Proc. Natl. Acad. Sci. USA
Vol. 85, pp. 8430–8434, November 1988
Biochemistry

cDNA sequence of rat liver fructose-1,6-bisphosphatase and evidence for down-regulation of its mRNA by insulin

(amino acid sequence/diabetes)

M. RAAFAT EL-MAGHRABI*, JO PILKIS*, ALISON J. MARKER*, ANN D. COLOSIA*, GUY D'ANGELO*, BLAIR A. FRASER†, AND SIMON J. PILKIS*

*Department of Physiology and Biophysics, State University of New York at Stony Brook, Stony Brook, NY 11794; and †Division of Biochemistry and Biophysics, Food and Drug Administration, Bethesda, MD 20892

Communicated by Charles R. Park, August 8, 1988

ABSTRACT **A coding-length clone of rat liver fructose-1,6-bisphosphatase (EC 3.1.3.11) was isolated by immunological screening of a cDNA library in λgt11. Its identity was verified by comparing the deduced amino acid sequence with that obtained by direct sequencing of a complete set of CNBr and proteolytic peptides from the purified protein. The enzyme subunit is composed of 362 amino acids and has *N*-acetylvaline as the amino-terminal residue. The cDNA, 1255 base pairs (bp) long, consisted of 1086 bp of coding region, 15 bp of 5′ untranslated sequence, and 154 bp at the 3′ untranslated end. The 3′ untranslated sequence contained a polyadenylylation signal (AATAAA) followed after 30 bp by a stretch of 7 adenines at the end of the clone. The deduced amino acid sequence was identical to the primary sequence of the protein and confirmed the alignment of five nonoverlapping peptides. It also confirmed the 27-residue extension, unique to the rat liver subunit, ending with a carboxyl-terminal phenylalanine. RNA blot analyses using the radiolabeled liver cDNA as a probe revealed a single band of fructose-1,6-bisphosphatase mRNA, 1.4 kilobases long, in liver and kidney but not in nongluconeogenic tissues. Fructose-1,6-bisphosphatase mRNA was increased 10-fold in livers from diabetic rats and was reduced to control levels after 24 hr of insulin treatment, suggesting that the changes in enzyme activity observed in diabetes and after insulin treatment are due to alterations in mRNA abundance.**

The hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate, catalyzed by fructose-1,6-bisphosphatase (Fru-1,6-P_2ase; EC 3.1.3.11), is an essential step in the gluconeogenic pathway (1) and is under both acute and long-term hormonal control. Acute hormonal regulation occurs primarily by modulation of the potent inhibitor fructose 2,6-bisphosphate (Fru-2,6-P_2) (2, 3), which has been postulated to bind either to the active site (2, 4) or to a separate allosteric site (3, 5). Most of the studies on allosteric regulation of Fru-1,6-P_2ase by Fru-2,6-P_2 have been carried out on the rat liver enzyme, but identification of the Fru -2,6-P_2 binding site has been hampered both by lack of primary sequence and x-ray crystallographic information and by lack of a cDNA clone with which to do site-directed mutagenesis studies. Yeast (6) and rat liver (7) Fru-1,6-P_2ase have also been reported to be regulated by cAMP-dependent phosphorylation. Phosphorylation of the rat liver enzyme at a site(s) located on a carboxyl-terminal extension unique to this form (8, 9)‡ has been reported to increase enzyme activity (7, 10), but the changes have not been universally observed (8). Phosphorylation of the yeast enzyme, on the other hand, at an amino-terminal extension (11) results in a marked inhibition of activity (6). Liver Fru-1,6-P_2ase activity has been reported to increase during diabetes and starvation (12, 13), suggesting that the enzyme is subject to long-term regulation at the levels of transcription and/or translation.

Since the DNA encoding mammalian enzyme had not been cloned and there was no information on the size, distribution, or concentration of its mRNA, we addressed these issues and report here the isolation and nucleotide sequence of a coding-length cDNA of rat liver Fru-1,6-P_2ase, the complete amino acid sequence of the enzyme subunit, and data showing hormonal regulation of its mRNA.§

EXPERIMENTAL PROCEDURES

Materials. Fru-1,6-P_2ase was purified to homogeneity from rat liver (7) and antibody to the purified enzyme was raised in New Zealand White rabbits (14). Additional antisera to the rat and mouse liver enzymes, from rabbits and goats, were kind gifts of Bernard Horecker (Cornell University) and Frank Marcus (Chicago Medical School), respectively. A 1305-base-pair (bp) fragment of rat liver cytosolic phospho*enol*pyruvate carboxykinase (PEPCK) cDNA was a kind gift of Daryl Granner (Vanderbilt University). Rat livers were a kind gift of Thomas Claus (Lederle Laboratories). *Achromobacter* protease I (lysyl endoproteinase) was a gift of T. Masaki (Ibaraki University, Japan). L-1-Tosylamido-2-phenylethyl chloromethyl ketone-treated trypsin (TPCK-trypsin) and carboxypeptidase Y were from Sigma. The rat liver cDNA library in λgt11 was from Clontech. Restriction enzymes and the M13 universal primer for dideoxy sequencing were from New England Biolabs. Deoxy- and dideoxynucleoside triphosphates were from Pharmacia; DNA polymerase Klenow fragment, acrylamide, and the DNA-sequencing apparatus were from International Biotechnologies; and [α-[^{35}S]thio]dATP and the Multiprime DNA-labeling kit were from Amersham. Nylon membranes (GeneScreen) were from DuPont.

Immunological Screening of Rat Liver cDNA in λgt11. A λgt11 recombinant cDNA rat liver library plated on *Escherichia coli* Y1090 cells was screened with Fru-1,6-P_2ase antibody at a 1:1000 dilution of the rabbit antisera or a 1:500 dilution of the goat antiserum, essentially as described by Young and Davis (15). After plaque purification of immunopositive clones, preliminary determination of the size of the insert(s) was obtained from the molecular mass of the fusion protein(s) (16, 17).

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. §1734 solely to indicate this fact.

Abbreviations: Fru-1,6-P_2ase, fructose-1,6-bisphosphatase; Fru-2,6-P_2, fructose 2,6-bisphosphate; PEPCK, phospho*enol*pyruvate carboxykinase.

‡Rat kidney Fru-1,6-P_2ase also lacks the carboxyl-terminal extension containing the cAMP-dependent phosphorylation site (M.R.E.-M. and S.J.P., unpublished results).

§The sequence reported in this paper is being deposited in the EMBL/GenBank data base (IntelliGenetics, Mountain View, CA, and Eur. Mol. Biol. Lab., Heidelberg) (accession no. J04112).

DNA Sequencing. *Eco*RI restriction fragments of the cDNA were subcloned into the *Eco*RI site of the plasmid vector pEMBL18 (18) and sequenced by the chain-termination method (19) using [α-[^{35}S]thio]dATP. Both strands of cDNA were sequenced at least three times.

Preparation and Sequencing of Carboxamidomethylated Fru-1,6-P_2ase Peptides. The purified enzyme was [^{14}C]carboxamidomethylated and cleaved at methionine with CNBr, and the peptides were purified by HPLC on Spherogel-TSK SW columns and by reverse-phase HPLC as described for rat liver 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase (20). Tryptic or lysyl endoproteinase cleavage peptides of the enzyme were prepared and purified by reverse-phase HPLC (20) and their amino acid sequences were determined according to Hunkapillar *et al.* (21) with an Applied Biosystems model 470A gas-phase sequencer. The carboxyl-terminal residues of the enzyme were determined by carboxypeptidase Y digestion (20).

RNA Blot Hybridization Analyses. Total cytoplasmic RNA, extracted from tissues with guanidinium isothiocyanate (22), was denatured in the presence of formaldehyde and formamide and resolved by electrophoresis in 1% agarose gels in the presence of 18% formaldehyde before transfer to nylon membranes (23). The membranes were dried briefly, heated at 80°C in a vacuum oven for 2 hr, and then prehybridized for 6 hr at 42°C in 50% formamide/20 mM piperazine-*N*,*N*′-bis(2-ethanesulfonate), pH 6.4/80 mM NaCl/2 mM EDTA/0.5% $NaDodSO_4$ containing sonicated salmon sperm DNA at 100 μg/ml. cDNA restriction fragments were ^{32}P-labeled according to the random-primer method (24) by using a Multiprime kit (Amersham). The labeled probe was hybridized with the membranes at 42°C for 12–24 hr, after which the membranes were washed with four 500-ml washes of 15 mM NaCl/1.5 mM sodium citrate, pH 7.4/0.1% $NaDodSO_4$ (30 min per wash) at 50°C. The membranes were then exposed to x-ray film for 12–24 hr with an intensifying screen at −70°C.

RESULTS

Nucleotide Sequence of a Coding-Length cDNA. Two cDNAs for rat hepatic Fru-1,6-P_2ase were obtained by screening a rat liver cDNA library in λgt11 with antisera raised against the purified enzyme. Immunoblot analyses of the expressed fusion proteins (data not shown) indicated that clone I (≈134-kDa fusion peptide) coded for approximately half the enzyme subunit, whereas clone II (≈160-kDa fusion peptide) was large enough to contain the entire enzyme subunit (≈40 kDa). *Eco*RI digestion of clone II (≈1300 bp) gave three fragments, ≈650, ≈500, and ≈150 bp long (data not shown). Each *Eco*RI fragment was subcloned into the plasmid vector and sequenced. Clone II was found to be a nearly full-length cDNA (full coding-length) and its complete nucleotide and deduced amino acid sequences are shown in Fig. 1. The smallest restriction fragment (117 bp) included 12 bp of 5′ untranslated region, the initiation codon, and the coding sequence for the first 34 amino acids. The medium-sized restriction fragment (472 bp) coded for amino acids 35–191. The largest fragment (666 bp) included codons for amino acids 192-362 (carboxyl terminus), the termination codon, and 151 bp of 3′ untranslated region. The latter contained a polyadenylylation signal (AATAAA) followed by a stretch of 7 adenines at the end of the clone. To confirm the authenticity of the isolated clone, we compared the deduced amino acid sequence with that obtained from direct sequencing of a complete set of peptides generated by CNBr cleavage at methionine or by trypsin and lysyl endoproteinase digestion of the purified protein (Fig. 2). The cDNA and protein sequences were in total agreement. In addition, the nucleotide-deduced sequence confirmed the alignment of five non-overlapping peptides: those between lysyl endoproteinase peptides 3 and 4 (Fig. 2, K3 and K4), 7 and 8, 8 and 9, 13 and 14, and 17 and 20.

Identification of the Blocked Amino Terminus of Fru-1,6-P_2ase. Attempts to sequence the intact enzyme subunit or the first lysyl endoproteinase peptide did not yield any phenylthiohydantoin derivatives, confirming that the amino terminus of the rat liver enzyme is blocked (25). The first lysyl endoproteinase peptide (Fig. 2, K1) was analyzed by fast atom bombardment mass spectrometry (20). The $(M+H)^+$ ion calculated from the amino acid sequence of K1 would be 2661.4 mass units; the $(M+H)^+$ measured experimentally was 2703.6 mass units (data not shown). This difference of ≈42 mass units was attributed to N-acetylation of the peptide at the amino-terminal valine. Several fragment ions were noted at masses less than 2703.6 mass units, consistent with this proposed structure. Similar analysis of the first tryptic peptide [residues 1–16; calculated $(M+H)^+$ of 1703 mass units] indicated an $(M+H)^+$ of 1745 mass units (data not shown).

Identification of the Carboxyl Terminus of Fru-1,6-P_2ase. Rittenhouse *et al.* (8) reported that the carboxyl-terminal sequence of the rat liver enzyme was Asp-Glu-Leu, which is one residue shorter than the sequence we found from sequencing the last lysyl endoproteinase peptide (Asp-Glu-Leu-Phe; Fig. 2, K19) and from the nucleotide-deduced residues preceding the termination codon (Fig. 1). Substantiation of the sequence at the carboxyl terminus was obtained by digestion of the protein with carboxypeptidase Y; the first residue released was Phe, followed by Leu, Glu, and Asp (data not shown).

Tissue Distribution of Fru-1,6-P_2ase mRNA and Effect of Diabetes. The distribution of the enzyme mRNA in various rat tissues was carried out by probing a gel blot of size-fractionated cytoplasmic RNA from liver, kidney, brain, heart, and skeletal muscle with a radiolabeled fragment of the rat liver cDNA. A single species of Fru-1,6-P_2ase mRNA, about 1.4 kilobases (kb) long, was detected in liver (Fig. 3*A*, lane 1) and kidney (lane 2) RNA extracts but not in extracts from brain, heart, or skeletal muscle (lanes 3–5), even when hybridizations were done with 5 times the amount of RNA or at lower stringencies (data not shown).

Since Fru-1,6-P_2ase activity is elevated in diabetes and decreased by insulin treatment (12, 13), it was of interest to determine whether these changes are caused by changes in mRNA abundance. Fig. 3*B* shows the effect of alloxan-induced diabetes and insulin treatment on rat liver Fru-1,6-P_2ase mRNA. There was a 10-fold increase in the level of enzyme mRNA from diabetic livers (lane 7) over control levels (lane 6) that was restored to normal only after 24 hr of insulin treatment (lane 9). The increase in Fru-1,6-P_2ase mRNA paralleled that of the mRNA for PEPCK (26, 27) (lanes 6–9). However, insulin acted more rapidly to reduce PEPCK mRNA levels, which were reduced to control levels after only 6 hr of insulin treatment (lane 8). The changes in Fru-1,6-P_2ase mRNA were coincident with changes in the enzyme's activity seen in extracts from livers of diabetic and insulin-treated diabetic rats (data not shown) (12, 13).

DISCUSSION

The primary sequence of rat liver Fru-1,6-P_2ase was deduced from the nucleotide sequence of a near-full-length clone and confirmed by direct sequencing of the purified protein (Figs. 1 and 2). Fast atom bombardment mass spectrometry revealed that the amino-terminal valine was acetylated. There have been very few other reports of acetylated valine as the blocked amino-terminal residue (28). Sheep liver and pig kidney bisphosphatases have acetylated threonine at their amino termini (29, 30). A comparison of the amino-terminal sequences, putative allosteric and active-site regions, and

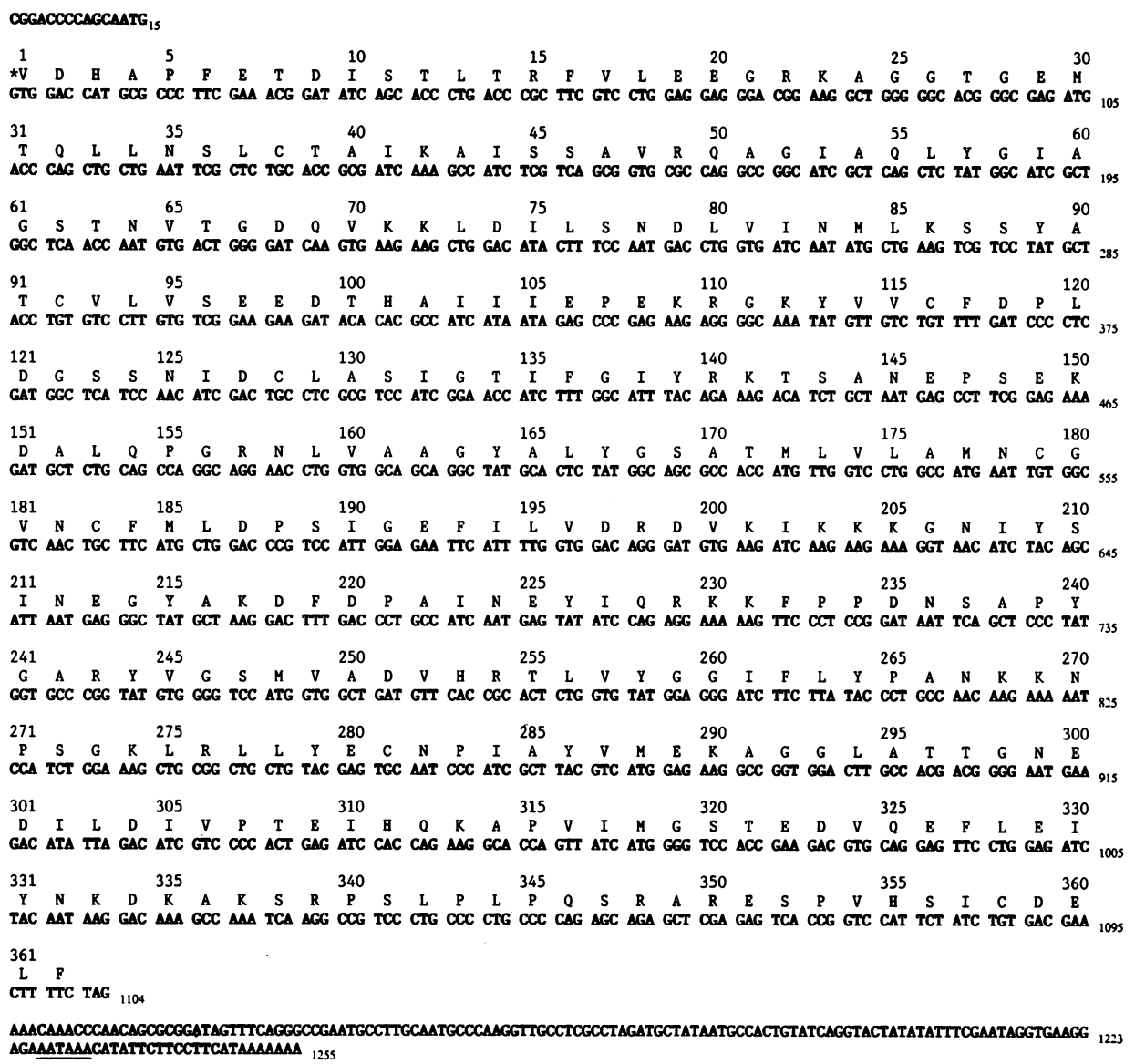

FIG. 1. Nucleotide and deduced amino acid sequences of Fru-1,6-P_2ase cDNA. The nucleotide sequence of the coding strand of the cDNA is given in bold type and numbered in subscript. The internal *Eco*RI restriction sites are after bases 117 and 589, and the polyadenylylation signal is underlined. Shown above the nucleotide sequence is the deduced amino acid sequence (given in one-letter symbols and numbered above the residues), starting with the acetylated valine (*) and ending with phe-362 preceding the termination codon.

carboxyl-terminal sequences of Fru-1,6-P_2ases from rat liver, pig kidney, and yeast (Fig. 4) reveals a high degree of conservation, with an overall identity of ≈85% among the mammalian sequences. The similarity is even greater at the active and allosteric regions. For example, Lys-274, implicated in substrate binding (30, 31), is conserved in all three primary sequences (refs. 30–33 and Fig. 4), as are 92% of the amino acids in this active-site region of the enzyme subunit from all sources (residues 240–290) (30, 31, 33). Lys-141 and Cys-128, reported to be involved in AMP binding and other allosteric behavior of the pig and rabbit enzymes (30, 31), are also present in the rat liver form but not in that of yeast (32) (Fig. 4). The retention of allosteric inhibition of yeast Fru-1,6-P_2ase by AMP (33) without conservation of these residues casts some doubt on their role in allosteric behavior of mammalian enzyme forms (34). Our findings suggest that the secondary structure of rat liver Fru-1,6-P_2ase may not be that different from those of other tissues and species (34), but identification of allosteric and active-site domains with certainty must await crystallographic data.

The major difference between the rat liver and other mammalian enzymes is at the carboxyl terminus. The 27-residue carboxyl-terminal extension, unique to the rat liver enzyme, contains the cAMP-dependent phosphorylation site (Ser-341) (7, 8) as well as a second cAMP-dependent phosphorylation site (Ser-356) identified by Chatterjee *et al.* (9). As suggested by Rittenhouse *et al.* (8), the absence of the entire carboxyl-terminal extension in the pig kidney and rabbit and sheep liver enzymes explains the inability of cAMP-dependent protein kinase to phosphorylate them.‡ It is interesting that glycolytic, gluconeogenic, and glycogen-metabolizing enzymes whose activities have been shown to be unequivocally affected by changes in their phosphorylation states have amino-terminal phosphorylation sites (35–38), including yeast Fru-1,6-P_2ase (33), whereas those reported to undergo essentially "silent" phosphorylations, such as rat liver Fru-1,6-P_2ase (39) and 6-phosphofructo-1-kinase (39), have carboxyl-terminal phosphorylation sites. X-ray crystallographic examination of the spatial relationship between the phosphorylation sites in these enzymes and the remainder of their respective subunits is necessary, however, before any generalized conclusion can be made.

An ≈1.4-kb species of Fru-1,6-P_2ase mRNA was detected by hybridization with a fragment of the liver cDNA in liver

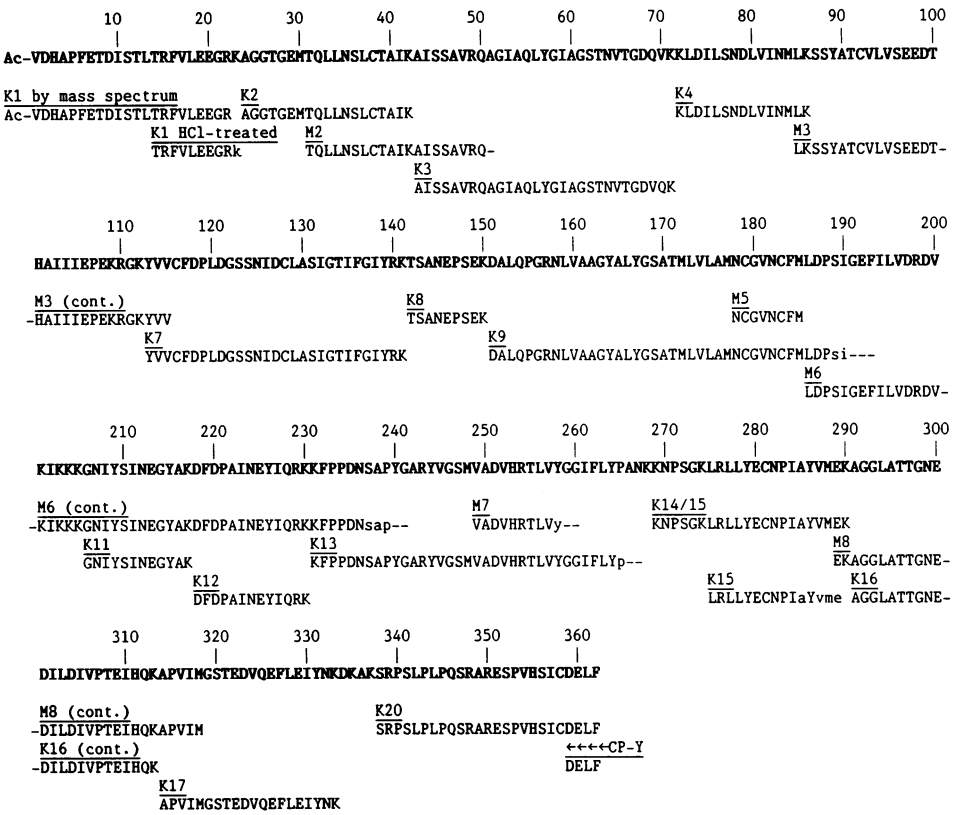

FIG. 2. Proof of the primary sequence of rat liver Fru-1,6-P_2ase. The amino acid sequence of the rat liver enzyme is given in bold type. The names of the peptides are underlined and are designated by letters denoting the method used to generate them: K, lysyl endoproteinase cleavage; M, cleavage at methionine with CNBr. Carboxypeptidase Y digestion (CP-Y) was used to determine the 4 carboxyl-terminal residues. Residues (one-letter symbols) determined with certainty are given in uppercase letters and tentatively identified residues are in lowercase. Dashes indicate incomplete sequencing of peptides. Ac, acetyl.

and kidney RNA extracts (Fig. 3*A*) but not in extracts from skeletal muscle, heart, or brain. The absence of a signal in the extracts from these tissues could either be due to the low abundance of Fru-1,6-P_2ase mRNA or be due to the presence of a different form of the enzyme. However, the inability of anti-liver Fru-1,6-P_2ase serum to crossreact with brain Fru-1,6-P_2ase (40) and the finding of significant levels of Fru-1,6-P_2ase activity in muscle (41) favor the latter explanation. The

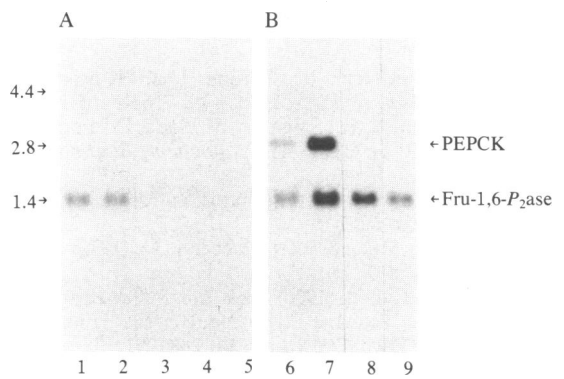

FIG. 3. Blot hybridization analysis of RNA from normal liver, kidney, muscle, and brain and diabetic liver. Cytoplasmic RNA (5 μg per lane) from normal fed rat liver (lanes 1 and 6), kidney (lane 2), brain (lane 3), heart (lane 4), or skeletal muscle (lane 5) and from alloxan-diabetic rat livers with no insulin treatment (lane 7) or after 6 hr (lane 8) or 24 hr (lane 9) of insulin was denatured in formaldehyde/formamide, resolved in agarose/formaldehyde gel, transferred to nylon membrane, and probed with Fru-1,6-P_2ase [^{32}P]cDNA (666-bp fragment; 1–3 × 10^6 cpm/ng) (*A* and *B*) and with PEPCK [^{32}P]cDNA (1305-bp fragment; 1 × 10^6 cpm/ng) (*B*). An RNA "ladder" (3 μg each) from Bethesda Research Laboratories and PEPCK mRNA (2800 bp) were used as the size markers (lengths in kb at left). Rats were made diabetic by intravenous injection of freshly prepared alloxan (50 mg/kg of body weight) and were used 3 days later if their blood glucose levels were ≥450 mg/dl. Insulin treatment consisted of an initial bolus of 10 units followed by 4 units every 4 hr.

liver cDNA probe hybridized with the kidney mRNA in spite of the differences in the length and sequence at the carboxyl terminus of the respective enzymes (Fig. 4 and ‡), suggesting that the two forms arose either by alternative RNA splicing or from different genes. In addition, posttranslational modification cannot be ruled out. On the other hand, Fru-1,6-P_2ases from nongluconeogenic tissues such as brain and skeletal muscle are probably products of genes distinct from those found in liver and kidney. Isolation of a single Fru-1,6-P_2ase gene species from a rat genomic library (M.R.E.-M. and S.J.P., unpublished work) by using the liver cDNA as a probe has confirmed the finding that other gene forms of the enzyme are not homologous with the liver cDNA.

Rat hepatic Fru-1,6-P_2ase activity has been reported to increase in diabetes and starvation (12, 13). Insulin administration to diabetic rats lowered liver Fru-1,6-P_2ase activity to normal or subnormal levels (12). Similar responses have been reported for liver cytosolic PEPCK and have been attributed to cAMP-dependent induction and insulin-dependent repression of its gene expression (26, 27). cAMP acts to increase PEPCK mRNA by increasing the rate of gene transcription (26, 27) as well as decreasing the rate of mRNA degradation (41). The results shown in Fig. 3*B* strongly suggest that the changes in Fru-1,6-P_2ase activity result from changes in the amount of its cytoplasmic mRNA. These changes may be due to changes in transcription rates or in mRNA processing or degradation. The increase in Fru-1,6-P_2ase mRNA in diabetes may result (*i*) from a decrease in insulin levels, which would otherwise repress mRNA synthesis or promote its degradation, or (*ii*) from an increase in cAMP concentration leading to induction of enzyme mRNA or decreased mRNA degradation. The slow decrease in Fru-1,6-P_2ase mRNA upon insulin administration, relative to PEPCK mRNA, suggests that the half-life of the former may be longer. Insulin has been shown to affect mRNA abundance of a number of proteins involved in the hepatic gluconeogenic/glycolytic pathway, including PEPCK (26, 27, 42), glucokinase (43), and pyruvate kinase (44). The results reported here add Fru-1,6-P_2ase to the list of enzymes whose mRNA is regulated by insulin, and may

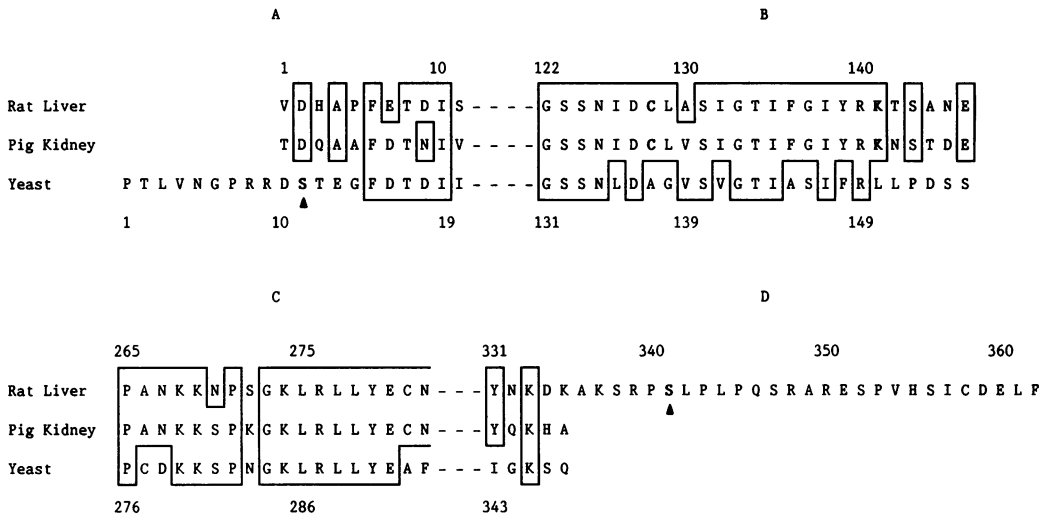

FIG. 4. Comparison of the amino- and carboxyl-terminal sequences and putative allosteric and active-site regions of Fru-1,6-P_2ases from mammalian tissues and yeast. The pig kidney sequence is from ref. 30 and the yeast sequence is from ref. 32. Solid triangles denote the cAMP-dependent phosphorylation sites.

provide another model for the study of insulin regulation of gene expression.

The availability of a coding-length cDNA for rat liver Fru-1,6-P_2ase will not only facilitate studies on the mechanism of hormonal regulation of this key regulatory enzyme's gene expression but will also make possible the use of site-directed mutagenesis to investigate structure–function problems such as the location of the Fru-2,6-P_2 binding site.

This research was supported by Grant DK3835402 from the National Institutes of Health and Grant DMB 8608989 from the National Science Foundation.

1. Horecker, B. L., Melloni, E. & Pontremoli, S. (1975) *Adv. Enzymol.* **42,** 193–226.
2. Pilkis, S. J., El-Maghrabi, M. R., McGrane, M. M., Pilkis, J. & Claus, T. H. (1981) *J. Biol. Chem.* **256,** 8016–8020.
3. Van Schaftingen, E. & Hers, H.-G. (1981) *Proc. Natl. Acad. Sci. USA* **78,** 2861–2863.
4. Kitajima, S. & Uyeda, K. (1983) *J. Biol. Chem.* **258,** 7352–7357.
5. Meek, D. W. & Nimmo, H. G. (1983) *FEBS Lett.* **160,** 105–109.
6. Müller, D. & Holzer, H. (1981) *Biochem. Biophys. Res. Commun.* **103,** 926–933.
7. Riou, J. P., Claus, T. H., Flockhardt, D. A., Corbin, J. D. & Pilkis, S. J. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 4615–4619.
8. Rittenhouse, J., Chatterjee, T., Marcus, F., Reardon, I. & Heinrikson, R. L. (1983) *J. Biol. Chem.* **258,** 7648–7652.
9. Chatterjee, T., Rittenhouse, J., Marcus, F., Reardon, I. & Heinrikson, R. L. (1984) *J. Biol. Chem.* **259,** 3831–3833.
10. Ekdahl, K. N. & Ekman, P. (1985) *J. Biol. Chem.* **260,** 14173–14179.
11. Rittenhouse, J., Harrsch, P. B. & Marcus, F. (1986) *J. Biol. Chem.* **261,** 3939–3943.
12. Weber, G., Singhal, R. L. & Srivastava, S. K. (1965) *Proc. Natl. Acad. Sci. USA* **53,** 96–104.
13. Mazzotta, M. Y. & Veneziale, C. M. (1980) *Biochim. Biophys. Acta* **611,** 156–167.
14. El-Maghrabi, M. R., Correia, J., Heil, P., Pate, T. M., Cobb, C. & Pilkis, S. J. (1986) *Proc. Natl. Acad. Sci. USA* **83,** 5005–5009.
15. Young, R. A. & Davis, R. W. (1983) *Science* **222,** 778–782.
16. Morrison, D. K., Carter, J. K. & Moyer, R. W. (1985) *J. Virol.* **55,** 670–675.
17. Towbin, H., Staehelin, T. & Gordon, J. (1979) *Proc. Natl. Acad. Sci. USA* **76,** 4350–4354.
18. Dente, L., Cesareni, G. & Cortese, R. (1983) *Nucleic Acids Res.* **11,** 1645–1656.
19. Sanger, F., Nicklen, S. & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 5463–5467.
20. Lively, M. O., El-Maghrabi, M. R., Pilkis, J., D'Angelo, G., Colosia, A. D., Ciavola, J., Fraser, B. A. & Pilkis, S. J. (1988) *J. Biol. Chem.* **263,** 839–849.
21. Hunkapillar, M. W. & Hood, L. E. (1983) *Methods Enzymol.* **91,** 486–493.
22. Chirgwin, J. M., Przybyla, A. E., MacDonald, R. J. & Rutter, W. J. (1979) *Biochemistry* **18,** 5294–5299.
23. Maniatis, T., Fritsch, E. F. & Sambrook, J. (1982) *Molecular Cloning: A Laboratory Manual* (Cold Spring Harbor Lab., Cold Spring Harbor, NY).
24. Feinberg, A. P. & Vogelstein, B. (1983) *Anal. Biochem.* **132,** 6–13.
25. Tejwani, G. A., Pedrosa, F. O., Pontremoli, S. & Horecker, B. L. (1976) *Arch. Biochem. Biophys.* **177,** 255–264.
26. Granner, D. K. & Andreone, T. L. (1985) *Diabetes/Metab. Rev.* **1,** 139–170.
27. Cimbala, M. A., Van Lelyveld, P. & Hanson, R. W. (1980) *Adv. Enzyme Regul.* **19,** 205–214.
28. Persson, B., Flinta, C., von Heijne, G. & Jörnvall, H. (1985) *Eur. J. Biochem.* **152,** 523–527.
29. Fisher, W. K. & Thompson, E. O. P. (1980) *Aust. J. Biol. Sci.* **33,** 665–674.
30. Marcus, F., Edelstein, I., Reardon, I. & Heinrikson, R. L. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 7161–7165.
31. Xu, G.-J., Datta, A. G., Singh, V. N., Suda, H., Pontremoli, S. & Horecker, B. L. (1981) *Arch. Biochem. Biophys.* **210,** 98–103.
32. Rogers, D. T., Hiller, E., Mitsock, L. & Orr, E. (1988) *J. Biol. Chem.* **263,** 6051–6057.
33. Funayama, S., Molano, J. & Gancedo, C. (1979) *Arch. Biochem. Biophys.* **197,** 170–177.
34. Martinez, J. & Cid, H. (1986) *Arch. Biol. Med. Exp.* **19,** 77–83.
35. Bergström, G., Ekman, P., Dahlquist, U., Humble, E. & Engström, L. (1975) *FEBS Lett.* **56,** 288–291.
36. Cohen, P., Watson, D. C. & Dixon, H. (1975) *Eur. J. Biochem.* **51,** 79–92.
37. Fischer, E. H., Graves, D. J., Snyder Crittenden, E. R. & Krebs, E. G. (1959) *J. Biol. Chem.* **231,** 65–71.
38. Soderling, T. H. (1976) *J. Biol. Chem.* **251,** 4359–4364.
39. Pilkis, S. J., Claus, T. H., Kountz, P. D. & El-Maghrabi, M. R. (1986) in *The Enzymes*, eds. Boyer, P. & Krebs, E. (Academic, New York), Vol. 17, pp. 3–46.
40. Majumder, A. L. & Eisenberg, F. (1977) *Proc. Natl. Acad. Sci. USA* **74,** 3222–3225.
41. Tejwani, G. A. (1983) *Adv. Enzymol. Relat. Areas Mol. Biol.* **54,** 121–194.
42. Hod, Y. & Hanson, R. W. (1988) *J. Biol. Chem.* **263,** 7747–7752.
43. Sibrowski, W. & Seitz, H. J. (1984) *J. Biol. Chem.* **259,** 343–346.
44. Noguchi, T., Inoue, H. & Tanaka, T. (1985) *J. Biol. Chem.* **260,** 14393–14397.