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Complete nucleotide sequence coding for rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase derived from a cDNA clone

M.I. Darville, K.M. Crepin, J. Vandekerckhove*, J. Van Damme*, J.N. Octave[+], M.H. Rider, M.J. Marchand, L. Hue and G.G. Rousseau

Hormone and Metabolic Research Unit, International Institute of Cellular and Molecular Pathology, [+] Unité de Biologie et de Pharmacologie Cellulaires, Louvain University Medical School, B-1200 Brussel and **Laboratory of Genetics, Rijksuniversiteit, B-9000 Gent, Belgium*

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cDNA clones for 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase were isolated from rat liver expression libraries in λgt11 by antibody, oligonucleotide, and cDNA screening. One 1860 bp long clone contained a full-length nucleotide sequence coding for the 470 amino acids of each of the two identical subunits of the bifunctional enzyme. This clone also contained untranslated sequences, one 173 bp long upstream from the ATG start codon and one 271 bp long downstream from the TGA stop codon. The clone was terminated by a poly(A) tail of 29 nucleotides.

cDNA; 6-Phosphofructo-2-kinase; Fructose-2,6-bisphosphatase; (Rat liver)

1. INTRODUCTION

Fructose 2,6-bisphosphate has been identified in all eukaryotic tissues and is a potent stimulator of 6-phosphofructo-1-kinase. Its synthesis and degradation are catalysed by 6-phosphofructo-2-kinase (EC 2.7.1.105; PFK-2) and fructose 2,6-bisphosphatase (EC 3.1.3.46; FBPase-2), respectively. In rat liver, both reactions are catalysed at separate sites on the same 55 kDa polypeptide chain, two of which make up the native bifunctional enzyme (reviews [1,2]). In purified enzyme preparations, the PFK-2/FBPase-2 activity ratio of the heart enzyme is at least one order of magnitude greater than that of the liver enzyme, whereas the muscle enzyme contains more bisphosphatase than kinase activity. Indeed, these and other data suggest the existence of isozymes [3,4]. Liver PFK-2/FBPase-2 is a substrate of the cAMP-dependent protein kinase which activates the bisphosphatase and inactivates the kinase as a result of protein phosphorylation. In HTC cells, PFK-2 activity is increased by glucocorticoids [5], while in chick embryo fibroblasts, PFK-2 is activated by growth factors, phorbol esters [6], and tyrosine-specific oncogenic protein kinases [7]. As a first step in the understanding of the structure-activity relationships for the PFK-2/FBPase-2 isozymes and to study their regulation we have undertaken the present work. We report here the complete nucleotide sequence of the rat liver bifunctional enzyme derived from cDNA clones.

Correspondence address: G.G. Rousseau, UCL-ICP 7529, avenue Hippocrate 75, B-1200 Brussels, Belgium

The nucleotide sequence presented here has been submitted to the EMBL/GenBank database under the accession number Y00702

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   1  CAAAAGAGGGAGCTGGAGATAATCTGTGAGAGGCTCCGAAGCCCAGTACATCCCCCTGACTCAGC
  66  CCTGCCCACCTGCTGCTGTGACTACAGCAACAGACAAGACAGCTAGGAAGATAGGAAGTGAGGTCC
 132  AATACCTTGTGGGCAGTGGTGTCATTAGCTGCAACGCCTAAGATGTCTCGAGAGATGGGAGAACTC
                                          S  R  E  M  G  E  L      7
 198  ACTCAAACCAGGTTACAGAAGATCTGGATTCCACACAGCAGCAGTAGCAGCGTGCTGCAACGGCGA
      T  Q  T  R  L  Q  K  I  W  I  P  H  S  S  S  S  S  Y  L  Q  R  R     29
 264  AGGGGCTCCTCCATACCACAGTTCACTAATTCTCCCACGATGGTGATCATGGTGGGTTTACCAGCT
      R  G  S  S  I  P  Q  F  T  N  S  P  T  M  V  I  M  V  G  L  P  A     51
 330  CGAGGCAAGACCTACATCTCTACGAAGCTCACACGCTATCTCAACTGGATAGGAACACCAACTAAA
      R  G  K  T  Y  I  S  T  K  L  T  R  Y  L  N  W  I  G  T  P  T  K     73
 396  GTGTTTAATTTAGGTCAGTATCGACGAGAGGCAGTGAGTTACAGGAACTATGAATTCTTTCGCCCA
      V  F  N  L  G  Q  Y  R  R  E  A  V  S  Y  R  N  Y  E  F  F  R  P     95
 462  GACAACACAGAGGCCCAGCTTATCAGGAAGCAGTGTGCTCTAGCAGCCCTAAAGGATGTCCATAAG
      D  N  T  E  A  Q  L  I  R  K  Q  C  A  L  A  A  L  K  D  V  H  K    117
 528  TATCTCAGCCGCGAGGAAGGTCATGTTGCGGTTTTTGATGCCACCAACACTACCAGAGAACGACGA
      Y  L  S  R  E  E  G  H  V  A  V  F  D  A  T  N  T  T  R  E  R  R    139
 594  TCGTTGATTCTACAGTTTGCTAAGGAACATGGTTATAAGGTCTTCTTTATTGAGTCTATTTGTAAT
      S  L  I  L  Q  F  A  K  E  H  G  Y  K  V  F  F  I  E  S  I  C  N    161
 660  GACCCCGAAATCATTGCAGAAAACATCAAGCAAGTGAAACTTGGTAGTCCTGATTACATAGACTGT
      D  P  E  I  I  A  E  N  I  K  Q  V  K  L  G  S  P  D  Y  I  D  C    183
 726  GACCAAGAAAAGGTTTTGGAAGACTTTCTAAAGAGAATAGAGTGCTATGAGATCAACTACCAACCT
      D  Q  E  K  V  L  E  D  F  L  K  R  I  E  C  Y  E  I  N  Y  Q  P    205
 792  TTGGATGAGGAATTGGACAGCCACCTGTCCTACATCAAGATCTTCGACGTGGGCACACGCTACATG
      L  D  E  E  L  D  S  H  L  S  Y  I  K  I  F  D  V  G  T  R  Y  M    227
 858  GTAAATCGAGTGCAGGACCACGTTCAGAGCCGTACAGCCTACTACCTCATGAACATCCATGTCACA
      V  N  R  V  Q  D  H  V  Q  S  R  T  A  Y  Y  L  M  N  I  H  V  T    249
 924  CCTCGATCTATCTACCTATGCCGCCATGGTGAGAGTGAACTCAACCTTAGAGGCCGCATTGGAGGT
      P  R  S  I  Y  L  C  R  H  G  E  S  E  L  N  L  R  G  R  I  G  G    271
 990  GACTCTGGCCTCTCAGCTCGGGGCAAGCAGTATGCCTATGCACTAGCCAACTTCATCCGGTCTCAA
      D  S  G  L  S  A  R  G  K  Q  Y  A  Y  A  L  A  N  F  I  R  S  Q    293
1056  GGCATCAGCTCCCTGAAAGTATGGACTAGCCACATGAAGAGGACCATTCAGACCGCTGAAGCCCTA
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     G  I  S  S  L  K  V  W  T  S  H  M  K  R  T  I  Q  T  A  E  A  L      315
1121 GGTGTCCCCTATGAACAGTGGAAGGCCCTGAATGAGATTGATGCGGGTGTCTGTGAAGAGATGACC
     G  V  P  Y  E  Q  W  K  A  L  N  E  I  D  A  G  V  C  E  E  M  T      337
1188 TATGAAGAAATTCAGGAACACTACCCTGAGGAATTTGCACTACGGGACCAGGATAAATATCGTTAC
     Y  E  E  I  Q  E  H  Y  P  E  E  F  A  L  R  D  Q  D  K  Y  R  Y      359
1254 CGCTATCCCAAGGGAGAGTCCTATGAGGATCTGGTTCAGCGTCTTGAACCAGTTATAATGGAGCTA
     R  Y  P  K  G  E  S  Y  E  D  L  V  Q  R  L  E  P  V  I  M  E  L      381
1320 GAACGGCAAGAAAATGTACTGGTGATCTGTCACCAGGCTGTCATGCGGTGCCTCCTGGCATACTTC
     E  R  Q  E  N  V  L  V  I  C  H  Q  A  V  M  R  C  L  L  A  Y  F      403
1386 CTGGATAAAAGTTCAGATGAGCTGCCCTATCTCAAGTGTCCTCTGCATACTGTGCTCAAACTCACA
     L  D  K  S  S  D  E  L  P  Y  L  K  C  P  L  H  T  V  L  K  L  T      425
1452 CCTGTGGCTTATGGCTGCAGAGTGGAGTCCATCTACCTGAATGTGGAGGCTGTGAACACACACCGG
     P  V  A  Y  G  C  R  V  E  S  I  Y  L  N  V  E  A  V  N  T  H  R      447
1518 GACAAGCCTGAGAATGTGGACATCACCCGTGAAGCTGAGGAAGCCTTGGACACTGTACCTGCCCAT
     D  K  P  E  N  V  D  I  T  R  E  A  E  E  A  L  D  T  V  P  A  H      469
1584 TACTGAGCCCTTTTCAAGTGATCAGATTGTCTCTGTTCTCACCCCCTTCCTCCTGTAGGAGCTGCT
     Y                                                                      470
1650 GCCCTTGTTCTCCTTAAGCAGACTCTGGCTATGGCCTGAGAGTGCCCTACCTCCAGTGAAGAAGTC
1716 CGTAGCAGCTCCCAAACAGGTCTCAATTCCTAGCCACAACTAAGGCACCTACCTAGTTGTGGATAA
1782 AGTTCCTTTTAAATATTCTCTGATAAATAAAGATGTTTATTTCTGCCTATA(29)
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Fig.1. Nucleotide sequence of clone RL2K-22C and amino acid sequence derived for rat liver PFK-2/FBPase-2. Each amino acid is aligned with the first letter of the corresponding codon. Numbers on the left refer to nucleotides, those on the right to amino acids. The polyadenylation signal and the sequenced tryptic peptides are underlined. Boxes indicate the translation initiation and stop codons. The nucleotide sequence shown has been obtained either from both cDNA strands of appropriate fragments, or from one strand and confirmed by sequencing the corresponding fragments of clones RL2K-4C and (or) -5C. The 100 bp long sequence at the 5′-end of the clone has been obtained in the 5′ to 3′ direction.

2. EXPERIMENTAL

PFK-2/FBPase-2 was purified as described [8,9] from the livers of 25 male Wistar rats. The method includes two precipitation steps with polyethylene glycol followed by chromatography on DEAE-Trisacryl and Blue Sepharose. This yielded 2.3 mg protein (42 mU/mg) and showed one major 55 kDa band after SDS-PAGE. Rabbits were injected with the material eluted from this band and polyclonal antibodies against denatured PFK-2/FBPase-2 were raised. 300 μg PFK-2/FBPase-2 were digested with trypsin and the fragments separated by reversed-phase HPLC on a C-4 column. Nine peptides were selected for microsequencing using a gas-phase sequenator (Applied Biosystems, USA). Oligonucleotides based on the microsequence were purchased from Eurogentec (Liège, Belgium). Two rat liver cDNA expression libraries in λgt11 were screened. One, from a

Sprague-Dawley female rat, was purchased from Clontech. The other library (JNO library) was prepared from male Wistar rat poly(A)-containing RNA by oligo(dT) priming as in [10]. Phage and plasmid DNA was prepared [11] and M13 dideoxy sequencing was performed as described [12] using a kit from Amersham. Enzymes were purchased from BRL or Boehringer. ^{32}P-nucleotides were from Amersham. 1 × SSC solution contained 0.15 M NaCl and 0.015 M sodium citrate.

3. RESULTS AND DISCUSSION

3.1. *Isolation of a partial cDNA clone*

The antiserum used (IRL-5) was specific for PFK-2. First, it gave at 1:1500 dilution a positive ELISA signal with 1 ng native or denatured rat liver PFK-2 spotted onto nitrocellulose. Second, by Western blotting, it detected a single band at 55 kDa after SDS-PAGE of rat liver cytosol and a single spot after isoelectrofocusing in the second dimension. Third, this antiserum precipitated PFK-2 activity of rat liver cytosol in the presence of protein A-Sepharose. None of these effects were observed with nonimmune rabbit serum. The Clontech library was titrated in strain Y1090 and 5.6×10^5 recombinant clones were screened [13] with antiserum IRL-5 at 1:600 dilution preadsorbed on a Y1090 lysate, and revealed with a peroxidase-coupled anti-rabbit IgG antibody (BioRad). Three clones remained positive at the tertiary screening. The three clones (henceforth called RL2K-8) were identical because they had the same length (about 1100 bp), cross-hybridized in Southern blots, and displayed the same restriction pattern with *Bgl*II, *Pst*I and *Rsa*I.

Microsequencing of PFK-2/FBPase-2 yielded nine peptides. Based on codon usage frequency [14] a 16 amino acid long peptide was used to construct a 47-mer oligonucleotide probe (5′-TTCCACTGCTCATATGGCACGCCCAGGGC-CTCAGCTGTCTGGATGGT). This served to confirm the identity of clone RL2K-8 by in situ hybridization of bacteriophage plaques [11]. The 47-mer probe hybridized with RL2K-8 DNA in conditions (hybridization in 2 × SSC, 50°C; washings in 1 × SSC, 50°C) under which it did not hybridize with nonrecombinant DNA or with four unrelated clones. Further evidence that RL2K-8 was specific for PFK-2/FBPase-2 stemmed from the finding (see below) that it was identical to the PFK-2 clone obtained recently by Colosia et al. [15] from the same cDNA library.

3.2. *Characterization of a full-length cDNA*

Since clone RL2K-8 was too short to code for the entire PFK-2/FBPase-2 sequence, more clones were searched for by screening another rat liver cDNA library (JNO) with RL2K-8 after recloning in the *Eco*RI site of pBR322. From 5.4×10^5 recombinant clones, 11 were positive at the tertiary screening. Two of them, RL2K-4C and -5C, are currently being characterized. Clone RL2K-22C, which is 1860 bp long, was recloned in pBR322 and digested with *Eco*RI, *Pst*I and *Bgl*II to yield fragments suitable for subcloning in the M13 vector [16], and sequencing. The sequence of clone RL2K-22C is shown in fig.1. It extends from 173 bp upstream from the translation initiation site to a 29-mer poly(A) tail located 242 bp downstream from the TGA stop codon. The poly(A) tail is preceded by a poly(A) signal AATAAA 20 bp upstream. The ATG start site is preceded by a sequence compatible with the (C)(C)A(C)(C)ATG(G) consensus proposed [17] for translation initiation signals. The 1086 bp clone RL2K-8 spans from nucleotide 675 (amino acid 167) to 1760 (171 bp beyond the stop signal) of clone RL2K-22C. The 1410 bp translated sequence of clone RL2K-22C codes for a 470 amino acid protein of M_r 54570. The corresponding nucleotide sequence is identical to that of Colosia et al. [15] except for nucleotide 1262 which is C instead of G. All nine peptides, 113 amino acids altogether, derived from the sequence are found within this protein. Their position is shown in fig.1. The 47-mer probe corresponded to amino acids 308–323 and was 83% homologous to the actual nucleotide sequence. A search of the EMBL databank (release no.11, April 1987) revealed no homology greater than 60% with known nucleotide sequences. Northern blot analysis of poly(A)-containing RNA from rat liver yielded a major band corresponding to an mRNA about 2150 nucleotides long. Thus, the 5′-untranslated portion of PFK-2 mRNA is about 500 bp long.

The amino acid sequence obtained from the purified protein by Colosia et al. [15] is identical to that deduced from the nucleotide sequence shown in fig.1, except for amino acids 134 (Tyr instead of

Thr) and 137 (Phe instead of Glu). Whether this is due to a difference in rat strain (Wistar in fig.1 vs Sprague-Dawley in [15]) is not known. As the NH_2-terminal residue was previously identified as a blocked serine, the initiator methionine is removed prior to the final blocking of the protein. Three functional domains of the protein can be tentatively identified (see [15] for discussion). The PFK-2 domain contains the three cysteines at amino acid positions 160, 183 and 198. These three cysteinyl residues are clustered together in a 38-residue CNBr fragment which may be important in maintaining the conformation of the PFK-2 sugar phosphate binding site [18]. The FBPase-2 domain contains the critical histidyl residue 258. The third domain, near the amino-terminus, contains the site of phosphorylation (Ser 32) by the cAMP-dependent protein kinase. This site is immediately preceded by the sequence Arg-Arg-Gly which is typical [19] for conferring specific recognition by this kinase. Rat liver PFK-2 contains no consensus sequence [20] for recognition by protein kinase C, an enzyme that phosphorylates heart, but not liver PFK-2 [3]. However, the sequence 352–359 contains residues (Arg.X.X.Asp.X.X.X.Tyr) that could confer specific recognition by tyrosine-specific protein kinases [20]. The RL2K-22C clone should allow one to identify the transcription initiation site, to characterize the PFK-2 isozymes and mRNAs, to clone the corresponding gene(s), and to study their regulation.

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