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# Systematic Analysis of Molecular Defects in the Ferrochelatase Gene from Patients with Erythropoietic Protoporphyria

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## Summary

**Erythropoietic protoporphyria (EPP; MIM 177000) is an inherited disorder caused by partial deficiency of ferrochelatase (FECH), the last enzyme in the heme biosynthetic pathway. In EPP patients, the FECH deficiency causes accumulation of free protoporphyrin in the erythron, associated with a painful skin photosensitivity. In rare cases, the massive accumulation of protoporphyrin in hepatocytes may lead to a rapidly progressive liver failure. The mode of inheritance in EPP is complex and can be either autosomal dominant with low clinical penetrance, as it is in most cases, or autosomal recessive. To acquire an in-depth knowledge of the genetic basis of EPP, we conducted a systematic mutation analysis of the FECH gene, following a procedure that combines the exon-by-exon denaturing-gradient-gel-electrophoresis screening of the FECH genomic DNA and direct sequencing. Twenty different mutations, 15 of which are newly described here, have been characterized in 26 of 29 EPP patients of Swiss and French origin. All the EPP patients, including those with liver complications, were heterozygous for the mutations identified in the FECH gene. The deleterious effect of all missense mutations has been assessed by bacterial expression of the respective FECH cDNAs generated by site-directed mutagenesis. Mutations leading to a null allele were a common feature among three EPP pedigrees with liver complications. Our systematic molecular study has resulted in a significant enlargement of the mutation repertoire in the FECH gene and has shed new light on the hereditary behavior of EPP.**

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## Introduction

Erythropoietic protoporphyria (EPP; MIM 177000) is an inherited disorder of heme biosynthesis, first recognized by Magnus et al. (1961). EPP is caused by a partial deficiency of ferrochelatase (FECH; E.C.4.99.1.1), the terminal enzyme of the heme biosynthetic pathway that catalyzes the insertion of ferrous iron into protoporphyrin IX to form heme. In EPP patients, the accumulation of free protoporphyrin because of FECH deficiency takes place principally in the erythropoietic tissue. Clinically, an excess amount of free protoporphyrin accumulates in the skin, causing an extremely painful photosensitivity in patients, starting at early childhood. In <5% of patients, the accumulation of protoporphyrin in the liver leads to liver injury characterized by cholestasis and unpredictable terminal liver failure (Kappas et al. 1995).

The cDNA sequence of 423 amino acid residues that encodes human FECH has been published (Nakahashi et al. 1990 [GenBank D00726]), together with the structure and location of the FECH gene on chromosome 18q21.3 (Whitcombe et al. 1991; Taketani et al. 1992). The FECH gene contains 11 exons and spans ~45 kb. To date, 23 different molecular defects in the FECH gene have been described, coming from a number of reports mostly based on FECH cDNA analysis (for references, see table 1). However, no molecular-analysis strategy has yet been developed for examining the FECH gene mutations systematically.

The mode of inheritance of EPP is mainly autosomal dominant with incomplete penetrance but, rarely, can be autosomal recessive (Lamoril et al. 1991; Cox 1997). In the dominant type of EPP, different degrees of enzyme deficiency can be seen in patients versus asymptomatic gene carriers—that is, symptomatic patients usually have <50% of the normal activity, whereas asymptomatic gene carriers show ~50% of normal activity (Bonkowsky et al. 1975; Deybach et al. 1986; Norris et al. 1991). This indicates that factors in addition to the mutations are involved in the phenotypic manifestations of EPP. In one family, "low expression" of an apparently

## Table 1

**Mutations in the Human FECH Gene**

| Exon and Mutation | Sequence Modification[a] | Relationship to Liver Disease[b] | Reference(s) |
|---|---|---|---|
| 1: | | | |
| del $G^{40}$ | Frameshift | | Todd et al. (1993) |
| 2: | | | |
| $G^{163}\rightarrow T$ | G55C | | Lamoril et al. (1991) |
| $C^{175}\rightarrow T$ | Q59X | Present/absent | Schneider-Yin et al. (1994*a*)/present study |
| 3: | | | |
| $IVS3^{+2}$, t→g | SD (del exon 3) | | Sarkany et al. (1994*b*) |
| $T^{212}\rightarrow A$ | I71K | | Present study |
| ins $T^{213}$ | Frameshift | Present in P5, absent in P6 | Present study |
| $C^{286}\rightarrow T$ | Q96X | | Henriksson et al. (1996) |
| 4: | | | |
| $C^{343}\rightarrow T$ | R115X | Absent/present | Henriksson et al. (1996)/present study |
| $T^{451}\rightarrow C$ | S151P | | Present study |
| G463C | SD (del exon 4) | | Wang (1996) |
| $IVS4^{+1}$, g→c | SD (del exon 4) | | Present study |
| 5: | | | |
| $T^{557}\rightarrow C$ | I186T | | Imoto et al. (1996) |
| $T^{571}\rightarrow C$ | Y191H | | Present study |
| $C^{574}\rightarrow A$ | P192T | | Present study |
| del $TACAG^{580}$ | Frameshift | | Schneider-Yin et al. (1995); present study |
| 7: | | | |
| $IVS7^{+1}$, g→a | SD (del exon 7) | | Nakahashi et al. (1993*a*) |
| $IVS7^{+5}$, g→a | SD (del exon 7) | | Wang et al. (1994) |
| del $T^{709}$ | Frameshift | Present | Present study |
| del $GAGGA^{751}$ | Frameshift | | Henriksson et al. (1996) |
| $G^{801}\rightarrow A$ | M267I | | Lamoril et al. (1991) |
| 8: | | | |
| $C^{848}\rightarrow T$ | T283I | | Present study |
| $T^{863}\rightarrow A$ | M288K | | present study |
| del $TG^{899}$ | Frameshift | | Schneider-Yin et al. (1994*a*); present study |
| $G^{903}\rightarrow A$ | W301X | | Present study |
| $IVS8^{+1}$, g→t | SD (del exon 8) | | Wang et al. (1995) |
| 9: | | | |
| $IVS8^{-2}$, a→g | SA (del exon 9) | | Present study |
| $IVS9^{+1}$, g→a | SD (del exon 9) | Present | Nakahashi et al. (1993*b*) |
| 10: | | | |
| $IVS9^{-2}$, a→g | SA (del exon 10) | | Present study |
| $IVS10^{+1}$, g→t | SD (del exon 10) | | Present study |
| $IVS10^{+1}$, del g | SD (del exon 10) | | Gouya et al. (1996) |
| $IVS10^{+3}$, a→g | SD (del exon 10) | Present | Sarkany et al. (1994*a*) |
| $C^{1001}\rightarrow T$ | P334L | | Present study |
| $T^{1088}\rightarrow G$ | SD (del exon 10) | Present | Sarkany et al. (1994*a*) |
| del $T^{1122}$ | Frameshift | | Henriksson et al. (1996) |
| $A^{1135}\rightarrow T$ | K379X | | Wang et al. (1993); present study |
| 11: | | | |
| $T^{1250}\rightarrow C$ | F417S | | Brenner et al. (1992) |
| del $CTT^{1248}$ | del F417 | | Present study |
| Complete deletion of one FECH allele (del 18q) | | | Magness et al. (1994) |

[a] SD = splice donor–site mutation; and SA = splice acceptor–site mutation.

[b] A virgule (/) indicates that the mutation has been given two, different classifications, which correspond, respectively, to the two studies shown in the "References" column.

normal FECH allele in combination with a mutant allele has been proposed as being responsible for the EPP phenotype (Gouya et al. 1996). Whether this phenomenon is generally operative and would explain the puzzling aspects of dominant EPP remains to be confirmed.

There is currently no known index that predicts the onset or severity of the disease. A systematic analysis of the FECH gene mutations is a prerequisite for investigating the inheritance patterns of EPP, for seeking genotype-phenotype relationships, and for providing further insight into the genetic mechanisms responsible for decreased FECH activity in EPP. In this study, we developed a two-step screening strategy using denaturing gradient gel electrophoresis (DGGE) followed by direct sequencing, in order to rapidly identify FECH gene mutations in all 11 exons plus intron/exon boundaries. This

strategy was applied to a large Swiss and French EPP cohort and identified a number of novel mutations in this gene.

## Subjects and Methods

### *Patients and Specimens*

Twenty-nine unrelated EPP patients of French and Swiss origin were studied. The diagnosis of EPP was established unequivocally, on the basis of the clinical symptoms of photosensitivity, increased protoporphyrin concentrations in red blood cells (RBCs) and feces, and/or partial reduction of FECH activity (table 2). Peripheral blood samples were collected from the patients and their family members, with informed consent. Lymphocyte FECH activity was measured fluorometrically on the basis of zinc-mesoporphyrin formation, according to the method described by Li et al. (1987). FECH measured in most of the patients show an average residual activity that was 32% of the normal level (the mean ± 2 SD was 1.6 ± 0.5 in patients vs. 5.0 ± 1.5 in controls), a level characteristic of symptomatic EPP patients. Two patients (P7 and P15) underwent liver transplantation before the study, and a third patient (P5) had a brother who died of liver failure. The main clinical and biochemical data of EPP patients are summarized in table 2.

### *In Vitro Amplification of Genomic DNA*

Genomic DNA was isolated by use of either a procedure described by Loparev et al. (1991) or a QIAamp Blood kit (QIAGEN). Each of the 11 exons of the FECH gene was amplified by PCR using primer sets listed in the Appendix (table A1). The PCR fragments cover the respective exons and their flanking intronic sequences (two overlapping fragments, in the case of exons 4 and 11). PCR conditions were as follows: a 30-$\mu$l reaction mixture containing 0.2–1 $\mu$g of genomic DNA, 100 pmol of each primer, 10 mM Tris-HCl, pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, and 200 $\mu$M of each dNTP was incubated at 94°C for 5 min. Then, 20 $\mu$l of a second reaction mixture was added ("hot start"), containing 10 mM Tris-HCl, pH 8.3, 1.5 mM $MgCl_2$, and 3.5 U of High-Fidelity *Taq* polymerase (Boehringer Mannheim). The reactions were performed in 40 amplification cycles, each of 1 min at 94°C, 1 min at the annealing temperatures given in the Appendix (table A1), and 1 min of elongation at 72°C.

### *Reverse-Transcription PCR (RT-PCR)*

Total RNA was extracted from isolated peripheral blood mononuclear cells by means of a standard technique (Chomczynski and Sacchi 1987). cDNA was obtained by reverse transcription of total RNA, with oligo(dT) used as a primer. FECH cDNA was amplified as described elsewhere (Gouya et al. 1996).

### *DGGE Analysis of the FECH Gene*

Aliquots (30 $\mu$l) of the amplified fragments were analyzed by DGGE in a D GENE System (BioRad-Hercules). Two electrophoretic parameters—namely, the denaturant concentration (100% denaturant corresponds to 7 M urea and 40% deionized formamide) and the running time—were selected for the individual fragment by computer analyses using Melt 87 and SQHTX software (Lerman and Silverstein 1987). Linear gradients of the denaturant were formed in 7.5% polyacrylamide gels (acrylamide:bisacrylamide ratio 37.5:1) with a size of 16 × 16 cm. Electrophoreses were performed at a constant 120 V in TEA buffer (40 mM Tris acetate, 20 mM sodium acetate, and 1 mM EDTA, pH 7.4). The DNA fragments were photographed under a UV transilluminator after ethidium bromide staining. For each EPP patient, all 11 exons and their intron-flanking sequences were systematically analyzed by DGGE, to eliminate the possibility of another sequence variation and to ensure that the detected base substitution could not be due to a polymorphism. However, since DGGE patterns of exon 1 were not always conclusive, exon 1 was sequenced in all EPP patients. DGGE conditions for the FECH exons were optimized by running the samples with already well-characterized mutations in the respective sequences (including known polymorphisms). One to three of the so-called positive controls were used for each exon–mobility-shift assay, and some of these control DGGE profiles are presented in figure 1.

### *Sequencing of Genomic DNA*

DNA fragments with abnormal DGGE patterns were directly sequenced by use of the fMol DNA sequencing kit (Promega-Biotech) with $^{35}$S-dATP and primers listed in the Appendix (table A2).

### *Construction and Prokaryotic Expression of Normal and Mutated Human FECH cDNA*

The normal human FECH cDNA was expressed by means of the pGEX-2T expression vector (Pharmacia LKB Biotechnology), according to a procedure described by Lamoril et al. (1995). To study missense mutations and deletions, site-directed mutagenesis was performed, with the normal cloned FECH cDNA (pGEX-FECH) used as template. We used the transformed site-directed mutagenesis kit (Clontech Laboratories) based on the long-primer unique site–elimination mutagenesis method described by Deng and Nickoloff (1992). In brief, long primers were generated by PCR. The 5′-phosphorylated sense oligonucleotide (mutagenic primer) introduced the mutation. A 5′-phosphorylated antisense

**Table 2**

**Clinical and Biochemical Data and Mutations Identified in the FECH Gene of EPP Patients**

| Patient (Origin) | Age (Sex) | Clinical Symptom(s)[a] | Protoporphyrin in RBCs[b] ($\mu$M/liter) | FECH Activity in Lymphocytes at 37°C[c] (nmol zinc-mesoporphyrin/h/mg protein) | Exon Affected[d] | FECH Gene Mutation | | Sequence Modification[f] |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Novel | Known[e] | |
| P1 (Swiss) | 32 (M) | PS | 40.6 | … | 2 | | $C^{175}\rightarrow T$ | Q59X |
| P2 (Swiss) | 41 (F) | PS | 38.5 | … | 2 | | $C^{175}\rightarrow T$ | Q59X |
| P3 (Swiss) | 38 (F) | PS | 11.7 | … | 2 | | $C^{175}\rightarrow T$ | Q59X |
| P4 (Swiss) | 41 (F) | PS | 44.3 | … | 3 | $T^{212}\rightarrow A$ | | I71K |
| P5 (Swiss) | 32 (M) | PS, ALF | 88.0 | … | 3 | ins $T^{213}$ | | Frameshift |
| P6 (Swiss) | 7 (F) | PS | 46.0 | … | 3 | ins $T^{213}$ | | Frameshift |
| P7 (French) | 49 (F) | PS, LT | 25.6 | 1.5 | 4 | | $C^{343}\rightarrow T$ | R115X |
| P8 (Swiss) | 15 (F) | PS | 13.2 | … | 4 | $T^{451}\rightarrow C$ | | S151P |
| P9 (French) | 51 (M) | PS | 29.5 | 1.6 | 4 | $IVS4^{+1}$, g→c | | SD (del exon 4) |
| P10 (French) | 13 (M) | PS | 4.0 | 2.5 | 5 | $T^{571}\rightarrow C$ | | Y191H |
| | | | | | 9 | $IVS8^{-2}$, a→g | | SA (del exon 9) |
| P11 (French) | 65 (F) | PS, PG | 17.8 | 1.8 | 5 | $C^{574}\rightarrow A$ | | P192T |
| P12 (Swiss) | 44 (F) | PS | 14.6 | … | 5 | | del TACAG580 | Frameshift |
| P13 (Swiss) | 42 (F) | PS | 17.6 | … | 5 | | del TACAG580 | Frameshift |
| P14 (French) | 20 (M) | PS | 23.4 | 1.3 | 5 | | del TACAG580 | Frameshift |
| P15 (French) | 35 (F) | PS, LT | 97.0/28.6[g] | .8 | 7 | del $T^{709}$ | | Frameshift |
| P16 (French) | 19 (M) | PS | 41.6 | 1.8 | 8 | $C^{848}\rightarrow T$ | | T283I |
| P17 (French) | 59 (M) | PS | 7.8 | 1.6 | 8 | $T^{863}\rightarrow A$ | | M288K |
| P18 (Swiss) | 18 (M) | PS, ALF | 26.0 | … | 8 | | del $TG^{899}$ | Frameshift |
| P19 (Swiss) | 31 (M) | PS | 12.5 | … | 8 | | del $TG^{899}$ | Frameshift |
| P20 (Swiss) | 8 (M) | PS | 23.5 | … | 8 | | del $TG^{899}$ | Frameshift |
| P21 (French) | 54 (F) | PS, PG | 10.8 | 1.1 | 8 | $G^{903}\rightarrow A$ | | W301X |
| P22 (French) | 15 (F) | PS | 3.4 | 2.2 | 10 | $IVS9^{-2}$, a→g | | SA (del exon 10) |
| P23 (French) | 23 (M) | PS | 26.4 | 1.1 | 10 | $IVS10^{+1}$, a→g | | SD (del exon 10) |
| P24 (French) | 24 (M) | PS | 22.3 | 1.4 | 10 | $C^{1001}\rightarrow T$ | | P334L |
| P25 (French) | 51 (F) | PS | 43.4 | 1.2 | 10 | | $A^{1135}\rightarrow T$ | K379X |
| P26 (French) | 16 (F) | PS | 19.9 | 1.6 | 11 | del $CTT^{1248}$ | | del F417 |
| P27 (French) | 6 (F) | PS | 13.2 | 1.0 | ND | … | … | … |
| P28 (French) | 25 (M) | PS | 24.4 | 2.4 | ND | … | … | … |
| P29 (Swiss) | 34 (M) | PS | 19.9 | … | ND | … | … | … |

[a] PS = photosensitivity of the skin; ALF = abnormal liver function, evaluated in serum by standard clinical chemistry tests (transaminases, alkaline phosphatase, and gamma glutamyl transpeptidase); LT = liver transplantation; and PG = protoporphyrin gallstone.

[b] Normal <1.9.

[c] Normal (mean ± SD) = 5.0 ± 1.5. An ellipsis (…) denotes that data were not available.

[d] ND = not detected.

[e] For references, see table 1.

[f] SD = splice donor–site mutation; and SA = splice acceptor–site mutation.

[g] Before/after liver transplantation.

oligonucleotide, which mutates a unique *Bsa*AI restriction site in the pGEX-FECH plasmid (selection primer), was used. The sequences of these primers are given in the Appendix (table A3). Standard DNA elongation, ligation, and two-step digestion/transformation of mutated plasmids in mutS *Escherichia coli* and *E. coli* DH5$\alpha$ strains were performed according to the supplier's recommendations. The entire sequence of the mutated plasmid was verified by sequencing.

### *FECH Activity in Crude Bacterial Extracts*

The recombinant bacteria (*E. coli* DH5$\alpha$) were grown, and FECH activity in bacteria lysates was determined, by the same procedure as was used for measurement of lymphocyte FECH activity (see Patients and Specimens subsection).

## Results

### *Fifteen Novel mutations in the FECH Gene*

A total of 20 different mutations, including 15 "new" mutations (i.e., not described in previous studies), have been identified in the FECH gene of 26 of 29 EPP patients studied. DGGE gels from 10 exonic fragments with different mobility shifts are shown in figure 1. The subsequent sequencing of the samples with an abnormal

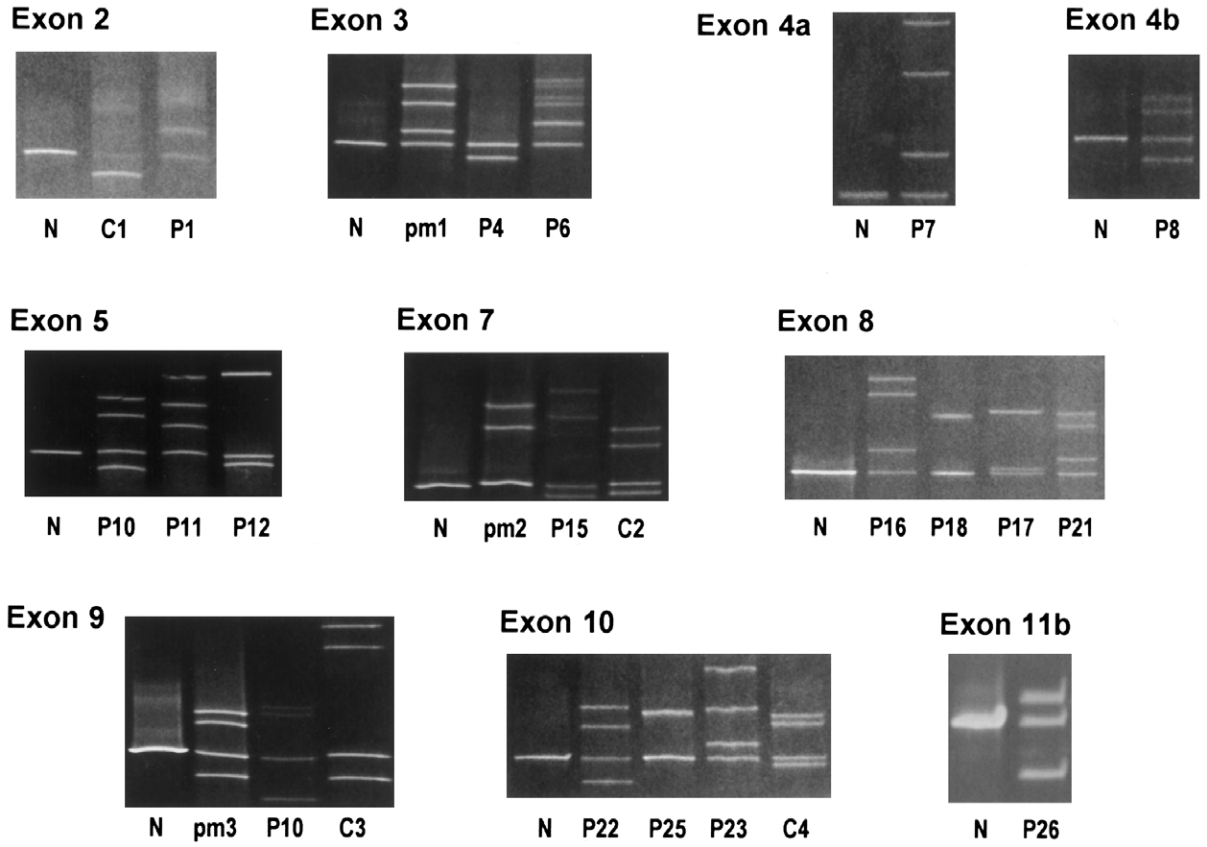


**Figure 1** DGGE analysis of nine exons of the FECH gene. Lane N, Normal controls (i.e., samples with absence of either heterozygosity for mutations or polymorphisms, which appeared as a single band on the DGGE gels). Lanes P1–P26, Samples from different EPP patients with abnormal DGGE patterns (i.e., double, triple, or quadruple bands with different migration rates). The descriptions of the mutations, revealed by subsequent direct sequencing, are given in table 2. Lanes pm1–pm3, Three polymorphisms—$^{287}$A/G, $^{798}$G/C, and $^{921}$A/G, respectively. Patterns from four of the positive control samples (samples with already known mutations) used in the study are presented: C1, del AA$^{168}$; C2, T$^{790}$C; C3, del AG$^{970}$; and C4, IVS10$^{+1}$, del g.

DGGE pattern revealed the causative mutation (table 2). Eight of the 15 new mutations are point mutations that lead to substitution of a single amino acid in the FECH protein. Four additional point mutations lie inside intronic sequences, predicting anomalous RNA splicings of exons 4, 9, and 10. In these cases, the skipping of the respective exons was verified by RT-PCR and cDNA electrophoresis (figs. 2 and 3C). A 1-bp insertion, ins T$^{213}$, and a 1-bp deletion, del T$^{709}$, causing frameshifts with stop signals occurring, respectively, 9 and 6 codons downstream, may result in formation of a truncated protein. A 3-bp deletion, del CTT$^{1248}$, removes a phenylalanine residue at position 417 (ΔF417), near the carboxyl-terminus of FECH. No mutations were found in three patients (P27, P28, and P29), either by DGGE screening or by further sequencing of the 11 exons and the respective exon/intron boundaries. Patient P27 was shown to have only one FECH allele expressed, on the basis of haplotyping and polymorphism study. In this patient, the heterozygosity with respect to the FECH gene polymorphisms in the genomic DNA was absent in cDNA, and, as expected, the steady-state mRNA level was half of normal. Sequencing of the promoter region (500 bp upstream from the initial codon) failed to detect any abnormalities. In the two remaining patients (P28 and P29) from whom only genomic DNA was available, DGGE screening and sequencing of the corresponding DNA fragments were unable to identify any sequence variations. Study of seven known intragenic polymorphic sites ($^{-251}$a/g, IVS1$^{-23}$c/t, IVS2 dinucleotide repeat, $^{287}$A/G, $^{798}$G/C, $^{921}$A/G, and $^{1520}$C/T) spreading over the FECH gene, from the promotor to the 3′ UTR, indicated that both FECH alleles were present and that the FECH locus was largely intact in both patients.

During this study, three known exonic polymorphisms—$^{287}$A/G, $^{798}$G/C, and $^{921}$A/G—have been discerned in DGGE gels of exons 3, 7, and 9, respectively (fig. 1). The allelic frequencies of these three polymorphic sites, as determined by their specific DGGE patterns in samples from 50 normal subjects, were 9% versus

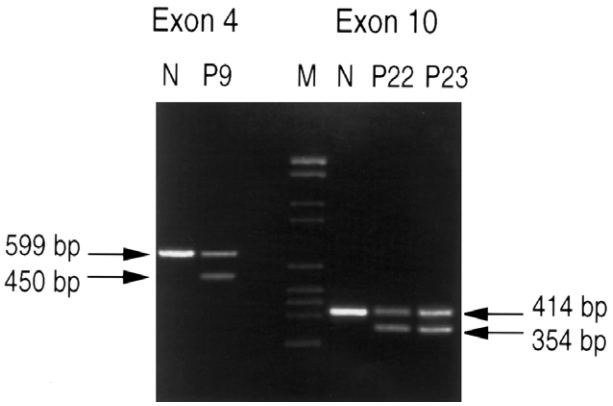


**Figure 2** Agarose gel electrophoresis (ethidium bromide staining) of RT-PCR products from patients with splicing defects: exon 4 in patient P9 and exon 10 in patients P22 and P23. The sizes of PCR products are indicated. The size differences between normal cDNA fragments (*upper bands*) and cDNA fragments lacking the respective exon are 149 bp (exon 4 skipping) and 60 bp (exon 10 skipping). Lanes N, Normal control. Lane M, Molecular-weight markers.

91%, for $^{287}$A/G; 70% versus 30%, for $^{798}$G/C; and 72% versus 28%, for $^{921}$G/A. Allelic frequencies of these polymorphisms in our EPP cohort were similar to those of the normal subjects.

### *Recurrent Mutations among Swiss EPP Patients*

A relatively high incidence of recurrent mutations was observed among the Swiss EPP patients. The family history of each Swiss EPP case was recorded, to $\geqslant$3 generations prior to the index patient. None of the Swiss families included in this study appeared to be related, on the basis of the data that were available. Table 3 lists four mutations that were identified in at least two unrelated Swiss patients; three of these mutations had been published previously, and one is new. The number of Swiss EPP patients having one of these four mutations constituted 86% (12/14) of the total Swiss EPP cases studied so far. Mutations del TACAG$^{580}$ and del TG$^{899}$ were also found in patients P14 and P18, from France.

### *In Vitro Expression of Mutated FECH Genes*

To examine the impact of the mutations on FECH activity, seven new point mutations (I71K, S151P, Y191H, P192T, T283I, M288K, and P334L), as well as the 3-bp in-frame deletion CTT$^{1248–1250}$, were expressed in a bacterial system. The nonsense mutation G$^{903}$→A, leading to substitution of tryptophan-301 by a stop codon, is likely to generate an inactive enzyme and, therefore, was not expressed. All expression constructs except pGEX-Y191H showed dramatic reduction in FECH activity—that is, <19% that of the normal enzyme (table 4). As shown in the sequence alignment of six different FECHs (fig. 4), all substitutions occurred at amino acid residues that are highly conserved, at least among mammalian species. The T$^{571}$→C transition that led to the Y191H substitution (which retains 72% of normal activity) was found to be associated with a second mutation (a splice-site disruption, IVS8$^{-2}$, a→g; see below), located on the same FECH allele, and is likely to completely inactivate the enzyme.

### *Two Mutations on the Same Allele of the FECH Gene*

Twenty-five of the 26 positively identified EPP patients were simple heterozygotes for their respective mutations. The exception was patient P10. In this patient, DGGE screening and the subsequent sequencing unveiled two distinct mutations (Y191H and IVS8$^{-2}$, a→g; table 2) in the FECH gene. By DGGE screening of exons 5 and 9, these two mutations were identified in the patient's mother but not in his father, suggesting that, in this particular EPP family, the two mutations reside on the same allele of the FECH gene (fig. 3*A* and *B*). The presence of an anomalous cDNA species lacking exon 9, as

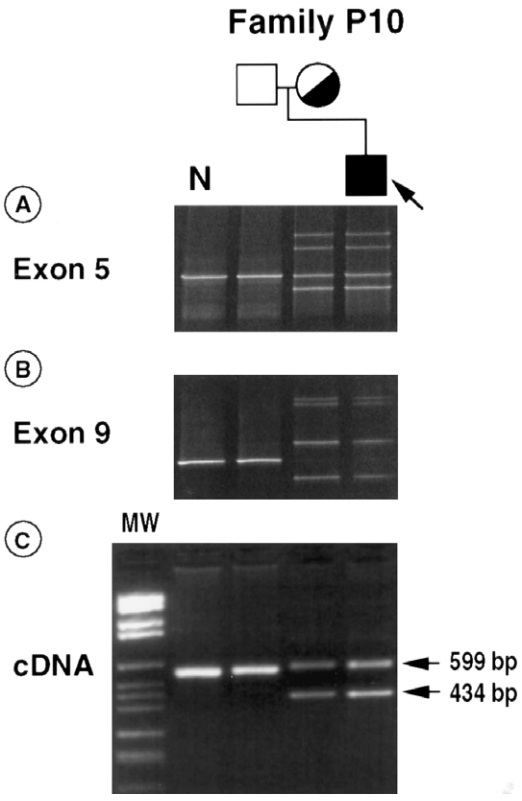


**Figure 3** Genomic and cDNA analyses of EPP family P10 . *A*, Exon 5 DGGE profile of FECH genomic DNA (mutation Y191H). *B*, Exon 9 DGGE profile of FECH genomic DNA (IVS8$^{-2}$, a→g). *C*, Agarose gel electrophoresis of normal FECH cDNA fragment (599 bp) and exon 9–skipped fragment (434 bp). Lane N, Normal control. Lane MW, Molecular-weight markers.

**Table 3**

**Recurrent Mutations among Swiss EPPs**

| Exon | Patients | Mutation | Frequency |
|---|---|---|---|
| 2 | RS[a], P1, P2, P3, | $C^{175}\rightarrow T$ | 4/14 |
| 3 | P5, P6 | ins $T^{213}$ | 2/14 |
| 5 | DV[b], P12, P13 | del $TACAG^{580}$ | 3/14 |
| 8 | MB[a], P19, P20 | del $TG^{899}$ | 3/14 |

[a] Source: Schneider-Yin et al. (1994*a*).
[b] Source: Schneider-Yin et al. (1995).

a result of the $IVS8^{-2}$, a→g mutation, was confirmed in both the patient and his mother, by RT-PCR and cDNA electrophoresis (fig. 3*C*).

### *Family Studies of EPP Patients with Liver Complications*

Patients P7 and P15 suffered from severe liver complications and underwent liver transplantation, which corrected cholestasis and normalized the liver-function tests. The erythrocyte protoporphyrin has remained <30 μM/liter, for 4 years in patient P7 and for 2 years in patient P15, after the liver transplantations. A third patient, P5, whose brother died of EPP-related liver disease, displayed abnormal liver-function tests. Three different mutations—a single base insertion (ins $T^{213}$, in patient P5), a single base deletion (del $T^{709}$, in patient P15), and a nonsense mutation (R115X, in patient P7)—were identified in the FECH genes of these three patients (table 2). No other mutations were found in the FECH DNA sequences. The transmission of these single mutations within the affected pedigrees (fig. 5) was followed by DGGE screening.

## Discussion

We have conducted the first systematic mutation study of 29 EPP patients, using a strategy combining a DGGE screening method with targeted sequencing—namely, a sequencing of only those exons or DNA fragments that have abnormal DGGE patterns. In most of the previously published EPP cases, mutation detection was performed by molecular analysis of the FECH cDNA (1.3 kb). DGGE as a screening method has been shown to be highly sensitive in the mutation study of the porphobilinogen deaminase gene in acute intermittent porphyria (Puy et al. 1997; MIM 176000). In our study, DGGE screening of EPP patients has achieved an overall detection rate of 90% (26/29 patients). The undetected abnormalities in three EPP cases may involve mutations in unexplored parts of the gene, as has been reported for other porphyrias (Puy et al. 1997). However, the size (45 kb) of the FECH gene precludes the use of a complete analysis of gene defects as a routine analysis in EPP patients.

Our finding of 15 new mutations expands to 38 the list of all known mutations in the FECH gene (table 1). In general, mutations in the FECH gene are highly heterogeneous and often are family specific, as is the case among the French EPP patients whom we studied. Certain prevalent mutations, however, were observed among the Swiss EPP patients whom we studied. The "recurrent" mutations probably reflect, to a certain extent, the genetic make-up of the relatively isolated Swiss population.

As shown in table 1, the FECH gene mutations are spread over 10 exons. So far, no mutations have been found in exon 6. Although no "hot spots" for mutation

**Table 4**

**Expression of Mutated FECH in *E. coli***

| Patient | Construct | Specific Activity of FECH at 37°C[a] (nmol zinc-mesoporphyrin/h/mg lysate protein) | Residual Activity of Human FECH[b] (%) |
|---|---|---|---|
| | pGEX | .0 | .0 |
| | pGEX-FECH | 16.0 | 100.0 |
| P4 | pGEX-I71K | .1 | <.5 |
| P8 | pGEX-S151P | .1 | <.5 |
| P10 | pGEX-Y191H | 11.3 | 72.0 |
| P11 | pGEX-P192T | .1 | <.5 |
| P16 | pGEX-T283I | .45 | 2.8 |
| P17 | pGEX-M288K | .1 | <.5 |
| P24 | pGEX-P334L | 3.0 | 19.0 |
| P26 | pGEX-del CTT1248 | .1 | <.5 |

[a] Mean of four experiments.
[b] As calculated from the reference value of the normal human FECH cDNA construct (pGEX-FECH).

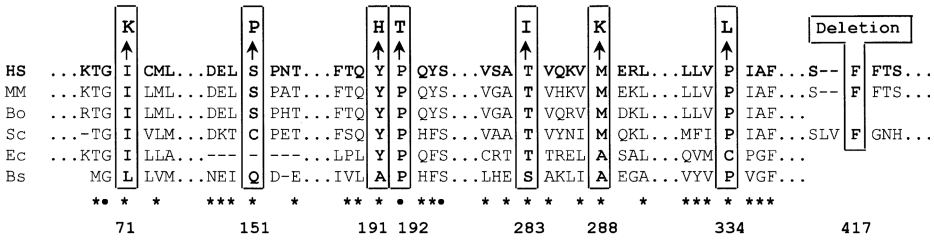


**Figure 4** Comparison of amino acid sequences deduced from nucleotide sequences of human (HS), mouse (MM), bovine (Bo), *Saccharomyces cerevisiae* (Sc), *E. coli* (Ec), and *Bacillus subtilis* (Bs). Periods (.) indicate amino acids that are identical in all FECHs; and asterisks (*) indicate amino acids that are identical in mammalian FECHs. The numbers and the boxes indicate the position of the amino acids substituted in the human sequence.

can be clearly localized on the basis of the currently available data, exon 10 of the FECH gene seems to be a more frequent target for mutations than are other exons. The predominant type of mutations are single-nucleotide substitutions in the coding region of the FECH gene (42%), followed by exon skippings (34%) and small deletions and insertions (21%). For the first time, a base-pair insertion (ins $T^{213}$) has been found in the FECH gene. A complete deletion of one FECH allele, due to loss of chromosome 18q, has been reported, so far, in one patient (Magness et al. 1994).

In earlier publications on mutation analyses in the FECH gene, exon skipping was the dominant type of mutation in the spectrum. That might reflect certain bias due to the fact that deletions of large fragments of RNA, as results of the exon skippings, were easily recognizable and therefore selectively captured when cDNAs were analyzed. DGGE screening of the genomic DNA, on the other hand, detects mutations indiscriminately.

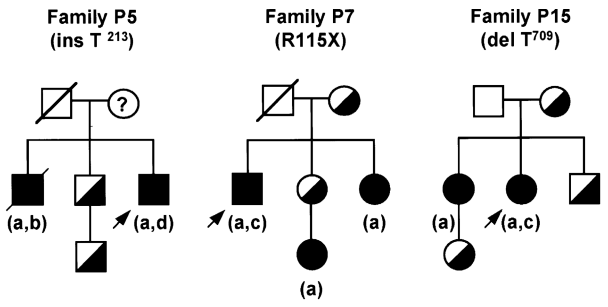


**Figure 5** Pedigree of three EPP families with severe liver disease. A nonsense mutation ($C^{343}$→T) and frameshift mutations (ins $T^{213}$ and del $T^{709}$) were identified in P7, P5, and P15, respectively. Among individuals who carry the mutation, overt and asymptomatic EPP patients are indicated by completely blackened and half-blackened symbols, respectively. Unblackened symbols denote normal individuals. The unblackened circle containing a question mark denotes an individual who was not tested. The clinical conditions of the EPP patients is indicated in paraphrases: photosensitivity (a), liver failure (b), liver transplantation (c), and abnormal liver-function tests (d).

So far, limited data are available on the structural features and catalytic mechanism of human FECH, because its tertiary structure is not yet resolved. Mutation analysis provides important information for study of the function of individual amino acids in the overall catalytic process. F417, located 6 amino acid residues away from the C-terminus, has been extensively studied. The first naturally occurring mutation found to affect F417 was the $T^{1250}$→C transition, converting phenylalanine-417 to serine (F417S; Brenner et al. 1992). In vitro expression of mutant F417S showed <2% of normal activity. To further examine the role of F417, a number of other mutants—F417L, F417Y, and F417W—were constructed, by means of site-directed mutagenesis (Dailey et al. 1994). All showed little or no measurable enzyme activity. Combining the experimental results from the human FECH, as well as from yeast and bacterial FECHs, Dailey et al. (1994) proposed that F417 is directly involved in maintaining the enzyme stability. Our patient P26 presented a new genotype—namely, the F417 deletion in the protein. The mutant FECH devoid of F417 was totally inactive when expressed in vitro, confirming F417's importance for complete enzyme activity.

The inheritance of EPP is rather complex. The FECH mutations in the majority of EPP patients are transmitted as an autosomal dominant trait, however, with incomplete penetrance and variable clinical expression. There have been two confirmed cases shown to be compound heterozygotes for distinct mutations identified on both alleles of the FECH gene (Lamoril et al. 1991; Sarkany et al. 1994*a*). Recessive inheritance was demonstrated by coinheritance of the two disabling alleles from asymptomatic parents. In our study, we found only one FECH mutation in genomic DNA from each of the 25 patients, suggesting that compound heterozygosity is a rare event in EPP. In the EPP families that we examined, all mutations were transmitted as an autosomal dominant trait. Two mutations identified in patient P10, however, aligned on the same allele of the FECH gene. The two

mutations were clearly not equally deleterious to the enzyme: whereas exon 9 skipping (as the result of the point mutation in the acceptor site of intron 8) inactivated the enzyme, the Y191H substitution (as the result of $T^{571}$→C transition) caused only a slight reduction of the enzyme activity (72% of normal) when it was expressed in a bacterial system. The expression results, together with the fact that the Y191H mutation has never been reported in EPP, suggest that this mutation may play either a minor or no role in FECH deficiency and might be a rare polymorphism.

Liver failure is a rare, albeit life-threatening, complication of EPP. There have been three published cases on the genetic analysis of EPP with liver complications (Nakahashi et al. 1993*a;* Sarkany et al. 1994*a;* Schneider-Yin et al. 1994*a,* 1994*b*). We report here three new cases—P5, P7, and P15. Although the total number of studied cases is still small, EPP patients with liver complications do exhibit certain unique features that differentiate them from EPP patients having only symptoms of photosensitivity: (1) In the three EPP pedigrees reported here, there is an unusual high clinical penetrance of the disease, with at least two symptomatic EPP patients (with or without liver complications) in one generation (fig. 5). (2) So far, all known molecular defects in EPP patients with liver complications are either frameshifts or nonsense mutations. In the six studied EPP cases (table 1), no missense mutation was found to be associated with liver complications. In other words, EPP patients with nonsense mutations, deletions, insertions, and exon skipping might be at higher risk of developing liver disease than are EPP patients with missense mutations. (3) In most of these cases, a single mutation is the sole abnormality in the FECH gene. Only one confirmed EPP case with liver complications involved two mutations on both alleles of the gene (Sarkany et al. 1994*a*).

Incomplete penetrance and variable clinical symptoms, ranging from mild photosensitivity to severe liver injury, are the puzzling features of EPP. Although our present knowledge cannot provide definitive answers to questions concerning the etiology of EPP, there is a strong indication that multiple factors play a collective role in the outcome of the disease. The FECH gene mutations leading to partial deficiency of FECH activity are certainly the primary cause of the EPP condition. A low expression of the normal FECH allele has been shown to account for the inheritance of protoporphyria in one pedigree, which might explain the lower than expected, 50% FECH residual activity in this EPP patient (Gouya et al. 1996). However, in all protoporphyric patients reported here, there is no strict correlation between genetic defects and either the erythrocyte protoporphyrin levels, the residual FECH activities, or the disease severity (table 2). So far, neither heterozygosity nor homozygosity (compound heterozygosity) for FECH gene mutations has been shown to be directly associated with distinct clinical presentations—that is, photosensitivity versus liver complication. Finally, genetic background of individuals is strongly suggested to contribute to the variable disease expression. This hypothesis is supported by the various clinical manifestations, ranging from mild skin photosensitivity to terminal liver failure, among the four unrelated Swiss patients (patients RS, P1, P2, and P3) who carry the same point mutation, $C^{175}$→T, Q59X (table 3). In these four patients, the $C^{175}$→T transition is the only mutation identified in the FECH gene, indicating that other genetic and/or environmental factors may affect the severity of EPP. A similar phenomenon has also been observed in a mouse EPP model. The same mutation, $Fech^{m1Pas}$ ($T^{293}$→A, M98K), in the homozygous state led to a severe phenotype with rapid liver injury (cytolysis and cholestasis) in the BALB/c strain (Tutois et al. 1991; Boulechfar et al. 1993) but led to a much milder clinical presentation (absence of cytolysis and mild cholestasis) when expressed in the C57BL/6 strain (J. C. Deybach, unpublished data).

Undoubtedly, a systematic analysis of the molecular defects in the FECH gene among EPP patients and their family members is the initial and important step toward understanding of the inheritance and pathophysiology of EPP, a step that is the basis for a proper genetic counseling. Our study has shown that a DGGE-based strategy is suitable as a way to perform, with high efficiency and sensitivity, mutation analysis in the FECH gene.

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# Appendix

## Table A1

**PCR Primers and Conditions for DGGE Analysis of FECH Exons**

| Exon | Primer Sequence[a] | | Annealing Temperature (°C) | Denaturant (%) | Running Time (h) |
|---|---|---|---|---|---|
| | Forward | Reverse | | | |
| 1 | 5′-CACTGCTGGGCGGACACCTG-3′ | 5′-ggacagacccacttacGCGG-3′ | 57 | 59–99 | 8 |
| 2 | 5′-gatttcatgcgagcacttta-3′ | 5′-tcttatttgtacctgatgtt-3′ | 53 | 38–78 | 6 |
| 3 | 5′-aaccgttgtattttatttta-3′ | 5′-tgtcaatgtagtgccaaggt-3′ | 53 | 15–59 | 6 |
| 4a | 5′-aatccacttacctgtatgtt-3′ | 5′-TTGGAAGTCCATATCTTGAT-3′ | 52 | 34–74 | 6 |
| 4b | 5′-GATTCAAGAGCAGTACCGCA-3′ | 5′-ttcgaaagaactaatctaat-3′ | 53 | 31–71 | 4 |
| 5 | 5′-tccacatgtctcttcctaat-3′ | 5′-cgtatctaacctttccactg-3′ | 50 | 28–68 | 8 |
| 6 | 5′-gcatctactaagtggtctct-3′ | 5′-gacaggtagctggcaagcac-3′ | 55 | 44–84 | 12 |
| 7 | 5′-tcctctttatcctcctttct-3′ | 5′-cacccaatcctctatcacta-3′ | 50 | 25–65 | 6 |
| 8 | 5′-caggtgtttaatggctgaac-3′ | 5′-taaattttaccaataagagc-3′ | 48 | 37–77 | 6 |
| 9 | 5′-tccacttagcgattcgtcta-3′ | 5′-tggaaaaaggcagatgggtg-3′ | 53 | 28–68 | 6 |
| 10 | 5′-aatatatctgtgtttttcct-3′ | 5′-ctctggtatgttctactaaa-3′ | 53 | 15–59 | 4 |
| 11a | 5′-accctgtgcttctcatgcgt-3′ | 5′-GCTGGCTGGTGAAGAAGGAT-3′ | 53 | 38–78 | 6 |
| 11b | 5′-GCTGTCCGCTCTGTGTCAAT-3′ | 5′-ACATCGGAGGTATCTGGAGG-3′ | 53 | 44–84 | 8 |

[a] Exon sequences are shown as uppercase letters; and intron sequences are shown as lowercase letters. A 40-bp GC-clamp (5′-GGGGCGGGGGCGGCGGGACGGGGCGCGGGGGCGGGGGCG-3′) was attached to the 5′-end of the reverse primers.

## Table A2

**Sequencing Conditions**

| Exon | Sequencing Primer | | Optimal Annealing Temperature (°C) |
|---|---|---|---|
| | Sense | Antisense | |
| 1 | GTGGTGGGGAGCGGGCTTCT | GATCCTGGCCCTGGCGGC | 55 |
| 2 | TTACCTGCCTGCAGAGAAATGC | GCTGGGCTGTTTCTGTGGTG | 55 |
| 3 | AAGTGTGACAAATCAACCGTTG | TTGAGAGACACACATGTCAATG | 50 |
| 4 | AACGTTTCTCAGGTTGCTAAGCT | TTCATAACTACTTCGAAAGAACTA | 52 |
| 5 | TAATAGCCCCTCACAAATAC | ATTTTTGCCAGCACCGTATC | 50 |
| 6 | AGCAGGAGTCTGTTTTTATTTCAC | ATGAGAAGCTGATTCACACTAGA | 52 |
| 7 | TTTCCTTCCATTTCTTTCTTCC | GAAATCACCCAATCCTCTATCA | 52 |
| 8 | CAGGTGTTTAATGGCTGAAC | AAATAAATTTTACCAATAAGAG | 49 |
| 9 | GGGGTACTACAGACCGTCAG | GATGGAAAAAGGCAGATGG | 53 |
| 10 | AAAAAGGAAAATAAAATATATC | TTCTCAGAGGATTACTCTCT | 46 |
| 11 | TGTGGAGTTGAAAACATCAGAAGAG | CGGGGTCCACCGGCGGGGG | 58 |

## Table A3

**Primers for FECH Expression Study**

| Primer Type | Primer Sequence[a] |
|---|---|
| Selection | 5′-ACACTCCGCTATCGCTCCGCGACTGGGTCATGGCT-3′ |
| Mutagenic: | |
| I71K | 5′-AGGAAGCCGAAAACTGGAAAATTAATGCTAAACAT-3′ |
| S151P | 5′-GAATTGCCCCCCAACACAGCCCCTCAC-3′ |
| Y191H | 5′-AGGGCTATTGCTTTCACACAGCATCCACAGTACAG-3′ |
| P192T | 5′-AGGGCTATTGCTTTCACACAGTATACACAGTACAG-3′ |
| T283I | 5′-GAGGTAAGCGCCATTGTCCAAAAAGTCATG-3′ |
| M288K | 5′-AAAGTCAAGGAAAGGCTGGAGTACTGCAACCCCTA-3′ |
| P334L | 5′-GTTCTGATAGCATTTACCAGTGACCATATTGAAAC-3′ |
| delCTT[1248] | 5′-TGCAGGGAGACTAAATCCT*TCACCAGCCAGCA-3′ |

[a] Mutated bases that either abolish the unique *Bsa*AI restriction site or introduce the desired mutations are underlined; and the asterisk (*) indicates the position of the deletion.

## Electronic-Database Information

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

GenBank, http://www.ncbi.nlm.nih.gov/Web/Genbank (for human FECH [D00726])

Online Mendelian inheritance in man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for erythropoietic protoporphyria [MIM 177000] and acute intermittent porphyria [MIM 176000])

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