Expression of mouse phosphofructokinase-M gene alternative transcripts: evidence for the conserved two-promoter system

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Molecular cloning of the 5′ part of mouse phosphofructokinase-M cDNA was performed. In the 46 cDNA clones isolated, there were two classes of 5′ untranslated sequences. One had an *Eco*RI site within its 5′ untranslated sequence. This showed 83.0% similarity with human type B mRNA for phosphofructokinase-M. The other lacked an *Eco*RI site, showing 92.9% similarity with human type C mRNA. Using the reverse-transcription PCR technique, we found that the transcript with an *Eco*RI site was exclusively expressed in cardiac and skeletal muscles, while that without an *Eco*RI site was expressed in all the mouse tissues examined. The results suggested that the mouse phosphofructokinase-M gene was transcribed through alternative splicing by the multiple promoters. This transcription mechanism was considered to be evolutionarily conserved. The level of phosphofructokinase-M gene expression in mouse cardiac and skeletal muscles decreased in the ketotic diabetic state. Although the regulatory mechanism and the physiological significance are not fully known, this would indicate that phosphofructokinase-M gene transcripts are affected during the diabetic state.

INTRODUCTION

Phosphofructokinase (ATP: D-fructose 6-phosphate 1-phosphotransferase, EC 2.7.1.11, PFK) contributes to the regulation of the intermediary step of glycolysis (Uyeda, 1979; Vora, 1982). Three isoenzymes of PFK have been reported. They are L (liver), M (muscle) and P (platelet) types in human and L, M and C types in rodents (Kahn et al., 1979; Dunaway et al., 1986; Simpson and Fothergill-Gilmore, 1991; Eto et al., 1994). The structural and functional features of human PFK-M gene are the tissue-specific double promoter system and the alternative splicing mechanism (Nakajima et al., 1990a,b; Sharma et al., 1990; Yamasaki et al., 1991). To elucidate the regulation of PFK genes, we have cloned and reported human PFK-M cDNA (Nakajima et al., 1987), human PFK-M gene (Yamasaki et al., 1991) and rat PFK-L cDNA (Hotta et al., 1991). In the rat liver, neither refeeding nor insulin induced PFK-L gene expression appreciably (Hotta et al., 1991). The glucose flux at the PFK step *in vivo* is mainly controlled by fructose 6-phosphate 2-kinase/fructose-2,6-bisphosphatase (Pilkis and Claus, 1991). In the other important steps of hepatic glycolysis, glucokinase and L-type pyruvate kinase are regulated at their gene levels by insulin and glucagon (Noguchi et al., 1982; Iynedjian et al., 1988). Whether PFK-L gene is susceptible to control by insulin is yet to be clarified. Although skeletal muscles are one of the major organs which utilize glucose as the main fuel, it is questionable whether the PFK-M gene is regulated by nutritional and/or hormonal conditions. In this study, we have isolated mouse PFK-M cDNA clones with special reference to its heterogeneity in 5′ untranslated sequences (5′UTs). In addition to the analysis of the tissue-specific expression of the heterogenous gene transcripts, mRNA content in muscle tissues was examined in diabetic mice.

MATERIALS AND METHODS

Materials

Except for Moloney murine leukaemia virus reverse transcriptase (GIBCO BRL Life Technologies, Gaithersburg, MD, U.S.A.), *AmpliTaq* (Perkin–Elmer Cetus, Norwalk, CT, U.S.A.), Deep $Vent_R$ DNA polymerase (New England Biolabs, Beverly, MA, U.S.A.), Taq DyeDeoxy Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, U.S.A.), [α-^{32}P]dATP (111 TBq/mmol, DuPont–New England Nuclear, Boston, MA, U.S.A.) and Multiprime DNA labelling system (Amersham International, Amersham, Bucks., U.K.), all materials used in the molecular biological procedures, including restriction endonucleases, modifying enzymes and pUC119 vector, were purchased from Takara Shuzo, Japan. Hybond-N was obtained from Amersham. Oligonucleotide primers were synthesized on a Model 381A DNA synthesizer (Applied Biosystems). The sequences of the primers are shown in Figure 1. Five-week-old male ICR mice were supplied by JCL, Japan. The intermediate-acting insulin (Lente MC) and the short-acting insulin (Actrapid MC-human) were products of Novo Industri A/S, Copenhagen, Denmark. All other chemical reagents used were of the highest grade.

Isolation of mouse PFK-M cDNA

For PCR reactions, *AmpliTaq* was used throughout unless otherwise specified and the thermal cycling was principally performed at 95 °C for 1 min, 60 °C for 1 min and 72 °C for 1 min. Primers RM7 and P650 were synthesized according to the human PFK-M cDNA positions 1–21 and 639–667 (Nakajima et al., 1987) respectively, for reverse-transcription (RT)-PCR in the

Abbreviations used: PFK (-M), (M-type) phosphofructokinase; RT-PCR, reverse-transcription PCR; 5′UT, 5′ untranslated sequence; RACE, rapid amplification of cDNA ends.

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The nucleotide sequences reported have been submitted to the GSDB/DDBJ/EMBL/NCBI databases under the accession numbers D21865, D21867, D21868 and D21869.

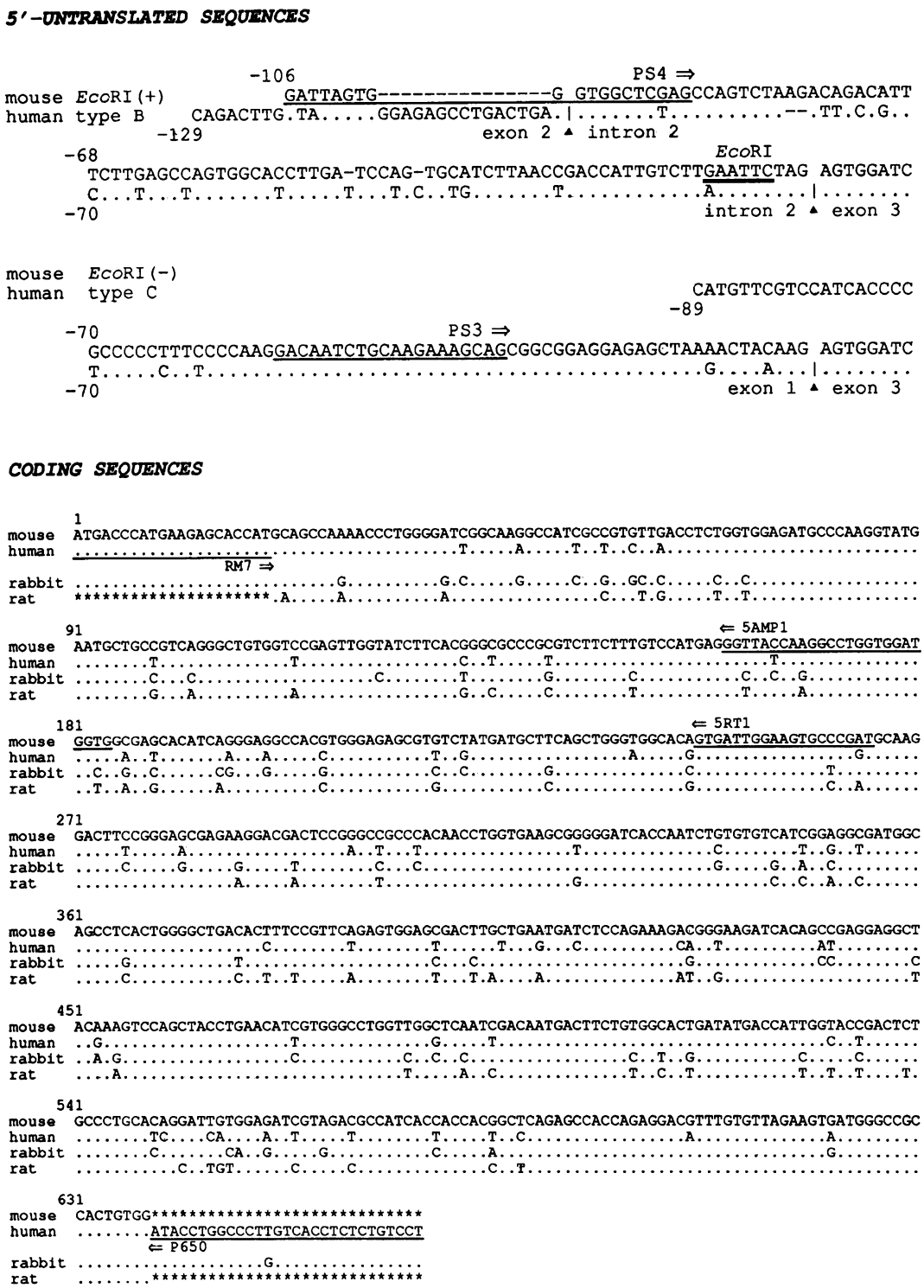

Figure 1 Nucleotide sequences of cloned cDNAs representing the mouse PFK-M mRNA variants and the alignments with the mammalian PFK-M sequences

Mouse PFK-M mRNA 5′UTs are compared with the human counterpart sequences (Nakajima et al., 1987; Yamasaki et al., 1991). Coding sequences for mouse, human, rabbit (Lee et al., 1987) and rat (PCR-cloned) are also paralleled. Symbols indicate gaps (–), splice junctions in the human PFK-M (¦), identical residues to the upper sequences (.) and primer-incorporated sequences (*). PCR primers used in this study are underlined and their directions of extension are shown by arrows. Double underlining shows the specific *Eco*RI site.

mouse and rat skeletal muscle RNA as reported (Nakajima et al., 1990b). Amplified products were subcloned into the *Hin*cII site of pUC119 and were sequenced. From the mouse sequence, 5RT1 and 5AMP1 for 5′-RACE (rapid amplification of cDNA ends) were synthesized. According to the method of Frohman et al. (1988), 5′-RACE was performed on mouse skeletal muscle RNA using Deep Vent$_R$ DNA polymerase. The RACE products were ligated to the *Hin*cII site of pUC119 vector. From this

library 46 cDNA clones for mouse PFK-M were isolated. Sequence determinations were made on both senses analysed by the automated DNA-sequencing system (Model 373A, Applied Biosystems).

Analysis of PFK-M mRNA expression in mouse tissues

Mice were kept under specific pathogen-free conditions and fed ordinary laboratory chow *ad libitum*. They were made diabetic by an intraperitoneal injection of streptozotocin (200 mg/kg body weight). Mice having glucosuria and ketonuria for at least two consecutive days before killing were defined as ketotic. Some of the ketotic mice were treated with subcutaneous injections of intermediate-acting insulin (30 units/kg) for 3 days. The intermediate-acting insulin (15 units/kg, subcutaneous) and the short-acting insulin (15 units/kg, intraperitoneal) were injected 5 h before killing. From the brain, heart, lung, liver, kidney, muscle and testis of the control mouse, RNA was extracted by the method of Chomczynski and Sacchi (1987). RNA (20 μg) was separated on a 0.8% agarose/2.2 M formaldehyde gel and transferred to Hybond-N. The filter was hybridized with ^{32}P-labelled human PFK-M cDNA (residues 1656–2380) under high stringency conditions as recommended by the manufacturer. Autoradiography was performed at −80 °C with an intensifying screen for 7 days. The alternative transcripts were examined by RT-PCR using the common primer 5AMP1 and variant specific primers PS4 and PS3 for the detection of *Eco*RI (+) and *Eco*RI (−) mRNAs respectively. Thermal cycling of 30 and 40 cycles was performed on the first-strand cDNA of various mouse tissues. Cardiac and skeletal muscle RNA were prepared from the control, ketotic diabetic and insulin-treated diabetic mice. The RNA (10 μg) was stained with 50 μg/ml ethidium bromide and, after electrophoresis as described above, the visualized 28S and 18S ribosomal RNA bands were photographed with a u.v. transilluminator to ensure that the RNA was intact and evenly loaded. After Northern hybridization, the amount of PFK-M mRNA was measured using a Bioimaging Analyser (BAS-2000 system, FUJIX, Japan).

RESULTS

By RT-PCR, using RM7 and P650, mouse and rat PFK-M cDNA fragments were amplified. The sequences contained 667 bp of N-terminal coding regions of the PFK-M mRNA (Figure 1). Similarities of mouse versus human, rabbit and rat PFK-Ms were 90.4%, 89.5% and 95.8%, excluding the primer sequences, respectively. Amino acid sequence similarities were 96.7%, 96.7% and 99.0% for mouse versus human, rabbit and rat respectively. Cloning of 5′UTs of the mouse PFK-M mRNA was performed by 5′-RACE using Deep Vent$_R$ DNA polymerase instead of *Taq* DNA polymerase to prevent PCR errors (Dunning et al., 1988; Lohff and Cease, 1991). There was no obvious misincorporation. The isolated cDNAs represented the two classes of mRNA sequences. The 5′UT of the longest cDNA clone in each class is shown in Figure 1. One contained an *Eco*RI site at the −12 base within the 5′UT and the other contained no *Eco*RI site having a different 5′UT. The sequence from the −8 position to the coding region was the same in the two mRNA species. Sequence similarities to human PFK-M type-B and -C mRNAs were estimated with respect to the 5′UT. The similarities were 83.0% and 92.9% between the mouse *Eco*RI (+) mRNA and human type-B mRNA and between the mouse *Eco*RI (−) mRNA and human type-C mRNA respectively. Putative splice junctions could be assigned at the −8 position just the same as in human alternative transcripts (Yamasaki et al., 1991).

Expression of the mouse PFK-M mRNA variants

Northern-blot analysis revealed that expression of PFK-M mRNA was dominant in cardiac and skeletal muscles (Figure 2). Very low expression was also observed in the brain and kidney. To determine the mode of expression of the alternative transcripts in the various mouse tissues, RT-PCR was performed using variant specific primers, PS4 and PS3. The *Eco*RI (−) mRNA species gave a 238 bp PCR product and was widely distributed in all the tissues examined, including brain, heart, lung, liver, kidney, skeletal muscle and testis. The expression of the *Eco*RI (+) mRNA species (290 bp) was restricted only to the cardiac and skeletal muscles. When the amplification was increased from

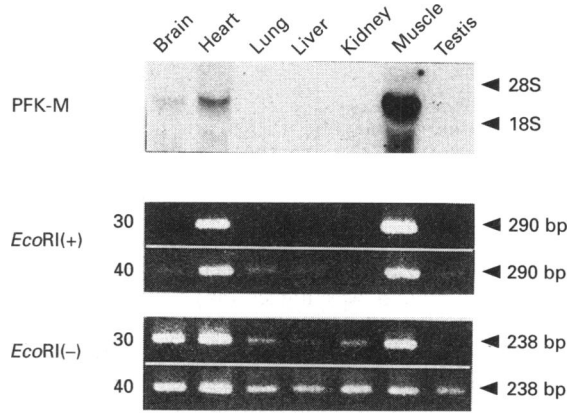

Figure 2 Expression of PFK-M gene transcripts in normal mouse tissues

Northern-blot analysis for 20 μg of RNA from the indicated tissues is shown in the upper panel. The results of the variant-specific RT-PCR are shown in the middle [the *Eco*RI (+), primers PS4 and 5AMP1] and in the lower panels [the *Eco*RI (−), primers PS3 and 5AMP1]. Numbers indicate the PCR cycles. Migration of ribosomal RNA (28S and 18S) and the sizes of the PCR products are marked to the right.

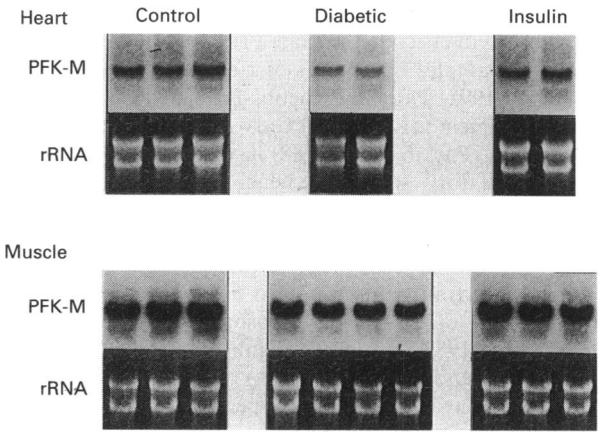

Figure 3 PFK-M mRNA expression during diabetes and an insulin treatment

Ten micrograms of cardiac (upper) and skeletal (lower) muscle RNA from the control, ketotic diabetic and insulin-treated mice was analysed by Northern hybridization. The amount of loaded RNA was equal as obviously seen by the ethidium bromide-stained ribosomal RNA (rRNA) indicated beneath each panel.

30 to 40 cycles, faint amplification of *Eco*RI (+) mRNA was also observed in the rest of the tissues (Figure 2).

PFK-M gene expression in muscle tissues in diabetic mice

The cardiac and skeletal muscle RNA prepared from the control, ketotic diabetic and insulin-treated diabetic mice were analysed by Northern-blot hybridization (Figure 3). The probe used was the C-terminal coding region of human PFK-M cDNA. Cross-hybridization with the other PFK isoenzyme mRNA was considered to be negligible by the use of this cDNA fragment in a high-stringency condition. The total PFK-M mRNA in muscle and heart decreased in ketotic diabetes to 62.2% (2) and 51.8% (4) of that of the control mice, which recovered by insulin treatment to 85.9% (2) and 76.5% (3) of the control level respectively. The mean value for the three control mice was assumed to be 100%. The value in parentheses represents the number of mice used in calculating the mean.

DISCUSSION

It is notable that eukaryotic PFKs are double the size of prokaryote ones (Evans et al., 1981). Poorman et al. (1984) proposed a hypothesis suggesting that the mammalian PFK genes have evolved from the ancestral bacterial one by internal gene duplication. As shown in this study, the amino acid sequence similarity between PFK-M N-terminal coding regions of mouse, rat, rabbit and human was higher (approx. 90%) than that between the corresponding regions of human PFK-M and -L (68.4%). This suggests that the establishment of the isoenzyme genes preceded the speciation of animal species during evolution.

When the variant specific primers PS4 and PS3 were used, two mRNA sequences were reproducibly amplified. This indicates that the heterogenous 5′UT isolated from the RACE library does not include artifacts. By a preliminary Southern-blot analysis using the cDNA probe common to the two mRNA variants [residues −16 to +184 of the *Eco*RI (+) cDNA], we observed that the mouse PFK-M gene existed as a single copy per haploid genome (results not shown). Thus the two mRNA sequences presented here represent alternative splicing products transcribed from a single PFK-M gene.

The physiological significance of these mRNA variants, whose translation products code the same PFK-M protein, needs to be evaluated. Examples like 5′UTs of ribosomal protein mRNAs (Levy et al., 1991; Patel and Jacobs-Lorena, 1992), *Drosophila* heat shock protein mRNAs (McGary and Lindquist, 1985) and c-*myc* mRNA (Parkin et al., 1988) have been shown to contain putative regulatory sequences which affect their translation efficiencies. The oligopyrimidine tract just downstream of the cap site is also proposed to contribute to the translation efficiency (Patel and Jacobs-Lorena, 1991). By our inspection, the 5′UT of the *Eco*RI (+) transcript contained a brief similarity (47.2%) with the 5′UT of *Drosophila* ribosomal protein A1 mRNA (Quian et al., 1987). An oligopyrimidine tract ($C_5T_3C_4$) was found in the 5′-end of the *Eco*RI (−) transcript. These observations suggest that mRNA variant-specific translational control might play a part in adjusting the level of enzyme protein expression.

We have reported that the structural and functional features of the human PFK-M gene are the tissue-specific two promoter system and alternative splicing events (Nakajima et al., 1990b; Yamasaki et al., 1991). Exon 1 is promoted by an upstream housekeeping promoter to constitutively express type-C mRNA. Within intron 1, an alternate promoter exists. Motifs of CAAT- and TATA-boxes and muscle-specific M-CAT enhancers contribute to the muscle tissue-specific expression of type-A/B mRNAs. Type-A and type-B mRNAs are alternatively spliced. When intron 2 is retained in the 5′UT, type-B mRNA is produced (Yamasaki et al., 1991). In this study, the mouse PFK-M cDNA corresponding to the human type-A mRNA was not cloned. Sequences of splice junctions and an 89-base intron similar to the human type-B 5′UT were found within the mouse *Eco*RI(+) mRNA 5′UT. However, the putative donor sequence showed a relatively low identity with the consensus sequence (Mount, 1982) and the expected 5′ exon would be as short as 9 bases. Since 5′-RACE method gives a good estimation of the transcription start site (Frohman et al., 1988), the length of the 5′UT would be as long as those shown in Figure 1. Thus, splicing efficiency of the 89-base intron sequence would be low. For these reasons, a mouse homologue of the human type-A mRNA would be hardly observed.

Li et al. (1990) reported that a single housekeeping promoter initiated the multiple transcription and produced alternative transcripts with heterogeneous 5′UTs in the rabbit PFK-M gene. There may be a species difference and the results presented here suggest that the mouse PFK-M gene would be controlled by the two-promoter system. When the excessive thermal cycling was performed (over 40 cycles), the *Eco*RI (+) transcript was also detected faintly in the non-muscle tissues. Considering the sensitivity of the method, such a trace amount of the transcript could hold less importance in the physiological significance. The two-promoter system and the tissue-specific expression would be evolutionarily conserved features in the mouse PFK-M gene also.

Dunaway et al. (1986) showed that the PFK-M enzyme activity in diabetic rats was decreased in cardiac muscles. However, there has been no evidence that PFK-M gene expression is affected by diabetes. In the present study, Northern-blot analysis revealed the decrease of total PFK-M mRNA in the cardiac and skeletal muscles of ketotic diabetic mice. After treatment with insulin, this decrement was recovered to some extent. Although the mechanism and the physiological significance are not fully elucidated, this would be a novel observation in studies of PFK-M genes. In our preliminary observations using the semi-quantitative RT-PCR method, the contents of *Eco*RI (+) mRNA (%) in total PFK-M mRNA were 58.1 ± 0.93, 48.3 ± 1.51 and 49.6 ± 2.42 in the control, diabetic and insulin-treated mice respectively. The ketotic diabetic mice showed a decrease ($P < 0.01$, Student's *t*-test) of *Eco*RI (+) transcript in their cardiac muscles. The *Eco*RI (+) mRNA content (%) in skeletal muscles was essentially unchanged, at 76.2 ± 1.05, 74.2 ± 3.51 and 75.4 ± 0.46 in the control, diabetic and insulin-treated mice respectively. All data represent the mean ± S.E.M. of three mice. The splicing pattern of PFK-M in the diabetic state would be an interesting focus in studying insulin action on RNA processing. However, the changes by diabetes were very small and insulin treatment had no effect on the splicing pattern in this study. Indeed, diabetes may cause these effects, but it is not clear whether it is insulin deficiency *per se*, or one of the many other factors happening consequently to make animals diabetic, that affects the transcription and splicing. Further investigations into the regulatory mechanism of PFK-M gene would be worthwhile in elucidating the metabolic derangement of cardiac and skeletal muscles in the diabetic state.

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