Molecular Cloning of DNA Complementary to Rat L-type Pyruvate Kinase mRNA

NUTRITIONAL AND HORMONAL REGULATION OF L-TYPE PYRUVATE KINASE mRNA CONCENTRATION*

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Rat liver L-type pyruvate kinase mRNA was enriched from total polysomes by immunoprecipitation with a specific antibody and Staphylococcus aureus cells. Double-stranded cDNA synthesized from the enriched mRNA was inserted into the 

Patl site of pBR322, and the resultant recombinant DNA molecules were used to transform Escherichia coli. Three clones containing DNA complementary to L-type pyruvate kinase mRNA were identified by colony hybridization, hybrid-selected translation, and dot blot hybridization. A partial restriction endonuclease map of these clones was constructed covering about 1.86 kilobase pairs. The cDNA insert of recombinant plasmid pLPK-14 was used as a hybridization probe to quantitate L-type pyruvate kinase mRNA in rat liver after various treatments. The level of hybridizable L-type enzyme mRNA was markedly increased by a high carbohydrate diet. Diabetes greatly reduced the mRNA level in the liver of rats maintained on a high carbohydrate diet. However, insulin administration resulted in restoration of the mRNA level to normal within 24 h. These changes were approximately proportional to the changes in the level of translatable L-type pyruvate kinase mRNA. Thus, we conclude that nutritional and hormonal regulation of synthesis of hepatic L-type pyruvate kinase occurs at the pretranslational level.

The L-type isozyme of pyruvate kinase (ATP:pyruvate 2-O-phosphotransferase, EC 2.7.1.40) is the major form in liver (1) and is believed to play a role in regulation of carbohydrate metabolism. The activity of this enzyme is markedly altered by dietary and hormonal stimuli (1-3). For example, a high carbohydrate diet increases the enzyme activity, while starvation or diabetes decreases the activity, and insulin administration to diabetic rats results in restoration of the enzyme activity to normal. Recent studies indicated that these alterations in the L-type pyruvate kinase activity were due to changes in the rate of enzyme synthesis (4, 5) and could be explained primarily by changes in the level of translatable mRNA of this enzyme (6, 7). For further study of the regulatory mechanisms involved in these changes in mRNA activity, it was necessary to construct a DNA complementary to L-type pyruvate kinase mRNA. In this work, we isolated three recombinant plasmids containing L-type pyruvate kinase cDNA sequences and used one of them as a hybridization probe to quantitate L-type pyruvate kinase mRNA in rat liver after various treatments. Our results indicate that alterations in the L-type pyruvate kinase mRNA activity are consistent with the changes in the level of hybridizable L-type enzyme mRNA sequences.

EXPERIMENTAL PROCEDURES

Materials—Streptozotocin, protein A-Sepharose C1-4B, Stnuclease, and ribonuclease inhibitor were obtained from Sigma. [3H]dCTP and [32P]dCTP were purchased from Amersham Corp. [3S]Methionine and ENHANCE from New England Nuclear. Terminal deoxynucleotidyl transferase, oligo(dT)-cellulose (type 3) was obtained from Collaborative Research, Staphylococcus aureus Cowan strain I (Pansorbin) from CalbiochemBehring, and avian myeloblastosis virus reverse transcriptase from Dr. J. W. Beard, Life Sciences, Inc., St. Petersburg, FL. Calf liver tRNA, yeast tRNA, poly(A), and BglI were purchased from Boehringer Mannheim, and other restriction endonucleases and DNase I were products of Takara Shuzo. X-ray film was obtained from Eastman Kodak Co.

Animals—Male Sprague-Dawley rats (170-190 g of body weight) were made diabetic by intravenous injection of streptozotocin (55 mg/kg) after starvation for 20 h (8). Rats with blood glucose levels of over 300 mg/dl were used for experiments, 7 to 10 days after streptozotocin treatment. Insulin (50 units/kg) was injected subcutaneously 24 and 48 h before killing. High carbohydrate diet containing 10% casein, 10% sucrose, and 71% dextrin (2) was given to normal rats that had been starved for 2 days, and to diabetic rats from 2 days before insulin administration.

RNA Extraction—Total RNA was extracted from frozen tissues with phenol/chloroform as described previously (9). Poly(A)+ mRNA was isolated on oligo(dT)-cellulose (10).

Enrichment of L-type Pyruvate Kinase mRNA—Total polysomes from 50 to 60 g of liver from rats refed on high carbohydrate diet for 1 day were prepared by magnesium precipitation as described (11). Polysomes synthesizing L-type enzyme were immunoprecipitated by the method of Gough and Adams (12), as described by Boule et al. (13). Rabbit anti-L-type pyruvate kinase antibody (7) was made ribonuclease-free by affinity chromatography on protein A-Sepharose C1-4B column (14). Four micrograms of immunoglobulin and 3 μl of 10% (w/v) S. aureus cells were used per 1.3 ml of unit of polysomes. Total RNA was extracted from immunoprecipitated polysomes with phenol/chloroform and poly(A)+ mRNA was isolated by a single passage over oligo(dT)-cellulose.

Construction of Recombinant Plasmids and Transformation—L-
type pyruvate kinase-enriched Poly(A)^+ RNA (2 μg) was used as a template for the synthesis of double-stranded cDNA by reverse transcriptase, essentially as described (15), except that ribonuclease inhibitor (250 units/ml) was included in the mixture for synthesis of single-stranded cDNA (16). Double-stranded cDNA was made blunt-ended by incubation with S1 nuclease (15), and tailed with dCTP and terminal transferase (17). The dC-tailed cDNA was size-fractionated on 5% polyacrylamide gel. DNA greater than 600 bp was recovered as a template for the synthesis of double-stranded cDNA by reverse transcriptase. After size fractionation, the double-stranded cDNA was inserted into the PstI site of pBR322 by the dG/dC-tailing technique. The resultant recombinant plasmids were used to transform E. coli strain HB 101. From 2 μg of the enriched poly(A)^+ RNA, 46 ng of dC-tailed, double-stranded cDNA was obtained, which produced about 2000 colonies. About 85% of these colonies were ampicillin-sensitive and were screened for L-type pyruvate kinase cDNA by colony hybridization procedure, using the 760-bp PstI fragment of cloned M2-type pyruvate kinase cDNA (pM2-PK-33) as a probe. Clone pM2-PK-33 is one of the M2-type pyruvate kinase clones isolated in our laboratory, and the 760-bp PstI fragment of this plasmid showed a considerable degree of cross-hybridization with L-type enzyme mRNA under conditions of low stringency. Plasmid DNA was isolated by CsCl density gradient centrifugation (21).

Hybrid-selected Translation—Samples of 25 μg of recombinant plasmid DNA or pBR322 were linearized with EcoRI and spotted onto nitrocellulose filters as described by Kafatos et al. (22). The filters were incubated for 16 h at 45°C with 500 μg of poly(A)^+ RNA isolated from the livers of diabetic rats treated with insulin for 24 h. The reaction mixture contained 50% formamide, 20 mM piperazine-N,N'-bis(2-ethanesulfonic acid) (pH 6.4), 0.5 M NaCl, 0.2% SDS, 100 μg/ml of yeast tRNA, and 100 μg/ml of poly(A). The filters were then washed with 0.1 SSC containing the bound mRNA was eluted as described by Ricciardi et al. (23). The RNA was precipitated with ethanol after addition of 8 μg of calf liver tRNA and translated in a reticulocyte lysate system as described below.

Translational Analysis—Messenger RNA was translated in a nuclelease-treated rabbit reticulocyte lysate system (24). The conditions for translation, analysis of translational products, and immunoprecipitation of L-type pyruvate kinase have been described (7).

Dot Blot Hybridization Assay—RNA was spotted onto a nitrocellulose filter that had been pretreated with 20 × SSC (25). The filter was baked at 80°C for 2 h, and then prehybridized for at least 4 h at 50°C in 20% formamide, 5 × SSC, 50 μM sodium phosphate (pH 6.8), 5 × Denhardt's solution, 0.1% SDS, and sonicated, denatured salmon testis DNA (250 μg/ml). The hybridization was carried out for 20–22 h at 50°C in the same solution except that the concentration of Denhardt's solution was reduced to 2×, and 0.3% dextran sulfate and 32P-labeled cloned L-type enzyme cDNA insert was included. The nitrocellulose filter was then washed with 0.1 × SSC containing 0.1% SDS twice at room temperature, and three times at 55°C. RNA spots were located by autoradiography, and areas of identical size containing the RNA spots were cut out and counted in a liquid scintillation counter. Values were corrected for the background by subtracting the radioactivity of contiguous areas of the same filter not containing bound RNA.

Nick Translation—Cloned L-type pyruvate kinase cDNA insert was purified on gel by the method of Drezten et al. (26), and nick-translated with [α-32P]dCTP (800 Ci/mmol) to a specific activity of 4.8 × 10^6 cpm/μg (27).

RESULTS AND DISCUSSION

Construction and Identification of L-type Pyruvate Kinase cDNA—Since the maximally induced levels of L-type pyruvate kinase mRNA represent only about 0.3% of the total hepatic mRNA, as measured by in vitro translational assay, we enriched the enzyme mRNA by immunoprecipitation of polysomes with specific antibody and S. aureus cells. As shown in Fig. 1, a major protein synthesized under direction of the enriched mRNA co-migrated exactly with the authentic L-type pyruvate kinase subunit (lane 1). This clearly indicated that the RNA preparation was specifically enriched in the L-type enzyme mRNA, as compared with translational products from total polysomal mRNA (lane 2). The amount of the L-type subunit synthesized in vitro was estimated by immunoprecipitation and SDS-polyacrylamide gel electrophoresis as described before (7). The results indicated that the L-type enzyme mRNA constituted about 6.5% of the enriched mRNA, as compared with 0.28% of the total polysomal mRNA. No M2-type enzyme, which is a minor form of pyruvate kinase in liver, was detected in translational products from the enriched mRNA (data not shown).

The enriched mRNA was used to synthesize double-stranded cDNA with reverse transcriptase. After size fractionation, the double-stranded cDNA was inserted into the PstI site of pBR322 by the dG/dC-tailing technique. The resultant recombinant plasmids were used to transform E. coli strain HB 101. From 2 μg of the enriched poly(A)^+ RNA, 46 ng of dC-tailed, double-stranded cDNA was obtained, which produced about 2000 colonies. About 85% of these colonies were ampicillin-sensitive and were screened for L-type pyruvate kinase sequences by the colony hybridization procedure, using the 760-bp PstI fragment of cloned M2-type pyruvate kinase cDNA (pM2-PK-33) as a probe. Clone pM2-PK-33 is one of the M2-type pyruvate kinase clones isolated in our laboratory, and the 760-bp PstI fragment of this plasmid showed a considerable degree of cross-hybridization with L-type enzyme mRNA under conditions of low stringency. Since M2-type enzyme mRNA activity was not detected in the enriched mRNA used for cloning, clones selected by this method were likely to contain DNA sequences complementary to L-type enzyme. Of about 500 colonies examined, 18 colonies gave strong signals. Only three of the 18 gave a positive reaction on restesting.

The plasmid DNAs from these three colonies, named pLPK-1, -11, and -14, were each purified and characterized further by hybrid-selected translation. For assessment of the specificity of hybrid selection, pBR322 and pX were also each bound to a nitrocellulose filter. pX is an unidentified recombinant plasmid with a 1.5-kb long cDNA insert which did not give a positive reaction when screened by colony hybridization. Three recombinant DNAs, pLPK-1, -11, and -14, selected mRNA which directed synthesis of a peptide of identical molecular size to the L-type subunit (Fig. 2, lanes 4–6).

![Fig. 1. Cell-free translation of immunopurified L-type pyruvate kinase mRNA. Poly(A)^+ RNA was isolated from rat liver polysomes before and after immunoprecipitation of polysomes with anti-L-type pyruvate kinase antibody. Cell-free translation was carried out in a 20-μl reticulocyte lysate system with RNA from immunoprecipitated polysomes (lane 1), with RNA from total polysomes (lane 2), or with no added RNA (lane 3). Translational products were analyzed by SDS-polyacrylamide gel electrophoresis and fluorography. L-PK, L-type pyruvate kinase.](image-url)
This peptide was identified as the L-type pyruvate kinase subunit by immunoprecipitation with antibody against the L-type enzyme (Fig. 2, lanes 9–11). The minor band visible just below the L-type subunit was probably that of an incomplete L-type subunit generated by premature termination of translation of the L-type enzyme mRNA, since this peptide was also immunoprecipitated with anti-L-type enzyme antibody (a faint band can be seen in the fluorograph). On the other hand, no band of the L-type subunit was detected in translation products synthesized from RNA selected by pBR322 (lanes 2 and 7) or pX (lanes 3 and 8). pX also did not select any specific mRNA. The explanation for this may be that the RNA preparation used for hybrid selection contained an undetectable amount of mRNA hybridizable to this plasmid. These results indicate that the three clones, pLPK-1, -11, and -14, specifically selected the L-type pyruvate kinase mRNA. However, the results did not exclude the slight possibility that these cloned cDNAs are complementary not to L-type enzyme but to M-type pyruvate kinase mRNA, since these clones were selected using M-type enzyme cDNA as a probe. This possibility was tested by dot blot hybridization. Poly(A)+ RNA was isolated from AH-130 Yoshida ascites hepatoma cells and skeletal muscle, which express only M-, and M1-type pyruvate kinase, respectively (9, 28), and from the liver of rats given high carbohydrate diet, and was spotted onto three sets of nitrocellulose filters. The filters were hybridized with cloned L-type enzyme cDNA inserts nick-translated with [α-32P]dCTP, washed under conditions of high stringency, and subjected to autoradiography. Radioactivity was found only in the spot of hepatic mRNA on all three filters (data not shown). Longer exposure revealed very weak signals in hepatoma cells and muscle RNAs on all filters, as expected from the cross-hybridization of M-type cDNA to L-type mRNA described above. Thus, we conclude that the three clones, pLPK-1, -11, and -14, contain DNA sequences complementary to L-type pyruvate kinase mRNA.

Characterization of L-type Pyruvate Kinase cDNA Clones—The cDNA inserts could be excised with PstI and did not have an internal PstI site (Fig. 3). The inserts in pLPK-1, -11, and -14 were about 1.7, 0.83, and 1.3 kb in length, respectively. For determination of the relation of these cDNA inserts, whole plasmids or gel-purified cDNA inserts were digested with one or two of various restriction endonucleases. The resultant DNA fragments were analyzed by electrophoresis on 5% polyacrylamide gel or 1% agarose gel with HindIII-digested pBR322, and HindIII-digested λDNA as molecular size markers. No cleavage sites for EcoRI, HindIII, PstI, BamHI, or BglII were found.
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Procedures" (data not shown). When a high carbohydrate diet was given for 1 day to normal rats that had been starved for 2 days, the relative sequence abundance of L-type enzyme mRNA was much higher than that in control rats maintained on laboratory chow. This increased level of mRNA was reduced to the control level in diabetic rats fed on a high carbohydrate diet. Insulin administration to diabetic rats resulted in about 10.5- and 8-fold increases in the level of hybridizable L-type enzyme mRNA sequences were approximately proportional to the changes in level of translatable mRNA of this enzyme as reported previously (7). Thus, we conclude that synthesis of L-type pyruvate kinase is regulated at a point prior to mRNA translation.

The availability of specific cDNA probe to L-type enzyme will facilitate the analysis of regulatory mechanisms of the expression of its gene.

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