Mapping and functional analysis of an instability element in phosphoenolpyruvate carboxykinase mRNA

OMAR F. LATERZA1, LYNN TAYLOR1, SHASHIKALA UNNITHAN2, LY NGUYEN1, AND NORMAN P. CURTHOYS1

1Department of Biochemistry and Molecular Biology, Colorado State University, Ft. Collins 80523-1870; and 2Department of Natural, Applied, and Environmental Sciences, Front Range Community College, Fort Collins, Colorado 80526

Received 20 September 1999; accepted in final form 19 July 2000

Laterza, Omar F., Lynn Taylor, Shashikala Unnithan, Ly Nguyen, and Norman P. Curthoys. Mapping and functional analysis of an instability element in phosphoenolpyruvate carboxykinase mRNA. Am J Physiol Renal Physiol 279: F866–F873, 2000.—Phosphoenolpyruvate carboxykinase (PEPCK) is a key regulatory enzyme of renal gluconeogenesis. The 3'-nontranslated region of the PEPCK mRNA contains an instability element that facilitates its rapid turnover and contributes to the regulation of PEPCK gene expression. Such processes are mediated by specific protein-binding elements. Thus RNA gel shift analysis was used to identify proteins in rat renal cortical cytosolic extracts that bind to the 3'-nontranslated region of the PEPCK mRNA. Deletion constructs were then used to map the binding interactions to two adjacent RNA segments (PEPCK-6 and PEPCK-7). However, competition experiments established that only the binding to PEPCK-7 was specific. Functional studies were performed by cloning similar segments in a luciferase reporter construct, pLuc/Zeo. This analysis indicated that both PEPCK-6 and PEPCK-7 segments were necessary to produce a decrease in luciferase activity equivalent to that observed with the full-length 3'-nontranslated region. Thus the PEPCK-7 segment binds a specific protein that may recruit one or more proteins to form a complex that mediates the rapid decay of the PEPCK mRNA.

The cytosolic isoform of phosphoenolpyruvate carboxykinase (PEPCK) catalyzes the rate-limiting reaction in both hepatic and renal gluconeogenesis. However, this activity is not regulated by allosteric mechanisms or by covalent modifications (9). Instead, it is regulated primarily by mechanisms that affect the level of the PEPCK mRNA and subsequently determine the level of the PEPCK protein. This is accomplished through changes in either the rate of synthesis or the rate of degradation of the PEPCK mRNA. Regulation of PEPCK gene expression occurs primarily at the level of transcription, a process that has been characterized extensively (10). In contrast, the mechanism that mediates the turnover of PEPCK mRNA is poorly understood even though the latter process also plays an important role in the physiological regulation of PEPCK gene expression.

The time required for an mRNA to change from one steady-state level to another is largely proportional to its half-life (11). Thus the rapid induction of an mRNA after activation of transcription is feasible only if the mRNA also has a rapid turnover. Previous studies indicate that the PEPCK mRNA is degraded with a half-life of 1 h in rat kidney cortex (9) and of 4 h in LLC-PK1-F* cells (14). The turnover of the PEPCK mRNA in liver and hepatoma cells occurs even more rapidly (13). The mRNAs that encode various cytokines and the proteins of immediate early genes are typically degraded with half-lives of <1 h (3). The latter mRNAs usually contain AU-rich elements, including AUUUA or UUAUUUAUU sequences, that function as instability elements. However, very little is known about the sequence of potential elements that may mediate the turnover of an mRNA that occurs at the intermediate rate characteristic of the PEPCK mRNA in kidney cells. To characterize the mechanism of mRNA turnover, it is necessary to initially determine the cis-acting elements present in an mRNA molecule that are involved in protein recognition. The elucidated sequences can then be used to purify and characterize the RNA-binding proteins and to identify related mRNAs whose stability may be determined by the same mechanism.

RNA electrophoretic mobility shift assays were used to detect a protein in the cytosolic fraction of rat hepatoma FTO-2B cells that bound to multiple oligoribonucleotides derived from the 3'-nontranslated region of the PEPCK mRNA (24). The observed binding was not sequence specific, but it may have required a stem-loop structure. The presence of another PEPCK mRNA-binding protein in cytosolic extracts of rat hepatocytes (4, 5) has also been described. This binding occurred primarily within the last 256 nucleotides of the PEPCK mRNA, but additional protein-binding sites were detected in the remainder of the 3'-nontranslated region. A protein with the same electrophoretic motility was

Address for reprint requests and other correspondence: N. Curthoys, Dept. of Biochemistry and Molecular Biology, Colorado State Univ., Ft. Collins, CO 80523-1870 (E-mail: NCurth@lamar. ColoState.edu).

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observed to bind to the 3'-ends of the histone H1 mRNA and the arylsulfatase A mRNA. More recent studies indicate that the light chain of rat ferritin (12) binds to various RNAs, including the 3'-nontranslated region of the PEPCK mRNA. Thus none of the previously identified PEPCK mRNA-binding proteins exhibit specific binding.

In the present study, a specific binding interaction between the 3'-nontranslated region of the PEPCK mRNA and a cytosolic protein expressed in rat kidney cortex was characterized. The specific binding interaction was mapped to a 50-base segment (PEPCK-7). However, functional studies suggest that an adjacent element (PEPCK-6), which may function as a low-affinity protein binding site, is also required to constitute a functional instability element. Thus binding of a specific protein to the PEPCK-7 segment may recruit one or more proteins to the PEPCK-6 element to form a complex that mediates the rapid decay of the PEPCK mRNA.

MATERIALS AND METHODS

Materials. [α-32P]UTP (specific activity 3,000 Ci/mol) was purchased from ICN Biochemicals or Amersham. Restriction enzymes, T4 DNA ligase, T7 RNA polymerase, and RNase T1 were acquired from Boehringer Mannheim and New England Biolabs. RNasin and the Dual-Luciferase Reporter Assay System were obtained from Promega. The PCR site-directed mutagenesis kit and the PCR-Script Amp cloning kit were purchased from Stratagene. All other biochemicals were purchased from Sigma.

Construction of transcription vectors. Various plasmids (Fig. 1) were synthesized to utilize T7 RNA polymerase to transcribe the RNAs necessary to map the binding site of proteins that interact with the 3'-nontranslated region of the PEPCK mRNA. The PEPCK/pCR-Script plasmid contains 593 bp of the 3'-nontranslated region of the PEPCK cDNA that extends from 2,003 to 2,595 bp. It was synthesized by using the oligonucleotides 5'-ATCAGCTAGCTGTAATCACC-3' (forward) and 5'-TTTCACTAGTGGTCAAGGAT underlined letters designate partial Asp 718 and the mutated bases are shown in bold letters. The oligonucleotides are underlined, and the mutated bases are shown in bold letters. The resulting PCR fragment was subsequently cloned into the SfII site of pPCR-Script Amp SK(+). Restriction analysis was used to identify a PEPCK/pPCR-Script plasmid in which the 5'-end of the PEPCK sequence was positioned near the Kpn I site of the multicloning site. The desired plasmid was restricted with Asp718 and Nhe I, and the resulting vector fragment was religated to produce the PEPCK-1 plasmid. The deletion eliminated the intervening muticloning sites between the T7 RNA polymerase promoter and the sequence that encodes the 3'-nontranslated region of the PEPCK mRNA.

The PEPCK-3 plasmid was synthesized by restricting the PEPCK/pPCR-Script plasmid with Kpn I and Sph I. The ends of the restricted plasmid were blunted with T4 DNA polymerase and ligation with T4 DNA ligase. The PEPCK-4 plasmid was derived from PEPCK-3 by creating a Hind III site at position 2,505 bp with the QuickChange Site-Directed Mutagenesis Kit from Stratagene. The complementary primers 5'-GTATATAACTAGAGGTGC-3' (forward) and 5'-GGCAGACGTACGTCTTCTTAGTAC-3' (reverse) were used for the PCR site-directed mutagenesis. The sequences that encode the Hind III site are underlined, and the mutated bases are shown in bold letters. The construction of this site was necessary due to the lack of a convenient restriction site in this region.

The PEPCK-5 plasmid was constructed by annealing two oligonucleotides and ligationing them into pBlueScript II SK(−) (Stratagene), which was previously restricted with Asp718 and Spe I. The oligonucleotides were 5'-GTACCGTGCTTT- GTTGCTAGTGATATGA-3' (forward) and 5'-CTAGTGATACATCTAGCAACG-3' (reverse). The underlined letters designate partial Asp718 and Spe I sites. Similarly, the PEPCK-6 plasmid was constructed by annealing the oligonucleotides 5'-GTACCGTGCTTTTATATTTTTTTAATATTTTTTTTAGCTACGTCTAAG-3' (forward) and 5'-CTAGGGTATACCTAAAATAATTAAAAATACG-3' (reverse) and inserting them into pBlueScript II SK(−) that had been previously digested with...
Asp718 and XbaI sites. The finally, the PEPC-7 plasmid was synthesized by annealing the oligonucleotides 5′-GCTAGCTGTAATCCCGATGG-3′ (forward) and 5′-CTAGTG-TGCAAGGATCTATTTGCTATGTAAGAGGTTAA-GAAAGGCACTT-3′ (reverse) and inserting them into pBlueScript II SK(−) that had been previously digested with Asp718 and SpeI. The underlined letters designate partial Asp718 and SpeI sites.

Generation of DNA templates. The pPCR-Script Amp SK(+) and pBlueScript II SK(−) vectors contain a BssH II site immediately upstream of the T7 promoter. Thus DNA templates are normally obtained by restricting either expression vector with BssH II and a second restriction enzyme. However, for many of the constructs described above, it was not possible to restrict with BssH II because of the presence of this site within the 3′-nontranslated region of the PEPCK cDNA. Thus a Pvu II site that is located just upstream of the BssH II site had to be used in the construction of some of the DNA templates. The PEPC-1 template was a Pvu II/BssH II fragment of the PEPCK-1 plasmid. The PEPC-2 template was obtained by restricting the PEPC-1 plasmid with BssH II. The PEPC-3 and PEPC-4 templates were Pvu II/Spe I and Pvu II/Hind III fragments that were excised from the PEPC-3 and PEPC-4 plasmids, respectively. The PEPC-5, PEPC-6, and PEPC-7 templates were obtained by restricting the respective plasmids with BssH II and either XbaI (PEPC-6) or SpeI (PEPC-5 and PEPC-7).

In vitro transcription. Transcription reactions were performed using a slight modification of the standard method (23). A 10-μl reaction mixture containing 10 ng/μl DNA template, 40 μCi of [α-32P]UTP, 0.2 mM ATP, CTP, and GTP, 50 μM unlabeled UTP, 20 units of RNasin, and 10 mM dithiothreitol was incubated at 37°C for 1 h. Next, 1 unit of RNase-free DNase was added, and the reaction mixture was incubated at 37°C for an additional 15 min. The final reaction volume was adjusted to 50 μl with diethyl pyrocarbonate (DEPC)-treated water, and the mixture was then centrifuged at 14,000 g at 4°C to pellet mitochondrial RNA. The supernatant was then centrifuged at 21,000 g at 4°C to pellet mitochondrial RNA. The cortex was dissected from the papilla and medulla, cut into small pieces, weighed, and placed in an equal volume of 40 mM HEPES, pH 7.4, containing 100 mM potassium acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, 10 μM leupeptin, 10 μM antipain, and 10 μg/ml phenylmethylsulfonyl fluoride. The tissue was incubated on ice for 40 min using a Dounce homogenizer. An aliquot of the homogenate was examined microscopically for released nuclei. The sample was then centrifuged at 1,000 g for 10 min at 4°C to pellet intact cells and nuclei. The supernatant was centrifuged at 10,000 g for 10 min at 4°C to pellet membranes and organelles and polyribosomes. The final supernatant was separated into aliquots and was frozen at −70°C. Protein concentration was determined by the Lowry assay using BSA as the standard (21).

Construction of luciferase vectors. The pLuc/Zeo plasmid was designed to facilitate the rapid detection and mapping of mRNA instability elements (O. F. Laterza, C. Cole, W. Liu, L. Taylor, and N. P. Curthoys, unpublished observation). It contains the cytomegalovirus (CMV) promoter, the entire coding region of the firefly luciferase gene, a segment of the 3′-nontranslated region of the Simina virus 40 containing a mRNA instability element (O. F. Laterza, C. Cole, W. Liu, L. Taylor, and N. P. Curthoys, unpublished observation). The PEPC-1 plasmid, pLuc/Zeo, was digested with a BssH II/Hind III site within the multicloning site. The pLuc/Zeo plasmid contains two Hind III sites. Thus a partial digest was performed to generate the vector fragment that was restricted only at the Hind III site within the multicloning site.

Various segments of the 3′-nontranslated region of the PEPCK cDNA were also cloned into the pLuc/Zeo plasmid (Fig. 1). The initial plasmid, pLuc-PEPCK-1/Zeo, contained the 593-bp segment of the 3′-nontranslated region of the PEPCK gene that was initially cloned into pPCR-Script Amp SK(−). The resulting plasmid (PEPCK/pPCR-Script) was digested with BamHI and Not I restriction enzymes, and the excised PEPC-1 fragment was cloned into the corresponding sites within pLuc/Zeo. The pLuc/Zeo plasmid was used to generate two Hind III sites. Thus a partial digest was performed to generate the vector fragment that was restricted only at the Hind III site within the multicloning site.

The insert was amplified from PEPCK/pPCR-Script using the oligonucleotides 5′-GCTAGCTGTAATCCCGATGG-3′ (forward) and 5′-AGCTCAGTATGTTCTTTGTCG-3′ (reverse) and was cloned into pPCR-Script. The resulting plasmid was digested with BamHI and Not I, and the desired fragment was cloned into the corresponding sites within pLuc/Zeo. The insert for pLuc-PEPCK-3/Zeo was PCR amplified from the GP plasmid (24) and was cloned into pPCR-Script. The forward oligonucleotide was 5′-AGTTCTTACCGTTACCTGC-3′ while the reverse oligonucleotide was 5′-TTCACCTAGTGTGTCAGGAT-3′. A Not I/Ala I fragment...
from PEPCk-3/pPCR-Script was then cloned into pLuc/Zeo. pLuc-PEPCk-6/Zeo was assembled by digesting the PEPCk-6 plasmid with Asp718 and Not I and cloning the desired product into the corresponding sites in pLuc/Zeo. pLuc-PEPCk-6/7/Zeo was constructed by initially annealing the two 85 bp complementary oligonucleotides 5'-GGCCGGGTATGTTAAAATTTTTTATAC-CTGGCCCTTTTCTTACCTTTTACATATTGAAATAGGTATCCTGACCAGCTAGC-3' (forward) and 5'-GGCCGGTACGTGTGTCAGTATCTTTAATTCAATTTGTAAAAGAGT-GTAAGAAGGCGGCTGTATAAAAATAATT-AAAACATACGC-3' (reverse). Partial Not I sites are noted by underlining, and an Nhe I site is noted by italics. The annealed oligo was cloned directly into the Not I site of pLuc/Zeo. The Nhe I site was included to facilitate verification of the correct orientation of the inserted oligonucleotide sequence. pLuc-PEPCk-7/Zeo was constructed by initially annealing the oligonucleotides used to produce the PEPCk-7 plasmid to form partial Asp718 and Spe I sites. The annealed oligos were then inserted into pLuc/Zeo that had been previously restricted with Asp718 and Xba I. pLuc/Zeo contains two Xba I sites. Thus a partial digest was used to produce the fragment that was restricted only at the Xba I site within the multicloning site.

Luciferase assay. LLC-PK1-F- cells were grown on 10-cm plates using a 50:50 mixture of DMEM and Ham's F-12 medium containing 5 mM glucose. At 3 days postsplitting in six-well plates, the cells were transiently cotransfected by calcium phosphate precipitation (2) of 1.0 μg of the various pLuc/Zeo plasmids and 0.2 μg of pRL-TK (Promega). Approximately 24 h later, the transfection media were removed, and fresh media were added. The cells were cultured for an additional 24 h and washed twice with 2 ml of PBS, and cell extracts were prepared and assayed using the reagents contained in the Dual-Luciferase Reporter Assay System (Promega). The firefly luciferase activities obtained from the various pLuc/Zeo plasmids were standardized versus the corresponding Renilla luciferase activities measured with the parent construct was normalized to the mean of the standardized luciferase activity from the various pLuc/Zeo plasmids. For competitive purposes, the mean of the standardized luciferase activities measured with the parent construct was normalized to the mean of the standardized luciferase activity of the corresponding Renilla luciferase. The firefly luciferase activities obtained from the various pLuc/Zeo plasmids were normalized to the mean of the standardized luciferase activity from the various pLuc/Zeo plasmids. The firefly luciferase activities obtained from the various pLuc/Zeo plasmids were normalized to the mean of the standardized luciferase activity from the various pLuc/Zeo plasmids. The firefly luciferase activities obtained from the various pLuc/Zeo plasmids were normalized to the mean of the standardized luciferase activity from the various pLuc/Zeo plasmids. The firefly luciferase activities obtained from the various pLuc/Zeo plasmids were normalized to the mean of the standardized luciferase activity from the various pLuc/Zeo plasmids.

RESULTS

Mapping of protein binding sites. RNA gel shift analysis was performed to detect the presence of proteins in cytosolic extracts of rat kidney cortex that bind to the 3'-nontranslated region of the PEPCk mRNA. The PEPCk-1 RNA that contains 593 nucleotides of the 3'-nontranslated region of the PEPCk mRNA was used as the initial probe. When the 32P-labeled PEPCk-1 RNA was incubated with cytosolic extracts from rat kidney cortex, digested with RNase T1, and separated by electrophoresis on a native polyacrylamide gel, two well-resolved bands that had a slower mobility than the digested probe were observed (Fig. 2, lane 2).

Additional RNAs (PEPCk-2 through PEPCk-7) were synthesized to further map the binding sites of these proteins (Fig. 1). Both binding activities observed with the PEPCk-1 RNA were also detected with the PEPCk-3 RNA (Fig. 2). However, no binding activity was observed with the PEPCk-2 RNA. Thus the binding observed with the PEPCk-1 RNA must occur within the last 241 bases of the 3'-nontranslated region. Four additional RNAs were tested to further map the binding site of the two proteins. The first RNA tested was PEPCk-4, which is 150 nucleotides in length and extends from base 2,355 to base 2,505. It showed no apparent binding activity. The three other RNA fragments extended over the remainder of the 3'-nontranslated region. The two smaller RNAs, PEPCk-5 and PEPCk-6, contain 20 and 23 nucleotides, respectively, whereas PEPCk-7 contains 50 nucleotides. Again, no binding activity was detected with the PEPCk-5 transcript. However, the band with higher mobility was reproduced with the PEPCk-6 RNA and the band with lower mobility mapped to the PEPCk-7 RNA. This observation further supports the conclusion that rat renal cortical extracts may contain at least two distinct proteins that bind to the 3'-nontranslated region of the PEPCk mRNA.

Competition studies. The previously identified binding interactions of various proteins with the 3'-nontranslated region of the PEPCk mRNA occurred with ambiguous specificity (4, 5, 12). Thus competition studies were performed to determine the specificity of the interaction of the binding proteins detected in the rat renal cortical cytosolic extracts. Samples containing 40 fmol of 32P-labeled PEPCk-6 RNA were incubated with 3 μg of extract in the presence of increasing amounts (10, 30, and 100-fold excess) of unlabeled PEPCk-5, PEPCk-6, PEPCk-7, GA R-2I, and (AUUU)5A RNAs (Fig. 3). The GA R-2I RNA is a 29-base segment of the
3’-nontranslated region of glutaminase mRNA that functions as a pH-responsive element (18). The (AUUU)$_5$A RNA contains five reiterations of an AUUUA sequence. This is a common recognition sequence for proteins that bind to AU-rich elements (3). The band observed with the labeled PEPCK-6 RNA was effectively competed with increasing amounts of unlabeled PEPCK-7, GA R-2I, and (AUUU)$_5$A RNAs, and it was only slightly competed with increasing amounts of unlabeled PEPCK-5 and PEPCK-6 RNAs (Fig. 3). The observation that unrelated RNAs [PEPCK-7, R-2I, and (AUUU)$_5$A] compete more effectively than the PEPCK-6 RNA suggests that the binding interaction produced with this segment of the PEPCK mRNA is nonspecific. Interestingly, the RNAs that compete most effectively contain a high percentage of A and U residues. This is particularly true for the GA R-2I and (AUUU)$_5$A RNAs. Thus the PEPCK-6 RNA may bind various proteins that interact with AU-rich elements.

A similar experiment was performed to determine the specificity of the binding observed with the PEPCK-7 transcript. Again, the binding interaction observed with the PEPCK-7 RNA and cytosolic extracts from rat renal cortex was competed with increasing amounts (10-, 30-, and 100-fold excess) of unlabeled PEPCK-5, PEPCK-6, PEPCK-7, GA R-2I, and (AUUU)$_5$A RNAs (Fig. 4). In contrast to the PEPCK-6 RNA, only the unlabeled PEPCK-7 RNA significantly competed with the $^{32}$P-labeled PEPCK-7 RNA. The observed competition was evident with only a 10-fold excess of unlabeled PEPCK-7 RNA, and it was almost complete when a 100-fold excess was used. Only a slight competition was observed with a 100-fold excess of the GA R-2I and (AUUU)$_5$A RNAs. Thus binding to the PEPCK-7 RNA is specific.

Mapping of the instability element. The pLuc/Zeo plasmid was used to determine if the specific binding of a protein to the PEPCK-7 segment mediates the inherent instability of the PEPCK mRNA. This plasmid contains a chimeric gene that includes the coding sequence of the firefly luciferase and the 3’-nontranslated region and polyadenylation signal of the very stable bovine growth hormone mRNA. The plasmid also contains a large multicloning site within the 3’-nontranslated region to facilitate the cloning of cDNA fragments that encode potential mRNA instability elements. The insertion of short sequences within the 3’-nontranslated region is unlikely to affect transcription from the CMV promoter or translation of the chimeric mRNAs but may decrease the stability of the resulting transcript. Thus differences in the luciferase activities produced from the pLuc/Zeo plasmid and a derived construct will reflect the relative abundance of the two mRNAs that in turn should primarily reflect their relative stability.

To illustrate the reliability of this approach, a 507-bp segment of the rabbit $\beta$-globin cDNA was cloned in the...
Luciferase activity produced from the pLuc/Zeo plasmid was not significantly different from the activity produced from the parent plasmid (Fig. 5). In contrast, transfection with pLuc-PEPCK-1/Zeo containing the 593-bp segment that constitutes the 3'-nontranslated region of the PEPCK cDNA produced only 42% of the luciferase activity observed with the pLuc/Zeo plasmid. Additional constructs (Fig. 1) were tested to map the location of the instability element. Insertion of the 5'-half of the 3'-nontranslated region of the PEPCK mRNA (pLuc-PEPCK-2/Zeo) had no effect on the luciferase activity. However, nearly all of the decrease in luciferase activity observed with the pLuc-PEPCK-1/Zeo plasmid was retained in the pLuc-PEPCK-3/Zeo plasmid that contains the 3'-half of the 3'-nontranslated region. Thus two additional constructs were prepared to determine separately the effect of the PEPCK-6 and PEPCK-7 segments. Neither of these constructs exhibited the same decrease in luciferase activity as observed with pLuc-PEPCK-3/Zeo plasmid. Surprisingly, of the two constructs, only the pLuc-PEPCK-6/Zeo plasmid, which contains the nonspecific protein binding site, produced a slight but significant decrease in luciferase activity. Therefore, an additional construct containing both the PEPCK-6 and PEPCK-7 segments was synthesized and tested. The pLuc-PEPCK-6:7/Zeo plasmid produced the same decrease in luciferase activity as observed with the pLuc-PEPCK-3/Zeo plasmid. Therefore, both the PEPCK-6 and PEPCK-7 segments are required to constitute a functional instability element within the PEPCK mRNA.

**DISCUSSION**

Cytosolic proteins that bind to the 3'-nontranslated region of the PEPCK mRNA are likely to play an important role in mediating the degradation of this mRNA. Various protein/PEPCK mRNA interactions were previously characterized using cytosolic extracts of hepatoma cells or rat hepatocytes (4, 5, 12, 24). However, the function of the characterized interactions was never tested directly. Furthermore, none of the previously characterized interactions was found to be specific. The detected binding interactions occurred with multiple segments of the PEPCK mRNA and with unrelated RNAs. The first direct evidence for the presence of a destabilizing cis-acting element within the 3'-nontranslated region of the PEPCK mRNA was obtained from experiments that measured the half-life of various chimeric β-globin mRNAs expressed in LLC-PK₁-F cells. The insertion of the complete 3'-nontranslated region of the PEPCK mRNA produced a chimeric mRNA that decayed with a half-life of ~5 h compared with a half-life >30 h for the parent construct (8).

In the present study, potential binding to the entire 3'-nontranslated region was tested using cytosolic extracts of rat kidney cortex, and the two observed binding activities were then mapped to two unique RNA segments. Competition studies demonstrated that binding to the PEPCK-7 RNA exhibits a high degree of specificity. Thus the experiments reported in the present study are the first to demonstrate a specific interaction of a cytosolic protein with a discrete segment of the 3'-nontranslated region of the PEPCK mRNA. The PEPCK-7 RNA (Fig. 6A) has a stretch of 21 nucleotides in which 19 are either A or U residues. However, the remainder of the PEPCK-7 RNA is not

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**Fig. 5.** Mapping of the instability element within the 3'-nontranslated region of the PEPCK mRNA. LLC-PK₁-F cells were transiently transfected with a pLuc/Zeo plasmid containing either no inserted DNA (--) or inserted β-globin (BG) or PEPCK (segments 1 through 6:7 as shown in Fig. 1) cDNA and with the pRL-TK plasmid. The resulting firefly luciferase activities were standardized vs. the corresponding Renilla luciferase activities and normalized so that the activity produced from the pLuc/Zeo plasmid was set equal to 1. Each bar represents the mean ± SE of data derived from 4 separate experiments in which each analysis was performed in triplicate (n = 12). *Con structs where the normalized activity is significantly less than that of the pLuc/Zeo plasmid (P < 0.01).

**Fig. 6.** Analysis of the protein-binding sites within the 3'-nontranslated region of the PEPCK mRNA. A: PEPCK-7 contains a direct repeat of an 8-base sequence that is separated by a single nucleotide. This sequence is shown in capital letters and is underlined. Analysis using the RNAdraw software (22) predicted a relatively stable stem-loop structure that consists of an 8-bp stem with two mismatches and an 11-nucleotide loop. B: sequence of the PEPCK-6 RNA.
enriched for AU nucleotides. Sequence analysis revealed a direct repeat of 8 bases that are separated by a single nucleotide and a potential secondary structure that contains an 8-base stem with 2 mismatches and an 11-base loop (22). The free energy for the formation of this structure was predicted to be $-12.7 \text{ kJ}$ at $37^\circ \text{C}$. It is unknown whether one or more of these potential elements constitute the site that binds a specific protein from rat kidney cortex.

The interaction of another rat kidney protein was also detected with the PEPCK-6 segment that is also rich in AU nucleotides (Fig. 6B). However, competition with different RNAs demonstrated that this binding was nonspecific. This protein may have a weak affinity for AU-rich sequences, since it was preferentially competed by RNAs that contain a high percentage of A and U nucleotides. Thus the binding observed may represent a nonspecific or low-affinity interaction with one or more of the broad range of AU-rich RNA-binding proteins (3).

The results of the mapping experiments performed with the pLuc/Zeo plasmid indicate that neither the PEPCK-6 nor the PEPCK-7 segment is sufficient to mediate the instability inherent in the full-length 3′-nontranslated region of the PEPCK mRNA. However, incorporation of both segments in the pLuc/Zeo plasmid produced a decrease in luciferase activity that was nearly equal to that observed with the pLuc-PEPCK-1/Zeo plasmid. Thus the high affinity and specific binding of a protein to the PEPCK-7 sequence are not sufficient to mediate the turnover of the PEPCK mRNA. Instead, the initial binding of a protein to the PEPCK-7 segment may recruit or enhance the binding of one or more proteins that interact in part with the AU-rich PEPCK-6 sequence to form a complex. It is likely that this complex is what mediates the turnover of the PEPCK mRNA. This hypothesis is also supported by the RNA gel shift data shown in Fig. 2. When incubated with a rat renal cortical cytosolic extract, both the PEPCK-1 and the PEPCK-3 RNAs produce two discrete bands in an RNA gel shift. In both cases, the more intense band has the slower mobility. It is the protein binding represented by this band that maps to the PEPCK-6 segment. However, with the shorter PEPCK-6 segment, protein binding is substantially reduced. This observation suggests that the binding observed with the longer segments is facilitated by interaction with the protein that binds specifically to the PEPCK-7 segment. The high affinity and specificity of the binding observed with the PEPCK-7 RNA suggest that it should be feasible to use this sequence as an affinity ligand to purify the associated binding protein. Once identified, the PEPCK-7 RNA binding protein may be used to identify the additional proteins that are necessary to mediate the turnover of the PEPCK mRNA.

RNA-protein interactions are also critical in mediating physiological changes in mRNA turnover that contribute to the regulation of gene expression (26). Various hormones and effectors have been reported to affect the stability of the PEPCK mRNA. For example, treatment with glucocorticoids (25) or cAMP (20) caused an increase in the half-life of the PEPCK mRNA in liver. It was initially observed that the increase in PEPCK mRNA levels after treatment with cAMP persisted after the activation of transcription had returned to basal levels (17, 27). Subsequently, the effect of cAMP was characterized further by direct measurement of the half-life of the PEPCK mRNA in hepatoma cells (13). The addition of cAMP caused a four- to sixfold increase in the half-life of the PEPCK mRNA (13, 20). Similar observations suggest that acidosis may increase the stability of the PEPCK mRNA in kidney (15, 16), whereas treatment of LLC-PK1-F cells with phorbol esters caused a decrease in the half-life of the PEPCK mRNA (19).

Preliminary experiments indicate that LLC-PK1-F+ cells also express a protein that exhibits a high affinity and specific binding interaction with the PEPCK-7 segment of the PEPCK mRNA. This interaction was increased in extracts of LLC-PK1-F+ cells that were treated with either acidic media or with cAMP. However, neither treatment produced a change in luciferase activity in cells transiently transfected with the pLuc-PEPCK-1/Zeo construct that was significantly different from the change observed with the parent pLuc/Zeo construct. Thus the observed changes in luciferase activity were due primarily to the effects of cAMP or of acidic media on the activity of the CMV promoter that drives expression of the luciferase gene. Therefore, it will be necessary to develop alternative approaches to investigate whether the identified instability element also contributes to the mechanism by which the half-life of the PEPCK mRNA is altered in response to chronic treatment with different physiological stimuli.

This research was supported in part by National Institutes of Diabetes and Digestive and Kidney Diseases Grant DK-43704 awarded to N. P. Curthoys.

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