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Molecular Epidemiology and Diagnosis of PBG Deaminase Gene Defects in Acute Intermittent Porphyria

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Summary

Acute intermittent porphyria (AIP) is the major autosomal dominant form of acute hepatic porphyrias. The disease is due to mutations in the gene encoding for porphobilinogen (PBG) deaminase and is characterized by life-threatening neurovisceral attacks, often precipitated by drugs, fasting, cyclical hormonal changes, or infectious diseases. This report describes a prospective study on the molecular epidemiology of PBG deaminase gene defects in AIP. It uses a sensitive, reliable, and easy-to-handle method for routine AIP molecular diagnosis and family study based on an exon-by-exon denaturing gradient gel electrophoresis (DGGE) strategy followed by direct sequencing. Fifteen genomic DNA fragments, including all the coding sequence and covering 3.35 kb of the PBG deaminase gene, were investigated in 405 subjects from 121 unrelated French Caucasian AIP families who had not been screened previously at the DNA level. PBG deaminase gene mutations were identified in 109 families, but only 78 were of different type, and each of them had a prevalence rate <5%. Among these mutations, 33 had not been published previously. Sixty percent of these 78 mutations were located in only three exons (exons 10, 12, and 14), 44% were missense, 18% were splice defect, 19% were frameshift, and 16% were nonsense. In addition, two de novo mutational events were characterized. The evaluation of the efficiency of the standard PBG deaminase enzymatic screening method for gene-carrier detection indicated 95% of concordancy with the molecular-based diagnosis.

Introduction

Acute intermittent porphyria (AIP) is an autosomal dominant disorder caused by a 50% reduction in the activity of PBG deaminase (E.C.4.3.1.8), the third enzyme in the heme biosynthetic pathway. AIP is the most common of the acute hepatic porphyrias, and the prevalence of the disease has been estimated at 1/10,000 in different countries (Bonkowsky 1982; Kappas et al. 1995).

PBG deaminase is encoded by a single 11-kb gene located in chromosomal region 11q24.1-11q24.2 (Namba et al. 1991). The gene contains 15 exons with a size range of 39–438 bp (Yoo et al. 1993). Two distinct mRNAs are produced by the alternative splicing of two primary transcripts arising from two promotors. The upstream promotor is active in all tissues, and the housekeeping transcript initiated by this promoter is encoded by exons 1 and 3–15. The other promotor lies 3 kb downstream in intron 1 and is active only in erythroid cells. Its utilization produces a transcript encoded by exons 2–15 (Grandchamp et al. 1987). In classical AIP the housekeeping and the erythroid-specific enzymes both have half-normal activities in erythroid and nonerythroid tissues, whereas in the variant, nonerythroid form of the disease the enzymatic defect is present only in nonerythroid cells and is caused by molecular defects in exon 1 (Mustajoki 1981; Mustajoki and Desnick 1985; Wilson et al. 1986; Grandchamp et al. 1989*b*, 1989*c*).

Clinical onset of the disease is characterized by intermittent attacks of neurological dysfunction, including abdominal pain and neuropsychiatric symptoms. Diagnosis of AIP in patients is readily done by detection of the urinary overproduction of porphyrin precursors, δ aminolevulinic acid (ALA) and PBG (Lamon et al. 1977). The clinical expression shows intra- and interindividual variability. Presymptomatic diagnosis of AIP gene carriers is essential to prevent acute, life-threatening neurological attacks, by avoidance of known precipitating factors such as drugs, alcohol, and fasting (Kappas et al. 1995). When the patients are informed and precipitating factors are avoided, only 10%–20% of the patients present acute attacks, yet 50% have milder symptoms during their lifetime (Kauppinen and Mustajoki 1992). Asymptomatic patients do not consistently excrete excess amounts of ALA and PBG, and the usual screening method in AIP families is based on measurement of erythrocyte PBG deaminase activity (Meyer et al. 1972). Diagnosis by this enzymatic assay is not un-

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ambiguous, because of a significant overlap between carrier values and control values (Sassa et al. 1974; Bonaiti-Pellie et al. 1984). In addition, subjects with a nonerythroid variant form of AIP cannot be detected, because they have a normal erythrocyte PBG deaminase activity (Mustajoki 1981).

DNA analysis is a potentially useful alternative method for establishing the status of individuals for whom enzyme measurements give equivocal results. However, the molecular diagnosis of AIP is complicated by the large allelic heterogeneity, with 81 different mutations in the PBG deaminase gene reported to date (for review, see Deybach and Puy 1995; also see Kauppinen et al. 1995; Lundin et al. 1995; Morita et al. 1995; Schreiber at al. 1995; Ong et al. 1996; Puy et al. 1996). In The Netherlands and Sweden, a limited number of mutations occur in most AIP families (Lee and Anvret 1991; Gu et al. 1993, 1994). However, in Finland a high heterogeneity of AIP mutations has been reported (Kauppinen et al. 1995).

In this study, we prospectively investigated 121 French Caucasian AIP families including 405 members seen at the Centre Français des Porphyries during 1992–95 who were clinically and biochemically well characterized. The sensitivity and specificity of the denaturing gradient gel electrophoresis (DGGE) method for screening mutations in all the 15 exons and exon/intron boundaries of the PBG deaminase gene were evaluated. The molecular epidemiology of the Caucasian AIP cohort was determined, and the efficiency of the molecular-biology method for AIP diagnosis was compared with that of the standard enzymatic method.

Patients and Methods

Patients

All the AIP families included in the study were diagnosed in the Centre Français des Porphyries during 1992–95, and patients from each AIP family were followed up clinically in the center. A total of 405 subjects from 121 unrelated French AIP families of Caucasian origin were subjected to both erythrocyte PBG deaminase activity measurement and molecular analysis of the PBG deaminase gene. These families represented approximately one-third of the French AIP population known in 1995. The diagnosis of AIP was based on typical clinical symptoms, with increased urinary excretion of the heme precursors ALA and PBG, normal fecal porphyrin excretion, and (except in two AIP families with a nonerythroid form of the disease) a well-documented decrease in PBG deaminase activity in erythrocytes (Stein and Tschudy 1970). This AIP cohort included four patients whose mutations already had been reported in a previous "short communication" (Puy et al. 1996).

PBG Deaminase Assay

The assay measures the kinetic development of uroporphyrin fluorescence produced from PBG, as described elsewhere (Meyer et al. 1972), with slight modifications. In brief, the erythrocytes from EDTA-anticoagulated blood were washed in 8.5% saccharose. The packed cells were lysed by addition of 10 vol of 0.1 mol Tris-HCL buffer pH 8.0/liter, containing 0.2% Triton X-100. The hemoglobin concentration was measured by the cyanmethemoglobin method (Boehringer Mannheim). The reaction mixture contained 25 μl of lysate, 200 μl of 0.1 mmol Tris-HCL buffer pH 8.0/liter, and 25 μl of 1 mmol PBG/liter. After incubation in the dark for 60 min at 37°C, the reaction was stopped by addition of 1 ml of 10% trichloracetic acid.

After centrifugation at 10,000 *g* for 5 min, the supernatant fluorescence emission (excitation at 405, emission at 655) was measured on a Shimadzu RF-540 spectrofluorometer fitted with a red-sensitive photomultiplier and was converted to picomoles of uroporphyrin, with uroporphyrin I (Sigma) used as a standard. The enzymatic activity was expressed as picomoles of uroporphyrin formed per hour per milligram of hemoglobin (Hb) at 37°C. Mean ± SD PBG deaminase activity in controls ($n = 150$) was 125 ± 20 pmol of uroporphyrin/h/mg Hb at 37°C. The intraassay coefficient of variation was 3% ($n = 30$), and the interassay coefficient of variation was 6% ($n = 25$). The enzyme activity cutoff for the AIP gene carrier has been established at 85 pmol uroporphyrin/h/mg Hb at 37°C (mean normal value minus 2 SD) (Bonaiti-Pellie et al. 1984). According to previous studies, children <1 year old, as well as samples with reticulocyte count >3%, were excluded from the enzymatic screening (Sassa et al. 1974; Bonaiti-Pellie et al. 1984; Astrin and Desnick 1994).

Detection of PBG Deaminase Gene Sequence Variation by DGGE

Genomic DNA was prepared from peripheral leukocytes or lymphoblastoid cells as described elsewhere (Higuchi 1990). DNA was amplified by PCR in an automated DNA thermal cycler (Kontron) by use of *Taq* polymerase (Beckman). Fifteen different PCR products for 15 DGGE analyses were designed to cover the whole coding sequence of the PBG deaminase gene and >50 bp upstream and downstream of each exon/intron boundary. This exon-by-exon strategy allowed screening of 3.35 kb of the 11 kb of the chromosomal PBG deaminase gene. DGGE was performed according to the method of Myers et al. (1987). Computer analyses were performed with the MELT 87 and SQHTX programs written and kindly provided by Lerman and Silverstein (1987). These programs were used to select the positions of PCR primers and the optimal experimental conditions for DGGE analysis. One of the two amplification prim-

ers of each pair bore an additional GC-rich sequence at its 5′ extremity (table 1), in order to create an artificial high-temperature melting domain in the PCR product (Sheffield et al. 1989). For each patient, all 15 exons and their surrounding intron sequences were systematically analyzed by DGGE, to eliminate the possibility of another sequence variation and to ensure that a detected base substitution could not be due to a polymorphism. Aliquots (10 ml) of each amplified DNA were separated by electrophoresis in an 8% polyacrylamide gel with a linearly increasing denaturant gradient (100% denaturant: 7 mol/liter urea/40% formamide). For each exonic fragment explored by DGGE, we systematically loaded on the gel a homozygous normal control and a heterozygous mutated control for the DNA sequence under study (see legend to fig. 1). This was done to avoid any technical problem during either the formation of gel gradients or the electrophoretic migration. On the DGGE gels, migration of alleles from a homozygous normal subject gave a single band representing the normal homoduplex

Table 1

Sequence of Oligonucleotide Primers and Conditions Used in PCR and DGGE Analysis of All PBG Deaminase Exons

Exon(s) and Primer	5′-Sequence[a]	Size (bp)	PCR Conditions		DGGE Conditions[b]
			$MgCl_2$ (mM)	Temperature (°C)	
1:					
Human PBG deaminase exon 01 sense clamped	$(GC)_{30}$-CGTGTCCCCGGTACTCGCC	203	1.5	60	50%/80%, 6 h
Human PBG deaminase intron 01 antisense	CTCGTCCAGAAGCCCAAAGTGTG				
2:					
Human PBG deaminase intron 02 sense	CCACTGACAACTGCCTTGGTCAA	217	1.0	60	50%/80%, 3 h
Human PBG deaminase intron 02 antisense clamped	$(GC)_{50}$-GAAATACTCCAAGGCTGGTCCT				
3:					
Human PBG deaminase intron 02 sense clamped	$(GC)_{50}$-GCCAGTGATTCTGGTTCTTGGAC	257	1.5	57	45%–75%, 3 h
Human PBG deaminase intron 03 antisense	TCCCCCAATTTGTGATGCTGT				
4:					
Human PBG deaminase intron 03 sense	AAGAGTCTGAGCCGTGGCTGG	233	1.0	57	50%/80%, 3½ h
Human PBG deaminase intron 04 antisense clamped	$(GC)_{35}$-GTTGTGTTCTCTCCTCTCGGGG				
5 and 6:					
Human PBG deaminase intron 04 sense	AAATGCTGATCAATAATGAGCACCT	308	1.0	55	40%/90%, 4 h
Human PBG deaminase intron 06 antisense clamped	$(GC)_{40}$-CTGGGAGGGCACCACACTCTCC				
7:					
Human PBG deaminase intron 06 sense clamped	$(GC)_{45}$-TGGCTGCTCATACCCTTTCTCT	215	1.0	56	40%/90%, 3 h
Human PBG deaminase intron 07 antisense	ATGATGCCTACCCCTGCCCA				
8:					
Human PBG deaminase intron 07 sense clamped	$(GC)_{45}$-AATCGAGAGAGAATAGAGGTGAT	254	1.0	56	50%/80%, 3 h
Human PBG deaminase intron 08 antisense	TCCCATCCCTGCATCTTCTGG				
9:					
Human PBG deaminase intron 08 sense	ATCTCATTGTAACTTCTCTCTGGGC	215	1.0	55	50%/80%, 3½ h
Human PBG deaminase intron 09 antisense clamped	$(GC)_{40}$-CTTGTCTTTTTCCTTGGCTGCT				
10:					
Human PBG deaminase intron 09 sense	CCGACACTGTGGTCCTTAGCAA	250	1.0	56	50%/80%, 3 h
Human PBG deaminase intron 10 antisense clamped	$(GC)_{45}$-TGGGGATGACTGTAAGGCAGA				
11:					
Human PBG deaminase intron 10 sense	CTGCCAGGTGCTTTTAGACA	185	1.0	58	55%/90%, 3 h
Human PBG deaminase intron 11 antisense clamped	$(GC)_{45}$-AGGCATATGTCACCATGTGG				
12:					
Human PBG deaminase intron 11 sense clamped	$(GC)_{50}$-CAGGCCTGATGTCCTAGGATGT	235	1.0	55	40%/70%, 3¼ h
Human PBG deaminase intron 12 antisense	TCCCCAGCCCTCCACAGGT				
13:					
Human PBG deaminase intron 12 sense clamped	$(GC)_{45}$-TTGTGTATGGATAGGACCAG	298	1.0	55	40%/90%, 3 h
Human PBG deaminase intron 13 antisense	CTACCTAGAAACCTGGGATG				
14:					
Human PBG deaminase intron 13 sense clamped	$(GC)_{45}$-TAGTCCCCTCTCAGACTGTG	220	1.0	55	55%/90%, 3½ h
Human PBG deaminase intron 14 antisense	ATGCACTCTTGTTTATTACCCC				
15:					
Human PBG deaminase intron 14 sense	TCACCAAATCCCACCTCCTTCCC	315	1.0	61	45%/75%, 3½ h
Human PBG deaminase exon 15 antisense clamped	$(GC)_{40}$-GAATGGGGCACTGAGGCCCG				

[a] Sequences of GC clamps are as follows: $(GC)_{30}$, CGCCCGCCGCGCCCCGCGCCCGTCCCGCCG; $(GC)_{35}$, CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCC; $(GC)_{40}$, CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG; $(GC)_{45}$, CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCCCCC; and $(GC)_{50}$, CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCCCCCGCCCG.

[b] Percentages are of denaturant (100% = 7 M urea and 40% [v/v] formamide), and time is of electrophoresis (at 150 V and 60°C).

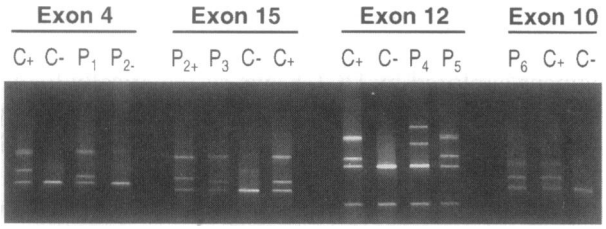

Figure 1 DGGE as a screening procedure. In this example, DGGE analysis was used to screen mutations in four exons of the PBG deaminase gene from six patients (P1–P6) of newly diagnosed AIP families. For each exon screened, a negative control with a normal homozygous DNA sequence (C−) and a positive control with a known mutated or polymorphic heterozygous DNA sequence (C+) were loaded onto the gel and were run under the same experimental conditions as were used for patients (primers and DGGE procedure are indicated in table 1). The course of mutation analysis for each patient sample was as follows: DNA extraction, separate amplification of the 15 exons, DGGE of the 15 exons, and direct sequencing of fragments with abnormal DGGE pattern.

whereas, that from heterozygous mutated subjects exhibited three or four bands; the two lower bands corresponded, respectively, to the normal and mutated homoduplexes, and the upper bands corresponded to the two types of mutant/normal heteroduplexes (fig. 1). The electrophoretic migration profile produced by each specific heterozygous sequence variation in a given amplified DNA fragment was reproducible between runs, with small variations in the distance between homo and heteroduplexes. The primer sequences, hybridization temperature for each PCR, and the electrophoretic conditions used for DGGE analysis are given in table 1.

Genomic DNA Sequencing

Abnormal DNA homodimeric bands were isolated from the DGGE gels and were directly sequenced by use of ^{35}S-dATP and the fMol DNA^{TM} sequencing kit (Promega-Biotech). The mutations were confirmed further by sequencing of amplified products from genomic DNA, with different primers as used for DGGE analysis. In addition, to limit the risk that the detected base change could be a rare polymorphism, ≥200 French control samples were screened by DGGE, to avoid similar abnormal migration pattern in normal subjects.

Reverse-Transcription PCR

When a splice-site disruption was detected, RNA isolated from lymphoblastoid cells was studied by reverse-transcription PCR, to confirm exon skipping (Higuchi 1990).

DNA Microsatellite Analysis

In suspected de novo AIP cases, nonpaternity was excluded by genotyping at six highly polymorphic loci (D12S89, D12S364, D12S358, D12S320, D21S11, and D21S226). Sequence information and PCR conditions were obtained from Genome Data Base (Gyapay et al. 1994).

Results

DGGE Analysis of the PBG Deaminase Gene

All coding sequences and exon/intron boundaries of the human PBG deaminase gene could be screened by DGGE. The mutation study in AIP families was conducted by use of a two-step strategy: all 15 exons were first explored in one patient from each newly diagnosed AIP family whose mutation had not yet been determined (fig. 1). When a sequence variation was detected by DGGE and, by direct sequencing, was characterized further as a mutation, all the family members were investigated by DGGE analysis of the affected exon (fig. 2). In each DNA fragment that presented an abnormal DGGE pattern, a sequence variation (either a mutation or a polymorphism) was always characterized by further direct sequencing. Among the 121 unrelated AIP families studied, a PBG deaminase gene mutation could be identified in 109 families. However, in 12 remaining AIP patients from 12 unrelated families, the DGGE screening was completely normal; and all the exons and exon/intron junctions were sequenced, and no base change or sequence alteration was found.

Molecular Epidemiology of PBG Deaminase Gene Defects in AIP

In 109 AIP families, a total of 78 different types of mutation were characterized, 33 of which had not been

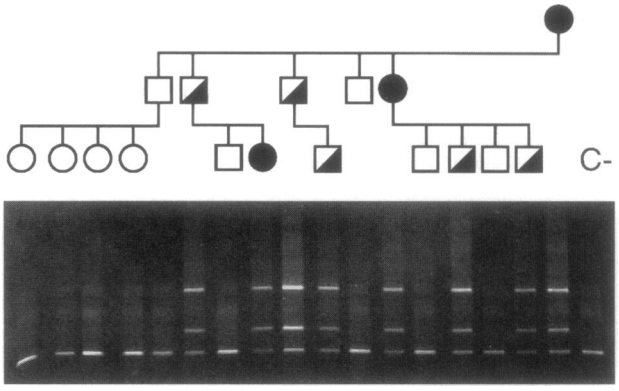

Figure 2 DGGE as a diagnostic procedure. After characterization of the mutation by direct sequencing, DGGE analysis was used to detect gene carriers in a family with the nonerythroid form of AIP. Normal individuals showed the normal homodimeric band, whereas a pattern of three bands was observed in patients and asymptomatic gene carriers. Completely blackened symbols denote patients with overt AIP; and half-blackened symbols denote asymptomatic carriers. The sequential steps for each sample analysis were as follows: DNA extraction and one PCR followed by one DGGE of the affected exon.

reported elsewhere. These 78 different mutations are listed in table 2.The mutations were spread over all exons (except exon 2) of the PBG deaminase gene. Most of them were "family specific"—that is, were found in only one pedigree; however, four were found to have a slightly higher prevalence (~5%): two missense mutations (R167W and E250K), one nonsense mutation (R149X), and one splice-site disruption in exon 14 (a G→A transition at position 912+1). Two yet-undescribed families had a mutation in exon 1, leading to a nonerythroid form of AIP: one had a mutation already described in a Finnish patient (i.e., an exon 1 splicing defect due to G→T transversion at position 33; Grandchamp et al. 1989*b*); and the other mutation was similar to the Dutch type (exon 1 splicing defect due to G→A transition at position 33+1; Grandchamp et al. 1989*c*). In the classical form of AIP, 60% of the mutations were located in exons 10, 12, and 14. Of the 78 mutations, 61 were single base substitutions (35 missense mutations, 14 splice defects, and 12 nonsense mutations), and 17 were base deletions or insertions (15 were frameshifts [10 of which were deletions and 5 of which were insertions], 1 was a 21-bp in-frame duplication, and 1 was a 30-bp deletion). Point mutations affected a G or a C in 84% of cases, and CpG dinucleotides were involved in 52% of cases (table 2). Among the 35 missense mutations found, 10 were newly described; 8 of these 10 involved evolutionarily invariant amino acid residues, and the remaining 2 (H256Y and A270D) were likely to change local hydrophobicity and charge pattern. In addition, all of them cosegregate with erythrocyte PBG deaminase activity in the affected families, through at least two generations. These results strongly suggest that these gene defects were directly causative of disease. Arginine or glutamic acid were replaced in 16 of the 35 missense mutations (table 2). The glutamic acid at position 250 was implicated in several mutations (E250Q, E250K, E250V, and E250A), as were the arginines at positions 149, 167, and 173 (R149X, R149Q, and R149L; R167W and R167Q; and R173W and R173Q). The functional consequences of the 14 mutations modifying splice sites were confirmed by the demonstration of a shortened mRNA (data not shown): in 10 of these mutations, exon skipping resulted from donor splice-site mutations, and in the 4 others it resulted from acceptor splice-site mutations. No new polymorphism was found in any coding or intronic sequence explored by DGGE.

De Novo Case

DGGE analysis of the DNA from one AIP patient gave an abnormal mobility pattern only in exon 3. Direct sequencing of the corresponding genomic DNA fragment showed only a G→A transition at position 77, leading to replacement of an arginine by histidine in the protein (R26H). The patient was the only member of the family who was known to have AIP symptoms. Neither of the patient's parents was a carrier of the mutation, and their urinary and enzymatic analyses were also normal. Nonpaternity was excluded by DNA microsatellite analysis (see Patients and Methods).

Molecular Diagnosis versus Enzymatic Screening in AIP

When a mutation in the PBG deaminase gene was characterized in one AIP patient, relatives within the family were screened by use of a single PCR amplification followed by a single DGGE of the mutated exon (fig. 2). This strategy allowed us to determine the molecular status of 385 members from the 109 characterized AIP families and to accurately classify them into three groups (patients, asymptomatic gene carriers, and normal subjects). The molecular diagnosis was then compared with the previous, enzyme-based diagnosis. The PBG deaminase activities of the members of the three groups are plotted in figure 3. It appeared that, in 95% of cases (240/252; fig. 3), the AIP gene–carrier status based on the PBG deaminase enzymatic activity was confirmed by the molecular diagnosis. Indeed, the two families with the nonerythroid variant form of AIP (23 individuals), whose carrier status could be established only by DNA analysis, were excluded from the concordance study.

Discussion

Before this study, a total of 81 different mutations in the PBG deaminase gene had been reported as causing AIP. To date, only one systematic and extensive study has been performed on a large scale, in Finland: 28 AIP families were screened by SSCP, and 19 different mutations were reported (Kauppinen et al. 1995).

Using DGGE, we screened 15 DNA segments covering one-third of the chromosomal PBG deaminase gene, including the complete coding sequence. Among the 121 unrelated AIP families analyzed, mutations were identified in 109. In 12 families, DGGE screening of patients' genomic DNA was negative, and direct sequencing failed to detect any mutation in the corresponding sequences. However, in all these cases, there was a well-documented PBG deaminase enzyme deficiency, which segregates through three generations (not shown); therefore, the causative mutation probably lies in an unexplored part of the gene. When these later cases are taken into account, the sensitivity of the DGGE method is $\geq$90% when applied to a large heterogeneous AIP cohort. Alternative methods—SSCP and heteroduplex analysis—also have been applied to PBG deaminase gene mutation screening (Kauppinen et al. 1995; Schreiber et al. 1995). SSCP requires the use of multiple

Table 2

Mutations in PBG Deaminase Gene in 109 French AIP Families

Position	Mutation[a]	Sequence Modification[b]	Restriction Enzyme	Reference
Exon 1:				
33	GCG→GCT	SD (del exon 1)	*Hph*I	Grandchamp et al. (1989*b*)
Intron 1:				
33 +1	gtg→atg	SD (del exon 1)	...	Grandchamp et al. (1989*c*)
Exon 3:				
64	CGG→TGC	R22C	...	Ong et al. (1996)
70	GGT→AGT	G24S	...	Present study
76	CGC→TGC	R26C	*Bsp*MI	Kauppinen et al. (1995)
77	CGC→CAC	R26H	...	Llewellyn et al. (1993)
83	AGC→AAC	S28N	...	Present study
Exon 4:				
91	CGCT→CACT	A31T	...	Gu et al. (1994)
100	CAG→AAG	Q34K	...	Mgone et al. (1992)
108	GAC→GAT	D36D	...	Present study
125	TTG→TAG	L42X	...	Puy et al. (1996)
158	ins A	Frameshift (stop→cod +12)	...	Present study
Intron 4:				
160 +1	gtg→ttg	SD (del exon 4)	...	Present study
Exon 5:				
163	GCT→TCT	A55S	...	Gu et al. (1994)
174	del C	Frameshift (stop→cod +40)	...	Gu et al. (1994)
182	ins G	Frameshift (stop→cod +5)	...	Gu et al. (1994)
206–207	del CT	S69X	...	Present study
Intron 5:				
210 +1	gta→ata	SD (del exon 5)	*Mae*III	Gu et al. (1994)
210 +2	ins G	SD (del exon 5)	...	Present study
Exon 6:				
218–219	del AG	Frameshift (stop→cod +9)	...	Gu et al. (1994)
Exon 7:				
291	del G	Frameshift (stop→cod +37)	...	Present study
331	CGGA→CAGA	G111R	*Nla*IV	Gu et al. (1993)
342	TGC→TGA	C114X	...	Present study
Exon 8:				
346	CGG→TGG	R116W	...	Lee et al. (1991)
371	GTC→GAC	V124D	...	Present study
Exon 9:				
445	CGA→TGA	R149X	...	Kauppinen et al. (1995)
446	CGA→CAA	R149Q	*Mbo*II	Delfau et al. (1991)
446	CGA→CTA	R149L	*Mbo*II	Gu et al. (1994)
Exon 10:				
499	CGG→TGG	R167W	...	Gu et al. (1992)
500	CGG→CAG	R167Q	...	Delfau et al. (1990)
500	del G	Frameshift (stop→cod +98)	...	Present study
517	del 17 bp	Frameshift (stop→cod +30)	*Msp*I	Present study
517	CGG→TGG	R173W	*Msp*I	Lee and Anvret (1991)
518	CGG→CAG	R173Q	*Msp*I	Delfau et al. (1990)
530	CTG→CGG	L177R	...	Mgone et al. (1992)
532	GAC→AAC	D178N	...	Present study
576–595	del 19 bp	Frameshift (stop→cod +58)	...	Present study
593	TGG→TAG	W198X	*Nhe*I	Lee and Anvret (1991)
612	CAG→CAT	SD (del 9 bp exon 10)	*Bam*HI	Delfau et al. (1991)
Intron 10:				
612 +2	gta→gca	SD (del exon 10)	...	Present study
613 −31	cac→cgc	SA (del exon 11)	...	Present study
Exon 11:				
625	GAG→AAG	E209K	*Mnl*I	Grandchamp et al. (1989*a*)
639	TAT→TAG	Y213X	...	Present study
651	CAG→CAT	Q217H	...	Present study

(*continued*)

Table 2 (continued)

Position	Mutation[a]	Sequence Modification[b]	Restriction Enzyme	Reference
Intron 11:				
652 −1	del G	SA (del exon 12)	...	Present study
652 −1	cag→cac	SA (del exon 12)	...	Present study
Exon 12:				
667	GAA→AAA	E223K	...	Gu et al. (1994)
673	CGA→TGA	R225X	...	Kauppinen et al. (1995)
691–721	del 30 bp	Truncated protein (?)	*Bbv*I	Present study
713	CTG→CGG	L238R	*Bbv*I	Kauppinen et al. (1995)
715–716	del CA	Frameshift (stop→cod +9)	...	Puy et al. (1996)
716	ins C	Frameshift (stop→cod +10)	...	Present study
723	Duplication 21 bp	7-amino-acid repeat	...	Present study
730–731	del CT	Frameshift (stop→cod +6)	...	Mgone et al. (1993)
734	CTT→CGT	L245R	...	Delfau et al. (1991)
740	TGC→TTC	C247F	*Fnu*4HI	Chen et al. (1994)
742	ins 8 bp (TTCGCTGC)	Frameshift (stop→cod +10)	...	Gu et al. (1994)
748	GAA→CAA	E250Q	...	Lundin et al. (1995)
748	GAA→AAA	E250K	...	Gu et al. (1994)
749	GAA→GTA	E250V	...	Present study
749	GAA→GCA	E250A	...	Present study
755	GCC→GTC	A252V	*Eco*NI	Mgone et al. (1993)
766	CAC→TAC	H256Y	...	Present study
771	CTG→CTA	SD (del exon 12)	*Bst*NI	Grandchamp et al. (1989*a*)
Intron 12:				
771 +1	gta→ata	SD (del exon 12)	...	Puy et al. (1996)
772 −1	cag→caa	SA (del exon 13)	...	Present study
Exon 13:				
799	CGTG→ATG	V267M	...	Present study
809	GCT→GAT	A270D	...	Present study
823	CAA→TAA	Q275X	...	Present study
Exon 14:				
848	TGG→TAG	W283X	...	Mgone et al. (1994)
849	TGG→TGA	W283X	...	Schreiber et al. (1995)
854–855	del TA	Frameshift (stop→cod +4)	...	Present study
863	TCA→TAA	S288X	...	Present study
900	del T	Frameshift (stop→cod +15)	...	Delfau et al. (1991)
Intron 14:				
912 +1	gta→ata	SD (del exon 14)	*Rsa*I	Gu et al. (1993)
Exon 15:				
913	ins C	Frameshift (stop→cod +1)	*Nla*III	Puy et al. (1996)
973	CGA→TGA	R325X	*Nla*III	Petersen et al. (1995)
1004	GGC→GAC	G335D	...	Present study

[a] Mutations occurring at the CpG dinucleotide are underlined.

[b] SD = donor splice-site mutation; SA = acceptor splice-site mutation; del = deletion; ins = insertion; and (stop→cod +*X*) = stop codon occurs *X* codons downstream.

electrophoretic conditions, to increase the sensitivity to as much as 89% (Kauppinen et al. 1995). Moreover, the preliminary DGGE modelization step has the benefit of leading to a predictible result for the DNA fragment under study, in contrast to SSCP or heteroduplex analysis. But, whatever the molecular screening method used, it must be stressed that all the 15 exons and exon/intron boundaries must be explored systematically, even if a sequence variation is found in the course of the screening study. This is necessary in order to eliminate both the possibility of a second sequence variation and the possibility that a detected base change is a rare polymorphism. To date, 10 intragenic polymorphisms have been described, and it is noteworthy that no new discernible polymorphism could be found by DGGE screening in the 3.35 kb of the PBG deaminase gene from 405 Caucasian subjects.

This study underlines the considerable heterogeneity of molecular defects in AIP in France. A total of 78 different mutations were identified, including 33 that

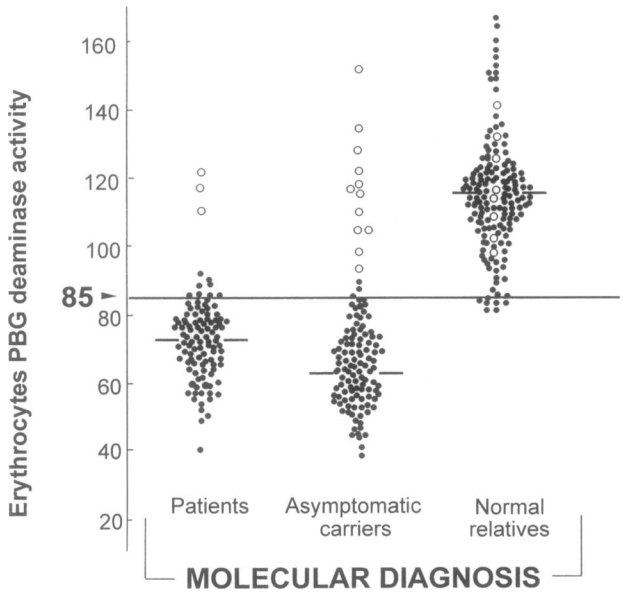

Figure 3 Molecular versus enzymatic diagnosis in AIP. AIP gene–carrier/–noncarrier status was compared for each relative, by use of erythrocyte PBG deaminase activity and DNA analysis. Unblackened circles denote members of families who have the nonerythroid variant form of AIP; and black dots denote members of families who have the classical form of AIP. Mean ± SD erythrocyte PBG deaminase activity in controls (n = 150) was 125 ± 21 pmol of uroporphyrin/h/mg Hb at 37°C. The enzyme-activity cutoff for AIP carriers was set at 85 pmol of uroporphyrin/h/mg Hb at 37°C. When data from subjects with the nonerythroid form of AIP (n = 23) were excluded, mean ± SD PBG deaminase activities in AIP patients (n = 110), asymptomatic AIP gene carriers (n = 107), and normal relatives (n = 145) were 72 ± 8, 64 ± 10, and 118 ± 21, respectively. Concordance was found in 240/252 (95%) of AIP relatives.

had not been described elsewhere. Of these 33 new mutations, 7 were splice-site disruptions further confirmed by the presence of a shortened mRNA; of the 10 newly described missense mutations, 8 affected strictly conserved amino acid residues and were located in regions with a high homology between man, mouse, and *Escherichia coli*. Direct or translated premature stop codons were created by the 16 remaining new mutations. Most of the mutations were family specific, and only mutations R167W, E250K, and W283X and the exon 14 donor splice-site mutation had a slightly higher incidence, of as much as 5%. These AIP families with the same mutation could be followed though at least three generations; usually they came from different parts of France. This finding differs from those of studies in Sweden and in The Netherlands, where suspected founder effects have been reported (Lee and Anvret 1991; Gu et al. 1993). The 78 mutations identified were distributed in all the exons or exon/intron boundaries of the PBG deaminase gene, except for exon 2; a molecular defect in exon 2 would be expected to lead to PBG deaminase deficiency restricted to its erythroid isoform and without consequent pathological features (Grandchamp et al. 1989*c*). Two of the 78 mutations occurred in the exon 1/intron 1 junction border, as elsewhere had been described in a Finnish and a Dutch family with a variant nonerythroid form of AIP (Grandchamp et al. 1989*b*, 1989*c*). A third mutation causing the nonerythroid form of AIP—a G→A transition in the initiation codon of the housekeeping transcript—has been characterized recently (Chen et al. 1994) but was not found in any AIP family in the present study.

Sixty percent of the mutations occurred in exons 10, 12, and 14. This finding could be helpful for the diagnosis of new AIP families, since analysis of these three exons would have a high probability of success. In previous studies in other countries, exons 10 and 12 were also often affected (at frequencies of 25% and 30%, respectively) (for review, see Deybach and Puy 1995).

The distribution of the different types of mutations shows a predominance of small mutational events responsible for AIP. Although the distribution of each nucleotide has been reported to be homogeneous (20%–29%; Yoo et al. 1993) along the PBG deaminase coding sequence, in this study a G and a C are involved, respectively, in 56% and 28% of single base substitutions. This disequilibrium could be explained by the high frequency (10/14) of mutated G's in consensus splice sites and by CpG dinucleotides, the latter of which are known hot spots for mutations (Cooper and Youssoufian 1988). Twenty-three mutations are due to the deamination of a methylated cytosine, resulting in a G→A transition in 17 cases and in a C→T transition in 6 cases.

Missense mutations were involved in 44% of cases. However, data from previous multiple-case reports (more often based on cDNA analysis) showed missense mutations in 60% of the cases (for review, see Astrin and Desnick 1994). This indicates the need for a prospective, exhaustive, and standardized method using genomic DNA to accurately describe the molecular epidemiology of AIP in different populations.

On the basis of the *E. coli*–deduced PBG deaminase structure, it has been argued that the polypeptide chain of the human enzyme is folded into three domains of approximately the same size (Jordan and Woodcock 1991; Brownlie et al. 1994; Wood et al. 1995). Figure 4 shows the human PBG deaminase secondary structure, with the position of all missense mutations found in the French Caucasian AIP families studied. Interestingly, most of the mutations affecting exon 12 were in an α-helix that links domains 1 and 3.

Two de novo events were also identified in the course of the study. One patient had a G→A transition at position 77 (amino acid substitution R26H), which already had been described in an AIP family with a classical dominant inheritance (Llewellyn et al. 1993). In another family, a healthy relative was found to have a silent

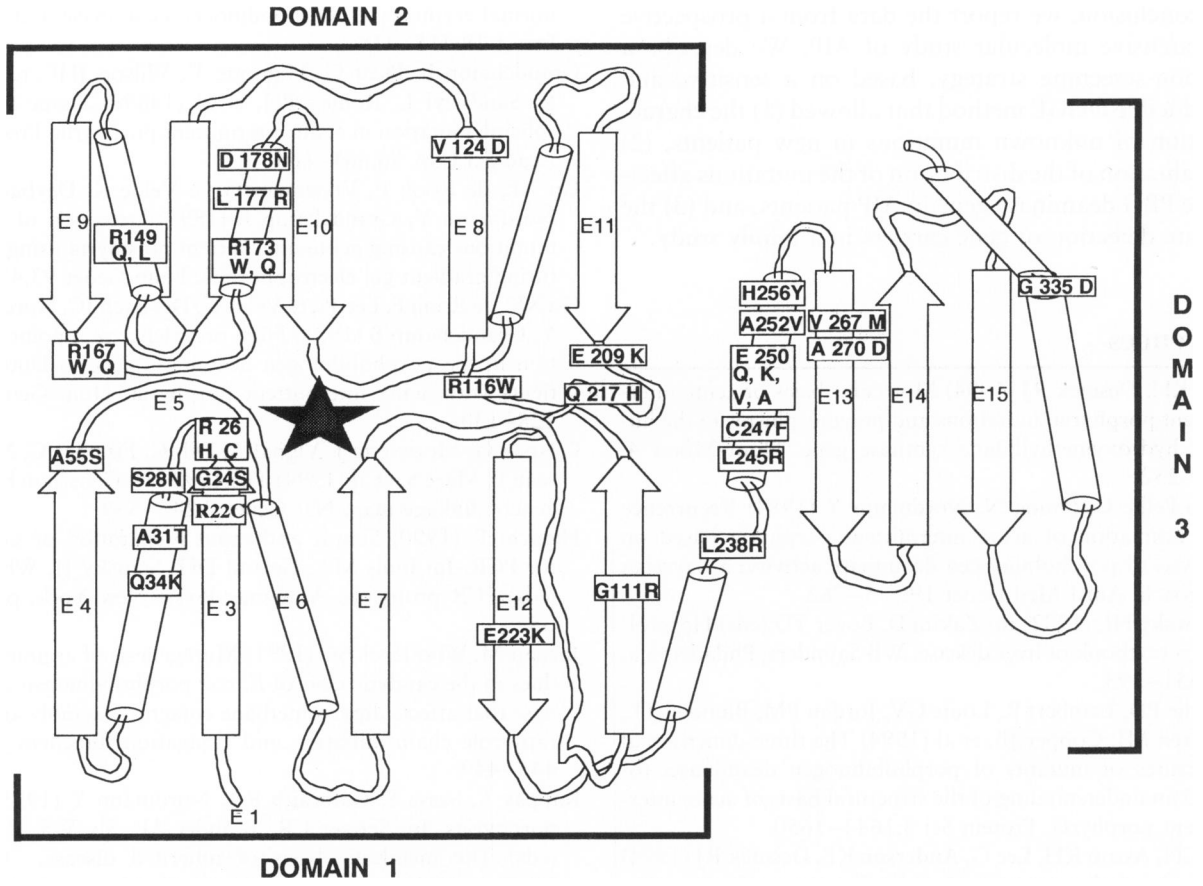

Figure 4 Schematic representation of PBG deaminase secondary structural elements, based on crystal structure of *E. coli* enzyme. Helices are shown as cylinders, and the β sheets are shown as planes (in the manner of Jordan and Woodcock 1991; Brownlie et al. 1994; Wood et al. 1995). The positions of the missense mutations characterized in French Caucasian AIP families, as determined by homology comparisons, are indicated; note that most of them occur near the active site and affect arginine residues. Nine mutations, of the 35 missense mutations reported, affect exon 12 at the α-helix hydrophobic interface between domains 1 and 3.

mutation affecting the third base of a GAC codon, at position 108 (D36D; table 2); this de novo event was also confirmed by the normal genomic DNA sequence in both parents and by paternity investigation. Two de novo mutations have been reported elasewhere, one in Finland (R195C; Kauppinen et al. 1995) and one in the United Kingdom (R173W; Whatley et al. 1995).

Since an early and accurate recognition of asymptomatic carriers of AIP is essential for efficient prevention of acute attacks, this study demonstrates the high efficiency of molecular-biology techniques in the diagnosis of AIP, and it provides the opportunity to evaluate the efficiency of erythrocyte PBG deaminase measurement for AIP gene–carrier detection. Results from AIP relatives indicate that, despite its intrinsic probabilistic diagnostic value, the erythrocyte PBG deaminase assay is a sensitive test for detection of asymptomatic gene carriers (since it has 95% concordance with the molecular diagnosis; fig. 3). However, one must emphasize that the 5% discrepancy concerned 12 isolated subjects from 12 AIP families with a different PBG deaminase gene mutation. Although we excluded unsuitable samples (e.g., high reticulocyte levels and children <1 year old), in these 12 subjects PBG deaminase activities were within a narrow range (79–87) around the chosen cutoff value of 85 pmol uroporphyrin/h/mg Hb for AIP gene carriers (fig. 3). This suggests that one must be cautious in the interpretation of the enzymatic data in the overlap range between high heterozygote and low normal values. In family studies, the detection of a known mutation in the PBG deaminase gene is technically easy and reliable. For patients with the nonerythroid AIP variant, the genetic test is superior to the erythrocyte assay, and it is the only specific diagnostic method at the asymptomatic stage in classic AIP cases; thus it should be employed whenever possible. However, the genetic approach may fail to detect the causal mutation in some cases (e.g., in the 12 classic AIP patients in this study), whereas the enzymatic assay would still have a predictive value for finding carriers in their respective families.

In conclusion, we report the data from a prospective and extensive molecular study of AIP. We describe a mutation-screening strategy, based on a sensitive and reproducible DGGE method that allowed (1) the characterization of unknown mutations in new patients, (2) the evaluation of the distribution of the mutations affecting the PBG deaminase gene in AIP patients, and (3) the accurate detection of gene carriers in a family study.

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