Molecular basis of essential fructosuria: molecular cloning and mutational analysis of human ketohexokinase (fructokinase)

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Essential fructosuria is one of the oldest known inborn errors of metabolism. It is a benign condition which is believed to result from deficiency of hepatic fructo-kinase (ketohexokinase, KHK, E.C.2.7.1.3). This enzyme catalyses the first step of metabolism of dietary fructose, conversion of fructose to fructose-1-phosphate. Despite the early recognition of this disorder, the primary structure of human KHK and the molecular basis of essential fructosuria have not been previously defined. In this report, the isolation and sequencing of full-length cDNA clones encoding human ketohexokinase are described. Alternative mRNA species and alternative KHK isozymes are produced by alternative polyadenylation and splicing of the KHK gene. The KHK proteins show a high level of sequence conservation relative to rat KHK. Direct evidence that mutation of the KHK structural gene is the cause of essential fructosuria was also obtained. In a well-characterized family, in which three of eight siblings have fructosuria, all affected individuals are compound heterozygotes for two mutations Gly40Arg and Ala43Thr. Both mutations result from G→A transitions, and each alters the same conserved region of the KHK protein. Neither mutation was seen in a sample of 52 unrelated control individuals. An additional conservative amino acid change (Val49Ile) was present on the KHK allele bearing Ala43Thr.

INTRODUCTION

In mammals, dietary fructose is primarily metabolized through a pathway distinct from that responsible for glucose metabolism. This pathway utilizes three specialized enzymes, fructokinase (ketohexokinase, KHK, EC 2.7.1.3), aldolase B (fructose-1-phosphate aldolase, EC 4.1.2.13) and triokinase (EC 2.7.1.28), which convert fructose into intermediates of the glycolytic and gluconeogenic pathways. KHK is found predominantly in liver, kidney, and small intestine, and catalyses the conversion of fructose to fructose-1-phosphate. It is also active using other ketose sugars as substrate (for review, see ref. 1).

Human KHK has been purified from liver, to a specific activity which suggests it constitutes about 0.07% of liver protein (2).

Both the human and bovine enzymes appear to be dimers (2,3), with an estimated subunit molecular weight of 39 000. Human KHK has an apparent K_m for fructose of 0.86 mM (2); its high V_{max} allows very rapid metabolism of dietary fructose via the specialized fructose pathway, which bypasses the usual major site of glycolytic regulation (phosphofructokinase). After parenteral fructose administration, this can result in severe lactic acidosis even in normal individuals (for review, see ref. 1).

Essential fructosuria (MIM number 229800), although not one of the four disorders discussed by Garrod, can claim to be one of the earliest described inborn errors of metabolism, having been first recognized almost 120 years ago. It is a benign condition characterized by the intermittent appearance of fructose in the urine. In affected subjects, ingestion of dietary fructose, sucrose or sorbitol is followed by an abnormally large and persistent rise in blood fructose concentration, and by excretion of 10–20% of the ingested load in the urine (1). Essential fructosuria appears to be inherited as an autosomal recessive trait (see Discussion). In one affected individual an indirect enzyme assay was used to demonstrate a deficiency of hepatic fructokinase (4), but the molecular basis for essential fructosuria has remained otherwise undefined.

No molecular genetic studies of human KHK have been previously undertaken. The primary structure of rat liver KHK was, however, recently described (5). This work demonstrated that KHK is structurally unlike other mammalian hexokinases, and does not show significant homology with other known mammalian protein families. However, short sequence motifs shared with some bacterial phosphotransferases, which like KHK have a furanose sugar substrate, did suggest the possibility of an ancestral furanose kinase, from which these proteins evolved.

RESULTS

Isolation of human KHK cDNA clones

Since no other information was available, as to which residues of KHK might be conserved between mammals, we chose to screen for human KHK by low-stringency hybridization with the entire rat KHK coding region, rather than by polymerase chain reaction (PCR) with degenerate oligonucleotide pools. Three cDNA clones > 1 kb in size, isolated in this way from a cDNA library of the hepatoblastoma cell HepG2, were completely sequenced. The nucleotide sequence and translated open reading frame of the longest of these (pHKHK3a) are shown in Figure

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GTAGCCTC •

ATGGAAGAGAAAGCAGATCCTGTGCGTGGGGCTAGTGGTGCTGGACGTCATCAGCCTGGTGGACAAGTACCT •0
MetGluGluLysGlnIleLeuLeuCysValGlyLeuValValLeuAspValIleSerLeuValAspLysTyrPro 24

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GCTGACTTCCTG
AlaAspPheLeu

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LeuAspAspLeuArgArgTyrSerValAspLeuArgArgTyrThrValPheGlnThrThrGlySerValProIle 96
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ValAlaAspPheArgArgArgGlyValAspValSerGlnValAlaTrpGlnSerLysGlyAspThrProSer

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SerAlaThrAspPheGluLysValAspLeuThrGlnPheLysTrpIleHisIleGluGlyGlyArgAsnAlaSer 144

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GluGlnValLysMetLeuGlnArgIleAspAlaHisAsnThrArgGlnProProGluGlnLysIleArgVal 168

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CACTCGGATGCTTTCCCGCCCACCCCGCGTGGTGGATACACTGGGAGCTGGAGACACCTTCAATGCCTCCGTC 800
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ATCTTCAGCCTCTCCCAGGGAGGAAGCTGCAGAAGACACTGAGATTCGGGTGCCAGGTGGCCGGCAAGAAG 872
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TTGGGGCCAACTCCAATATAGGGTGGGTAAGGCCTTATAATGTAAAGAGCATATAATGTAAAGGGCTTTAGA 1736

GTGAGACAGACCTGGATTCAAATCTGCCATTTAATTAGCTGCATATCACCTTAGGGTACAGCACTTAACGCA 1808

ATCTGCCTCAATTTCTTCATCTGTCAAATGGAACCAATTCTGCTTGGCTACAGAATTGTGAGGATAAAA 1880

ATCATATATAAAAAAAAAA 1900

Figure 1. The sequence of *KHK* cDNA. The sequence of the longest clone (pHKHK3a) is shown. The shorter clones pHKHK3d and pHKHK1-2 lie within this sequence; their 5' limits are indicated by I and U, respectively. Both of these shorter clones end at the upstream polyadenylation site indicated by *. The consensus and variant polyadenylation signals are underlined. The alternatively spliced exon (C) encoded in clone pHKHK1-2 is shown in italics below the main sequence. This exon corresponds closely to the sequence of the rat *KHK* cDNA (5; see also Figure 2). Matching residues between the two alternative splice forms of human KHK are underlined. The precise limits of the alternatively spliced exon cannot be stated at present since genomic clones have yet to be analysed. The Gly40Arg and Ala43Thr changes are caused by the mutations indicated in bold type at nt 126 and 143. The other sequence variants observed are also indicated in the same way. Arg159Gly was found only in clone pHKHK3d, and it is unclear at present whether it represents a cloning artefact or a true polymorphic variant. The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank and DDBJ Nucleotide Sequence Databases under the accession numbers X78677 and X78678.

1. The positions of the sequence variants detected in other clones (discussed below) are also shown.

Alternative splicing and polyadenylation

The most notable feature of pHKHK3a is its long (984 nt) 3' untranslated region. This is in contrast to the 186 nt 3' untranslated region of the published rat *KHK* mRNA. The other two human clones (pHKHK3d, pHKHK1-2), however, are derived from mRNA which has utilized an upstream polyadenylation site, closer to the position of that in the rat (giving a 3' untranslated region of 218 nt).

The sequences of pHKHK3a, pHKHK3d and pHKHK1-2 are collinear except for nt 218 to 352 in Figure 1. Here, pHKHK1-2 differs over its first 140 bp from the sequence of the other two clones. This appears to result from alternative splicing to include either of two exons. The published rat cDNA sequence (5) corresponds in this region to the splice variant found in pHKHK1-2, referred to subsequently as form C. The other splice variant is referred to below as form A. Closer inspection (Figure 1) shows that these two alternatively spliced exons can be aligned with each other to reveal conservation of several amino acid residues. This suggests that an intragenic duplication event may have given rise to the alternative exons in this region of the *KHK* gene.

The predicted molecular weight of the human KHK A subunit is 32 734. The initiator ATG shown in Figure 1 is presumptive, since no in-frame upstream stop codon is present in the clones. However, this assignment is based on the position of the initiator codon in the rat mRNA (5) which was verified by the agreement of the predicted protein M_r with the M_r directly determined by mass spectrometry.

A single amino acid variant was found among the three independent HepG2 *KHK* cDNA clones. Arg159 is altered to Gly by an A→G transition in clone pHKHK3d. The alignment in Figure 2 shows that unlike the two residues mutated in essential fructosuria patients (see below) Arg159 is not conserved between human and rat, and indeed lies at the centre of the most poorly conserved region of KHK.

Tissue distribution of *KHK* mRNA

KHK activity is found principally in liver, kidney, and small intestine (2); pancreatic islet cells also possess a similar activity (6). Because of this tissue specificity, it was initially unclear whether analysis of *KHK* RNA in patients with essential fructosuria would be possible. To investigate this, reverse transcription and PCR (RT−PCR) of the 5' coding region (nt 1−410, primers KHK1/KHK3) were performed on RNA from human fetal tissues. As expected, strong signals were obtained from liver, kidney, gut and pancreas, as well as (unexpectedly) from spleen. However, we were encouraged to find lower yields of PCR product also from adrenal, muscle, brain and eye, as well as from human diploid fibroblasts and from an EBV-transformed lymphoblastoid cell line (data not shown). This may be in keeping with previous observations of low level KHK activity in some other tissues (2).

Mutations causing essential fructosuria

Next, we investigated the postulate that mutation of the human *KK* gene underlies the rare metabolic disorder, essential fructosuria. The family analysed has been the subject of previous metabolic studies which have demonstrated excessive fructosaemia and fructosuria after oral or intravenous fructose, sorbitol, or sucrose (7−9). After an intravenous fructose bolus (200 mg/kg) the liver concentrations of fructose-1-phosphate, ATP, and phosphate, as determined by ^{31}P magnetic resonance spectroscopy in one affected family member (RK), remained unchanged, in contrast to controls, confirming that fructokinase was indeed inactive (10,11). In this Swiss family, three of eight siblings have fructosuria. Since the parents are third degree cousins, it was anticipated that homozygosity for a single mutation would underlie the fructosuria. As the intron−exon structure of *KHK* remains to be defined, analysis of genomic DNA is difficult.
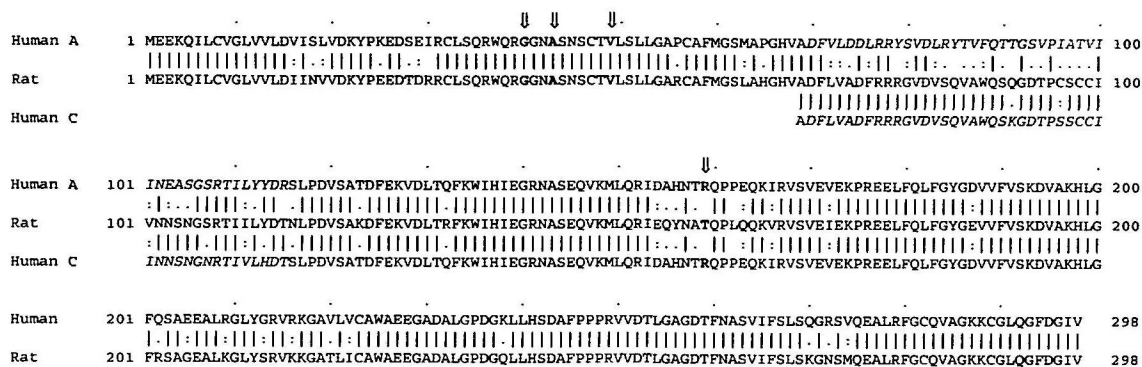
Figure 2. Alignment of rat and human KHK amino acid sequences. The human A form is shown above the rat (middle line) and the human C form beneath it (partial sequence only, the 3′ region being identical to the A form). The positions of the human sequence variants Gly40Arg, Ala43Thr, Val49Ile (found in the essential fructosuria family) and of Arg159Gly (found in clone pHKHK3d) are indicated by bold type and by ⇓ above the human sequence. It can be seen that whereas the region containing Gly40, Ala43 and Val49 is well conserved, Arg159 lies within the most poorly conserved region of the protein. The residues in italics in the human sequences indicate the minimum extent of the alternatively spliced exons. Identical residues are indicated by | between adjacent residues, and conservative or semi-conservative substitutions by : and . , respectively.

However, the finding of *KHK* mRNA in lymphoblastoid cells suggested the feasibility of analysing RNA. Therefore, RT−PCR of RNA from a lymphoblastoid cell line from one patient (AK) was performed, amplifying the coding region in two overlapping segments. The PCR products were cloned into the pCRScriptSK+ plasmid vector (Stratagene, Inc.) and single overlapping clones were sequenced. The sequence of the entire coding region thus obtained revealed two single-base substitutions. The first, A→G at nt 587, is a silent change at the third base of Lys193. The other, G→A at nt 126, changes the Gly40 codon to Arg. This mutation has occurred at a CpG dinucleotide, within a well-conserved region (Figure 2); it destroys a *Bst*UI site (CGCG) at nt 123−126.

A PCR assay on genomic DNA was then used to analyse for the nt 126 G→A change in a sample of 52 normal individuals. None contained the putative mutation, which is therefore unlikely to be a normal polymorphic variant. Next, the same assay was used to type all 10 members of the fructosuria family, by *Bst*UI digestion of the 94 bp PCR product (Figure 3a). Surprisingly, this showed that the three fructosuric individuals were heterozygous for Gly40Arg, inherited from their mother. The initial supposition of homozygosity by descent for a single mutation in this consanguineous family was therefore erroneous. Fortuitously though, the other *Bst*UI site predicted in the PCR product (at nt 134−137) was found to be absent from one allele in the father in this family (Figure 3a, lane 1). All three fructosuric patients had also inherited this second sequence variant (Figure 3a, lanes 6, 8, 9). Since the *Bst*UI site at 134−137 is also part of an *Mlu*I site (ACGCGT), this second mutation was more clearly demonstrated by *Mlu*I digestion of the PCR product (Figure 3b). To define the exact nature of the second mutation, the 94 bp product was digested with *Mlu*I and the *Mlu*I-resistant 94 bp fraction cloned and sequenced. This revealed two further G→A changes, at nt 135 and 153. The first of these causes the non-conservative substitution Ala43Thr and the second the conservative change Val49Ile. Like Gly40Arg, the Ala43Thr mutation was not observed in any of the 52 control DNA samples analysed by *Bst*UI digestion, and hence is unlikely to be a polymorphic variant. Separate analysis of the Val49Ile substitution showed the father in this family to be homozygous for Ile49 and
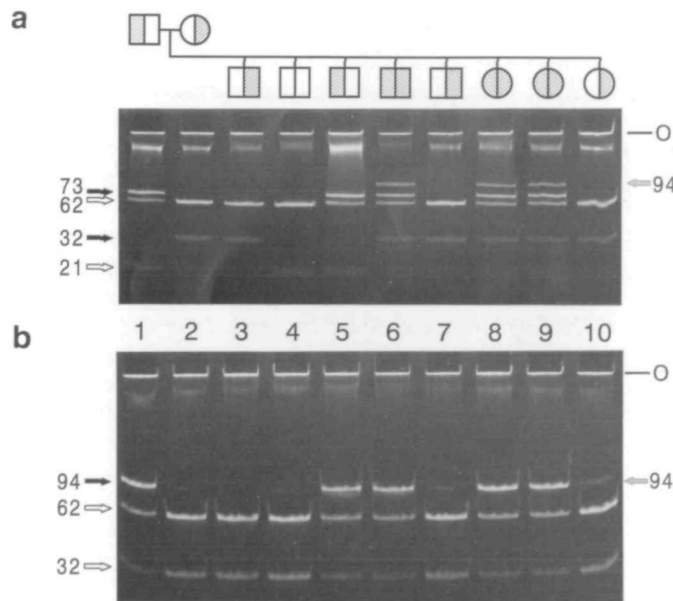

Figure 3. DNA analysis of a family with essential fructosuria. On the pedigree, *KHK* mutations are indicated by shading of the right (Gly40Arg) or left (Ala43Thr) half of the symbol. (a) 21% polyacrylamide minigel showing ethidium bromide-stained *Bst*UI digests of a 94 bp PCR product (KHK14−KHK15) containing the Gly40Arg and Ala43Thr mutation sites. The grey arrow to the right indicates the position of undigested product (not present on this gel) and of the uncut heteroduplex bands in the affected individuals in lanes 6, 8 and 9. To the left, white arrows indicate the normal digestion products (62, 21 and 11 (not seen) bp) and black arrows the fragments of increased size resulting from loss of one or other *Bst*UI site (32 bp for Gly40Arg, 73 bp for Ala43Thr). (b) Fifteen percent polyacrylamide minigel showing ethidium bromide-stained *Mlu*I digests of the same PCR products. The normal digestion products (white arrows) are 62 and 32 bp, the Ala43Thr mutation prevents cleavage (94 bp, black arrow). The faint 94 bp bands in lanes 7 and 10 are due to incomplete *Mlu*I digestion, which is difficult to overcome with this small fragment. O = origin.

the mother homozygous for Val49 (not shown). Thus a total of four *KHK* haplotypes can be defined within the family; Gly40 Ala43 Ile49 (normal paternal haplotype), Gly40 Ala43 Val49 (normal maternal haplotype), Gly40 Thr43 Ile49 (mutant paternal

haplotype) and Arg40 Ala43 Val49 (mutant maternal haplotype). All six possible combinations of the four haplotypes are seen in the family, but only Gly40 Thr43 Ile49/Arg40 Ala43 Val49 results in fructosuria.

DISCUSSION

In this report, we demonstrate that *KHK* structural gene defects underlie essential fructosuria. The family analysed has been the subject of previous metabolic studies (7–11). However, because of the tissue specificity of *KHK* expression, deficiency of hepatic KHK appears only to have been proven in one fructosuric individual, by enzyme assay on a liver biopsy (4). Essential fructosuria is certainly rare. Lasker estimated its incidence at less than 1 in 130 000, but plausibly suggested this was an overestimate (12). In four of her five families there was parental consanguinity, and the proportion of such families was used to suggest a true frequency nearer one in a million, as well as to support autosomal recessive inheritance. We were surprised, in light of this, to find that in the family we report here the parental consanguinity appears to be coincidental, with the affected individuals being compound heterozygotes for the mutations Gly40Arg and Ala43Thr.

Since two amino acid substitutions were found together on the paternal *KHK* allele in our family, we cannot be completely confident that Ala43Thr alone (in the absence of Val49Ile) is a null mutation. To prove this would require introduction of each change separately into the normal *KHK* sequence by *in vitro* mutagenesis and expression. Furthermore, to prove absolutely that Gly40Arg or Ala43Thr are null mutations would require the expression of each mutation in the context of each of the two (A and C) splice variants. None the less, several lines of evidence suggest that Gly40Arg and Ala43Thr are indeed responsible for the fructosuric phenotype. They lie in a conserved region of the protein, produce non-conservative amino acid changes, and are not present in > 100 control alleles. Val49Ile, on the other hand, does not produce fructosuria when present in the homozygous state, and is a common polymorphism in normal Europeans (data not shown). Finally, under the rather unlikely null hypothesis that essential fructosuria could result from a mutation unlinked to *KHK*, the concordant segregation between the mutations and the fructosuria in this family has a probability of only $(\frac{1}{4})^2 (\frac{3}{4})^5$ (excluding one proband) ≈ 1 in 67.

The most important new finding from these studies of human *KHK* is that of alternative splicing, due to inclusion of one or other of two similar exons, which we presume arose by an intragenic duplication, though clarification of this must await analysis of genomic *KHK* clones. It is not known at present whether both splice forms of human KHK are enzymatically active. However, the rat cDNA, which *is* known to be active when expressed in isolation (13), is close in sequence to the human C form, suggesting strongly that the latter at least will encode a functional enzyme. Since both the human and bovine KHK appear to be dimers in their native state, at least three different KHK isozymes (A-A, A-C and C-C) could exist *in vivo*. Both mutations we have identified lie in a conserved region, common to both KHK splice forms, so that each could potentially ablate function of all KHK isozymes.

KHK is believed to be synthesized largely in liver, renal cortex, and small intestine, but one previous report also demonstrated KHK-like activity in pancreas (6). Our preliminary RT–PCR experiments are not quantitative but tend to confirm these

observations at the RNA level. The distribution of different KHK splice forms in individual tissues requires analysis by RNase protection, and is currently under study. However, preliminary RT–PCR data suggest that the C-type mRNA may be confined to those tissues expressing *KHK* at high level (liver, kidney, gut, pancreas). It is interesting to note that the gene for glucokinase, another hexokinase displaying a restricted tissue distribution which includes liver and pancreatic islet cell, is subject to cell-type specific alternative splicing in both humans and rat (14,15). Characterization of the details of *KHK* alternative splicing and tissue-specific promoter function await the isolation of *KHK* genomic clones.

It is uncertain whether the rat *KHK* gene is also alternatively spliced. However, the considerable divergence between human A and C exons, compared with the close similarity between rat and human C exons, suggests that the duplication which produced the alternative exons is ancient, and pre-dated the divergence of rodent and primate lineages.

MATERIALS AND METHODS

Isolation of human *KHK* cDNA clones

The rat liver *KHK* cDNA construct pUC-KHK-G7 (5) was digested with *Bgl*II to excise a 0.9 kb coding region fragment, which was labelled by random priming and used to screen a library (constructed by Dr D.Simmonds, Oxford University) of HepG2 (hepatoblastoma cell line) cDNA in the vector pCDM8 (16), propagated on the *supF*-selecting strain MC1061/P3. Final washing conditions for the cross-species hybridization were: 6×SSC, 0.1% SDS. Screening of 50 000 colonies yielded 13 positive clones, of which three (pHKHK3a, pHKHK3d and pHKHK1-2; insert sizes 2.0, 1.15 and 1.1 kb) were completely sequenced directly on double-stranded templates, using synthetic internal or flanking primers.

Cell culture and RT–PCR

Lymphoblastoid cells were grown in RPMI1640 medium, with 10% fetal calf serum, 2 mM L-glutamine, and 25 μg/ml gentamicin. Total cell RNA was prepared by the AGPC method (17). Reverse-transcription of 1 μg total RNA was performed for 1 h at 37°C in a 30 μl reaction mixture containing random hexadeoxynucleotide primers, 10 mM TrisHCl, pH 8.3, 50 mM KCl, 6.5 mM MgCl₂, 10 mM dithiothreitol, 1 mM each dNTP, 15 U MuLV reverse transcriptase (BRL-Gibco). For PCR, 15 μl of cDNA synthesis reaction mixture was added directly to 35 μl RT–PCR mix (10 mM TrisHCl pH 8.3, 50 mM KCl, 1.5 mM MgCl₂, 285 nM each PCR primer, 1 mM EDTA, 2U recombinant *Taq* polymerase). The *KHK* coding region was amplified in two overlapping segments using primer pairs KHK1/KHK3 and KHK2/KHK11. In each case, 40 cycles of 1, 1, 2 min at 94, 63, 72°C were performed in a Perkin-Elmer Cetus Thermal Cycler. Products were gel-purified prior to cloning.

Genomic DNA analysis

Genomic DNA was isolated from frozen whole EDTA-blood by Triton X-100 lysis and proteinase K digestion (18). For typing of the Gly40Arg and Ala43Thr mutations, primers KHK14 and KHK15 were used for 30 cycles of PCR under conditions as above except for a 60°C annealing temperature. The 94 bp product is cleaved by *Bst*UI (CGCG) to 21 + 11 + 62 bp (Gly40 Ala43), to 32 + 62 bp (Arg40 Ala43) or to 21 + 73 bp (Gly40 Thr43). The small size of the PCR product was dictated by the apparent positions of splice junctions and the unknown genomic sequence flanking the exons, and by the proximity of the two *Bst*UI sites. The *Bst*UI digestion products were analysed on 21% polyacrylamide gels.

Oligonucleotide sequences

KHK1: GTAGCCTCATGGAAGAGAAGC; KHK2: GTGTCTGCTACAGA-CTTTGAG; KHK3: CTTGAACTGGGTCAGATCAAC; KHK11: AGC-TTGCATCTGTCCCCTGAA; KHK14: TTTGTCCCAGAGATGGCAGCG; KHK15: CATTGAGCCCATGAAGGC.

ACKNOWLEDGEMENTS

ABBREVIATIONS

KHK, ketohexokinase; K_m, Michaelis constant; MIM, *Mendelian Inheritance in Man*; M_r, relative molecular mass; PCR, polymerase chain reaction; SDS, sodium dodecyl sulphate; SSC, salt/sodium citrate (0.15 M NaCl, 15 mM sodium citrate, pH 7.0).

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