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Identification of Three Novel Mutations in Non-Ashkenazi Italian Patients with Muscle Phosphofructokinase Deficiency

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Summary

We have identified three novel mutations in four non-Ashkenazi Italian patients with muscle phosphofructokinase (PFK-M) deficiency (Tarui disease). Patient 1 was homozygous for an A-to-C substitution at the 3′ end of intron 6 of the PFK-M gene, changing the consensus splice-junction sequence AG to CG. The mutation leads to activation of two cryptic splice sites in exon 7, resulting in one 5 bp– and one 12 bp–deleted transcript. An affected brother was also homozygous, and both parents were heterozygous, for the splice-junction mutation. Patient 2 was homozygous for a G-to-C substitution at codon 39, changing an encoded arginine (CGA) to proline (CCA). Patient 3 was heterozygous for an A-to-C substitution at codon 543, changing an encoded aspartate (GAC) to alanine (GCC); the PFK-M gene on the other allele was not expressed, but sequencing of the reported regulatory region of the gene did not reveal any mutation.

Introduction

Phosphofructokinase (PFK) (ATP; D-fructose-6-phosphate 1-phosphotransferase; E.C.2.7.1.11) catalyzes the ATP-dependent phosphorylation of fructose-6-phosphate to fructose-1,6-biphosphate in the glycolytic pathway (Uyeda 1979). Three subunits—PFK-M (muscle), PFK-L (liver), and PFK-P (platelet)—compose tetrameric isozymes (Vora 1982). Mature human muscle contains only the homotetramer of PFK-M, and erythrocytes contain various tetrameric combinations of PFK-M and PFK-L.

Hereditary PFK-M deficiency (glycogenosis type VII, or Tarui disease) is characterized by myopathic features —exercise intolerance, cramps, and myoglobinuria— together with signs of compensated hemolytic anemia and hyperuricemia (Tarui et al. 1965; Kono et al. 1986; Rowland et al. 1986). The gene encoding PFK-M has been assigned to chromosome 1 (Vora et al. 1982); both the sequence of the full-length cDNA and the structure of the genomic DNA have been reported (Nakajima et al. 1987; Yamasaki et al. 1991). Alternative splicing generates three types of PFK-M mRNA (A, B, and C), which have the same coding region but different 5′ UTR sequences. However, type B, containing exon 2 and "intron 2," is the form predominantly expressed in muscle (Nakajima et al. 1990*a*, 1990*c*). Exon 9 can also be partially skipped by alternative splicing in various normal cell lines or tissues (Nakajima et al. 1990*a*; Sharma et al. 1990).

A splice-junction mutation has been described in the original Japanese family with PFK-M deficiency (Nakajima et al. 1990*b*), and a second splice-junction mutation (Raben et al. 1993*a*), a single-nucleotide deletion, and a missense mutation (Raben et al. 1993*b*) have been reported in Ashkenazi Jewish families. The molecular genetic basis of PFK-M deficiency in other ethnic populations has not been explored. We report three novel mutations in four non-Ashkenazi Italian patients with PFK-M deficiency.

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Subjects and Methods

Patients and Controls

Patient 1.—A 17-year-old man complained, since childhood, of myalgia and cramps after intense exercise. He had had no episode of myoglobinuria. His peripheral red-blood-cell count and hemoglobin were normal, but he had reticulocytosis (6.1%). Serum creatine kinase was 2,355 IU/liter (normal 30–230 IU/liter); LDH was 575 IU/liter (normal 230–460 IU/liter); and uric acid was 8.1 mg/dl (normal 3.5–7 mg/dl). Muscle biopsy showed myopathic changes with subsarcolemmal glycogen accumulation. Residual PFK activity in muscle was 1.8% of normal, and glycogen was 2.1% (normal <1%). His brother was also affected. There was no parental consanguinity, but the family lived in a small village in southern Italy.

Patient 2.—A 35-year-old man complained, since adolescence, of exercise intolerance, exercise-related myalgia, and cramps and had experienced a few episodes of myoglobinuria after intense exercise. He was first seen by an internist, for mild jaundice. Serum bilirubin was 1.8 mg/dl (normal <1.0 mg/dl); creatine kinase was 7,800 IU/liter (normal <190 IU/liter); lactate dehydrogenase was 900 IU/liter (normal 80–200 IU/liter); glutamic oxaloacetic transaminase was 200 IU/liter (normal 8–50 IU/liter); and glutamic pyruvic transaminase was 45 IU/liter (normal 8–50 IU/liter). Serum uric acid level, peripheral red-blood-cell count, and hemoglobin concentration were normal, but he had reticulocytosis (4.1%). A forearm ischemic-exercise test caused no increase of venous lactate. Muscle biopsy showed subsarcolemmal and intermyofibrillar glycogen accumulations, and residual PFK activity was 3.3% of normal. The parents were first cousins.

Patient 3.—A 43-year-old man had noticed, since childhood, that he could not keep up with his peers in physical activities. At age 33 years, he developed proximal weakness, myalgia, and exercise intolerance. He also had had an episode of hematuria with urate stones. He was jaundiced, and serum bilirubin was 14.70 μmol/liter (normal <6.84 μmol/liter). Because he had no sign of hemolysis, Gilbert syndrome was diagnosed. Serum creatine kinase was 1,208 IU/liter (normal <190 IU/liter); aldolase was 32 IU/liter (normal <7.6 IU/liter); and uric acid was normal. Forearm ischemic exercise resulted in a flat lactate response. Muscle biopsy showed fiber size variability and vacuoles, mainly in type 2 fibers; some vacuoles were optically empty, while others were filled with glycogen. Residual PFK activity in muscle was 6% of normal.

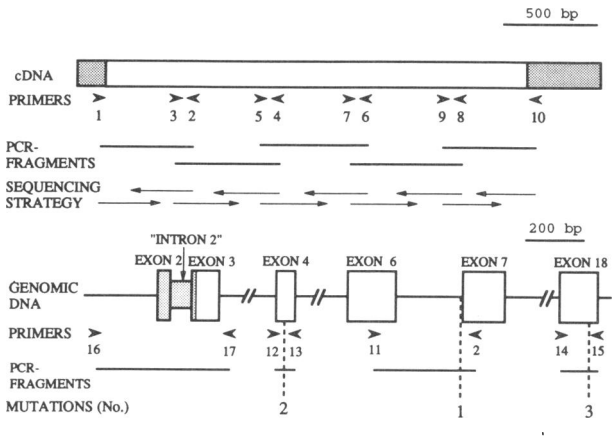

Figure 1 Primers and PCR fragments of PFK-M cDNA (*top*) and genomic DNA (*bottom*). Shaded boxes indicate untranslated regions. The narrow box indicates "intron 2," which is not spliced out in the more commonly expressed type B mRNA in muscle.

Control muscle consisted of biopsies obtained for diagnostic purposes from individuals ultimately deemed to be free of neuromuscular diseases. Control genomic DNAs were extracted, as described below, from muscle or white blood cells of 47 normal individuals and 9 patients with various metabolic myopathies: 2 with myoclonic epilepsy and ragged-red fibers (MERRF); 2 with mitochondrial encephalomyopathy, lactic acidosis, and strokelike episodes (MELAS); 2 with myophosphorylase deficiency; 1 with phosphoglycerate kinase deficiency; and 2 with muscle phosphoglycerate mutase deficiency.

RNA Extraction, PCR Amplification, and Sequencing

Total RNA was extracted by a modified cesium chloride centrifugation method (Glisin et al. 1974), from muscle tissues of patients and of a normal control. Five DNA fragments, encompassing the entire coding region of the PFK-M cDNA, were directly amplified from total RNA by using primers 1–10 (fig. 1 and table 1) and the Gene Amp Thermostable rTth Reverse Transcriptase RNA PCR kit (Perkin Elmer Cetus), by following the manufacturer's specifications. Primer 1 was designed in "intron 2" to amplify a PCR fragment from a commonly expressed type (type B) of PFK-M mRNA, because "intron 2" is not spliced out in type B mRNA (Nakajima et al. 1990*c*). The PCR products were electrophoresed through 2% NuSieve agarose gels (FMC Bioproducts), purified by a GeneClean kit (BIO101), and sequenced using the same primers and the dsDNA Cycle Sequencing System kit (GIBCO BRL Life Technologies), by following the manufacturer's specifica-

Table 1

Primers Used in the Present Study

No.	Primer[a]	Sequence[b]	Position
1	S	5′-CTGCTGTGTCTTAACTGACC-3′	"Intron 2"
2	A	5′-TGTCAATTGACCCAACCAGG-3′	Exon 7
3	S	5′-AAGATCACAGATGAGGAGGC-3′	Exon 7
4	A	5′-GCCATCACTGCTTCCACACC-3′	Exon 12
5	S	5′-GGGGTGGGACGCCATCAGCC-3′	Exon 11
6	A	5′-TGTTCAAAGCTCTTCTTGGG-3′	Exon 17
7	S	5′-GCTGGACTGGCCAAGGTGGC-3′	Exon 16
8	A	5′-TGGTATAGTTCTCATTGCAC-3′	Exon 21
9	S	5′-AAAAGATGAAAACAACTGTG-3′	Exon 20
10	A	5′-ATGATCAGGTAATCTATTCC-3′	Exon 24
11	S	5′-CACTGGGGCTGACACCTTCCG-3′	Exon 6
12	S	5′-tgtccctcctttcagGTATGA-3′	Intron 3–exon 4
13	A	5′-accaacCTCATGGACAAAGAA-3′	Intron 4–exon 4
14	S	5′-ttcttagGCTTACACAGGGGG-3′	Intron 17–exon 18
15	A	5′-ctcacTGTGCAGATAGTATTG-3′	Intron 18–exon 18
16	S	5′-ctttttctcctccctttct-3′	Intron 1
17	A	5′-caccagccatgtttttgtc-3′	Intron 3

[a] S = sense primer; and A = antisense primer.

[b] Sequences corresponding to introns are indicated in lowercase letters, except for primer 1, because "intron 2" is not spliced out in the more commonly expressed type (type B) of PFK-M mRNA in muscle (Nakajima et al. 1990*a*, 1990*c*).

tions. DNA fragments were subjected to a cycle-sequencing PCR program in the presence of 2 pmol of [γ-^{32}P]ATP 5′ end–labeled primer. The PCR products were electrophoresed through a 6% polyacrylamide/7 M urea gel. The gel was vacuum-dried for 30 min and exposed to Kodak XAR film for 12 h. PCR fragments were again amplified, using primers 11 and 2 from the PCR fragments of patient 1 and of a control, by using primers 1 and 2; were electrophoresed through a 15% polyacrylamide gel; were purified as described elsewhere (Maxam and Gilbert 1977); and were sequenced. *Taq* polymerase and reagents were purchased from Boehringer-Mannheim. PCR conditions were as follows: 30 cycles of denaturation, 94°C for 1 min; annealing, 65°C for 1 min; and extension, 72°C for 2 min. Sequences were compared both with those of PCR fragments obtained from a normal control and with the previously reported cDNA sequence (Nakajima et al. 1987).

Genomic DNA Extraction, PCR Amplification, Sequencing, and Restriction-Endonuclease Cleavage Test

Genomic DNAs were extracted from white blood cells of patients, their family members, and normal controls as described elsewhere (Shanske et al. 1987). PCR fragments, including the entire intron 6, which contains mutation 1 (table 2), were amplified from genomic DNAs of patient 1 and of a normal control by using primers 11 and 2 (fig. 1 and table 1). PCR conditions were as described above. The PCR fragments were directly sequenced, as described above, using the same primers. To simplify the detection of mutation 1, the PCR fragments were digested by the restriction endonuclease *Hpa*II, which cleaves the fragment in the presence of mutation 1 (fig. 2). *Hpa*II was purchased from Boehringer-Mannheim. Restriction digests were analyzed on a 2% NuSieve agarose gel.

PCR fragments containing mutations 2 (table 2) were amplified from genomic DNAs of patient 2 and of a normal control by using primers 12 and 13 (fig. 1 and table 1). PCR conditions were as follows: two cycles of denaturation, 94°C for 1 min; annealing, 60°C for 1 min; and extension, 72°C for 1 min; this was followed by 38 cycles of denaturation, 91°C for 1 min; annealing, 65°C for 1 min; and extension, 72°C for 1 min. To simplify the detection of mutation 2, the PCR fragment was digested by the restriction endonuclease *Nla*IV, which cleaves the fragment at the mutant site (fig. 3). *Nla*IV was purchased from New England Biolabs. Restriction digests were analyzed on a 15% polyacrylamide gel.

Table 2

Molecular Genetic Defects in PFK-M Deficiency

Reference	Nucleotide Change (codon or location)	Deduced Effect
Nakajima et al. 1990*b*	GT→TT (5′ end of intron 15)	75-bp in-frame deletion in transcript
Raben et al. 1993*a*	GT→AT (5′ end of intron 5)	Skipping of exon 5, in-frame deletion
Raben et al. 1993*b*	CGA→CTA (39)	Arg→Leu
Raben et al. 1993*b*	C→deletion (exon 22)	Frameshift
Present report (mutation 1)	AG→CG (3′ end of intron 6)	Two deleted transcripts
Present report (mutation 2)	CGA→CCA (39)	Arg→Pro
Present report (mutation 3)	GAC→GCC (543)	Asp→Ala

PCR fragments containing mutation 3 (table 2) were amplified from genomic DNAs of patient 3 and of a normal control by using primers 14 and 15 (fig. 1 and table 1). PCR conditions were identical to those described for primers 12 and 13. The PCR fragments were directly sequenced, as described above, using primer 14.

PCR fragments containing a downstream portion of intron 1 that includes the regulatory region of type B mRNA (Yamasaki et al. 1991) were amplified, from genomic DNAs of patients and of a normal control, using primers 16 in intron 1 and 15 in intron 3 (fig. 1 and table 1). PCR conditions were identical to those used for primers 11 and 2, except that 55°C was the annealing temperature. The PCR fragments were directly sequenced, as described above, using the same primers.

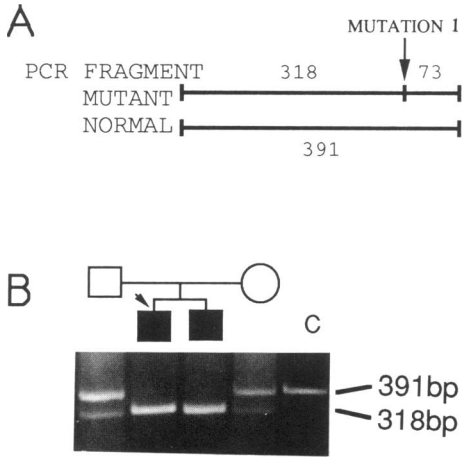

Figure 2 *A*, Schematic representation of PCR fragments amplified using primers 11 and 2. Numbers indicate lengths (in bp) of segments cleaved by *Hpa*II in mutant and control DNA. *B*, Pedigree of patient 1, and PCR fragments electrophoresed through a 2% NuSieve agarose gel after digestion with *Hpa*II. The arrowhead indicates patient 1. Lane C, Normal control.

Results

We amplified five partially overlapping DNA fragments encompassing the entire coding region of the PFK-M cDNA from RNA extracted from three unrelated non-Ashkenazi Italian patients with PFK-M deficiency.

A Splice-Junction Mutation at the 3′ End of Intron 6 (Mutation 1)

In patient 1, electrophoresis of these PCR fragments through a 2% NuSieve agarose gel showed that a fragment amplified from primers 1 and 2 appeared different in length from the corresponding control fragment. Se-

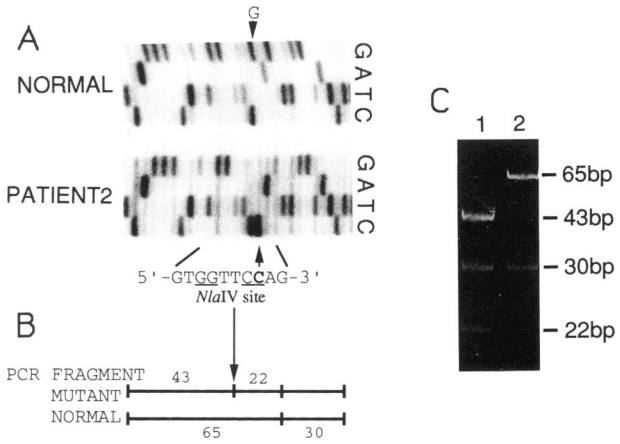

Figure 3 *A*, Sequences of PCR fragments amplified using primers 1 and 2 from RNAs of patient 2 and a control. Mutation 2 (boldface "C") makes an *Nla*IV site. *B*, Schematic representation of PCR fragments amplified using primers 12 and 13. Numbers indicate lengths (in bp) of segments cleaved by *Nla*IV in mutant and control DNA. *C*, PCR fragments amplified from genomic DNA of patient 2 (lane 1) and of a control (lane 2), by using primers 12 and 13, and electrophoresed through a 15% polyacrylamide gel after digestion with *Nla*IV.

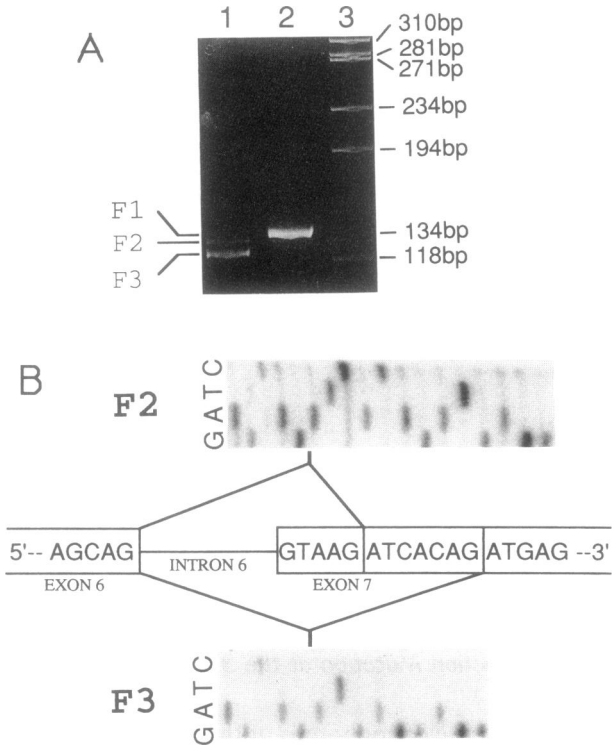

Figure 4 *A*, PCR fragments resulting from use of primers 11 and 2 from RNAs of patient 1 (lane 1) and a control (lane 2), electrophoresed through a 15% polyacrylamide gel. F1 = normal length fragment; and F2 and F3 = truncated fragments. Lane 3, DNA size marker, ΦX174 digested with *Hae*III. *B*, Sequences of the two truncated fragments (F2 and F3), showing abnormal splicings.

quence analysis was ambiguous and suggested the presence of more than one fragment. Therefore, we amplified a shorter PCR fragment by using primers 11 and 2, and the PCR product amplified with primers 1 and 2 as a template. Electrophoresis of this fragment through a 15% polyacrylamide gel showed a faint normal band and two shorter bands (fig. 4*A*). Each band was purified and sequenced. Of the two truncated fragments, one harbored a 5-bp deletion and the other a 12-bp deletion, and the breakpoints of both deletions corresponded to the junction between exons 6 and 7 (fig. 4*B*). All other fragments showed no difference in sequence from that of a normal control.

Next, we amplified, from genomic DNA of patient 1, a PCR fragment containing the entire intron 6, using primers 11 and 2. Analysis on a 2% NuSieve agarose gel showed that the size of this fragment was normal, a result implying that there was no deletion in genomic DNA. However, thorough sequence analysis of this fragment identified a homozygous A-to-C substitution at the penultimate nucleotide of intron 6 (fig. 5*A*), while the rest of the sequence was normal (fig. 5*B*).

To confirm mutation 1 and to develop a simple screening procedure, we digested the PCR fragment from genomic DNA by *Hpa*II. In the presence of mutation 1, the PCR fragment (391 bp) was cleaved by *Hpa*I into two fragments, one of 318 bp and one of 73 bp (fig. 2). This method confirmed that patient 1 was homozygous for mutation 1 and showed that his affected brother was also homozygous and that both parents were heterozygous (fig. 2). Screening of 47 normal and 9 disease controls showed that none of them had mutation 1.

A Missense Mutation at Codon 39 (Mutation 2)

In patient 2, we identified a G-to-C substitution, changing an encoded arginine (CGA) to proline (CCA) at codon 39 in exon 4 (fig. 3*A*). The rest of the sequence of the coding region, as well as the regulatory region, was identical to that of a control.

A PCR fragment amplified from genomic DNA by using primers 12 and 13 (fig. 1 and table 1) can be used

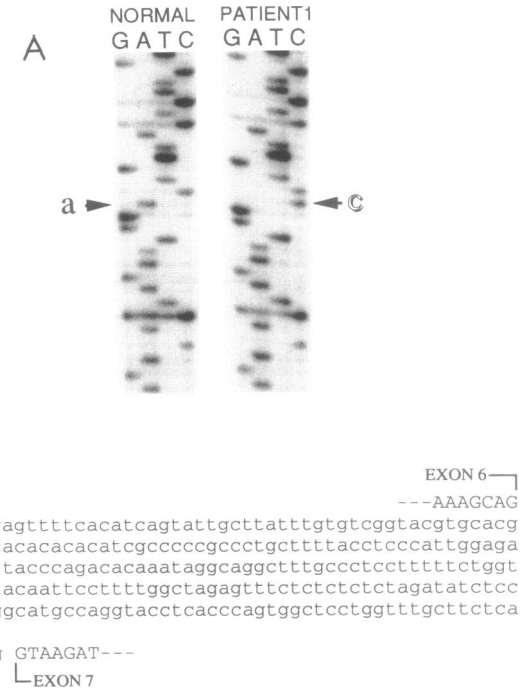

Figure 5 *A*, Sequences of PCR fragments amplified using primers 11 and 2 from genomic DNAs of patient 1 and a control. Highlighted "c" indicates mutation 1. *B*, Sequence of full-length intron 6. Mutation 1 is indicated by the arrowhead.

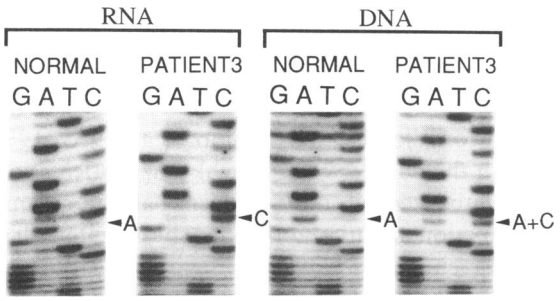

Figure 6 Sequences of PCR fragments, amplified using primers 7 and 8 from RNAs of a control and of patient 3, and sequences of PCR fragments amplified from their genomic DNAs by using primers 14 and 15.

to detect the mutation. As the nucleotide change creates a new *Nla*IV site, the PCR fragment (95 bp) containing mutation 2 is cleaved into three segments (43, 22, and 30 bp) by *Nla*IV, while the normal fragment is cleaved into two segments (65 and 30 bp) (fig. 3*B* and *C*). This method showed that patient 2 was homozygous for mutation 2; screening of 47 normal and 9 disease controls showed that none of them had this mutation.

A Missense Mutation at Codon 543 (Mutation 3)

In patient 3, we identified an A-to-C substitution, changing an encoded aspartate (GAC) to alanine (GCC) at codon 543 in exon 18 (fig. 6). The rest of the sequence was identical to that of a control.

A PCR fragment amplified from genomic DNA by using primers 14 and 15 (fig. 1 and table 1) was used to detect this mutation (mutation 3). However, sequencing of this fragment from genomic DNA showed that the patient was heterozygous for mutation 3, while sequencing from RNA showed only one band corresponding to the mutant C (fig. 6). This phenomenon was confirmed using restriction endonuclease *Bso*FI, which cleaves at the site of mutation 3 (data not shown). Restriction-enzyme analysis also showed that the patient's mother, brother, and daughter were heterozygous for mutation 3, while his son and nephew were negative. Screening of 47 normal and 9 disease controls failed to show mutation 3.

In an effort to understand why the PFK-M gene on the second allele was not expressed, we amplified a PCR fragment containing a downstream portion of intron 1, which is believed to be the regulatory region for the expression of type B mRNA (Yamasaki et al. 1991), using primers 16 and 17 (fig. 1 and table 1). Sequencing of this fragment, however, showed no differences from normal.

A polymorphism had been reported by Sharma et al. (1989) at nucleotide 516 in codon 172, consisting of a T or a C. In our studies, we found that all three patients as well as our control had a C.

Discussion

We have identified three novel mutations in four patients with PFK-M deficiency from three non-Ashkenazi Italian families. Patient 1 was homozygous for an A-to-C substitution at the penultimate nucleotide of intron 6 (mutation 1). Every intron has a consensus sequence AG at the 3′ end, a sequence that is thought to be essential for RNA splicing (Breathnach and Chambon 1981; Mount 1982). The AG was changed to CG by mutation 1, and, as a result, two cryptic splice sites in exon 7, TAAG/ATCA and ACAG/ATGA, were activated instead, generating two mRNAs, one with a 5-bp deletion and the other with a 12-bp deletion (fig. 4). Of the two mRNA species, the species with the 12-bp deletion was more prevalent (fig. 4*A*). In addition to these two abnormal mRNAs, analysis of the PCR product on a polyacrylamide gel showed a small amount of normal mRNA (fig. 4*A*). Many mutations in the consensus sequence AG at the 3′ end of introns have been reported in human diseases, causing exon skipping or activation of cryptic splice sites (Krawczak et al. 1992). The finding, in patient 1, of some normally spliced fragment is surprising because the AG is 100% conserved (Breathnach and Chambon 1981; Mount 1982) and was destroyed by the mutation. This finding may be explained by the high sensitivity of "nested" PCR. While the 5-bp deletion leads to a frameshift with premature termination of transcription, the more common 12-bp deletion leads only to an in-frame deletion of four amino acids in the peptide, and there is no evidence that these residues are located in active sites (Poorman et al. 1984). Still, we believe that mutation 1 is responsible for PFK-M deficiency, because (1) we failed to detect any other nucleotide difference in the coding region of the PFK-M cDNA of patient 1; (2) an affected brother of patient 1 was also homozygous for the same mutation, and both parents were heterozygous; and (3) 47 normal and 9 disease controls did not have the mutation.

Patient 2 was homozygous for a G-to-C substitution (mutation 2), changing an encoded arginine (CGA) to proline (CCA) at codon 39 in exon 4. This arginine residue was considered to be an ATP-binding site in a

crystallographic study of PFK from *Bacillus stearothermophilus* (Evans et al. 1981; Poorman et al. 1984). It is interesting that one of the mutations previously reported in Ashkenazi Jewish patients (Raben et al. 1993*b*) was a G-to-T substitution at the same nucleotide, but rather than changing the encoded arginine to proline, the mutation changed it to leucine (CTA). This coincidence may suggest that the arginine residue is important for enzyme activity.

Patient 3 was heterozygous for an A-to-C substitution (mutation 3), changing an encoded aspartate (GAC) to alanine (GCC) at codon 543 in exon 18. The aspartate residue does not appear to be located at an active site (Poorman et al. 1984).

The two residues changed by mutations 2 and 3 are, however, conserved in two subunits and in several species, including *B. stearothermophilus* PFK (Poorman et al. 1984); rabbit PFK-M (Lee et al. 1987); and human (Levanon et al. 1989), rat (Gehnrich et al. 1988), and mouse PFK-L (Hotta et al. 1991). Since only the 3′-half sequence of the human PFK-P cDNA has been reported (Simpson and Fothergill-Gilmore 1991), the presence of an arginine residue at codon 39 is hypothetical, but the aspartate residue at codon 543 is also conserved in this subunit. Moreover, the following reasons suggest that mutations 2 and 3 are pathogenic: (1) there was no other nucleotide difference in the sequence of the entire coding region of the PFK-M cDNA and in the regulatory region from either patient; and (2) 47 normal and 9 disease controls had neither mutation 2 nor mutation 3.

Because the PFK-M gene on the other allele of patient 3 was not expressed (fig. 6), we looked for a mutation in the regulatory region of the PFK-M gene of this patient. A downstream sequence of intron 1 has been shown to act as a positive regulator for type B mRNA (Yamasaki et al. 1991). We amplified and sequenced this region but found no difference from a control. There may be a mutation in another still-unknown positive regulatory element farther upstream from the region that we studied. It is also possible that a nonsense mutation, not detected by our method, may reduce mRNA level from the other allele, as reported in several human diseases (Mashima et al. 1992; Belgrader et al. 1993).

While PFK-M deficiency is a rare inherited disorder, several pathogenic mutations have been reported (table 2), especially in Ashkenazi Jews (Raben et al. 1993*a*, 1993*b*), who represent most of the U.S. patients. No mutations have been described in other populations, except for a splice-junction mutation in a Japanese family (Nakajima et al. 1990*b*). In three non-Ashkenazi Italian families, we found three different mutations, suggesting a high degree of genetic heterogeneity, similar to what has been reported in myophosphorylase deficiency (McArdle disease) (Tsujino et al. 1993, 1994). Although the number of non-Ashkenazi patients studied is relatively small, none of them had the common splice-junction mutation found in Ashkenazi Jewish patients (Raben et al. 1993*a*, 1993*b*), a lack that may explain why PFK-M deficiency appears to be less common in non-Ashkenazi populations. Examples of clinical heterogeneity have been reported in PFK-M deficiency, including a fatal infantile form and a late-onset form (Servidei et al. 1986; Vora et al. 1987), but the relation between molecular genetic lesions and clinical phenotypes is not yet established. Molecular genetic studies in patients with clinical variants may provide further evidence of genetic heterogeneity.

Acknowledgments

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