Molecular Study of Pyruvate Kinase Deficient Patients with Hereditary Nonspherocytic Hemolytic Anemia

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Abstract

DNA analysis was performed on 30 unrelated patients with hereditary nonspherocytic hemolytic anemia (HNSHA) who had been found to be pyruvate kinase (PK) deficient by enzyme assay. 19 different mutations were identified among 58 of the 60 alleles at risk. 13 of these were missense mutations that caused single amino acid changes. Included were the following nucleotide substitutions: 401A, 464C, 993A, 1022C, 1076A, 1178G, 1179A, 1373A, 1378A, 1456T, 1484T, 1493A, 1529A. The remaining six mutations were as follows: two nonsense mutations, 721T and 808T; a nucleotide deletion, 307C; a nucleotide insertion, 1089GG; a three nucleotide in frame deletion, 391-392-393 and a deletion of 1149 bp from the *PKLR* gene that resulted in the loss of exon 11. All the patients were studied for two polymorphic sites, nucleotide (nt) 1705 A/C and a microsatellite in intron 11, to better understand the origin of the mutations. The 1529A mutation, which is the most common mutation in the European population, was found in 25 alleles. With a single exception this mutation was in linkage disequilibrium with both of the polymorphic markers, i.e., found with 1705C and 14 repeats in the microsatellite. This finding is consistent with a single origin of this common mutation. Other mutations occurring more than once were of much lower frequency than the 1529A mutation. (*J. Clin. Invest.* 1995. 95:1702–1709.) Key words: mutation • polymorphism • microsatellite • haplotype

Introduction

Pyruvate kinase (PK)[1] is a ubiquitous enzyme that plays an important role in the glycolytic pathway. Two different PK genes are present in mammals, *PKLR* coding for the liver (L) and red cell (R) isoenzymes and *PKM*, coding for the M1 and M2 isoenzymes (1). The *PKLR* gene is organized into 12 exons. The first and the second exons are specifically transcribed respectively to the R and the L-type PK mRNA by two different tissue-specific promoters (2).

In the erythrocyte only one of the two products of the *PKLR* gene is normally expressed, the R-type (3). PK deficiency is the single most common cause of hereditary nonspherocytic hemolytic anemia (HNSHA). HNSHA due to pyruvate kinase deficiency was first described in 1961 (4). By 1983 more than 300 cases had been reported (5). The clinical symptoms are variable, ranging from cases of pronounced neonatal jaundice requiring multiple exchange transfusions, to a fully compensated hemolytic anemia. In some cases PK deficiency can be fatal in early childhood. Splenectomy is effective in decreasing hemolysis in most of the patients (1).

In 1979 the International Committee for Standardization in Hematology established standard methods for characterizing PK variants so that it would be possible to compare results from different laboratories (6). However, many difficulties still arise when enzymologic methods are used for either diagnosis or enzyme characterization. Because of the low activity and stability of the mutant enzyme, more blood may be required than is readily available. In transfusion-dependent patients samples are contaminated with transfused, normal erythrocytes. The product of the *PKM* gene present in leukocytes may also confuse the results. Interpretation of the biochemical properties of the residual enzyme is made difficult or impossible in compound heterozygotes by the formation of hybrid enzymes with different biochemical properties.

The cloning of the R-type cDNA has enabled us to study this enzyme deficiency at the DNA level (7, 8). Sequence analysis has also made it possible to establish diagnoses and to determine to what extent variants occurring in different patients actually differed. Previously, we determined the sequence of the introns flanking all 12 exons of the *PKLR* gene and using a PCR-based technology we documented eight mutations in PK deficient patients from genomic DNA (9). In this investigation we describe the results in all thirty patients affected with PK deficiency that we have studied to date. Some of these have recently been reported in abstracts (10, 11).

Methods

Patients. Patients with HNSHA were screened by assay of erythrocyte enzymes, as reported previously (12). Table I presents the ethnic origin and enzymologic data pertaining to the PK deficient patients. Patients 6 and 27 were transfused shortly before blood was drawn. In spite of the transfusion, the red cells of patient 6 had low PK activity, but those of patient 27 had high activity and the diagnosis of PK deficiency was established only after performing red cell PK assays on the mother and sister of the patient. The results of mutation analysis in some of these patients have been reported earlier (9), as indicated in Table I. They are included in the present report to provide a more comprehensive account of the mutations we have identified.

Nomenclature. The different nomenclatures that have been used to identify the exons of the *PKLR* gene are shown in Fig. 1. Mutations are designated using the type-R cDNA sequence, with the A of the initiation ATG being assigned number 1. Amino acids are numbered from the first methionine of the deduced R-type enzyme.

1. *Abbreviations used in this paper:* HNSHA, hereditary nonspherocytic hemolytic anemia; PK, pyruvate kinase.

Table I. Characteristics of Patients with Hemolytic Anemia Due to PK Deficiency

Patient	Ethnic origin	PK activity, V_{max} in IU per g of Hb	Low S % of V_{max}[‡]	Low S + FDP % of V_{max}[§]	Mutation (cDNA nt)	Amino acid substitution	nt 1705	Microsatellite ATT repeat
1	U.S. white*	4	51.9	57.6	1484C → T/1529G → A	Ala495 → Val/Arg510 → Gln	C/C	15/14
2	U.S. white	4.28	31.2	31.2	1456C → T/1178A → G	Arg486 → Trp/Asn393 → Ser	C/C	14/14
3	U.S. white*	2.5	22.8	40	1529G → A/1529G → A	Arg510 → Gln/Arg510 → Gln	C/C	14/14
4	U.S. white*	2.8	38.5	64.5	1529G → A/1529G → A	Arg510 → GlnArg510 → Gln	C/C	14/14
5	U.S. white*	3.8	18.4	42.9	1529G → A/464T → C	Arg510 → Gln/Leu155 → Pro	C/C	14/12
6	Gypsy	9.1	9.3	39	del exon 11/del exon 11	NA/NA	C/C	15/15
7	U.S. white*	1.9	4	16	1484C → T/1529G → A	Ala495 → Val/Arg510 → Gln	C/C	15/14
8	U.S. white*	2.71	11.4	47.1	1456C → T/401T → A	Arg486 → Trp/Val134 → Asp	C/C	15/16
9	Gypsy	2.55	30.8	50	del exon 11/del exon 11	NA/NA	C/C	15/15
10	U.S. African*	4.1	15.6	56.9	1076G → A/[‖]	Arg359 → His/[‖]	A/A	12/13
11	Canadian white*	1.9	28.6	42.9	1529G → A/1089G → GG	Arg510 → Gln/NA	C/A	14/13
12	U.S. white*	7.6	7.6	25.4	391–393 del/721G → T	Ile131 → del/Glu241 → End	C/C	15/14
13	U.S. white	4.06	25.74	53.47	1529G → A/721G → T	Arg510 → Gln/Glu241 → End	C/C	14/14
14	U.S. white	1.91	21	21	307C del/1378G → A	NA/Val460 → Met	A/C	12/16
15	U.S. white	5.18	25.6	38.6	1456C → T/1493G → A	Arg486 → Trp/Arg498 → His	C/A	14/13
16	U.S. white	6.85	14.19	48.62	1529G → A/[‖]	Arg510 → Gln/?	C/C	14/15
17	U.S. white	5.75	18.2	45.5	1529G → A/1529G → A	Arg510 → Gln/Arg510 → Gln	C/C	14/14
18	U.S. white	7.73	14.4	46.5	1456C → T/1179T → A	Arg486 → Trp/Asn393 → Lys	A/C	14/14
19	U.S. white	6.22	24.6	61.4	1529G → A/1373G → A	Arg510 → Gln/Gly458 → Asp	C/C	14/13
20	U.S. white	8.4	15.1	42	1529G → A/721G → T	Arg510 → Gln/Glu241 → End	C/C	14/14
21	U.S. white	5.86	7.78	39.28	1529G → A/808C → T	Arg510 → Gln/Arg270 → End	C/C	14/14
22	U.S. white	2.51	40	60	1529G → A/1529G → A	Arg510 → Gln/Arg510 → Gln	C/C	14/14
23	U.S. white	2.57	26.3	47.4	1529G → A/1529G → A	Arg510 → Gln/Arg510 → Gln	C/C	14/15
24	U.S white	2.8	29.7	66.2	1529G → A/993C → A	Arg510 → Gln/Asp331 → Glu	C/C	14/14
25	U.S. white	1.6	10	45	1484C → T/1529G → A	Ala495 → Val/Arg510 → Gln	C/C	14/15
26	U.S. white	2.5	23.3	50	1529G → A/1529G → A	Arg510 → Gln/Arg510 → Gln	C/C	14/14
27	U.S. white	16.6	8.42	45.31	1456C → T/1022G → C	Arg486 → Trp/Gly341 → Ala	C/C	14/14
28	Hispanic	1.65	16.66	51.3	1378G → A/1378G → A	Val460 → Met/Val460 → Met	C/C	16/16
29	Gypsy	2.21	36.4	50	del exon 11/del exon 11	NA/NA	C/C	15/15
30	U.S. white	1.25	29.4	47.1	1529G → A/1529G → A	Arg510 → Gln/Arg510 → Gln	C/C	14/14
Normal	mean±SD	15.0±2.0	16.6±7.9	45.8±7.9				

* Reported previously (9). ‡ Assay carried out at low substrate concentration (12). § Assay carried out at low substrate concentration and added fructose diphosphate (12). ‖ Whole coding sequence normal. *NA*, not applicable.

DNA preparation. Genomic DNA was purified from peripheral blood using the Applied Biosystems (Foster City, CA) DNA extractor.

Search for common mutations by restriction enzyme analysis. In our first report (9), mutations 1529A, 1456T and 1484T seemed to occur more often than others in the U.S. Caucasian population. Therefore, patients from this ethnic group were initially screened for these mutations.

Single-stranded conformation polymorphism and sequence analysis. When the more common mutations were not found, single-stranded conformation polymorphism analysis (13) of the coding region, and not
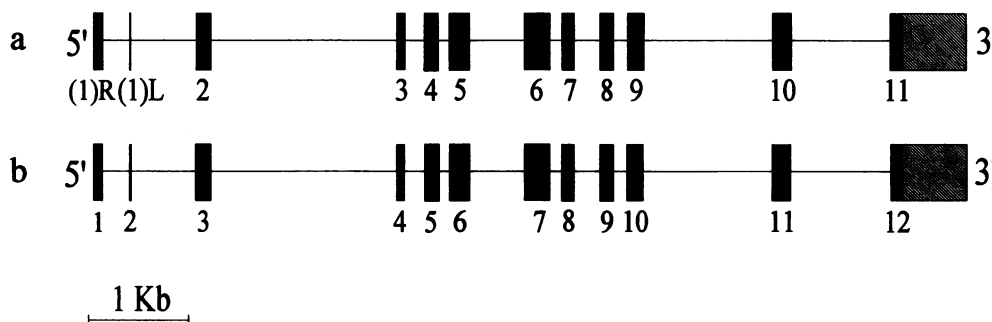
PKLR gene

Figure 1. The *PKLR* gene. The closed boxes represent the exons, the shaded boxes represent the non-coding region. *a* shows the nomenclature that we used previously for the exons of the *PKLR* gene (9). 1(R) is the first exon of the red blood cell cDNA, 1(L) is the first exon of the liver cDNA. *b* shows the nomenclature employed in this paper, numbering each exon consecutively.

including the promoter, was performed, followed by direct sequencing of the coding region of exons in which band shifts were observed (9). Direct sequencing was carried out on DNA of the remaining patients until mutations were identified.

Sequencing of intron 10 and intron 11 was carried out to determine the boundaries of the putative deletion in the *PKLR* gene of patients 6, 9, and 29, where exon 11 was missing. This was done using the oligonucleotides listed in Table II and shown in Fig. 2.

Confirmation of the mutations by restriction enzyme analysis. All the mutations were confirmed using restriction enzyme analysis except for the PK Gypsy. When a new mutation did not create or destroy a restriction endonuclease site a mismatched oligonucleotide primer that created an informative restriction site was made in order to confirm the mutation. Oligonucleotides and the restriction endonucleases used are listed in Table III.

Determination of the polymorphic sites. Two polymorphic sites were studied in all the patients to better understand the origin of the mutations. The polymorphic site at nt 1705 (14) was examined using restriction enzyme analysis as previously reported (15). A microsatellite (variable number tandem repeat), recently identified in intron 11 by Lenzner et

Figure 2. Sequence of introns 10 (*a*) and 11 (*b*). Exon sequences are in uppercase letters, intron sequences are in lowercase letters. Oligonucleotides listed in Table II are underlined. The bold sequence in *b* represents the microsatellite identified by Lenzner et al (16), here shown with 12 ATT repeats. The approximate region of the *Alu* sequence is italicized.

Table III. Oligonucleotides and Restriction Enzymes Used to Detect the Mutations in the PKLR Gene

Mutation	Exon	Sense oligonucleotide	Antisense oligonucleotide	Size of PCR fragments	Enzyme	Size of normal fragments	Size of mutated fragments
307C del	4	A	B	78 nor.–77 mut.	StuI+	78	23 + 54
391–393 del	5	C	D	232 nor.–229 mut.	MwoI+	9 + 223	9 + 64 + 156
401T → A	5	C	D	232	BsiEI+	232	78 + 154
464T → C	5	C	D	232	NciI+	79 + 153	60 + 79 + 93
721G → T	7	E	F	411	"AflIII+"	411	17 + 394
808C → T	7	E	F	411	DdeI+	411	108 + 303
993C → A	7	E	F	411	"StuI+"	411	19 + 392
1022G → C	8	G	H	252	StyI–	101 + 151	252
1076G → A	8	G	H	252	HhaI–	92 + 160	252
1089G → GG	8	G	H	252 nor.–253 mut.	MwoI+	252	76 + 177
1178A → G	9	I	J	289	BsrI+	94 + 195	45 + 94 + 150
1179T → A	9	I	J	289	AluI+	5 + 12 + 126 + 146	5 + 12 + 50 + 96 + 126
1373G → A	10	K	L	181	SfaNI+	181	38 + 143
1378G → A	10	K	L	181	"NsiI+"	181	18 + 163
1456C → T	11	M	N	326	BsmAI–	73 + 253	326
1484C → T	11	M	N	326	MwoI–	25 + 68 + 96 + 137	68 + 121 + 137
1493G → A	11	M	N	326	BsrBI–	109 + 217	326
1529G → A	11	M	N	326	StyI+	326	143 + 183

Sense oligonucleotides	Antisense oligonucleotides
A = 5'-GCCAGCATCTCGCTCCGTGG-3'	B = 5'-TGGGAGAAGTTGAGTCGCGC-3'
C = 5'-CGAGGTCCTGGCCACCTTCC-3'	D = 5'-GCGCCGCCTTTCCGGCCCTG-3'
E = 5'-GAGGGACTGGTGACCCACGT-3'	F = 5'-CCCCCGTGCCACCATGAGGC-3'
G = 5'-CACCTTTCTTCTCCTGCCTG-3'	H = 5'-CAGGTGTCCCTAAAACCCAC-3'
I = 5'-GATTACCAAGCCCCGGTCAA-3'	J = 5'-CGTAGCTCCTCAAACAGCTG-3'
K = 5'-CCTTCCATACCCCAGTGCCC-3'	L = 5'-CAGCACTTGAAGGCAGCATGCA-3'
M = 5'-CTCGTTCACCACTTTCTTGC-3'	N = 5'-GAGGCAAGGCCCTTTGAGTG-3'

The bold font in the oligonucleotides represents a mismatched nucleotide. Quotes indicate that an artificial restriction site has been introduced by using a mismatched oligonucleotide.

al (16) (see Fig. 2), was examined in the normal population and in the patients according to the procedure described by Lenzner et al (16) using the oligonucleotides 5'-GGCTGGTTCTCGTTACAG-3', and 5'-GGAGAATCGCTTGAACTCAG-3' and the PCR buffer described by Kogan et al. (17).

Studies of cDNA prepared from reticulocytes of patients with the putative exon 11 deletion. Whole blood was passed through a column of microcrystalline cellulose and α-cellulose (Sigma Chem. Co., St. Louis, MO) to remove leukocytes and platelets (18). Reticulocytes

were isolated by centrifugation through a Percoll gradient (19). Total RNA was extracted from the reticulocytes using Trizol Reagent (total RNA isolation reagent. GIBCO BRL, Gaithersburg, MD). First strand cDNA was made from the total RNA, using oligo(dT) and reverse transcriptase as described (20). DNA extending from cDNA nt 1362 to cDNA nt 1694 was amplified by 35 PCR cycles using 250 ng of sense oligonucleotide 5'-CACCGCCATTGGTGCTGTGG-3' and antisense 5'-GTGTAGCCGGAGCCAGGTCG-3'. The PCR product was purified with phenol/chloroform, ethanol-precipitated and redissolved in water. The approximate size of the PCR product was established by electrophoresis in a 10% polyacrylamide gel using molecular marker (pBR322 DNA MspI digest) after staining with ethidium bromide. The same PCR product was also sequenced with the fmol DNA Sequencing
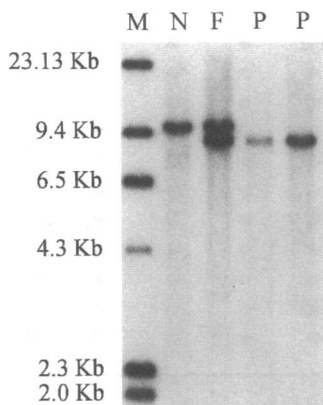
Figure 3. Detection of the PK Gypsy deletion by restriction analysis and Southern blotting of the PKLR gene. M, marker ^{32}P-labeled λ DNA-HindIII digest; N, normal subject; F, father of patient 6; P, patient 6. The father's DNA shows two distinct bands. The top band is also present in DNA from the normal subject and represents a 10-kb fragment obtainable with an EcoRI digestion. The lower band is present in DNA from the patient and the different size is due to the missing portion of the gene.
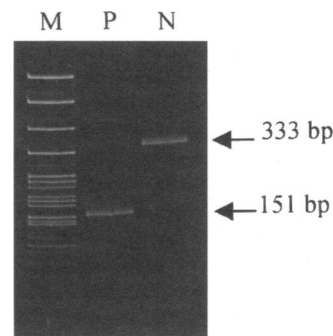
Figure 4. Detection of the deletion of exon 11 in the mRNA of the Gypsy patient. M, marker pBR322 DNA-MspI digest; P, patient 9; N, normal subject. The size difference of the two fragments (333 bp normal and 151 bp patient 9) obtained from the similar PCR is due to the absence of exon 11 (182 bp) in the patient.

Figure 5. Nucleotide sequences across the deletion junction of the PK Gypsy and corresponding intron 10 and intron 11 sequences of the normal gene. The area of the crossing over is in the box. Dots denote matches between the three sequences.

Results

Mutations. The mutations present in the thirty patients are summarized in Table I. In two patients the second mutation could not be found in the coding region.

Three patients of gypsy extraction appeared to be homozygous for the same mutation. It was designated PK Gypsy. The first evidence that a unique, homozygous mutation existed in these patients was found by PCR. We were able to amplify exons 10 and 12 but not exon 11 (data not shown). Southern blotting revealed a major deletion in the *PKLR* gene estimated to be over 1 kb in size. Fig. 3 is a Southern blot that shows that patient 6 is homozygous for this deletion and that the father is heterozygous. Fig. 4 shows the size of the PCR products made using total RNA from patient 9 and from a normal subject. The size difference in this cDNA fragment is ~ 182 bp, the length of exon 11. Subsequent sequencing of these two PCR fragments confirmed these observations. Using the oligonucleotides shown in Table II we were able to determine the sequence

System (Promega Corp. Madison, WI) using two end-labeled primers 5'- CTGCCTTCAAGTGCTGTGCT - 3' and 5'- CACCACAATCAC - CAGGTCTC-3'.

Restriction analysis and Southern blotting of the PKLR gene. A PCR fragment of 1101 base pairs made from R-type cDNA extending from nt 702 to nt 1802 served as a probe in Southern blotting studies. The probe was labeled with [^{32}P]dATP using a Stratagene Inc. (La Jolla, CA) Prime-it Random Primer Kit. The electrophoresis and Southern blot were performed using the Neat System (Oncor Inc., Gaithersburg, MD). 10 µg of genomic DNA were incubated with 300 units of EcoR I (New England Biolabs, Beverly, MA) at 37°C overnight to ensure complete digestion. After electrophoresis in 0.7% agarose for 16 h at 3.3 V/cm, the DNA was transferred to a nylon membrane (Sure Blot., Oncor Inc.) and hybridized in 10% Dextran Sulfate, 50% deionized formamide, 1% SDS, 200 µg/ml denatured sonicated/sheared salmon sperm DNA and 900 mM NaCl with ~ 1 µCi of probe per 100 cm^2 of filter at 42°C overnight. It was washed with 0.1× SSC and 0.1% SDS at 55°C, and visualized using XAR x-ray film. A ^{32}P-labeled HindIII digest of λ DNA provided size markers.

of introns 10 and 11 (see Fig. 2) in a normal subject and in the Gypsy patients. Fig. 5 illustrates the sequence beginning in intron 10 and skipping to intron 11. This deletion, 1149 bp in length, caused the absence of exon 11 in the mRNA resulting in a subsequent frameshift.

Polymorphisms. Table I documents the polymorphisms at nt 1705 and at the microsatellite ATT repeat in intron 11 in these patients. The allele frequency of the intron 11 microsatellite in the Caucasian population was estimated by a study of 100 chromosomes of unrelated subjects. Eight different sequences were identified among the 50 individuals examined, the sequence containing the 14 ATT repeats was found to be the most common (see Table IV). Six homozygotes for the 1529A mutation all manifest the C/C genotype at nt 1705 and the 14/14 genotype at the microsatellite. The seventh homozygous patient (23) had a C/C genotye but a 14/15 genotype. The genotype was confirmed by study of the patient's parents. In seven patients heterozygous for the 1529A mutation at least one 14 repeat was present in each patient.

Discussion

Among the 60 alleles at risk in the 30 PK deficient patients studied, 58 mutations were found in the coding region. Although it is possible that a mutation was present and was missed for technical reasons, these mutations may represent substitutions in the promoter region, in the 3' untranslated region (affecting mRNA stability), or in an intron, creating a new splice site.

Table IV. Population Frequency of the Intron 11 Microsatellite ATT Repeat of the PKLR Gene in 50 Subjects of European Origin

ATT repeat numbers	Population Frequency
12	0.16
13	0.05
14	0.49
15	0.19
16	0.05
17	0.03
18	0.02
19	0.01

Table V. Conservation of Amino Acids among Several Species

Mutation	Amino acid substitution	Species				
		Human	Cat	Chicken	Rat	Yeast
				Isoenzyme		
		R	M2	M1	M1	R
401 T → A	134Val → Asp	●	●	●	Ile	Ala
464 T → C	155Leu → Pro	●	●	●	●	●
993 C → A	331Asp → Glu	Tyr	●	●	●	●
1022 G → C	341Gly → Ala	●	●	●	●	●
1076 G → A	359Arg → His	●	●	●	●	Lys
1178 A → G	393Asn → Ser	●	●	●	●	●
1179 T → A	393Asn → Lys	●	●	●	●	●
1373 G → A	458Gly → Asp	●	●	●	●	Pro
1378 G → A	460Val → Met	●	●	●	●	●
1456 C → T	486Arg → Trp	●	●	●	Gln	Lys
1484 C → T	495Ala → Val	●	●	●	●	Leu
1493 G → A	498Arg → His	●	●	●	●	●
1529 G → A	510Arg → Gln	●	●	●	●	●

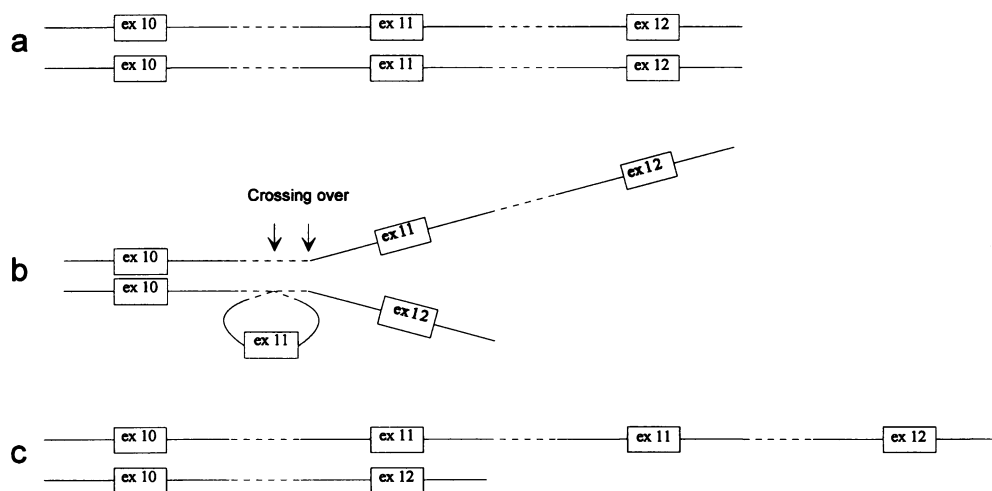The black dot represents the conserved amino acids.

Figure 6. Schematic representation of misaligned chromatids before crossover. This may cause the PK Gypsy deletion. *a* shows the two chromatids. The broken line represents the two regions with high homology located in intron 10 and in intron 11. *b* shows the misalignment of the two chromatids. Any crossover in the region between the arrows will result in a loss of exon 11 in one chromatid and the acquisition of exon 11 in the other as shown in *c*.

We found 19 different mutations among the 58 PK mutations identified. Five of these cause either a stop codon or a frameshift and are therefore undoubtedly the cause of the deficiency. One mutation causes the deletion of an amino acid, which presumably results in the enzyme deficiency. However, the cause-and-effect relationship between a single amino acid substitution and the enzyme defect is less certain. Whenever a new nucleotide substitution was found, the whole coding region was sequenced. If no other nucleotide substitution was found we considered the mutation we had detected to be the cause of the deficiency. We also compared the mutated triplets with the corresponding PK amino acid sequence in other species. Each of the 12 mutated amino acids was one that was well conserved among the species examined, further strengthening the presumption that the change would decrease enzyme activity (Table V).

Of the 12 amino acids changes documented, only a few are correlated with the putative binding site determined by crystallographic studies of the cat muscle PK (21). The 464C mutation changes one of the amino acids located in the domain A in the β-sheet 3, the 993A and 1022C mutations change the amino acids close to the binding site located in the A domain β-sheet 6 and α-helix 6. Significantly, 5 of the 8 mutations that are in domain C, believed to play a role in intersubunit contacts, involve loss of arginine residues and could cause enzyme instability by weakening salt bridges that hold the subunits together. These and the remaining mutations may make the enzyme more vulnerable to proteolysis, explaining the reduced quantity of red cell PK antigen in some patients (22). Alternatively, they may be at other sites that are proximate to the active sites of the enzyme in its three dimensional structure.

Since heterozygotes for PK deficiency have no known selective advantage, we might have expected that most cases would be represented by new mutations rather than selection of one particular mutation. In 1993 we first reported that we found one mutation, 1529A, to be more common than the others in a group of 10 patients (9), an observation that has recently been confirmed by Lenzner et al. (23). This mutation occurred a disproportionate twenty-five times in our 30 patients and represents 41.6% of all the alleles studied. This mutation always seems to be associated with a cytosine at the polymorphic site at nt 1705 and with a single exception (patient 23) with the 14 ATT repeat in the microsatellite, located in intron 11. Lenzner has also observed (personal communication) the same haplotype in association with the 1529A mutation in the European population. These findings suggest a single origin of this mutation, a finding that is compatible with the existence of the 1529A mutation as a balanced polymorphism. Other mutations also were present more than once: 1456T five times, 1486T three times, 1378A three times, and 721T three times. Patient 28 was homozygous for the 1378A mutation. Since the frequency of this mutation is low it is likely that either the parents of patient 28 are related or this mutation is more common in the Hispanic population than in the European population. It is notable that all of these mutations appear to be always in the same haplotype. There is only one case that is not consistent with this observation. In patient 8 the 1456T mutation was found both with the 15 or the 16 ATT repeat rather than the 14ATT repeat. This situation, as in the case of patient 23, could be explained by the fact that the microsatellites seem to be unstable and that a change occurred in these chromosomes after the original mutation arose. Alternatively, the association of these mutations with a different haplotype could be the result of a crossover in one of the patients' ancestors. The fact that these mutations, particularly 1529A, seem to have a single origin can best be explained by a possible advantage for the heterozygote and, as we suggested in the past (24), the advantage could be that the high concentration of 2,3-bisphosphoglycerate in carrier erythrocytes reduces erythrocyte oxygen affinity.

PK Gypsy is the mutation that results in the most severe damage to the *PKLR* gene. If any protein survives intracellular proteolysis, the C domain, which seems to be responsible for the inter-subunit contact, would be missing. We would expect any residual protein to have a very low activity and it is possible that, as has already been reported by others (23, 25), the activity that is observed is due to expression of M2 isoenzyme, not the mutant R isoenzyme. PK Gypsy probably originated from a misalignment of the two alleles during crossing over in meiosis, as has been described frequently (26–29). We found two regions in intron 10 and 11 that are nearly identical, both matching an Alu sequence, and that might have caused this misalignment of the two chromatids. Fig. 6 shows how an unequal crossover would result in two different products: one from which exon 11 is absent and one where two exon 11's are present. There is no doubt that this mutation has a single origin and this is confirmed by the fact that we found the six PK Gypsy alleles always to be in the same haplotype.

Since PK deficiency can be a devastating disease, parents who already have an affected child often request prenatal diag-

Table VI. Mutations Known to Cause Pyruvate Kinase Deficiency

Name	cDNA number from upstream ATG	Base substitution	Amino acid number	Amino acid substitution	Exon	Reference
*	Ivs3 (−1)	g → a	Not applicable	Not applicable[‡]	Ivs3	(23)
*	307	C → Del	Not applicable	Not applicable[§]	4	This paper
*	391, 392, 393	ATC → Del	131	Ile → Del	5	(9)
*	401	T → A	134	Val → Asp	5	(9)
*	464	T → C	155	Leu → Pro	5	(9)
Linz	487	C → T	163	Arg → Cys	5	(32)
*	721	G → T	241	Glu → End	7	(9)
*	808	C → T	270	Arg → End	7	This paper
Hong Kong	941	T → C	314	Ile → Thr	7	(34)
Kowloon	Ivs7 (+1)	g → t	Not applicable	Not applicable[‡]	Ivs7	(34)
*	993	C → A	331	Asp → Glu	8	This paper
*	994	G → A	332	Gly → Ser	8	(23)
*	1006	G → T	336	Ala → Ser	8	(23)
*	1022	G → C	341	Gly → Ala	8	This paper
*	1060, 1061, 1062	AAG → Del	354	Lys → Del	8	(23)
*	1076	G → A	359	Arg → His	8	(9)
*	1081	A → G	361	Asn → Asp	8	(23)
*	1089	G → GG	Not applicable	Not applicable[§]	8	This paper
Osaka	1102	G → T	368	Val → Phe	8	(25)
Tokyo, Beirut, Nagasaki	1151	C → T	384	Thr → Met	9	(8, 14, 32)
*	1174	G → A	392	Ala → Thr	9	(23)
*	1178	A → G	393	Asn → Ser	9	This paper
*	1179	T → A	393	Asn → Lys	9	This paper
*	1203	C → CAGC	Not applicable	Cys → CysSer	9	(23)
Fukushima, Maebashi, Sendai	1261	C → A	421	Gln → Lys	9	(14)
Saporo	1277	G → A	426	Arg → Gln	10	(33)
*	1373	G → A	458	Gly → Asp	10	This paper
*	1378	G → A	460	Val → Met	10	This paper
Amish	1436	G → A	479	Arg → His[‡]	10	(35)
Gypsy	1437–1618	182 nt Del	Not applicable	Not applicable[‖]	11	This paper
*	1456	C → T	486	Arg → Trp	11	(9)
*	1484	C → T	495	Ala → Val	11	(9)
*	1493	G → A	498	Arg → His	11	This paper (23)
*	1529	G → A	510	Arg → Gln	11	(9, 23)
*	1594	C → T	532	Arg → Trp	11	(23)

* The variant has no name. ‡ Splice site. § Frameshift. ‖ Deletion of 60 amino acids and frameshift.

nosis. In the past we had the opportunity to make a diagnosis during a second pregnancy, for the parents of patient 20. In this case the mutations present in the affected child were already known therefore we could establish the diagnosis by direct analysis of the fetal DNA (30). When mutations that affect a family are not known, analysis of polymorphisms linked with the *PKLR* gene provides a helpful alternative. For this purpose the microsatellite reported by Lenzner et al (16) can be very useful. In our study of a normal Caucasian population we found eight different alleles at this locus. Even though some of these alleles are fairly common, using this marker along with the polymorphism at nt 1705 A/C of the *PKLR* gene (14) and the Pv1.1 polymorphism of the tightly linked glucocerebrosidase gene (15, 31) provides a greatly improved probability of obtaining an informative pedigree.

The number of PK mutations known, including the ones in this report, is now 35 (8, 9, 11, 14, 23, 25, 32–35) (Table VI). Only three of these mutations have been detected by more than one group of investigators. Mutation 1151T was first found in the Japanese population (8) and soon after in a Lebanese family (32). Subsequent reports suggest that this mutation may be the most common in the Japanese population (14). Two other mutations initially documented by us and also found by others are the 1493A mutation which has been reported once as a heterozygote (23) and the 1529A mutation which has been found seven times in a group of 12 patients (23). The 1529A mutation was found in the same haplotype with respect to the polymorphic site at nt 1705 and the same low residual PK activity (23). During the last two years there has been a rapid increase of identified mutations that cause PK deficiency. The large number of mutations that we now know and in particular the high frequency of the 1529A mutation, are very useful for

diagnosis, especially prenatal diagnosis and in transfusion-dependent patients.

Acknowledgments

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