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Mutations in the Liver Glycogen Phosphorylase Gene (*PYGL*) Underlying Glycogenosis Type VI (Hers Disease)

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Summary

Deficiency of glycogen phosphorylase in the liver gives rise to glycogen-storage disease type VI (Hers disease; MIM 232700). We report the identification of the first mutations in *PYGL,* the gene encoding the liver isoform of glycogen phosphorylase, in three patients with Hers disease. These are two splice-site mutations and two missense mutations. A mutation of the 5′ splice-site consensus of intron 14 causes the retention of intron 14 and the utilization of two illegitimate 5′ splice sites, whereas a mutation of the 3′ splice-site consensus of intron 4 causes the skipping of exon 5. Two missense mutations, N338S and N376K, both cause nonconservative replacements of amino acids that are absolutely conserved even in yeast and bacterial phosphorylases. We also report corrections of the *PYGL* coding sequence, sequence polymorphisms, and a partial *PYGL* gene structure with introns in the same positions as in *PYGM,* the gene of the muscle isoform of phosphorylase. Our findings demonstrate that *PYGL* mutations cause Hers disease, and they may improve laboratory diagnosis of deficiencies of the liver phosphorylase system.

Introduction

Glycogen phosphorylase catalyzes the degradation of glycogen to glucose-1-phosphate by the phosphorylytic cleavage of α-1,4-glycosidic bonds. In concert with debranching enzyme, phosphorylase is crucial for the utilization of glycogen as a storage form of glucose in virtually all animal cells. Glycogen is most abundant in the liver, where it serves as a buffer reservoir of glucose units for the maintenance of constant blood-glucose levels, and in muscle, where it can be rapidly broken down to provide energy for contraction. Phosphorylase activity is regulated by various allosteric ligands and through phosphorylation by phosphorylase kinase, which, in turn, is controlled by neural and hormonal signals. Three phosphorylase isoforms are known—the muscle (M), liver (L) and brain (B) isoforms. They are encoded by separate genes—*PYGM, PYGL,* and *PYGB,* respectively—which have been mapped to chromosomes 11, 14, and 20, respectively. The existence of isoforms with distinct regulatory properties probably reflects the different functional roles and modes of regulation of glycogen metabolism in different cell types (Crerar et al. 1995, and references therein).

Heritable deficiencies of phosphorylase cause glycogen-storage disease (Chen and Burchell 1995). A defect of the muscle isoform underlies glycogenosis type V (McArdle disease), which, in most cases, is characterized by exercise intolerance (early fatigue, pain, and cramps on exertion) and juvenile to adult onset of symptoms. Numerous mutations in *PYGM* have been identified in recent years (Bartram et al. 1995; Vorgerd et al. 1998; also see references in the latter). Phosphorylase deficiency in the liver (glycogenosis type VI; Hers disease [Hers 1959]; MIM 232700) manifests in infants, primarily with hepatomegaly and growth retardation. It typically takes a benign course, with remission of symptoms as the children grow up. No mutations underlying liver phosphorylase deficiency have been reported.

Of the three isoforms of glycogen phosphorylase, expression of the L isoform seems to predominate in the liver, both as protein and as mRNA (David and Crerar 1986; Newgard et al. 1988). Therefore, mutations in *PYGL* would be suspected to be responsible for Hers disease. Since liver phosphorylase deficiency can be difficult to diagnose biochemically and, in particular, difficult to differentiate from the much more common deficiency of the activating enzyme, phosphorylase kinase, mutation analysis may aid in the laboratory diagnosis

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of deficiencies of the liver phosphorylase system. The present study identifies, for the first time, mutations in *PYGL* in patients with liver phosphorylase deficiency.

Subjects, Material, and Methods

Patient 1 (BA.B), now 4 years 6 mo old, is the son of Israeli Arab Bedouin parents who are first cousins. The parents, four sisters, and two brothers are healthy. The patient presented, at age 2 years, with hepatomegaly and growth retardation (3d percentile) but had no clinical history of fasting hypoglycemia. Serum transaminases, triglycerides, and cholesterol were moderately elevated. Total leukocyte phosphorylase activity was 12.5% of that in a normal control, and phosphorylase *a* activity was 11% of that in the control.

Patient 2 (SC.A) is the son of unrelated, healthy parents of Suriname Hindustani ethnic background. He has no siblings. Now 5 years 9 mo old, he presented, at age 2 years, with hepatomegaly and severe growth retardation. Transaminases were intermittently elevated, whereas plasma lipids were normal. Hypoglycemia was measured after an 8-h fast. Severe glycogen storage in hepatocytes was observed by both light microscopy and electron microscopy. In a liver biopsy, moderately decreased activities were determined for total phosphorylase (13.5 μmol/min/g protein [control range 24–44 μmol/min/g protein]) and phosphorylase *a* (6 μmol/min/g protein [control range 15–30 μmol/min/g protein]). In erythrocytes, both activities were not significantly decreased. Phosphorylase kinase was normal, both in erythrocytes and in liver.

Patient 3 (TS.M), now 3 years 4 mo old, is the daughter of consanguineous Turkish parents. At age 1 year she presented with hepatomegaly. Body length was at the 50th percentile, and weight was at the 10th percentile. Transaminases, triglycerides, and cholesterol were elevated. In a liver biopsy, glycogen was strongly elevated (15% [normal range 2%–6%]), whereas borderline low values were determined for total phosphorylase (22 μmol/min/g protein [normal range 20–110 μmol/min/g protein]) and phosphorylase *a* (9 μmol/min/g protein [normal range 10–50 μmol/min/g protein]). Liver phosphorylase kinase was also low (1 μmol/min/g protein [normal 3–30 μmol/min/g protein]). Total phosphorylase and phosphorylase *a* in leukocytes were in the normal range, as were glycogen and phosphorylase kinase in erythrocytes.

Total RNA and genomic DNA were purified from frozen whole blood by conventional methods, and amplification and direct sequencing were performed as described in detail elsewhere (Burwinkel et al. 1996). For PCR between different exons of *PYGL,* the long-template PCR system of Boehringer Mannheim was employed. Glycogen phosphorylase activity was determined according to the method of Lederer et al. (1975) and Lederer and Stalmans (1976).

Results

Mutations in PYGL*, in Three Patients*

Since the structure of *PYGL* has not been determined, we based our mutation search on the amplification of the *PYGL* coding sequence (Newgard et al. 1986), in four overlapping intervals (A–D), by reverse transcriptase–PCR (RT-PCR), from RNA isolated from whole blood. PCR products were analyzed by direct sequencing, which employed the amplification primers and additional sequencing primers (table 1).

Patient 1 is the son of consanguineous parents and was therefore suspected to be homozygous for a single mutation. RT-PCR of RNA from this patient yielded an aberrant pattern of three bands, instead of one, in interval D (fig. 1). Sequencing showed that the main, longer-than-normal PCR product contains an insert of 119 nucleotides (nt) in codon R589, resulting in a frameshift and introducing a stop codon after five missense codons. The minor band at the bottom represents a deletion of 67 nt that reaches from codon V567 to R589 and that also causes a frameshift (fig. 2). The band in the middle represents the heteroduplex of these two products, plus a third sequence with a 36-nt insertion, in codon R589, containing an in-frame stop codon. In *PYGM,* there is a short intron—intron 14—of 265 nt, in the position corresponding to codon R589 (Burke et al. 1987). We suspected that the exon-intron architecture of *PYGL* is similar and could amplify, with primers LP7 and LP11 (table 1 and fig. 3)—which, by analogy with *PYGM,* are predicted to lie in exons 14 and 15, respectively—the corresponding gene segments from genomic DNA of the patient and a normal control. Sequencing showed that the patient's insert is indeed an intron, presumably intron 14, but with a G→A replacement in the GT consensus dinucleotide of the 5′ splice site (figs. 2 and 3). This splice-site mutation thus leads to the retention of intron 14 (the main product, the top band) and two aberrant splice products employing neighboring GT dinucleotides (doubly underlined in fig. 3) in exon 14 and in intron 14, respectively, as illegitimate 5′ splice sites. Both parents were confirmed to be heterozygous for the mutation.

RT-PCR of RNA of patient 2, who had no known parental consanguinity, produced an aberrant band pattern in interval B, displaying a normal-sized top band and two additional bands below (fig. 1). The top band represents the full-length sequence, whereas the bottom band results from an in-frame deletion of 132 nt (44

Table 1

Primers for Amplification and Analysis of the *PYGL* Coding Sequence

PCR Interval	Primer	Position (Direction)	Sequence
RT-PCR:			
A	LP1	−51 (→)	5′-CCG CAC TTC CAG CTC TCT G-3′
A	LP2	559 (←)	5′-CAT ATC TGA GCC AAT CAT CTG-3′
B	LP3	476 (→)	5′-GCA TTC GGT ATG AAT ATG GGA-3′
B	LP4	1124 (←)	5′-TAG GCG AAG GTC TTC TGG TT-3′
C	LP5	1038 (→)	5′-GAT CCC TGA GCT GAT GAG G-3′
C	LP6	1704 (←)	5′-CAC CTG GAC ATC AAA CAT GG-3′
D/E	LP7	1628 (→)	5′-AGC TGA AGT TTT CTC AGT TCC-3′
D	LP8	2592 (←)	5′-ATG TTC AAG TTC AGT AAG AAG C-3′
S	LP9	2109 (→)	5′-AGC TGG GGA AGA GAA CCT G-3′
S	LP10	2188 (←)	5′-TTG CCT CGT ACC CTT TCT TG-3′
Gene analysis:			
E (intron 14)	LP11	1825 (←)	5′-TAC CAC CAA TGA TAA CTG TCC-3′
F (intron 5)	LP12	741 (←)	5′-AGC AGA CCA GAG GCG CAT G-3′
F	LP13	594 (→)	5′-GCT GCC TGT GCA CTT CTA TG-3′
G (intron 4)	LP14	660 (←)	5′-TTG AGT GTC AAT CCA CTT GG-3′
G	LP15	456 (→)	5′-ACT TGC AGC CTA TGG ATA CG-3′
S	LPi1	Exon 5–116 (→)	5′-CAG TAT TCA TAA GTT GTT TCA G-3′

codons) between codons Q175 and V220 (fig. 2); the middle band is a heteroduplex of the top and bottom bands. The deletion corresponds to exon 5 of *PYGM*. Therefore, we again suspected a splice-site mutation and could amplify the *PYGL* region between putative exons 4 and 6 from normal genomic DNA, although intron 4 proved to be >10,000 nt long (fig. 3), as opposed to only 327 nt in *PYGM*. We then amplified and sequenced the corresponding regions from the DNA of patient 2. His sequences around the 5′ end of intron 4 and both ends of intron 5 were normal, whereas he was heterozygous for a G→C substitution in the AG consensus of the 3′ splice site of intron 4, which causes the skipping of exon 5 (figs. 2 and 3). The only other sequence abnormalities in the *PYGL* coding sequence of patient 2 were a V221I missense mutation and a N338S missense mutation, both also in interval B. The sequence of the normal-sized RT-PCR product of interval B (fig. 1) is homogeneous for these two replacements, whereas the short RT-PCR product of interval B, lacking exon 5, has normal V221 and N338 codons (also see the legend to fig. 2). From this, we conclude that the splice-site mutation and the two missense mutations are on separate alleles, although parental samples were not available for analysis, and that together they account for the patient's enzyme deficiency. The finding that the full-length splice product represents exclusively the allele with the point mutations also indicates that the splice-site mutation on the other allele is fully abortive and allows no notable background of normal transcript splicing. Codon N338 is absolutely conserved in all three isoforms of glycogen phosphorylase and even in plant, yeast, and bacterial phosphorylases (fig. 2). The N338S mutation is therefore very likely the second disease mutation. The V221I replacement is a conservative substitution; valine 221 is conserved in mammalian and yeast phosphorylases but, in phosphorylases from slime mold, potato, and *Escherichia coli,* is replaced by isoleucine, leucine, or methionine (Hudson et al. 1993). This sequence variant may therefore be a polymorphism, and it is listed as such in table 2.

Patient 3 is the daughter of consanguineous parents. The only sequence abnormality in her entire *PYGL* coding sequence is an apparently homozygous N376K missense mutation. N376 is absolutely conserved in the

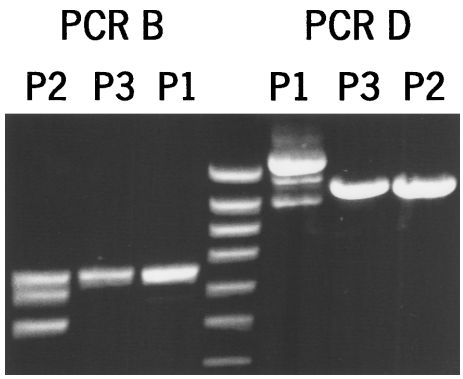

Figure 1 RT-PCR products indicating aberrant mRNA processing due to splice-site mutations in patients 1 and 2. Sequence intervals B and D were amplified from RNAs of patients 1–3, and the products were resolved by agarose gel electrophoresis and were stained with ethidium bromide. The central lane carries a 100-nt molecular-size ladder (*top band;* 1,000 nt).

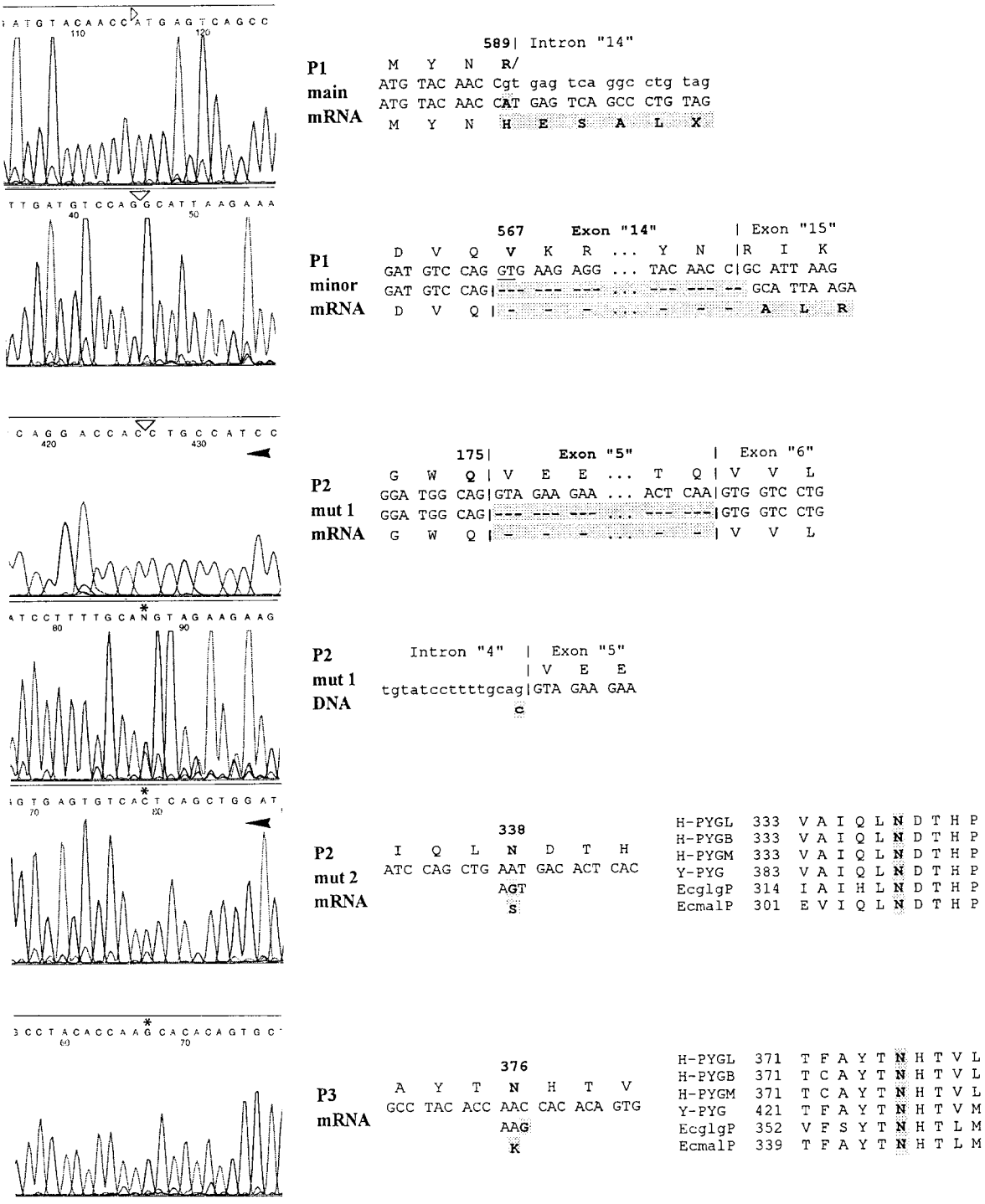

Figure 2 Identification of *PYGL* mutations in patients 1–3. The left column shows the sequencing electropherograms documenting the mutations. Triangles denote insertions or deletions; asterisks (*) denote nucleotide replacements; and arrowheads indicate where sequences have to be read in complementary fashion from right to left. On the right, normal and mutant sequences are shown in comparison, with mutant sequence positions highlighted in boldface type and shaded. Patients 1 and 3 are homozygous for their respective mutations. The two mutant alleles of patient 2 give homogeneous sequences if determined from RT-PCR products, because both lie in interval B, so that the RT-PCR products derived from the two alleles have different lengths and are separated by electrophoresis before being sequenced (fig. 1). Amplified from genomic DNA, the splice-site mutation (mut 1) of patient 2 is seen together with the normal nucleotide of the second allele in this position. Numbering of exons and introns is tentative, by analogy with the numbering of corresponding exons in *PYGM*. The GT dinucleotide in exon 14 (*underlined*) in patient 1 serves as an illegitimate 5′ splice site. The third aberrant-splicing product of patient 1, employing a GT in intron 14 (see fig. 3), is not illustrated. For the missense mutations of patients 2 and 3, high phylogenetic conservation of the mutated amino acids is illustrated by alignment of corresponding partial sequences from the three human glycogen phosphorylase isoforms, from yeast glycogen phosphorylase, and from both *E. coli* glycogen phosphorylase and maltodextrin phosphorylase. The sequences are taken from the work of Hudson et al. (1993), where an alignment with additional phosphorylase sequences from slime mold and potato (N338 and N376 being conserved also in these species) and an assignment of sequence positions to crystal structural features can also be found.

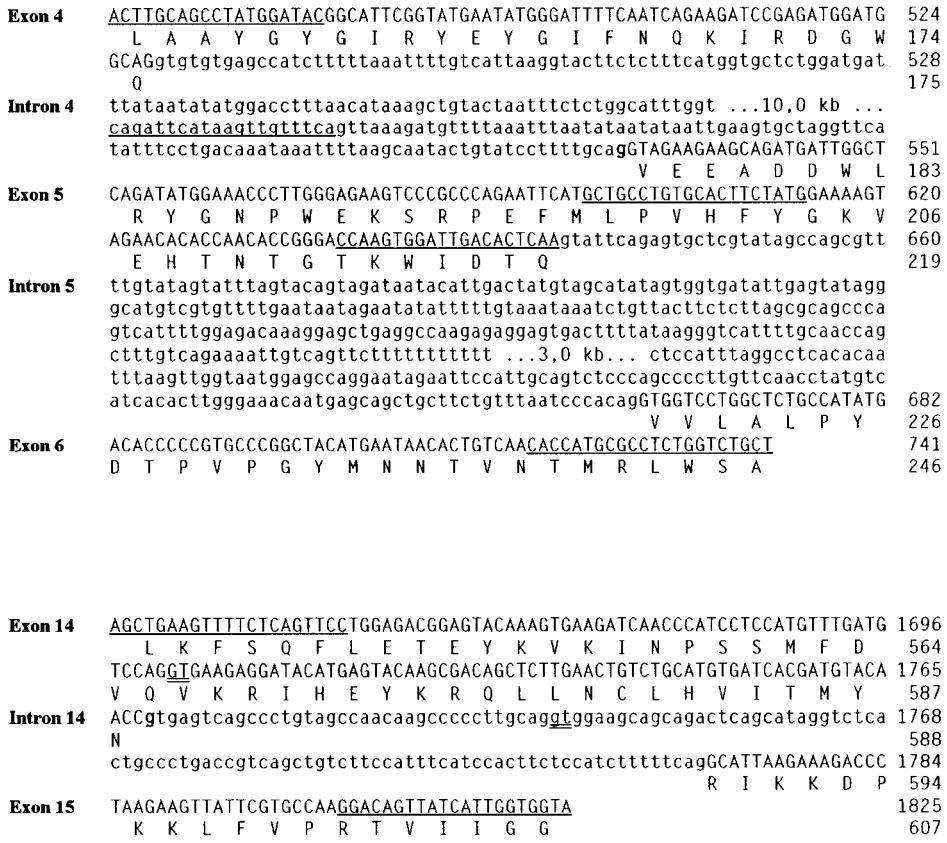

Figure 3 Partial structure of the human *PYGL*. Exons are denoted by uppercase letters, and introns are denoted by lowercase letters. Primers LP15, LPi1, LP13, LP14, LP12, LP7, and LP11 (*from top to bottom*), which were employed for amplification and sequencing, are underlined. The g residues that are mutated at the end of intron 4 and at the beginning of intron 14, in patients 2 and 1, respectively, are highlighted in boldface type. The GT dinucleotides in exon 14 and intron 14 that function as illegitimate *5′* splice sites in patient 1 are doubly underlined. Nucleotide numbering and amino acid numbering refer to the cDNA sequence. The sequences have been submitted to the database (accession numbers Y15229–Y15232).

three mammalian phosphorylase isoforms and also in plant, yeast, and bacterial phosphorylases and is therefore a convincing deficiency mutation (fig. 2).

Partial PYGL *Structure*

As described above, parts of the human *PYGL,* between exons 4 and 6 and between exons 14 and 15, could be amplified and sequenced directly from genomic DNA (fig. 3). Introns 4 and 14 are found in positions precisely corresponding to those of *PYGM*. Intron 5 is shifted upstream in phase, by 1 nt, in *PYGL,* relative to *PYGM*. Similarly precise conservation of intron positions but variation of intron lengths has been observed between the genes of other protein isoforms (e.g., see Aperlo et al. 1996; Burwinkel et al. 1998). These observations suggest that the rest of *PYGL* also will be accessible for mutation analysis in an analogous way.

Corrected PYGL *cDNA Sequence and Identification of Polymorphisms*

In all seven *PYGL* chromosomes analyzed by us, 13 codons of the *PYGL* coding sequence differed from the published cDNA sequence (table 2). Three nucleotide replacements are synonymous, but the others imply changes in the encoded amino acids. In most of these instances, the amino acids predicted by our sequence are identical or more similar to their counterparts in the M and B isoforms than are those in the published cDNA sequence (table 2). Therefore, we believe that the sequence determined by us is the correct one, and we have submitted it to the GenBank/EMBL sequence database (accession number Y15233). In four other codons, we have found single-nucleotide replacements between different chromosomes, three of them synonymous and one causing a conservative amino acid substitution, and we believe them to be polymorphisms (table 2).

Table 2

Sequence Corrections and Polymorphisms in the *PYGL* Coding Sequence

A. Amino Acid Replacements in All Seven Chromosomes Analyzed		
Amino Acid Replacement	Nucleotide Replacement	Corresponding Amino Acids in M/B Phosphorylase
Gly1Ala	GGC→GCG	Ser/Gly
Glu2Lys	GAA→AAG	Arg/Glu
Glu82Val	GAA→GTA	Ile/Ile
Glu230Val	GAG→GTG	Val/Val
Gln322Ala	CAA→GCA	Val/Val
Arg343Ala	CGC→GCA	Ser/Ala
Ile344Leu	ATC→CTC	Leu/Leu
Asn368Thr	AAC→ACC	Thr/Thr
Pro424Arg	CCT→CGT	Arg/Arg
Ser714Arg	AGC→AGG	Arg/Arg
B. Synonymous Nucleotide Replacements in All Seven Chromosomes Analyzed		
Amino Acid Affected	Nucleotide Replacement	Corresponding Codons in M/B Phosphorylase
Pro3	CCG→CCC	CCC/CCG
Thr5	ACA→ACG	TCA/ACG
Arg60	CGG→CGC	CGC/CGC
Polymorphisms		
Asp50	GAC, six times	GAT, once
Val221	GTC, six times	ATC, once (=Ile)
Asp339	GAT, twice	GAC, five times
Thr671	ACC, twice	ACT, five times

Discussion

In the present study, we have reported the identification of the first mutations in *PYGL,* the gene encoding the L isoform of glycogen phosphorylase, demonstrating that *PYGL* is the gene responsible for glycogenosis type VI (Hers disease). The splice-site mutation of patient 1 leads to three aberrant splicing products, all with a disrupted reading frame. The splice-site mutation of patient 2 causes the in-frame deletion of a highly conserved segment of 44 amino acids, including sites implicated in dimerization and glycogen binding (Hudson et al. 1993). The two missense mutations both cause nonconservative replacements of residues absolutely conserved between mammals, plants, yeast, and bacteria. N338 is, according to the crystal structure of muscle glycogen phosphorylase, buried in the interior of the protein, whereas N376 is directly adjacent to the H377 residue implicated in glucose interaction at the active site (Hudson et al. 1993). It still is possible that the mutant proteins affected by the in-frame deletion and the missense mutations retain some enzymatic activity, which might account for the relatively high residual activity values measured in the livers of patients 2 and 3. Background activity in the liver, which also is seen in patient 1, may also be due to coexpression of the B isoform (Newgard et al. 1988).

Mutation identification based on amplification of the *PYGL* coding sequence from blood RNA or DNA may help to improve the diagnosis of type VI glycogenosis. Some laboratories find that it is difficult to determine, by biochemical analysis, whether a defect in the liver phosphorylase system is due to a deficiency of phosphorylase itself or to a deficiency of phosphorylase kinase. This is attributed to the fact that phosphorylase activity is influenced by multiple allosteric effectors and humoral and neural signals that are difficult to control. Moreover, phosphorylase kinase deficiency is typically accompanied also by decreased total phosphorylase activity (Lederer et al. 1975; Maire et al. 1991). Determination of phosphorylase activity can be performed in leukocytes and other peripheral blood cells, but the L, B, and M isoforms seem to be expressed in different proportions in different blood cell types (Koster et al. 1976; Dahan et al. 1988), and details of blood-cell fractionation may influence the possibility of detecting a deficiency of the L isoenzyme (see data on patients 2 and 3, in the Subjects, Material, and Methods section above). Some laboratories therefore consider it necessary to perform a liver biopsy to establish the diagnosis, and the possibility of being able to identify *PYGL* mutations from blood RNA or DNA may help to avoid such biopsies.

The biochemical data on patient 3 were ambiguous because both phosphorylase and phosphorylase kinase were low in a liver biopsy but were normal in blood cells, and the patient was not available to allow us to repeat the enzyme determinations. Since patient 3 is a daughter of consanguineous parents, an autosomal mode of inheritance was suspected, and the autosomal phosphorylase kinase genes that can give rise to liver glycogenosis—that is, *PHKB* and *PHKG2* (Maichele et al. 1996; Burwinkel et al. 1997*a,* 1997*b,* 1998; van den Berg et al. 1997)—therefore were considered along with *PYGL.* However, the entire *PHKG2* coding sequence and most of *PHKB* were analyzed, without any sequence abnormalities being found, whereas a plausible N376K missense mutation was found in *PYGL.* The example of patient 3 thus illustrates that mutation analysis may substantiate a diagnosis of type VI glycogenosis when biochemical analysis remains insufficient.

A functional deficiency of glycogen phosphorylase in the liver can be caused by mutations in four genes: either in *PYGL* or in *PHKB, PHKA2,* or *PHKG2*—the genes encoding, respectively, the β subunit and the L isoforms of the α and γ subunits of the activating enzyme, phosphorylase kinase. With the identification of *PYGL* mutations, patients with mutations in any of these four genes have now been described. Mutations in *PHKA2, PHKB,* and *PHKG2* produce similar clinical phenotypes

and generally have a good prognosis (Kilimann 1997), progression to cirrhosis being very rare and occurring mainly in association with *PHKG2* mutations (Burwinkel et al. 1998). The patients with *PYGL* mutations who have been described here are clinically indistinguishable from patients with liver phosphorylase kinase deficiency, confirming that direct deficiency of phosphorylase does not have more-severe functional consequences than those produced by a deficiency of its regulatory protein kinase.

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