

## The molecular defect of ferrochelatase in a patient with erythropoietic protoporphyria

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**ABSTRACT** The molecular basis of an inherited defect of ferrochelatase in a patient with erythropoietic protoporphyria (EPP) was investigated. Ferrochelatase is the terminal enzyme in the heme biosynthetic pathway and catalyzes the insertion of ferrous iron into protoporphyrin IX to form heme. In Epstein-Barr virus-transformed lymphoblastoid cells from a proband with EPP, enzyme activity, an immunochemically quantifiable protein, and mRNA content of ferrochelatase were about one-half the normal level. In contrast, the rate of transcription of ferrochelatase mRNA in the proband's cells was normal, suggesting that decreased ferrochelatase mRNA is due to an unstable transcript. cDNA clones encoding ferrochelatase in the proband, isolated by amplification using the polymerase chain reaction, were found to be classified either into those encoding the normal protein or into those encoding an abnormal protein that lacked exon 2 of the ferrochelatase gene, indicating that the proband is heterozygous for the ferrochelatase defect. Genomic DNA analysis revealed that the abnormal allele had a point mutation, C → T, near the acceptor site of intron 1. This point mutation appears to be responsible for the post-transcriptional splicing abnormality resulting in an aberrant transcript of ferrochelatase in this patient.

EPP is an inherited disorder of porphyrin metabolism characterized clinically by photosensitivity and biochemically by a marked increase of protoporphyrin IX in erythrocytes, plasma, and feces (1). It is the most common form of erythropoietic porphyria (2). In humans, EPP is thought to be an autosomal dominant disease, but the frequency of its clinical expression is highly variable (2). Previous studies have shown that, in patients with EPP, the activity of ferrochelatase (protoheme ferro-lyase, EC 4.99.1.1) is decreased to ≈50% compared with normal levels, in all tissues and isolated cell preparations so far examined: e.g., bone marrow (3), liver (4), cultured skin fibroblasts (5), and lymphoblasts (6). A deficiency of ferrochelatase activity is consistent with the marked increase in protoporphyrin IX, a substrate for the enzyme, observed in erythrocytes, plasma, and liver of patients with EPP, and accounts for cutaneous photosensitivity in this disorder.

Among the seven enzymes in the heme biosynthetic pathway that are implicated in inherited defects of heme biosynthesis, molecular defects have been analyzed in four: i.e., δ-aminolevulinic acid dehydratase (7), porphobilinogen deaminase (8, 9), uroporphyrinogen III cosynthase (10), and uroporphyrinogen decarboxylase (11). Molecular analysis has not yet been reported for the remaining three enzymes, including the ferrochelatase defect in EPP. We have investigated the molecular defect of ferrochelatase in a patient with EPP, by amplifying ferrochelatase mRNA from lymphoblastoid cells using PCR. In this communication, we report that a single base substitution, C → T, near the acceptor site of

intron 1 appears to be responsible for exon 2 skipping in this patient, which results in an unstable mRNA for the enzyme.

### MATERIALS AND METHODS

**Patient.** The patient was a 12-yr-old white girl, with the cutaneous photosensitivity characteristic of EPP. Protoporphyrin concentrations in her erythrocytes, plasma, and stool were markedly elevated (Table 1), and erythrocyte porphyrins consisted almost exclusively of free, not Zn-chelated, protoporphyrin (13). Ferrochelatase activity, as determined by an assay based on the ability of lymphocytes to form protoporphyrin from its precursor, δ-aminolevulinic acid (6), indicated that the proband's enzyme activity was about one-half (46.5%) the normal level (Table 1). These findings in the proband's cells are compatible with the diagnosis of EPP. The proband's parents were light-skinned but clinically unaffected. Ferrochelatase assays indicated that the mother had a normal enzyme activity (92.6%), while the father had an abnormally decreased enzyme activity (52.9%) as compared with normal controls. The proband's lymphoblastoid cells had normal activity of porphobilinogen deaminase (data not shown). Although the parents were available for ferrochelatase assays in an early study, they and several other members of this family were no longer available for the molecular studies on the ferrochelatase defect, for reasons beyond our control. Partial ferrochelatase deficiency (≈50%) is also known to occur in both asymptomatic carriers and clinically affected patients in other EPP families (6, 14). Thus the occurrence of ≈50% ferrochelatase deficiency both in the father and in the daughter is similar to that in other EPP families and is compatible with an autosomal dominant inheritance of EPP in this family.

**Cell Cultures.** Isolation of lymphocytes and transformation with Epstein-Barr virus were carried out as described previously (15).

**Western Blot Analysis.** Cross-reactive immunological material (CRIM) was examined by Western blot analysis using mitochondria isolated from  $3 \times 10^7$  lymphoblastoid cells of the proband and from a normal subject. The antibody used in this study was a rabbit antiserum raised against homogeneously purified bovine ferrochelatase (16). Detection of the specific immune complex was made using an enhanced chemiluminescence assay (17), and quantitation was carried out by densitometry using an LKB Ultrosan XL enhanced laser densitometer.

**Northern Blot Analysis.** Human ferrochelatase cDNA, λHF2-1 (18), was inserted into the pGEM4z vector (Promega), and a radiolabeled riboprobe was prepared according to the method of Melton *et al.* (19). Total RNA was isolated from  $3 \times 10^7$  cells by the method of Cathala *et al.* (20), and 20 μg of total RNA was analyzed by Northern blot analysis (21). Hybridized Zeta probe filters were treated with RNase

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Abbreviations: EPP, erythropoietic protoporphyria; CRIM, cross-reactive immunological material; bp, base pair.

Table 1. Biochemical findings of a patient with EPP

	Patient	Normal	EPP
Erythrocyte protoporphyrin, $\mu\text{g}/\text{dl}$	734-979	62.76 $\pm$ 18.80 (mean $\pm$ SD, $n = 160$ )	805 $\pm$ 374 (mean $\pm$ SEM, $n = 32$ ) (12)
Stool porphyrins, $\mu\text{g}/\text{g}$ of dry weight	606	<100	132 $\pm$ 84 (mean $\pm$ SEM, $n = 10$ ) (12)
Plasma porphyrins, $\mu\text{g}/\text{dl}$	25.8	0	25.4 $\pm$ 14.7 (mean $\pm$ SEM, $n = 12$ ) (12)
Protoporphyrin accumulation from ALA, pmol/mg of protein per 24 h	626	291.5 $\pm$ 16.5 (mean $\pm$ SEM, $n = 8$ )	687.3 $\pm$ 53.4 (mean $\pm$ SEM, $n = 9$ )

As an indirect assay for ferrochelatase, protoporphyrin IX formation by lymphocytes was determined as described previously (6). Lymphocytes were incubated with phytohemagglutinin (PHA) and pokeweed mitogen (PWM) for 3 days and then with 0.6 mM  $\delta$ -aminolevulinic acid (ALA) for 24 h. Protoporphyrin IX accumulation found by this assay has been shown to be inversely correlated with ferrochelatase activity (6). Both PHA/PWM-stimulated lymphocytes and Epstein-Barr virus-transformed lymphoblastoid cells give similar results with this assay (unpublished observation).

A (1  $\mu\text{g}/\text{ml}$ ) for 15 min at 37°C and then washed under a highly stringent condition [i.e., 15 mM NaCl/1.5 mM sodium citrate, pH 7 (0.1 $\times$  SSC), 58°C, for 30 min]. mRNA concentrations were quantitated by densitometry.

**Southern Blot Analysis.** An  $\approx$ 400-base-pair (bp) fragment upstream of the *Bam*HI site in  $\lambda$ HF2-1 cDNA was obtained, and the fragment was radiolabeled using random oligonucleotide primers (17). Protein-free DNAs from lymphoblastoid cells of the proband and a normal individual were prepared by a standard method for high molecular weight DNA isolation (17). Southern blot analysis was carried out as described previously (17).

**Nuclear Runoff Transcription Assay.** Nuclei were isolated from  $5 \times 10^7$  cells from the proband and from a normal individual. Runoff transcription assays were carried out using nuclear lysates in the presence of 200  $\mu\text{Ci}$  of [ $\alpha$ - $^{32}\text{P}$ ]UTP (1 Ci = 37 GBq; New England Nuclear) as described previously (17).

**Synthesis and Amplification of Ferrochelatase cDNA of the Proband.** cDNA synthesis and amplification were carried out as described previously (7). cDNA was synthesized from 5  $\mu\text{g}$  of total cellular RNA by using SuperScript RNase H<sup>-</sup> reverse transcriptase (GIBCO/BRL) and (dT)<sub>12-18</sub> (Pharmacia), and then used for amplification by PCR.

Two oligomers—5'-GCGGCCCGGGCCGAGGCTGCCAGG-3' and 5'-GGACGAGCTCCACCGGCGGGGG-3'—were prepared for use as PCR amplification primers based on their sequence specificity to  $\lambda$ HF2-1 by the addition of *Xma*I and *Sac*I sites, respectively, at 5' terminus of the probe. One oligomer thus corresponded to the 5'-untranslated and the other to the 3'-untranslated region of  $\lambda$ HF-2. PCR amplifications were carried out six times, and further steps were processed separately (7). PCR products were digested with *Xma*I and *Sac*I and cloned into the pGEM4z vector to transform JM109 cells.

**Amplification of Ferrochelatase Genomic DNA of the Proband.** Analysis of a genomic DNA fragment that harbors exon 2 and its flanking regions was carried out as follows: total DNA from the proband's cells was digested with *Eco*RI and *Bam*HI. The samples were then amplified by PCR, using two oligomers—5'-GGATCCAGTGGGGAAGGAGGG-3' and 5'-GGAGGGCAGAAGGAGACTGATCG-3'—corresponding, respectively, to the intron 1 sequence at 0.6 kilobase (kb) upstream from exon 2 and to the intron 2 sequence at 0.8 kb downstream from exon 2. Amplifications were performed six times independently. After amplification, the PCR products were subjected to phosphorylation and blunt-end formation and ligated to the pUC18 vector to transform JM109 cells.

**Nucleotide Sequence Analysis of DNA.** Appropriate restriction fragments of cDNA and genomic DNA were subcloned into M13mp18 or pUC18. DNA sequence analyses were carried out by the dideoxynucleotide chain-termination

method (17) using genetically engineered T7 DNA polymerase (United States Biochemical).

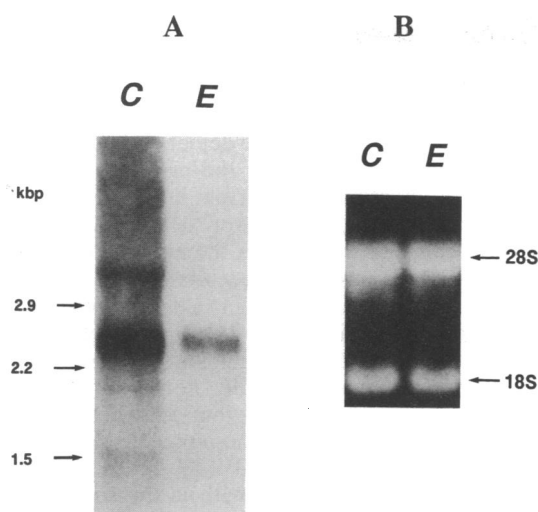
## RESULTS

**Ferrochelatase Content in the Proband's Cells.** To determine whether the decreased enzyme activity is due to a decreased concentration of ferrochelatase, immunochemical quantification of the ferrochelatase protein was performed using Western blot analysis. Both in normal control and in EPP cells, ferrochelatase was immunochemically blotted as a 42-kDa band, consistent with earlier findings (18, 22). Ferrochelatase content in the proband's cells, determined by densitometry of the 42-kDa band on x-ray films, was  $\approx$ 50% compared with that in normal lymphoblastoid cells (data not shown). This finding indicates that the observed decrease in ferrochelatase activity in the proband's cells is due to a decreased concentration of the normal enzyme and that the ferrochelatase deficiency in the proband is CRIM negative.

**Ferrochelatase mRNA Level in the Proband's Cells.** CRIM-negative deficiency may be due either to a decrease in the amount of mRNA encoding the protein or to a protein with an extremely short half-life. To distinguish these possibilities, Northern blot analysis of mRNA encoding ferrochelatase was performed. Two mRNAs for ferrochelatase of 1.6 (the minor species) and 2.5 kilobase pairs (kbp) (the major species) were detected both in normal control and in EPP cells (Fig. 1), consistent with earlier findings (18). The level of ferrochelatase mRNA in the proband's cells was 30%–60% compared with that of cells from three normal control subjects. These results suggest that the observed decrease in immunodetectable ferrochelatase is due to a decreased content of mRNA encoding ferrochelatase.

**Newly Synthesized Ferrochelatase mRNA.** To determine whether the observed decrease in mRNA concentration in the proband is due to a decrease in the rate of transcription or to an abnormally short half-life of the transcript, a runoff transcription assay was performed. The results showed that there was no difference in the transcription rates of the ferrochelatase gene, as well as those of the  $\beta$ -actin gene (used as a reference control) between the proband's cells and control cells (Fig. 2).

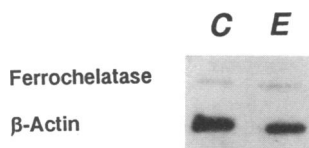
**Cloning and Sequencing the Proband's cDNA.** To examine the nature of the deficiency of ferrochelatase mRNA, ferrochelatase cDNA was synthesized from total RNA prepared from the proband's lymphoblastoid cells. A total of 19 clones were isolated, 17 of which were found by nucleotide sequencing to have the normal sequence. The remaining 2 clones, termed pEPP1, however, were found to have an abnormal sequence in which a 127-bp fragment was deleted, corresponding to nucleotides 68–194 of normal ferrochelatase cDNA. Since these two clones had been isolated independently, and both clones had the identical mutation, as dis-



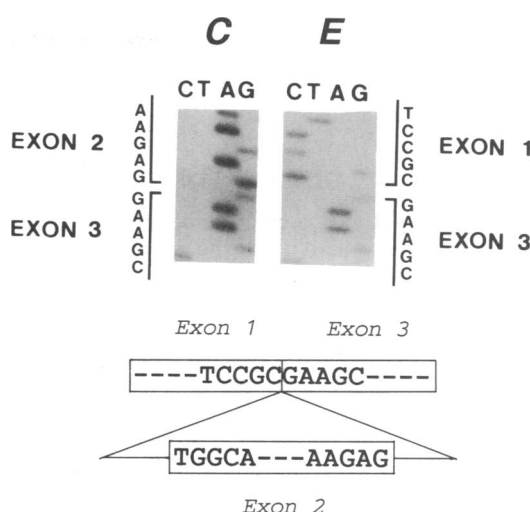
**FIG. 1.** Northern blot analysis of ferrochelate mRNA. Twenty-microgram samples of total RNA isolated from lymphoblastoid cells of the proband and of a normal subject were analyzed as described in *Materials and Methods*. (A) Northern blot analysis. Bands at 1.6 and 2.5 kbp correspond to mature ferrochelate mRNA; two other bands reflect immature mRNA. (B) Ethidium bromide-stained agarose gels containing total RNA. Lanes: C, control; E, EPP (the proband).

cussed below, it is unlikely that the observed mutation is an error of the *Thermus aquaticus* (*Taq*) polymerase reaction during the amplification process. Comparison of the sequence of pEPP1 with that of normal genomic DNA of ferrochelate (unpublished data) showed that the missing 127-bp fragment corresponded to the entire region of exon 2 (Fig. 3). This splicing mutation in pEPP1 also accompanied a frameshift with a premature stop codon (TAA) at 215 bp in the normal sequence. Thus, the aberrant mRNA in the proband would encode a truncated protein having only 29 amino acids (MRS LGANMAAALRAAGVLLRDPRSRKLEY; the abnormal sequence is underlined). An aberrant protein product encoded by pEPP1 mRNA must be an extremely minor product, since the frequency of pEPP1 mRNA is  $\approx 10\%$  compared with the normal mRNA. In addition, even if such an abnormal product is formed, it is unlikely that the product could be detected, since it would have an abnormally short half-life and may lack a normal epitope necessary for recognition by the antibody.

**Genomic DNA Analysis.** To examine whether the pEPP1 mutation is due to a large deletion or to a rearrangement of the ferrochelate gene, genomic Southern blot analysis was performed. Since the deletion in pEPP1 is the fragment corresponding to nucleotides 68–194 in the normal cDNA sequence, a cDNA fragment that covers the region upstream of the *Bam*HI site at nucleotide 385 of the normal cDNA was radiolabeled and used as a probe. There was no difference in the restriction fragment probed by the cDNA (Fig. 4), indicating that the pEPP1 mutation is likely to be due to a point



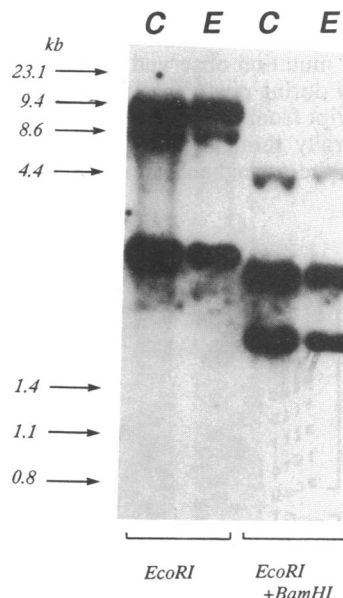
**FIG. 2.** Runoff transcription analysis of ferrochelate. Nuclei were isolated from  $5 \times 10^7$  lymphoblastoid cells of the proband and of a normal subject, and runoff transcription assays were carried out as described in *Materials and Methods*. Lanes: C, control; E, EPP (the proband).



**FIG. 3.** Nucleotide sequence analysis of ferrochelate cDNAs from the proband's lymphoblastoid cells. cDNAs were isolated from lymphoblastoid cells of the proband. Of 19 independently isolated clones, 17 had the normal ferrochelate sequence (C), while 2 had an identical abnormal sequence with the absence of exon 2 (E).

mutation, or to a minor deletion, that is below the detection level by Southern blot analysis.

In pEPP1, exon 1 is precisely joined to exon 3 without any addition or omission of nucleotide(s) between the exons. It is therefore unlikely that a mutation(s) responsible for exon 2 skipping is located either in the donor site of intron 1 or in the acceptor site of intron 2. To define the exact site of mutation, a genomic DNA fragment of the proband's cells harboring the acceptor site of intron 1 and the donor site of intron 2 was isolated. Eleven independently isolated clones were used, and sequence analyses were performed for a region encompassing from position 140 upstream of exon 2 to position 180 downstream of exon 2. Of the 11 clones, 7 were found to have the normal sequence, while 4 were found to have a C  $\rightarrow$  T mutation in intron 1, 23 bp upstream relative to the first base pair in exon 2 (Fig. 5). No mutation(s) was detected in the



**FIG. 4.** Southern blot analysis of the ferrochelate gene. Genomic DNA fragments from lymphoblastoid cells of the proband and of a normal subject were digested with *Eco*RI or *Eco*RI/*Bam*HI, and Southern blot analysis was carried out as described in *Materials and Methods*. Lanes: C, control; E, EPP (the proband).

donor site of intron 2 in any of these clones. The ratio of frequency of the normal gene to the mutant gene was also confirmed to be  $\approx 1$ , by direct sequence analysis of a genomic DNA fragment (data not shown).

### DISCUSSION

We have described a molecular defect of ferrochelatase in EPP. Ferrochelatase activity in the proband's lymphoblastoid cells was  $\approx 50\%$  of that in normal cells (Table 1), which accounts for the marked accumulation of protoporphyrin IX in various tissues, the excessive fecal excretion of protoporphyrin IX, and the cutaneous photosensitivity in the patient. Immunochemical study indicated that decreased ferrochelatase activity in the proband's cells is due to a CRIM-negative deficiency of the enzyme protein (Fig. 1).

Our findings also demonstrated that the decrease in ferrochelatase protein is approximately proportional to the reduction in ferrochelatase mRNA levels in the proband's cells (Fig. 1). The rate of transcription of ferrochelatase mRNA was, however, unaltered in the proband's cells, as judged by runoff transcription assays (Fig. 2). In contrast, the frequency of pEPP1 was substantially lower than that of the normal cDNA (2:17), suggesting that the aberrant mRNA has an abnormally short half-life. Sequence analysis of cDNA demonstrated that pEPP1 has exon 2 skipping. pEPP1 also exhibited a frameshift mutation that would result in a truncated protein. Since the existence of such a premature termination codon in a mRNA is known to significantly reduce its stability (23–26), it is conceivable that the aberrant ferrochelatase transcript in the proband's cells also has a decreased stability. Thus it is likely that the observed decrease in ferrochelatase mRNA content in the proband's cells is the result of decreased stability of the abnormal transcript.

Both the normal and the aberrant transcripts of ferrochelatase were found in the proband's cells. Thus our proband represents a heterozygous gene carrier for the ferrochelatase defect. Sequence analysis of a genomic DNA fragment from the proband's cells harboring exon 2 also demonstrated both the normal sequence and a sequence with a point mutation near the acceptor site of intron 1 (Fig. 5). The ratio of frequency of the normal gene to that of the aberrant gene was close to 1. Since no other mutations were detected, it is likely that the C  $\rightarrow$  T mutation observed is indeed responsible for exon 2 skipping during posttranscriptional processing of the primary transcript from the mutant allele.

EPP is generally thought to be an autosomal dominant disease (2). On the other hand, Went and Klasen (27) have

concluded that EPP is an autosomally recessive disorder, because only a few patients were found in a large number of family members of index cases. The findings in this study, however, indicate that our proband represents a heterozygous carrier of the ferrochelatase defect and that EPP in this family is a dominant disease. If the proband had inherited another as yet undefined defect from her mother that might have contributed to the clinical expression of EPP, the mode of inheritance of EPP in this case, though unlikely, would be compatible with an autosomal recessive trait.

It should be pointed out that in patients with congenital erythropoietic porphyria (CEP), the activity of uroporphyrinogen III cosynthase is close to nil, and it is  $\approx 50\%$  of normal in both parents who are clinically normal (28). It is also known that there is a point mutation(s) in each allele of the cosynthase gene: i.e., either as homozygous or doubly heterozygous, in patients with CEP (10). Thus the mode of inheritance of CEP is compatible with an autosomal recessive trait. In families with EPP, however, ferrochelatase activity in patients is not nil but rather is  $\approx 50\%$  of normal, and only one of the parents in this family and others (6) had an enzymatic deficiency ( $\approx 50\%$  of normal). These considerations support the idea that the EPP in our proband's family is an autosomal dominant trait. Why some heterozygous carriers of the enzyme deficiency develop EPP, while others do not, remains an enigma, but this condition is also characteristic of other dominant forms of the porphyrias: e.g., acute intermittent porphyria, hereditary coproporphyria, and variegate porphyria (2).

It should be noted that the C  $\rightarrow$  T mutation observed did not occur within the consensus donor-splice junction sequence of an intron (29). Several possible mechanisms may be considered for the C  $\rightarrow$  T mutation leading to exon 2 skipping (Fig. 6). First, the site of the C  $\rightarrow$  T substitution at -23 (assuming the first base of exon 2 as +1) is located between the 3'-splice site consensus sequence starting at -19 and a potential branch point at -26. Thus it is possible that this point mutation may alter the affinity of the acceptor site of intron 1, resulting in an alternative splicing between exon 1 and exon 3. Second, an altered sequence in the mutant mRNA—e.g., UGU\*GA (-25 to -21)—could form an abnormal base-paired stem with UCACA (-6 to -2), as well as an unpaired loop. This aberrant stem-loop structure (-25 to -2) could potentially interfere with normal splicing (30, 31). Third, the C  $\rightarrow$  T mutation at -23 could affect the interaction between the putative branch site, 5'-AUUUCAUGC-3' (-31 to -23), and the U2 small nuclear ribonucleoprotein, 3'- $\Delta$ UG· $\Delta$ UG-5' (base-paired nucleotides are underlined), which may inhibit the normal splicing reaction between exon 1 and exon 2 (32–35). Since both intron 1 and intron 2 are extremely long (8 kb and 7 kb, respectively), it may be possible to prepare a truncated precursor RNA for analysis of the mechanism of the splicing defect in this patient, since this method has been successful in the case of other mRNAs (31, 35–37).

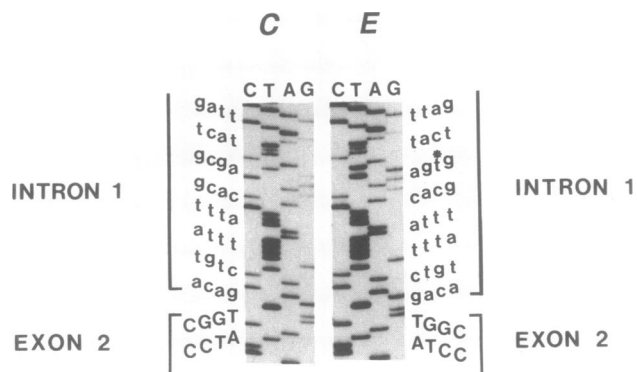


FIG. 5. Nucleotide sequence analysis of ferrochelatase genomic DNA harboring the acceptor site of intron 1. A genomic DNA fragment, harboring exon 2 and its flanking regions, was isolated. Four of 11 clones had a C  $\rightarrow$  T mutation 23 bp upstream of the start site of exon 2. No mutations were found in the donor site of intron 2. (C) Normal sequence. (E) Mutant sequence.

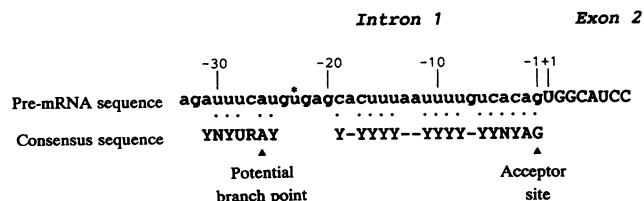


FIG. 6. Nucleotide sequence of the mutant ferrochelatase pre-mRNA and the consensus sequence for the splice junction. Pre-mRNA sequence, the intron sequence is shown in lowercase letters and the exon sequence is shown in uppercase letters. The mutated point (at position -23) is indicated by an asterisk and corresponding bases are indicated by small dots.

Heterogeneous types of mutation have been described in other forms of human porphyria, indicating that various mutations may be responsible for a single clinical disease entity. It is also likely that other mutations may be found in EPP, as more cases are examined at the molecular level. The availability of cloned cDNAs encoding the normal and the aberrant ferrochelatases should facilitate the detection of other molecular forms of the inherited defect of this enzyme.

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