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Identification of a novel *Pfkfb1* mRNA variant in rat fetal liver

Jesús Cosin-Roger [a,1], Santiago Vernia [a,1], Maria Soledad Alvarez [a], Carme Cucarella [a], Lisardo Boscá [b,c], Paloma Martin-Sanz [b,c], Ana Julia Fernández-Alvarez [a,*], Marta Casado [a,c,*]

[a] Instituto de Biomedicina de Valencia, IBV-CSIC, Jaime Roig 11, 46010 Valencia, Spain
[b] Instituto de Investigaciones Biomédicas Alberto Sols, CSIC-UAM. Madrid. Arturo Duperier 4, 28029 Madrid, Spain
[c] Centro de Investigación Biomédica en Red de Enfermedades Hepáticas y Digestivas (CIBERehd), Córcega 180, 08036 Barcelona, Spain

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ABSTRACT

The bifunctional enzyme 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (PFK-2/FBPase-2) catalyzes the synthesis and degradation of fructose-2,6-bisphosphate, a key metabolite in the glucose homeostasis. Four genes, *Pfkfb1–4*, have been characterized in mammals that code for several isoforms generated by alternative splicing through the control of several promoters and 5′ non-coding exons. Here, we characterize in fetal rat liver new mRNA variants which are transcribed from a new *Pfkfb1* gene promoter. The long variant codes to a new isoform (FL-PFK-2) that would be of relevant function to modulate the transition of fetal to adult liver metabolism.

1. Introduction

Fructose-2,6-bisphosphate (Fru-2,6-P_2) is the most potent allosteric activator of 6-phosphofructokinase-1 (PFK-1), as well as being an inhibitor of the fructose-1,6-bisphosphatase (FBPase-1) [1]. In addition, high levels of Fru-2,6-P_2 also favor glycolysis and suppress gluconeogenesis at gene expression level. Fru-2,6-P_2 has been recently demonstrated to have an important role in the transcriptional regulation of glycolytic and lipogenic enzymes, thereby regulating glucose homeostasis [2].

Fru-2,6-P_2 concentration depends on the balance between the activities of 6-phosphofructo-2-kinase (PFK-2) and fructose-2,6-bisphosphatase (FBPase-2), which functions as a homodimeric protein (PFK-2/FBPase-2) with these two activities located at separate sites on each protein subunits [3]. Four genes, designated *Pfkfb1–4*, encode at least seven different isoenzymes of PFK-2/FBPase-2 in mammalian tissues, which share the catalytic site but differ in the N-terminal and C-terminal extension length which contain the phosphorylation sites that regulate the kinase and biphosphatase activities [4].

Pfkfb1 gene (60 kb, 17 exons), which codes for the liver (L) and skeletal muscle (M) isoforms, gives rise, from distinct promoters, to the mRNA transcripts denominated F, M and L that differ by their 5′ end and share 13 consecutive exons (2–14) [5]. The isoforms L and M contain the exon 1L and 1M respectively. The F mRNA variant, present in several rat derived cell lines like FTO2B and in proliferating tissues, contains two non-coding exons (denominated 1aF and 1bF) upstream from, and spliced with part of, the exon 1M. [6].

Fetal liver is metabolically characterized by high glycolytic and pentose phosphate pathway activities [7]. Three days before born, an increased glycogen synthesis is observed, which will assure sufficient energetic fuel for the neonate during the first hours in the post-uterine life until the gluconeogenic process starts to function [8]. Our previous work demonstrated that fetal rat liver expresses a PFK-2/FBPase-2 form that differs from the adult liver enzyme in the inhibition by phosphorylation by PKA and in the recognition by an antibody specific for the NH_2 terminal domain of the adult liver enzyme. Experimental evidence suggests that this fetal form is replaced by the adult form after birth [9,10]. Here, we characterize a new *Pfkfb1* splice variant present in fetal rat liver, analyzing its expression during liver development in order to assess its physiologic relevance.

Abbreviations: Fru-2,6-P_2, fructose-2,6-bisphosphate; PFK-2/FBPase-2, 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase; FL, fetal liver; AS, alternative splicing; PKA, protein kinase A; Bp, base pairs.

* Corresponding authors. Address: Instituto de Biomedicina de Valencia, IBV-CSIC, Jaime Roig 11, 46010 Valencia, Spain (M. Casado). Fax: +34 96 3690800.

E-mail addresses: anajfernandezalvarez@gmail.com (A.J. Fernández-Alvarez), mcasado@ibv.csic.es (M. Casado).

[1] Both authors contributed equally.

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2. Materials and methods

2.1. Cell cultures and tissue extraction

Human embryonic kidney 293T (HEK293T) and human liver hepatocellular HepG2 cells were maintained in Dulbecco's modified Eagle's medium (Lonza, Basel, Switzerland) containing 25 mM glucose, conventional antibiotics and 10% FBS. Rat hepatocytes in primary culture were prepared from adult rats as previously described [11].

Adult and fetal tissues were obtained from Wistar rats feeded *ad libitum* with a standard diet and treated according to the Institutional Care Instructions (Bioethical Commission from Consejo Superior de Investigaciones Científicas, CSIC, Spain). Fetuses were obtained by cesarea at 18, 19, 20 and 21 days post coitum (dpc).

2.2. Total RNA extraction, RT-PCR and qPCR

Total RNA was isolated from cells or tissues by using TRIzol reagent (Life Technologies, Carlsbad, CA, USA). First-strand cDNA was synthesized from 1 μg of total RNA using random hexamers and expand reverse transcriptase (Roche). The cDNA was used as a template for conventional or quantitative PCR using specific primers for the determination of *Pfkfb1* mRNA variants (sequences available on demand). *Gapdh* or β-actin genes were used as internal control. PCR fragments were generated with a 2-min melting step at 94 °C, followed by 35 cycles of amplification (94 °C for 10 s, 55 °C for 30 s, and 68 °C for 45 s) and a terminal 7 min extension at 68 °C. Real-time quantitative PCR using Sybr Green reagent (Life Technologies) and specific FL primers were carried out following cycling parameters: 95 °C 5 min, followed by 40 cycles of 95 °C 15 s and 60 °C 1 min. The replicates were then averaged, and fold induction was determined in a ΔΔCt based fold-change calculations using rat *Gapdh* as control gene.

2.3. Race

To get the full 5′ sequence of fetal rat liver *Pfkfb1* cDNA, total liver RNA was treated and amplified with FirstChoice® RNA ligase-mediated rapid amplification of cDNA ends (RLM-RACE) kit (Life Technologies) according to the manufacturer's protocol, and using external and nested primers corresponding to the 5′-RACE adaptor sequence or complementary to *Pfkfb1* (exon 2 reverse primer 5′-TTTAGTTGGTGTTCCTATCCAGTTGA-3′ and, for the second round amplification, exon 2 reverse primer 5′-ACCATGATCACCATCACCATCGTGGGA-3′).

2.4. Plasmid constructs and luciferase assay

A 3359 bp PCR fragment of the 5′ upstream from the FL exon of the rat *Pfkfb1* gene was cloned into the MluI/BglII restriction sites of the pGL3-basic vector (Promega, Wisconsin, USA) to obtain the FL-prom construction. For the FL-PFK-2 expression vector, a 1503 bp (FL long) or 1473 bp (FL short) fragment containing the complete cDNA coding sequence (FL exon – exons 2–14) was cloned into the HindII/EcoRV restriction sites of the pcDNA3.1+ vector (Life Technologies). All constructs were verified by sequencing.

Transfection assays were performed using Lipofectamine 2000 (Life Technologies) in six-well culture dishes with 3.8 μg of ProFL-basic reporter plasmid and 200 ng of Renilla luciferase vector (pRL-TK) 6 h after seeding. Luciferase activities were measured 48 h after transfection in a Wallac 1420 VICTOR luminometer according to the technical manual for the Dual-Luciferase reprorter assay system (Promega).

2.5. Protein extraction, in vitro expression and immunoblotting

Extracts from rat tissues were obtained as previously described [12]. *In vitro* protein expression was performed from FL PFK-2 constructs described previously using TnT Quick Coupled Transcription/Translation Systems kit (Promega) according to the manufacturer's protocol. The products of reactions were analyzed by Western blot as previously described [11] using an anti-PFK-2 antibody (sc-48828, Santa Cruz Biotechnology Santa Cruz, CA, USA). PVDF membranes were revealed using the Blotting Luminol Reagent (Santa Cruz) in a FujiFilm LAS-3000.

2.6. Enzyme assay

The measure of PFK-2 activity is based on the formation of Fru-2,6-P_2 from ATP and Fru-6P, using the method described by Bartrons and col. [13]. Briefly, the active form of PFK-2 was assayed at pH 6.6 in the presence of 1 mM Fru-6-P, 5 mM MgATP, and 1 mM potassium phosphate. Fru-6-P was in a 1:3 ratio with glucose 6-phosphate. Protein levels were determined with Bradford reagent (Bio-Rad, Hercules, CA, USA).

3. Results

3.1. Characterization of the 5′ end of the rat fetal liver Pfkfb1 mRNA variants

To analyze the presence of a fetal specific mRNA variant in rat liver, we used total mRNA from rat fetuses to perform a 5′RACE assay using 5́adapter-specific primers (outer and inner primer) and specific primers in exon 2, taking into account that all previously mRNA variants described present a common structure starting on exon 2. The PCR fragments obtained after the nested PCR assay were cloned for sequencing. We discovered transcripts that initiated at the exon 1L and 1M as well as transcripts that contained a 126 bp sequence that mapped inside the first intron of *Pfkfb1*, and several clones presented an extra 38 bp extension in the 3′ end. These results suggested the presence of a new exon in rat *Pfkfb1*, named exon FL, probably with an alternative processing, giving rise to 2 variants: FL-long and FL-short. The genomic localization of the newly discovered sequence corresponds to a region that extends between the 1L exon and the exon 2 separated by 3247 bp and 11,355 bp, respectively (Fig. 1A).

Next, a study was performed at the transcriptional level by RT-PCR in rat fetal liver and other adult tissues to detect the new transcripts of *Pfkfb1* gene to verify that the results were not artifacts of RLM-RACE technique. As shown in Fig. 1B, positive expression of FL-PKF-2 variant was highly detected both in fetal and adult liver, with an important expression in pancreas and a very low expression detected in the other analyzed tissues.

To further analyze the presence of two newly described splicing variants of the FL exon, the expression of short and long FL-PFK2 mRNAs was determined in fetal and adult liver using primers specific for each variant (Fig. 2A). Both FL-short and FL-long were present in fetal and adult liver, in muscle tissue, and slightly expressed in testis, heart and brain. As derived from the data shown in Fig. 2A, it seemed that although both short and long variants were expressed in fetal and adult liver, the relative amounts of each isoform were quite different. To assess this item we performed a quantitative PCR using the Sybr green technology together with specific primer to quantify the amount of FL-short and long variants. FL-long exon displayed a more than 30-fold expression level in the fetal liver as compared to the expression in the adult tissue while the FL-short was similarly expressed in both stages (Fig. 2B).

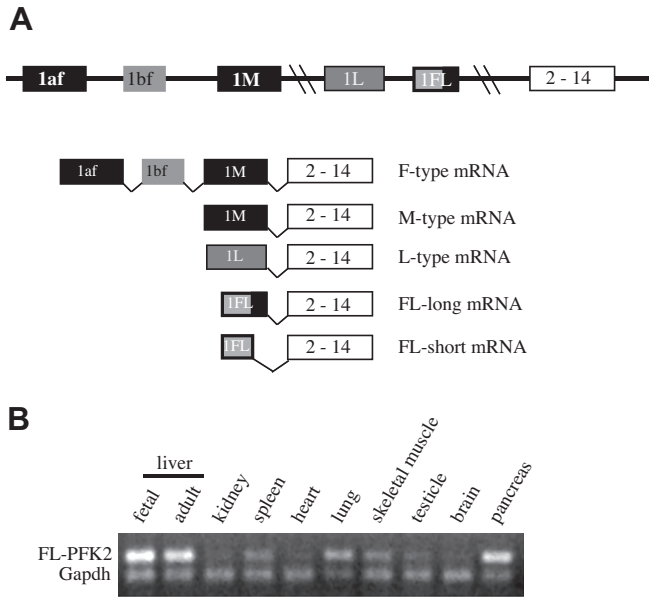

Fig. 1. Identification of new Pfkfb1 mRNA variants. (A) Genomic localization of the novel transcribed exon (FL exon) identified by RACE. All previously described exons are also shown. The organization of the different mRNA variants are drawn. (B) Expression of the new FL-PFK2 isoform in different rat tissues. Total RNA (1 μg) isolated from rat tissues was reverse transcribed and cDNA was amplified using FL-PFK2 isoform-specific primers. *Gapdh* was used as a control for RT reaction. A representative blot out of four is shown.

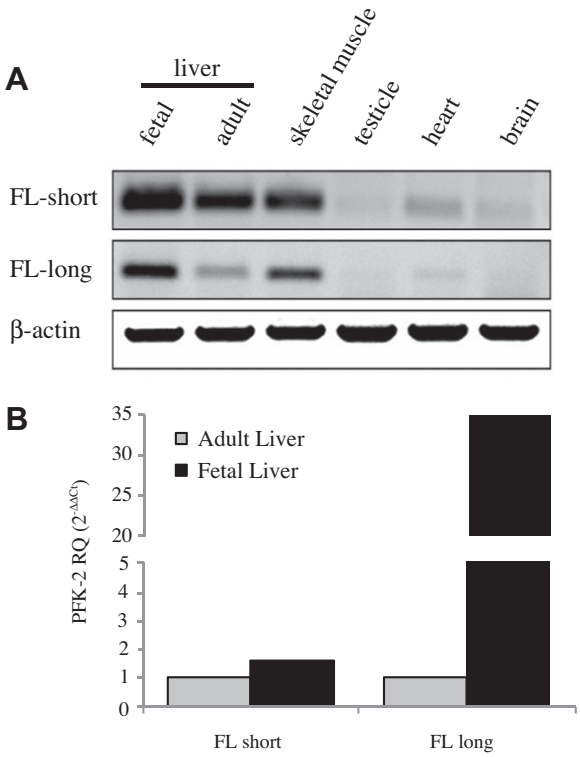

Fig. 2. Differential tissue expression of the FL-short and FL-long PFK-2/FBPase-2 mRNA. (A) Total RNA from 18-day-old fetal liver and several adult tissues were analyzed by RT-PCR using specific primers recognizing the alternative splicing of the FL exon. Amplification of β-actin was used as a control. (B) Relative quantification of the FL-short and FL-long variants in fetal and adult rat liver using the Sybr Green method. Quantification of *Gapdh* was used as endogenous control. Results are representative of 3 experiments and are expressed as fold induction relative to the expression of each isoform in the adult liver.

3.2. In silico and in vitro identification of FL promoter

Although software tools were not able to predict a promoter region upstream or downstream of the FL exon, we detected, by the use of *in silico* tools the presence of transcription initiation elements. Although a TATA element was absent, as it occurs in F promoter, we could determine the presence of an Inr element (TCACTCA) together with a DPE element (TGGCTTC) at positions +28 and +33 from transcription start site (Fig. 3A). Moreover, we could also identify two conserved adenine residues at positions +24 and +27 [14]. To asses if the 5′ upstream region could be responsible of the FL exon transcription, we cloned 3359 pb from the end of exon L to FL exon in a luciferase reporter vector and we performed a luciferase assay by transient transfection in HEK293T and HepG2 cells as well as in primary rat liver hepatocytes. The putative promoter region stimulated 6 times the expression of the luciferase in HEK293T cells, 1.2 times in HepG2 and 1.6 times in hepatocytes (Fig. 3B). These results demonstrate that this region can act *in vitro* as a promoter.

3.3. Expression and functionality of the new FL PFK-2 variants

Once we have determined that FL exon arose from *Pfkfb1* gene through the control of a third promoter located 3.5 kb from exon L, we decided to analyze if the predicted mRNA was capable of producing *in vitro* a functional protein. The predicted aminoacidic sequences using the Transeq tool revealed an untranslated ATG corresponding to a non-coding FL exon. To examine whether FL-variants give rise to translated proteins, identical amounts of *in vitro* synthesized FL-variants were translated in rabbit reticulocyte lysates. Western blotting with an antibody mapping within an internal region of PFK-2, revealed immunoreactive band corresponding to full length PFK-2 only in translated FL-long variant, with a similar profile to that detected in fetal or adult rat liver extracts (Fig. 4A).

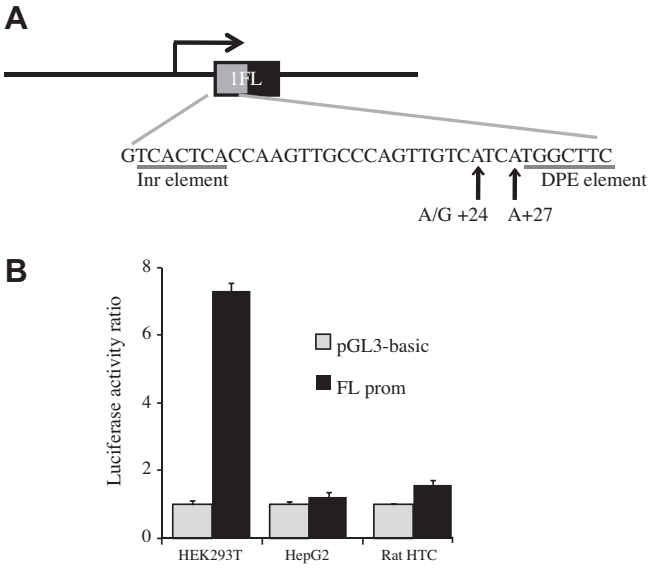

Fig. 3. Identification of the active promoter region associated with FL-exon. (A) Schematic representation of FL exon organization. Common regulatory elements recognized are shown in the sequence. (B) The predicted promoter sequence was cloned in a luciferase reporter vector and the luciferase was assayed in transient transfected cells. Luciferase expression was analyzed 48 h post-transfection. Results are expressed as fold induction relative to the empty vector considered as 1. A Renillla Luciferase expression vector was used as a transfection efficiency control. Data are reported as means + SD of four independent experiments.

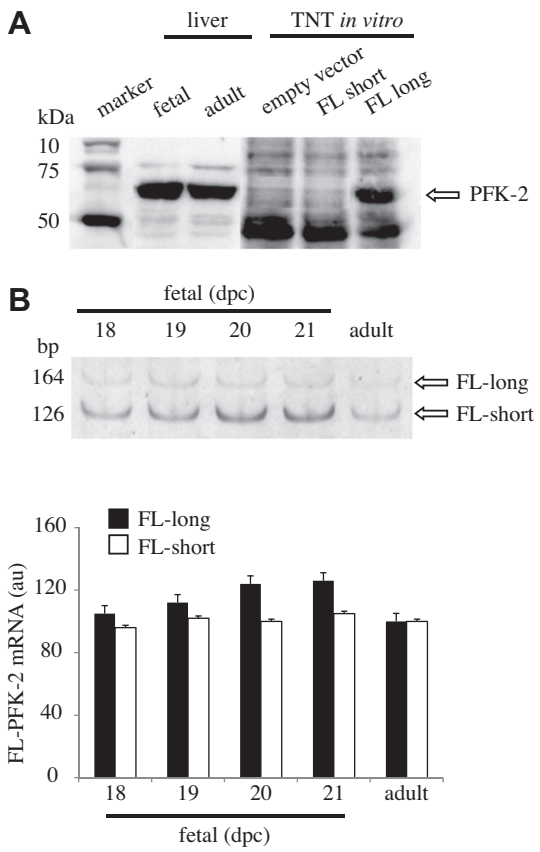

Fig. 4. *In vitro* and *in vivo* expression of the putative FL-short and FL-long PFK-2/FBPase-2 isoforms. (A) Sixty micrograms of total protein extract from 18-days-old fetal liver, adult liver and 5 μl *in vitro* translated proteins from FL-expression vectors were analyzed by Western blot using common liver PFK-2 antibody. A molecular mass marker was loaded as shown in the left lane. A representative western blot out of four is shown. (B) Total RNA was obtained from rat fetal liver at different days post coitum (dpc), reverse transcriptased, and PCR amplified with specific primers to analyze the expression pattern of the FL variants during fetal liver development. PCR products were electrophoresed on 12% polyacrylamide (29:1) gel, developed by silver staining. Results are expressed as arbitrary units after densitometric analysis of FL-PFK-2 expression relative to adult tissue considered as 100%.

The results obtained support the existence of a new PFK-2/FBPase-2 isoform translated from FL-long mRNA. We next used the *in vitro* synthetized protein to analyze the functional activity of the resulting enzyme. Kinase activity was assayed at pH 6.6 as described in Section 2. The results obtained were 5.67, 8.9 and 303.7 pmol/min and mg protein for adult, fetal and *in vitro* synthetized protein, respectively. These results indicate that the *in vitro* synthesized protein presents kinase activity.

3.4. Developmental expression pattern for FL-PFK-2

Finally, to address the developmental expression pattern of FL variants, we determined the presence of the FL-PFK-2 mRNA by RT-PCR during the perinatal transition. FL-long mRNA levels remained constant during this period, while the short variant showed a peak of expression around birth, and reached stability in adulthood (Fig. 4B).

4. Discussion

Transition from the fetal to the neonatal period is accompanied by important changes in liver carbohydrate metabolism induced by hormonal and nutritional factors [8,15] and by modifications in the expression pattern of enzymes and tissue-specific isoenzymes [16]. The bifunctional enzyme PFK-2/FBPase-2 plays a fundamental role in the modulation of the levels of Fru-2,6-P_2, and thus, it participates in the regulation of carbohydrate metabolism adapting it to the requirements in each specific tissue, and each metabolic state.

In liver, a previous work demonstrated important differences between the adult and the fetal form of PFK-2/FBPase-2 at the protein level [10], but there was some controversy about the form of PFK-2/FBPase-2 mRNA present in fetal liver [9]. Dupriez et al. characterized a third mRNA and its promoter of the *Pfkfb1* gene already known to encode the L and M isoenzymes [6]. The sequence of the protein translated from the F-type mRNA is not expected to differ from that of muscle isoenzyme. The kinetic properties of the M isoenzyme resemble those of the phosphorylated L isoenzyme since the skeletal muscle form has a phosphatase activity 5–10 times greater than that of kinase [17]. However, FBPase-2 activity of the fetal enzyme is lower than that of the adult tissue, so kinetic properties of characterized fetal form not correspond to the isoform codified by F-promoter [10]. In the present study we demonstrate the presence of two mRNA variants of PFK-2/FBPase-2 in fetal rat liver that we denominate FL-short and FL-long-PFK-2. Both variants were determined by RACE but the quantitative analysis of the cDNAs suggests important differences in the relative amounts of each variant mRNA levels during fetal development. Moreover, we could prove that only FL-long variant encoded a functional protein whose reading frame encompasses exons 2–14 of the *Pfkfb1* gene. This protein had the same sequence as the L and M isoforms except for the N-terminus.

The computational study of the 5′UTR sequence of the new discovered FL exon in the *Pfkfb1* gene reveals the presence of a potential regulatory region that consists of a TATA-less promoter. A study performed by Suzuki and col. [18], showed that almost all putative promoter regions contained GC boxes and initiator motifs, and both characteristics were found in the FL promoter predicted region. It has been recently demonstrated that not only the metabolism changes during fetal to adult liver transition but also the transcriptional apparatus, including the core promoter [19]. The presence of different isoforms in each developmental stage could be in part due to these changes.

Alternative splicing (AS) and alternative transcription initiation (ATI) are the primary sources of 5′UTR transcript diversity, and several reports have conjectured that these mechanisms might play an important role in orchestrating complex regulatory mechanisms within the 5′UTRs. It has been previously reported that AS affect both the aminoacidic structure of proteins and the regulation of translation by the production of mRNA with different secondary structures depending on the transcribed exon [20,21]. Thus, we can hypothesize that the production of two FL variants of PFK-2 in fetal liver is a mechanism that regulate translation during the fetal to adult transition. The appearance of the untranslated FL-short variant with growing levels until 21 dpc would be an inhibitory signal for the fetal form expression thus giving rise to the adult form expression.

Recently, it has become increasingly clear that the 5′UTR of eukaryotic mRNA is a key site of multiple forms of post-transcriptional regulation of gene expression, especially those containing at least one AUG codon (uAUG) upstream of the main open reading frame (ORF) [22,23]. In some cases, the uAUG have been demonstrated to have a negative effect over the true ORF transcription [20]. As we show in Fig. 3, the first new exon denominated FL, presents an untranslated AUG that could form a transcriptional regulatory signal for the expression of FL-PFK-2.

In the present work, we have identified a novel mRNA of PFK-2/FBPase-2 specific of fetal liver that differs to L isoform by exon 1 L

that contains sites for phosphorylation by PKA and gives rise to a new isoenzyme (FL-PFK-2) that may favor Fru-2,6-P_2 synthesis. Glucose supply during fetal life is continuously ensured by the transplacental input; therefore, fetal liver is prepared to do an intense glycolysis and FL-PFK-2 will promote the flux through PFK-1. However, L-type PFK-2 isoenzyme, regulated by PKA phosphorylation, must accumulate around/birth to allow gluconeogenesis to proceed when glycogen stores are depleted and plasma glucose levels decrease, a situation that applies to neonatal liver. The presence of the two isoforms containing the FL non-coding exon during the last days of fetal liver could be a transcriptional regulatory signal for the strict regulation of glycolytic changes during the fetal to adult transition. Further analysis of the activity of this mRNA and its uAUGs will be of great interest to study the mechanisms involved.

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