Am. J. Hum. Genet. 47:1002–1007, 1990

A Termination Mutation Prevalent in Norwegian Haplotype 7 Phenylketonuria Genes

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Summary

RFLPs in the phenylalanine hydroxylase (PAH) gene locus were determined in 47 Norwegian nuclear families that had at least one child with phenylketonuria (PKU). The PKU haplotype distribution differed somewhat from that of other European populations. Mutant haplotype 7 is relatively rare in other populations but constituted 20% of all mutant haplotypes in Norway. In 14 of the 17 mutant haplotypes 7, a previously unreported deletion of the *Bam*HI restriction site in exon 7 of the PAH gene was observed. The abrogation of the *Bam*HI site was shown to be due to a G-to-T transversion, changing Gly 272 to Ter 272 in exon 7 of the gene, thus directly identifying the PKU mutation. Unlike the families of the other PKU patients, the families with this mutation clustered along the southeastern coast of Norway, suggesting a founder effect for this mutation.

Introduction

Deficiency of human phenylalanine hydroxylase (PAH) results in phenylketonuria (PKU), an autosomal recessive disorder that causes severe mental retardation in untreated children. Molecular genetic investigations of PKU were made possible by the cloning of a full-length human PAH cDNA probe (Kwok et al. 1985). Restriction-site-polymorphism haplotype analysis, followed by characterization of their mutations, has revealed several single-base mutations; some of them are in linkage disequilibrium with particular haplotypes. Some common single-base substitutions have been described in association with haplotypes 1 (Abadie et al. 1989; Okano et al. 1990), 2 (DiLella et al. 1987; John et al. 1989), 3 (DiLella et al. 1986*b*), and 4 (Dwormiczak et al. 1989; Wang et al. 1989; Okano et al. 1990). In addition, some rare or private mutations also have been found (Lichter-Konecki et al. 1988; Lyonnet et al. 1989; Avigad et al. 1990).

Received February 12, 1990; final revision received August 9, 1990.
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0002-9297/90/4706-0018$02.00

During a preliminary search for new restriction-site polymorphisms in the PAH gene in unrelated Norwegian blood donors, a new *Bam*HI polymorphism was detected and recorded in 1988 (allele system Ma; Howard Hughes Medical Institute-Yale, HGML data base). The present investigation of PAH haplotypes in Norwegian families who have PKU revealed an unprecedentedly high frequency of mutant haplotypes 7, most of which possessed the new polymorphic *Bam*HI restriction fragment. We report here the Norwegian PKU haplotype frequencies and the detection of a novel haplotype 7 mutation prevalent in Norway.

Patients and Methods

Patients

Forty-seven index cases were recruited, on a volunteer basis, from the PKU patients diagnosed and followed at the Department of Pediatric Research, National Hospital, Oslo. The diagnosis was made in the neonatal period either through Norway's compulsory neonatal screening program for PKU (33 cases), because the subjects were younger sibs of PKU patients (four cases), or, before the screening program was effective, on the basis of clinical symptoms of PKU (10 cases). The diagnosis of classical PKU was based on both a maxi-

mum neonatal serum phenylalanine level above 1,200 μmol/liter and the presence of secondary metabolites in urine. Dietary restriction was required for all affected children. Information on the birthplace of the grandparents of the patients showed that the families originated from all parts of the country, except for the most northerly county. Most subjects (56%) were from the southeastern part of Norway, where the population density also is highest (39% of the population). No known consanguinity between parents was established. Except for affected sibs, no other PKU patients were known in these kindreds. The median age of the PKU patients was 9 years (range 1–19 years); 27 were females, and 20 were males.

DNA Haplotype Analysis

Initially the index cases and their parents were tested. When necessary for the assignment of haplotypes, DNA from sibs were also investigated (12 families). Genomic DNA was extracted from venous blood samples and was digested with the restriction enzymes *Bgl*II, *Pvu*II, *Eco*RI, *Msp*I, *Xmn*I, *Hin*dIII, *Eco*RV, and *Bam*HI. Southern analysis was performed as described by Vandenplas et al. (1984), with a ^{32}P-labeled insert of the recombinant plasmid phPAH247, containing the entire PAH cDNA (Kwok et al. 1985).

Assignment of haplotypes was possible in 43 (91%) of the 47 PKU families studied, giving a total of 171 haplotypes, 86 mutant and 85 normal (one father was unavailable for analysis, and the haplotype of his normal chromosome could not be inferred). The enumeration of the haplotypes was according to the recommendations of Woo (1988). Comparisons of haplotype frequencies were made by χ^2 contingency tests.

Polymerase Chain Reaction (PCR) Amplification and Restriction-Enzyme Analysis

The sense primer A (5′-TTCATCCCAGCTTGCACTGG-3′), which binds 10 bp upstream of exon 7, and the antisense primer B (5′-CAGTACTCACGGTTCGGGGGT-3′), which binds 10 bp downstream of exon 7, were used to amplify 156 bp of the PAH exon 7 region. Amplification of the genomic DNA by PCR (Saiki et al. 1986) was accomplished in a 100-μl reaction mixture containing 1 μg genomic DNA, 10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.12 mM of each dNTP, 0.6 μM of each primer, and 2.5 units *Taq* polymerase (Perkin Elmer–Cetus). Amplification was performed for 30 cycles, each consisting of DNA denaturation at 92°C for 1 min, annealing at 55°C for 1 min, and primer extension at 72°C for 1 min. A 40-μl sample of the PCR reaction was subjected to *Bam*HI restriction-enzyme digestion for 4 h in the appropriate buffer. Agarose-gel analysis of the products was performed in 3% Nusieve agarose (FMC Co.).

Asymmetric PCR and DNA Sequencing

Exon 7 of the PAH gene, plus its flanking intronic regions, were amplified by PCR as above but by using primers 7A and 7B as described by Okano et al. (1990). The resulting 291-bp product was purified by agarose-gel electrophoresis followed by adsorption to glass beads (Geneclean; Bio101, Inc.). A second PCR amplification to generate single-stranded DNA was performed as above with the same 7A and 7B primers at a ratio of 1:50 (Gyllensten and Erlich 1988). The amplification primers were removed by centrifugation through a Millipore UF-MC filter. DNA sequencing was performed using the Sequenase kit (USB Co.), using 40% of the retentrate.

Results

RFLP analysis revealed 13 different haplotypes. The haplotype frequencies in normal and mutant chromosomes are listed in table 1. For comparison, data from Scotland (Sullivan et al. 1989), which has a predominantly Celtic population (Saugstad 1975), and from Denmark (Chakraborty et al. 1987) are included. The haplotype frequencies of the normal chromosomes did not differ significantly between these populations ($P > .25$). For the PKU chromosomes there was no overall haplotype heterogeneity between Norway and Scotland ($P = .1$). In comparison with Denmark, however, there was a clear heterogeneity ($P < .01$), mainly because of both the relatively high frequency of mutant haplotype 7 and the low frequencies of haplotypes 2 and 3 in the Norwegian population.

Haplotype 7 was found in 17 (20%) of 86 mutant chromosomes but in only six (7%) of 85 normal chromosomes. In 14 of the 17 mutant haplotype 7 chromosomes but in none of the normal chromosomes, an unusual 12.4-kb *Bam*HI restriction fragment was detected. The remaining chromosomes carried a 7.8-kb fragment plus a 4.6-kb fragment. Unfortunately, the 4.6-kb DNA fragment comigrates with a second, constant 4.6-kb band in Southern analyses. Therefore, the single 7.8-kb fragment was used to identify the normal allele. Mendelian inheritance of alleles is shown in figure 1.

It is interesting that the haplotype 7 chromosomes having the novel *Bam*HI restriction fragment showed

Table 1

Number (%) of Normal and Mutant RFLP Haplotypes at the PAH Locus in Three European Countries

	Norway		Scotland		Denmark	
Haplotype	Normal	Mutant	Normal	Mutant	Normal	Mutant
1	24 (28)	18 (21)	10 (32)	10 (30)	23 (35)	12 (18)
2	6 (7)	9 (11)	1 (3)	3 (9)	3 (5)	13 (20)
3	3 (4)	15 (17)	2 (6)	6 (18)	2 (3)	25 (38)
4	23 (27)	8 (9)	4 (13)	2 (6)	21 (32)	9 (14)
5	12 (14)	5 (6)	3 (10)	1 (3)	7 (11)	0
6	2 (2)	0	1 (3)	0	0	2 (3)
7	7 (8)	17 (20)	3 (10)	0	7 (11)	1 (1)
8	0	5 (6)	3 (10)	1 (3)	1 (2)	0
9	2 (2)	1 (1)	0	1 (3)	0	1 (2)
10	1 (1)	0	0	0	1 (2)	0
11	2 (2)	1 (1)	0	0	1 (2)	1 (2)
12	2 (2)	7 (8)	0	1 (3)	0	2 (3)
32	1 (1)	0	0	0	0	0
Other	0	0	4 (13)	8 (24)	0	0
Total	85	86	31	33	66	66

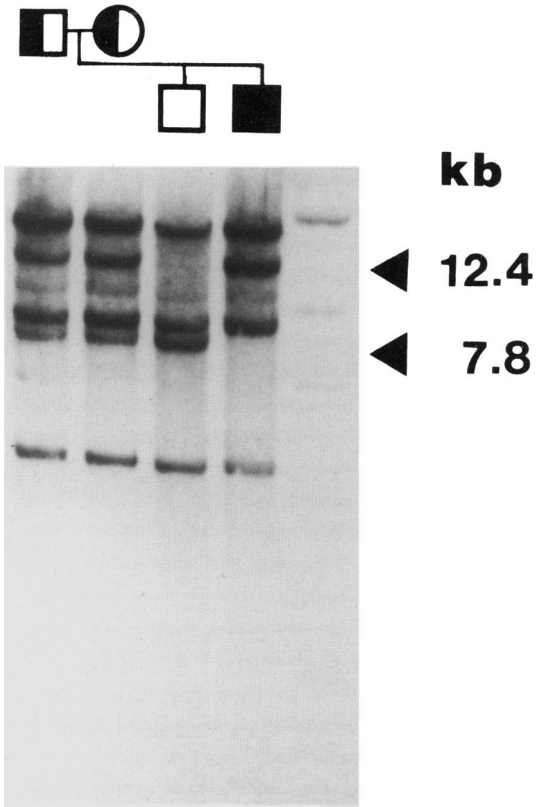

Figure 1 Southern analysis of the PKU family, showing loss of *Bam*HI site in exon 7 of haplotype 7. Genomic DNA was digested with the restriction enzyme *Bam*HI and was subjected to Southern analysis using the cDNA insert of phPAH247 as the hybridization probe. The novel 12.4-kb allele is passed from each of the two heterozygous parents to the PKU child homozygous for the 12.4-kb allele. The unaffected sibling is homozygous for the 7.8-kb allele.

a restricted geographical distribution. The native districts of individual parents shown to be heterozygous for haplotype 7 mutations are indicated in figure 2. The geographical distribution of the origin of the individual haplotypes 7 having the novel *Bam*HI fragment was different from that of the remaining haplotype 7 chromosomes.

Extended restriction mapping by Southern blot analyses using different partial fragments of the full-length probe suggested that the polymorphic *Bam*HI restriction site was the known *Bam*HI site in exon 7 (110 bp downstream in the exon) (Kwok et al. 1985; DiLella et al. 1986*a*). A previously unreported constant *Bam*HI site in intron 6, in combination with the polymorphic one, would explain the origin of the fragment lengths observed (fig. 3).

To confirm the results of the Southern analyses, we amplified exon 7 of the PAH gene from genomic DNA of the 17 families that had mutant haplotype 7. The amplification products were then cut with *Bam*HI (fig. 4). The results were consistent with the Southern analyses.

Eventually, DNA sequencing of exon 7 from normal

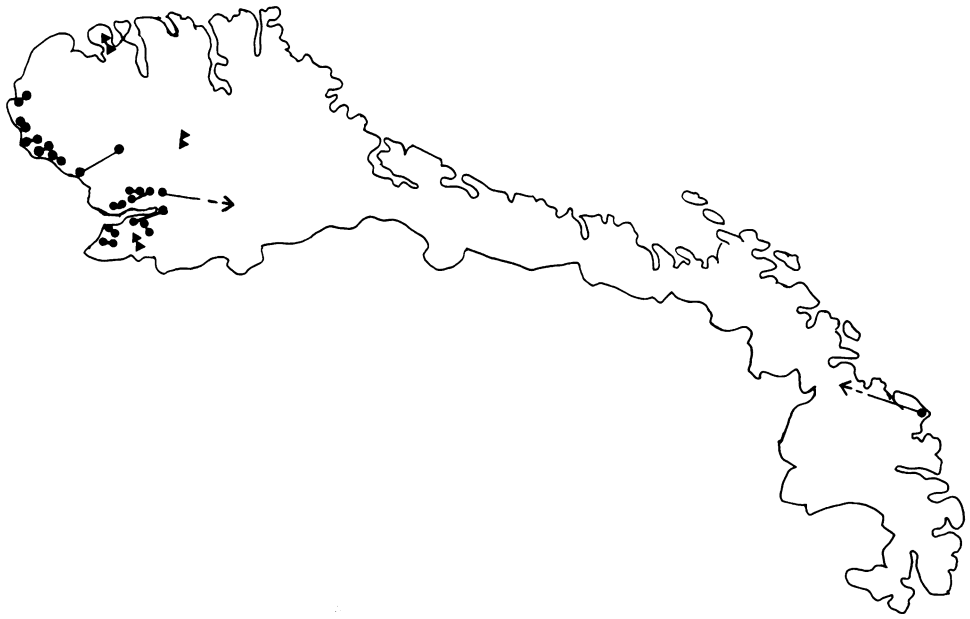

Figure 2 Map of Norway, showing birthplaces of grandparents of PKU patients who have mutant haplotype 7. Only the birthplaces for the relevant pairs of grandparents are shown (one of whom contributed the mutant haplotype 7). Black circles (●) denote mutant haplotypes 7 that have the codon 272 mutation; black triangles (▲) denote mutant haplotypes 7 that do not have this mutation.

and mutant alleles, reamplified by asymmetric PCR, identified a G-to-T single-base transversion at the first base of codon 272. This indicates a substitution of Gly (GGA) by a stop codon (TGA) (fig. 5). The base substitution also abrogates the *Bam*HI restriction site by altering the first base of its hexanucleotide recognition site. The same single-base mutation was detected, by DNA PCR amplification and *Bam*HI digestion, on all 14 mutant haplotype 7 chromosomes having the *Bam*HI 12.4-kb allele in Southern analyses but was not detected on the three other mutant haplotypes 7. On the basis of Southern analyses, this mutation also was not present on any other chromosomes in the present study.

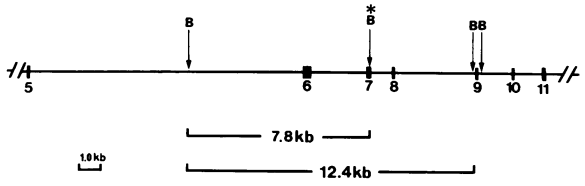

Figure 3 Restriction map of local part of PAH gene, showing the generation of 12.4-kb *Bam*HI fragment. The normal 7.8-kb fragment is also indicated. The differential 4.6-kb fragment comigrates with another, constant 4.6-kb band in Southern analyses and is not used to identify the normal alleles. Exons 5–11 are shown as blackened boxes. B = *Bam*HI site. The mutated *Bam*HI restriction site in exon 7 is indicated by an asterisk (*).

Discussion

The distribution of normal PAH haplotypes is quite similar among European populations. In contrast, the frequencies of haplotypes carrying PKU mutations vary considerably (Sullivan et al. 1989).

The most striking feature of the haplotype distribution in the Norwegian population was the high frequency of a previously undescribed haplotype 7 having an abrogated *Bam*HI site and termination mutation. This mutation was present on most (but not all) PKU haplotype 7 genes. Furthermore, it was only detected on chromosomes having this particular haplotype.

The geographical clustering of the native districts of the relevant grandparents of the PKU children having this mutation—i.e., the fact that all these grandparents were from the southeastern coast of Norway—may suggest a common genetic origin. The mutation has since been observed once in Denmark in a patient of Norwegian ancestry (F. Güttler, personal communication), a finding supporting a possibly "Norwegian" origin of this gene. In Sweden, three of four patients who have this mutation, discovered independently, have their familial origins close to the Norwegian southeastern coast (Svensson et al., in press; E. Svensson, personal communication).

There is no known interrelationship between Nor-

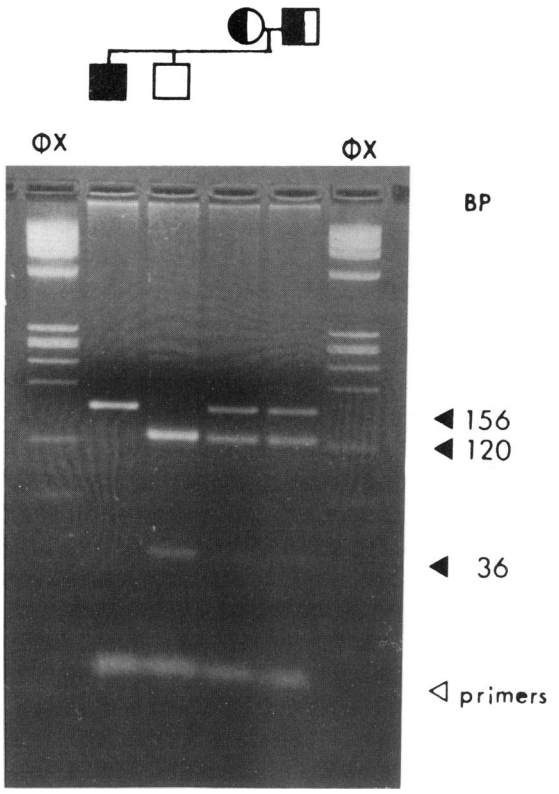

Figure 4 Demonstration of *Bam*HI restriction-site abrogation on PKU haplotype 7, by restriction-enzyme analysis of PCR-amplified fragments. Amplification of a 156-bp DNA fragment (containing exon 7 and a 10-bp intronic sequence on each end) of the four family members shown in fig. 1 was followed by *Bam*HI digestion. DNA from the affected child was not cleaved by the enzyme, whereas DNA from the healthy sibling displayed two fragments, one of 120 bp and one of 36 bp. The father and the mother are heterozygous.

wegian PKU families. However, a common gene source seems likely, considering (*a*) the rarity of the codon 272 mutation in other populations and (*b*) that the total Norwegian population after the Black Death in 1349–50 numbered only 100,000–150,000 people.

No haplotype 7 was found in Scotland (Sullivan et al. 1989), and none with an abrogation of this *Bam*HI site was found in France (F. Rey, personal communication). Thus, our findings contribute no support for the hypothesis of a Celtic origin (Saugstad 1975) for this particular gene.

The data above provide evidence that the codon 272 mutation is a severe PKU mutation. A stop codon in position 272 leads to a deletion of approximately 40% of the PAH polypeptide. None—or a very low—enzyme activity would be expected from the resulting polypeptide. The single PKU child homozygous for this mutation, as well as children (n = 8) who are heterozygous for haplotypes 7 and 1 and for haplotypes 7 and 3, as well as for haplotypes 7 and 8, had severe, classical PKU with very high neonatal serum phenylalanine levels.

The newborn incidence of PKU in Norway is close to 1/15,000 (L. Skjelkvåle, personal communication). Under the assumption of genetic equilibrium, the total frequency of all PKU genes is .00816. If the present sample of PKU patients is representative of all PKU patients in Norway, the new haplotype 7 mutation constitutes 14/86 (16.3%) of that frequency (.00133). Thus, as many as 1/376 of the general population may be heterozygous for this PKU gene. Presumably this gene frequency is much higher along the southeastern coast of Norway.

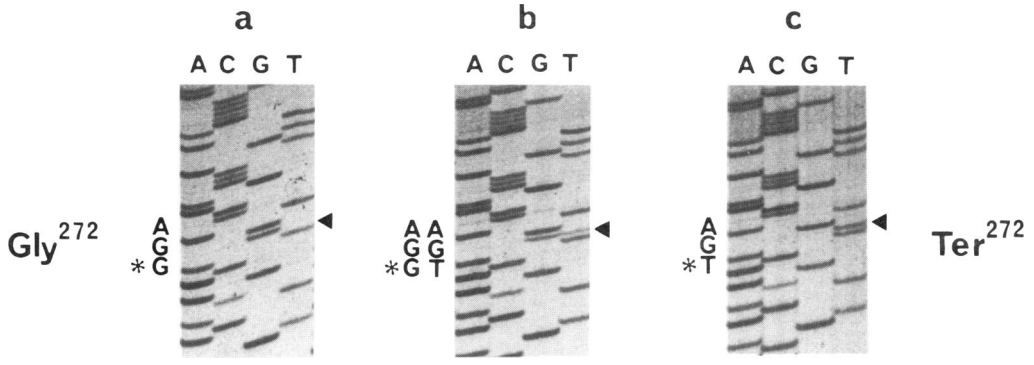

Figure 5 DNA sequence analysis detecting termination mutation in exon 7 of human PAH gene. DNA from each member of the PKU family shown in fig. 1 was subjected to asymmetric PCR and direct genomic sequencing of exon 7, as described in the text. Partial sequence ladders for exon 7 from the unaffected sibling (a), the father (b), and the homozygous PKU child (c) are shown. An asterisk (*) indicates the altered base. DNA from the mother (not shown) was identical to that of the father. The GGA-to-TGA transversion changes a codon for glycine at position 272 to a stop codon.

This haplotype 7 mutation was initially seen in DNA from a healthy, anonymous blood donor. It is likely that this person is heterozygous for PKU.

Acknowledgments

This work was supported by a grant from the Norwegian Research Council for the Sciences and the Humanities. We wish to thank professor Savio L. C. Woo for cooperation, including putting the PAH cDNA probe at our disposal and sharing, prior to publication, information on primer sequences.

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