MOLECULAR GENETICS OF ACUTE INTERMITTENT PORPHYRIA IN FINLAND

SAMI MUSTAJOKI

Helsinki 1999

Division of Endocrinology, Department of Medicine, University of Helsinki and

Department of Human Molecular Genetics, National Public Health Institute, Finland

MOLECULAR GENETICS OF ACUTE INTERMITTENT PORPHYRIA IN FINLAND

SAMI MUSTAJOKI

To be presented, with the permission of
the Faculty of Medicine, University of Helsinki,
for public examination in auditorium 1,
Meilahti Hospital, on October 29th, 1999 at 12 noon.

Helsinki 1999

Supervisors Raili Kauppinen, M.D., Ph.D.
Department of Medicine, Division of Endocrinology
University of Helsinki, Finland

Professor Leena Peltonen-Palotie, M.D., Ph.D.
Department of Human Molecular Genetics
National Public Health Institute and
Department of Medical Genetics
University of Helsinki, Finland

Reviewers Professor Jean-Charles Deybach, M.D., Ph.D.
Hôpital L. Mourier and Faculté de Médecine X. Bichat
Université Paris 7, France

Professor Marja-Liisa Savontaus, Ph.D.
Department of Medical Genetics
University of Turku, Finland

Opponent Professor Juha Kere, M.D., Ph.D.
Finnish Genome Center
University of Helsinki, Finland

ISBN 951-45-8723-5 (PDF version)
Helsinki 1999
Helsingin yliopiston verkkojulkaisut

To Ulla

CONTENTS

ABBREVIATIONS 6

ORIGINAL PUBLICATIONS 7

1. INTRODUCTION 8

2. REVIEW OF THE LITERATURE 10
2.1. Heme biosynthesis 10
2.2. The porphyrias 12
2.3. Acute intermittent porphyria 14
2.3.1. History 14
2.3.2. Prevalence 15
2.3.3. Clinical features 16
2.3.4. Pathogenesis 18
2.3.5. Biochemical findings 19
2.3.6. CRIM subtypes 20
2.4. Porphobilinogen deaminase 20
2.4.1. Function 21
2.4.2. Structure 23
2.5. Molecular genetics of acute intermittent porphyria 23
2.5.1. The porphobilinogen deaminase gene 23
2.5.2. Mutations resulting in AIP 25

3. AIMS OF THE PRESENT STUDY 30

4. MATERIAL AND METHODS 31
4.1. Patients 31
4.2. DNA and RNA isolation, and cDNA synthesis 31
4.3. DNA amplification 31
4.4. Subcloning 32
4.5. Mutation screening 32
4.6. Sequencing 33
4.7. Solid-phase minisequencing 33
4.8. In vitro mutagenesis 34

4.9. COS-1 cell culture and DNA transfection 34
4.10. Enzyme activity assay 34
4.11. Western blot 35
4.12. Pulse-chase and immunoprecipitation 35
4.13. Immunofluorescence 35

5. RESULTS AND DISCUSSION 37
5.1. Identified mutations (I, II, III) 37
5.2. SSCP and DGGE in screening for novel mutations (I, II, III, IV) 41
5.3. Monitoring the effect of mutations to allelic transcript levels (V) 43
5.4. Expression of mutated polypeptides (III, VI) 47
5.5. Outcome of mutations 52
5.5.1. Mutations resulting in premature termination codon 52
5.5.2. Missense mutations 53
5.5.3. Splicing mutations 57
5.5.4. Other mutations 59
5.6. Molecular genetics of AIP in the Finnish population 61
5.5. DNA diagnosis of AIP 64

6. SUMMARY AND CONCLUSIONS 66

7. ACKNOWLEDGEMENTS 67

8. REFERENCES 69

ORIGINAL ARTICLES I-VI 79

ABBREVIATIONS

aa	Amino acid
AIP	Acute intermittent porphyria
ALA	Aminolevulinic acid
bp	Base pair
BSA	Bovine serum albumin
CYP	Cytochrome P-450
DGGE	Denaturing gradient gel electrophoresis
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
EDTA	Edetate disodium tetraacetic acid
IVS	Intervening sequence
kb	Kilobase
kD	Kilodalton
mRNA	Messenger ribonucleic acid
PBG	Porphobilinogen
PBGD	Porphobilinogen deaminase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
SDS	Sodium dodecyl sulfate
SSCP	Single-strand conformation polymorphism

Amino acids are abbreviated by one or three letter codes and nucleotides by one letter codes.

LIST OF ORIGINAL PUBLICATIONS

I Kauppinen R, Mustajoki S, Pihlaja H, Peltonen L, Mustajoki P: Acute intermittent porphyria in Finland: 19 mutations in the porphobilinogen deaminase gene. Hum Mol Genet 4:215-222, 1995.

II Mustajoki S, Pihlaja H, Ahola H, Petersen NE, Mustajoki P, Kauppinen R: Three splicing defects, an insertion, and two missense mutations responsible for acute intermittent porphyria. Hum Genet 102:541-548, 1998.

III Mustajoki S, Ahola H, Mustajoki P, Kauppinen R: Insertion of Alu element responsible for acute intermittent porphyria. Hum Mutat 13:431-438, 1999.

IV Nissen H, Petersen NE, Mustajoki S, Hansen TS, Mustajoki P, Kauppinen R, Hørder M: Diagnostic strategy, genetic diagnosis and identification of new mutations in acute intermittent porphyria by denaturing gradient gel electrophoresis. Hum Mutat 9:122-130, 1997.

V Mustajoki S, Kauppinen R, Mustajoki P, Suomalainen A, Peltonen L: Steady state transcript levels of the porphobilinogen deaminase gene in patients with acute intermittent porphyria. Genome Res 7:1054-1060, 1997.

VI Mustajoki S, Laine M, Lahtela M, Mustajoki P, Peltonen L, Kauppinen R: Acute intermittent porphyria: expression of mutant and wild type porphobilinogen deaminase in COS-1 cells. Manuscript submitted.

In addition, some unpublished data are presented.

1. INTRODUCTION

Heme is synthesized in every eukaryotic and prokaryotic cell and it carries out many important biologic functions. It is the prosthetic group of many hemeproteins. The best known function of heme is its oxygen binding and transport in hemoglobin, and erythroblasts synthesize 85% of the total body heme is the bone marrow (Berk et al. 1976). In addition, heme is involved in the mitochondrial respiratory chain where it transports electrons to cytochromes. CYP enzymes, which metabolize a large number of clinically important drugs as well as several endogenous and exogenous substances, contain heme. Tryptophan pyrrolase, which catalyses the oxidation of tryptophan, is also a heme-dependent enzyme.

Porphyrias are defects in heme biosynthesis. The heme biosynthesis pathway contains eight steps and dysfunction in seven of them is associated with a specific porphyria (Figure 1); defects in erythroid specific ALA synthase result in X-chromosome linked sideroblastic anemia.

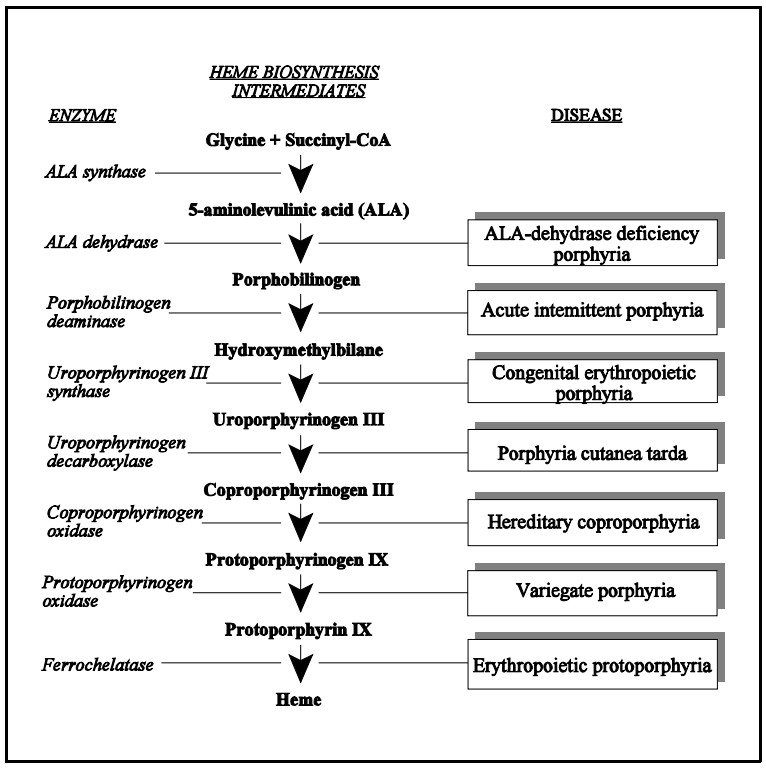


Figure 1.1. *Heme biosynthesis pathway.*

Acute intermittent porphyria (AIP) is caused by a defect in the third enzyme in the heme biosynthetic pathway, porphobilinogen deaminase (PBGD, also referred to as hydroxymethylbilane synthase and uroporphyrinogen I synthase, EC 4.3.1.8). It is the most common type of acute porphyria in Finland (Mustajoki and Koskelo 1976) and in most other countries (Sassa and Kappas 1981). AIP is characterized by potentially lethal acute attacks with abdominal pain, tachycardia and various other neuropsychiatric signs and symptoms (Kappas et al. 1995).

The gene coding for human PBGD was characterized in 1986 (Raich et al. 1986) which enabled the characterization of the molecular genetic background of AIP. The purpose of this series of investigations was to identify the molecular defects in Finnish AIP patients and, furthermore, to elucidate the mechanisms by which a mutation results in the deficient function of the enzyme.

2. REVIEW OF THE LITERATURE

2.1. Heme biosynthesis

All the enzymes and their genes in the heme biosynthetic pathway have been characterized (Kappas et al. 1995, Nishimura et al. 1995). The biosynthesis pathway is illustrated in Figure 2.1. The first and the last three of the enzymes in the pathway are localized in mitochondria; the intermediate enzymes are cytosolic. The precursors of heme are glycine and succinyl coenzyme A which are combined by aminolevulinic acid (ALA) synthase to form 5-aminolevulinic acid in the presence of the cofactor pyridoxal 5'-phosphate. Next, two molecules of ALA are condensed to a monopyrrole porphobilinogen (PBG) by ALA dehydratase.

Porphobilinogen deaminase (PBGD) assembles the four rings of PBG in a stepwise fashion in which the pyrrole ring A is first bound to the deaminase followed by rings B, C and finally D (Figure 2.2.). The dipyrromethane cofactor, which arises from the autocatalytic coupling of two molecules of PBG, is covalently linked to the enzyme. The cofactor functions as a primer to which the four substrate molecules are sequentially attached but is not itself incorporated into the product, hydroxymethylbilane.

The fourth enzyme, uroporphyrinogen III synthase, converts the highly unstable hydroxymethylbilane to uroporphyrinogen III and in this reaction the linear tetrapyrrole molecule is closed to form a ring. In the absence of uroporphyrinogen III synthase, hydroxymethylbilane may non-enzymatically close to uroporphyrinogen I. In normal circumstances, this isoform is present only in minute amounts.

The four carboxylic groups of the acetic acid chains in uroporphyrinogen are removed in the reaction catalysed by cytosolic uroporphyrinogen decarboxylase. In the presence of oxygen coproporphyrinogen oxidase catalyses the removal of the carboxyl group and the two hydrogens from the propionic groups of the pyrrole rings and forms vinyl groups at these positions resulting in protoporphyrinogen IX.

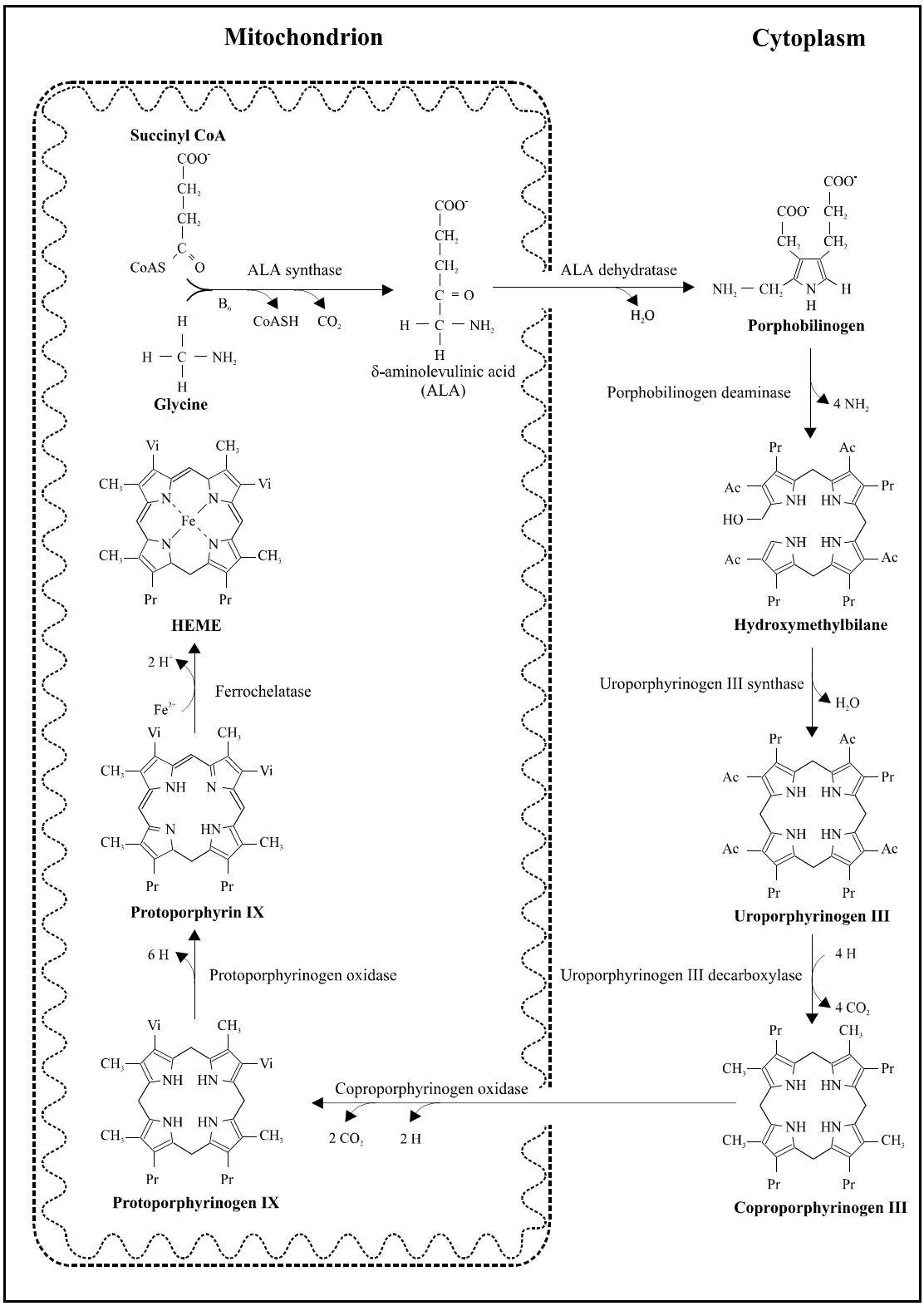


Figure 2.1. *Enzymes and intermediate products in the heme biosynthesis pathway.*
Ac= $-CH_2COOH$; Pr= $-CH_2CH_2COOH$; Vi= $-CH=CH_2$

In the seventh step of heme biosynthesis, six hydrogen atoms are removed from protoporphyrinogen IX to form protoporphyrin IX, a reaction catalysed by protoporphyrinogen oxidase. The oxidation of protoporphyrinogen may also occur non-enzymatically.

Finally, iron (Fe^{2+}) is incorporated into protoporphyrin IX by a mitochondrial ferrochelatase, also known as heme synthase.

The heme biosynthesis is regulated in distinctive manners in erythroid and non-erythroid tissues but ALA synthase is the rate-limiting enzyme of heme biosynthesis in all tissues (Kappas et al. 1995). Two different genes code for ALA synthase: the nonspecific ALA synthase is ubiquitous whereas the erythroid ALA synthase is expressed only in erythroid tissues (Kappas et al. 1995). Biosynthesis of heme in the liver and in other non-erythroid tissues is regulated by a feedback mechanism in which the end product heme regulates the synthesis of non-specific isoform of ALA synthase at the transcriptional and translational level (Moore et al. 1987). The activity of nonspecific ALA synthase may be also induced by various chemicals, drugs, lead, and alcohol and reduced by glucose. The erythroid-specific isozyme of ALA synthase is, in contrast to the nonspecific ALA synthase, upregulated by increased heme concentration (Fujita et al. 1991). The ALA synthase activity is also regulated by many other factors, for example by erythropoietin and by the availability of iron (May et al. 1995).

More detailed information about heme biosynthesis is available in the literature (e.g. Moore et al. 1987, Kappas et al. 1995).

2.2. The porphyrias

The porphyrias are a heterogeneous group of metabolic disorders. They have been divided into two groups, erythropoietic and hepatic porphyrias (Moore et al. 1987) according to the tissue where the excess porphyrins or their precursors are mainly synthesized. In general, the clinical manifestations of porphyrias are either skin photosensitivity due to

Table 2.1. *Classification of porphyrias**

Classification	Deficient enzyme	Manifestations	Inheritance
Erythropoietic			
Congenital erythropoietic porphyria	Uroporphyrinogen III synthase	Photosensitivity	Autosomal recessive
Erythropoietic protoporphyria	Ferrochelatase	Photosensitivity	Autosomal dominant/recessive
Hepatic			
ALA dehydratase deficiency porphyria	ALA dehydratase	Chronic neurologic symptoms	Autosomal recessive
Acute intermittent porphyria	Porphobilinogen deaminase	Acute attacks	Autosomal dominant
Hereditary coproporphyria	Coproporphyrinogen oxidase	Acute attacks and/or excessive skin fragility	Autosomal dominant
Variegate porphyria	Protoporphyrinogen oxidase	Acute attacks and/or excessive skin fragility	Autosomal dominant
Porphyria cutanea tarda	Uroporphyrinogen decarboxylase	Excessive skin fragility	Autosomal dominant/ sporadic
Hepatoerythropoietic porphyria	Uroporphyrinogen decarboxylase	Photosensitivity	Autosomal recessive

* Modified from Kappas et al. 1995

photoreactivity of porphyrins, neurovisceral dysfunction (acute attacks), or both (Kappas et al. 1995). Most of the porphyrias are inherited dominantly in autosomes. ALA dehydratase deficiency and congenital erythropoietic porphyria have, however, autosomal recessive inheritance. Furthermore, a significant number of patients with porphyria cutanea tarda are sporadic cases (de Verneuil et al. 1978). Hepatoerythropoietic porphyria is a rare homozygous form of the familial type of porphyria cutanea tarda (Toback et al. 1987). A summary of different porphyria types is given in Table 2.1.

2.3. Acute intermittent porphyria

2.3.1. History

The modern history of acute porphyrias, reviewed extensively elsewhere (Goldberg and Rimington 1962, Dean 1963, Tschudy et al. 1975), begins at the end of the 19th century, when an increasing number of patients with abdominal pain and neurological symptoms was described. The typical attacks of acute porphyria were associated already in these early days with drug treatment.

Most of the porphyrin nomenclature is based on Hans Fischer's, a Nobel laureate in 1930, studies on the heme biosynthesis in the 1920's. In 1937 Jan Waldenström, who had previously worked in Fischer's laboratory, described over one hundred patients with acute porphyria, most of whom originated from a small village in Northern Sweden (Waldenström 1937). In this study the Mendelian dominant mode of inheritance in AIP was demonstrated for the first time. Later, he proposed that porphyrias could result from enzyme defects in the heme pathway and was also the first to use the term 'acute intermittent porphyria' (Waldenström 1957). A screening test for porphobilinogen was introduced in 1941 by Watson, who also was Fischer's co-worker (Watson and Schwartz 1941). He also introduced hematin for treatment of acute porphyrias (Watson et al. 1978). In the 1960's Granick discovered that ALA synthase, the first step in heme biosynthesis, is induced by chemicals which cause acute porphyria (Granick 1963, Granick 1966). This observation led to the concept that overproduction of ALA may represent the primary genetic defect in all acute porphyrias (Granick 1966), but this hypothesis was soon

found to be false because increased activity of this enzyme by itself could not account for the distinct patterns of porphyrin precursors and porphyrin excretion. The biochemical background of AIP was confirmed when it was demonstrated that the disease is due to a defect in PBGD (Miyagi 1970, Meyer et al. 1972).

The characterization of the porphobilinogen deaminase gene (Raich et al. 1986, Grandchamp et al. 1987, Chretien et al. 1988, Lee 1991a, Namba et al. 1991) initiated multiple investigations of the molecular genetic background of AIP, and today several mutations responsible for AIP have been identified (see chapter 2.5.2).

The first Finnish patient with acute porphyria was reported in 1928 (Langenskiöld 1928). The first comprehensive study of the prevalence and clinical characteristics of acute porphyrias in Finland was reported in 1976 by Pertti Mustajoki and Pentti Koskelo (Mustajoki and Koskelo 1976). The prognosis, precipitating factors and associated diseases in Finnish patients with acute porphyrias have been investigated thoroughly by Raili Kauppinen and Pertti Mustajoki (Kauppinen et al. 1992).

2.3.2. Prevalence

AIP is the most common type of acute porphyria in most countries (Kappas et al. 1995), only in the Republic of South Africa is the prevalence of variegate porphyria higher than that of AIP. The prevalence of AIP in Finland has been estimated to be 3:100000 (Mustajoki and Koskelo 1976). In other countries the reported prevalence figures are similar or slightly higher: 5-10:100000 in the United States of America, in Western Australia 3:100000 (Tschudy et al. 1975). The highest prevalence of AIP has been described in northern Sweden where the prevalence is 100:100000 and, furthermore, in two small municipalities the prevalence is as high as 0.5-2%. However, the overall prevalence of AIP in Sweden is only 10:100000 (Andersson 1997). In a study of healthy Finnish blood donors the prevalence of low PBGD activity was 1:500 (Mustajoki et al. 1992) and similar figures were obtained in a study which consisted of healthy French blood donors (Nordmann et al. 1997). This implies that the prevalence of AIP, though asymptomatic, may be much higher than previously estimated.

2.3.3. Clinical features

Approximately 10-20% of the affected individuals experience acute attacks during their lifetime and 50% of patients have milder symptoms typical for AIP (Kauppinen and Mustajoki 1992). Half of the subjects with decreased PBGD activity remain, thus, asymptomatic throughout their lifetimes. Acute attacks are extremely rare before puberty and the first acute attack usually manifests at an age between 15 and 40 years (Mustajoki and Koskelo 1976).

Table 2.2. *Symptoms and signs of an acute attack**

Abdominal pain	90%
Dark or red urine	89%
Vomiting	61%
Constipation	56%
Extremity and/or back pain	53%
Muscle weakness	53%
Mental symptoms	46%
Hypertension (diast. ≥100 mmHg)	42%
Tachycardia	40%
Fever	35%
Sensory loss	19%
Convulsions	14%
Respiratory muscle paralysis	14%
Diarrhea	9%

*Total of 774 patients. Data combined from (Waldenström 1957, Goldberg 1959, Stein and Tschudy 1970, Wetterberg 1974, Mustajoki and Koskelo 1976, Andersson 1997)

The symptoms during the acute attacks are mainly related to neurological dysfunctions. The incidence of symptoms and physical signs are listed in Table 2.2. Abdominal pain is almost invariably present and in severe cases it may mimic 'acute surgical abdomen'. Dark or red urine is also frequently observed but the presence of other symptoms varies considerably which sometimes causes difficulties in making the right diagnosis. Unless treated, the symptoms of an acute attack may proceed to lethal respiratory paralysis. Nowadays, however, life-threatening symptoms during acute attacks seldom occur because of increased knowledge of precipitating factors and improved therapy (Kauppinen and Mustajoki 1992). The course of an acute attack is variable and porphyric symptoms may last from a few days to several weeks (Kappas et al. 1995). Some patients may have symptoms almost continuously, but the majority of

symptomatic patients have a few well-delineated attacks with relatively asymptomatic intervening remissions (Kappas et al. 1995).

The porphyric attacks are often precipitated by environmental or endogenous factors, such as changes in hormonal state (menstrual cycle and pregnancy), several therapeutic drugs (e.g. barbiturates and sulphonamides), alcohol, infections, stress, and inadequate caloric intake (Kauppinen and Mustajoki 1992, Kappas et al. 1995, Andersson 1997). According to a Finnish survey, at present the most common cause for an acute attack is the menstrual cycle in females and excessive alcohol consumption in males (Kauppinen and Mustajoki 1992). Avoiding the precipitating factors usually decreases the incidence of attacks. Women at fertile age may need hormonal therapy which suppresses their endogenous sex hormone production.

Some diseases are more common in patients with acute porphyria than in the normal population. The risk for hepatocellular carcinoma is increased 61 to 114-fold (Kauppinen and Mustajoki 1992, Andersson et al. 1996, Linet et al. 1999) and is the cause of death in about one-quarter of AIP patients (Andersson et al. 1996). Chronic hypertension has been suggested to be more common in patients with manifest AIP than in those with latent AIP or in healthy controls in Swedish material of one large AIP family (Andersson and Lithner 1994): 56% of the patients with manifest AIP had hypertension. However, the prevalence of chronic hypertension was much lower (~24%) in the Finnish material which consisted of both AIP and variegate porphyria patients from 53 families and, furthermore, the occurrence of hypertension did not correlate with clinical manifestations (Kauppinen and Mustajoki 1992). When Finnish AIP patients were compared to the general Finnish population, the prevalence rates of hypertension with medication were higher only in two age-specific subgroups (Kauppinen and Mustajoki 1992). Cardiovascular mortality was not higher among AIP patients (Kauppinen and Mustajoki 1992, Andersson et al. 1996). The prevalence of chronic renal failure has been suggested to be higher among AIP patients (Kauppinen and Mustajoki 1992, Andersson and Lithner 1994).

2.3.4. Pathogenesis

Under normal conditions, the heme biosynthesis pathway is able to fulfill its task inspite of decreased PBGD activity. It is widely presumed that increased metabolic demand for heme in the liver leads to an induction of ALA synthase expression (Kappas et al. 1995, Elder et al. 1997). This results in accumulation of intermediates of the heme biosynthesis pathway, namely delta-aminolevulinic acid and porphobilinogen, since the defective enzyme is not able to convert all the porphobilinogen to hydroxymethylbilane. The resulting deficiency of the final product, heme, leads to further induction of ALA synthase provoking *circulus vitiosus*, an escalating metabolic chain reaction.

About 65 percent of the aminolevulinic acid produced in the rat liver is utilized for the formation of CYP isoenzymes (Sassa and Kappas 1981). Some drugs, alcoholic beverages and steroid hormones, i.e. substances which are known to precipitate porphyric symptoms, are known to induce or increase the inducibility of hepatic ALA synthase and may also increase the intrahepatic requirement for heme by inducing the synthesis of CYP isoenzymes (Elder et al. 1997). This leads to the accumulation of delta- aminolevulinic acid and porphobilinogen because the deficient PBGD cannot fulfill the requirements of the accelerated heme biosynthesis.

The symptoms of AIP result from neurologic dysfunction and acute attacks are known to be caused by the induction of ALA synthase. However, the details of molecular pathogenesis are still obscure. Three major hypothesis has been proposed to explain the symptomatology of an acute attack (Moore et al. 1987, Kappas et al. 1995):

1) depletion of heme in the cells of the central nervous system (Lindberg et al. 1996, Lindberg et al. 1999),
2) neurotoxicity of accumulated intermediates, especially aminolevulinic acid (Bonkovsky 1993), and
3) neurotransmitter disturbance secondary to the deficiency of heme and tryptophan pyrrolase (a heme dependent enzyme) in the liver (Litman and Correia 1983).

Although all these theories are supported by some experimental findings, none of them can explain all the various symptoms during an acute attack, and the pathogenetic mechanisms of an acute attack remain, thus, unsolved.

The development of a transgenic porphobilinogen deaminase deficient mouse (Lindberg et al. 1996, Lindberg et al. 1999) is the first experimental model for acute porphyrias. Studies with PBGD (-/-) mice suggest that heme deficiency and consequent dysfunction of hemeproteins cause chronic and progressive neuropathy leading to impairment of motor coordination and muscle weakness (Lindberg et al. 1999). However, the mouse model more resembles the chronic neuropathy seen in some patients and it has not yet been able to elucidate the pathogenetic mechanisms of acute attacks.

2.3.5. Biochemical findings

During an acute attack AIP patients always excrete increased amounts of the porphyrin precursors, delta-aminolevulinic acid and porphobilinogen, in urine (Bissel 1982). Qualitative tests, e.g. Watson-Schwartz and Hoesch tests (Bissel 1982), have been developed for rapid confirmation of diagnosis, but sensitivity or specificity of these tests limit their usefulness (Lamon et al. 1977). A quantitative assay (Mauzerall and Granick 1956) has been used for more precise diagnosis. During the asymptomatic phase more than one half of AIP patients do not excrete PBG in urine and these patients are less susceptible to acute attacks (Kauppinen and Mustajoki 1992).

Biochemical diagnosis of acute intermittent porphyria is based on measurement of erythrocyte porphobilinogen deaminase activity (Magnussen et al. 1974, Ford et al. 1980). In this assay, porphobilinogen is used as a substrate and the uroporphyrin I formed is measured by spectrofluorometry. In general, the erythrocyte PBGD activity in AIP patients is ~50% of that found in normal subjects. Due to alternative splicing of the erythroid and wild type isoform (Grandchamp et al. 1987), however, the erythrocyte PBGD activity is normal in the variant form of AIP (Mustajoki 1981). Furthermore, there are wide individual variations of PBGD activity among healthy controls (Mustajoki et al. 1992) and the normal and pathological values

have an overlapping zone. In addition, many diseases - for example renal insufficiency, iron deficiency anemia and various malignancies - may affect PBGD activity (Moore et al. 1987). These confounding factors limit the usefulness of biochemical methods in the diagnosis of AIP.

2.3.6. CRIM subtypes

Before the DNA era, AIP was divided into two major subtypes according to the ratio of enzyme activity and the amount of immunoreactive protein in erythrocytes (Anderson et al. 1981, Desnick et al. 1985, Lannfelt et al. 1989a). When the amount of immunoreactive protein reflects the amount of enzymatically active protein, the patient is classified as cross-reactive immunologic material (CRIM) negative. CRIM negative patients have been further divided into two groups according to the PBGD activity in their erythrocytes: in type 1 the enzyme activity is 50% of the normal activity whereas in type 2 the erythrocyte activity is normal. Several explanations have been proposed for the absence of detectable mutant protein, i.e. CRIM negativity (Mustajoki and Desnick 1985): instability of mutant mRNA, insufficient translation, rapid intracellular decay of the mutant polypeptide, or due to an altered structure, the antibody fails to detect the mutant polypeptide.

Enzymatically inactive but immunologically detectable protein is detected in erythrocytes of CRIM positive patients. Two CRIM positive subtypes have been identified. In type 1 the ratio of the protein and enzyme activity is ~1.6, i.e. the amount of inactive enzyme is 60-80% of that of the active enzyme. In type 2 the CRIM/activity ratio is ~5.7, i.e. the amount of inactive enzyme is 5.7-fold that of the active enzyme. In both CRIM positive subtypes the amount of enzyme intermediates is increased (Anderson et al. 1981, Desnick et al. 1985), suggesting that the enzyme protein is synthesized normally but it cannot catalyze deamination or elongate the pyrrole chain normally.

2.4. Porphobilinogen deaminase

PBGD is a monomeric enzyme and in humans two isoforms are present: the 44 kD house-keeping enzyme and the 42 kD erythroid-specific enzyme. The ubiquitous polypeptide, which

consists of 361 amino acid residues, is encoded by a single gene on chromosome 11q, and the two isoforms differ only in their N-terminal amino acid sequences; the first 17 amino acids of the wild type enzyme are not present in the erythroid isoform (Grandchamp et al. 1989c). Since, the deaminases show exceptional heat stability, their characterization has been very convenient (Shoolingin-Jordan 1995).

2.4.1. Function

PBGD catalyzes the tetramerization of porphobilinogen. The reaction produces a highly unstable 1-hydroxymethylbilane (preuroporphyrinogen) intermediate which is followed by the formation of cyclic tetramere uroporphyrinogen by uroporphyrinogen III synthase. PBGD assembles the four rings of PBG in a step-wise fashion, in which the pyrrole ring A is first bound to the deaminase followed by rings B, C, D (Figure 2.2.) The dipyrromethane cofactor anchors the substrate molecules at the catalytic center and directs the construction of the tetrapyrrole (Jordan and Warren 1987). The cofactor is formed by autocatalytic coupling of two molecules of PBG, the same molecule which also acts as the substrate for the reaction.

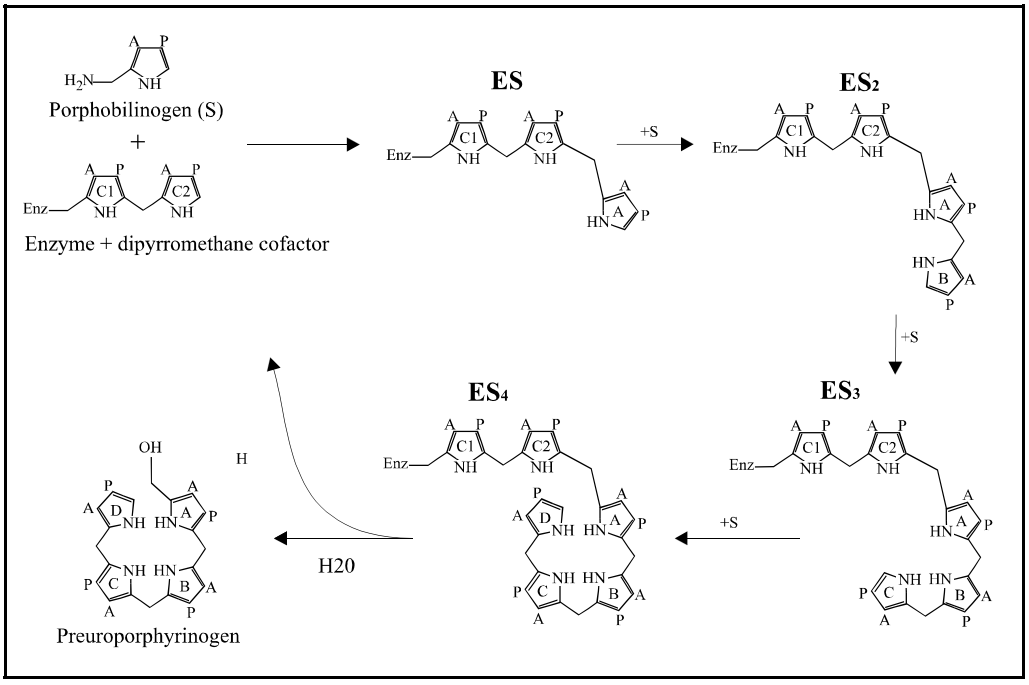


Figure 2.2. *Reaction cascade catalyzed by porphobilinogen deaminase*

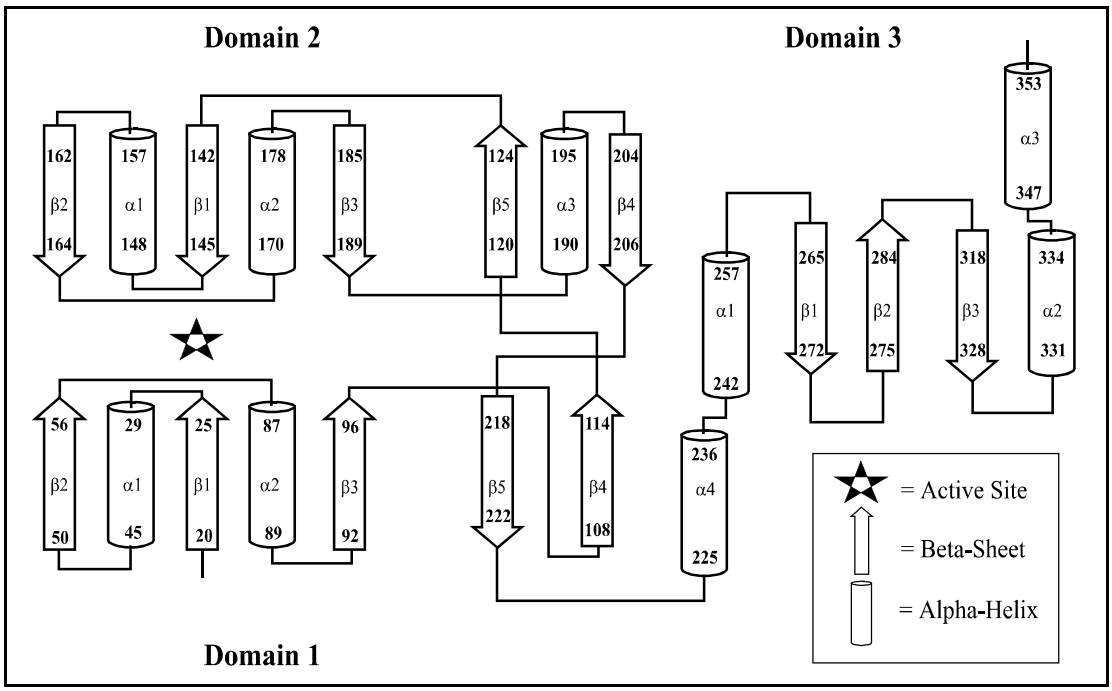


Figure 2.3. *Schematic representation of human PBGD secondary structural elements. Based on a crystal structure of E. coli enzyme (Louie et al. 1992) and on a model of human PBGD (Brownlie et al. 1994).*

The enzyme-intermediate complexes have been isolated and studied further (Warren and Jordan 1988). Of these, the first intermediate complex (i.e. the enzyme with one substrate molecule bound) is the most labile and the second intermediate the most stable at 37ºC. The last enzyme-intermediate complex, in which all four porphobilinogen rings are attached to the enzyme via the dipyrromethane cofactor, does not accumulate at all, presumably due to rapid release of the tetrapyrrole from the enzyme.

PBGD has, thus, the ability to perform multiple chemical reactions despite its small size. The enzyme catalyzes the repetitive condensation of PBG units with an acceptor pyrrole chain and is able to 'count' precisely and terminate the reaction when the tetrapyrrole chain has been assembled. In addition, the apoenzyme installs its own dipyrromethane cofactor to form the holoenzyme.

2.4.2. Structure

The structure of human PBGD has been predicted using the three-dimensional structure of PBGD purified and crystallized from *E. coli* (Louie et al. 1992, Brownlie et al. 1994). The molecule is composed of three domains of similar sizes linked together by flexible hinge regions (Figure 2.3.). The enzyme possesses a single active site that is used for each porphobilinogen condensation (Warren and Jordan 1988, Louie et al. 1992). In *E. coli* (the corresponding human residues are in parenthesis), the dipyrromethane cofactor is covalently bound to cysteine-242 (Cys261). In addition, residues aspartate-84 (Asp99), arginines 131, 132, 149, 155 (Arg149, 150, 167, 173), lysine-83 (Lys98), as well as some other residues form salt-bridge and hydrogen bond interactions with the cofactor (Brownlie et al. 1994). The positively charged side chains of Arg11, 132, and 155 (Arg26, 150, 173) are thought to interact with the substrate (Lambert et al. 1994).

Fifty-eight residues in the PBGD polypeptide are invariant according to amino acid sequence deduced from the nucleotide sequence determination from a wide range of organisms, and the conserved amino acids are clustered in hydrophobic core of the molecule, in other conformationally important locations, or at the active site. This suggests a similar structure and, furthermore, similar mechanism of catalytic activity in organisms with different phylogenetic age (Brownlie et al. 1994).

2.5. Molecular genetics of acute intermittent porphyria

2.5.1. The porphobilinogen deaminase gene

The gene coding for PBGD, which has been identified and thoroughly characterized (Raich et al. 1986, Grandchamp et al. 1987, Chretien et al. 1988, Lee 1991a, Namba et al. 1991, Yoo et al. 1993), is assigned to chromosome 11q24 (Namba et al. 1991). The size of the gene is 10 kb of which 1.3 kb represents coding sequence. The genomic sequence is divided into 15 exons ranging from 39 and 438 bp and 14 introns ranging from 87 to 2913 bp (Figure 2.4.).

Two isoforms, arising from different promoters, are transcribed (Chretien et al. 1988). The mRNA of the housekeeping (non-erythropoietic) isoform contains exons 1 and 3 to 15 coding for an enzyme of 361 amino acids, whereas the erythroid isoform is encoded by exons 2 to 15 (Figure 2.4). The translation initiation codon for the housekeeping isoform is located in exon 1 and for the erythroid-specific isoform in exon 3. The erythroid isoform, thus, lacks the first 17 amino acids of the amino terminus.

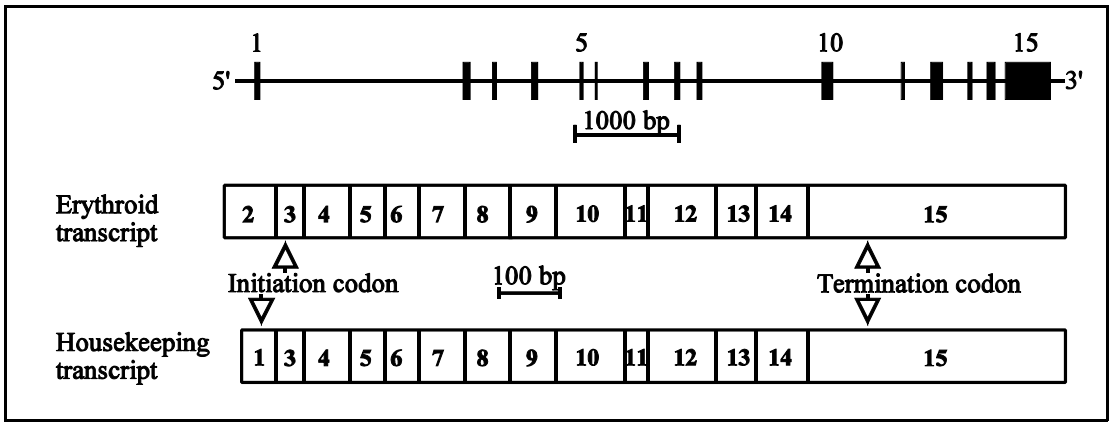


Figure 2.4. *The porphobilinogen deaminase gene and two mRNAs transcribed by alternative splicing.*

The erythroid promoter region is located in intron 1 and its structure is very similar to that of the β-globin gene (Chretien et al. 1988). The erythroid-specific transcript, thus, appears to be regulated by similar set of transcription factors, suggesting that both genes are similarly regulated during erythroid differentiation (Mignotte et al. 1989). The promoter region for the housekeeping transcript is located upstream of exon 1 (Chretien et al. 1988, Yoo et al. 1993). The minimal promoter sequence has been identified by deletion mapping (Lundin and Anvret 1997) and it is located between -243 and -115 nucleotides from the translation initiation codon. This region contains two Sp1 consensus recognition sequences, a 13 bp repeat sequence, and an AP1 consensus recognition sequence. In addition, sequence elements that have negative regulatory function are located closer to the translational initiation site (Lundin and Anvret 1997).

To date, thirteen intragenic polymorphisms have been identified in the PBGD gene (Lee and Anvret 1987, Llewellyn et al. 1987, Lee et al. 1988, Gu et al. 1991, Lee 1991b, Picat et al. 1991, Daimon et al. 1993a, Yoo et al. 1993, Law et al. 1999, Whatley et al. 1999). One of the polymorphisms is exonic, located in exon 10, but it does not alter the amino acid sequence. Nine polymorphisms are located in intron regions of the gene; four of them are found in intron 1, the longest (2.9 kb) intron in the PBGD gene. In addition to the intragenic polymorphisms, two polymorphisms have been identified in the non-erythroid promoter region (Picat et al. 1991, Schreiber et al. 1992, Lundin and Anvret 1997).

Six Alu elements have been found in the intronic sequences of the PBGD gene. Alu elements are derived from 7SL RNA by internal deletions or point mutations of the 7SL sequence followed by dimerization (Makalowski et al. 1994). They integrate throughout the primate genomes via a process called retroposition (Rogers 1985) which involves generation of an RNA polymerase III transcript, reverse transcription, and integration at staggered nicks within AT-rich regions of the genome (Jagadeeswaran et al. 1981). A typical Alu element is 282 nucleotides long and it is composed of two homologous but distinct subunits. Alu repeats can be divided into various subfamilies based on the presence of commonly shared diagnostic mutations (Jurka 1993). Two of the Alu sequences in the PBGD gene are located in the 5' flanking region, three in intron 1. The sixth Alu element is in intron 9 and is the only Alu in sense orientation. Five of the Alu sequences belong to the relatively modern Sa subfamily whereas one is homologous to the older J subfamily (Yoo et al. 1993).

2.5.2. Mutations resulting in AIP

Up to July 1999, 149 different mutations resulting in AIP had been identified in the PBGD gene worldwide (Table 2.3.).

Of the mutations, 56 (38%) are substitutions of one amino acid and 37 (25%) mutations affect splicing. Fifteen mutations result in an immediate termination codon and, furthermore, 36 mutations cause frame shifts which have been predicted to lead to a premature termination codon after a variable number missense amino acids. One mutation hits the translation initiation

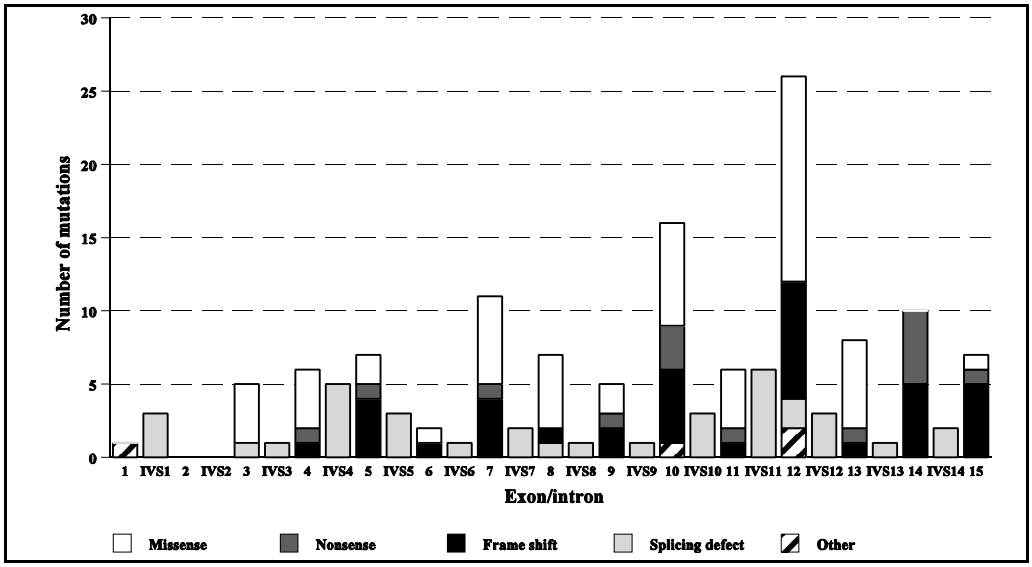


Figure 2.5. *Location and type of identified mutations in the PBGD gene*

codon and the remaining three mutations are deletions or insertions, which do not cause frame shift.

Figure 2.5. shows the distribution of the mutations by location and mutation type. No mutations causing AIP have been identified in exon 2 and its flanking sequences. This was somewhat expected, because exon 2, transcribed only to the erythroid mRNA, does not code for polypeptide since the initiation codon of this isoform is located in exon 3. Mutations responsible for AIP are dispersed quite evenly in the PBGD gene and there is no hot spot for the mutations. Twenty-nine (19%) of the mutations are located in exon 12, which is clearly the highest number of all exons, but this exon the second longest exon in the PBGD gene and it is almost twice as long as the majority of other exons (Figure 2.4.). Notably, six of eight residues known to be crucial for the enzyme activity (R26, K93, D94, R149, R150, R167, R173, and C261) are substituted resulting in AIP, only at positions K93 and R150 there are no reports of mutations so far.

In the light of the mutations published in the PBGD gene so far, the molecular genetic background is highly heterogeneous despite the rather uniform clinical manifestations of the disease. Although studies of geno/phenotype comparisons have not yet been published, nothing

implies that the type or the location of the mutation affects the clinical manifestations of the disease. Furthermore, it seems that most of the mutations in the PBGD gene dramatically decrease the enzymatic activity of the polypeptide encoded by the mutant allele.

Table 2.3. *Characterized mutations in the PBGD gene.*

Mutation	*Outcome*	*Ref.**	*Mutation*	*Outcome*	*Ref.**	*Mutation*	*Outcome*	*Ref.**
Exon 1			*Intron 5*			446G T	R149L	(11)
3G T	translation defect	(1)	IVS5+1G A	del exon 5	(11)	463C T	Q155X	(25)
33G T	splicing defect	(2)	IVS5+2insG	del exon 5	(9)	470insA	frame shift	(26)
Intron 1			IVS5+2T C	del exon 5	(12)	489insTCCT	frame shift	(12)
IVS1+1G A	splicing defect	(3)	*Exon 6*			*Intron 9*		
IVS1+2T A	splicing defect	(4)	218delAG	frame shift	(11)	IVS9-1G A	del exon 10	(27)
IVS1+5C G	splicing defect	(4)	254T G	L85R	(12)	*Exon 10*		
Exon 3			*Intron 6*			499C T	R167W	(28)
64C T	R22C	(5)	IVS6+1G C	splicing defect	(17)	500G A	R167Q	(29)
66C G	del exon 3	(6)	*Exon 7*			500delG	frame shift	(9)
70G A	G24S	(7)	269T G	V90G	(12)	517C T	R173W	(30)
77G A	R26H	(8)	277G T	V93F	(1)	517del17bp	frame shift	(9)
83G A	S28N	(9)	287C T	S96F	(18)	518G A	R173Q	(29)
Intron 3			291delG	frame shift	(7)	530T G	L177R	(13)
IVS3+1G A	splicing defect	(10)	295G C	D99H	(18)	532G A	D178N	(9)
Exon 4			314insC	frame shift	(16)	541C T	Q181X	(12)
91G A	A31T	(11)	323insT	frame shift	(12)	576del 19bp	frame shift	(9)
91G C	A31P	(12)	331G A	G111R	(19)	589del 17bp	frame shift	(31)
100C A	Q34K	(13)	340insT	frame shift	(12)	593G A	W198X	(32)
125T A	L42X	(14)	342C A	C114X	(9)	601C T	R201W	(27)
125T C	L42S	(12)	*Intron 7*			604delG	frame shift	(26)
158insA	frame shift	(7)	IVS7+33G T	del exon 7	(12)	610C T	Q204X	(22)
Intron 4			IVS7-1G A	del exon 8	(20)	612G T	del 3 aa	(24)
IVS4+1G A	del exon 4	(12)	*Exon 8*			*Intron 10*		
IVS4+1G T	del exon 4	(9)	346C T	R116W	(21)	IVS10+2TAGGG CCCTA		
IVS4-6C G	del exon 5	(12)	347G A	R116Q	(22)		splicing defect	(15)
IVS4-2A C	del exon 5	(12)	356C T	P119L	(10)	IVS10+2T C	del exon 10	(9)
IVS4-1G C	splicing defect	(15)	365C G	A122G	(23)	IVS10-31A G	del exon 11	(9)
Exon 5			371T A	V124D	(9)	*Exon 11*		
163G T	A55S	(11)	del 704 bp	frame shift	(23)	625G A	E209K	(33)
168delGT	frame shift	(16)	421delG	del exon 8	(12)	629delA	frame shift	(34)
174delC	frame shift	(11)	*Intron 8*			639T G	Y213X	(9)
181G A	D61N	(12)	IVS8+1G T	splicing defect	(10)	646G A	G216D	(17)
182insG	frame shift	(11)	*Exon 9*			650A T	Q217L	(35)
184delAA	frame shift	(12)	446G A	R149Q	(24)	651G T	Q217H	(9)
206delCT	S69X	(9)						

Mutation	*Outcome*	*Ref.**	*Mutation*	*Outcome*	*Ref.**	*Mutation*	*Outcome*	*Ref.**
Intron 11			749A C	E250A	(9)	*Intron 13*		
IVS11+1G C	splicing defect	(15)	754G C	A252T	(37)	IVS13+1G A	del exon 13	(6)
IVS11+2T C	ins intron 11	(12)	754G A	A252T	(36)	*Exon 14*		
IVS11+3C G	splicing defect	(6)	755C T	A252V	(36)	847delTG	frame shift	(17)
IVS11-2A G	splicing defect	(15)	761T C	L254P	(23)	848G A	W283X	(22)
IVS11-1G C	del exon 12	(9)	766C A	H256N	(13)	849G A	W283X	(16)
IVS11-1delG	del exon 12	(9)	766C T	H256Y	(9)	854delTA	frame shift	(9)
Exon 12			771insT	frame shift	(38)	863C A	S288X	(9)
656C A	A219D	(12)	771G A	del exon 12	(39)	863C G	S288X	(15)
667G A	E223K	(11)	771G C	del exon 12	(40)	866delATAG	frame shift	(12)
678insAA	frame shift	(12)	*Intron 12*			874C T	Q292X	(35)
691del30bp	truncated protein?	(9)	IVS12+1G A	del exon 12	(14)	900insT	frame shift	(41)
715delCA	frame shift	(14)	IVS12+1G T	del exon 12	(7)	900delT	frame shift	(24)
716insC	frame shift	(31)	IVS12-1G A	del exon 13	(9)	*Intron 14*		
721delC	frame shift	(12)	*Exon 13*			IVS14+1G T	del exon 14	(12)
723ins21bp	7 aa repeat	(9)	782G A	C261Y	(23)	IVS14+1G A	del exon 14	(19)
730delCT	frame shift	(36)	794insAGCC	frame shift	(12)	*Exon 15*		
734T G	L245R	(24)	799G A	V267M	(7)	913insC	frame shift	(14)
739T C	C247R	(36)	806C T	T269I	(22)	973C T	R325X	(42)
742ins8bp	frame shift	(11)	809C A	A270D	(9)	982delG	frame shift	(12)
748G C	E250A	(10)	809C G	A270G	(31)	986insT	frame shift	(15)
748ins8bp	frame shift	(12)	820C A	G274R	(22)	1004delG	frame shift	(5)
748G A	E250K	(11)	823C T	Q275X	(9)	1004G A	G335D	(9)
749A T	E250V	(9)				1062insC	frame shift	(43)

* 1, Chen et al. 1994; 2, Grandchamp et al. 1989c; 3, Grandchamp et al. 1989b, 4, Puy et al. 1998; 5, Ong et al. 1998; 6, Llewellyn et al. 1996; 7, Rosipal et al. 1997; 8, Llewellyn et al. 1993; 9, Puy et al. 1997b; 10, Lundin et al. 1995; 11, Gu et al. 1994; 12, Whatley et al. 1999; 13, Mgone et al. 1992; 14, Puy et al. 1996; 15, Petersen et al. 1998; 16, Schreiber et al. 1995a; 17, Lundin et al. 1997; 18, Deybach and Puy 1995; 19, Gu et al. 1993a; 20, Schreiber et al. 1994b; 21, Lee et al. 1990; 22, Mgone et al. 1994; 23, Brownlie et al. 1994; 24, Delfau et al. 1991; 25, Scobie et al. 1990; 26, Schreiber et al. 1994a; 27, Lundin et al. 1994; 28, Gu et al. 1992; 29, Delfau et al. 1990; 30, Lee 1991a; 31, Lee and Anvret 1991; 32, Puy et al. 1997a; 33, Grandchamp et al. 1992; 34, Lee et al. 1994; 35, Schneider-Yin et al. 1999; 36, Mgone et al. 1993; 37, article IV; 38, Ong et al. 1996; 39, Grandchamp et al. 1989a; 40, Daimon et al. 1993b; 41, Schreiber et al. 1995b; 42, Petersen et al. 1996; 43, Daimon et al. 1994

3. AIMS OF THE PRESENT STUDY

In the beginning of this study in 1993, the specific mutation resulting in AIP was identified only in 9 (23%) of the 40 known Finnish AIP families. Furthermore, at that time very little was known about the molecular mechanisms of how a mutation in the PBGD gene results in a defective enzyme.

The aims of this study were:

1) To identify the gene defects resulting in AIP among Finnish patients.

2) To develop a method for screening of the PBGD gene for novel mutations and to develop methods for DNA diagnostics of AIP for Finnish families.

3) To characterize the consequences of selected AIP mutations at the mRNA or polypeptide and cellular level.

4. MATERIAL AND METHODS

4.1. Patients

A register for Finnish porphyria patients was established in 1966 and to date it comprises of approximately 250 AIP patients belonging to 40 different families (Kauppinen and Mustajoki 1992). In the classical form of AIP the diagnosis has been based on typical clinical manifestations or on increased excretion of porphobilinogen in urine (Mauzerall and Granick 1956) and on low PBGD activity in erythrocytes (Ford et al. 1980). In the majority of cases, the pedigrees have been traced up to the 19th century and in some cases up to the 17th century (Mustajoki and Koskelo 1976). According to this analysis, the families are not related to each other. A DNA sample, and in most cases also a RNA sample, was available from at least one patient from each family. In addition, DNA samples of healthy family members and healthy unrelated controls have been available. The study protocol has been approved by the Ethics Committee of the Third Department of Medicine, University of Helsinki.

4.2. DNA and RNA isolation, and cDNA synthesis

Leukocyte DNA was extracted from venous blood samples using the method described in (Sambrook et al. 1989) or DNA was released from leukocytes as described by Higuchi (1989). Total RNA was prepared from lymphoblastoid cells using the guanidium isothiocyanate method (Chirgwin et al. 1979). Complementary DNA was synthesized from 2-5 μg of total RNA using M-MuLV Reverse Transcriptase (New England Biolabs, ME, USA), RNase inhibitor (RNAsin^R Promega, WI, USA), dNTPs, and random hexanucleotide mix (Boehringer Mannheim, Germany) or a specific primer for 3' untranslated region of the PBGD gene.

4.3. DNA amplification

Isolated DNA (final concentration 2-5 ng/μl), lysed leucocyte sample (1:10 v/v), or RT product (3:20 v/v) was used as the template for polymerase chain reaction (PCR, Mullis and Faloona 1987). The PCR reaction mixture contained 16-23-mer primers (0.2-1 mM), dNTPs at 0.2 mM

concentrations and 0.03 U/µl of DNA polymerase (Dynazyme^{R}, Finnzymes, Finland) in enzyme buffer. The temperature profile for the PCR reactions was 2 min at 94°C for the first denaturation step, followed by 30-60 s at 94°C, 30-60 s at 54-60°C, and 30-90 s at 72°C for 30 cycles. The other primer was biotinylated if the PCR product was used in sequencing or minisequencing reactions. The primer sequences are given in original articles I-V.

4.4. Subcloning

When the consequences of mutations in the exon/intron boundaries (IVS1+3G T and IVS13-2A G) were studied, the normal allele interfered with direct sequencing and both alleles were studied separately by subcloning. In the case of Alu insertion, the normal allele and poly T region interfered with direct sequencing of the branch points, and the 318 bp and 651 bp fragments including exon 5 were amplified from a patient's genomic DNA sample. After amplification, digestion and purification, the fragments were ligated into the corresponding restriction sites of the pUC18 vector (Pharmacia, Sweden), transformed in *E. coli* and both alleles were sequenced separately.

4.5. Mutation screening

Mutations were examined using two different screening methods: single-strand conformation polymorphism (SSCP, Orita et al. 1989) and denaturing gradient gel electrophoresis (DGGE, Myers et al. 1987, Nissen et al. 1995). Prior to SSCP, the ^{32}P-labeled PCR products, which covered exon 1 and exons 3 to 15 of the PBGD gene, were digested into shorter fragments when longer than 500 bp. The primer sequences and restriction enzymes used are given in article I. The samples were diluted 1:5 in 1% sodium dodecyl sulfate (SDS)/10mM edetate disodium tetraacetic acid (EDTA), mixed with an equal volume (3 µl) of 95% (vol/vol) formamide/20 mM EDTA containing 0.05% bromophenol blue and 0.05% xylene cyanol, and denatured by heating. The diluted and denatured sample was electrophoresed in a 5% bis/acrylamide (60:1) gel using different glycerol and TBE-buffer concentrations at 4°C or room temperature. After drying the gels were autoradiographied at -70°C for 12-48 h.

For DGGE, to the 5' end of one of the PCR primers was attached a 40-50-bp-long stretch of guanosine and cytidine nucleotides (GC-clamp) to increase the sensitivity of DGGE (Sheffield et al. 1989). The GC-clamped PCR-products were run on linearly increasing denaturing gradient polyacrylamide gels of 20%-60% denaturant at 60ºC for 5 hours. After electrophoresis, the gel was stained with ethidium bromide and visualized with ultraviolet translumination.

4.6. Sequencing

The PCR products were purified using the Qiagen Gel Purification Kit (Qiagen, CA, USA) or Fluoricon avidin-polystyrene-assay particles (Idexx Laboratories, ME, USA), which was used if PCR was performed with biotinylated primers. The DNA was sequenced in both sense and antisense directions using the dideoxynucleotide chain termination method (Sanger et al. 1977) with commercial kits (Sequenase 2.0 Sequencing Kit, USB, OH, USA or Amplicycle Sequencing Kit, Perkin Elmer, CT, USA) according to the manufacturers' instructions.

4.7. Solid-phase minisequencing

The solid-phase minisequencing method (Syvänen et al. 1993) was used to identify the variable nucleotide in the sample. Figure 1 in article V shows schematically the steps of solid-phase minisequencing. The method is suitable for screening a previously identified mutation, and it may also be used for quantitative measuring.

PCR was performed as described above with the exception that the PCR mixture contained biotinylated and unbiotinylated primers in proportion of 1:5. For each minisequencing reaction 10 µl of the PCR product and 40 µl of sodium phosphate buffer were added to streptavidin-coated microtiter wells and incubated at 37ºC for 90 minutes. The wells were washed three times and denatured twice with sodium hydroxide at room temperature. To each well was added 50 µl mixture containing specific primer, DNA polymerase, and [^{3}H]dNTP. The labeled nucleotide was hybridized next to the minisequencing primer during the 10-minute incubation at 50ºC. The nucleotides not incorporated were washed out as described above. The primer

hybridized with labeled nucleotide was denatured with sodium hydroxide. The radioactivity eluted with sodium hydroxide was counted in a liquid scintillation counter.

4.8. In vitro mutagenesis

Mutagenesis was performed using a Chameleon Double-Stranded Site-Directed Mutagenesis Kit (Stratagene, CA, USA) according to the manufacturer's instructions. The full-length coding region of the PBGD cDNA (Grandchamp et al. 1989a), used as a template, was ligated in between the *Hind*III and *Eco*RI sites of mammalian expression vector SVpoly (Stacey and Schnieke 1990). The oligonucleotides used in each mutagenesis reaction are given in publication VI. The selection primer was located in the polylinker region of SVpoly which removed the cleavage site for *Xba*I. Mutant clones were confirmed by sequencing.

4.9. COS-1 cell culture and DNA transfection

COS-1 cells were maintained in Dulbecco's modified Eagle's medium (Gibco-BRL, NY, USA) supplemented with 10% fetal heat-inactivated bovine serum and antibiotics. For transfection, the cells were seeded on a 3 cm 6-well plate at $4*10^5$ cells/well and grown overnight. The cells were transfected with 1.5 µg of the plasmid construct by lipofection (Felgner et al. 1987) using FuGENE 6 transfection reagent (Boehringer Mannheim, Germany). Forty-eight hours post-transfection the cells were either harvested for the PBGD activity and Western blot analyses, or pulse-chase experiments were performed.

4.10. Enzyme activity assay

One hundred µl of the cell lysate in 1.0 ml of 1% Triton X-100 was pre-incubated at +37ºC for 15 minutes prior to adding 500 µl of 1.4 mM porphobilinogen in phosphate-citrate buffer (Ford et al. 1980). The mixture was incubated for 30 minutes at +37ºC and the reaction was stopped with 2.5 ml of 10% trichloride-acetatic acid. The fluorescence of the supernatant was measured using 100 µg/l uroporphyrin I standard at the excitation wavelength of 407 nm and the emission wavelength of 598 nm. Protein concentration in a cell lysate was determined by a dye-binding

reaction (Bio-Rad Protein Assay, CA USA). An expression vector control was used to monitor the background expression of PBGD in COS-1 cells.

4.11. Western blot

A mixture of 20 µl of cell lysate in phosphate-buffered saline (PBS) containing 1% Triton X-100, 5 µl of 5 x Laemmli's reagent (Laemmli 1970), 1 µl dithiothreitol , and 1 µl of 20% SDS was run a 14% SDS-polyacrylamide gel. The gel was electroblotted on a nitrocellulose filter. The filter was filled with 5 % bovine milk proteins and reacted with a 1:2000 dilution of polyclonal rabbit anti-human-PBGD antibody (Lannfelt et al. 1989b). Anti-rabbit IgG-Alkaline Phosphatase conjugate (1:7500, Promega) was used as a secondary antibody and the proteins were visualized using 0.66% nitro blue tetrazolium and 0.33% 5-bromo-4-chloro-3-indolyl-1-phosphate (Promega).

4.12. Pulse-chase and immunoprecipitation

The cells were starved in a cysteine- and methionine-free medium for 60 min before labeling with 200 µCi/ml Promix (^{35}S-Cys + ^{35}S-Met, Amersham, UK). A one-hour pulse was followed by 1-23 hour chase in normal medium without bovine serum. After the chase period the cells were harvested and resuspended in PBS containing 1% Triton X-100 and lysed by freeze-thawing three times.

The PBGD proteins were immunoprecipitated from the cell lysates using polyclonal rabbit PBGD antibodies (1:2000) and formalin-fixed *Staphylococcus aureus* cells (Pansorbin cells, Calbiochem, CA, USA, Proia et al. 1984). The labeled and precipitated proteins were separated by 14% SDS-polyacrylamide gel and visualized by autoradiography.

4.13. Immunofluorescence

For immunofluorescence staining the transfected COS-1 cells were grown on 12 mm coverslips and fixed in 3% paraformaldehyde for 30 min at room temperature. After fixation the cells were

washed in PBS and permeabilized in PBS containing 0.2% saponin, 0.5% bovine serum albumin (BSA) for 30 min at room temperature. This was followed by incubation with a 1:2000 dilution of PBGD antibody for 30 min at room temperature. The cells were washed in PBS containing 0.2% saponin and 0.5% BSA and incubated with 1:150 dilution of rhodamine-conjugated secondary antibody against rabbit immunoglobulins (Immunotech, MA, USA) in the same buffer for 45 min at room temperature. The cells were washed in PBS and mounted on slides in 87% glycerol.

5. RESULTS AND DISCUSSION

Prior to this study 4 mutations in 9 Finnish AIP families had been identified (Kauppinen 1992a). The search for an underlying genetic defect in the remaining 31 families was begun using SSCP analysis. The PCR fragments were designed to cover exons 1 and 3 to 15 of the PBGD gene. Exon 2 was not analyzed, since it encodes only erythroid isoform. If a mobility shift was detected in SSCP analysis, the corresponding PCR product was direct sequenced. The identified mutations and their outcome are discussed in Chapters 5.1. and 5.5.

The sensitivity and specificity of the DGGE method for identifying mutations in the PBGD gene was tested. Because two different screening methods - SSCP and DGGE - were used in this series of investigation, their efficiency has been compared. However direct comparison was not possible since the DGGE method was tested blindly and prospectively and the overall sensitivity of SSCP method was analyzed retrospectively (Chapter 5.2.).

The steady state transcript levels of six different mutant alleles were accurately determined using solid-phase minisequencing. In addition, whenever RNA isolated from the patient's lymphoblastoid cell line was available, amplified cDNA was sequenced, which provides a rough estimate of the transcript level of the mutant allele (Chapter 5.3.).

To study further the consequences of mutations in the PBGD gene, seven different mutant cDNA constructs were produced and expressed in eukaryotic COS cells. The properties of mutant proteins were investigated by PBGD activity measurements, and pulse-chase and immunofluorescence studies (Chapter 5.4.).

5.1. Identified mutations (I, II, III)

The series of investigations presented here have revealed 22 additional defects in the PBGD gene resulting in AIP (Table 5.1., Figure 5.1.). Combined with previously identified genetic defects the 26 characterized mutations cover 38 (95%) of a total of 40 unrelated Finnish AIP families.

The mutation was always confirmed by direct sequencing the sample in the other direction or by restriction enzyme analysis when available. When a mutation was identified and its segregation with the disease was confirmed in the family, no further sequencing was performed. In each patient, thus, one to 14 exons were sequenced before a mutation was identified. To exclude a rare polymorphism, the nucleotide sequence of exons 12 and 14 in 50 Finnish unrelated healthy controls was determined and this analysis revealed no sequence variations. When performing the DGGE analysis of the patients' genomic DNA samples, all mutations, except the large insertion in exon 5, resulted in a deviated pattern in the corresponding exon. None of the DGGE patterns similar to these mutations were observed when the DNA samples of more than 50 Danish AIP patients were analyzed. In addition, a variable number of unrelated healthy controls and healthy family members were analyzed for each mutation. Thus, a rare polymorphism was considered very unlikely.

As in most other populations (Table 2.3.), also in Finland the AIP mutations are highly heterogeneous both in their type and location. Furthermore, almost all mutations are family specific. Thirteen (50%) mutations are located in exons 10, 12, and 14. Of the twelve missense mutations (46% of all identified mutations), six are substitutions of an arginine residue. A substitution of one base pair causes a nonsense codon in five cases. In addition, two deletions and two insertions result in a frame shift leading to a premature termination codon after a variable number of missense codons. Four mutations affect the splicing of the primary transcript. As a consequence of two splicing mutations (IVS+3G T, IVS13-2A G), the intron sequence is retented into the transcript and a premature termination codon is introduced. One splicing mutation (86A T) was shown to result in exon skipping and a truncated protein, but in the case of a splicing defect in exon 1 (33G T) no mutant transcript could be detected (Kauppinen 1992b). Deletion of one nucleotide in the last exon results in a frame shift which removes the normally utilized termination codon and no additional termination codon is available before the polyadenylation signal.

In two (5% of all Finnish AIP families) AIP patients the genetic defect causing AIP remains unknown. In these patients, the diagnosis was based on typical clinical manifestations and on increased excretion of porphobilinogen in urine and/or on low PBGD activity in erythrocytes.

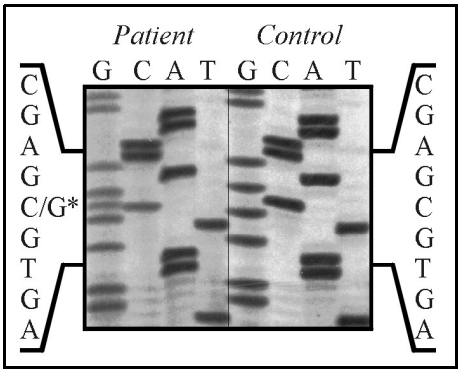


Figure 5.1. *Identifying the mutation by direct sequencing. As an example mutation 673C G.*

Both patients are the only affected individuals in their families. In the one patient's sample there was a mobility shift in exon 11 when DGGE analysis was performed, whereas the other patient's DGGE analysis was negative. The entire coding sequence and flanking intronic regions were sequenced and no nucleotide alteration was found in the genomic DNA samples of these patients. Neither did the amplification of the PBGD gene in long fragments (3.4-4.5 kb) reveal additional information.

The genetic defect remained unsolved in some cases also in a French study, where the mutations were systematically searched for using DGGE analysis and sequencing (Puy et al. 1997b). The patient material comprised of 405 patients from 121 unrelated AIP families, and in 12 (10%) families the DGGE screening was normal and no base changes were identified despite sequencing all exons and exon/intron junctions. Based on the data from Finland and France, sequencing only the coding areas and flanking intronic regions of the PBGD gene does not seem to guarantee the detection of all mutations. In these patients the underlying defect may be the in promoter region, in the middle of intronic sequence, or it may be a large insertion or deletion which remains non-detectable with the methods used in these studies. Because the enzyme activity measurement has wide overlapping zone and urinary PBG and dALA are elevated also in other porphyrias, the subjects may also have been misdiagnosed as AIP patients.

Table 5.1. *Identified mutations in Finnish AIP patients*

	Mutation	Location	Outcome	cDNA[a]	CRIM[b]	Enzyme[c]	No. of families	Ref.[d]
1	33G T	1	splicing defect	-	-	*Hph*I	1	(1)
2	IVS1+3G T	intron 1	intron 1 retention	+	-	*Hph*I	1	II
3	76C T	3	Arg26 Cys	+	+	*BspM*I	1	I
4	86A T	3	exon 3 skipping	+	-	*BstX*I	1	II
5	97delA	4	frame shift	+	-		3	I
6	100C T	4	Gln34 Stop	+	-		1	I
7	Ins 332 bp	5	frame shift	-	-		1	III
8	293A G	7	Lys98 Arg	n.d.	-	*Nla*IV	1	I
9	346C T	8	Arg116 Trp	-	-		1	(2)
10	417insCA	8	frame shift	n.d.	-		1	II
11	445C T	9	Arg149 Stop	-	-		2	I
12	499C T	10	Arg167 Trp	+	+		5	(3)
13	517C T	10	Arg173 Trp	+	-/(+)	*Msp*I	2	(4)
14	518G A	10	Arg173 Gln	+	+	*Msp*I	4	(5)
15	583C T	10	Arg195 Cys	n.d.	-	*Hha*I	1	I
16	593G A	10	Trp198 Stop	-	-	*Nhe*I	1	(6)
17	664G A	12	Val222 Met	+	-		1	II
18	673C G	12	Arg225 Gly	+	-	*Nla*IV	1	I
19	673C T	12	Arg225 Stop	-	-		2	I
20	713T G	12	Leu238 Arg	-	-	*Bbv*I	1	I
21	740G T	12	Cys247 Phe	+	-	*Fnu4H*I	1	I
22	IVS13-2A G	intron 13	intron 13 retention	+	-	*Msp*I	1	II
23	833T C	14	Leu278 Pro	+	-	*Bsl*I	1	II
24	838G A	14	Gly280 Arg	-	-	*Mae*I	1	I
25	886C T	14	Gln296 Stop	-	-		1	I
26	1073delA	15	frame shift	-	-	*Mse*I	1	I

[a] +, mutation seen in cDNA; - mutation not seen in cDNA; n.d., not done.
[b] CRIM, cross-reactive immunologic material; +, inactive protein detected; -, no inactive protein detected.
[c] Mutation specific restriction enzyme.
[d] First described by: 1, Grandchamp et al. 1989b; 2, Lee et al. 1990; 3, Gu et al. 1992; 4, Lee 1991a; 5, Delfau et al. 1990; 6, Lee and Anvret 1991.

5.2. SSCP and DGGE in screening for novel mutations (I, II, III, IV)

Mutations were searched for using two different screening methods: single-strand conformation polymorphism (SSCP, Orita et al. 1989) and denaturing gradient gel electrophoresis (DGGE, Myers et al. 1987, Nissen et al. 1995). SSCP analysis is based on the mobility shifts of a single-stranded DNA under renaturing conditions. During electrophoresis the single-stranded DNA rehybridizes and the migration position is determined by the secondary structure, which is influenced by the sequence variants. In DGGE analysis the amplified DNA samples are run on a gel, in which the amount of denaturant is gradually increased. Sequence variation in the sample has an effect on the melting point which alters the migration. Previously, both SSCP (Kauppinen et al. 1992, Chen et al. 1994, Schreiber et al. 1994a, Schreiber et al. 1994b, Schreiber et al. 1994c, Schreiber et al. 1995b) and DGGE (Gu et al. 1992, Gu et al. 1993b, Gu et al. 1994, Petersen et al. 1996, Puy et al. 1996, Nordmann et al. 1997, Puy et al. 1997a, Puy et al. 1997b, Rosipal et al. 1997, Petersen et al. 1998, Puy et al. 1998) have been used in searching for mutations responsible for AIP.

The overall sensitivity of SSCP in screening mutations of the PBGD gene was 90%, i.e.18 of the 20 identified mutations could be detected using this method when five different electrophoretic conditions (glycerol concentration was 0%, 5%, or 10% and the gel was run at +4°C or at room temperature) were used. However, the sensitivity of SSCP was significantly lower if only a single electrophoretic condition was used: gel electrophoresis at room temperature with 5% and 10% glycerol detected 12/20 (60%) and 14/19 (74%) of the mutations, respectively, while gel electrophoresis at +4°C without glycerol detected 15/19 (79%) of the mutations. The length of the analyzed PCR fragment had a variable effect on the sensitivity of the analysis: in the case of mutation 97delA both an increase and a decrease in the size of the fragment (510 bp←446 bp 324 bp or 189 bp) from the original resulted in a shift and in SSCP analysis of mutation 1073delA even minor changes in the size of the fragment (192 bp or 197 bp←211 bp 228 bp) caused disappearance of the mobility shift. The specificity of the SSCP method was not evaluated in this study.

The efficacy of the DGGE method in detecting mutations causing AIP was evaluated by analyzing blindly the DNA samples of 22 AIP patients and 6 healthy controls. As a result, the correct mutation carrying region was found in samples of all 22 patients, but in two samples two potential regions were initially highlighted. In both cases, the false positive pattern was located in exon 7 and flanking intron sequencing, and sequence analysis revealed a previously unknown polymorphism in intron 6. Common polymorphisms in the PBGD gene could easily be distinguished from mutations because the number of analyzed samples was relatively high and the location of polymorphic sequence variations was known. The DGGE patterns in all healthy controls were normal, only common polymorphisms could be identified. In addition six mutations described in article II, which were not included in the study with a blinded design, resulted in a mobility shift in the corresponding region.

However, DGGE analysis could not identify the 333 bp Alu insertion located in exon 5. This was expected since the length of the normal fragment covering exons 5 and 6 in DGGE analysis is 393 bp and in the patient sample the PCR product is almost twice as long, 726 bp. The allele with the insertion migrates more slowly and most probably will not reach the analytical area in the gel by the end of the electrophoresis. When the results of these studies are combined, the sensitivity of DGGE analysis was 96% (25/26 mutations could be identified with the method) and the specificity was 94% (two false positive mobility shifts in 35 samples).

The DGGE and SSCP methods have previously been evaluated both in the screening of the PBGD gene and several other genes, and the earlier results are in good agreement with the data obtained from our experiments. When several sets of electrophoretic conditions is used, SSCP detects 70-95% of mutations but using only one condition reduces the sensitivity considerably (Michaud et al. 1992, Grompe 1993, Moyret et al. 1994). Furthermore, the sensitivity of this method has been reported to be highest when the fragment size is below 200 bp (Grompe 1993), which limits the usefulness of the method when large genes are screened for mutations. The sensitivity of DGGE has been estimated to be as high as 95-100% in several studies (Grompe 1993, Moyret et al. 1994, Macek et al. 1997, Puy et al. 1997a, Puy et al. 1997b) and single base differences can be detected with high accuracy in PCR products of up to 600 bp in length.

DGGE appears, thus, to be superior in sensitivity when compared with SSCP. However, the setup of the DGGE method is laborious: the running conditions of each fragment needs to be optimized separately but tuning up of the system may be assisted by computer analysis. Once conditions are finalized, the DGGE analysis proceeds rapidly. SSCP method has gained popularity because of its simple and straightforward implementation. Although it is a powerful method, the sensitivity of SSCP has not reached the same level as DGGE. Furthermore, the method lacks guidelines for its optimization for a defined DNA sequence (Michaud et al. 1992, Moyret et al. 1994) and it usually requires the use of radioactive nucleotides to label the fragments to be analyzed.

5.3. Monitoring the effect of mutations to allelic transcript levels (V)

The steady state level of allelic transcripts in lymphoblasts of eight AIP patients were analyzed using solid-phase minisequencing. The patients had the following mutations: 517C T (R173W), 518G A (R173Q), 673C G (R225G), 673C T (R225X), 713T G (L278P) and 1073delA (frame shift). All mutations, except the missense mutation 517C T in exon 10, affected the steady state transcript levels of the mutant allele (Table 5.2.). The mutant mRNA levels in lymphocytes varied from 5 to 95% of the corresponding wild type allele levels. In contrast to the CRIM negative mutation 517C T, the CRIM positive mutation in the same codon 518G A resulted in reduction of the steady state transcript level of the mutant allele to 65% of that of the normal allele. Interestingly, the mutant transcript of the patient with mutation 517C T seemed to be more unstable than the normal transcript, because mutant transcript seemed to degrade more rapidly during storage at -80ºC. However, this preliminary finding was not studied further.

Two of the mutations, 673C G or T (R225G or X), affecting the same nucleotide in exon 12 differed considerably in their effect on allelic transcript: the transcript level of the allele with a missense mutation was decreased to 80% of that of the normal allele whereas the nonsense mutation at the same position resulted in a dramatic decrease (5-fold) in the levels of the mutant transcript. The mRNA samples of three subjects with the same nonsense mutation 673G T (R225X) were also analyzed. Although clinical manifestations have been different in these

patients, the steady state transcript levels were almost identical: 23%, 20%, and 24% of the normal allele.

Even though the data showed variations between the levels of mutant transcript in AIP patients, the mutant transcript level did not correlate to the CRIM class, to the location of the disease causing mutation in the PBGD gene, or to the clinical phenotype of AIP.

Nonsense codons have been reported to dramatically decrease the mRNA levels of the mutant allele and, typically, the closer the mutation is to 5' end of the transcript, the more dramatic is the effect (Cooper 1993). Direct sequencing of amplified cDNA product gives in most cases a rough estimation of the steady state transcript level of the mutant allele, since the mutant allele is detected if its level is at least 30% of the normal allele. In our material the mutant allele could be detected in sequenced cDNA in the case of four nonsense mutations: IVS1+3G T, 97delA, 100C T, and IVS13-2A G (Table 5.1.). Total RNA from the patient with mutation 417insCA was not available, and the remaining five nonsense mutations were not detected when the corresponding cDNA was sequenced. This suggests that mutant allele gets transcribed at least to some extent in the case of four nonsense mutations. Three of these four mutations are located in exons 1 or 3 and one in intron 13. Those mutations which could not be detected by sequencing of cDNA are located in exons 1, 5, 8, 10, 12 and 14 (Table 5.1.). Thus, our results are not in agreement with the data obtained from mutations of other genes, since in those mutant alleles that are transcribed the mutations are located closer to the 5' end of the PBGD gene than those mutant alleles that are not transcribed. However, the low number of mutations and the lack of accuracy of this method prevents us from drawing any definitive conclusions concerning this finding.

Mutations resulting in premature termination codons do not decrease the rate of gene transcription (Urlaub et al. 1989, Cheng and Maquat 1993). However, in the majority of cases the levels of mutant mRNA, imposed by premature termination codon, are decreased (Cooper 1993, Peltz et al. 1994, Maquat 1995). Previously, it was anticipated that mRNA decay would take place in the cytoplasm, because termination codon recognition requires cytoplasmic ribosomes. Several pieces of evidence have, however, implied that the low levels of mutant

transcripts are due to nuclear events (Urlaub et al. 1989, Baserga and Benz 1992, Cheng and Maquat 1993, Cooper 1993, Simpson and Stolzfus 1994, Carter et al. 1996)

The mechanisms of how premature termination codons reduce mutant mRNA levels are obscure. Carter and co-workers (Carter et al. 1996) demonstrated that at least two signals are required to trigger the down-regulation of mutant mRNA: a nonsense codon and a spliceable downstream intron. According to this result, thus, premature termination codons in the last exon would not affect the mutant RNA level.

The translational-translocation model (Urlaub et al. 1989) proposes that during mRNA export from the nucleus to the cytoplasm, the 5' end of a transcript is scanned by cytoplasmic translational machinery while the 3' end is still undergoing mRNA splicing, which is a nuclear event. The premature nonsense codon in the nuclear pore-associated mRNA, scanned by a cytoplasmic ribosome, would ultimately result in the decay of the mRNA. The length of the nuclear pore has been estimated to be 15 nm or ~45 nucleotides of linear mRNA (Alberts et al. 1994). According to this model, hence, a nonsense codon should be at least 60 nucleotides (an additional 15 nucleotides are required for the termination codon to be positioned at the P site of the ribosome) from a downstream intron to be recognized in the cytoplasm before the intron is spliced out in the nucleus. The studies on the T-cell receptor β-gene, however, suggested that the border for nonsense-mediated mRNA down-regulation is below 10 nucleotides from the exon/intron junction (Carter et al. 1996), and these data are inconsistent with the translational-translocation model.

In the marker model (Carter et al. 1996) a downstream intron could act as a second signal to trigger down-regulation. This model is based on the presumption that after splicing the previous location of intron is marked on mature mRNA. The potential marker is a specific SR protein, which remains bound to exons after RNA splicing, or the marker may be RNA which has undergone post-transcriptional modification. This model allows the triggering of mRNA decay before it has completely exited the nucleus, even if the premature termination was scanned by a cytoplasmic termination codon.

So far, the only known cellular structure that can scan codons is a cytoplasmic ribosome. However, a model in which a nuclear component would recognize nonsense codon has been proposed (Urlaub et al. 1989, Carter et al. 1996). Although there is no direct evidence for a nuclear scanner, the model accounts for all the important features characterized in experiments: mutant mRNA is destroyed in the nucleus, a spliceable intron acts as a second signal to trigger mRNA down-regulation, and a nonsense codon may be located only 10 nucleotides upstream of the last intron to trigger mRNA decay.

Unlike nonsense mutations, the effect of missense mutations on transcript levels has characteristically been reported to be non-significant (Mustafa et al. 1995, Karttunen et al. 1996, Ploos van Amstel et al. 1996). In contrast, our results demonstrate that also missense mutations can reduce the abundance of mutant mRNA. In our experiment we have used Ebstein-Barr -virus infected lymphoblasts and the transcript levels in immortalized lymphoblasts may differ from those in cells *in vivo*. However, an allele specific effect would be unlikely and consequently we have here emphasized the relative ratios of mutant and wild type allelic transcripts.

Table 5.2. *Consequences of selected mutations on the steady-state transcript level and enzyme activity after eukaryotic expression.*

Mutation	Outcome	CRIM	Mutant mRNA as a ratio of wild type mRNA	Enzyme activity ±S.E. nmol/mg prot/h [b]
76C T	R26C	+		105±17 (0%)
Alu insertion	Trunc. protein	-		[270±80 (0%)][c]
499C T	R167W	+		273±66 (5%)
517C T	R173W	-	0.97	173±33 (2%)
518G A	R173Q	+	0.66	140±11 (0%)
673C G	R225G	-	0.82	626±78 (16%)
673C T	R225X	-	0.22	142±9 (0%)
713T G	L278P	-	<0.05	
1073delA [a]	frame shift	-	0.72	1660±174 (50%)
Wild type				3173±507 (100%)

Control COS	127±7

[a] *Normally utilized termination codon abolished. The polypeptide synthesized by the mutant construct differs from the predicted polypeptide in patients with this mutation.*
[b] *Mean value of four independent measurements. Percentage of mean normal activity corrected with the endogenous COS-1 cell PBGD activity in parenthesis.*
[c] *Enzyme activity is not comparable because of separate assay.*

5.4. Expression of mutated polypeptides (III, VI)

To analyze further the consequences of mutations on the function of PBGD enzyme, eight mutations were introduced to PBGD cDNA and the mutated constructs were expressed in eukaryotic COS cells. Six of the tested mutations (R26C, Alu insertion, R167W, R173W, R173Q, R225X) resulted in the reduction of PBGD activity to the background level (0-5%). These results confirm the assumed causality of the amino acid substitution and the low PBGD activity measured in the patients' erythrocytes. In two cases (R225G, 1073delA), however, the enzyme activity was only partially disturbed and 16-50% residual activity could be detected (Table 5.2.).

Western blot analysis shows that the mutant constructs R26C, R167W, R173W, R173Q, and R225G expressed polypeptides which reacted with the antibodies directed against human PBGD and the migration pattern was comparable with that of the normal enzyme (Figure 5.2.). As predicted, the construct with mutation 1073delA removing the normally used termination codon, produced a longer polypeptide. The constructs with R225X and Alu insertion, both of which are predicted to result in a truncated protein, produced no detectable polypeptide in Western blot analysis, suggesting that either the polyclonal antibody did not recognize the truncated polypeptide or the polypeptide is very unstable.

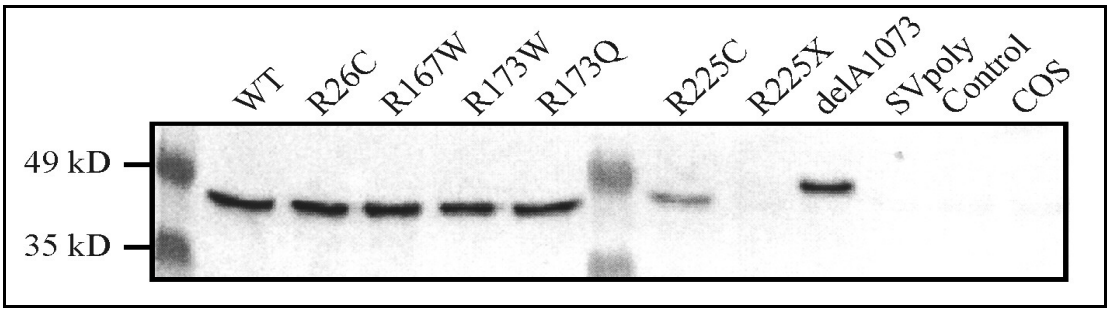


Figure 5.2. *Western blot after expression of mutated PBGD constructs in COS cells.*

The stability of mutant polypeptides R173W, R173Q, R225C, R225X, and 1073delA were studied using a pulse-chase experiment. The construct with the mutation R225X did not produce detectable polypeptide. The other mutated constructs were found to encode a polypeptide with a stability comparable to the wild type polypeptide, since the signals of all mutated polypeptides remained similar with the wild type polypeptide at all time points (Figure 5.3.). When polypeptides were chased for 23 hours, both mutant and wild type PBGD were degraded based on a weaker signal in immunoprecipitation.

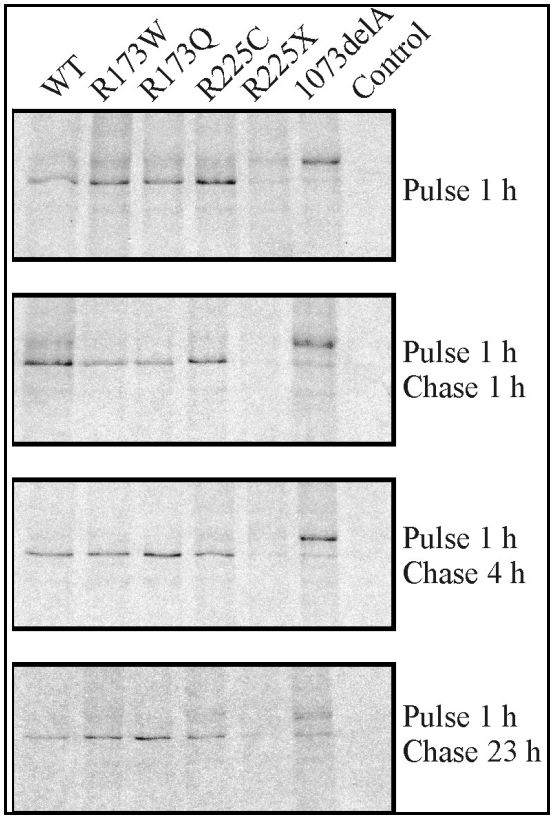


Figure 5.3. *Pulse-chase experiment of wild type and mutated PBGD*

The half-life of the erythroid form of PBGD is approximately 24 hours if sufficient porphobilinogen is present to form tetrapyrrole-bound intermediates which are less susceptible to degrade than the free enzyme (Beaumont et al. 1986). When the amount of porphobilinogen is inadequate, the polypeptide degrades more rapidly: half of the polypeptide is lost within 10 hours (Beaumont et al. 1986). The housekeeping isoform of PBGD appeared to be more stable in the absence of substrate than the erythroid isoform, but the half-life of the housekeeping isoform was not accurately determined (Beaumont et al. 1986). In COS-1 cells transfected with PBGD cDNA, the enzyme is expressed without regulation and the amount of endogenous porphobilinogen is probably not sufficient to bind to every molecule formed. In these circumstances, thus, it is presumed that unbound PBGD is more prevalent than enzyme-intermediate complex, leading to more instable polypeptide. This hypothesis supports the results of our experiment, in which the PBGD were clearly degraded after a 23-hour chase.

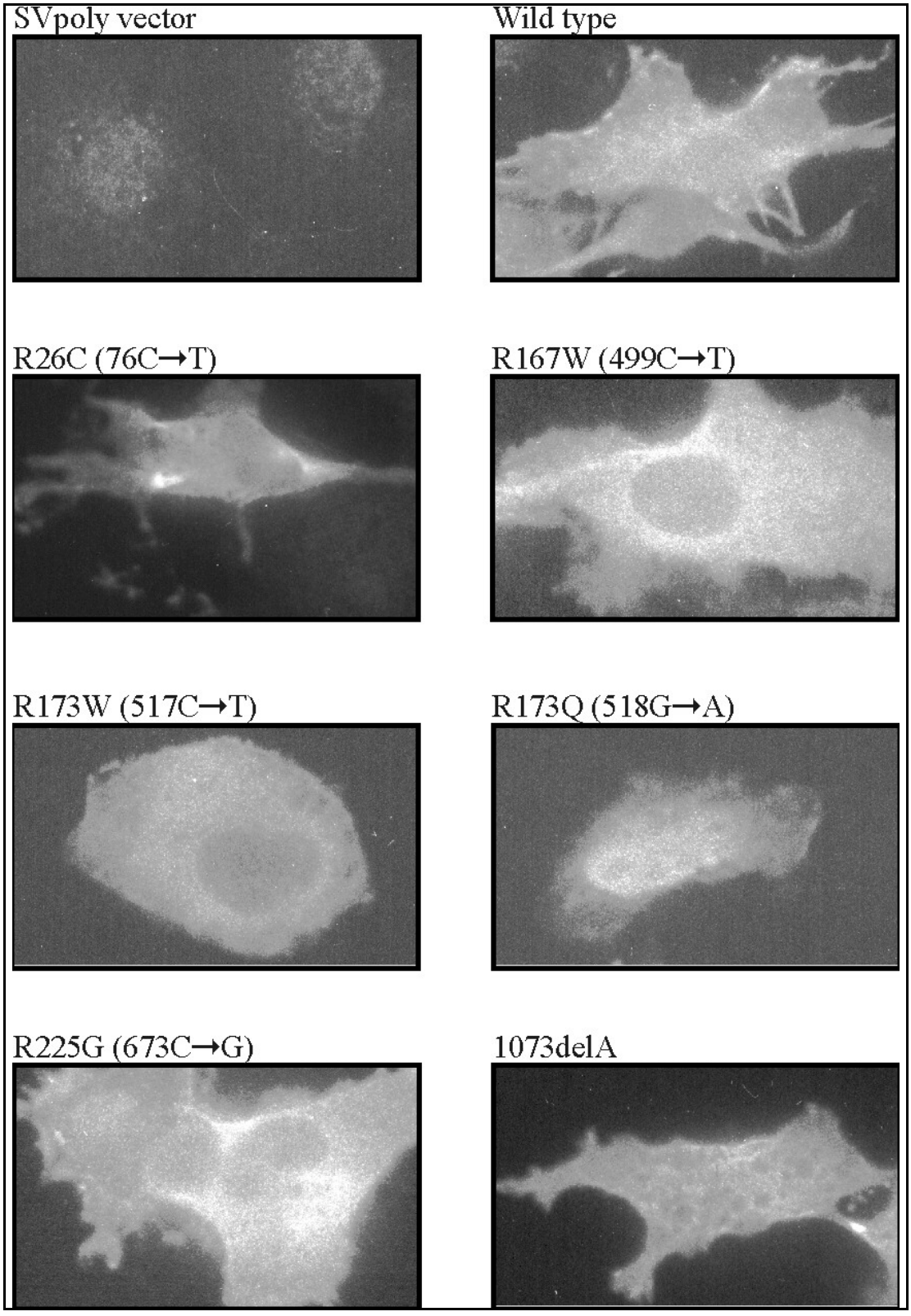


Figure 5.4. *Immunofluorescence of mutated and wild type PBGD after expression.*

Based on the immunofluorescence staining, the transfection efficiency using lipofection method was approximately 20% for each mutated cDNA studied (data not shown), and thus, it was independent of the construct composition. The construct with the nonsense mutation R225X was excluded from this experiment, since in the Western blot analysis or in the immunoprecipitation assay no PBGD polypeptide could be detected. Figure 5.4. shows that in the immunofluorescence staining the wild type polypeptide is diffusely dispersed in the cytoplasm. The localization of the mutated polypeptides was indistinguishable from that of the wild type. Thus, no abnormal accumulation or targeting of the mutated polypeptides was observed. Moreover, the mutations representing different CRIM classes did not differ from each other.

While this study is the first in which the properties of mutant PBGD were analyzed using *in vitro* expression in eukaryotic cells, the effect of mutations in the PBGD gene on the enzyme activity has been studied in three previous reports (Delfau et al. 1990, Chen et al. 1994, Ong et al. 1997) These experiments were performed by expressing the mutated cDNAs of human PBGD in *E. coli*. In these studies all mutations resulted in decreased enzyme activity and the properties of mutant polypeptides were further analyzed by measuring the enzyme activity in various pH environments (Delfau et al. 1990) or by determining the thermostability of the polypeptides (Chen et al. 1994). Mutant PBGD appeared to have similar enzyme activities after eukaryotic and prokaryotic expression, which was expected, since PBGD is a cytosolic enzyme and is not further processed post-translationally.

Based on the studies of human PBGD mutants (Delfau et al. 1990, Chen et al. 1994, Ong et al. 1997), E. coli mutants (Jordan and Woodcock 1991, Lander et al. 1991), and the three-dimensional model of the enzyme (Louie et al. 1992, Brownlie et al. 1994), mutations altering the residues in the vicinity of the active site have always detrimental effect on the enzyme activity. Amino acid substitutions located elsewhere in the polypeptide have variable consequences. These mutations are thought either to destabilize the polypeptide or to alter the protein structure in a way which prevents the substrate reaching the active site. In the present study the mutant enzyme molecules were as stable as the normal polypeptide after pulse-chase experiment. However, Chen et al. (1994) showed that mutations C247F and R201W lost half of

the enzyme activity in less than 30 minutes at 65°C while the $T_{1/2}$ of normal PBGD was 120 minutes. Although this finding cannot be generalized to all the mutations in the PBGD gene, it suggests that at least some amino acid substitutions actually both destabilize the polypeptide and decrease enzymatic activity. Interestingly, four mutations located in domain 3 of the polypeptide have been expressed and all of them had relatively high residual activity of 10-50% (Chen et al. 1994, VI) after *in vitro* expression.

5.5. Outcome of mutations

The type and location of mutations resulting in AIP differ from each other considerably and most of the mutations appear to have a detrimental effect on the normal function of the enzyme. There is a wide range of mechanisms leading to an inactive enzyme and in this chapter the potential mechanisms are discussed.

5.5.1. Mutations resulting in premature termination codon

In the case of ten mutations the predicted outcome would be the introduction of a premature termination codon: IVS1+3G T (see chapter 5.5.3), 97delA, 100C T, insertion of 332 bp in exon 5 (see chapter 5.5.4.), 417insCA, 445C T, 593G A, 673C T, IVS13-2G A (see chapter 5.5.3.), and 886C T. The consequences of a premature termination codon on the function of the enzyme are often dramatic. It is probable that the polypeptide encoded by the mutant allele is enzymatically totally inactive, since many structures essential for the enzymatic activity are not translated, and the stability of the polypeptide is probably also influenced by the missing residues (Brownlie et al. 1994). In addition, the transcript level of the mutant allele is often decreased (see chapter 5.2.). All nonsense mutations described here are found in CRIM negative patients, which suggests that mutant polypeptide is not produced or the polypeptide is unstable (Mustajoki and Desnick 1985).

The outcome of the nonsense mutation R225X (673C T) was studied in detail. The solid-phase minisequencing method was used to determine the steady state transcript level of the mutant allele, which was reduced to one fifth of that of normal allele (Table 5.2.). Furthermore, the mutant transcript levels of the three clinically different patients with this mutation were

similar. One of the patients has had acute attacks, her sister has experienced milder symptoms, and their mother has always been asymptomatic. Thus, there was no correlation between the phenotype and the mutant mRNA levels in patients' lymphoblasts. To study the consequences of the mutation on protein level, the mutation was introduced in the human PBGD cDNA and expressed in COS cells. The mutant enzyme was not detected by PBGD antibody (Figure 5.2.) and it had no enzyme activity (Table 5.2.). In conclusion, the mutant transcript level is remarkably decreased and even if mutant transcript was translated, the polypeptide would be enzymatically inactive.

5.5.2. Missense mutations

Thirteen base changes were predicted to result in an amino acid substitution. The substituted residues are highly conserved in different species (Grandchamp et al. 1984, Raich et al. 1986, Thomas and Jordan 1986, Beaumont et al. 1989, Petricek et al. 1990, Keng et al. 1992, Mohr et al. 1994, Abdelhak et al. 1997). Of the substituted residues all six arginines and lysine-98 are invariant and thus completely conserved in the nine species studied (human, murine, rat, feline, *Saccharomyces cerevisiae*, *Escherichia coli*, *Pseudomonas aeruginosa*, *Bacillus subtilis*, and *Euglena gracilis*), and the remaining five residues are identical at least in mammalian species. The invariance of the residues would suggest that they are crucial for the proper function of the enzyme. The consequences of selected mutations were further characterized 1) by determining the steady transcript level of the mutant allele and 2) in vitro expression of the mutagenized cDNA construct and subsequent analyses of the polypeptide chains.

The three-dimensional model of PBGD polypeptide enables comparison of the mutant residues with their corresponding normal residues in the *E. coli* crystal structure (Louie et al. 1992) and provides information about the structural and functional consequences of the mutations in the PBGD polypeptide (Brownlie et al. 1994). In addition, the mutagenesis studies of the *E. coli* PBGD have provided valuable information about the function of the enzyme (Jordan and Woodcock 1991, Lander et al. 1991, Woodcock and Jordan 1994). The consequences of the missense mutations based on the information obtained from these studies are discussed below.

R26C (76C T). The arginine-26 is located in domain 1 of the PBGD (Figure 2.3.) in the vicinity of the active cleft. The positively charged side chains of the residue are thought to interact with the substrate (Lambert et al. 1994). In our expression studies the mutant C26 displayed no enzymatic activity (Table 5.2.). This result is consistent with the mutagenesis studies in *E. coli* PBGD: substituting the corresponding residue (R11) results in complete inactivation of the enzyme (Jordan and Woodcock 1991, Lander et al. 1991), because the attachment of the first porphobilinogen ring of the substrate (E ES) is inhibited (Jordan and Woodcock 1991). According to the pulse-chase experiment (Figure 5.2.) the mutant polypeptide is as stable as the wild type enzyme, which is in agreement with the CRIM positive status of the patients carrying this mutation.

R167W (499C T). Arginine167 (R149 in *E. coli*) interacts with the side chain of the cofactor. Its modification to H149 in the *E. coli* enzyme leads to an accumulation of the ES intermediate (enzyme with a single porphobilinogen attached) (Jordan and Woodcock 1991) and a similar perturbation might also be expected for this mutation (Brownlie et al. 1994). In our expression studies this mutation displayed a minimal residual activity of 5% (Table 5.2.). This mutation is found in CRIM positive patients, which is consistent with the pulse-chase experiment, in which the mutant enzyme was as stable as the wild type enzyme and folded normally, but lacking function.

R173W (517C T) and R173Q (518G A). The side chain of arginine-173 interacts with the cofactor and the residue also participates in substrate binding (Shoolingin-Jordan 1995). The mutants W173 and Q173 were expressed in COS cells and the polypeptides synthesized had no enzymatic activity even though both polypeptides were detected in the Western blot analysis by the antibody and in pulse-chase experiments their stability was comparable to the wild type PBGD (Table 5.2., Figures 5.2 and 5.3.). The mutagenesis studies in *E. coli* R155 (equivalent to human R173) also led to severe impairment in substrate binding as well as the release of the tetrapyrrole product (Jordan and Woodcock 1991, Lander et al. 1991). The mutant transcript levels compared to the wild type allele in the case of mutations R173W and R173Q were 97% and 66%, respectively (Table 5.2.).

According to expression studies and determination of transcript levels, thus, the outcome of mutations substituting R173 are quite similar. The Finnish patients carrying the mutation R173W have previously been classified as CRIM negative or CRIM positive and patients with the mutation R173Q as CRIM positive (Mustajoki and Desnick 1985). Our data suggests that patients carrying the mutation R173W are weakly CRIM positive, i.e. inactive polypeptide is present, but its amount is so low that it is hardly detectable by rocket immunoelectrophoresis. This would give us satisfactory explanation for the different CRIM classes in different patients with the same mutation.

R225G (673C G). Invariant arginine-225 is located on the outer surface of the enzyme (α_4-helix of the subunit 1) and its side chain forms hydrogen bonds to other side chains and also hydrogen bond to main-chain carbonyl (Brownlie et al. 1994). Furthermore, substituting arginine with glycine probably destabilizes the protein structure, since, unlike arginine, glycine does not usually form α-helix structures (Branden and Tooze 1991). The transcript level of the mutant allele was also determined and it was only slightly decreased (80% of the level of the wild type allele). In *in vitro* expression the stability of the mutant polypeptide did not differ from the wild type PBGD (Figure 5.3.) and, furthermore, the mutated enzyme displayed a residual activity of 16% (Table 5.2.). Patients with this mutation are CRIM negative, i.e. there is no inactive enzyme in patients' erythrocytes. The absence of inactive enzyme could be explained by disruption of interactions and incomplete α-helix formation, which might destabilize the polypeptide.

*K98R (293A G).*This highly invariant lysine interacts with dipyrromethane cofactor together with the neighboring aspartate-99 and four arginines (R149, R150, R167, and R173). Substitution of the corresponding lysine (K83) by various amino acids in *E. coli* resulted in an enzyme that retains less than 1% of its activity (Woodcock and Jordan 1994) and presumably the consequence of this mutation similar.

R116W (346C T). This substitution is the most common mutation causing AIP in the Dutch population (Gu et al. 1993b). The arginine residue, located on a short strand linking domains 1 and 2, forms a salt bridge with glutamic acid in position 250 but is not involved in interactions

with the substrate or the cofactor. This mutation has been expressed in *E. coli* and the residual PBGD activity was 3.4% (Ong et al. 1997). In addition, the mutagenesis study of the mutant R101L in *E. coli* (equivalent to human position 116) displayed 37% residual activity and the mutation had little effect on either the kinetic parameters of the enzyme or on the formation of the substrate enzyme complexes (Lander et al. 1991). The patients with this mutation have CRIM negative status, which is thought to have resulted from the loss of stabilizing interactions to the glutamic acid residue in position 250 (Brownlie et al. 1994).

R195C (583C T). The patient carrying this mutation is the only affected individual in her family. Neither parent was a carrier of the mutation and paternity was confirmed using DNA fingerprint analysis, thus suggesting a *de novo* mutation. Substituting this arginine inhibits the assembling of the third porphobilinogen unit to the growing chain (Lander et al. 1991).

C247F (740G T). The cysteine residue is buried in the hydrophobic core of the PBGD polypeptide and its substitution is speculated to cause a steric disruption of the polypeptide backbone (Brownlie et al. 1994).

V222M (664G A), L238R (713T G), L278P (833T G) and G280R (838G A). These residues are conserved only in mammalian species and, furthermore, they do not appear to interact with other amino acids in the polypeptide (Brownlie et al. 1994). Thus, the mechanism of how these mutations cause inactivation of the enzyme is not thoroughly clarified. These substitutions are, however, considered as causative agents for AIP, since 1) no other nucleotide variation could be identified in the coding region of the PBGD gene by direct sequencing and/or 2) the DNA analyses of the family members were in agreement with the pedigree analysis and the results from biochemical analyses.

All mutations identified in CRIM positive patients were arginine substitutions. These arginine residues form interactions with the substrate or the cofactor and are essential for enzymatic activity. Minor changes in the polypeptide structure among CRIM positive patients were expected, since CRIM positivity requires inactive but stable polypeptide which is detected by a specific antibody. A truncated polypeptide, caused by a premature termination codon or larger

disruption in the nucleotide sequence, would presumably be unstable. However, the possibility exists that the antibody used in the immunological assays may not recognize it.

5.5.3. Splicing mutations

Four mutations interfere with the normal splicing pattern resulting in aberrant splicing. The consensus sequence for RNA splicing is known (Table 5.3.) but mechanisms for a splicing defect have not been thoroughly clarified. However, studies of different types of mutations at the splice sites have provided useful information about aberrant splicing (Krawczak et al. 1992, Nakai and Sakamoto 1994)

Table 5.3. *Consensus sequences for RNA splicing.*[a]

5'---$^{C}_{A}$AGgu$^{a}_{g}$agt	------a------	uuuuuuuuuuuu$^{c}_{u}$ag$^{G}_{A}$---3' cccccccccccn
5' splice site (donor site)	*branch point A*	*3' splice site (acceptor site)*

[a] Adapted from (Alberts et al. 1994). Intronic sequence in lowercase and exonic sequence in uppercase letters. *n*, any nucleotide

Three of the splicing mutations are located at the 5' donor splice site. In the case of the mutation IVS1+3G T, the cryptic splice site is present 67 nucleotides away from the authentic splice site. This finding is in agreement with the previous survey, in which most of the cryptic splice sites activated by mutations are located relatively near, usually within 100 nucleotides, to the authentic splice site (Nakai and Sakamoto 1994). The rest of the gene is transcribed normally, but due to the uneven amount of nucleotides in the inserted fragment, a frame shift appears at the beginning of exon 3 introducing a premature termination codon in exon 5. The length of the predicted polypeptide is 73 amino acids and only the first exon would be translated normally. Because the mutant polypeptide is dramatically truncated, it can be predicted to be enzymatically inactive.

The mutation 33G T hits the last nucleotide of exon 1. The substitution does not modify the amino acid and, although definitive proof is missing, it is thought to be responsible for abnormal splicing (Grandchamp et al. 1989b, Kauppinen 1992b). Due to the tissue-specificity,

the erythrocyte PBGD activity in patients with mutations 33G T and IVS1+3G T is normal and these mutations cause the variant form of AIP.

Exon skipping is the most frequent consequence of a splice mutation at either the 5' or 3' splice site (Nakai and Sakamoto 1994), and it is also the predicted outcome of the mutation 86A T. Interestingly, this base change would also result in the amino acid substitution Q29L if the normal splicing was not affected. If no alternative sites with sufficient homology to the consensus sequence exist in the vicinity, either exon skipping or greatly reduced production of normal transcripts seem to be the predominant phenotypic consequences (Krawczak et al. 1992). In this case exon skipping is probably due to a lack of the potential cryptic splice site in the vicinity of the 5' splice site of intron 3 and, as a result, 18 amino acids encoded by exon 3 are missing from the predicted PBGD polypeptide. The consequence of a missing exon 3 for the function of the PBGD enzyme is most probably detrimental, since the exon codes for residues (for example R26) which interact with the substrate (Jordan and Woodcock 1991, Brownlie et al. 1994).

At the 3' splice site, the adenine at position -2 is highly conserved and this nucleotide is significant for splicing events, since substitutions of this nucleotide are more frequent than theoretical models predict (Krawczak et al. 1992). Intron retention, however, is the most infrequent pattern of aberrant splicing (Nakai and Sakamoto 1994). According to the 'exon definition hypothesis', a model derived from experimental analyses (Niwa et al. 1992), intron retention is permitted when the intron is small enough to satisfy the exon length limitation. In the case of the mutation IVS13-2A G, the total length of exon 13, intron 13, and exon 14 includes only 204 nucleotides, which agrees well with this hypothesis. A premature termination is introduced in intron 13 and the predicted polypeptide contains 275 normally translated amino acids followed by 26 amino acids encoded by intron 13. In the PBGD polypeptide, exons 13 and 14 code for residues in the subunit β2 of the third domain (Brownlie et al. 1994). Similarly, the nonsense mutation in exon 14 (W283X) deletes 78 residues from domain 3 and results in a truncated polypeptide but yet retains ~10% of the normal expressed PBGD activity in *E. coli* (Chen et al. 1994). The consequence of mutation IVS13-2A G on the enzyme properties may be similar.

5.5.4. Other mutations

Insertion of an Alu element. In SSCP analysis covering exons 5 to 7 a mobility shift was identified in a sample of a patient belonging to a large AIP family (Figure 5.5.). The band migrating more slowly than normal DNA strands was excised from the SSCP gel, diluted, and reamplified. The PCR product was sequenced revealing a sequence homologous to an Alu sequence. An extra fragment of 1019 bp in size could also be demonstrated in PCR covering exons 5 to 7 from genomic DNA samples of five patients known to have low erythrocyte PBGD activity, suggesting that an Alu insertion of ~350 bp in exon 5 of the PBGD gene was responsible for AIP in this family. Simple amplification of exons 5 to 7 can easily be used in the molecular diagnosis of this insertion (Figure 5.5.). It is of note that this additional fragment could also be demonstrated by amplification of exon 5 alone but not in PCR reaction over exons 3 to 5.

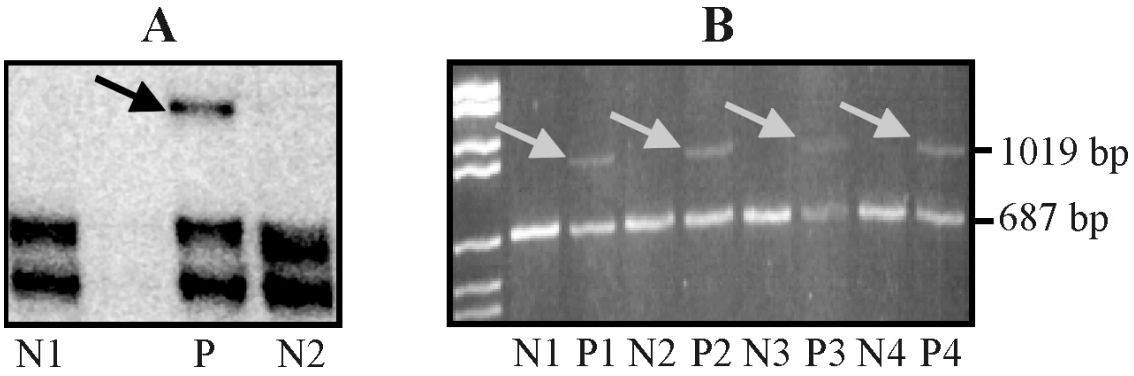


Figure 5.5. *A) SSCP analysis showing an insertion which results in a mobility shift (arrow). B) PCR covering exons 5 to 7. Genomic DNA of the patients with Alu insertation demonstrate both normal (687 bp) and inserted (1019 bp) alleles, whereas healthy controls demonstrate a normal allele. Leftmost lane, DNA molecular weight maker VI (Boehringer Mannheim, Germany). N, healthy control, P, AIP patient.*

A 333 bp Alu insertion in exon 5 was characterized after subcloning and sequencing the two alleles of different size separately. The mutant allele included a 13 bp target site duplication and 3' terminal poly(A) tract in the antisense orientation. The sequence comparison of the inserted Alu element with a database for repeated elements (Pythia, Jurka 1993) showed that it belongs to the Ya5 subfamily. This subgroup is one of the youngest and currently most active Alu subfamilies in evolution. There are no earlier reports of Alu insertions resulting in AIP and

none of the six Alu repeats located in the intronic areas of the PBGD gene belong to the same subfamily (Yoo et al. 1993).

The predicted polypeptide contains 64 normally translated amino acids followed by 13 amino acids not normally found in the PBGD polypeptide. A premature termination codon, which is introduced at the beginning of the insertion, is located at the first codon after the 42 thymidines which form the polyadenine tail of the Alu element. Amplification and sequencing of the reverse transcribed total RNA extracted from a patient's lymphoblast cell line exposed only the wild type allele.

The expression of the mutated and wild type PBGD constructs in COS-1 cells showed that the insertion of the Alu element resulted in a total loss of the enzyme activity, which was expected since nearly all structures crucial for the enzymatic function of the PBGD are missing (Shoolingin-Jordan 1995). The *Tfi*I restriction site located in exon 3 was removed from the construct for the cloning purpose. This base change, however, did not alter the amino acid sequence. The PBGD activity produced by this construct was close to normal, i.e. 88% of the wild type activity. When the polyclonal rabbit anti-human-PBGD antibody was used to identify the PBGD polypeptide in Western analysis, no mutant polypeptide could be detected. In contrast, the wild type PBGD as well as the PBGD construct, in which the *Tfi*I restriction site was removed, produced a detectable polypeptide of normal size (42 kD).

Insertion of an Alu element into the coding sequence of a gene is an infrequent phenomenon: only 17 coding sequences have been previous reported to be disrupted by an Alu repeat (Makalowski et al. 1994). Furthermore, only eight of these Alu insertions have been associated with a disease or a loss of biological activity. The disruption is most commonly caused by a mutation splicing an intronic Alu sequence into the coding region of mRNA. Retroposition of an Alu element into an exon occurs more rarely, and in that case, the Alu cassette is usually in the sense orientation (Makalowski et al. 1994).
However, the PBGD Alu cassette is incorporated directly via retroposition into exon 5 in the antisense orientation.

1073delA. A deletion of one nucleotide is located 11 base pairs upstream of the termination. The consequence of this deletion is a frame shift which removes the normally utilized termination codon and does not create a new one before the polyadenylation signal. If the translation stops at the polyadenylation signal, the length of the polypeptide would be 448 amino acids instead of the 361 of the wild type polypeptide. The first 357 amino acids of the mutant polypeptide are predicted to be identical with the wild type enzyme and, thus, it contains all the components known to be essential for the function of the PBGD.

When the transcript level of the mutant allele was determined, the results suggested that the mutant allele is transcribed normally (Table 5.2.). However, the mutant mRNA appeared to get degraded more rapidly than the transcript from the wild type allele during storage at -80ºC. This mutation was studied further by expressing the mutant PBGD-cDNA in COS cells. The mutant clone displayed 50% residual activity (Table 5.2.). The non-coding region of the construct, however, differed from the human PBGD gene. In the mutated construct the last 46 residues of the synthesized polypeptide were encoded by the 3'-UTR region of the PBGD cDNA and the vector sequence and a termination codon is introduced after 404 amino acids. This polypeptide encoded by the construct is, thus, 44 amino acids shorter than the predicted mutant polypeptide of the patient. The stability of the polypeptide encoded by the mutant construct was comparable to the normal polypeptide in the pulse-chase experiment. However, this finding does not exclude the possibility that mutant polypeptide would actually be more unstable *in vivo*.

5.6. Molecular genetics of AIP in the Finnish population

The population of Finland has been isolated during the past centuries and many diseases which are rare in other countries are relatively common in Finland (de la Chapelle 1993). Furthermore, there is an obvious founder effect in the case of many diseases; one mutation is prevalent and is found in almost every affected individual. Studies from Sweden, the Netherlands and Switzerland, suggest that a founder effect is present in these countries also in the case of AIP (Lee and Anvret 1991, Gu et al. 1993b, Schneider-Yin et al. 1999). However, the molecular defects are heterogeneous in the Finnish AIP patients, most of the identified mutations are family specific (Table 5.1.). The R173Q mutation was the underlying reason for

AIP in four families. The pedigree of these families have been traced back for at least three generations and no links could be identified between ancestors. However, three of the families, originate from the same municipality (R173Q, number 14 in Figure 5.6.), implying that they most probably are related to each other. In addition, the two families with mutation R149X are originally from neighboring villages, which also suggests a common ancestor. Thus, there is a minor founder effect among the Finnish AIP families. Also in France and in the United Kingdom the molecular genetic background is highly heterogeneous and no founder effect has been identified in these countries (Puy et al. 1997b, Whatley et al. 1999).

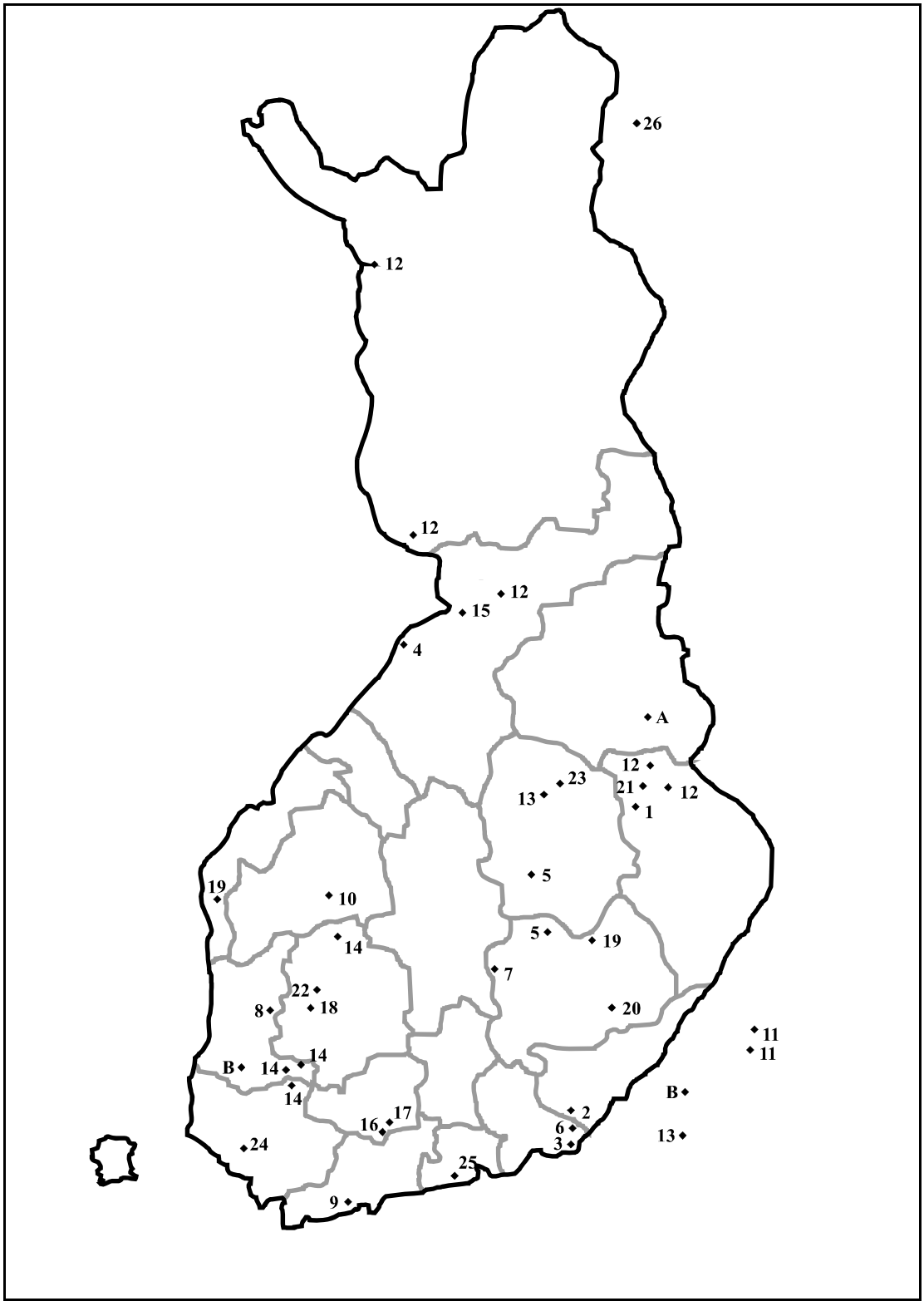


Figure 5.6. *The origin of the Finnish AIP families. The numbers refer to mutations in Table 5.1. A and B: Origin of AIP families with yet unidentified genetic defect.*

The mutation 1073delA was found in a patient who belongs to the Skolt community. The Skolts are Saamians who live in Northern Lappland. They have been very isolated for centuries and form a homogenic population. Thus, it was possible, that the prevalence of AIP among Skolts would be higher than in the rest of the Finnish population. To test this hypothesis, 94 DNA samples from members of the Skolt community were screened for this mutation using the minisequencing method (data not shown). As a result, the mutation could be identified in two additional samples. These two individuals belonged to the same family as the index patient and, furthermore, they were previously classified as AIP patients based on biochemical analyses. All other samples were negative for this mutation and, thus, the conclusion was that prevalence of AIP was not considerably higher in the Skolt community compared to the rest of the Finnish population.

5.5. DNA diagnosis of AIP

The identification of underlying genetic defects now allows a reliable DNA-based diagnostics of AIP in each family, which is in routine use when AIP status of asymptomatic family members is determined. At least two separate analyses are carried out for each DNA sample. Digestion with a restriction enzyme is used whenever possible, direct sequencing is performed in both directions and minisequencing is also available in the case of some mutations.

The possibility of determining the genetic defect has also enabled the evaluation of the value of biochemical methods in AIP diagnosis. Detailed screening for a mutation was tested in three families representing different subtypes of AIP (Table 5.4.). The samples of a total of 81 family members, among them 28 individuals with mutations were analyzed using both DNA and biochemical methods. Biochemical methods (erythrocyte PBGD activity, urinary PBG and ALA) failed to detect 25% (7/28) of patients. Furthermore, four out of 53 (8%) healthy family members were falsely diagnosed as AIP carriers, but this may be due to the relatively low PBGD activity from both parents' erythrocytes although the patients' father is apparently healthy.

Table 5.4. *Evaluation of biochemical methods in AIP diagnosis*

Mutation [a]	PBGD activity [b]	Total number of analyzed family members	No. of mutation carriers	False negative result in biochemical tests	False positive result in biochemical tests
33G T (-)	N	30	10	2	0
499C T (+)	↓	28	12	3	4
673C T (-)	↓	23	6	2	0

[a] CRIM status in parenthesis
[b] PBGD activity in erythrocytes; N, normal activity; ↓, low activity

The feasibility of enzymatic screening in AIP diagnosis has also been evaluated in large material of French AIP patients and their family members (Puy et al. 1997b). As a result, determination of PBGD activity gave wrong diagnosis in 16% (45/280). However, if patients with variant AIP were excluded from the analysis, the AIP gene-carrier status was correctly stated in 240/252 (95%) cases. Thus, the biochemical methods have their limitations and DNA analysis should be used whenever available in AIP diagnosis during the asymptomatic phase.

6. SUMMARY AND CONCLUSIONS

In the present work the molecular genetics of Finnish AIP patients was characterized. The results of the studies and their conclusions can be summarized as follows:

- Twenty-two mutations were identified in the PBGD gene resulting in AIP. Together with the previously identified genetic defects, the 26 characterized mutations cover 38 (95%) of a total of 40 unrelated Finnish AIP families. Despite the heterogeneity at the molecular level, the clinical manifestations of AIP are relatively uniform and no evidence emerged for genotype-phenotype correlation.
- A specific diagnosing method based on DNA techniques was developed for each mutation. The DNA diagnosis should always be used in screening of family members, since biochemical methods fail to detect 10-20% of AIP patients.
- Mutations in the PBGD gene were screened using two different methods: DGGE and SSCP. Both methods were useful and were able to detect most genetic defects, but the sensitivity of DGGE was considerably higher.
- The steady state mRNA levels of six mutations were analyzed using solid-phase minisequencing. The mutant mRNA levels in lymphocytes varied from 5 to 95% of the corresponding wild type allele levels and all mutations, except the missense mutation R173W in exon 10, affected the steady state transcript levels of the mutant allele. The decreased mutant transcript level did not correlate either to CRIM class, to the location of the disease causing mutation in the PBGD gene, or to the clinical phenotype of AIP.
- Using *in vitro* expression in COS-1 cells, eight mutations identified previously in Finnish AIP patients were analyzed. All mutations resulted in a decrease in PBGD activity, and six of the mutated enzymes displayed only minimal residual activity of 0-5%, confirming the causality of the amino acid substitution and the low PBGD activity. The mutated polypeptides were as stable as the normal PBGD, with the exception of truncated polypeptide (mutation R225X) which could not be detected with the polyclonal antibody. Furthermore, no abnormalities in the intracellular location of mutated polypeptides could be observed by immuno-fluorescence.

7. ACKNOWLEDGEMENTS

This study was carried out during years of 1993-1999 at the Department of Medicine, Division of Endocrinology, University of Helsinki and at the Department of Human Molecular Genetics, National Public Health Institute. Professor Marja-Riitta Taskinen, M.D., the Head of the Division of Endocrinology is highly acknowledged for providing me excellent laboratory facilities and for continuous support in my efforts.

I express my greatest gratitude to my supervisors Raili Kauppinen and Leena Palotie-Peltonen. Raili has patiently taught me all the laboratory skills and the scientific way of thinking, although I was not always an especially quick learner. Her overwhelming enthusiasm towards porphyrias and molecular genetics has been contagious. Leena amazes me time and time again with her incredible memory and thorough knowledge of molecular genetics. Even though her schedule is very tight, she always found a time for me in her diary. After a meeting with Leena all problems whatsoever seemed much smaller.

Professors Marja-Liisa Savontaus and Jean-Charles Deybach reviewed this thesis. They gave constructive criticism and noted important weaknesses in it. I appreciate their careful examination of this manuscript and most of all their positive attitude towards my work.

I am more than grateful to my uncle Pertti Mustajoki, M.D., who originally introduced me to the world of porphyrias. Pertti's exceptionally wide perspective over the disorders of the heme metabolism is admirable. His supportive attitude towards my research has encouraged me repeatedly. Furthermore, our discussions have greatly influenced my way of working not only as a researcher but also as a practicing physician.

I would like to warmly thank all my collaborators Anu Wartiovaara (Suomalainen), M.D., Minna Laine (Peltola), M.D., Maija Lahtela-Kakko, Ph.D., and the members of the Danish Porphyria Research Laboratory: Henrik Nissen, Ph.D., Niels Erik Petersen, Ph.D., Torben Hansen, Ph.D., and Mogens Hørder, Ph.D. Their excellent knowledge and skills have considerably helped me during this study. Professor Bernard Grandchamp, M.D. is acknowledged for providing us with the PBGD cDNA and Dr. Ylva Floderus, PhD., provided us with PBGD antibody, which is also highly appreciated.

I warmly thank Ms. Hannele Pihlaja and Ms. Helena Ahola for their excellent technical assistance and always positive and flexible attitude. The intensive moments of laboratory work were made very pleasant when I could engage in interesting conversations concerning almost everything in the whole wide world. Although I did not have any direct collaboration with Dr. Kaisa Timonen, M.D., I found her presence in the porphyria research group very pleasant. Professor Raimo Tenhunen, M.D. is acknowledged for providing the facilities for enzyme activity measurements.

The colleagues and laboratory personnel in the Scientific Laboratory of the Helsinki University Central Hospital are warmly acknowledged for giving relaxing and hilarious company during the coffee-breaks, which too often were extended to coffee-hours. The atmosphere in the coffee-room of the Scientific Laboratory was exceptional, and that repeatedly cheered me up after an unsuccessful experiment. Especially I want to thank Ms. Sirkka-Liisa (Kikka)

Runeberg, whose sense of humor and exceptional ability to listen have made a deep and everlasting impact on me.

I want to express my warmest gratitude to my other uncle professor Arto Mustajoki, Ph.D. The weekly squash matches, followed by the 'hair-drying', i.e. beer or two in the pub nearby, are unforgettable. Although the world did not become any better place to live despite our serious efforts, our conversations offered me fresh perspectives to the scientific world, especially from the linguistic and administrative point of view.

I have spent many breathtaking moments with the running team of the Scientific laboratory. The gentle pressure of the other regular members of the team, Satu Vehkavaara, Tatu Miettinen, Jaakko Kaukonen, Nina Lindbohm, and Heikki Relas, made me to take care of my physical wellbeing. Besides, no one else but a young researcher can really understand the difficulties of a young researcher, and our conversations during the weekly runs were often very therapeutic. Furthermore, I am pleased that the Scientific Losers Club was finally founded, although the two founder members, Jaakko and I, do not actually meet the criteria for the membership any more.

I have a wonderful family. My parents Erkki Mustajoki and Päivi Lindstedt have given me all the love and support I have ever needed. The importance of my two sisters, Henriikka Clarkeburn and Iida-Maaria Lindstedt, in my life cannot be exaggerated. The supportive attitude of Risto Lindstedt, Jeffrey Clarkeburn and my parents-in-law, Anna-Liisa and Olli Laurila, is also highly appreciated.

Finally, I am more than grateful to my wife Ulla. She has been there during all the phases of this work. She has patiently tolerated me when all went wrong in the lab and with her I have shared the moments of success. No words are sufficient to express my love to her.

This work was supported by grants from University of Helsinki, Academy of Finland, the Research Funds and the Clinical Research Institute of the Helsinki University Central Hospital, Emil Aaltonen Foundation, the Paulo Foundation, the Instrumentarium Foundation, the Science and Research Foundation of Farmos Medical Company, the Aarne Koskelo Foundation, and the Finnish Medical Society Duodecim.

Helsinki, October 1999

8. REFERENCES

Abdelhak S, Kalatzis V, Heilig R, Compain S, Samson D, Vincent C, Levi-Acobas F, Cruaud C, Le Merrer M, Mathieu M, König R, Vigneron J, Weissenbach J, Petit C, Weil D. Clustering of mutations responsible for branchi-oto-renal (BOR) syndrome in the *eyes absent* homologous region (eyaHR) of EYA1. Hum Mol Genet 6:2247-2255, 1997.

Alberts B, Bray D, Lewis J, Raff M, Roberts K, Watson JD. Molecular Biology of the Cell. Garland Publishing Inc., New York, 1994.

Anderson PM, Reddy RM, Anderson KM, Desnick RJ. Characterization of the porphobilinogen deaminase deficiency in acute intermittent porphyria. J Clin Invest 68:1-12, 1981.

Andersson C, Lithner F. Hypertension and renal disease in patients with acute intermittent porphyria. J Intern Med 236:169-175, 1994.

Andersson C, Bjersing L, Lithner F. The epidemiology of hepatocellular carcinoma in patients with acute intermittent porphyria. J Int Med 240:195-201, 1996.

Andersson C. Acute intermittent porphyria in Northern Sweden. A population-based study. Academic dissertation. Departments of Family Medicine and Medicine, University of Umeå, Sweden. 1997.

Baserga SJ, Benz EJJ. Beta-globin nonsense mutation: deficient accumulation of mRNA occurs despite normal cytoplasmic stability. Proc Natl Acad Sci USA 90:482-486, 1992.

Beaumont C, Grandchamp B, Bogard M, de Verneuil H, Nordmann Y. Porphobilinogen deaminase is unstable in the absence of its substrate. Biochim Biophys Acta 882:384-388, 1986.

Beaumont C, Porcher C, Picat C, Nordmann Y, Grandchamp B. The mouse porphobilinogen deaminase gene. J Biol Chem 264:14829-14834, 1989.

Berk PD, Blaschke TF, Scharschmidt BF, Waggoner JG, Berlin NI. A new approach to quantitation of the various sources of bilirubin in man. J Lab Clin Med 12:389-390, 1976.

Bissel L. Laboratory evaluation in porphyria. Semin Liv Dis 2:100-107, 1982.

Bonkovsky HL. Advances in understanding and treating 'The little imitator', acute porphyria. Gastroenterology 105:590-594, 1993.

Branden C, Tooze J. Introduction to protein structure. Garland Publishing Inc., New York, 1991.

Brownlie PD, Lambert R, Louie GV, Jordan PM, Blundell TM, Warren MJ, Cooper JB, Wood SP. The three-dimensional structures of mutants of porphobilinogen deaminase: Toward an understanding of the structural basis of acute intermittent porphyria. Prot Sci 3:1644-1650, 1994.

Carter MS, Li S, Wilkinson MF. A splicing-dependent regulatory mechanism that detects translation signal. EMBO J 15:5965-5975, 1996.

de la Chapelle A. Disease gene mapping in isolated human populations: the example of Finland. J Hum Genet 30:857-865, 1993.

Chen C-H, Astrin KH, Lee G, Anderson KE, Desnick RJ. Acute intermittent porphyria: identification and expression of exonic mutations in the hydroxymethylbilane synthase gene. J Clin Invest 94:1927-1937, 1994.

Cheng J, Maquat LE. Nonsense codons can reduce the abundance of nuclear mRNA without affecting the abundance of pre-mRNA or the half-life of cytoplasmic mRNA. Mol Cell Biol 10:5215-5225, 1993.

Chirgwin JM, Przbyla AE, McDonald RJ, Rutter WJ. Isolation of biologically active ribonucleic acid from sources enriched in ribonuclease. Biochemistry 18:5294-5299, 1979.

Chretien S, Dubart A, Beaupain D, Raich N, Grandchamp B, Rosa J, Goossens M, Romeo PH. Alternative transcription and splicing of the human porphobilinogen deaminase results either in tissue-specific or in house-keeping expression. Proc Natl Acad Sci USA 85:6-9, 1988.

Cooper DN. Human gene mutations affecting RNA processing and translation. Ann Med 25:11-17, 1993.

Daimon M, Morita Y, Yamatani K, Igarashi M, Fukase N, Ohnuma H, Sugiyama K, Ogawa A, Hideo M, Manaka H, Sasaki H. Two new polymorphisms in introns 2 and 3 of the human porphobilinogen deaminase gene. Hum Genet 92:549-553, 1993a.

Daimon M, Yamatani K, Igarashi M, Fukase N, Morita Y, Ogawa A, Tominaga M, Sasaki H. Acute intermittent porphyria caused by a G to C mutation in exon 12 of the porphobilinogen deaminase gene that results in exon skipping. Hum Genet 92:549-553, 1993b.

Daimon M, Yamatani K, Igarashi M, Fukase N, Morita Y, Ogawa A, Tominaga M, Sasaki H. Acute intermittent porphyria caused by a single base insertion of C in exon 15 of the porphobilinogen deaminase gene that results in a frame shift and premature stopping of translation. Hum Genet 93:533-537, 1994.

Dean G. The porphyrias. A story of inheritance and environment. Pitman Medical Publishing Co., London, 1963.

Delfau M, Picat C, de Rooij F, Hamer K, Bogard M, Wilson J, Deybach J, Nordmann Y, Grandchamp B. Two different point G to A mutations in exon 10 of the porphobilinogen deaminase gene are responsible for acute intermittent porphyria. J Clin Invest 86:1511-1516, 1990.

Delfau M, Picat C, de Rooij F, Voortman G, Deybach J, Nordmann Y, Grandchamp B. Molecular heterogeneity of acute intermittent porphyria: identification of four additional mutations resulting in the CRIM-negative subtype of the disease. Am J Hum Genet 49:421-428, 1991.

Desnick RJ, Ostasiewicz LT, Tishler PA, Mustajoki P. Acute intermittent porphyria: characterization of a novel mutation in the structural gene for porphobilinogen deaminase. J Clin Invest 76:865-874, 1985.

Deybach J-C, Puy H. Porphobilinogen deaminase gene structure and molecular defects. J Bioenerg Biomembr 27:197-205, 1995.

Elder GH, Hift RJ, Meissner PN. The acute porphyrias. Lancet 349:1613-1617, 1997.

Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielsen M. Lipofection: a highly efficient, lipid mediated DNA-transfection procedure. Proc Natl Acad Sci USA 84:7413-7417, 1987.

Ford RE, Ou C-N, Ellefson RD. Assay of erythrocyte uroporphyrinogen I synthase activity, with porphobilinogen as substrate. Clin Chem 26:1182-1185, 1980.

Fujita H, Yamamoto M, Hayashi N, Sassa S. Erythroleukemia differentiation. Distinctive responses of the erythroid-specific and the nonspecific δ-aminolevulinate synthase mRNA. J Biol Chem 266:17494-17502, 1991.

Goldberg A. Acute intermittent porphyria: A study of 50 cases. Q J Med 28:183-209, 1959.

Goldberg A, Rimington C. Diseases of porphyrin metabolism. Charles C. Thomas Springfield, Illinois, 1962.

Grandchamp B, Romeo PH, Dubert A, Raich N, Rosa J, Nordmann Y, Goossens M. Molecular cloning of a cDNA sequence complementary to porphobilinogen deaminase mRNA from rat. Proc Natl Acad Sci USA 81:5036-5040, 1984.

Grandchamp B, de Verneuil H, Beaumont C, Chretien S, Walter O, Nordmann Y. Tissue-specific expression of porphobilinogen deaminase: two isoenzymes from a single gene. Eur J Biochem 162:105-110, 1987.

Grandchamp B, Picat C, de Rooij F, Beaumont C, Wilson P, Deybach J-C, Nordmann Y. A point mutation G-A in exon 12 of the porphobilinogen deaminase gene results in exon skipping and is responsible for acute intermittent porphyria. Nucleic Acids Res 17:6637-6649, 1989a.

Grandchamp B, Picat C, Kauppinen R, Mignotte V, Peltonen L, Mustajoki P, Romeo PH, Goossens M, Nordmann Y. Molecular analysis of acute intermittent porphyria in a Finnish family with normal erythrocyte porphobilinogen deaminase. Eur J Clin Invest 19:415-418, 1989b.

Grandchamp B, Picat C, Mignotte V, Wilson JHP, TeVelde K, Sandkuyl L, Romeo PH, Goossens M, Nordmann Y. Tissue-specific splicing mutation in acute intermittent porphyria. Proc Natl Acad Sci USA 86:661-664, 1989c.

Grandchamp B, Picat C, de Rooij F, Delfau MH, Gu XF, Deybach J-C, Wilson JPH, teVelde K, Nordmann Y. Porphobilinogen deaminase gene abnormalities in acute intermittent porphyria. International meeting on porphyrin metabolism and iron metabolism, Papendal, the Netherlands, 1992.

Granick S. Induction of the synthesis of 5-ALA synthetase in liver parenchymal cells in culture by chemicals inducing acute porphyria. J Biol Chem 238:2247-2250, 1963.

Granick S. The induction *in vitro* of the synthesis of delta-aminolevulinic acid synthetase in chemical porphyria: A response to certain drugs, sex hormones and foreign chemicals. J Biol Chem 241:1359-1375, 1966.

Grompe M. The rapid detection of unknown mutations in nucleic acids. Nature Genet 5:111-117, 1993.

Gu XF, Lee JS, Delfau MH, Grandchamp B. PCR detection of a G/T polymorphism at exon 10 of the porphobilinogen deaminase gene (PBG-D). Nucleic Acids Res 19:1966, 1991.

Gu XF, de Rooij F, Voortman G, TeVelde K, Nordmann Y, Grandchamp B. High frequency of mutations in exon 10 of the porphobilinogen deaminase gene in patients with a CRIM-positive subtype of acute intermittent porphyria. Am J Hum Genet 51:660-665, 1992.

Gu XF, de Rooij F, de Baar E, Bruyland M, Lissens W, Nordmann Y, Grandchamp B. Two novel mutations of the porphobilinogen deaminase gene in acute intermittent porphyria. Hum Mol Genet 2:1735-1736, 1993a.

Gu XF, de Rooij F, Lee J-S, Te Velde K, Deybach JC, Nordmann Y, Grandchamp B. High prevalence of a point mutation in the porphobilinogen deaminase gene in Dutch patients with acute intermittent porphyria. Hum Genet 91:128-130, 1993b.

Gu XF, de Rooij F, Voortman G, TeVelde K, Deybach J-C, Nordmann Y, Grandchamp B. Detection of eleven mutations causing acute intermittent porphyria using denaturing gradient gel electrophoresis. Hum Genet 93:47-52, 1994.

Higuchi R. Simple and rapid preparation of samples for PCR. In: Ehrlich HA, eds. PCR Technology. Principles and Applications for DNA Amplification. New York: Stockton Press. pp. 31-38, 1989.

Jagadeeswaran P, Forget BG, Weissman SM. Short interspersed repetitive DNA elements in eucaryotes: transposable DNA elements generated by reverse transcription of RNA pol III transcripts? Cell 26:141-142, 1981.

Jordan PM, Warren MJ. Evidence for a dipyrromethane cofactor at the catalytic site of *E. coli* porphobilinogen deaminase. FEBS Lett 225:87-92, 1987.

Jordan PM, Woodcock SC. Mutagenesis of arginine residues in the catalytic cleft of Escherichia coli porphobilinogen deaminase that affects dipyrromethane cofactor assembly and tetrapyrrole chain initiation and elongation. Biochem J 280:445-449, 1991.

Jurka J. A new subfamily of recently retroposed human Alu repeats. Nucleic Acids Res 21:2252, 1993.

Kappas A, Sassa S, Galbraith RA, Nordmann Y. The porphyrias. In: Scriver CR, Beaudet A, Sly WS, Valle D, eds. The metabolic and molecular bases of inherited diseases. New York: McGraw-Hill. pp. 2116-2127, 1995.

Karttunen L, Lönnqvist L, Godfrey M, Peltonen L, Syvänen A-C. An accurate method for comparing transcript of two alleles or highly homologous genes: application to fibrillin transcripts in Marfan patients' fibroblasts. Genome Res 6:392-403, 1996.

Kauppinen R. Prognosis of acute porphyrias and molecular genetics of acute intermittent porphyria in Finland. Academic dissertation. Department of Human Molecular Genetics, National Public Health Institute and Third Department of Medicine, University of Helsinki, Finland. 1992a.

Kauppinen R. Single-strand conformation polymorphism (SSCP) analysis applied to the diagnosis of acute intermittent porphyria. Mol Cell Probes 6:527-530, 1992b.

Kauppinen R, Mustajoki P. Prognosis of acute intermittent porphyria: occurrence of acute attacks, precipitating factors and associated disease. Medicine 71:1-13, 1992.

Kauppinen R, Peltonen L, Pihlaja H, Mustajoki P. CRIM-positive mutations of acute intermittent porphyria in Finland. Hum Mutat 1:392-396, 1992.

Keng TC, Richard C, Larocque R. Structure and regulation of yeast HEM3, the gene for porphobilinogen deaminase. Mol Gen Genet 234:233-243, 1992.

Krawczak M, Reiss J, Cooper DN. The mutational spectrum of single base pair substitutions in mRNA splice junctions of human genes: causes and consequences. Hum Genet 90:41-54, 1992.

Laemmli U. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685, 1970.

Lambert R, Brownlie PD, Woodcock SC, Louie GV, Cooper JB, Warren MJ, Jordan PM, Blundell TL, Wood SP. Structural studies on porphobilinogen deaminase. In: Chadwick DJ, Ackrill K, eds. The Biosynthesis of the tetrapyrrole pigments. Ciba Foundation Symposium 180. Chichester: Wiley. pp. 97-110, 1994.

Lamon JM, Frykholm BC, Tschudy DP. Screening tests in acute porphyria. Arch Neurol 34:709-712, 1977.

Lander M, Pitt AR, Alefounder PR, Bardy D, Abell C, Battersby AR. Studies on the mechanism of hydroxymethylbilane synthase concerning the role of arginine residues in substrate binding. Biochem J 275:447-452, 1991.

Langenskiöld F. Om hematoporfyri med ileusliknande symtom. Finska Läk-Sällsk Handl 70:241, 1928.

Lannfelt L, Wetterberg L, Gellerfors P, Lilius L, Floderus Y, Thunell S. Mutations in acute intermittent porphyria detected by ELISA measurement of porphobilinogen deaminase. J Clin Chem Clin Biochem 183:857-862, 1989a.

Lannfelt L, Wetterberg L, Lilius L, Thunell S, Gellefors P. ELISA for measuring porphobilinogen deaminase in human erythrocytes. Clin Chim Acta 183:227-238, 1989b.

Law WK, Choy KW, Lam CW. Novel single nucleotide polymorphism (9678G -> A) for linkage analysis of acute intermittent porphyria. Clin Chem 45:308-309, 1999.

Lee GY, Astrin KH, Desnick RJ. Acute intermittent porphyria: A single-base deletion and a nonsense mutation in the human hydroxymethylbilane synthase gene, predicting truncations of the enzyme polypeptide. Am J Med Genet 58:155-158, 1994.

Lee JS, Anvret M. A PstI polymorphism for the human porphobilinogen deaminase gene (PBG). Nucleic Acids Res 15:6307, 1987.

Lee JS, Anvret M, Lindsten J, Lannfelt L, Gellerfors P, Wetterberg L, Floderus Y, Thunell S. DNA polymorphisms within the porphobilinogen deaminase gene in two Swedish families with acute intermittent porphyria. Hum Genet 79:379-381, 1988.

Lee J, Grandchamp B, Anvret M. A point mutation of the human porphobilinogen deaminase gene in a Swedish family with acute intermittent porphyria. Am J Hum Genet 47[Suppl]:A162, 1990.

Lee J. Molecular genetic investigation of the human porphobilinogen deaminase gene in acute intermittent porphyria. Academic dissertation. Karolinska Institute, Stockholm, Sweden. 1991a.

Lee JS. Alternative dideoxy sequencing of double-stranded DNA by cyclic reactions using *Taq* polymerase. DNA Cell Biol 10:67-73, 1991b.

Lee J, Anvret M. Identification of the most common mutation within the porphobilinogen deaminase gene in Swedish patients with acute intermittent porphyria. Proc Natl Acad Sci USA 88:10912-10915, 1991.

Lindberg RLP, Porcher C, Grandchamp B, Ledermann B, Bürki K, Brandner S, Aguzzi A, Meyer UA. Porphobilinogen deaminase deficiency in mice causes a neuropathy resembling that of human hepatic porphyria. Nature Genet 12:195-199, 1996.

Lindberg RLP, Martini R, Bamgartner M, Erne B, Borg J, Zielasek J, Ricker K, Steck A, Toyka KV, Meyer UA. Motor neuropathy in porphobilinogen deaminase-deficient mice imitates neuropathy of human acute porphyria. J Clin Invest 103:1127-1134, 1999.

Linet MS, Gridley G, Nyren O, Mellemkjaer L, Olsen JH, Keehm S, Adami HO, Fraumeni JF. Primary liver cancer, other malignancies, and mortality risks following porphyria: A cohort study in Denmark and Sweden. Am J Epidem 149:1010-1015, 1999.

Litman DA, Correia MA. L-tryptophan: A common denominator of biochemical and neurological events of acute hepatic porphyria. Science 222:1031, 1983.

Llewellyn DH, Kalsheker NA, Elder GH, Harrison PR, Chretien S, Goossens M. A MspI polymorphism for the human porphobilinogen deaminase gene. Nucleic Acids Res 15:1349, 1987.

Llewellyn D, Whatley S, Elder G. Acute intermittent porphyria caused by an arginine to histidine substitution (R26H) in the cofactor-binding cleft of porphobilinogen deaminase. Hum Mol Genet 2:1315-1316, 1993.

Llewellyn DH, Scobie GA, Urquhart AJ, Whatley SD, Roberts AG, Harrison PR, Elder GH. Acute intermittent porphyria caused by defective splicing of porphobilinogen deaminase RNA: a synonymous codon mutation at -22 bp from the 5' splice site causes skipping of exon 3. J Med Genet 33:437-438, 1996.

Louie GV, Brownlie PD, Lambert R, Cooper JB, Blundell TL, Wood SP, Warren MJ, Woodcock SC, Jordan PM. Structure of porphobilinogen deaminase reveals a flexible multidomain polymerase with a single catalytic site. Nature 359:33-39, 1992.

Lundin G, Wedell A, Thunell S, Anvret M. Two new mutations in the porphobilinogen deaminase gene and a screening method using PCR amplification of specific alleles. Hum Genet 93:59-62, 1994.

Lundin G, Hashemi J, Floderus Y, Thunell S, Sagen E, Lægreid A, Wassif W, Peters T, Anvret M. Four mutations in the porphobilinogen deaminase gene in patients with acute intermittent porphyria. J Med Genet 32:979-981, 1995.

Lundin G, Anvret M. Characterization and regulation of the nonerythroid porphobilinogen deaminase promoter. Biohem Biophys Res Comm 231:409-411, 1997.

Lundin G, Lee JS, Thunell S, Anvret M. Genetic investigation of the porphobilinogen deaminase gene in Swedish acute intermittent porphyria families. Hum Genet 100:63-66, 1997.

Macek MJ, Mercier B, Macková A, Miller PW, Hamosh A, Férec C, Cutting GR. Sensitivity of the denaturing gradient gel electrophoresis technique in detection of known mutations and novel Asian mutations in the CFTR gene. Hum Mutat 9:136-147, 1997.

Magnussen CR, Levine JB, Doherty JM, Cheeseman JO, Tschudy DP. A red cell enzyme method for the diagnosis of acute intermittent porphyria. Blood 44:857-868, 1974.

Makalowski W, Mitchell GA, Labuda D. Alu sequences in the coding regions of mRNA: a source for protein variability. Trends Genet 10:188-193, 1994.

Maquat LE. When cells stop making sense: effects of nonsense codons on RNA metabolism in vertebrate cells. RNA 1:453-465, 1995.

Mauzerall D, Granick S. The occurrence and determination of delta-aminolevulinic acid and porphobilinogen in urine. J Biol Chem 219:435-436, 1956.

May B, Dogra S, Sadlon T, Bhasker C, Cox T, Bottomley S. Molecular regulation of heme biosynthesis in higher vertebrates. Prog Nucleic Acid Res Mol Biol 51:1-51, 1995.

Meyer UA, Strand LJ, Doss M, Rees C, Marver HS. Intermittent acute porphyria - demonstration of genetic defect in porphobilinogen metabolism. New Engl J Med 286:1277-1282, 1972.

Mgone C, Lanyon W, Moore M, Connor J. Detection of seven point mutations in the porphobilinogen deaminase gene in patients with acute intermittent porphyria, by direct sequencing of in vitro amplified cDNA. Hum Genet 90:12-16, 1992.

Mgone C, Lanyon W, Moore M, Louie G, Connor J. Detection of high mutation frequency in exon 12 of the porphobilinogen deaminase gene in patients with acute intermittent porphyria. Hum Genet 92:619-622, 1993.

Mgone C, Lanyon W, Moore M, Louie G, Connor J. Identification of five novel mutations in the porphobilinogen deaminase gene. Hum Mol Genet 3:809-811, 1994.

Michaud J, Brody LC, Steel G, Fontaine G, Martin LS, Valle D, Mitchell G. Strand-separating conformational polymorphism analysis: efficacy of detection of point mutations in the human ornithine δ-aminotransferase gene. Genomics 13:389-394, 1992.

Mignotte V, Wall L, deBoer E, Grosveld F, Romeo P-H. Two tissue-specific factors bind the erythroid promoter of human porphobilinogen deaminase gene. Nucl Acids Res 17:37-54, 1989.

Miyagi K. Deficiency of hepatic porphobilinogen deaminase in acute intermittent porphyria. J, Kyushu Haematol Soc 20:190-203, 1970.

Mohr CD, Stonsteby SK, Deretic V. The *Pseudomonas aeruginosa* homologs of hemC and hemD are linked to the gene encoding the regulator of mucoidy AlgR. Mol Gen Genet 242:177-184, 1994.

Moore MR, McColl KEL, Rimington C, Goldberg A. Disorders of porphyrin metabolism. Plenum Publishing Corporation, New York, 1987.

Moyret C, Theillet C, Puig PL, Molés J-P, Thomas G, Hamelin R. Relative efficiency of denaturing gradient gel electrophoresis and single strand conformation polymorphism in the detection of mutations exons 5 to 8 of the p53 gene. Oncogene 9:1739-1743, 1994.

Mullis KB, Faloona F. Specific synthesis of DNA in vitro via a polymerase-catalyzed chain reaction. Methods Enzymol 155:335-350, 1987.

Mustafa S, Pabinger I, Mannhalter C. Protein S deficiency type I: identification of point mutations in 9 of 10 families. Blood 86:3444-3451, 1995.

Mustajoki P, Koskelo P. Hereditary hepatic porphyrias in Finland. Acta Med Scand 200:171-178, 1976.

Mustajoki P. Normal erythrocyte uroporphyrinogen I synthase in a kindred with acute intermittent porphyria. Ann Intern Med 95:162-166, 1981.

Mustajoki P, Desnick RJ. Genetic heterogeneity in acute intermittent porphyria: characterization and frequency of porphobilinogen deaminase in Finland. Brit Med J 291:505-509, 1985.

Mustajoki P, Kauppinen R, Lannfelt L, Koistinen J. Frequency of low porphobilinogen deaminase activity in Finland. J Intern Med 231:3889-3395, 1992.

Myers RM, Maniatis T, Lerman LS. Denaturation and localization of single base changes by denaturing gradient gel electrophoresis. Methods Enzymol 155:501-527, 1987.

Nakai K, Sakamoto H. Construction of a novel database containing aberrant splicing mutations of mammalian genes. Gene 141:171-177, 1994.

Namba H, Narahara K, Tsuji K, Yokoyama Y, Seino Y. Assignment of human porphobilinogen deaminase to 11q24.1 - q24.2 by *in situ* hybridization and gene dosage studies. Cytogenet Cell Genet 57:105-108, 1991.

Nishimura K, Taketani S, Inokuchi H. Cloning of a human cDNA for protoporphyrinogen oxidase by complementation in vivo of a hemG mutant of Escherichia coli. J Biol Chem 270:8076-8080, 1995.

Nissen H, Hansen AB, Guldberg P, Petersen NE, Larsen ML, Haghfelt T, Kristiansen K, Hørder M. Cellular and molecular cardiology: Genetic diagnosis with the denaturing gel electrophoresis technique improves diagnostic precision in familial hypercholesterolemia. Circulation 91:1641-1646, 1995.

Niwa M, MacDonald CC, Berget SM. Are vertebrate exons scanned during splice-site selection? Nature 360:277-280, 1992.

Nordmann Y, Puy H, da Silva V, Simonin S, Robreau AM, Bonaiti C, Phung LN, Deybach JC. Acute intermittent porphyria: prevalence of mutations in the porphobilinogen deaminase gene in blood donors in France. J Int Med 242:213-217, 1997.

Ong PM, Lanyon WG, Hift RJ, Halkett J, Moore MR, Mgone CS, Connor JM. Detection of four mutations in six unrelated patients with acute intermittent porphyria. Mol Cell Probe 10:57-61, 1996.

Ong PML, Lanyon WG, Graham G, Hift RJ, Halkett J, Moore MR, Connor JM. Acute intermittent porphyria: the *in vitro* expression of mutant hydroxymethylbilane synthase. Mol Cell Probe 11:293-296, 1997.

Ong PM, Lanyon WG, Hift RJ, Halkett J, Cramp CE, Moore MR, Connor JM. Identification of two novel mutations in the hydroxymethylbilane synthase gene in three patients from two unrelated families with acute intermittent porphyria. Hum Hered 48:24-29, 1998.

Orita M, Suzuki Y, Sekiya T, Hayashi K. Rapid and sensitive detection of point mutations and DNA polymorphisms using the polymerase chain reaction. Genomics 5:874-879, 1989.

Peltz SW, He F, Welch E, Jacobson A. Nonsense-mediated decay in yeast. In: eds. Progress in Nucleic Acids Research and Molecular Biology. New York: Academic Press. pp. 271-298, 1994.

Petersen NE, Nissen H, Hansen TS, Rasmussen K, Brock A, Hørder M. R235X mutation in exon 15 of the porphobilinogen deaminase gene identified in two Danish families with acute intermittent porphyria. Clin Chem 42:106-107, 1996.

Petersen NE, Nissen H, Hørder M, Senz J, Jamani A, Schreiber WE. Mutation screening by denaturing gradient gel electrophoresis in North American patients with acute intermittent porphyria. Clin Chem 44:1766-1768, 1998.

Petricek M, Rutberg L, Schroder I, Hederstedt L. Cloning and characterization of the hemA region of the *Bacillus subtilis* chromosome. J Bacteriol 172:2250-2258, 1990.

Picat C, Bourgeois F, Grandchamp B. PCR detection of a C/T polymorphism in exon 1 of the porphobilinogen deaminase gene (PBGD). Nucleic Acids Res 19:5099, 1991.

Ploos van Amstel JK, Bergman AJIW, van Beurden EACM, Roijers JFM, Peelen T, van der Berg IET, Poll-The BT, Kvittingen EA, Berger R. Hereditary tyrosinemia type 1: novel missense, nonsense and splice consensus mutations in the human fumarylacetoacetate hydrolase gene variability of the genotype-phenotype relationship. Hum Genet 97:51-59, 1996.

Proia R, D'Azzo A, Neufeld EF. Association of alfa- and beta-subunits during biosynthesis of beta-hexosaminidase in cultured fibroblasts. J Biol Chem 259:3350-3354, 1984.

Puy H, Deybach J-C, Robreau AM, Lamoril L, Nordmann Y. Detection of four novel mutations in the porphobilinogen deaminase gene in French Caucasian patients with acute intermittent porphyria. Hum Hered 46:177-180, 1996.

Puy H, Aquaron R, Lamoril J, Robreau AM, Nordmann Y, Deybach JC. Acute intermittent porphyria: rapid molecular diagnosis. Cell Mol Biol 43:37-45, 1997a.

Puy H, Deybach J-C, Lamoril J, Robreau AM, Da Silva V, Gouya L, Grandchamp B, Nordmann Y. Molecular epidemiology and diagnosis of PBG deaminase gene defects in acute intermittent porphyria. Am J Hum Genet 60:1373-1383, 1997b.

Puy H, Gross U, Deybach JC, Robreau AM, Frank M, Nordmann Y, Doss M. Exon 1 donor splice site mutations in the porphobilinogen deaminase gene in the non-erythroid variant form of acute intermittent porphyria. Hum Genet 103:570-575, 1998.

Raich N, Romeo PH, Dubart A, Beaupain D, Cohen-Solal M, Goossens M. Molecular cloning and complete primary sequence of human erythrocyte PBG deaminase. Nucleic Acids Res 14:5955-5968, 1986.

Rogers J. The origin and evolution of retroposons. Int Rev Cytol 93:187-279, 1985.

Rosipal R, Puy H, Lamoril J, Martasek P, Nordmann Y, Deybach JC. Molecular analysis of porphobilinogen (PBG) deaminase gene mutations in acute intermittent porphyria: first study in patients of Slavic origin. Scand J Clin Lab Inv 57:217-224, 1997.

Sambrook J, Fritsch EF, Maniatis T. Molecular cloning: A laboratory manual. In: eds. Cold Spring Harbor, New York: Cold Spring Harbor Laboratory Press. pp. 9.16-19.19, 1989.

Sanger F, Nicklen S, Coulson AR. DNA sequencing with chain-termination inhibitors. Proc Natl Acad Sci USA 74:5463-5467, 1977.

Sassa S, Kappas A. Genetic, metabolic, and biochemical aspects of the porphyrias. In: Harris H, Hirschhorn K, eds. Advances in Human Genetics. New York: Plenum. pp. 121-231, 1981.

Schneider-Yin X, Bogard C, Rüfenacht UB, Puy H, Nordmann Y, Minder EI, Deybach J-C. Identification of a prevalent nonsense mutation (W283X) and two novel mutations in the porphobilinogen deaminase gene of Swiss patients with acute intermittent porphyria. Hum Hered in press, 1999.

Schreiber WE, Jamani A, Ritchie B. Detection of a T/C polymorphism in the porphobilinogen deaminase gene by polymerase chain reaction amplification of specific alleles. Clin Chem 38:2153-2155, 1992.

Schreiber W, Fong F, Jamani A. Frameshift mutations in exons 9 and 10 of the porphobilinogen deaminase gene produce a cross reacting immunological material (CRIM)-negative form of acute intermittent porphyria. Hum Genet 93:552-556, 1994a.

Schreiber WE, Fong F, Jamani A. Molecular diagnosis of acute intermittent porphyria by analysis of DNA extracted from hair roots. Clin Chem 40:1744-1748, 1994b.

Schreiber WE, Rozon C, Fong F, Jamani A. Detection of polymorphisms and mutations in the porphobilinogen deaminase gene by nonisotopic SSCP. Clin Chem 40:1982-1983, 1994c.

Schreiber WE, Fong F, Nassar BA, Jamani A. Heteroduplex analysis detects frameshift and point mutations in patients with acute intermittent porphyria. Hum Genet 96:161-166, 1995a.

Schreiber WE, Jamani A, Armstrong JG. Acute intermittent porphyria in a native North American family. Biochemical and molecular analysis. Am J Clin Pathol 103:730-734, 1995b.

Scobie G, Llewellyn D, Urquhart A, Smyth S, Kalsheker N, Harrison P, Elder G. Acute intermittent porphyria caused by a C-T mutation that produces a stop codon in the porphobilinogen deaminase gene. Hum Genet 85:631-634, 1990.

Sheffield VC, Cox DR, Lerman LS, Myers RM. Attachment of a 40 base G + C rich sequence (GC-clamp) to genomic DNA fragments by the polymerase chain reaction results in improved detection of single base changes. Proc Natl Acad Sci USA 86:232-236, 1989.

Shoolingin-Jordan PM. Porphobilinogen deaminase and uroporphyrinogen III synthase: structure, molecular biology, and mechanism. J Bioenerg Biomembr 27:181-195, 1995.

Simpson SB, Stolzfus CM. Frameshift mutations in the v-src gene of avian sarcoma virus act in *cis* to specifically reduce v-src mRNA levels. Mol Cell Biol 14:1835-1844, 1994.

Stacey A, Schnieke A. SVpoly: a versatile mammalian expression vector. Nucleic Acids Res 18:2829, 1990.

Stein JA, Tschudy DP. Acute intermittent porphyria: A clinical and biochemical study of 46 patients. Medicine 49:1-16, 1970.

Syvänen A-C, Sajantila A, Lukka M. Identification of individuals by analysis of biallelic DNA markers using PCR and solid-phase minisequencing. Am J Hum Genet 52:46-59, 1993.

Thomas SD, Jordan PM. Nucleotide sequence of the hemC locus encoding porphobilinogen deaminase of *Escherichia coli* K12. Nucleic Acid Res 14:6215-6226, 1986.

Toback AC, Sassa S, Poh-Fitzpatrick MB, Schechter J, Zaider E, Harber LC, Kappas A. Hepatoerythropoietic porphyria: Clinical, biochemical and enzymatic studies in a three-generation family lineage. N Engl J Med 316:645-650, 1987.

Tschudy DP, Valsamis M, Magnussen CR. Acute intermittent porphyria: Clinical and selected research aspects. Ann Intern Med 83:851, 1975.

Urlaub G, Mitchell PJ, Ciudad CJ, Chasin LA. Nonsense mutations in the dihydrofolate reductase gene affect RNA processing. Mol Cell Biol 9:2868-2880, 1989.

de Verneuil H, Aitken G, Nordmann Y. Familial and sporadic porphyria cutanea: Two different diseases. Hum Genet 44:145-151, 1978.

Waldenström J. Studien über porphyrie. Acta Med Scand Suppl 82:1-254, 1937.

Waldenström J. The porphyrias as inborn errors of metabolism. Am J Med 22:758-773, 1957.

Warren MJ, Jordan PM. Investigation into the nature of substrate binding to the dipyrromethane cofactor of *Escherichia coli* porphobilinogen deaminase. Biochemistry 27:9020-9030, 1988.

Watson CJ, Schwartz S. Simple test for urinary porphobilinogen. Proc Soc Exp Biol Med 47:393-394, 1941.

Watson CJ, Pierach CA, Bossenmaier I, Cardinal R. Use of haematin in the acute attack of the "inducible" hepatic porphyrias. Adv Intern Med 23:265-285, 1978.

Wetterberg L. Akut intermittent porfyri. Läkartidningen 71:2461-2465, 1974.

Whatley SD, Woolf JR, Elder GH. Comparison of complementary and genomic DNA sequencing for the detection of mutations in the HMBS gene in British patients with acute intermittent porphyria: identification of twenty five novel mutations. Hum Genet 104:505-510, 1999.

Woodcock SC, Jordan PM. Evidence for participation of aspartate-84 as a catalytic group at the active site of porphobilinogen deaminase obtained by site-directed mutagenesis of the hemC gene from Escherichia coli. Biochemistry 33:2688-2695, 1994.

Yoo HW, Warner CA, Chen CH, Desnick RJ. Hydroxymethylbilane synthase: complete genomic sequence and amplifiable polymorphisms in the human gene. Genomics 15:21-29, 1993.