

Variegate Porphyrria in Western Europe: Identification of *PPOX* Gene Mutations in 104 Families, Extent of Allelic Heterogeneity, and Absence of Correlation between Phenotype and Type of Mutation

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Summary

Variegate porphyria (VP) is a low-penetrance, autosomal dominant disorder characterized clinically by skin lesions and acute neurovisceral attacks that occur separately or together. It results from partial deficiency of protoporphyrinogen oxidase encoded by the *PPOX* gene. VP is relatively common in South Africa, where most patients have inherited the same mutation in the *PPOX* gene from a common ancestor, but few families from elsewhere have been studied. Here we describe the molecular basis and clinical features of 108 unrelated patients from France and the United Kingdom. Mutations in the *PPOX* gene were identified by a combination of screening (denaturing gradient gel electrophoresis, heteroduplex analysis, or denaturing high-performance liquid chromatography) and direct automated sequencing of amplified genomic DNA. A total of 60 novel and 6 previously reported mutations (25 missense, 24 frame-shift, 10 splice site, and 7 nonsense) were identified in 104 (96%) of these unrelated patients, together with 3 previously unrecognized single-nucleotide polymorphisms. VP is less heterogeneous than other acute porphyrias; 5 mutations were present in 28 (26%) of the families, whereas 47 mutations were restricted to 1 family; only 2 mutations were found in both countries. The pattern of clinical presentation was identical to that reported from South Africa and was not influenced by type of mutation. Our results define the molecular genetics of VP in western Europe, demonstrate its allelic heterogeneity outside South Africa, and show that genotype is not a significant determinant of mode of presentation.

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Introduction

Variegate porphyria (VP [MIM 176200]) is a disorder of heme biosynthesis and results from partial deficiency of protoporphyrinogen oxidase (*PPOX* [E.C.1.3.3.4]) (Brenner and Bloomer 1980; Kirsch et al. 1998). As implied by its name, it presents in different ways—either with skin fragility and bullae, with acute neurovisceral crises, or with both together (Kirsch et al. 1998). Symptoms are very rare before puberty. The disorder is inherited in an autosomal dominant pattern with low clinical penetrance. Approximately three-quarters of those affected remain asymptomatic but are at risk for development of acute neurovisceral crises if they are exposed to certain drugs, alcohol, or other provocative factors (Jenkins 1996; Elder et al. 1997; Kirsch et al. 1998). Detection of asymptomatic affected individuals—so that they can be advised to avoid provoking agents—is an important part of the management of families with VP. Biochemical methods for their detection are technically complex or insensitive (Long et al. 1993; Da Silva et al. 1995).

The 5-kb *PPOX* gene on chromosome 1q 22-23 contains 1 noncoding and 12 coding exons (Nishimura et al. 1995; Roberts et al. 1995; Taketani et al. 1995; Puy et al. 1996). Mutations in this gene have been shown to cause VP (Deybach et al. 1996; Lam et al. 1996, 1997; Meissner et al. 1996; Warnich et al. 1996; de Rooij et al. 1997; Frank et al. 1997a, 1997b, 1998a, 1998b, 1998c, 1998d; Kauppinen et al. 1997; Corrigall et al. 1998; also see Human Gene Mutation Database) and the rare related condition, homozygous VP (Meissner et al. 1996; Frank et al. 1998e; Roberts et al. 1998). VP is common in South Africa, where an estimated 20,000 descendants of a Dutch couple who married at the Cape in 1688 have inherited the same mutation (R59W) in the *PPOX* gene (Dean 1971; Meissner et al. 1996; Warnich et al. 1996; Groenewald et al. 1998). Outside South Africa, the disease is less common but is increasingly becoming recognized, as confusion with porphyria cutanea tarda or other acute porphyrias is resolved by ap-

plication of more-precise diagnostic methods (Poh-Fitzpatrick 1980; Long et al. 1993). Apart from one series of 57 patients from Finland (Mustajoki 1980), of whom only 18 had symptoms, only small numbers of cases have been reported outside South Africa (Armas et al. 1992; Kirsch et al. 1998). *PPOX* mutations that cause VP have recently been identified in a few families (Deybach et al. 1996; Lam et al. 1996, 1997; de Rooij et al. 1997; Frank et al. 1997a, 1997b, 1998a, 1998b, 1998c, 1998d; Kauppinen et al. 1997) from Europe and the United States, but neither the extent of allelic heterogeneity, the distribution of mutations within the *PPOX* gene, nor genotype-phenotype correlations have yet been studied in a large group of patients. Here we report an investigation of the *PPOX* gene in 108 apparently unrelated patients with VP who are from France and the United Kingdom.

Patients and Methods

Patients

We studied 108 apparently unrelated patients with VP who presented sequentially for mutational analysis. Of these, 103 patients had current or past symptoms of VP; 5 patients had never had symptoms but were referred because of a family history of either acute or cutaneous porphyria and were found, by biochemical investigation, to have VP. In all 108 patients, the diagnosis was established by fecal porphyrin measurement, *PPOX* assay, or fluorescence-emission spectroscopy of plasma (Long et al. 1993; Da Silva et al. 1995). The mode of presentation (skin lesions alone, acute attack alone, or both together) was recorded on all symptomatic patients. Unrelatedness was determined by family inquiries; none of the patients were known to be related. Of the 108 patients, 51 (34 female and 17 male) came from France and 57 (42 female, 15 male) came from the United Kingdom. Patients resided throughout the United Kingdom and France; all were white.

Identification of Mutations

DNA was extracted from peripheral blood leukocytes or Epstein-Barr virus-transformed lymphoblasts. Exons 1–13 of the *PPOX* gene, along with 45–160 bp of their flanking regions, were amplified by means of the primers and conditions listed in table 1 and were screened for mutations, by either heteroduplex analysis (HDA) (exons 1–7, 12, and 13) or denaturing gradient gel electrophoresis (DGGE) (exons 8–11). Preliminary computer analyses of genomic *PPOX* sequence were performed with the MELT 87 and SQHTX programs, written and kindly provided by Lerman and Silverstein (1987). They predicted optimal experimental conditions for DGGE only in exons 8–11. For these exons, DGGE analyses

were performed according to the method of Myers et al. (1987). HDA was used as an alternative mutation-screening method for those exons not analyzable by DGGE. The gels used for HDA were prepared with a polyacrylamide-derived commercial matrix, MDE[®] (mutation-detection enhancement; FMC Bioproducts). Regions showing abnormal patterns were sequenced to identify mutations and polymorphisms.

When no abnormality was detected by DGGE or HDA, exons 4, 7, 8, and 10–13, along with 55–256 bp of their flanking regions, were screened by denaturing high-performance liquid chromatography (RP-HPLC), as described elsewhere (O'Donovan et al. 1998), and 520 bp 5' to exon 1, exons 1–3, 5, 6, and 9 and their flanking regions were sequenced. For those patients in whom no abnormality was found by HPLC, exons 4, 7, 8, and 10–13 and their flanking regions were sequenced.

For DNA sequencing, the *PPOX* gene was amplified in four fragments—exons 1–4 with 520 bp 5' to exon 1 (1,565 bp), exons 3–7 (2,014 bp), exons 7–9 (1,071 bp), and exons 10–13 (851 bp)—by means of the primers and conditions listed in table 1. PCR-amplified double-stranded DNA was purified from agarose gels by means of a Qiaquick gel-extraction kit (Qiagen) and was cycle sequenced by means of fluorescent ddNTPs (AmplitaqFS, Rhodamine, or BigDye) and an ABI 377 automated sequencer (PE Biosystems). Sequencing primers were chosen to include 58–250 bp of flanking regions. Sequences were analyzed by visual inspection. The presence of mutations was confirmed either by sequencing of both DNA strands or by digestion, with the appropriate restriction enzyme, of amplified DNA.

RNA Analysis

For investigation of splicing of exon 8, RNA was obtained by extraction from phytohemagglutinin (PHA)-transformed lymphocytes and was reverse transcribed as described elsewhere (Roberts et al. 1998). cDNA for *PPOX* was amplified by PCR with a sense primer in exon 6 (5'-AGGTCCTGCTTTCCCAGTCTCTTC) and an antisense primer in exon 13 (5'-TATTTTCATGAATGAGAGTTGGGGATC). The product was purified from a 2% agarose gel by means of a Qiaquick gel-extraction kit, and both strands were sequenced as described above.

Polymorphisms

The frequency of known and suspected single-nucleotide polymorphisms (SNPs) in the *PPOX* gene were determined by study of either 105 unrelated subjects (65 French and 40 U.K.) or 78 nonporphyric white families from the Centre d'Etude du Polymorphisme Humain (Paris), by means of either direct sequencing, restriction-enzyme digestion, DGGE, or HDA (table 2). Homozygosity or heterozygosity at intragenic polymorphic sites

Table 1**Sequence of Oligonucleotide Primers and Conditions Used in PCR and DGGE or HDA Analysis of All PPOX Exons**

Exon and Primer	5'-Sequence ^a	Size (bp)	Conditions			
			PCR		DGGE ^b	HDA ^b
			MgCl ₂ (mM)	Temperature (°C)		
1: PV 617 S PV 956 AS	CCAAGTCCCGCCAATCCAGAT ACGGATGGGCTGGTGGAAACAG	360	1.5	61	...	500 V for 10 h
2: PV-MDE-2S PV-MDE-2AS	CTGGACGGGGACCTCGCTGTT GTAGGGAGGGGTAAGAGGCATATT	355	1.0	60	...	500 V for 10 h
3: PV-MDE-3S PV-MDE-3AS	CCCTCTGAATATGCCTCTTAC AACATACTTCCCTCCCTAAAC	305	1.0	56	...	500 V for 10 h
4: PV 1544-S PV 1785-AS	GGGAATAGAGTTTAGGGGAGGA GAAGAAAGAAATGGAAGGCATA	272	1.5	56	...	500 V for 10 h
5: PV-MDE-5S PV-MDE-5AS	TGGAGCTGGGGAGGTATGTC CTGGTTTAGGAGGGAAGAGG	234	1.0	56	...	500 V for 10 h
6: PV-MDE-6S PV-MDE-6AS	CCCACCCTCATTCCCTACCA AGCACCCCTTGTCCCCACTC	307	1.0	56	...	500 V for 10 h
7: PV 2196-S PV 2485-AS	CCTAAAGTGCTGGGATTACA TGGATAAAGATGGAAACACTGA	316	1.5	56	...	500 V for 10 h
8: PV 3774-S-Cl PV 4051-AS	(GC) ₅₀ CAATCTCTTCATCCTGGGTCAG AGTTCAGGGGCATGGTGTGGT	348	1.5	56	40%–90%, 150 V for 6 h	...
9: PV 4051-S-Cl PV 4370-AS	(GC) ₅₀ ACCACACCATGCCCTGAAC GCCAGGCTGGTCTCGAACTC	370	1.0	59	40%–90%, 150 V for 6 h	...
10: PV 4538-S-Cl PV 4849-AS	(GC) ₅₀ ATTACACTCCAGCCTGGGTGA AAGGAGGGAATATAGCACTGAT	383	1.5	56	40%–90%, 150 V for 6 h	...
11: PV 4762-S PV 5121-AS-Cl	TCAGAGTGACTGTGAGGAGGAG (GC) ₅₀ AGGATGCAGGGAAAAGTTTATTAT	433	1.5	56	40%–90%, 150 V for 6 h	...
12 and 13: PV 5074-S PV 3851-AS	AGACTGGAACATTTGTCCTGT AAGCCAAGCCAAGCAATTTT	401	1.5	54	...	500 V for 10 h
1–4: V3169-S A6166-AS	GAGGTTATGTACTGGGAGGA CACGTAGAGGAACCTGTTCTGGGCAGC	1565 ^c	1.5	64		
4–7: A6167-S A6168-AS	TTGTGGGATGTCTAGGAGAGGTTG GTAGCCCATGTCTAAGTAGCTTCTAAG	2014	1.5	64		
7–9: P2052-S V8508-AS	AGTGGTCACTTCGTGGAG CGAACTCCTGACCTTGTTATC	1071	1.5	64		
10–13: T2713-S W7097-AS	GAGAGACAGCCTCAGCTAG TATTTTCATGAATGAGAGTTGGGGATC	851	1.5	61		

^a The sequence of GC clamp is as follows: (GC)₅₀ = CGCCCGCCGCGCCCGCGCCCGTCCCGCCGCCCGCCCGCCCGCCCGCCCG.

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

^c Includes 520 bases of 5' UTR.

Table 2
SNPs in the Human *PPOX* Gene

Exon/Intron and Position ^a	Screening Method	Allele (Frequency)
Exon 1:		
-253	Direct sequencing	G (1.00) C (0.0)
-247	Direct sequencing	A (.46) C (.54)
-246	Direct sequencing	G (1.00) T (.00)
-151	Direct sequencing	G (.93) T (.07)
-118	Direct sequencing	C (.84) G (.16)
Intron 2:		
IVS2-47	HDA	G (.61) C (.39)
Intron 4:		
IVS4-327	<i>HinfI</i> digestion	G (.94) A (.06)
Intron 6:		
IVS6-150	<i>SfiI</i> digestion	T (.96) C (.04)
IVS6-237	Direct sequencing	A (.73) C (.27)
Exon 7:		
767 (P256R) ^b	HDA	C (.95) G (.05)
Intron 8:		
IVS8+57 ^b	DGGE	A (.96) C (.04)
Exon 9:		
911 (R304H)	DGGE	G (.61) A (.39)
Intron 10:		
IVS10-22 ^b	DGGE	G (.97) C (.03)

^a Nucleotides are numbered according to the *PPOX* cDNA sequence (see "Numbering System" subsection in text).

^b Previously unrecognized SNPs.

was determined for all patients with mutations that were present in four or more families; partial haplotypes were deduced by comparison of the profiles of polymorphic variation for individuals who shared a mutation.

Numbering System

Nucleotides are numbered according to the cDNA sequence derived from the *PPOX* genomic sequence (Puy et al. 1996) (HGMP GenBank accession number X99450), in which the A of the ATG initiation codon is numbered "+1."

Results

Identification of Mutations

Initially, exons 1-13 and their flanking regions were amplified from genomic DNA from all 108 unrelated

patients with VP. Amplified fragments were screened by either HDA or DGGE, and any showing abnormal patterns were sequenced. This approach identified 54 mutations in 82 patients and 3 SNPs in addition to the 10 SNPs reported elsewhere (Puy et al. 1996; Warnich et al. 1996; Lam et al. 1997; Kotze et al. 1998) (table 2). A total of 13 mutations were identified in 22 of the other 26 patients, by either direct sequencing (9 mutations) or HPLC (4 mutations). Sequencing of all exons, their flanking regions, and 520 bp upstream from exon 1 failed to detect any base change in the four remaining patients. Each of these four patients was heterozygous for one intragenic SNP (-247A/C, -118C/G, IVS2-47C/G, or 911A/G).

Overall, analysis of exons 1-13 by HDA or DGGE identified 54 (77%) of 70 mutations, if it is assumed that the four patients with undetected mutations all had different mutations. All 11 SNPs found in the population that we studied gave abnormal patterns on HDA or DGGE, facilitating screening of sufficient subjects to establish allele frequencies (table 2). Direct automated DNA sequencing detected 66 mutations in 104 (96%) of our 108 patients, giving this method a sensitivity of 94% (66 of 70 mutations detected) (95% confidence interval 90%-99%).

Distribution and Type of Mutation

Mutations were distributed throughout the coding region of the *PPOX* gene, with no exon having >10 or <2 (fig. 1 and table 3); 24 (36%) of the mutations were small insertions or deletions that introduced a frameshift leading to a stop codon; 25 (38%) were missense, including one codon deletion (841-843delCAC [H281del]) and three that altered the initiation codon; 9 (14%) changed invariant nucleotides at splice sites; and 7 (11%) produced stop codons (fig. 1 and table 3). In one patient, the only base change identified was adjacent to the splice-acceptor site of exon 8 (IVS7-9T→G). Reverse transcription-PCR of cDNA

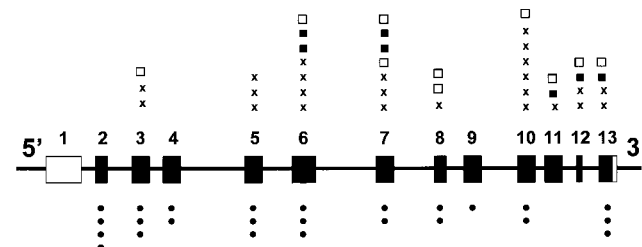


Figure 1 Distribution of mutations in the human *PPOX* gene. Missense mutations (•) are shown below the gene diagram, with chain termination and splice mutations (frameshift [×], nonsense [■], and splice defects [□]) above it. Unshaded areas of exons denote noncoding regions.

Table 3**Mutations in the PPOX Gene in VP**

Exon/Intron and Mutation	Effect	Screen	Restriction-Enzyme Site	Country	No. of Families
Exon 2:					
1A→G	M1V	HDA	<i>Nla</i> III	France	1
1A→C	M1L	HDA	<i>Nla</i> III	France	1
3G→C	M1I	HDA	<i>Nla</i> III	France	1
45G→C	L15F	HDA	<i>Eae</i> I	United Kingdom	7
Exon 3:					
113G→C	R38P	...	<i>Bsi</i> II	France	1
119G→A	G40E	HDA	<i>Mnl</i> II	France	1
157–160delATCT	Stop+13	HDA		United Kingdom	1
199–200insT	Stop+10	HDA	<i>Bgl</i> II	France	1
218T→C	L73P	...	<i>Bsa</i> JI	United Kingdom	1
Intron 3:					
IVS3–1G→C	Deletion of exon 4	...	<i>Mae</i> III	France	1
Exon 4:					
251T→G	V84G	HDA	<i>Cvi</i> JI	United Kingdom	1
254T→C	L85P	HDA		France	2
Exon 5:					
363–364insC	Stop+21	HDA		United Kingdom	2
376–377delCT	Stop+16	HDA	<i>Mnl</i> II	United Kingdom	1
428A→T	D143V	HDA		France	1
454C→T ^a	R152C	...	<i>Hba</i> I	France	1
460del23	Stop+15	HDA		United Kingdom	1
461T→C	L154P	HDA	<i>Eco</i> RI	France	1
Exon 6:					
472G→A	V158M	HDA		France	1
503G→A ^b	R168H	HDA	<i>Nco</i> I	France and United Kingdom	2
515C→T	A172V	...		France	1
528–529insT	Stop+1	HDA		France	1
538–539delAT ^c	Stop+10	HDA	<i>Mva</i> I	United Kingdom	2
542–556del15	Stop+291	HDA		France	1
565delC	Stop+44	HDA		United Kingdom	1
565C→T	E189X	HDA		United Kingdom	4
593T→G	L198X	HDA		France	1
Intron 6:					
IVS6+1G→T	Deletion of exon 6	HDA		France	1
IVS6–1G→T	Deletion of exon 7	HDA	<i>Pst</i> I	France	1
Exon 7:					
672G→A	W224X	HDA and DGGE	<i>Nla</i> IV	France	2
694G→C ^d	G232R	DGGE	<i>Mnl</i> II	France	2
695G→C	G232R	HDA and DGGE	<i>Mnl</i> II	France	1
745–746insC ^d	Stop+31	HDA and DGGE	<i>Dde</i> I	France	1
745delG	Stop+23	HDA and DGGE		France and United Kingdom	2
745–746insG	Stop+31	HDA and DGGE	<i>Taq</i> I	France	1
803G→A	W268X	HDA and DGGE	<i>Mae</i> I	United Kingdom	2
Intron 7:					
IVS7+2T→C	Deletion of exon 7	HPLC		United Kingdom	1
IVS7–9T→G	8 bp into cDNA	DGGE	<i>Nla</i> III	United Kingdom	1
Exon 8:					
841–843delCAC	H281del	HDA and DGGE		France	3
845T→A	V282D	HPLC	<i>Mae</i> III	United Kingdom	2
856delA	Stop+20	HDA and DGGE		France	1
868G→C	V290L (deletion of exon 8)	DGGE	<i>Mae</i> III	United Kingdom	1
Exon 9:					
884T→C	L295P	DGGE		United Kingdom	6
Intron 9:					
IVS9–1G→C	Deletion of exon 10	DGGE	<i>Eco</i> RII	United Kingdom	1

(continued)

Table 3 (continued)

Exon/Intron and Mutation	Effect	Screen	Restriction-Enzyme Site	Country	No. of Families
Exon 10:					
1004T→G	V335G	DGGE		France	1
1048T→C	S350P	DGGE	<i>HinfI</i>	United Kingdom	1
1053–1054insT	Stop+3	HDA and DGGE		United Kingdom	1
1081–1082insG	Stop+19	HDA and DGGE	<i>BglI</i>	United Kingdom	2
1082–1083insC	Stop+18	HDA and DGGE	<i>BglI</i>	France	6
1083delT ^c	Stop+3	HDA and DGGE	<i>BglI</i>	France	1
1083–1084insG	Stop+18	HDA and DGGE	<i>BglI</i>	France	1
1090–1091delAG	Stop+15	HDA and DGGE	<i>AhoNI</i>	United Kingdom	1
Intron 10:					
IVS10–1G→T	Deletion of exon 11	DGGE	<i>DdeI</i>	France	1
Exon 11:					
1119G→A	W373X	DGGE	<i>ApyI</i>	France	1
1147–1148delGT	Stop+50	HPLC		France	1
Exon 12:					
1274–1275delGT	Stop+7	HDA	<i>MaeIII</i>	United Kingdom	1
1281G→A	W427X	...	<i>BstI</i>	United Kingdom	1
1287delA	Stop	HDA and DGGE		France	2
Intron 12:					
IVS12+1delG	Deletion of exon 12	HDA	<i>SpeI</i>	France	2
IVS12–2A→G	Deletion of exon 13	HDA	<i>MspI</i>	United Kingdom	1
Exon 13:					
1292–1293delAG	Stop+2	...	<i>HinfI</i>	United Kingdom	2
1303C→T	Q435X	HPLC		United Kingdom	5
1331T→C	L444P	HDA		United Kingdom	1
1357G→A	G453R	...		United Kingdom	1
1358G→T	G453V	...		United Kingdom	1
1384–1385delAG	Stop+13	HDA and DGGE		France	1

^a Previously reported by Frank et al. (1998c).

^b Previously reported by Frank et al. (1998b).

^c Previously reported by Corrigall et al. (1998).

^d Previously reported by Deybach et al. (1996).

^e Previously reported by Frank and Christiano (1997).

for PPOX (exons 6–13) from this patient gave a product (~900 bp) of the size (918 bp) predicted for the normal allele. Sequencing of this product showed a mixture of similar quantities of two cDNA species—one from the normal allele and one with addition of 8 bp to the 5' end of exon 8—indicating that this mutation creates an additional splice-acceptor site (GTTTTCAG[GCTCTCAG-----]) that is used in preference to the normal site.

Allelic Heterogeneity

Five mutations were present in four to seven families (table 3), accounting for a total of 28 (26%) of our 108 families. All other mutations were present in three or fewer families, with 47 being restricted to a single family (table 3).

The disease was less heterogeneous in the United Kingdom than in France. Of the 37 mutations identified in French families, only 1 (1082insC) was found in more

than three families, being present in six families (12% of the 47 families) (table 3). In contrast, 4 of the 31 mutations in U.K. patients (i.e., mutations L15F, E189X, L295P, and Q435X) were found in 22 (39%) of the families from that country (table 3). Analysis of intra-genic polymorphisms showed that the L15F and Q435X mutations were each associated with at least two different haplotypes (mutation L15P—haplotypes [–247A, –151G, IVS2–47G, 911A] and [–247A, –151G, IVS2–47G, 911G]; and mutation G435X—haplotypes [–151G, –181C, IVS2–47C, 911G] and [–151G, –181C, IVS2–47G, 911G]), indicating that neither mutation is likely to have been inherited from a single common ancestor. For the three other mutations, this analysis did not exclude common ancestry.

Only two infrequent mutations (503G→A [R168H] and 745delG) were found in both countries (table 3), and both occur at potentially hypermutable sites: a CpG dinucleotide on the antisense strand (R168H) and a polyG tract (745delG) (Cooper and Krawczak 1993).

Phenotype-Genotype Relationship

VP presents clinically in three distinct ways: with skin lesions alone, with an attack of acute porphyria without skin lesions, or with both together. The numbers of our patients in each category are shown in table 4. Percentages did not differ significantly between France (59% cutaneous) and the United Kingdom (58% cutaneous) and are very close to those reported elsewhere for a large series from South Africa (Eales et al. 1980; Kirsch et al. 1998).

Table 5 shows the relationship between mode of presentation and type of mutation. Although truncating mutations (frameshift, nonsense, or splice site) appear to be associated with presentation with skin lesions alone, the difference was not statistically significant ($\chi^2 = 5.33$; $P = .07$). Four of the five mutations that were present in five or more families were associated with more than one type of clinical presentation; all patients with the 1082insC mutation presented with skin lesions alone.

Discussion

Here we have documented the clinical features and molecular basis of VP in 108 unrelated patients from western Europe. Mutations were identified in genomic DNA by a combined screening and sequencing approach. DGGE, which has been shown to be highly effective for the detection of mutations in the other acute porphyrias (Puy et al. 1997; Rosipal et al. 1999), cannot be applied to the whole of the *PPOX* gene, and HDA is known to lack sensitivity (Tchernitchko et al. 1999). However, all the mutations that we identified could be detected by direct automated sequencing of exons and their flanking regions, and this would appear to be the method of choice for mutational analysis of VP.

Mutations were not identified in four patients. Complete deletions of the *PPOX* gene were excluded by showing that each of these patients was heterozygous for an intragenic SNP. All had an unequivocal diagnosis of VP, and it is probable that the causative mutations

Table 4

VP Clinical Features

CLINICAL FEATURE(S)	NO. (%) IN SUBJECTS FROM	
	Present Study ($n = 103$) ^a	South Africa ^b ($n = 269$)
Skin lesions alone	61 (59%)	156 (58%)
Acute attack alone	20 (20%)	51 (19%)
Both together	22 (21%)	62 (23%)

^a Figures for France and the United Kingdom were not significantly different. Clinical information was available for 103 of the 108 unrelated patients.

^b Data are from Eales et al. (1980).

Table 5

VP Clinical Features and Type of Mutation

CLINICAL FEATURE(S) ^a	NO. OF PATIENTS (%) OF TOTAL WITH MUTATION TYPE		
	Missense ($n = 38$)	Splice Site ($n = 11$)	Nonsense or Frameshift ($n = 51$)
Skin lesions alone	18 (47%)	5 (45%)	35 (69%)
Acute attack alone	12 (32%)	1 (10%)	7 (14%)
Both together	8 (21%)	5 (45%)	9 (17%)

^a At presentation, in the index case from 100 unrelated families. Differences between groups are not significant ($\chi^2 = 5.33$; $P = .07$).

either lie outside the gene regions that we sequenced or were partial deletions or insertions not detectable by our PCR-based methods.

Two patients had both a chain-termination or splice mutation (IVS6+1G→T or 1054insT, respectively) and a missense mutation (767C→G [P256R]) that were shown, by family studies, to be on the same allele. The P256R mutation has previously been reported to be one of two mutant *PPOX* alleles in a compound heterozygote with homozygous VP (Kauppinen et al. 1997). Investigation of 156 normal alleles showed that this mutation, which affects a residue that is poorly conserved between species (fig. 2), is a polymorphism present in ~10% of the French population (table 2). Two previously unreported intragenic SNPs were also identified (table 2). Five single-base changes in exon 1 have recently been reported in VP and have been interpreted as evidence that this UTR of the gene is particularly mutation prone (Kotze et al. 1998). We sequenced exon 1 from 45 patients with VP and were unable to confirm this finding; two of the SNPs found by Kotze et al. (1998) in only three normal controls (i.e., mutations -253G/C and -246G/C) were not present in our cohort (table 2).

We have identified 66 different mutations in the *PPOX* gene in VP (table 3), 60 of which are novel, bringing the total number of mutations reported in this condition to 79 (Deybach et al. 1996; Lam et al. 1996, 1997; Meissner et al. 1996; Warnich et al. 1996; de Rooij et al. 1997; Frank et al. 1997a; Frank et al. 1997b; Frank et al. 1998a, 1998b, 1998c, 1998d; Kauppinen et al. 1997; Corrigall et al. 1998; also see Human Gene Mutation Database), whereas a further 9 mutations have been identified only in its homozygous variant (Meissner et al. 1996; Kauppinen et al. 1997; Frank et al. 1998e; Roberts et al. 1998). Of the mutations in our patients, 31 (7 nonsense and 24 frameshift) introduce premature stop codons (table 3) and are likely to abolish *PPOX* activity, primarily by accelerating mRNA decay (Culbertson 1999). The 10 splice-site mutations that we identified (table 3) either change invariant bases at the donor

	F	P	E	P	G	P	V	C	P	M	H	V	R	R	del	D	P	H	G	P	P	V/R
	15	38	40	73	84	85	143	152	154	158	168	172	232	256	281	282	295	304	335	350	444	453
1	L	R	G	L	V	L	D	R	L	V	R	A	G	P	H	V	I	R	V	S	L	G
2	L	R	G	L	V	L	D	R	L	V	R	A	G	P	H	V	I	R	V	S	L	G
3	L	R	G	L	A	V	D	R	F	Y	R	G	P	H	L	K	V	S	V	S	L	G
4	L	R	G	A	-	D	R	L	A	T	A	G	R	L	S	L	D	A	S	L	G	G
5	L	R	G	Q	V	N	D	R	V	V	S	A	G	K	S	V	L	L	I	N	V	G
6	L	R	G	A	Q	L	D	D	F	V	S	A	G	A	V	V	A	V	S	L	G	G
7	L	H	G	K	V	I	D	Q	L	I	G	G	G	A	V	L	R	L	S	W	G	G
8	L	R	G	T	-	E	R	L	V	S	A	G	K	S	V	L	N	I	S	L	G	G
9	L	R	G	T	L	V	E	R	L	V	S	A	G	S	I	L	D	H	S	L	G	G
10	L	K	G	F	Q	Q	H	R	F	V	A	G	S	A	V	A	N	V	S	L	G	G
	*	*	*			*	*			*	*			*	*			*	*	*	*	*

Figure 2 Missense mutations and intraexonic SNPs in human PPOX. The amino acid sequence for the human enzyme (1) was compared with those of the mouse (2), *Saccharomyces cerevisiae* (3), *Mycococcus xanthus* (4), *Bacillus subtilis* (5) *Mycobacterium tuberculosis* (6), *Propionibacterium freundenreichii* (7), *Nicotiana tabacum I* (plastid) (8), *Nicotiana tabacum II* (mitochondria) (9), and *Arabidopsis thaliana* (10). Comparisons are shown only for mutated residues; numbers refer to the human sequence; letters above numbers denote mutant residues. Intraexonic polymorphisms (P256R [256] and R304H [304]) are in italics. A pair of vertically stacked asterisks (**) denotes a conserved residue, and a single asterisk (*) denotes a highly conserved residue. Sequences were aligned by Clustal W1.7 (Thompson et al. 1994).

or acceptor site (Cooper and Krawczak 1993) or introduce an alternative splice site that produces a frameshift in mRNA for PPOX (table 3) and are also likely to substantially reduce activity.

Twenty-five mutations altered the coding sequence for PPOX (table 3). Three of these altered the translation-initiation codon (table 3). Similar mutations in other genes are known to decrease steady-state mRNA concentrations, through failure of initiation with premature termination (Cooper and Krawczak 1993). The consequences of the 22 other missense mutations that we have identified are more difficult to predict, because little is known about the structure/function of PPOX. The human PPOX gene encodes a 51-kD protein that functions as a flavin- and oxygen-dependent protoporphyrinogen oxidase associated with the inner mitochondrial membrane (Deybach et al. 1985; Nishimura et al. 1995; Dailey and Dailey 1996). Sequence analysis shows both a dinucleotide-binding motif, in the amino-terminal region, that is highly conserved among flavin adenine dinucleotide (FAD)-binding proteins (Nishimura et al. 1995; Dailey and Dailey 1998) and an adjacent 60-residue-long region that is similar throughout a superfamily of FAD-binding proteins (Dailey and Dailey 1998). There is no identifiable transmembrane domain (Dailey and Dailey 1996), and the absence of a mitochondrial targeting presequence suggests the presence of an internal targeting signal that remains to be identified. Other than the initial methionine, 12 of the 20 residues that are changed by missense mutations are highly conserved

between mammals, bacteria, and plants (fig. 2) and are therefore likely to be essential for PPOX function, stability, or transport. One of these (L15F) lies in the FAD-binding site, whereas two others (R38P and G40E) are in the adjacent region shared by the FAD-binding protein superfamily (Dailey and Dailey 1998). Seven (35%) of the residues are substituted by proline, with five of them being leucine-to-proline mutations, and a similar mutation (S450P) has been reported elsewhere (Frank et al. 1998d). The high frequency of mutations of proline is unusual and, since this residue is known to decrease protein flexibility (Tian et al. 1998) and to interrupt α -helical regions, may suggest that PPOX is very sensitive to small structural changes. Two other missense mutations (V282D and 281delH) introduce major changes (charge alteration or deletion) at adjacent residues that are partially conserved (fig. 2).

We have recently shown that missense PPOX mutations that preserve 10%–25% of wild-type activity are present on at least one allele in all patients with the rare homozygous form of VP (Roberts et al. 1998). None of these were found in our study, and it has yet to be shown that such mild mutations ever cause clinically overt VP in heterozygotes. It seems likely that such mutations represent one end of a functional spectrum of PPOX missense mutations, with mutations that abolish activity—and that therefore are lethal in homozygotes and thus only found in VP—at the other end. The frameshift, stop, and at least some of the missense mutations that we have identified are likely to abolish activity. It seems probable that mutations with intermediate effect on catalytic activity also exist and may cause VP, but with a low clinical penetrance. Further studies may show that some of the missense and splice-defective mutations in our patients fall into this category.

VP shows less allelic heterogeneity in western Europe than is shown by other acute hepatic porphyrias. In France and the United Kingdom, all the mutations identified in the HBMS (hydroxymethylbilane synthase) gene in 166 unrelated patients with acute intermittent porphyria have a prevalence of <5%—apart from one mutation (R173W) in the United Kingdom, which has a prevalence of 10% (Puy et al. 1997; Whatley et al. 1999). Hereditary coproporphyria is similarly heterogeneous in both France and the United Kingdom (Rosipal et al. 1999; authors' unpublished data). In contrast, five of the mutations that we have identified in VP have prevalences, in these two countries, of 7%–12%. Analysis of intragenic SNPs showed that at least two of these mutations are likely to have arisen on more than one occasion, so their relatively high frequency cannot be explained solely by their presence in the population of extended families. The apparent difference, in heterogeneity, between France and the United Kingdom (table 3) requires further investigation, but neither country

shows the predominance of a single mutation seen in South Africa and, to a lesser extent, Finland (Kauppinen et al. 1997). In addition, even though the frequencies of SNPs were similar, there was an almost complete separation between the types of mutation found in France and those found in the United Kingdom, with only two infrequent mutations (R168H and 745delG) being present in both populations; both of these occur at hypermutable sites, and one of them (R168H) has been found in other countries (de Rooij et al. 1997; Frank et al. 1998b). This separation suggests a relatively recent origin for the mutations in our families. The R59W South African founder mutation was not present in any of our patients. To date, it has been identified only in Afrikans families of Dutch descent (Kirsch et al. 1998) and in the Netherlands (De Rooij et al. 1997).

Previous reports of VP outside South Africa (Mustajoki 1980; Arnas et al. 1992; Kirsch et al. 1998) have included too few symptomatic patients for any firm conclusions to be drawn about whether the clinical features reported from South Africa (Eales et al. 1980; Kirsch et al. 1998) are special to that country or typical of the disease in general. Here we have shown that the clinical features in a large, molecularly heterogeneous series from western Europe are identical to those seen in South Africa, where almost all patients share the same mutation (Meissner et al. 1996; Groenewald et al. 1998). This finding suggests that VP in South Africa is clinically representative of the disease elsewhere and that allelic heterogeneity does not substantially alter the clinical pattern—an observation that would be predicted if the majority of PPOX mutations that cause VP lead to near-complete or total loss of activity, as does the R59W mutation in South Africa (Meissner et al. 1996). Measurements of PPOX activity in South African and other patients with VP have consistently shown a 50% decrease, consistent with complete haplodeficiency (Brenner and Bloomer 1980; Da Silva et al. 1995; Kirsch et al. 1998). More detailed analysis of our findings also fails to identify any major relationship between clinical features and type of mutation. Of the mutations that we identified, 31 (47%) produced premature-termination codons and are likely to have abolished all PPOX activity. Yet the distribution of the associated clinical features was similar to that found in the group as a whole (tables 4 and 5), and there was no significant correlation between type of mutation and clinical presentation (table 5). In addition, four of the five relatively frequent mutations that we found were associated with two or more clinical presentations. Overall, therefore, the genotype does not appear to be a significant determinant of clinical severity, if skin disease alone is regarded as a mild manifestation and if an acute attack is regarded as a severe one. Environmental influences and genetic effects from other loci are likely to be more important in this respect.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Human Gene Mutation Database, <http://www.uwcm.ac.uk/uwcm/mg/hgmd0.html> (for mutations in PPOX gene)
 Human Genome Mapping Project, <http://www.hgmp.mrc.ac.uk> (for accession numbers for PPOX genomic/cDNA sequences: X99450 [human], U25114 [mouse], U18778 [*Saccharomyces cerevisiae*], M73709 [*Myxococcus xanthus*], M97208 [*Bacillus subtilis*], AL021186 [*Mycobacterium tuberculosis*], D85417 [*Propionibacterium freudenreichii*], Y13467 [*Nicotiana tabacum I*], Y13466 [*Nicotiana tabacum II*], and D83139 [*Arabidopsis thaliana*])
 Online Mendelian Inheritance in Man (OMIM), <http://www.ncbi.nlm.nih.gov/Omim> (for VP [MIM 176200])

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