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GT to AT transition at a splice donor site causes skipping of the preceding exon in Phenylketonuria

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ABSTRACT

Classical Phenylketonuria (PKU) is an autosomal recessive human genetic disorder caused by a deficiency of hepatic phenylalanine hydroxylase (PAH). We isolated several mutant PAH cDNA clones from a PKU carrier individual and showed that they contained an internal 116 base pair deletion, corresponding precisely to exon 12 of the human chromosomal PAH gene. The deletion causes the synthesis of a truncated protein lacking the C-terminal 52 amino acids. Gene transfer and expression studies using the mutant PAH cDNA indicated that the deletion abolishes PAH activity in the cell as a result of protein instability. To determine the molecular basis of the deletion, the mutant chromosomal PAH gene was isolated from this individual and shown to contain a GT-->AT substitution at the 5' splice donor site of intron 12. Thus, the consequence of the splice donor site mutation in the human liver is the skipping of the preceding exon during RNA splicing.

INTRODUCTION

Classical Phenylketonuria (PKU) is an autosomal recessive human genetic disorder caused by a deficiency of hepatic phenylalanine hydroxylase (PAH, phenylalanine 4-monooxygenase, EC 1.14.16.1). In the normal human liver, PAH catalyzes the hydroxylation of phenylalanine to tyrosine. Absence of PAH activity causes an accumulation of serum phenylalanine, resulting in hyperphenylalaninemia and a depletion of its normal catabolites. Without detection at birth followed by rigid implementation of a restricted synthetic diet low in phenylalanine, the abnormalities associated with phenylalanine metabolism in PKU patients will cause postnatal brain damage and severe mental retardation. PKU is the most common inborn error in amino acid metabolism. Its incidence among Caucasians is about 1:10,000, and the mutant gene frequency predicts that 1 in 50 individuals is a carrier of the disease trait (for reviews, see ref. 1,2).

To understand the molecular basis of PKU, we previously isolated PAH cDNA clones from two independent human liver cDNA libraries (3, 4). The original

liver cDNA library was constructed in pBR322 (5) and provided several partial-length PAH cDNA clones (3) representing the 3'-half of PAH mRNA. The second cDNA library was constructed in λgt11 from a different liver source, from which a full-length 2.4 kb PAH cDNA (phPAH247) was obtained (4). Clone phPAH247 has been sequenced (4) and shown to code for enzymatically active PAH upon transfection into mammalian cells (6). When we characterized the partial-length human PAH cDNA clones from the original library by restriction mapping and sequence analysis, normal and mutant clones were identified. The normal cDNA was comparable to hPAH247 and contained a 588 bp HindIII fragment in the peptide coding region, while the mutant cDNA contained an internal 472 bp HindIII fragment instead. In this paper we report that this fragment size difference in the mutant cDNA resulted from a deletion of the entire exon 12 sequence (coding for amino acids #401-438 of PAH) during mRNA processing. This deletion results in a frameshift mutation that abolishes PAH stability and enzymatic activity. The presence of normal and mutant PAH mRNAs in the liver of the "apparently normal" individual used in this study demonstrated that this individual was a PKU carrier. The molecular cloning and characterization of the corresponding mutant chromosomal PAH gene demonstrated a GT-->AT transition at the 5'-donor splice site of intron 12, which is identical to the one we recently reported for the most prevalent PKU allele among Caucasians of Northern European decent (7). The cloning of a mutant PAH cDNA from a PKU carrier in this report enabled us to show that the intron 12 splice donor site mutation in PKU results in skipping of exon 12 during mRNA processing *in vivo*.

MATERIALS AND METHODS

Enzymes and Reagents

Restriction endonucleases and T4 DNA ligase were purchased from New England Biolabs. *Escherichia coli* DNA polymerase I (Klenow fragment) was purchased from Boehringer Mannheim. T4 polynucleotide kinase and the M13 cloning/sequencing kit was obtained from PL Biochemicals. [α-^{35}S]dATP (650Ci/mmol) for sequencing and [^{14}C] phenylalanine (600 Ci/mmol) for PAH activity assays were purchased from Amersham Corporation, and [γ-^{32}P]ATP (7000 Ci/mmol, crude fraction) was purchased from ICN.

Isolation and Characterization of Human PAH cDNA Clones

We previously constructed a human liver cDNA library (comprised of 40,000 independent transformants) cloned into the PstI site of pBR322 by the dG-dC tailing method (5). The human cDNA library was screened using a previously

cloned rat PAH cDNA (9) and a total of eight PAH cDNA clones were isolated from this library (3). Restriction enzyme mapping of the cDNA clones and selected DNA fragments reported in this paper was carried out as described by Kwok et al (4).

DNA Sequence Analysis

The internal 588 bp HindIII fragment isolated from PAH cDNAs were inserted into M13mp18(10), and sequenced by the dideoxynucleotide chain-termination method (11). The entire sequence of the HindIII fragment was determined by using M13 clones containing inserts in both orientations. The remaining sequence of the PAH cDNA was determined by the chemical modification method of Maxam and Gilbert (12) using the sequencing strategy previously reported for hPAH247 (4). The plasmids were digested with the appropriate restriction endonuclease and either 5' end labelled with polynucleotide kinase and [γ-^{32}P]ATP or 3' end labelled with DNA polymerase I (Klenow fragment) and [α-^{32}P]dNTP. After digestion with a second restriction enzyme, single end labelled fragments were isolated from 5% polyacrylamide gels for DNA sequence analysis.

Plasmid Constructions

Standard cloning techniques were used for plasmid constructions. Eukaryotic expression vector p91023(B) (13) was linearized with EcoRI and treated with calf intestinal alkaline phosphatase (2.5 units/50 pmol of 5' termini). This vector contains the SV40 origin of replication, which enables propogation of recombinant clones in monkey kidney (COS) cells (14). To construct the normal PAH cDNA expression clone, phPAH247 was restricted with EcoRI and the full-length cDNA insert was isolated by preparative low melting temperature agarose gel electrophoresis (International Biotechnologies, Inc.). PAH cDNA insert (10ng) was ligated to p91023(B) (90ng) in a 20 µl reaction at 8^{o}C overnight. Ligated DNA was used to transform E. coli HB101, and bacterial transformants were selected for resistance to tetracycline (7.5 µg/ml of growth medium). DNA was isolated from transformants by the alkaline lysis procedure (15) and the orientation of PAH cDNA insert in the p91023(B) vector was determined by restriction enzyme mapping (4).

To construct the full-length mutant PAH cDNA expression clone, the 588 bp internal HindIII fragment of hPAH247 was replaced by the corresponding HindIII fragment (containing the 116 bp deletion) from the partial-length mutant PAH cDNA (Fig. 3A). The orientation of the mutant HindIII fragment within the cDNA was then determined by restriction endonuclease mapping (4). The resulting full-length mutant PAH cDNA fragment was ligated into the EcoRI site of p91023(B) as described above for the normal PAH cDNA.

Gene Transfer Experiments

Normal and mutant PAHcDNA/p91023B constructs were used to transfect COS cells in suspension by the Chu and Sharp method (16). Briefly, cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% bovine calf serum. Cells from three 15 cm plates (about 10^8 cells) were transfected in 2 ml of DNA-calcium phosphate cocktail (16) (containing 50 µg of recombinant plasmid) for 15 minutes at room temperature in a 50 ml polypropylene tube, followed by the addition of 40 ml of DMEM supplemented with 6.25 mM $CaCl_2$. Ten ml aliquots of the suspended cells were then plated onto each of four 15 cm plates. After 5 hours at 37°C, the cells were shocked in 25% glycerol, and then incubated in growth medium for approximately 36 hours.

PAH mRNA and Protein Analysis

Transfected cells from three 15 cm plates were harvested by scraping in 30 ml of phosphate buffered saline (PBS), centrifuged, and resuspended in 400 µl of PBS. PAH activity assays and Western blot analysis were carried out on cell extracts as previously described (6). RNA was isolated from cells from one 15-cm plate by phenol extraction and spotted directly on nitrocellulose using a slot blot manifold (Schleicher and Schuell). The filter was hybridized to labelled hPAH247.

Genomic DNA Cloning

Frozen liver tissue (0.5g) was ground to a fine powder in liquid nitrogen using a mortar and pestle. After the liquid nitrogen evaporated, 10ml of lysis buffer (0.1 M NaCl, 1.0 mM EDTA, 0.05 M Tris-HCl (pH7.5), 100 µg/ml proteinase K, and 0.5% SDS) was immediately added, and DNA was isolated as described (17). Genomic DNA (100 µg) was digested to completion with EcoRI and size fractionated on a 1.25-5 M NaCl gradient (17). Lambda (λ) bacteriophage CH4A cloning arms were prepared by ligation of the cohesive termini and digestion with EcoRI as described (18). Contaminating stuffer fragments (6.6 kb and 7.8 kb) were removed from the cloning arms (31 kb) by NaCl gradient centrifugation. Size-fractionated genomic DNA (0.45 µg; 10-20 kb) was ligated to λCH4A arms (1.3 µg) in a 16 µl reaction at 12°C overnight. The resulting concatenated DNA molecules were packaged with extracts prepared by the procedure of Grosveld et al. (19), and the infectious bacteriophage particles were used to transduce E. coli LE392. The genomic library was screened by the amplification method of Woo (20) using hPAH247 as the hybridization probe.

Oligonucleotide Hybridization Analysis of Genomic Clones

DNA was isolated from positive bacteriophage plaques as previously described (4). EcoRI plus HindIII digests of the recombinant clones were electrophoresed on 1% agarose gels, stained with ethidium bromide, and processed for direct-gel hybridization (21). The dried gel membranes were hybridized overnight at 37°C in 6 x NET (0.9 M NaCl, 6 mM EDTA, 0.5% SDS, and 0.09 M Tris, pH7.5) containing 0.2 mg of salmon sperm DNA and 2 x 10^6 cpm of oligonucleotide probe per ml of hybridization solution. The gel membranes were then washed in tetramethylammonium chloride at T_D-5°C (21) and then autoradiographed between two Quanta III intensifier screens (Dupont) at -80°C.

S1 Endonuclease Mapping

^{32}P-end labelled DNA probe (50,000 cpm) was co-precipitated with human liver cytoplasmic RNA (50 µg), pelleted by centrifugation, and resuspended in 30 µl of hybridization buffer (30 mM piperazine-N-N'-bis (2-ethanesulfonic acid) (pH6.4), 1mM EDTA (pH8.0), 300 mM NaCl, and 70% deionized formamide). After hybridization, 320 µl of ice-cold S1 nuclease buffer (300 mM NaCl, 50 mM Na-acetate [pH4.5], 2 mM zinc acetate [pH4.5]) containing 2,000 units of S1 nuclease (Miles Laboratories, Inc.) was added to the reaction, and the sample was incubated at 37°C for 2 hours. The reaction was then stopped by the addition of 14 µl of stop buffer (0.5 M Tris-HCl, 0.25 M EDTA (pH7.5)), extracted once with an equal volume of phenol/chloroform/isoamyl alcohol (24:24:1) and then ethanol precipitated. The S1-nuclease resistant DNA-RNA hybrid was collected by centrifugation, dried under vacuum, and dissolved in 10 µl of loading buffer (50 mM NaOH, 1mM EDTA, 2.5% Ficoll, and 0.025% bromocresol green). The sizes of the protected fragments were determined by alkaline-agarose gel electrophoresis and autoradiography.

RESULTS

Identification of an Internally Deleted Human PAH cDNA Clone

We previously reported the isolation of several partial-length PAH cDNA clones representing the 3' half of PAH mRNA from a human liver cDNA library constructed in pBR322 (3). Restriction endonuclease analysis of these recombinants established the overlapping regions of the inserts and revealed normal and mutant cDNA types (Fig. 1). The normal cDNA (lane 1) contained an internal 588 bp HindIII fragment that was longer than the corresponding 472 bp

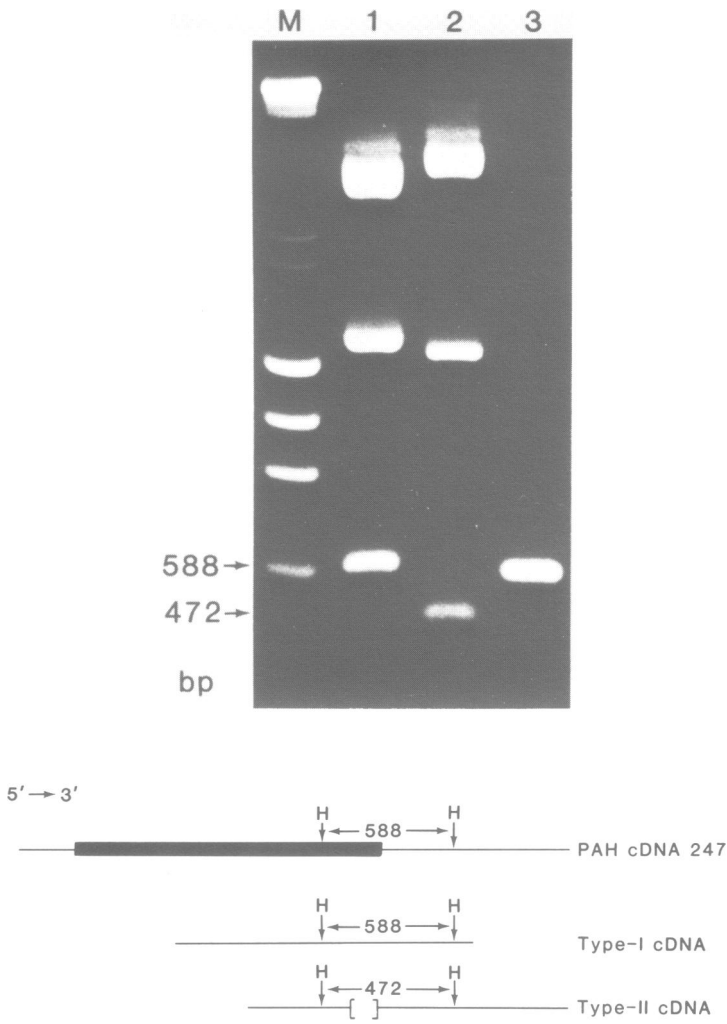

Figure 1. Identification of normal and mutant PAH cDNA clones.
Lane M, bacteriophage λ-HindIII and QX174-HaeIII restricted DNA size markers; lane 1, normal cDNA clone restricted with HindIII; lane 2, mutant cDNA clone restricted with HindIII; lane 3, purified 588 bp HindIII fragment derived from hPAH247 (4). The solid box in hPAH247 represents the coding region, and the solid lines represent the untranslated regions. Normal and mutant cDNA's are partial length PAH clones that were isolated from a human liver cDNA library constructed in the PstI site of pBR322(3). The overlapping regions of the cDNA clones and the size comparison of the internal HindIII fragments are shown schematically on the right. Brackets denote the deleted region of the mutant PAH cDNA. H, HindIII.

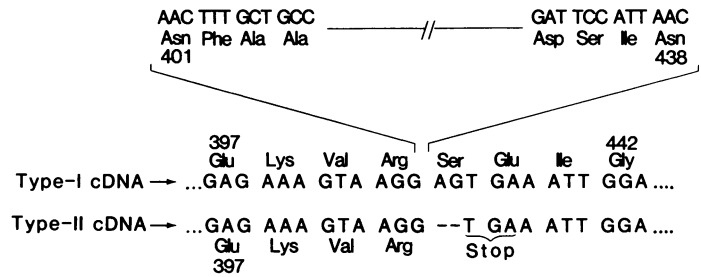

Figure 2. Alignment of homologous nucleotide sequences of normal and mutant cDNA's in the regions flanking the deletion. Nucleotides 1423 to 1536 encoding amino acids 401 to 438 of PAH (4), in addition to nucleotides 1537 and 1538 of the Ser (#439) codon (dashes), are deleted from the mutant cDNA.

HindIII fragment from the mutant cDNA (lane 2). The internal HindIII fragment purified from hPAH247 (4) (lane 3) comigrated with the corresponding HindIII fragment of the normal cDNA (lane 1), suggesting that the mutant cDNA phenotype resulted from a deletion within the internal HindIII fragment. The upper two bands in lanes 1 and 2 correspond to pBR322 and cDNA sequence flanking the internal PAH HindIII fragment.

The precise location of the deletion in the mutant cDNA was defined by sequence analysis. The nucleotide sequence of the internal HindIII fragment derived from the normal cDNA was identical to that previously reported for hPAH247, which corresponds to nucleotides 1309 to 1902 of the cDNA (4). The sequence derived from the mutant HindIII fragment was identical to the normal cDNA except for a 116 bp internal deletion of contiguous DNA spanning nucleotides 1423 to 1538, which includes the 3'-coding sequence of PAH cDNA (Fig. 1, open bracket). Interestingly, the 116 bp deleted region corresponded precisely to exon 12 of the human chromosomal PAH gene (8). The nucleotide sequences flanking the internal HindIII fragments of both normal and mutant cDNA's were identical to the corresponding regions of hPAH247 (data not shown).

Exon 12 deletion abolishes PAH enzymatic activity and stability

Figure 2 shows the alignment of the nucleotide sequences of the normal and mutant cDNA's in the immediate 5' and 3' regions flanking the deletion. The deletion includes 114 bp encoding amino acids 401 to 438 of PAH and an AG dinucleotide deletion in the Ser #439 codon, resulting in a frameshift mutation that introduces a premature termination codon (UGA) following Arg 400 in the mutant cDNA. Since this mutation would result in a PAH product truncated by 52 amino acids at the carboxyl-terminus, we determined its effect on PAH activity by gene transfer and transient expression experiments.

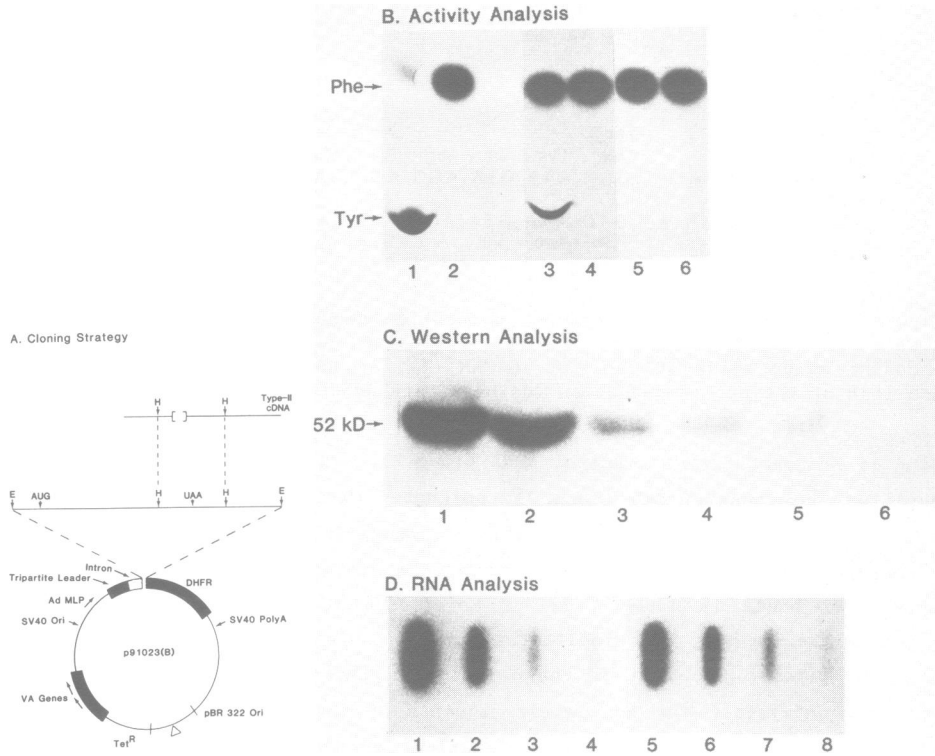

Figure 3. Biochemical analysis of COS cells transfected with normal and mutant PAH cDNA's. A). Cloning strategy: the full-length PAH cDNA insert was isolated from phPAH247 by EcoRI restriction. The start codon (AUG) and stop codon (UAA) define the boundaries of the coding region of the cDNA. A full-length mutant PAH cDNA was generated by replacing the HindIII fragment of hPAH247 with the corresponding HindIII fragment from mutant cDNA. Normal and mutant PAH cDNA's were cloned into the EcoRI site of the eukaryotic expression vector p91023(B). B). PAH activity assay: assays were carried out as previously described (6) in the presence (odd numbered lanes) or absence (even numbered lanes) of the synthetic cofactor tetramethylhydropterin. Lanes 1 and 2, normal human liver extract; lanes 3 and 4, extracts from COS cells transfected with normal PAH cDNA; lanes 5 and 6, extracts from COS cells transfected with mutant PAH cDNA. C). Western blot analysis of PAH in cells transfected with normal and mutant PAH cDNA. Lane 1, normal human liver extract (100µg); lanes 2, 3 and 4, extracts from normal PAH cDNA-transfected cells (100, 5, and 0.5 µg, respectively); lane 6, extracts from mutant PAH cDNA-transfected cells (100µg). D). Slot-blot hybridization analysis of PAH mRNA content in cells transfected with normal and mutant PAH cDNA. Lanes 1-4, total RNA isolated from normal PAH cDNA-transfected cells (10, 5, 1, and 0.5 µg, respectively); lanes 5-8, total RNA isolated from mutant PAH cDNA-transfected cells (10, 5, 1, and 0.5µg, respectively).

A full-length PAH cDNA bearing the deletion was constructed by replacing the normal 588 bp HindIII fragment of hPAH247 with the 472 bp HindIII fragment isolated from the mutant cDNA (Fig. 3A). The normal and internally deleted recombinant cDNAs were then cloned into the EcoRI site of the eukaryotic expression vector p91023(B) (13), downstream of the adenovirus major late promoter (Ad MLP). PAH activity assays measuring the conversion of [^{14}C]-phenylalanine to [^{14}C]-tyrosine were performed using extracts from cells transfected with the normal and mutant PAH cDNA constructs. PAH activity was present in extracts from control human liver (Fig. 3B, lane 1) and in extracts from cells transfected with the normal PAH cDNA/p91023(B) construct (Fig. 3B, lane 3). However, PAH activity was not detected in extracts from cells transfected with the mutant PAH cDNA/p91023B construct (Fig. 3B, lane 5), or in assays carried out in the absence of cofactor (Fig. 3B, lanes 2, 4 and 6). The complete absence of PAH activity is indicative of the Classical PKU phenotype and can be attributed specifically to the deletion of the exon 12 coding sequence in the mutant mRNA.

Western blot analysis using a PAH-specific antibody (6) demonstrated a 52-kd protein present in normal human liver extract (Fig. 3C, lane 1). Extracts from transfected cells expressing normal PAH cDNA also contained abundant levels of immunoreactive PAH (Fig. 3C, lane 2) which was identical in size to that present in the control liver. When the Western analysis was carried out under conditions that could detect less than 1% of the normal PAH levels (Fig. 3C, lane 5), no immunoreactive protein was detected in extracts from cells transfected with the mutant PAH cDNA construct (Fig. 3C, lane 6). Hybridization analysis of RNA isolated from transfected cells demonstrated that both the normal (Fig. 3D, lanes 1-4) and mutant (Fig. 3D, lanes 5-8) cDNA constructs expressed the respective mRNAs at comparable levels.

Characterization of the mutation causing exon 12 deletion

Nucleotides 1423 to 1538 deleted from the mutant PAH cDNA correspond to exon 12 of the PAH gene (8). Since sequences encoded by exons 11 and 13 were present in the mutant clone, the molecular basis of this mutation could result either from an exon 12 deletion in the mutant PAH allele or skipping of exon 12 during processing of the mutant PAH mRNA transcript. To distinguish these possibilities, we constructed a genomic DNA library from the human liver sample from which the mutant cDNA was obtained. We screened the library with hPAH247 and isolated the corresponding genomic DNA clones. Since the mutant cDNA deletion corresponded to exon 12, we focused our analysis on this region

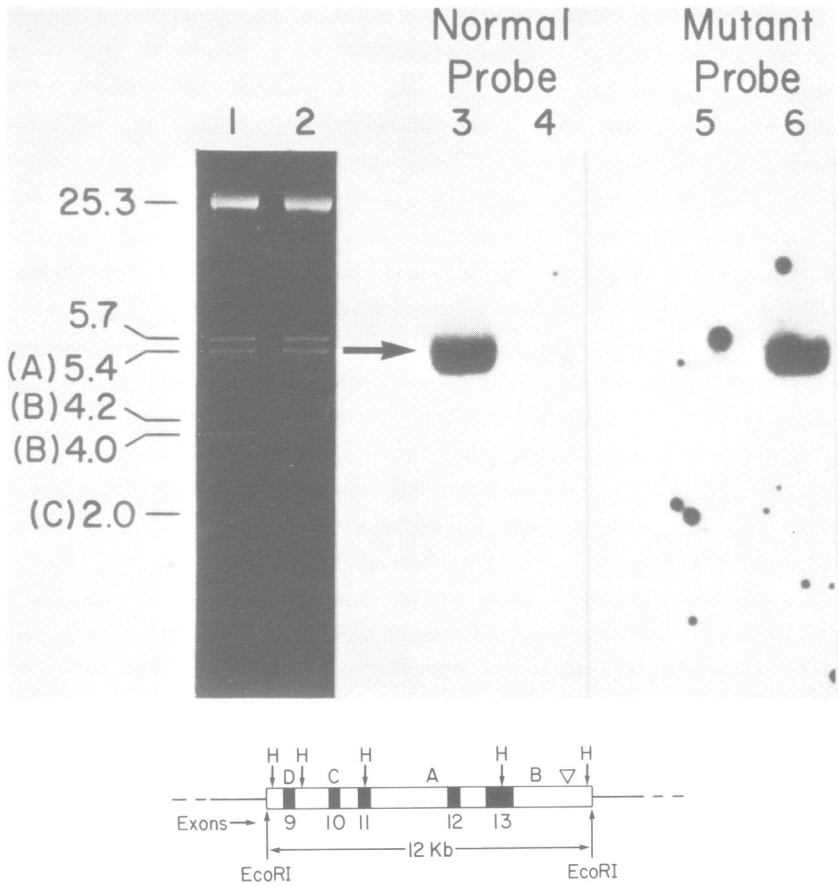

Figure 4. Oligonucleotide hybridization analysis of normal and mutant PAH genomic clones. The 12-kb EcoRI fragment (box) containing exons 9 to 13 and 3'-flanking region of the PAH gene was cloned into the EcoRI site of λCH4A vector (solid lines). HindIII (H) plus EcoRI restriction of the recombinant clones were electrophoresed on 1% agarose gels, stained with ethidium bromide (lanes 1 and 2), and processed for direct-gel hybridization as previously described (7). Dried gels were hybridized to normal (5'-AAATTACTTACTGTTAATGGA-3') (lanes 3 and 4) and mutant (5'-TCCATTAACAATAAGTAATTT-3") (lanes 5 and 6) specific intron 12 donor splice site oligonucleotide probes (7). Exon 12 is contained in a 5.4-kb HindIII fragment (A). The HindIII insertion polymorphism (inverted triangle) is located in the 3'-flanking region of the PAH gene in HindIII fragment B (8). The HindIII fragment D (1 kb) containing exon 9 is not shown on this gel. The upper most fragment (25.3 kb) contains the 20 kb left λCH4A arm annealed at the cos site to the 5.3 Kb right arm fragment. The 5.7 Kb frament corresponds to the remaining portion of the λCH4A right arm after enzyme digestion.

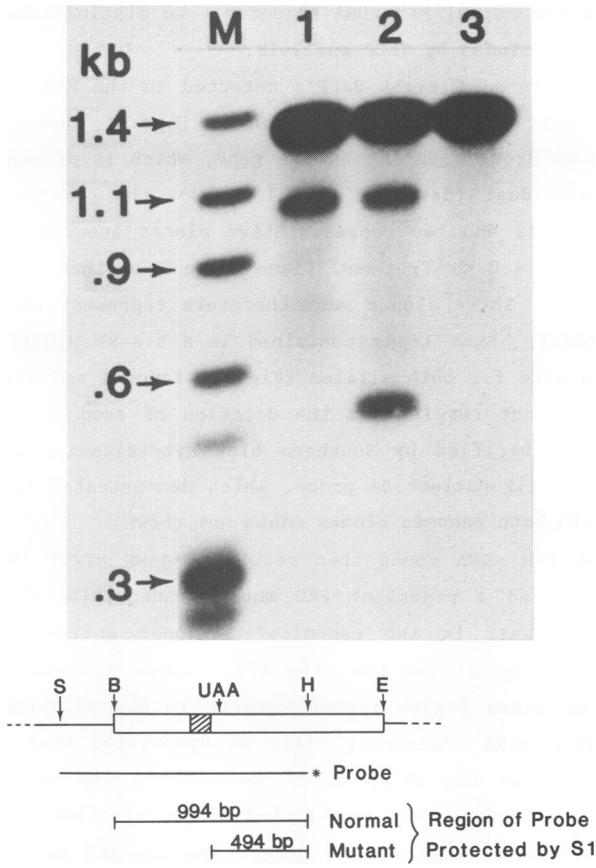

Figure 5. S1-nuclease analysis of human liver RNA.
A 1.4-kb (BamHI(B)-->EcoRI(E)) 3'-fragment derived from hPAH247 was sub-cloned directly into pBR322. The recombinant plasmid was restricted at a unique HpaI (H) site in the 3'-untranslated region at nucleotide #2032 of the PAH cDNA (4). Following end-labelling with [γ^{32}P]-ATP and T4 kinase, the DNA was then restricted at a unique SalI (S) site in pBR322. The 1269 bp end-labelled PAH probe, containing 994 bp of PAH cDNA and 275 bp of pBR322, was then analyzed by S1-nuclease digestion after annealing to 50µg of total RNA isolated from control (lane 1) and mutant carrier (lane 2) liver. Lane 3, S1 nuclease digested probe after annealing to 50 µg of tRNA to measure the extent of self-annealing of the complementary DNA strands and the level of resistance of the blunt ends to S1-nuclease. M, end-labelled QX174-HaeIII restricted DNA size markers. The hatched box corresponds to the cDNA region encoded by exon 12. UAA refers to the stop codon position in PAH mRNA.

of the gene. Exons 9 to 13, including 3-kb of 3'-flanking sequence, are contained in a 12-kb EcoRI fragment of the PAH gene (7) (Fig. 4). In addition to a mutant PAH allele, the genomic library should also contain a second

allele encoding the normal PAH cDNA sequence. We distinguished the normal and mutant allelic PAH clones by RFLP analysis.

The map positions of eight RFLP's detected in the PAH gene have recently been reported (8). There is a 4.2/4.0 kb HindIII insertion polymorphism within this 12-kb EcoRI fragment of the gene, which is present in the genomic DNA of this individual (data not shown). Thus, the genomic DNA clones were digested by HindIII, and two representative clones are shown in Fig. 4. One clone generated a 4.0-kb fragment (lane 1) and another generated a 4.2-kb fragment (lane 2). These clones must therefore represent the two PAH alleles in this individual. Exon 12 is contained in a 5.4-kb HindIII fragment which is identical in size for both alleles (Fig. 4, lanes 1 and 2), suggesting that the mutation does not result from the deletion of exon 12 in the mutant PAH allele. This was verified by Southern blot hybridization analysis using an exon 12 specific oligonucleotide probe, which demonstrated that this exon was indeed present in both genomic clones (data not shown).

The mutant PAH mRNA could then result from an error in RNA processing. We recently reported a prevalent PKU mutation resulting from a single base substitution (GT- ->AT) in the canonical 5'-donor splice site at the exon 12/intron 12 junction of the PAH gene (7). Since a similar substitution in intron 2 of the human β-globin gene results in the skipping of exon 2 from the corresponding mRNA transcript (31), we speculated that the PKU splicing mutation might cause the skipping of exon 12 in the mRNA. To test this hypothesis, we used previously constructed oligonucleotide probes specific for the normal and mutant sequences to detect the GT- ->AT mutation of intron 12 (7). Using the hybridization conditions previously reported (7, 21), the normal oligonucleotide probe hybridized specifically to the 5.4-kb HindIII fragment of the PAH clone containing the 4.0-kb polymorphic HindIII fragment (Fig. 4, lane 3), while the mutant oligonucleotide probe hybridized specifically to the 4.2-kb allele (Fig. 4, lane 6). The data suggested strongly that the exon 12 skipping mutation identified at the PAH mRNA level resulted from a GT- >AT transition at the 5'-splice donor site of intron 12 of the mutant allele. This exact mutation was subsequently verified by sequence analysis of the cloned mutant gene (data not shown).

S1 Nuclease analysis of normal and mutant PAH RNA transcripts

In order to estimate the relative frequency of aberrant splicing among RNA transcripts from the mutant PAH allele in the human liver, we performed S1 nuclease protection experiments using total RNA isolated from the subject liver sample (Fig. 5). When the 1.3-kb PAH cDNA probe containing exons 7-13

plus part of the 3'-untranslated region was annealed to control human liver RNA, a distinct S1 nuclease resistant 994-bp fragment was detected (lane 1). This fragment represented complete protection of the PAH cDNA (spanning nucleotides 1038 to 2032) by the normal PAH mRNA transcript (Fig. 5, schematic). An S1 nuclease protection assay on the subject liver RNA sample showed a 994-bp fragment protected by the normal PAH gene transcript, plus a 494-bp fragment of similar intensity (lane 2). The latter fragment represented protection of the PAH cDNA by a PAH mRNA species lacking the exon 12 sequence (Fig. 5, schematic). The data showed that the steady-state levels of the normal and mutant PAH mRNA transcripts in the human liver are comparable, suggesting that the predominant splicing event associated with the mutant allele transcript in the human liver is exon 12 skipping.

DISCUSSION

We previously reported the isolation of eight partial-length PAH cDNA clones from a human liver cDNA library (3). We were surprised to find that three of these cDNA clones contained an exon 12 sequence deletion. The deletion of exon 12 sequence in the cDNA caused a frameshift mutation that resulted in a premature stop codon which causes the deletion of 52 amino acids from the C-terminus of PAH. Gene transfer and expression studies using the mutant PAH cDNA demonstrated that it results in an unstable protein, thereby abolishing PAH activity in the cell. The complete absence of PAH activity is indicative of the Classical PKU phenotype and can be attributed specifically to the loss of the C-terminal portion of PAH encoded by exon 12. The data is comparable to the lack of immunoreactive protein in PKU liver biopsy specimens reported by other laboratories (26-28).

The GT to AT mutation at the 5' splice donor site observed in this study was the same as the one we reported recently to be associated with mutant

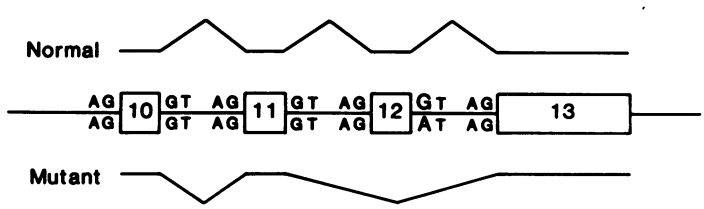

Figure 6. A schematic representation of the exon 12-skipping event in the splicing of a mutant precursor PAH RNA transcript containing a GT to AT transition at the 5' splice donor site of intron 12.

haplotype-3 alleles (7) which is the most prevalent among PKU alleles in Caucasians. Thus, the subject individual was a PKU carrier without a family history of PKU, and contained a common mutant PAH allele. We recently reported that PKU patients with mutant RFLP haplotype combinations 2//2, 2//3, and 3//3 have the highest phenylalanine level in blood and follow a severe clinical course (25). Having established at the molecular and biochemical levels that the mutations in the PAH gene associated with haplotype-2 (29) and haplotype-3 (this manuscript) result in no detectable enzymatic activity, it is understandable why these patients exhibit such severe biochemical and clinical phenotypes.

S1 nuclease protection experiments demonstrated that the levels of normal and mutant PAH mRNA's were comparable, suggesting that the level of expression and processing of both alleles into the respective mRNA products were similar in the human liver cell. Thus, the possibility of mRNA instability being the underlying cause of the PKU mutation can be ruled out. Furthermore, the exon 11-exon 13 splice appeared to be the major product derived from the mutant allele, with no cryptic splice sites being used to generate other aberrantly spliced mRNA products in vivo. This aberrant splicing event utilizes the 5' donor splice site of intron 11 and the 3'-acceptor splice site of intron 12 (Fig. 6).

What causes the exon 12 deletion in PKU? Normal intron splice sites in eukaryotic genes have conserved GT and AG dinucleotides at the 5'-donor and 3'-acceptor sites, respectively (22,23). β-thalassemia splicing mutations have provided definitive evidence for the importance of these splice sites in accurate RNA processing (24). In the human β-globin gene, a G to A splice donor site mutation in intron 2 completely inactivates the 5'-splice site of this intron, and causes skipping of exon 2 in mature β-globin mRNA in the patient's bone marrow cells (31). Since there are only 3 exons in the β-globin gene, exon 3 can only splice with exon 1 if skipping of the preceding exon is the major consequence of the mutation in vivo. In the mutant PAH gene, however, exon 13 could have been spliced with any of the preceding 11 exons. The fact that the major mRNA product from this allele only lacks exon 12 would suggest that this exon is disregarded by the splicing machinery as a result of the GT to AT mutation at the splice donor site of intron 12. Similarly, in the hamster dihydrofolate reductase gene, a GT to CT transition at the splice donor site of intron 5 causes skipping of exon 5, and only exon 4 to exon 6 splice products were detected (30).

Whatever mechanism is involved in the exon skipping event, it must take

into account that the 3' acceptor splice site of the preceding intron (i.e., intron 11) is perfectly normal but is not utilized effectively in splicing (Fig. 6). Mutant cDNA clones representing other aberrantly spliced products were not present in the liver cDNA library suggesting exon 12 skipping is indeed the major splicing event in the mutant RNA. There may be cooperative interactions between the small ribonucleoprotein particles at the intron/exon boundaries, such that the ribonucleoprotein particles cannot bind efficiently to the 3' acceptor site of intron 11 if no interacting ribonucleoprotein particles are present at the 5' donor site of intron 12. Under these circumstances, exon 12 would have been regarded as part of an intron and removed during splicing by joining together exons 11 and 13. This hypothesis would suggest that not only introns, but also exons, are looped out during RNA slicing in a splicesome (32).

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