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Cloning and expression of novel isoforms of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase from bovine heart***

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Distinct 6-phosphofructo-2-kinase (PFK-2)/fructose 2,6-bisphosphatase (FBPase-2) cDNAs were cloned from bovine heart, showing that PFK-2/FBPase-2 gene B, which contains 16 exons, codes for at least five mRNAs. Three of them (B1, B2, B4) could encode the 58,000-M_r isozyme. In B2 mRNA, exon 15 encodes four more residues than in B1. In B4 mRNA, exon 15 encodes six more residues than in B1, but exon 16 (20 residues) is missing. B3 mRNA corresponds to the 54,000-M_r isozyme. It lacks exon 15 and also differs from the other mRNAs in the 5′ noncoding region. B5 mRNA encodes a truncated form. When expressed in *E. coli*, the recombinant isoforms corresponding to all these mRNAs except B5 exhibited PFK-2 activity.

Fructose-2,6-bisphosphatase; Fructose 2,6-bisphosphate; Glycolysis; 6-Phosphofructo-2-kinase; Splicing; Bovine heart

1. INTRODUCTION

The synthesis and degradation of fructose-2,6-bisphosphate, a potent stimulator of 6-phosphofructo-1-kinase, are catalyzed by 6-phosphofructo-2-kinase (PFK-2, EC 2.7.1.105) and fructose-2,6-bisphosphatase (FBPase-2, EC 3.1.3.46), respectively. These two activities are borne by each subunit of the homodimeric bifunctional enzyme PFK-2/FBPase-2. The existence of several PFK-2/FBPase-2 isozymes that differ in tissue-specific expression and response to hormonal signals [1] provides a mechanism for the local and temporal control of glycolysis. Changes in heart glycolysis in response to substrates such as glucose and ketone bodies, to epinephrine and insulin, and to workload correlate with changes in fructose-2,6-bisphosphate concentration [2–5]. Bovine heart PFK-2/FBPase-2 is phosphorylated on multiple sites by cAMP-dependent protein kinase (PKA), which stimulates PFK-2 activity, by protein kinase C (PKC), and by Ca^{2+}-calmodulin kinase [5–7]. Analysis of purified bovine heart PFK-2/FBPase-2 by SDS/PAGE revealed two isozymes with M_r values of 58,000 and 54,000 [8]. These isozymes derive from the same gene, called gene B [9], by alternative splicing [10,11], the smaller isozyme lacking a peptide that contains sites for phosphorylation by PKA and PKC. This peptide corresponds to exon 15 of the rat gene, which contains 16 exons [9]. A cDNA fully coding for bovine heart PFK-2/FBPase-2 and containing exon 15 has been described [12]. This cDNA predicts a subunit of 60,679 Da. Removal of exon 15 would yield a subunit of 53,909 Da. We decided to clone the mRNAs encoding the 54,000- and 58,000-M_r isozymes and to express them separately. In the course of this work, we discovered that bovine heart actually contains at last five PFK-2/FBPase-2 mRNAs that differ in coding sequence.

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***The following accession numbers have been assigned to the sequences mentioned in this article: X74563 applies to *B. taurus* (clone 24) mRNA for 6-phosphofructo-2-kinase, exon 1; and X74564 applies to *B. taurus* (clone 15-2) mRNA for 6-phosphofructo-2-kinase, exon 1.

Abbreviations. FBPase-2, fructose-2,6-bisphosphatase; ORF, open reading frame, PCR, polymerase chain reaction; PFK-2, 6-phosphofructo-2-kinase; PKA, cyclic AMP-dependent protein kinase; PKC: protein kinase C.

2. EXPERIMENTAL

2.1. *Materials*

Bacterial media were from Gibco-BRL. Hybond N^+ membranes, [α-^{32}P]dCTP, [^{35}S]dATPαS, T7 DNA polymerase and T4 DNA ligase were from Amersham. The pBluescript (KS)II$^+$ phagemid and the *E. coli* cloning host XL1-Blue were from Stratagene. The helper phage R408 was from Promega. The expression vector pET3a and the *E. coli* hosts BL21(DE3), BL21(DE3)pLysE and BL21(DE3)pLysS, were gifts from Dr. Studier [13]. Restriction enzymes were from Boehringer and synthetic oligonucleotides were from Pharmacia LKB Biotechnology Inc. Other reagents were from Boehringer or Sigma.

2.2. *Library screening and sequencing of bovine heart PFK-2/FBPase-2 clones*

The λgt11 bovine heart cDNA library (from Clontech) was screened by hybridization to a rat heart cDNA probe (RH1–9) corresponding

to exons 1 to 9 of PFK-2/FBPase-2 gene B [9]. The probe was labeled with [α-^{32}P]dCTP using a random oligo-labeling kit (Amersham) and was hybridized overnight at 65°C in 3.5 × SSC, 0.5% SDS, 2 mM EDTA, 25 mM sodium phosphate and 1 × Denhardt's solution, in the presence of herring sperm DNA at 100 μg/ml [14]. Phage plaques were purified and cDNA inserts were subcloned in pBluescript and sequenced using the reagents from US Biochemicals, except that Sequenase was replaced by T7 DNA polymerase. Preparation of total bovine heart RNA, reverse transcription and polymerase chain reaction (PCR) were performed [11] with the oligonucleotides described in the text.

2.3. *Directed mutagenesis and construction of the expression plasmids*

Fully coding cDNAs corresponding to each isoform were reconstituted in the (+) strand of pBluescript. Clone 18-2 was chosen to provide the left half of all the isoforms i.e. from the ATG initiation codon in exon 2 to the *Eco*RI site located 438 nucleotides downstream from it. Clone 18-2 was first mutated [15] with an Amersham kit to insert *Hin*dIII/*Nde*I sites just before the initiation codon. The coding sequence (exons 2–6) was isolated after *Hin*dIII–*Eco*RI digestion and inserted in pBluescript in the (+) orientation, yielding the MUT18-2 phagemid. Clones corresponding to the right half of the isoforms, i.e. from *Eco*RI to the 3'-end of the cDNAs, were mutated to insert a *Bam*HI site just downstream from the stop codon. The fragments containing the coding sequence were excised by *Eco*RI–*Bam*HI digestion and inserted into MUT18-2 to produce the fully coding phagemids. All mutagenesis and subcloning procedures were verified by sequencing. The inserts corresponding to the fully coding sequence of each isoform were purified after restriction by *Nde*I and *Bam*HI and ligated individually in the pET3a plasmid. *E. coli* of the BL21 strain were transformed with these expression vectors and recombinant protein expression was induced with isopropyl β-D-thiogalactopyranoside.

3. RESULTS

3.1. *Characterization of the mRNAs*

At the tertiary screening of the bovine heart cDNA library with the RH1–9 probe 28 positive out of 1.3×10^6 clones were obtained. The positive clones were classified by hybridization with oligonucleotide probes, one specific for exon 5, one for exon 15, and one for exon 16. Seven clones were positive with the three probes, two with the probes for exons 5 and 16, six with the probes for exons 5 and 15, and thirteen with the probe for exon 5. Subcloning and sequencing of clones representative of these four families showed that, besides the mRNA cloned by Sakata and Uyeda [12], there exist at least four other types of heart PFK-2/FBPase-2 mRNAs. The data in Fig. 1 show how the novel mRNAs identified here (B2 to B5) differ from the one (B1) characterized earlier [12]. A comparison of their cDNA sequence with that of gene B [9] showed that they all derive from gene B, hence the nomenclature proposed.

B2 mRNA is identical to B1 except for 12 nucleotides at the junction between exons 15 and 16 (Fig. 1). This results from the use of a more distal splice site in exon 15 (Fig. 2), which is therefore called 15a in B1 mRNA and 15b in B2 mRNA. Thus, B2 mRNA encodes a peptide (H61) four residues longer than that (H60) coded by B1 mRNA (530 residues).

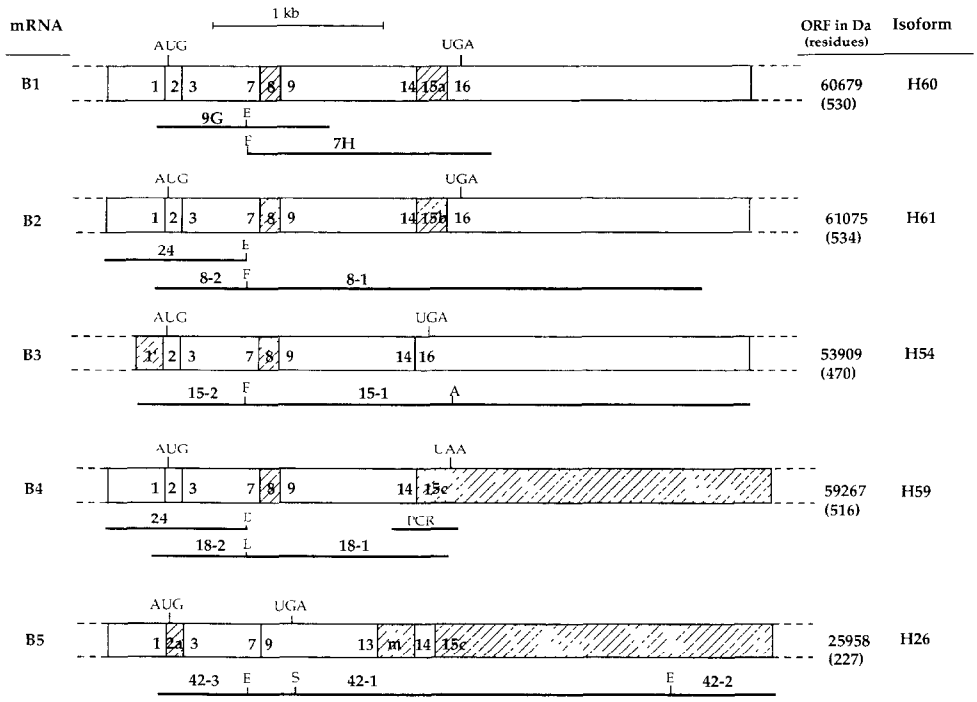

Fig. 1. Structure of the mRNAs for the heart isoforms of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase. The boxes, in which numbers designate the exons, correspond to the mRNAs. Hatched zones and lower case letters refer to sequences that differ between the mRNAs; m, intron. The lines between the boxes correspond to the cDNA clones. Clones 9G and 7H were described earlier [12]. E, *Eco*RI; A, *Ava*I; S, *Stu*I. The number of residues and molecular mass of peptides encoded by the ORFs discounts the initial methionine.

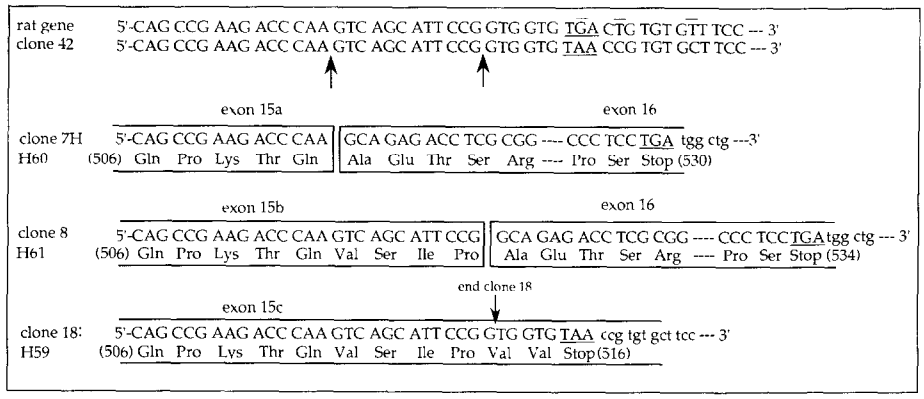

Fig. 2. C-terminal organization of the H60, H61 and H59 isoforms in the coding region. The upper line refers to the rat gene sequence in the exon 15 region, where the mismatches with the sequence of clone 42 are overlined. Arrows indicate the position of the splice sites. Numbers in parentheses refer to position of the residue. To save space, 13 residues have been omitted within the sequence of exon 16. The sequence of clone 7H is from [12]

B3 mRNA does not contain exon 15, reducing the predicted peptide (H54) to 470 residues. Its mass (53,909 Da) fits with the subunit M_r of 54,000 determined for the smaller heart isozyme. Its sequence indeed contains the peptides that we identified by microsequencing of the 54,000-M_r isozyme [11]. B3 mRNA also differs from the other mRNAs at the 5′ end, upstream from the 21 nucleotides that precede the AUG initiation codon. Thus, B3 mRNA contains an alternative 5′ noncoding exon (exon 1′), so that it could arise from a different primary transcript of gene B through use of another promoter. Alternatively, all the mRNAs described here could derive from a single primary transcript starting with (a) common noncoding exon(s), not present in our cDNAs, spliced with either exon 1 or exon 1′.

The sequence of the cDNA corresponding to B4 mRNA was identical to that of B2 mRNA except that it ended on the 3′ side one nucleotide (G) beyond exon 15b (Fig. 2). Four out of the six clones that were positive with the probes for exons 5 and 15 were sequenced; all terminated at this same nucleotide. The presence of the *Eco*RI linker (CCCGAATTC) used to construct the library just after this G indicated that these clones ended prematurely because of a cloning artifact. We investigated whether this G was the first of a broken exon 16, or was followed by a novel coding sequence, or else belonged to an extended version of exon 15b, in which case gene B predicts a stop codon six nucleotides downstream (Fig. 2). The latter possibility was examined for three reasons. First, it predicted a C-terminal sequence (VSIPVV) identical to that of a PFK-2/FBPase-2 derived from a rat heart cDNA [16]. Second, the predicted peptide had a size (59,267 Da) close to that of the 58,000-M_r isozyme. Third, it fitted with the sequence of our clone 42, which extends further downstream than clone 18 and is devoid of exon 16. To test these hypotheses we amplified by PCR a cDNA obtained on total heart RNA by reverse transcription from a primer (A in Fig. 3) complementary to a sequence of clone 42 located 66 nucleotides downstream from the putative TAA stop codon. Amplification between primer C, corresponding to a sequence in exon 13, and primer B, located just 5′ to primer A, yielded the expected band of 374 nucleotides and a band of 113 nucleotides (Fig. 3). These PCR products were cloned and sequenced. The 113-nucleotide band was nonspecific, while the sequence of the 374 nucleotide-long product perfectly matched the expected one. This supports the existence, in bovine heart, of a mRNA (B4) coding for a PFK-2/FBPase-2 isoform that contains an extended version of exon 15 and is devoid of exon 16. The stop codon is located in exon 15 (Fig. 2), which is longer than in B2 mRNA and is therefore called here 15c (Figs. 1 and 2). The peptide (H59) predicted from B4 mRNA is 516 residues long and is 59,267 Da. This is closer to the 58,000-M_r isozyme described in bovine heart than the longer peptides predicted from B1 and B2 mRNAs.

The sequence of clone 42 predicted an mRNA similar to that of B4 mRNA, i.e. with exon 15c and without exon 16. However, exon 8 was missing, leading to a change of reading frame in exon 9 where a stop codon is encountered after 177 nucleotides. In addition, clone 42 lacked a TGT codon, which corresponds to Cys^{28} at the end of exon 2. This deletion, which does not change the ORF, probably results from the choice of another splice site, exon 2 being replaced by a slightly shorter version called here exon 2a. The ORF resulting from the lack of exon 8 predicts a peptide (H26) of 227 residues (25,958 Da) whose sequence is identical to the first 168 residues of the other isoforms described above, without Cys^{28}, followed by an unrelated sequence of 59 residues: (169)GSFFHQGDKRGPEISREQSPGLHPEQDRLLPHEYPRPPSHHLPLPAWRERVQPLGEDWG(227). This truncated isoform is expected to lack PFK-2 and FBPase-2 activity. Indeed, some of the residues that are essential for PFK-2 activity are located between positions 168 and 250, while the FBPase-2 domain starts

beyond position 250 [17]. Finally, the 3′ noncoding sequence of clone 42 was interrupted between exons 13 and 14 (Fig. 1) by 252 nucleotides that correspond to intron *m* of gene B [9].

3.2. *Expression of the isoforms*

To express the different isoforms as recombinant proteins, the relevant parts of the cDNA clones were isolated from pBluescript and fully coding cDNAs were reconstituted in the (+) strand to obtain at the same time a tool for future site-directed mutagenesis of the isoforms. Phagemid B2 was constructed by inserting the coding sequence of the *Bam*HI-mutated clone 8-1 into the MUT18-2 phagemid (see Section 2). Phagemid B1 was obtained in the same way except that clone 8-1 underwent a second directed mutagenesis with a 29-mer oligonucleotide to delete the 12 bp that make the difference between exon 15b and exon 15a. For phagemid B3, the mutagenesis failed. Instead of introducing a *Bam*HI site, we therefore used the *Ava*I site in clone 15–1, located 123 nucleotides downstream from the stop codon. Phagemid B4 was constructed by inserting into MUT18-2 clone 18-1 to which the 5 nucleotides and the stop codon missing at the end of exon 15c, followed by a *Bam*HI site, were added by mutagenesis using a 40-mer oligonucleotide. In phagemid B5, we inserted the cDNA sequence encoding the truncated peptide by cloning in MUT18-2 a fragment of clone 42-1 extending from the *Eco*RI site to a *Stu*I site located 5 nucleotides beyond the stop codon of the ORF.

BL21 bacteria were transformed with the appropriate pET3a vectors and expression of the different isoforms was induced. PFK-2 activity, which is absent in untransformed bacteria, was assayed in bacterial lysates prepared as described [18]. The results of Table I show that

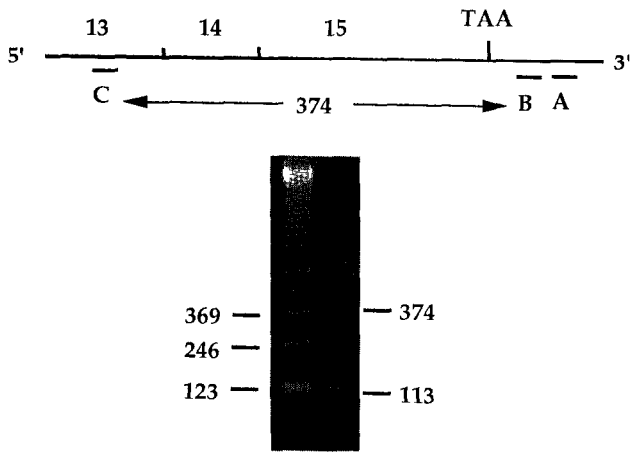

Fig. 3. Characterization by PCR of the C-terminus of the H59 isoform. Ethidium bromide staining of the 2% agarose gel electrophoresis of the PCR products with the nucleotide ladder on the left. The relevant region of mRNA B4 is depicted, with exons 13, 14 and 15c indicated. A, B and C correspond to the oligonucleotide primers used. The number between arrows refers to the number of nucleotides expected from the cDNA sequence

Table I

PFK-2 activity in the supernatant of lysates of *E. coli* transformed with the recombinant heart isoforms

Isoform	Activity	Specific activity	Recovery in supernatant	PFK-2 per l of culture*	
	munit	munit/mg protein	%	unit	mg
H60	111	4.6	80	2.4	48
H61	102	4.5	87	2.3	46
H54	17	0.8	18	0.4	8
H59	78	2.5	85	1.7	34
H26	0	0	0	0	0

Measurements were made on 45 ml of culture. One unit corresponds to the formation of 1 μmol of product per min.

*Calculated assuming the specific activity of pure enzyme is 50 mU/mg [8,11]

all the isoforms, except H26, exhibited PFK-2 activity as expected from their predicted sequence. The presence of the H26 isoform in the lysate was confirmed by Western blotting using an antibody directed against a synthetic peptide corresponding to residues 181–193, which are located in the specific region of this isoform (data not shown). Almost all the PFK-2 activity was recovered in the supernatant of the lysates for the H61, H60 and H59 isoforms, while that of the H54 isoform was mainly found in inclusion bodies. This could result from the decrease in overall charge of the protein (−2 for H54 vs. +5 for H61 and H60 and +4 for H59, at pH 7), due to the absence in H54 of the 60 residues coded by exon 15. Purification of these isoforms in order to determine their individual kinetic properties is in progress.

4. DISCUSSION

Biochemical studies have defined in bovine heart two PFK-2/FBPase-2 isozymes with subunit M_r of 58,000 and 54,000 [8]. The cDNA cloned earlier from that tissue, which corresponds to mRNA B1, predicts a peptide (H60) of 60,679 Da [12] and is encoded by 16 exons of gene B [9]. We report here a novel cDNA predicting a mRNA (B2) and a peptide (H61) with four additional residues because of the use of distinct splice sites at the end of exon 15. We also demonstrate by PCR that bovine heart contains a mRNA (B4) in which none of these splice sites is used and is therefore devoid of exon 16. This mRNA encodes a peptide (H59) of 59,267 Da. Thus, at least three peptides (H61, H60, H59), which probably originate from the same primary transcript, may account for the 58,000-*Mr* isoform of bovine heart PFK-2/FBPase-2. Which of these three isoforms is expressed in vivo is unknown. H59 is the more likely candidate because its molecular mass is close to that of the purified protein and its C-terminal sequence corresponds to that of rat heart PFK-2/FBPase-2 [16]. Be-

cause of splicing with exon 16, the sequence coded by exon 15 is shorter (by 2 residues) in H61, and even shorter (by 6 residues) in H60, than in the H59 isoform. Although these deletions do not contain the phosphorylation sites, the absence of Pro^{514} in H60 might influence the flexibility of the peptide in this region. It is also of interest that the 3′ trailer of the mRNAs bearing exon 16 (B1, B2 and B3) differs from that of B4 mRNA. Because the 3′ end of the mRNA contains signals that control mRNA stability, translation and/or location within the cell [19], this difference might influence the expression of the 58,000-*Mr* isoform of PFK-2/FBPase-2 in heart.

Using PCR and microsequencing strategies, the 54,000-*Mr* isozyme was identified earlier as a product of gene B but lacking exon 15, which results in the deletion of 60 amino acids [10,11]. We have now cloned the cDNA corresponding to the mRNA (B3) coding for this short isozyme (called here the H54 isoform) and confirmed that it results from the splicing of exon 14 with exon 16. This probably leads to differences in sensitivity to regulatory signals between H54 and the other isoforms. Indeed, exon 15 codes for Ser^{466} which is phosphorylated by PKA and PKC, for Thr^{475} which is phosphorylated by PKC, and for Ser^{483} which is phosphorylated by PKA [7,8,11]. Thus, the H54 isoform lacks all these sites although it still shares with the H59, H60 and H61 isoforms Ser^{84}, which is phosphorylated by PKC [7]. The difference in C-terminal sequences also bears on the overall subunit charge (see above), which is liable to change upon phosphorylation.

The structure of B5 mRNA, originating from the same primary transcript as the H61, H60 and H59 isoforms, predicts a truncated peptide called here H26. This peptide entails exons 2 to 9 with loss of exon 8 and of residues that are essential for PFK-2 activity. The recombinant protein indeed lacked PFK-2 activity. We have detected a B5-type mRNA in rat heart and other tissues (unpublished).

Although none of our cDNA clones ends with a poly(A) tail, the overall length of the mRNAs derived from them (3.9 kb) is close to that (about 4 kb) determined by Northern blot analysis of bovine heart RNA [12]. These clones now provide about 1.65 kb of additional sequence in the identical 3′ trailer of the mRNAs B1, B2 and B3, and almost all (1.9 kb) the 3′ trailer of the mRNAs B4 and B5. A comparison of their sequence with that of the rat gene B [9] failed to reveal a 3′ coding exon that would match the ORF found beyond exon 15 in that gene. Likewise, no ORF corresponding to the bovine exon 16 has been identified so far in the rat gene. This comparison also shows the occurrence in bovine heart PFK-2/FBPase-2 mRNAs of a 5′ noncoding exon as is the case in the rat. The clones described here extend the known sequence of exon 1 from 5 bp [12] to 330 bp and yield 41 bp of the novel exon 1′. They also provide tools to investigate the short-term and long-term regulation of PFK-2/FBPase-2 in heart. Glycolysis in this tissue may require regional control within the organ [20]. The different PFK-2/FBPase-2 isoforms characterized here might participate in this control.

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