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A rat gene encoding heart 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase

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There are at least 3 isozymes of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase, a bifunctional enzyme which catalyzes the synthesis and degradation of fructose 2,6-bisphosphate. A 22-kb rat gene that encodes the heart isozyme has been identified and compared with the 55-kb rat gene encoding the liver and muscle isozymes which had been described earlier. Although these 2 genes include 12 successive similar exons, they contain dissimilar exons at both ends, consistent with the occurrence of different regulatory domains at the N- and C-termini in the 3 isozymes.

Fructose-2,6-bisphosphatase; Fructose-2,6-bisphosphate; Glycolysis; 6-Phosphofructo-2-kinase; Gene fusion; Rat heart

1. INTRODUCTION

Fructose 2,6-bisphosphate is a ubiquitous stimulator of 6-phosphofructo-1-kinase. Its synthesis and degradation are catalyzed by the bifunctional enzyme 6-phosphofructo-2-kinase (PFK-2, EC 2.7.1.105)/fructose 2,6-bisphosphatase (FBPase-2, EC 3.1.3.46). Three PFK-2/FBPase-2 isozymes have been identified by biochemical and immunological criteria, namely the liver (L), the muscle (M), and the heart (H) types (reviewed in [1]). We have cloned the L-type [2] and the M-type [3] cDNAs and have characterized a 55-kb rat gene (henceforth called gene A) that encodes the corresponding mRNAs by alternative use of 2 promoters [4]. The elucidation of the origin of the H-isozyme [5] is important because it appears to replace the L-isozyme in regenerating liver [6] and in some hepatoma cells [7]. Moreover, the three PFK-2/FBPase-2 isozymes differ by their sensitivity to cyclic AMP-dependent protein kinase (PKA). PKA-dependent phosphorylation inactivates PFK-2 and activates FBPase-2 in the L-isozyme, while it activates PFK-2 in the H-isozyme. The M-isozyme is insensitive to PKA. In gene A, exon 1′ (L-isozyme) includes the PKA phosphorylation site while exon 1 (M-isozyme) lacks it. We now describe a PFK-2/FBPase-2 rat gene (gene B) that codes for the H-isozyme.

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Abbreviations: bp, base pairs; FBPase-2, fructose-2,6-bisphosphatase; ORF, open reading frame; PFK-2, 6-phosphofructo-2-kinase; PKA, protein kinase A

2. EXPERIMENTAL

A rat genomic library in λEMBL3 was kindly provided by W. Schmid and G. Schütz (Heidelberg). cDNA probe RL2K-8 corresponds to nucleotides 674 (exon 6) to 1759 (exon 14) of L-type rat mRNA [2]. A cDNA probe RH1-9, i.e. including exons 1–8 and part of exon 9 (811 bp) of gene B, was constructed by reverse transcription and polymerase chain reaction amplification of rat heart poly(A)-rich RNA (Lebeau et al., in preparation). RH2-3 cDNA and AB247 oligonucleotide probes correspond to nucleotides 117–310 and 6–25 of RH1-9, respectively. JNO1 is a 27-mer oligonucleotide based on a peptide [8] from bovine heart PFK-2/FBPase-2 conserved in exon 4 of gene A. JNO1 was 89% identical to nucleotides 417–443 of RH1-9.

The rat genomic library was screened as described [4]. Southern blots were performed according to [9] and Southern Cross Restriction Mapping (NEN) according to the manufacturer's instructions. Restriction fragments from genomic clones extracted from agarose gels ([10] or GeneClean, Westburg) were subcloned in M13mp8/9, BlueScript or PTZ18/19 vectors and sequenced by the chain termination method [11] using a Sequenase DNA sequencing kit (USB) or a Kilobase sequencing system (BRL) with universal primers and successive synthetic oligonucleotides.

3. RESULTS AND DISCUSSION

When screening the rat genomic library for gene A [4], four overlapping clones (λ2, 3, 15 and 16) detected with probe RL2K-8 yielded restriction fragments that were incompatible with the map of gene A and were no longer seen in Southern blots performed at high stringency. The restriction map of λ3 was established by Southern Cross (Fig. 1) and the other clones were aligned with λ3 on the basis of their restriction fragments. The sequencing over 6 kb of λ3 fragments that hybridized with RL2K-8 showed 6 open reading frames (ORF) (corresponding to exons 9–14, see below) that had exactly the same length as, and had a sequence similar to, exons 8–13 of gene A which entail the FBPase-2 domain

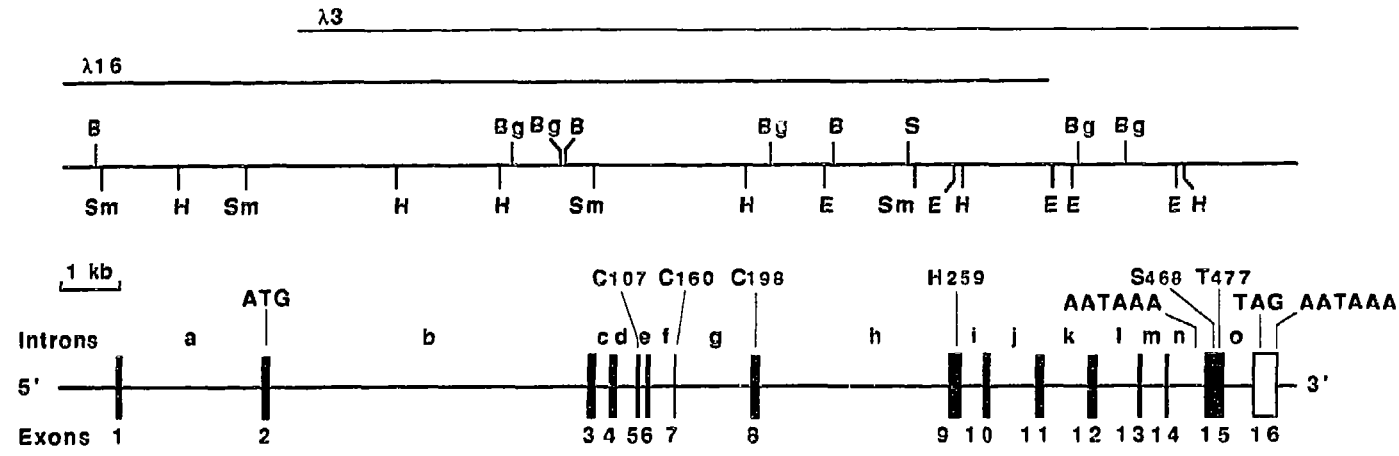

Fig. 1. PFK-2/FBPase-2 gene B. Upper horizontal lines refer to the rat genomic clones indicated. The restriction map shows sites for *Bam*HI (B), *Bgl*II (Bg), *Eco*RI (E), *Hin*dIII (H), *Sal*I (S) and *Sma*I (Sm). The position in the exons of the residues that are critical for activity or are phosphorylated by PKA (Ser 468) or protein kinase C (Thr 477) is indicated. The exact location of exon 2 with respect to its flanking *Hin*dIII sites is not known.

(Table I). Beyond these ORF's, there was another one (i.e. exon 15) that includes a peptide of 17 amino acids (Fig. 2) described earlier in the bovine H-isozyme [12]. This peptide contains the sites for phosphorylation by protein kinases A and C that are typical for this isozyme. We concluded that clone λ3 corresponds to at least part of the gene encoding the H-isozyme.

In gene A, the PFK-2 domain is encoded by exons 2–7. Since the H-isozyme also displays a PFK-2 activity, it was likely that gene B extended further upstream. The missing portion was therefore searched for in clone λ16. *Hin*dIII, *Bam*HI, and *Eco*RI restriction fragments containing putative exons were identified by Southern blotting using probes RH1-9, RH2-3, AB247, and JNO1. The positive fragments were subcloned and their sequencing was pursued until the entire sequence of RH1-9, namely the cDNA upstream from the first ORF that had been identified in λ3, was accounted for. There was no discrepancy between the genomic and cDNA sequences, making it unnecessary to sequence all the genomic fragments in both directions. The eight exons (1–8) of gene B, from which RH1-9 cDNA was derived, were thereby identified.

While this work was in progress, the sequence of a fully-coding cDNA for bovine PFK-2/FBPase-2 became available [13]. A comparison of this sequence with the rat genomic fragments sequenced here allowed the assignment of the ORF's to defined exons and showed that the rat H-isozyme is encoded by at least 15 exons (Table II). The exon–intron junctions conform to the rodent consensus [14]. At the 5′ end of the gene delineated by comparison with RH1-9, there is a noncoding exon. Exon 2, which bears no relationship with exons 1 or 1′ of gene A, contains an ATG which is in a context favorable (ACTACCATGT) for translation initiation [15]. The twelve exons (3–14) that encompass the PFK-2 and FBPase domains follow. They share an overall identity of 66% with the nucleotides and of 71% with the amino acids of exons 2–13 of gene A (Table I). In genes A and B, the 3 cysteines that are important for PFK-2 activity and the histidine that is phosphorylated in the FBPase-2 reaction (Fig. 1) are located in the corresponding exons [4]. This conserved region of gene B is followed by a 3′ end that contains several interesting features (Fig. 2). First one finds, in intron *n*, an 80-bp noncoding sequence that corresponds, except for insertions and deletions, to a vestigial 5′ end of exon 14 of gene A. Exon 15 contains a PKA phosphorylation site which, in gene A, is located in exon 1′. Intron *o* contains a 50-bp GT repeat. This intron is followed by a putative exon encoding ORF 16. However, this ORF bears no relationship with the peptide AET-

Table I

Comparison of PFK-2/FBPase-2 genes A and B

Gene A exons[a]	1	1′	2	3	4	5	6	7	8	9	10	11	12	13	14	
Gene B exons (ORF)	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	(16)
Identical nucleotides (%)	nr	nr	60	52	55	65	68	59	68	69	70	80	71	74	nr	-
Identical amino acids (%)	nr	nr	71	72	54	72	63	57	72	69	80	88	81	73	nr	-

[a]In gene A, the first 2 exons were called 1 and 1′ because they are separated by the L-type promoter and therefore belong to different (i.e. M- or L-) primary transcripts, all the other exons being present in both transcripts [4]. nr, not related.

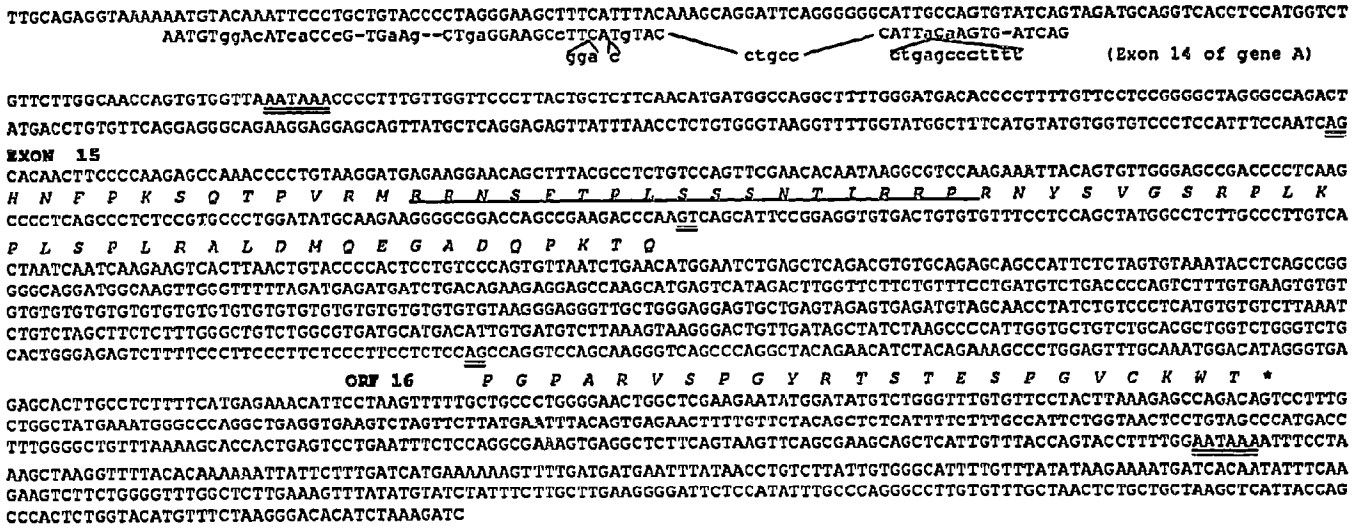

Fig. 2. 3′ End of gene B. Nucleotides underlined twice indicate the intron/exon junctions and 2 potential polyadenylation signals. Residues underlined in exon 15 correspond to a peptide described in [12], with the phosphorylated serine (4th residue) and threonine (13th residue). ORF16 differs from the C-terminus of the bovine H-isozyme (see text). The star indicates a stop codon. The 5′ part of exon 14 of gene A is aligned below the gene B sequence. Insertions and deletions are included to maximize homology. Nucleotides in lower case in exon 14 indicate divergences.

SRAAHRLPSPAPPTSPS that follows the amino acid sequence corresponding to exon 15 in the bovine H-isozyme [13]. Finally, there are 2 polyadenylation signals, one of which is in intron *n*. This raises the possibility that gene B encodes mRNA(s) lacking the phosphorylation sites. It is remarkable that the intronic part of gene B is much smaller than that of gene A, reducing the length of gene B to about half that of gene A. The latter has been considered as originating from the fusion of 2 ancestral genes, one evolutionarily related to the 6-phosphofructo-1-kinase gene, which resulted in the PFK-2 domain (exons 2–7), and one evolutionarily related to the phosphoglycerate mutase and acid phosphatase genes, which resulted in the FBPase-2 domain (exon 8–13) [4,16]. The organization of gene B is consistent with this view and supports the hypothesis [17] that many genes result from the assembly of exons originating from distinct genomic regions. Indeed, the portion containing exons 3–14 of gene B appears to result from a duplication of an

Table II

Localization and sequence of exon-intron junctions in PFK-2/FBPase-2 gene B

Exon	Position in cDNA		5′ splice site		Intron	3′ splice site		
1	1–81		agg	GTGAGC	*a*	CCTTCTTTCCCCAG	gtg	
2	82–195	(Ser-31)	gct	GTGAGT	*b*	CTTTTTTTTTTTAG	cat	(Ser-31)
3	196–321	(Val-73)	aag	GTATGC	*c*	TGTTTTTGCTGCAG	tgt	(Val-73)
4	322–418	(Lys-105)	caa	GTGAGT	*d*	TGTTCTCATGTCAG	aca	(Glu-106)
5	419–485	(Ala-127)	gcg	GTAAGC	*e*	TGCTTTCTGTGTAG	gtg	(Val-128)
6	486–560	(Lys-152	aag	GTGGGT	*f*	CTCTCCATGCCCAG	gta	(Val-153)
7	561–617	(Leu-171)	ctg	GTGAGT	*g*	TCCTGTGATTTCAG	gag	(Glu-172)
8	618–742	(Lys-213)	taa	GTAAGA	*h*	TCCCCCTGTCCCAG	gga	(Lys-213)
9	743–950	(Gln-282)	cag	GTGAGC	*i*	TTTCCAAACCCCAG	ttt	(Phe-283)
10	951–1097	(Ala-331)	gct	GTGAGT	*j*	ATGGCTCTTTGTAG	ggc	(Gly-332)
11	1098–1202	(Glu-366)	gag	GTGAGT	*k*	ACTTCCCCTCAAAG	tca	(Ser-367)
12	1203–1332	(Asp-410)	cag	GTGCCA	*l*	CCTCCTTCTTTCAG	atg	(Asp-410)
13	1333–1395	(Gly-431)	atg	GTAACT	*m*	TCCTGTTTTTTCAG	gtt	(Gly-431)
14	1396–1460	(Thr-452)	act	GTAAGT	*n*	CCATTTCCAATCAG	cac	(His-453)
15	1461–1640	(Gln-512)	caa	GTCAGC	*o*	CCCTTCCTCTCCAG	cca	(Pro-513)
(16)	1641–1708	(Thr-535)	gac					
	Rodent consensus		c ag a	A GT AGT G		TTTTTTTTTT T N AG CCCCCCCCCC C	g	

Nucleotides in exons are lowercase letters and in introns are capital letters. N, any nucleotide. Exon 1 is noncoding. The codons underlined are those for the amino acids indicated.

ancestral gene that also yielded gene A, followed by recruitment, at the 5′ and 3′ ends, of other, regulatory, exons that bear no relationship with those at the ends of gene A. While gene A is on the X chromosome in rat [4] and man [18], gene B has been assigned, as a single copy gene, to chromosome 13 in rat, and chromosome 1 in man [18]. The identification of a PFK-2/FBPase-2 gene on an autosome now explains the presence [19] of fructose 2,6-bisphosphate in sperm, where the X chromosome is inactive [20], and indeed transcripts from gene B are expressed in the testis (Lebeau et al., in preparation).

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