

## RAPID COMMUNICATION

## Mutations in the Pyruvate Kinase L Gene in Patients With Hereditary Hemolytic Anemia

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We have completely sequenced the introns of the human L-type pyruvate kinase (PK) gene using the published cDNA sequence. Subsequently, DNA from 12 unrelated PK deficiency (PKD) patients of Central European origin was investigated for mutations in this gene by solid-phase sequencing. We detected 10 different mutations, 9 of which result in single amino acid alterations, whereas the tenth destroys a splice site. Eight of the 10 mutations have not been described before. We found 7 missense mutations: G<sup>994</sup> → A (Gly-332 → Ser), G<sup>1006</sup> → T (Ala-336 → Ser), A<sup>1061</sup> → G (Asn-361 → Asp), G<sup>1174</sup> → A (Ala-392 → Thr), G<sup>1493</sup> → A (Arg-498 → His), G<sup>1529</sup> → A (Arg-510 → Gln), C<sup>1594</sup> → T (Arg-532 → Trp), one in-frame triplet deletion (del) as well as one insertion (ins): del AAG<sup>1060-62</sup> (del Lys-354), ins AGC after C<sup>1203</sup> (ins Ser

after Cys-401), and one splice-site mutation at the border of intron A to exon 3: g/G<sup>283</sup> → a/G. Although the enzymatic properties are substantially changed in all PK mutations, only two affected amino acid positions are in or close to the active site. Mutations C<sup>1594</sup> → T, G<sup>994</sup> → A, del AAG<sup>1060-62</sup> and the splice-site mutation g/G<sup>283</sup> → a/G have been detected in two different patients each. Mutation G<sup>1529</sup> → A was found in five different alleles. Haplotype analysis with the A/C polymorphism at position 1705 gave evidence for a single origin of this most frequent mutation in PKD as suggested by Baronciani and Beutler (*Proc Natl Acad Sci USA* 90:4324, 1993). Carrier detection and prenatal diagnosis are now feasible for the affected families.

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THE MOST COMMON cause for congenital nonspherocytic hemolytic anemia among glycolytic enzyme defects is a deficiency of the enzyme pyruvate kinase (PK). In humans as in other mammals, four isozymes exist: PK-L and PK-R are expressed in liver and blood cells, respectively, by the use of different promoters of the PK-L gene,<sup>1</sup> whereas PK-M<sub>1</sub> (muscle and brain) and PK-M<sub>2</sub> (fetal and most adult tissues) are produced by alternative splicing during transcription of the PK-M gene.<sup>2</sup> Mutations in the PK-L gene have been described to cause PK deficiency (PKD). Although the deficiency has been known at the protein level since 1961,<sup>3</sup> the PK-L cDNA was cloned and sequenced only in 1988.<sup>4</sup>

No more than five mutations have been described in PK deficiency (PKD) patients of Lebanese, Turkish, and Japanese origin within the last 3 years.<sup>5-9</sup> Only recently, 12 additional mutations have been published by Baronciani and Beutler<sup>10,11</sup> and 2 by Kanno et al.<sup>12</sup> Apart from one nonsense, two frameshift, and one splice-site mutation, all mutations found so far are single amino acid substitutions. They are scattered throughout the coding sequence. However, a certain prevalence has been observed of the C<sup>1151</sup> → T mutation in one Lebanese and two Japanese families, and a strong prevalence of the G<sup>1529</sup> → A mutation (10 of 26 alleles) in US patients of caucasian origin.<sup>10,11</sup>

We have previously reported the biochemical characterization of PKD patients from Central Europe.<sup>13,14</sup> To determine the mutations causing the PKD in these patients we completely sequenced the human PK-L gene introns and designed primers that cover 100% of the coding sequence including the R-specific exon. With this polymerase chain reaction (PCR)/sequencing approach, we analyzed the DNA of twelve unrelated patients suffering from PKD and detected putative disease-causing mutations in 18 of the 24 alleles investigated. For some of the affected families, we developed carrier diagnostics on the basis of PCR amplification combined with restriction enzyme digestion and allele-specific oligonucleotide hybridization (ASO).

## MATERIALS AND METHODS

**Patients and DNA.** Twelve unrelated patients with hemolytic anemia caused by PKD of Czech, Slovak, German, and English

origin were investigated. For most of the patients, the enzyme deficiency was characterized according to the International Committee for Standardization in Haematology.<sup>15</sup> The most important hematologic and enzyme-kinetic parameters of these patients are listed in Table 1.

Only patients 5 and 11 were presumed to be heterozygous for the PKD because of their mild disease course and reticulocytosis and only slightly reduced enzyme activity. They were selected mainly for their abnormal enzyme-kinetic behavior (high Km-PEP variants, ie, variants with a high Michaelis constant for phosphoenal pyruvate). No hematologic data were available for patient 12, but he showed similar severe clinical symptoms as did patient 2, who is also homozygous for the same mutation.

For most of the variant enzymes, a reduced intracellular stability, an altered electrophoretic mobility, and an increased sensitivity against proteolytic degradation (trypsination) were observed. In patients 1, 3, 6, and 9, a low but detectable PK-M activity was measured at up to 10% to 15% of a normal PK-R activity. DNA was isolated from frozen total blood samples according to Kunkel et al.<sup>16</sup>

**Determination of intronic sequences.** From the published human PK-L cDNA<sup>4</sup> and the PK-L-gene promoter sequence,<sup>17</sup> six pairs of oligonucleotide primers were constructed and used in PCRs to amplify fragments containing introns R-J (Fig 1). Their sequences were as follows: PR: 5'-AGC TAA CTT CAG TAA AGT AC-3'; E3\*: 5'-

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Table 1. Genetic, Enzymatic, and Hematologic Characteristics of PKDs

Patient No.	Reticulocytes (%)	Mutation (allele 1/allele 2)	Amino Acid Substitution	Vmax/K ( $\mu\text{mol/L/mL}$ RBC xhx 37°C)	S <sub>0.5</sub> PEP ( $\mu\text{mol/L}$ )
1	60-80	ins AGC after C <sup>1203</sup> /G <sup>1529</sup> → A	ins Ser after Cys-401/Arg-510 → Gln	20	172
2	25	G <sup>1529</sup> → A/G <sup>1529</sup> → A	Arg-510 → Gln/Arg-510 → Gln	29	156
3	20-40	C <sup>1594</sup> → T/G <sup>994</sup> → A	Arg-532 → Trp/Gly-332 → Ser	37	206
4	53-80	intron A 3'term.g → a/G <sup>1493</sup> → A	splice site mutation/Arg-498 → His	72	387
5	4-5	G <sup>1006</sup> → T/?	Ala-336 → Ser/?	262	1650
6	60-90	del AAG <sup>1060-62</sup> /C <sup>1594</sup> → T	del.Lys-354/Arg-532 → Trp	30	62
7	8-10	A <sup>1081</sup> → G/?	Asn-361 → Asp/?	58	281
8	5-7	intron A 3'term.g → a/?	splice site mutation/?	78	393
9	30-90	G <sup>994</sup> → A/?	Gly-332 → Ser/?	73	100
10	9	del.AAG <sup>1060-62</sup> /?	del.Lys-354/?	60	242
11	15	G <sup>1174</sup> → A/?	Ala-392 → Thr/?	146	1270
12	20-30	G <sup>1529</sup> → A/G <sup>1529</sup> → A	Arg-510 → Gln/Arg-510 → Gln	ND	ND
Control	0-1			342 ± 32	707 ± 89

Abbreviations: RBC, red blood cell; PEP, phosphoenol pyruvate; ND, not determined; ?, no mutation detected in the second allele.

AGG CAG AGG TGT TCC AGG-3'; E3: 5'-CAG CTG CCA GCT GCT ATG-3'; E5\*: 5'-GTC CCT TGG TGT CCA GG-3'; E5: 5'-GAG AGC TTT GCA GGT TCC-3'; E7\*: 5'-GAA GCG CAG GTC TCG GAC-3'; E7: 5'-AGG GAC TGG TGA CCC AAG-3'; E10\*: 5'-AGT GGG ATC ACG GCT TAG-3'; E9: 5'-CAA TGC TGT GCT GGA TGG-3'; E11\*: 5'-CAT CAT CTG CCC AGA TGG-3'; E11: 5'-CAG CCC AGC TTC TGT CTC-3'; E12\*: 5'-GCT GGA GAA CGT AGA CTG-3. Each primer pair contained one biotinylated primer (marked by an asterisk) that was used to produce single-stranded PCR products after conjugation with streptavidin magnetic beads (DynaL Corp, Oslo, Norway) and alkali denaturation for solid-phase sequencing. Sequencing reactions were done by the dideoxynucleotide chain termination method using T7 polymerase (Pharmacia, Uppsala, Sweden) or Sequenase (US Biochemical, Cleveland, OH).

**Mutation analysis.** All mutations were analyzed by direct genomic sequencing of exons 1(R) to 12. In exon 12, only the coding sequence was analyzed. The position of the primers are shown in Fig 1, their sequences were as follows: PR: 5'-AGC TAA CTT CAG TAA AGT AC-3'; IR\*: 5'-GAT GTG GAT CAT TTA TGC-3'; IR: 5'-TAG GCA GGC CAG AGC ACA G-3'; IB\*: 5'-AGA TGA AGA AGC ACC TCA AG-3'; IB: 5'-CGT TCT GAG AAT GGT AAT GG-3'; IE\*: 5'-CTG ATG GGG GAG CCA AGG-3'; IE: 5'-ACC GCA GCT GGC TCT TTC-3'; IG\*: 5'-GAC ACC CAC ACT CTC AG-3'; IG: 5'-CAG TGT GAG TCC TAC AAC-3'; II\*: 5'-CTG ACC CAA AGC TCC ATC-3'; II: 5'-AGT GAC ACC TGG AAC TGG-3'; IJ\*: 5'-GAT ATC TCA GTC TTA GTG-3'; IJ: 5'-CCT TGG CTT CCC AAA GTG-3'; E12\*: 5'-GCT GGA GAA CGT AGA CTG-3'. The PCR products were sequenced using the solid-

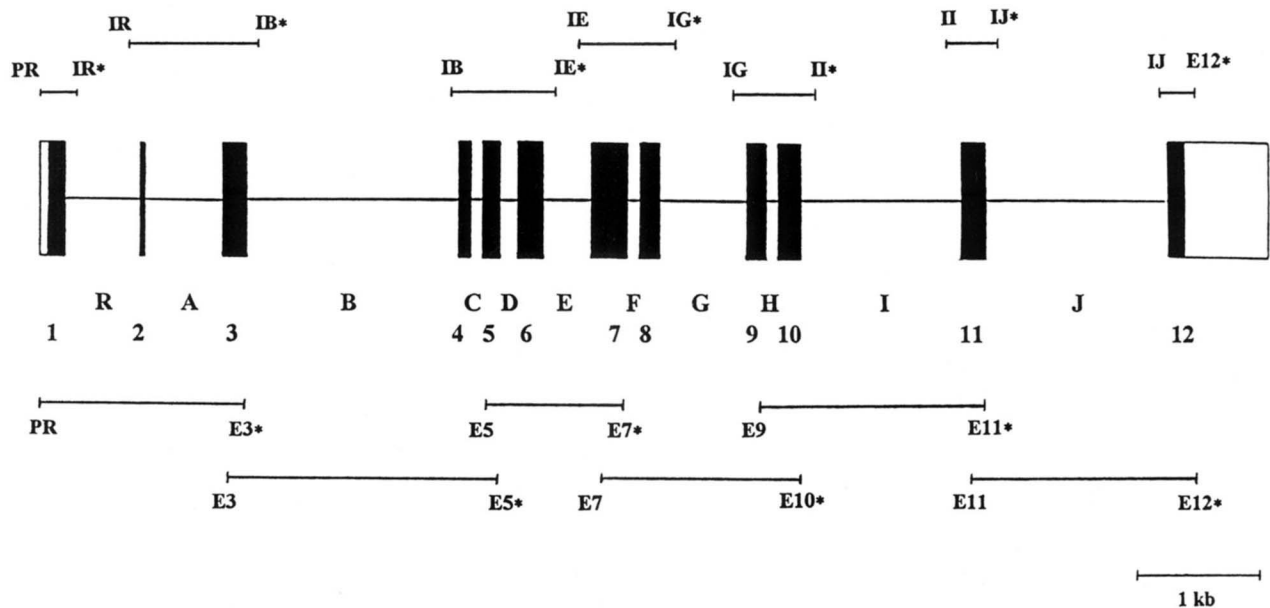


Fig 1. Sequencing strategy of the PK gene is shown. Oligonucleotide primers for the determination of PK-L-gene intronic sequences were constructed on the basis of the sequences reported by Tani et al<sup>4</sup> and Kanno et al.<sup>15</sup> Their position and the amplified fragments are shown underlining the gene. The primers designed on the basis of the determined intron sequences and the amplified PCR-fragments used for mutation analysis by sequencing overline the gene. For sequencing, the longer PCR products additional internal primers were used (see Materials and Methods).

phase technique described above. For sequencing the longer PCR products obtained by primers IR-IG\*, IB-IE\*, IE-IG\*, and IG-II\*, additional internal primers were designed.

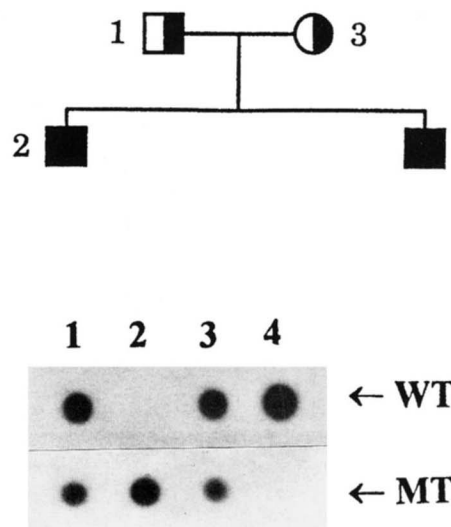
**ASO.** Genomic DNA of patient 2 ( $G^{1529} \rightarrow A$ ) was amplified with primers E11/E12\* and PCR products were blotted onto a nylon membrane (Hybond N, Amersham, Arlington Heights, IL) and hybridized with P-32 kinase labeled either wild type (WT-E11) or mutant type (MT-E11) ASOs according to standard protocols.<sup>17</sup> Filters were washed at a final stringency of  $2 \times$  SSC at 56°C for oligonucleotide WT-E11 or at 54°C for MT-E11 respectively. Oligonucleotides used were as follows: WT-E11, 5'-CACTTATGCCGA-GGAGTC-3'; and MT-E11, 5'-CACTTATGCCAAGGAGTC-3'.

**Restriction enzyme analysis of PCR fragments.** Genomic DNA of patients 1 (insertion AGC after C<sup>1203</sup>) and 6 (deletion of AAG<sup>1060-62</sup>) was amplified by PCR with primers 9H/10\* (patient 1) or 8H/9\* (patient 6) and digested with the restriction endonucleases *Pst* I and *Hae*III, respectively, according to the manufacturers recommendations (Boehringer, Mannheim, Germany). Specific fragments were separated by 10% nondenaturing polyacrylamide gel electrophoresis (PAGE) and stained by ethidium bromide. Oligonucleotides were as follows: 8H, 5'-GAT CCC AGC AGA GAA GG-3'; 9\*, 5'-CAC AGC ATT GGC GAC ATC-3'; 9H, 5'-CAA TGC TGT GCT GGA TGG-3'; 10\*, 5'-AGT GGG ATC ACG GCT TAG-3'.

## RESULTS

**PK-L gene introns.** The published sequence of the human PK-L-cDNA<sup>4</sup> and the R-type-specific exon R<sup>17</sup> was used to design a set of primers for amplification and sequencing of all PK-L-gene introns. At present, only the sequence of the rat PK-L gene is available.<sup>1,18</sup> The human PK-L-gene introns do not differ markedly in length from the rat PK-L gene introns. The overall size of the human gene is about 8,600 bp (Fig 1) compared with the rat gene that comprises 8,360 bp.<sup>17</sup> The sequence of the complete gene will be published elsewhere.<sup>19</sup> From the intronic sequences, primers were chosen to amplify and completely sequence all 12 exons of the patients (Fig 1).

**PK gene mutations.** The results of the mutation analysis by PCR-sequencing of the DNA of 12 unrelated PKD patients are summarized in Table 1. Ten different putative disease-causing mutations were detected on a total of 18 chromosomes. For 6 chromosomes, no mutation could be detected. Of the 10 mutations found, 9 were single amino acid changes (7 substitutions, 1 del and 1 ins). Eight of these mutations have not been described before. We also detected the mutation Arg-510  $\rightarrow$  Gln previously reported by Baronciani and Beutler<sup>10,11</sup> to occur in three patients, two homozygous, and one compound heterozygous. Interestingly, as in their study, this is the most prevalent mutation in our population. All these mutations are on the same C haplotype when analyzed for the A/C polymorphism at position 1705, suggesting a common origin of this mutation. The other yet-known mutation is the Arg-498  $\rightarrow$  His substitution, also described by Baronciani and Beutler in a US caucasian patient.<sup>11</sup> A mutation not detected before in PKD is the splice-site mutation at the border of intron A to exon 3 (5'-tag/GGC-3' into 5'-taa/GGC-3'). The only other splice-site mutation associated with PKD which affected the +1 position of intron 7 was shown by Kanno et al.<sup>12</sup> Comparison of our sequencing data of normal and PKD persons with the published cDNA sequence of Tani et al.<sup>4</sup> showed the same



**Fig 2.** ASO detecting  $G^{1529} \rightarrow A$  mutation of PKD patient 2 is shown. ASO probes were constructed differing only in the mutated nucleotide. (WT, wild type; MT, mutant type; see Materials and Methods). Dot blots of the DNA of patient 2 (lane 2), of his father (lane 1), his mother (lane 3), and of a normal person (lane 4) hybridized specifically to the appropriate oligonucleotides. A homozygous brother of patient 2 shown in the pedigree was not included in the study.

five differences as Baronciani and Beutler<sup>10</sup> noticed: In more than 50 sequences, we always found A<sup>388</sup> to be a T, A<sup>393</sup>  $\rightarrow$  C, G<sup>694</sup>  $\rightarrow$  A, and CG<sup>1267/68</sup> to be GC. The G<sup>694</sup>  $\rightarrow$  A and the CG<sup>1267/68</sup>  $\rightarrow$  GC changes have already been corrected by Kanno et al in the partial genomic sequence,<sup>3</sup> suggesting a cloning artefact or sequencing error in the originally published sequence. Whether the differences at nucleotide (nt) positions 388 and 393 represent polymorphic sites between Japanese and European populations or are sequencing errors remains to be determined.

**Family studies.** For the purpose of family studies and prenatal diagnostics, we applied some standard DNA analysis techniques to selected PKD cases. Allele-specific probes were designed that differed only in the mutated nt G<sup>1529</sup> (see Materials and Methods). In a dot-blot assay the probes WT (wild type) and MT (mutant type) hybridized specifically to the normal or mutated allele under stringent hybridization conditions (Fig 2). As the family analysis shows, homozygotes (patient 2) can easily be differentiated from heterozygotes (father and mother to patient 2) as well as from normal persons.

Where the mutation creates or destroys the recognition site of a restriction enzyme, the PCR amplification can be combined with a restriction enzyme analysis. This is shown in Fig 3. The insertion of an AGC after nt 1203 creates a new *Pst* I site. In DNA carrying the mutation, a 197-bp *Pst* I fragment is cut into two new 161- and 36-bp fragments. As Fig 3 shows, patient 1 is heterozygous for the mutation (lane 5) which he inherited from his mother (lane 7). His father (lane 6) is not a carrier of this mutation, but of the substitution  $G^{1529} \rightarrow A$ . A nephew (lane 2) and the son (lane

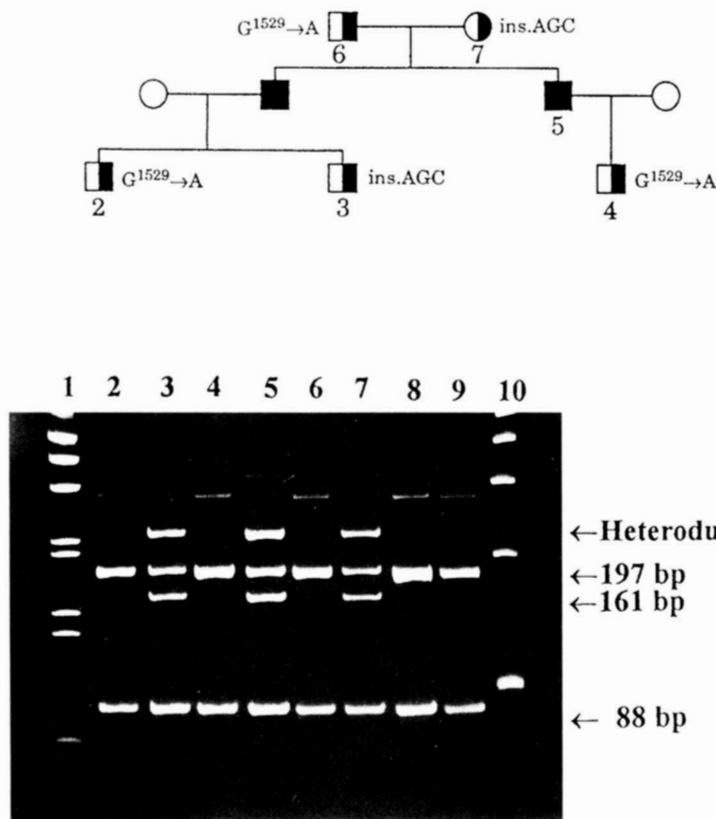


Fig 3. Detection of the AGC insertion between nt 1203 and 1204 with restriction enzyme analysis is shown. Exon 9 DNA fragments of patient 1 and members of his family were amplified by PCR with the aid of primers flanking the mutation (see Materials and Methods), digested with *Pst* I and separated by nondenaturing PAGE. The AGC insertion creates a new recognition site. Wild type, 197 and 88 nt; mutant type, 161, 88, and 36 bp; Lanes: 1 and 10, MW markers; 2, nephew 1 of patient 1; 3, nephew 2 of patient 1; 4, son of patient 1; 5, patient 1; 6, father of patient 1; 7, mother of patient 1; 8 and 9, normal persons. The 36-bp fragment is not shown. A double heterozygous brother of patient 1 was not included in the study (see pedigree). A heteroduplex is visible in heterozygous individuals.

4) do not have the insertion; however, the second nephew (lane 3) carries it (see pedigree).

The PCR amplification of genomic DNA fragments with the aid of oligonucleotide primers flanking the mutated site of interest can also be applied to analyze directly some of the mutations described in Table 1. The 3-bp deletion AAG<sup>1060-62</sup> can be detected in the family of patient 6 by the length difference of an amplified *Hae*III fragment representing this mutation (Fig 4).

#### DISCUSSION

In this report, we present 10 mutations in the PK-L gene associated with nonspherocytic hemolytic anemia, 8 of which have not been described before. All, with the exception of one splice-site mutation, cause single amino acid substitutions in the R/L-type peptide. One of these changes is an insertion of an additional amino acid, Ser, after Cys-401. All other changes concern amino acids highly conserved in PK isoenzymes among vertebrates during evolution.<sup>20</sup> This and the fact that all substituted amino acids differ markedly in their physicochemical properties from original residues substantiates the assumption that they all represent disease-producing mutations. Moreover, none of the mutations associated with PKD have been detected in 18 control persons.

In 6 of 12 patients, we did not find a mutation in the second allele. In 4 of them, because of the course of the disease and the low-enzyme activity (see Table 1), a com-

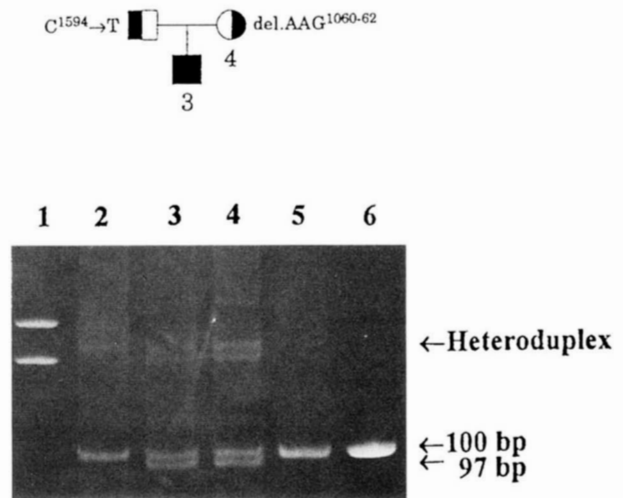
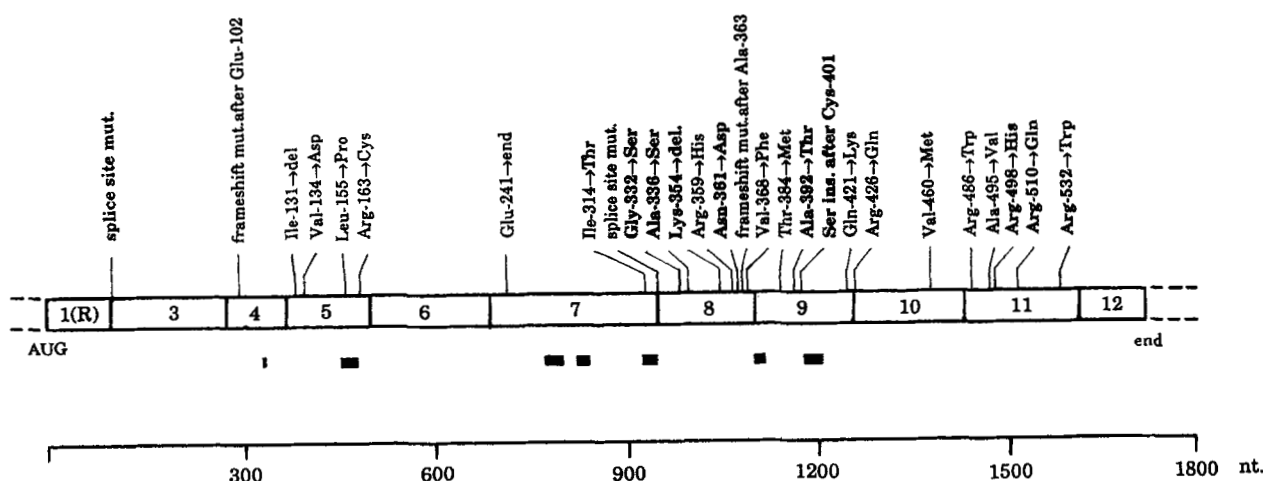


Fig 4. Detection of the deletion AAG<sup>1060-62</sup> by PCR amplification and direct comparison of the length of restriction fragments is shown. DNA of patient 6 and his mother was amplified by PCR with oligonucleotide primers flanking the site of mutation (see Materials and Methods). The specific fragments were digested with *Hae*III and separated by nondenaturing PAGE. The deletion is characterized by a 97-bp band instead of a 100-bp band for the normal allele. Lanes: 1 and 6, MW markers; 2 and 5, normal persons; 3, patient 6; 4, mother of patient 6. A heteroduplex is visible in heterozygous individuals.

PK-R-cDNA



**Fig 5. Summary of current data on mutations in PKDs is shown. Mutations that have been determined in our laboratory are shown in bold case letters above the PK-L cDNA; published data of other groups<sup>5-12</sup> are printed in normal type letters. Amino acids of the active site or close to it, deduced from the tertiary structure of cat muscle PK,<sup>19</sup> are marked by bars below the cDNA.**

pound heterozygous defect is very probable (patients 7, 8, 9, and 10). Patients 5 and 11, with enzyme activities of about 80% and 50% of normal, are most likely heterozygous. They were included for their abnormal enzyme kinetics (high Km PEP variants), but do not show any serious clinical abnormalities. We sequenced both DNA strands from each exon; however, we cannot completely exclude that we missed the second mutation for technical reasons. But it seems to us more likely that these alleles have mutations in regulative noncoding regions of the gene. If these four patients possess mutations in regulatory regions of the PK gene, their defects must be clarified by analyzing expression at the RNA and/or protein level. In all cases analyzed, the hematologic and biochemical phenotype matched the putative PKD genotype. In Fig 5, we summarize our and all previously published data on mutations in the PK-L gene. A wide distribution over the whole coding sequence is apparent, but there is a tendency for mutations to accumulate mainly in exons 5, 8, 9, and 11. Although in nearly all cases the enzyme-kinetic properties of the mutated enzymes were substantially changed (Vmax, Km PEP, activation by F6P, and inhibition by adenosine triphosphate, but never the adenosine diphosphate dependence), only a few amino acids in or close to the active site, according to Muirhead et al,<sup>19</sup> are affected (Leu-155 → Pro, Arg-163 → Lys, Ala-336 → Ser and possibly the insertion of Ser after Cys-401). The substitution of Ala-336 in the active site by a Ser (patient 5) does not change the stability of the enzyme, but alters the substrate binding affinity of phosphoenolpyruvate considerably (Table 1). It is noticeable that seven of twenty-one amino acid substitutions involve arginines, indicating that salt bridges play an important role in the integrity of the PK structure. The correspondence of the high prevalence of the Arg-510 → Gln mutation in our population (5 of 24 alleles) with the high frequency of the same mutation in the population investigated by Baronciani and Beutler (10 of 26 alleles) supports

the idea of a common single origin of this mutation. The three patients carrying the mutation are of German and English origin, whereas all Czech or Slovak patients analyzed so far do not bear it. A further argument for the single-origin hypothesis is the finding that all these mutations are on the same C haplotype when analyzed for the A/C polymorphism at position 1705.

A type of mutation only detected once before in PKD is a splice-site mutation.<sup>12</sup> In contrast to the mutation of the +1 position of intron 7, which Kanno et al described, the DNA of our two patients is mutated at position -1 in intron 3 (patients 4 and 8, Table 1). Currently, we do not have data on the functional consequence of this mutation, such as cDNA sequences of the affected patients. But as an analysis of published splice-site sequences shows, this intron 3' terminal guanosine is absolutely invariant. With no exception in all introns of about 1,550 splice junctions, this position has always been found to be a g,<sup>21</sup> so that we can assume that this mutation results in a splicing variant. How it influences expression remains to be determined.

The application of intronic oligonucleotide primers with the powerful PCR/genomic sequencing methodology allows a reliable molecular analysis of putative PKDs. ASO hybridization and PCR-amplification/restriction enzyme analysis are more convenient alternatives, especially for the tracing of a known defect in family studies and prenatal diagnosis, as we show with three selected PKD families.

In summary, with the description of 10 putative disease-causing mutations in the PK-L gene, the overall number of published mutations is now 27. This relatively high number allows the conclusion that various point mutations leading to amino acid substitutions are the most common cause of PKD. This situation is very similar to that found in glucose-6-phosphate dehydrogenase or triosephosphate isomerase deficiencies.<sup>22,23</sup> Nevertheless, there is one mutation that accounts for more than a quarter of all cases, the substitution of Arg-510 by Gln.

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