)cggtagcatccaaggtct-3
11B	7014-7258	5'- <u>catctcactgccaggtg</u> -3'	5'- <u>GCCGCC</u> (GAATTC)cggtagcatccaaggtct-3
12A	7333-7554	5'- <u>GCCGCC</u> (GAATTC)cctgatgtcctagatgtt-3'	5'- <u>GCCGCC</u> (GAATTC)gtaagaaatcttccctgcc-3'
12B	7281-7554	5'- <u>agtagatagaggtggtccca</u> -3'	5'- <u>GCCGCC</u> (GAATTC)gtaagaaatcttccctgcc-3'
13A	7641-7848	5'- <u>GCCGCC</u> (GAATTC)cagtgatgtcctcacagtctg-3'	5'- <u>GCCGCC</u> (GAATTC)tacatagaacctggatgc-3'
13B	7641-7840	5'- <u>GCCGCC</u> (GAATTC)cagtgatgtcctcacagtctg-3'	5'- <u>ctacatagaacctggatg</u> -3'
14-15A	7830-8061	5'- <u>GCCGCC</u> (GAATTC)gcatcccagggttctaggt-3'	5'- <u>GCCGCC</u> (GAATTC)ctgtgccccacaacca-3'
14-15B	7830-8560	5'- <u>GCCGCC</u> (GAATTC)gcatcccagggttctaggt-3'	5'- <u>aaggccttctgtttgtcc</u> -3'

\* A, Primer sets for SSCP analysis; B, primer sets for solid phase direct sequencing, dot indicates biotinylated nucleotide. <sup>†</sup> HMB-synthase genomic sequences in lower case letters, EcoRI recognition sites in parentheses, underlined nucleotides represent additional non-HMB-synthase nucleotides to facilitate restriction enzyme cleavage. Genomic sequence position from Yoo et al. (10).

flanking intronic regions were synthesized using biotinylated phosphoramidites in a synthesizer (model 380B; Applied Biosystems, Inc.) (Table I). After amplification of genomic DNA as described above, an aliquot (40  $\mu$ l) of the amplified product was denatured and the biotinylated single strands were isolated by affinity capture using streptavidin-coated magnetic beads (Dynal, Inc., Great Neck, NY) (59), and then the biotinylated single-stranded PCR products were sequenced as described above.

**Mutation confirmation in genomic DNA.** Nucleotide changes detected by direct sequencing were confirmed in genomic DNA isolated from peripheral blood leukocytes or lymphoid cells (53, 54) from the patients and appropriate family members by restriction endonuclease analyses or by dot-blot hybridization with allele-specific oligonucleotides (ASOs). Computer-assisted analyses revealed that five of the mutations obliterated restriction sites, permitting rapid analysis of the appropriate exons amplified from genomic DNA with the indicated biotinylated primer set as described above (Table I). For restriction analyses, an aliquot (10  $\mu$ l) of each amplification product was digested for 4 h with the appropriate restriction endonuclease (New England Biolabs Inc., Beverly, MA) and electrophoresed in agarose or polyacrylamide gels as follows: M11: digested with NlaIII at 37°C, electrophoresed in 8% polyacrylamide gels; V93F: BstNI at 65°C, 2% agarose gels; R201W: MspI at 37°C, 2% agarose gels; C247F: Fnu4HI at 37°C, 4% agarose gels; and W283X: HinfI at 37°C, 12% polyacrylamide gels. The gels were stained with ethidium bromide (0.5  $\mu$ g/ml) and then visualized under a long-wave ultraviolet light. The R116W mutation was confirmed by dot-blot hybridization of exon 8 amplified from genomic DNA, with the normal (5'-GTTTTCCCGCTGGGGG-3') and mutation-specific (5'-GTTTTCCCACTGGGGG-3') oligonucleotides that were end-labeled with [ $\gamma$ -<sup>32</sup>P]ATP (60) as described previously (61). The over-

night hybridization and washes were performed at 56°C. Membranes were exposed to Kodak XAR-5 film with an intensifying screen for ~ 6 h.

**Prokaryotic expression and characterization of HMB-synthase mutations.** The normal and mutant HMB-synthase alleles were expressed in *Escherichia coli* using the pKK233-2 vector (Pharmacia LKB Biotechnology Inc., Piscataway, NJ) as described previously (62). To introduce each of the mutations into the pKK-HMB-synthase (pKK-HMBS) expression construct, the "megaprimer" method for site-directed mutagenesis was used (63). In this mutagenesis strategy, three oligonucleotide primers were used to perform two rounds of PCR. The product of the first amplification, which incorporated the mutation, was used as a megaprimer for the second amplification, which generated a larger product containing the required cDNA sequence and appropriate restriction sites for cassette subcloning. All amplifications were performed for 30 cycles with denaturation at 94°C for 1 min, annealing at 60°C for 1 min, and extension at 72°C for 1 min. For the V93F mutation, sense and antisense primers MP1 (5'-GCGGCGGAATTCATGGCTGGTAACGGCAATGC-3') and MP2 (5'-GAACAAACAGGTCCACTTC-3', mutation underlined) were used to generate the 132-bp megaprimer product. The amplification product was gel-purified and used as the sense primer to amplify a 1,132-bp fragment in a reaction with the antisense primer MP3 (5'-GCCGCCGCAAGCTTCTGTGCCCA-CAAACCA-3') and pKK-HMBS DNA as template. The second amplification product was digested with KpnI and NsiI, and the KpnI/NsiI fragment was ligated as a cassette into the corresponding sites in pKK-HMBS, generating the mutant construct pKK-HMBS-V93F. The R116W mutation was introduced into the expression construct using sense primer MP1 and antisense primer MP4 (5'-TCCCACTTGCAG-

ATGGCTC-3') to amplify the 363-bp megaprimer. The gel-purified megaprimer was then used as the sense primer, together with antisense primer MP5 (5'-CCCATCCTTCATAGCTG-3') and pKK-HMBS as template DNA, to amplify an 832-bp product. The 832-bp PCR product was digested with NsiI and KpnI. The NsiI/KpnI fragment was cassette-ligated into pKK-HMBS, generating the mutant construct pKK-HMBS-R116W. For the R201W mutation, the sense primer MP1 and antisense primer MP6 (5'-CACAAGCATACATGCATTCCTCAGGGTGCA-GGATCTGCCCAACCCAGTTGTGCCA-3' containing an NsiI site) were used to amplify a 658-bp product which was digested with KpnI and NsiI and cassette-ligated into pKK-HMBS, producing pKK-HMBS-R201W. For the C247F mutation, sense primer MP7 (5'-GTTCAGTGC-CATCATCTG-3') and antisense primer MP8 (5'-TCAGCG-ATGAAGCGAAGCAG-3') were used to amplify a 205-bp megaprimer. The purified megaprimer was used as the sense primer to generate a 575-bp PCR product with antisense primer MP4, which was then digested with NsiI and XbaI and cassette-ligated to corresponding cloning sites of pKK-HMBS, yielding pKK-HMBS-C247F. For the W283X mutation, sense primer MP9 (5'-GGAGGAGTCTGAAGTCTAGACC-3') and antisense primer MP4 were used to generate a 282-bp megaprimer. The purified megaprimer was used as the antisense primer to produce a PCR product with sense primer MP10 (5'-TGTGGTGGG-AACCAGCT-3'). The 697-bp PCR product was digested with NsiI and XbaI and cassette-ligated into pKK-HMBS, resulting in pKK-HMBS-W283X. The entire coding region of each expression construct was sequenced to confirm its authenticity. After transfection of *E. coli*, single colonies were isolated and the inserts were sequenced to confirm the presence of the desired mutation. Transfection, bacterial growth, isopropylthiogalactoside induction, and HMB-synthase assays were performed as described previously (62). For enzyme stability studies, samples from the bacterial lysates, equalized for enzymatic activity, were incubated at 65°C for 180 min. Aliquots were removed at timed intervals and placed on ice, and the HMB-synthase activity was determined as described.

*In vitro coupled transcription/translation of normal and mutant cDNA.* The normal housekeeping and erythroid-specific cDNAs encoding HMB-synthase were subcloned into the prokaryotic expression vectors pET5a and pET11a (Novagen, Inc., Madison, WI), which were designated pET-HMBSH and pET-HMBHe, respectively. The V93F, R116W, R201W, C247F, and W283X mutations were introduced into pET-HMBSH by digesting the respective pKK-HMBS expression construct with KpnI and XbaI and cassette-ligating the KpnI/XbaI fragment into the pET-HMBSH vector. The M1I mutation was introduced into the pET-HMBS vector by the megaprimer site-directed mutagenesis strategy (63). The sense primer MP11 (5'-TTAATACGACTCACT-ATAGG-3', the 20-nt pET vector T7 promoter) and the antisense primer (5'-TACCAGATATATGTATCTCC-3') were used to generate a 92-bp megaprimer which contained a XbaI site between the T7 promoter and the HMB-synthase initiation codon. The megaprimer was purified and used as sense primer, together with antisense primer MP12 (5'-GTC-CTTCAAGGAGTAA-3') to amplify a 379-bp product, which was digested with XbaI and KpnI and cassette-ligated into pET-HMBS generating pET-HMBS-M1I. Each pET expression construct was confirmed by sequencing. The normal and mutant HMB-synthase constructs were transcribed and translated *in vitro* using the TNT™ Coupled Reticulocyte Lysate System according to the manufacturer's instructions (Promega Corp.). Aliquots (5 µl) of the translated radiolabeled products were denatured at 100°C for 5 min and then analyzed by gel electrophoresis in an 8% SDS-PAGE. The gel was fixed with 5% methanol and 5% acetic acid for 15 min and incubated in autofluor (National Diagnostics, Inc., Somerville, NJ) for 30 min to enhance the signal. The gel was dried and exposed to Kodak XAR-5 film for 10 h.

## Results

*Identification of HMB-synthase V93F, R116W, R201W, and C247F mutations by SSCP.* Of the original 10 unrelated AIP

heterozygotes screened for 8 known mutations (29–35), 2 had R167Q (proband 1 and 2) and 1 had R167W (proband 3). For identification of the mutations in the remaining 7 unrelated AIP patients, each of the 15 HMB-synthase-coding exons was amplified from lymphoblast genomic DNA for SSCP analysis using primers designed to include the intron/exon boundaries (10). The SSCP profiles for proband 4 (V93F), probands 5 and 6 (R116W), proband 7 (R201W), and proband 8 (C247F) revealed mobility shifts in exons 7, 8, 10, and 12, respectively (Fig. 2; R201W and C247F not shown). When each of these exons was amplified from the respective proband's genomic DNA and sequenced, single point mutations were identified which predicted amino acid substitutions (Fig. 3, B–E). Proband 4 had a G to T transversion of nucleotide 277 in exon 7 which predicted a valine to phenylalanine substitution at residue 93 (designated V93F). Probands 5 and 6 had a C to T transition of nucleotide 346 in exon 8 which would cause an arginine to tryptophan substitution in residue 116 (R116W). Proband 7 had a C to T transition at nucleotide 601 in exon 10 predicting an arginine to tryptophan substitution in residue 201 (R201W). Proband 8 had a G to T transversion of nucleotide 740 in exon 12 which would change a cysteine to phenylalanine in residue 247 (C247F). SSCP analysis of all other exons in the five probands studied did not reveal any mobility shifts resulting from coding region mutations. However, three additional mobility shifts were detected (10) that resulted from intronic polymorphisms in genomic positions 3581A/G (BsmA1), 7064C/A (HinfI), and 7998G/A (MnII).

*Identification of M1I and W283X by direct solid-phase sequencing.* All 15 HMB-synthase exons and flanking regions from probands 9 and 10 were amplified with 12 biotinylated primer sets and were sequenced directly using a solid-phase strategy. In proband 9, a G to A transition of nucleotide 3 in exon 1 predicted a methionine to isoleucine substitution (M1I) in the initiation of translation codon (Fig. 3 A). In proband 10, a G to A transition of nucleotide 848 in exon 14 predicted a tryptophan to stop substitution (W283X) (Fig. 3 F). No other changes were detected in the amplified products from these probands. Initial confirmation of the above mutations was made by demonstrating these lesions in genomic DNA from the respective probands and their available family members by restriction endonuclease digestion or by dot-blot hybridization with ASOs. As shown in Fig. 4, the M1I mutation which obliterated the only NlaIII site in exon 1 was demonstrated in amplified genomic DNA containing exon 1 from proband 9 and several first degree relatives. The proband, her mother, brother, and daughter each had the undigested 250-bp fragment representing the mutant allele, as well as the digested 133- and 117-bp fragments from the normal allele, confirming the inheritance of this allele in these symptomatic individuals. Analogously, the V93F, R201W, C247F, and W283X mutations were each confirmed in the respective exon amplified from genomic DNA, by digestion with a specific restriction enzyme whose cleavage site was obliterated by the mutation. For each mutation, the restriction enzyme and fragment sizes were: V93F, BstN1 (mutant 159-, normal 116- and 43-, and constant 114-bp fragments); R201W, MspI (mutant 162-, normal 84- and 78-, and constant 107-bp fragments); C247F, Fnu4HI (mutant 144-, normal 118- and 88-, and constant 101-bp fragments); and W283X, HinfI (mutant 154-, normal 147- and 7-, and constant 99-bp fragments). The R116W mutation was confirmed by amplification of exon 8

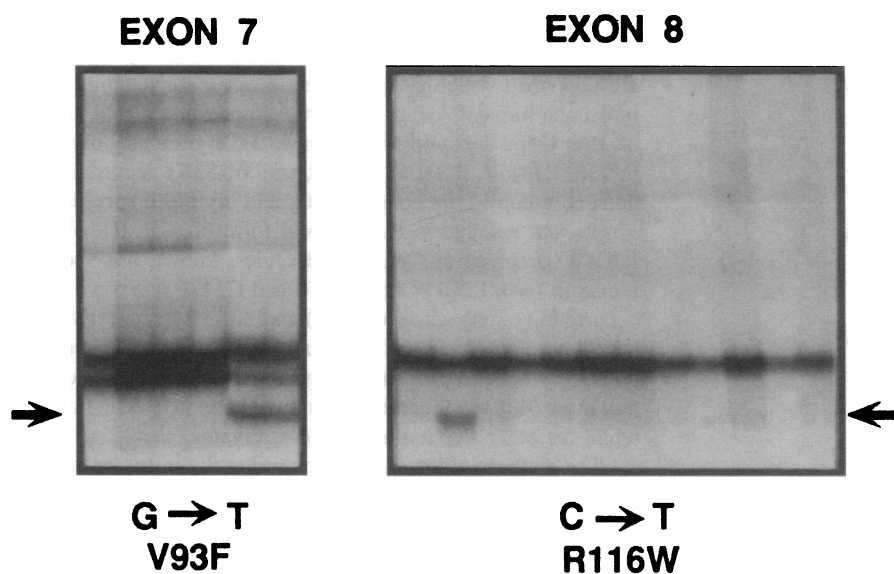


Figure 2. SSCP analysis of HMB-synthase coding exons 7 and 8 from unrelated AIP probands 4 and 5. Arrows indicate the mobility shifts. See text for details.

from genomic DNA of probands 5 and 6 (respective first degree relatives) and dot-blot hybridization using ASOs (data not shown). In each of these families, the respective mutation was identified in genomic DNAs from the proband, from other affected family members, and from previously undiagnosed relatives (in certain families).

**Prokaryotic expression of the HMB-synthase mutations.** To further characterize the HMB-synthase mutations, pKK-HMBS expression vectors for each of the mutant alleles were constructed, expressed in *E. coli*, and the enzymatic activity and stability of the recombinant proteins were determined. Table II shows the HMB-synthase activities of the expressed normal allele and the V93F, R116W, R201W, C247F, and W283X alleles after transformation and isopropylthiogalactoside induction. Note that the R201W, C247F, and W283X mutations expressed enzymes with significant residual activity (10–42% of the normal mean), while the activities of the V93F and R116W mutations were < 2% of the mean level expressed by the normal allele. Fig. 5 compares the relative stabilities of the expressed R201W, C247F, W283X, and normal HMB-synthase proteins when incubated at 65°C, pH 8.2. The half-life of the normal enzyme was ~ 145 min, whereas the half-lives for both the R201W and C247F enzymes were ~ 25 and 20 min, respectively, indicating that the mutant proteins had less than one-sixth the stability of the normal enzyme under these conditions. The R201W enzyme, which had > 40% of normal mean activity when expressed in *E. coli*, was rapidly inactivated to levels < 5% of initial activity at 180 min. The C247F enzyme, which had ~ 10% of normal mean activity when expressed in *E. coli*, retained ~ 10% of initial activity at 180 min of heat inactivation. Of interest, the truncated W283X enzyme had a normal thermostability profile.

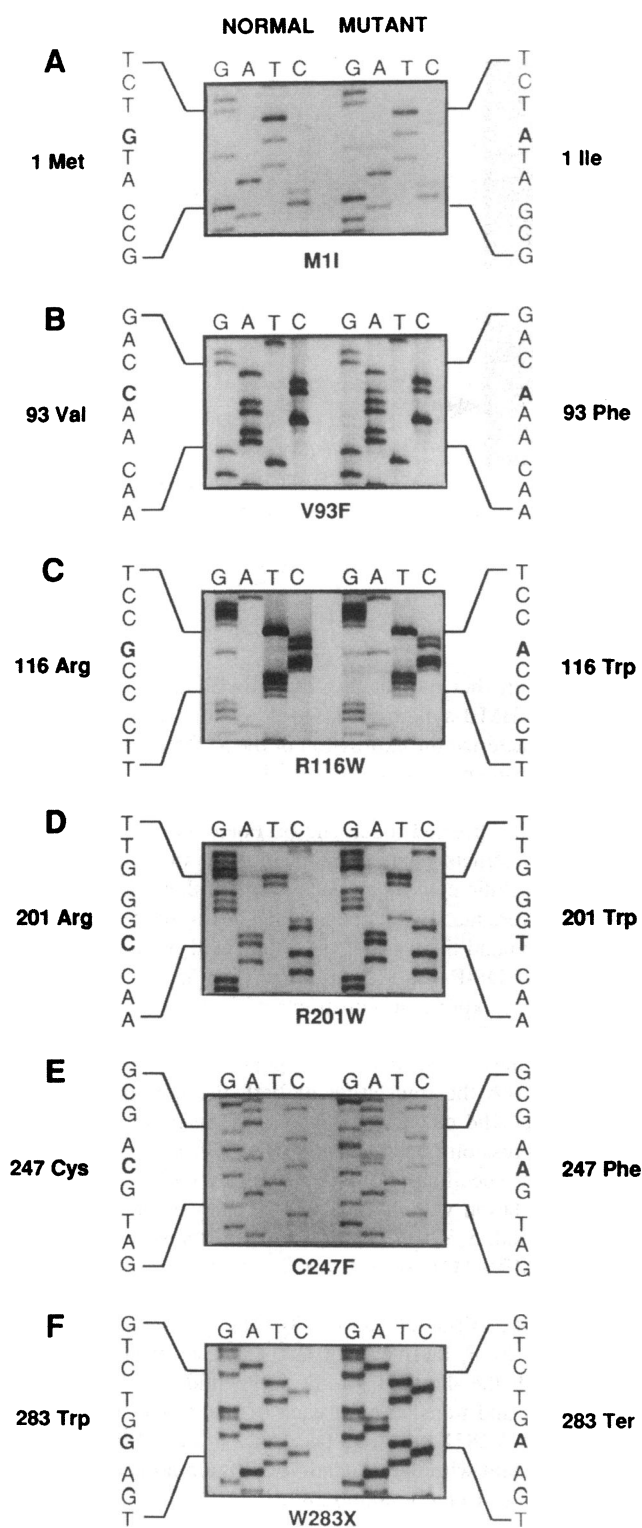
**Characterization of the M11 mutation causing variant AIP.** Erythrocytes from proband 9 with AIP and her available first degree relatives were assayed for HMB-synthase. Fig. 6 shows the enzymatic activities in unrelated heterozygotes with classical AIP, proband 9 and available family members, and unrelated normal individuals. Notably, the proband and those with the

M11 mutation (her mother, brother, and daughter) had normal erythrocyte HMB-synthase activities.

To characterize the expression of the M11 mutation, particularly to determine its effect on initiation of translation, the M11 mutation was introduced into the pET prokaryotic expression vector by site-directed mutagenesis. For comparison, pET expression constructs containing the normal housekeeping and erythroid-specific cDNAs and each of the other HMB-synthase mutations were made. Each construct was transcribed and translated in vitro and the translated radiolabeled products were assessed by SDS-PAGE. As shown in Fig. 7, the normal housekeeping expression construct, pET-HMBS<sub>h</sub>, which contains the initiation codons for both the housekeeping and erythroid enzymes encoded two polypeptides of 42 and 40 kD, consistent with the translation of both the housekeeping and erythroid-specific enzymes. In contrast, the normal erythroid-specific expression construct, pET-HMBS<sub>e</sub>, expressed the 40-kD erythroid-specific protein as well as a 33.5-kD polypeptide, whose translation was initiated by the next downstream ATG (at cDNA codon 56) in the pET-HMBS<sub>e</sub> construct. Notably, the pET-HMBS-M11 construct only expressed the 40-kD polypeptide whose translation was initiated by the downstream erythroid-specific ATG (at codon 18). The pET constructs containing the V93F, R116W, R201W, and C247F mutations expressed both the 42- and 40-kD polypeptides, demonstrating the stability and translation of each of the mutant mRNAs. In contrast, the W283X construct encoded 31- and 29-kD polypeptides, consistent with the truncation of 78 amino acids after the aberrant stop triplet at codon 283.

## Discussion

The identification and characterization of the mutations in the HMB-synthase gene causing AIP have increased our understanding of the molecular basis of variant AIP (22, 23), identified the molecular heterogeneity underlying both the classical and variant forms (29–41), permitted the precise diagnosis of asymptomatic heterozygotes (22, 23, 29–41), and provided the



**Figure 3.** Identification of exonic mutations in the HMB-synthase gene causing AIP. Partial DNA sequences of the PCR-amplified products from genomic DNAs of unrelated AIP patients showing: (A) the G to A transition of nucleotide 3 predicting a methionine to isoleucine substitution at residue 1 (M11); (B) a C to A transversion of nucleotide 277 encoding an alanine to phenylalanine replacement at residue 93 (V93F); (C) a G to A transition of nucleotide 346 resulting in an arginine to tryptophan change at residue 116 (R116W); (D) the C to T transition

opportunity for structure–function correlations of the human enzyme. Here, six new mutations in the HMB-synthase gene are described which further increase our understanding of the molecular basis of AIP and the synthesis, structure, and function of this heme biosynthetic enzyme. Five of these mutations (V93F, R116W, R201W, C247F, and W283X) occurred in codons common to the housekeeping and erythroid-specific transcripts and resulted in the classical form of AIP. R116W and R201W occurred at CpG dinucleotides, known hot spots for mutation (64). R201W in exon 10 and C247F in exon 12 each occurred in exons where 6 (25%) and 8 (33%), respectively, of the 24 previously known AIP mutations had been located (65). R116W, which was recently found to be a common Dutch AIP mutation (40), was identified in two unrelated AIP families, while the other five mutations were private.

The sixth mutation (M11) occurred at the initiation methionine of the housekeeping transcript and caused the variant form of AIP. Affected members of this family had normal erythrocytic HMB-synthase activity (Fig. 6) and presumably had half-normal activity of the housekeeping isozyme which was responsible for the disease manifestations in liver, kidney, brain, and other tissues. Consistent with this concept, the coupled *in vitro* transcription/translation of the M11 allele resulted in the synthesis of only the 40-kD erythroid-specific enzyme, whereas the normal housekeeping sequence encoded both the 40-kD erythroid-specific and the 42-kD housekeeping isozymes (Fig. 7). These *in vitro* findings support the prediction that the RNA polymerase fails to recognize the mutated housekeeping translation start codon *in vivo*. Thus, M11 is the third mutation identified that causes variant AIP, the other two lesions being RNA splicing defects at the exon 1/intron 1 boundary (22, 23).

These six new mutations were identified by two different strategies: (a) SSCP screening of PCR-amplified exonic and flanking intronic sequences followed by solid-phase direct sequencing; and (b) solid-phase direct sequencing of single-stranded PCR products amplified from genomic DNA with biotinylated primers. SSCP analysis revealed mobility shifts in exons 2, 7, 8, 10, and 12 in amplified genomic DNA from five of the seven unrelated AIP patients studied. The nucleotide changes causing these shifts were identified by solid-phase direct sequencing. In addition to four detected mutations (V93F, R116W, R201W, and C247F), a common new polymorphism (3119 g/t), which caused the mobility shift in exon 2 PCR product, was found in the 5' region of intron 2. However, it should be noted that the SSCP technique does not detect all mutations (e.g., reference 66). To detect the remaining two mutations (M11 and W283X), single-stranded solid phase direct sequencing of the amplified products from genomic DNA using biotinylated primers proved successful. Thus, for mutation analysis in the HMB-synthase gene, detection techniques such as SSCP and denaturing gradient gel electrophoresis are useful (41, 66); however, it may be more efficient to directly sequence all amplified codons and their intron/exon boundaries. More-

of nucleotide 601 predicting an arginine to tryptophan substitution at residue 201 (R201W); (E) a C to A transversion of nucleotide 740 encoding a cysteine to phenylalanine replacement at residue 247 (C247F); and (F) a G to A transition of nucleotide 849 resulting in a tryptophan to a stop codon at residue 283 (W283X).

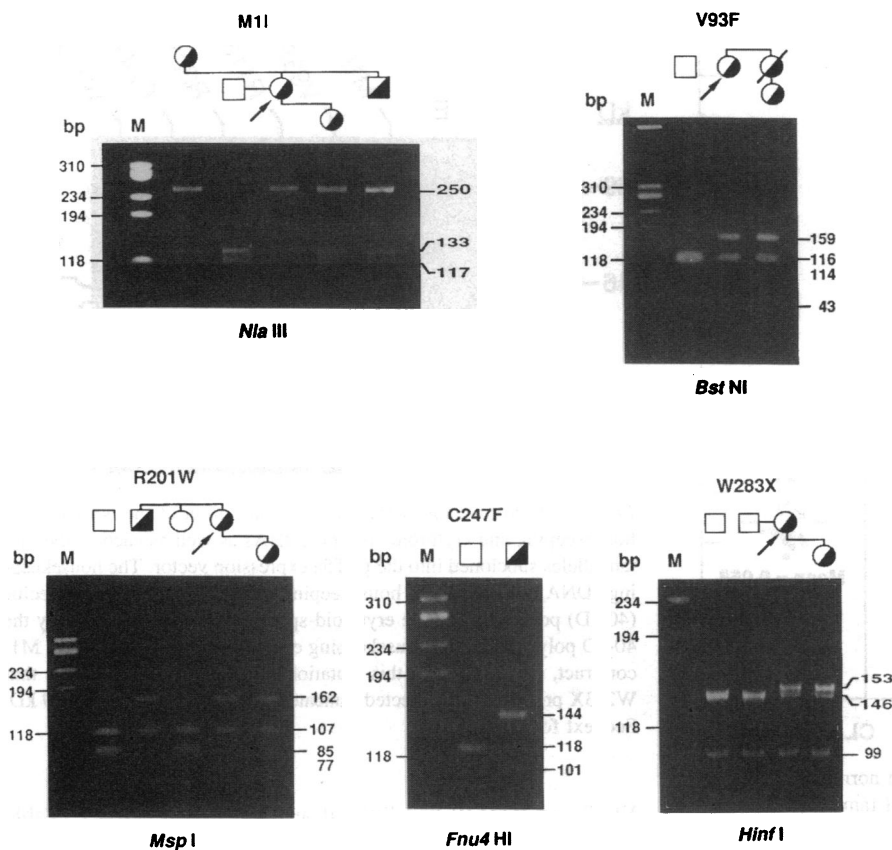


Figure 4. Amplification assays used to confirm the HMB-synthase M11, V93F, R201W, C247F, and W283X mutations. For each mutation, the ethidium bromide-stained agarose (V93F, R201W, C247F) or polyacrylamide (M11, W283X) electrophoretic gel shows digestion with the indicated restriction endonuclease of the respective normal or mutant sequence in amplified genomic DNA of AIP heterozygotes and unaffected family members. Arrows indicate probands; M, DNA base pair markers. See text for details.

over, even if a mutation is detected by SSCP or denaturing gradient gel electrophoresis, it is necessary to sequence the remainder of the coding region and flanking intronic sequences to eliminate the possibility of a second mutation as well as to exclude the possibility that the detected base substitution is a polymorphism.

Prokaryotic expression of the HMB-synthase mutant alleles revealed that the V93F and R116W mutations produced little, if any, enzymatic activity (Table II). In contrast, the R201W and C247F mutant alleles produced ~ 40 and 10% of the activity expressed by the normal allele, respectively. Heat inactivation studies indicated that both proteins were relatively unstable,

Table II. Expression of HMB-Synthase Mutations in *E. coli*

Construct	HMB-Synthase activity		Percentage of mean normal activity
	Mean	Range*	
	U/mg	%	
pKK233-2	1.5	1.20–1.90	0
pKK-HMBS	194.0	186.00–205.00	100
pKK-HMBS-V93F	2.3	1.98–2.58	1.1
pKK-HMBS-R116W	2.8	2.40–3.32	1.4
pKK-HMBS-R201W	80.7	67.00–88.70	41.6
pKK-HMBS-C247F	21.3	10.80–29.20	11.0
pKK-HMBS-W283X	20.0	18.80–22.20	10.3

\*Range represents results of three independent experiments.

losing over 90% of control activity after heating at 65°C. Presumably, their half-life in heme-producing erythroid cells and in hepatocytes also would be significantly reduced (Fig. 5). Interestingly, W283X produced a mutant protein in which the carboxy-terminal 78 residues were deleted, but retained ~ 10%

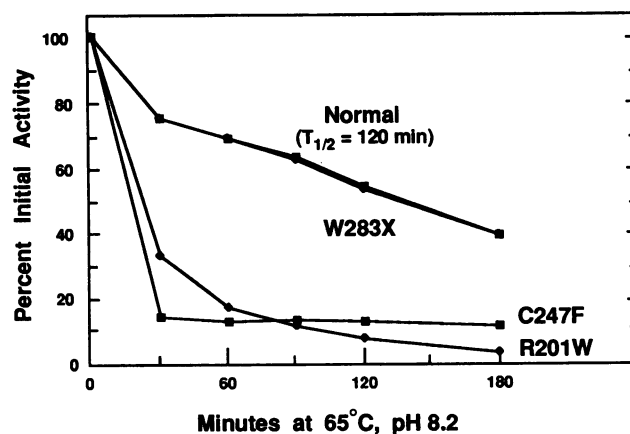


Figure 5. Thermostability of the enzymes expressed in *E. coli* by pKK-HMBS (normal allele) and pKK-HMBS-R201W, pKK-HMBS-C247F, and pKK-HMBS-W283X. Cell extracts were incubated at 65°C and pH 8.2 for the indicated times, and the HMB-synthase enzyme activities were determined. The results are expressed as the percentage of initial activity based on the mean of three independent assays for each mutation.

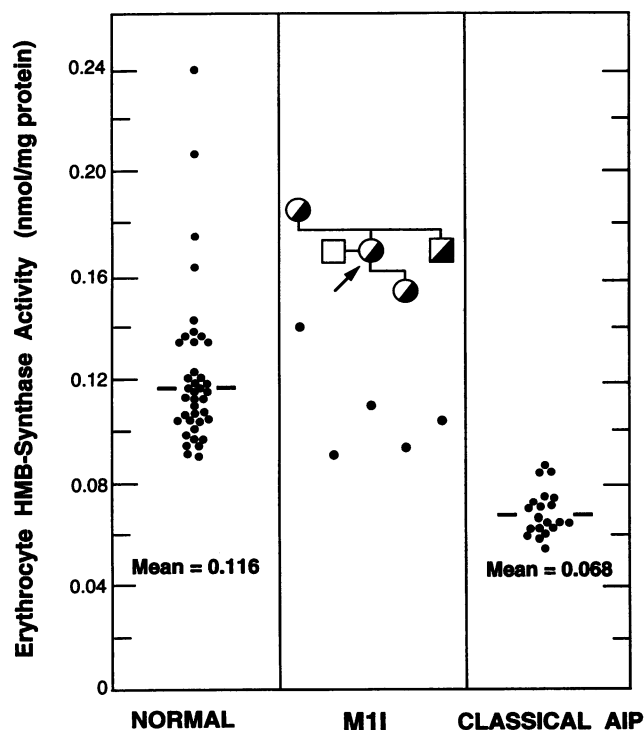


Figure 6. Erythrocyte HMB-synthase activity in normal individuals ( $n = 35$ ), classical AIP patients ( $n = 25$ ), and M1I family members as indicated in the pedigree. Note that the HMB-synthase activities of affected M1I family members are within the normal range, consistent with the M1I mutation causing variant AIP.

of the expressed normal activity and was as stable as the normal enzyme in vitro (Fig. 5).

Recently, HMB-synthase purified from *E. coli* was crystallized to a 1.9 Å resolution (67). The crystal structure revealed three domains, domains 1 and 2 were structurally identical to the group II periplasmic binding proteins and the duplicated lobe of the bilobal transferrins. The dipyrromethane cofactor was bound to the sulfur atom of cystine 242 in domain 3. The cofactor's acetate and propionate side chains were in a deep cleft between domains 1 and 2 and formed salt-bridge and hydrogen bond interactions with invariant arginine residues (R11, R132, and R155) and adjacent amino acids in domains 1 and 2 (e.g., F62, D84, S129, T127). Since the *E. coli* and human HMB-synthase amino acid sequences have 45% homology, it is possible to infer the location and structure/function relationship of certain human HMB-synthase mutations (35, 37). For example, Llewellyn et al. (37) described a cross-reacting immunologic material-positive AIP patient with a missense mutation of arginine codon 26 (R26H), which corresponds to *E. coli* R11, an invariant arginine which forms a salt bridge with the acidic side chain of the second pyrrole ring of the dipyrrolic cofactor (67). Notably, site-specific mutagenesis of the homologous *E. coli* arginine (R11 to H11) resulted in a enzyme protein with < 1% of normal activity that could not bind substrate or catalyze chain elongation (68). Presumably, the R26H mutation similarly altered the human enzyme's structure and function.

Additional structure/function correlations can be inferred from the mutations described here (Fig. 8). Human mutations

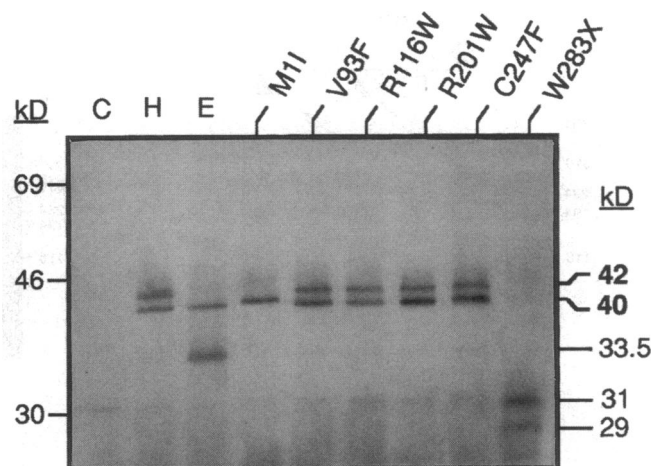


Figure 7. In vitro coupled transcription/translation of the normal housekeeping and erythroid-specific cDNAs as well as each of the mutant alleles subcloned into the pET5a expression vector. The housekeeping cDNA produced both housekeeping (42 kD) and erythroid-specific (40 kD) polypeptides. The erythroid-specific cDNA expressed only the 40-kD polypeptide. No housekeeping enzyme was produced by the M1I construct, consistent with this mutation causing variant AIP. Note that W283X produced the expected truncated polypeptides of 31 and 29 kD. See text for details.

V93F and R116W had little, if any, expressed activity (Table II). The human V93F mutation occurred at the corresponding *E. coli* variant residue A78 in domain 2 located in a  $\beta$ -sheet near a surface loop. Presumably, the replacement of this small, hydrophobic residue with a bulky, aromatic amino acid rendered the enzyme inactive and/or unstable due to alteration of the active site conformation and/or the interface with the adjacent  $\alpha$ -helix (Fig. 8). Human mutation R116W occurred at a position corresponding to an invariant arginine in *E. coli* (R101) that forms a salt bridge with Glu 231 in domain 3, one of a series of polar interactions positioning domain 3 alongside domain 1 (67). Site-specific mutagenesis of *E. coli* R101 inhibited enzymatic activity (68), consistent with the finding that the homologous human R116W mutation had essentially no expressed activity.

Human mutations R201W and C247F both had residual enzymatic activity, but were unstable. The arginine of human mutation R201W corresponds to *E. coli* R182, a residue that occurs at a surface loop joining an  $\alpha$ -helix and  $\beta$ -sheet in domain 1. Expression of the human R201W mutant enzyme in *E. coli* resulted in ~40% of the activity produced by the normal allele, consistent with the fact that the mutant residue was in a surface loop. However, the mutant enzyme was particularly unstable (Fig. 5) due to the replacement of the polar arginine with the bulkier and more hydrophobic tryptophan which presumably altered the loop's turn configuration and the enzyme's stability. Human residue C247 corresponds to *E. coli* V228, a variant residue in domain 3 involved in a hydrophobic interface with domain 1. The human C247F mutant enzyme had ~10% of normal expressed activity, but was markedly unstable, presumably due to the substitution of the bulky, aromatic residue which interfered with the hydrophobic interactions between domains 1 and 3. Similarly, C247R, another mutation in this codon identified in a classical AIP patient, introduced a large, polar



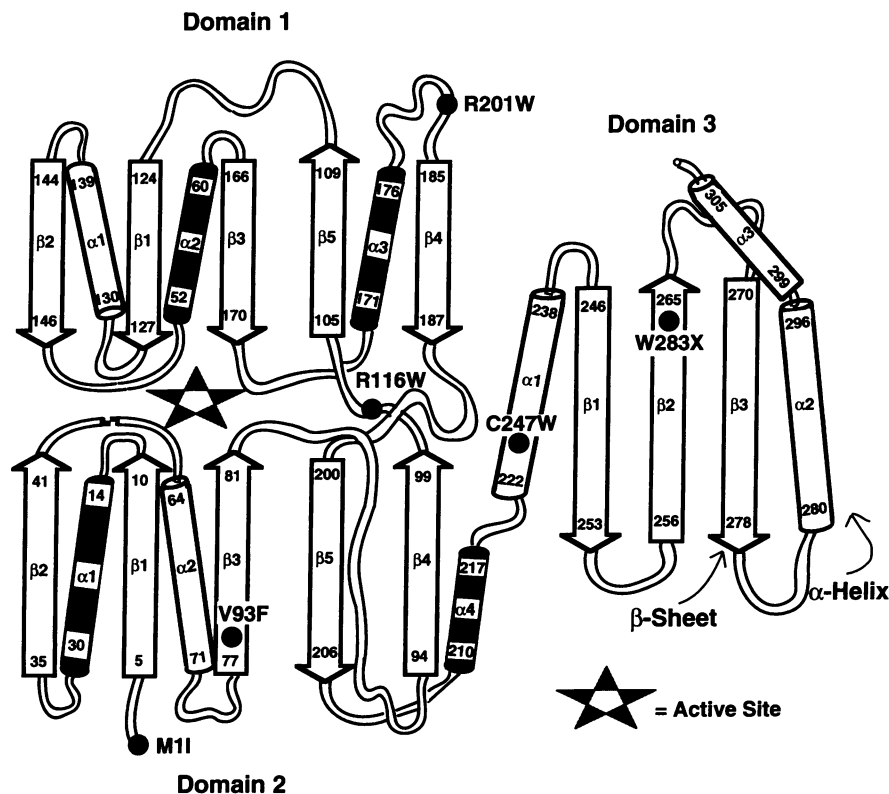


Figure 8. Schematic representation of the secondary structural elements based on the crystal structure of *E. coli* HMB-synthase. Helices are shown as cylinders and are shaded if behind the plane of the  $\beta$ -sheet (after Louie et al., reference 67). The positions of the M11, V63F, R116W, R201W, C247F, and W283X mutations, as determined by homology comparisons, are indicated. Note that V93F and R116W, which expressed < 2% of normal activity, occurred near the active site, while the R201W and C247F mutations that encode unstable proteins with residual activity occurred in a surface loop (R201W) and in an hydrophobic interface between domains 2 and 3 (C247F). The W283X mutation, which expressed a stable enzyme with ~ 10% residual activity, was located in domain 3.

arginine, which also interrupted the hydrophobic interactions between domains 1 and 3 (35).

Human mutation W283X, corresponding to *E. coli* G264, deleted 78 residues from domain 3, yet retained normal stability and ~ 10% of normal expressed activity. Domain 3 of the human HMB-synthase polypeptide has 29 more amino acids than the corresponding *E. coli* domain. Extra residues were inserted into the human sequence between corresponding *E. coli* codons G274 and A279 which occur in the loop following the  $\beta$ -sheet  $\beta_3$ , in domain 3. Since these 29 residues are highly conserved in humans, mice, and rats, they may be important in positioning the terminal invariant residues and their flanking sequences for extension of the growing pyrrole and/or release of the linear hydroxymethylbilane product (68).

In summary, six new mutations have been identified in the HMB-synthase gene which cause AIP. Not only did these lesions permit precise carrier detection in their respective AIP families, but they revealed a new molecular mechanism for variant AIP and provided further delineation of the genetic heterogeneity underlying classical and variant AIP. Moreover, comparison of the mutated residues with the position of their corresponding residues in the *E. coli* crystal structure provided insight into the structure/function relationships of human HMB-synthase.

## Acknowledgments

The authors thank Lisa Bland and Annie Hernandez for preparation of the manuscript and the following physicians for referral of patients and/or diagnostic specimens: Dr. Russell Graham (Altamonte Springs, FL), Dr. Thomas W. Sparks (Baton Rouge, LA), Dr. Joseph Hirsch (Louis-

ville, KY), Dr. Mary J. Hodak (York, PA), and Dr. Sylvia Bottomley (Oklahoma City, OK).

This research was supported in part by a grant (5 RO1 DK26824) from the National Institutes of Health, a grant (1-0853) from the March of Dimes Birth Defects Foundation, grants (5 MO1 RR00071 and 2 MO1 RR0073) from the National Center for Research Resources, National Institutes of Health for the General Clinical Research Centers at the Mount Sinai School of Medicine and the University of Texas Medical Branch, respectively, a grant (5 P30 HD28822) from the National Institutes of Health to the Mount Sinai Child Health Research Center, and a grant (FD-R-00710) from the Food and Drug Administration Office of Orphan Product Development.

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