Vol. 198, No. 1, 1994
January 14, 1994

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 335-340

MOLECULAR CLONING AND TISSUE SPECIFIC EXPRESSION OF FRUCTOSE 6-PHOSPHATE,2-KINASE:FRUCTOSE 2,6-BISPHOSPHATASE OF RAT BRAIN[1]

Fusao Watanabe, Akiko Sakai*, Eisuke Furuya*, and Kosaku Uyeda

Department of Veterans Affairs Medical Center and The University of Texas Southwestern Medical Center at Dallas, 4500 South Lancaster Rd., Dallas, TX 75216

*Department of Chemistry, Osaka Medical College, 2-41 Sawaraki-cho, Takatsuki, Osaka 569 Japan

Received November 23, 1993

A cDNA clone (3591 base pairs) encoding an isozyme of Fructose 6-phosphate, 2-kinase:Fructose 2,6-bisphosphatase was isolated from a rat brain cDNA library. The 5' sequence of this clone (1241 base pairs) was identical to that of the heart type isozyme cDNA (except for 7 base pair mismatches), but the 3' nucleotide sequence (2024 base pairs) was completely different. Its deduced amino acid sequence showed that the enzyme lacked a regulatory domain which contained phosphorylation sites for protein kinase A and C. The results of Northern blotting and polymerase chain reaction demonstrated that the mRNA, 7.4 kilobases long, was expressed also in heart, testis, liver, and skeletal muscle.

Fructose 2,6-bisphosphate is the most potent activator of phosphofructokinase and plays an important role in regulation of glycolysis (1). Its synthesis and degradation are catalyzed by a bifunctional enzyme, Fructose 6-phosphate,2-kinase:Fructose 2,6-bisphosphatase (Fru 6-P,2-kinase:Fru 2,6-Pase). The intracellular concentration of Fructose 2,6-bisphosphate is determined by the relative activities of the kinase and the phosphatase of the bifunctional enzyme. Thus far, four major isozymes of mammalian enzymes, including liver (2), skeletal muscle (3), heart (4), and testis (5) have been characterized.

[1]This work was supported by the Department of Veterans Affairs, NIDDK (DK 16194), and the Ministry of Education, Science and Culture of Japan (04680201).

Abbreviations: Fru 6-P 2-kinase:Fru 2,6-Pase, fructose 6-phosphate 2-kinase:fructose 2,6-bisphosphatase; PCR, polymerase chain reaction; bp, base pairs; kb, kilobase pairs.

0006-291X/94 $5.00

Recently, Ventura *et al.* (6,7) reported the occurrence of a unique isozyme in bovine and rat brain whose enzyme activities are not affected by protein kinase A or protein kinase C. They isolated an isozyme from bovine brain with a subunit M_r of 120,000 (6), which is 2× larger than the other known mammalian bifunctional enzymes.

Our earlier immunological studies[2] showed the presence of a heart type isozyme with subunit M_r=54,000 in rat brain. In order to characterize the brain-specific Fru 6-P,2-kinase:Fru 2,6-Pase further, molecular cloning of rat brain isozyme(s) was performed. In this paper, we present the identification of a new isozymic form in brain which was a product of a rat heart gene by an alternative splicing process. The results of cDNA and amino acid sequence determinations as well as tissue-specific expression are presented.

EXPERIMENTAL PROCEDURES

Materials - Reagents were obtained as follows: cDNA synthesis kit from Amersham Corporation (Arlington Heights, IL); restriction endonucleases from Toyobo (Tokyo, Japan) and New England BioLabs (Beverly, MA); SuperScript RNase H$^-$ reverse transcriptase from Gibco-BRL (Gaithersburg, MD), Taq DNA polymerase from Perkin-Elmer Cetus Instruments (Norwalk, CT).

Synthesis and PCR Amplification of cDNA - The oligo(dT)-primed cDNA was synthesized from poly(A)$^+$-RNA (1 μg), using 200 units SuperScript RNaseH$^-$ reverse transcriptase in 20 μl of reaction mixture according to the manufacturer's instructions. An aliquot (1 μl) of the reaction mixture was amplified in 100 μl of 10 mM Tris-HCl buffer (pH 8.3), containing 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (w/v) gelatin, 0.2 mM of each deoxyribonucleotide, 2.5 unit Taq DNA polymerase, and 1 μM oligonucleotide primers, 5'-CTCCACATCATCTTCAA-3', corresponding to #1447-1463 of RB10 cDNA and 5'-TGACAGCGTCAACAGGC-3', corresponding to #2512-2528 (8). The polymerase chain reaction (PCR) consisted of 30 cycles of denaturation at 94°C for 30 sec, primer annealing 53°C for 20 sec, and primer extension at 72°C for 1 min, with an additional 2 min primer extension included after the final cycle.

RESULTS AND DISCUSSION

Molecular Cloning of Fru 6-P 2-kinase:Fru 2,6-Pase cDNA from Rat Brain - cDNA was synthesized with oligo(dT) primer using rat brain poly(A)$^+$-RNA (5 μg) as a template. The cDNA library containing 3×10^6 independent clones was constructed using λgt10 phage vector and screened using as probes Fru 6-P,2-kinase:Fru 2,6-Pase cDNAs from bovine heart, human liver, and frog skeletal muscle (manuscript in preparation). Several

[2]Unpublished results of R. Sakakibara, M. Taniyama, and K. Uyeda.

screenings yielded five positive clones which harbored cDNA inserts ranging from 0.5 kilobase pairs (kb) to 3.4 kb. Four of these clones were found to share common DNA sequences by restriction enzyme mapping and partial nucleotide sequencing. RB10 containing the 3.4 kb insert was chosen for complete analysis. The fifth clone, RB7, contained a 0.9 kb insert that was not identical by restriction mapping to the other clones. None of the clones contained a large enough insert to encode the enzyme similar to that reported by Ventura *et al.* (6).

cDNA Sequence and Deduced Amino Acid Sequence of Rat Brain Fru 6-P, 2-kinase:Fru 2,6-Pase - The 5' region (308-1548 bp) of RB10 was identical to that of one of the heart isozyme cDNAs previously reported (5) except for 7 base mismatches (Fig. 1, underlined). This common 5' region includes the well-conserved catalytic domains and corresponds to a genomic sequence derived from rat heart exon 3 to exon 14 (9). It seems likely that this clone (derived from Sprague-Dawley rats in Japan) and the rat heart isozyme (derived from S.D. rats in US) represent the same gene, and the 7 base mismatches are probably due to differences in rat sources. However, there was no homology between the 3' regions (1549-3572 bp) of the clone and the rat heart cDNA. This disparate region of the rat heart cDNA is derived from exon 15 and 16 of the gene (9), and it encodes a polypeptide containing Ser and Thr which are known to be phosphorylated by cAMP-dependent protein kinase and protein kinase C (10). The nucleotide sequence analysis of the other three clones revealed that they also lacked this peptide. The 3' sequence of RB10 showed high homology with exon 16 of the bovine heart isozyme gene (manuscripts in preparation), but not with exon 16 of the rat heart gene (9). These results suggest that RB10 may arise by a tissue-specific alternative splicing process using an as yet unidentified exon of the rat heart gene.

The fifth clone, RB7, did not harbor a full length cDNA but contained only a coding region whose deduced amino acid sequence was 70-80% identical to the known mammalian bifunctional isozymes, including heart. These results suggested the existence of multiple isozymes of Fru 6-P,2-kinase:Fru 2,6-Pase in rat brain.

Northern Blotting of RB10 mRNA - To study the *in vivo* expression of RB10 DNA. Northern blotting hybridization was carried out with poly(A)$^+$-RNA isolated from various rat tissues. As shown in Figure 2, a single intense signal corresponding to 7.4 kb was clearly detected with mRNA from brain, heart, testis, and liver. The intensity of these bands was approximately the same, but that of skeletal muscle was too weak to be

```
gcctcactcgggtggtggcagtgttcaagaggactaactcttgtcatccggattccagtt   60
cgattcctcggatccaattaggaagacactccatcatgcgattggagtcctcgtcgtggc  120
ccagagatagaaagacggacggggaaggggagacctaaccagatagctaggtgcctcctga 180
agaactaccATGTCTGAGAATAGTACATTTTCCACAGAAGACAGCAGCAGCAGCAGCTAT  240
          M  S  E  N  S  T  F  S  T  E  D  S  S  S  S  S  Y
          1                          10
AAACCCCACGCCTCAAACCTCCGAAGGGCAGGGAAGAAATGCTCATGGGCTTCCTACATG  300
 K  P  H  A  S  N  L  R  R  A  G  K  K  C  S  W  A  S  Y  M
       20                         30
ACCAACTCCCCAACACTCATTGTTATGATTGGCTTGCCAGCCCGGGGTAAGACTTATGTG  360
 T  N  S  P  T  L  I  V  M  I  G  L  P  A  R  G  K  T  Y  V
       40                         50
TCCAAGAAATTAACACGCTACCTGAACTGGATTGGAGTACCTACCAAAGTGTTTAATCTT  420
 S  K  K  L  T  R  Y  L  N  W  I  G  V  P  T  K  V  F  N  L
       60                         70
GGTGTATATCGACGGGAAGCAGTCAAGTCCTATAAGTCCTATGACTTCTTTCGACATGAC  480
 G  V  Y  R  R  E  A  V  K  S  Y  K  S  Y  D  F  F  R  H  D
       80                         90
AATGAAGAGGCCATGAAGATCCGCAAACAGTGTGCCCTGGTGGCACTGGAAGATGTGAAG  540
 N  E  E  A  M  K  I  R  K  Q  C  A  L  V  A  L  E  D  V  K
      100                        110
GCCTACTTTACTGAAGAGAGTGGGCAGATCGCGGTGTTTGATGCCACCAATACCACTCGG  600
 A  Y  F  T  E  E  S  G  Q  I  A  V  F  D  A  T  N  T  T  R
      120                        130
GAGAGGAGGGACATGATTTTGAACTTTGCCAAGCAGAATGCCTTCAAGGTATTCTTTGTG  660
 E  R  R  D  M  I  L  N  F  A  K  Q  N  A  F  K  V  F  F  V
      140                        150
GAATCTGTGTGTGATGATCCTGATGTCATTGCTGCCAATATTCTGGAGGTAAAAGTGTCA  720
 E  S  V  C  D  D  P  D  V  I  A  A  N  I  L  E  V  K  V  S
      160                        170
AGCCCTGACTACCCCGAAAGGAATAGGGAGAATGTGATGGAGGACTTCCTGAAGAGAATT  780
 S  P  D  Y  P  E  R  N  R  E  N  V  M  E  D  F  L  K  R  I
      180                        190
GAGTGCTACAAGGTCACTTACCAGCCCCTTGACCCAGACAACTATGATAAGGACCTCTCG  840
 E  C  Y  K  V  T  Y  Q  P  L  D  P  D  N  Y  D  K  D  L  S
      200                        210
TTCATAAAGGTGATGAATGTAGGCCAGAGGTTTCTGGTCAACAGAGTTCAGGACTACATC  900
 F  I  K  V  M  N  V  G  Q  R  F  L  V  N  R  V  Q  D  Y  I
      220                        230
CAGAGTAAGATTGTCTACTACCTGATGAACATCCATGTCCATCCTCGCACCATCTATCTG  960
 Q  S  K  I  V  Y  Y  L  M  N  I  H  V  H  P  R  T  I  Y  L
      240                        250
TGCCGGCACGGAGAGAGCGAATTCAATCTTTTGGGAAAGATTGGGGGTGACTCTGGCCTT 1020
 C  R  H  G  E  S  E  F  N  L  L  G  K  I  G  G  D  S  G  L
      260                        270
TCGTTGCGAGGAAAGCAGTTTGCTCAGGCTCTGAAGAAGTTTCTGGAGGAACAGGAGATC 1080
 S  L  R  G  K  Q  F  A  Q  A  L  K  K  F  L  E  E  Q  E  I
      280                        290
CAGGACCTCAAAGTGTGGACAAGCCAGTTGAAGAGGACAATTCAGACTGCTGAGTCTCTT 1140
 Q  D  L  K  V  W  T  S  Q  L  K  R  T  I  Q  T  A  E  S  L
      300                        310
GGGGTGACCTATGAGCAATGGAAGATCCTAAATGAGATTGATGCTGGCGTGTGTGAGGAG 1200
 G  V  T  Y  E  Q  W  K  I  L  N  E  I  D  A  G  V  C  E  E
      320                        330
```

Figure 1. cDNA sequence of RB10 and its deduced amino acid sequence. Nucleotides are numbered in the 5' to 3' direction. The deduced amino acid residues are given in one-letter code and are indicated below the nucleotide triplets. Nucleotides representing the proposed coding region are capitalized. The nucleotides of 5'-region (308-1548 bp) of RB10 which show mismatch with rat heart cDNA are underlined.

shown in an autoradiogram. However, the same band was detected with skeletal muscle upon longer exposure. Ventura *et al.* (7) also reported a similar band at 6.8 kb in rat brain and heart using the rat liver bifunctional enzyme cDNA as a probe.

Tissue Specific Alternative Splicing of RB10 - In order to determine whether the mRNA that encodes the peptide containing the phosphorylation sites of the heart type enzyme

```
ATGACTTATTCGGAGATCGAACAACGGTATCCAGAGGAATTTGCACTTCGAGATCAAGAG 1260
 M  T  Y  S  E  I  E  Q  R  Y  P  E  E  F  A  L  R  D  Q  E
     340                             350
AAGTATCTGTATCGATATCCTGGTGGGGAGTCATACCAGGACCTGGTGCAGCGGCTGGAG 1320
 K  Y  L  Y  R  Y  P  G  G  E  S  Y  Q  D  L  V  Q  R  L  E
     360                             370
CCTGTGATCATGGAGCTGGAGCGGCAAGGCAACGTCCTCGTTATCTCTCACCAGGCTGTC 1380
 P  V  I  M  E  L  E  R  Q  G  N  V  L  V  I  S  H  Q  A  V
     380                             390
ATGCGCTGCCTCCTGGCCTACTTCTTGGATAAAGGCGCAGATGAGTTGCCGTACTTGAGG 1440
 M  R  C  L  L  A  Y  F  L  D  K  G  A  D  E  L  P  Y  L  R
     400                             410
TGCCCCCTCCACATCATCTTCAAACTTACTCCTGTGGCCTATGGTTGCAAAGTGGAAACA 1500
 C  P  L  H  I  I  F  K  L  T  P  V  A  Y  G  C  K  V  E  T
     420                             430
ATTACACTGAATGTGGAAGCTGTGGACACACATCGTGACAAGCCAACTGAAGTGGAGAAT 1560
 I  T  L  N  V  E  A  V  D  T  H  R  D  K  P  T  E  V  E  N
     440                             450
GTGCTGGCTAAGCATAGACGCCCCTCAATGGCATCCCTCACTTTGCTCTCCtgatgtgag 1620
 V  L  A  K  H  R  R  P  S  M  A  S  L  T  L  L  S
     460                             470         474
gctgaggccagacctctccgaggaactggatctaccaaaaagcttggcatgccagcatcc 1680
ctgaggctagaacaggaagttaagtgggaacgtctcatttgaggctaccaacagtaaggc 1740
gtgggaaaccttcactgcatttctacccctcatgcccaacaggaacattactacattgtg 1800
cctcaaaaagtcgtcaacagcctgagcaagttagttcttgtctcttttgaaccaaatttg 1860
caaatgccattctaacagaggcattcagcattctacctgtggccgactgactgcatcctt 1920
agctggagatttgcctaatgtgtgaatgcactgcttcagaattggtttctagatgagggt 1980
gaacttcaggaagccttttctgctccatgagagctgctcttttcaccggacggaggagag 2040
cagggtgaagtgcagtctgagtgctagtggtcgcctcctgcagtgtgggagatgagacag 2100
agcaccaggtgaatggagggcgtcggaaactaagctcgcaagtgctcttctctggtagca 2160
gaagagtagtgtccatggttcccttcccacatagagaagttttccttgttccatatcctt 2220
tggtgcagggtctggctgctggacttccccaaaggaccaacccttacattctctcgtagc 2280
tactgttttctctctgaggcttgtaactcacacagatagacaggactgaaccagtgcctc 2340
ctgaggagttgggactgaagggaccatctccaacccatgaagaaatagagatggcttctt 2400
ttttttttaaatttcaaaataggcagaagcagccatgcccctgtgtctagttgatgtcg 2460
tctgtggaaagctgtgcctgtgcctgcgggagcagtgggcctatgctcggtgcctgttga 2520
cgctgtcactcctgtgatcagcaggtctcccacagcacgtggcagatgcggcaaagccgt 2580
ggggcatctatgagtgagtgtctccttcagaagaatccgattgctgtccgcaggtcactg 2640
agtgtctgatgcatactcactattaccatgagggcacccctagtgttacttctgaagaag 2700
aatgcagaagccacaaagtgtactgtacctcaccagtgttactgtggttctttgttccat 2760
ttcactgaagctgcacacttgttgggcagaaagataagtagcaagaaaagtaataaaatg 2820
tgctttttctaaatgttctttcatactgtagacaatactggagactgttacccaaggagg 2880
atgtgatgagctgctggcagttcactgactcccaggctgctgcttgctttctatgtatgc 2940
atgtccctggagagaggctcctcctccctctgcctgggacaggcgtttccccgtggctg 3000
atgcagcatactgtacacagggttttttttcttgtggtataggatgctctctgcattgaa 3060
gtgcacccctttctgatgatcagatggtatttttatgaagctatttattttgtgtgagtt 3120
tgcatgtatttatcagaatgtcatttctgttctcagtgttctgctagcattgtaggcggc 3180
ttgaacgctgagcctaatgtctatagtattaccttaagttaaatgtgtagagcgtaaatg 3240
tttgtatatgtcaaagaatggtaactgttatgactgtagatttttcaatttttttttctta 3300
ttgattttattataaaccaagaaatgtatttctctggaaaacaggttctctcttagataa 3360
gtaaaaccatgctaaaatatctcaacaacatatactgcttttgtaataagaaaataaagt 3420
tatttttaattaaaaggtacaatgaaatttttcaattcgcatgcaaaaacacatcatttga 3480
tacattacatatgcacaaaatattaacattttccaaaatgtacttgaaagtttattaagg 3540
atgtttgaaattattaaatggtatctgtttccaaaaaaaaaaaaaaaaaaa          3591
```

Figure 1 - Continued

occurs in other rat tissue, mRNAs from brain, testis, liver, and skeletal muscle were analyzed by PCR. The cDNAs synthesized from those mRNAs were amplified with synthetic oligonucleotide primers corresponding to #1447-1463 and to #2512-2528 nucleotide residues of RB10. The PCR amplification detected a band (1.1 kb) in all lanes, the size expected if derived from mRNAs which lack sequences encoding the phosphorylation domain of the enzyme in these tissues (Fig. 3). An additional band of 1.3 kb was detected in rat heart and skeletal muscle (lane 2 and lane 5 in Fig. 3). The additional 0.2 kb DNA of the 1.3 kb band may correspond to the phosphorylation site peptide.

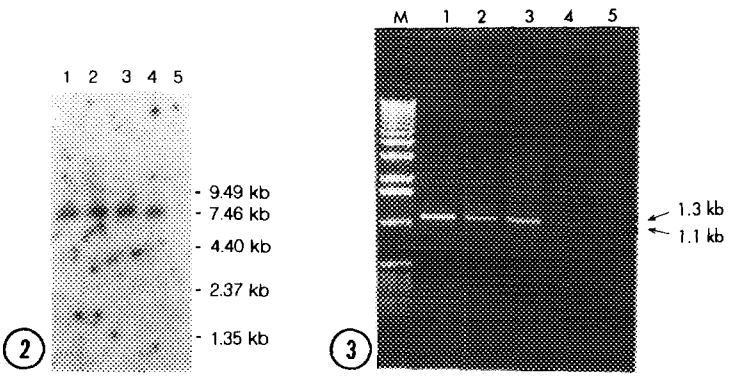

Figure 2. Tissue-specific expression of Fru 6-P,2-kinase:Fru 2,6-Pase mRNA in rat tissues. Poly(A)$^+$-RNA (10 μg) from various rat tissues was fractionated by electrophoresis and hybridized with ^{32}P-labeled RB10 cDNA. The origins of mRNA were as follows: 1, brain; 2, heart; 3, testis; 4, liver; and 5, skeletal muscle.

Figure 3. Analysis of RB10 mRNA by PCR. The synthesis of cDNA and PCR amplification were carried out as described in **EXPERIMENTAL PROCEDURES**. The reaction mixture (10 μl) was subjected to 1% agarose gel electrophoresis. The origins of mRNA were as follows: 1, brain; 2, heart; 3, testis; 4, liver; and 5, skeletal muscle; M, molecular size standard.

Thus, we conclude that the heart type isozyme of Fru 6-P,2-kinase:Fru 2,6-Pase lacking the phosphorylation domain peptide occurs in rat brain, testis, and liver. The enzyme in these tissues is not responsive to regulation by cAMP. Rat heart and skeletal muscle contain both types of the heart bifunctional isozyme, one of which is subject to regulation by cAMP. Furthermore, the heart type isozyme appears to be the most abundant form among the isozymes in rat brain. We were unable to detect any additional brain-specific isozyme such as that reported by others (6).

REFERENCES

1. Uyeda, K., Furuya, E., Richards, C.S., and Yokoyama, M. (1982) Mol. Cell Biochem. 48:97-120
2. Sakakibara, R., Kitajima, S., and Uyeda, K. (1984) J. Biol. Chem. 259:41-46
3. van Schaftingen, E. and Hers, H.G. (1986) Eur. J. Biochem. 159:359-365
4. Kitamura, K. and Uyeda, K. (1988) J. Biol. Chem. 263:9027-9033
5. Sakata, J., Abe, Y., and Uyeda, K. (1991) J. Biol. Chem. 266:15764-15770
6. Ventura, F., Rosa, J.L., Ambrosio, S., Pilkis, S.J., and Bartrons, R. (1992) J. Biol. Chem. 267:17939-17943
7. Ventura, F., Rosa J.L., Ambrosio, S., Gil, J., and Bartrons, R. (1991) Biochem. J. 276:455-460
8. Saiki, R.K., Gelfand, D.H., Stoffel, S., Scharf, S.J., Higuchi, R., Horn, G.T., Mullis, K.B., and Erlich, H.A. (1988) Science, 239:487-491
9. Darville M.I., Chikri, M., Lebeau, E., Hue, L., and Rousseau, G.G. (1991) FEBS Lett. 288:91-94
10. Kitamura, K., Kangawa, K., Matsuo, H., and Uyeda, K. (1988) J. Biol. Chem. 263:16796-16801