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Functional Expression of Human Mutant Phosphofructokinase in Yeast: Genetic Defects in French Canadian and Swiss Patients with Phosphofructokinase Deficiency

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Summary

Human phosphofructokinase (PFK) is a tetrameric enzyme, encoded by muscle, liver, and platelet genes. Deficiency of muscle PFK (PFK-M), glycogenosis type VII (Tarui disease), is an autosomal recessive disorder characterized by an exertional myopathy and hemolytic syndrome. Several disease-causing mutations have been identified in the PFK-M gene in Japanese, Ashkenazi Jewish, and Italian patients. We describe the genetic defects in French Canadian and Swiss patients with the disease, and we use a genetically well-defined yeast system devoid of endogenous PFK for structure-function studies of the mutant PFKs. A G-to-A transition at codon 209–in exon 8 of the PFK-M gene, changing an encoded Gly to Asp, is responsible for the disease in a homozygous French Canadian patient. Gly-209–mutated protein is completely inactive in the yeast system. The Swiss patient is a genetic compound, carrying a G-to-A transition at codon 100 in exon 6 (Arg to Gln) and a G-to-A transition at codon 696 in exon 22 (Arg to His). The mutants expressed in yeast generate functional enzyme with modest changes in thermal stability. The advantages and limitations of the yeast system for expression of human mutant PFKs are discussed.

Introduction

A major rate-limiting step of glycolysis, phosphorylation of fructose-6 phosphate to fructose-1,6 bisphosphate, is catalyzed by phosphofructokinase (PFK; ATP, fructose-6-phosphate 1-phosphotransferase; E.C.2.7.1.11). The mammalian PFK is a tetrameric enzyme and is subject to allosteric regulation (Bloxham and Lardy 1973; Dunaway 1983; Kemp and Foe 1983; Dunaway and Kasten 1987). Three structural loci on chromosomes 1, 21, and 10 code for muscle (PFK-M), liver (PFK-L), and platelet (PFK-P) subunits (Vora et al. 1982, 1983; Van Keuren et al. 1986), respectively, which are variably expressed in different tissues. The PFK isoenzymes randomly aggregate to form homotetramers or heterotetramers, depending on the relative abundance of the subunits in a particular tissue. PFK-M is the sole subunit in muscle; the red-cell PFK contains a different combination of PFK-L and PFK-M subunits.

An inherited deficiency of PFK, glycogenosis type VII (Tarui et al. 1965; Layzer et al. 1967), results in a complete block in muscle glycolysis, leading to intolerance to exercise, cramps, myoglobinuria, and compensated hemolysis (Rowland 1986). The observed clinical symptoms reflect the lack of PFK in muscle and partial reduction of the enzyme in erythrocytes.

The PFK-M gene has been cloned and sequenced (Nakajima et al. 1987; Sharma et al. 1989; Valdez et al. 1989; Yamasaki et al. 1991), and three types of mRNA (A, B, and C) that are transcribed from the gene have been identified (Nakajima et al. 1990*a*, 1990*c*). These transcripts share the sequence of the coding region but diverge at the 5′UTR, which contains two additional exons. Muscle expresses almost exclusively types A and B mRNA. cDNA-A is formed by a splicing event which removes an 89-bp intron located upstream of the ATG in exon 3; cDNA-B is a predominant form in muscle and retains this intron, and cDNA-C is formed by removing exon 2 and both introns in the 5′ UTR. Alternatively spliced mRNAs differentially expressed in various tissues suggested the existence of two different promoters and tissue-specific transcription factors. The skipping of exon 11 (exon 9, based on the nomenclature for the rabbit PFK-M gene) is another alternative splicing event, described by Sharma et al. (1990).

Several mutations have been identified in patients with PFK deficiency: splicing defects, a nucleotide deletion, and point mutations (Nakajima et al. 1990*b*; Raben et al. 1993; Hamaguchi et al. 1994; Sherman et al. 1994; Tsujino et al. 1994). It is interesting that two of the reported point mutations in unrelated individuals occurred at the same nucleotide in exon 4, changing Arg-39 either to Leu in an

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Ashkenazi Jewish patient or to Pro in an Italian patient, suggesting the importance of this amino acid for the activity of the enzyme. The effects of the mutations have not been tested in cultured mammalian cells, mainly because of the presence of endogenous PFK in these cells.

In this study we use the genetically well-defined yeast system, in which the endogenous PFK can be selectively and completely inactivated (Heinisch 1993), to express three novel mutations (in a French Canadian patient and a Swiss patient) as well as the previously reported mutation in exon 4.

In yeast, PFK is composed of 4α and 4β subunits, which are encoded by *PFK1* and *PFK2* genes, respectively. Yeast carrying mutations in either of the two genes do not show any detectable PFK activity in vitro but retain the ability to grow on glucose; double mutants, however, are glucose negative (Heinisch 1986). Thus, the activity of the expressed PFK can be detected in two ways: by an in vitro assay and by the ability to restore the glucose-positive phenotype of yeast double mutants. We discuss the application of this system for studying the effect of structural PFK mutations on the enzyme function.

Subjects, Material, and Methods

Subjects

FC is a 57-year-old male of French Canadian descent. He had a childhood history of nausea with extreme exercise but denied having any limitations in physical activity. He first complained of exercise intolerance at the age of 45 years and has subsequently developed fixed muscle weakness. His parents were first cousins. The Swiss patient is a 25-year-old female with a typical presentation of the disease. Diagnoses in both cases were confirmed by muscle biopsies, which showed no detectable PFK activity in FC and ~8% activity in the Swiss patient. The two patients and family members provided informed consent for genetic evaluation.

RNA PCR Amplification

Total RNA was extracted from frozen muscle biopsy specimen by lysis in guanidium thiocyanate and extraction with phenol-chloroform (Chomczynski and Sacchi 1987). RNA was denatured by heating at 70°C for 10 min and reverse-transcribed into single-stranded cDNA at 42°C for 60 min in 20 μl of 10 mM Tris (pH 8.3), 50 mM $MgCl_2$, 1 mM each deoxynucleotide triphosphate, 50 U of RNasin, oligo(dT) primer, and 2 U of AMV reverse transcriptase (Boehringer-Mannheim). Two microliters of the reaction product were used to amplify three overlapping fragments encompassing the 2.4-kb coding region of the PFK-M gene. The sequences of the primers are as follows: fragment I (bp −66–958) 5′-gccgttcctttagctagtggcatcttg (sense)/5′-cttccacacccatcctgctgccca (antisense); fragment II (bp 642–1769) 5′-cctggcccttgtcacctctctgtc (sense)/5′-gccccagctgccagtccagccat (antisense); and fragment III (bp 1668–2381) 5′-caagcagtcagcagctggcaccaa (sense)/5′-catgatcaggtaatctattcccct (antisense). Amplifications were performed using Cetus *Taq* polymerase buffer with 1.5 mM $MgCl_2$ for 35 cycles of the following: annealing at 55°C for 1 min; extension at 72°C for 1 min 30 s; and denaturation at 95°C for 1 min.

Genomic DNA Amplification

Genomic DNA was isolated from peripheral blood as described elsewhere (Higuchi 1989*a*). The primers for PCR were located in the introns and encompassed each of the 24 exons and splice junctions of the PFK-M gene; the upstream primers contained a GC-rich sequence attached to the 5′ end of the specific sequence. Genomic DNA was amplified with AmpliTaq (Perkin Elmer Cetus) at the following conditions: 95°C for 30 s, 55°C for 30 s, and 72°C for 50 s, for 35 cycles, with a final extension of 5 min at 72°C. The sequences of the intronic primers for exons 6, 8, and 22 amplifications are as follows: exon 6, 5′-ctggggagctgacttctacc(sense)/5′-taagcaatactgatgtgaaaact (antisense); exon 8, 5′-ggactgtgtcatatgtcta (sense)/5′-ggaataaatgggtcagaatgagg (antisense); and exon 22, 5′-cctctgtaatttttatgttt (sense)/5′-atagagggcactcgctctcaccc (antisense). The sequences of the other intronic primers are available on request.

Denaturing Gradient Gel Electrophoresis (DGGE)

The amplified genomic fragments (~200 ng DNA) were subjected to electrophoresis on a vertical gel containing a linearly increasing gradient of denaturant, parallel to the direction of the electrophoresis as described elsewhere (Myers et al. 1985, 1987). The gels were run at 80 V for 16 h at 60°C and stained with ethidium bromide to visualize the bands.

Southern Analysis

Ten micrograms of DNA were digested to completion with *Eco*RI, *Hin*dIII, and *Bam*HI restriction enzymes, were fractionated on a 0.8% agarose gel, and were transferred to a Nytran membrane. The blots were hybridized with a PFK-M cDNA probe ^{32}P labeled by random priming. The membrane was washed with 2 × SSPE, 0.1% SDS for 30-min at room temperature, followed by a 30-min wash at 50°C and a 30-min wash at 65°C.

Direct Sequence Analysis

Amplified cDNA fragments and genomic PCR products which showed altered mobility on DGGE were directly sequenced. Single-stranded DNA for sequencing was generated with nested primers in a two-stage PCR protocol, as we described elsewhere (Nichols and Raben 1994). The sequencing reactions were performed with Sequenase 2.0 according to the manufacturer's directions (U.S. Biochemical).

Northern and Western analyses

Total yeast RNA was prepared as described elsewhere (Heinisch 1986). RNA (10 μg) was fractionated on 1% agarose gels containing 6.3% formaldehyde, was transferred to positively charged membranes (Boehringer-Mannheim), and hybridized nonradioactively at 68°C in the presence of 20% SDS as described elsewhere (Engler-Blum et al. 1993). A 1.8-kb human PFK-M cDNA probe was labeled with DIG deoxigenin (Boehringer-Mannheim). Western analysis was performed as described elsewhere (Jacoby et al. 1993). For some experiments, the "western-light protein detection kit" (Tropix) was used according to the recommendations of the manufacturer.

Determination of PFK Activity

Crude extracts were prepared from yeast cells (A_{600} = 2.0–2.5) with glass beads (Breitenbach-Schmitt et al. 1984). PFK activity was assayed as described (Tarui et al. 1965). The reaction mixture in 50 mM K-phosphate buffer (pH 7.2) contained 10 μM fructose-2,6-biphosphate as an allosteric activator, 2 mM DTT, 10 mM $MgCl_2$, 1 mM ATP, 0.2 mM NADH, and 1 U of each of the following enzymes: aldolase, triosephosphate isomerase, and glycerophosphate dehydrogenase. The reaction was started with fructose-6-phosphate at a final concentration of 5 mM; oxidation of NADH was measured at 340 nm at 25°C. Bovine serum albumin was used as a standard for protein determination. For heat inactivation studies, crude extracts containing 0.7–2.4 mg protein/ml were placed at 60°C at time 0 and aliquots were removed for enzymatic determinations at the intervals indicated. Activities at time 0 were set at 100% for each strain tested.

Plasmids and Site-Specific Mutagenesis

pJJH71 vector with a constitutively expressed yeast PFK2 promoter was used for expression experiments. The PCR fragment corresponding to the 1,276-bp yeast *PFK2* promoter (Heinisch et al. 1991) was cloned into the *Eco*RI/*Bam*HI site of the high-copy-number yeast/*Escherichia coli* shuttle vector YEp352, with *URA3* as a selectable marker. YEp351 vector with another selectable marker (*LEU2*) was used for some experiments (Hill et al. 1986). Two recipient strains were used: a mutant derivative of HD56-5A in which yeast *pfk1* gene has been deleted by substitution with a *HIS3* marker (Arvanitidis and Heinisch 1994); and HD114-8D, the isogenic derivative carrying deletions in both *pfk1* and *pfk2* genes. Media and carbon sources used for growth have been described elsewhere (Arvanitidis and Heinisch 1994).

A full-length human PFK-M cDNA was amplified from pARV2PFKM vector (provided by A. McLachlan [Scripps Clinic and Research Foundation, La Jolla, California], to introduce *Bam*HI restriction sites at the ends of the cDNA and to remove the 5′ noncoding sequence. The *Bam*HI sequence in the upstream primer was followed by the ATG codon. The 2.4-kb cDNA fragment was gel purified, was subcloned into the pJJH71 vector, and was sequenced to ensure that no errors were introduced by PCR. The resulting nonmutant construct, termed "pJJH71PFK," was used for expression in yeast and as a template to introduce point mutations. (One of the clones, pJJH71PFK-Val, which contained a PCR-introduced error, resulting in Ala-778 to Val, was used as an additional control for mutant constructs.)

To generate constructs containing mutations in exons 6, 8, or 22 we used the recombinant PCR technique (Higuchi 1989*b*). Each of the newly recognized mutations was introduced into the complementary oligonucleotides (inside primers), which were used in combination with either sense or antisense oligonucleotides (outside primers) to generate two overlapping primary PCR fragments spanning the mutation site. The fragments were gel purified, were mixed together (50 ng each), were denatured, and were allowed to reanneal; the recombinants with recessed 3′ ends were subjected to extension with *Taq* DNA polymerase, followed by regular PCR with the outside primers, containing convenient restriction sites for cloning into pJJH71PFK.

pJJH71PFK was digested with *Sac*I, and the released fragment was substituted with the mutated template, containing exon 22–altered sequence (pJJH71PFK-22). For constructs with the exon 6 mutation (pJJH71PFK-6) the nonmutant plasmid was digested with *Spe*I and *Mun*I, releasing two fragments, since PFK-M cDNA contains two *Mun*I sites; to subclone the exon 6–altered template, the first *Mun*I site at position 491 was destroyed by introducing a silent base change. Exon 8 mutated template was subcloned into *Mun*I-digested nonmutant plasmid, giving rise to pJJH71PFK-8. Since the base change in exon 4 is located at close proximity to the convenient restriction site, mutated template was generated by single PCR, and the product was subcloned into *Spe*I/*Age*I sites, giving rise to pJJH71PFK-4. Two other constructs, pJJH71PFK5′UT-n and pJJH71PFK5′UT-m, contain a 110-bp sequence (normal or with a base change at the transcription start point [TSP]) upstream from the ATG, in addition to the coding sequence. The mutations and the integrity of the inserts were confirmed by sequencing.

Results

Detection of Abnormal Alleles

We selected DGGE as a screening method because previously it allowed us to identify all mutations in this gene, in nine families with PFK deficiency (Raben et al. 1993; Sherman et al. 1994). The GC-clamped amplified products from genomic DNA were resolved on gels with 25%–75% or 40%–80% denaturant. Amplified fragments that showed altered mobility on DGGE were directly sequenced.

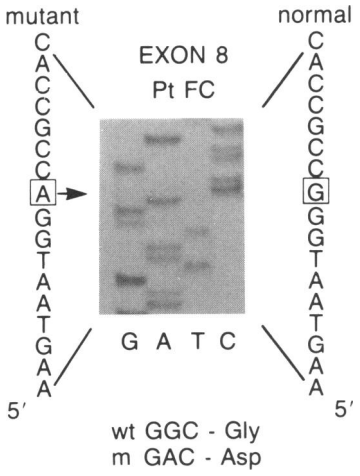

Figure 1 Partial sequence of exon 8 of the PFK-M gene. Direct sequencing of the PCR-amplified DNA showing a G-to-A transition, resulting in a substitution of Asp for Gly-209 in a homozygous French Canadian patient.

Genetic Defect in a French Canadian patient.—DNA fragments corresponding to exon 7 and exon 8 had altered DGGE patterns, compared with those in the control. Exon 7 contained a previously reported polymorphism, a silent C-to-T base change at position 516 of the PFK-M gene. The DGGE migration pattern for exon 8 was consistent with heterozygosity in the mother and homozygosity in the patient. Sequence analysis revealed a G-to-A transition resulting in a substitution of Asp for Gly-209 (fig. 1). The mutation is located in the evolutionarily conserved domain implicated in substrate binding and therefore is likely to be of functional importance.

Genetic defect in a Swiss patient.—Two polymorphic regions were detected by DGGE: exon 22 and exon 2 in the 5′ UTR. Direct sequencing revealed a G-to-A transition at position 2087 in exon 22, resulting in a substitution of His for Arg-696. Analysis of the family showed that the mutation was inherited from her father (fig. 2*a*). PCR amplification and sequencing of the cDNA region spanning exon 22 demonstrated the presence of both G and A (fig. 2*b*), indicating that both alleles are expressed.

Heterozygosity in the exon 2 region resulted from a g-to-t transversion in intron 2 (fig. 3, I*A*); this base change is located at one of the TSPs of the B-type mRNA. The substitution creates a *Bsm*AI restriction site; digestion of the genomic PCR-amplified DNA from family members (fig. 3, I*B*) showed that this base change in the patient and in the unaffected brother is inherited from the mother. We next screened 30 unrelated subjects (of similar background) for this substitution and detected it in four heterozygous individuals and one homozygous unaffected control, suggesting that the transversion is a polymorphism, unrelated to the PFK phenotype.

Having found the exon 22 mutation in one allele of the PFK-M gene, we sought a mutation in the second allele. Since gross gene deletions would escape detection by DGGE, we screened for these by Southern analysis. No band of abnormal size was found after digestion of genomic DNA with *Eco*RI, *Bam*HI, and *Hin*dIII restriction enzymes and hybridization with PFK-M cDNA probe (not shown).

Although there are examples of disease-causing mutations in the noncoding region (Crossly and Brownlee 1990; Hobbs et al. 1992; Vidaud et al. 1993), it seemed very unlikely that a substitution at the TSP was associated with PFK phenotype, because (i) the same base change was found in homozygous and heterozygous unaffected control subjects, and (ii) the PFK-M gene, like other housekeeping genes, has multiple transcription start sites, and two major TSPs for types A and B mRNA are located in exon 2 of the gene (Yamasaki et al. 1991).

To identify a mutation on the second allele, the RNA was reverse transcribed, and the cDNA was amplified with three pairs of primers, generating overlapping fragments spanning the entire coding region of the PFK-M gene. Direct sequencing of the PCR products revealed a G-to-A

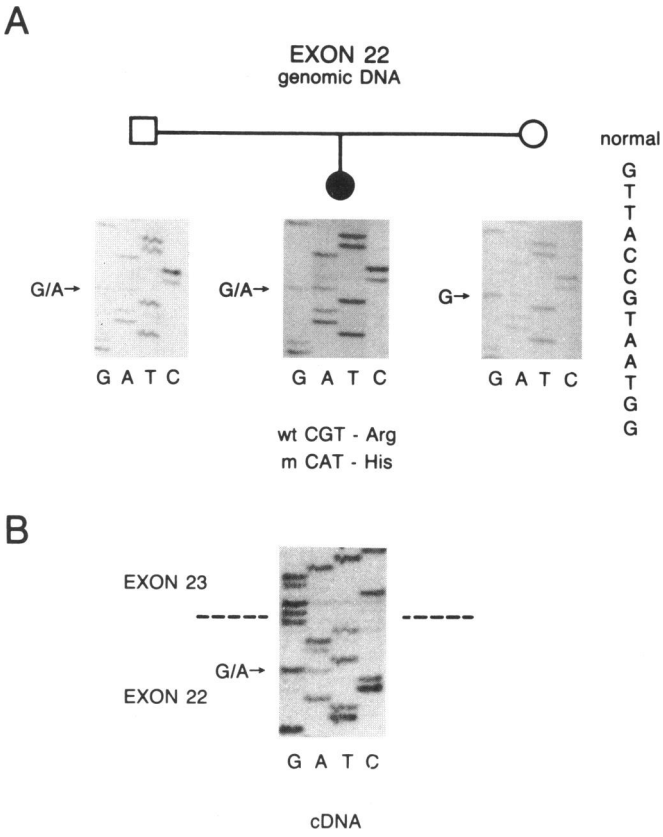

Figure 2 Partial sequence of exon 22 of he PFK-M gene. Direct sequencing of the PCR-amplified genomic DNA (*A*) or cDNA (*B*), showing a G-to-A transition, resulting in a substitution of His for Arg-696 in a compound heterozygous Swiss patient. The mutation is inherited from the father; the mother has normal sequence in this region of the gene.

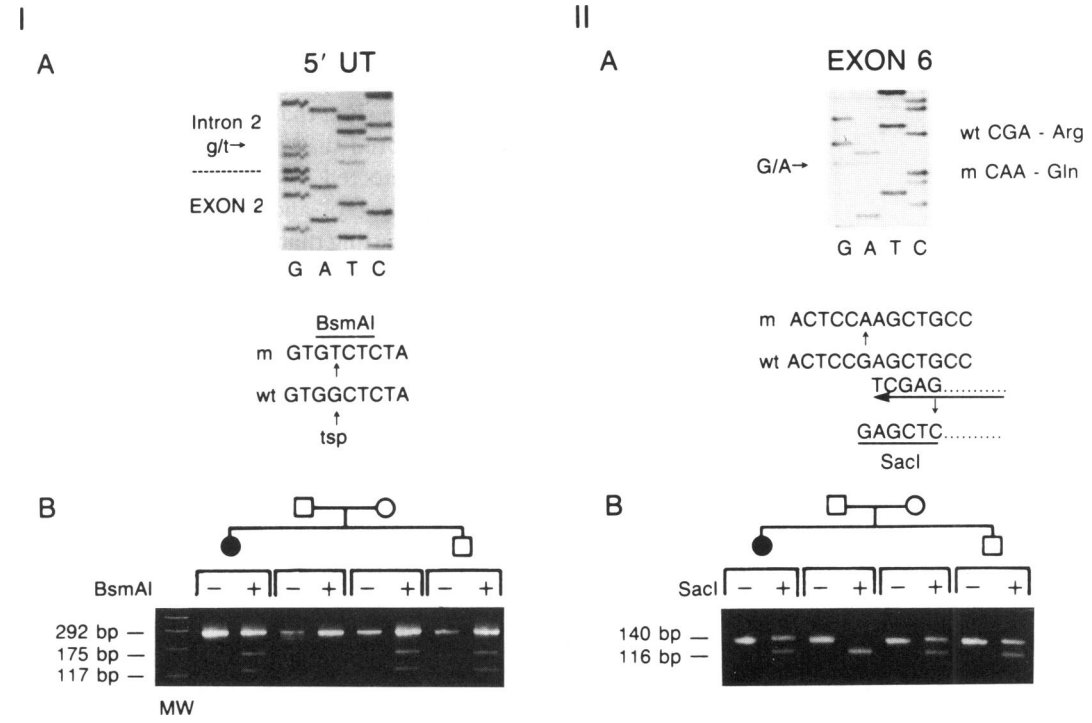

Figure 3 Partial sequences of the exon 2/intron 2 junction at the 5′ UTR and of the exon 6 of the PFK-M gene. A G-to-A transition in exon 6 results in a substitution of Gln for Arg-100 in a compound heterozygous Swiss patient (IIA). The mutation resides on the maternal allele, as shown by restriction digestion of the PCR products amplified with a mismatched primer (IIB). The primer creates a *Sac*I site only with the wild-type sequence. A g-to-t base change in the intron 2 (IA) creates a *Bsm*AI restriction site (IB). Both the mutation in exon 6 and the base change in intron 2 reside on the same allele.

transition at position 299 in exon 6, resulting in a substitution of Gln for Arg-100 (fig. 3, IIA). A silent G-to-A transition at the third position of codon 82 was identified in exon 6; this variant does not result in an amino acid change and therefore represents a polymorphism. To confirm the presence of the exon 6 mutation and to screen the family members we designed a mismatched primer that created a *Sac*I restriction site in the PCR fragment from the normal but not from the mutated sequence. The restriction digest (shown in fig. 3, IIB) established that the patient, mother, and unaffected brother are heterozygous for the exon 6 mutation. Screening of 30 unrelated control subjects revealed no alleles with the exon 6 mutation.

To estimate the level of expression of the exon 6–mutated allele, which also has a polymorphic base change at the TSP in intron 2, we took advantage of this polymorphism and amplified the cDNA and genomic regions encompassing the TSP with primers in exon 2 and intron 2 (fig. 4). The resulting fragments were digested with *Bsm*AI restriction enzyme. Relative levels of RNA were estimated by comparing the ratio of digested/undigested PCR products of PFK-M transcripts with that of genomic DNA. As is shown in figure 4, the relative amount of digested product was much lower in cDNA than in genomic DNA, suggesting that the maternal allele is underexpressed.

Thus, the patient is a genetic compound, carrying the substitution of Arg-696 for His in exon 22 on the paternal allele and Arg-100 for Gln in exon 6 on the maternal allele. Neither of these mutations is located in the evolutionarily conserved region of the protein, and it is difficult to predict the effect of the changes on protein structure.

Yeast Expression Studies

pJJH71PFK constructs, containing newly identified mutations as well as a previously described mutation in exon 4 in an Ashkenazi patient, were transformed in either the HD56-5A strain carrying a deletion of the yeast *pfk1* gene or the HD114-8D strain carrying *pfk1 pfk2* double deletions. It has been previously established that double-deletion mutants grow only on such nonfermentable carbon sources as glycerol and ethanol, while single-deletion mutants retain the ability to grow on glucose but lack detectable PFK activity in vitro (Heinisch 1993; Arvanitidis and Heinisch 1994). Therefore, HD114-8D transformants were selected on plates with synthetic medium containing glycerol and ethanol but lacking uracil. Of >100 colonies growing on each plate, ≥3 were picked onto fresh medium and replica-plated onto both rich medium and synthetic medium containing 2% glucose as a carbon source. Transformants carrying pJJH71 (the vector without human PFK cDNA) and pJJH71PFK-8 did not complement the glucose-negative phenotype of the recipient strain, sug-

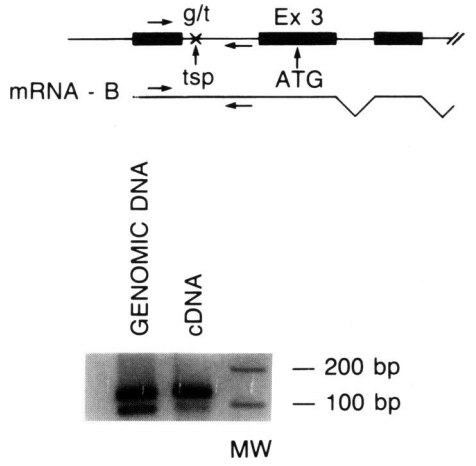

Figure 4 Estimation of the level of expression of the exon 6 mutated allele. The region spanning the exon 2/intron 2 junction was amplified from the genomic DNA or from mRNA. The position of the primers is indicated by the arrows. Nucleotide t in intron 2 is the marker for the maternal allele, which also harbors the mutation in exon 6. The fragments were digested with *Bsm*AI, which was created by a g-to-t base change. The relative level of digested/undigested product is lower in the cDNA, compared with that in the genomic DNA, suggesting that this allele is underexpressed.

gesting that the mutation in exon 8 is located in a catalytic site of the enzyme. All other plasmids conferred growth on glucose. Growth kinetics of the glucose-positive transformants indicated that all grow slightly slower than a wild-type yeast strain transformed with plasmid alone (table 1). The data indicate that mutations in exons 4, 6, and 22 generate functional proteins. Transformants with clones containing 5′ UTR showed a significant increase in generation time, suggesting that the upstream sequence inhibits expression in yeast. It is interesting that a g-to-t base change (pJJH71PFK5′UT-m) apparently confers better expression in yeast.

Evidence for expression of the mutant alleles in yeast.—Northern and western analysis provided direct evidence that the human gene is expressed in yeast (figs. 5 and 6). Northern blots were performed for selected transformants grown either on glucose or on glycerol and ethanol; mRNA of ~3 kb was detected in the transformants (fig. 5). A yeast strain transformed with the vector alone served as a negative control for northern analysis. To ensure that the protein is made, we performed western analysis with polyclonal antibodies to mammalian PFK (provided by G. Dunaway [Springfield, IL]). A highly homologous PFK from rabbit muscle (Boehringer-Mannheim) was used as a positive control. In contrast to a wild-type yeast strain, all glucose-positive transformants of HD114-8D, grown on synthetic medium with 2% glucose, showed a band of the expected size (fig. 6*A*). Since transformants of this strain with pJJH71PFK-8 do not grow on glucose, the plasmid containing the exon 8 mutation was transformed into HD56-5A carrying a single *pfk1* deletion. Western blot showed that a protein of the same size is produced in such transformants, although in significantly lower amounts (fig. 6*B*), suggesting that the mutant protein is unstable. It should be noted that at pH 6.4 smaller-size bands (~50 kD) were detected in western blots from transformants grown on glycerol and ethanol as carbon sources (not shown), suggesting that a yeast protease may cleave human PFK under these conditions.

Determination of specific PFK activities and heat inactivation studies.—Glucose-positive transformants of HD114-8D (*pfk1::HIS3 pfk2::HIS3 ura3 leu2*) were grown on synthetic medium with 4% glucose lacking uracil for the preparation of crude extracts. In a first experiment, three independent transformants of each clone were grown to late logarithmic phase. To minimize variation in copy numbers one transformant of each clone was used to establish the growth behavior similar to that described in table 1. The transformants showed only slight variations in specific PFK activities, as compared with the wild-type human PFK (table 2*A*). Thus, pJJH71PFK and pJJH71PFK-22 showed similar activities—pJJH71PFK-4 having slightly reduced and pJJH71PFK-6 having slightly elevated PFK activities. Note that strains carrying YEp351 derivatives (with *LEU2* as a selection marker) show significantly lower specific activities compared with that in pJJH71, presumably because of a lower copy number of the vector.

Clones tested in HD56-5A carrying a deletion only in yeast *pfk1* gave similar results (table 2*B*). No activity is conferred by pJJH71PFK-8, again indicating that fructose-6-phosphate binding is affected.

Transformants carrying clones with the 5′ UTR sequence showed significantly reduced specific activities, with the one carrying the original sequence being most strongly affected. The data confirmed previous findings (Heinisch 1993) and indicated that the expression in yeast is inhibited by the 5′ sequence and is dependent on the GC-content of the leader sequence.

To gain further insight into the structure of the mutated protein still showing PFK activity when expressed in yeast, we determined the kinetics of heat inactivation of different transformants. As is shown in figure 7, pJJH71PFK-4 encodes an enzyme that is highly unstable, compared with the wild-type human PFK. The mutant protein loses 90% of its activity within the first 10 min and all of it after 20 min, while the wild-type human PFK loses only 15% and 40% activity at the respective intervals. pJJH71PFK-6 shows an intermediate stability, while pJJH71PFK-22 does not seem to be affected at all. To mimic the heterozygosity of the Swiss patient in the yeast system, we also expressed both the PFK-6 and the PFK-22 alleles in HD114-8D (*pfk1::HIS3 pfk2::HIS3 ura3 leu2*) from two different multicopy vectors within the same cells. The double transformants gave intermediate results in heat inactivation experiments (fig. 7). It should be noted that the two

Table 1

Generation Time of Yeast Transformants

Strain	Relevant Genotype	Plasmid	Generation Time (min)
HD56-5A	*PFK1 PFK2 ura3-52*	pJJH71	120
HD114-8D	*pfk1::HIS3 pfk2::HIS3 ura3-52*	pJJH71PFK	132
HD114-8D	*pfk1::HIS3 pfk2::HIS3 ura3-52*	pJJH71PFK-4	141
HD114-8D	*pfk1::HIS3 pfk2::HIS3 ura3-52*	pJJH71PFK-6	132
HD114-8D	*pkf1::HIS3 pfk2::HIS3 ura3-52*	pJJH71PFK-22	132
HD114-8D	*pfk1::HIS3 pfk2::HIS3 ura3-52*	pJJH71PFK5'UT-n	216
HD114-8D	*pfk1::HIS3 pfk2::HIS3 ura3-52*	pJJH71PFK5'UT-m	165

NOTE.—Cells were grown in synthetic complete medium lacking uracil and containing 4% glucose as the sole carbon source. Growth was measured over a period of 10 h at OD_{600}, and generation time was determined from the slope during logarithmic growth.

multicopy plasmids led to an increase in specific PFK activity, compared with strains carrying only one of the plasmids (table 2*B*).

Thus, the yeast expression studies provided direct evidence that Gly-209 (a mutation site in exon 8 in a French Canadian patient) is located in the region involved in the substrate-binding site of the human PFK; the deficiency in a homozygous patient is caused by the production of catalytically inactive protein.

The mechanism of PFK deficiency caused by other point mutations is less clear. In the yeast system the mutation in exon 22 does not lead to any appreciable change in the protein. On the other hand, the mutations in exons 4 and 6 produce enzymes that are more sensitive to heat inactivation, indicating that the overall structure of the homotetrameric protein may be altered.

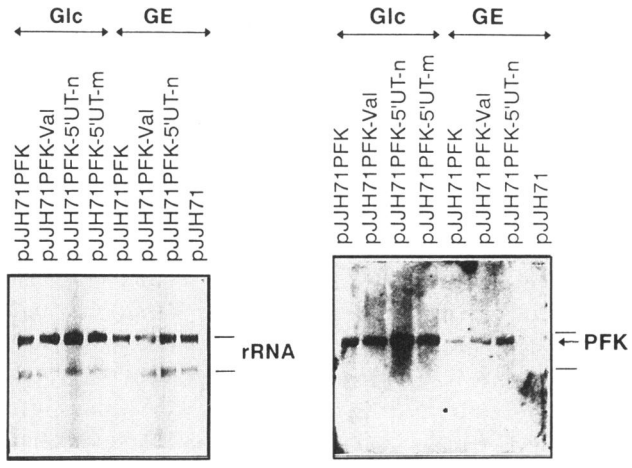

Figure 5 Northern blot of selected yeast transformants. Strain HD114-8D, carrying deletions in both yeast *PFK* genes, was transformed with plasmids indicated. The transformants were grown either on rich media containing glycerol (Glc) or synthetic media containing glycerol and ethanol (GE) as carbon sources. *Left panel,* Ethidium bromide–stained gel (loading control). *Right panel,* 30-min exposure of the nonradioactive northern blot.

Discussion

Several pathogenic mutations have been identified in patients with PFK deficiency, establishing the genetic heterogeneity of the disease. Splicing defects (mutations at the donor site of intron 15 and intron 19) are responsible for PFK deficiency in two unrelated Japanese patients (Nakajima et al. 1990*b*; Hamaguchi et al. 1994). We recently identified mutations in a group of Ashkenazi patients who share two common mutations: (i) a splicing defect, which involves the donor site of intron 5 and results in exon 5 skip, and (ii) a C nucleotide deletion at position 2003 (exon 22), which results in a frameshift and premature stop codon (Raben et al. 1993; Sherman et al. 1994). One of the compound heterozygous Ashkenazi patients had a missense mutation, changing Arg-39 to Leu on one of the alleles. Yet another splicing mutation, which involves the acceptor site of intron 6, and two missense mutations (Arg-39 to Pro and Asp-543 to Ala) have been identified in four Italian patients with the disease (Tsujino et al. 1994). It is curious that Arg-39 was mutated in both Ashkenazi and Italian patients with the disease.

We now describe three novel mutations in one French Canadian patient and one Swiss patient with Tarui disease. The French Canadian patient is homozygous for a G-to-A transition in exon 8, resulting in a substitution of evolutionarily conserved Gly-209 by Asp. The patient did not recount a history of exercise intolerance; he served in the army and developed the symptoms only in his 40s. In general, late-onset PFK deficiency has been considered a doubtful nosologic form because, in a few described cases, the symptoms of easy fatigability and inability to keep up with others appeared in early childhood (Hays et al. 1981; Vora et al. 1987; Danon et al. 1988). Late-onset fixed muscle weakness developing in these patients may represent a

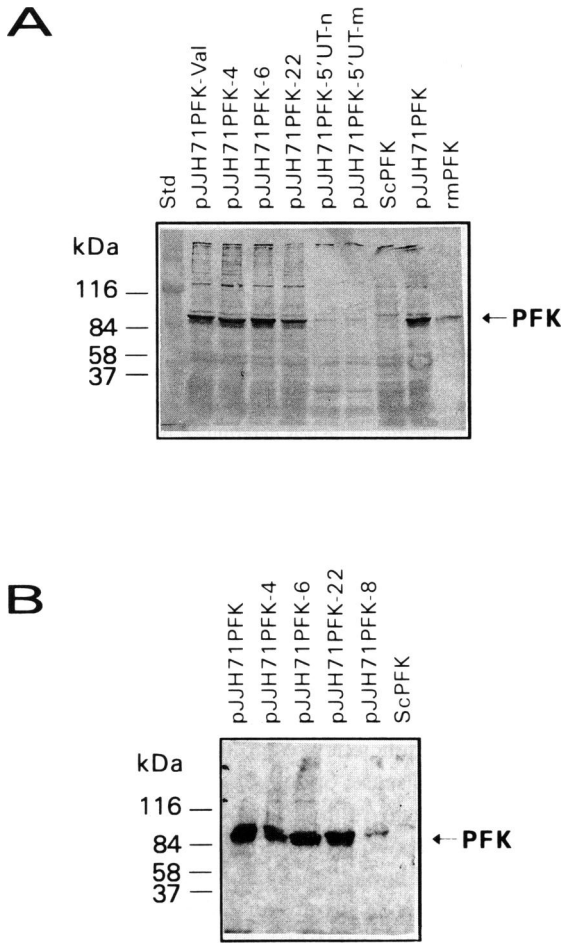

Figure 6 Western blot analysis of yeast transformants. *A*, Strain HD114-8D, carrying deletions in both yeast *PFK* genes, transformed with the plasmids indicated. Crude extracts were prepared for immunological detection by using standard procedures (see Subjects, Material, and Methods). An extract from a wild-type yeast strain HD56-5A (Sc PFK) and purified rabbit muscle PFK were used as controls. Strains were grown on synthetic medium with 2% glucose lacking uracil. *B*, Transformants of a *pfk1*-deletion derivative of HD56-5A, grown on glucose media lacking uracil.

natural course of the disease, rather than a distinct clinical entity (Argov et al. 1994).

The Swiss patient, who suffered from muscle PFK deficiency of the classic type, is a genetic compound: a G-to-A transition in exon 6, inherited from her mother, would predict a substitution of Gln for Arg-100, and a G-to-A transition in exon 22, inherited from her father, would predict a substitution of His for Arg-696. (It should be noted that the mutation in exon 6 had been missed by the DGGE analysis for no obvious reason, indicating the limitations of this screening technique.) The exon 6 mutated allele is underexpressed, as is shown by restriction digestion of the RNA/PCR fragment spanning a polymorphic g-to-t base change at the TSP in intron 2. The reason for the lower level of transcription from this allele is not clear. It could be due to the mutation itself and/or the effect of the base change at the TSP. However, there might be another mutation farther upstream that affects PFK expression in the Swiss patient.

The effect of the missense mutations (identified in this study as well as in previous reports) and their relevance to the PFK phenotype is not obvious, since some of them are located in evolutionarily conserved regions of the gene, while others are not. Furthermore, the mutational analysis of *Escherichia coli* PFK demonstrated that some of the mutations in the conserved region had only modest effect on the enzyme activity (Kundrot and Evans 1991). It has been also shown that mutation of Arg following Gly-209 in the yeast enzyme does not result in any appreciable changes in the phenotype, even though it is highly conserved among the species (J. Heinisch, unpublished results). These data emphasize the importance of the expression experiments for structure-function studies of the mutant PFK.

The disease-causing mutations are expected to occur either at the catalytic site of the PFK molecule or at the subunit interaction site, since the ability to form polymers is critical for the enzyme function and the smallest active form of PFK in vitro is a tetramer. The described tertiary structure and location of the catalytic and effector sites of the bacterial enzyme (Evans et al. 1981) provided the basis for predictions concerning the structural organization of mammalian PFKs, since they share significant homology at the amino acid level. Mammalian enzymes are thought to have evolved by duplication of a prokaryotic gene, as shown by clear homology among the N- and C-halves of rabbit and bacterial enzymes (Poorman et al. 1984).

Gly-209, the site of mutation in a homozygous French Canadian patient, is located at the evolutionarily highly conserved domain present in all known PFK isoforms. Both duplicated halves of the human PFK-M contain this domain, and, on the basis of the structure of the bacterial enzyme, the region is a part of the active site involved in substrate binding. Therefore, one would expect a loss of function of the protein encoded by the exon 8–mutated gene. Arg-39 mutated in previously described Ashkenazi (Sherman et al. 1994) and Italian (Tsujino et al. 1994) patients is also conserved among the species; it is present in both parts of the duplicated gene and is predicted to be part of the ATP binding site. In contrast, Arg-100 and Arg-696, the sites of mutations in exons 6 and 22 in the Swiss patient, are not located in evolutionarily conserved regions and are not duplicated in the protein. Furthermore, Arg-696 in the C-terminal part lies within the stretch of amino acids that stand out as "extra residues" when the two halves of the human muscle kinase are aligned with the bacterial sequence. Although these changes would predict a minimal effect on protein structure, we think that the mutations are pathogenic, because (i) the entire coding region, as well as the upstream regulatory region, was sequenced, and no other abnormalities were found; and (ii)

Table 2

Specific PFK Activities in Yeast Transformants

Strain	Relevant Genotype	Plasmid	Activity[a] (mean)	% Normal	Activity[a] OD_{600} = 2	% Normal
A:						
HD56-5A	*PFK1 PFK2 ura3-52*	pJJH71	536	...	525	...
HD114-8D	*pfk1::HIS3 pfk2::HIS3 ura3-52*	pJJH71PFK	571	100	747	100
HD114-8D	*pfk1::HIS3 pfk2::HIS3 ura3-52*	pJJH71PFK-4	466	82	545	73
HD114-8D	*pfk1::HIS3 pfk2::HIS3 ura3-52*	pJJH71PFK-6	838	147	932	125
HD114-8D	*pfk1::HIS3 pfk2::HIS3 ura3-52*	pJJH71PFK-22	572	100	737	99
HD114-8D	*pfk1::HIS3 pfk2::HIS3 ura3-52*	pJJH71PFK5'UT-n	154	27	189	25
HD114-8D	*pfk1::HIS3 pfk2::HIS3 ura3-52*	pJJH71PFK5'UT-m	296	54	504	67
B:						
HD56-5A	*pfk::HIS3 PFK2 ura3-52 leu2*	pJJH71PFK	751			
HD56-5A	*pfk::HIS3 PFK2 ura3-52 leu2*	pJJH71PFK-8	BD			
HD56-5A	*pfk::HIS3 PFK2 ura3-52 leu2*	pJJH71PFK-22	760			
HD56-5A	*pfk::HIS3 PFK2 ura3-52 leu2*	YEp351PFK-6	287			
HD56-5A	*pfk::HIS3 PFK2 ura3-52 leu2*	YEp351PFK-22	296			
HD56-5A	*pfk::HIS3 PFK2 ura3-52 leu2*	pJJH71PFK-6 + YEp351PFK-22	885			
HD56-5A	*pfk::HIS3 PFK2 ura3-52 leu2*	pJJH71PFK-22 + YEp351PFK-22	863			

[a] Specific activities are given in mU/mg protein and were determined in crude extracts prepared from cells grown on synthetic complete medium lacking uracil and/or leucine, as required. Mean activities were determined from three (A) or two (B) independent transformants grown overnight to late logarithmic phase (BD = below detection; the lower limit of detection is ≈5 mU/mg protein).

30 control subjects contained normal sequences in exons 6 and 22.

The function of human mutant PFK was studied by complementation of the engineered yeast strains devoid of the endogenous *PFK* activity. The system seemed especially attractive, because the wild-type human PFK was shown to restore the enzymatic activity of yeast single mutants (with one *PFK* gene deleted) and to restore the glucose-positive phenotype of yeast double mutants (with both *PFK* genes deleted; Heinisch 1993). The results of the expression experiments fit comfortably with the prediction based on the location of the mutations in gene. Gly-209-mutated PFK expressed in yeast single mutants did not show any detectable enzyme activity and did not complement the glucose-negative phenotype of the double-mutant recipient strain. The inability of pJJH71PFK-8 to confer growth on glucose strongly suggests that the expressed protein is catalytically inactive.

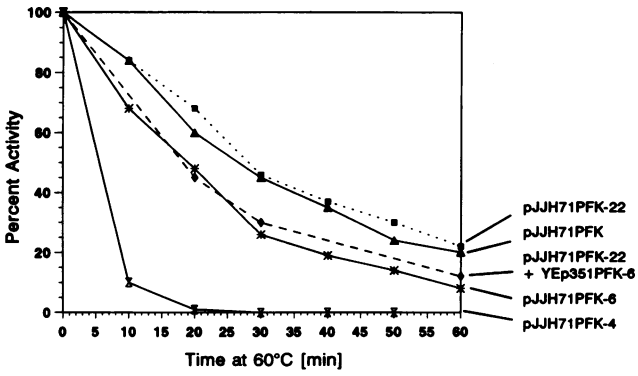

Figure 7 Heat inactivation of mutant human PFK enzymes expressed in yeast. Yeast transformed with the indicated plasmids were grown on synthetic medium with glucose lacking uracil and leucine as required. Crude extracts were placed in a water bath at 60°C, and the enzymatic activities were determined at the indicated time points (the activity for each strain was taken to be 100% at time 0).

All other mutants that did not affect presumed substrate binding sites generated functional enzymes in yeast. As is shown by heat inactivation experiments, the substitution of Leu for Arg-39, identified previously in an Ashkenazi patient, resulted in a significant loss of enzyme stability, suggesting that the conformational changes in the protein may be responsible for the total lack of enzyme activity detected in the muscle biopsy. It should be noted that the Ashkenazi patient was a compound heterozygote, carrying a splicing defect on the second allele, which would predict generation of a protein lacking 26 amino acids. The mechanism of the deficiency in this case could also involve the complete inactivation of the truncated protein and the inability of the mutated subunits to either tetramerize or polymerize in vivo to form an active enzyme. Mammalian PFKs are known to self-aggregate into polymers rendering enzymatically active protein (Aaronson et al. 1972; Lad et al. 1973).

The genotype-phenotype relationship in a Swiss patient is less clear, because Arg-100 (exon 6) and Arg-696 (exon

22) mutants expressed in yeast resulted in only modest changes in the stability of the enzyme. These mutants produce functional enzyme in yeast but apparently not in humans. The difference could be attributed either to different conformational states of the tetrameric enzyme in human and yeast cells or to different stability of the enzyme in the extracts for enzyme assay in both systems. It is interesting that the patient has residual PFK activity in muscle biopsy (9.3 mU/mg; ~8% normal), indicating that small amounts of functional enzyme are produced in vivo as well. In several reported cases with measurable amounts of residual PFK, the clinical syndrome was not atypical (Rowland et al. 1986). It is also important to emphasize that in vivo the tissue-specific regulation of the enzyme is controlled by a complex of mechanisms, including modulation by the effectors, phosphorylation, and control of synthesis and degradation, conditions that cannot be faithfully reproduced in a surrogate yeast host.

Thus, the yeast system is suitable to assess the effect of mutations, in the human PFK gene, on the *catalytic activity* of the enzyme. Both the stability and the conformational changes of some of the mutants can also be tested in this system. The system may also prove useful for production of high amounts of protein for crystallographic studies.

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