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Molecular genetics of acute intermittent porphyria and studies of the human cytosolic heme biosynthetic enzymes

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MOLECULAR GENETICS OF ACUTE INTERMITTENT PORPHYRIA AND STUDIES OF THE HUMAN CYTOSOLIC HEME BIOSYNTHETIC ENZYMES

by

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ABSTRACT

MOLECULAR GENETICS OF ACUTE INTERMITTENT PORPHYRIA AND STUDIES OF THE HUMAN CYTOSOLIC HEME BIOSYNTHETIC ENZYMES

by

Chia-Hsiang Chen

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Acute intermittent porphyria (AIP), an autosomal dominant disease, results from the defective heme biosynthetic enzyme, hydroxymethylbilane synthase (EC 4.3.1.8; HMBS). Asymptomatic heterozygotes are induced to manifest symptoms by various factors. We cloned and determined the complete sequences of human HMBS gene. Four new polymorphic sites were identified by the single strand conformation polymorphism analysis, a common one in intron 2 (3119 G/T), one common *Bsm*AI site in intron 3 (3581 A/G), another common *Hinf*I in intron 10 (7064 C/A), and a rare *Mnl*I site in intron 14 (7998 G/A). Six exonic mutations causing AIP were identified: an initiating methionine to isoleucine substitution (M1I), which causing variant AIP. Four missense mutations, V93F, R116W, R201W, C247F; and a nonsense mutation W283X, which cause classical AIP. Expression studies revealed that R201W, C247F, and W283X had residual activity.

Large quantities of recombinant human HMBS isozymes were purified by using immobilized metal affinity chromatography. Purified HMBS isozymes have similar

physical and kinetic properties.

To determine if HMBS and UROS form a complex, polyclonal anti-HMBS antibodies were used to immunoprecipitate HMBS from human HepG2 and K562 cells labeled with [^{35}S]-methionine. UROS did not coprecipitate with HMBS. Furthermore, a two-hybrid protein interaction assay shows negative result for the presence of enyzyme complex between HMBS and UROS.

In summary, these studies have provided further delineation and understanding of the molecular genetics and cellular biochemistry of human HMBS, a critical cytosolic enzyme in the heme biosynthetic pathway.

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The Metabolic Pathway. Heme (ferroprotoporphyrin IX), is an essential molecule for life, and is the prosthetic group of various proteins including, hemoglobin, mitochondrial and microsomal cytochromes, catalase, peroxidase and tryptophan pyrrolase. Therefore, heme is critical to a variety of physiological functions. Alterations or defects in the enzymes required for heme biosynthesis result in profound metabolic and clinical disturbances known as porphyrias (1-4).

Heme biosynthesis requires eight enzymatic steps (Figure 1-1). The first and the last three steps take place in the mitochondrion, the remaining steps occur in the cytosol. The first committed precursor in heme biosynthesis is δ-aminolevulinic acid (ALA). In mammals and photosynthetic bacteria, this aminoketo acid is synthesized from succinyl CoA and glycine by the pyridoxal phosphate requiring enzyme, ALA-synthase (ALAS). This enzyme is the first and rate-limiting step of heme biosynthesis, and this pathway is known as the C4 pathway (5, 6). However, in higher plants and many prokaryotic systems, ALA is synthesized from glutamate; this pathway is called the C5 pathway (7).

In both of these pathways, two molecules of ALA are condensed by the enzyme δ-aminolevulinic acid dehydratase (ALAD) to yield porphobilinogen (PBG), the building block for tetrapyrrole biosynthesis. Four molecules of PBG are required for the formation of the tetrapyrrole, uroporphyrinogen III. This occurs by a two step process catalyzed by the third and fourth enzymes in the pathway, hydroxymethylbilane synthase (HMBS) and

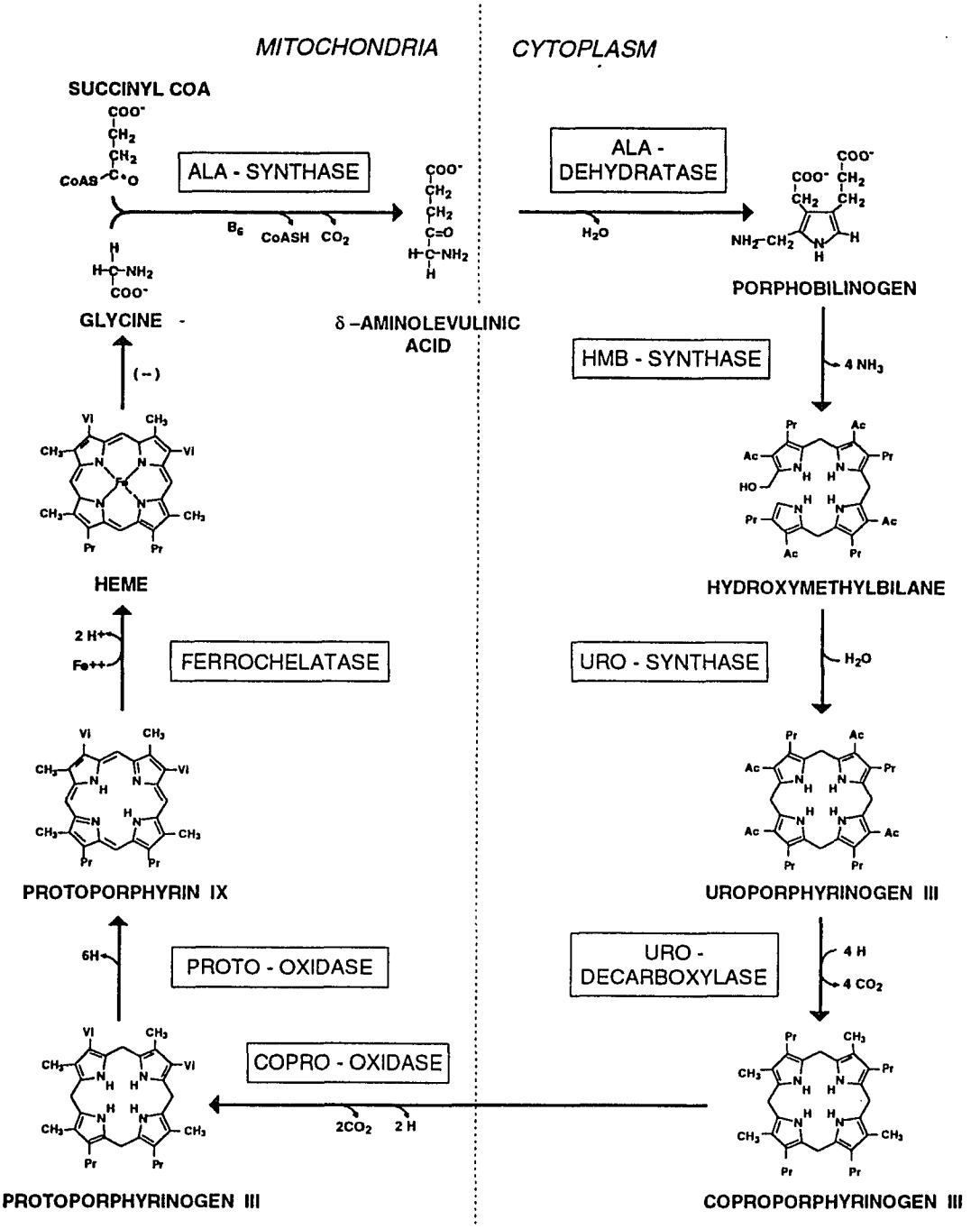


Figure 1-1. Heme biosynthesis pathway

uroporphyrinogen III synthase (UROS). HMBS catalyzes the head to tail condensation of four PBG molecules by a series of deamination steps to form the linear tetrapyrrole, hydroxymethylbilane (HMB). Uroporphyrinogen III synthase (UROS) catalyzes the rearrangement and rapid cyclization of HMB to form the asymmetric, physiologic, octacarboxylate porphyrinogen, uroporphyrinogen III. Uroporphyrinogen III is not only the precursor of heme, it is also the precursor of vitamin B_{12} and chlorophyll.

The fifth enzyme in the pathway, uroporphyrinogen decarboxylase (UROD), catalyzes the sequential removal of the four carboxyl groups from the acetic acid side chains of uroporphyrinogen III to form the methyl groups of coproporphyrinogen III, a tetracarboxylate porphyrinogen. This compound then enters the mitochondrion where coproporphyrinogen oxidase, the sixth enzyme, catalyzes the decarboxylation of two of the four propionic acid groups to form the two vinyl groups of protoporphyrinogen IX, a dicarboxylate porphyrinogen. Next, protoporphyrinogen oxidase catalyzes the oxidation of protoporphyrinogen IX to protoporphyrin IX. The final step in the heme biosynthesis is the insertion of ferrous iron into protoporphyrin IX to form heme. This reaction is catalyzed by the eighth enzyme in the pathway, ferrochelatase (8-10).

Each of the heme biosynthetic enzymes is encoded by a nuclear gene. To date, the full-length human cDNAs for seven enzymes, including those for the erythroid and housekeeping forms of ALAS, have been isolated and sequenced, and the chromosomal locations of all the heme biosynthetic genes have been assigned (11).

Tissue-Specific Regulation of Heme Biosynthesis. In man, about 85% of heme

is synthesized in erythroid cells to provide heme for hemoglobin. Most of the remaining heme is produced in the liver where it is used primarily as the prosthetic group for the cytochrome P-450 enzymes, as well as for other hemoproteins. The regulation of heme biosynthesis in erythroid cells is different from that in liver (7, 12). In liver, heme biosynthesis is under negative feedback control. The concentration of free heme in the liver regulates the synthesis and mitochondrial translocation of hepatic ALAS encoded by its housekeeping gene. Heme represses the synthesis of the housekeeping ALAS mRNA and can also interfere with the transport of the enzyme from the cytosol to the mitochondria. ALAS is inducible by many of the same chemicals that induce cytochrome P-450 enzymes in the endoplasmic reticulum of the liver. Because most of the heme synthesized in the liver is used for the synthesis of cytochrome P-450 enzymes, the induction of hepatic ALAS and cytochrome P-450 occur in a coordinated fashion.

In erythroid cells, the massive production of heme for hemoglobin is under another regulatory mechanism (13). The erythroid-specific ALAS gene on X-chromosome is expressed at much higher levels than the ALAS housekeeping gene on chromosome 3. An additional erythroid-specific control mechanism for ALAS synthesis is the regulation of iron transport into the cells for incorporation into heme.

2. Acute Intermittent Porphyria (AIP):

The inherited porphyrias are disorders resulting from the deficient activity of specific heme biosynthetic enzymes. These disorders are classified as either hepatic or erythroid, depending on the primary site of expression of the specific defect in each

disorder. The classification of the porphyrias, including their respective clinical, biochemical and genetic features have recently been reviewed in a treatise by Moore et al. (1)

Among the porphyrias, acute intermittent porphyria (AIP) is one of the most common. This hepatic porphyria is inherited as an autosomal dominant trait and results from the half-normal level of HMBS activity (14-16). This disease is panethnic, but more common in Scandinavia, and perhaps Britain. The enzyme deficiency can be demonstrated in most heterozygous individuals who inherit a mutant allele (17).

Clinical Manifestations of AIP. Clinical expression of AIP is highly variable. Activation of the disease is clearly related to ecogenic factors which can precipitate acute manifestations of the disease. The majority of heterozygotes for AIP remain clinically asymptomatic or "latent" unless exposed to the factors that increase the production of porphyrins. The most common precipitating factors are endogenous steroid hormones, porphyrinogenic drugs and a low caloric diet, usually instituted in an effort to lose weight. The major drugs known to be harmful in AIP are barbiturates and sulfonamide antibiotics. Attacks also can be provoked by infections and the stress of major surgery. Treatment of acute attacks with hematin or heme derivatives has proven effective. Attacks can be prevented by avoiding known precipitating factors.

Because the neurovisceral symptoms of this disease rarely occur before puberty and are often nonspecific, a high index of suspicion is needed to suggest the proper diagnosis. The disease can be disabling, but is only occasionally fatal. Abdominal pain, the most common symptom, is usually steady and poorly localized, but may be cramping.

Ileus, abdominal distention and decreased bowel sounds are common. However, increased bowel sounds and diarrhea may occur. Abdominal tenderness, fever, and leukocytosis are usually absent or mild because the manifestations are neurologic rather than inflammatory. Nausea, vomiting, constipation, tachycardia, hypertension, mental symptoms, pain in the limbs, head, neck or chest, muscle weakness, sensory loss, dysuria and urinary retention also are characteristic. Tachycardia, hypertension, restlessness, tremors, and excess sweating are attributed to sympathetic overactivity.

The peripheral neuropathy in this disease is due to axonal degeneration (rather than demyelinization) and affects primarily motor neurons. Clinically significant neuropathy does not occur with all acute attacks; abdominal symptoms are usually more prominent. Motor neuropathy usually affects the proximal muscles initially, and more often in the shoulders and arms than in the legs. Sensory changes such as paresthesis and loss of sensation are less prominent than motor involvement.

Mental symptoms such as anxiety, insomnia, depression, disorientation, hallucinations and paranoia can accompany acute attacks. Seizures can be a neurological manifestation of an acute attack or be a result of hyponatremia.

3. Molecular Biology of Human HMBS:

HMBS from human erythrocytes has been purified to homogeneity and its physical and kinetic properties have been determined (18). The monomeric enzyme is encoded by a single gene localized to the chromosomal region 11q24.1→q24.2 (19, 20). Subsequent characterization showed that the 10 kb chromosomal gene for HMBS has 15 exons and

that tissue specificity for this enzyme results from alternative splicing. A single chromosomal gene encodes both the 42 kD housekeeping enzyme and the 40 kD erythroid-specific enzyme, which differ only in their N-terminal amino acid sequences. The human cDNAs encoding the housekeeping and erythroid isoforms have been isolated and sequenced (21). The chromosomal gene has been shown to have two distinct promoters that generate the housekeeping and erythroid-specific transcripts by alternative splicing (22, 23). The housekeeping promoter is in the 5' flanking region of the gene and its transcript is encoded by exons 1 and 3 through 15. The housekeeping enzyme has 361 amino acids. The erythroid-specific promoter is in intron 1 and its transcript is encoded by exon 2 through 15. The erythroid-specific form contains 344 amino acids. 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 globin gene promoters. Recent studies have shown that the erythroid-specific promoter contains cis-acting regions, which bind the NF-E1 and NF-E2 transacting erythroid-specific factors and that theses factors are required for transcriptional regulation of the HMBS erythroid promoter (24, 25).

Studies of *E. coli* HMBS revealed that there is a cofactor bound to this enzyme, the cofactor contains two molecules of PBG covalently linked as a dipyrromethane cofactor (26). This cofactor is bound to the enzyme and is responsible for the covalent binding of the substrate molecules during the assembly of the tetrapyrrole, but is not subject to catalytic turnover (27). Further investigation has revealed that the

dipyrromethane cofactor is not just confined to the *E. coli* enzyme but that it is present in HMBS from animals, dicotyledonous and monocotyledonous plants as well as in *R. sphaeroides.*

Although a genomic clone for HMBS has been isolated, and partial sequences of this gene have been reported, the intron/exon boundaries and most intronic sequences have not been reported. In order to facilitate the investigation of the structure and regulation of the HMBS gene, and identification of the mutations causing AIP, it is very important to isolate the HMBS gene, and to determine its complete genomic sequence.

4. Biochemical and Molecular Studies of AIP:

Classical and Variant AIP. Two major subtypes of AIP have been identified: classical AIP and variant AIP, the latter representing 5 to 10% of AIP families (28). During acute attacks, both classical and variant AIP patients are easily diagnosed by their elevated levels of urinary ALA and PBG. However, in asymptomatic patients, urinary ALA and PBG levels are usually in the normal range. The diagnosis of asymptomatic heterozygotes with classical AIP relies on the demonstration of half-normal levels of erythrocyte HMBS activity. However, there is a significant overlap between normal and obligate heterozygote activities, resulting in a large percentage of inconclusive results. In addition, the enzymatic diagnosis of asymptomatic heterozygotes with variant AIP is not possible in erythrocytes, since they have normal erythrocyte HMBS activity (29-31). This is due to the fact that the HMBS gene has two promoter regions which regulate the transcription of the two alternatively-spliced transcripts encoding the housekeeping and

erythroid-specific forms of this enzyme. In variant AIP, splicing mutations have been identified which render the housekeeping transcript non-functional, but do not affect the alternatively-spliced erythroid transcript. The molecular heterogeneity of the mutations responsible for AIP is indicated by immunological studies.

Immunologic Studies of HMBS Mutations Causing AIP. Four major classes of mutations causing AIP have been identified (32, 33). The majority of AIP heterozygotes with classic disease (half-normal levels of HMBS) had no cross-reactive immunological material (CRIM) produced by the mutant allele (designated CRIM-negative type 1). The possible molecular lesions responsible for such CRIM-negative mutations include complete or partial gene deletions as well as point mutations or insertions that alter the processing or stability of the mRNA, create stop codons, or appreciably alter the conformation or stability of the mutant protein. A much less common CRIM-negative type (designated CRIM-negative type II) has been identified in variant AIP families who have half normal HMBS levels in non-erythroid tissues, but normal HMBS enzymatic activity in erythrocytes. This variant form of AIP was first described in Finland (29). Two types of CRIM-positive mutations (designated type I and II) have been characterized. In both types, the mutant alleles were expressed, but the enzyme proteins had altered kinetic and/or stability properties. Presumably, most of the CRIM-positive mutations result from missense mutations in the coding region of the HMBS gene.

HMBS Mutations Causing Variant AIP. In variant AIP families, the enzymatic deficiency is restricted to non-erythroid cells. The defect could result from a mutation

located either in the non-erythroid specific sequences of HMBS gene or in a distinct gene, the product of which is important in the transregulation of HMBS expression in non-erythroid cells. Linkage studies of RFLPs and AIP in a large Dutch family and in a Finnish family established a tight linkage between the phenotype and the HMBS gene. When the HMBS gene from the Dutch kindred was sequenced, a G to A transition was observed at the first nucleotide of intron 1 (IVS1+1) (34). This mutation modified the normal splice consensus sequence GT to AT. Since the first intron interrupts the sequence coding for the non-erythroid isoform of HMBS, it would be expected that abnormal splicing would lead to no or decreased expression of the housekeeping gene product. In contrast, in erythroid cells, transcription of the gene starts 2.8 kb downstream from the identified mutation and so this mutation has no consequence on the transcription of erythroid-specific HMBS sequence in these cells. In the Finnish AIP family, a different point mutation was found within the non-erythroid exon: at the last nucleotide of exon 1, a single base substitution (CG to CT) lead to a splicing defect, and the failure to express the housekeeping transcript (35). These two cases provide examples of mutations located within an ubiquitously expressed gene, which affect its function in certain tissues only, due to differential transcription and splicing.

HMBS Mutations Causing Classical AIP. In classical AIP, many defects in the HMBS gene causing AIP have been identified. These defects include a small deletion leading to a frame shift (36), a nonsense mutation resulting in a truncated protein (37) and missense mutations generating no or low HMBS activity (38-40). These mutations confirmed the genetic heterogeneity of AIP. Exon 10 is the region in which mutations

occurred most often, representing a hot spot for mutations in the HMBS gene (41). Most of the known mutations were private, found only in few unrelated AIP families. However, some mutations were common in certain populations. A base substitution (G to A) in exon 10 lead to nonsense mutation in codon 198 (W198X) and produced a truncated protein. This lesion is the most common mutation in Swedish families (42). This mutation supports the hypothesis of a "founder effect" in the families originating from Lapland.

Due to the fact that AIP is clinically" latent", molecular diagnosis of heterozygotes in AIP family members is essential to avoid acute attacks. Linkage studies of RFLPs in informative families would be helpful in the molecular diagnosis of AIP. To date, six RFLPs have been identified, *Msp*I, *Pst*I, *Bst*NI and *Apa*LI in intron 1, an *Apa*I site in exon 1 and an exon 10 polymorphic site (43-46). Although there is marked linkage disequilibrium for the four intron 1 RFLPs in northern European populations (47), it is helpful in detection of AIP heterozygotes in informative families (48). More useful RFLPs would be helpful in the molecular diagnosis of AIP heterozygotes. Identification of new mutations would not only be diagnostic of AIP heterozygotes, but also would help understand the molecular pathogenesis underlying this disorder of porphyrin metabolism.

5. Protein Structure and Function of HMBS:

Recombinant DNA techniques have facilitated the study of protein function and structure. Large quantities of proteins can be produced by cloned genes, and purified to homogeneity using advanced purification technologies. The purified protein can be used

for characterization of its kinetic and physical properties; moreover, the three-dimensional structure of the purified protein can be determined by X-ray diffraction analysis of its crystals. Site-directed mutagenesis is also an important technique to study protein structure and function. Amino acids in the protein can be replaced at any position and the importance of the amino acid of interest can be addressed.

HMBS occurs ubiquitously with over 45% amino acid sequence identity among the proteins from *E. coli, Bacillus subtilis, Euglena gracilis,* rat, mouse and man. The enzyme generates enzyme-intermediate complexes ES1, ES2, ES3 and ES4 during the course of the tetrapolymerization reaction. These complexes are stable and can be isolated using ion exchange chromatography (18). Hydrolytic release of the product HMB from ES4, regenerates the holoenzyme, and occurs by reversal of the initial coupling reaction. Biochemical evidence suggests that all of the PBG-coupling steps occur at a single catalytic site.

Recently, the three-domain structure of HMBS from *E. coli* has been defined by X-ray analysis at 1.9 Å resolution (49). Two of the domains structurally resemble the transferrins and periplasmic binding proteins. The dipyrromethane cofactor is covalently linked to domain 3 but is bound by extensive salt-bridges and hydrogen-bonds within the cleft between domain 1 and 2 at a position corresponding to the binding sites for the small-molecule ligands in the analogous proteins. The X-ray structure and results from site-directed mutagenesis provide evidence for a single catalytic site. Interdomain flexibility may aid elongation of the polypyrrole product in the active-site cleft of the enzyme (39).

A number of amino-acid replacements in human HMBS have been reported that give rise to AIP. With the crystal structure determination of *E. coli* HMBS, the possible structural and functional effects of these mutations can be interpreted. For example, the evolutionarily invariant amino acid residues, Gln 19, Arg 149, and Arg 155, are involved in a network of hydrogen-bonding interactions within the active-site cleft. Most notably, the two arginine residues interact directly through salt-bridges with the acidic side groups of the cofactor, and may form part of the binding site for the PBG substrate. Leu 159, also invariant, is packed in the hydrophobic core of the protein molecule, and replacement by arginine would be structurally deleterious (39).

Despite the 45% homology of amino acid sequences between the human and *E. coli* HMBS proteins, the human enzyme is about 60 residues longer. Moreover, there are two isoforms of human HMBS. Thus, it is essential to produce large quantities of the human HMBS proteins and to purify them in order to characterize the respective kinetic and physical differences of the two isoforms. In addition, the three-dimensional structure of human HMBS can be resolved by X-ray diffraction from the purified recombinant proteins. This would be helpful in understanding the structural changes and functional consequences of mutations in HMBS causing AIP.

6. Cytosolic Heme Biosynthetic Enzyme Complex:

There are well-characterized examples of enzymes involved in a metabolic pathway that are organized in multienzyme complexes. Such examples include enzymes in DNA biosynthesis, Krebs TCA cycle, and fatty acid oxidation (50-53). These

complexes provide an extremely efficient organization for the kinetics and regulation of the pathway, particularly if certain enzymes are rate-limiting or affected by product inhibition. With respect to heme biosynthesis, it is notable that four of the enzymes are located in the mitochondrion, while the other four are in the cytosol. Notably, heme is produced in the mitochondrion and in that organelle environment exerts its regulatory effect on the synthesis of ALA by the housekeeping ALAS enzyme. Although the study of each heme biosynthetic enzyme has elaborated the pathway for heme biosynthesis, to fully understand its organization, it would be of interest to determine if the four cytosolic enzymes are structurally organized in a cytosolic complex. There is now evidence, both experimental and theoretical that most of intermediary metabolism takes place in organized states. The mode of organization of component enzymes may entail one or more of the following: (1) protein-protein complexes, (2) association with (or in) a membrane, and (3) attachment to fibrocytoskeletal elements. In some cases the organization is strong, such that the system can be extracted from cells and studied by standard analytic-enzymological methods (54). For others, however, the interaction is weak (or transient) and readily disrupted by extraction, dilution, etc., in which case special analytical techniques and indirect methods must be used to demonstrate the existence of a complex..

"Metabolons" are enzyme complexes that carry out a series of sequential reactions in a biosynthetic pathway where the intermediates are considered to be out of diffusion equilibrium with identical molecules in the bulk phase of the same compartment of the cell. This process is referred to as channelling. Multienzyme complexes have been

described in prokaryotes as well as eukaryotes. There have been a number of advantages identified for the existence of multienzyme complexes: (1) If the substrates can be transferred directly from one active site to another so that the substrate's concentration in the microenvironment of the second site is kept relatively high compared to the average bulk concentration of that substrate, then one spares the limited solvation capacity of cellular water. (2) In such situations, high concentrations of substrates are achieved with fewer substrate molecules. (3) When the input to a sequence of metabolic reactions is changed, then the transient time for the attainment of a new steady state is reached faster for a multienzyme complex than for comparable enzyme activities free in solution. (4) If substrates are unstable in aqueous environments or if they can be acted on by other enzymes, then direct transfer to a successive active site is a mechanism for their preservation. (5) Interactions between sequential proteins have been shown to have allosteric (thus regulatory) effects on the activities involved. (6) Such complexes, if they are attached to or are part of the cellular structural elements or macromolecules, would have the advantage that the diffusion of their enzymatic components could possibly take place in one or two dimensions rather than three dimensions (50).

In the heme biosynthetic pathway, the four enzymes present in the cytosol are ALAD, HMBS, UROS and UROD. The fact that HMB, generated by HMBS, is rapidly metabolized to uroporphyrinogen III by UROS (previously known as cosynthase) argues that HMBS and UROS may be physically adjacent. Several investigators have suggested that the two enzymes exist in a complex (55, 56). In fact, it is possible that the four cytosolic enzymes exist in a complex, or even a cytosolic complex attached to the

mitochondria. The availability of purified UROS and HMBS should permit definitive investigation of this possibility as well as the characterization of the precise mechanism by which PBG is converted to uroporphyrinogen III in humans. Moreover, it is intriguing to speculate that all four cytosolic enzymes may function in a complex, thereby permitting the rapid and efficient conversion of ALA, synthesized in the mitochondria and transported to the cytosol, to coproporphyrinogen, which then returns to the mitochondria for conversion to heme. Such a cytosolic complex would facilitate the efficiency of sequential reactions and produce the "vital" heme required for the cell functions.

Chapter 2. Hydroxymethylbilane Synthase: Complete Genomic Sequence and Amplifiable Polymorphisms In The Human Gene

Summary

Acute intermittent porphyria (AIP), an autosomal dominant inborn error of heme biosynthesis, results from the half-normal activity of the heme biosynthetic enzyme, hydroxymethylbilane synthase (HMBS). Heterozygous individuals are prone to life-threatening acute neurologic attacks which are precipitated by certain drugs and other metabolic, hormonal and nutritional factors. Since the biochemical diagnosis of heterozygous individuals has been problematic, recent efforts have focused on the identification of mutations and diagnostically useful restriction fragment length polymorphisms (RFLPs) in the HMBS gene. To facilitate these endeavors, the human HMBS gene, including 1.1 kb of the 5' flanking region, was isolated and completely sequenced in both orientations. The 10,024 bp gene contained 15 exons ranging in size from 39 bp to 438 bp and 14 introns ranging from 87 bp to 2913 bp. All intron/exon boundaries conformed to the GT/AG consensus rule. There were six *Alu* repetitive elements; 1 of the J and 5 of the Sa subfamilies. Analysis of the 1.1 kb 5' flanking region revealed putative regulatory elements for the housekeeping promoter including AP1, AP4, SP1, TRE, ENH and CAC. This region contained 10 *Hpa*II sites and had an overall GC content of 54%. Intron 1, which contained the erythroid-specific promoter, had putative regulatory motifs for NF-1, NF-E1, NF-E1(b), NF-E2, AP1, AP4, TOPO, CAAC, CAC, CAAT, and TATA. The locations and variant nucleotides for the known RFLPs in intron 1 were identified [*Msp*I, nucleotide (nt) 1345 G/A; *Pst*I, 1500 C/T;

*Apa*LI, 2377 C/A; and *Bst*NI, 2479 G/A] and improved polymerase chain reaction (PCR)-based detection methods for each were established. Three new polymorphic sites were identified by the single strand conformation polymorphism (SSCP) technique, a common *Bsm*AI site in intron 3 (3581 A/G), a common *Hinf*I RFLP in intron 10 (7064 C/A) and a rare *Mnl*I site in intron 14 (7998G/A). The allele frequencies of five previously known and the new polymorphic sites in a normal Caucasian population indicated that the intron 1 and intron 3 RFLPs were in linkage disequilibrium; however, the *Hinf*I site segregated independently. The availability of the entire HMB-synthase genomic sequence and of improved and new amplifiable assays for the intragenic RFLPs should facilitate mutation detection and genotype prediction in AIP families in which the specific mutations have not been identified.

Introduction

Hydroxymethylbilane synthase (HMBS, formerly known as porphobilinogen deaminase or uroporphyrinogen I synthase; E.C.4.3.1.8), the third enzyme in the heme biosynthetic pathway, catalyzes the head to tail condensation of four monopyrrole porphobilinogen (PBG) molecules to form the linear tetrapyrrole hydroxymethylbilane (HMB). The enzyme from human erythrocytes has been purified to homogeneity and its physical and kinetic properties have been determined (1). The monomeric enzyme is encoded by a single gene localized to the chromosomal region 11q24.1→q24.2 (2, 3). The chromosomal gene encodes the 44 kDa housekeeping and 42 kDa erythroid-specific enzymes which differ only in their *N*-terminal amino acid sequences. cDNAs encoding the erythroid and housekeeping isoforms have been isolated and sequenced (4, 5) and the chromosomal gene has been shown to have two distinct promoters which generate the housekeeping and erythroid-specific transcripts by alternative splicing (5, 6). The housekeeping promoter is in the 5' flanking region and its transcript is encoded by exons 1 and 3 through 15. The erythroid-specific promoter is in intron 1 and its transcript is encoded by exons 2 through 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 β-globin gene promoter (6-8).

The half-normal activity of HMB-synthase is the enzyme defect in acute intermittent porphyria (AIP), an autosomal dominant disease. This disorder is characterized by episodic, life-threatening acute neurologic attacks, which are precipitated

by certain drugs and a variety of metabolic and hormonal factors that increase heme biosynthesis (9, 10). Thus, the diagnosis of AIP heterozygotes is crucial, as the primary form of medical management is the avoidance of such inducing factors. Symptomatic heterozygotes, who excrete increased levels of porphyrin precursors, δ--aminolevulinic acid (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 (11-14) and the occurrence of rare AIP heterozygotes with normal erythrocyte values (15, 16). 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.

Previous biochemical and immunologic studies revealed marked genetic heterogeneity of the mutations causing AIP (17, 18). The recent finding of 10 different mutations which were private or found only in a few unrelated families (19-25) emphasized the molecular heterogeneity of the mutations causing this disease and the need for diagnostically useful RFLPs. To date, six intragenic RFLPs have been identified, *Msp*I, *Pst*I, *Bst*NI and *Apa*LI sites in intron 1 (20, 26-28), an exon 1 *Apa*I site (29) and an exon 10 polymorphic site (30). Though there is marked linkage disequilibrium for the four intron 1 RFLPs in northern European populations (28, 31), these polymorphisms have proven useful for heterozygote diagnosis in informative families whose mutations have not been determined.

Previously, Chrietien and co-workers (1988) isolated an HMBS genomic clone and reported that it contained 15 exons. They determined 305 bp of 5' flanking sequence and 310 bp of intron 1 sequence upstream from the housekeeping and erythroid-specific transcription initiation sites, respectively. However, these investigators did not report the intron/exon boundaries or any other intronic sequence. In this communication, the complete sequence of the human HMBS gene, including 1100 nucleotides (nt) of 5' flanking and 686 nt of 3' flanking sequence is presented. This sequence permitted the identification of additional putative regulatory elements for the housekeeping and erythroid-specific promoters and the occurrence of *Alu* repetitive elements in and adjacent to the gene. In addition, improved assays were developed for the amplification of the four intron 1 RFLPs and three newly identified polymorphic sites. Of note, the newly identified intron 10 *Hinf*I polymorphic site segregated independently, whereas the intron 1 and 3 RFLP haplotypes were in linkage disequilibrium.

Materials and Methods

Construction of Synthetic Oligonucleotides

Unique oligonucleotides for sequencing the human HMBS gene, for use as polymerase chain reaction (PCR) primers, and for allele-specific hybridization probes were synthesized on an Applied Biosystems Model 380B oligonucleotide synthesizer using phophoramidite chemistry (32).

Isolation and Characterization of Genomic Clones

A human genomic library (average insert size ~10 to 15 kb) was constructed in the phage vector EMBL 3 (Promega) and kindly provided by Dr. Ruth Kornreich (Mount Sinai School of Medicine, New York, NY). The library was screened at a density of ~10,000 plaques/150 mm petri dish using a full-length hepatic HMBS cDNA, pHMBS-1. This cDNA was recloned (A. Roberts and R.J. Desnick, unpublished results) from a human liver cDNA library, kindly provided by Dr. Stuart Orkin (Harvard Medical School, Cambridge, MA), using oligonucleotides based on the erythrocyte HMBS cDNA sequence (4). The cDNA was radiolabeled with [α-^{32}P]CTP (~3000 Ci/nmol; Amersham) using a random primed labeling kit according to the manufacturer's instructions (Amersham). A total of 360,000 independent recombinants were screened. After three rounds of plaque purification, DNA was isolated by the lysate method (33) from two putative positive clones (gHMBS-1 and gHMBS-2). As shown in Figure 2-1, clone gHMBS-1 had an ~15 kb insert and Southern hybridization analyses using radiolabeled oligonucleotide probes from the 5'- and 3'- untranslated regions of the cDNA indicated that this insert contained the entire HMBS gene. The other clone, gHMB-S2, had an ~18 kb insert, which

contained only the last exon, the 3' untranslated region of the gene and 3' flanking sequence. Limited restriction mapping indicated that the two overlapping genomic clones spanned a 30 kb region that included about 5 kb and 15 kb of 5' and 3' flanking sequences, respectively. *Eco*RI restriction fragments of 7 and 6.7 kb from the gHMBS-1 insert which contained the 5' and 3' regions of the gene, respectively, were subcloned into pGEM 7Z plasmid vector (designated HMBS1-5' and HMBS-1-3', respectively) for sequencing.

DNA Sequencing and Computer-Assisted Analysis

Double-stranded DNA sequencing of the subcloned genomic *Eco*RI fragments was performed in both orientations by the dideoxy method (34) using universal or HMBS cDNA- specific synthetic oligonucleotide primers and Sequenase (U.S. Biochemical Corp., Cleveland, OH). Searches for nucleotide and amino acid sequence similarities and for *cis*-acting promoter elements were carried out using the MacVector program (version 3.5) with the nucleic acid subsequence database (International Biotechnologies, Inc.). Sequence alignments and contig generation were performed with the Microgenie DNA analysis program (Beckman).

Amplification and Detection of the HMBS Intragenic Polymorphisms

The primer sets for amplification of genomic DNA fragments containing the *Msp*I and *Pst*I sites, the *Bst*NI and *Apa*LI sites and the exon 10 G/T polymorphic site are indicated in Table 2-1. Each primer was constructed with nucleotides corresponding to the indicated genomic sequence and most had an additional 12 nucleotides which included an *Eco*RI site for subcloning. Amplification of each fragment was performed essentially

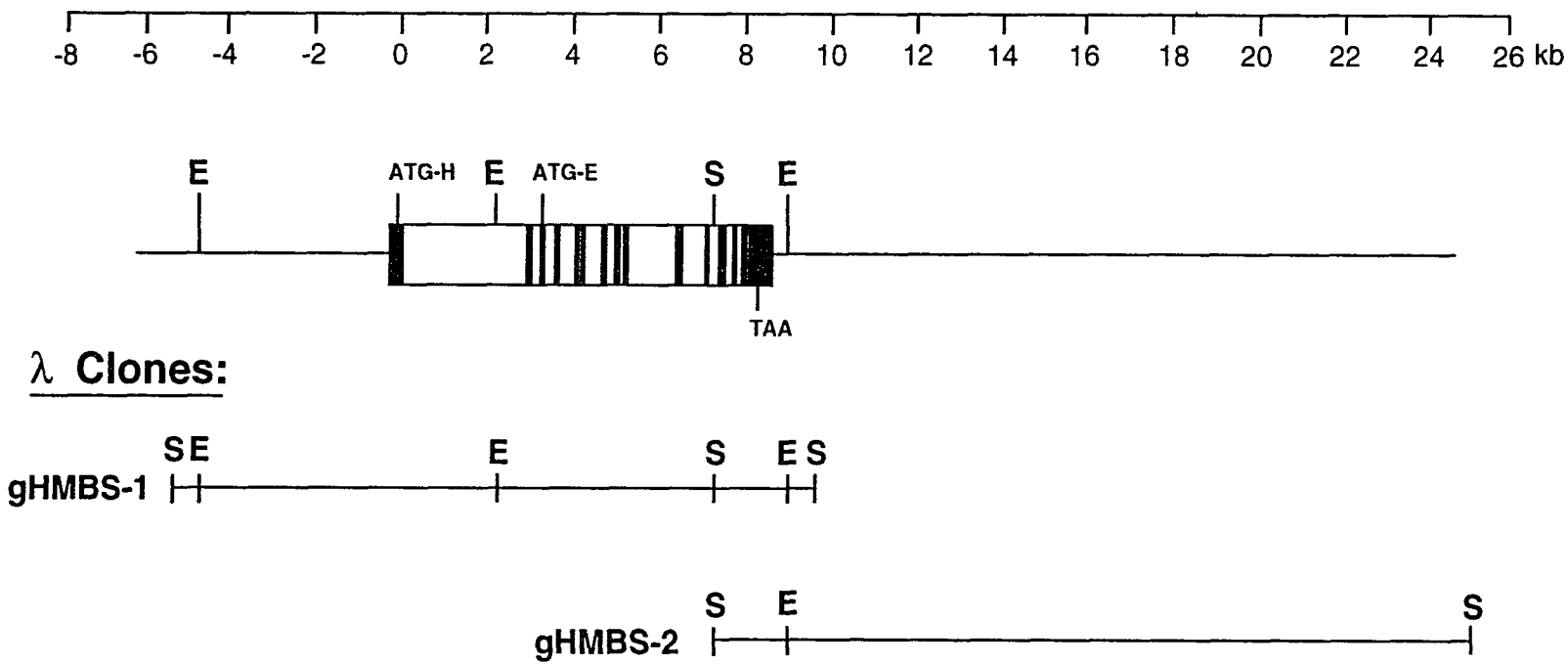


Figure 2-1.. Parial restriction maps of the genomic clones for human HMBS.

as described (35) using the GeneAmp DNA Amplification Reagent Kit (Perkin Elmer/Cetus, Norwalk, CT). The standard PCR mixture contained 0.5 μg of genomic DNA, 1 μM each of the sense and antisense primers, 50 mM KCl, 10 mM Tris-HCl, pH 8.8, 1.5 mM $MgCl_2$, 0.1% Triton-X100, 0.2 mM dNTPs and 2.5 units of *Taq* DNA polymerase. After an initial denaturation at 94 °C for 5 min, amplification of either the 1157 bp genomic region containing the *Msp*I and *Pst*I sites or the 891 bp genomic region containing the *Bst*NI and *Apa*LI sites was performed for 30 cycles consisting of denaturation at 94 °C for 1 min, annealing at 58 °C for 2 min, and extension at 72 °C for 1 min. The 437 bp PCR product containing the exon 10 G/T site was amplified for 30 cycles consisting of denaturation at 94 °C for 1 min, annealing at 52 °C for 1.5 min and extension at 72 °C for 1.5 min. Aliquots (10-20 μl) of the 1157 bp *Msp*I/*Pst*I and the 891 bp *Bst*NI/*Apa*LI amplification products were digested with the appropriate restriction endonuclease for 2 h and the lengths of the digestion products of each reaction were determined by electrophoresis in an 1.8% agarose gel using *Hae*III-digested ΦX174 DNA as length standards after staining with ethidium bromide (0.5 μg/ml). The electrophoretic gel was photographed under longwave ultraviolet light with a Polaroid MP6 apparatus and Polaroid Type 57 film. The exon 10 G/T polymorphism was detected in the 437 bp amplification product by hybridization with allele-specific oligonucleotides (ASOs) using a dot-blot apparatus and Zeta-Probe filters (Bio-Rad Labs, Richmond, CA) as previously described (36). Hybridization of amplified DNA with the 5' end-labeled normal (5'-CAACCGGGTTGGGCAGGTA-3') or mutation-specific (5'-CAACCGGGTGGGCAGGTA-3') oligonucleotide was performed at 53 °C overnight

in 0.15% sodium dodecylsulfate, 6X SSPE (1X SSPE is 0.15 M NaCl, 10 mM NaH_2PO_4, 1 mM EDTA, pH 7.4), and 10X Denhardt's solution (1X Denhardt's is 0.1% Ficoll, 0.1% polyvinylpyrrolidene, 0.1% bovine serum albumin). The filters were washed twice at 53 °C for 30 min in 6X SSC (1X SSC is 0.15 M NaCl, 0.015 M sodium citrate, pH 7.0). The hybridization signals were detected by autoradiography using Kodak XAR-5 film with an intensifying screen for about 3 h.

SSCP Detection and Amplification of New Intragenic Polymorphisms by SSCP

In the course of analyses for HMBS mutations in genomic DNAs from unrelated AIP heterozygotes (C-H. Chen et al., unpublished results), additional intragenic RFLPs were detected by SSCP using the method of Orita et al. (37) as modified by Michaud et al. (38). All exons and adjacent intronic regions were amplified. Each 20 μl amplification mixture contained 100 ng of genomic DNA, 10 pmol of each primer, 31.25 μM dNTPs, 5 μCi [α-^{35}S]dATP, 10 mM Tris-HCl, pH 8.8, 50 mM KCl, 1.5 mM $MgCl_2$, 0.1% Triton X-100 and 2.5 units of *Taq* polymerase (Perkin Elmer/Cetus). Amplification (30 cycles) was performed with denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 1 min. The amplification products were diluted 1:4.5 in 0.1% sodium dodecylsulfate, 10 mM EDTA, and then mixed with 4.5 μl of formamide loading dye. Samples (3 μl) 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 then subjected to direct nucleotide sequencing (39) using the dideoxy chain termination method (34).

The *Bsm*AI, *Hinf*I and *Mnl*I polymorphic sites were analyzed by PCR amplification of genomic DNA using the respective primer sets in Table 2-1. The standard reaction mixtures in a total of 100 μl were the same as described above for amplification of the intron 1 PCR products. For amplification, an initial denaturation step was carried out at 94 °C for 5 min, then 30 amplification cycles were performed with denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min and extension at 72 °C for 1 min. Aliquots (10 μl) were digested with the appropriate enzyme for 2 h and the lengths of the *Bsm*AI and *Hinf*I products were determined by electrophoresis in 2% agarose gels, whereas the *Mnl*I products were electrophoresed in 8% polyacrylamide gels. The products were visualized after staining with ethidium bromide (0.5 μl/mg).

Polymorphic Allele Frequency and Segregation of Haplotypes

Heparinized blood was obtained by venipuncture from over 100 normal individuals and 10 AIP heterozygotes with informed consent. DNA was extracted by the rapid procedure (40) and used directly for the amplification assays described above. The allele frequencies for each polymorphism were determined. Information from individuals whose allelic haplotypes could be unambiguously determined were used for haplotype analyses. Polymorphic sites were analyzed two at a time to determine if each intragenic polymorphism segregated independently or if they were in linkage disequilibrium. The Chi-square test with three degrees of freedom was used to determine the probability that the two alleles segregated independently.

Results and Discussion

Organization of the Human HMBS Gene

The entire 10,024-nt HMB-synthase genomic sequence including 1100 bp upstream from housekeeping gene initiation of translation site and 686 nt of 3' flanking sequence was determined and has been deposited in GeneBank (Accession No. M95623). The genomic sequence was divided into 15 exons ranging from 39 to 438 bp and 14 introns ranging from 87 to 2913 bp. The translation initiation codons for the housekeeping and erythroid-specific sequences (designated ATG-H and ATG-E, respectively) were located at nt 1-3 in exon 1 (Figure 2-2A) and at nt 3233-3235 in exon 3, respectively. The coding sequences for housekeeping and erythroid-specific transcripts were identical to those previousely reported (4, 5), except for the occurrence of two base substitutions in exon 10 at nucleotide 6437 (G) and nucleotide 6479 (G/T). The 6437 G, which created a BstNI site, was demonstrated in over 100 alleles from unrelated normal individuals (data not shown), whereas the 6479 G/T was a polymorphic site in exon 10 (see below). As shown in Table 2-2, all intron/exon boundaries conformed to the GT/AG rule (41), and were consistent with the 5' and 3' consensus sequences for splice junctions of transcribed genes (42). Putative lariat sequences were identified between -39 and -20 nt from the 3' splice junction for all 14 introns by homology to the consensus sequence (C/T)N(C/T)T(A/G)A(C/T) (43). All three codon phases were observed at the exon junctions of the gene (Table 2-2).

Analysis of the HMBS Housekeeping and Erythroid-Specific Promoter Elements

The determination of 945 bp upstream from the housekeeping initiation of

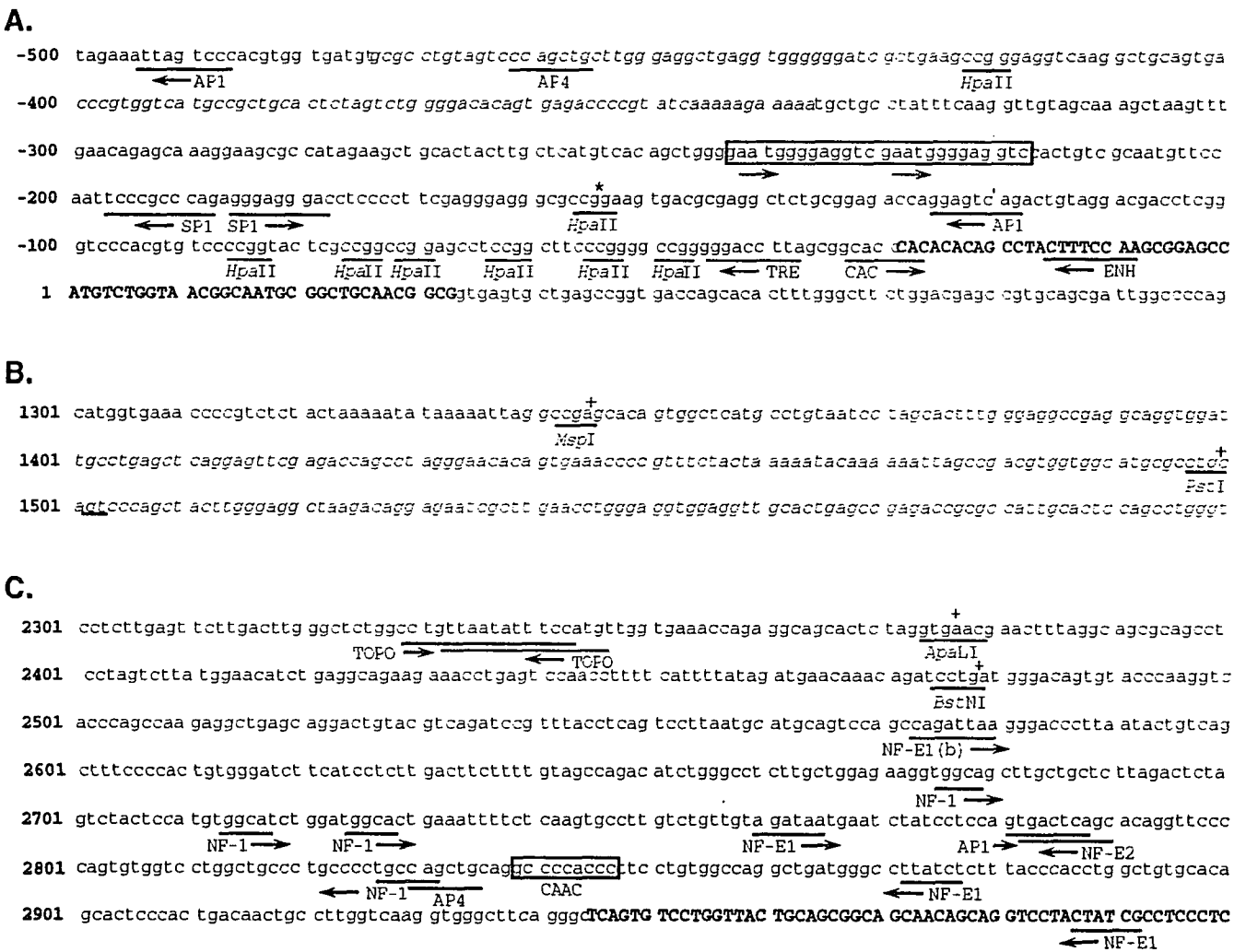


Figure 2-2. Partial sequences of human HMBS gene.

Table 2-1. Nucleotide Sequence of the Intron/Exon Boundaries in the Human PBGD Gene

Exon number and size (nt)		CDNA position Housekeeping	(Erythroid)	5' Splice donor	Intron number and size (nt)		3' Splice acceptor	Codon phase
							5' ttagcggcaccca CACACAGC	
1	(62)	-29 - 33		CAACGGCG gtgagt	1	(2913)	agcactcccactgacaactgccttggtcaaggtgggcttcagggc TCAGTGTC	0
2	(94)		(-112 - -19)	GGCAGAAG gtactg	2	(178)	acactagaaacagaggggactgtgacctggggactttttctgcag GAAGAAAA	
3	(54)	34 - 87	(-18 - 36)	AGAGCCAG gtgggt	3	(328)	gccgtggctgggaagggcaggactaatccaaatctctacccgcag CTTGCTCG	0
4	(73)	88 - 160	(37 - 109)	TGAAATCA gtgagt	4	(376)	atgctgatcaataatgagcacctgattgattgactctctcctcag TTGCTATG	I
5	(50)	161 - 210	(110 - 159)	TCTCTAAG gtaaca	5	(87)	tttccttccctgaagggattcactcaggctctttctgtccggcag ATTGGAGA	0
6	(56)	211 - 266	(160 - 215)	AAGAATGA gtaagt	6	(412)	gctgctcataccctttctctttgccccctctcccatctctatag AGTGGACC	II
7	(78)	267 - 344	(216 - 293)	ATCTGCAA gtaaga	7	(231)	acttaaatctcttccctcattctgtgcccttccctcctcccccag GCGGGAAA	II
8	(78)	345 - 422	(294 - 371)	GAGAAGAG gtaagt	8	(124)	tgactgcctagtgttaaaatctcattgtaacttctctctgggcag TGTGGTGG	II
9	(76)	423 - 498	(372 - 447)	GGAGTATT gtatcc	9	(1148)	ggccccagattgcccgacactgtggtccttagcaactctccacag CGGGGAAA	0
10	(114)	499 - 612	(448 - 561)	TGGGGCAG gtaggg	10	(599)	acacccccgtgtccacccttttgactccctgttccgcctccacag ATCCTGCA	0
11	(39)	613 - 651	(562 - 600)	TGGGCCAG gtacac	11	(242)	ttggggaaagatcaggcctgatgtcctaggatgtttttccatcag GGGGCCTT	0
12	(120)	652 - 771	(601 - 720)	GGCACCTG gtaggg	12	(236)	gtggtgttaagagcccttgcagctcacaagaacttcttgttacag GAAGGAGG	0
13	(54)	772 - 825	(721 - 774)	ATGGGCAA gtaagt	13	(125)	tagtcccctctcagactgtgctgaggcaactgttttcttccccag CTGTACCT	0
14	(87)	826 - 912	(775 - 861)	CTGCCCAG gtacca	14	(90)	ataatctcttgttctcaccaaatcccacctccttccctcatacag CATGAAGA	0
15	(438)	913 -1350	(862 -1299)	CTGAAGGTgttgtg-3'				
		Consensus sequences		donor: $^{A}_{C}$AG gt$^{a}_{g}$agt			lariat: C$^{C}_{t}$n$^{C}_{t}$t$^{a}_{g}$a$^{C}_{t}$ acceptor: $^{cccccccccc}_{ttttttttt}$ncag	

transcription site and of the entire 2713-bp of intron 1 permitted the identification of additional putative regulatory elements, since the previously reported elements in the housekeeping and erythroid-specific promoter regions were based on the analysis of only 305 bp of the 5' flanking region and only 310 bp upstream from exon 2, the erythroid cap site (6). Computer-assisted analysis of 1100 bp upstream from the initiation of translation site for the housekeeping gene identified the presence of 10 *Hpa*II sites (overall GC content of 54%), two AP1 binding sites (both in the antisense orientation at nt -491 to -487 and nt -126 to -119), an AP4 binding site (sense nt -461 to -456), a thyroid responsive element (TRE) binding site (antisense nt -45 to 38), and two core enhancer element (ENH) binding sites (both antisense at nt -828 to -821 and nt -16 to -9), in addition to the reported two SP1 elements (nt -201 to -191 and -190 to -181) and the adjacent 13 base repeats (-243 to -217) (Fig. 2A). Although CAAT- and TATA-like promoter elements were not present, a CAC box was identified at nt -33 to -29 in the sense orientation.

The erythroid-specific promoter had the previously reported AP1 binding site (sense orientation at nt 2781 to 2788), a CAAC motif (sense at nt 2839 to 2847), a CAAT-like box (sense at nt 2855 to 2863), a TATA-like element (sense at nt 2913 to 2918), two NF-E1 binding sites (sense at nt 2760-2765 and antisense at nt 2782-2790), and one NF-E2 binding site (antisense at nt 2782-2790) (6, 8, 44, 45). Computer-assisted analysis of the additional 2403 bp of intron 1 reported here revealed other putative regulatory elements including four NF-1 binding sites (three in the sense orientations at nt 2675–2679, 2713–2717, 2724–2728; the other, antisense at nt 2827–2831) two

topoisomerase (TOPO) binding sites (sense at nt 2332–2346 and antisense at nt 2329–2343), one AP4 binding site (sense at nt 2830–2835), and a NF-E1(b) binding site (sense at nt 2573–2580) (Figure 2-2C). The functional significance of the additional putative housekeeping and erythroid regulatory elements requires future evaluation.

Alu Repetitive Elements

Six *Alu* repetitive elements were identified in or adjacent to the HMBS gene. Of interest, the 10 kb gene was relatively *Alu*-rich, as these repetitive elements occur on the average every 4 kb in the genome (46, 47). The gene had two *Alu* elements in the 5' flanking region, three in intron 1, and one in intron 9. All except the intron 9 repeat element were in the forward orientation. The *Alu*-2 and *Alu*-4 elements contained only the right half of the repeat element corresponding to residues 152 to 293 and 125 to 298 of the consensus sequence, respectively. When categorized into subfamilies based on the classification of Jurka and Smith (48), *Alu*-2 was homologous to the older J subfamily, while the other five *Alu* elements were assigned to a branch of the more modern Sa subfamily. When each of thease repeat elements was aligned with the *Alu* consensus sequence (48), the percentage identity ranged from 76.2 to 90.6%. Of the 24 CpG dinucleotides in the consensus sequence, the number in each of the six HMB-synthase *Alu* elements that presumably had undergone spontaneous deamination of 5-methylcytosine to thymidine ranged from 10 to 16, with Alu elements 4, 5, and 6 each having 66.7% mutated CpGs.

Intragenic Polymorphic Sites and Improved Amplification Assays

Of the six known polymorphic sites in the HMBS gene (26, 27, 29, 30, 39, 49),

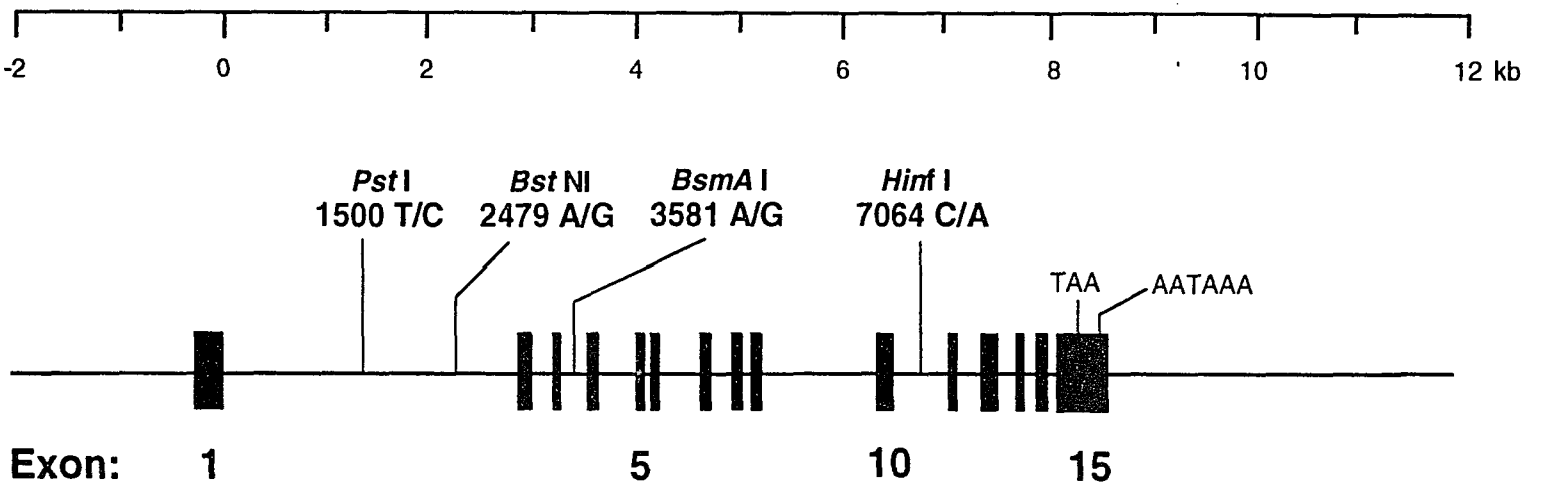
the genomic sequence permitted the precise location of the *Msp*I, *Pst*I, *Bst*NI and *Apa*LI sites in intron 1 (Figure 2-2B and Figure 2-3). The allelic nucleotides for each of these RFLPs was determined by sequencing amplified PCR products from individuals who were homozygous for the respective allele. The variant nucleotides and their genomic sequences were *Msp*I, nt 1345 G/A; *Pst*I nt 1500 C/T; *Apa*LI nt 2377 C/A; and *Bst*NI nt 2479 G/A. In addition, the finding of a base difference at genomic position 6479 with the reported cDNA sequence (5) led to the identification of a polymorphic site in exon 10. While these studies were in process, Lee (36) also detected the same allelic sequences for the *Apa*LI and *Bst*NI polymorphisms in individuals of Swedish extraction and Gu et al. (30) reported the exon 10 6479 G/T polymorphism. Improved conditions for the PCR-based amplification and analysis of these polymorphic sites were established. As shown in Figure 2-4, the *Msp*I and *Pst*I sites were conveniently amplified from genomic DNA in a single 1.1 kb product which could be directly digested with each enzyme and visualized by agarose gel electrophoresis and ethidium bromide staining. Similarly, an 891 bp intron 1 product containing both the *Apa*LI and *Bst*NI sites was readily amplified for direct digestion with the respective endonuclease (Figure 2-4). This method was a significant advance over the previous multistep procedure to PCR amplify the intron 1 polymorphic sites. For these polymorphic sites involved the sometimes difficult amplification of a 3.3 kb product, gel purification of the PCR product, followed by sequential digestions with *Eco*RI, then *Msp*I, *Pst*I or *Bst*NI for resolution of the polymorphism after a second agagrose gel electrophoresis (49, 50). While these studies were in progress, Lee et al. (36) also reported a procedure using a different set of PCR

primers to amplify a 0.9 kb product that improved detection of the *Apa*LI site. Although their PCR product also contained the polymorphic *Bst*NI site, the PCR product also had other *Bst*NI sites which hindered analysis of this polymorphism since the size of a constant band (247 bp) was similar to the 241 bp fragment from the *Bst*NI+ allele.

In the course of mutation detection studies by SSCP analysis of amplified regions of the gene from unrelated AIP patients and normal individuals, three different mobility shifts were identified (Figure 2-5). Sequencing of the PCR products from these regions revealed that these mobility shifts were due to polymorphic sites in intron 3 at nt 3581 (A or G), intron 10 at nt 7064 (C or A), and intron 14 at nt 7998 (G or A). The polymorphic nucleotides either created (nt 3581 G, *Bsm*AI) or deleted (nt 7064A, *Hinf*I; nt 7998A, *Mnl*I) a restriction site (Figure 2-3), and PCR-based amplification assays were developed for each as illustrated in Figure 2-6.

Polymorphic Allele Frequencies and Haplotypes

The allele nces for the five known polymorphisms (*Msp*I, *Pst*I, *Apa*LI, *Bst*NI and the 6479 G/T) and the three new polymorphisms (*Bsm*AI, *Hinf*I and *Mnl*I) were determined in over 90 alleles from a normal American Caucasian population using the newly developed PCR-based assays (Table 2-3). Each polymorphic site had two alleles, and the frequencies of their common alleles ranged from 0.58 to 0.75, with the exception of the intron 14 *Mnl*I- (7998A) allele which was 97%. For each polymorphism the distribution of homozygotes and heterozygotes was in Hardy-Weinberg equilibrium. Since the adjacent intron 1 RFLPs had been shown to be in strong linkage disequilibrium in Swedish (39, 50), Finnish (51) and Welsh (31) populations, analyses were performed



	χ^2	N	p
979	13.7	66	<0.01
1102	28.3	54	<0.001
2081	16.2	68	<0.01
4102	4.7	74	NS
4585	3.0	70	NS
5564	2.6	82	NS

Figure 2-3. Segregaation analysis of HMBS intronic polymorphisms

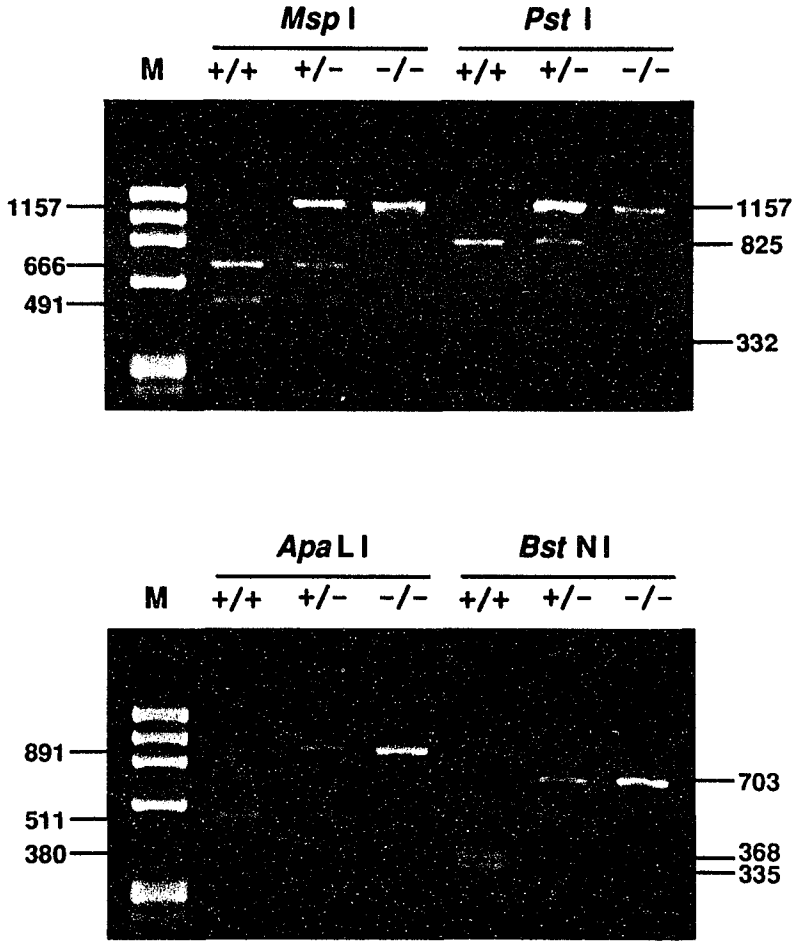


Figure 2-4. Improved PCR assay of Intron 1 polymorphisms

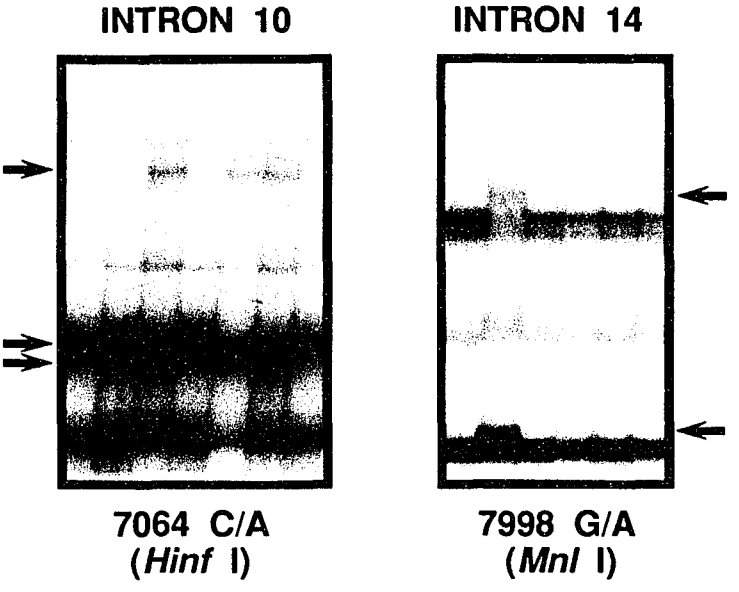


Figure 2-5. SSCP identification of HMBS intronic polymorphisms

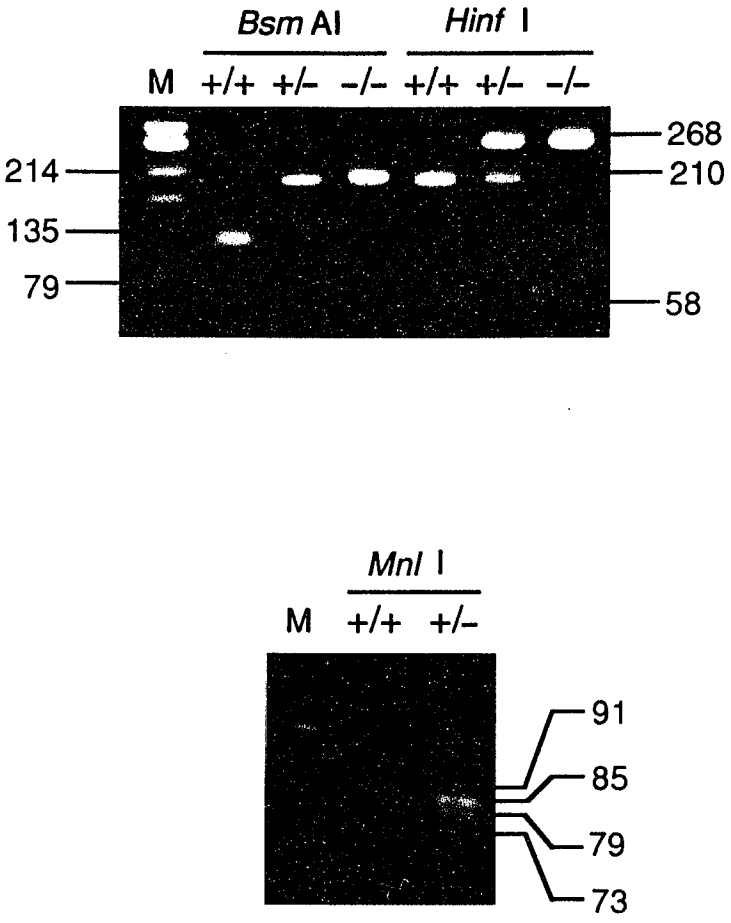


Figure 2-6. Amplification assay for HMBS BsmAI, HinfI and MnlI intronic polymorphisms

TABLE 2-2

Allele Frequencies of HMB-Synthase Polymorphisms in Normal American Caucasians

Genomic Position	Location	Restriction Site		N	Allele Frequency
1345 G	Intron 1	*Msp*I	+	104	0.58
A			−		0.42
1500 T	Intron 1	*Pst* I	−	106	0.62
C			+		0.38
2377 C	Intron 1	*Apa*LI	+	100	0.54
A			−		0.46
2479 A	Intron 1	*Bst*NI	−	104	0.62
G			+		0.38
3581 A	Intron 3	*Bsm*AI	−	96	0.59
G			+		0.41
6479 G	Exon 10			100	0.69
T					0.31
7064 C	Intron 10	*Hinf* I	+	92	0.75
A			−		0.25
7998 G	Intron 14	*Mnl* I	+	92	0.99
A			−		0.01

to determine if the intron 3 *Bsm*AI and intron 10 *Hinf*I RFLPs also were in linkage disequilibrium with the intron 1 polymorphisms. The *Pst*I/*Bst*NI, *Pst*I/*Bsm*AI, *Bst*NI/*Bsm*AI, *Pst*I/*Hinf*I, *Bst*NI/*Hinf*I, *Bsm*AI/*Hinf*I haplotypes were assigned for subjects who were not heterozygous at both loci. Figure 3B shows the nucleotide distance between the polymorphic sites, the number of informative haplotypes analyzed, and the calculated Chi-square and probability values for the independent segregation of each haplotype. Consistent with the European studies, the two intron 1 RFLPs (*Pst*I and *Bst*NI) were in marked linkage disequilibrium, (χ^2 = 13.7; p,0.004); however, all four haplotypes were observed, in contrast to the finding of only two in the Welsh population (31). Interestingly, the newly identified *Bsm*AI polymorphism was in strong linkage disequilibrium with both intron 1 RFLPs, PstI (χ^2 = 28.3, p<0.001); *Bst*NI (χ^2 = 16.2; p,0.002). Thus, the determination of an informative intron 1 or 3 RFLP would be equivalent to and preclude the determination of the other RFLPs in this region.

In contrast, the exon 10 *Hinf*I polymorphism was not in linkage disequilibrium with the intron 1 and 3 RFLPs which were over 4 kb upstream. The χ^2 values for the *Hinf*I haplotypes with *Pst*I, *Bst*NI or *Bsm*AI were not significant, indicating that the *Hinf*I site segregated independently from the intron 1 and 3 polymorphisms. Thus, the common *Hinf*I RFLP should prove diagnostically valuable in AIP families whose specific HMBS mutations have not been determined.

In summary, the determination of the complete genomic sequence for HMBS, including the identification of additional housekeeping and erythroid-specific promoter elements, should facilitate further studies of the regulation of this novel gene. In addition,

the development of improved and new amplification assays for the intragenic polymorphic sites, and especially the identification of the independently segregating *Hinf*I RFLP, should prove valuable for the diagnosis of presymptomatic AIP heterozygotes in families whose specific HMBS mutations have not been identified.

Chapter 3. Acute Intermittent Porphyria: Identification and Expression of Exonic Mutations in the Hydroxymethylbilane Synthase Gene.

Summary

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; HMB-synthase). 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 HMB-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), which precluded translation of the housekeeping, but not the erythroid-specific isozyme; four missense mutations, V93F, R116W, R201W, C247F; and a nonsense mutation W283X which truncated the housekeeping and erythroid-specific isozymes. Each mutation was confirmed in genomic DNA from family members. Only the W283X lesion was found in another unrelated AIP family. Expression of each mutation in *E. 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. Of interest, aberrant HMB-synthase transcripts due to abnormal splicing occurred frequently, in part accounting for low enzymatic activity in normal individuals and the provoked expression of disease in heterozygotes with half normal activity. These mutations provide insight into the molecular pathology of classic and variant AIP and facilitate molecular diagnosis

in AIP families.

Introduction

Acute intermittent porphyria (AIP), an autosomal dominant inborn error of metabolism, results from the half-normal activity of hydroxymethylbilane synthase (EC 4.3.1.8; HMBS), 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 δ-aminolevulinic acid synthase activity, the first and rate-limiting enzyme of heme biosynthesis, thereby increasing the production of the porphyrin precursors, δ-aminolevulinic acid (ALA) and PBG (5-7). The half-normal hepatic HMBS activity in AIP patients is insufficient to prevent the pathological accumulation of these porphyrin precursors, which precipitate 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.

HMBS 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). The gene contains 15 exons and two distinct promoters that generate housekeeping and erythroid-specific transcripts by

alternatively splicing (11, 12). The housekeeping promoter is in the 5' flanking region and its transcript contains exons 1 and 3 through 15. The erythroid-specific promoter is in intron 1 and its transcript is encoded by exons 2 through 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 β-globin gene promoter (11, 13, 14). cDNAs encoding the 42-kD housekeeping and 40-kD erythroid-specific isozymes, which differ only in their N-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-specific enzymes both have half-normal activities, whereas in variant AIP (representing about 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 HMBS gene.

To date, 24 different mutations in the HMBS gene have been detected. Most of these mutations are either private or found only in a few unrelated families, emphasizing the molecular heterogeneity of the mutations causing this disease (29-40). 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 restriction fragment length polymorphisms (RFLPs) have been identified which have proven useful for heterozygote diagnosis in informative families whose mutations have not been determined (41-47). However, there is marked linkage disequilibrium for the intron 1 and intron 3 RFLPs in northern European and American populations (48-50), with the exception of a recently identified common intron 10 *Hinf*I site (10).

The availability of the entire HMBS genomic sequence (10) has facilitated mutation identification by the single-strand conformational polymorphism 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. In

addition, abnormal housekeeping transcripts were detected by reverse transcription of total lymphocyte RNA indicating that the normally low level of enzymatic activity is in part due to aberrant splicing, thereby making this enzyme more rate-limiting in the pathway and more vulnerable to disease expression.

Materials and Methods

Patient Specimens, Enzyme Assays and Molecular Screening. Peripheral blood samples were collected from 10 unrelated AIP patients and their family members with informed consent. Lymphoid cell lines were established using cyclosporin A and Epstein-Barr virus as previously described (51). 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, Grand Island, NY). From each proband erythrocyte lysates were assayed for HMBS activity (21) and genomic DNA was extracted from peripheral blood (52, 53) for detection of known HMBS mutations ($IVSI^{-1}$, $IVSI^{+1}$, Q155X, R167Q, R167W, R173Q, W198X and $IVS12^{-1}$) by analysis of polymerase chain reaction (PCR)-amplified genomic DNA by dot-blot hybridization with allele specific oligonucleotides or digestion with specific endonucleases as previously described (29-35).

cDNA amplification and sequencing of mutant HMBS alleles. Total RNA was isolated from cultured lymphoblasts using standard techniques (54). 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. (55) 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 min, and extension

at 72 °C for 2 min. The sense primer (5'-GCGGCGGAATTCCATGGCTGGTAACGGCAATGC-3') corresponded to nt -2 to 20 of the HMBS housekeeping cDNA (12) with a T to G substitution at nt 4 (underlined) to introduce an *Nco*I site and an additional 12 nt. The antisense primer (5'-GCCGCCGCCAAGCTTCTGTGCCCCACAAACCA-3') corresponded to nt 1088 to 1105 with an additional 12 nt including a *Hind*III site (underlined). These primers were synthesized on an Applied Biosystems Model 380B Synthesizer (Foster City, CA). After amplification, the 1132 bp PCR product, which included the entire coding sequence, was extracted with phenol/chloroform, precipitated with isopropanol, and digested with *Nco*I and *Hind*III. The digested PCR products were purified by electrophoresis in an 1% agarose gel and then extracted by DEAE-impregnated filters as described (55). The purified digested amplification products were ligated into the corresponding cloning sites of the pKK233-2 expression vector (Pharmacia Fine Chemical, Uppsala, Sweden) and the vector construct (designated pKK-HMBS) was transfected into *E. coli* DH5α (56). Single colonies were selected and subcultured in Luria broth. An aliquot (3 ml) of an overnight culture was centrifuged in a microfuge for 3 min, the pellet was washed with Tris-HCl buffer (0.1 M Tris, pH 8.2 and 0.1 mm DTT), and then resuspended in 0.5 ml of the Tris-HCl buffer. The cell suspensions were lysed by sonication for 45 sec, and centrifuged in a microfuge at 4 °C for 15 min. An aliquot (100 μl) of the supernatant was incubated with 200 μl of 0.5 mm porphobilinogen and 450 μl of Tris-HCl buffer at 37 °C for 30 min. The reaction was stopped by adding 250 μl of 50% trichloracetic acid, allowed to stand for 20 min at 23 °C, and then the product was visualized after oxidation by long

wave ultraviolet light. Subclones whose expressed products did not produce fluorescence (or had reduced fluorescence) were sequenced by the dideoxy chain termination method (57) using Sequenase according to manufacturer's instructions (United States Biochemical Corp., Cleveland, OH). Computer-assisted sequence analyses were performed with Microgenie software (Beckman Instruments, Inc., Palo Alto, CA).

Detection of HMB-synthase mutations by SSCP. To detect mutations in HMBS, the single-strand conformation polymorphism technique (SSCP) was used as described (58) and modified (59). The twelve primer sets used for amplification of each exon and their flanking intronic sequences are indicated in Table 3-1, note that exons 2 and 3, 5 and 6, and 14 and 15 were amplified as single PCR products which included the respective intervening introns. Each 20 μl reaction mixture contained 100 ng of genomic DNA; 10 pmol of each primer; 31.25 mM of each dNTP; 5μCi[α-^{35}S] dATP; 10 mM Tris-HCl, pH 8.8; 50 mM KCl; 1.5 mM $MgCl_2$; 0.1% Triton X-100 and 2.5 units 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 sec. To amplify exon 1, the annealing temperature was raised to 66 °C. Aliquots (1 μl) of the amplified DNAs were diluted with 4.5 μl of 0.1% SDS containing 10 mM EDTA and 4.5 μl of 95% formamide loading dye. Samples (10 μl) were denatured at 100 °C for 5 min, and then snap-frozen in ice water. After centrifuging for 30 sec at 4 °C, Aliquots (3 μl) were electrophoresed in 6% polyacrylamide gels containing 10% glycerol at 5 to 30 W and 4 °C. The gels were dried and exposed to Kodak XAR-5 film for 1

to 2 days. Amplified fragments showing mobility shifts were subjected to solid phase direct sequencing as described below.

Detection of HMBS 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 flanking intronic regions were synthesized using biotinylated phosphoramidites in an Applied Biosystems Model 380B Synthesizer (Table I). Following amplification of genomic DNA as described above, an aliquot (40 μl) of the amplified product was mixed with 25 μl of prewashed streptavidin beads (Dynal Inc., Great Neck, NY) and the mixture was incubated for 30 min at 23 °C, and then centrifuged for 30 sec in a microfuge. The supernatant was removed and the pelleted beads were washed with 40 μl of 10 mM Tris-HCl buffer, pH 7.5, containing 1 mM EDTA and 2.0 M NaCl, and then were incubated for 10 min with 40 μl of 0.1M NaOH for strand separation. Following centrifugation, the pelleted beads were successively washed with 50 μl 0.1M NaOH, 50 μl of the above Tris-HCl buffer and then with 50 μl of TE buffer (10 mM Tris-HCl, pH 7.4 containing 1 mM EDTA). The pelleted beads were resuspended in 7 μl of H_2O and sequenced by the dideoxy chain termination method using Sequenase 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) 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

Table3- 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‡	Oligonucleotide primers	
		Sense	Antisense
1A	-130-120	5'-gagaccaggagtcagactgt-3'	5'-agacgactgaggatggcaac-3'
1B	-131-120	5'-gagaccaggagtcagactgt-3'	5'-agacgactgaggatggcaac-3'
2-3A	2911-3309	5'-GCCGCC(GAATTC)tgacaactgccttggtc-3'	5'-GCCGCC(GAATTC)ctgttctgacaaacctcct-3'
2-3B	2911-3360	5'-cccacccttcctgtggccag-3'	5'-GCCGCC(GAATTC)ctgttctgacaaacctcct-3'
4A	3520-3710	5'-GCCGCC(GAATTC)gcctaacctgtgacagtct-3'	5'-GCCGCC(GAATTC)actgggcttcccattagct-3'
4B	3520-3770	5'-GCCGCC(GAATTC)gcctaacctgtgacagtct-3'	5'-agcgggacgggctttagcta-3'
5-6A	4012-4298	5'-GCCGCC(GAATTC)gagcacctgattgattgac-3'	5'-GCCGCC(GAATTC)agacctagcatactagg-3'
5-6B	3951-4298	5'-gcctctgtccccatcatgaa-3'	5'-GCCGCC(GAATTC)agacctagcatactagg-3'
7A	4546-4796	5'-GCCGCC(GAATTC)ggctccaccactgaagtag-3'	5'-GCCGCC(GAATTC)tcaggccccaaagggaaag-3'
7B	4546-4796	5'-ggctccaccactgaagt-3'	5'-GCCGCC(GAATTC)tcaggccccaaagggaaag-3'
8A	4881-5119	5'-GCCGCC(GAATTC)cgagagagaatagaggtga-3'	5'-GCCGCC(GAATTC)acactaggcaggtcactgtt-3'
8B	4881-5119	5'-GCCGCC(GAATTC)cgagagagaatagaggtga-3'	5'-aacactaggcagtcactgt-3'
9A	5099-5293	5'-GCCGCC(GAATTC)aacagtgactgcctagtgt-3'	5'-GCCGCC(GAATTC)tgtctttttccttggctgc-3'
9B	5061-5293	5'-tgtgcccagaagatgcaggg-3'	5'-GCCGCC(GAATTC)tgtctttttccttggctgc-3'
10A	6295-6539	5'-GCCGCC(GAATTC)aaagacagactcaggcaga	5'-GCCGCC(GAATTC)taaggcagaaaggagatgc-3'
10B	6295-6590	5'-GCCGCC(GAATTC)aaagacagactcaggcaga	5'-atcgctttcacacaatcata-3'
11A	7014-7258	5'-GCCGCC(GAATTC)catctcactgccaggtgct-3'	5'-GCCGCC(GAATTC)cggtagcatcccaaggtct-3
11B	7014-7258	5'-catctcactgccaggtgc-3'	5'-GCCGCC(GAATTC)cggtagcatcccaaggtct-3
12A	7333-7554	5'-GCCGCC(GAATTC)cctgatgtcctaggatgtt-3'	5'-GCCGCC(GAATTC)gtaagaaatcttccctgcc-3'
12B	7281-7554	5'-agtagatagaggtggtccca-3'	5'-GCCGCC(GAATTC)gtaagaaatcttccctgcc-3'
13A	7641-7848	5'-GCCGCC(GAATTC)cagtgatgtcctcacagtctg-3'	5'-GCCGCC(GAATTC)tacctagaaacctgggatgc-3'
13B	7641-7840	5'-GCCGCC(GAATTC)cagtgatgtcctcacagtctg-3'	5'-ctacctagaaacctgggatg-3'
14-15A	7830-8061	5'-GCCGCC(GAATTC)gcatcccaggtttctaggt-3'	5'-GCCGCC(GAATTC)tctgtgccccacaaacca-3'
14-15B	7830-8560	5'-GCCGCC(GAATTC)gcatcccaggtttctaggt-3'	5'-aagggctttgtgtttgttcc-3'

* A=Primer sets for SSCP analysis, B=Primer sets for solid phase direct sequencing, dot indicated biotinylated nucleotide.

‡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).

restriction sites, permitting rapid analysis of the appropriate exon amplified from genomic DNA with the indicated biotinylated primer set as described above (Table 3-I). For restriction analyses, an aliquot (10 μl) 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: M1I: digested with *Nla*III at 37 °C, electrophoresed in 8% polyacrylamide gels; V93F: *Bst*NI at 65 °C, 2% agarose gels; R201W: *Msp*I at 37 °C, in 2% agarose gels; C247F: *Fnu*4HI at 37 °C, 4% agarose gels; and W283X: *Hinf*I at 37 °C, 12% polyacrylamide gels. The gels were stained with ethidium bromide (0.5 μg/ml) and then visualized under a long wave ultraviolet light. The R116W mutation was confirmed by dot-blot hybridization of genomic DNA with ASOs. The amplification product for exon 8 (40 μl) was denatured in 360 μl of 0.4 M NaOH containing 25 mM EDTA for 5 min, and then 180 μl aliquots were transferred to duplicate Zeta Probe membranes (Bio-Rad, Richmond, CA) using a Minifold I dot blot apparatus (Schleicher and Schuell, Keene, NH). For allele-specific hybridization, the normal (5'-GTTTTCCCGCCTGGGGG-3') and mutation-specific (5'-GTTTTCCCACCTGGGGG-3') oligonucleotides were end-labelled with [γ-^{32}P]ATP (56) and the radiolabelled ASOs were added to the hybridization solution at a concentration of 1 x 10^6 cpm/ml. Prehybridizations for 3 h in 6X SSPE (1X SSPE is 0.15 NaCl, 1 mM EDTA), 10X Denhardt's (1X Denhardt's is 0.1% Ficoll/0.1% polyvinylpyrrolidone/0.1% BSA) and overnight hybridization was performed at 56 °C. 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 normal or mutation-specific oligonucleotide probe. Membranes were exposed to Kodak XAR-5 film with an intensifying screen for approximately 6 h.

Prokaryotic expression and characterization of HMB-synthase mutations. The normal and mutant HMB-synthase alleles were expressed in *E. coli* using the pKK233-2 vector (Pharmacia, Piscataway, NJ) as previously described. To introduce each of the mutations into the pKK-HMBS expression construct, the "megaprimer" method for site-directed mutagenesis was used (60). 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'-GCGGCGGAATTCCATGGCTGGTAACGGCAATGC-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 1132 bp fragment in a reaction with the antisense primer MP3 (5'-GCCGCCGCCAAGCTTCTGTGCCCCACAAACCA-3') and pKK-HMBS DNA as template. The second amplification product was digested with *Kpn*I and *Nsi*I, and the *Kpn*I/*Nsi*I 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'-TCCCACTTGCAGATGGCTC-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 a 832 bp product. The 832 bp PCR product was digested with *Nsi*I and *Kpn*I. The *Nsi*I/*Kpn*I 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'-ACAAGCATACATGCATTCCTCAGGGTGCAGGATCTGCCCAACCCAGTTGTGCCA-3', containing an *Nsi*I site were used to amplify a 658 bp product which was digested with *Kpn*I and *Nsi*I and cassette ligated into pKK-HMBS, producing pKK-HMBS-R201W. For the C247F mutation, sense primer MP7 (5'-GTTCAGTGCCATCATCCTG-3') and antisense primer MP8 (5'-TCAGCGATGAAGCGAAGCAG-3') were used to amplify a 205 bp meeaprimer. The purified megaprimer was used as the sense primer to generate a 575 bp PCR product with antisense primer MP4, which then was digested with *Nsi*I and *Xba*I and cassette ligated to corresponding cloning sites of pKK-HMBS, yielding pKK-HMBS-C247F. For the W283X mutation, sense primer MP9 (5'-GGAGGAGTCTGAAGTCTAGACG-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'-TGTGGTGGGAACCAGCT-3'). The 697 bp PCR product was digested with *Nsi*I and *Xba*I 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, IPTG induction and HMBS assays were performed as previously described (61). 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, placed on ice, and the HMBS activity was determined as described.

In vitro coupled transcription/translation of normal and mutant cDNA. The normal housekeeping and erythroid-specific cDNA encoding HMBS were subcloned into the prokaryotic expression vectors pET5a and pET11a (Novagen, Inc., Madison, WI), which were designated pET-HMBSh and pET-HMBSe, respectively. The V93F, R116W, R201W, C247F and W283X mutations were introduced into pET-HMBSh by digesting the respective pKK-HMBS expression construct with *Kpn*I and *Xba*I and cassette ligated the *Kpn*I/*Xba*I fragment into the pET-HMBSh vector. The M1I mutation was introduced into the pET-HMBSh vector by the "megaprimer" site-directed mutagenesis strategy (51). The sense primer MP11 (5'-TTAATACGACTCACTATAGG-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 *Xba*I site between the T7 promoter and the HMBS initiation codon. The megaprimer was purified and used as sense primer, together with antisense primer MP12 (5'-GTCCTTCAAGGAGTAA-3') to amplify a 379 bp product, which was digested with *Xba*I and *Kpn*I and cassette ligated into pET-HMBSh, generating pET-HMBS-M1I. Each pET expression construct was confirmed by

sequencing. The normal and mutant HMBS constructs were transcribed and translated *in vitro* using the TNT™ Coupled Reticulocyte Lysate System according to the manufacturer's instructions (Promega Corp., Madison, WI). 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, 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 HMBS mutations and alternative splicing by cDNA amplification and sequencing. Of the original 10 unrelated AIP heterozygotes screened for eight known mutations, two had R167Q and one had R167W. Initial efforts to identify exonic lesions causing AIP focused on two unrelated patients (Probands 1 and 2) who had about 50% of mean normal erythrocytic HMBS activity, disease manifestations, and positive family histories. Total RNA was isolated from cultured lymphocytes from each proband, reverse-transcribed into cDNA, and then the entire HMBS coding region was PCR-amplified. The amplified products were subcloned into the pKK233-2 expression vector and 20 individual subclones from each proband were expressed in *E. coli* and the lysates assayed for HMBS activity. Subclones which had no or markedly reduced fluorescence (Figure 3- 1) were sequenced. No consistent mutation was detected in the non-fluorescing subclones from either proband. Among these subclones, approximately 45% had different single point mutations, 5% had two base substitutions, and the remaining sequences were alternatively spliced. Twelve different single point mutations which resulted in amino acid substitutions were identified (data not shown). None of these mutations was detected in amplified genomic DNA from the respective probands indicating that these activity-negative mutations were PCR errors. Even more surprising, about half of the subclones had alternatively spliced transcripts. A shown in Figure 3-2, about 75% of the alternatively spliced transcripts resulted from skipping exon 3 or the deletion of 18 bp in exon 5 due to the use of a cryptic 3' acceptor splice site. Alternatively spliced transcripts missing both exon 4, exon 5, or both exons 3 and 5 occurred with similar lower

frequencies. The use of an alternative 5' donor splice site in intron 1 which inserted 91 additional nucleotides into the coding sequence occurred rarely. All of the normal 5' donor and 3' acceptor sequences were identical or similar to the respective consensus sequences. The exon 5 (tcagTTG) (10) normal and cryptic 3' acceptor sequences (acagGGG) did not provide an obvious explanation for the high frequency of alternative splicing at this site. In addition, the sequences of the normal (GCGgtgagt) and cryptic 5' donor (TTGgtcaga) sites of intron 1 were similar to these of the consensus sequence, with the exception of the C in the +3 position of the cryptic site.

Identification of V93F, R116W, R201W and C247F mutations by SSCP. Based on the location of the intron/exon boundaries (10), each of the 15 coding exons from 5 unrelated AIP patients was amplified for SSCP analysis. The SSCP profiles for Proband 1 (V93F), Proband 2 (R116W), Proband 3 (R201W) and Proband 4 (C247F) revealed mobility shifts in exons 7, 8, 10 and 12, respectively (Figure 3-3; 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 (Figure 3-4B,3-4C, 3-4D, and 3-4E). Proband 1 had a G to T transversion of nt 277 in exon 7 which predicted a valine to phenylalanine substitution at residue 93 (designated V93F). Proband 2 had a C to T transition of nt 346 in exon 8 which would cause an arginine to tryptophan substitution in residue 116 (R116W). Proband 3 had a C to T transition at nt 601 in exon 10 predicting an arginine to tryptophan substitution in residue 201 (R201W). Proband 4 had a G to T transversion of nt 740 in exon 12 which would change a cystein to phenylalanine in residue 247 (C247F). SSCP analysis

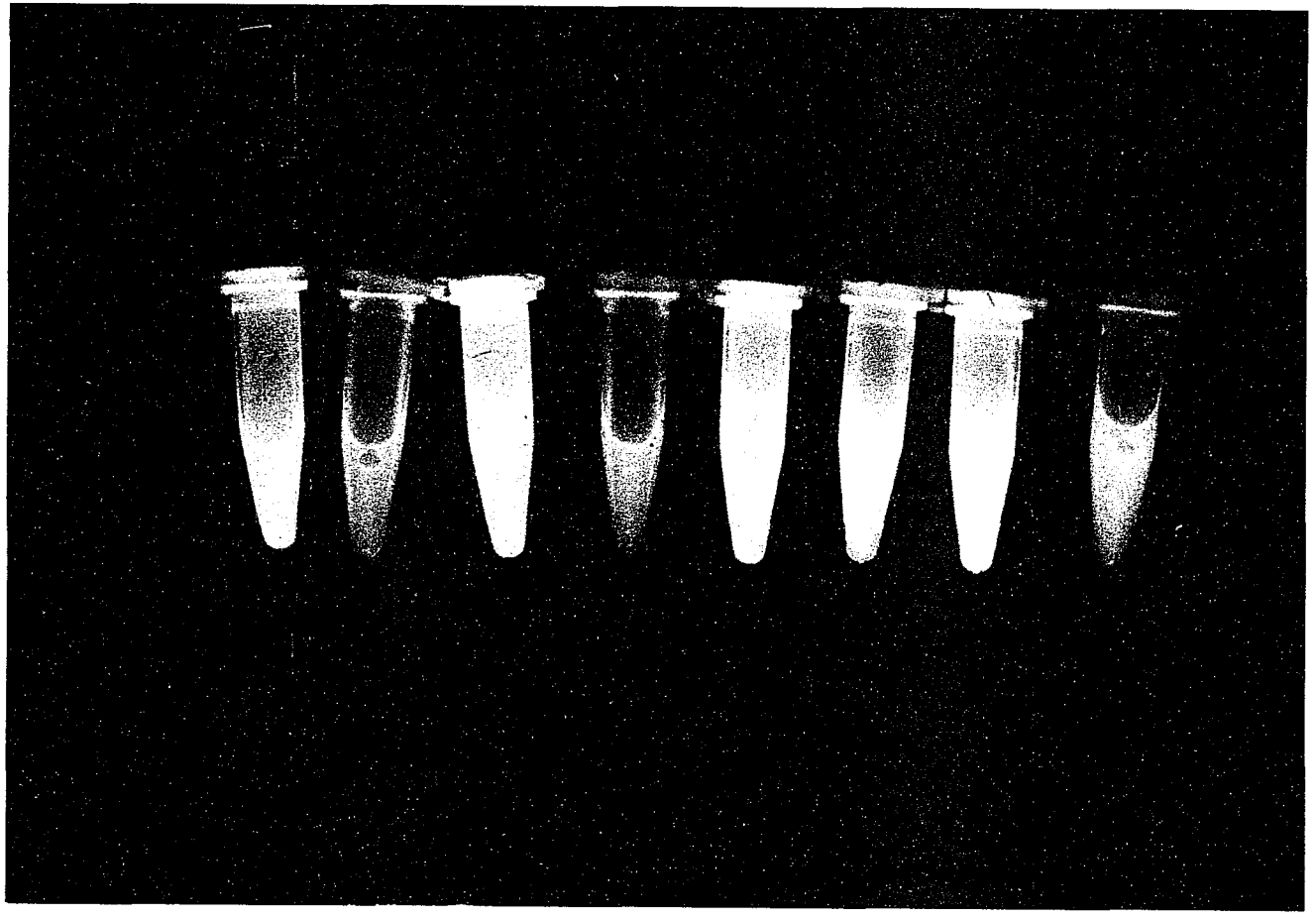


Figure 3-1. HMBS assay for RT-PCR clones with functional and nonfunctional activities.

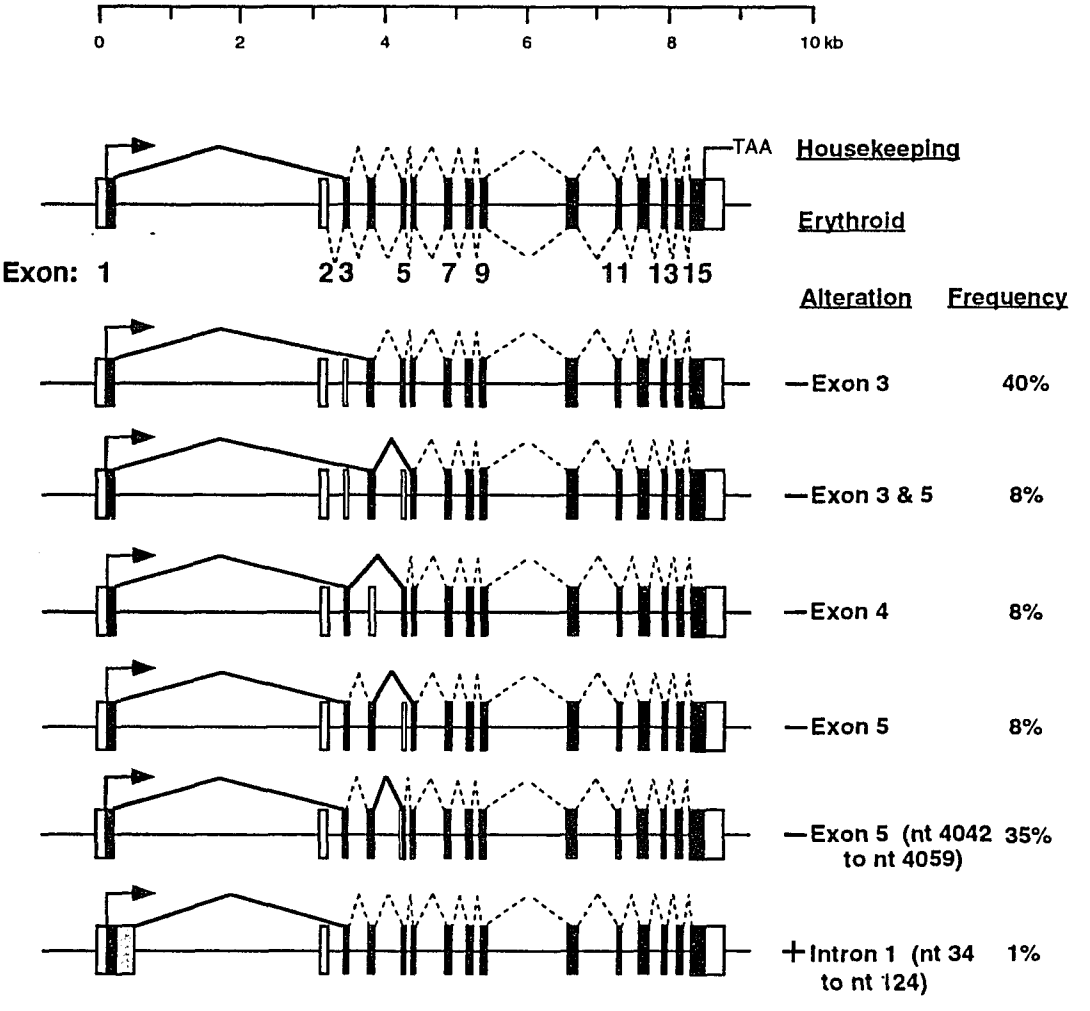


Figure 3-2. Aberrantly splicing of HMBS transcripts.

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 (Hinf1) and 7998G/A (Mn1I).

Identification of M1I and W283X by direct solid-phase sequencing. All 15 HMBS exons and flanking regions from Probands 6 and 7 were amplified with 12 biotinylated primer sets and were sequenced directly using a solid-phase strategy. In Proband 6, a G to A transition of nt 3 in exon 1 predicted a methionine to isoleucine substitution (M1I) in the initiation of translation codon (Figure 3-3A). In Proband 7, a G to A transition of nt 848 in exon 14 predicted a tryptophan to stop substitution (W283X) (Figure 3-4F). 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 Fiure 3-5, the M1I mutation which obliterated the only *Nla*III site in exon 1, was demonstrated in amplified genomic DNA containing exon 1 from Proband 6 and several first degree relatives. The Proband, her mother, brother and daughter each had the undigested 250 bp fragment represented 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 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

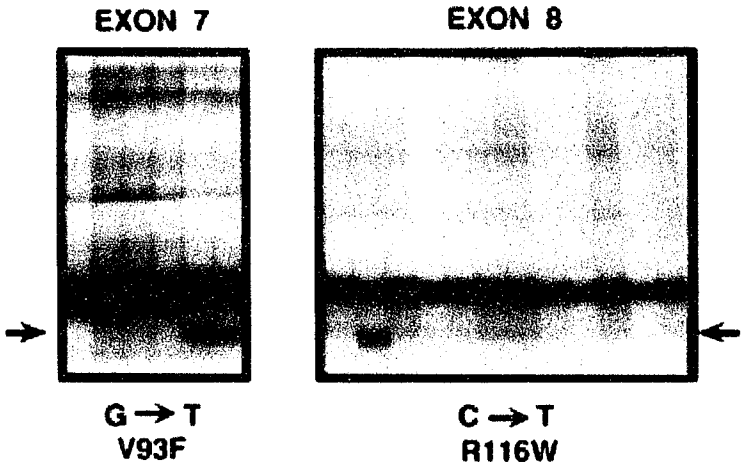


Figure 3-3. Exonic mutations of HMBS gene with SSCP mobility shift.

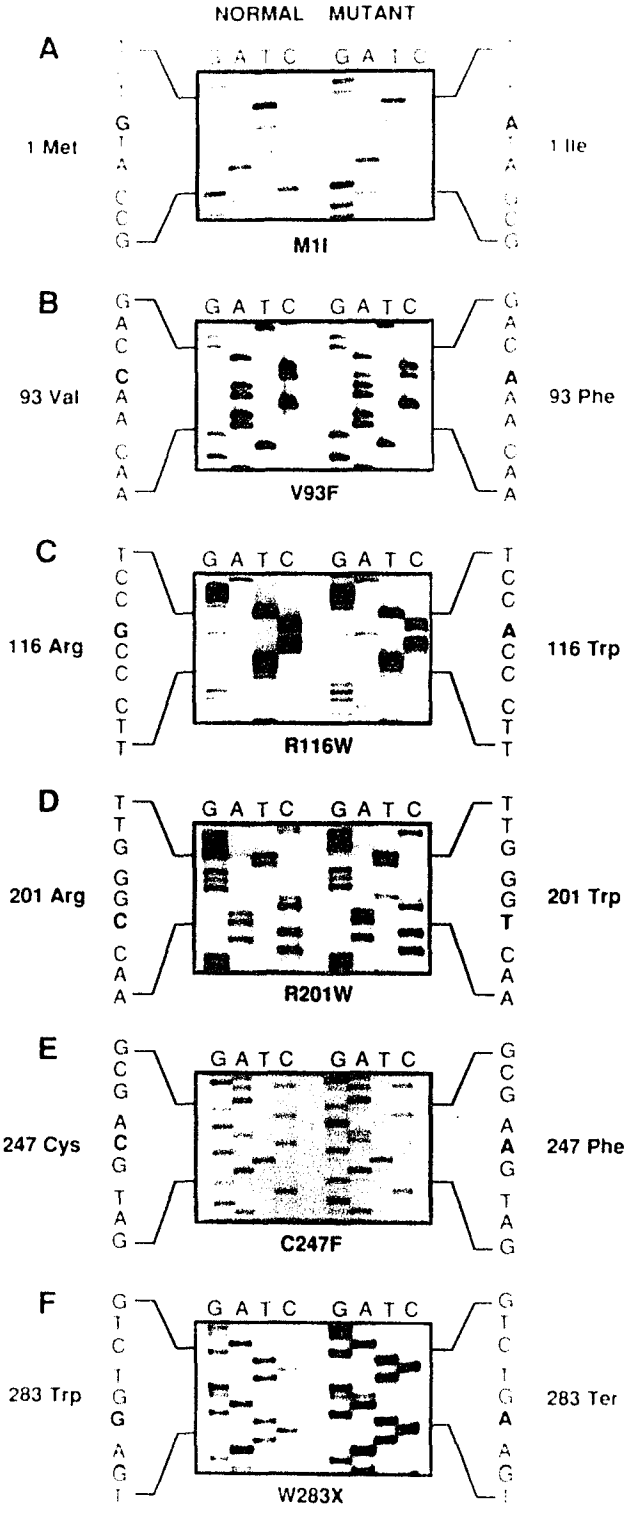


Figure 3-4. Sequence variants of mutations in HMBS gene causing AIP.

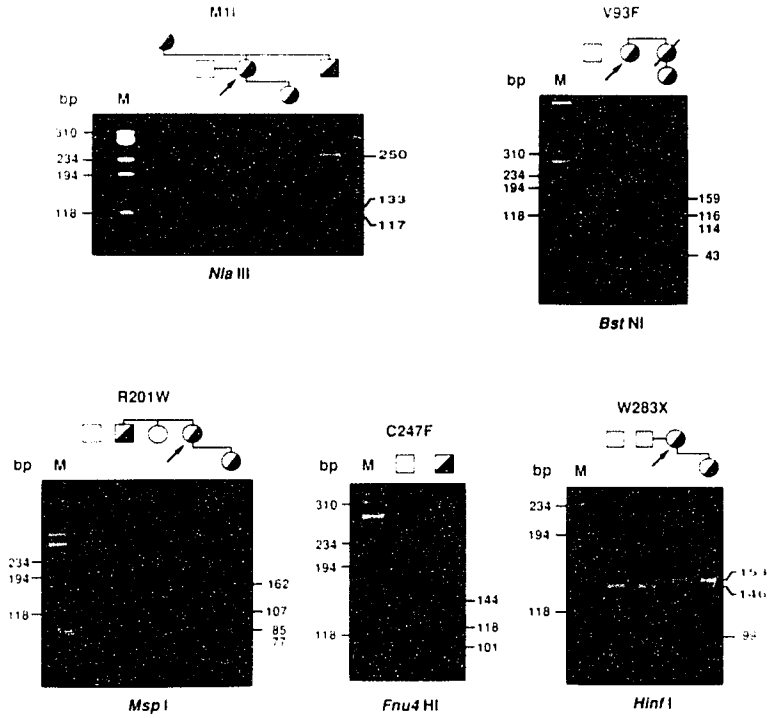


Figure 3-5. Confirmation of mutations in AIP families by restriction analysis

each mutation, the restriction enzyme and fragment sizes were: V93F, *Bst*N1 (Mutant fragment 159 bp, normal, 116 and 43 bp, and constant 114 bp fragments); R201W, *Msp*I (mutant 162 bp, normal 84 and 78 bp, constant 107 bp); C247F, *Fnu*4HI (mutant 144 bp, normal 118 and 88, and constant 101 bp fragments) and W283X, *Hinf*I (mutant 154, normal 147 and 7 bp, constant 99 bp fragments). The R116W mutation was confirmed by amplification of exon 8 from genomic DNA of Proband 3 and her mother by dot-blot hybridization using ASOs (date 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 HMBS mutations. To further characterize the HMBS 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 3-II shows the HMBS activities of the expressed normal allele and the V93F, R116W, R201W, C247F and W283X alleles following transformation and IPTG 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 less than 2% of the mean level expressed by the normal allele. Figure 6 compares the relative stabilities of the expressed R201W, C247F, W283X and normal HMBS proteins when incubated at 65 °C and pH 8.2. The half-life of the normal enzyme was about 145 min, whereas that for both the R201W and C247F enzymes were about 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 over 40% of normal mean activity when expressed in *E. coli*, was rapidly inactivated to levels less than 5% of initial activity at 180 min. The C247F enzyme, which had about 10% of normal mean activity when expressed in *E. coli*, retained about 10% of initial activity at 180 min of heat inactivation. Of interest, the truncated W283X enzyme had a normal thermostability profile.

Characterization of the M1I mutation causing variant AIP. Erythrocytes from Proband 6 with AIP and her available first degree relatives were assayed for HMBS. Figure 3-7 shows the enzymatic activities in unrelated heterozygotes with classical AIP, Proband 6 and available family members and unrelated normal individuals. Notably, the proband, and those with the M1I mutation (her mother, brother and daughter) had normal erythrocyte HMBS activity.

To characterize the expression of the M1I mutation, particularly to determine its effect on initiation of translation, the M1I 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 HMBS mutations were made. Each construct was transcribed and translated *in vitro* and the translated radiolabelled products were assessed by SDS-PAGE. As shown in Figure 3-8, the normal housekeeping expression construct, pET-HMBSh, 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-HMBSe, expressed the 40 kD erythroid-specific protein as well

Table 3-2. EXPRESSION OF HMB - SYNTHASE MUTATIONS IN E. coli

Construct	HMB-Synthase Activity		Percent of Mean Normal Activty
	Mean	Range*	
	(U/mg)		(%)
pKK233-2	1.5	1.20 -1.90	0
pKK-HMBS	194	186 - 205	100
pKK-HMBS-V93F	2.3	1.98 - 2.58	1.1
pKK-HMBS-R116W	2.8	2.4 - 3.32	1.4
pKK-HMBS-R201W	80.7	67.0 - 88.7	41.6
pKK-HMBS-C247F	21.3	10.8 - 29.2	11.0
pKK-HMBS-W283X	20.0	18.8 - 22.2	10.3

* Range represents results of three independent experiments.

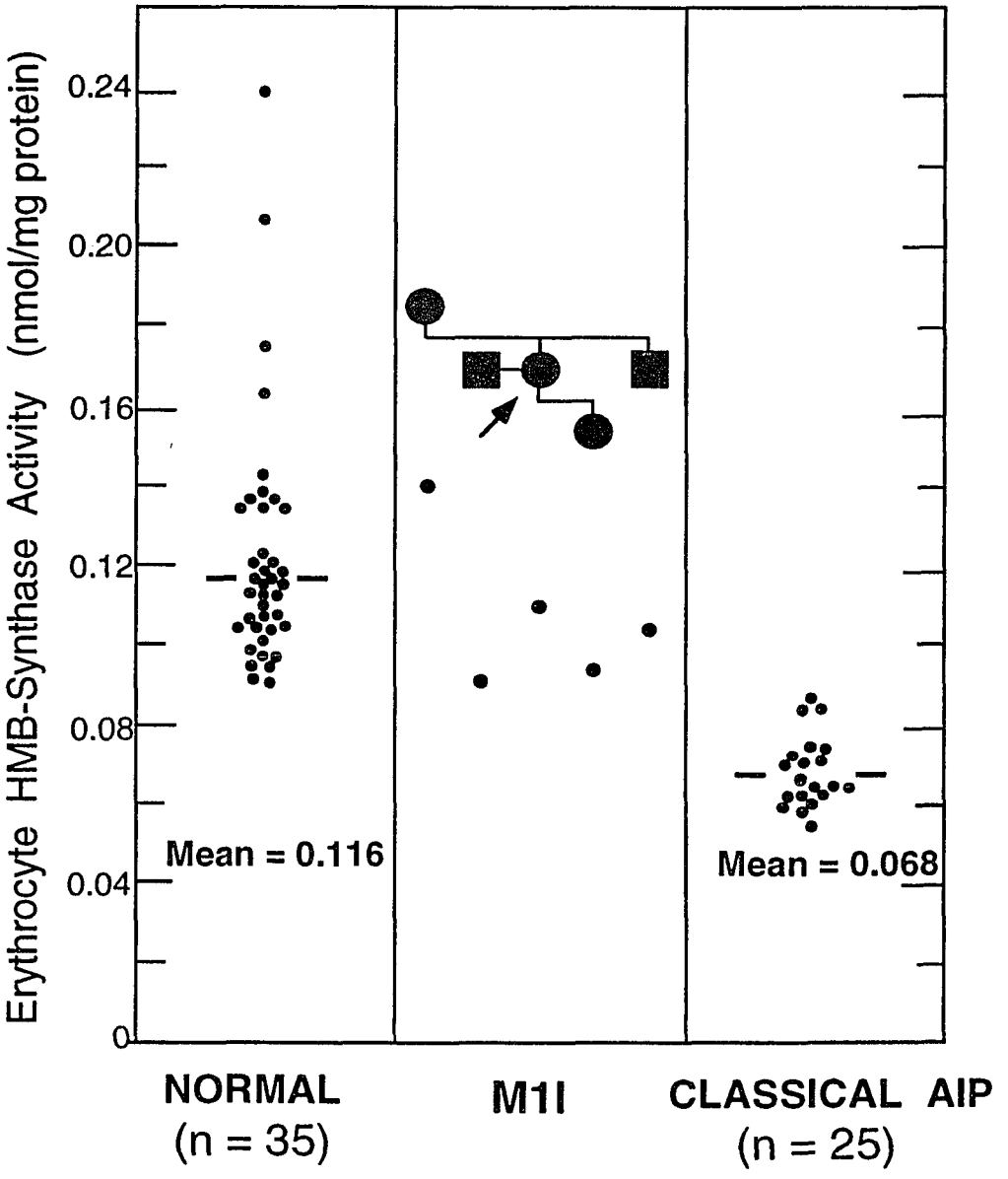


Figure 3-6. Erythrocyte HMBS activity in M1I variant AIP family.

as a 33.5 kD polypeptide, whose translation was initiated by the next downstream ATG (at codon 18) in the pET-HMBSe construct. Notably, the pET-HMBS-M1I construct only expressed the 40 kD polypeptide whose translation was initiated by the erythroid-specific ATG. 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 mutant stop codon at codon 283.

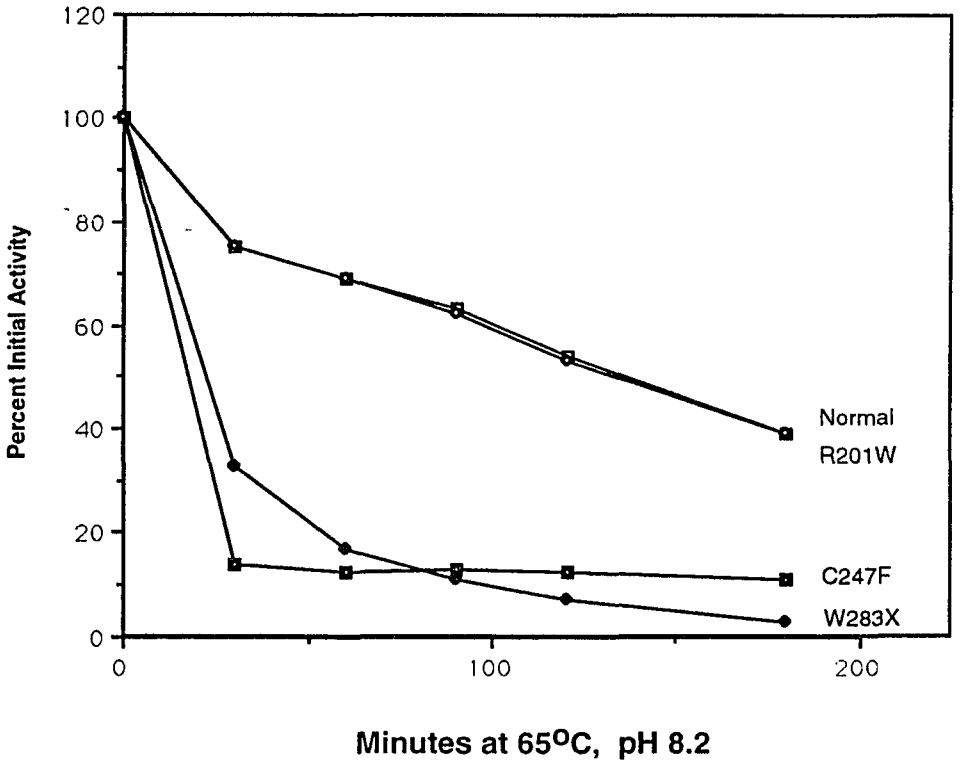


Figure 3-7. Heat inactivation study of HMBS mutant enzymes.

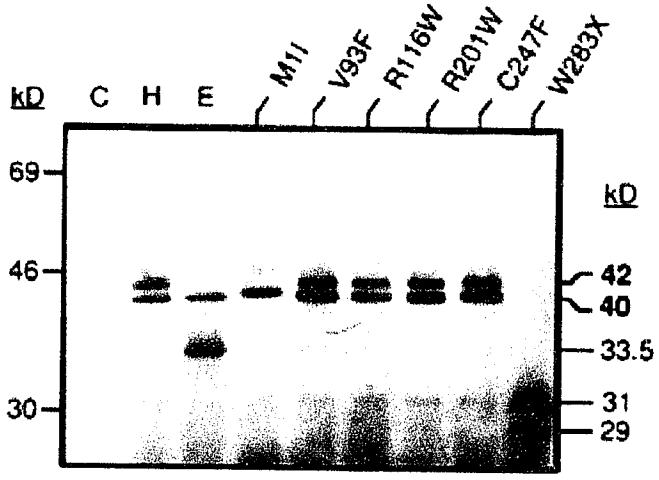


Figure 3-8. In vitro transcription/translation of HMBS cDNA and mutant alleles

Discussion

The identification and characterization of the mutations in the HMBS gene causing AIP have increased our understanding of the molecular basis of variant AIP (22, 23), the molecular heterogeneity underlying both classical and variant forms (29-40), improved diagnosis, and the opportunity for structure-function correlations of the human enzyme. Here, six new mutations in the HMBS 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 (62). R201W in exon 10 and C247F in exon 12 each occurred in exons exons where six (25%) and eight (33%) of the 24 known AIP mutations had been previously located, respectively (65). R116W, which was recently found to be a common Dutch AIP mutation (40), and W283X were each identified in two unrelated AIP families, while the other four mutations were private. R116W was recently found to be the most common Dutch AIP mutation (40).

The sixth mutation (M1I) occurred at the initiation methionine of the housekeeping transcript and caused the variant form of AIP. Affected members of this family had normal erythroid HMBS activity (Fig. 7), and presumably had half-normal activity of the housekeeping isozyme in the liver and other tissues, which was responsible for the disease manifestations. Consistent with this concept, the coupled *in vitro* transcription/translation of the M1I allele resulted in the synthesis of only the 40 kD erythroid-specific enzyme,

whereas the normal sequence encoded both the 40kD erythroid-specific and the 42 kD housekeeping isozymes (Fig. 8). These *in vitro* findings support the concept that the RNA polymerase fails to recognize the mutated housekeeping translation start codon *in vivo*. Thus, M1I is the third mutation identified that causes variant AIP, the other two lesions being RNA splicing defects at the exon 1/intron1 boundary (22, 23).

These six new mutations were identified by three different strategies: 1)RT-PCR of total lymphoblast RNA followed by subcloning and sequencing, 2) SSCP screening of PCR-amplified exonic and flanking intronic sequences followed by solid-phase direct sequencing, and 3) solid-phase direct sequencing of the PCR-amplified products from genomic DNA. Using the RT-PCR strategy, the amplified HMBS cDNAs were subcloned into a prokaryotic vector and expressed. The cDNA clones with no or low activity were easily identified by their inability to produce the fluorescent product, uroporphyrin I (Fig. 1), were sequenced, thus eliminating the normal HMBS alleles. However, all of the detected point mutations occurred in single clones and were not present in genomic DNA from the patients indicating that they were PCR errors, the error rate being about 1 per 500 nucleotides. Although these mutations resulted from non-optimal PCR conditions for Taq polymerase, they were not discarded as they provided with with little or no HMBS activity for future structure/function analyses. In addition, aberrantly spliced cDNAs were found in some of the sequenced RT-PCR clones with no HMBS activity. These and subsequently sequenced RT-PCR clones generated from lymphoblast RNA isolated from both AIP patients and normal individuals had alternatively splice transcript and no mutations in the coding or intron flanking sequences. Although alternative splicing is

required to generate the housekeeping transcript by skipping the erythroid-specific exon 2, the finding that about 50% of the aberrant HMBS transcripts had skipped exon 3 or exons 3 and 5, suggested that the fidelity of the splicing mechanism to join exon 1 to exon 3 (thereby deleting the 3 ~kb sequence containing introns 1 and 2 and exon 2) was not totally efficient, at least in lymphoblasts. If HMBS is spliced aberrantly in other tissues, this may account in part for the low level of HMBS activity in normal tissue, and serve as a negative mechanism to regulate the expression of HMBS. Comparison of 5' donor and 3' acceptor sequences, 1,2,3,5 did not reveal any weak sequence. The aberrant transcripts may have resulted from "illegitimate transcription" as described by Chelly et al (62).

In amplified genomic DNA from different patients, SSCP analysis revealed mobility shifts in exons 7, 8, 10 and 12 and the nucleotide changes causing these shifts were identified by solid-phase direct sequencing. In addition to four detected mutations (V93F, R116W, R201W, C247F), a common new polymorphism (3119 g/t) was found in intron 2. However, it should be noted that the sensitivity of mutation detection by SSCP varies (e.g. 63), and to identify mutations that do not cause a mobility shift, it is necessary to sequence the entire cDNA and intron/exon boundaries of the mutant allele. Thus, for mutaion analysis in the HMBS gene, detection technique such as SSCP and denaturing gradient gel electrophoresis or or direct sequencing of amplified condon and the intron/exon boundary is preferred over RT-PCR techniques.

Prokaryotic expression of the HMBS mutations revealed that V93F and R116W had little (1-2%), if any normal activity, while R201W and C247F had 40% and 10% of

the activity expressed by the normal allele, respectively. Heat inactivation studies indicated that both proteins were relatively unstable, losing over 90% of control activity after heating at 65 °C. Presumably, their half-life in heme-producing erythroid cells also would be significantly reduced (Fig 6). Interestingly, W283X produced a mutant protein in which the carboxy terminal 79 residues were deleted, but retained about 10% normal activity and was as stable as the normal enzyme in vitro (Fig. 6). Thus, patients with the severe V93F and R116W might be expected to have more frequent and severe attacks while patients whose mutant allele expressed residual activity may have milder, less frequent attacks.

Recently, HMBS purified from *E. coli* was and crystallized to 1.9 Å resolution (66). The crystal structure revealed three domains, two of which structurally resembled the *E. coli* periplasmic binding proteins and the transferrins. Since there is about 45% homology between the *E. coli* and human HMBS amino acid sequences, efforts have been directed to model the structure of the human sequence and to infer the location and structure/function relationship of certain human HMBS mutations. Analysis of E. coli HMBS identified conserved arginine residues (R11, R116, R131, R149, R155) which form salt bridges with the acid side chains of the dipyrrolic cofactor (66). All these arginines have been shown by site-directed mutagenesis to be required for full HMBS activity (67). Two arginines (codons 149, 150 in exon 10) of the predicted human sequence appear to be involved in cofactor assembly and correct folding of the enzyme (66). Three other arginines (R26, R267, and R273 in the human enzyme) appear to be involved in substrate binding and chain elongation. Four of the five CRIM positive

mutations (R26H, R167W, R267Q) involve mutation of these important arginines. The substitution of these arginines markedly reduces catalytic activity but apparently does not severely alter the stability and/or conformation of HMBS enzyme, thus accounting for the CRIM-postive phenotype of these mutations (37).

Based on the location of homologous residues in the *E. coli* HMBS crystal structure, V93F which had the formation of active site was located at β-4 in domain 1, R116W was located in a loop between β-1 and β-5 in domain 2, which is part of the active site. R201W, which altered the enzyme's structural stability and activity, was located at β-5 in domain 2. C247F was located in the β-1 of domain 3, although this cystein was not the cofactor-dipyrromethane binding site in *E. coli*. It must be important for the proper folding and/or stability of human HMBS, as the C247F had only 1-2% normal activity.

In summary, six new mutations have been identified in the HMBS gene that 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, these mutations and the PCR artifacts that markedly reduced or destroyed enzymatic activity are useful for molecular modeling studies to gain insight into the structure-function relationships of human HMBS.

Chapter 4. Purification and Properties of Recombinant Human Housekeeping and Erythroid-specific Hydroxymethylbilane Synthase *Isozymes*

Summary

Human hydroxymethylbilane synthase catalyzes the third step of the human heme biosynthetic pathway. An immobilized metal affinity chromatography (IMAC) was employed to simplify the purification of recombinant human housekeeping and erythroid-specific HMB-synthase expressed in *E. coli*. Two cDNAs encoding housekeeping and erythroid specific hydroxymethylbilane synthase were inserted to a gene fragment encoding a stretch of 10 histidines and a protease factor Xa recognition sequences at their N-terminus. The fusion proteins bind to immobilized metal ion affinity column, and were purified to homogeneity by elution with high concentration of imidazole. The expressed HMB-S was up to 20% of the total bacterial protein. One half of the overexpressed proteins are soluble and active, while the other half of the expressed proteins were insoluble aggregates. From 1 liter of cell culture, 20-30 mg of pure and active recombinant protein can be obtained. The fusion protein was also cleaved with protease factor Xa to produce active protein with only an extra histidine at N-terminus of initiating methionine.

The fusion proteins and cleaved protein for both the housekeeping and erythroid-specific HMBS isozymes had similar physical properties, including pH optima and thermostability. The specific activities and Km values for both purified isozymes were the same. Large amount of each active recombinant isozymes were prepared for crystallization and X-ray diffraction analysis.

Introduction

Human hydroxymethylbilane synthase (HMBS, formerly known as porphobilinogen deaminase or uroporphyrinogen I synthase; EC 4.3.1.8) is the third enzyme in the heme biosynthetic pathway and catalyzes the head-to-tail condensation of four monopyrrole porphobilinogen (PBG) molecules to form the linear tetrapyrrole hydroxymethylbilane (HMB) (1). HMB-synthase is encoded by a single gene localized to the chromosomal region 11q24.1→q24.2 (2). Both a 42 kD housekeeping and a 40 kD erythroid-specific proteins are encoded by the HMB-synthase gene. The gene has been isolated and its complete sequences have been determined (3). The 10 Kb gene contains 15 exons. The chromosomal gene has been shown to have two distinct promoters that generate the housekeeping and erythroid-specific transcripts by alternative splicing (4). The housekeeping promoter is in the 5' flanking region and its transcript is encoded by exon 1 and 3 through 15. The erythroid-specific promoter is in intron 1 and its transcript is encoded by exon 2 through 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 tissue and has regulatory elements similar to those of the β-globin gene promoter (5-7).

HMB-synthase has been purified from spinach (8), *Rhodopseudomonas spheroids* (9, 10) and *E.coli*. HMB-synthase has also been purified to homogeneity from mammalian sources, including bovine erythrocytes (11), rat liver (12), murine erythrocytes and human erythrocytes (13). Several forms of stable enzyme-substrate intermediates were identified during purification of HMB-synthase from human erythrocytes from our

laboratory (14).

A deficiency of HMB-synthase activity is the enzyme defect in acute intermittent porphyria (AIP), an autosomal dominant disease. This disorder is characterized by episodic, life-threatening acute neurologic attacks, which are precipitated by certain drugs and a variety of metabolic and hormonal factors that increase heme biosynthesis (15). Biochemical and immunological investigations showed the genetic heterogeneity of defects of HMBS causing AIP (16). Recent interest in the human enzyme has been prompted by the identification of several molecular defects in the HMB-synthase gene causing AIP (17).

Study of the structural and functional relationship of HMB-synthase will facilitate understanding of the molecular pathogenesis of AIP, and will also would facilitate the study of the molecular mechanism of mammalian tetrapyrrole biosynthesis and regulation. In order to study structure/function aspects of human HMB-synthase, it is necessary to obtain sufficient amount of pure enzyme for crystallization and X-ray diffraction analysis.

Recombinant DNA technology was to obtain large amount of pure human HMBS isozymes. Both housekeeping and erythroid-specific form of human HMBS full length cDNA were subcloned to a prokaryotic expression vector pET16b and were expressed in *E. coli*. This expression vector is regulated by a strong T7*lac* promoter (18), and produces a fusion protein containing a leader sequence with ten histidines and a protease factor Xa cleaving site at the N-terminal of the target protein. The histidine residuals can bind to the immobilized metal ion column and can be eluted with high concentration of imidazole. This method results in a purification procedure with high yields (19).

In this communication, we report the overexpression, purification and characterization of human recombinant housekeeping and erythroid-specific HMB-synthase isozymes, Our purification method obtained large amount of active form of both isozymes. This permitted characterization of the physical and kinetic properties of both isozymes and facilitated the crystallization of these enzymes for X-ray diffraction analysis.

Materials and Methods

Materials - E. coli strain DH5α was used as host cells for cloning and sequencing of both housekeeping and erythroid-specific HMBS cDNA constructs. The prokaryotic expression vector pET16b and expression host cells *E. coli* strain BL21(pLys) were purchased from Novagen. BL21(pLys) was used for induction and expression of recombinant human HMBS. Isopropyl-1-thio-β-D-galactopyranoside (IPTG), nickel sulfate and Imidazole were supplied from Sigma. Metal ion chelating resin matrix Sepharose 6B Fast Flow was purchased from Pharmacia LKB Biotechnology Inc. Protease factor Xa was purchased from New England Biolabs.

Construction of the Expression Vectors - Two primers, sense primer (5'-GCCGCCCATATGTCTGGTAACGGCAATGC-3') and antisense primer (5'-GCCGCCCATATGTCTGTGCCCCACAAACCA-3'), both containing a *Nde*I site at linker sequences were used to PCR amplify the full length housekeeping HMBS cDNA using pET-HMBSh as template described previously. Two other primers, sense primer (5'-GCCGCCCATATGAGACTGATTCGCGTGGGT-3') and antisense primer (5'GCCGCCCATATGTCTGTGCCCCACAAACCA-3'), also containing *a Nde*I site at linker sequences were used to PCR amplify full length erythroid-specific HMBS cDNA. The PCR reaction was carried as following: after an initiatial denaturation at 94 °C for 5 min, 30 cycles of PCR reaction was performed with a denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C 1 min. The PCR amplified full length housekeeping and erythroid-specific HMBS cDNA was digested with *Nde*I at 37 °C overnight and gel purified. The *Nde*I digested PCR product was cloned to the

pET16b using standard procedure. The authenticity of the expression constructs were confirmed by sequencing. The manipulation of the plasmids was carried out in *E. coli* strain DH5α. The constructs of pET16b with housekeeping and erythroid-specific HMBS cDNAs were designated pET16b-HMBSh and pET16b-HMBSe, respectively.

Expression and Purification of Proteins - Both pET16b-HMBSh and pET16b-HMBe were purified from *E. coli* strain DH5α, and used to transfect *E. coli* strain BL21(pLys). Overnight cultures were prepared from single colony using LB medium and incubated at 37 °C with shaking. Next day, the overnight culture was inoculated to 1 liter LB flask. The culture was grown to O.D. 0.4 at A_{600} nm at 37 °C with shaking, and IPTG was added to the cell culture at final concentration of 1 mM to induce the expression of recombinant proteins. The cultures were grown at room temperature with shaking overnight. Next day, the cells were harvested by centrifugation at 5,000 *g* for 20 min. Supernatant was decanted, the pellets were resuspended in 30 ml ice-cold binding buffer (20 mM Tris buffer, pH 8.2, NaCl 500 mM). The cell suspensions were sonicated on ice until the extracts no longer viscous. The cell lysates were digested with DNase in cold room for 4 hours. After digestion, the lysates were centrifugated at 10,000g for 20 min to remove the debris, the post-centrifugation supernatant was filtered through a 0.45 micron membrane before loading to the preequilibrated nickel ion affinity column.

Immobilized Metal Affinity Chromatography - Metal ion chelating resin Chelating Sepharose 6B Fast Flow (Pharmacia) was used in the experiments to bind the His·Tag fusion proteins. The resin has bound iminodiacetic acid group which can efficiently coordinate divalent metal ion. The gel was used according to the recommendations of the

supplier. The column was equilibrated with 5 mM nickel sulfate and thereafter the excess of the mental ions was removed by washing with binding buffer (5 mM imidazole, 500 mM naCl, 20 mM Tris-HCl, pH 8). The NaCl was included in the buffer to eliminate possible ion-exchange effect. After loading the cell extract, washing and elution, the column can be regenerated by stripping with 10 volumes of 50 mM EDTA, 500 mM NaCl, in 20 mM Tris-HCl, pH 8, and subsequently washed with water. The washed column can be reequilibrated with nickel sulfate solution. The immobilized metal ion affinity column was prepared according to the manufacturer's instruction. After loading the smaples, the column was washed 10 volumns of 1X binding buffer, and subsequently with 10 volumes of 100 mM imidazole in 20 mM Tris-HCl buffer, pH 8 containing 500 mM NaCl, and 10 volumes of 200 mM imidazole in 20 mM Tris-HCl, pH 8 containing 500 mM NaCl. The bound fusion proteins were eluted with 300 mM imidazole, 20 mM Tris-HCl buffer, pH 8, and 500 mM NaCl. After elution, the purified was dialyzed against 20 mM Tris-HCl buffer, pH 8 in the cold room overnight to remove imidazole and high salt. After dialysis, the purified protein was concentrated by ultrafiltration using Amicon membrane with cutoff of 30 kD. The purification processed was monitored by checking the HMBS enzyme activity and by protein gel electrophoresis.

Protease Factor Xa Digestion - Purified fusion protein was digested with factor Xa with a ratio of 1 mg recombinant protein to 20-50 μg of factor Xa in 50 mM Tris-HCl buffer, pH 8, 100 mM NaCl and 1 mM $CaCl_2$ at room temperature for 8-16 hours. After digestion, the reaction mixture was mixed with preequilibrated nickel ion resin to remove the undigested fusion protein, which will bind to the chelating resin. The cleaved protein

was collected and concentrated for further characterization.

Enzyme Assay of HMB-Synthase - The enzyme was assayed by quantitation of uroporphyrin fluorescence using a fluorometer equipped with a 405 nm excitation filter and aa 595 nm emission filter. For routine assay, the standard reaction mixture contained 200 µl 0.5 mM porphobilinogen, 100 µl diluted enzyme, and 450 µl 0.1 M Tris-HCl buffer (containing 0.1 mM DTT). the solution was mixed and incubated in the dark at 37 °C for half hour and the reaction was stopped by adding 250 µl 50% trichloroacetic acid. The rection was exposed to light to oxidize the uroporphyrinogen to uroporphyrin. One unit of HMBS activity represented 1 nmol of uroporphyrin produced per hour at 37 °C. Specific activities were calculated after determination of protein by the method of Bradford using bovine serum albumin as a standard.

Gel Electrophoresis--The monitor of expression and purification of the recombinant proteins from metal ion affinity column, and the determination of the molecular weight were performed by SDS-PAGE gel electrophoresis (10%) according to Laemmli (20). The gels were stained by Coomassi blue. Molecular-weight standards used were Rainbow markers (from Amershan), including bovine serum albumin (68 kd), creatine kinase (40 kd), carbonic anhydrase (29 kd) and cytochrome c (12kd).

Characterization of Fusion and Cleaved HMBS Isozymes - Effect of pH on the enzyme activity of HMBS isozymes was carried out in 50 mM Tris-HCl buffer, pH values varied from 6 to 10 with 0.2 interval difference. Thermostability of the fuison and cleaved HMBS isozymes was carried out in romm temperature, 37 °C and 55 °C, HMBS enzyme activity was determined with different incubation time of 30, 60, 90, and 120

minutes. Km was determined by measuring the HMBS activity in varying concentration of freshly prepared porphobilinogen. The data obtained from the fluoroescence HMB-synthase assay were plotted as the reciprocal of the initial velocity versus reciprocal of variable substrate concentrations.

Results

Human HMBS levels expressed in E. coli - During the exponential phase of growth, *E. coli* containing the expression construct was induced using 1 mM IPTG to initiate the expression of human HMBS. The cultures were then incubated at room temperature overnight. The levels of the expressed proteins were estimated to be up to 20% of total bacterial proteins as determined by SDS-PAGE. Half of the HMBS proteins were in soluble fraction of cell lysates, the other half became insoluble aggregates. The insoluble aggregates formed inclusion bodies which were visible under microscope. The soluble fraction containing the enzyme activities were used for further purification. In the uninduced cell culture, there was only low level HMBS enzyme activity, indicating little leaky control of the T7*lac* promoter.

Purification of Recombinant HMBS - After several pilot studies to optimize the concentration for washing and eluting fusion protein binding to the nickel affinity column, the optimal concentration of imidazole to elute both the recombinant human housekeeping and erythroid-specific HMBS isozymes was determined to be 300 mM imidazole in 50 mM Tris buffer pH 8, with 500 mM NaCl. From 1 liter cell culture, 20-30 mg pure recombinant protein can be recovered from the column. The specific activity, yield and purification for both isozymes were listed in Table 4-1. SDS-PAGE analysis of HMB-synthase before and after induction, expression and immobilized metal ion affinity chromatography purification are shown in Figure 4-1. From the gel, recombinant fusion proteins of HMBS isozymes were purified to homogeneity in one step. The yield is about 10-15%, and purification is about 5 fold. After elution with high concentration of

Table 4-1

IMMOBILIZED METAL AFFINITY CHROMATOGRAPHY (IMAC) OF RECOMBINANT HUMAN HMB-SYNTHASE ISOZYMES

Step	Volume	Total Activity	Specific Activity	Protein	Yield	Purification
	(ml)	(U)	(U/mg)	(mg)	(%)	(- fold)
Housekeeping Isozyme						
Supernatant	30	69,530	200	220	100	1
IMAC*	50	24,500	980	25	35	4.9
Erythroid Isozyme						
Supernatant	30	98,100	280	280	100	1
IMAC*	50	30,600	1,020	26	31	3.6

*After IMAC, sample dialyzed against 20 mM Tris buffer, pH 8.2, and concentrated by ultracentrifugation.

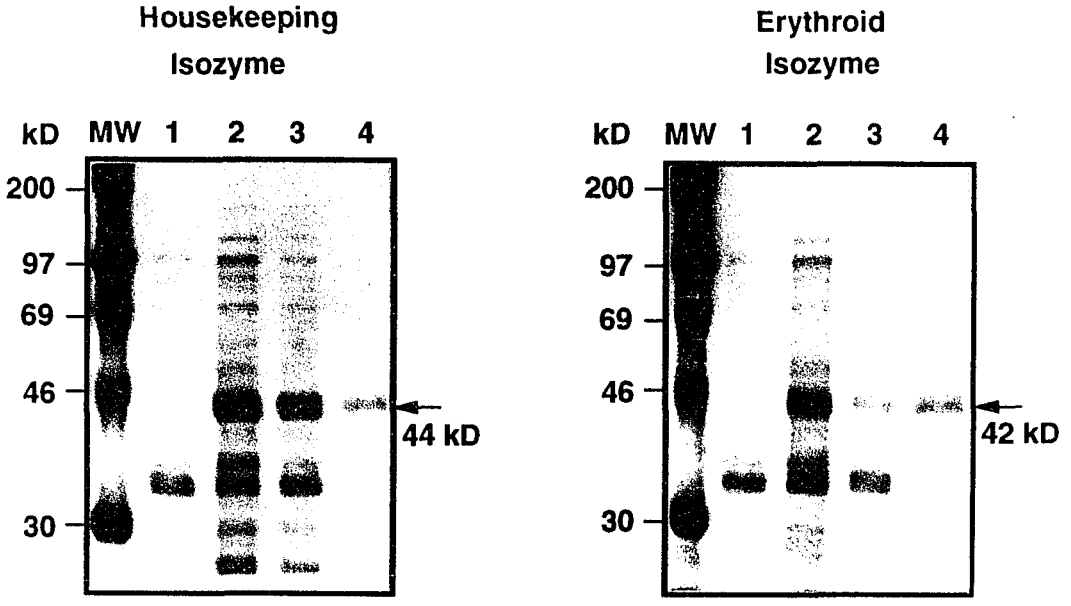


Figure 4-1. SDS-PAGE analysis of purification of recombinant human HMBS.

imidazole, the eluted recombiant HMBS fusion proteins were dialyzed against 20 mM Tris buffer, pH 8 in the cold room overnight to remove the imidazole and salt. After dialysis, the protein was concentrated by ultrafiltration to the concentration of 1 mg/ml, the dialyzed protein was used for protease factor Xa digestion and for further characterization.

Properties of purified HMBS - The molecular weight of purified HMBS was determined by SDS-PAGE. The recombinant housekeeping fusion protein has molecular weight about 44 kD, and erythroid isozyme has molecular weight around 42 kD. To determine the physical and biochemical properties of purified HMBS isozymes, time course of the HMBS activity was determined first. The production of uroporphyrin was in linear proportion to incubation period within 30 minutes using certain amount of protein (data not shown). The pH effect on both houskeeping and erythroid HMBS isozymes revealed that the optimal pH for each form of protein was the same, pH 8.2. The data was shown in Figure 4-2. Thermostability studies of the recombinant fusion HMBS isozymes were stable in room temperature and 37 °C, the half-life at 55 °C were longer that 3 hours, the data were shown in Figure 4-3 and 4-4. The half life of erythoid isozyme is longer than that of houskeeping isozyme.

Kinetic Properties of HMBS - For kinetic study, different concentrations of substrate and HMBS were tested to find linear range of initial velocity. In steady-state kinetics, the Km of housekeeping HMBS fusion protein was 45 μM and the Km of the factor Xa cleaved housekeeping HMBS was 34 μM. The Km of erythroid HMBS fusion protein was 40 μM, and the Km of the protease factor Xa cleaved erythroid HMBS was

35 μM. The specific activity and the Km data of the recombinant HMBS isozymes of fusion protein and cleaved proteins are listed in table 2. The cleaved protein has higher specific activty and higher substrate affinity than that of fusion protein in both HMBS isozymes, however, the difference is negligible. Also notably, there is no significant difference of specific activity and Km values between housekeeping and erythroid HMBS isozymes.

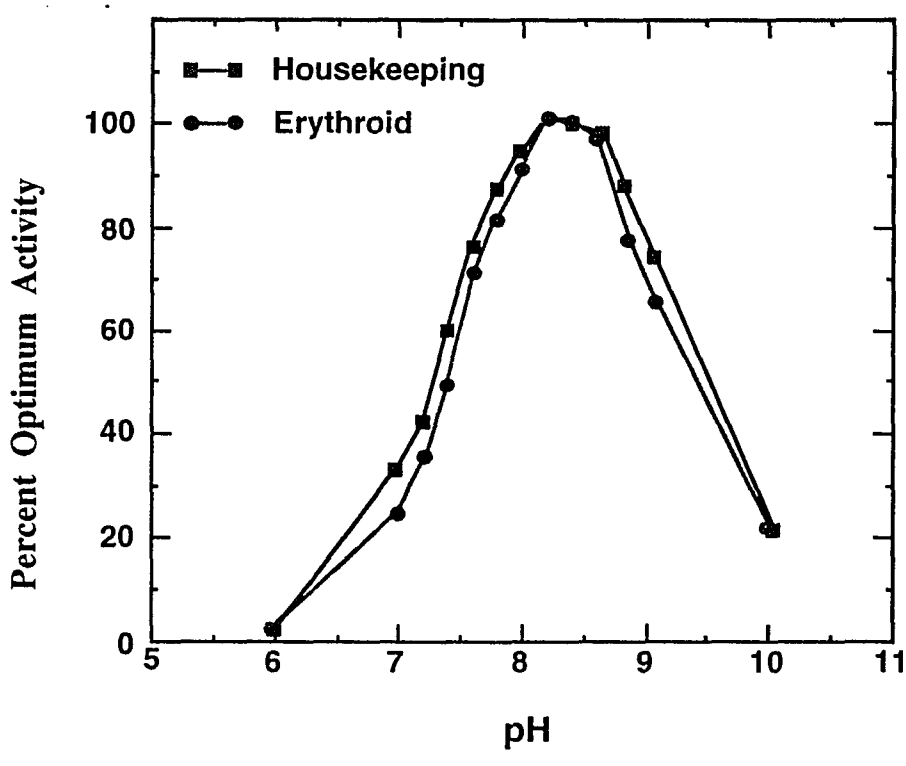


Figure 4-2. Profile of effect of pH on the purified recombinant human HMBS isozymes.

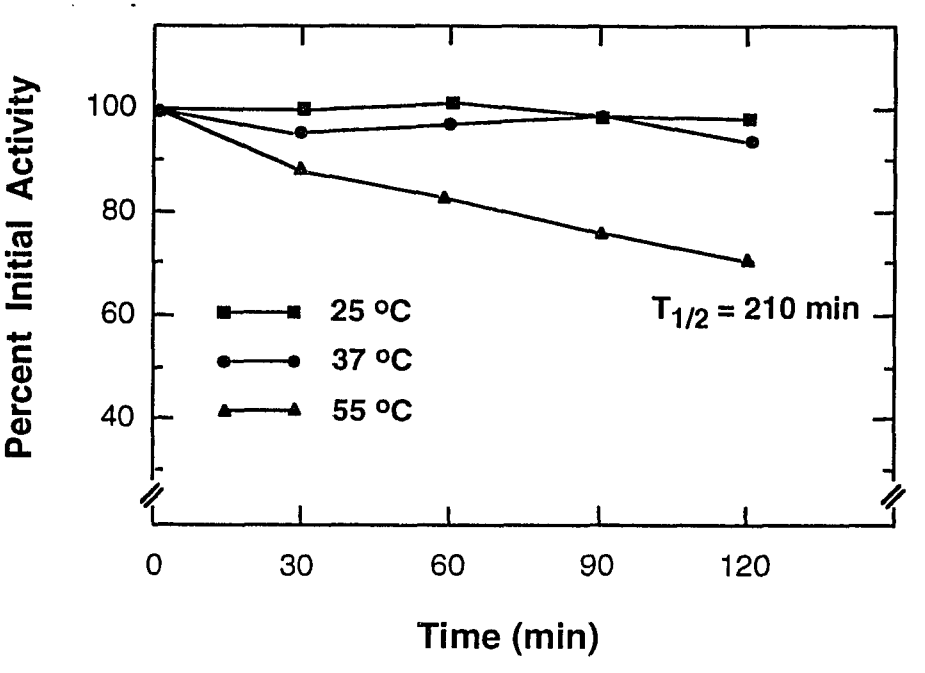


Figure 4-3. Thermostability of recombinant human housekeeping HMBS.

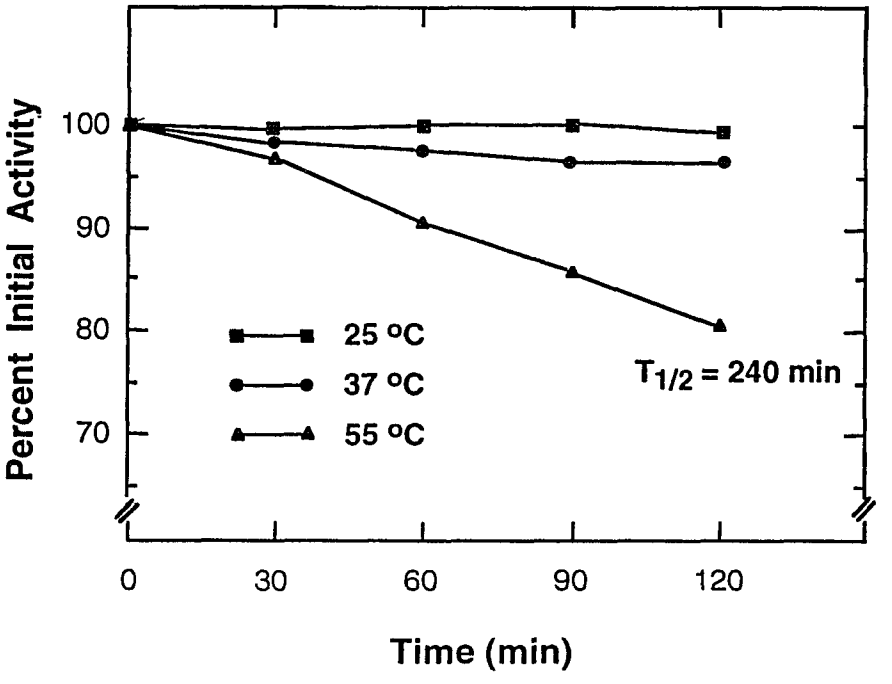


Figure 4-4. Thermostability of recombinant human erythroid-specific HMBS.

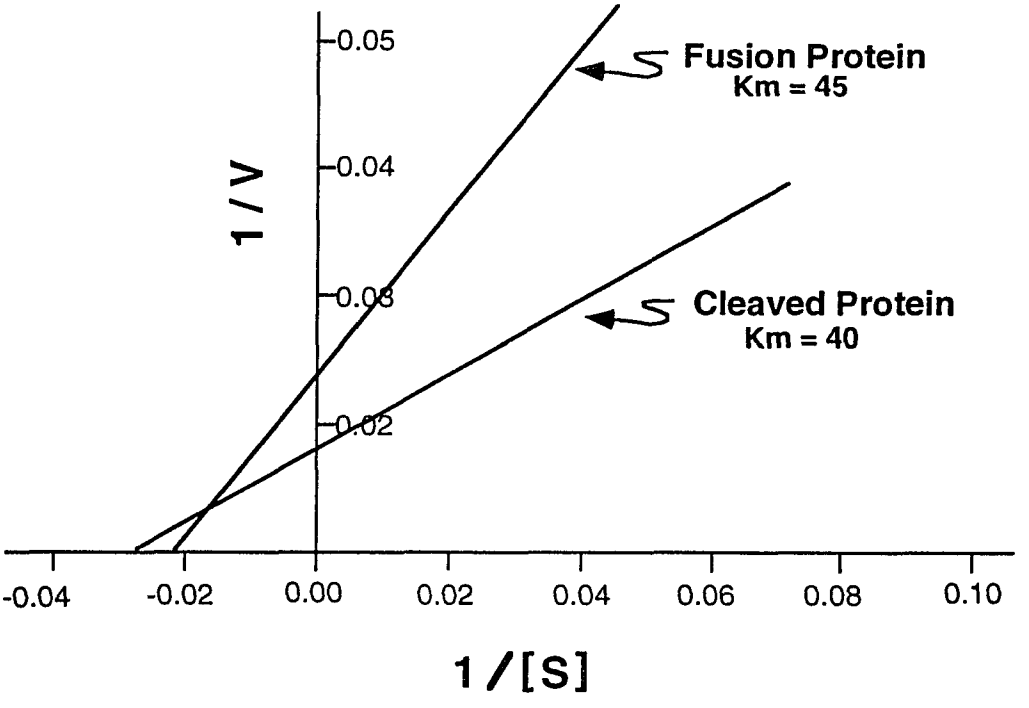


Figure 4-5. Lineweaver-Burk plot of housekeeping HMBS recombinant fusion protein and protein cleaved with factor Xa.

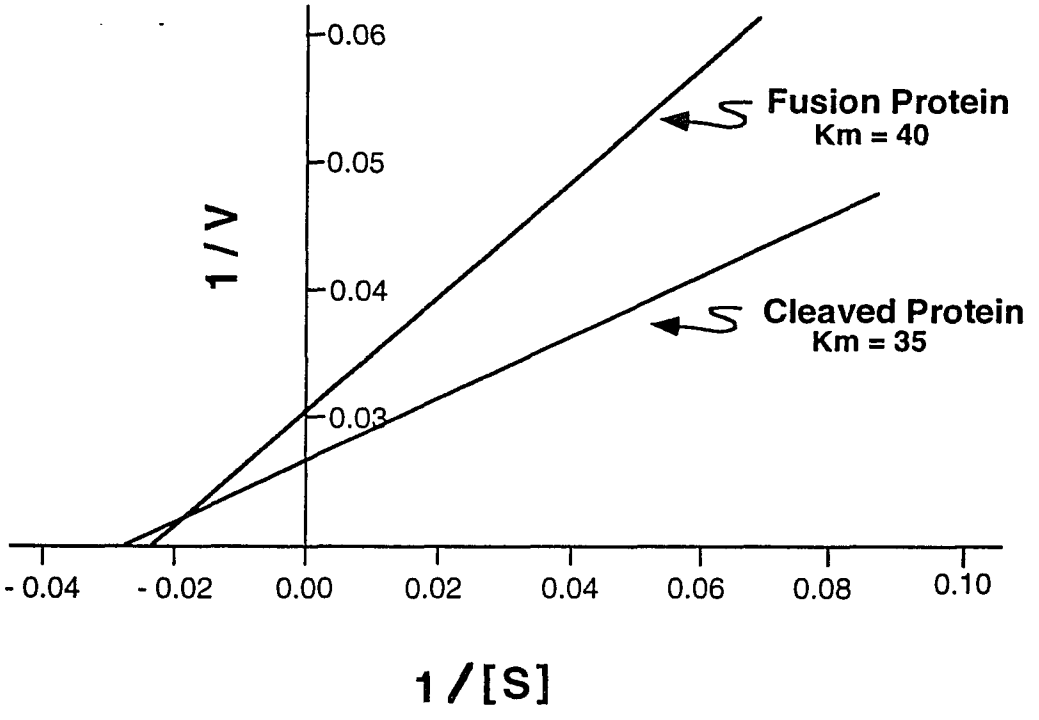


Figure 4-6. Lineweaver-Burk plot of erythroid-specific HMBS recombinant fusion protein and protein cleaved with factor Xa.

Table 4-2

KINETIC PROPERTIES OF PURIFIED RECOMBINANT HUMAN HMB-SYNTHASE ISOZYMES

Isozymes	Km	Specific Activity
	(mM)	(U[#]/ mg)
Housekeeping Isozyme :		
Fusion Protein	45	980
Cleaved Protein[*]	34	1,033
Erythroid Isozyme:		
Fusion Protein	40	1,020
Cleaved Protein[*]	35	1,180

* Fusion protein cleaved with factor Xa.

One unit (U) equals 1 nmole of uroporphyrin produced per hour at 37 ^{o}C.

Discussion

In this communication, we described a simple and fast one-step purification method, immobilized metal affinity chromatography (or IMAC), to purify human housekeeping and erythroid-specific HMB-synthase expressed in *E. coli.* Both housekeeping and erythroid-specific HMBS cDNAs were subcloned to the *Nde*I site of pET16b, respectively. The manipulation and construction of both expression plasmids were carried out in *E. coli* strain DH5α. DH5α does not contain T7 polymerase gene, therefore it does not express HMB-synthase. *E. coli* strain BL21(DE3) was used as host cells to perform the expression experiments. The expressed human HMBS has a stretch of leader sequences at its N-terminus. This leader sequences contain a segment of 10 consecutive histidine, and a factor Xa recognition sequence. The total length of this stretch of leader sequences is 21 amino acids and the ten histidine stretch was used to facilitate one-step purification. When the fusion protein of human HMBS was cleaved with factor Xa, only one extra amino acid, a histidine, was left over in front of first methionine codon. This is main rationale that pET16b was used in this communication. In our study, no differences of the HMBS activities before and after digestion with factor Xa (21) was found. Apparently, the leader sequence of the fusion construct does not affect the folding and activity of HMBS.

One of the major concerns in using fusion constructs to facilitate purification of protein is that the extra stretch of proteins may affect the correct folding of the expressed protein. In GST-system, the stretch of leader sequences is very long, which may have more interference in the folding of fusion protein (22). In comparison to GST-system,

the stretch of the leader sequences in pET16 vector is very short. In addition, our data shows no difference of enzyme activity of purified recombinant HMBS and HMBS purified from human erythrocytes. Moreover, the IMAC purification is labor and time saving and high yield of pure protein can be obtained to facilitate further structure study.

However, there is some drawbacks of IMAC system. Thioreducing agent can not be used in buffer during cell extraction and purification since these agents will oxidize the metal ions, which will precipitate, and ruin the IMAC. Proteins needing disulfide bond to retain the enzyme activity can not be purified using IMAC system.

After initiation of expression of HMBS in *E.coli* using IPTG, if the cell cultures were incubated at 37 °C temperature, 90% of the expressed proteins became insoluble aggregates, and lost their enzyme activities. However if the cell cultures were incubated at room temperature, 50% of the expressed proteins were in soluble fraction, and still retained the enzyme activities. The suboptimal growth temperature helps the correct folding of the overexpressed protein and increases the yield. This phenomenon was well known and employed in many instances of overexpression of recombinant proteins (23). The underlying mechanism is unknown, but it was proposed that at suboptimal temperature, the newly made proteins have enough time to fold in correct order, and avoid from becoming insoluble aggregates due to overcrowding of expressed proteins. However, it is not only the suboptimal growth temperature that determines the solubility of overexpressed protein. We have subcloned HMBS cDNA into pET5a and overexpressed it. When grew at 37 °C, 50% of expressed proteins are in soluble fraction. However, when grew at room temperature, 90% of the expressed proteins are in soluble

fraction, indicating that in pET16b construct, the leader sequences of the fusion protein affect the solubility of expressed protein. In addition, other studies found when some proteins were overexpressed, more than 95% are insoluble aggregates, suggesting that protein structure itself also affects the solubility.

In this communication, we found no difference of HMBS activity and physical properties between recombinant fusion protein and the native protein proteins purified from human erythrocytes, suggesting structural functional similarity between these two proteins. This experiment offers a rapid and efficient method to obtain enough amount of HMBS for crystallization study.

Chapter 5. The Human Cytosolic Heme Biosynthetic Enzymes Hydroxymethylbilane Synthase and Uroporphyrinogen III Synthase do not Interact in an Enzyme complex.

Summary

Hydroxymethylbilane synthase (HMBS), the third enzyme in human heme biosynthetic pathway, catalyzes the head to tail condensation of four molecules of porphobilinogen by deamination to form the linear tetrapyrrole, hydroxymethylbilane (HMB). Uroporphyrinogen III-synthase (UROS), the fourth enzyme, catalyzes the rearrangement and cyclization of the linear HMB to uroporphyrinogen III (URO III). Since the HMB, generated by HMBS, is rapidly converted to URO III, physical association between HMBS and UROS was proposed by several investigators. To identify the putative multienzyme complex of these cytosolic heme biosynthetic enzymes, polyclonal antibodies against human HMBS were employed in an attempt to coimmunoprecipitate the human UROS and HMBS. Cell extracts from [^{35}S]-methionine labelled human hepatocellular carcinoma cell line, HepG2 or human erythroleukemic cell line, K562, were used for immunoprecipitation experiments in the presence or absence of a thio-sensitive cross-linking agent, DSP. No evidence for physical association between HMBS and UROS. In addition, using a two-hybrid system in which GAL4 DNA binding domain fused to UROS, and the GAL4 transcription activation domain was fused to HMBS. No interaction between these two fusion proteins, suggesting no physical association between HMBS and UROS. These results suggest that HMBS and UROS do not form a cytosolic multienzyme complex and the transformation of HMB into URO III probably proceeds via a dissociative mechanism.

Introduction

Human HMBS and UROS catalyze two sequential heme biosynthetic reactions in the cellular cytoplasms. HMBS, the third enzyme in the heme biosynthetic pathway, catalyzes the head to tail condensation of four molecules of PBG by deamination to form the linear tetrapyrrole, HMB. In the presence of UROS, the HMB is rapidly converted to URO III by a reaction mechanism which involves the intramolecular rearrangement of the D-pyrrole group and ring closure (1). In the absence of UROS, HMB is nonenzymatically cyclized to form uroporphyrinogen I (URO I), an isomer which is detrimental to cellular function. Deficient activity of UROS in homozygotes with congenital erythropoietic porphyria (CEP) results in the accumulation of URO I, the primary pathologic compound in this inherited human disorder (2, 3).

In the metabolic pathways of cells, there are many instances known that the pathway intermediates are transferred from one enzyme to another without complete equilibration with the surrounding medium. This process is called metabolite channeling or "facilitated substrate delivery" (4, 5). Metabolite channeling process is achieved through some sort of structural organization for the pathway components, e.g. multifunctional enzymes, multienzyme complexes, dynamic enzyme associations or microcompartmentation. There are several advantages of metabolite channeling: (1) If the substrates can be transferred directly from one active site to another, the substrate's concentration in the microenvironment of the second site is kept relatively high compared to the average bulk concentration of that substrate and the limited solvation capacity of cellular water is avoided. (2) High concentrations of substrates with fewer substrate

molecules can be achieved. (3) When the input to a sequence of metabolic reactions is changed, the transient time for attainment of a new steady state is reached faster for a multienzyme complex than for comparable enzyme activities free in solution. (4) If substrates are unstable in aqueous environments or if they can be acted on by other enzymes, then direct transfer to a successive active site is a mechanism for preservation of the substrate. (5) Interactions between the sequential proteins may have allosteric (thus regulatory) effects on the activities involved. (6) Complexes, if they are attached to or are part of the cellular structural elements or macromolecules, would have the advantage that the diffusion of their enzymatic components could possibly take place in one or two rather than three dimensions (6).

In the heme biosynthetic pathway, both HMBS and UROS are cytosolic enzymes and catalyze sequential reactions. Several investigators have proposed that HMBS may be physically associated with UROS, such a cytosolic enzyme complex would facilitate efficiency of the sequential reactions and protect the porphyrinogen intermediates from being oxidized to nonmetabolizable porphyrins.

Based on these considerations, we explored whether HMBS physically associates with UROS. Two strategies were used to investigate the putative physical association. First, coimmunoprecipitation combined with crosslinking was employed using human hepatocellular carcinoma cell line, HepG2 and human erythroleukemic cell line, K562 as experiment systems. Rabbit against human HMBS polyclonal antibodies were used to immunoprecipitate [^{35}S]-methionine labeled HepG2 and K562 cells treated with DSP or without DSP cross-linking. There are several successful examples by using this approach,

including study of glycoprotein topology on intact human red blood cells (7), molecular identification of receptors for vasoactive intestinal peptide in rat intestinal epithelium (8). Second, we used a yeast expression system designed to detect protein-protein interactions as a result of their ability to reconstitute the transactivating function of GAL4 protein. Interacting fusion proteins combine to form a DNA-binding and transcriptional activation dimer that induces synthesis from a β-galactosidase reporter gene in an appropriate host (Fields and Song, 1989). This approach has been tested and demonstrated for many pairs of interacting proteins including yeast SNF1 and SNF4 (9), maize B and C1(10) mammalian Jun and Fos (11), Ras and Raf (12, 13), p53 and T-antigen (14, 15).

In this communication, we report our studies showing no evidence for physical association or positional proximity between two cytosolic heme biosynthetic enzymes, HMBS and UROS. Therefore, the formation of uroporphyrinogen from HMB probably proceeds via diffusion mechanism, rather than metabolites channeling.

Materials and Methods

Cell Cultures--Two cell lines were used in this experiment. A human erythroleukemic cell line, K562 cells, was a gift from Dr. David F. Bishop (Department of Human Genetics, Mt. Sinai Medical Center, New York); the other one is human hepatocellular carcinoma cell line, HepG2, a gift from Dr. Cederbaum (Department of Biochemistry, Mt. Sinai Medical Center, New York). K562 cells were was grown in RPMI 1640 culture media, supplemented with 10% fetal calf serum, 100 units penicillin, streptomycin 0.1 mg/ml and 2 mM glutamine in 5% CO_2 at 37 °C. The cells were subcultured twice a week. Cells were grown to a density of 1 x 10^6/ml for all experiments. HepG2 cells were grown in T-75 flasks containing minimum essential medium supplemented with 10% heat inactivated fetal calf serum, L-glutamine, and penicillin-streptomycin. The cells were grown to confluency when they had a density of 1.5 to 2 x 10^5 cells/cm^2.

Cell labeling and chemical cross-linking--K562 cells were pelleted by centrifugation at 250*g*, washed with PBS and resuspended in methionine-free RPMI 1640 medium supplemented with 10% dialyzed fetal cal serum. The cells (4 x 10^6/ml) were preincubated for 30 min 37 °C, and then [^{35}S]methionine (50 μCi/ml) was added, and incubation continued overnight. The incorporation of [^{35}S]methionine into proteins was terminated by the addition of 0.7 mM nonradioactive methionine and centrifugation of the cell suspensions at 250 x *g* for 10 min at 4 °C. The cells were washed with phosphate buffer saline (PBS) at 4 °C. The thio-cleavable homobifunctional crosslinking agent DSP (dithiobis succinimidyl propionate, Pierce Europe B.V., Oud-Beijerland, The Netherlands)

was dissolved at 10 mg/ml in DMSO immediately before use and added to the cell K562 suspension to a final concentration to a final concentration of 0.5 mM and allowed to react with the cells for 30 min at room temperature. The reaction was quenched by the addition of 1/10 volume of 1 M Tris-HCl, pH 8.0. The cells were pelleted and lysed in a 0.5 ml buffer containing 0.5% SDS, 5mM Tris-HCl, pH 8.0 and 5 mM EDTA. HepG2 subconfluent cultures were labeled with 250 μCi of [^{35}S]methionine per 5 ml in methionine-free DMEM containing 10% dialyzed fetal calf serum overnight. The flasks were washed free of medium with PBS and incubated with gentle shaking for 30 min at room temperature with the DSP at 0.5 mM diluted in PBS from 0.1 M solution in dimethyl sulfoxide (DMSO). The reaction was quenched with 0.1 M Tris-HCl at room temperature 5 min. Subsequently, the cells were treated with Trypsin and EDTA, and pelleted by centrifugation. The pellet was resuspended in PBS at a concentration of 1 x 10^7 cells/ml

Immunoprecipitation analysis--The 0.5 ml K562 and/or HepG2 cell suspensions were sonicated and boiled for 5 min. 150 μl of 1M Tris-HCl, pH 8.0 and 167 μl 10% Triton X-100 were added and the lysates were cleared by centrifugation 14.000*g* for 5 min at 4 °C. The supernatant was transferred to a new tube, and 20 μl antiserum was added and incubated at room temperature with rocking for 1 hour. The reaction mixture was centrifuged at 14,000*g* for 5 min at cold room, the supernatant was transferred to a new tube, and mixed with 30 μl protein A Sepharose (Pharmacia, Sweden) slurry (50%, v/v in PBS). The mixture was incubated at room temperature with rocking for 1 hour. After incubation, the beads were collected by centrifugation, and washed 5 times with

buffer containing 2.5% Triton X-100, 0.5% SDS, 0.25 M Sucrose, 0.05 M Tris (pH 8.0), 5 mM EDTA, and 2 times with 10 mM Tris-HCl buffer pH 8.0 and 0.1 M NaCl. The beads was sucked dry, and boiled for 5 min in reducing sample buffer (2% SDS, 0.1 M Tris-HCl, pH 6.8, 10% glycerol, 0.02% bromphenol blue and 5% β-mercaptoethanol) and separated by 10% SDS-PAGE gel. The gel was fixed and dried before exposing to X-ray film with the film being kept at -70 °C overnight. The radioimage was enhanced by using Amplifier (Bio-Rad, USA).

Construction of Plasmids for Two-Hybrid Analysis--The plasmids used in the reports were purchased from Clontech Laboratory Inc. pGBT9 is a 5.4 kb DNA-binding domain hybrid cloning vector, used to generate fusion protein of the UROS with the GAL4 DNA-binding domain. Two primers (sense: 5'-GCCGCCGAATTCATGAAGGTTCTTTTACTG-3', antisense: 5'-GCCGCCGAATTCTCAGCAGCAGCCATGGGG-3') with *EcoR*I restriction site at linker sequences were used to PCR amplify full length UROS cDNA using pKK-UROS as template described previously. The PCR products were digested with *EcoR*I, gel purified and subcloned into the pGBT9 *EcoR*I cloning site. The UROS cDNA is in frame down stream of GAL4 DNA binding domain. The authenticity of this expression construct was confirmed by sequencing. This construct was designated as pGBT9-UROS. pGAD424 is a 6.6 kb activation domain hybrid cloning vector, used to generate fusion protein of the HMBS with GAL4 activation domain. Two primers (sense: 5'-GCCGCCGAATTCATGAGAGTGATTCGCGTG-3', antisense: 5'-GCCGCCGAATTCTTAATGGGCATCTTGAAG-3') with *EcoR*I restriction site at linker

sequences were used to PCR amplify full length HMBS cDNA using pET-HMBSe as template described previously. The PCR products were digested with *EcoR*I, gel purified and subcloned into the pGBT9 *EcoRI* cloning site. The HMBS cDNA is in frame down stream of GAL4 activation domain. The authenticity of this expression construct was also confirmed by sequencing, and the construct was designated as pGADA424-HMBS. pVA3 is a 6.4 kb positive control plasmid which encoding a murine p53/GAL4 DNA-binding domain hybrid in pGBT9. pTD1 is a 15 kb positive control plasmid encoding an SV40 large T-antigen/GAL4 activation domain hybrid in pGAD424. pCL1 is a 15.3 kb positive control plasmid that encodes and expresses the full-length, wild type GAL4 protein. The manipulations of all plasmids were carried out in *E. coli* strain DH5α.

Yeast Strain and Transformation Procedure--All experiments in this study used yeast Y153 (MATa gal4 gal80 his3-200 trp1-901 ade2-101 ura3-52 leu2-3,-112+URA3::GAL-->lacZ, LYS2::GAL-HIS3). Cells were grown in YPD cultures to prepare competent cells. The method for preparation of competent cells and transformation were performed according to the manufacturer's instruction (16, 17). In brief, yeast competent cells were prepared by the lithium acetate method, cotransformation was carried out by incubating 0.1 μg of each type of plasmid DNA, together with 100 μg of salmon sperm carrier DNA, and 100 μl yeast competent cell. 0.6 ml PEG/LiAc solution was added to each tube with gentle mixing, and the mixture was incubated for 30 min at 30 °C with shaking (200 rpm). 70 μl DMSO was the added to the cells which were then subject to heat shock at 42 °C for 15 min. The cells were pelleted and washed with TE once and plated to appropriate selective minimal medium plate. Cotransformation

of pGAD424-HMBS and pGBT9-UROS, pGAD424 and pGBT9, pVA3 and pTD1 were plated on synthetic minimal medium plate with the dropout of leucine and tryptophan as selective markers.

Color Development Assays--Yeast harboring both GAL4 DNA binding domain and transcription activation domain fusion proteins were monitored for β-galactosidase activity using plate assay methods. Yeast transformants were transferred to nitrocellulose filtered, permeabilized in liquid nitrogen, and placed on Whatman No. 1 filter paper that had been soaked in Z-buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM $MgCl_2$, 50 mM β-mercaptoethanol) containing 1.0 mg/ml 5-bromo-4-chloro-3-indolyl β-D-galactoside at 30 °C. Positive colonies appeared in 1 to 10 h.

Results

Immunoprecipitation Using Anti-HMBS with Chemical Cross-linking - In order to determine whether there is physical interactions between HMBS and UROS, polyclonal anti-human HMBS antibodies were used to immunoprecipitate the cell extracts from either HepG2 or K562 cells. The antibodies were raised from rabbits using purified recombinant human housekeeping HMBS described elsewhere. Before immunoprecipitation, the HepG2 and K562 cells were treated with thiocleavable cross-linker, DSP. The control cells were only treated with the solvent without DSP. The data were shown in Figure 5-1. Lane 1 and 2 are results from HepG2 cells, lane 3 and 4 are results from K562 cells. Lane 2 and 4 are cells treated with DSP 0.5 mM before immunoprecipitation, lane 1 and 3 are cells without treatment of DSP. In HepG2 cells, single band approximately 42 kD was resolved, corresponding to the position of housekeeping HMBS. However, there is no band resolved in 29-30 kD position, corresponding to the position of UROS. Same results were found when using K562 cells as experiment system. In K562 cells system, two bands approximately 42 and 40 kD were resolved, corresponding to the housekeeping and erythroid-specific HMBS. This result supports that two isozymes exist in K562 cells, also from the same intensity of the two bands, these two isozymes are approximately the same amount. There is no need to induce the differentiation of K562 cells in order to obtain erythroid-specific HMBS. These experiments did not indicate the presence of protein-protein interactions between HMBS and URO-synthase.

Two-hybrid protein-protein interactions Assay - Since there is no appropriate

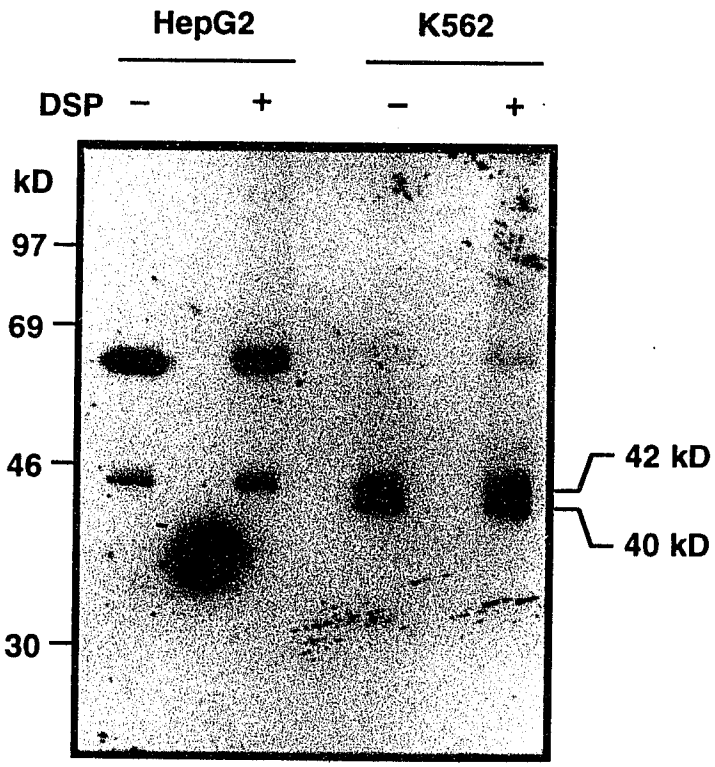


Figure 5-1. Immunoprecipitation assay of HMBS.

positive control in immunoprecipitation studies mentioned above, there is question raised about if the cross-linker, DSP, works appropriately. In order to further clarify the question, we determined to use the two-hybrid system to pursue this question. Human erythroid-specific HMBS cDNA was expressed in conjugation with GAL4 transcription activation domain, UROS was expressed in conjugation with GAL4 DAN-binding domain Figure 5-2. These plasmids with these two fusion constructs were cotransfected to the yeast strain 153, which has a reporter gene *lac*Z with GAL1 promoter. Positive control were yeast cotransformed with pVA3 and pTD1, negative control were cells cotransformed with shuttle vectors, pGAD424, and pGBT9, as described in method section Filter β-galactosidase assay showed the results that positive control colonies turned blue in 2 hours, negative control remained colorless after 30 hours reaction with Z-buffer. The cells cotransformed with HMBS and UROS fusion constructs showed negative results, suggesting no interactions between HMBS and UROS (Figure 5-3).

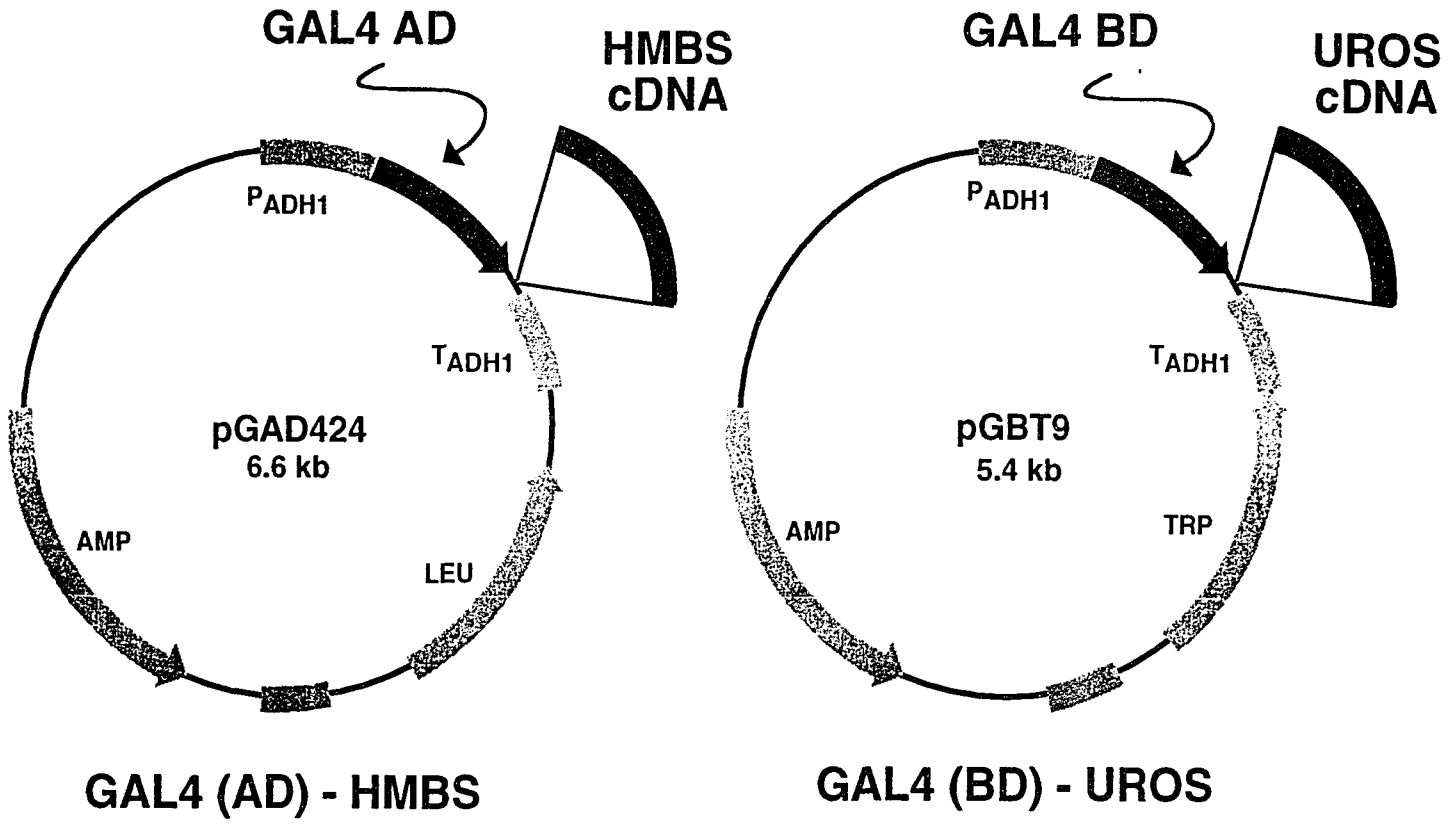


Figure 5-2. Constructs for two-hybrid protein interaction assay.

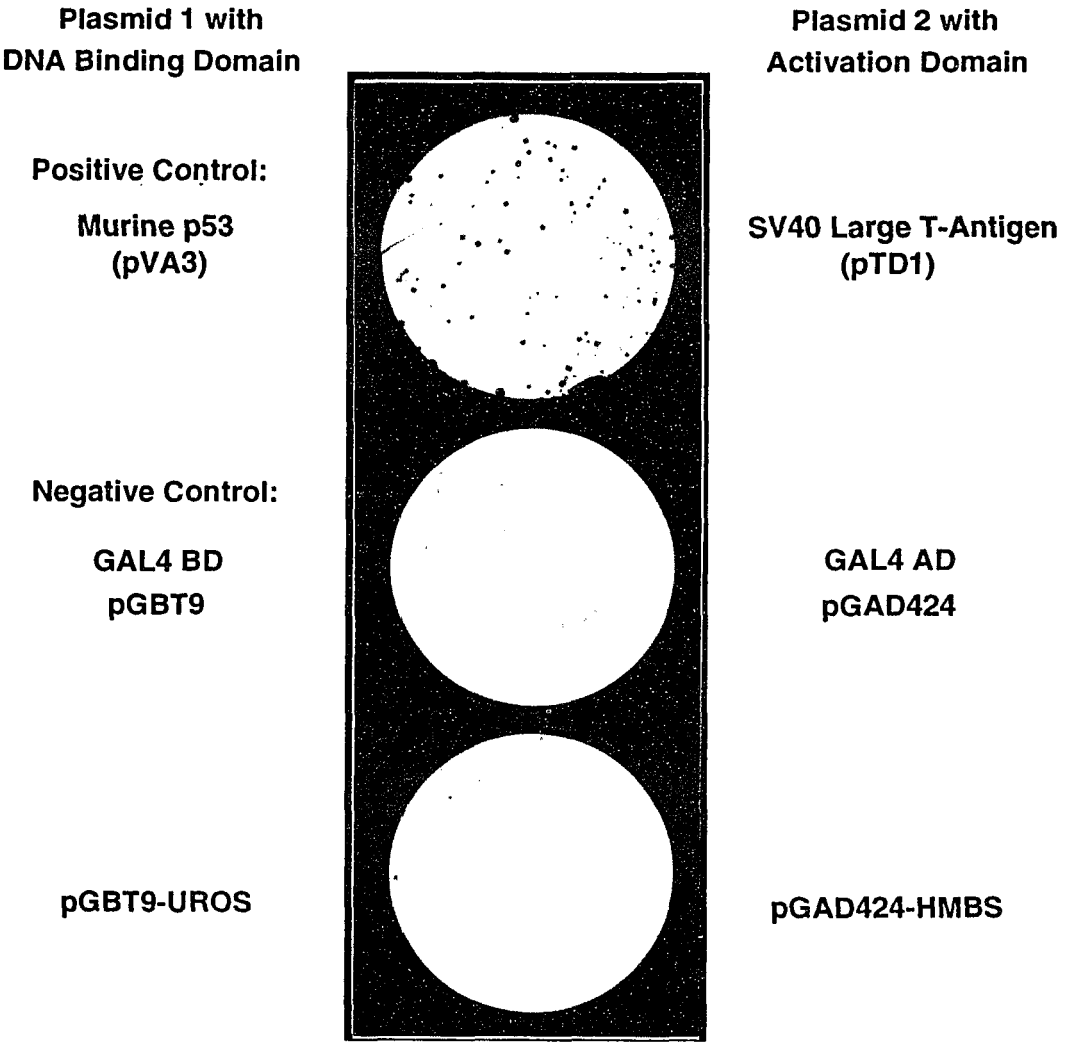


Figure 5-3. Results of two-hybrid protein interaction assay.

Discussion

It is intriguing to speculate that HMBS and UROS may function in a complex, thereby permitting the rapid and efficient conversion of PBG to URO III (18-20), the first cyclic tetrapyrrole and the physiologic precursor of vital heme containing macromolecules including hemoglobins, cytochromes, chlorophylls, and cobalamins. Nevertheless, our results demonstrate that no evidence for such a physical association between HMBS and UROS. In the immunoprecipitation studies, antihuman HMBS polyclonal antibodies precipitated the housekeeping form of HMBS in radiolabeled human hepatocellular carcinoma cells, HepG2. In human erythroleukemic cell lines, K562, two isoforms of HMBS were immunoprecipitated, corresponding to housekeeping and erythroid-specific HMBS. These results indicate the antihuman HMBS antibodies recognize two isoforms of HMBS. In these experiments, thiocleavable homobifunctional crosslinker DSP was used to crosslink the putative enzyme complex. The disulfide bond of DSP can be reduced by thioreducing agents, dithiothreitol (DTT) or β-mercaptethanol, and the crosslinked complex can be resolved by SDS-PAGE. If the HMBS physically associated with UROS, the enzyme complex could be immunoprecipitated and resolved by SDS-PAGE electrophoresis after treating the DSP with reducing agents. However, using anti-HMBS antibodies, we do not find protein corresponding to the position of UROS that coprecipitates with HMBS. In essence, using the coimmunoprecipitation combined with chemical crosslinking approach, we showed no evidence for physical proximity or juxtaposition of HMBS and UROS in human HepG2 and K562 cell cultures..

To further support our immunoprecipitation findings, two-hybrid system for

detecting protein-protein interactions was adapted to explore the presumable interactions between HMBS and UROS. The two-hybrid assay system is based on the fact that the yeast transcriptional factor GAL4 consists of two physically separable molecular domains: one acts as the DNA-binding domain, while the other functions as the transcriptional activation domain. The DNA-binding domain localizes the transcription factor to specific DNA sequences present in the upstream region of genes that are regulated by this factor, while the activation domain contacts other components of the transcription machinery required to initiate transcription. Both domains are required for normal activation function, and normally the two domains are part of the same protein. However, it has been shown that a functional activator can be assembled in vivo from separated domains of the same or unrelated transcription factors via recombinant DNA technology. By using this approach, human erythroid-specific HMBS cDNA was subcloned to a shuttle vector pGAD424, which contains the transcription activation domain in the upstream region of the inserted erythroid-specific HMBS cDNA. This construct generated the fusion protein with transcription activation domain at N-terminal and HMBS at C-terminal. The UROS cDNA was subcloned to another shuttle vector pGBT9, this construct produced fusion protein with DNA-binding domain at N-terminal and UROS at C-terminal. If HMBS and URO-synthase form multienzyme complex, their physical association or positional juxtaposition will reconstitute the transcription activity of GAL4, and trigger the reporter gene lacZ which encodes enzyme β-galactosidase to be expressed. Yeast strain Y153 was cotransfected with these two hybrid plasmids, if the interactions exist, the transformed yeast clones will turn blue by using filter β-galactosidase assay, otherwise there will be

no color detected. By using this method, we could not find blue colonies indicating association between HMBS and UROS. This result supports our previous findings of immunoprecipitation study, there is no evidence suggesting the existence of multienzyme complex between HMBS and UROS.

Two-hybrid system to detect the protein-protein interactions became popular recently and several protein-protein interactions were demonstrated by this method, however, there are still some limitations in its applications. If the two-hybrid assay is negative, it is difficult to determine whether the negative result is false negative. There is no proof to show that the fusion construct still retains the appropriate biological function as its native enzyme. In our experiment, we can not test if the fusion proteins that contain HMBS and UROS still have the appropriate enzymatic activities when they were transfected into the yeast cells. In other words, we are not sure if the fusion proteins fold correctly to retain the normal properties.

There are well-characterized instances of enzymes involved in a metabolic pathway that are organized in multienzyme complexes, and support the metabolites channeling phenomena. These include tryptophan synthase (21), enzymes involved in DNA biosynthesis, glycogen biosynthesis, fatty acid oxidation (6, 22, 23). Such a cellular organization would be efficient in terms of the kinetics and the regulation of the pathway, particularly if certain enzymes are rate limiting or affected by product inhibition. Still, there are many cases that intermediate metabolites from metabolic pathways were transferred to the next enzyme by the mechanism of diffusion. Previously, the existence of the metabolic intermediates channelling effects were indicated by the kinetic

parameters. However, there is some limitations in methods of determining the kinetic parameters, furthermore, the interpretation of these kinetic data is not proof of controversy. Therefore, physical evidence for the protein-protein interactions is more convincing. Nevertheless, in some cases the association is strong, such that the system can be extracted from cells and studied by standard analytic-enzymological methods. For others, however, the interaction is weak (or transient) and readily disrupted by extraction, dilution, etc., in which case physical evidence is difficult. In the case of HMBS and UROS, in our experiments, strong interactions or steady-state interactions find no support. Still, we can not exclude the possibility of dynamic interaction or transient interactions in the reaction of heme biosynthesis.

In summary, our efforts to detect the putative multienzyme complex using immunoprecipitation combined with chemical cross-linking, and using in vivo two-hybrid protein-protein interaction assay, found no evidence for such interactions. Our results support that the linear tetrapyrrole HMB formed by the HMBS proceed to next enzyme, UROS, via diffusion. Although, transient interaction or dynamic channelling can not be excluded from our experiments.

Chapter 6. Conclusions

In this thesis, the following conclusions were obtained:

(1). Cloning and isolation of human HMBS gene. The complete genomic sequences of human HMBS gene were determined, including the identification of additional housekeeping and erythroid-specific promoter elements. This should facilitate further studies of regulation of this novel gene. In addition, four new intragenic polymorphisms were identified, especially the identification of the independently segregating HinfI RFLP, should prove valuable for the diagnosis of presymptomatic AIP heterozygotes in families whose specific HMBS mutations have not been identified.

(2). Six new mutations causing AIP were identified. The identification of the precise molecular lesions in families with AIP permits precise carrier detection. Counseling of family members with mutations in their HMBS gene could prevent the development of attacks of AIP. The identification of a novel M1I mutation demonstrates a new genetic heterogeneity causing variant AIP. Of interest, aberrant HMBS transcripts due to abnormal splicing occurred frequently, resulting in decreased active enzyme in AIP heterozygotes and normal individuals. These mutations provide insight into the molecular pathology of classic and variant AIP.

(3). Both housekeeping and erythroid-specific HMBS have been purified using recombinant DNA techniques combined with immobilized metal affinity chromatography (IMAC). The physical and kinetic properties of the purified HMBS were characterized,

there is no obvious difference detected in comparison with the HMBS from human erythrocytes. The simple and quick method of obtain large amount of pure HMBS should facilitate the crystallization of this enzyme, also, should be helpful in studying the structural and functional aspects of this enzyme.

(4). Evidences for lack of physical association or positional proximity between HMBS and UROS were demonstrated using immunoprecipitation approach and two-hybrid protein-protein interactions assay system. The result is contrary to previous suggestion that these two enzymes may form multienzyme complex in the cytoplasm of cell. Our experiments also support that the formation of uroporphyrinogen III from the precursor hydroxymethylbilane proceeds via diffusion mechanism, rather than metabolite channeling.

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