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A point mutation G→A in exon 12 of the porphobilinogen deaminase gene results in exon skipping and is responsible for acute intermittent porphyria

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ABSTRACT

We have determined the mutation in a patient with acute intermittent porphyria. The mRNA coding for porphobilinogen deaminase was reverse transcribed then the cDNA was enzymatically amplified in vitro. Upon sequencing of a polymerase chain reaction product of abnormal size we found that this fragment lacked exon 12 of the gene. We analysed a genomic fragment containing exon 12 and determined that the patient was heterozygous for a point mutation G A at the last position of exon 12. We propose that this base change is responsible for an abnormal processing of the mutant allele such that exon 12 is missing in the mature mRNA. The resulting abberant mRNA encodes a truncated protein which is inactive but stable and can be detected using antibodies directed against the normal enzyme.

INTRODUCTION

Acute intermittent porphyria (AIP) results from a partial deficiency of the third enzyme of heme biosynthesis, porphobilinogen deaminase (PBG-D porphobilinogen ammonia-lyase (polymerizing), EC 4.3.1.8) that is inherited as an autosomal dominant trait (1,2). In affected individuals the mean activity of the deficient enzyme is 50% normal in keeping with the heterozygous state of these subjects. However, most of the people with this genetic enzyme deficiency remain asymptomatic. Clinical manifestation of the disease is usually linked to environmental factors such as drugs, alcohol, and caloric deprivation (2).

The molecular heterogeneity of the mutations responsible for AIP is indicated by immunological studies of the defective enzyme. Different investigators documented the existence a normal amount as well as an excess amount of cross reacting immunological material indicated respectively (CRIM)-negative and CRIM-positive mutations (3,4). Quantitative differences in the amount of CRIM have been found between with CRIM-positive mutations (3,4). Two normal human PBG-D cDNA differing by their 5' extremity were

cloned and sequenced (5,6). The human PBG-D gene has been isolated and its organization characterized (7). This gene has two promoters, one house-keeping and one erythroid-specific and gives raise to two different mRNAS having a specific first exon and 13 common exons (7). The determination of the mutations responsible for different subtypes of AIP has recently started and we have reported two independant point mutations leading to a defective splicing of intron 1. These mutations lead to a rare subtype of AIP characterized by the restriction of the enzymatic defect to non-erythropoietic tissues (8,9). In the more commun forms of AIP with a PBG-D deficiency in all cell types, so far, no mutation has been reported accounting for the enzymatic abnormality.

In one patient with a CRIM negative mutation and a generalized deficiency in the enzyme activity, a preliminary report (10) indicated that a post-transcriptional splicing abnormality may be responsible for the enzyme defect.

In the different subtypes of CRIM positive AIP, no data have been reported so far from DNA or RNA studies. However, because there is evidence for a structurally abnormal protein, it can be assumed that a pathological mRNA is also present in nucleated cells from these patients and that the mutations lie within the coding sequence of the mRNA.

We have undertaken a study of the molecular abnormalities responsible for AIP in different families using the polymerase chain reaction (PCR) to amplify in vitro the PBGD mRNA extracted from lymphoblastoid cell lines. Here we report that a single base substitution G → A at the last position of exon 12 leads to exon skipping and that the shortening of the mRNA produces an abnormal but immunoreactive protein.

MATERIAL AND METHODS

Case report

Subjects consisted of six patients from different families with acute intermittent porphyria. The diagnosis was deduced from clinical and biological abnormalities and measurement of PBG-D activity in red blood cells (11). The enzymatic defect was confirmed on lymphoblastoid cells.

Quantification of cross-reacting immunological material

CRIM was determined in lymphoblastoid cells, derived from EBV transformed B-lymphocytes, of both AIP patients and control individuals according to a method adapted from the Rooij et al. (12). Cells were cultured in RPMI 1640 medium containing 20% fetal calf serum (200.10^6 cells/32 ml) and

collected by centrifugation (10' x 850 g). The cell pellets were washed twice in phosphate buffered saline (Dulbecco's PBS, Flow laboratories) and the final pellet was frozen at -70°C. For CRIM detection the pellet was thawed in 100µl Tris buffer (50 mmol/L Tris.HCl pH 8.0, 20 mmol/L histidine, 10 g/L bovine serum albumin, 0.02 % NaN_3) and subsequent cell disruption was carried out using a vibro-mixer (2 x 45 sec, Model E1 from Chem. Ap. A. G. Mannedorf Z.H., Switzerland) fitted with a glass rod. The CRIM detection was performed in the following way: 50 ul of a protein-A Sepharose suspension (Pharmacia) was placed in microtitration wells, then either 50 ul diluted Rabbit anti-Human PBG-D antiserum (P) or 50 ul diluted control serum (C) was added and incubated for 1 hour at room temperature. 50 ul of the disrupted lymphoblastoid cell suspension was added to the wells, and incubated 1 hour at room temperature. The plates were centrifuged and the residual (unbound) PBG-D activity was determined in the supernatant according to de Rooij et al (13) but using a Tris buffer in addition containing 1 mmol/L EDTA and 1% Tween 20. The CRIM is the amount of antibody that bind 100 units of PBG-D activity (1 unit of PBG-D activity produces 1 pmol of uroporphyrinogen per hour at 37°C) and was calculated by substraction the P value from the corresponding C value of a sample. A increase in CRIM found in AIP samples compared to control individuals illustrates the presence of excess, non-catalytic PBG-D in patients.

RNA and DNA extraction

DNA was prepared from lymphoblastoid cells as described by Kunkel et al (14). Total cellular RNA was prepared according to Adrian and Hutton (15).

In vitro amplification

One ug of total cellular RNA was reverse transcribed in 20 ul of a reaction mixture containing 50 mM Kcl, 20 mM TRIS-HCl at pH 8.4 2.5 mM $MgCl_2$, 100 ug/ml bovine serum albumin, 1 mM dATP 1 mM dCTP, 1 mM dGTP, 1 mM TTP, 0.1 ug oligo dT and 200 units of murine moloney leukemia virus reverse transcriptase. This mixture was incubated for 30 min at 42°C. The reaction was stopped and the RNA-cDNA hybrid were denatured by heating at 100° for 2 minutes then quenched in ice. cDNA sequences were then amplified as described (16) with minor modifications. Briefly 100 ul of reaction mixture contained the cDNA mixture in 50 mmol/L KCl, 20 mmol/L TRIS (pH 8.4), 2 mmol/L $MgCl_2$, 200 mmol/L of each dNTP, 50 pmol of each primer and 2 units of Taq polymerase (Perkin-Elmer Cetus). The two oligonucleotide primers used (phe1 and phe15 are presented in figure 1). The reactions were performed using a DNA thermocycler (Perkin-Elmer Cetus) as follows: cycles of 1 min

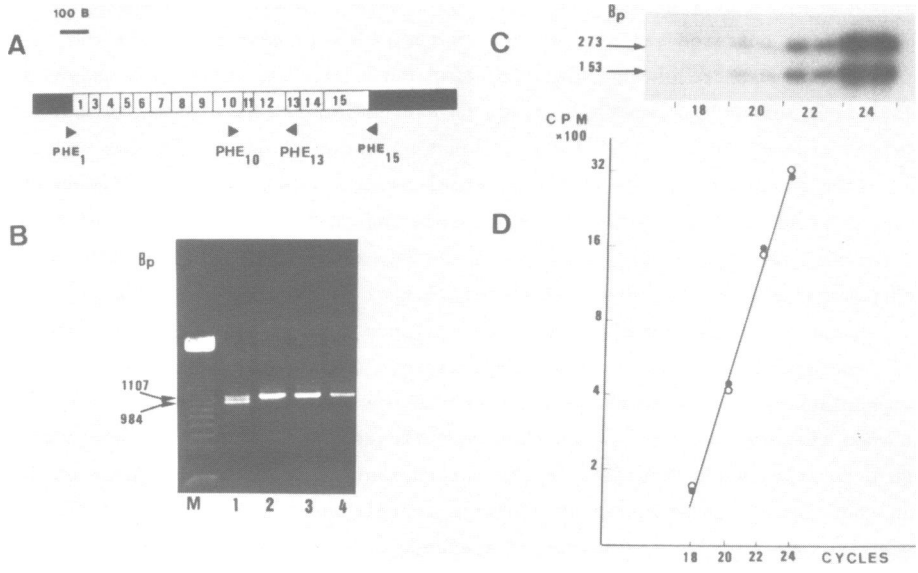

Figure 1. In vitro amplification of PBG-D mRNA

a. Schematic representation of the PBG-D mRNA. Exon are numbered 1 to 15. Exon 1 is linked to exon 3 in the PBG-D mRNA from non-erythroid tissues (7). Dark boxes represent the 5' and 3' non coding regions of exons 1 and 15 respectively. The triangles indicate the position of the oligonucleotides used for the PCR. The scale is indicated in bases. The size of the exons is drawn according to reference 7. The exact exons boundaries and intron-exon junctions are unpublished data (P.H. Romeo, personnal communication).

b. Analysis of the amplification products. PBG-D mRNAs from lymphoblastoid cells of AIP patients with CRM-positive mutations (1,2,3) and normal control (4) were amplified as described in the methods section. The sequence of oligonucleotide primers used for the PCR reaction are: phe 1: CATGTCTGGTAACGGCAATGCGG, phe 15: CTGTGCCCCACAAACCAG, M = 123 bp ladder markers.

Fragments are shown after ethidium bromide staining and migration in a 1.5% agarose gel in TBE buffer. The numbers on the left indicate the estimated size of the two bands observed for patient 1 by comparison with the markers.

c. Amplification of two $cDNA_S$ fragments from patient 1. PCR was conducted using oligonucleotides phe_{10} and phe_{13} from 0.2 μg of patient's RNA. The conditions were as described in the method's section for phe_1 and phe_{15} except that 5 Ci of ^{32}PdCTP were added in each reaction and that the concentration of $MgCl_2$ was 2.5 mM. After the indicated number of cycles, 10 μl aliquots of each reaction were run in duplicate on a 2% agarose gel. The DNA was fixed with 8% acetic acid then the gel was dried and autoradiographed. The arrows indicate the sizes of the amplified fragments. The sequence of the oligonucleotides is as follows:
phe_{10}: GGAAGCTGGACGAGCAGCAGGA
phe_{13}: CTACTGGCACACTGCAGCCTC

denaturation of 95°C, 1 min annealing at 55°C and 1.5 min elongation at 72°C were repeated 30 times, the elongation time of the last cycle being 5 minutes.

Direct sequencing of the PCR products

After resolution in a 1.5% low gelling temperature agarose (Sigma type VII) the abnormal band was excised and recovered by electroelution and ethanol precipitated. 100 ng of the fragment in 7 ul of water was used for direct DNA sequencing. 2 ul of 5 x sequenase buffer (200 mmol/l TRIS HCl pH 7.5, 50 mmol/l $MgCl_2$, 250 mmol/l NaCl), 5 pmol of the sequencing primer was added. This mixture was heat-denatured at 100°C for 3 minutes and quenched in ice. The labelling and sequencing reactions were carried out using ^{35}SdATP (Amersham) and the sequenase sequencing kit (USB Corporation) according to the instruction of the manufacturer, except that the annealing step was omitted.

In vitro amplification of the genomic DNA

Oligonucleotides hybridizing to coding strand of exon 13 (phe_{13}) to the minus strand of intron 11 (phi 11) are described of figure 3. They were used for amplifying a 426 bp fragment from genomic DNA containing exon 12. The PCR conditions were identical to that described for cDNA amplification except that the hydridization temperature was 60°C.

Cloning and sequencing of PCR products

The PCR products obtained from genomic DNA as described above were purified from low gelling temperature agarose. The purified DNAs were phosphorylated and then blunt-ligated into Sma 1 digested, phosphatase treated PGEM 7Z (Promega Biotech) vector. Clones DNA was then sequenced using the SP6 and T7 promoter primers using a sequenase sequencing kit.

Analysis of amplified products by hybridization

Amplified fragments from genomic DNA were electrophoresed in a 2% agarose gel, blotted to Zeta-probe membrane under alkaline conditions, then hybridized to allele-specific oligonucleotides (figure 4). For hybridization analysis, 10 ul of amplified sample was processed as described. Zeta-Probe

d. Quantification of the two amplified fragments. The radioactivity incorporated in the fragments visualized in (c) was determined by scintillation counting and plotted on a semi-logarithmic scale against the number of cycles. The efficiency factor of the amplification was calculated according to Chelly et al (17). Open and closed circles represent the radioactivity incorporated in the 273 bp and the 153 bp fragments respectively.

Table 1.

Cell line	- IgG pmol	+ IgG pmol	delta pmol	CRM L IgG/100 units	CRM ratio
Patient 1	16.32	4.97	11.35	5.28	1.85
Patient 2	16.58	3.56	13.02	4.61	1.61
Patient 3	17.74	8.46	9.28	6.47	2.26
Control	29.16	8.24	20.93	2.86	1

Cross reacting immune material in lymphoblastoid cells from AIP patients of various families and a control individual. PBG-D activity is determined either in the presence or absence of rabbit anti-human PBG-D antibodies. Experimental procedures are described in the method section.

nylon filters were prehybridized in 5 x SSEP/5 x Denhardt's solution/0.5% $NaDodSO_4$ for 1 hr at 55°C and then hybridized at the same temperature for 2-4 hr with radiolabeled oligonucleotide at 10^6 cmp/ml. (1 x SSEP = 0.15 M NaCl/0.01 M NaH_2Po_4/0.001 M EDTA, ph 7.4; 1 x Denhardt's solution = 0.02% polyvinylpyrrolidone/0.02% Ficoll/0.02% bovine serum albumin). The probe was labeled at the 5' end with γ ^{32}-P ATP to a specific activity of 10^9 cpm/ug. Blots were washed at room temperature in 2 x SSEP for 30 min and then in 5 x SSEP/0.1% $NaDodSO_4$ at 68°C for 4 min and autoradiographed at -70°C with one Dupont Cronex Lightning Plus intensifying screen.

RESULTS

In order to investigate the nature of the mutations in patients with AIP we set up the conditions for amplifying in vitro the coding sequence of the PBG-D cDNA starting from patients mRNA.

In vitro amplification of PBG-D cDNA was carried out after oligo T primed reverse transcription of total cellular RNA. The primers used for the PCR allowed the amplification of the total length of the coding sequence (figure 1) and, after 30 cycles, a clearly detectable band could be visualized by ethidium bromide staining of agarose gels (figure 1b).

From six patients under investigation three had clearly CRIM positive mutations with a CRIM ratio between 1.61 and 2.26 (table 1) and three had CRIM negative mutations. Analysis of the PCR products after in vitro amplification revealed for all patients a band of normal size and for only one of them (with a CRIM positive mutation) an additional band corresponding to an amplified product of smaller size than normal. The difference being estimated to about 120 bp. Since AIP patients are heterozygous for a

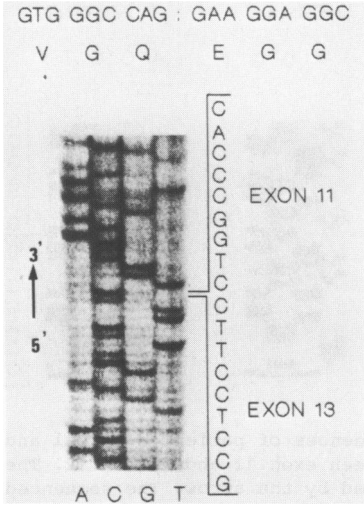

Figure 2. Nucleotide sequence of the patient's aberrant cDNA.
Top, nucleotide and aminoacid sequences of parts of exons 11 and 13 as they have been reported (5,6). The exon boundaries are symbolized by: The abnormal fragment from amplification of patient's 1 mRNA was isolated and sequenced using a oligonucleotide primer phe_{14}: CATGGTAGCCTGCATGGTCTC hybridizing to the coding strand of exon 14. The antisens strand is read in the 5'→ 3' direction.

defective PBG-D allele it was likely that the shorter fragment reflected the presence in patient's cells of the transcript from the mutant gene in addition to the normal one. From the size of the different exons we suspected that the absence of either exon 10 or exon 12 in the abnormal product might account for its smaller size. To test this hypothesis we isolated the abnormal fragment. After electrophoresis by electroelution and ethanol precipitation 100 ng of the fragment was then directly sequenced using a primers hybridizing to exon 14. From the sequences it became evident that exon 12 was missing and that exon 11 was precisely linked to exon 13 (figure 2). The ratio between the amount of the two $mRNA_S$ was determined by coamplifying the corresponding $cDNA_S$ in the presence of labelled dCTP for different numbers of cycles. Under the described conditions (figure 1c) we estimated that the two fragments, respectively 273 bp and 153 bp in size, were amplified exponentially between 18 and 24 cycles and that the efficiency factor (17) was 0.7 for both of them (figure 1d). Since the ratio between the radioactivity incorporated into the two amplified fragments was always 1 we

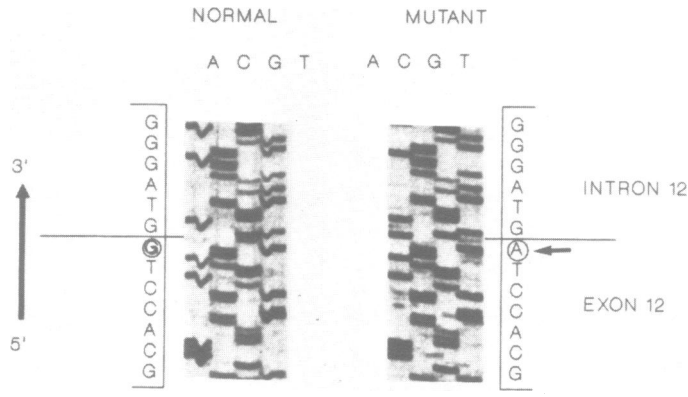

Figure 3. Nucleotide sequences of patient's normal and mutant alleles around the splice junction between exon 12 and intron 12. The base change in the mutant allele is indicated by the arrow. The sequenced fragments were obtained as described in the legend of figure 4 and subcloned into a plasmid vector.

can conclude that the RNA_S from which they derive are present in approximatly equal amounts in patient's cells.

In order to precisely determine the nature of the mutation leading to skipping of exon 12 in the mutant mRNA, we chose a set of primers, one matching the + strand of intron 11 and the other the - strand of exon 13. This set of primers was used to amplify in vitro exon 12 and the flanking sequences yielding an amplified fragment of 426 bp. Attempts to directly sequence this fragment suggested a mutation at the last position of exon 12 but several ambiguities within the sequence prevented us to draw a definitive conclusion. We therefore decided to clone the amplified fragment into the plasmid vector PGEM 7Z (Promega Biotech) and sequenced four independant clones two of which displayed a G→ A transition within the donor splicing site of intron 12. The normal sequence CTG/GTAGGG being changed to CTA/GTAGGG (figure 3). We then confirmed that this base change was not an error of the Taq polymerase during the amplification process by hybridization of the uncloned amplified fragment with allele-specific oligonucleotides. One of the oligonucleotides matched the normal sequence and the other matched the mutated one. As expected, the amplified fragment from the patient hybridized to both probes in agreement with the fact that he is heterozygous for the mutation, although amplified DNA from normal controls only hybridized with the oligonucleotide of normal sequence (figure 4). Since no other abnormality was detected in the sequence of the mutant allele it is extremely likely that

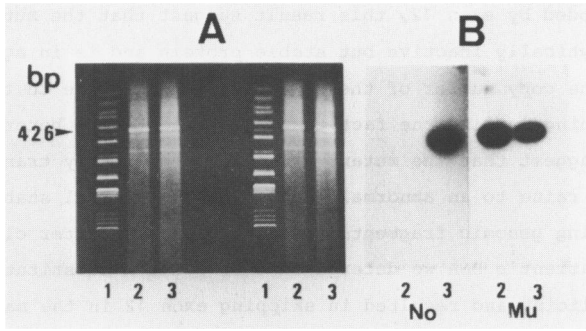

Figure 4. Direct detection of the mutation by hybridization of PCR products with allele-specific oligonucleotides. Genomic DNA from patient's and control's cells was submitted to PCR with the oligonucleotides phi 11: TTGGGGAAAATCAGGCCTGATG and the phe 13: CTACTGGCACACTGCAGCCTC.

a. 10 ul of each PCR was electrophoresed in duplicate on a 1.5% agarose ethidium-bromide stained gel 1 = size markers, 2 = patient, 3 = normal control

b. The same gel was southern blotted and hybridized to allele-specific oligonucleotides.
M_u = mutated probe GAGGCACCTAGTAGGGCCT
N_o = normal probe GAGGCACCTGGTAGGGCCT

the observed mutation is indeed responsible for skipping of exon 12 during post-transcriptional maturation of the primary transcript from the mutated allele.

DISCUSSION

Here we describe the isolation and characterization of a mutation responsible for acute intermittent porphyria. We have investigated a patient with a CRIM positive mutation by analysing the expression of the mutant allele. Using in vitro amplification of the patient's RNA isolated from lymphoblastoid cells we evidenced the presence of two amplified fragments: one of normal size and the other of smaller size. By directly sequencing the abnormal fragment we found that exon 12 of the gene was absent. This was considered to reflect the presence in patient's cells of a mutant mRNA coexisting with the normal one (figure 1b). A more quantitative estimation of the ratio between the two RNA_S strongly suggest that they are present in similar amounts. Since junction of exon 11 to exon 13 does not disrupt the reading frame the mutant mRNA was expected to be translated into a truncated protein missing the 40 amino acid residues encoded by exon 12. Immunological studies using a polyclonal rabbit antiserum against PBG-D demonstrated a CRIM ratio of about 2. Assuming that no major antigenic determinant is present in

the peptide encoded by exon 12, this result suggest that the mutant mRNA encodes a catalytically inactive but stable protein and is in agreement with the idea that the copy number of the mutant in RNA is close to that of the normal one. Keeping in mind the fact that AIP patients are heterozygous, these results suggest that the mutant allele is efficiently transcribed and spliced to give raise to an abnormal form of mRNA of normal stability.

By sequencing genomic fragments containing exon 12 after cloning PCR products from patient's DNA we determined that a G → A substitution caused aberrant RNA splicing and resulted in skipping exon 12 in the mature mRNA. This point mutation is located at position -1 relative to the 5' splice site of intron 12 and this was confirmed by direct hybridization of amplified products to allele-specific oligonucleotides. The observed base change suppress a $BstN_1$ site; although the mutation could theoritically be detectable by Southern blot analysis of unamplified genomic DNA, the sizes fo the expected fragments (83 bp and 137 bp for the normal allele) are too small to allow an easy detection of the abnormality using this approach. The role of specific bases in the splicing of higher eukaryote pre-mRNAs has been extensively studied by both mutagenesis of cloned genes and analysis of naturally occuring mutations (18). Moreover a systematic analysis of known RNA splice function sequences of eucaryotic protein coding genes was carried out (18). These studies confirmed the Chambon's rule (20) that the 5' and 3' - splice sites conform consensus sequences in which GT and AG dinucleotides are absolutely conserved. Indeed, mutations at these sites abolish normal splicing and there are several examples for which alteration of the GT dinucleotide at a splice donor site results in the skipping of the preceeding exon (21,22). Until now, only very few mutations have been reported in the exon residues of the 5' splice site (23,24) whereas several point mutations have been detected in the intron residues (19,21).

The functional consequence of the mutation described here may appear surprising since the mutated 5' splice site still contains a 5 nucleotides match with the consensus. When analyzed according to Shapiro and Senapathy the mutated sequence scored 71 instead of 83.4 for the normal 5' splice site, for comparison the normal sequence of the 5' splice site of intron 11 scores 75 (not shown) and authentic intron donor sites have been described with scores less than 70 (19). However, since pairing to U_1 snRNA seems to be a requirement for splicing, the position of the bases matching the consensus relative to each other may be a determining factor. It is noteworthy in this respect that the mutation that we describe changes one of the four

Table 2. Normal donor sites of intron 11 and 12 in the human PBG-D gene and mutated site of intron 12

Exon-intron bundary	Sequence	Score	ΔG kcal/mol
12 normal	CTG/GTA GGG	83,4	- 3,4
12 mutated	CTA/GTA GGG	71	+ 1,4
consensus	CAG/GTA AGT	100	- 10,9

The bases matching the consensus sequence are underlined. The scores (between 0 and 100 were calculated according to Shapiro and Senapathy (18). The ΔG values were calculated according to Freir et al. (27) for the 5' splice region-U_1 Sn RNA interaction.

consecutive bases able to pair with the U_1 snRNA. The ΔG value for the 5' splice region - U_1 snRNA interaction is therefore much decreased (Table 2). Our present results emphasize the importance of the contribution of the last nucleotide of the 5' exon for normal splicing.

Previous work at the protein level (3,4) and RFLP studies (26,27) indicated that acute intermittent porphyria is a heterogeneous disease at the molecular level and that point mutations rather than large deletions are likely to account for most of the cases (26). Here we used a newly developped approach to determine the molecular abnormality responsible for CRIM positive mutations, namely the in vitro amplification of mRNA. It is noteworthy that from three patients with the subtype of AIP studied here only one had a RNA with an abnormal size. A previous report demonstrated the presence of a shortened RNA in a patient with a CRIM negative mutation (10). From the size of the RNA "deletion" it is likely that a different exon from exon 12 was absent from the mutant RNA. These results emphasize the fact that even in phenotypically homogeneous subtypes of AIP, different mutations may be responsible for the disease and that, on the contrary, similar mechanisms may lead to different consequences on the mutant protein. It is likely that in vitro amplification of DNA or cDNA followed by sequencing of the amplified product will play an important role for delineating the spectrum of the mutations responsible for AIP and for analyzing the molecular epidemiology of these mutations.

The knowledge of the mutation of CRIM positive AIP subtypes in combination with the details about protein-substrate interaction will perhaps

reveal the exact enzymatic mechanism of the coupling of porphobilinogen molecules to 4-hydroxymethylbilane.

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