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ORIGINAL INVESTIGATION

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# A silent mutation induces exon skipping in the phenylalanine hydroxylase gene in phenylketonuria

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**Abstract** An A→T substitution in cDNA nucleotide 1197 (c.1197A/T) of the human phenylalanine hydroxylase (PAH) gene has been regarded as a silent mutation, because both the wild-type (GUA) and the mutant (GUU) alleles encode a valine residue at codon 399 (V399 V). The nucleotide c.1197 is located at the 3'-end of exon 11at position –3 of the exon-intron junction. To explore whether the substitution exerts any effects on the processing of the PAH mRNA, illegitimate PAH transcripts from lymphoblast cultures of a phenylketonuria (PKU) patient heterozygous for c.1197A/T were analyzed by the polymerase chain reaction following reverse-transcription (RT-PCR). mRNAs with an exon 11 deletion were revealed. Furthermore, by using an R408 W mutation in the paternal allele as a marker, sequence analysis of the RT-PCR products indicates that virtually all PAH transcripts from the maternal allele with the c.1197A/T substitution do not contain exon 11. To address whether this substitution is the main determinant for exon skipping, PAH minigenes with or without the substitution were constructed and transfected to a human hepatoma cell line. Analysis of the transcription products by S1 nuclease mapping clearly indicated that such exon 11 skipping was directly associated with the c.1197A/T substitution. Thus, this study demonstrates that the c.1197A/T substitution in the PAH gene is not just a neutral polymorphism but a mutation that induces post-transcriptional skipping of exon 11 leading to a PKU phenotype.

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## Introduction

Classical phenylketonuria (PKU; MIM 261600; Online Mendelian Inheritance in Man, http://www.ncbi.nlm.nih.gov/Omim) is a human genetic disorder resulting from deficiency of phenylalanine hydroxylase (PAH) (Jervis 1953; Udenfriend and Bessman 1953; Kaufmans 1976). To date, more than 300 PAH mutations have been reported (Nowacki et al. 1998; Online PAH data base, http://www.mcgill.ca/pahdb/), most of which occur in the coding sequence. Whereas the majority of the mutations result in alterations in amino acid residues, a small number of mutations occur in the third position of a codon and are classified as silent mutations. Silent mutations have been reported to cause diseases mainly by affecting RNA processing. For instance, activation of splice sites mediated by exonic silent mutation in the genes encoding β-globin, the growth hormone receptor and fibroblast growth factor receptor 2 has been reported to cause $\beta^+$-thalassemia, Laron dwarfism and Crouzon syndrome, respectively (Goldsmith et al. 1983; Berg et al. 1992; Li et al. 1995). In addition, silent mutations may have functional consequences and lead to a disease state by disrupting exonic splicing enhancers that act to enhance the use of the specific splice site (for a review, see Cooper and Mattox 1997). During the screening of PAH mutations in Taiwan, we encountered three PKU families each carrying, in one of the PAH alleles, an A→T transversion at cDNA nucleotide (nt) 1197 (c.1197A/T) and no other mutations. This substitution results in a codon 399 change from GUA to GUU. Both codons encode a valine (V399 V). This A→T transversion at cDNA nt 1197 has been reported to occur at a frequency of 0.005 and 0.09 in normal and mutant PAH genes, respectively, in the Chinese population (Huang et al. 1991). Nucleotide c.1197 is located at the 3'-end of exon 11 at position –3 of the exon-intron junction (Fig. 1A). Whereas the sequence between –2 to +6 of the splice-donor site is regarded to be highly conserved, no particular preference has been assigned to nucleotide –3 at the splice-donor site (Mount 1982; Fig. 1A).

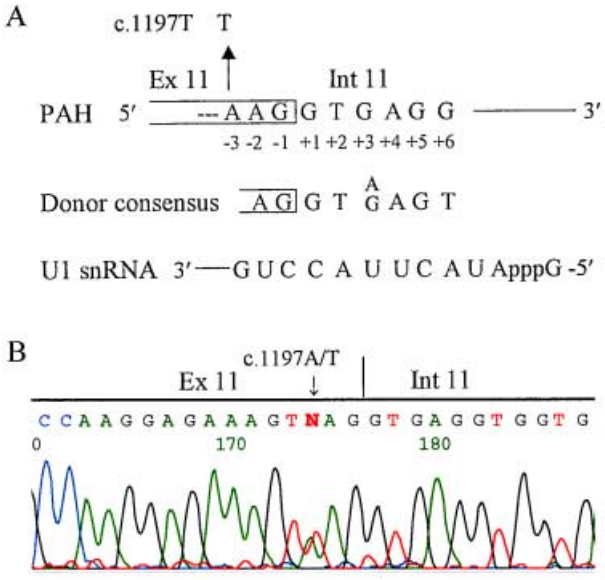


**Fig. 1** **A** Schematic presentation of splice-donor site of intron 11 of the PAH gene. The consensus splice-donor sequence and the 5'-end of U1 snRNA that base-paired with the splice-donor sequence on the pre-mRNA are also shown. The c.1197A/T substitution occurring at nucleotide position –3 of the PAH intron 11 is indicated. **B** Direct sequencing of the genomic DNA of PKU proband U. A 295-bp DNA fragment containing PAH exon 11 and its flanking intron sequence was amplified from lymphocyte DNA of the proband of PKU family U. The amplified products were sequenced directly. An A→T transversion corresponding to cDNA nt 1197 (c.1197A/T) was identified in one of the PAH alleles, changing codon 399 from GUA to GUU, both of which encode the valine residue.

Thus, it remains to be determined whether the c.1197A/T substitution has any effects on expression of the PAH gene. In this study, we show that c.1197A/T is indeed a mutation that leads to the skipping of exon 11 during the pre-mRNA processing step that thereby results in a PKU phenotype.

## Materials and methods

### Cell line

Lymphoblast cell lines were established from PKU probands by using an Epstein-Barr virus transformation protocol (Chan et al. 1986). RPMI 2650 (Su et al. 1981) was obtained from the American Type Culture Collection. HuH-7 is a human hepatoma cell line (Nakabayashi et al. 1982). Cells were cultured in Dulbecco's modified Eagle's medium with 10% fetal bovine serum.

### Polymerase chain reaction, reverse transcription/polymerase chain reaction, and direct DNA sequencing

To analyze the DNA sequence of the splice-donor junction of intron 11 of the human PAH gene, a 295-bp DNA fragment containing the PAH exon 11 and its flanking intron sequences was obtained by polymerase chain reaction (PCR) amplification of genomic DNA of the proband by using primers AP255 and AP264 as described by Dworniczak et al. (1991). To analyze PAH mRNA, total RNA isolated from lymphoblast or other cell lines was reverse-transcribed into single-stranded cDNA by using primer P6 on exon 13 as described by Okano et al. (1994). The cDNA sequence covering exons 7–13 was PCR-amplified by using primers P5 and P6 (Okano et al. 1994). The exon 10–12 segment was re-amplified by using primers P178 and P179 residing in exons 10 and 12, respectively. Primer P178, 5'-ccgggtacctctagaTGTGGAGTTTGGGCTCTGCAAACAAGGAGACT-3', spans cDNA nt 984–1015 (uppercase letters) and carries a heterologous linker sequence of *Kpn*I and *Xba*I recognition sites at the 5'-end (lowercase letters). Primer P179, 5'-ccggatatcgaattcGCCAAAATCTTAAGCTGCTGGGTATTGTCCAAG-3', spans nt 1301–1269 with the linker sequence of *Eco*RV and *Eco*RI at its 5'-end. The restriction enzyme recognition sites at the ends were included to facilitate cloning of the PCR products. The PCR or reverse transcription/PCR (RT-PCR) products were sequenced using the BigDye Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Cetus) with an ABI Prism 377 DNA Sequencer (PE Biosystems).

### Plasmid construction

To construct the PAH mini-gene carrying either nucleotide A or T at position c.1197, a genomic fragment including exons 10–12 was obtained by PCR amplification with primers P178 and P179. The template used was a genomic DNA preparation from proband U who is heterozygous at c.1197A/T. The amplified products were cloned into pSVpoly vector (Stacey and Schnieke 1990). To assure that nucleotide c.1197 is the only difference between the mini-gene constructs of the wild-type [pPAH(w)] and the mutant [pPAH(m)] alleles, pPAH(m) was first derived from the cloning process. pPAH(w) was subsequently constructed by an A→T nucleotide substitution at c.1197 in the pPAH(m) clone by site-directed mutagenesis (Promega). The presence of only the mutation being analyzed in these two constructs was confirmed by DNA sequencing.

### Transfection, RNA isolation, and S1 nuclease mapping

The HuH-7 cell line seeded at $1\times10^7$ cells per 150-mm-diameter culture dish was transfected with 30 μg DNA of wild-type pPAH(w), mutant pPAH(m), or vector pSVpoly, respectively, by the calcium-phosphate co-precipitation method (Spandidos and Wilkie 1984). After transfection (72 h), cells were harvested for RNA isolation by the guanidium/cesium chloride method (Glisin et al. 1974). S1 nuclease analysis was performed by using appropriate restriction fragments labeled at the 5'-end with T4 polynucleotide kinase (Su et al. 1983).

## Results

### Association of exon 11 skipping with allele carrying the c.1197A/T transversion

PAH gene is expressed exclusively in liver. Since liver tissue was not readily available for this study, an analysis of illegitimate PAH transcripts from lymphoblasts of PKU proband U was carried out as described by Okano et al. (1994). In addition to the c.1197A/T substitution in maternal allele (Fig. 1B), this patient also carries a paternal allele with a nucleotide C→T transition (c.1222C→T) in exon 12 resulting in an arginine to tryptophan substitution at codon 408 (R408 W; Fig. 2A). The segment of the PAH transcript consisting of exons 10–12 was amplified by RT-PCR. Besides a normal size cDNA of 348 bp, a shorter cDNA fragment of 214 bp in size was detected (Fig. 2B, lane 1). Sequence analysis showed that the shorter cDNA

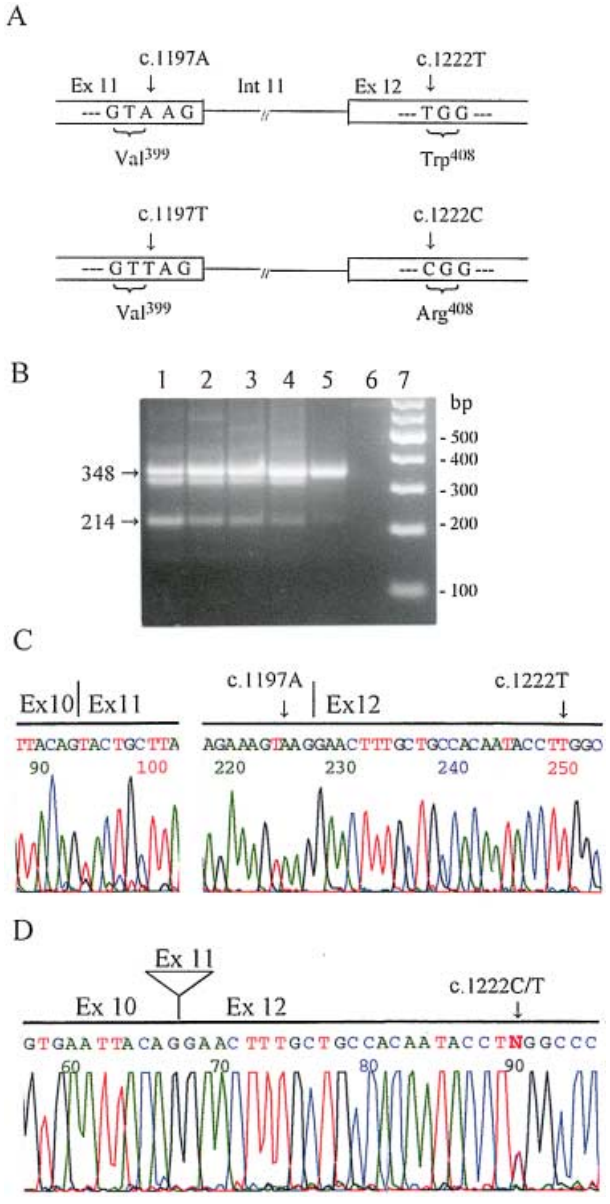


**Fig. 2A–D** Association of exon 11 skipping with the allele carrying the c.1197A/T transversion. **A** The genotypes of the paternal (*top*) and maternal alleles (*bottom*) of PKU proband U are schematically illustrated. **B** Analysis of PAH mRNA by RT-PCR. For cDNA synthesis, total RNAs isolated from a lymphoblast cell line from proband U and heterozygous at cDNA nt 1197 (c.1197A/T; *lane 1*), two independent lymphoblast cell lines carrying the wild-type c.1197A allele (*lanes 2*, *3*), a human hepatoma cell line HuH-7 (*lane 4*), and a human epithelium cell line RPMI 2650 (*lane 5*) were used. cDNA covering exons 7–13 was amplified by using primers P5 and P6. The sequence containing exons 10–12 was re-amplified with primers P178 and P179. *Lane 6* A cDNA synthesis control in the absence of reverse transcriptase and with the same RNA template as in *lane 1*. The amplified products were analyzed on a 3% agarose gel stained with ethidium bromide. *Lane 7* A 100-bp DNA ladder. **C, D** Direct sequencing of the 348-bp and 214-bp RT-PCR products. The 348-bp (**C**) and 214-bp (**D**) cDNAs from proband U were isolated from agarose gel and the sequences determined

was a product arising from transcripts skipping exon 11. Interestingly, the 214-bp cDNA was also detectable at a low level in two lymphoblast cell lines (Fig. 2B, lanes 2 and 3) and a human hepatoma cell line HuH-7 (Fig. 2B, lane 4), all of which are homozygous at c.1197 (c.1197A), whereas such a band was undetectable in cDNA derived from a human epithelium cell line, RPMI 2650 (Fig. 2B, lane 5). The possibility that other DNA polymorphisms such as c.1194A/G (Benit et al. 1994) and c.1198A/C (Takarada et al. 1993) located near the 3'-end of exon 11 were responsible for the observed exon 11 skipping was ruled out, since these sites have been shown to be normal in these cell lines (data not shown). A faint band below the wild-type-sized cDNA was also detected in all the samples, except in RPMI 2650. This minor DNA species was probably a DNA heteroduplex formed between cDNA strands of the wild-type and exon 11 deletion mutant as has been observed previously (Eckhart et al. 1999).

To investigate the allelic origin of the aberrant transcript, both the 348-bp and 214-bp cDNAs derived from the proband were sequenced. The data showed that the 348-bp cDNA was associated exclusively with the paternal allele bearing the c.1222T and the wild-type c.1197A sequence (Fig. 2C). This suggests that mRNA transcribed from the maternal allele with the c.1197T sequence could not be processed properly. On the other hand, cDNAs with the exon 11 deletion were associated with both c.1222C and c.1222T (Fig. 2D), in agreement with the observation that exon 11 skipping could occur even in the wild-type PAH allele (Fig. 2B).

## The c.1197A/T substitution is the major determinant for exon 11 skipping

To evaluate the effect of c.1197A/T on pre-mRNA splicing, we constructed a PAH mini-gene that spans exons 10–12 and harbors c.1197A/T for expression analysis in the human hepatoma cell line HuH-7 (Fig. 3). The PAH genomic fragment of about 4 kb in size was expressed by using an SV40-based expression vector. The wild-type pPAH(w) and mutant pPAH(m) constructs have an identical background except for the c.1197 nucleotide position. The plasmid DNAs together with the vector control were transiently transfected into HuH-7 cells. The structure of the resulting PAH mini-gene RNAs was determined by S1 nuclease mapping. Mapping with a 502-bp 5'-end labeled probe spanning cDNA exons 7–12 would generate a fully protected product of 337 nt in length corresponding to a mRNA species with a contiguous segment with exons 10–12 (Fig. 3A). However, in mRNA with a disruption between exons 10 and 12, such as deletion of exon 11, a protected product of 121 nt would be expected. To avoid detection of the endogenous PAH mRNA, the probe was end-labeled at a *Bam*H1 restriction site that was present only within the transfected gene constructs. The protected DNA fragments were displayed by gel electrophoresis and quantitated with a PhosphorImager (Molecular Dynamics). The data showed that, in the transfection experi-

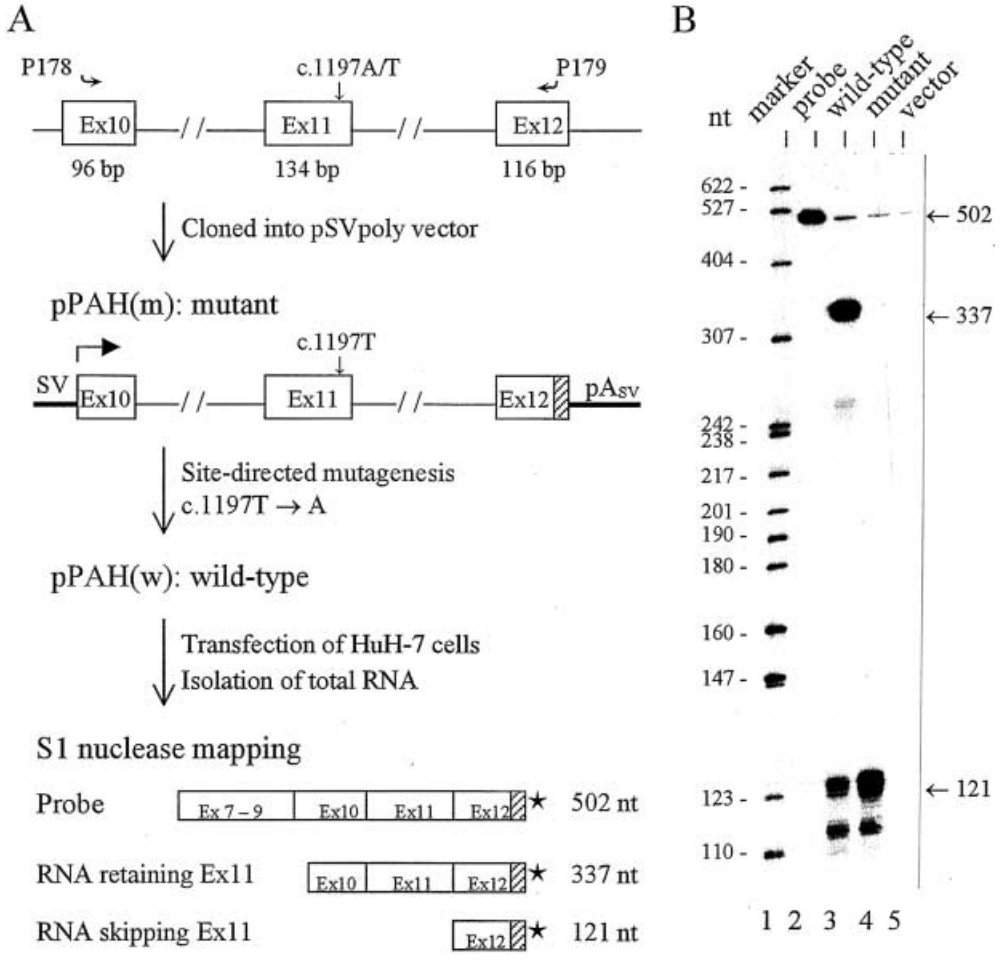


**Fig. 3A, B** Demonstration of the c.1197A/T transversion as the major determinant for exon 11 skipping by S1 nuclease mapping. **A** Strategy for analyzing the splicing abnormality in the PAH gene carrying c.1197T. The genomic PAH sequence was amplified from the genomic DNA of proband U who was heterozygous at c.1197A/T by PCR with primers P178 and P179. The mini-gene containing c.1197A [pPAH(w)] or c.1197T [pPAH(m)] was under the transcriptional control of a SV40 early promoter (SV) and its polyadenylation signal ($pA_{SV}$). The *shaded box* represents the vector sequence joined to exon 12 of the PAH gene in the constructs. The structure of mRNA in the transfected cells was analyzed by S1 nuclease mapping. The configuration of the S1 probe and the expected size of the fragments (in nt) protected by RNAs of normal or aberrant splicing are schematically represented. *Stars* $^{32}$P 5'-end labels. **B** Polyacrylamide gel electrophoresis of protected products. Total RNAs from plasmid DNA-transfected HuH-7 cells were subjected to S1 nuclease mapping analysis. Lanes labeled *wild type*, *mutant*, and *vector* represent analysis performed with RNAs isolated from pPAH(w), pPAH(m), and pSVpoly-transfected cells, respectively. *Marker* End-labeled *Msp*I-digested pBR322 DNA, *probe* undigested DNA probe

ment with the wild-type construct, the fully protected 337-nt band accounted for 65% of all protected products (Fig. 3B, lane 3). However, only 1% of RNA derived from transfection with the mutant construct was the fully protected species (Fig. 3B, lane 4). Instead, the major species was the 121-nt band, which represented the mRNA species lacking exon 11. The appearance of a small amount of 121-nt mutant protected fragment in the wild-type transfectants was consistent with the results of RT-PCR analysis of endogenous PAH RNAs transcribed from the wild-type PAH gene (Fig. 2B). In addition, a band smaller than 121 nt in size also appeared in wild-type and mutant transfectants but not in the case of the vector control. The band could be a product derived from mRNA by means of a cryptic splice-donor site in exon 12 or it might an artifact attributable to S1 nuclease over-digestion. The data thus clearly indicate that the c.1197A/T substitution is the main determinant for the skipping of exon 11 during PAH pre-mRNA processing.

## Discussion

By analyzing aberrant PAH transcripts from lymphoblasts and by transient transfection assay, we show that transcription from a PAH allele carrying the c.1197A/T substitution results in mRNA skipping of exon 11. The translation reading frame is altered since this mRNA has 134 fewer nucleotides than normally spliced mRNA. The PAH protein translated from such an mRNA would eliminate the C-terminal 97 amino acids (residues 356–452), which contain a part of the catalytic domain and the entire

tetramerization domain of the PAH protein (for a review, see Hufton et al. 1995). Thus, both the function and stability of the truncated protein are severely affected (Fusetti et al. 1998). The c.1197A/T mutation has been identified in three unrelated PKU families in this study. All probands are compound heterozygotes with the c.1197A/T mutation being associated with the R408 W mutation, a small deletion in exon 3 (unpublished data) and an unknown mutation, respectively. In agreement with the biochemical phenotype of the c.1197A/T mutation, probands of these PKU families show a severe clinical phenotype (data not shown). In addition to the production of truncated protein, aberrantly spliced mRNA is usually unstable because the frequent appearance of premature stop codons in such mRNA species often leads to activation of the nonsense-mediated mRNA decay pathway (for a review, see Maquat 1996). The exon-11-skipping event induced by the c.1197A/T mutation results in a frame-shift with the replacement of the C-terminal 97 residue segment with 21 missense codons followed by a premature stop codon in exon 12. A study of the human triosephosphate isomerase gene has shown that nonsense codons located less than 50–55 bp upstream of the 3'-most intron fail to mediate mRNA decay (Zhang et al. 1998). The premature stop codon in the aberrant PAH mRNA is located 50 bp upstream of intron 12, which is the 3'-most intron of the human PAH gene. Conceivably, effects of the nonsense-mediated mRNA decay pathway on the stability of the aberrant PAH mRNA species are minimal. The unavailability of liver mRNA from the same patient precludes examination of such a possibility. However, the finding that RT-PCR products from the allele carrying c.1197A/T can easily be detected in lymphoblasts of proband U seems to support such a notion. In addition, the detection of aberrantly spliced mRNA transcribed from the wild-type allele of the PAH gene may in part attribute to the stability of this transcript. It is noteworthy that, even if such aberrantly spliced PAH mRNAs do bypass RNA surveillance by the nonsense-mediated mRNA decay pathway, truncated protein translated from the transcripts would not be expected to exert a dominant-negative effect, since PAH protein lacking the tetramerization domain is known to be unstable (Fusetti et al. 1998). In addition, such protein loses its oligomerization ability.

Based on the analysis of 104 independent splice site mutations, Ketterling et al. (1999) have proposed a „5–6 hypothesis“ to predict the severity of defects in splice-donor site. They postulate that splice-donor mutations not involving the invariant GT doublet at the splice-donor junction would result in abolishment of more than 99% of normal splicing if the splice-donor sequence matches the consensus in only five or six bases of the eight-base consensus sequence (see Fig. 1A). On the other hand, such mutations result in normal or moderately reduced splicing activity if the donor matches the consensus at seven or eight bases. Since splice-donor site of PAH intron 11 matches seven out of eight consensus bases (Fig. 1A), this site is expected to be insensitive to splice-donor site mutations if a GT-invariant is not involved. However, our data show that splicing is deleterious when the A→T substitution occurs at the –3 position. This event was observed at a low but detectable level even in the wild-type PAH allele. Base pairing between the splice-donor site sequence on the pre-mRNA and the U1 small-nuclear RNA (snRNA) is important for a splicing reaction (Mount et al. 1983; Krainer and Maniatis 1985). In this regard, the authentic sequence of the splice-donor site of PAH intron 11 is aAG\GugAGg (Fig. 1), where the lowercase letters at positions -3, +3, and +6 represent discordant nucleotides that do not base-pair with U1 snRNA. On the other hand, only position +6 does not match with the consensus sequence of the splice-donor site (AG/GURAGU, R = purine; Fig. 1). Shapiro and Senapathy (1987) have developed a consensus value to calculate the similarity of a given splice site to the consensus sequences. The splice-donor site of intron 11 has a high value of 0.916. This value is further increased to 0.922 when the nucleotide at position –3 is not included. Apparently, the splice-donor sequence alone is not sufficient to assure fidelity of splicing. Additional *cis*-elements, such as the splice-acceptor sequence in the preceding intron and exonic splicing enhancers, are also important for exon recognition (Berget 1995; Blencowe 2000). In this respect, the splice-acceptor sequence of intron 10 has a low consensus value of 0.772 because of the poor pyrimidine content at pyrimidine positions (Shapiro and Senapathy 1987). It is possible that the low degree of conservation in the splice-acceptor sequence of intron 10 has contributed to the aberrant splicing of the native gene. In such a context, decreasing the consensus value of the splice-donor sequence of intron 11 from 0.916 in the wild-type allele to 0.881 in the c.1197A/T mutant has understandably resulted in an unfavorable situation for exon recognition during pre-mRNA processing. On the other hand, many exonic mutations that are associated with diseases are known to disrupt the exonic splicing enhancer (ESE; for a review, see Cooper and Mattox 1997). The best recognized feature of ESEs is that most are purine-rich. The nucleotides surrounding c.1197 (AAGGAGAAAGTAAG with c.1197A underlined) are indeed purine-rich. It is possible that the effect of the c.1197A/T mutation on splice-site selection is not attributable to changes in the consensus sequences at the splice sites but is a consequence of affected ESE. Further studies are required to elucidate the mechanism involving exon 11 recognition during pre-mRNA processing of the human PAH gene.

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