Molecular cloning and tissue-specific expression of mouse kidney 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase

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Abstract A 1932 bp cDNA clone encoding a novel isozyme of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) was isolated from a mouse kidney cDNA library. The sequence encodes 519 amino acids and, based on homology to rat heart genomic sequence, appears to be the product of alternative splicing from PFK-2/FBPase-2 gene B with an extended version of exon 15. Northern blot analysis indicated that this clone corresponds to an 8 kb mRNA expressed in multiple tissues, with the strongest signal in kidney, and detects several additional transcripts which may be alternatively spliced from gene B.

Key words: Alternative splicing; Fructose-2,6-bisphosphatase; Fructose 2,6-bisphosphate; Glycolysis; 6-Phosphofructo-2-kinase; Mouse kidney

1. Introduction

Fructose 2,6-bisphosphate (Fru-2,6-P_2) plays a key role in the regulation of glycolysis by acting as a potent allosteric activator of the rate-determining enzyme, phosphofructokinase (PFK-1; EC 2.7.1.11). Unlike other regulatory elements of PFK-1, such as ATP, which are synthesized directly in the glycolytic pathway, Fru-2,6-P_2 is synthesized and broken down by a separate, specific enzyme, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2; EC 2.7.1.105/FBPase-2; EC 3.1.3.46). The bifunctional enzyme functions as a homodimer with two distinct catalytic sites on each identical polypeptide chain [1,2].

Studies to date have revealed a complex system of genetic organization, with five mammalian isozyme forms identified, referred to as liver [3], skeletal muscle [4], heart [5], testis [6], and brain [7,8], based on their tissue prevalence. The isozymes are encoded by at least two distinct genes which have been characterized (genes A and B), and possibly as many as four. In rat, gene A is a 60 kb X-linked locus encoding the liver, muscle and fetal isozyme transcripts [9,10], while gene B is a 22 kb autosomal locus encoding the heart isozyme [11]. PFK-2/FBPase-2 is one of only five known bifunctional enzymes which catalyze opposing reactions and is unique because it is the only one whose target is a metabolite rather than a protein [12].

The cellular concentration of Fru-2,6-P_2 depends on the balance between kinase and phosphatase activities. This balance has been shown to be regulated by a number of metabolic and hormonal signals which act through differential phosphorylation and dephosphorylation of the enzyme. The isozymes differ in their tissue-specific expression and response to hormonal signals, and a combination of isozymes derived from both of the identified PFK-2/FBPase-2 loci may be simultaneously present as determined by the distinct metabolic needs of a particular tissue [13]. The related role of the enzyme in kidney, as compared to liver, would suggest various isozyme types to be present in this tissue; however, which PFK-2/FBPase-2 loci are expressed in kidney has remained ambiguous. A study in which 40% of kidney PFK-2/FBPase-2 was precipitated by antiserum to the liver enzyme [14] indicated a close relationship between the isozyme types present in these tissues. In addition, dot-blot experiments on total RNA indicated that liver-type isozyme was present at a low level in kidney, while muscle-type isozyme was present at a fairly substantial level [15]. On the other hand, Northern analysis has suggested that gene A is not at all expressed in kidney [16], and this premise has been used as the basis for an experiment on tissue-specific promoters [17]. Here we report the screening of a mouse kidney cDNA library in order to study the PFK-2/FBPase-2 isozymes present in this tissue, and the isolation of a novel full-length heart-type isozyme which appears to be the product of alternative splicing from gene B and is preferentially expressed in kidney.

2. Materials and methods

2.1. Materials

Bacterial media were from Difco. Hybond N+ membranes, [α-^{32}P]dCTP, [α-^{35}S]dATP, and dNTPs were from Amersham. Klenow DNA polymerase and T4 DNA ligase were from Gibco-BRL. Sequenase version 2.0 enzyme and kit were from USB. Wizard lambda minipreps and Taq polymerase were from Promega. The λgt10 mouse kidney cDNA library and multiple tissue mouse Northern blot were from Clontech. One Shot competent cells and TA cloning kit were from Invitrogen. A mouse brain cDNA preparation made by oligo(dT) priming and PCR amplification was the gift of Dr. J. Bristulf (Genetics Laboratory, Oxford). All other materials were reagent grade and obtained from commercial sources.

2.2. Isolation and sequencing of cDNA clones

A λgt10 mouse kidney cDNA library was screened with a 1.25 kb *Eco*RI fragment derived from the 3′ portion of human liver PFK-2/FBPase-2 cDNA [18]. Labeling was by random priming with [α-^{32}P]dCTP [19]. Hybridization was carried out at 65°C in Church buffer [20], and washing at the same temperature was to a final stringency of 40 mM sodium phosphate, pH 7.2/0.1% SDS. Filters were exposed to Kodak XAR film with Kodak intensifying screens at −70°C. Inserts were subcloned into pUC9 using standard techniques [21]. Plas-

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Abbreviations: bp, base pairs; FBPase-2, fructose-2,6-bisphosphatase; Fru-2,6-P_2, fructose 2,6-bisphosphate; kb, kilobases; PFK-2, 6-phosphofructo-2-kinase

The nucleotide sequences presented here have been submitted to the EMBL/Genbank database under the accession numbers X98847 (2kbC5) and X98848 (500bC6).

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mids were sequenced on both strands using the dideoxy method [22] using universal and reverse primers as well as primers derived from the obtained sequence.

2.3. Sequence analysis

All sequence analysis was performed using the GCG (Madison, WI) software package [23]. Multiple sequence alignments were carried out using the PILEUP program with default parameter settings.

2.4. Confirmation of 3′ sequence of 2kbC5 by PCR

Primers were designed which spanned from exon 15 to sequence previously identified as intronic [11]. RAG mouse genomic DNA, a cDNA preparation made from mouse brain, the original cDNA clone, and water controls were used as templates. As a control, primers were designed from exons 7 and 8 which spanned a 1 kb intron. Primer details were as follows (5′ starting position is given with respect to nucleotide sequence in Fig. 2; 'reverse' primers are complementary to coding strand): exon 15, forward (20mer, starting at nucleotide 1486), extended exon 15, reverse (20mer, 1874), exon 7, forward (22mer, 502), exon 8, reverse (20mer, 671). Conditions were: 200 mM dNTPs, 10 mM Tris-HCl, 5 mM KCl, 1.5 mM $MgCl_2$, 1 mM each primer, Taq polymerase. Cycling parameters were 94°C, 5 min ('hot start'); 94°C, 30 s; 62°C, 30 s; 74°C, 105 s; 32 cycles. Products were TA cloned according to the kit manufacturer's instructions and sequenced.

2.5. Northern-blot analysis

A multiple tissue mouse Northern blot was prehybridized for 6 h at 42°C in 5×SSPE, 10×Denhardt's solution, 2% SDS, 50% freshly deionized formamide and 100 μg/ml denatured salmon sperm and hybridized overnight at the same temperature with fresh solution containing ^{32}P-labeled 2kbC5 cDNA insert. The filter was washed for 30 min at room temperature with several changes of 2×SSC/0.5% SDS, followed by 40 min at 50°C with one change of 0.1×SSC/0.1% SDS, and then exposed to Kodak XAR film.

3. Results

3.1. Isolation and characterization of cDNA clones

Tertiary screening of the mouse kidney library with the cDNA encoding human liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase [18] yielded two positive clones containing inserts sized approx. 0.5 and 2 kb, and designated 500bC6 and 2kbC5, respectively. The different hybridization patterns of the two clones on genomic and hybrid DNA gave a preliminary indication that 500bC6 was derived from an X-linked locus and 2kbC5 was autosomal in origin (data not shown). Precise mapping of the loci is in progress. Sequencing reinforced this hypothesis as nucleotides 2–215 of 500bC6 are predicted to encode 72 amino acids which show extremely high homology to the 3′ ends of rat liver (96% nucleotide, 100% amino acid homology) [24] and human liver (82% nucleotide, 96% amino acid homology) [25] isozyme sequences; the fragment is therefore predicted to encode the mouse homologue (sequence submitted to EMBL/Genbank databases, accession no. X98848). Since the 3′ end is common to liver and skeletal muscle isozymes [9], it is not clear at present which of these isozymes corresponds to the isolated transcript; however, a previous study has shown the skeletal muscle isoform to be expressed at a higher level in kidney [15].

Sequence analysis of the 1932 bp clone 2kbC5 predicts a putative open reading frame (ORF) extending from nucleotides 37 to 1596 in reading frame +1 and terminating with a TAA. This would encode a protein of 519 amino acids (Fig. 1). The 5′ end of the 2kbC5 from nucleotides 1 to 1395 identifies with published coding sequence of rat brain isozyme, which has been hypothesized to be encoded by an alternatively spliced message from gene B [8]. The 3′ end from bases 1396 to 1571 is homologous to the bovine heart-type isozyme, but also includes a region previously identified as intronic (bases 1572–1932) in the genomic sequence of the rat heart gene B [11]. An homologous 3′ sequence has been observed in a partial sequence of a rat heart-type cDNA isolated from testis [6]. These homologies suggest that the 1932 bp clone is a heart-type isozyme alternatively spliced from PFK-2/FBPase-2 gene B.

Further comparison of the sequence to that of the rat genomic heart sequence (Genbank accession nos: X65953, X65954, X65955, X65956, X65957, X65958, X61956) indicates that it includes 76 bp of non-coding exon 1, exons 2–14, an extended version of exon 15, and since it fails to use exon 15 splice sites, is devoid of exon 16.

3.2. Confirmation of 2kbC5 3′ sequence by PCR

The presence of sequence previously identified as intronic at the 3′ end of the 2kbC5 cDNA clone was tested to determine whether it was representative of an actual transcript or was a cloning artifact. PCR primers were designed which spanned from exon 15 (prior to the putative splice sites) to 335 bp beyond the start of 'intronic' sequence. These primers were used to amplify from genomic DNA, a cDNA preparation made from mouse brain (another tissue which would be expected to have a high representation of heart-type isozyme), and the original cDNA clone. If the intronic sequence were normally spliced out and 2kbC5 represented an aberrant clone, the predicted product of size 389 bp would amplify only from genomic and the plasmid template and no product would be expected from an independent cDNA library (unless it were contaminated with genomic DNA) (Fig. 2, top). As a control, primers were designed from exon 7 and 8 which spanned the 1 kb intron between them; on the same templates, these primers would be expected to give a much larger product on the genomic template (1106 bp) than on processed cDNA (171 bp). The presence of two bands from the library template would indicate contamination by genomic DNA.

This experiment yielded identical bands of the expected size (389 bp), from genomic DNA, the mouse brain cDNA preparation, and the original clone, using the primers including intronic sequence from the 3′ end of 2kbC5 (Fig. 2, bottom). These PCR products were cloned and sequenced and found to match the expected sequence derived from 2kbC5. This indicates that this variant of exon 15, including the 'intronic' sequence, is present in an independent cDNA preparation and does not represent a cloning artifact. Amplification using the control primers yielded a band of the expected 1106 bp size on genomic template as well as a 171 bp product derived from the processed sequence on the cDNA library and 2kbC5 clone templates. No contamination from genomic DNA was

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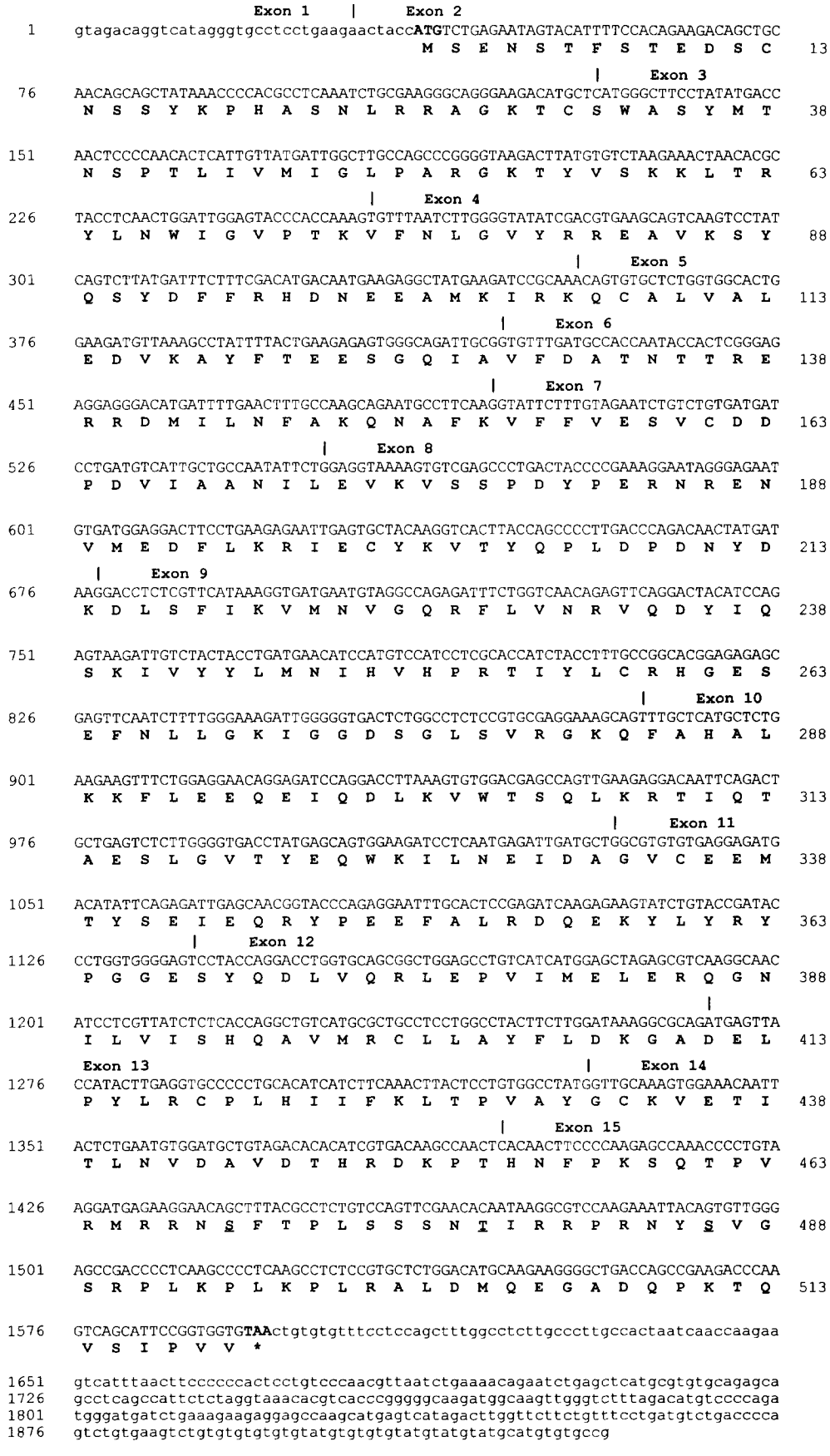
Fig. 1. Complete nucleotide and predicted amino acid sequence of 2kbC5. Nucleotides representing proposed coding region are capitalized. Numbers on the left refer to nucleotide sequence while those on the right refer to deduced amino acid sequence given in single-letter code below the nucleotide sequence. Translation start and stop sites are in bold. Potential phosphorylation sites are underlined: two sites for cAMP-dependent protein kinase (PKA) at Ser-469 and Ser-486 and a protein kinase C (PKC) phosphorylation site at Thr-478. These sites correspond to regulatory sites previously characterized in rat and bovine heart-type isozymes [11,26,28]. Exon prediction is based on homology to rat heart genomic sequence [11].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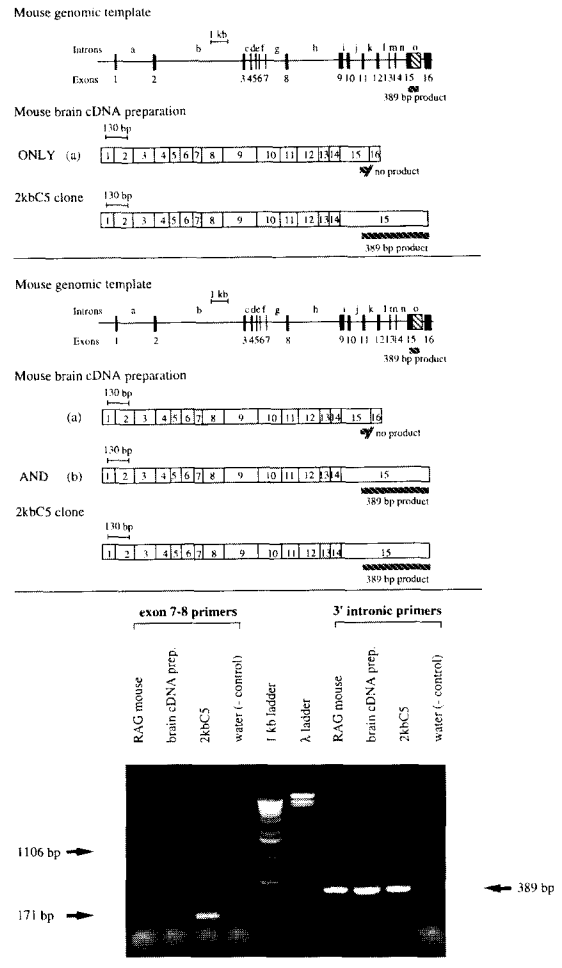

Fig. 2. Test of 2kbC5 3′ sequence by PCR. (Top) Schematic representation of experiment. (i) Expected PCR products if 2kbC5 is an aberrant clone; (ii) expected PCR products if 2kbC5 represents an actual transcript. Genomic structure (with extended exon 15 suggested by this study, indicated by striped box) and mouse cDNA (a) are based on the rat heart gene [11]. (Bottom) PCR products separated on a 1% agarose gel and stained with ethidium bromide. PCR amplification on genomic mouse template (RAG), an independent mouse brain cDNA preparation, and the original 2kbC5 clone was tested using both exon 7–8 control primers and 3′ intronic primers. The control reaction using exon 7–8 primers yielded bands of the expected size in genomic (1106 bp) and spliced cDNA (171 bp) templates and showed that the cDNA preparation was not contaminated with genomic DNA. Products of the expected 389 bp size were obtained in all samples using the 3′ intronic primers.

observed in the cDNA preparation as only one processed band was present (Fig. 2, bottom).

3.3. Comparison of predicted protein sequences of isozymes

The predicted amino acid sequence of 2kbC5 encoded by the complete ORF exhibits significant homology to previously characterized PFK-2/FBPase-2 isozymes (Fig. 3). The 2kbC5 predicted protein is 82% homologous to rat liver [24], 83% homologous to rat testis [6], 96% similar to rat brain [8], 99% homologous to a partial rat heart sequence [6] and 93% homologous to bovine heart [26]. It therefore appears to represent a mouse heart-type isozyme. The active sites of the bifunctional enzyme are conserved between the various isozymes, while the sequences differ at the 5′ and 3′ ends due to differences in regulatory domains.

3.4. Pattern of expression and size of transcript

A Northern blot of poly-A+ enriched mRNA from various mouse tissues was probed with 2kbC5 (Fig. 4, top). A transcript of about 8 kb was detected in multiple tissues, with the strongest signal in kidney, which most likely corresponds to the heart-type isozyme encoded by 2kbC5. The same probe detected two additional transcripts in multiple tissues, sized 4 kb, 6 kb, as well as a larger 11 kb transcript in brain and skeletal muscle only, which may correlate with other alternatively spliced transcripts from the heart gene. In testis only, an additional 3 kb transcript was detected. Since a 3.3 kb mRNA has previously been observed in this tissue which is not derived from the testis-specific isozyme [6], this may correspond to the heart-type transcript specifically expressed in testis. The liver- and muscle-type isozymes had been observed to be smaller (2.1–2 kb and 1.9 kb, respectively) in previous studies [16,15,27] and no bands of corresponding sizes were detected by 2kbC5.

4. Discussion

In spite of the presumed similarity of its roles in kidney and liver, PFK-2/FBPase-2 expression has not been definitively studied in renal tissue. Screening of a kidney-specific adult mouse cDNA library with human liver PFK-2/FBPase-2 isolated two distinct cDNA clones, 500bC6 and 2kbC5. Preliminary hybridization results and sequence homology to previously characterized isozymes in other species suggested that these were derived from the X-linked and autosomal loci, respectively. The isolation of a transcript apparently derived from PFK-2/FBPase-2 gene A was an interesting finding since expression of the X-linked locus in kidney had been a matter of controversy. This disagrees with the findings [16,17] that gene A is not expressed in kidney, and supports the results which found liver and skeletal muscle isozymes present in renal tissue [14,15]. The isolation of distinct isozyme forms from separate loci indicates the complexity of the enzymatic organization in kidney and is striking in light of the enzyme's regulatory rather than functional role.

Sequence and homology data of the 1932 bp mouse clone (2kbC5) indicated that it spans the entire open reading frame of a previously uncharacterized heart-type isozyme derived from gene B with a longer form of exon 15. The differential splicing implied by this result is supported by earlier characterization of the homologous bovine autosomal locus which has been shown to encode at least five mRNAs [28]. Comparison of the mouse heart-type isozyme encoded by 2kbC5 with

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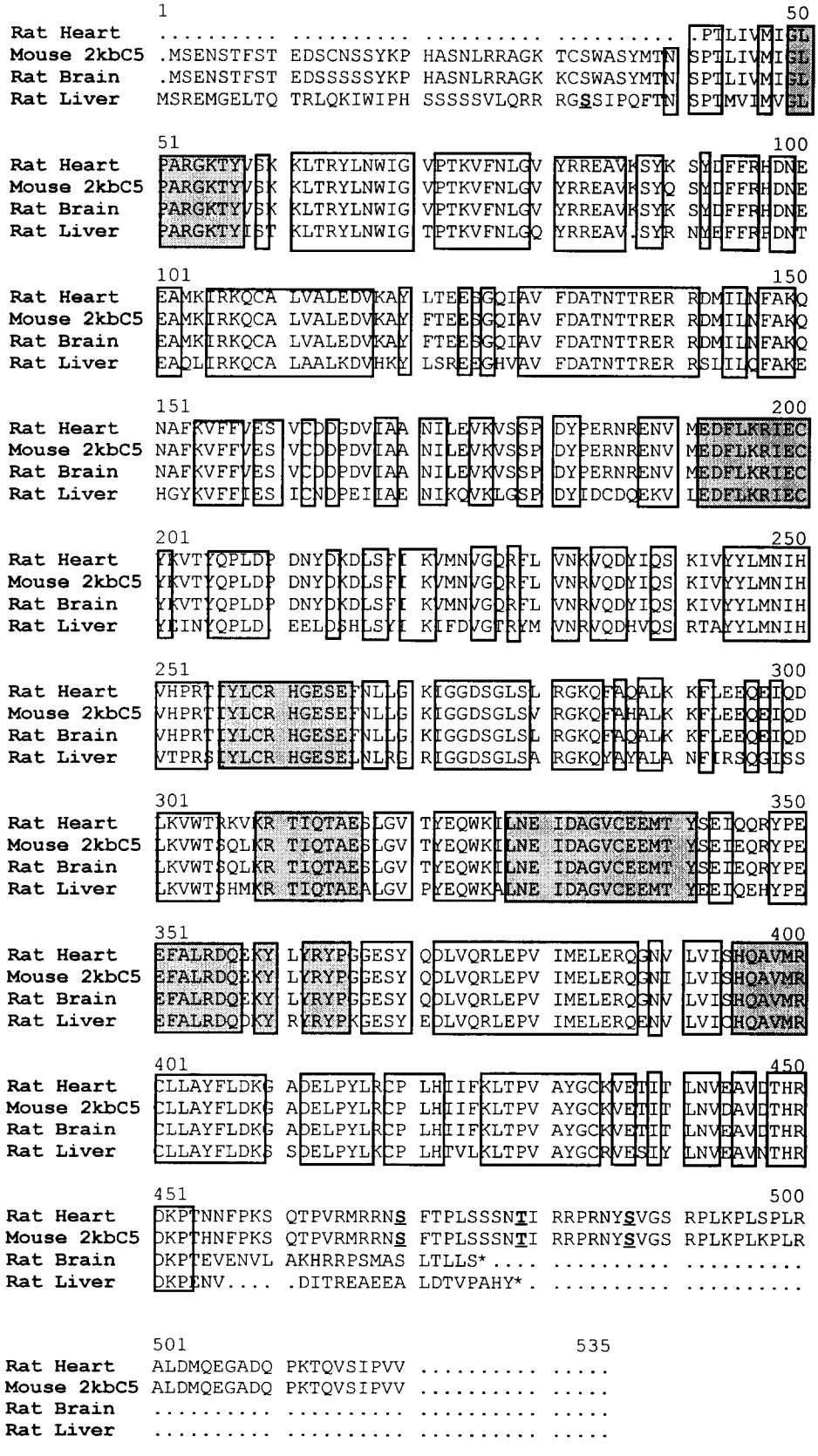
Fig. 3. Comparison of protein sequence of mouse 2kbC5 to rat PFK-2/FBPase-2 isozymes. Alignment is shown for PFK-2/FBPase-2 isozymes: partial rat heart-type sequence [6]; mouse 2kbC5; rat brain [8] and rat liver [24]. Amino acids which are identical between all four isozymes are enclosed in boxes. Shaded boxes contain conserved catalytic and substrate binding sites [12]. Using numbering based on rat liver sequence, these sites include: Arg-352, Lys-356, and Arg-360, residues shown to bind substrate and/or product which are found on a surface loop of all mammalian FBPases; His-258, Glu-327 and His-392, a trio of catalytic residues in rat liver [30,12]; Arg-195, a key residue involved in Fru-6-P binding [31]; Arg-257 and Arg-307, which associate with the reactive C-2 phospho group of Fru-2,6-P_2 [30,32]; and the ATP binding site, Gly-Leu-Pro-Ala-Arg-Gly-Lys-Thr [33]. Underlined residues represent potential phosphorylation sites.

the general information available for the bovine clones indicates that a similar transcript without exon 16 and an extended version of exon 15 has been observed, although the predicted protein from the bovine sequence was 517 amino acids in comparison to the 519 amino acids predicted by the mouse clone (including initiation methionines).



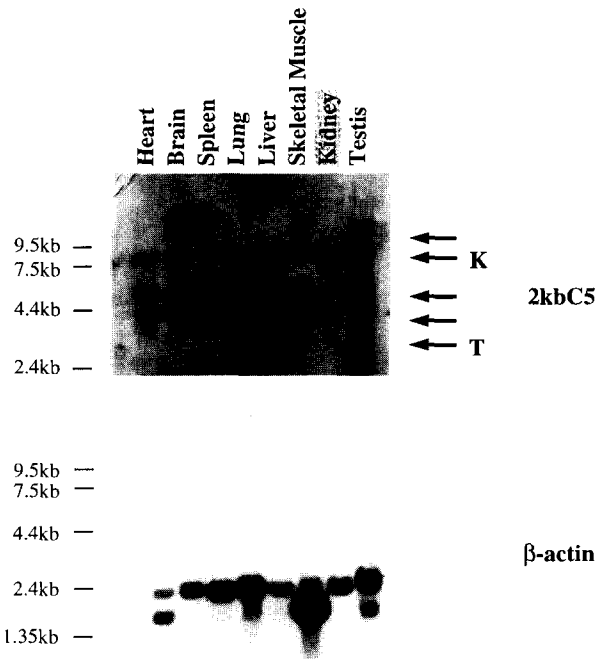


Fig. 4. Northern blot analysis of poly-A-enriched RNA isolated from multiple mouse tissues using mouse 2kbC5 cDNA probe (top) and β-actin control probe (bottom). Prominent bands are indicated by arrows. 2kbC5 appears likely to have been derived from the ~8 kb transcript (indicated as K) which has the strongest signal in kidney and may be the heart-type isozyme preferentially expressed in this tissue. T indicates a 3 kb band observed in testis only. Probing with β-actin cDNA control probe revealed that loading of the skeletal muscle track was significantly higher than in others. The actual representation of transcripts in this tissue may therefore be weaker than exhibited on the blot. Positions and sizes in kilobases of RNA ladder marker are indicated on the left.

Comparison of the protein sequence of the mouse heart-type isozyme with those of other PFK-2/FBPase-2 isozymes indicates that the active site residues are highly conserved between the isozymes with different regulatory domains at the N- and C-termini (Fig. 3). PFK-2/FBPase-2 catalytic and substrate binding site residues have been identified in rat liver [12] and the majority of these sites were found to be conserved throughout the mammalian isoforms, including the newly isolated mouse heart-type clone. Comparison of the predicted amino acid sequence of mouse heart-type clone to previously characterized PFK-2/FBPase-2 isozymes in other species reveals interesting homologies with respect to evolution of the enzyme. The conservation and assembly of separate domains evidenced by the various isozyme forms makes a strong case to support the exon shuffling hypothesis [11,29]. Future characterization of tissue-specific expression mechanisms for PFK-2/FBPase-2 should yield fascinating insights into the evolution of the enzyme.

The detection of multiple bands in Northern blots probed with the heart-type clone 2kbC5 is consistent with the identification of at least five mRNAs derived from gene B [28]. The entire PFK-2/FBPase-2 heart-type transcript predicted to correspond to 2kbC5 (due to its intense signal in kidney) detected on the Northern blot appeared to be approx. 8 kb, even though the coding region is confined to ~1.6 kb. This size discrepancy suggests a large untranslated region of the transcript. 76 bp of untranslated exon 1 as well as 336 bp of 3′ untranslated region have been isolated, yet the poly-A+ tail has not yet been reached nor has any polyadenylation signal (AATAAA) been identified at the 3′ end. Further characterization of the untranslated regions may provide interesting insights into tissue-specific control of expression.

Further studies of PFK-2/FBPase-2 expression in kidney are required to characterize thoroughly the multiple isozyme forms present in this tissue. This in turn should help to elucidate the overall organization of this complex regulatory system.

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