Am. J. Hum. Genet. 46:18–25, 1990

Missense Mutations Associated with RFLP Haplotypes 1 and 4 of the Human Phenylalanine Hydroxlase Gene

Yoshiyuki Okano,* Tao Wang,* Randy C. Eisensmith,* Beat Steinmann,†
Richard Gitzelmann,† and Savio L. C. Woo*

*Howard Hughes Medical Institute, Department of Cell Biology and Institute of Molecular Genetics, Baylor College of Medicine, Houston; and †Department of Pediatrics, Kinderspital, Zurich

Summary

We report missense mutations associated with haplotype 1 and haplotype 4 alleles of the human phenylalanine hydroxylase (PAH) gene. Individual exon-containing regions were amplified by polymerase chain reaction from genomic DNA of a PKU patient who was a haplotype 1/4 compound heterozygote. The amplified DNA fragments were subcloned into M13 for sequence analysis. Missense mutations were observed in exons 5 and 7, resulting in the substitution of Arg by Gln at residues 158 and 261 of the enzyme, respectively. Expression analysis in heterozygous mammalian cells after site-directed mutagenesis demonstrated that the Arg^{158}-to-Gln^{158} mutation is a PKU mutation, whereas the Arg^{261}-to-Gln^{261} mutation is apparently silent in the assay system. Hybridization analysis using allele-specific oligonucleotide probes demonstrated that the Arg^{158}-to-Gln^{158} mutation is present in two of six mutant haplotype 4 alleles among the Swiss and constitutes about 40% of all mutant haplotype 4 alleles in the European population. The mutation is not present in normal alleles or in any mutant alleles of other haplotypes. The results provide conclusive evidence that there is linkage disequilibrium between mutation and haplotype in the PAH gene and that multiple mutations have occurred in the PAH gene of a prevalent haplotype among Caucasians.

Introduction

Classical phenylketonuria (PKU) is an autosomal recessive disorder caused by an inborn error of amino acid metabolism, with an incidence of about 1/10,000 Caucasian births. The disease results from the deficiency of hepatic phenylalanine hydroxylase (PAH) and causes severe mental retardation unless the child is rigorously maintained on a low-phenylalanine diet (for reviews, see Scriver et al. 1988, 1989). Although neonatal screening for PKU (Guthrie and Susi 1963) is routinely carried out in Western countries, prenatal diagnosis by enzyme assays of amniotic cell extracts was not feasible, as the enzyme is expressed specifically in the liver. The study of PKU at the molecular level started with the construction of a full-length and functional human PAH complementary DNA (hPAH 247) (Kwok et al. 1985; Ledley et al. 1985). We used this cDNA to identify and map eight RFLPs at the human PAH locus (Woo et al. 1983; Lidsky et al. 1985*b;* DiLella et al. 1986*a*), which permitted prenatal diagnosis for PKU by DNA analysis (Lidsky et al. 1985*a;* Ledley et al. 1988).

RFLP haplotype analysis of the human PAH locus has revealed the presence of at least 43 different haplotypes (Woo 1988). About 90% of the PKU alleles in the northern European population are confined to haplotypes 1–4. Haplotypes 2 and 3 are especially frequent in this population, constituting the majority of mutant alleles (Chakraborty et al. 1987; Aulehla-Scholz et al. 1988; Lichter-Konecki et al. 1988*b;* Rey et al. 1988; Riess et al. 1988; Daiger et al. 1989; Sullivan et al. 1989). The mutation associated with haplotype 3 is caused by a single base substitution at the exon 12/intron 12 boundary (DiLella et al. 1986*b;* Marvit et al. 1987), while the mutation associated with haplo-

Received June 30, 1989; revision received September 18, 1989.
Address for correspondence and reprints: Savio L. C. Woo, Howard Hughes Medical Institute, Baylor College of Medicine, One Baylor Plaza, Houston, TX 77030.
© 1990 by The American Society of Human Genetics. All rights ıeserved.
0002-9297/90/4601-0004$02.00

type 2 is caused by an Arg^{408}-to-Trp^{408} substitution in exon 12 of the mutant PAH genes (DiLella et al. 1987). These mutations are tightly linked to the respective haplotypes among mutant alleles, providing strong evidence for linkage disequilibrium (Woo 1988). In the present paper, we report missense mutations associated with haplotypes 1 and 4 in the Swiss population.

Materials and Methods

1. *Amplification of Genomic DNA*

Genomic DNA was isolated from white blood cells of a Swiss PKU patient bearing haplotype 1 and haplotype 4 mutant alleles. The 13 exon-containing regions of the 90-kb PAH gene were individually amplified by polymerase chain-reaction (PCR) for 40 cycles as described elsewhere (DiLella et al. 1988). Phosphorylated oligonucleotides intronic to the gene were purchased from Genetic Designs, Inc. (Houston) and were used as amplification primers.

2. *M13 Cloning and Sequencing of PCR Products*

Amplified exonic DNA fragments were purified by ultrafiltration using Centricon 10 microconcentrators (Amicon). One microgram purified DNA and 0.6 μg *Sma*I and alkaline phosphatase–treated (Boehringer Mannheim) M13 mp18 vector were ligated in 50 μl of the standard ligation buffer. The ligation products were transformed into competent *Escherichia coli* TG-1 cells and plated on IPTG/X-GAL agar plates (Messing 1983). The resulting plaques were lifted onto nitrocellulose filters (Millipore Co.) for hybridization screening using ^{32}P-labeled hPAH247 DNA. Multiple independent recombinant clones were picked, and single-strand M13 DNA was prepared (Messing 1983). Dideoxy sequencing was performed with a universal primer by using the Sequenase DNA sequencing kit. (United States Biochemical Co.).

3. *Dot-Blot Hybridization*

Amplified DNA samples were applied directly onto Zeta-probe membranes (Bio Rad) by using a dot-blot manifold (Schleicher & Schuell) and analyzed by hybridization with allele-specific oligonucleotide probes (19-mer) for the normal and mutant alleles, as described elsewhere (DiLella et al. 1988).

4. *Site-directed Mutagenesis of PAH cDNA*

Specific base substitution was generated in a full-length PAH cDNA fragment by site-directed mutagenesis in a M13 vector according to a method described by Zoller and Smith (1983). Plaques containing the desired mutant sequence were identified by screening with the ^{32}P-end-labeled oligonucleotide utilized in the mutagenesis procedure, described elsewhere (DiLella and Woo 1987). The authenticity of the mutant clone was established by DNA sequence analysis of the mutagenized site.

5. *Expression Analysis of the Mutant Recombinant*

Double-stranded RF DNA was prepared according to the method of Messing (1983), followed by *Eco*RI excision of the 2.4-kb inserts from both the mutant and normal hPAH247 recombinants. The mutant and normal PAH cDNAs were separately subcloned into the eukaryotic expression vector p91023(B) (Wong et al. 1985). These recombinant clones were used for transfer into monkey kidney COS cells. PAH RNA, protein, and enzymatic activity were determined in the cellular extracts according to a method described elsewhere (Ledley et al. 1985).

Results

Amplification of Exons 5 and 7 of the PAH Gene

Figure 1*A* shows the primers for PCR amplification of exons 5 and 7 plus the flanking intronic regions of the human PAH gene. With these amplification primers, specific bands of 260 bp and 291 bp are expected from exon 5 and exon 7 regions, respectively, of the PAH gene. The amplified DNA fragments were electrophoresed on 2% NuSieve agarose gels to confirm the expected sizes (fig. 1*B*). After amplification for various cycles, strong bands of 260 bp containing exon 5 (lanes 1–4) and of 291 bp containing exon 7 (lanes 5–8) are present. Lane 9 contained patient's genomic DNA without amplification. Southern blotting and hybridization using a labeled hPAH247 probe confirmed that the amplified DNA frgments contained exonic PAH sequences (fig. 1*C*).

Identification of Missense Mutations in the PAH Gene

The amplified DNA fragments were cloned into M13, and 5–10 individual recombinant plaques for each exon were selected for sequence analysis. The results revealed two mutations. The first mutation is a G-to-A transition at the second base of codon 158, resulting in the substitution of Arg (CGG) by Gln (CAG) in exon 5 (fig. 2). This mutation was present in four independent clones. The second mutation is also a G-to-A transition at the second base of codon 261, resulting in the substitution of Arg (CGA) by Gln (CAA) in exon 7 (fig.

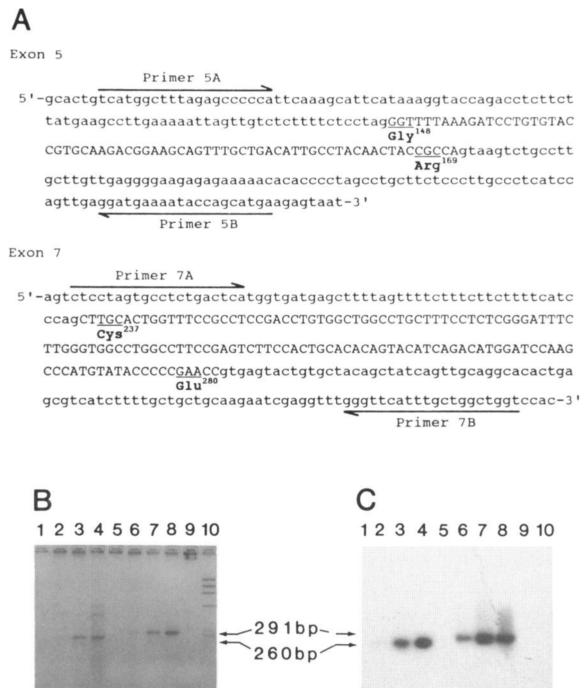

Figure 1 *A*, Sequences of exon 5 and of exon 7 plus flanking introns of the human PAH gene. The sequences (capital letters denote exons, and small letters denote introns) were determined from fragments of the exon 5– and exon 7–containing region in a human genomic DNA clone (DiLella et al. 1986*a*). Exon 5 encodes amino acid residues 148–169 in the enzyme, while exon 7 encodes amino acid residues 237–280 in the enzyme. The locations of the PCR primers are shown by the arrows. Primer 5A (5′-TCATGGCTTTAGAGCCCCCA-3′) is the sense strand of intron 4, 74–93 nucleotides 5′ of exon 5. Primer 5B (5′-TCATGCTGGTATTTTCATCC-3′) is complementary to the sense DNA strand of intron 5, 80–99 nucleotides 3′ of exon 5. Primer 7A (5′-CTCCTAGTGCCTCTGACTCA-3′) is the sense strand of intron 6, 52–61 nucleotides 5′ of exon 7. Primer 7B (5′-ACC-AGCCAGCAAATGCAACCC-3′) is complementary to the sense DNA strand of intron 8, 75–84 nucleotides 3′ of exon 7. *B*, Agarose-gel electrophoresis of the PCR-amplified product. *C*, Southern hybridization with hPAH247 probe of the amplified products. Lanes 1–4, 0.1 μg patient's genomic DNA after 10, 20, 30 and 40 cycles, respectively, of PCR amplification for exon 5. Lanes 4–8, same amplification for exon 7; lane 9, 1.0 μg patient's genomic DNA without amplification; lane 10, *Hae*III-digested ϕx174 DNA marker.

2). This mutation was present in three independent clones.

Mandelian Segregation of the Mutant Alleles in PKU Kindreds

Genomic DNA samples from the proband, both parents, and an unaffected sibling were independently amplified by PCR and analyzed by dot-blot hybridization using allele-specific oligonucleotide probes. The mutant-158 and -261 probes are complementary to the sense strand of the normal exonic sequences, except for a G-A mismatch at the mutation site. The normal-158 and -261 probes are complementary to the sense strand of the normal gene sequence. Both normal probes hybridized with DNA from all four members of the nuclear family, indicating successful amplification of all DNA samples (fig. 3, family A). Both mutant probes hybridized with the proband sample, demonstrating that the missense mutations are present in the patient's genomic DNA and were not artifacts of PCR (fig. 3, family A). The mutant-158 probe also hybridized with DNA samples of the father and sibling, who are carriers of the mutant haplotype 4 allele, but not with the DNA sample from the mother, who bears a mutant haplotype 1 allele. Conversely, the mutant-261 probe hybridized with the maternal sample but not with the paternal or sibling samples. Thus, the codon 158 and codon 261 mutations were derived, respectively, from the haplotype 4 and haplotype 1 mutant alleles in this family.

Two additional Swiss PKU families bearing the missense mutant alleles were similarly analyzed (fig. 3, families B and C). In family B, both parents are carriers of mutant haplotype 1 alleles bearing the codon 261 mutation, while the proband is homozygous for this mutant allele. In family C, the father is the carrier of the mutant haplotype 4 allele bearing the codon 158 mutation, and the mother is the carrier of the mutant haplotype 1 allele bearing the codon 261 mutation. The proband is a compound heterozygote of the two mutant alleles. Failure of the mutant-158 probe to hybridize with the DNA of the sibling indicates that the sibling's haplotype 4 allele is normal, and thus the sibling is not a carrier.

Mutation Verification by Mutagenesis and Expression Analysis

The respective mutations were introduced into normal PAH cDNAs in a eukaryotic expression vector, and expression analysis was performed in mammalian cells. Similar steady-state levels of normal and mutant PAH mRNAs were present in the transfected cells as determined by northern hybridization and dot-blot analysis of serially diluted RNA samples (data not shown). The PAH protein contents in the transfected cells were measured by Western blot analysis using a goat anti–human PAH antibody preparation (Robson et al. 1982). The content of human PAH in 10, 50, and 100 μg of extracts of cells transfected with the normal PAH cDNA (fig. 4*A*, lanes 2–4) was compared with that in cells

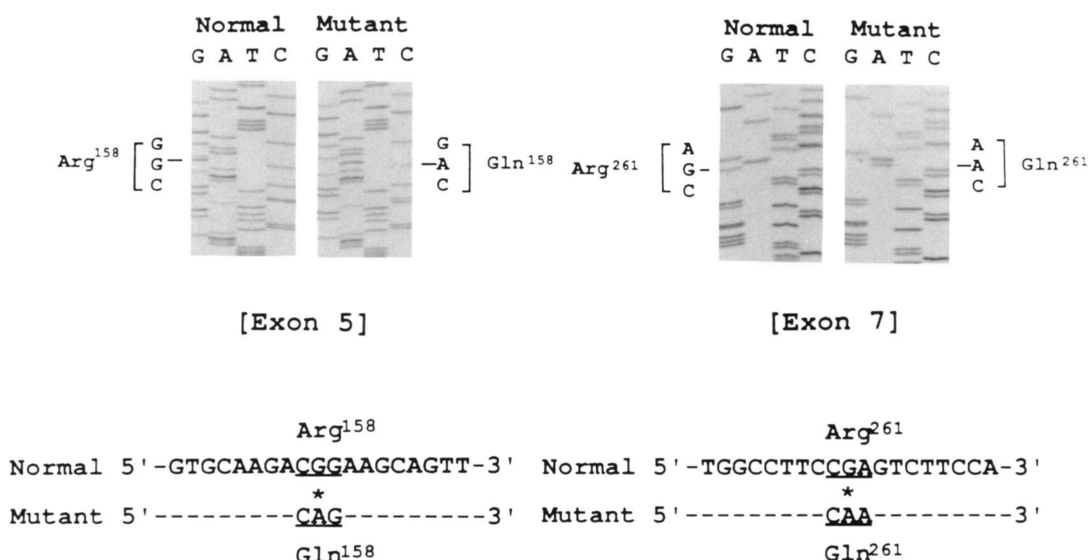

Figure 2 Sequence analysis of missense mutations in exons 5 and 7 of a normal and a mutant PAH allele. The G-to-A transition in exon 5 results in the substitution of Arg^{158} by Gln^{158}. The G-to-A transition in exon 7 results in the substitution of Arg^{261} by Gln^{261}.

transfected with the mutant-158 (lanes 5–7) and mutant-261 (lanes 8–10) contructs. The results demonstrated that the steady-state levels of the normal mutant PAH proteins are comparable in the respectively transfected cells. PAH enzymatic activity was then determined in 0, 10, 20, 50, 100, and 250 μg (fig. 4*B*, lanes 1–6) of extracts from cells transfected with the normal and mutant PAH cDNAs. While the Arg^{158}-to-Gln^{158} mutant protein contains clearly reduced enzymatic activity, it is surprising that the Arg^{261}-to-Gln^{261} mutant protein contains almost full enzymatic activity. In an attempt to identify additional mutations in the patient's DNA, we performed DNA amplification and sequence analyses of all exon-containing regions of the PAH gene. No additional mutation was found.

Linkage Disequilibrium between the Missense Mutations and Haplotypes 1 and 4

Dot-blot hybridization analysis was performed on 17 Swiss PKU families with allele-specific oligonucleotide probes to determine whether there is linkage disequilibrium between the missense mutations and the respective haplotypes, which are the most prevalent among PAH alleles in the Swiss population (Sullivan et al. 1989). Of 18 mutant haplotype 1 alleles, 13 bear the codon 261 mutation; of six mutant haplotype 4 alleles, two bear the codon 158 mutation (table 1). Both mutations are not present in any normal alleles as well as in alleles of other haplotypes.

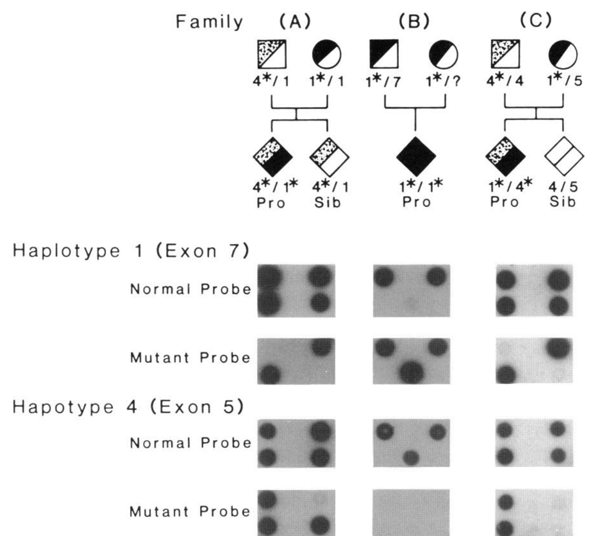

Figure 3 Transmission of the missense mutant alleles in three different Swiss PKU families as analyzed by PCR amplification followed by dot-blot hybridization using specific oligonucleotide probes. The probes used to detect the substitution at codon 158 in exon 5 were as follows: the normal probe (5′-AACTGCTTCCGTCCTTGCCAC-3′) is the antisense DNA strand, and the mutant probe (5′-AACTGCTTCTGTCCTTGCCAC-3′) is the antisense DNA strand. The probes used to detect the substitution at codon 261 in exon 7 were as follows: the normal probe (5′-TGGAAGACTCGGAAGGCCA-3′) is the antisense DNA strand, and the mutant probe (5′-TGGAAGACTTGGAAGGCCA-3′) is the antisense DNA strand. Genomic DNA was isolated from leukocytes of different family members. Exon 5– and exon 7–containing regions (260 and 291 bp, respectively) were amplified by PCR, dot-blotted onto z-probe membrane, and hybridized with allele-specific oligonucleotide probes.

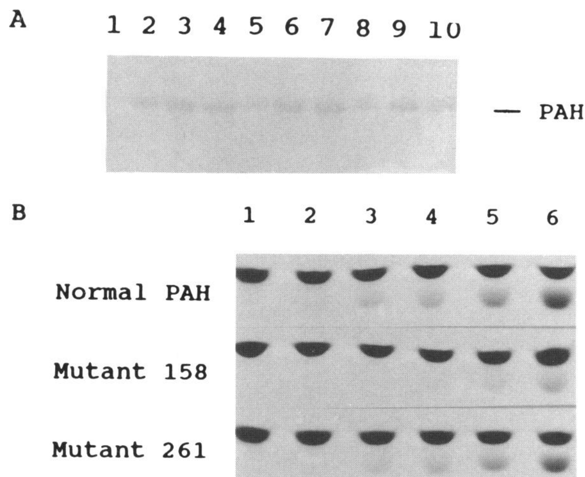

Figure 4 Analysis of PAH in COS cells transfected with normal and mutant hPAH247 cDNAs. *A,* Western blot analysis. Lanes 1–4, 0, 10, 50, and 100 µg, respectively, of protein extract from COS cells transfected with the normal PAH cDNA construct. Lanes 5–7, 10, 50, and 100 µg respectively, of protein extract from cells transfected with the codon 158 mutant cDNA. Lanes 8–10, 10, 50, and 100 µg, respectively, of protein extract from the cells transfected with codon 261 mutant cDNA. *B,* PAH enzymatic assay of cellular extracts, measuring the conversion of ^{14}C-Phe to ^{14}C-Tyr Lanes 1–6, 0, 10, 20, 50, 100, and 250 µg, respectively, of protein extracts in the assay.

Correlation between PAH Genotypes and PKU Phenotypes

The biochemical phenotypes of the probands in the three families analyzed are shown in figure 3, while the clinical phenotypes of these three individuals, as well as those of three others, are shown in table 2. Both patients who are compound heterozygotes of the mutant-158 and -261 alleles (families A and C) exhibited a relatively mild PKU phenotype (Güttler 1980). Their serum phenylalanine levels were 8 and 17 mg/dl at 5–6 d of age, and, on intervention by moderately restricted diet, the levels were maintained at 2–20 mg/dl. Their phenylalanine tolerance was 11–24 mg/kg/d at 2–8 years of age. These results suggest that there is residual enzymatic activity in the livers of these patients, which could have resulted from productive expression of either or both mutant alleles.

To assess the in vivo effect of the mutant allele containing the Arg^{261}-to-Gln^{261} mutation, two patients who are homozygous for this mutant allele (families B and D) were analyzed and observed to exhibit even milder phenotypes. Serum phenylalanine was 10–15 mg/dl at 4–5 d of age and was reduced to 2–8 mg/dl on postdiet treatment. Their phenylalanine tolerance levels were above 20 mg/kg/d at 2–8 years of age. The clinical data strongly suggest that this is a mild mutant allele, which is supported by the biochemical and clinical phenotypes of two additional patients who are compound heterozygotes bearing Arg^{261}-to-Gln^{261} and an Arg^{408}-to-Trp^{408} mutant alleles (families E and F). Since the latter mutant allele confers a null phenotype (DiLella et al. 1987), the patients can be considered hemizygous for the mutant-261 allele. These patients still exhibited a relatively mild phenotype, although they did have somewhat higher serum phenylalanine levels both pre- and posttreatment relative to patients in families B and D, who bear two such mutant alleles.

Table 1

Association of Mutant-158 and -261 with Mutant Haplotype 1 Alleles and with Mutant Haplotype 4 Alleles in Switzerland

	No. of Hybridizing Alleles/ No. of Alleles Analyzed			
	Mutant-158		Mutant-261	
Haplotype	Normal	PKU	Normal	PKU
1...........	0/12	0/18	0/12	13/18
2...........	0/3	0/4	0/3	0/4
3...........	0/0	0/2	0/0	0/2
4...........	0/7	2/6	0/7	0/6
Others	0/10	0/4	0/10	0/4

Discussion

PKU is a heterogenous metabolic disorder at the clinical level (for reviews, see Scriver et al. 1988, 1989). We previously reported that PKU patients who are either homozygotes or compound heterozygotes of both the mutant haplotype 2 alleles and the mutant haplotype 3 alleles follow a severe clinical course, whereas patients who carry mutant alleles of either haplotype 1 or haplotype 4 exhibit a milder clinical phenotype in general (Güttler et al. 1987). Because the mutations in the PAH gene that are associated with haplotypes 2 and 3 result in undetectable enzymatic activity (DiLella et al. 1986*b,* 1987), it is not surprising that patients who are either homozygotes or compound heterozygotes of these two mutant haplotypes follow a severe clinical course. We have identified a new missense mutation in a haplotype 4 allele of a Swiss PKU patient. The mutation is characterized by a single nucleotide substitution in exon 5 of the PAH gene, encoding amino acid residue number 158. The specific enzymatic activity of the Arg^{158}-to-Gln^{158} mutation is less than 10%

Table 2

Correlation between PAH Genotypes and PKU Phenotypes

Family	Patient Initials	Haplotypes	Mutations	Serum Phenylalanine (mg/dl)		Phenylalanine Tolerance (age) (mg/kg/d)
				Pretreatment	Posttreatment	
A	A.R.	1/4	261/158	8 (5 d)	2–20	27 (9 mo) 24 (2 years) 16 (4 years) 11 (6 years)
B	M.S.	1/1	261/261	10 (4 d)	2–8	19 (6 mo) 18 (1 year) 22 (2 years)
C	P.A.	1/4	261/158	17 (6 d)	2–15	30 (6 mo) 27 (1 year) 21 (3 years) 12 (8 years)
D	P.E.	1/1	261/261	15 (5 d)	3–8	38 (5 mo) 29 (1 year) 22 (3 years) 16 (5 years) 21 (8 years)
E	A.G.	1/2	261/408	21 (3 years)	2–16	20 (6 years) 18 (8 years)
F	R.S.	1/2	261/408	17 (5 d)	10–13	43 (7 mo) 23 (1 year) 20 (3 years) 14 (5 years) 15 (8 years)

of the normal, and the residual enzyme activity is consistent with the milder clinical phenotype of the patient.

The codon 158 mutation in exon 5 is present on two independent haplotype 4 alleles in Switzerland. Using allele-specific probes to analyze DNA samples of PKU kindreds from various European countries, we have obtained preliminary evidence that the codon 158 mutation is rather prevalent in other European populations. Not only is the association between mutation and haplotype maintained, but it also comprises about 40% of all haplotype 4 mutant alleles in Europe (Y. Okano, unpublished results). Thus, the data suggest the existence of linkage between the Arg^{158}-to-Gln^{158} mutation and mutant haplotype 4 alleles and support the hypothesis of the spread of a single chromosome bearing this mutation among the Caucasians by a founder effect. The data also suggest that there must be multiple mutations associated with the haplotype 4 mutant alleles. This could be attributed to the fact that haplotype 4 is prevalent among normal PAH alleles and may therefore have a greater chance to sustain independent mutational events during evolution.

With regard to the Arg^{261}-to-Gln^{261} mutation, it is surprising that it appears to be silent. It should be noted, however, that our expression analysis deals only with the missense mutation at the steady-state protein level in a heterozygous mammalian cell and does not address the possibility that the nucleotide substitution may manifest itself as a PKU allele at some other biochemical level. This may be a possibility, as the mutant allele appears to have a mild effect in patients who are homozygous for this allele or who are compound heterozygotes bearing an additional null allele (table 2). Nevertheless, the mutation in exon 7 is well represented among mutant haplotype 1 alleles in the Swiss population (13 of 18 alleles) and constitutes about 25% of all mutant haplotype 1 alleles among Europeans (Y. Okano, unpublished results). It is obviously in linkage disequilibrium with the mutant haplotype 1 allele. If indeed it is not the cause of PKU, the nucleotide substitution must have occurred on a mutant haplotype 1 background. Furthermore, since it has never been found on any normal alleles, it can be readily used as a specific probe for detection of the corresponding mutant haplo-

type 1 allele, which will be applicable in prenatal diagnosis and carrier detection of PKU in the Caucasian population.

The two newly identified nucleotide substitutions in the PAH gene are both G-to-A transitions. A C-to-T transition has been identified in exon 12 of the PAH gene in Danish PKU patients (DiLella et al. 1987). A Pro^{311}-to-Leu^{311} mutation was identified in exon 9 of a rare German PKU allele (Lichter-Konecki et al. 1988*a*), and a Glu^{280}-to-Lys^{280} mutation in exon 7 was reported from the northern African population (Lyonet et al. 1989). More recently an Arg^{111}-to-Ter^{111} mutation, which is in linkage disequilibrium with a haplotype 4 allele among the Orientals (Wang et al. 1989), has been identified in exon 3 of the PAH gene. Most of these missense mutation sites involve CpG dinucleotides in either the sense strand or the antisense strand. CpG is the most common site of methylation in mammalian DNA. Thus, deamination of 5-methylcytosine, leading to a C-to-T transition (Silva and White 1988), may be the most common cause of PKU.

Acknowledgments

This work was supported in part by NIH grant HD-17711 to S.L.C.W., who is also an Investigator of the Howard Hughes Medical Institute.

References

Aulehla-Scholz C, Vorgerd M, Sautter E, Leupold D, Mahlmann R, Ullrich K, Olek K, et al (1988) Phenylketonuria: distribution of DNA diagnostic patterns in German families. Hum Genet 78:353–355.

Chakraborty R, Lidsky AS, Daiger SP, Güttler F, Sullivan S, Dilella AG, Woo SLC (1987) Polymorphic DNA haplotypes at the human phenylalanine hydroxylase locus and their relationship with phenylketonuria. Hum Genet 76:40–46

Daiger SP, Chakraborty R, Reed L, Fekete G, Schuler D, Berenssi G, Nasz I, et al (1989) Polymorphic DNA haplotypes at the phenylalanine hydroxylase (PAH) locus in European families with phenylketonuria (PKU). Am J Hum Genet 45:310–318

DiLella AG, Huang WM, Woo SLC (1988) Screening for phenylketonuria mutations by DNA amplification with the polymerase chain reaction. Lancet 1:497–499

DiLella AG, Kwok SCM, Ledley FD, Marvit J, Woo SLC (1986*a*) Molecular structure and polymorphic map of the human phenylalanine hydroxylase gene. Biochemistry 25: 743–749

DiLella AG, Marvit J, Brayton K, Woo SLC (1987) An amino-acid substitution involved in phenylketonuria is in linkage disequilibrium with DNA haplotype 2. Nature 327:333–336

DiLella AG, Marvit J, Lidsky AS, Güttler F, Woo SLC (1986*b*) Tight linkage between a splicing mutation and a specific DNA haplotype in phenylketonuria. Nature 322:799–803

DiLella AG, Woo SLC (1987*b*) Hybridization of genomic DNA to oligonucleotide probes in the presence of tetramethylammonium chloride. Methods Enzymol 152:447–451

Guthrie R, Susi A (1963) A simple phenylalanine method for detecting phenylketonuria in large populations of newborn infants. Pediatrics 32:338–343

Güttler F (1980) Hyperphenylalaninemia: diagnosis and classification of the various types of phenylalanine hydroxylase deficiency in childhood. Acta Pediatr Scand [Suppl] 280:1–80

Güttler F, Ledley FD, Lidsky AS, DiLella AG, Sullivan SE, Woo SLC (1987) Correlation between polymorphic DNA haplotypes at phenylalanine hydroxylase locus and clinical phenotypes of phenylketonuria. J Pediatr 110:68–71

Kwok SCM, Ledley FD, DiLella AG, Robson KJH, Woo SLC (1985) Nucleotide sequence of a full-length complementary DNA clone and amino acid sequence of human phenylalanine hydroxylase. Biochemistry 24:556–561

Ledley FD, Grenett HE, DiLella AG, Kwok SCM, Woo SLC (1985) Gene transfer and expression of human phenylalanine hydroxylase. Science 228:77–79

Ledley FD, Koch R, Jew K, Beaudet A, O'Brien WE, Bartos DP, Woo SLC (1988) Phenylalanine hydroxylase expression in liver of a fetus with phenylketonuria. J Pediatr 113: 463–468

Lichter-Konecki U, Konecki DS, DiLella AG, Brayton K, Marvit J, Hahn TM, Trefz FK et al (1988*a*) Phenylalanine hydroxylase deficiency caused by a single base substitution in an exon of the human phenylalanine hydroxylase gene. Biochemistry 27:2881–2885

Lichter-Konecki U, Schlotter M, Konecki DS, Labeit S, Woo SLC, Trefz FK (1988b) Linkage disequilibrium between mutation and RFLP haplotype at the phenylalanine hydroxylase locus in the German population. Hum Genet 78: 347–352

Lidsky AS, Güttler F, Woo SLC (1985*a*) Prenatal diagnosis of classical phenylketonuria by DNA analysis. Lancet 1:549–551

Lidsky AS, Ledley FD, DiLella AG, Kwok SCM, Daiger SP, Robson KJH, Woo SLC (1985*b*) Extensive restriction site polymorphism at the human phenylalanine hydroxylase locus and application in prenatal diagnosis of phenylketonuria. Am J Hum Genet 37:619–634

Lyonnet S, Caillaud C, Rey F, Berthelon M, Frézal J, Rey J, Munnich A (1989) Molecular genetics of phenylketonuria in Mediterranean countries: a mutation associated with partial phenylalanine hydroxylase deficiency. Am J Hum Genet 44:511–517

Marvit J, DiLella AG, Brayton K, Ledley FD, Robson KJH, Woo SLC (1987) GT to AT transition at a splice donor site causes skipping of the preceding exon in phenylketonuria. Nucleic Acids Res 15:5613–5628

Messing J (1983) New M13 vectors for cloning. Methods Enzymol 101:21–78

Rey F, Berthelon M, Caillaud C, Lyonnet S, Abadie V, Blandin-Savoja F, Feingold J, et al (1988) Clinical and molecular heterogeneity of phenylalanine hydroxylase deficiences in France. Am J Hum Genet 43:914–921

Riess O, Michel A, Speer A, Meiske W, Cobet G, Coutelle C (1988) Linkage disequilibrium between RFLP haplotype 2 and the affected PAH allele in PKU families from the Berlin area of the German Democratic Republic. Hum Genet 78:343–346

Robson KJH, Chandra T, MacGillivray RTA, Woo SLC (1982) Polysome immunoprecipitation of phenylalanine hydroxylase mRNA from rat liver and cloning of its cDNA. Proc Natl Acad Sci USA 79:4701–4705

Scriver CR, Kaufman S, Woo SLC (1988) Mendelian Hyperphenylalaninemia. Annu Rev Genet 22:301–321

——— (1989) The hyperphenylalaninemias. In: Scriver CR, Beaudet A, Sly W, Valle D (eds) The metabolic basis of inherited disease, 6th ed. McGraw-Hill, New York pp 495–546

Silva AJ, White R (1988) Inheritance of allelic blueprints for methylation patterns. Cell 54:145–152

Sullivan SE, Moore SD, Connor JM, King M, Cockburn F, Steinmann B, Gitzelmann R, et al (1989) Haplotype distribution of the human phenylalanine hydroxylase locus in Scotland and Switzerland. Am J Hum Genet 44:652–659

Wang T, Okano Y, Eisensmith R, Huang S-Z, Zeng Y-T, Lo WHY, Woo SLC (1989) Molecular genetics of phenylketonuria in Orientals: linkage disequilibrium between a termination mutation and haplotype 4 of the phenylalanine hydroxylase gene. Am J Hum Genet 45:000–000

Wong GG, Witek JS, Temple PA, Wilkens KM, Leary AC, Luxenberg DP, Jones SS, et al (1985) Human GM-CSF: molecular cloning of the complementary DNA and purification of the natural and recombinant proteins. Science 228:810–815

Woo SLC (1988) Molecular basis and population genetics of phenylketonuria. Biochemistry 28:1–7

Woo SLC, Lidsky AS, Güttler F, Chandra T, Robson KJH (1983) Cloned human phenylalanine hydroxylase gene allows prenatal diagnosis and carrier detection of classical phenylketonuria. Nature 306:151–155

Zoller MJ, Smith M (1983) Oligonucleotide-directed mutagenesis of DNA fragments cloned into M13 vectors. Methods Enzymol 100:468–500