MOLECULAR GENETICS OF UROPORPHYRINOGEN III SYNTHASE AND CONGENITAL ERYTHROPOIETIC PORPHYRIA

by

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ABSTRACT

MOLECULAR GENETICS OF UROPORPHYRINOGEN III SYNTHASE AND CONGENITAL ERYTHROPOIETIC PORPHYRIA

by

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Uroporphyrinogen III synthase (URO-S; EC 4.2.1.75), the fourth enzyme in the heme biosynthetic pathway, is responsible for the conversion of hydroxymethylbilane to the cyclic tetrapyrrole, uroporphyrinogen III, and deficient URO-S is the enzymatic defect in congenital erythropoietic porphyria (CEP). Studies were undertaken to characterize the molecular genetics of UROS and the nature of the genetic defects which cause CEP. These studies included: 1) identification of UROS mutations in unrelated CEP patients which established the molecular genetic heterogeneity of this disease; 2) isolation of the murine counterpart of the human UROS cDNA and recognition of two murine and two human UROS transcripts by alternative splicing from the same gene, and 3) further characterization of the genomic structure, including the first identification of a regulatory mutation in two human CEP patients.

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Chapter 1. Introduction

1. Heme Biosynthesis:

Heme is central to biological oxidation processes and is also an essential component of oxygen carriers (Moore et al., 1987). It is the prosthetic group of various proteins (e.g., myoglobin, hemoglobin, catalase, peroxidase, and cytochrome C), and is important to a variety of physiological functions. Deficiencies in the heme biosynthetic pathway result in a series of metabolic defects known as porphyrias (Moore et al., 1987; Kappas et al., 1989; Jordan, 1990). The heme biosynthetic pathway is shown in Figure 1. While all the eight enzymes involved in the pathway are encoded by nuclear genes, the first and last three function in the mitochondria, and the rest four are cytosolic. Except for protoporphyrinogen oxidase, the full-length cDNAs encoding all the other seven human heme biosynthetic enzymes have been isolated (Raich et al., 1986; Romeo et al., 1986; Wetmur et al., 1986; Grandchamp et al., 1987; Bishop 1990; Tsai et al., 1988; Nakahashi et al., 1990; Bawden et al., 1992; Martasek et al., 1994) and the corresponding genes have been mapped to respective chromosomes (Wang et al., 1981; Grandchamp et al., 1983; Wang et al., 1985; Dubert et al., 1986; potluri et al., 1987; Sutherland et al., 1988; Bishop et al., 1990; Astrin et al., 1991; Namba et al., 1991; Whitcombe et al., 1991; Taketani et al., 1992; Brenner et al., 1992).

The first step in the biosynthesis of heme is the condensation of glycine with an activated succinyl coenzyme A to form δ-aminolevulinate (ALA). The reaction is catalyzed by δ-aminolevulinate synthase (ALAS), a

pyridoxal phosphate requiring enzyme. This is the rate-limiting step and is tissue-specifically regulated. Erythroid-specific and non-erythroid house-keeping forms of ALA-synthase are encoded by separate genes (Bawden et al., 1987; Bishop, 1990), and two isoforms of the erythroid-specific enzyme are encoded by alternatively spliced transcripts from the same gene (Conboy et al., 1992). Decreased erythroid-specific ALA-synthase activity has been reported in several types of sideroblastic anemia (Aoki et al., 1979; Bttomley 1992) and mutations in the erythroid-specific gene have been identified in patients with pyridoxine responsive X-linked sideroblastic anemia (Cotter et al., 1992; Cotter et al., 1994).

Two molecules of δ-aminolevulinate then condense to form the pyrrole derivative porphobilinogen (PBG). This dehydration reaction is catalyzed by δ-aminolevulinate dehydratase. Eight identical subunits combine to form the active enzyme . Zinc binds to the enzyme at the ratio of eight ions per octamer and is needed both for optimum activity and for the structural role which facilitates the enzymatically active confirmation. Erythroid-specific and house-keeping transcripts with mutually exclusive exon 1 but identical coding sequence were generate by utilization of two distinct promoters and alternative splicing mechanism. Deficient ALA-dehydratase activity has been shown to be the enzymatic defect in inherited hepatic porphyria.

The formation of uroporphyrinogen III from four molecules of porphobilinogen consists of two steps, catalyzed by hydroxymethylbilane synthase (HMB-synthase) and uroporphyrinogen III synthase (URO-

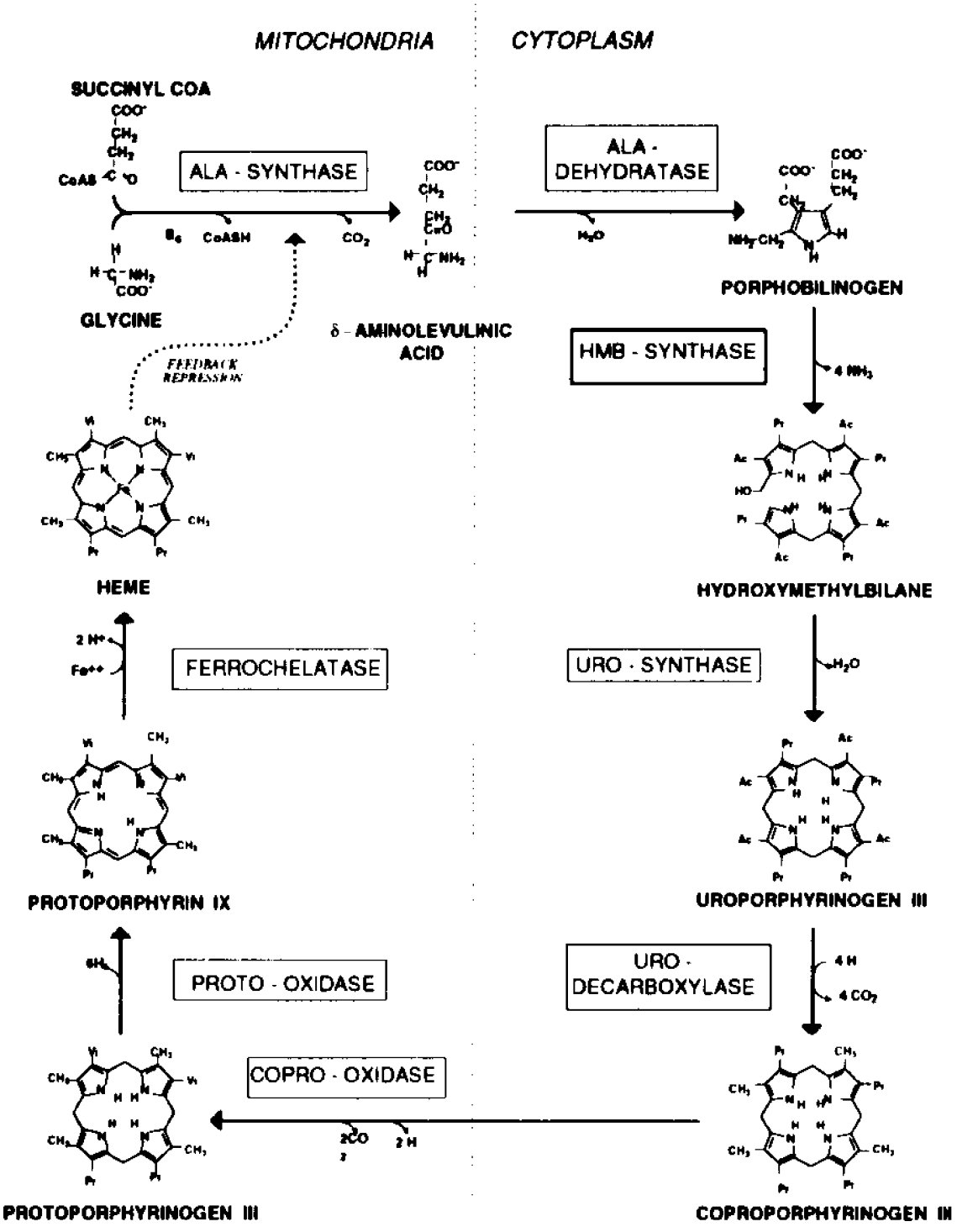

Figure 1-1. Current Concept of Human Heme.

synthase), respectively. Four PBG molecules condense head-to-tail sequentially by a series of deamination steps to form the linear tetramer, hydroxymethylbilane, which then rearranges and cyclizes to form the asymmetric porphyrinogen. Both erythroid and house-keeping forms of HMB-synthase isozymes are monomers and are encoded by two alternatively spliced transcripts from a single gene. In acute intermittent porphyria, half-normal HMB-synthase activity leads to excess accumulation of PBG. The erythroid and hepatic forms of URO-synthase has been found to be identical (Tsai et al., 1988). The genomic organization of URO-synthase has not been reported. In congenital erythropoietic porphyria, URO-synthase activity is markedly deficient, leading to the spontaneous formation and accumulation of photo-toxic I series porphyrin isomers. HMB-synthase and URO-synthase may exist as a cytosolic enzyme complex (Tsai et al., 1988).

The skeleton of porphyrin has been produced by this step. In the subsequent steps, the side chains and degree of saturation of the porphyrin ring are changed by a series of carboxylations and oxydations to form proto-porphyrin. The first four decarboxylation steps are catalyzed by uroporphyrinogen decarboxylase (URO-decarboxylase), the fifth enzyme in the pathway. In the reaction 4 carboxyl groups are removed from the octacarboxylic uroporphyrinogen to form the tetracarboxylic coproporphyrinogen. Although URO-decarboxylase is not stereo-specific and catalyzes the decarboxylation of both series I and series III isomers, series III isomer is preferred over series I isomer as substrate (Smith and Francis, 1979, 1981). URO-decarboxylase has been purified to homogeneity from human erythrocytes and was found to be a monomeric

protein with a molecular weight of 42 kDa (de Verneuil et al., 1983). A single species of mRNA encoding URO-decarboxylase has been found till now, suggesting that there are no tissue-specific isozymes (Romeo et al., 1986a, b). Deficient URO-decarboxylase activity is the enzymatic defect in porphyria cutanea tarda and hepatoerythropoietic porphyria (de Verneuil et al 1978; Elder et al., 1981).

Coproporphyrinogen oxidase (COPRO-oxidase), which is located between the inner and outer membrane of mitochondria (Elder and Evans, 1978; Grandchamp et al., 1978), catalyzes the oxidation and decarboxylation of two propionyl groups, on ring A and B, respectively, of coproporphyrinogen to two vinyl groups to yield the dicarboxyl porphyrinogen protoporphyrinogen IX. COPRO-oxidase can only use molecular oxygen as hydrogen acceptor (Sano and Granick, 1961). The enzyme can use the series III and IV isomers of coproporphyrinogen as substrate, but not the series I and II isomers (Batlle et al., 1965; Porra and Falk, 1964). COPRO-oxidase has been purified from bovine liver and has been shown to be a monomeric protein with a molecular weight of 71.6 kDa (Yoshinaga and Sano, 1980). The mouse and human cDNAs encoding COPRO-oxidase have been isolated and sequenced (Kohno et al., 1993; Martesek et al., 1994). The half-normal activity of COPRO-oxidase is the enzymatic defect in hereditary coproporphyria (HCP) (Elder et al., 1976). Homozygous forms of HCP and harderoporphyria (a variant of HCP) are associated with more severe deficiencies of COPRO-oxidase activity (Grandchamp and Nordmann 1977; Nordmann et al 1983).

The next step in the pathway is the oxidation of protoporphyrinogen IX to protoporphyrin IX, and is catalyzed by protoporphyrinogen oxidase (PROTO-oxidase). The enzyme is embedded in the inner membrane of mitochondria (Deybach et al., 1985). The enzyme is inhibited by the presence of bilirubin, as demonstrated in Gilbert's syndrome and the Gunn rat (McColl et al., 1985, 1986). The murine PROTO-oxidase has been purified and shown to be a monomeric protein with a molecular weight of 65 kDa (Dailey and Karr, 1987). The half-normal activity of this enzyme is the enzymatic defect in variegate porphyria (Brenner and Bloomer, 1980).

The final step in the heme biosynthetic pathway, the insertion of ferrous form of iron into protoporphyrin IX to form heme, is catalyzed by ferrochelatase (Goldberg et al., 1956). This enzyme is firmly attached to the inner side of the inner mitochondrial membrane (McKay et al., 1969; Jones and Jones, 1969). The bovine ferrochelatase has been purified and has been shown to have a monomeric molecular weight of about 40 kDa (Dailey and Fleming, 1983). The enzyme may function as a dimer (Straka et al., 1991). The full-length cDNA encoding the human ferrochelatase has been isolated and sequenced (Nakahashi et al., 1990). Deficient activity of ferrochelatase is the enzymatic defect in erythropoietic protoporphyria (Bonkowsky et al., 1975; Bottomley et al., 1975).

2. Congenital Erythropoietic Porphyria:

Congenital erythropoietic porphyria (CEP), a rare autosomal recessive disease, also known as Günther's disease, is the first human porphyria to be related to a specific enzymatic defect (Romeo and Levin,

1969). A characteristic abnormality of the disease is a more than 80% decrease in the activity of uroporphyrinogen III synthase, the fourth enzyme of the heme biosynthetic pathway (hydroxymethylbilane hydrolase [cyclizing]; EC. 4.2.1.75.) (Romeo and Levin, 1969; Deybach et al., 1981). Uroporphyrinogen III is the first cyclic tetrapyrrole in the pathway (Battersby et al., 1990). Although the mechanism of uroporphyrinogen III formation is still not fully understood (Battersby et al., 1990; Batlle and Rossetti, 1977; Rossetti et al., 1980), it is known that under the catalysis of URO-synthase, hydroxymethylbilane is quickly turned over to uroporphyrinogen III isomer by ring closure and intramolecular rearrangement of ring D. In CEP patients, the enzymatic defect results in the accumulation of hydroxymethylbilane that is nonenzymatically converted to uroporphyrinogen I isomer (Fig. 2) (Tsai et al., 1987a; Battersby et al., 1979, 1982a, 1982b, 1983; Jordan et al., 1979; Anderson and Desnick et al., 1980), which is then oxidized to uroporphyrin I (URO I), a nonphysiologic and photo-toxic compound (Kappas et al., 1989). The accumulation of URO I in erythrocytes leads to hemolysis, and the released porphyrin I isomer is deposited in tissues and bones and excreted in the urine and feces. URO I is a photocatalytic compound, and exposure of the skin to sunlight, other forms of ultraviolet light, or trauma results in blistering and vesicle formation (Bickers et al., 1987). Ruptured vesicles are prone to secondary infection. URO I also causes blood vessel damage which lead to local ischemia and tissue necrosis (Moore et al., 1987). The disease is phenotypically heterogeneous: severely affected patients are transfusion-dependent, and have secondary hypersplenism and

disfiguring cutaneous involvement, whereas mildly affected patients may

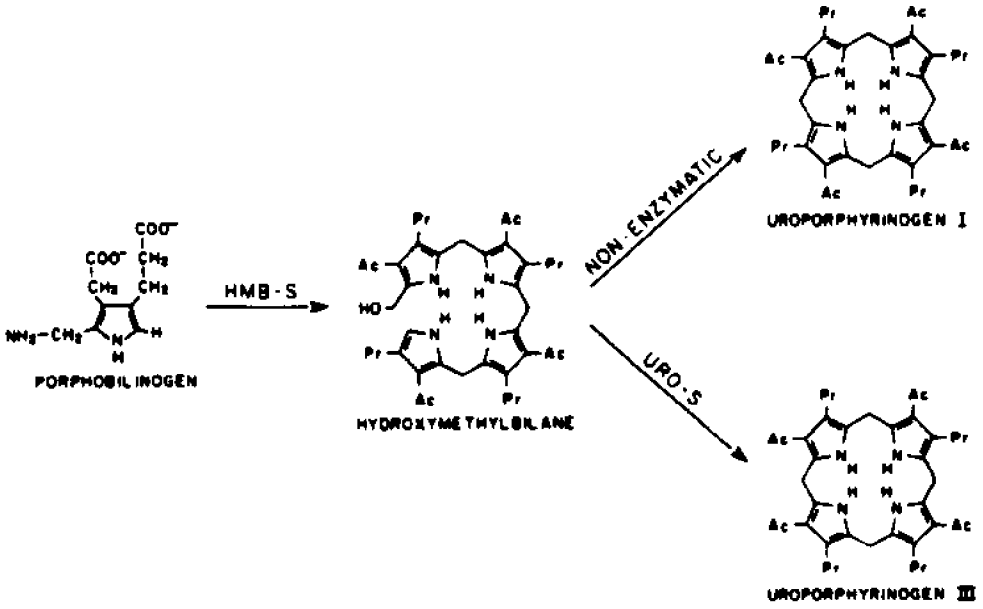

Figure 1-2. The enzymatic defect in congenital erythropoietic porphyria (Courtesy of Dr. K.H. Astrin).

have only cutaneous involvement (Nordmann and Deybach, 1982). Later-onset adult patients with mild disease symptoms have also been described (Deybach et al., 1981; Horiguchi et al., 1989).

Since heme is essential for survival, it is expected that the two mutant alleles in CEP patients should expresses sufficient URO-synthase activity to maintain the minimal level of heme biosynthesis required for life. It has been hypothesized that the amount of residual activity might determine the severity of the phenotype. In other words, while environmental factors and genetic backgrounds could affect the phenotype of the disease, the difference in URO-synthase residual activity may contribute significantly to the observed phenotypical heterogeneity of CEP (Desnick and Anderson, 1991). This hypothesis is consistent with the results of mutation analysis

among CEP patients, which has shown that the molecular lesions causing CEP are heterogeneous (Warner et al., 1992; Xu et al., 1993), and characterization of the CEP mutations on the mutant URO-synthase will provide insight into the possible correlation between genotype and phenotype in CEP.

3. Molecular Biology Of URO-Synthase:

Human URO-synthase has been purified to homogeneity from erythrocytes, and shown to be a monomeric protein with an apparent molecular weight of 29.5 kD (Tsai et al., 1987b). The purified enzyme had a specific activity of over 300,000 units/mg, an isoelectric point of 5.5, and was remarkably thermolabile ($t_{1/2}$ at 60 °C ~ 1 min). Molecular weight estimates of URO-synthase were ~30,000 and 29,500 by gel filtration and analytical SDS-PAGE, respectively, indicating that URO-synthase was a monomeric enzyme (Tsai et al., 1987b). Using hydroxymethylbilane as substrate, the purified enzyme formed uroporphyrinogen III in the absence of hydroxymethylbilane synthase or other cofactors. The pH *versus* activity curve for URO-synthase was broad, with optimal activity observed at about 7.4 (Tsai et al., 1987b). The *K*m for hydroxymethylbilane was 5-20 μM. The enzyme was activated by Na^+, K^+, Mg^{2+}, and Ca^{2+} and was inhibited by Cd^{2+}, Cu^{2+}, Hg^{2+}, and Zn^{2+} (Tsai et al., 1987b). Like all human cytosolic heme biosynthetic enzymes, URO-synthase was strongly inhibited by p-Chloromercuribenzoate (pCMB) and enhanced by the presence of thiol reducing agents such as Dithiothreitol (DTT), implicating the importance of reduced cysteine residue(s) in the active sites (Tsai et al., 1987b).

The full-length cDNA encoding the human URO-synthase polypeptide of 265 amino acids has been isolated, sequenced, and expressed in *Escherichia coli* (Tsai et al., 1988). Using the cDNA as a probe, a single URO-synthase gene has been regionally assigned to a narrow region of chromosome 10q25.3 → q26.3 (Astrin et al., 1991). Genomic clones have been obtained, and it has been shown that the ~42 kb URO-synthase gene has ten exons, with only exon one being a noncoding exon. All exon-intron boundaries have been determined (Warner et al., 1990).

The catalytic efficiency of URO-synthase is remarkably high (Jordan et al., 1980). The turnover number for URO-synthase is about 30 to 250 times greater than those for the other three cytosolic heme biosynthetic enzymes. To account for such rapid conversion, it has been hypothesized that HMB-synthase and URO-synthase may exist in a complex so that hydroxymethylbilane, product of the first enzyme, can be directly transferred to the second enzyme as substrate (Bogorad, 1958; Hart and Battersby, 1985; Sancovich et al., 1969; Llambias and Batlle, 1971; Frydman and Feinstein, 1974; Higuchi and Bogorad, 1975). Moreover, it has been suggested that all the four cytosolic enzymes may function in a complex, thereby permitting the rapid and efficient conversion of δ-aminolevulinic acid, 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 enhance the efficiency of the sequential reactions and prevent the porphyrinogen intermediates from being oxidized to nonmetabolizable and potentially toxic porphyrins (Tsai et al., 1987b).

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Chapter 2

CONGENITAL ERYTHROPOIETIC PORPHYRIA: IDENTIFICATION AND EXPRESSION OF TEN MUTATIONS IN THE UROPORPHYRINOGEN III SYNTHASE GENE

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Abstract

To investigate the molecular basis of the phenotypic heterogeneity in congenital erythropoietic porphyria (CEP), the mutations in the uroporphyrinogen III synthase (URO-synthase) gene from unrelated patients were determined. Six missense (L4F, Y19C, V82F, V99A, A104V, and G225S), a nonsense (Q249X), a frameshift (633insA), and two splicing mutations ($IVS2^{+1}$ and $IVS9^{\Delta A+4}$) were identified. When L4F, Y19C, V82F, V99A, A104V, 633insA, G225S, and Q249X were expressed in *E. coli*, only the V82F, V99A, and A104V alleles expressed residual enzymatic activity . Of note, the V82F mutation, which occurs adjacent to the 5' donor site of intron 4, resulted in about 54% aberrantly spliced transcripts with exon 4 deleted. Thus, this novel exonic single base substitution caused two lesions, a missense mutation and an aberrantly spliced transcript. Of the splicing mutations, the $IVS2^{+1}$ allele produced a single transcript with exon 2 deleted, whereas the $IVS9^{\Delta A+4}$ allele was alternatively spliced, about 26% being normal transcripts and the remainder with exon 9 deleted. The amount of residual activity expressed by each allele provided a basis to correlate genotype with disease severity, thereby permitting genotype/phenotype predictions in this clinically heterogeneous disease.

Introduction

Congenital erythropoietic porphyria (CEP), also known as Günther disease, is an inborn error of heme biosynthesis resulting from the markedly deficient activity of the fourth enzyme of the heme biosynthetic pathway, uroporphyrinogen III synthase (URO-synthase; EC 4.2.1.75), and the accumulation of the non-physiologic uroporphyrin I (URO I) isomer (Kappas et al 1989, Desnick & Anderson 1991, Romeo & Levin 1969). In patients with this autosomal recessive disease, the clinical manifestations are markedly heterogeneous, ranging from non-immune hydrops fetalis to milder, later-onset forms which have only cutaneous lesions in adult life (Kappas et al 1989;, Desnick & Anderson 1991, Romeo & Levin 1969, Nordmann & Deybach 1982, Deybach et al 1981, Horiguchi et al 1989). Severely affected patients are transfusion-dependent throughout life, have secondary hypersplenism and are usually disfigured due to the cutaneous involvement. Additional manifestations may include hypertrichosis, alopecia and erythrodontia. Although milder patients have some residual activity to synthesize sufficient heme for hematopoiesis and for other essential heme-requiring proteins, the amount of residual activity appears to be correlated with clinical severity (Warner et al 1990, Warner et al 1992).

URO-synthase normally catalyzes the conversion of the linear tetrapyrrole, hydroxymethylbilane (HMB), to uroporphyrinogen III, the physiologic cyclic isomer which is metabolized in subsequent enzymatic steps to heme (Battersby et al 1982a, b, Figure 2-1). The markedly deficient activity of URO-synthase results in the non-enzymatic conversion of HMB to the uroporphyrinogen I isomer, which is then oxidized to the non-physiologic and

pathogenic compound, uroporphyrin I (URO I). Excessive URO I in erythrocytes leads to hemolysis, and the released porphyrin isomer is deposited in tissues and bones and is excreted in the urine and feces. Light activates the photocatalytic URO I resulting in tissue damage and the formation of bullous lesions which rupture, often becoming infected leading to bone resorption and cutaneous deformity (Moore et al 1987, Poh-Fitzpatrick 1986, Kramer et al 1965, Kaufman et al 1967).

Human URO-synthase has been purified to homogeneity from erythrocytes and shown to be a monomeric protein with an apparent molecular weight of 29.5 kD (Tsai et al 1987)). Recently, the full-length cDNA encoding the human URO-synthase polypeptide of 265 amino acids was isolated, sequenced, and expressed in *Escherichia coli* (Tsai et al 1988). Using the cDNA as a probe, a single URO-synthase gene was assigned to the narrow chromosomal region, 10q25.3 → q26.3 (Astrin et al 1991). Analysis of the URO-synthase genomic sequence revealed 10 exons, and the sequence of each exon-intron junction has been determined (Warner et al 1990). The availability of the URO-synthase cDNA permitted investigation of the molecular lesions causing CEP. To date, six missense mutations (Warner et al 1990, Warner et al 1992, de Verneuil et al 1989, Deybach et al 1990, Boulechfar et al 1992), an 80 bp insertion, and a 98 bp deletion (del148-245) (Boulechfar et al 1992) have been identified in unrelated CEP patients. Of note, only the C73R lesion was common, occurring in about one-third of the CEP alleles studied, whereas the other mutations were detected only in one to three unrelated families (Warner et al 1990, Warner et al 1992, de Verneuil et al 1989, Deybach et al 1990, Boulechfar et al 1992). Each of the missense mutations have been expressed in *E. coli* (Warner et al 1992, Boulechfar et al 1992), and several were found to

have significant residual activity which appeared to correlate with milder phenotypes (Warner et al 1990, Warner et al 1992, Boulechfar et al 1992).

In this communication, a rapid sequencing technique was used to analyze all ten exons from 20 unrelated CEP patients. Ten new mutations (L4F, Y19C, V82F, V99A, A104V, G225S, Q249X, 633insA, IVS2^{+1}, and IVS9$^{\Delta A+4}$) and four of the previously reported missense mutations were identified. Expression of the new missense, nonsense, and frameshift mutations revealed that only V82F, V99A, and A104V had detectable residual activity, and that IVS2^{+1}, IVS9$^{\Delta A+4}$, and V82F altered splicing of the normal transcript. Notably, the levels of residual activity expressed by both mutant alleles were correlated with disease severity. These studies document the molecular heterogeneity in CEP and demonstrate the usefulness of expression studies for genotype/phenotype correlations.

Methods

Patient specimens and severity classification. Peripheral blood samples were collected from 20 unrelated CEP patients and their family members with informed consent. Lymphoid cell lines were established using cyclosporin A and Epstein-Barr virus as previously described (Anderson et al 1984). Cells were maintained by standard procedures in RPMI 1640 media supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin, and 1 mg/ml of streptomycin (Gibco Laboratories, Grand Island, NY). Patients who developed non-immune hydrops fetalis and/or were transfusion-dependent at any age were classified as severe. Transfusion-independent patients were classified as moderate or mild depending on their age, hematologic indices, splenic size, and/or extent and severity of their cutaneous lesions.

Detection of URO-synthase mutations. Genomic DNA was extracted from lymphoblasts (Sambrook et al 1989) and each exon, including its intron/exon boundaries, was amplified by the polymerase chain reaction (Saki et al 1993) using primer sets in which one primer was biotinylated as indicated in Table 2-I. Each 50-ul amplification reaction contained 2 ug of genomic DNA, 20 pmol of each primer, 10 nM of each dNTP, 50 mM Tris-HCl, pH 9.0, 50 mM NaCl, 10 mM $MgCl_2$, and 2 units of *Taq* polymerase (Promega Biotec). After an initial 5 min incubation at 94 °C, amplification (30 cycles) was performed with denaturation at 94 °C for 1 min, extension at 72 °C for 0.5 min, and annealing at the indicated conditions for each primer set (Table 2-I). An aliquot (40 ul) of each amplification product was incubated with 40 ul of streptavidin-coated paramagnetic beads (Dynabeads M-280 Streptavidin, Dynal, Inc., Lake

Success, NY) for 30 min with occasional gentle mixing. Beads with bound biotinylated PCR products were separated with a magnet as described (Dynabeads 1993, Figure 2-2), the strands were denatured in 10 ul of 0.1 M NaOH for 30 min, and the non-biotinylated strands were eluted with two 50 ul washes of 0.1 M NaOH. The biotinylated strands were washed with 50 ul of the binding & washing buffer (10 mM Tris-HCl, pH 7.5, containing 1.0 mM EDTA and 2.0 M NaCl) and then with 50 ul of TE. The biotinylated strands were resuspended in 7 ul of H_2O and used as templates for dideoxy chain sequencing (Sanger et al 1977). The sequencing gels were loaded with the C (and then T, A, and G) reactions from a series of patients in adjacent wells so that the nucleotide sequence could be rapidly read by pattern recognition and mutations could be easily identified (see Fig. 2-3).

Prokaryotic expression and characterization of URO-synthase mutations. The normal and mutant URO-synthase alleles were expressed in *E. coli* using the pKK223-3 vector (Pharmacia LKB Biotechnology, Inc., Piscataway, NJ) as previously described (Warner et al 1992, Tsai et al 1988). pKK-UROS-V99A, pKK-UROS-A104V, pKK-UROS-633insA, and pKK-UROS-G225S were constructed by cassette substitution of the cloned mutant cDNA into the pKK-UROS vector (Warner et al 1992), while the pKK-UROS-L4F, pKK-UROS-Y19C, pKK-UROS-V82F, pKK-UROS-Q249X constructs were engineered by mega-primer PCR mutagenesis (Gobinds et al 1990) and then were cassette-substituted into the pKK-UROS vector.

For construction of pKK-UROS-V99A, antisense primer CW153 corresponding to nt 385 to 294 of the URO-synthase cDNA with the underlined T to C transition at nt 296 (5'-tatattctgcaagcttttctgcatttccacaggtttctccttctgtatccagg

ccaattttactcactagaagcagtagcatttccagcc-3') and sense primer CW71 corresponding to nt 381 to 357 of URO-synthase cDNA (5'-ttctgcaagcttttctgcatttcca-3') were used to amplify pKK-UROS. The amplification product was digested with *Hind*III and the 225-nt *Hind*III fragment was used as a cassette to replace the corresponding fragment in pKK-UROS. pKK-UROS-A104V was similarly constructed except that primer CW155, which corresponded to nt 385 to 310 with the underlined C to T transition at nt 311 (5'-tatattctgcaagcttttctgcatttccacaggtttctccttctgtatccaggccaattttactcactagagaaac-3'), was used in place of primer CW153. For pKK-UROS-633insA, sense primer CW131 (5'-ggcgcggaattcccggggatccgtcgacctgcagccaagctgtcccgcgagtgccc-3'), which corresponded to 35 bp of the polycloning site of the pKK223-3 vector, the adjacent 15 bp of the URO-synthase cDNA and an additional 6 bp non-pKK-UROS sequence at its 5' end, was used with antisense primer WX11 (5'-aacttaatttgatcgatattgtcaccagattaactcctgaatg-3'), which correspond to nt 642 to nt 621 of URO-synthase cDNA and had the underlined insertion of an A after nt 633, was used to amplify pKK-UROS. The amplification product was digested with *Cla*I and then *Eco*RI, and the 717 nt fragment was cassette inserted into pKK-UROS. pKK-UROS-G225S was made by replacing the *Cla*I- *Bsu*36I fragment with a cassette generated by amplification of pKK-UROS using sense primer WX12, corresponding to nt 628 to 673 with the underlined G to A transition at nt 673 (5'-gagttatctggtgacaatatcgatcaaattaagtttgcagccatca-3'), and antisense primer WX18 (5'-accgcttctgcgttctgatt-3') which annealed to the pKK223-3 vector sequence downstream of the *Hind*III subcloning site.

The mega-primer procedure (Gobinds et al 1990) was used to engineer the following expression constructs. For pKK-UROS-L4F, the sense primer CW131 and antisense primer CW71 were used to amplify a 422 nt URO-

synthase cDNA sequence containing the L4F mutation. The 422 nt PCR product was gel purified and used as a "mega-primer" to amplify the URO-synthase cDNA with antisense primer WX15, which corresponded to nt 728 to 701 of the URO-synthase cDNA (5'-gcagtgcagcttacaggaaggccctggg-3'). The final 769 nt PCR product was digested with *Cla*I and *Eco*RI, and the resulting 717 nt fragment was cassette inserted into pKK-UROS. For pKK-UROS-Y19C, sense primer CW131 and antisense primer CW163, which corresponded to nt 102 to 55 with the underlined A to G transition at nt 56 (5'-agggatcaaagtggcttcaagtccatataatcctaattccctga-tac-3'), were used to amplify the URO-synthase cDNA to generate the mega-primer, which was then used to amplify the URO-synthase cDNA with antisense primer WX15. The final PCR product was digested with *Cla*I and *Eco*RI, and the 717-nt fragment was cassette inserted into pKK-UROS. For pKK-UROS-V82F, the megaprimer was generated by amplification of URO-synthase cDNA with sense primer WX4, which corresponded to nt 222 to 246 with the underlined G to T transversion at nt 244 (5'-ggagcagcaaaacaataaaactgaattc-3') and antisense primer CW71. The mega-primer was used with sense primer CW131 to amplify the URO-synthase cDNA, and the 422 nt product was digested with *Hind*III. The liberated 225-nt fragment was then cassette inserted into pKK-UROS.

For expression, each construct was used to transform of *E. coli* strain JM109. Single colonies were isolated and the inserts were completely sequenced to confirm the engineered sequence and the absence of PCR errors. Bacterial growth, isopropylthiogalactoside induction, and URO-synthase assays were performed as previously described (Warner et al 1992, Tsai et al 1988). For enzyme stability studies, samples from the bacterial lysates, equalized for enzymatic activity, were incubated at 37 °C in the presence of 0.5

mg/ml of bovine serum albumin (Sigma Chemical Co.) for 60 min. Aliquots were removed at timed intervals, placed on ice, and the URO-synthase activity was determined by the coupled assay (Tsai et al 1988).

Analysis of splicing mutations. To determine the presence of stable, aberrantly spliced transcripts for the IVS2^{+1}, IVS9$^{\Delta A+4}$, and V82F alleles, mRNA was isolated from the respective lymphoid cell line using the FastTrack^{TM} mRNA Isolation Kit version 3.6 (Invitrogen, San Diego, CA) and reverse-transcribed with M-MuLV reverse transcriptase (RT) according to manufacturer's instructions (New England Biolabs, Inc., Beverly, MA), using as primer the antisense oligonucleotide CW129 (5'-atgcctggctccatccag-3'), which is complementary to nt 866 to 849 of the URO-synthase cDNA. The 937 bp URO-synthase RT product was then PCR-amplified (24) with sense primer WX169 (5'-gcagccttttcgctgggactgcgcgacacc-3') and antisense primer WX174 (5'-atggggccagcgctaggtggctgac-3'), which annealed to nt -114 to nt -84 and nt 823 to nt 799 of the URO-synthase cDNA, respectively. The amplification reaction (100 µl) contained 2 ml of the URO-synthase RT-PCR product, 100 pmol of each primer, 10 nmol of each dNTP, 50 mM Tris-HCl, pH 9.0, 50 mM NaCl, 10 mM $MgCl_2$, and 2 units of *Taq* polymerase (Promega Biotec.) After an initial 5 min incubation at 94 °C, 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 RT-PCR products were electrophoresed in 1 % low melting point agarose gels. The normal and abnormally-sized bands were gel purified using the Wizard^{TM} PCR Preps DNA purification system (Promega Biotec) and sequenced with an Exo(-) *Pfu* Cyclist^{TM} DNA Sequencing Kit (Stratagene, La Jolla, CA), according to the respective manufacturer's instructions.

The relative amount of the normally and abnormally-spliced transcripts for the IVS2^{+1}, IVS9$^{\Delta A+4}$ and V82F alleles were determined as follows. For the IVS2^{+1}/L4F genotype, the URO-synthase RT-PCR products were amplified using antisense primer WX3 (5'-caggatgagaaagcttctca-3'), which anneals to nt 141 to 160 of URO-synthase cDNA, and sense primer WX173 (5'-tgccctataaggactgccaggcaataatgaaggtc-3'), which annealed to nt -26 to nt 9 of URO-synthase cDNA, and contained the underlined T to C mismatch that created an *Ava*II restriction site in the PCR product derived from the normal transcript. The 186 bp PCR product was gel purified as above, digested with *Ava*II (New England Biolabs, Inc., Beverly, MA), and electrophoresced in a 2% agarose gel. For the IVS9$^{\Delta A+4}$/IVS9$^{\Delta A+4}$ genotype, the RT-PCR reaction was performed as described above using sense and antisense primers WX169 and WX174, respectively, except that 10 mCi of a-^{32}P-dCTP was added to each amplification reaction. The normal and abnormal bands were individually gel purified, the mean radioactivity of each (normalized for the number of cytosine residues) was determined using a LKB 1219 RackBeta Liquid Scintillation Counter (Pharmacia LKB, Gaithersburg, MD). For the L4F/V82F genotype, the URO-synthase RT-PCR product was amplified in the presence of 10 mCi of a-^{35}S-dATP with sense primer WX19 (5'-ttatatggacttgaagccac-3') and antisense primer WX17 (5'-ggttcccttggattcctggg-3'), which anneal to nt 73 to nt 92 and nt 519 to nt 538 of the URO-synthase cDNA, respectively. The radioactive PCR products were purified as above, digested with *Eco*RI (New England Biolabs, Inc., Beverly, MA), which recognizes only the V82F allele, and then electrophoresed in a 1.5% agarose gel. The PCR product and digestion fragments were gel purified, and the radioactivity of each was measured and normalized for the number of adenine residues as above.

Results

Identification of the URO-synthase mutations. Each of the URO-synthase exons and their respective intron/exon junctions were amplified from the genomic DNAs of 20 unrelated CEP patients using biotinylated primers for rapid strand separation and single-strand solid phase sequencing. As shown in Fig. 2-3, the individual dideoxy chain sequencing reactions (e.g., C, A, T, of G) for a series of patients were run side by side, permitting rapid identification by deviations from the normal pattern of point mutations (e.g., Fig. 2-3, exon 2) as well as small or large insertions and deletions (e.g., Fig. 2-3, exon 9). Using this technique, URO-synthase mutations in each of the 20 unrelated patients were identified (Table2-2). The ten new mutations included: six missense mutations, a C to T transition of nt 10 that predicted a leucine to phenylalanine substitution at residue 4 (designated L4F), an A to G transition of nt 56 that predicted a tyrosine to cysteine replacement at residue 19 (Y19C), a G to T transversion of nt 244 that predicted a valine to phenylalanine substitution at residue 82 (V82F), a T to C transition of nt 296 that predicted a valine to alanine replacement at residue 99 (V99A), a C to T transition of nt 311 that predicted a alanine to valine substitution at residue 104 (104V), and a G to A transition of nt 673 that predicted a glycine to serine replacement at residue 225 (G225S); a nonsense mutation, a C to T transition of nt 745 that predicted a glutamine to termination signal in codon 249 (Q249X); a frameshift mutation, the insertion of an adenine after cDNA nt 633 in codon 212 that caused a frameshift and predicted a premature stop at codon 214 (633insA); two splicing mutations, a gt to at transition of the 5' donor splice site of intron 2 ($IVS2^{+1}$) and the deletion of an adenine in the +3 or +4 5' donor splice site (gtaag to gta-g) of intron 9 (designated $IVS9^{\Delta A+4}$).

In addition to the new mutations, four previously reported missense mutations were identified, T62A, A66V, C73R, and T228M. A total of 34 of the 40 mutant alleles in the 20 CEP patients studied were detected (Table 2-2). In 14 of the 20 patients, both mutant alleles were identified. Presumably, the six undetected mutations involved lesions such as gene deletions, promoter mutations or other splicing defects. The allele frequencies of the 14 mutations in the 20 patients studied here, as well as among all reported CEP patients are shown in Table 2-3. The C73R and L4F mutations were the most frequent with 32.5% and 10.0%, respectively, of the 40 alleles studied here, and 26.6% and 9.4% of the 64 CEP alleles reported here and elsewhere (Warner et al 1990, Warner et al 1992, de Verneuil et al 1989, Deybach et al 1990, Boulechfar et al 1992).

Prokaryotic expression of the URO-synthase mutations. To further characterize the URO-synthase mutations, pKK-UROS expression vectors for each of the newly identified exonic mutations were constructed, sequenced, and expressed in *E. coli.* Table 2-4 shows the mean and range of URO-synthase activity for the expressed normal allele and each mutant allele. Only the missense mutations V82F, V99A, A104V had mean residual activities that were greater than 3% of the expressed mean normal activity. Notably, the V82F allele expressed 35.8% of that expressed by the normal allele. However, incubation of the expressed activity at 37 °C revealed that the V82F activity was less stable than the normally expressed enzyme, the half-lives being 30.5 and 49.5 min, respectively (Fig. 2-4). The thermostability of the V82F enzyme at 37 °C and 60 min was similar to that of the previously reported V66A allele which expressed significant residual activity (15% of normal) in *E. coli* (Warner et al, 1992).

Analysis of the URO-synthase splicing mutations. The putative splicing mutations (IVS2^{+1}, IVS9$^{\Delta A+4}$, and V82F) were characterized by sequencing the URO-synthase RT-PCR products and determining their relative abundance in the respective lymphoid cell lines. Two RT-PCR products were present in the IVS2^{+1}/L4F lymphoid cells, a normal product of 937 bp, and a smaller 848 bp product in which the entire exon 2 was deleted (Fig. 2-5A). That the IVS2^{+1} allele did not produce any normal-sized URO-synthase transcripts was indicated by the absence of detectable *Ava*II digestion fragments. Two URO-synthase RT-PCR products were present in the IVS9$^{\Delta A+4}$/IVS9$^{\Delta A+4}$ lymphoid cells, a normal product of 937 bp, and a smaller 838 bp product in which sequencing revealed that the entire exon 9 was deleted (Fig. 2-5B). Radiolabeling of the RT-PCR products from three independent experiments revealed that 73.5% (range 69.0 - 77.9%) of the IVS9$^{\Delta A+4}$/IVS9$^{\Delta A+4}$ RT-PCR products had the exon 9 deletion. Two URO-synthase RT-PCR products were present in L4F/V82F lymphoid cells, a normal product of 937 bp, and a smaller 840 bp product in which sequencing revealed that the entire exon 4 was deleted (Fig. 2-5C). Radiolabeling of the *Eco*RI-digested RT-PCR products from three independent experiments revealed that 53.8% (range 48.7 to 56.2%) of the RT-PCR products from the V82F allele had the exon 4 deletion.

Genotype-phenotype correlations in unrelated CEP patients. Identification and expression of both URO-synthase mutations in 13 of the 20 CEP patients permitted the estimation of their relative residual activities for genotype-phenotype comparisons. Each patient was classified as mild, moderate, or severely affected based on a review of their clinical history and/or a recent examination by one of us (RJD). Homoallelism for the most common allele, C73R, was associated with non-immune hydrops fetalis and/or

transfusion dependency from birth, as evidenced by the phenotypes of patients 5, 19, and 20. Heteroallelism of C73R with a mutation that expressed little, if any, residual activity (Warner et al 1992 and Table 2-4) also resulted in a severe or moderately severe phenotype (e.g., patients 2, 7, and 15). In contrast, patients heteroallelic for mutations that expressed residual activity (e.g., C73/A66V, L4F/V82F, and T62A/Q249X) were milder, even if one of the alleles expressed no activity (Warner et al 1992).

Discussion

In this communication, the ten new and four previously reported mutations in the URO-synthase gene were detected in genomic DNA from 20 unrelated CEP patients using biotinylated primers to amplify each exon and adjacent exon-intron junctions, and separate single-strands for direct solid phase sequencing. To facilitate mutation detection, each of the four sequencing reactions for each exon from a series of patients was electrophoresed in adjacent lanes so that mutations could be readily detected by pattern recognition. This mutation detection strategy identified ten new lesions including six missense mutations, a nonsense mutation, a one base insertion, and two splicing defects. The point mutations were readily detected as isolated single bands in different sequencing reactions, whereas the insertion and deletion altered the position of the nucleotide in all four reactions (see Fig. 2-3D). In this way, the entire coding region and all intron/exon boundaries from each patient were rapidly sequenced and any lesions were detected by pattern recognition, rather than by completely reading the nucleotide sequence for each patient.

Since all exons and intron/exon boundaries must be sequenced to identify all possible disease-causing mutations in each allele, the direct sequencing strategy provided a single, efficient approach for mutation analysis and sequencing. This direct sequencing strategy proved more rapid and efficient for mutation detection in this large gene containing 10 exons encoding 265 residues than mutation detection methods such as the single strand conformation polymorphism (SSCP) technique (Orita et al 1989), heteroduplex analysis (White et al 1992), or the use of denaturing gradient gel

electrophoresis (Lerman et al 1984), each of which requires one round of mutation detection followed by sequencing (e.g., Boulechfar et al 1992). This amplification and sequencing strategy has proven efficient for mutation analysis in this gene, as well as for others studied in this laboratory (Chen et al 1994, Eng et al 1993).

Eight of the ten new mutations were single base substitutions. Only the G225S lesion occurred at a CpG dinucleotide, a mutational "hot spot" due to deamination of methylcytosine to thymidine (Coulondre et al 1978). During the course of this investigation, the L4F mutation was reported independently by Boulechfar et al 1992. Mutations L4F, Y19C, V82F and G225S markedly altered the nature of the encoded amino acid (e.g., charge, size, etc), whereas mutations V99A and A104V substituted isofunctional residues. Of note, expression of the missense mutations revealed that those that altered charge, size or hydrophilicity had no activity, or very low levels of residual activity, whereas isofunctional substitutions had detectable residual activities. Of interest, the V82F lesion expressed about 36% of expressed normal activity. However, the V82F residual activity was inactivated *in vitro* at 37 °C at a rate about twice that of the normal expressed enzyme, presumably reflecting the decreased stability of this mutant protein *in vivo*. The nonsense lesion Q249X predicted premature truncation of the normal 265 amino acid polypeptide , deleting the terminal 17 residues of the enzyme. Expression of this mutation resulted in little, if any, detectable activity. The 633insA frameshift occurred in codon 212 and predicted different amino acids in residues 212 and 213, and premature truncation of the remaining 52 carboxy-terminal amino acids. Expression of the 633insA lesion resulted in very little, if any, activity.

The IVS2^{+1}, IVS9$^{\Delta A+4}$ and V82F mutations all involved the 5' donor splice consensus sequence, G/gtaag (Shapiro et al 1987, Senapathy et 1990). The IVS2^{+1} lesion occurred at invariant position 1 of intron 2, a mutation that typically results in exon skipping (Sillence et al 1991). Analysis of the RT-PCR products from the IVS2^{+1}/L4F patient by *Ava*II digestion (which would only cleave the normal sequence) did not digest any normally sized transcripts, indicating that all the transcripts encoded by the IVS2^{+1} allele were aberrant (Fig. 3A). In contrast, the adenine deletion in the +3 or +4 position of the intron 9 consensus sequence resulted in two URO-synthase transcripts (Fig. 2-5B). The shorter, exon 9 deleted transcript represented about 73.5% of the radiolabeled RT-PCR products encoded by this allele. Thus, the IVS2^{+1} allele expressed no functional enzyme, whereas the IVS9$^{\Delta A+4}$ allele produced about 25% normal transcripts encoding the active enzyme monomer. Of note, the V82F mutation, which substituted a C to G in the most 3' nucleotide of exon 4, altered the 5' splice consensus sequence and resulted in abnormal splicing in about 54% of the transcripts encoded by this allele. Thus, the V82F single base substitution resulted in two lesions, about half the transcripts were aberrantly spliced, while the remainder encoded a missense mutation which had about 35% of normal activity (albeit thermolabile) when expressed in *E. coli*. A similar exonic lesion which caused alternative splicing of the a5(IV) collagen gene was previously identified in a patient with Alport syndrome (Nomura et al 1993).

The identification of these mutations further expands the molecular heterogeneity of the URO-synthase mutations causing CEP. To date, a total of 14 different mutations have been identified in studies of 32 unrelated CEP patients. The C73R mutation was the most common CEP allele, occurring in 26.6% of all CEP alleles studied (Warner et al 1990, Warner et al 1992). The

L4F allele also was common, occurring in about 10% of all CEP patients studied. Expression of these mutations in *E. coli* provided the opportunity to correlate genotype, predicted residual activity and disease severity. Of the 20 patients studied, both alleles were identified in 14, permitting initial genotype/phenotype correlations. The three CEP patients who were homoallelic for C73R were severely affected, having hydrops fetalis and requiring intrauterine transfusions (patient 20), being stillborn (patient 19) or transfusion dependent, expiring at five months of age (patient 5). The clinical phenotypes of these three patients suggest that homoallelism for C73R produces insufficient activity to sustain life, consistent with the fact that the C73R mutation had little, if any, activity when expressed in *E. coli.* (Warner et al 1992, Boulechfar et al 1992). In contrast, homoallelism for the IVS9$^{\Delta A+4}$ mutation resulted in a mild phenotype, since about 25% of the transcripts encoded by this allele were normal.

As no other patients were homoallelic for their lesions, the respective phenotype depends on the amount of residual activity contributed by each mutant allele. Since the C73R mutation encodes negligible activity, the four patients heteroallelic for C73R provide information on the effects of the L4F, A66V, A104V, and T228M alleles. The C73R/L4F patient was not transfusion dependent, but was moderately severe, consistent with the fact that the L4F allele expressed relatively little residual activity in *E. coli* (Table 2-4). In contrast, the patients heteroallelic for the A66V, A104V, and T228M alleles had moderate to mild (C73R/A66V) phenotypes, consistent with the expression of sufficient residual activity to produce adequate heme to avoid transfusion dependence. Similarly, patients with a single allele that expressed significant activity in *E. coli* might be expected to have milder phenotypes. However, the

expressed residual activity must be assessed for its relative stability. For example, the patient with the L4F/V82F might be expected to be mild, since the V82F allele had about 36% of expressed normal activity. However, when the V82F residual activity was incubated at 37 °C, the mutant enzyme proved unstable. Thus, heteroallelism for L4F and V82F was consistent with the moderately severe phenotype of this patient. Thus, the presence of residual activity with relatively normal stability of the expressed enzyme would be consistent with a milder phenotype. Further analysis of the URO-synthase genotype of these and other CEP patients should further delineate the molecular heterogeneity underlying CEP and provide additional genotype/phenotype correlations.

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Table 2-1. Conditions and Primer Sets for Amplification and Sequencing of UROS-Synthase Exons.*

Primer Set	URO-synthase cDNA sequence amplified	Oligonucleotide primer	Annealing condition (°C, min)
1	-196-(-27)	a. 5'-Bgcgcagccacccacgagaccccagtctgaggtg-3'	58 ; 1.0
		b. 5'-actgtgtcacacaccgccggccgggcccct-3'	
		c. 5'-ccgcgggcttcggcgtcgta-3'	
2	-26-63	a. 5'-Bgataggtctcttccacaag-3'	51 ; 1.0
		b. 5'-ACTACT(GAATTC)tttattctgcgtcagttcc-3'	
		c. 5'-gttttgcaaaacccatcc-3'	
3	64-147	a. 5'-Bggttttgcaaaacctcaga-3'	51 ; 1.0
		b. 5'-ACTACT(GAATTC)gtaagttatgctgccatgt-3'	
		c. 5'-ACTACT(GAATTC)gtaagttatgctgccatgt-3'	
4	148-244	a. 5'-ttggagctgccacgcggtgg-3'	58 ; 1.0
		b. 5'-Bctgggagtttaggagatag-3'	
		c. 5'-gctgcagaattctgagtctt-3'	
5	245-319	a. 5'-Batagtttgctttgctcacag-3'	50 ; 1.0
		b. 5'-ACTACT(GAATTC)ctgcattcttatcagtag-3'	
		c. 5'-taaactgagttaaactgtt-3'	
6	320-394	a. 5'-Bataccctgtgtatttgcacgt-3'	50 ; 0.5
		b. 5'-ACTACT(GAATTC)caagaatgcactgaggaaa-3'	
		c. 5'-ACTACT(GAATTC)caagaatgcactgaggaaa-3'	
7	395-475	a. 5'-Bctgagtcctagaagcagag-3'	50 ; 0.5
		b. 5'-ACTACT(GAATTC)cacccacttctatcactgc-3'	
		c. 5'-cacttctatcactgcaa-3'	

Table 2-1. Conditions and Primer Sets for Amplification and Sequencing of URO-Synthase Exons (continued).*

8	476-561	a. 5'-Baggagggtggtgctgtatt-3'	52 ; 1.0
		b. 5'-ACTACT(GAATTC)gggacagtgaaaccacata-3'	
		c. 5'-acatctatcagctcgtgcccttgaccct-3'	
9	562-660	a. 5'-Bttggtggcctgtggcttga-3'	52 ; 1.0
		b. 5'-ACTACT(GAATTC)taaggcacctgctaggcca-3'	
		c. 5'-ggcttgaggtcttgatgcggat-3'	
10	661-1083	a. 5'-Bcgtcacatgagcagtaacg-3'	52 ; 1.0
		b. 5'-ACTACT(GAATTC)atagtctcctccctg-3'	
		c. 5'-atgcctggctccatccag-3'	

*_Eco_R I recognition sites are in parentheses. Underlined nucleotides represent additional non-URO-synthase sequence to facilitate restriction enzyme cleavage. B indicates biotinylated nucleotide. Oligonucleotides a, b, and c indicate sense and antisense amplification primers and sequencing primer, respectively.

Table 2-2
Genotype-Phenotype Correlations in Unrelated CEP Patients

Proband*	Age	Sex	Ancestry	Disease severity‡	Genotype§
1	17 yr	M	Scottish, Irish / German	Mild	C73R / A66V
2	17 yr	M	Romanian	Moderately severe	C73R / T228M
3	19 yr	M	Japanese	Mild	T62A / Q249X
4	58 yr	F	African Black	Moderate	V99A / 633InsA
5	5 mo	F	Cree Indian	Severe/Deceased, HF	C73R / C73R
6	9 yr	F	English	Severe, TD, TR	C73R /
7	23 yr	M	English	Moderately severe	L4F / C73R
8	13 yr	F	Alaskan Indian	Moderate	C73R / A104V
9	9 yr	M	English	Moderate	C73R /
10	14 yr	F	German, Hungarian / Swedish	Moderate	C73R /
11	40 yr	F	English, Irish / Polish	Mild	G225S / T228M
12	28 yr	M	English	Moderate	G225S /
13	9 yr	F	Italian	Mild	L4F / V82F
14	10 yr	M	English	Severe, TD, TR	L4F /
15	3 yr	F	Northern European	Moderate	L4F / IVS2+1

Table 2-2 Genotype-Phenotype Correlations in Unrelated CEP Patients (Continued).

16	18 yr	M	Indian	Mild	$IVS9^{\Delta A+4}$ / $IVS9^{\Delta A+4}$
17	49 yr	F	Swedish	Very mild	$IVS2^{+1}$ /
18	15 yr	M	English / Irish	Severe, TD	**Y19C / G225S**
19	Stillborn	F	Belgian	Severe, HF	**C73R / C73R**
20	1 yr	M	Puerto Rican	Severe, HF, TD	**C73R / C73R**

*Probands 1-11 were previously reported (8) and the clinical and biochemical features of proband 19 were described (39). No proband was known to be consanguineous. ‡TD = transfusion dependent, HF = hydrops fetalis, TR = chronic transfusion therapy to suppress erythropoiesis. §Mutations described in text in boldface type. When known, ancestry of mutation indicated by back-slash corresponding to respective genotype .

Table 2-3
Allele Frequency of URO-Synthase Mutations Causing CEP

Type of Mutation	Number (and %) of Alleles of 40 CEP Alleles Studied		% of All CEP Mutant Alleles Studied* (64 Alleles)
	(No.)	(%)	
Missense Mutations:			
C73R	13	32.5	26.6
L4F	4	10	9.4
G225S	3	7.5	4.7
T228M	2	5	4.7
Y19C, T62A, A66V, V82F, V99A, ,A104V, Q249X (each)	1	2.5	1.6
Frameshift Mutation:			
633insA	1	2.5	1.6
Splicing Defect Mutations:			
IVS2^{+1}	2	5	3.1
IVS9$^{\Delta A+4}$	2	5	3.1
Unknown Alleles:	6	15	17.2

*References Warner et al 1990, 1992, de Verneuil et al 1982, Deybach et al 1990, Boulechfar et al 1992

Table 2-4
Expression of URO-Synthase in *E. coli*

Construct	URO-synthase activity (U/mg)*		% of Mean normal level
	Mean	Range	
pKK223-3	1.91	0.00-3.47	---
pKK-UROS	73.68	63.18-87.10	100
pKK-UROS-L4F	3.38	3.39-4.48	1.8
pKK-UROS-Y19C	2.69	1.62-3.46	1.1
pKK-UROS-V82F	27.58	18.34-39.36	35.8
pKK-UROS-V99A	5.93	3.61-8.23	5.6
pKK-UROS-A104V	7.44	3.63-10.55	7.7
pKK-UROS-633insA	2.78	1.47-3.79	1.2
pKK-UROS-G225S	2.79	0.55-4.25	1.2
pKK-UROS-Q249X	2.71	1.65-3.31	1.1

*Mean and range of activities for duplicate assays of three independent experiments, induced for 3 h with 5 mM isopropylthiogalactoside.

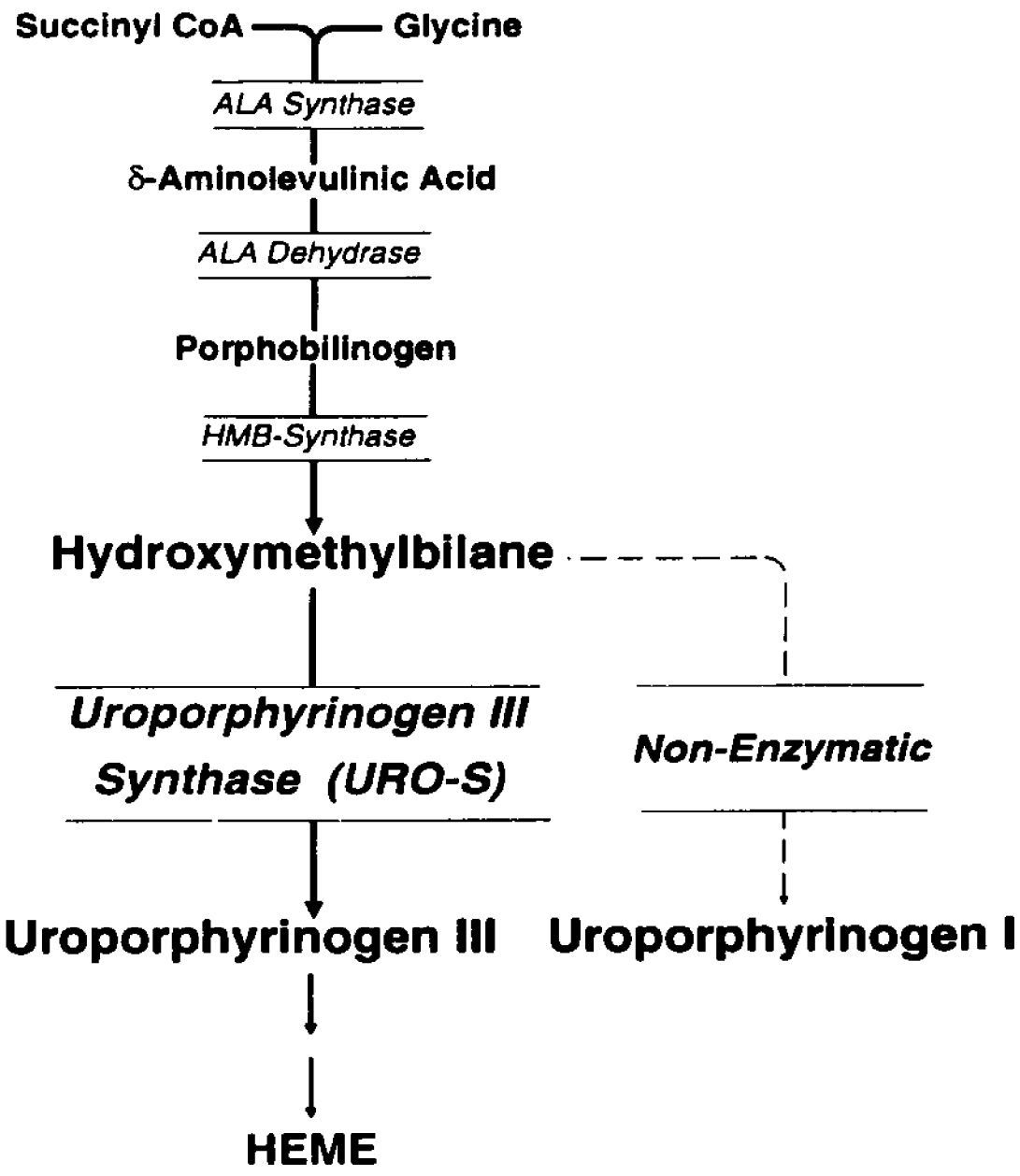

Figure 2-1 Metabolic Defect in Congenital Erythropoietic Porphyria

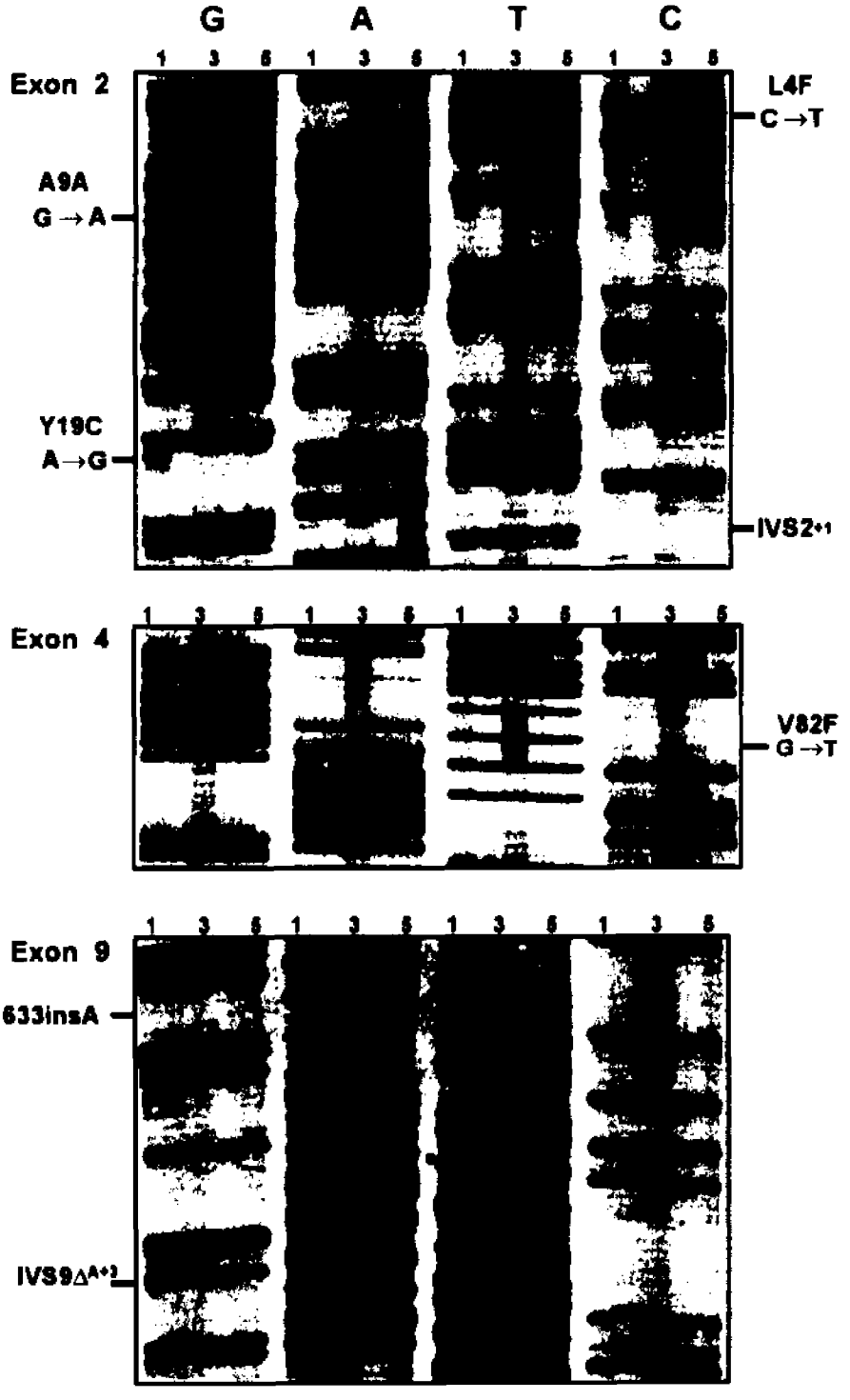

Figure 2-2. Rapid identification of mutations in the URO-synthase gene by pattern recognition.

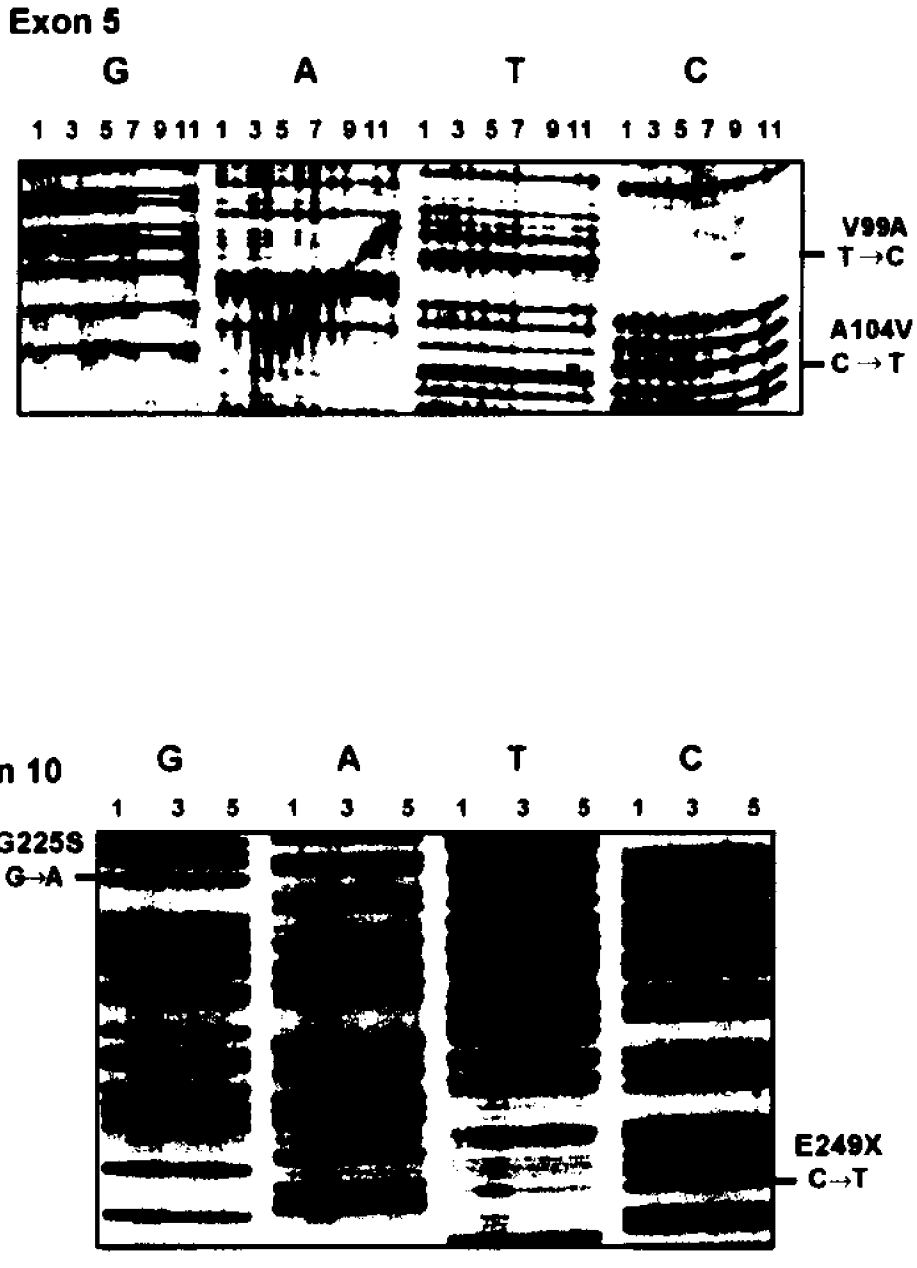

Figure 2-3. Rapid identification of mutations in the URO-synthase gene by pattern recognition.

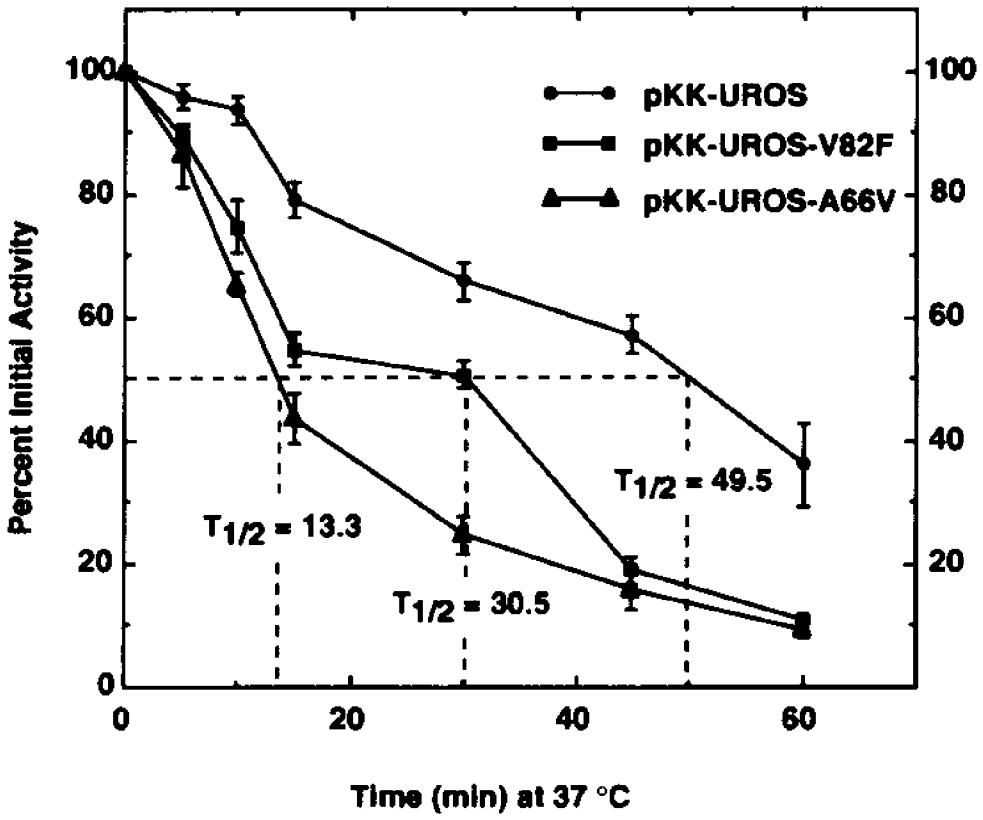

Figure 2-4. Thermostability of the URO-synthase activity expressed in pKK-UROS, pKK-UROS-V82F and pKK-UROS-A66V in *E. coli*.

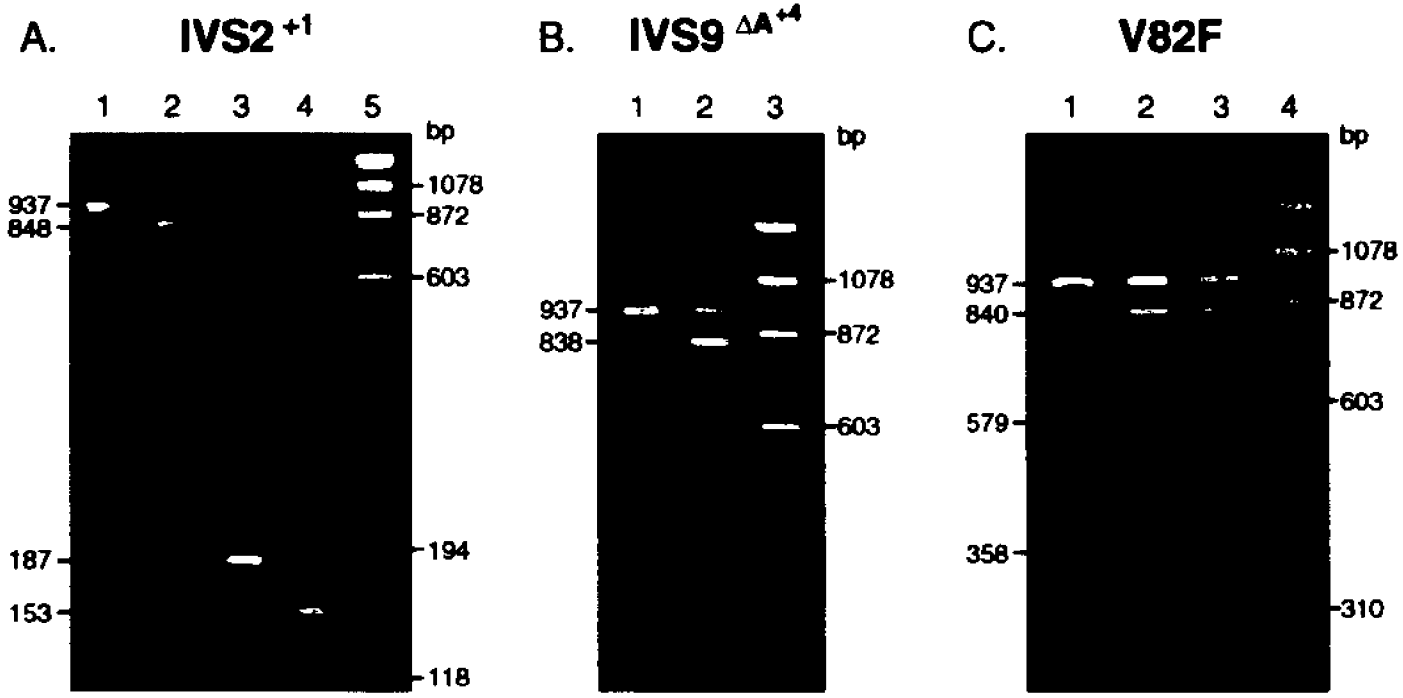

Figure 2-5. Analysis of the Splicing Mutations. Agarose gel electrophoresis and ethidium bromide staining of URO-synthase RT-PCR products from total mRNA of patients with the mutations described in the text.

Chapter 3

UROPORPHYRINOGEN III SYNTHASE: MOLECULAR CLONING, NUCLEOTIDE SEQUENCE, EXPRESSION OF A MOUSE FULL-LENGTH cDNA AND ITS LOCALIZATION ON MOUSE CHROMOSOME 7

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Summary

Uroporphyrinogen III synthase (URO-S; EC 4.2.1.75), the fourth enzyme in the heme biosynthetic pathway, is responsible for the conversion of hydroxymethylbilane to the cyclic tetrapyrrole, uroporphyrinogen III. The deficient activity of URO-S is the enzymatic defect in congenital erythropoietic porphyria (CEP), an autosomal recessive disorder. For the generation of a mouse model of CEP, the human URO-S cDNA was used to screen 2 x 10^6 recombinants from a mouse adult liver cDNA library. Ten positive clones were isolated and dideoxy sequencing of the entire 1.6 kb insert of clone pmUROS-1 revealed 5' and 3' untra bp, respectively, and an open reading frame of 798 bp encoding a 265 amino acid polypeptide with a predicted molecular mass of 28,501 Da. The mouse and human coding sequences had 80.5 and 77.8% nucleotide and amino acid identity, respectively. The authenticity of the mouse cDNA was established by expression of the active monomeric enzyme in *E. coli*. In addition, the analysis of two multilocus genetic crosses localized the mouse gene on Chromosome (Chr) 7, consistent with the mapping of the human gene to a position of conserved synteny on chromosome 10. The isolation and, expression, and chromosomal mapping of this full-length cDNA should facilitate studies of the structure and organization of the mouse genomic sequence and the development of a mouse model of CEP for characterization of the disease pathogenesis and evaluation of gene therapy.

Introduction

Uroporphyrinogen III synthase [URO-synthase; hydroxymethylbilane hydro-lyase (cyclizing), EC 4.2.1.75] is the heme biosynthetic enzyme responsible for the conversion of the linear tetrapyrrole, hydroxymethylbilane (HMB) to uroporphyrinogen III, the first cyclic tetrapyrrole and the physiologic precursor of heme (Bogard and Granick, 1953) The enzyme functions both as an isomerase and a cyclase as it catalyzes the intramolecular rearrangement of ring D and ring closure, respectively (Battersby et al., 1982a, b). In the absence of URO-synthase activity, HMB is nonenzymatically cyclized to form the nonphysiologic uroporphyrinogen I isomer, which is then oxidized to uroporphyrin I, a nonphysiologic and pathogenic compound. The enzyme has been purified to homogeneity from human erythrocytes (Tsai et al., 1987b and bovine liver (Sancovich et al., 1969), and each was shown to be a monomeric protein, the human enzyme having an apparent molecular weight of 29.5 kDa. Recently, the human full-length cDNA was isolated, characterized and expressed in *E. coli* (Tsai et al., 1988). The 1.3 kb cDNA had 5' and 3' untranslated sequences of 196 and 288 bp, respectively, and encoded a polypeptide of 265 amino acids with a predicted molecular mass of 28,607 Da. A single human gene encoding URO-synthase has been regionally mapped to chromosome 10q25.2→26.3 (Astrin et al., 1991). In addition, cDNA sequences encoding this enzyme have been isolated from *C. vibrioforme* (Majumdar and Wyche, 1992), *E. coli* (Sasarman et al., 1987), and *P. aeruginosa* (Mohr et al., 1994).

The deficient (but not absent) activity of URO-synthase is the enzymatic defect in the human disorder, congenital erythropoietic porphyria (CEP) (Romeo

and Levin, 1969.) This inborn error of heme biosynthesis is inherited as an autosomal recessive trait and is clinically heterogeneous (For review, see Desnick and Anderson, 1991; Kappas et al., 1989). To date, a variety of mutations have been identified in the human URO-synthase gene which cause this disease (e.g., Boulechfar et al., 1992; Warner et al., 1992; Xu et al., 1994; Xu *et al.*, in review). The variation in disease severity has been correlated with the amount and stability of the residual activity expressed by both mutant alleles in affected patients. Animal analogs of human CEP have been reported in cattle and pigs (Fourie, 1936; Clare and Stephen s, 1944), but no known mouse or rodent models of the disease have been identified, with the exception of the fox squirrel which normally has a low level of URO-synthase activity (Turner, 1937; Levin and Flyger, 1971). The availability of a mouse model for CEP would permit studies of disease pathogenesis as well as the development and assessment of various strategies to treat this disease, including enzyme replacement, bone marrow transplantation and gene therapy. Therefore, efforts were undertaken to isolate the full-length mouse URO-synthase cDNA. In this communication, the isolation and complete nucleotide sequence of a full-length cDNA encoding mouse URO-synthase are described. The authenticity of the mouse cDNA was demonstrated by prokaryotic expression of catalytically active enzyme. In addition, the gene encoding URO-synthase, *Uros*, was regionally mapped to mouse Chr 7, consistent with the assignment of the human gene to a region of conserved synteny on chromosome 10.

Materials and Methods

Reagents. Radioisotopes were from Amersham Corporation Life Science Division, Arlington Heights, IL. The low water extractable HATF filters used in plaque screening/hybridization were purchased from Millipore Co., Bedford, MA. Reagents for DNA sequencing were obtained from U.S. Biochemicals, Cleveland, OH. Agarose was from GIBCO BRL, Inc., Gaithersburg, MD. Restriction enzymes, T_4 DNA ligase and the random priming kit were purchased from New England Biolabs, Inc., Beverly, MA. The *Taq* polymerase was obtained from Promega Co., Madison, WI. The pKK223-3 expression vector was from Pharmacia Biotech Inc., Piscataway, NJ.

cDNA library screening. A total of 2 X 10^6 recombinants from the B6/CBAF1J mouse liver cDNA library (Strategene Cloning System, La Jolla, CA, catalogue no. 935302) constructed in the lambda ZAP vector were screened with radiolabeled human URO-synthase cDNA as described previously (Sambrook et al., 1989). The filters were prehybridized at 50 °C for 4 hr with 0.1% $NaDodSO_4$/6 X SSC/5 X Denhardt's solution and 100 µl/ml denatured salmon sperm DNA (1 X SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0; 1 X Denhardt's solution = 0.02% Ficoll/0.02% polyvinylpyrrolindone/ 0.02% bovine serum albumin). Hybridization of human cDNA (1 X 10^6 cpm/ml) was carried out at 50 °C for 16 hr. Washing was performed at 50 °C with 2 X SSC/0.1% $NaDodSO_4$ for 2 hr. Putative positive plaques were subcloned and rescreened as above until they were homogeneous.

Sequencing. The cDNA inserts from the positive clones were excised *in vivo* from the lambda ZAP vector in the pBluescript phagemid with the

ExAssist^{TM}/SOLAR^{TM} System according to manufacturer's protocol (Strategene Cloning System, La Jolla, California). Dideoxy chain termination sequencing of each putative positive clone was performed to determine authenticity of the cDNA inserts (Sanger et al., 1977)

Prokaryotic expression. The coding region from the mouse URO-synthase cDNA was subcloned into the pKK223-3 prokaryotic expression vector (Pharmacia Biotech Inc., Piscataway, NJ) by the following procedure. The 798 bp coding region was amplified by the polymerase chain reaction (PCR) (x) with sense primer WX177 (5'-GCGCGCgaattc*ataaggactgccaggcaata*atgaaggttctctta ctaaa-3') and antisense primer WX160 (5'-GCCGCCgaattctcagcaacagtggtttggct -3'). The italicized sequence is from the 5' untranslated region of the human URO-synthase cDNA and contained an AGGA sequence which was identical to the *E. coli* Shine-Dalgarno consensus sequence. Note that the *Eco*RI recognition sites are underlined, and the capitalized nucleotides represent additional non-URO-synthase sequence to facilitate restriction enzyme cleavage. The PCR product was digested with *Eco*RI and the 824 bp fragment was subcloned into the *Eco*RI site of pKK223-3 expression vector. The construct, designated pKK-mUROS, was confirmed by dideoxy DNA sequencing, and then was used to transform *E. coli* strain JM109. The expressed URO-synthase activity was assayed as previously described (Tsai et al., 1987a).

Genetic mapping. Two multilocus genetic crosses were analyzed for the inheritance of URO-synthase: (NFS/N or C58/J X*Mus musculus musculus*) X *M. m. musculus* (Kozak et al., 1990) and (NFS/N X *Mus spretus*) X *M. spretus* or C58/J (Adamson et al., 1991). DNA from the progeny of these crosses were

typed for approximately 700 markers which map to all 19 autosomes and the X chromosome, and included the Chr 7 markers *Zp2* (zona pellucida protein 2), *Oat* (ornithine aminotransferase), *Cyp2e1* (cytochrome P450 2e1), *Hras1* (Harvey *ras* oncogene 1), *Fgf3* (fibroblast growth factor 2, formerly *Int2*), and *Mtv35* (mammary tumor virus 35). The probes and enzymes used to type *Zp2, Oat, Cyp2e1, Fgf3,* and *Hras1* have been described previously (Lunsford et al., 1993; Lunsford et al., 1990; and Ramesh et al., 1992zdv). *Mtv35* was typed as a 12.7 kb *Eco*RI *spretus* fragment using as probe a 1.4 kb *Pst*I fragment of the C3H MMTV (X). Data were stored and analyzed using the program LOCUS developed by C. E. Buckler (NIAID, Bethesda, MD). Percent recombination and standard errors between specific loci were calculated from the number of recombinants according to Green (1981). Loci were ordered by minimizing the number of double recombinants.

Results

Library screening and characterization of positive cDNA clones. Of the 24 clones initially selected, 10 remained positive after purification. The 10 cDNAs ranged in size from ~1.2 to ~1.6 kb. The pBluescript phagemid constructs containing the cDNA inserts were designated pmUROS-1 - 10; pmUROS-1 contained the largest insert of 1.6 kb.

Nucleotide and protein sequence analyses. Dideoxy nucleotide sequencing of all 10 clones revealed that each contained the entire 798 bp coding region, but varied in the lengths of the 5' and 3' untranslated regions. The sequence of the 1.6 kb pmUROS-1 is shown in Fig. 3-1. The 798 bp open reading frame encoded a polypeptide of 265 amino acids with a predicted molecular mass of 28,501 Da. Of note, the mouse pmUROS-1 cDNA had a novel 21 bp polyadenylation cleavage signal region, AATAAATAAATAAATAATAAA, which contained three overlapping AATAAA sequences and an adjacent fourth cleavage signal one bp downstream. Presumably the first AATAAA sequence was functional as it was positioned 19 bp upstream from the poly(A) tract. The consensus recognition sequence (CACTG) for the U4 small nuclear ribonucleoprotein (Berget, 1984) was located 172 bp upstream from the poly(A) tract.

Comparison of the human and mouse URO-synthase cDNA coding regions revealed that they had 80.5 and 77.8% nucleotide and predicted amino acid identity, respectively (Fig.3-2). The regions of nucleotide and predicted amino acid homology were distributed throughout the entire open reading frame, disrupted only by short stretches of no more than four nonhomologous

nucleotides or three different amino acids. In contrast, there was little, if any, amino acid homology between the mouse or human sequences with the URO-synthase coding region from *Chlorobium vibroforme* (25.1% with 9 gaps of 1-5 amino acids) (Majumdar and Wyche,1992), *Escherichia coli* (13.0% with 7 gaps of 1-9 amino acids) (Sasar,am et al.,1987) and *Pseudomonas aeruginosa* (14.7% with 7 gaps of 1-6 amino acids) (Mohr et al., 1994). There were no remarkable regions of homology shared between the mammalian and viral or bacterial sequences. However, there were isolated residues that occurred in the same position in all five species (i.e., four L, four G, one S, one T, and one Y) (Fig. 3-2). Similarly, several amino acids (i.e., four L, three A, three E, two V, one P, and one W) were in the same position in four of the five species. With four exceptions, these residues were distributed randomly in the polypeptide sequence.

Prokaryotic expression of mouse URO-synthase. In contrast to the successful expression of the human URO-synthase cDNA using the pKK223-3 vector, initial efforts to subclone the mouse URO-synthase cDNA in this vector proved unsuccessful. No correctly oriented clones were obtained (data not shown), presumably due to the lethality of such clones expressing the enzyme at high levels. Therefore, the -1 to -20 nt sequence from the 5' untranslated region of human URO-synthase cDNA was engineered upstream of the mouse URO-synthase open reading frame. This construct positioned the mouse start codon 30 bp downstream from the Shine-Dalgarno sequence (AGGA) in the vector (De Boer et al., 1983), and 12 bp downstream from an AGGA sequence present in the 5' untranslated human URO-synthase cDNA sequence. This construct permitted expression of the mouse URO-synthase in *E. coli* without induction, presumably due to insufficient endogeneous repressor synthesis by

the *E. coli* host cell. As shown in Table 3-1, the average endogenous URO-synthase activity in untransduced *E. coli* cells was less than 2 U/mg protein whereas the mean activity was increased 12-fold in cells transduced with the expression construct. When induced with isopropyl ß-D-thiogalactopyranoside (IPTG), the mean increase in activity over the endogenous level was about 47-fold.

Genetic mapping. *Sst*I digestion of parental NFS/N and *M. spretus* DNAs identified major UROS-reactive fragments of 18.8 and 13.0 kb, respectively. NFS/N and *M. m. musculus* DNAs produced *Eco*RI fragments of 28.0 and 12.4, and 17.5 and 13.2 kb, respectively. The inheritance of these fragments was typed in both genetic crosses and compared with the inheritance of over 700 markers previously typed and mapped in these crosses. As shown in Fig. 3-3, the gene encoding URO-synthase, mapped to mouse Chr 7. Closest linkage was observed with *Oat* for which no recombinants were identified in the 196 mice typed for both markers. This indicates that, at the 95% confidence level, *Uros* and *Oat* are within 1.5 cM. The human homolog of *Oat* has been mapped to 10q26 (Barret et al., 1987). Thus, these results are consistent with the previous assignment of the human URO-synthase gene to 10q25.2→26.3 (Astrin et al., 1991).

Discussion

A full-length mouse cDNA encoding URO-synthase was isolated by screening a mouse liver cDNA library with the radiolabeled human URO-synthase cDNA. Authenticity of the full-length cDNA was established by the over 75% identity of the predicted mouse amino acid sequence with that predicted by its human counterpart (Fig. 3-2) and by microbial expression of active recombinant enzyme (Table 3-1). In addition, the mouse gene was localized on Chr 7 to a region of conserved synteny with human chromosome 10. This map position was consistent with the regional assignment of the human gene to 10q25.2→26.3 (Astrin et al., 1991).

High levels of mouse URO-synthase activity were expressed as the native enzyme in bacteria transformed by the pKK-mUROS construct. Notably, this expression vector had a strong *tac* promoter and two Shine-Dalgarno tetranucleotide (AGGA) consensus sequences, one in the vector 30 bp upstream from the URO-synthase initiation codon, and an identical sequence in the 5' untranslated sequence of human URO-synthase cDNA which was placed 13 bp upstream from the initiation codon. Since the optimal location of the ribosome binding site is 5-9 bp from the initiation codon (Kozak, 1983), the plasmid presumably used the human URO-synthase AGGA sequence for ribosome binding and efficient translation. Two lines of evidence support this concept. First, initial efforts to express the mouse cDNA involved a construct in which the PCR-amplified open reading frame (without additional 5' untranslated sequence) was subcloned into the pKK223-3 vector such that the initiation ATG was 10 bp downstream from the vector's Shine-Dalgarno sequence. However, most (>95%) of the recombinants recovered after ligation and transformation of

the host cells had the cDNA insert in the antisense orientation. It was presumed that the construct in the sense orientation was readily expressed, resulting in high levels of uroporphyrinogen and subsequent porphyrin metabolites which were toxic and poisoned these cells, even when 2 % glucose was present in the medium to suppress the *tac* promoter and the recombinants were grown in the dark to minimize phototoxicity of elevated level of porphyrins. Interestingly, the remaining constructs in the sense orientation had PCR errors. Second, the human URO-synthase expression construct with the AGGA sequence 13 bp from the initiation ATG was effectively expressed. Thus, the mouse pKK-mUROS vector was designed to be analogous to the human URO-synthase expression vector. Of note, the expression of the mouse and human URO-synthase constructs was comparable (Tsai et al., 1988).

An interesting and novel finding in the mouse URO-synthase cDNA was the presence of a series of four polyadenylation cleavage signals which began 25 bp upstream from the poly (A) tract. While two or more polyadenylation signals in the same gene may cause alternative polyadenylation, the occurrence of overlapping signals has been identified only in a single gene, the human a-spectrin gene which had five polyadenylation consensus sequences in a 3' untranslated region of 229 bp, with two overlapping and two other polyadenylation signals adjacent to each other (Hassoun *et al.*, 1994). In the mouse URO-synthase cDNA, a 21 bp sequence contained three overlapping AATAAA consensus cleavage signal sequences followed by a fourth sequence one bp downstream which preceded the poly (A) tract by 2 bp. The presence of four polyadenylation cleavage signals in a short region which included three overlapping sequences has not been previously reported in any mammalian gene. Presumably the first polyadenylation sequence which begins 25 bp

upstream of the poly (A) tract is functional. The significance of this "polyadenylation cleavage signal region" is unknown and could be investigated by site-specific mutagenesis and expression studies.

Although the mouse predicted amino acid sequence was highly homologous to the human sequence (77.8%), there was little, if any homology with the three available bacterial sequences (Fig. 3-2). This finding was consistent with the fact that the homology between mammalian and bacterial heme biosynthetic enzyme sequences also was low. For example, the percent identity for the human and *Bacillus subtilis* d-aminolevulinate dehydratase sequences was 20.2% (Wemur et al., 1986; Petricek et al., 1990). However, six of the 11 known human missense mutations causing CEP (i.e., L4F, A66V, V99A, A104V, G225S, and T228M) occurred at residues that were conserved in the mouse and at least one bacterial URO-synthase sequence, indicating that certain amino acid residues have been conserved among diverse species.

In summary, the availability of the mouse full-length cDNA encoding mouse URO-synthase should facilitate the study of the its gene structure, the understanding of mouse heme biosynthesis, and the isolation of the mouse genomic sequence for investigation of the regulation of this gene and for the construction of a mouse model of CEP by homologous recombination.

Note in Proof

After submission of this work, Bensidhoum and co-workers published the isolation of a 1487 bp cDNA encoding mouse UROS and its assignment to a region between *D7 Pas 2* and *Fgf 3* on mouse chromosome 7 (Mamm. Genome

5:728, 1994). The sequence published here had 67 additional 5' nucleotides, an additional G and an additional AGG after nucleotides 1179 and 1394 in their sequence, respectively; otherwise both sequences were identical.

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Table 3-1

Expression of Mouse URO-synthase in *E. coli*

	Specific activity* (U per mg protein)			
	Uninduced		Induced‡	
Plasmid	Mean	Range	Mean	Range
pKK223-3	1.98	1.77-2.45	2.32	1.56-3.24
pKK-mUROS	24.4	17.9-35.5	110.7	93.0-143.5

*Mean activity of three independent clones determined in duplicate by the coupled-enzyme assay (Tsai *et al.*, 1987a).

Units (U) = nmoles of uroporphyrinogen III produced per hr.

‡Induced for 3 hr with 5 mM IPTG prior to assay.

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-144                                                                                                    CTAGC CGGGTGAGAG GCCGCTCTCG GTTATCTCGG  -110

-109  CGTGCAGGT TGTCCTGCCT CTTAGCCCGC GAGGAACAGT CTGAGATCTA AGTAAGATCC ATTTCCTCGC TCTGCCGCAG CAGCGTCTTT TAGAAGGACT GCCAGGCACA    -1

   1  ATG AAG GTT CTC TTA CTA AAA GAC GCC AAG GAG GAT GAC AGC GGC CTG GAT CCA TAT ATC CAG GAG CTG CGA TTG TGT GGC CTA GAA GCC     90
   1  Met Lys Val Leu Leu Leu Lys Asp Ala Lys Glu Asp Asp Ser Gly Leu Asp Pro Tyr Ile Gln Glu Leu Arg Leu Cys Gly Leu Glu Ala     30

  91  ACA CTG ATT CCT GTG CTG TCA TTT GAG TTT ATG TCT CTC CCC AGT TTG TCA GAA AAG CTG TCT CAT CCT GAA GGC TTT GGA GGA CTC ATT    180
  31  Thr Leu Ile Pro Val Leu Ser Phe Glu Phe Met Ser Leu Pro Ser Leu Ser Glu Lys Leu Ser His Pro Glu Gly Phe Gly Gly Leu Ile     60

 181  TTC ACC AGC CCC AGG GCA GTG GAA GCA GTG AAG CTG TGT TTG GAG AAG GAC AAT AAA ACT GAA GCC TGG GAG AAG TCT CTG AAA GAC AGA    270
  61  Phe Thr Ser Pro Arg Ala Val Glu Ala Val Lys Leu Cys Leu Glu Lys Asp Asn Lys Thr Glu Ala Trp Glu Lys Ser Leu Lys Asp Arg     90

 271  TGG AAT GCC AAG TCT GTG TAC GTG GTT GGA AGT GCC ACC GCT TCT CTA GTG AAT AAA ATT GGT CTG GAT GCA GAA GGA GCG GGC AGT GGA    360
  91  Trp Asn Ala Lys Ser Val Tyr Val Val Gly Ser Ala Thr Ala Ser Leu Val Asn Lys Ile Gly Leu Asp Ala Glu Gly Ala Gly Ser Gly    120

 361  AAT GCA GAA AAG CTT GCT GAA TAT ATT TGC TCA AAG CCA TCT TCA GAG CTG CCT CTT CTC TTT CCG TGT GGA ACT ATC AAA GGA GAT ACT    450
 121  Asn Ala Glu Lys Leu Ala Glu Tyr Ile Cys Ser Lys Pro Ser Ser Glu Leu Pro Leu Leu Phe Pro Cys Gly Thr Ile Lys Gly Asp Thr    150

 451  CTT CCA AAA ATG CTC AAG GAC AAA GGG ATC CCC ATG GAA AGC ATG CAT GTC TAT CAG ACA GTT CCA CAC CCT GGG ATC CAA GGG AGC CTG    540
 151  Leu Pro Lys Met Leu Lys Asp Lys Gly Ile Pro Met Glu Ser Met His Val Tyr Gln Thr Val Pro His Pro Gly Ile Gln Gly Ser Leu    180

 541  AAG AGC TAC TAT GAA GAT CAG GGT ATC CCA GCC AGC ATC ACG TTT TTC AGT CCC TCC GGC CTT AAA TAC AGC CTC GAG TAT ATT CAG GCG    630
 181  Lys Ser Tyr Tyr Glu Asp Gln Gly Ile Pro Ala Ser Ile Thr Phe Phe Ser Pro Ser Gly Leu Lys Tyr Ser Leu Glu Tyr Ile Gln Ala    210

 631  TTA TCT GGC AGC AGC TTT GAC CAG ATT AAG TTT ATA GCC ATT GGC CCC AGT ACA ACC CGT GCT ATG GCT GCT AAG GGC CTG CCT GTG AGC    720
 211  Leu Ser Gly Ser Ser Phe Asp Gln Ile Lys Phe Ile Ala Ile Gly Pro Ser Thr Thr Arg Ala Met Ala Ala Lys Gly Leu Pro Val Ser    240

 721  TGC ACT GCA GAG AGC CCC ACA CCA CAA GCC CTG GCT GCA GGC ATC AGG AAT GTG CTG AAG CCA AAC CAC TGT TGC TGA GGGCTGTGCA GAGC    813
 241  Cys Thr Ala Glu Ser Pro Thr Pro Gln Ala Leu Ala Ala Gly Ile Arg Asn Val Leu Lys Pro Asn His Cys Cys TER                265

 813  CAGCTC CTTCCCTGTG CACGGGACAG CTCACTGCCT CTAGGATGGA ACGCAGATTC GGAGAGATCT GGAGAAGCTG CCTGTGAGCA TGTCACCGTC CTCCATGGAG TC    930

 921  CAGCTTGG AGCCTACGCC AGGTTCACTG GCCATTAGGT CACTGCCATT CGACACAGGG GGAGAGAAAG CTTCCTGGGT CTAGAAAGAA GGAACTGAAA TCGACCATGT   1038

1029  GGTGACTTGT GCCCACTCTT TTACAAACTG TATTTCTTTT ATGTTTATCG GTGTCTTGTC TGCATGTACA TCTGTGTGCC CTGTGCATAG CCAGTGCCTG GAGAAGCAA   1137

1138  A GTAAGGGTGG CAGATCCCCT CGAACTAGAG TTAGAGACAT CTGTGAGCTG CCATCCAGGT ACTGGGAATT GAACCCAGGT CCTCTGAAAG AATAAGTACT CTCAACC   1245

1246  ACT GAGCCATAAC TCCAGCCTAC TATTATCATC TGTGGTTCTC AGTGCAAAGT GGTGTTCTGG GACAACAGAA CATCATAGGC TCTCTGATGC TGACCTGATA AGAAT   1353

1354  GATAA ATCGCTATGT GTGAAGTAGA TTTAGTCATC ATAAATTAAA TAAATAAATA AATAATAAAT AGGAAAAAAA AAAAAAAAAA AAAAAAAAAA AAAAAAAAA AAA   1461

1462  AAAAAAA AAAAAAAAA                                                                                                      1477
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Figure 3-1. Nucleotide and predicted amino acid sequence of the pmUROS-1 cDNA insert containing the complete coding region for mouse URO-synthase

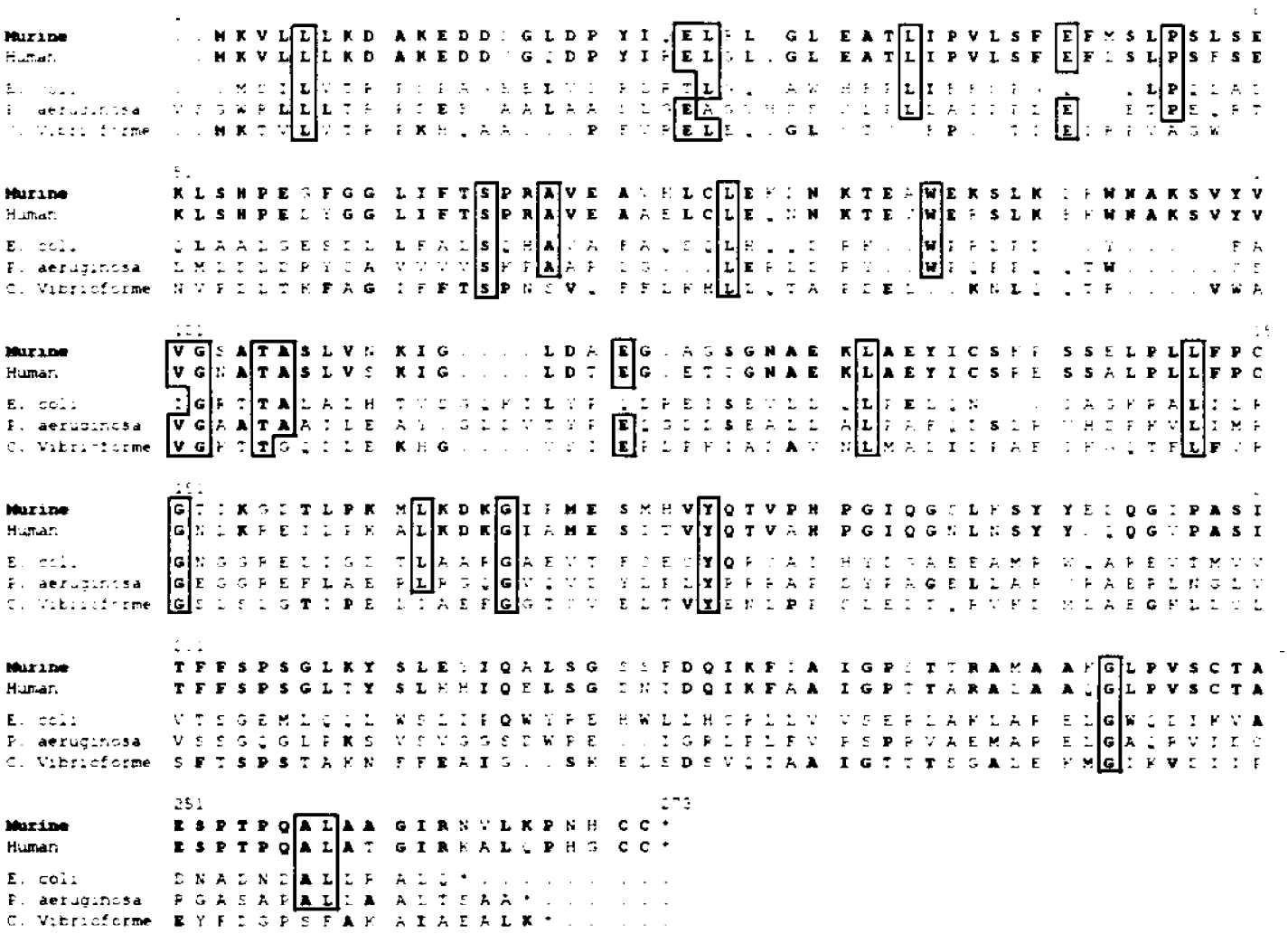

Figure 3-2. Comparison of the mouse URO-synthase predicted amino acid sequence with URO-synthase predicted sequence from other species.

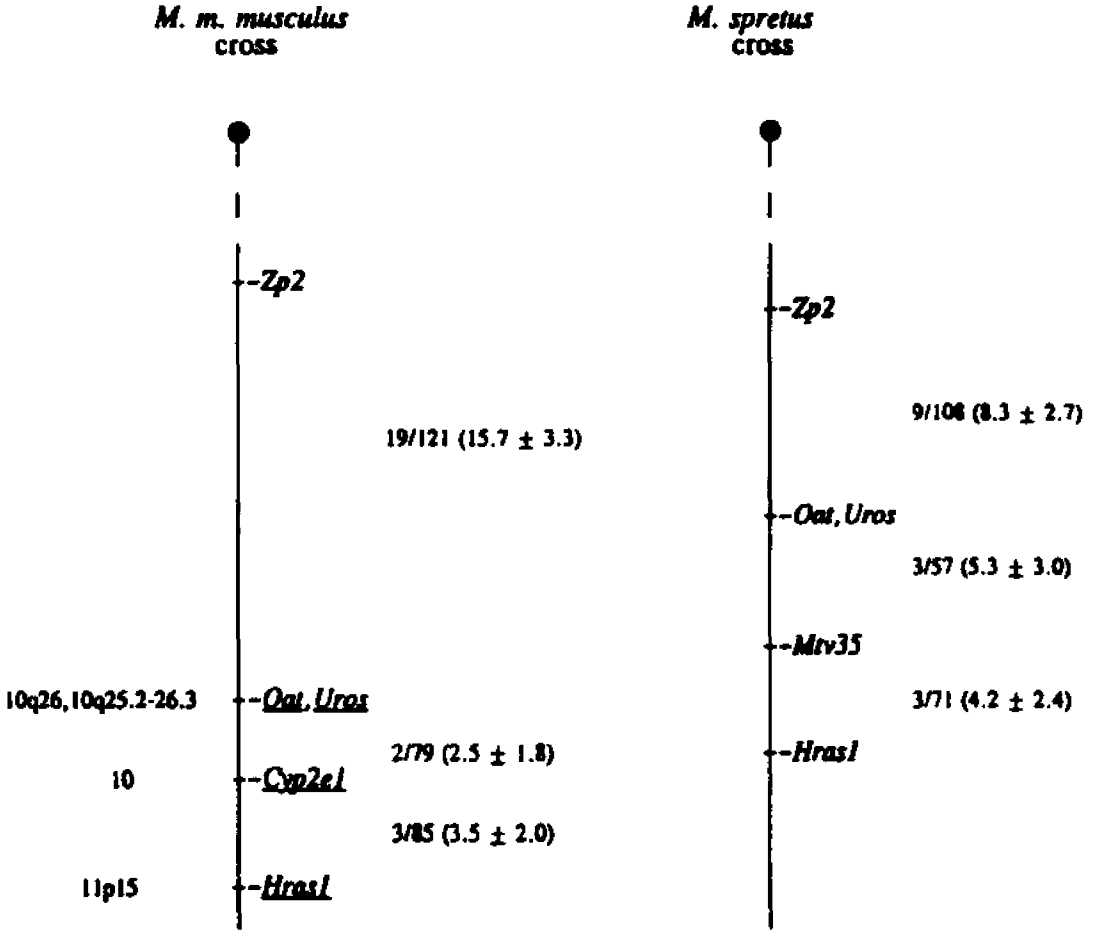

Figure 3-3 Abbreviated maps of mouse Chromosome 7 showing the map location of Uros.

Chapter 4

MUTATION UPDATE

Molecular Basis of Congenital Erythropoietic Porphyria: Mutations in the Human Uroporphyrinogen III Synthase Gene

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Abstract

Congenital erythropoietic porphyria (CEP) is an autosomal recessive inborn error of metabolism that results from the markedly deficient activity of the fourth enzyme in the heme biosynthetic pathway, uroporphyrinogen III synthase (URO-synthase). To date, 17 mutations have been described including 11 missense, one nonsense, two mRNA splicing defects, one deletion and two coding region insertions. Most mutations have been identified in one or a few unrelated families with the exception of C73R and L4F which occurred in 29.6% and 9.3% of the 54 mutant alleles studied, respectively. Interestingly, analysis of the mutant alleles identified only 83% of the causative mutations, suggesting that about 20% of the mutations causing CEP lie elsewhere in the gene. Of note, mutation V82F, resulting from a G to T transversion of the last nucleotide of exon 4, caused both a missense mutation and an aberrantly spliced RNA transcript. Prokaryotic expression of the mutant URO-synthase alleles identified those with significant residual activity, thereby permitting genotype/phenotype predictions for this clinically heterogeneous disease.

Introduction

Congenital erythropoietic porphyria (CEP), also known as Günther disease, is an inborn error of heme biosynthesis that results from the markedly deficient activity of uroporphyrinogen III synthase (URO-synthase; EC 4.2.1.75, hydroxymethylbilane hydrolase [cyclizing]) and the accumulation of the non-physiologic porphyrin isomer, uroporphyrin I (URO I) (Romeo and Levin, 1969; Desnick and Anderson, 1995; Kappas et al., 1995). Affected homozygotes with this autosomal recessive disease have markedly deficient URO-synthase activity. Sensitive assays have been developed to measure URO-synthase activity in erythrocytes and cultured cells; however, the erythrocyte assays are of limited value in transfused patients (Tsai et al, 1988). The URO-synthase activity is not totally deficient, even in severely affected homozygotes, since sufficient activity is required to synthesize uroporphyrin III, which is essential for heme production. The clinical manifestations of CEP are markedly heterogeneous, ranging from non-immune hydrops fetalis due to severe hemolytic anemia *in utero* to milder, later-onset forms, which have only cutaneous lesions in adult life (Deybach et al., 1981; Horiguchi et al., 1989; Desnick and Anderson, 1995; Kappas et al., 1995). Severely affected patients are transfusion-dependent throughout life, have secondary hypersplenism and are usually disfigured due to the cutaneous involvement. Other manifestations include hypertrichosis, alopecia and erythrodontia.

As shown in Figure 4-1, URO-synthase normally catalyzes the conversion of the linear tetrapyrrole, hydroxymethylbilane (HMB), by inversion of the pyrrole D ring and cyclization to uroporphyrinogen III, the physiologic cyclic isomer which is metabolized in subsequent enzymatic steps to heme

(Battersby et al., 1982a; Battersby et al., 1982b). The markedly deficient URO-synthase activity results in the non-enzymatic conversion of HMB to the uroporphyrinogen I isomer. This non-physiologic and pathogenic compound can be metabolized to coproporphyrinogen I, but further metabolism cannot proceed since the next enzyme in the pathway, coproporphyrinogen oxidase, is stereospecific for the III isomer. Uroporphyrinogen I and coproporphyrinogen I are then oxidized to the non-physiologic and presumably pathogenic porphyrins, URO I and coproporphyrin I, respectively.

Excessive URO I in erythrocytes leads to hemolysis, and the released porphyrin isomer is deposited in tissues and bones and is excreted in the urine and feces. Sunlight, and other forms of ultraviolet light, activate the photocatalytic URO I resulting in tissue damage and the formation of bullous lesions which rupture leading to scarring (Bickers and Pathak, 1987). Ruptured vesicles are prone to secondary infection leading to cutaneous scarring, bone resorption and deformities. Reviews of the clinical, biochemical and/or molecular aspects of CEP are available (Moore et al., 1987; Desnick and Anderson, 1995; Kappas et al., 1995).

Molecular Genetics of URO-Synthase

Human URO-synthase has been purified to homogeneity from erythrocytes and shown to be a monomeric protein with an apparent molecular weight of 29.5 kD (Tsai et al., 1987b). The purified enzyme had a specific activity of over 300,000 nmole/hr/mg, an isoelectric point of 5.5, and was thermolabile ($t_{1/2}$ at 60 ^{o}C ~ 1 min). The enzyme's pH optimum was 7.4 and the K_m for hydroxymethylbilane was 5-20 mM. The enzyme was activated by Na^+, K^+, Mg^{2+}, and Ca^{2+} and was inhibited by Cd^{2+}, Cu^{2+}, Hg^{2+}, and Zn^{2+}. The full-length cDNA encoding the human URO-synthase has been isolated, sequenced, and expressed in *Escherichia coli* (Tsai et al., 1988). The cDNA is 1296 bp with 5' and 3' untranslated regions of 196 bp and 302 bp, respectively, and an open reading frame of 798 bp that encodes a polypeptide of 265 amino acids. Using the cDNA as a probe, a single URO-synthase gene was assigned to the narrow chromosomal region, 10q25.3Æq26.3 (Astrin et al., 1991). Analysis of the URO-synthase genomic sequence revealed 10 exons; the sequence of each exon-intron junction has been determined (Warner et al., 1990). The availability of the full-length cDNA and genomic sequence has facilitated the identification of the molecular lesions causing CEP.

The mouse full-length cDNA encoding URO-synthase also has been isolated, sequenced and expressed (Bensidhoum et al., 1994; Xu et al., in press). The mouse cDNA had 5' and 3' untranslated regions of 144 and 623 bp, respectively, and had an open reading frame which encoded 265 amino acids. The murine gene was localized to a narrow region on mouse chromosome 7 within 1.5 cM of *Oat*, which is syntenic with the localization of the human gene on chromosome 10q25.3→q26.3. Comparison of the predicted

amino acid sequences of the human and mouse cDNAs revealed 80.5% nucleotide and 77.8% amino acid identity (Xu et al., in press). Interestingly, all but one (V82F) of the known CEP mutations occurred at amino acids that are conserved in both the mouse and human URO-synthase sequences.

Mutations in the URO-Synthase Gene

To date, 17 mutations have been identified in the URO-synthase gene causing CEP (Figure 4-2 and Table 4-1). These include: three gene rearrangements, a large deletion 148Δ98 (Boulechfar et al., 1992) and two insertions, a single base insertion 633insA (Xu et al., 1995) and a larger insertion 660ins80 (Boulechfar et al., 1992); three splicing mutations, $IVS2^{+1}$, $IVS9^{\Delta A+4}$ and V82F (Xu et al., 1995); and 12 single base substitutions, one nonsense and 11 missense mutations (de Verneuil et al., 1989; Deybach et al., 1990; Warner et al., 1990; Boulechfar et al., 1992; Warner et al., 1992a ; Xu et al., 1995). Of interest, six of these lesions occurred in exon 4, exon 10 having four lesions and exon 2 having three mutations. Of the 12 single base changes, T228M, G225S occurred at CpG dinucleotides, known hot spots for mutation (Barker et al., 1984; Cooper and Krawczak, 1990). Except for mutations C73R and L4F, these mutations have been found in only one or a small number of CEP families. As shown in Table 4-3, 15 mutations have been expressed in *E. coli* and only A66V, V82F, V99A and A104V had residual enzymatic activity, with A66V and V82F having activities greater than 10% of the mean normal expressed level.

Among these mutations, V82F was of particular interest since it was a single base substitution (G to T) in the last nucleotide of exon 4, at the 5' donor site for intron 4. To determine if this lesion also caused aberrant splicing, the URO-synthase transcripts in cultured lymphoblasts from a patient with the V82F lesion were characterized by sequencing the URO-synthase reverse transcribed- polymerase chain reaction (RT-PCR) products and by determining their relative abundance. Radiolabeling the *Eco*RI-digested RT-PCR products

revealed that 53.8% of the RT-PCR products from the V82F allele had deleted exon 4. Thus, this G to T transversion in the most 3' nucleotide of exon 4 altered the 5' splice consensus sequence and resulted in the abnormal splicing in about 50% of the transcripts. The other V82F transcripts had the missense mutation which expressed about 35% of mean normal expressed activity in *E. coli.* (Table III). Thus, V82F was both a missense mutation and a splicing defect.

The two identified splicing mutations, $IVS2^{+1}$ and $IVS9^{\Delta A+4}$, also involved 5' donor splice site sequences. RT-PCR studies of the $IVS2^{+1}$ mutation in lymphoid cells from a CEP patient whose genotype was $IVS2^{+1}$ /L4F revealed that all transcripts encoded by the $IVS2^{+1}$ allele were aberrant. In contrast, RT-PCR studies of the $IVS9^{\Delta A+4}$ mutation in lymphoid cells from a patient who was homoallelic for $IVS9^{\Delta A+4}$ revealed that exon 9 was deleted in about 75% of its transcripts (Xu et al., 1995).

The only deletion described to date was a 98 bp deletion (cDNA nt48del 98) which excised exon 4. This deletion resulted in a protein which was 50 amino acids shorter than the normal protein (33 amino acids were deleted and five different amino acids were inserted due to a frameshift) and had less than 3% of normal enzyme activity (Boulechfar et al., 1992.)

Two insertions have been identified. One (633insA) was an insertion of an adenine which caused a frameshift and resulted in termination at amino acid 214 (Xu et al., 1995). The second mutation, an insertion of 80 bases at position 660, also caused a frameshift and resulted in the synthesis of a polypepetide with the normal number of amino acids but encoded 45 different carboxy-terminal amino acids (Boulechfar et al., 1992.). Both of these insertion

mutations were expressed in *E. coli* and had activities of less than 2% of that expressed by the normal enzyme.

Frequency and Ancestry of the URO-Synthase Mutations Causing CEP

The frequency of the URO-synthase mutations which cause CEP is shown in Table 4-2. The only common mutations were C73R and L4F, which were found in 29.6% and 9.3% of the 54 alleles studied, respectively. The other URO-synthase mutations were identified in only one or two unrelated CEP patients with the exception of P53L, which was found in four unrelated patients, and G225S and T228M, which were each identified in three unrelated patients. Most patients were heteroallelic for the URO-synthase mutations with the exception of four affected individuals who were homoallelic. Three of these patients were homoallelic for C73R and one was homoallelic for IVS9$^{\Delta A+4}$. C73R, as well as the other URO-synthase mutations, were panethnic in origin, having been identified in such diverse racial and demographic groups as Japanese, Northern Europeans, Indians, Hispanics, African Americans, and Cree Indians. Notably, only 45 (83.3%) of the expected 54 mutations were detected in the 27 CEP patients studied. Of these, the lesions in 20 patients were detected by direct sequencing of the amplified genomic PCR products which contained the entire coding region and the intron/exon junctions (Warner et al., 1992; Xu et al., 1995) while the mutations in seven remaining patients were identified by sequencing subcloned RT-PCR products generated from cultured cells. Clearly, at least one, if not several cryptic mutations remain undetected, reminiscent of the experience with mutation analysis of the Factor VIII gene in patients with severe Hemophilia A (Lakich et al., 1993).

Genotype-Phenotype Correlations

The expression of the URO-synthase mutations in *E. coli* has permitted the estimation of their relative residual activities for genotype-phenotype comparisons. Table 4-3 shows the URO-synthase activities of mutations expressed in *E. coil* using the pKK223-3 vector. Following induction with IPTG, the mean activities ranged from essentially non-detectable to levels that were 14.5 to 35.8 percent of the mean activity expressed in *E. coli* by the normal cDNA. To assess the stability of the residual activities expressed by the A66V and V82F mutations, heat inactivation studies were performed. The mutant enzymes encoded by A66V and V82F were less stable than the normally expressed enzyme, with A66V and V82F having half-lives at 37 °C of 13.3 and 30.5 min, respectively, compared to 49.5 min for the expressed normal enzyme (Warner et al., 1992; Xu et al., 1995).

For genotype/phenotype correlations, a series of CEP patients were classified as very mild to severely affected based on age, and degree of hemolytic anemia, organomegaly, osteopenia, and cutaneous involvement (Table 4-4). Homoallelism for the most common allele, C73R (three patients), was correlated with the most severe phenotype, nonimmune hydrops fetalis and/or transfusion dependency from birth. Consistent with the severe phenotype of C73R homozygotes, expression of the C73R allele in *E. coli* resulted in the detection of less than 1 % of the activity expressed by the normal allele. Patients heteroallelic for C73R and a mutation that expressed little residual activity, such as T228M or A104V, also resulted in a severe or moderately severe phenotype. Patients heteroallelic for mutations that expressed residual activity such as V82F (35% of normal activity), A104V (7.7 %

of normal activity) and A66V (14.5% of normal activity) had milder forms of CEP, even if the other allele did not express detectable activity. For example, a teenage boy whose genotype was C73R/A66V only had mild cutaneous involvement and was not diagnosed until 15 years of age (Warner et al., 1992b). As additional mutations are identified and expressed, more information will become available to evaluate genotype/phenotype correlations.

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Table 4-1. Mutations in the URO-Synthase Gene of CEP Patients.

Exon / Intron	Allele designation	Nucleotide change (nt)	Effect on coding sequence	Reference
E2	L4F	CTT→TTT at 10	Leu→ Phe at 4	Deybach et al., 1990
	Y19C	TAT→TGT at 56	Tys→ Cys at 19	Xu et al,. 1995a
I2	IVS2+1	gt→at	5' Donor splice site mutation; deletion of exon 2	Xu et al., 1995a
E4	148del98	Deletion of 98 bases between 148 and 245 (exon 4)	Frameshift; results in protein 50 amino acids shorter than normal protein	Boulechfar et al., 1992
	P53L	CCT→CTT at 158	Pro→ Leu at 53	de Verneuil et al., 1989; Deybach et al., 1990
	T62A	ACC→GCA at 184	Thr→ Ala at 62	Warner at al., 1992
	A66V	GCA→GTA at 197	Ala→Val at 66	Warner et al., 1992
	C73R	TGT→CGT at 217	Cys→ Arg at 73	de Verneuil et al., 1989; Warner et al, 1992

Table 4-1. Mutations in the URO-Synthase Gene of CEP Patients (Continued).

	V82F	GTC→TTC at 243	Val→ Phe at 82	Xu et al., 1995a
E5	V99A	GTT→GCT at 296	Val→Ala at 99	Xu et al., 1995a
	A104A	GCT→GTT at 311	Ala→Val at 104	Xu et al., 1995a
E9	633insA	Insertion of A at 633	Frameshift; stop codon 28 codons downstream	Xu et al., 1995a
I9	IVS9DA^{+4}	gtaag→gtag	Deletion of an A; deletion of exon 9 in some mRNAs	Xu et al., 1995a
E10	660ins 80	Insertion of 80 bases	Frameshift at codon 221	Boulechfar et al., 1992
	G225S	GGC→AGC at 673	Gly→Ser at 225	Xu et al., 1995a
	T228M	ACG→ ATG at 683	Thr→Met at 228	Boulechfar et al., 1992; Warner et al., 1992
	E249X	CAA→TAA at 745	Glu→Ter at 249	Xu et al., 1995a

TABLE 4-2 Allele frequency of URO-Synthase Mutations causing CEP*.

Mutations	Number (of 54 CEP Alleles)	Percent (of 54 CEP Alleles)
Single Base Substitutions:		
C73R	16	29.6
L4F	5	9.3
P53L	3	5.6
G225S	3	5.6
T228M	3	5.6
Y19C	1	1.9
T62A	1	1.9
A66V	1	1.9
V99A,	1	1.9
A104V	1	1.9
Q249X	1	1.9
Gene Rearrangements:		
633insA	1	1.9
660ins80	1	1.9
148del98	2	3.7

TABLE 4-2. Allele frequency of URO-Synthase Mutations Causing CEP.*

RNA Processing Defects:		
$IVS2^{+1}$	2	3.7
$IVS9^{\Delta A+4}$	2	3.7
V82F	1	1.9
Unknown Alleles:	9	16.7

*Deybach et al., 1990; Boulechfar et al., 1992; Warner et al., 1992; Xu et al., 1995a.

TABLE 4-3. Expression of URO-Synthase Mutant Alleles in *E. Coli.*

Mutation	Residual Activity (Percent of expressed mean normal level)*	Reference
Normal	100	Warner et al., 1992
L4F	1.8	Xu et al., 1995a
Y19C	1.1	Xu et al., 1995a
P53L	<1.0	Warner et al., 1992
T62A	<1.0	Warner et al., 1992
A66V	14.5**	Warner et al., 1992
C73R	<1.0	Warner et al., 1992
V82F	35.8**	Xu et al., 1995a
V99A	5.6	Xu et al., 1995a
A104V	7.7	Xu et al., 1995a
633insA	1.2	Xu et al., 1995a
G225S	1.2	Xu et al., 1995a
T228M	<1.0	Warner et al., 1992
Q249X	1.1	Xu et al., 1995a
148Δ98	<2	Boulechfar et al., 1992
660ins80	<2	Boulechfar et al., 1992

* Each mutant URO-synthase allele was expressed using the pKK223-3 vector. Specific activity is expressed as the mean of 2-7 independent assays.

**Heat inactivation experiments demonstrated the residual activites to be markedly unstable when compared to the normal activity. See text for details.

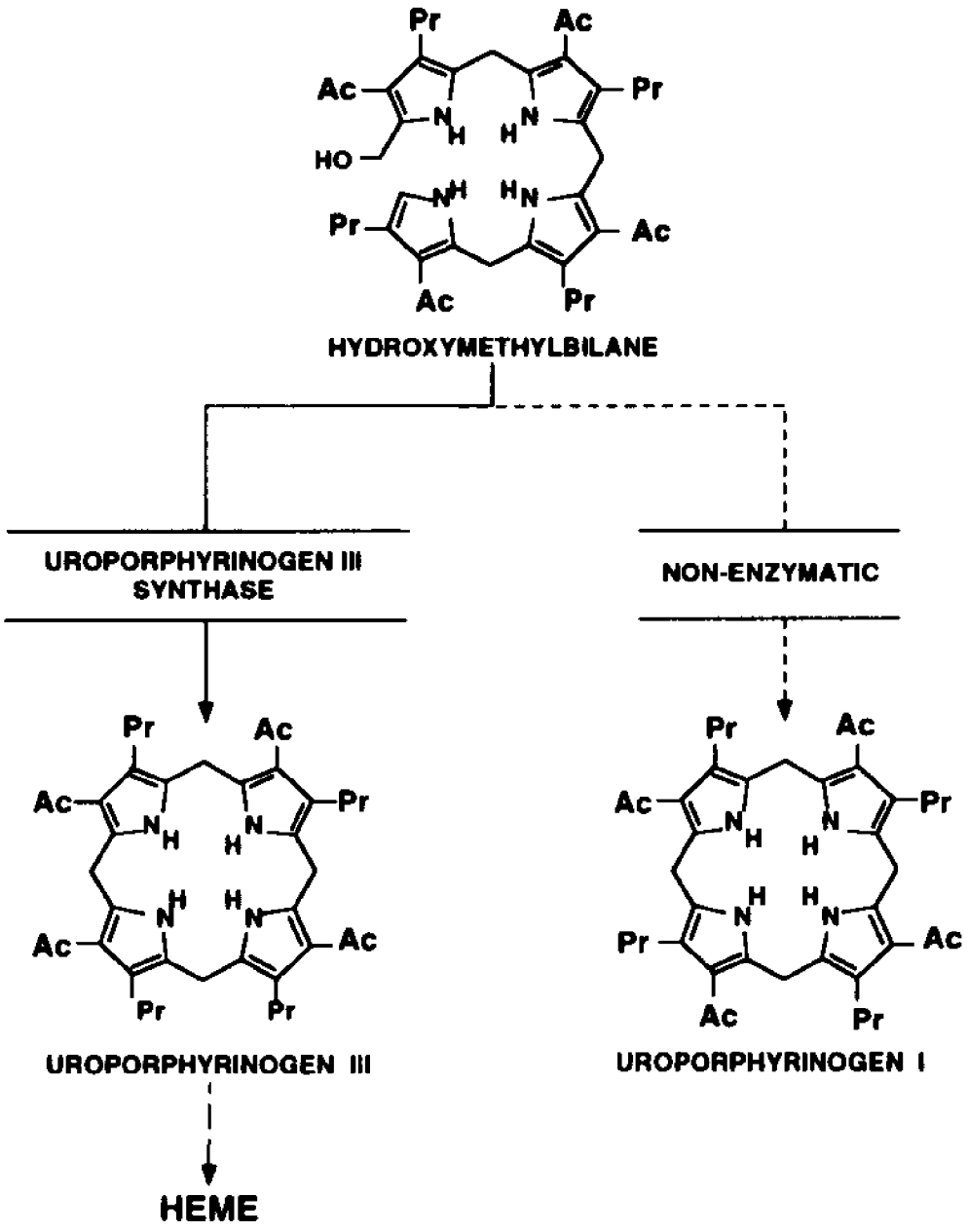

Figure 4-1. The Enzyme Defect in Congenital Erythropoietic Porphyria.

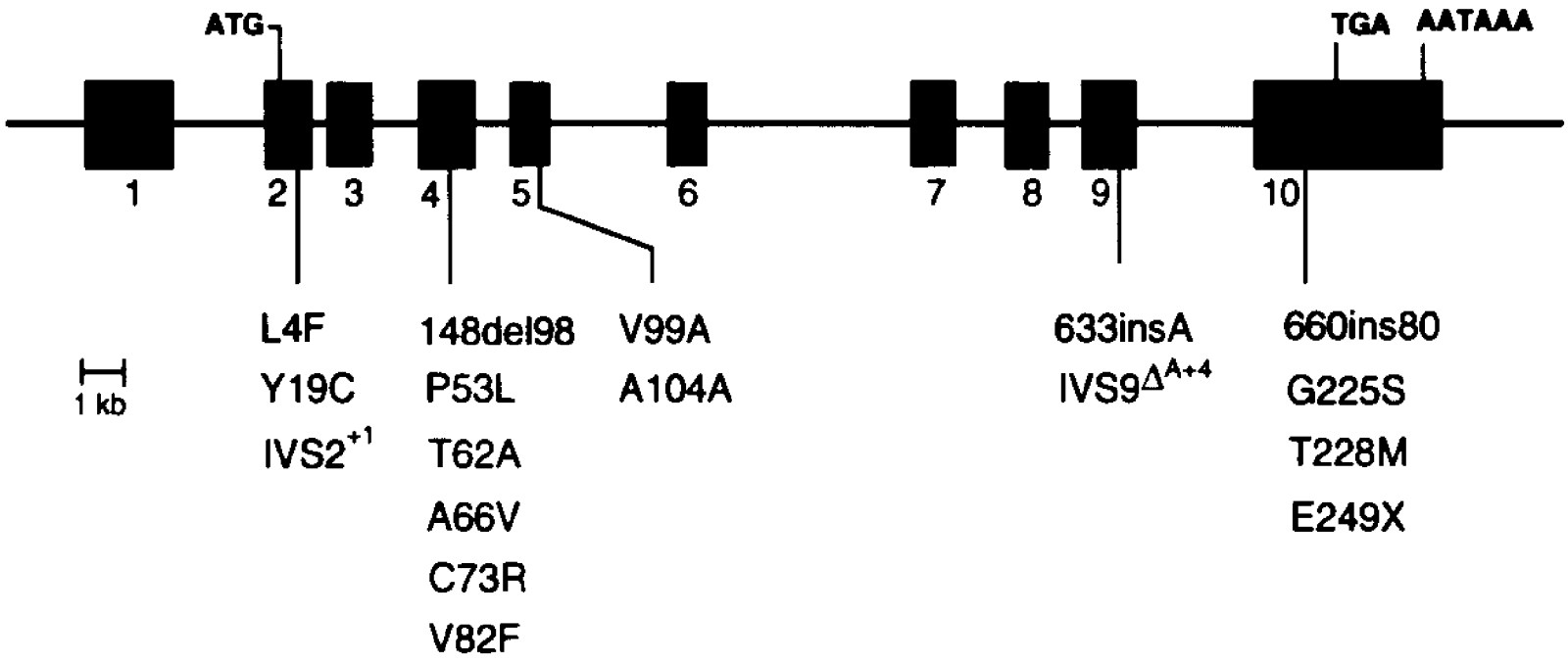

Figure 4-2. Mutations in the URO-Synthase Gene Causing CEP

Chapter 5

Organization of the Murine and Human Uroporphyrinogen III Synthase Genes: Evidence for Alternative Splicing from a Single Gene in Both Species

Weiming Xu and Robert J. Desnick

Abstract

Uroporphyrinogen III synthase (URO-synthase; EC 4.2.1.75), the fourth enzyme in the heme biosynthetic pathway, is responsible for the conversion of hydroxymethylbilane to the cyclic tetrapyrrole, uroporphyrinogen III, and the deficient activity of URO-synthase is the enzymatic defect in congenital erythropoietic porphyria (CEP). In the course of screening a mouse adult liver cDNA library (2 x 10^6 recombinants) with a human full-length URO-synthase cDNA probe, two groups of clones were identified which differed only in their most upstream 5' untranslated regions. The longest of the eight group 1 clones and six group 2 clones had 140 and 47 bp, respectively, unique 5' untranslated sequences followed by 80 bp of common sequence prior to the initiation ATG. Subsequent screening of a human fetal liver cDNA library (2 x 10^6 recombinants) also identified two groups of clones which differed only in their most 5' untranslated sequences. Of the 26 positive clones, two human group 1 cDNAs had the previously reported 5' untranslated sequence (8), while the longest of the other 24 positive clones had 107 different 5' untranslated nucleotides. Sequencing of the human URO-synthase genomic clone identified the 107 bp of 5' untranslated sequence immediately proceeding 26 bp of 5' untranslated sequence in exon 2. Computer assisted analysis of the adjacent intron 1 sequence revealed CAAT boxes, TATA boxes, GATA1 binding sites, GATA1-0 binding site, and NF-E2 binding site, consistent with a promoter region in intron 1. Of note, sequencing of the putative intron 1 promoter region in seven unrelated CEP patients identified a C to A transversion at -86 nt upstream of exon 2 in two patients who each had only one known URO-synthase mutation detected by sequencing the entire coding region and adjacent intron-exon boundaries. This -86 nt transversion was not detected in

23 unrelated CEP patients with two known mutations or in 85 unrelated normal individuals. These findings suggest that the human URO-synthase gene has two promoters, presumably for housekeeping and erythroid-specific expression. If so, the -86 nt transversion is the first regulatory mutation identified in any porphyria.

Introduction

Uroporphyrinogen III synthase [URO-synthase; hydroxymethylbilane hydrolyase (cyclizing), EC 4.2.1.75] is the heme biosynthetic enzyme responsible for the conversion of the linear tetrapyrrole, hydroxymethylbilane (HMB), to the first cyclic tetrapyrrole and the physiologic precursor of heme, uroporphyrinogen III (1). The enzyme functions both as an isomerase and a cyclase as it catalyzes the intramolecular rearrangement of ring D and ring closure, respectively (2, 3). The deficient (but not absent) activity of URO-synthase is the enzymatic defect in the human disorder, congenital erythropoietic porphyria (CEP) (4). This inborn error of heme biosynthesis is inherited as an autosomal recessive trait and is clinically heterogeneous (5, 6).

Previously, cDNAs encoding human URO-synthase were isolated from an adult liver library (8). The longest cDNA of 1.3 kb had 5' and 3' untranslated sequences of 196 and 288 bp, respectively, and encoded a polypeptide of 265 amino acids. This cDNA was expressed in *E. coli* (8). Using that cDNA as a probe, a single human gene encoding URO-synthase was regionally mapped to chromosome 10q25.2→26.3 (9). More recently, the URO-synthase genomic sequence was isolated (10). Analysis of the ~35 kb gene revealed 10 exons, and the sequence of each exon-intron junction was determined (10). Recently, a mouse full-length cDNA encoding URO-synthase was isolated, sequenced and expressed (11, 12). The mouse cDNA had 5' and 3' untranslated regions of 144 and 623 bp, respectively, and also had an open reading frame which encoded 265 amino acids. Comparison of the predicted amino acid sequences of the human and mouse cDNAs revealed 80.5% nucleotide and 77.8% amino acid identity (12). The murine gene was localized to a narrow region about 1.5

cM from *Oat* on mouse chromosome 7 (12), which was syntenic with the location of the human gene on chromosome 10q25.3→q26.3.

In this communication, we report that further characterization of mouse URO-synthase cDNAs from an adult mouse liver cDNA library revealed two groups of clones which differed only in their most upstream 5' untranslated sequences. The finding of two unique mouse URO-synthase transcripts suggested that the murine URO-synthase gene was either alternatively spliced, that two URO-synthase gene encoded almost identical transcripts, or that a single gene with two promoters encoded two transcripts, the later being analogous to the occurrence of housekeeping and erythroid-specific transcripts from a single gene for two other heme biosynthetic enzymes, d-aminolevulinate dehydratase (35, 36) and hydroxymethylbilane synthase (37). Subsequent cloning of human URO-synthase cDNAs from a fetal liver cDNA library revealed two cDNAs which also differed only in their most upstream 5' untranslated regions. These findings suggested that the human and mouse URO-synthase genes have two promoters and express tissue-specific transcripts encoding the same polypeptide. Sequence analysis revealed that the newly identified 107 bp upstream 5' translated region was located in intron 1, immediately adjacent to exon 2 in the human gene, and contained various putative erythroid promoter elements. The finding of a base substitution in this region in a CEP patient supported the concept that this region is important in the regulation of URO-synthase gene expression.

Experimental Procedures

Construction of Probes for Library Screening. For mouse cDNA library screening, sense and antisense primers CW131 (5'-ggcgcggaattcccggggatccgtcgacctgca-gccaagct**gtcccgcgagtgccc**-3' and CW129 (5'-atgcctggctccatccag-3'), which annealed to -36 to -22 nt and from 849 to 866 nt, respectively, of the human URO-synthase cDNA, were used to amplify the plasmid pKK223-3E-UROS-sense (8), and the resulting 942 bp polymerase chain reaction (PCR) (17) product was gel purified using the Wizard™ PCR Preps DNA purification system (Promega Biotec, Madison, WI). In CW131 only the bolded nucleotides were complementary to human URO-synthase cDNA. The ^{32}P-labeled probe was generated using the PCR product as template by random priming with the NEBlot™ Kit (New England Biolabs, Inc., Beverly, MA) according to manufacturer's instructions. For screening the human fetal liver cDNA library, the sense and antisense primers WX33 (5'-gaattaggattatatggacttgaa-3') and WX167 (5'-tcagcagcagccatggggctggagagc-3'), which annealed to nt 62 to 87 and nt 772 to 799, respectively, of the human URO-synthase cDNA, also were used to amplify the plasmid pKK223-3E-UROS-sense (8), and then the 737 bp PCR product was purified and ^{32}P-labeled as described above or previously (12).

Isolation of Mouse and Human cDNA Clones. A total of 2 X 10^6 recombinants from the B6/CBAF1J mouse adult liver cDNA library (Stratagene Cloning System, La Jolla, CA) constructed in the lambda ZAP vector were screened with the radiolabeled human URO-synthase cDNA as described previously (12). The filters (low water extractable HATF filters from Millipore Co., Bedford, MA) were prehybridized at 50 °C for 4 hr with 0.1% NaDod(SO_4)/6X SSC/5X

Denhardt's solution and 100 µl/ml denatured salmon sperm DNA (1 X SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7.0; 1 X Denhardt's solution = 0.02% Ficoll/ 0.02% polyvinylpyrrolindone/ 0.02% bovine serum albumin). Hybridization of the human cDNA (1 X 10^6 cpm/ml) was carried out at 50 °C for 16 hr. Washing was performed at 50 °C with 2 X SSC/0.1% $NaDodSO_4$ for 2 hr. Putative positive plaques were subcloned and rescreened as above until they were homogeneous. For isolation of human URO-synthase, 2 ¥ 10^6 recombinants from the human fetal liver 5'-stretch cDNA library (CLONTECH laboratories, Inc., Palo Alto, CA) constructed in the LambdaDR2 vector were screened with radiolabeled human URO-synthase cDNA as described above.

Analysis of Positive URO-Synthase Clones. The mouse cDNA inserts were excised from the positive clones *in vivo* from the lambda ZAP vector in the pBluescript phagemid with the ExAssist™/ SOLAR™ System according to manufacturer's protocol. For sizing, the mouse cDNA inserts were released by digestion with *Eco*RI followed by gel electrophoresis. The human cDNA inserts were excised from the positive clones in pDR2 plasmids by *in vivo* conversion in *E. coli* AM1 according to manufacturer's protocol. For sizing, the cDNA inserts were excised by *Bam*HI/*Xba*I double digestion followed by gel electrophoresis. Each of the mouse and human inserts were sequenced in both orientations by the dideoxy chain termination method (20) to determine authenticity of the cDNA inserts.

Computer Assisted Analysis. DNA sequencing films were read and information entered into MacVector™ 4.0 Program (International Biotechnologies, Inc., New Heaven, CT) by an IBI Gel Reader™. DNA sequence data analysis and DNA subsequence searches were carried out

using MacVector™ 4.0. Cis-acting transcriptional element searches were performed with the help of the transcriptional factor database compiled by Dr. David Bishop (Mount Sinai School of Medicine).

Analysis of Putative Normal and Mutant Promoter Regions. Genomic DNAs from unrelated CEP patients and from unrelated normal individuals were isolated from cultured lymphoblasts (16) or fresh blood collected in EDTA with informed consent. The putative promoter region upstream from exon 2 was amplified using sense and antisense primers WX110 (5'-btagatcacctgagctcatgagttcga-3') and WX207 (5'-cgcatccttcagtaaaagaaccttcat-3'). WX110 is a biotinylated primer that anneals to the region 679 to 654 nt upstream from the putative 5' end of exon 2. WX207 anneals the region from nt 27 to 1 in the previously reported human URO-synthase cDNA (8). The 100-ul amplification reaction contained 2 ug of genomic DNA, 100 pmol of each primer, 10 nM of each dNTP, 50 mM Tris-HCl, pH 9.0, 50 mM NaCl, 10 mM $MgCl_2$, and 2.5 units of *Taq* polymerase (Perkin-Elmer, Corp.). After an initial 5 min incubation at 94 °C, amplification (35 cycles) was performed with denaturation at 94 °C for 1 min, extension at 72 °C for 1 min, and annealing at 65 °C for 1 min. A final extension of 10 min at 72 °C was performed after the last cycle. An aliquot (40 ul) of each amplification product was incubated with 40 ul of streptavidin-coated paramagnetic beads (Dynabeads M-280 Streptavidin, Dynal, Inc., Lake Success, NY) for 30 min with occasional gentle mixing. Beads with bound biotinylated PCR products were separated with a magnet (18) and strand separation was performed as previously described (19) The biotinylated strands were resuspended in 7 ul of H_2O and used as templates for dideoxy chain sequencing (20). Antisense primers CW47 (5'-ccatgacttagcactaa-3') and CW 104 (5'-agggttgtatcaacaaca-3'), which anneal to

regions 19 to 36 nt and 192 to 210 nt upstream from the putative 5' end of exon 2, respectively, were used in addition to WX207 to sequence the ~600 bp promoter region in the PCR product.

Detection of the IVS1$^{86C \cdot A}$. The C to A transversion 86 bp upstream from the putative 5' end of exon 2 (Fig. 7) created a *Sau*3AI recognition site. Sense primer WX 109 (5'-tagatcacctgagctcatgagttcga-3'), which annealed to the region -679 to -654 nt upstream from the putative end of exon 2, and WX206 (5'-gcgacagagcaaggaaacagatcttaattagatctcaaag-3') which annealed to regions 104 to 66 nt nt downstream from the putative end of exon 2, respectively, were used to amplify genomic DNAs from unrelated normal individuals and CEP patients. PCR was carried out as described above and the products were purified with the Wizard™ PCR Preps DNA purification system. The 20 ul *Sau*3AI digestion contained 16.8 ul of purified PCR product, 2 ul of 10 x *Sau*3AI digestion buffer, 1ul of *Sau*3AI (New England BioLabs, Beverly, MA, 4,000 units/ml), 0.2 ul of 100 x acetylated bovine serum albumin. Following a incubation at 37 °C for at least 5 hr, the reaction mixture was electrophoresed in a 1.7% agarose gel. While only four fragments (4 bp, 11 bp, 21 bp and 748 bp) were expected from normal samples, five (4 bp, 11 bp, 21 bp, 158bp and 590 bp) and six (4 bp, 11 bp, 21 bp, 158bp, 590 bp and 748 bp) were generated from individuals homozygous and heterozygous, respectively, for the mutation.

Results

Isolation and Characterization of Positive Mouse URO-Synthase cDNAs. Of the 35 clones initially selected from the mouse adult liver cDNA library, 16 remained positive after purification. The 16 cDNAs ranged in size from ~1.4 to ~1.6 kb. All the clones were excised into pBluescript phagemid constructs and sequenced in both directions. While all the clones shared the same 3' untranslated sequence of 623 bp and an open reading frame of 798 bp encoding a 265 amino acid polypeptide, two different 5' untranslated sequences were found. The group 1 sequence, found in 8 clones, had up to 220 bp of 5' untranslated sequence; the group 2 sequence, found in 6 clones, had up to 127 bp of 5' untranslated sequence, of which the first 80 bp adjacent to the open reading frame were identical (Fig. 1). The other two clones had 5' untranslated regions less then 80 bp. These findings indicated that the murine URO-SYNTHASE gene was alternatively spliced in adult mouse liver, resulting in two transcripts which differed in their 5' untranslated regions. It also implied that there may be two different cis-acting promoter elements in the gene. Of interest, sequence 1 and 2 each contained a small open reading frame encoding a peptide of 22 and 18 amino acids, respectively (Fig. 2).

Isolation and Characterization of Positive Human URO-Synthase cDNAs. Of the 41 putative positive clones initially selected from the human fetal liver 5'-stretch cDNA library, 26 remained positive after purification. The cDNA inserts of the 26 positive clones ranged in size from ~1.2 to ~1.3 kb. All the clones were excises into pDR2 plasmid constructs and sequenced in both directions. While all the inserts shared the same sequence for exon 2 through exon 10, two different sequences were present in the 5' untranslated region encoded by

exon 1. Two clones had the same exon 1 sequence as the previously published human URO-synthase cDNA (8), while the other 24 clones had a novel 5' sequence of up to 107 bp (Fig. 2). There were no ATG triplets in either of the two 5' untranslated sequences. Comparison of the novel URO-SYNTHASE 5' untranslated sequence with the human genomic sequence revealed that the alternative exon 1 (exon 2) encoding the 107 bp is adjacent to the common exon 2. The organization of human URO-synthase gene and the alternative splicing of its transcripts is shown in Fig. 4.

Human URO-Synthase Promoters. Previously, the 5' flanking region upstream of exon 1 was determined in a normal genomic clone containing the URO-synthase gene (Tsai et al., unpublished data). Computer-assisted analysis of the promoter region (nt -1 to nt -528) upstream from the putative 5' end of exon 1 revealed a region containing several potential *cis*-acting regulatory elements, shown in boldface in Fig. 5. Putative transcription initiation sites were based on the longest cDNA clone sequences. Potential binding sites for transcription factors close to the putative transcription initiation site included two CACCC elements (22) at nt -31 and -155, respectively; two CAAT box-like elements (23) at nt -242 and -518, respectively; and four GATA1-0 binding sites (24), three in the reverse orientation at nt -103, -151 and -183, one in the forward orientation at nt -72.

Computer-assisted analysis of the promoter region (nt -1 to -700) upstream from the putative 5' end of exon 2 revealed the potential transcription elements shown in boldface in Fig. 6. Putative transcription initiation sites were based on the longest cDNA clone sequences. The potential transcriptional factor binding sites include: five GATA-1 binding sites (25, 26, 27), all in the

reverse orientation, at nt -63, -247, -270, -470 and -556; one GATA1-0 binding site, in the forward orientation at nt -13; one NF-E2 (28, 29, 30, 31, 32) binding site in the forward orientation at nt -31; one GATA1-0 binding site in the forward orientation at nt -13; four TATA box-like elements in the forward orientation at nt -411, -410, -412, and -585; and a CAAT box-like element in the forward orientation at nt -316.

Identification of Base Substitutions in the URO-Synthase Promoter Regions . Based on the finding of two putative promoters, efforts were directed to identify mutations in CEP patients who did not have both mutant alleles identified when the entire coding region and intron/ exon boundaries were analyzed. In one CEP patients, a C to A transversion was detected in the intron 1 promoter region upstream from exon 2 at nt -86, designated IVS1$^{-86C \cdot A}$. Each patient had one previously known mutant allele. One patient had the genotype IVS2^{+1}/IVS1$^{-86C \cdot A}$ and a mild phenotype; the other patient had the genotype C73R/IVS1$^{-86C \cdot A}$ and a moderately severe phenotype. To determine if this base substitution was a polymorphism, genomic DNAs of 85 normal individuals and 24 unrelated CEP patients were analyzed by PCR amplification and *Sau*3A1 digestion for the IVS1$^{-86C \cdot A}$ lesion. The mutation created a *Sau*3A1 site in gene permitting amplification of a 784 bp PCR product, a 748 bp *Sau*3A1 fragment of which when digested would be cleaved into 158 and 590 bp fragments when the sequence is mutant. As shown in Table I, the IVS1$^{-86C \cdot A}$ mutation was not present in the 170 normal alleles, was not detected in 36 alleles of CEP patients with two known or two unknown mutations, but was detected in one other CEP patient who had one previously known and one unknown allele.

Discussion

Human URO-synthase has been purified to homogeneity from erythrocytes and was shown to be a monomeric protein with an apparent molecular weight of 29.5 kDa (7). Using degenerate oligonucleotides based on partial amino acid sequences of the enzyme, eight positive clones were isolated from a human adult liver library, however, only the longest clone was characterized and expressed in *E. coli* (8).

To date the erythroid-specific expression of three of the eight heme biosynthetic genes has been demonstrated. These included d-aminolevulinate synthase (ALAS), d-aminolevulinate dehydratase (ALAD) and hydroxymethylbilane synthase (HMBS). In addition, evidence for two uroporphyrinogen decarboxylase isozymes in human erythrocytes has been presented by one group of investigators (33) but not by another (34). In this communication, the first evidence for the tissue-specific expression of URO-synthase is presented. The finding that there were two cDNAs for mouse URO-synthase may also be subject to transcriptional level regulations according to different tissues at different developmental stages by utilization of two promoters. Of interest, both mouse 5' untranslated regions contained small open reading frames as shown in Fig. 2. The occurrence of such Since the ATG triplet in the small opening frame in sequence 1 of mouse URO-synthase cDNA is in a better Kozak sequence than its counterpart in sequence 2, it is more likely for the small open reading frame in sequence 1 to start and then terminate a translation, thereby competing away to a higher extent the chance at which the downstream main open reading frame encoding the mouse URO-synthase peptide is translated.

While the demand for porphyrin varies with stages of development and from tissue to tissue, the significance of appropriate expression levels of heme biosynthetic enzymes may be more than being economic. While it has long been known that deficient URO-synthase leads to accumulation of the nonphysiologic and pathogenic uroporphyrin I (URO I) isomer and causes CEP (5, 13, 14), moderate level of mouse URO-synthase expression in *E. coli* has been shown to be lethal to the host cells even in total darkness (12). Similar phenomenon has also been observed for δ-aminolevulinate synthase (13), the first enzyme in the heme biosynthesis pathway. These findings suggested that like some other biological molecule, the quantity of porphyrins, irrespective of metabolizability, has to be controlled within a certain range *in vivo* for a healthy life.

Compared to the genes encoding ALAS, ALAD and PBGD, the organization of human URO-synthase gene resemble that of ALAD more than other two, since the two transcripts shared exon 2 through 10 and the same open reading frame. However, the organization of human URO-synthase gene is unique in that the so called exon 2 and exon 2 are essentially one exon, into which exon 1 is spliced in the other transcript.

As in human ALAD gene, both of the promoter sequences flanking exon 1 and 2 contained multiple erythroid specific transcription activation factors, therefore although it is reasonable to assume that as in other heme biosynthetic genes known to have two promoters, the two promoters in URO-synthase may serve the need of different transcription level, it is difficult to predict which one actively serve the more porphyrin-needy tissues, e.g., erythroid, or which one

mainly functions in cells that need low level of porphyrin for cytochrome and other hemoproteins. Further studies are needed to determine the degree of activity of the two promoters in different types of cells.

While the previous isolation and characterization of URO-synthase cDNA and genomic sequence have facilitated the identification of molecular lesions causing CEP, in some cases the entire coding region and mRNA processing elements on both alleles were examined, some 17% of mutant alleles remained unidentified in CEP homozygotes described in the literature (21). One possible explanation for the missing mutations is that they may be transcriptional defects, since no studies or examination of the transcriptional elements of the URO-synthase gene in either normal individuals or CEP patients have been reported. In this study, a C to A transversion 86 bp upstream from the putative 5' end of exon 2, which was present in two CEP homozygotes each with only one known mutation and was absent from both the 16 CEP patients with two known mutations and the 75 normal individuals screened. The allele frequency of this mutation among different populations is consistent with the possibility that it is disease causing, but the fact that this mutation did not affect any of the known transcription elements made it clear that further evaluation of the effect of this mutation on the function of the promoter region flanking exon 2 is needed.

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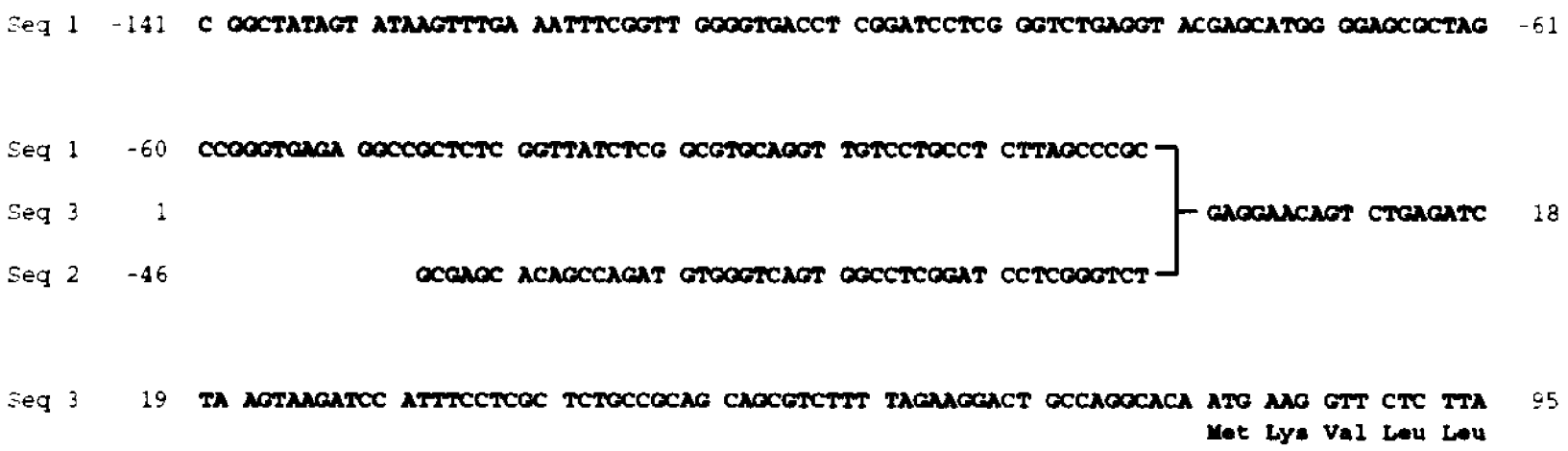

Figure 5-1. Mouse URO-synthase gene has two alternatively spliced transcripts differing in their 5' untranslated regions.

A. Group 1 Sequence

```
-220                                                                                                    -156
                                    CGGCTA TAGTATAAGT TTGAAATTTC GGTTGGGGTG ACCTCGGATC CTCGGGTCTG AGGTACGAG

-155                                                                                                     -81
   C ATG GGG AGC GCT ACG CGG GTG AGA GGC CGC TCT CGG TTA TCT CGG CGT GCA GGT TGT CCT GCC TCT TAG CCCGC
     Met Gly Ser Ala Thr Arg Val Arg Gly Arg Ser Arg Leu Ser Arg Arg Ala Gly Cys Pro Ala Ser ***

 -80                                                                                                1          9
   GAGGAACAGT CTGAGATCTA AGTAAGATCC ATTTCCTCGC TCTGCCGCAG CAGCGTCTTT TAGAAGGACT GCCAGGCACA ATG AAG GTT
                                                                                                      Met Lys Val
```

B. Group 2 Sequence

```
-127                                                                                                    -74
                                                    TGCGAGC ACAGCCAG ATG TGG GTC AGT GGC CTC GGA TCC TCG GGT CTG AGG AAC
                                                                     Met Trp Val Ser Gly Leu Gly Ser Ser Gly Leu Arg Asn

 -73                                                                                                1          9
   AGT CTG AGA TCT AAG TAA GATCC ATTTCCTCGC TCTGCCGCAG CAGCGTCTTT TAGAAGGACT GCCAGGCACA ATG AAG GTT
   Ser Leu Arg Ser Lys ***                                                                            Met Lys Val
```

Figure 5-2. The 5' untranslated regions of group 1 (A) and 2 (B) each contains a short open reading frame.

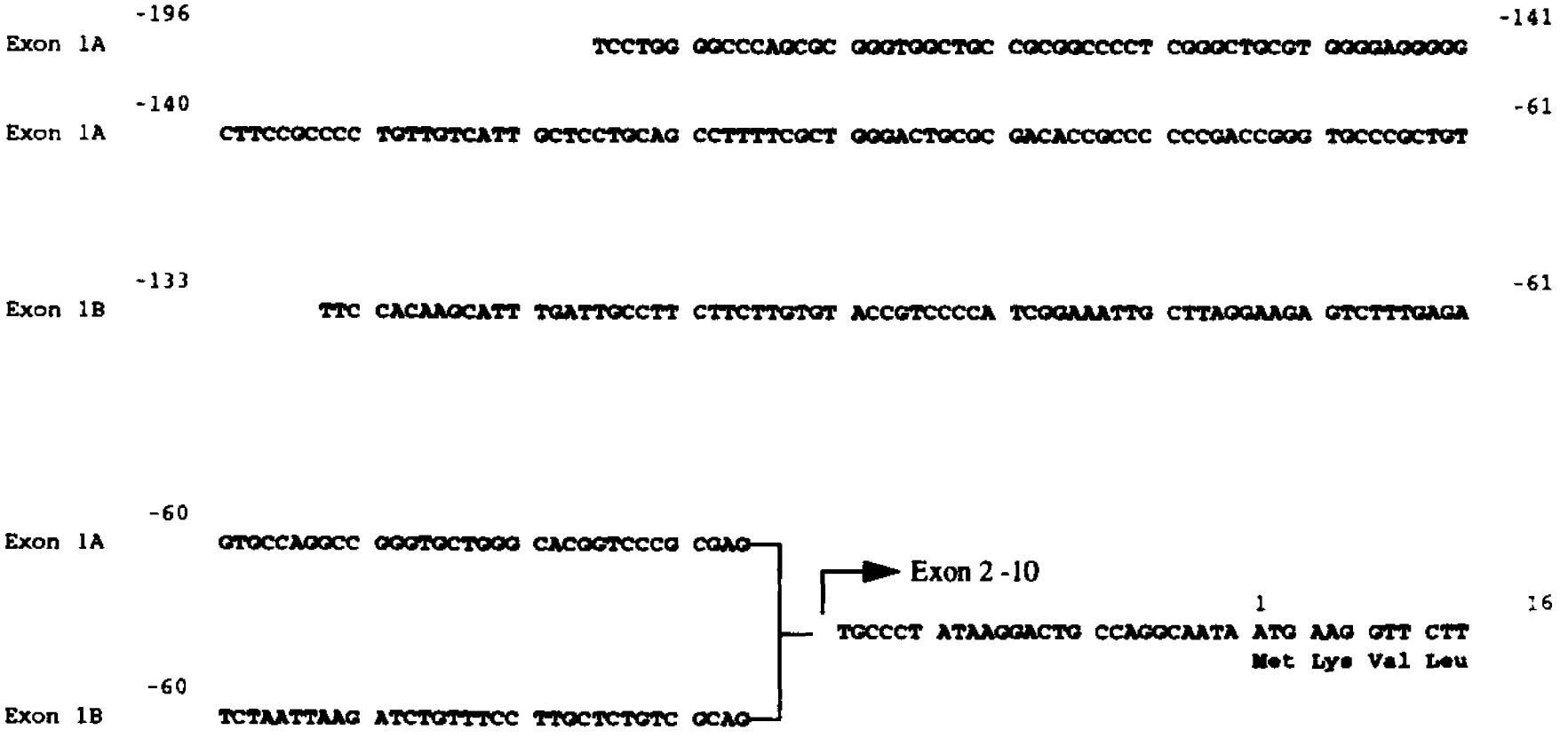

Figure 5-3. The two human URO-synthase gene transcripts differ in exon 1.

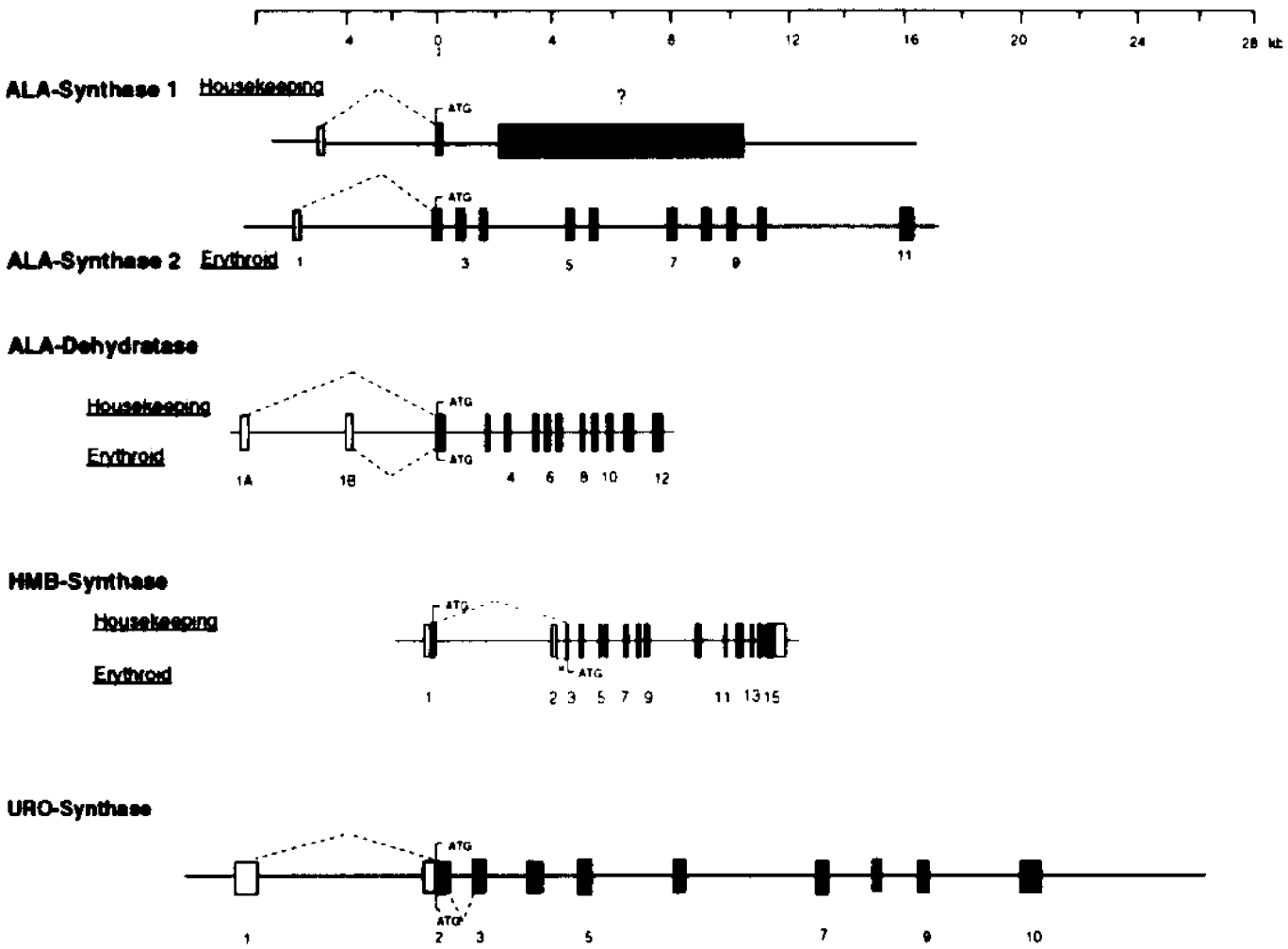

Figure 5-4. Gene Organization of the First Four Enzymes in the Heme Biosynthetic Pathway.

PROMOTER SEQUENCE:

```
  -528                                    TTCTCACC TCGTCAATGA CCTCGTCCTC
                                                    ›CAAT

  -500  TTCATCCTTT TCCTTGTCAC TGTCATCATC GTCTTCATCC TCATTTTCGA

  -450  CTTCTTTTTC CTCTTCCTCG TCGCGCTGGA CTGGGGGATC CGGCGGCTGC

  -400  GGAACCCCAC TTTCCACGGC ACGCCGCTTA GACCTGGACG CCATGTTGCC

  -350  GCTCACACTG CGCCTGCCGG CTTCCCCTTG CCTGACCTTC CGGACCAGCC

  -300  CATAGTAGCC AAGGGGGTGA GGAACTACGA AGAAGCTCGG CGCTGAGTGC

  -250  ACGTCAACCC CAATGCCCCT GCTGGATTCC GCAGCCATGA CCCCTTACAG
                 ·CAAT

  -200  TCACGTCCAG CTTGCGGTCC CATCTTGCTT TTTTCTTCCG CTTTCCACCC
                          · GATA1-0                        ·CAC

  -150  TCCATCACTC CTCCCAAAGC TTCCCCTCCC CTTCCCGTAG TGGTTCAACC
        · GATA1-0

  -100  CATCTAACCT CTGGCCTCAT TCCGTGGCCT GGATGGCGGT GGCATCGCGA
        · GATA1-0                              ·GATA1-0

   -50  GCGCCTACCT GCGCGCAGCC ACCCACGAGA CCCCAGTCTG AGGTGCGGGG
                              ·CAC
```

EXON 1:

```
     1  TCCTGGGGCC CAGCGCGGGT GGCTGCCGCG GCCCCTCGGG CTGCGTGGGG
```

Potential Regulatory Elements

1. "CAAT": GCCAAT
2. "CAC": CACCC
3. GATA1-0: SVNGATDGBB

B = G/T/C; D = G/A/T; N = G/A/T/C; S = C/G; V = G/A/C

Figure 5-5. The promoter region upstream from exon 1 of human UROsynthase gene.

```
PROMOTER SEQUENCE:

     -700  ATTTTGGGAG GCCGAGGCAG GTAGATCACC TGAGCTCATG AGTTCGAGAC

     -650  TAGCCTGGGC AACATGGTGA AACCCTGCCT CTAAAAAAAA AAAAAAAAAA

     -600  AAAAAAAAAT ACATCTATAA ACAAGAAGCT TCCCGTTGCT ATTAAGATAA
                           ›TATA BOX                          ‹GATA1

     -550  GAGCCACATT CCCTAATATG ACCCAACGAG ACCCAAAAGA ATTCTAGTGT

     -500  GAGCATCTTT ATGTTAATCT TTGGACACAT TTATCAGTAT TGCTTTAGTA
                                            ‹GATA1

     -450  TTCTTAGAAG TGAAATTACA GGAAGAAAGA AGATGAAGTA TATAAAGCTT
                                                  ›TATA BOX
                                                    ›TATA BOX

     -400  TTGATGTCAA CAAATCGTTT TCCATGTTGT TAGACTCTCT TATGATACTT

     -350  GACGTTTAGA AAACATCATT TAATAAGTAG AAGAGTCAAT ATAATTATTT
                                               ›CAAT
                                                    ›TATA BOX

     -300  CCCAAAGTGC TTATGAGCTA CTTAGCATAT TTATCTTGAA ATGGAAAAGC
                                            ‹GATA1

     -250  TATTTATCTC CTTAAAACAT AAGATATCAA TTTCTCTGTT TGTTGTTGAT
              ‹GATA1

     -200  ACAACCCTAA ATTTACATCA GTTCTCTTTC CGGAACCATA AACGTTTGGC

     -150  ATAGACACTT GGCTTTCACT AATGGTTTCA TCTGTCTTTC CAAGTGATAT

     -100  CAACTGCTAA CATGCTCTTT CTTGGCCTTA TCAGTGACAG GGGTCTTCAG
                                         ‹GATA1

      -50  AAAGAACAAG CCCATTAGTG CTAAGTCATG GTGTAGGAGG GATAGGTCTC
                                 ‹NF-E2               ›GATA1-0

EXON 2:

        1  TTCCACAAGC ATTTGATTGC CTTCTTCTTG TGTACCGTCC CCATCGGAAA

Potential Regulatory Elements:

        1. "CAAT":     GCCAAT
        2. GATA1:      WGATAR
        3. GATA1-0:    SVNGATDGBB
        4. TATA Box:   TATAWAW
        5. NF-E2:      GCTGAGTCA

           B = G/T/C; D = G/A/T; N = G/A/T/C; R = A/G; S = C/G;
           V = G/A/C; W = A/T
```

Figure 5-6. The promoter region upstream from exon 2 of human URO-synthase gene.

PROMOTER SEQUENCE:

-700 ATTTTGGGAG GCCGAGGCAG GTAGATCACC TGAGCTCATG AGTTCGAGAC

-650 TAGCCTGGGC AACATGGTGA AACCCTGCCT CTAAAAAAAA AAAAAAAAAA

-600 AAAAAAAAAT ACATC**TATAA AC**AAGAAGCT TCCCGTTGCT ATTA**AGATAA**
›TATA BOX ‹GATA1

-550 GAGCCACATT CCCTAATATG ACCCAACGAG ACCCAAAAGA ATTCTAGTGT

-500 GAGCATCTTT ATGTTAATCT TTGGACACAT **TTATCA**GTAT TGCTTTAGTA
‹GATA1

-450 TTCTTAGAAG TGAAATTACA GGAAGAAAGA AGATGAAG**TA TATAAAG**CTT
›TATA BOX
›TATA BOX

-400 TTGATGTCAA CAAATCGTTT TCCATGTTGT TAGACTCTCT TATGATACTT

-350 GACGTTTAGA AAACATCATT TAATAAGTAG AAGA**GTCAAT ATAATT**ATTT
‹CAAT
›TATA BOX

-300 CCCAAAGTGC TTATGAGCTA CTTAGCATAT **TTATCT**TGAA ATGGAAAAGC
‹GATA1

-250 TAT**TTATCT**C CTTAAAACAT AAGATATCAA TTTCTCTGTT TGTTGTTGAT
‹GATA1

-200 ACAACCCTAA ATTTACATCA GTTCTCTTTC CGGAACCATA AACGTTTGGC

-150 ATAGACACTT GGCTTTCACT AATGGTTTCA TCTGTCTTTC CAAGTGATAT

A
↑

-100 CAACTGCTAA CATGCTCTTT CTTGGCC**TTA TCA**GTGACAG GGGTCTTCAC
‹GATA1

-50 AAAGAACAAG CCCATTAGTG **CTAAGTCA**TG GTGTAGG**AGG GATAGGT**CTC
›NF-E2 ›GATA1-0

EXON 2:

1 TTCCACAAGC ATTTGATTGC CTTCTTCTTG TGTACCGTCC CCATCGGAAA

Potential Regulatory Elements:

1. "CAAT": GCCAAT
2. GATA1: WGATAR
3. GATA1-0: SVNGATDGBB
4. TATA Box: TATAWAW
5. NF-E2: GCTGAGTCA

B = G/T/C; D = G/A/T; N = G/A/T/C; R = A/G; S = C/G;
V = G/A/C; W = A/T

Figure 5-7. Potential mutation in the promoter region upstream from exon 2 of human URO-synthase gene.

Table 5-1. Frequency of the IVS$^{-86C \cdot A}$ Base Substitution in Normal and CEP Alleles.

Source	Number of Alleles Analyzed	Normal Alleles	Mutant Alleles
Normal Individuals:	170	170	0
CEP Homozygotes:			
-With Two Known UROS Mutations	32	32	0
-With One Known and One Unknown UROS Mutation	14	12	2
-With No Known UROS Mutations	4	4	0

Chapter 6 Conclusions

In summary, the studies of URO-synthase and CEP resulted in the following findings:

(1) Ten new coding region or splicing mutations causing CEP were identified. These included six missense mutations, one nonsense mutation, one frameshift mutation mutation, and two splicing mutations. The effect on URO-synthase activity was evaluated for each of the ten new mutations: those changing the peptide sequence by expression in *E. coli*, and those affecting normal mRNA splicing by quantification of different forms of splicing product. The identification and characterization of the new mutations provided a basis to correlate genotype with disease severity, thereby permitting genotype/phenotype predictions in this clinically heterogeneous disease.

(2) Cloning of a full-length mouse URO-synthase cDNA. A 1.6 kb mouse URO-synthase cDNA was isolated, sequenced, expressed in *E. coli*, and localized to mouse chromosome 7. The authenticity of this cDNA was established by the high identity of its predicted amino acid sequence with that of the human cDNA and by the expression of the active mouse enzyme in *E. coli*. The isolation, expression, and chromosomal mapping of this full-length cDNA should facilitate studies of the structure and organization of the mouse genomic sequence and the development of a mouse model of CEP for characterization of the disease pathogenesis and evaluation of gene therapy.

(3) Two URO-synthase transcripts from the same URO-synthase gene were identified. Further characterization of the mouse and human URO-synthase cDNA revealed evidence that both the mouse and human genes had two

promoters and their transcripts differed only at the most 5' untranslated region. Both forms of mouse transcripts had a small open reading frame in the 5' untranslated region of the URO-synthase gene. These short coding regions may down regulate translation of mouse URO-synthase. A distinct feature of the human URO-synthase gene was the occurrence of two transcripts resulting from two distinct promoters. Computer-assisted analysis revealed multiple erythroid-specific transcription activation elements in both promoter regions in the human URO-synthase gene. Moreover, sequencing of the intron 1 promoter region of CEP patients in which one of the mutant alleles was already identified revealed a C to A transversion 86 residues upstream from the initiation ATG in two unrelated patients. This finding represent the first regulatory mutation in any porphyria.

In conclusion, these studies have provided additional information on the nature of the mutations in the URO-synthase gene causing CEP as well as providing insight into the transcription of the mouse and human URO-synthase genes.

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Chapter 5

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