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Three Polymorphisms but No Disease-Causing Mutations in the Proximal Part of the Promoter of the Phenylalanine Hydroxylase Gene

Abstract

The proximal promoter region of the human phenylalanine hydroxylase (PAH) gene was analyzed for the presence of mutations in 122 European phenylketonuria (PKU) and hyperphenylalaninemia patients having altogether 187 uncharacterized mutant PAH alleles. This promoter fragment, which contained the most 5′ transcription start site and about 300 bp upstream, was sequenced directly from polymerase-chain-reaction-amplified genomic DNA. No disease-causing mutations but three neutral nucleotide substitutions were found. A −195 T-to-C transition was present on 1% of 441 normal and 0.3% of 653 mutant chromosomes. All chromosomes that carried this transition and to which a restriction fragment length polymorphism (RFLP) haplotype had been assigned were of haplotype 1. A −71 G-to-A change and a +7 C-to-T change were always observed together and were found on 1% of 425 normal and 4% of 681 mutant chromosomes. In addition, these two transitions were found in seven heterozygote samples where the phase could not be established due to incomplete family samples. In individuals where RFLP haplotyes were known and phase could be established, these linked substitutions were associated with RFLP haplotype 9. The relatively high frequency (10–20%) of these two polymorphisms on PKU chromosomes from Great Britain, Ireland and France may reflect a relative concentration of haplotype 9 alleles among PKU chromosomes from these countries compared to the rest of Europe. The absence of disease-causing mutations within a region of the PAH gene that possesses basal promoter activity suggests that transcriptional mutations are not likely causes of PKU in Caucasian populations.

Key Words
Phenylalanine hydroxylase
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Introduction

In phenylketonuria (PKU) and nonketonuric hyperphenylalaninemia (HPA), an inherited (autosomal recessive) deficiency of the liver-specific enzyme phenylalanine hydroxylase (PAH; phenylalanine 4-monooxygenase, EC 1.14.16.1) leads to elevated blood phenylalanine, which may cause mental retardation [1]. PKU and HPA are detected through neonatal screening programs in most developed countries and can therefore be treated from an early age with a special diet so as to prevent mental retardation.

Genetic characterization of the disease started with the cloning of a full-length PAH cDNA [2] and of the PAH gene [3]. More than 50 mutations have been reported [4]. Most of them are rare but, in a geographically defined population, a few relatively common mutations often account for the majority of PKU and HPA cases [4, 5]. Mutations in the PAH gene are usually strongly associated with specific haplotypes as defined by eight restriction fragment length polymorphisms (RFLPs) [5, 6]. Nearly all the reported mutations are located in exons (mainly missense and nonsense mutations) or flanking intron sequences (splicing mutations) [4, 5].

The PAH promoter has only recently become available for mutational studies. A 9-kb genomic fragment at the 5′ end of the PAH gene has been shown to contain all the necessary cis-acting elements to direct tissue- and development-specific expression of a fused chloramphenicol acteyltransferase (CAT) gene in transgenic mice [7]. Further characterization has revealed that the PAH promoter is TATA-less and has multiple transcription start (CAP) sites, the most 5′ of which is located 154 bp upstream of the first translation codon [8]. Experiments, in which the activity of deleted PAH promoter fragments driving a CAT gene has been assayed after transfection of hepatoma cell lines, have shown that full basal promoter activity resides in the most proximal 121 pb [Y. Wang, pers. commun.].

In an effort to find naturally occuring mutations in the PAH promoter, we chose to study its most proximal part, since this region alone is implicated as being essential for basal transcriptional activity. We selected European patients with one or two uncharacterized PAH alleles for analysis with direct sequencing of polymerase chain reaction (PCR)-amplified genomic DNA. Although a large number of patients were examined, no disease-causing mutations, but three rare polymorphisms were identified. Apparently, these three base substitutions are neutral, because they were found on both normal and mutant chromosomes.

Materials and Methods

Identification of Mutations

A total of 122 European patients (21 English, 33 French, 21 Italian, 39 Swedish and 8 Swiss) were selected for sequencing studies, since they had one or two uncharacterized PAH alleles. A total of 187 uncharacterized mutant PAH alleles (37 English, 55 French, 35 Italian, 50 Swedish and 10 Swiss) were thus examined. An 803-bp fragment covering exon 1 and the most 3′ region of the PAH promoter (fig. 1) was amplified from genomic DNA with Taq DNA polymerase (Promega) and Taq DNA polymerase × 10 buffer (Promega). The reactions also contained dATP, dCTP and dTTP at 200 μ*M* each and dGTP and 7-deaza-dGTP (Boehringer or Pharmacia) at 100 μ*M* each. Primers were (A) 5′-TTCAGGAGCAGTTGTGCGAATAGCTG-3′ and (B) 5′-TCTCTTTCTCTGGAGGCCCAAATTCC-3′ (fig. 1). After an initial cycle of 97 °C for 6 min, 57 °C for 90 s and 72 °C for 1 min, 35 cycles were carried out at 94 °C for 30 s, 57 °C for 90 s and 72 °C for 1 min, followed by 72 °C for 7 min. Single-stranded templates were generated from gel-purified amplification products [9, 10] and primer B, using the same PCR conditions as above. Sequencing was done as previously described [9, 10] with primers (C) 5′-TTCTTCCCTTTACCCAGGGCGGGC-3′ and (D) 5′-TGTTATACCAGACGGTCGCA-3′ (fig. 1).

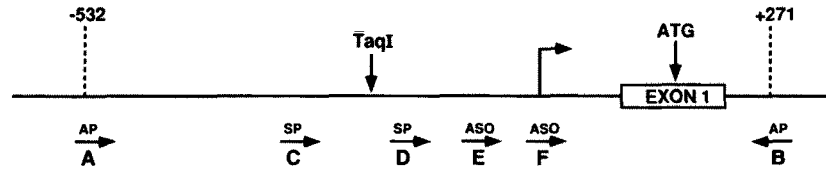

Fig. 1. Map of the 803-bp PCR-amplified PAH promoter fragment. AP = Amplification primer; SP = sequencing primer; ASO = oligonucleotide for allele-specific oligonucleotide hybridiziation. See text for primer and oligonucleotide sequences, and Konecki et al. [8] for the sequence of the entire region.

Population Screening

When the base changes described below had been identified through direct sequencing of the 122 patient samples, their frequencies among normal and mutant alleles in European populations were determined through analysis of all avaialble parental and patient samples from Czechoslovakia, Denmark, England, France, Hungary, Ireland, Italy, Norway, Scotland, Sweden and Switzerland. *Taq*I digestion of the 803-bp promoter fragment, which normally results in a 466-bp and a 337-bp fragment, was used to screen for the -195 T-to-C transition, since this substitution abolishes the only *Taq*I site in this fragment. Allele-specific oligonucleotide (ASO) hybridization was used to identify the -71 G-to-A change with oligonucleotides (E) 5′-GTCACCTGTCCAGGACG-3′ for the mutant and 5′-CGTCCTGGGCAGGTGAC-3′ for the normal sequence, and the +7 C-to-T change with primers (F) 5′-GCATCTCCACACAGTAA-3′ for the mutant and 5′-TTACTGTGCGGAGATGC-3′ for the normal sequence [10]. The presence (according to *Taq*I digestion or ASO hybridization) of any of the three base changes was always confirmed by sequencing.

Results

The -195 T-to-C Transition Is a Polymorphism Associated with Haplotype 1

A -195 T-to-C change (fig. 2) was identified on 4 of 441 (1%) normal and 2 of 653 (0.3%) mutant alleles (table 1). Two Swedish, 2 Danish and 1 Italian allele with this substitution were of haplotype 1 and 1 Italian allele was of unknown haplotype. Mendelian segregation was demonstrated in all 6 families. We considered the possibility that the alleged normal alleles with this substitution were in fact PKU alleles, in which case the -195 C-to-T change could be responsible for a reduced PAH activity and the parents carrying this substitution could have a mild form of HPA. However, this proved not to be the case, since 2 of the individuals with the -195 T-to-C change on a normal allele (2 Danish PKU mothers carrying a Y414C and an R408W mutation, respectively, on the mutant allele) had plasma phenylalanine levels compatible with normal or carrier state but not with HPA.

The -71 G-to-A and +7 C-to-T Transitions Are Polymorphisms Associated with Haplotype 9

A -71 G-to-A and a +7 C-to-T transition (fig. 2) were found to occur together on 4 of 425 (1%) normal and 25 of 681 (4%) mutant alleles (table 1). In addition, these two transitions were observed together in 7 parental samples, where the phase of the two transitions with respect to the normal and the mutant allele could not be established due to lack of patient samples. The observed frequencies

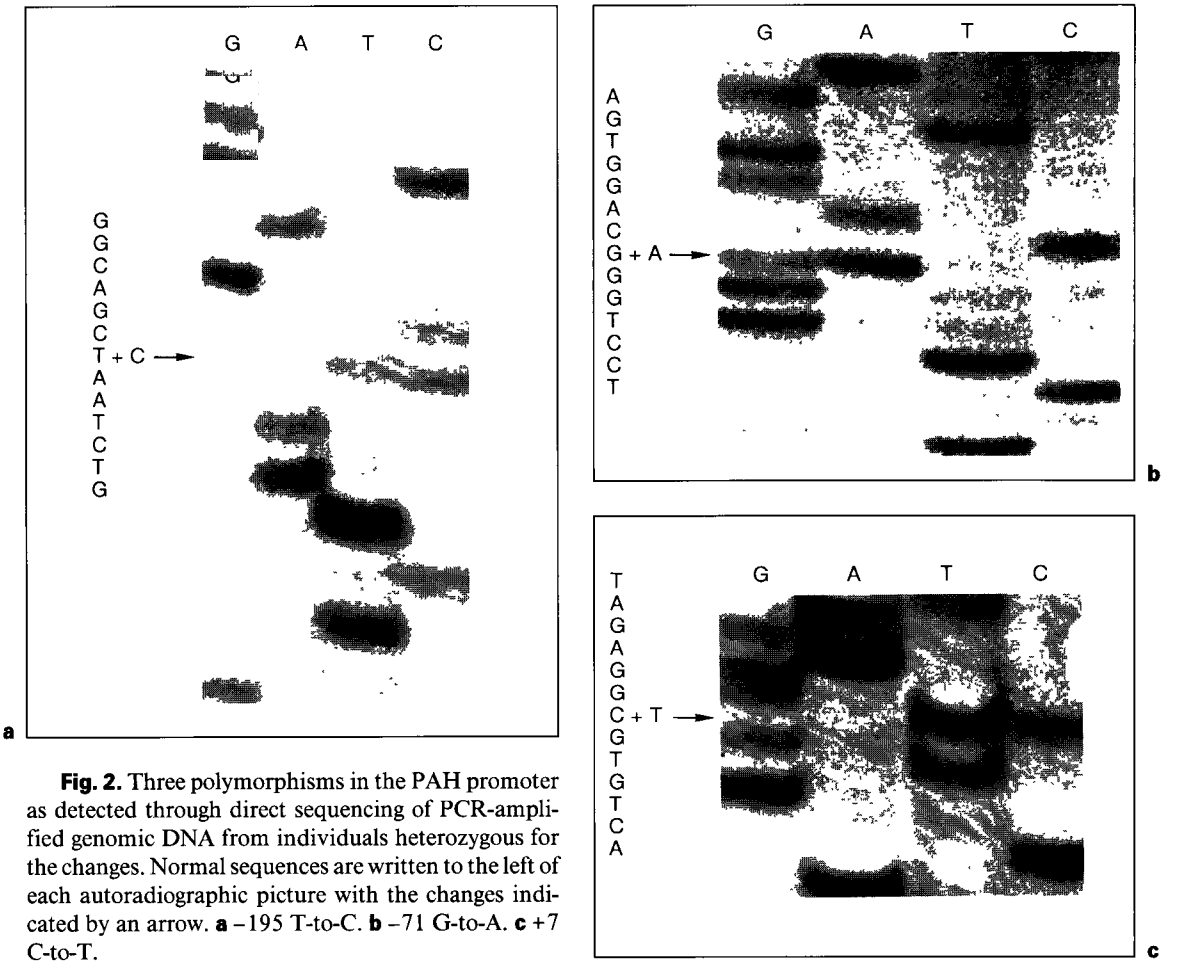

Fig. 2. Three polymorphisms in the PAH promoter as detected through direct sequencing of PCR-amplified genomic DNA from individuals heterozygous for the changes. Normal sequences are written to the left of each autoradiographic picture with the changes indicated by an arrow. **a** −195 T-to-C. **b** −71 G-to-A. **c** +7 C-to-T.

among normal chromosomes (1%) and mutant chromosomes (4%) are therefore underestimates. Furthermore, the difference in frequencies between mutant and normal chromosomes most likely reflects the greater contribution of mutant compared to normal chromosomes from populations with high prevalence: they were most frequent in Ireland, England and France, where they were present on 12–20% of the mutant alleles (no data for normal alleles) and Scotland, where they were present on 2.7–19% of the mutant alleles and 0–20% of the normal alleles (ranges are given because of the inability to establish the phase). They were also found in Scandinavia and Italy, but not in central Europe (table 1).

Eight of the 25 mutant and 2 of the 4 normal alleles carrying these transitions were of RFLP haplotype 9. A Mendelian segregation of the transitions with a mutant haplotype 9 allele could be shown in 3 families. The phase could not be determined in 2 additional patients who carried these transitions and had one haplotype 9 allele. The remaining alleles harboring these transitions were without RFLP haplotype assignment (RFLP haplo-

Table 1. Frequencies in European populations of three nucleotide substitutions in the PAH gene promoter

Country	Frequency of −195 T-to-C		Frequency of −71 G-to-A/+7 C-to-T	
	normal alleles	mutant alleles	normal alleles	mutant[a] alleles
Czechoslovakia	0/12	0/1	0/8	0/8
Denmark	2/89	0/100	1/75	2/82
England	0/60		6/50	
France	0/3	0/74	0/3	9/76
Hungary	0/33	0/34	0/31	0/68
Ireland	0/1	0/25		4/20
Italy	2/31	0/42	0/34	1/68 +1[b]
Norway	0/61	0/61	1/60	1/62
Scotland	0/32	0/41	0/30	1/37 +6[c]
Sweden	0/157	2/178	2/161	1/176
Switzerland	0/22	0/26	0/23	0/34
Total	4/441	2/653	4/425	25/681 +7

122 samples with at least one uncharacterized allele were analyzed through direct sequencing. In addition, all available European parental and patient samples were PCR-amplified for an 800-bp region of the PAH promoter and tested for the presence of base substitutions that had been identified in the 120 sequences samples. The −195 T-to-C change was analyzed with *TaqI* digestion and the −71 G-to-A/+7 C-to-T changes with ASO hybridization. The frequencies are given as the number of alleles containing the substitution over the total number of alleles analyzed. For technical reasons, the number of analyzed chromosomes vary for these substitutions.

[a] Of the mutant alleles carrying the −71 G-to-A and +7 C-to-T transitions, 2 Danish, 1 English, 2 Irish, and 1 Italian also carried the L348V mutation; 5 English, 6 French, 2 Irish, 1 Norwegian, 1 Scottish, and 1 Swedish did not carry the L348V mutation; 3 French could not be further analyzed due to limited amounts of DNA.

[b] Out of 2 Italian alleles carrying the −71 G-to-A and +7 C-to-T transitions, 1 was mutant and 1 was present in a carrier in whom the phase of these transitions with respect to the normal and the mutant allele could not be determined.

[c] Out of seven Scottish alleles carrying the −71 G-to-A and +7 C-to-T transitions, 1 was mutant and 6 were present in carriers in whom the phase of these transitions with respect to the normal and the mutant allele could no be determined.

types were unknown for all Irish, English, and Scottish alleles). No known haplotype 9 alleles did not have these two substitutions. Six of the mutant alleles carrying these substitutions (2 of haplotype 9 and 4 of unknown haplotype) also carried the L348V mutation, which is associated with haplotype 9 [11]. No known L348V alleles did not carry the two transitions. However, not all mutant alleles had been analyzed for the L348V mutation and no

alleles had been analyzed for the I65T [12, 13] and Δnt1043–11053→T278X [14] mutations, which are associated with Caucasian haplotype 9 alleles, and the Y356X mutation [15], which is associated with Oriental haplotype 9 alleles.

Discussion

The present study was undertaken to explore the hypothesis that some of the uncharacterized European PKU alleles may harbor disease-causing promoter mutations. However, no such mutations, but only neutral base changes, were found. A Swedish sample population consisting of 88 families with at least one PKU or HPA child had been examined with ASO screening for previously described mutations [16], and subsets of the same population had been analyzed with direct sequencing of selected PCR-amplified exons (exons 1, 4, 5, 7, 9, 10, 11, and 12) for novel mutations. The search for exonic and splicing PAH mutations in the Swedish population has thus not been completed, and 48 of the 178 mutant PAH alleles (27%) remain without an identified mutation [16]. The fraction of uncharacterized alleles varied among the other European populations that were included in the present study. However, for none of the studied patients had all exonic and flanking intronic regions been analyzed. Therefore, the 187 uncharacterized alleles that were analyzed for promoter mutations by direct sequencing may carry as yet unidentified exonic or splicing mutations. The fact that we did not find any disease-causing mutations within a fragment that possesses basal promoter activity does not exclude the possibility of mutations residing in more distant parts of the promoter maybe causing subtler changes in transcriptional activity. Such mutations would not, however, be expected to severely affect the transcription rate, since the region upstream of the most proximal 121 bp can be deleted without effect on basal transcriptional activity [Y. Wang, pers. commun.]. Recently, mutations that are likely to be disease-causing were found on 99% of mutant PAH alleles in a Danish sample population [F. Güttler, pers. commun.]. All these mutations were located in exons or flanking intronic regions. This finding, together with the lack of disease-causing mutations in the proximal PAH promoter, implies that there are no frequent PAH promoter mutations with a substantial effect on PAH activity as a cause of PKU and HPA in European patients.

The −195 T-to-C transition was associated with haplotype 1 in Scandinavia and Italy. Because haplotype 1 is common in these populations [4, 5, 17–19], this substitution may have occurred independently on haplotpye 1 alleles in them. It may also, however, have been spread through migration from a single origin. The presence of this alteration on normal as well as mutant chromosomes implies that it is a polymorphism without significant effect on PAH enzyme activity, and that it appeared before any disease-causing mutation occurred on the same allele. However, because this base change was found on only a small subset of haplotype 1 alleles, it most likely occurred after haplotype 1 evolved.

The −71 G-to-A and +7 C-to-T transitions were always observed together, and exclusively and inclusively associated with haplotype 9 in those studied populations for which we have RFLP haplotype data (Danish, French, Italian, Norwegian and Swedish). It is thus possible that the −71 G-to-A and +7 C-to-T transitions are part of the haplotype 9 framework. If so, the relative frequencies of these two polymorphisms should parallel the relative frequencies of haplotype 9 in various populations. The relative frequencies of haplotype 9 in Ireland and England are not pub-

lished, but is 9/103 (8.7%) of mutant and 2/80 (2.5%) of normal chromosomes in England [L. Tyfield, pers. commun.]. No haplotype 9 alleles have been identified in the Scottish population [17, 20], but recent analysis of the same samples for certain RFLPs in PCR-amplified DNA has revealed certain discrepancies with the original haplotype assignment [E. Svensson and R.C. Eisensmith, unpubl. data]. Haplotype 9 is found on 3% [17] to 7% [21] of French PKU chromosomes, and on about 1–2% of both PKU and normal chromosomes in Denmark [6], Norway [22], and Sweden [23]. The higher frequencies of the two polymorphisms than of haplotype 9 in those countries where they are most prevalent may suggest their presence on additional haplotypes. Alternatively, these two polymorphisms could be markers specifically for haplotype 9 and the discrepancies in terms of frequencies could be due to analysis of different subsets of the populations for RFLP haplotypes and the two polymorphisms.

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Announcement

European Concerted Action on Fanconi Anemia Research (EUFAR)

Starting January 1994 through December 1996, the European Community will sponsor a Biomed I concerted action to determine the clinical and molecular significance of the Fanconi anemia gene defects.

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