Am. J. Hum. Genet. 59:59–65, 1996

Glycogenosis Type VII (Tarui Disease) in a Swedish Family: Two Novel Mutations in Muscle Phosphofructokinase Gene (PFK-M) Resulting in Intron Retentions

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Summary

Phosphofructokinase (PFK) plays a major role in glycolysis. Human PFK is composed of three isoenzyme subunits (muscle [M], liver [L], and platelet [P]), which are encoded by different genes. Deficiency of muscle isoenzyme (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, Italian, French Canadian, and Swiss patients. We describe the genetic defect in a Swedish family with affected individuals in two generations. The patients are compound heterozygotes: two different mutations result in retention of intron 13 or intron 16 sequences into mRNA. A G1127A transition destroys the 5′ donor site of intron 13, resulting in a 155-nt retention of the intronic sequence. An a-to-g base change in intron 16 creates a new acceptor splice site, resulting in a 63-nt retention of intronic sequence. Both mutations are predicted to result in premature termination of translation. Some of the transcripts generated from the intron 16 mutated allele also contain intron 10 sequence unspliced.

Introduction

Phosphofructokinase (ATP: fructose-6-phosphate-1-phosphotransferase, EC.2.7.1.11; PFK) catalyzes a major rate-limiting step of glycolysis, phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate. Human PFK is composed of three isoenzymes, muscle (M), liver (L), and platelet (P), which are encoded by different genes on chromosomes 1, 21, and 10, respectively (Vora et al. 1982, 1983; Van Keuren et al. 1986). Mammalian PFK is a tetrameric enzyme subject to allosteric regulation (Dunaway 1983; Kemp and Foe 1983; Dunaway and Kasten 1987). The 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 cells; erythrocytes contain a hybrid of L and M subunits.

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). Three types of alternatively spliced mRNA are transcribed from the gene under the control of two different promoters and tissue-specific transcription factors (Nakajima et al. 1990*a*, 1990*c*, 1995). These transcripts share the sequence of the coding region but diverge at the 5′ UTR. The skipping of exon 11 is another described alternative splicing event that takes place in muscle and other tissues (Sharma et al. 1990).

An inherited deficiency of muscle PFK, glycogenosis type VII (Tarui et al. 1965; Layzer et al. 1967), results in a block in muscle glycolysis and glycogenolysis, leading to exercise intolerance, cramps, myoglobinuria, and hemolysis (Rowland 1986; Tarui 1995). The observed clinical symptoms reflect almost complete lack of muscle PFK activity and partial reduction of the enzyme in erythrocytes. The hemolysis is compensated since the muscle type contributes 50% of the total erythrocyte activity.

Several mutations have been identified in patients with PFK deficiency: splicing defects, a nucleotide deletion, point mutations, and a nonsense mutation (Nakajima et al. 1990*b*; Raben et al. 1993; Hamaguchi et al. 1994; Sherman et al. 1994; Tsujino et al. 1994; Vasconcelos et al. 1995; Raben et al. 1995*a*). We now describe two novel mutations in the PFK-M gene in a Swedish family with affected individuals in two generations. Despite considerable consanguinity in the family, the proband is a genetic compound.

Subjects, Material, and Methods

Subjects

The extended genealogical tree of a Swedish family who originates from the county of Vasterbotten in

Received November 3, 1995; accepted for publication April 10, 1996.

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0002-9297/96/5901-0010$02.00

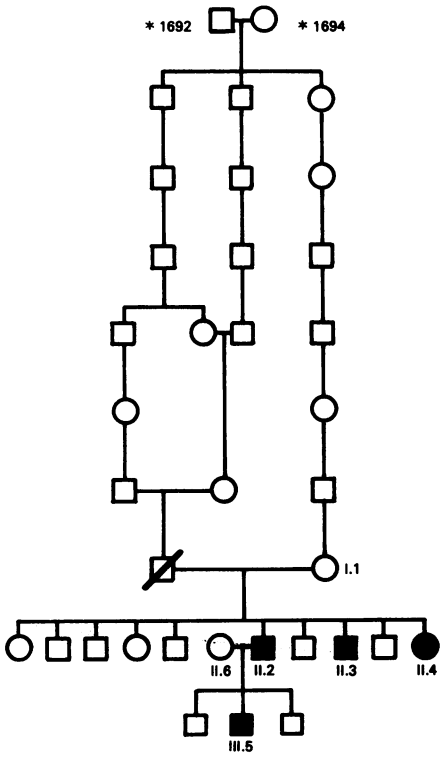

Figure 1 Complete pedigree of the Swedish family with PFK-M deficiency.

northern Sweden, with common ancestors dating to the 17th century, is presented in figure 1. We have studied a three-generation pedigree that included the unaffected grandmother (I.1), three affected individuals in the second generation (II.2, II.3, and II.4), and a 16-year-old grandson (III.5) who has been recently diagnosed with PFK-M deficiency. His parents (II.2 and II.6) reported no history of consanguinity.

The proband (II.3), a 39-year-old male, had a childhood history of exercise intolerance, nausea, vomiting, and muscle cramps after vigorous exercise. As he grew older he developed pronounced muscle weakness and gradually adopted a sedentary lifestyle. Laboratory data show increased reticulocytes and bilirubin levels. A 46-year-old brother (II.2) and a 34-year-old sister (II.4) have also suffered from limited tolerance for exercise, fatigability, and muscle pain after prolonged or vigorous exercise, since childhood. Both avoid any sports activity but feel well adjusted to everyday life.

The detailed clinical and laboratory data concerning this family are described (Rudolphi et al. 1995). The diagnosis in each case was confirmed by a muscle biopsy, which showed no detectable PFK activity. The patients and family members provided informed consent for genetic evaluation.

Genomic DNA Amplification

Genomic DNA was isolated from peripheral blood as described elsewhere (Higuchi 1989). The primers for PCR were located in the introns and encompassed each of the 24 exons and splice junctions of the PFK-M gene; one primer of each pair contained a 5′ 40-bp GC-rich sequence (GC-clamp) attached to the specific sequence. The sequences of the intronic primers are available on request. Genomic DNA was amplified at 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. Genomic amplification of the intron 10 and intron 16 regions were performed with primer pairs located in the adjacent exons (5′-ccaatcacctcagaagacatca/5′-gggtgtcatatcccagacgctt for intron 10 and 5′-tggactggccaaggtggct/5′-agttatattggcactgatctgt for intron 16).

Denaturing Gradient-Gel Electrophoresis (DGGE)

The amplified genomic fragments (~200 ng DNA) were electrophoresed on a 6.5% polyacrylamide gel containing a linearly increasing gradient of denaturant (100% denaturant = 7 M urea, 40% formamide) parallel to the direction of the electrophoresis, as described elsewhere (Myers et al. 1987). The gels were run at 80 V for 16 h at 60°C and stained with ethidium bromide to visualize the bands.

RNA PCR Amplification

Total RNA was extracted from frozen muscle biopsy specimens as described (Chomczynski and Saachi 1987). RNA was reverse-transcribed into cDNA with oligo(dT) primer and reverse transcriptase from avian myeloblastosis virus (Boehringer Mannheim) according to the manufacturer's instructions. The region of exon 13/exon 14 was amplified (primer pairs 5′-aggctgtgcgcctgccccctcat/5′-acagcagcattcatgcctgca) at 95°C for 1 min, 55°C for 1 min, and 72°C for 1 min (35 cycles). To identify a mutation on the second allele in which exon 13 is correctly spliced to exon 14, we used allele-specific primers to amplify two fragments encompassing the 2.4-kb coding region of the PFK-M gene (fragment I [bp −66 to +1145] 5′-gccgttcctttagctagtggcatcttg/5′-cagttgttcatgaagctccggcctc; fragment II [bp +1108 to +2381] 5′-cctgaagctgagaggccggagcttc/5′-catgatcaggtaatctattcccct). The antisense primer for fragment I and the sense primer for fragment II span the PFK-M exon 13/14 junction, and therefore they would not amplify the transcript with intron 13 retained. Amplifications were performed as described above, except that the extension time was increased to 2 min 30 s.

Direct Sequence Analysis

Amplified cDNA fragments and genomic PCR products that showed altered mobility on DGGE were directly sequenced (Nichols and Raben 1994). The sequencing reactions were performed with Sequenase 2.0 according to the manufacturer's directions (U.S. Biochemical).

Allele-Specific Oligonucleotide Hybridization

Amplified genomic DNA (~100–200 ng) encompassing the mutation site in intron 16 was applied in duplicate to a Nytran membrane using a slot-blot apparatus. Blots were probed with labeled oligonucleotides specific for either mutant (ttctcccccctcagttttcctgtctctt) or the wild-type sequence (ttctcccccctcaattttcctgtctctt) of intron 16. 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.

Results

To screen for mutations in the PFK-M gene, all 24 exons and splice junctions (including 10–15 nt of the intronic sequences) were amplified from genomic DNA, and the products were resolved on DGGE gels with 25%–75% or 40%–80% denaturant. The products with altered mobility were directly sequenced.

Identification of Exon 13 Mutation

DGGE analysis of patient II.2 revealed polymorphisms in exon 7 and exon 13. Exon 7 contained a previously reported polymorphism, a silent C516T base change (reviewed in Raben et al. 1995*b*). Exon 13 contained a G-to-A transition at the last nucleotide of the exon (position 1127). Figure 2*A* shows both a G and an A at the exon/intron 13 border, indicating that the patient is heterozygous for this mutation. The base change creates a new recognition sequence for *Bsr*I restriction endonuclease, and digestion of the PCR-amplified genomic DNA from family members (fig. 2*B*) shows that all affected individuals in two generations are heterozygous for the mutation. The unaffected grandmother has normal sequence at this site, indicating that patients II.2, II.3, and II.4 inherited the mutation from their father (the DNA was not available). The grandson (III.5) inherited the mutation from his father (II.2), since the mother (II.6) has normal sequence at this part of the gene.

To determine the effect of the mutation, we amplified the exon 13/exon 14 region from the cDNA with flanking primers. Two PCR products were found: the smaller fragment was identical to the published sequence, and the larger had a partial insertion (155 nt) of the intron 13 sequence (fig. 3*A*). Because the 5′ splice donor site of intron 13 is destroyed by the mutation, a cryptic site with a high degree of homology to the consensus sequence (fig. 3*C*) is activated within the intron. The insertion leads to an almost immediate termination codon (fig. 3*B*).

Intron 16 Mutation

The second allele was amplified in two overlapping fragments with allele-specific primers (see Subjects, Material, and Methods). PCR amplification of fragment I (bp −66–1145) yielded two products: a normal-size product and a larger one. Sequence analysis showed that both products were derived from the same allele, since they both contained an allele-specific T nucleotide at position 516. The larger product contained the entire intron 10 (252 bp) sequence (not shown) that introduces a premature termination codon. Intron 10 sequence and the splice junctions of exons 10 and 11 in the patient and in the normal control were identical.

Sequence analysis of fragment II (bp 1108–2381) revealed a single product with a 63-nt insertion at the junction of exons 16 and 17 (fig. 4). The insertion proved to be the 3′ part of intron 16 that when translated would result in an in-frame addition of 17 amino acids and premature termination. Amplification and sequence analysis of the patient's genomic DNA showed an A-to-G transition in intron 16 at −64 (fig. 4*B*). The same heterozygous mutation was identified in the affected sibs (II.3 and II.4) by sequence analysis (not shown). This mutation creates an AG dinucleotide in the intron that serves as an acceptor splice site. We have

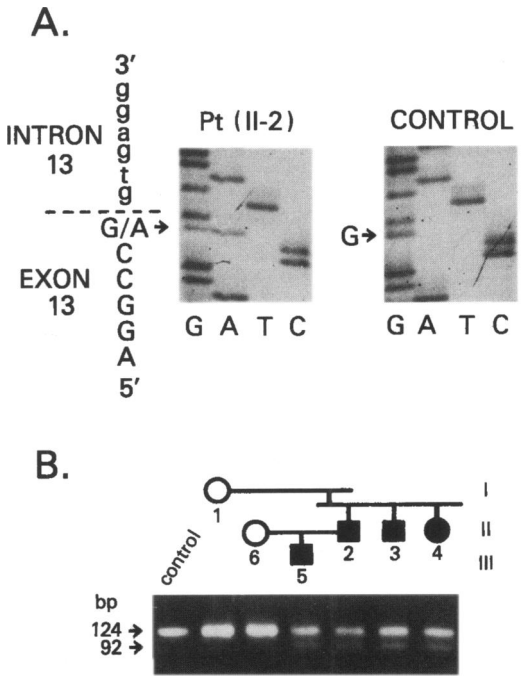

Figure 2 Exon 13 mutation. *A*, Identification of a G 1127 A transition in exon 13. Direct sequencing of the PCR amplified genomic DNA spanning the exon 13/intron 13 region showed both a G and an A at nucleotide position 1127. The patient is heterozygous for a nucleotide substitution that could destroy the 5′ splice site of intron 13. *B*, Use of restriction enzyme digestion to confirm the presence of exon 13 mutation in affected family members. The mutation creates a new *Bsr*I recognition sequence, yielding 92-bp and 34-bp products (the smaller product is not seen, because of its size). As predicted, all affected family members are heterozygous for this defect.

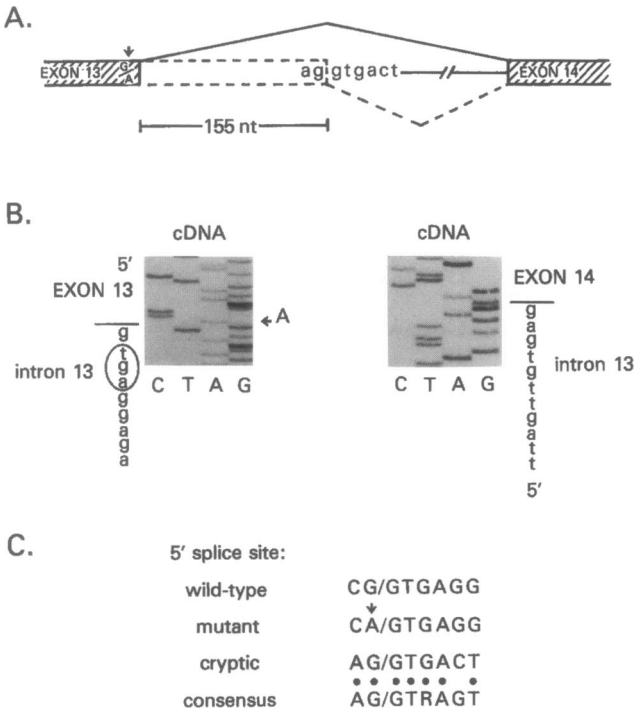

Figure 3 Alternative splicing created by exon 13 mutation. *A*, Schematic representation of normal splicing of exon 13 and exon 14 (*solid line*) and of aberrant splicing (*dashed line*) caused by the mutation at position 1127 in the PFK-M gene. Hatched box shows the 155-nt insertion into mRNA that occurs as a result of the alternative splicing. The arrow indicates the location of the G-to-A transition at the last nucleotide of exon 13. *B*, cDNA sequences at the 5′ (*left*) and the 3′ ends of the retained intron (*right*). *C*, Table showing that the new cryptic donor site within intron 13 has a high degree of homology to the consensus sequence with seven of eight identical nucleotides.

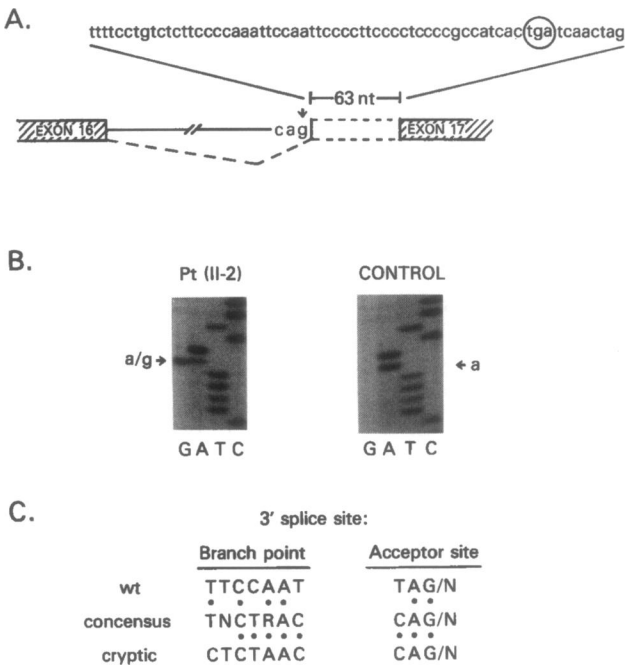

Figure 4 Alternative splicing created by intron 16 mutation. *A*, Schematic representation of aberrant splicing caused by the mutation in intron 16 at position −64. Dashed box shows the 63-nt insertion into mRNA that occurs as a result of the alternative splicing. The arrow indicates location of the a-to-g base change in the intron 16 of the PFK-M gene. *B*, Identification of an a-to-g mutation in intron 16 in the patient. Direct sequencing of the PCR-amplified genomic DNA showed that the patient is heterozygous for the base change. The mutation creates a new cryptic acceptor site within the intron and causes aberrant splicing. *C*, Table showing that the cryptic acceptor site in the intron 16 has a higher degree of homology to the consensus sequence than does the normal acceptor site.

not found any normally spliced mRNA generated from the second allele. The cryptic site is highly homologous to the consensus sequence at both the acceptor site and the branch point (fig. 4*C*).

Despite extensive consanguinity in the family the proband and two affected sibs are compound heterozygotes with two mutations, one that obliterates the 5′ donor site of the exon 13 and the other that creates a new acceptor site in the intron 16. The mutations result in insertion of intronic sequences into mRNA. Both mutations are predicted to result in premature termination of translation and thus account for the disease in affected individuals.

Allele-specific oligonucleotide hybridization showed that the unaffected grandmother (I.1) is heterozygous for the intron 16 mutation, since the DNA hybridized to both mutant (G) and wild-type (A) specific probes (fig. 5). DNA from 24 normal control individuals (17 were of the same ethnic background) as well as an unrelated PFK patient hybridized to the wild-type probe (seven controls are shown in fig. 5). Unexpectedly, both

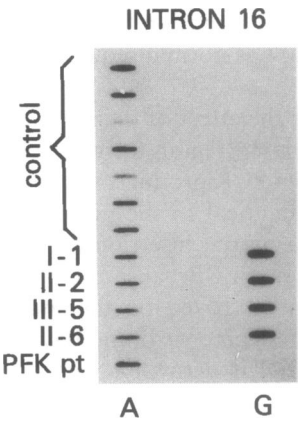

Figure 5 Detection of mutated and normal intron 16 sequence by allele-specific oligonucleotide hybridization. PCR-amplified products encompassing intron 16 were subjected to slot-blot analysis (in duplicate) with labeled probes that specifically recognize either the normal (A) or mutated (G) sequence.

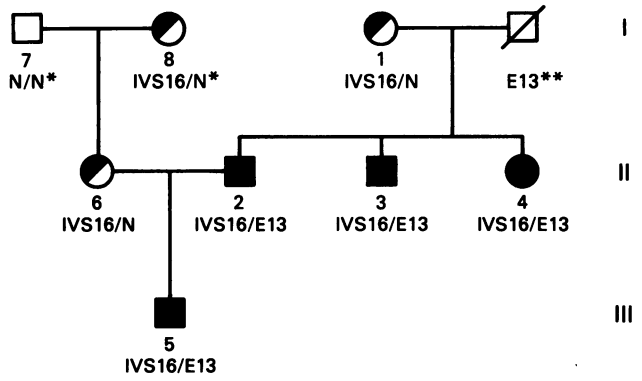

Figure 6 Genotype of the tested members of the family. Two additional individuals, I.7 and I.8, were screened for the intron 16 mutation to establish the inheritance of the defect in II.6. The data strongly indicate that I.8 and I.1 are related. IVS = intervening sequence; E = exon; one asterisk (*) = not tested; and two asterisks (**) = DNA not available.

the affected grandson (III.5) and his mother (II.6) were also heterozygous for the intron 16 mutation (fig. 5).

Thus, the affected individual in the third generation (III.5) and his father (II.2) share the same genotype: a G-to-A change in exon 13 on one allele and an A-to-G change in intron 16. The data indicated that there is an additional level of consanguinity in the family. To confirm the presence of the intron 16 mutation in II.6, we screened her parents (I.7 and I.8, fig. 6) by allele-specific hybridization and demonstrated that the mutation in II.6 is maternally inherited. The genotype of all tested family members is shown in figure 6.

Discussion

Several pathogenic mutations have been identified in patients with PFK deficiency, establishing the genetic heterogeneity of the disease. Thirteen different mutations have been reported to be associated with the disease in patients from five ethnic backgrounds (reviewed in Raben et al. 1995*b*; Vasconcelos et al. 1995). Most of the mutations have been identified in individual patients; only two defects (exon 5 skip and a nucleotide deletion in exon 22) are shared by several unrelated Ashkenazi patients (Raben et al. 1995*c*). We now report two novel mutations, both resulting in aberrant splicing and premature termination of translation. These include a base change at the last nucleotide of exon 13 (G1127A) resulting in partial intron 13 retention and a base change in intron 16 (G-64A) resulting in partial intron 16 retention. The two mutations we identified in a Swedish family result in major abnormalities, and no full-length product is expected to be translated from either of the alleles.

The exon 13 mutation destroys the 5′ splice donor site, and a cryptic site 155 bp downstream from the exon/intron 13 junction is used. Exon skipping is the most frequent phenotype associated with mutations at the 5′ splice site; intron retention is a rarely observed splicing pattern (Nakai and Sakamoto 1994). A mutation in patients with hereditary tyrosinemia type 1 is another example of aberrant splicing with partial intron retention caused by the mutation in the last nucleotide of exon 2 of the fumarylacetoacetase gene (Rootwelt et al. 1994).

The intron 16 mutation leads to the creation of an active 3′ acceptor splice site in intron 16 and to the insertion of 63 nt of intronic sequence into PFK-M transcripts. Normally spliced transcripts were not detected from this allele, even though the authentic acceptor splice site was not altered. Because the newly created 3′ splice site sequence (cag), in combination with its branch point sequence (ctctaac), more closely matches the consensus sequence than that of the authentic site, it appears that the authentic site is never used.

Some of the transcripts generated from the intron 16 mutated allele have intron 10 retained into mRNA. Sequence analysis of the intron, adjacent exons, and the splice sites did not reveal any abnormalities in this region. The same type of transcripts has been recently identified in another patient (of Ashkenazi origin) with PFK-M deficiency but not in normal controls (Vasconcelos et al. 1995). In both cases, intron 10–retained species arose on transcripts with premature termination, which may reflect a slow pre-mRNA processing of unstable transcripts. It is interesting that an alternatively spliced PFK-M transcript lacking the neighboring exon 11 sequence has been identified in multiple tissues in normal individuals (Sharma et al. 1990), suggesting a relative weakness of the splicing signals in this region of the gene.

All patients with described mutations, including the family we have studied, have classical symptoms of the disease: exercise intolerance, cramps, and myoglobinuria. However, the severity of the disease varies significantly, ranging from mild symptoms of easy fatigability to a progressive fixed muscle weakness. The genetic heterogeneity does not so far explain the clinical variability that characterizes PFK-M deficiency. Some of the previously identified mutations are located in evolutionarily conserved regions and affect the putative catalytic site or ADP/AMP and fructose 1,6-bisphosphate-binding sites, while others are not. The residual enzyme activity that was found in some patients but not the others also fails to correlate with the phenotypic variations of the disease.

The family we have studied is an excellent example of phenotypic diversity observed in association with the same mutation within a family. The three sibs (II.2, II.3, and II.4) with identical genotype exhibit different clini-

cal phenotypes. The proband (II.3) is much more severely affected by muscle weakness and by the hemolytic process than are his brother and sister. Since PFK deficiency is considered to be a single-gene disorder, the question remains whether the mutations are the sole determinants of the phenotype. The clinical variability of the disease indicates the impact of background genes and/or external factors on the presentation of the PFK deficiency.

An unusual feature of this family is the fact that the individuals in two generation are affected, which may give an impression of an autosomal dominant inheritance of the disease. A "pseudo-dominant" pattern of inheritance has been described with PFK deficiency in an inbred Ashkenazi family with a single mutation in the PFK-M gene (Vasconcelos et al. 1995). In the family reported here, by contrast, the "pseudo-dominant" pattern in the inbred community actually reflects the preservation of two rare alleles. It is interesting to note, therefore, that this community is located just across the Baltic Sea from the heart of the Jewish Pale of Settlement, the center for centuries of the Ashkenazi Jewish community, in which a substantial number of the world's cases of PFK deficiency have been described. It is possible that forces that have operated to preserve several mutations in lysosomal enzymes at anomalously high levels among Ashkenazi Jews (Diamond 1994) actually operate in Sweden as well, and are not limited to lysosomal mutations.

Acknowledgments

We are grateful to Dr. Peter Nilsson (Department of Clinical Chemistry, Umea University Hospital, Sweden) for providing the patient's DNA, Prof. Lars-Eric Thornell (Department of Anatomy, Umea University, Sweden) for determining PFK activity in muscle biopsies, and Mrs. Thea Halleberg for her excellent genealogical research.

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