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# Tissue-specific alternative splicing of rat brain fructose 6-phosphate 2-kinase/fructose 2,6-bisphosphatase

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**Abstract We have reported the occurrence of eight splice variants of rat brain fructose 6-phosphate 2-kinase/fructose 2,6-bisphosphatase (RB2K). In the present study, we quantified these splice variants in various tissues using a RNAse protection assay and found a tissue-specific pattern of alternative splicing of the RB2K gene. Splice variants containing exon F were specifically expressed in brain. Moreover, exons D and E were spliced in brain, skeletal muscle and heart. Consequently, eight, six, four and two splice variants were expressed in brain, skeletal muscle, heart and liver plus testis, respectively. These results suggest that distinct RB2K isoforms could be involved in regulation of glycolysis in a tissue-specific manner.**



*Key words:* Brain; Fructose 6-phosphate 2-kinase; Fructose 2,6-bisphosphatase; Alternative splicing; RNAse protection assay

## 1. Introduction

A bifunctional enzyme, fructose 6-phosphate 2-kinase (F6P2kinase)/fructose 2,6-bisphosphatase (F26Pase) (EC 2.7.1.105/EC 3.1.3.46), catalyzes the synthesis and degradation of fructose 2,6-bisphosphate, which is the most potent activator of phosphofructokinase and plays an important role in regulation of glycolysis in mammalian tissues [1–3]. Four mammalian genes of the tissue-specific isozymes of F6P2kinase/F26Pase have been identified in liver [4,5], heart [6–8], testis [9] and brain [10–12].

It was reported that the liver, heart and brain isozyme genes generate multiple isoforms by alternative splicing. The liver isozyme gene produces liver-type and muscle-type isoforms that share the same 13 exons and differ only by the first exon [5]. The first exon in the liver-type isoform contains a serine phosphorylated by protein kinase A but not that in the muscle-type. The F6P2kinase and F26Pase activities of the liver-type isoform are reciprocally regulated by the phosphorylation, which inhibits the former and activates the latter. On the other hand, the heart isozyme gene generates two isoforms (58 000 $M_r$ and 54 000 $M_r$ isoforms) through alternative splicing of exon 15, which also encodes a phosphorylation domain [13,14]. The phosphorylation of the 58 000 $M_r$ isoform by protein kinase A and C results in activation of the F6P2kinase without affecting the F26Pase activity [15,16]. However, the 54 000 $M_r$ isoform cannot be phosphorylated by either protein kinase. In this manner, an alternative splicing mechanism generates tissue-specific isoforms of which the enzymatic properties are different from each other, supporting the idea that the tissue-specific isoforms are expressed to adapt their enzymatic properties to metabolic urgency of a particular tissue [12]. Therefore, elucidating of a tissue-specific expression pattern of the isozyme is important for comprehension of the tissue-specific regulation mechanisms of glycolysis.

Previously, we reported that eight splice variants (rat brain F6P2kinase/F26Pase (RB2K) 1–8) are generated in rat brain from the single primary transcript of the rat brain isozyme gene [12]. Their variable region of the splice variants is composed of seven exons (exons A–G) and encodes the C-terminus containing consensus phosphorylation sites of protein kinase A and C. We also reported that the gene is expressed in skeletal muscle, heart, testis and liver. Therefore, we undertook a detailed analysis of the molecular diversity of the isoforms and their tissue distribution in order to gain new insights into the specific implication of this gene in the regulation of glycolysis.

In this study, we carried out quantitative analysis of the isoform mRNAs in brain, skeletal muscle, testis, heart and liver using a RNAse protection assay. We found tissue-specific regulation of the splicing events of the RB2K gene.

## 2. Materials and methods

### 2.1. RNA preparation

Total RNAs were purified from brain, skeletal muscle, testis, heart and liver of male Sprague-Dawley rats with guanidium thiocyanate [17] and poly(A)$^+$-RNAs were isolated by subjecting the total RNAs to oligo(dT)-cellulose column chromatography [18].

### 2.2. Reverse transcriptase polymerase chain reaction (RT-PCR)

RT-PCR experiments were carried out as described previously [12]. In brief, SuperScript II reverse transcriptase (Gibco BRL) and random primers were used to reverse transcribe 10 μg of poly(A)$^+$-RNA in a 40 μl reaction mixture. The RNA strand of a RNA-DNA hybrid was digested by the addition of 2 μl RNAse H. PCR was carried out in a total volume of 100 μl with 2.5 U of *Ex Taq* polymerase (Takara) according to the manufacturer, with 200 μM dNTPs, 200 nM of the appropriate sense and antisense primers and 1 μl of the first strand cDNA. The following primers were used for the amplification: P1, 5′-GAA CCC CTA CTT CAG CAT CAG-3′; P2, 5′-CTT CAA CAG GCA GGA GAC CAA-3′; P6, 5′-TAC TTG AAT GTA GAA TCA GTG AGC-3′ (Fig. 3). The samples were overlaid with 100 μl of mineral oil to prevent condensation, placed in a pre-heated DNA thermal cycler (Astec, Model PC-700) at 94°C and then subjected to 30 cycles consisting of a 30 s denaturation period at 94°C, a 30 s annealing period at 60°C and a 120 s period at 72°C for extension

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*Abbreviations:* F6P2kinase, fructose 6-phosphate 2-kinase; F26Pase, fructose 2,6-bisphosphatase; RB2K, rat brain F6P2kinase/F26Pase; nt, nucleotides; RT-PCR, reverse transcriptase polymerase chain reaction


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of the annealed primers. After amplification, the reaction products were electrophoresed on a 6% polyacrylamide gel.

*2.3. Construction of plasmids for protection assay*

PCR was performed with oligonucleotides 5′-GTA GGA TCC GTG AGC ACG CAT CGG-3′ (*Bam*HI sense primer) and 5′-GGA GAA TTC GGC ACC GCT CCG GGG-3′ (*Eco*RI antisense primer 1) with each of the RB2K1, RB2K2, RB2K3 and RB2K4 cDNAs [12] as a template to amplify a fragment consisting of the variable region of the RB2K flanked by the *Bam*HI and *Eco*RI sites. In the case of the synthesis of a fragment for RB2K7, an oligonucleotide 5′-GTG GAA TTC AAG CAA TGG AAT TAT-3′ (*Eco*RI antisense primer 2) was used instead of the *Eco*RI antisense primer 1. These amplified fragments were digested with *Bam*HI and *Eco*RI and ligated into the *Bam*HI/*Eco*RI sites of pSP72 (Promega). The resulting plasmids were linearized with *Bam*HI or *Eco*RI and used as templates to synthesize the antisense RNA probes or sense RNA standards, respectively.

*2.4. RNAse protection assays*

Antisense RNA probes and sense RNA standards were synthesized with T7 and SP6 polymerases, respectively. RNAse protection assays were carried out according to Sambrook et al. [18]. The radiolabelled probe was incubated with 5 μg of poly(A)$^+$-RNA or varying amounts of the sense RNA standard (0, 1, 5 or 10 pg) for 16 h at 45°C. Yeast tRNA was added to the sense RNA samples to bring the RNA total to 5 μg and the samples were then treated with RNAse A (40 μg/ml) (Sigma) and RNAse T1 (2 μg/ml) (Boehringer Mannheim). The protected fragments were isolated and electrophoresed on a 6% polyacrylamide sequencing gel.

## 3. Results

*3.1. Detection of tissue-specific alternative splicing of RB2K mRNA using RT-PCR*

To evaluate the extent of RB2K isoform diversity, we carried out a RT-PCR experiment using mRNAs isolated from various tissues. Three PCR primers were designed to amplify the cDNA coding the C-terminus of RB2K. A primer pair (P1 and P6) amplifies DNA fragments derived from the RB2K1–RB2K6 mRNAs. Another primer pair (P2 and P6) amplifies those of RB2K7 and RB2K8. The RT-PCR detected eight, four and two RB2K isoforms in brain, skeletal muscle and the other tissues, respectively (Fig. 1). RB2K1 and RB2K4, which contains exon F, were exclusively expressed in brain. RB2K2, RB2K5, RB2K7 and RB2K8 that use exon D occurred in both brain and skeletal muscle. RB2K3 and RB2K6 were found in brain, heart, liver and testis. These data indicate that the primary transcript of the gene is processed by tissue-specific alternative splicing.

*3.2. RNAse protection assay*

To assess the splicing efficiency of the alternative exons quantitatively, RNAse protection assays were performed with the isoform-specific antisense RNAs. Five RNA probes were chosen for these experiments so as to generate similar size protected fragments and to detect all possible coding region splice variants. Using the antisense RB2K1 probe, protected fragments of 395, 312, 311, 288 and 283 nucleotides (nt), which arise from the respective mRNAs of RB2K1, RB2K2, RB2K7, RB2K3 and RB2K4, were observed. RB2K1 was found in only brain and was not detected in all the other tissues examined (Fig. 2A). However, fragments of 312 nt as well as 311 and 288 nt as well as 283 nt co-migrated. To detect another brain-specific isoform (RB2K4), hybridization with the antisense RB2K4 probe was carried out. The assay resulted in a protected fragment of 308 nt found in only brain (Fig. 2B). These results demonstrate that RB2K1 and RB2K4 were expressed exclusively in brain.

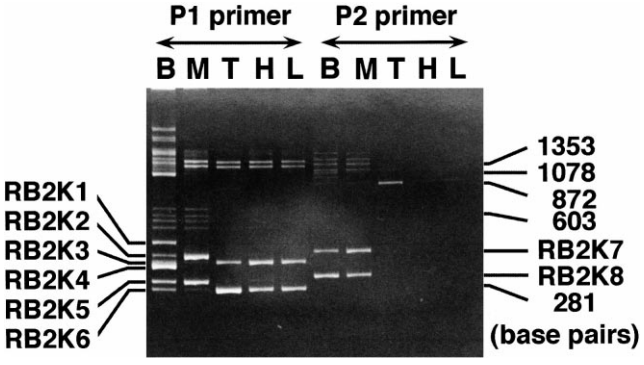



Fig. 1. RT-PCR analysis of alternatively spliced RB2K mRNAs. Poly(A)$^+$-RNA from each of the indicated rat tissues was reverse-transcribed and used for PCR analysis as described in Section 2. The products of the PCR reaction were separated by electrophoresis on a 6% polyacrylamide gel and stained with ethidium bromide. P1 and P2 primers were designed to amplify the C-terminal coding region of RB2K1–RB2K6 and RB2K7 plus RB2K8, respectively. The origins of the poly(A)$^+$-RNAs were as follows: (B) brain, (M) skeletal muscle, (T) testis, (H) heart and (L) liver.

The protected fragments of 342 and 230 nt corresponding to RB2K2 and RB2K5, respectively, were manifested in brain and skeletal muscle with the use of the antisense RB2K2 probe (Fig. 2C). These isoforms contain the sequence encoded by exon D. Even with prolonged exposures, no fragments were detected in heart, liver or testis.

The tissue distributions of the RB2K isoforms as determined by the RNAse protection analysis are so far in good agreement with the RT-PCR analysis. However, fragments of 319 and 207 nt protected by the antisense RB2K3 probe, which arise from the respective RB2K3 and RB2K6 mRNAs, were observed in all the tissues examined (Fig. 2D). The expression of RB2K3 and RB2K6 in skeletal muscle cannot be detected by the RT-PCR method (Fig. 1), although significant amounts of the mRNAs were detected by the RNAse protection assay. Moreover, 341 and 229 nt fragments, which correspond to the respective mRNAs of RB2K7 and RB2K8, were observed in not only brain and skeletal muscle but also in heart (Fig. 2E). The occurrence of RB2K7 and RB2K8 in heart also cannot be detected by RT-PCR. The discrepancy between the results obtained using the RT-PCR method and the RNAse protection assay to detect the expression of these isoforms reflects the difference in the detection limit of these two methods.

The amounts of the RB2K mRNAs were determined by quantitative scanning densitometry, within the linear range of film sensitivity of the autoradiographic signals of the RNAse protection assay (Table 1). All RB2K mRNAs were expressed at comparable levels to each other in these rat tissues. The total amount of RB2K isoforms in each tissue as determined by the RNAse protection assay is in good agreement with the tissue distribution of the RB2K analyzed by the Northern blot (cf. Fig. 1 of [12]).

## 4. Discussion

This study describes the tissue-specific alternative splicing events of the RB2K gene. As summarized in Fig. 3, exons D–

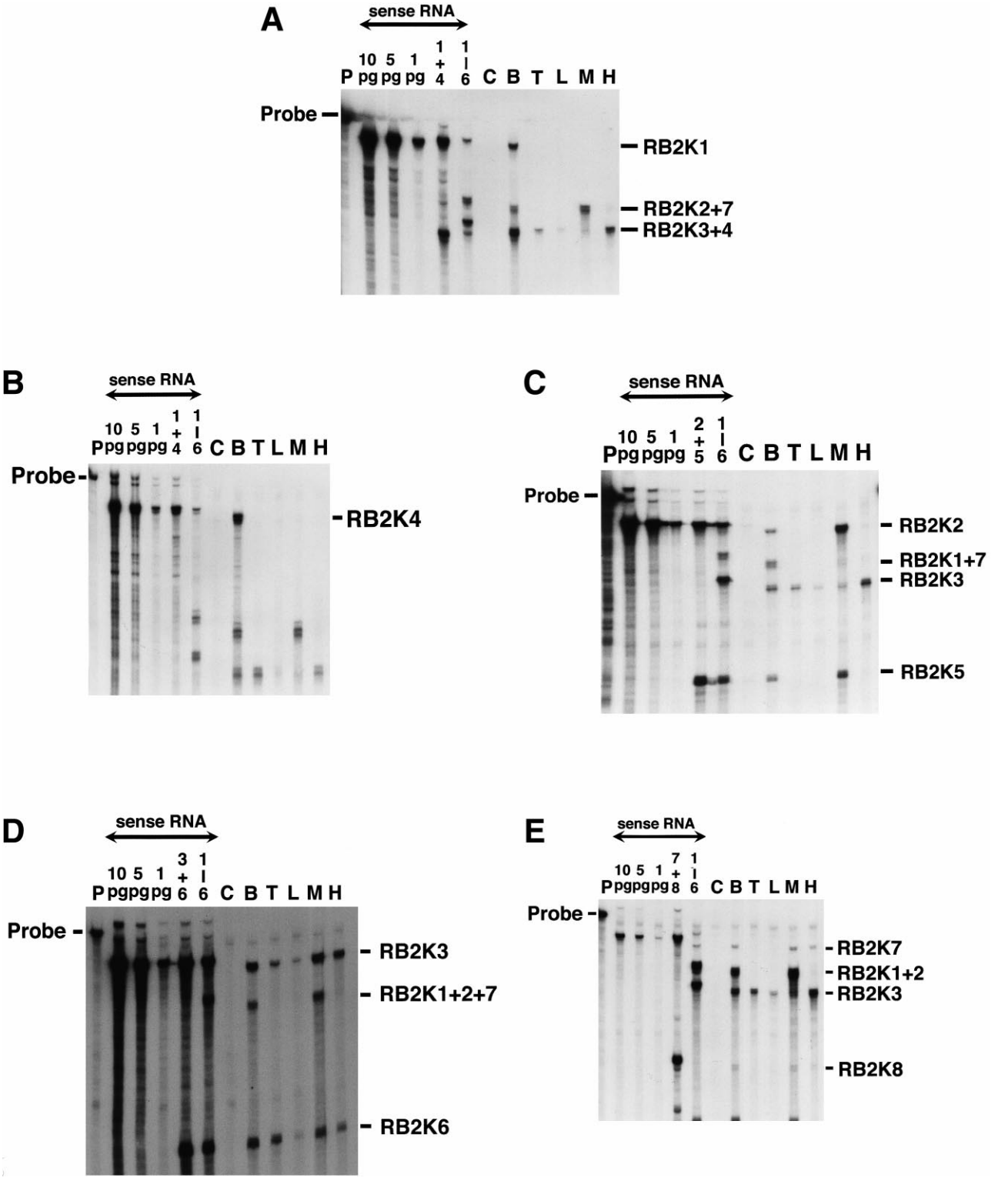
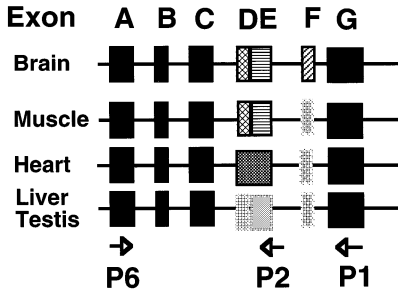



Fig. 2. RNAse protection analysis of RB2K expression in rat tissues. A: Poly(A)$^+$-RNA (5 μg) from rat tissues or sense standard RNA (1, 5 or 10 pg) was hybridized to a RB2K1-specific antisense RNA probe, treated with RNAse and separated by denaturing gel electrophoresis. The position of the RNA probe is indicated on the left and the positions of the tissue RNA-protected fragments are indicated on the right. RNAs in the lanes were as follows: (C) tRNA as negative control (5 μg), (B) brain, (T) testis, (L) liver, (M) skeletal muscle, (H) heart, (P) antisense RNA probe, (1, 5, 10 pg) sense RNA probes, (1+4) the mixture of sense RNAs of RB2K1 and RB2K4, (1–6) the mixture of sense RNAs of RB2K1–6. B–E: These are the same experiments with use of RB2K4-, RB2K2-, RB2K3- and RB2K7-specific probes, respectively. Size marker lanes containing sense RNAs are as follows: (1+4), (2+5), (3+6) and (7+8) are the mixtures of the sense RNAs of RB2K1 plus RB2K4, RB2K2 plus RB2K5, RB2K3 plus RB2K6 and RB2K7 plus RB2K8, respectively.




| | | | | | | | |
|---|---|---|---|---|---|---|---|
| RB2K1 | A | B | C | D | - | F | G |
| RB2K2 | A | B | C | D | - | - | G |
| RB2K3 | A | B | C | - | - | - | G |
| RB2K4 | A | - | C | D | - | F | G |
| RB2K5 | A | - | C | D | - | - | G |
| RB2K6 | A | - | C | - | - | - | G |
| RB2K7 | A | B | C | D | E | - | - |
| RB2K8 | A | - | C | D | E | - | - |

Fig. 3. Tissue-specific alternative splicing patterns of the RB2K gene. Upper panel: schematic diagram shows the intron-exon organization of the variable region of the RB2K gene in various tissues. The tissue-specific spliced exons are represented by hatched boxes. The exons that are not expressed in a particular tissue are represented by faded boxes. RT-PCR primers that were used in Fig. 1 are shown by arrows. Lower panel: splicing variants are represented by combinations of the exons.

F were spliced in a tissue-specific manner. Exon F was expressed in only brain. In the other tissues, it was spliced out from the primary transcript of the gene. Exon D and exon E are not divided by an intron structure. Moreover, a consensus sequence of a donor site of an intron is observed at the 5′-end of exon E [12]. These two exons were expressed not only in brain and skeletal muscle but also in heart (Fig. 2). In heart, however, the two alternative exons were always spliced together and behaved like a single exon. It implies that spliceosomes of heart cannot recognize the sequence of the donor site of exon E. These results suggest that tissue-specific factors interacting with specific *cis*-elements of the gene regulate the alternative splicing.

It has been reported that counterparts of the RB2K5 and RB2K6 isoforms are expressed in bovine and human tissues. Ventura et al. reported the partial cDNA sequence of bovine brain F6P2kinase/F26Pase that is a counterpart gene of RB2K [11]. The alignment analysis shows that the bovine cDNA sequence lacks exon B and terminates at the middle of exon C. Therefore, the cDNA of the bovine brain isozyme may be one of the counterparts of RB2K4, 5, 6 and 8. A counterpart of the RB2K6 isoform is expressed in human placenta and breast cancer cells [19,20]. Hamilton et al. reported that progestin induces the RB2K6 counterpart gene in human breast cancer cell lines [20]. They quantified the expression of the gene using a Northern blot with a cDNA probe, which is derived from the invariant region of the cDNA. Therefore, they did not observe the isoform-specific induction of the gene. Recently, Chesney et al. reported that a counterpart of RB2K5 is expressed in several human cancer cell lines and is induced by pro-inflammatory stimuli [21]. The induction of the counterparts of RB2K5 and RB2K6 in the cancer cell lines by the respective stimuli of the lipopolysaccharide and progestin is very interesting, implying coupling of enhanced glycolysis and cell proliferation.

Table 1
The amount of RB2K mRNAs in various tissues

| | Brain | Testis | Liver | Skeletal muscle | Heart |
|---|---|---|---|---|---|
| RB2K1 | 2.3 | – | – | – | – |
| RB2K2 | 1.4 | – | – | 4.9 | – |
| RB2K3 | 3.8 | 2.0 | 0.8 | 4.0 | 2.6 |
| RB2K4 | 9.1 | – | – | – | – |
| RB2K5 | 3.0 | – | – | 5.3 | – |
| RB2K6 | 6.4 | 4.5 | 0.9 | 5.1 | 2.0 |
| RB2K7 | 0.4 | – | – | 0.6 | 0.3 |
| RB2K8 | 1.0 | – | – | 1.3 | 0.4 |
| Total | 27.4 | 6.5 | 1.7 | 21.2 | 5.3 |

($\times 10^6$ copies/5 μg poly(A)$^+$-RNA).

RT-PCR is a sensitive and simple method to detect mRNA, although the methodology has a problem of poor reliability of quantitative measurement of mRNA. The method detected tissue-specific alternative splicing of the RB2K gene (Fig. 1). However, it failed to detect the occurrence of RB2K3 plus RB2K6 in skeletal muscle and RB2K7 plus RB2K8 in heart (Fig. 2D,E). There are several factors that affect the reliability of the method. In this case, it seems likely that the efficiency of the PCR amplification or the reverse transcription may become a limiting factor, because the undetectable fragments appeared as weak bands after RT-PCR with 40 amplification cycles instead of 30 (data not shown).

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