Isolation and Characterization of the Transcriptionally Regulated Mouse Liver (B-type) Phosphofructokinase Gene and Its Promoter*

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We have isolated and characterized a mouse gene encoding liver (B-type) phosphofructokinase, a key regulatory enzyme in glycolysis. The gene spans approximately 21.5 kilobase pairs and consists of 22 exons. Compared with the muscle (A-type) phosphofructokinase gene, the sizes of the introns are different although exon lengths are highly conserved. Two transcription start sites 10 bases apart were determined by primer extension experiments. The immediate 5' sequence does not possess a TATA or CCAAT box but contains multiple GC boxes (positions -10, -43, -50, -62, and +28 in the 5'-untranslated region) which may be Sp1-binding sites. An unusual feature of 200 base stretches of CT repeats is present at position -480 to -693. In addition, direct repeats of TCGGAAGGAG are found at positions -447 and -478. DNase I footprinting showed five regions where liver nuclear proteins may interact. Two proximal 5'-flanking regions spanning -1 to -20 and -50 to -70, which contain GC boxes. Also protected was a region spanning -70 to -90, which contains an AP-1 like sequence (TCAGTCA). The consensus AP-1 sequence, however, did not inhibit footprinting, indicating involvement of a distinct protein. Two distal regions spanning from -450 to -470 and from -500 to -520 were also protected. The former is positioned between the direct repeats and the latter is at the start of the CT repeats. The rate of transcription of the liver phosphofructokinase gene, as measured by run-on assays, increased 4-fold when previously starved mice were refed a high carbohydrate, fat-free diet. Further, this increase in mRNA level was blocked by 50% by the administration of dibutyryl cAMP. It will now be possible to define cis-acting elements responsible for the regulated expression of the mouse liver phosphofructokinase gene by fasting/refeeding and by cAMP. These results provide a useful system for the study of regulatory elements in liver phosphofructokinase gene transcription.

Phosphofructokinase (EC 2.7.1.11) catalyzes the phosphorylation of fructose 6-phosphate to fructose 1,6-bisphosphate and is a key regulatory enzyme in the glycolytic pathway. Phosphofructokinase activity is under allosteric regulation by various metabolites (1, 2). The smallest active form of the enzyme is a tetramer with a subunit M, of 80,000. Three types of subunits have been identified, i.e. muscle (A-type), liver (B-type), and brain (C-type) (3, 4). The genes encoding the three types of subunits are not known to be linked. Variable expression of these loci in different tissues leads to tissue-specific patterns of isozymes with differing physicochemical and regulatory properties (5–7). The phosphofructokinase isozymes isolated from muscle, liver, and brain show different sensitivity to ATP and citrate inhibition (8). In addition, the muscle isozyme has been reported to be better suited for binding to F-actin (8).

We have previously isolated cDNA sequences to both liver and muscle type phosphofructokinase (10, 11). Using these cDNA clones as probes in Northern blot analysis, we have observed that the muscle type phosphofructokinase expression was largely restricted to muscle tissue whereas liver-type phosphofructokinase was expressed not only in liver but also, although at lower levels, in tissues such as brain, kidney, and lung (10, 11). Comparison of the deduced amino acid sequences of mouse liver and muscle phosphofructokinase showed 67% homology, while mouse muscle phosphofructokinase showed 90% homology compared with the known rabbit muscle isozyme sequence, indicating higher conservation of amino acid sequence of isozymes between mammalian species than among different isozymes in the same species (10–12). We have also reported that the liver phosphofructokinase mRNA level was under hormonal and nutritional control (10). The liver phosphofructokinase mRNA level increased 4-fold when previously starved mice were refed a high carbohydrate, fat-free diet. Further, this increase in mRNA level was blocked by 50% by the administration of dibutyryl cAMP (11).

In order to study the molecular mechanisms underlying the regulation of liver phosphofructokinase gene expression by nutrients and hormones, we have isolated genomic clones coding for liver phosphofructokinase and analyzed the gene structure by defining the intron-exon positions and junction sequences. We have obtained the promoter and adjacent DNA sequences which contain a number of elements that may play roles in liver phosphofructokinase gene regulation. Several regions interact with liver nuclear proteins as shown by DNase I footprinting. We also show the transcriptional regulation of the mouse liver phosphofructokinase gene by fasting/refeeding and by cAMP. It will now be possible to define cis-acting elements responsible for the regulated expression of the liver phosphofructokinase gene under various physiological conditions.

EXPERIMENTAL PROCEDURES

Isolation and Characterization of Liver Phosphofructokinase Genomic Clones—A Charon 4A mouse genomic library constructed from partial EcoRI and HaellI/AluI digests of BALB/C sperm DNA (obtained from Dr. L. Hood, California Institute of Technology) and a BALB/C embryo library constructed from partial HaellI digests

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The nucleotide sequence(s) reported in this paper has been submitted to the GenBankTM/EMBL Data Bank with accession number(s) M81210.

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Liver Phosphofructokinase Gene and Its Promoter

Liver phosphofructokinase (PFK) is a key enzyme in the regulation of carbohydrate metabolism, catalyzing the conversion of fructose-6-phosphate to fructose-1,6-bisphosphate. The gene encoding PFK is located on chromosome 12 in humans and is highly expressed in liver and muscle tissue.

Isolation and Characterization of the Mouse Liver Phosphofructokinase Gene—We initially screened Charon 4A mouse genomic libraries with a previously cloned 2.7-kilobase full-length PFK cDNA sequence (10). Clone ALPFK-1 contained an 18.5-kbp insert of mouse DNA encompassing most of the liver phosphofructokinase gene. An overlapping clone (ALPFK-2) with an insert size of 10.8 kbp contained 130 bp of the 5'-flanking region of the liver phosphofructokinase gene. We further screened genomic libraries with the most 5'-1.4-kbp fragment, and isolated a genomic clone with an insert size of 18.2 kilobase (ALPFK-3) which contained the first 10 introns and approximately 10 kilobase of 5'-flanking region. Since the 3' most CDNA sequences were not contained in these genomic clones, the genomic libraries were also screened with the 730-bp PstI fragment from the 3' most region of the cDNA clone, pPK-2. Positive clone ALPFK-4 (14.6 kbp) contained the three most 3' exons and the 3'-flanking region of the liver phosphofructokinase gene.

To define the positions and boundaries of the exon blocks, the restriction fragments of the four clones were characterized by Southern blot analysis (Fig. 1). The EcoRI and XhoI fragments were subcloned into the plasmid Bluescript SK+.

RESULTS AND DISCUSSION

Isolation of 5'-Flanking Region of the Mouse Liver Phosphofructokinase Gene—We isolated a genomic clone that was 18.5 kbp in size, encompassing most of the 5'-flanking region of the liver phosphofructokinase gene. The clone contained 130 bp of sequence corresponding to nucleotides +62 to +87 of the noncoding strand of the gene.

Poly(A)+ RNA from mouse liver was obtained, and run-on transcription experiments were performed using this RNA as a template.

Preparation of Nuclear Extracts—Nuclear extracts were prepared from mouse liver by the methods of Nenhgais and Lubin (15). Protein concentrations were measured by the Bradford method using bovine serum albumin as the standard (16).

DNase I Footprinting Analysis—The DNAs used probes in the footprinting assays were prepared by end-labeling specific DNA fragments using the chain termination method using Sequenase (USB Biochemicals) and universal primers or oligonucleotides synthesized by standard phosphoamidite techniques on a BioSearch synthesizer according to the previously determined nucleotide sequence. Polymerase chain reaction amplification was utilized to determine the positions and sizes of the exons and introns in the gene. For intron/exon boundaries, oligonucleotides corresponding to exons according to the cDNA sequences and known rabbit muscle phosphofructokinase gene structure were synthesized and used for amplification by polymerase chain reaction utilizing the genomic and subcloned fragments as templates to verify the intron sizes. Polymerase chain reaction was carried out across not only one but in most cases additional intron sequences. Amplification products were analyzed by agarose gel electrophoresis.

Promoter Extension Analysis—A 2- to 4-kb P Cleavage to +87 of the noncoding strand of the mouse liver phosphofructokinase cDNA (11) was synthesized and was P-labeled with T4 polynucleotide kinase. The oligonucleotide was hybridized with 20 µg of poly(A) RNA preparation from mouse liver in 80% formamide, 40 mM PIPES, pH 6.4, 400 mM NaCl, and 1 µM EDTA at 60 °C. An autoradiogram was obtained by exposing the washed filters to x-ray film with an intensifying screen. The 5'-flanking region of the liver phosphofructokinase gene was synthesized. The oligonucleotide was labeled with 32P, and used for amplification by polymerase chain reaction utilizing the genomic and subcloned fragments as templates to verify the intron sizes.
Liver Phosphofructokinase Gene and Its Promoter

Fig. 1. Structure of the mouse liver phosphofructokinase gene. A, the four overlapping genomic clones and the restriction sites which were used to characterize the gene. E, EcoRI; X, XhoI, P, PstI; H, HindIII. The hatched box indicates the location of the liver phosphofructokinase structural gene. B, the organization of the liver phosphofructokinase gene and the comparison with the rabbit muscle phosphofructokinase gene. The solid boxes show the locations of the exons.

nucleotides. Both of the transcription initiation sites, which are 66 and 66 nucleotides upstream from the AUG translation initiation codon, were utilized with equal frequency as shown by the similar intensity of the two extension products shown in Fig. 1.

We have sequenced and analyzed 700 base pair upstream of the transcription initiation site (Fig. 3). A computer-assisted search of the 5′-flanking region of the mouse liver phosphofructokinase gene reveals elements that have potential roles in its transcription. The canonical polymerase II transcription elements TATA and CCAAT are lacking in the liver phosphofructokinase gene promoter. There are clusters of GC-rich domains with the consensus sequence for the Sp1-binding site at positions −10, −43, −50, and −62 as well as at +28 in the 5′-untranslated region of the liver phosphofructokinase gene (Fig. 3). These characteristics, i.e. absence of TATA and CCAAT and presence of multiple GC boxes, have been reported for the promoters of several genes which encode enzymes involved in metabolic reactions carried out in many cell types, and are thus named housekeeping genes (18–21). We have previously shown that whereas the muscle phosphofructokinase mRNA is present only in muscle tissue, mRNA for the liver isozyme was detected in all tissues examined, including liver, brain, lung, and kidney (11). Universal expression of liver phosphofructokinase suggests a housekeeping role, while muscle and brain isozymes may be expressed in a tissue-specific manner according to glycolytic needs. However, unlike other housekeeping genes, the liver phosphofructokinase mRNA is inducible in liver by refeeding of a high carbohydrate diet to previously fasted mice. This induction is blocked by 50% by the administration of dibutyryl cAMP at the start of refeeding (11). An unusual feature of the promoter region of the phosphofructokinase gene is the extended stretches at positions −480 to −693 of pyrimidine-rich (dC–dT) tracts, which have been associated with H form DNA (22, 23). Palindromes with a motif CCTC(N),CTCC are found in polyoma and SV40; in SV40 this sequence serves as a large T antigen-binding site. It has been reported that the alternating

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<th>Intron Size (kb)</th>
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to the oligonucleotide and extended by reverse transcriptase as described under "Experimental Procedures." The last four lanes (A, T, and C) represent sequence of the genomic fragment by dideoxy chain termination using the same primer as for the RNA analysis. Lane 1 shows the extension products with arrows indicating them. The start sites are also underlined in the adjacent nucleotide sequence. Essentially identical results were obtained from three separate experiments.

![Fig. 2. Primer extension analysis of the liver phosphofructokinase mRNA. 20 μg of poly(A) RNA from liver was hybridized to the oligonucleotide and extended by reverse transcriptase as described under "Experimental Procedures." The last four lanes (A, T, and C) represent sequence of the genomic fragment by dideoxy chain termination using the same primer as for the RNA analysis. Lane 1 shows the extension products with arrows indicating them. The start sites are also underlined in the adjacent nucleotide sequence. Essentially identical results were obtained from three separate experiments.](image)

The DNA sequence of the 5′-flanking region of the liver phosphofructokinase gene. The sequences of the first exon are represented by capital letters. The sequence is numbered in relation to the first transcription initiation site. There are two transcription start sites at +1 and +11. The translation initiator is overlined. The GC boxes are boxed. The AP-1 like sequences are underlined with heavy lines. The two light-dotted lines show the direct repeat sequences. The numbered heavy dotted lines with arrows indicate the oligonucleotides used in primer extension and for the probe synthesis in DNase I footprinting. The underlined sequences with letters indicate the regions protected from DNase I digestion. When the DNA fragment spanning -101 to -400 was used in the footprinting assay, no appreciable protection was detected in this middle 5′-flanking sequence of the gene. We could not detect any protection around another copy of an AP-1-like sequence present in this region at -273. In the distal 5′-flanking sequence of the gene, two regions of nucleotide -453 to -469 and -502 to -519 (Fig. 4b, panel A, boxes D and E), when DNA fragment spanning -351 to -699 was labeled on noncoding strand and used for footprinting. The competition experiments carried out with the 50-fold excess of oligonucleotides synthesized according to the sequences from -445 to -475 and from -485 to -522 eliminated the footprints to the respective regions completely (Fig. 4b, panels B and C). The former region is positioned between the direct repeat sequences described above. The latter region is the sequence at the start of the CT repeat sequences. The CT repeat sequences themselves did not show protection with the addition of nuclear extracts. In addition, binding of liver nuclear proteins produced distinct hypersensitive sites adjacent to the protected regions. In all cases, increasing amounts of liver nuclear extract caused higher degrees of protection. When we initially

![Fig. 3. The DNA sequence of the 5′-flanking region of the liver phosphofructokinase gene. The sequences of the first exon are represented by capital letters. The sequence is numbered in relation to the first transcription initiation site. There are two transcription start sites at +1 and +11. The translation initiator is overlined. The GC boxes are boxed. The AP-1 like sequences are underlined with heavy lines. The two light-dotted lines show the direct repeat sequences. The numbered heavy dotted lines with arrows indicate the oligonucleotides used in primer extension and for the probe synthesis in DNase I footprinting. The underlined sequences with letters indicate the regions protected from DNase I digestion. When the DNA fragment spanning -101 to -400 was used in the footprinting assay, no appreciable protection was detected in this middle 5′-flanking sequence of the gene. We could not detect any protection around another copy of an AP-1-like sequence present in this region at -273. In the distal 5′-flanking sequence of the gene, two regions of nucleotide -453 to -469 and -502 to -519 (Fig. 4b, panel A, boxes D and E), when DNA fragment spanning -351 to -699 was labeled on noncoding strand and used for footprinting. The competition experiments carried out with the 50-fold excess of oligonucleotides synthesized according to the sequences from -445 to -475 and from -485 to -522 eliminated the footprints to the respective regions completely (Fig. 4b, panels B and C). The former region is positioned between the direct repeat sequences described above. The latter region is the sequence at the start of the CT repeat sequences. The CT repeat sequences themselves did not show protection with the addition of nuclear extracts. In addition, binding of liver nuclear proteins produced distinct hypersensitive sites adjacent to the protected regions. In all cases, increasing amounts of liver nuclear extract caused higher degrees of protection. When we initially...](image)
FIG. 4. Binding of nuclear proteins to the mouse liver phosphofructokinase promoter region. DNA fragments containing the mouse liver phosphofructokinase promoter regions, +87 to −169 for section (a) and −351 to −699 for section (b) were utilized for footprinting. The binding assays were carried out with probes made by labeling noncoding or coding strands by T4 polynucleotide kinase, restriction digestion, low melting gel electrophoresis, and elution. Fifty thousand cpm of each probe were incubated with indicated amounts of liver nuclear extracts. DNase I footprinting reactions were performed as described under “Experimental Procedures” and analyzed on a 6% polyacrylamide/urea gel. The regions of protection from DNase I digestion are demarcated by boxes. Section a: left panel A; DNA fragment spanning +87 to −169 was labeled on noncoding strand; right panel A; DNA fragment spanning +87 to −169 was labeled on coding strand was employed for footprinting and indicated amounts (μg) of nuclear extract with no competing oligonucleotides were added, B; 60-fold excess of oligonucleotides (−65 to −97) added during preincubation, C; 60-fold excess of AP-1 consensus sequence (CGTGACTCAGCGCGC) added during preincubation. Section b: DNA fragment spanning −361 to −699 was labeled on noncoding strand and was employed for footprinting. A, no competing oligonucleotides were added; B, oligonucleotides (−445 to 475) added during preincubation; C, oligonucleotides (−485 to 522) added during preincubation. The regions of protection (A–E) are demarcated by the boxes on the side and are underlined with the designating letters in Fig. 3.

employed labeled DNA fragment spanning nucleotides +87 to −698 in the footprinting, similar results were obtained. At the present time, it is not known what functional roles the nuclease protected regions may play. However, these sequences may be important in isozyme-specific expression of the phosphofructokinase gene. We have cloned and sequenced the promoter region of the mouse muscle phosphofructokinase gene (data to be reported elsewhere). When compared with the promoter region of the mouse muscle phosphofructokinase gene, there is no sequence homology in the 5′-flanking regions of these two genes encoding liver and muscle isozymes.

Transcriptional Regulation of the Liver Phosphofructokinase Gene—We have carried out transcription run-on assays using nuclei prepared from the livers of previously fasted mice which were refed a high carbohydrate diet. As shown in Fig. 5, the transcription of the liver phosphofructokinase gene was barely detectable in fasted mouse liver. However, after 6 h of refeeding, the transcription rate increased approximately 5-fold. The increase in transcription rate in the livers of refed mice was completely abolished by the administration of cAMP. The actin gene transcription did not change appreciably during fasting, refeeding, and refeeding with cAMP treatment.
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**REFERENCES**


2. P. Rongnoparut and H. S. Sul, unpublished results.