Genetic Defect in Muscle Phosphofructokinase Deficiency

ABNORMAL SPlicing OF THE MUSCLE PHOSPHOFRUCTOKINASE GENE DUE TO A POINT MUTATION AT THE 5'-SPLICE SITE

Hiromu Nakajima, Norio Kono, Tomoyuki Yamasaki, Kikuko Hotta, Masanori Kawachi, Masamichi Kuwajima, Tamio Naguchi, Takehiko Tanaka, and Seichiro Tarui

From the Second Department of Internal Medicine, Osaka University Medical School, 1-1-50 Fukushima, Fukushima-ku, Osaka 553 and the Department of Nutrition and Physiological Chemistry, Osaka University Medical School, 4-3-57 Nakasone, Kita-ku, Osaka 530, Japan

The genetic defect in muscle phosphofructokinase deficiency (type VII glycogenosis, Tarui disease) was investigated. Six cDNAs for muscle phosphofructokinase, including a full-length clone, were isolated from a non-amplified library of muscle from a patient. By sequence analysis of these clones, a 75-base in-frame deletion was identified. The rest of the sequence was identical to that of the normal cDNA, except for a silent base transition at position 516 (ACT (Thr) to ACC (Thr)). The deletion was located in the 3'-terminal region of exon 13 (numbered with reference to the rabbit muscle phosphofructokinase gene (Lee, C.-P., Kao, M.-C., French, B. A., Putney, S. D., and Chang, S. H. (1987) J. Biol. Chem. 262, 4185–4189)).

The genomic DNA of the patient was amplified by polymerase chain reaction. Sequence analysis of the amplified DNA revealed a point mutation from G to T at the 5'-splice site of CAG:GTATGG to CAG:zTATGG. A cryptic splice site of ACT:GTGAGG located '75 bases upstream from the normal splice site was recognized.

DNA sequencing of this patient, were affected. The detailed clinical features and definite diagnosis of the enzyme defect in this patient have already been described in the first case report (5) of this disease, in which this patient was case 2. Normal muscle was obtained during surgical operation as reported previously (11). Informed consent to participate in the study was obtained from the patient, and the research was carried out in conformity with the declaration of Helsinki. Genomic DNAs of the patient and a normal subject were prepared from peripheral blood lymphocytes by a standard protocol (14).

Phosphofructokinase (ATP:D-fructose-6-phosphate 1-phosphotransferase, EC 2.7.1.11) is a tetrameric enzyme that plays a key role in the glycolytic pathway. There are three isozymes of phosphofructokinase, known as the muscle, platelet, and liver types. The platelet-type isozyme is also called the fibroblast type (1). The genes for the muscle, platelet (fibroblast), and liver types have been assigned on chromosomes 1, 10, and 21, respectively (2-4).

Hereditary phosphofructokinase deficiency in muscle (5) is classified as type VII glycogenosis and is also called Tarui disease (6). This disease has several heterogeneous symptoms (7), but typical clinical manifestations are intolerance to muscular exercise (5), increased hemolysis (5, 8), and hyperuricemia (9). Recently, partial cDNA cloning of human muscle phosphofructokinase (10), its full-length cDNA sequence (11), the expression of the full-length cDNA in mammalian cells (12), and the partial genomic sequence (13) have been reported. But there are no reports of studies on the genetic defect in patients with hereditary phosphofructokinase deficiency.

In this paper, we describe the identification of the genetic defect in a patient with Tarui disease by cDNA cloning and gene amplification.

MATERIALS AND METHODS

Source of Human Materials—A muscle biopsy specimen was obtained from a male patient (T. K.) with typical Tarui disease. His parents were first cousins, and three of the five siblings, including this patient, were affected. The detailed clinical features and definite diagnosis of the enzyme defect in this patient have already been described in the first case report (5) of this disease, in which this patient was case 2. Normal muscle was obtained during surgical operation as reported previously (11). Informed consent to participate in the study was obtained from the patient, and the research was carried out in conformity with the declaration of Helsinki. Genomic DNAs of the patient and a normal subject were prepared from peripheral blood lymphocytes by a standard protocol (14).

Construction of Full-length cDNA for Human Muscle Phosphofructokinase—The three overlapping cDNA clones for human muscle phosphofructokinase reported previously (11) were suitably digested with restriction enzymes and ligated to obtain a full-length cDNA. The ligated cDNA was introduced into the BamHI site of pUC119 and was sequenced to confirm that it was ligated correctly.

Cloning of Patient's cDNAs—Poly(A') RNA was prepared from the patient's muscle as described (14). Poly(A') RNA (1 μg) from the patient's muscle was fractionated through formaldehyde-agarose (0.8%) denaturing gel and transferred to a nylon membrane (Hybond N, Amersham, United Kingdom). The full-length human muscle phosphofructokinase cDNA and exon 4 of the human β-actin gene (19) (purchased from Nippon Gene, Toyama, Japan) were labeled with [a-32P]ATP by the random primer technique (16). The filter was hybridized with the phosphofructokinase cDNA, washed, and autoradiographed and then was reprobed with the β-actin probe to compare the amount of phosphofructokinase mRNA with that of a normal control. Hybridization, washing, and reprobing procedures were carried out according to the manufacturer's recommendations.

Cloning of Patient's cDNAs—Poly(A') RNA (1 μg) from the patient's muscle was primed with oligo(dT)12-18, and the double-stranded cDNA was synthesized by the method of Gubler and Hoffman (17). A cDNA library was constructed using λgt10 vectors as described (18). The cDNA library was constructed using λgt10 vectors as described (18). The clones were isolated and subcloned into the EcoRI site of pUC119 or pBluescript SKII(+). Sequence analysis from both ends of the clones using forward and reverse universal M13 primers revealed that three clones possessed the entire coding sequence. One of them contained the full-length sequence from the 5'-untranslated region to a poly(A') tract. These clones were sequenced extensively by making nested deletions.
unidirectional deletion mutants by controlled exonuclease III digestion as described (19). Nucleotide sequences were determined by the dideoxy chain termination technique of Sanger et al. (20) with modified T7 DNA polymerase (Sequenase, United States Biochemical Corp., Cleveland, OH).

Polymerase Chain Reaction (PCR) (21)—A set of 30-mer flanking the deletion site 5'-primer: 5'- AGT.GGT.TCG.CAC.ACA.GTG. GCT.GTG.ATG.AAC-3'; 3'-primer: 5'CAA.GTT.TAG.AGC.CAC. CTT.GCC.CAG.TCC.AGC-3') were synthesized in a DNA synthesizer (Model 381A, Applied Biosystems, Inc., Foster City, CA). The primer sites were selected so as to be located within exons 13 and 14, numbered with reference to the rabbit muscle phosphofructokinase gene (22). Amplification was performed with 1 ng of genomic DNA and 2.5 units of Taq DNA polymerase (AmpliTaq, Perkin-Elmer/Cetus, Norwalk, CT). Reaction conditions were essentially as recommended by the manufacturer. Amplification was performed by 25 cycles of denaturation, annealing, and polymerization at 94, 60, and 72 °C, respectively, as reported (23). Amplified DNA fragments were isolated after separation by polyacrylamide gel electrophoresis and subcloned into the HincII site of pUC119. They were sequenced using universal M13 and PCR primers. Multiple subclones were sequenced to overcome the possible statistical errors observed during polymerization with Taq DNA polymerase (24).

Nomenclature of Exon and Intron Numbers—Recently, the existence of a splice junction within the 5'-untranslated region of the human muscle phosphofructokinase cDNA was identified by analysis of the partial sequence of the human muscle phosphofructokinase gene (13). Its presence suggests that there is an extra noncoding exon and intron between exons 13 and 14. However, the exon and intron numbers of the original rabbit gene are tentatively applied to the human gene throughout this paper since the transcription initiation site and the promoter sequence of the gene have not yet been determined.

RESULTS

We examined the mode of expression of muscle phosphofructokinase mRNA in the patient's muscle by Northern analysis. The amount and apparent size of mRNA from the patient's muscle were not appreciably different from those from control muscle (data not shown). Next we cloned cDNAs of the patient's muscle phosphofructokinase and sequenced six independent clones. Taking the first adenine of the ATG initiation codon as position 1, we identified a silent base transition at position 516 (from ACT (Thr) to ACC (Thr)) directly connected in-frame to the downstream sequence, ATAGAG. The rest of the sequence was identical to that of the normal cDNA (11).

Since the deletion was located in mRNA corresponding to the 3'-terminal region of exon 13 of the rabbit muscle phosphofructokinase gene (22), the genomic sequence was determined by amplifying the relevant region by PCR. Gene amplification gave amplified products of the same size (~650 bp) from normal genomic DNA and from that of the patient, as evidenced by agarose gel electrophoresis of aliquots of the products. These products were introduced into the HincII site of pUC119 and sequenced using forward and reverse universal M13 and PCR primers. Sequence analysis of the subclones revealed that they had 150 bp of a coding sequence, a 438-bp intron, and 61 bp of a coding sequence. These sequences corresponded to exon 13, intron 13, and the 5'-part of exon 14, respectively, of the rabbit muscle phosphofructokinase gene (22). The boundaries between exon 13, intron 13, and exon 14 were found to be the same in the human and rabbit genes, except that the intron was longer in the human gene than in the rabbit gene (279 bp). A single base transition at position 516 in the patient's cDNA gave amplified products of the same size (~650 bp) from normal genomic DNA and from that of the patient, as evidenced by agarose gel electrophoresis of aliquots of the products. These products were introduced into the HincII site of pUC119 and sequenced using forward and reverse universal M13 and PCR primers. Sequence analysis of the subclones revealed that they had 150 bp of a coding sequence, a 438-bp intron, and 61 bp of a coding sequence. These sequences corresponded to exon 13, intron 13, and the 5'-part of exon 14, respectively, of the rabbit muscle phosphofructokinase gene (22). The boundaries between exon 13, intron 13, and exon 14 were found to be the same in the human and rabbit genes, except that the intron was longer in the human gene than in the rabbit gene (279 bp). A single base transition from G to T at the first base of the 5'-splice site of intron 13 was identified in all the subclones of the patient's DNA sequenced (Fig. 2). The normal splice site of CAG:GTATGG was changed by the transition to CAG:TTATGG in the patient's DNA. The rest of the sequence was identical to that of the normal control. A cryptic 5'-splice site of ACT:GTAGAG, located 75 bases upstream from the normal splice site, was used in the patient, resulting in a 75-base deletion in his mRNA (Fig. 3).

DISCUSSION

Since the first report on muscle phosphofructokinase deficiency (5), ~30 cases have been described (7). The mode of inheritance is autosomal and recessive. The heterogeneity in symptoms suggests that the genetic cause of this disease is not uniform. In this paper, the defect in one patient in the first family investigated was identified at the gene level. The base transition observed at position 516 in the patient's cDNA has also been found in the sequence of normal cDNA independently cloned by Sharma et al. (12). This base transition, which does not affect the amino acid, would be irrelevant to the disease. Downstream from this site, a 75-base in-frame deletion from residues 1267 to 1341 was identified. This abnormal mRNA sequence would code for a protein containing a deletion of 25 residues. There are reports of the existence of materials cross-reacting with antibodies to muscle phos-

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1 The abbreviations used are: PCR, polymerase chain reaction; bp, base pair(s).
the 5'-splice site of intron 13 in the normal gene. A transition from G to T at the first base of the 5'-splice site is indicated by an asterisk.

![FIG. 2. Point mutation in patient with Tarui disease.](image)

The sequence of the muscle phosphofructokinase gene around the 5'-splice site of intron 13 is shown. Subclones of gene amplification products were sequenced using the 5'-primer (see "Materials and Methods") as a sequencing primer. Nucleotides are thus for the coding strand. Identical nucleotides in the normal gene (left) and in that of the patient (right) are hyphenated. The sequence CAG:GTATGG is the 5'-splice site of intron 13 in the normal gene. A transition from G to T changed the sequence to CAG-TATGG in the patient, resulting in absence of splicing at this site.

**Fig. 2.** Point mutation in patient with Tarui disease. The sequence of the muscle phosphofructokinase gene around the 5'-splice site of intron 13 is shown. Subclones of gene amplification products were sequenced using the 5'-primer (see "Materials and Methods") as a sequencing primer. Nucleotides are thus for the coding strand. Identical nucleotides in the normal gene (left) and in that of the patient (right) are hyphenated. The sequence CAG:GTATGG is the 5'-splice site of intron 13 in the normal gene. A transition from G to T changed the sequence to CAG-TATGG in the patient, resulting in absence of splicing at this site.

![FIG. 3. Mutation in patient with Tarui disease.](image)

The sequence of the muscle phosphofructokinase gene corresponding to exon 13, intron 13, and exon 14 is shown. Sequences of 5'- and 3'-splice sites of the rabbit muscle phosphofructokinase gene (22) are also shown in parentheses. Lower-case letters indicate the nucleotide sequence of the intron. A single base transition from G to T at the first base of the 5'-splice site is indicated by an arrow. The cryptic splice site used in the patient is indicated by asterisks. Primer regions for PCR are underlined. The deletion of 75 bases (25 amino acids, indicated by one-letter abbreviations) found in the patient cDNA is boxed.

phosphofructokinase in extracts of muscle specimens from other patients (25-27). Studies are required to determine whether such abnormal protein is present in this case. Southern analysis of genomic DNA digested with EcoRI, BamHI, HindIII, and KpnI suggested the absence of restriction fragment length polymorphism in the patient. Thus, it is very unlikely that there is any other major rearrangement of a genomic structure besides the point mutation at the 5'-splice site identified in this study. Statistical errors, which are anticipated when Taq DNA polymerase is used for PCR (24), can be ruled out by the experimental results. Five independent subclones obtained after PCR were sequenced, and an equivocal point mutation was confirmed with no other base mismatch. Moreover, the point mutation identified fully explains the altered splicing found in the patient's mRNA.

Recently, abnormal splicing of the β-hexosaminidase β-chain gene transcript in one case of the juvenile form of Sandhoff disease has been reported (28). In this case, a single base transition from G to T within an intron created a new 3'-splice site and resulted in the in-frame addition of a 24-base intron sequence to the mRNA. The original 3'-splice site was not altered, but essentially no normally spliced transcript was detected. In the consensus sequence of the 5'-splice site ([(C/A)AG:GT(A/G)AGT], the first 2 bases of the donor sequence are reported to be highly restricted to GT (29). For example, a nucleotide substitution of A for the first G completely abolished the splicing at this site in one case of β-thalassemia (30). In our case, the base transition was G to T, which also inactivated the normal 5'-splice site. On the other hand, cryptic splice sites are known to be activated by such substitutions (31). The sequence of the cryptic site used in the patient was ACT-GTGAGG instead of the normal splice site CAG-GTGAGG (consensus bases underlined). Candidates for the alternative splice site [GT(NNN); N represents any base) were surveyed within exon 13 and intron 13 in the genomic sequence. Among the possible splice sites within this region, the cryptic site used had the highest degree of homology to the consensus sequence of the 5'-splice site. In addition, the sequence of cDNA clones from the patient suggested that the possibility that the other sites were spliced was very small.

Characterization of the abnormal gene product is crucial for understanding the normal physiological function of the enzyme protein as well as the pathophysiology of the disease with defective enzyme activity. Since mammalian phosphofructokinase is double the size of prokaryotic phosphofructokinase, gene duplication during evolution has been suggested (32). The primary structure of rabbit muscle phosphofructokinase shows internal homology, and the molecule can be considered to consist of homologous N- and C-terminal half-peptides, linked by a so-called "connecting peptide." Moreover, each half of the enzyme shows homology to prokaryotic phosphofructokinase (22, 32). If we assume that the mutant phosphofructokinase mRNA described here is translated, the 25 residues deleted would be located at positions 422-446. Data from crystallographic analysis of *Bacillus stearothermophilus* phosphofructokinase (33) and homology studies on rabbit muscle phosphofructokinase (32) indicate that this mutation would be within the C-terminal half-peptide, just downstream from the connecting peptide. In the tertiary structure of the rabbit enzyme, this area is considered to contribute in part to the allosteric sites of ADP/AMP activation and fructose 1,6-bisphosphate activation (32). One arginine residue (Arg423) in the active center of the ADP/AMP activation site is lost by this deletion. Moreover, the mutation would also eliminate two stretches of α-helices and a short β-sheet structure. Both the destruction of the allosteric sites and the kinking of the helical structure would result in drastic configurational change, leading to loss of catalytic activity. The consequences of this mutation would be very profound. Confirmatory data at the protein level for these deductions are, however, not yet available.

The fact that this patient is an offspring of consanguineous mating and our results support the idea of homozygosity of this patient. It is possible, however, that this case is a double-heterozygote, carrying two different mutant alleles of muscle

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phosphofructokinase at the same time. Further analyses of the other members of this family are necessary to determine the genetic lesion of this trait definitely. Moreover, the heterogeneous symptoms in phosphofructokinase deficiency suggest that several independent types of mutation may be involved. Therefore, investigations are also required on the genetic abnormalities in patients in other nonrelated families. Recently, the in vitro expression of normal phosphofructokinase has been reported (12). The expressions of various mutant phosphofructokinases constructed through site-directed mutagenesis would be useful for analyzing not only the pathophysiology of the disease, but also the structure-function relationship of this enzyme.

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