3'-NONTRANSLATED REGION OF PHOSPHOENOLPYRUVATE CARBOXYKINASE mRNA CONTAINS MULTIPLE INSTABILITY ELEMENTS THAT BIND AUFI*

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Running Title: AUF1 binds to PEPCK mRNA

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Phosphoenolpyruvate carboxykinase (PEPCK) catalyzes the conversion of oxaloacetate to phosphoenolpyruvate, a rate-limiting step in gluconeogenesis. However, this activity is not regulated by allosteric mechanisms or by covalent modifications (1). Instead, it is regulated by mechanisms that affect the level of the PEPCK mRNA and subsequently determine the level of the PEPCK protein. In liver, transcription of the PEPCK gene is inhibited by insulin and is activated by glucocorticoids, thyroid hormone, and glucagon, which acts through cAMP. Within the renal proximal tubule, parathyroid hormone and angiotensin II increase cAMP levels and cause an increased transcription of the PEPCK gene (2,3). The level of the PEPCK mRNA in rat kidney is also increased rapidly following the onset of metabolic acidosis (4). The latter adaptation is initiated within 1 h and reaches a 6-fold induction within 7 h. The 6-fold induced level of PEPCK mRNA is sustained in the kidneys of rats that are made chronically acidic (4).

The time required for an mRNA to change from one steady state to another is usually proportional to its half-life (5). Thus, rapid induction of an mRNA is feasible only if the mRNA has a rapid turnover. Previous studies have shown that the PEPCK mRNA is degraded rapidly in liver and in hepatoma cells (6), in rat kidney cortex (1), and in LLC-PK1-F+ cells (7), a line of porcine proximal tubule-like cells that were selected for their ability to grow in the absence of glucose (8). Previous studies have also demonstrated that the half-life of the PEPCK mRNA is increased in liver in response to cAMP (9) and glucocorticoids (10). Increased stability may also contribute to the sustained induction of renal PEPCK mRNA during chronic acidosis (4). Therefore, selective mRNA stabilization may play
an important role in the physiological regulation of PEPCK gene expression. However, the mechanisms that cause the rapid turnover and selective stabilization of the PEPCK mRNA are unknown.

The presence of a destabilizing cis-element within the 3'-UTR of the PEPCK mRNA was previously demonstrated by using 5,6-dichloro-1-β-D-ribofuranosylbenzamidazole (DRB), a polymerase II inhibitor, to determine the half-life of a chimeric β-globin-PEPCK mRNA expressed in LLC-PK₁-F⁺-cells (11). The parent β-globin (βG) mRNA decayed with a half-life of >30 h while the chimeric βG-PCK-1 mRNA, containing the complete 3'-UTR of PEPCK mRNA, decayed with a half-life of 5 h. RNA gel shift assays using a cytosolic extract of rat renal cortex identified two protein binding interactions, but only one exhibited properties characteristic of specific binding (12). The high affinity and specific interaction mapped to the PCK-7 segment of the PEPCK mRNA, while the low affinity and non-specific interaction mapped to the PCK-6 segment. However, functional studies using luciferase constructs suggested that both segments contribute to the rapid decay of the PEPCK mRNA (12).

In the present study, the contribution of individual segments of the 3'-UTR to the stability of the PEPCK mRNA were quantified by analyses of the half-lives of multiple βG-PCK reporter constructs expressed from a tetracycline-regulated promoter. Direct binding assays demonstrated that AUF1 binds to each of the segments that function as instability elements. Mutational analyses identified specific sequences within the PCK-6 and PCK-7 segments that bind AUF1. The results demonstrate that multiple AUF1 binding sites contribute to the rapid turnover of PEPCK mRNA.

**EXPERIMENTAL PROCEDURES**

**Materials** – Male Sprague-Dawley rats were purchased from Charles River. [α-³²P]UTP and [α-³²P]dCTP (3,000 Ci/mmole) were purchased from MP Biochemicals. The oligo labeling kit was from Ambion. Restriction enzymes, RNase T1, T7 RNA polymerase, and yeast tRNA were acquired from Roche, New England Biolabs or MBI Fermentas. GENECLEAN kits were obtained from Bio101, Inc and the PCRScript cloning kit was obtained from Stratagene. Micro Bio-spin columns and chemicals for acrylamide gels were purchased from Bio Rad Laboratories. RNAsin was obtained from Fischer. An RNA standard was purchased from Gibco-BRL, DMEM/F12 base medium was purchased from Sigma. Hybridase™ Thermostable RNase H was purchased from Epicentre. Geneticin (G418) and Hygromycin B were obtained from Mediatech. TRIzol® reagent was purchased from Invitrogen. Anti-AUF1 monoclonal antibody (5B9) was a gift from Dr. Gideon Dreyfuss. Polyclonal anti-AUF1 antibody was purchased from Upstate. Anti-CP1 and anti-CP2 antibodies were obtained from Dr. Stephen Liebhaber. Anti-HuR antibody was purchased from Santa Cruz Biotech. An expression plasmid that encodes the p40 isoform of AUF1 was obtained from Dr. Jeffrey Wilusz. All other biochemicals were purchased from Sigma.

**Construction of pBSSK-PCK transcription vectors and generation of DNA templates** – The various segments of the 3'-UTR of the PEPCK mRNA that were used in this study are illustrated in Fig. 1. The pBSSK-PCK-1, pBSSK-PCK-2, pBSSK-PCK-6, and pBSSK-PCK-7 plasmids and templates were prepared as described previously (12). The pBSSK-PCK-6/7 plasmid was constructed by annealing two oligonucleotides and ligating into pBlueScriptII-SK(-), which was previously digested with Asp718 and XbaI. The sequences of the forward and reverse oligonucleotides were 5’GTTACCGTATGTCTATTAATAGGTACCTGACCA3’ and 5’CTAGTGTCAGTATGTGTTGCAGGAGGAGTTGCAGGAGGAGGAGTCTATTATTTATACACTGCCCTTTCTTACCTTTTCTTTACATAATTGAAATAGGTATCCTGACCA3’, respectively. The underlined bases represent the forward or reverse oligonucleotides.
pBSSK-PCK-Mut-6, pBSSK-PCK-Mut-1, pBSSK-PCK-Mut-2, pBSSK-PCK-Mut-3, and pBSSK-PCK-Mut-4 were also constructed by annealing double stranded oligonucleotides and ligating them into pBlueScriptII-SK(-). The template for the pBSSK-PCK-Mut-6 construct was obtained by digesting the plamids with BssHII, SacI, and XbaI while the PCK-Mut-1, Mut-2, Mut-3 and Mut-4 templates were obtained by digestion of the plasmids with BssHII and XbaI.

**UV crosslinking of RNA-protein complexes**

The crosslinking experiments were performed with minor modifications of the procedure of You et al. (13). The samples were prepared as described for the RNA gel shift experiments, except that following RNase T1 digestion, they were transferred to a 96-well microliter plate and exposed to short wave (254 nm) radiation for 5 min in a UV Stratalinker 2400 (Stratagene). The samples were transferred to 1.5 ml microfuge tubes and an equal volume of 2x SDS sample buffer containing 10% (v/v) glycerol, 5% (v/v) β-mercaptoethanol, 3% (w/v) SDS, 184 mM Tris-Cl (pH 6.8), and 0.2% (w/v) bromophenol blue was added. The samples were heated in boiling water for 5 min and then resolved on a 10% SDS-polyacrylamide gel. The gel was dried and exposed to a PhosphorImager screen.

**In vitro Transcription** – In vitro transcription of 32P-labeled and unlabeled RNAs was performed as described previously (12) and the products were purified using Bio-Rad Micro Bio-Spin P30 columns. The 32P-labeled RNAs were quantified using extinction coefficients determined from the concentration of the transcripts was calculated by scintillation counting and DEPC-treated water was added to adjust the sample to the desired concentration. The absorbance of the unlabeled RNAs was measured at 260 nm and the concentration of the transcripts was calculated using extinction coefficients determined from the nucleotide composition.

**RNA Electrophoretic mobility shift (RNA-EMSA)** – An aliquot of rat renal cortical extract (12) containing approximately 1-8 µg of protein (14) or 40-400 ng of recombinant AUFl was pre-incubated for 10 min at room temperature in binding buffer containing 0.5% Nonidet P-40, 1 mM dithiothreitol, 2 µg yeast t-RNA, 40 units of RNAsin, 10% glycerol and 10-40 fmol of 32P-labeled RNA. A 30-, 200-, 300- or 500-fold excess of competitor RNA was added with the labeled RNA. The reaction mixture was incubated at room temperature for 20 min and then 1 µl of RNase T1 was added and the samples were incubated for 10 min at room temperature. The samples were separated on 5% native polyacrylamide gels and the gels were dried and exposed overnight to a PhosphorImager screen. In the supershift experiments, the various antibodies were preincubated with the rat renal cytosolic extract for 20 min at room temperature and then added to the binding buffer.

**Construction of TetβG-PCK vectors** – A tetracycline-regulated promoter system (15) was utilized to map the segments within the 3'-UTR of the PEPCK mRNA that function as instability elements. A chimeric β-globin/growth hormone gene was cloned into the pTRE2 plasmid to yield pTetβG. This construct contains a tetracycline (Tet)-responsive promoter, the coding region of rabbit β-globin (βG) gene, and the 3’-nontranslated region and polyadenylation site of bovine growth hormone (bGH) cDNA. The 3’-UTR of the rat PEPCK cDNA (corresponding to nucleotides 2008 to 2595) was PCR amplified using forward and reverse oligonucleotide primers that introduced SpeI and XbaI restriction sites at the 5’- and 3’-ends, respectively. The sequence of the forward primer was 5’GCACTAGTGGCGAATTGGGTACTAG3’ while that of the reverse primer was 5’CGTGGTCTAGATACCTATTTCAATTATGTAAAGAAAGG3’., where the underlined sequences are the SpeI and XbaI sites, respectively. The amplified sequence was then cloned into the SpeI site of pCRScript- SK(+) (Fig. 1). The resulting plasmid, pPCR-PCK-1, was then digested with SpeI and XbaI to yield a 604-bp fragment that was subsequently cloned into pTetβG at the SpeI site to yield pTβG-PCK-1. pTβG-PCK-1 was digested with BssHII and EcoRV restriction enzymes to release a 224-bp 3’-fragment. The remaining plasmid was blunted and religated to produce pTβG-PCK-2. The 224-bp fragment was blunted and inserted into pTetβG that was previously linearized with EcoRV to produce pTβG-PCK-3. Double stranded oligonucleotides containing the PCK-6, PCK-7 and PCK-6/7 sequences (12) were synthesized with SpeI and XbaI overhangs and inserted in pTet-βG that was previously digested with SpeI to yield pTβG-PCK-6, pTβG-PCK-7 and pTβG-PCK-6/7, respectively (Fig. 1). Thus, all of the
chimeric βG-PCK mRNAs retained the bGH 3' UTR that is contained in the control βG-PCK mRNA.

Creation of TβG-PCK stable cell lines - LLC-PK1-F' cells were obtained from Gerhard Gstraunthaler and cultured as previously described (8). Cell lines that stably express the TβG-PCK-1, TβG-PCK-2, TβG-PCK-3, TβG-PCK-6, TβG-PCK-7, and TβG-PCK-6/7 constructs were made by calcium phosphate cotransfection (16) of the pTβG-PCK plasmid and pcDNA 3.1/Hygro (Invitrogen) into 8C cells, a line of LLC-PK1-F' cells that over-express the tTA protein (2). At 24 h post-transfection, the medium was removed, the cells were washed twice with phosphate-buffered saline, and then fresh medium containing 0.2 mg/ml G-418 and 0.6 mg/ml Hygromycin B was added. After 14-21 days, individual colonies were isolated and expanded. After the cells were split onto 10 cm plates, the Hygromycin B concentration was reduced to 0.2 mg/ml. The cell lines were tested for Dox-responsiveness by maintaining the cells initially grown in medium minus Dox and then transferred to medium containing 50 ng/ml Dox for 48 h. This level of Dox was determined to be sufficient to completely shut-off transcription from the Tet-promoter. The cells were then washed two times with phosphate-buffered saline and grown for 3 h in fresh medium minus Dox to create a pulse of βG-PCK-1 mRNA. Subsequently, 1 µg/ml of Dox was added to inhibit transcription. RNA samples were isolated at various times following re-addition of Dox and subjected to Northern analysis to follow the decay of the newly synthesized βG-PCK-1 mRNA. Alternatively, the various TβG-PCK expressing LLC-PK1-F' cells were grown for 5-7 days in medium minus Dox. At time zero, 1 µg/ml Dox was added to each plate. At 0, 3, 6, 9, and 12 h post-Dox treatment, RNA was isolated and subjected to Northern analysis.

RNase H Treatment - RNase H treatment (17) was performed to selectively cleave the 3'-ends of the chimeric βG-PCK-1 mRNAs that were isolated from the pulse-chase experiments. The 21-nt PCK oligo, 5' GCCCAAGATT TTTT CTCCC 3', that encodes the template strand of the PEPCK cDNA from bp 2199-2219 was synthesized by Macromolecular Resources (Fort Collins, CO). A 30-mer of oligo(dT) was used to remove the poly(A) tail of the mRNA. The RNA samples were precipitated from Formazol by adjusting the final concentration of NaCl to 0.2 M and adding 4 volumes of ethanol. After 5 min at room temperature, the RNA was pelleted by centrifugation at 10,000 x g for 10 min. The pellets were resuspended in 25 µl of DEPC-treated water. The cleavage reaction was performed in 12.5 µl containing 10 µg RNA, 1.0 µl RNase H, and 50 pmole of the PCK oligo with or without addition of 50 pmole of oligo(dT). The mixture was incubated at 55 °C for 60 sec, then 1.5 µl of 10x RNase H buffer (0.5 M Heps, pH 7.4, 1.0 M NaCl, 20 mM MgCl2, and 10 mM dithiothreitol), pre-warmed to 62 °C, was added and the reaction was incubated at 62 °C for 15 min. An additional unit of RNase H (freshly diluted to 1 unit/µl with 1x RNase H buffer) was added and the sample was incubated at 62 °C for 15 min. Then, 29 µl of denaturing buffer (20 µl Formazol, 3 µl 10x MOPS, and 6 µl 37% formaldehyde solution) was added to stop the reaction. The sample was incubated at 55 °C for 5 min and then stored on ice. The RNase H-treated RNAs were subjected to Northern blot analysis as described previously (11), except that 10 µl of a RNase H gel loading buffer (50% glycerol, 1 mM EDTA, 0.25% bromophenol blue, and 0.25% xylene cyanol and 25 µl/ml of 10 mg/ml ethidium bromide) was added to the samples and a 1.2% agarose gel containing 20 mM MOPS, pH 7.0, 1 mM EDTA, and 1 mM sodium acetate was used. The blot was hybridized with the bGH probe.

Northern analyses - Total cellular RNA was isolated using the TR1zo1® reagent and the RNA concentration was determined by measuring the absorbance at 260 nm. A 507-bp fragment of rabbit β-globin cDNA was excised by restricting pRSV-βG (18) with HindIII and BglII. A 2.0-kb fragment of the 18S ribosomal RNA cDNA from Acanthamoeba castellanii was excised by restricting pAr2 (19) with HindIII and EcoRI. A 228-bp bovine growth hormone (bGH) fragment was excised from pPCRScript-bGH with SphI.
The fragments were separated on a 1% agarose gel, excised, and purified by using the GENE CLEAN kit. The synthesis of oligolabeled cDNA probes and Northern analysis were performed as described previously (11). The blots were exposed to a PhosphorImager screen and the intensity of the resulting digital image of each band was quantified using Molecular Dynamics Software. The level of the chimeric β-globin mRNA was divided by the corresponding level of 18S rRNA to correct for errors in sample loading. For half-life studies, the log of normalized data was then plotted versus the time after addition of doxycycline. The reported values are the mean ± S.E. of data obtained from triplicate samples. The line representing the best fit of the data points was determined by a KaleidaGraph program that weights each data point based upon its standard deviation.

RESULTS

Half-life analysis of βG-PCK-1 mRNA - A tetracycline-regulated promoter (Tet-Off system) was used to determine the half-life of the βG-PCK-1 mRNA. Stably transformed cells were maintained in the presence of 50 ng/ml of doxycycline (Dox) to suppress synthesis of the βG-PCK-1 mRNA. A transcriptional pulse was created by removing Dox from the medium for 3 h and subsequently chased by adding 1 µg/ml of Dox to selectively inhibit transcription of the TβG-PCK-1 mRNA. RNA samples were isolated from cells at various intervals following initiation of the chase and the levels of βG-PCK-1 mRNA and 18S rRNA were quantified by Northern analysis (Fig. 2A). This analysis demonstrated that degradation of βG-PCK-1 mRNA is initiated after a brief lag (30 min) and then proceeds with a very rapid half-life (t½ = 1.2 h).

Deadenylation of βG-PCK-1 mRNA - Rapid degradation of mammalian mRNAs is usually initiated by the binding of specific protein(s) to unique element(s) within the 3'-UTR (20,21). The RNA binding protein(s) subsequently recruit a poly(A)-specific ribonuclease (22) and the exosome (23) to remove the poly(A) tail and accomplish a rapid 3'→5' exonucleolytic degradation, respectively. RNase H treatment of the RNAs isolated from the βG-PCK-1 mRNA half-life analysis was performed to determine if deadenylation precedes the decay of the PEPCK mRNA. Incubation of the RNA isolated immediately after the 3 h pulse (0 h sample) with a complementary oligonucleotide and oligo(dT), followed by treatment with RNase H, produced a 315-nt fragment (Fig. 2B). This fragment corresponds to the expected length of the fully deadenylated 3'-end of the βG-PCK-1 mRNA. Digestion of the same RNA sample in the presence of only the complementary oligonucleotide produced larger fragments containing approximately 500-nt. Thus, the newly synthesized βG-PCK-1 mRNA contains a significant poly(A) tail. Identical treatment of RNAs isolated from the later time points demonstrated that the βG-PCK-1 mRNA undergoes a rapid deadenylation that occurs concomitant with the decay of the mRNA.

Mapping of the instability elements within the 3'-UTR of PEPCK - Further half-life analyses were performed using cells grown in the absence of Dox to maximally induce expression of the chimeric PEPCK mRNAs. Selective decay of the reporter mRNA was subsequently initiated by addition of 1 µg/ml Dox. Northern blot analysis was performed using RNAs isolated from 8C cells that stably express the parent TetβG construct. This analysis determined that the control βG mRNA is extremely stable and decays with a half-life of ~5 d (Fig. 3). Northern analysis also indicated that the βG-PCK-1 mRNA was degraded with a half-life of 1.8 h, consistent with the previous pulse-chase analysis.

Previous RNA gel shift analysis indicated that a protein in cytosolic extracts of rat kidney cortex binds specifically to PCK-7, a 50-nt segment of the 3'-UTR of the PEPCK mRNA (12). To test whether the PCK-7 segment is sufficient to cause the rapid destabilization of the PEPCK mRNA, the turnover of the βG-PCK-7 mRNA was determined. Northern blot analyses revealed that the βG-PCK-7 mRNA was degraded with a half-life of 17 h (Fig. 3). Therefore, insertion of just the PCK-7 segment only partially destabilized the chimeric mRNA and was not sufficient to produce the rapid turnover observed with βG-PCK-1 mRNA. Thus, additional sequences within the 3'-UTR may be needed to constitute the complete instability element of the PEPCK mRNA.

Sequence analysis of the 3'-UTRs of human, rat and mouse PEPCK mRNAs revealed the presence of a conserved 16-nt AU-sequence that is
located immediately upstream of the PCK-7 segment. This sequence is part of a 23-nt segment termed PCK-6 that binds a protein in a rat renal cytosolic extract with low affinity (12). Therefore, the PCK-6 segment was inserted into the parent Tetβ vector to generate pTβ-G-PCK-6. LLC-PK1-F⁵ cells were stably transfected with this construct and half-life analyses were performed. The βG-PCK-6 mRNA decayed with a half-life of only 6 h (Fig. 3).

To determine if the PCK-6 and PCK-7 segments act synergistically, cells that stably express pTβ-G-PCK-6/7 were subjected to half-life analysis. Northern blot analysis demonstrated that the βG-PCK-6/7 mRNA decayed with a half-life of 3.6 h (Fig. 4). This half-life was significantly lower than that observed with either βG-PCK-7 or the βG-PCK-6 mRNA but was still greater than that observed with the full-length PCK-1 segment. Therefore, additional sequences within the 3'-UTR may be needed to constitute the complete instability element of the PEPCK mRNA. To test this hypothesis, cells expressing constructs containing longer segments of the 3'-UTR of the PEPCK mRNA were generated.

The PCK-3 segment contains 224-nt from the 3'-end of the PEPCK mRNA that includes the PCK-6/7 segment (Fig. 1). Half-life analysis performed using RNAs isolated from cells expressing the βG-PCK-3 mRNA established that this mRNA decayed with a half-life of 3.6 h, the same as observed with the βG-PCK-6/7 mRNA (Fig. 4). Thus, the PCK-6/7 segment contains all of the instability elements within the PCK-3 segment.

The half-life of the βG-PCK-2 mRNA that contains the 5'-end of the 3'-UTR of the PEPCK mRNA was also determined. Previous RNA-gel shift assays with [32P]-labeled PCK-2 RNA and rat renal cytosolic extracts failed to demonstrate the formation of a RNA-protein complex (12). However, the βG-PCK-2 mRNA, when expressed in the porcine kidney cells, decayed with a half-life of 5.4 h (Fig. 4). Thus, the half-life analyses of the PCK-1, PCK-6, PCK-7 and PCK-2 constructs establish that the 3'-UTR of the PEPCK mRNA contains multiple instability elements that contribute to the rapid turnover that is observed with the full length chimeric mRNA.

AUFI1 binds within the 3'-UTR of the PEPCK mRNA - UV-crosslinking analysis was performed in order to identify the proteins within rat renal cytosolic extracts that bind within the PCK-6/7 segment. Proteins with apparent molecular weights that range from 40-100 kDa were found to be associated with the PCK-6/7 RNA (Fig. 5A). Identical patterns of labeled proteins were observed when PCK-1 and PCK-7 were incubated with the renal cytosolic extract and subjected to UV-crosslinking (data not shown).

To characterize the potential AUFI1 binding interactions, gel shift assays were performed using various PCK RNA segments. The recombinant 40-kDa isoform of AUFI1 exhibits high affinity and specific binding to the PCK-1 RNA (Fig. 6). The recombinant AUFI1 protein also forms identical complexes with the PCK-6 and PCK-7 RNAs. Interestingly, the [32P]-labeled PCK-2 RNA forms a weak complex with recombinant AUFI1. However, AUFI1 fails to form a complex with a 50-nt RNA transcribed from pBlueScript-SK(-) (data not shown).

A competition analysis was performed to demonstrate the specificity of AUFI1 binding to the various PCK RNAs. The interaction observed with PCK-1 RNA was blocked by addition of increasing amounts of unlabeled PCK-1 or PCK-7 RNAs but not by the addition of a 50-nt RNA transcribed from pBlueScript-SK(-) (Fig. 7A). A similar experiment demonstrated the specificity of AUFI1 binding to the PCK-6/7 RNA. A 30- to 500-fold excess of unlabeled PCK-6/7 RNAs, but not an unrelated RNA, effectively competed the binding (Fig. 7B). Even though, AUFI1 binds to the PCK-2 RNA weakly, the observed binding is also highly specific (Fig 7C). Therefore, AUFI1 exhibits specific binding to each segment of the PEPCK 3'-UTR that contains an instability element.
**Mutational analyses** - Mutational analyses were performed to identify the specific sequences within the PCK-6/7 RNA that bind AUF1. PCK-6 contains a UUAUUUUAU sequence (Fig. 8A) that is similar to the 9-nt AU-rich element in TNFα mRNA that binds AUF1 (26). When this sequence in PCK-6 RNA was mutated, by introducing multiple GC nucleotides, binding of recombinant AUF1 was abolished (Fig. 9).

PCK-7 contains a direct repeat of an 8-nt CU sequence separated by a single nucleotide, a potential secondary structure containing an 8-nt stem and an 11-nt loop, and a 22-nt stretch in which 18 of the nucleotides are A and U residues. To further map the AU-rich binding site, the PCK-7 RNA was divided into three sub-segments: PCK-8 that contains the CU repeats; PCK-9 RNA that consists of the AU-rich region; and PCK-10 RNA that forms the potential stem-loop structure (Fig. 8B). When incubated with a rat renal cytosolic extract, the PCK-8 and PCK-10 RNAs, but not the PCK-9 RNA, form a complex (data not shown). Similarly, the recombinant AUF1 binds to only the PCK-8 and PCK-10 RNAs (Fig 10A). Therefore, the CU-rich region and/or the potential stem loop structure might function as AU-rich binding sites. Three mutations of the PCK-7 RNA, termed PCK-7 mut-1, PCK-7 mut-2, and PCK-7 mut-3 were designed to test binding to the individual or combined CU repeats (Fig. 8C). A fourth mutant, PCK-7 mut-4, was designed to discriminate binding to the conserved sequence that constitutes the loop of the putative stem-loop structure. Recombinant AUF1 binds to PCK-7 RNA but not to any of the mutated constructs (Fig. 10B). Therefore, within the PCK-7 RNA, AUF1 binds to the CU-repeats, to the conserved loop sequence or to both elements.

**DISCUSSION**

Previous experiments using DRB, a specific inhibitor of RNA polymerase II, to measure the half-life of the single βG-PCK-1 mRNA in LLC-PK1-F+ cells demonstrated that the 3’-UTR of the PEPCK mRNA contains a cis-acting destabilizing element (11). In the current study, a tet-regulated promoter system was used to quantify the in vivo decay rates and to map the location of multiple instability elements within various chimeric βG-PCK mRNAs. Using this approach, the half-life observed for the βG-PCK-1 mRNA was significantly less than that measured in the previous experiments. Since DRB inhibits transcription of all polymerase II genes, it may block the continued synthesis of proteins that function in the normal process of mRNA turnover. In contrast, with the tet-regulated system, the transcription of the gene of interest is selectively inhibited by the addition of a sub-toxic level of doxycycline. Therefore, the observed half-life of the βG-PCK-1 mRNA of 1.2 to 1.8 h is a more reliable measure of its turnover in LLC-PK1-F+ cells. The analysis performed with multiple βG-PCK constructs indicates that the PEPCK mRNA contains multiple instability elements that are located within the PCK-2, PCK-6, and PCK-7 segments of the 3’-UTR. These findings are significant because the physiologically important adaptations in the levels of PEPCK mRNA that occur in response to various hormones and to changes in acid-base balance are made feasible by the rapid decay of the PEPCK mRNA.

The decay of mammalian mRNAs that contain AU-rich elements is frequently preceded by the removal of the poly(A) tail from the 3’-end of the mRNA (27). Deadenylation can proceed in either a synchronous or an asynchronous manner. Synchronous deadenylation results in the non-processive formation of a shortened poly(A) tail containing 30-60 nucleotides. With the c-fos mRNA (27), this process is completed before the decay of the body of the mRNA is initiated. In contrast, asynchronous deadenylation of the GM-CSF mRNA (27) results in the processive formation of a fully deadenylated mRNA that is then rapidly degraded. The PEPCK mRNA lacks a canonical AUUUA or UUAUUUAU element but contains a single related sequence (UUAUUUUAU) within the PCK-6 segment of its 3’-UTR. RNase-H assays indicate that the βG-PCK-1 mRNA undergoes a rapid and synchronous deadenylation that is complete within 2 h (Fig. 2B). However, approximately 50 percent of the newly synthesized βG-PCK-1 mRNA is already degraded within 2 h. Therefore, the degradation of the βG-PCK-1 mRNA may proceed by a combined mechanism in which a portion of the chimeric mRNA undergoes a rapid processive deadenylation that is complete within 30 min.
while the remainder undergoes a slower, but synchronous deadenylation.

The clonal lines expressing the remaining βG-PCK mRNAs produced only a low level of the chimeric mRNA during a 3 h transcriptional pulse. As a result, it was difficult to accurately quantify the decay of the other mRNAs using the pulse-chase protocol. Therefore, the half-lives of the various βG-PCK mRNAs containing shortened segments of the 3'-UTR of the PEPCK mRNA were determined by adding doxycycline to cells that were grown in the absence of doxycycline. A potential problem with this approach can occur if high levels of constitutive expression from the tet-regulated promoter saturate the cellular decay machinery and cause an inefficient decay of the mRNA (15). However, constitutive expression of the βG-PCK-1 mRNA exceeded that of the other mRNAs and the half-lives of the βG-PCK-1 mRNA determined by the two protocols were nearly identical. Hence, the half-lives obtained for the different βG-PCK constructs should accurately reflect the actual decay rates of the mRNAs. Without the pulse-chase protocol, it was not possible to characterize the mechanism of deadenylation of the various deletion constructs of the βG-PCK-1 mRNA.

Previous studies reported that cytosolic extracts of rat hepatoma cells (6) and hepatocytes (28) contain proteins that bind to multiple segments of 3'-UTR of PEPCK mRNA. While the observed binding interactions were affected by pretreatment of the cells with cAMP and insulin, neither study established the specificity of the observed interactions nor identified the involved binding elements. The subsequent use of affinity chromatography identified ferritin light chain as the protein in rat liver extracts that binds to the 3'-UTR of PEPCK mRNA (28). However, the physiological significance of this interaction remains to be confirmed. In the current study, AUF1, a known RNA binding protein, was shown to bind with high affinity and specificity to the PCK-2, PCK-6 and PCK-7, the same segments that contribute to the rapid turnover of the PEPCK mRNA. AUF1 binds to the 3'-UTRs of many unstable RNAs including c-myc (29), TNFα (30), GM-CSF (31), and COX-2 (32). AUF1 was also identified as hnRNP D (33), a protein that shuttles between the nucleus and the cytoplasm (34). Four isoforms of AUF1 (p37, p40, p42 and p45) are produced by alternative splicing of the initial AUF1 transcript (33). The p40 AUF1 isoform is predominantly cytoplasmic and hence was utilized in the reported experiments. AUF1 has also been shown to stabilize IL-3 RNA in NIH 3T3 cells (35) and destabilize it in K562 cells (36). Thus, its effect on mRNA stability depends on the target ARE and the auxiliary proteins expressed in a particular cell line (37).

Recruitment of ancillary factors by AUF1 on the target RNA is essential for the promotion of ARE-mediated mRNA decay. Yeast two-hybrid (38) and co-immunoprecipitation experiments (39) have shown that AUF1 interacts with a number of RNA binding proteins including NSAP-1, NSEP-1 and IMP-2. Interestingly, NSEP-1 exhibits an endoribonuclease activity in vitro, suggesting a possible role of these proteins in AUF1-mediated decay (38). Other proteins such as the poly(A) binding protein, the translation initiation factor eIF4G, the heat shock protein Hsp70 and the cognate heat shock protein Hsc70 also physically interact with AUF1 (40) and may regulate its activity. Thus, it will be interesting to determine if any of these proteins are also involved in decay of the PEPCK mRNA.

The high-affinity protein binding observed with rat renal cytoplasmic extracts maps to the PCK-7 segment of the 3'-UTR of the PEPCK mRNA (12). Thus, it was surprising to find that the PCK-7 segment did not produce a strong destabilizing effect compared to the full length 3'-UTR. Inclusion of the adjacent PCK-6 segment, which contains an UUAUUUAU motif, resulted in a decay rate nearly comparable to that observed with the full length 3'-UTR. Thus, binding of AUF1 at both sites may be necessary to recruit the proteins that mediate the rapid decay of the PEPCK mRNA. This hypothesis is supported by UV-crosslinking analysis of the full-length 3'-UTR incubated with rat renal cytoplasmic extracts. This analysis revealed the presence of multiple RNA-protein complexes (Fig. 5A), suggesting that other renal proteins might be binding to this RNA. However, super-shift analyses using antibodies against known RNA binding proteins including HuR, TTP and Hsp70 (data not shown) failed to detect these proteins in the PCK-1 RNA-protein complexes. Identification of additional binding
proteins involved in PEPCK mRNA turnover is currently under investigation.

Previous studies using a rat renal cytoplasmic extract failed to detect a complex with the PCK-2 RNA that constitutes the 5′-end of the 3′-UTR of PEPCK mRNA (12). However, this segment also binds recombinant AUF1 (Fig. 6) and exhibits a significant destabilizing effect when incorporated into the β-globin reporter mRNA. Sequence analysis revealed the presence of a stretch of alternating purine and pyrimidine nucleotides near the 5′-end of PCK-2. This region also contains a sequence that is highly conserved in the mouse, rat, and human PEPCK mRNAs (Fig. 1). AUF1 might bind within this conserved sequence. However, the affinity of the AUF1 binding observed with this segment is weak compared to that observed with the PCK-6/7 region. Thus, some other protein may bind to this segment and participate in the turnover of the PEPCK mRNA.

Sequence analysis of the mouse, rat, and human PEPCK mRNAs identified two fully conserved AU-rich sequences within the PCK-6/7 segments of their 3′-UTRs (Fig. 1). The first is an UUAAAUUAUUUAUACAC sequence that is located in the PCK-6 segment. Mutational analysis indicates that AUF1 binds to the AU-rich core within this conserved sequence (Fig. 9). The second is a CUUUACAUAAUUG sequence that is found within the PCK-7 segment. The latter sequence includes the entire loop of a stable stem-loop structure. The observation that AUF1 does not bind to the PCK-7 Mut-4 RNA suggests that it also recognizes the highly conserved sequence that constitutes the loop of the predicted stable stem-loop structure. The sequence of the PCK-7 Mut-4 RNA was carefully designed using RNAdraw software to ensure that the mutations introduced in the loop sequence would neither alter the ability to form the predicted stem-loop structure nor promote the formation of an alternative structure. The additional PCK-7 mutations also failed to form a complex with AUF1. The PCK-7 Mut-2 and Mut-3 constructs contain mutations in the second and in both of the CU-repeats, respectively. However, the two mutations would also disrupt the ability of the RNAs to form a stable stem-loop structure. Thus, the inability to bind to these mutations may indicate that the predicted stem loop structure is also necessary for AUF1 binding to the conserved sequence that constitutes the loop. However, the PCK-7 Mut-1 construct is predicted to form the same stem-loop structure, suggesting that the CU-repeats are also required for binding of the purified recombinant AUF1. This is the first demonstration that AUF1 binding may be affected by an adjacent CU-sequence.

The results of this study demonstrate that there are at least three destabilizing elements within the 3′-UTR of the PEPCK mRNA. The primary destabilizing elements are located within the last 73-nt of the 3′-UTR, while a weaker element resides within the initial 364-nt of the 3′-UTR. Based upon the reported observations, the following model for PEPCK mRNA degradation can be hypothesized. AUF1 and various ancillary factors recognize and bind to the AU- and CU-rich regions within the PCK6/7 segment. AUF1 and possibly other proteins also bind to the conserved sequence or the stretch of alternating purines and pyrimidines within the PCK-2 segment. The latter binding may occur simultaneously or may be facilitated by binding of AUF1 to the more distal elements. After the assembly of the final complex, AUF1 may recruit the polyadenine ribonuclease and the exosome, which subsequently degrade the PEPCK mRNA in the 3′→5′ direction.
FOOTNOTES

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1 Abbreviations are: βG, β-globin; PCK, phosphoenolpyruvate carboxykinase; bGH, bovine growth hormone; DRB, 5,6-dichloro-1-β-D-ribofuranosylbenzamidazole; AUF1, AU factor 1; TTP, tristetraprolin and Dox, doxycycline.

2 Unpublished data of J.M. Schroeder and N.P. Curthoys

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FIGURE LEGENDS

Fig. 1. The 3’-UTR of PEPCK mRNA. Panel A. Nucleotide sequence of the PCK segments. The UAA stop codon is italicized and underlined. The conserved sequences are shown in bold letters and the AU- and CU-rich sequences that are potential AU1 binding sites are underlined. Panel. B. Constructs used to map and to characterize the function of AU1 binding sites. The solid lines represent the full-length 3’-UTR and the portions of the 3’-UTR that were either transcribed as RNA probes or cloned into the β-globin reporter plasmid. The numbers indicate the corresponding positions within the PEPCK cDNA. The lengths of the PEPCK segments are indicated in nucleotides (nt).

Fig. 2. Pulse-chase analysis of the half-life and deadenylation of βG-PCK-1 mRNA. Cells were grown in medium containing 50 ng/ml Dox and transferred to medium minus Dox for 3 h. Transcription of the βG-PCK-1 gene was subsequently inhibited by addition of 1 µg/ml Dox and cells were harvested at various intervals. (Panel A) The levels of the chimeric mRNA and the 18S rRNA were quantified by Northern analysis. The levels of the βG-PCK-1 mRNAs were divided by the corresponding levels of 18S rRNAs to correct for errors in sample loading. The log of normalized data was then plotted against the time after Dox addition. The reported data are the mean ± S.E. of triplicate samples. (Panel B) A deoxyoligonucleotide (RNase H oligo), that is complementary to the 3'-end of the PEPCK mRNA, and RNase H were incubated with the isolated RNAs. A 30-mer of oligo-dT was also added to an aliquot of the RNA isolated at 0 h to produce a 315-nt fully deadenylated 3’-fragment of the βG-PCK-1 mRNA. The samples were then separated on a 2 % agarose gel and hybridized with a [32P]-labeled cDNA that binds specifically to the bGH sequence within the 3’-fragments. Lane 1 contains the fully deadenylated fragment of the βG-PCK-1 mRNA (Poly(A)). Lanes 2-7 contain the cleaved RNA fragments isolated at various times after addition of Dox.

Fig. 3. Comparison of the half-lives of βG, βG-PCK-1, βG-PCK-6 and βG-PCK-7 mRNAs. Cells were grown for 48 h in the absence of Dox and then 1 µg/ml Dox was added to inhibit transcription. RNA was isolated at various times after addition of Dox and the levels of the chimeric βG mRNAs and 18S rRNA were quantified by Northern analysis. The log of normalized data was then plotted against the time after Dox addition. The reported data are the mean ± S.E. of triplicate samples.
Fig. 4. Half-life analysis of βG-PCK-6/7, βG-PCK-3 and βG-PCK-2 mRNAs. Cells were grown for 48 h in the absence of Dox and then 1 µg/ml Dox was added to inhibit transcription. RNA was isolated at various times after addition of Dox and the levels of the βG-PCK-6/7, βG-PCK-3 and βG-PCK-2 mRNAs and the 18S rRNA were quantified by Northern analysis. The log of normalized data was then plotted against the time after Dox addition. The reported data are the mean ± S.E. of triplicate samples.

Fig. 5. Identification of AUF1 as a protein that binds to the instability elements within the 3'-UTR of the PEPCK mRNA. (Panel A) A rat renal cytosolic (RRC) extract was incubated in the absence and presence of [32P]-labeled PCK-6/7 RNA. The samples were subjected to UV radiation and then separated by 10 % SDS-PAGE. (Panel B) The RRC extract was preincubated in the absence or presence of the indicated antibodies. [32P]-labeled PCK-7 RNA was subsequently added and the resulting complexes were resolved on a native polyacrylamide gel. The two gels were imaged on a PhosphorImager.

Fig. 6. Recombinant AUF1 binds to [32P]-labeled PCK-1, PCK-7, PCK-6 and PCK-2 RNAs. The indicated ng amounts of recombinant p40 isoform of human AUF1 were incubated with the [32P]-labeled RNAs. The samples were treated with RNase T1 to digest the unbound probe and the resulting RNA-protein complexes were resolved on a native polyacrylamide gel and imaged on a PhosphorImager. The arrows designate the resulting RNA-protein complexes.

Fig. 7. Competition analysis of AUF1 binding to various PCK RNAs. (Panel A) The [32P]-labeled PCK-1 RNA was incubated in the absence or presence of recombinant AUF1. The samples in lanes 3-10 also contained the indicated fold excess (30X, 200X or 500X) of an unlabeled competitor RNA. pBS is a non-specific 50-nt RNA that was transcribed from pBlueScript-SK(-). The samples were treated with RNase T1 to digest the unbound probe and the remaining complexes were resolved on a native polyacrylamide gel and imaged on a PhosphorImager. The analysis was repeated using [32P]-labeled PCK-6/7 (Panel B) and PCK-2 RNAs (Panel C).

Fig. 8. Nucleotide sequence of the mutated and deleted RNAs derived from the PCK-6 and PCK-7 segments. (Panel A) Within the 22-nt PCK-6 segment, the 9 AU nucleotides (capital letters) were mutated to produce the mut-6 RNA. (Panel B) The PCK-8, PCK-9 and PCK-10 RNAs correspond to portions of the PCK-7 segments that contain the CU-repeats, the AU-rich region, and the stem-loop structure respectively. (Panel C). The capital letters indicate nucleotides that were mutated within PCK-7 to produce the Mut-1, Mut-2, Mut-3 and Mut-4 RNAs. The mutated segments selectively alter the first, second and both CU-regions and the sequence that constitutes the loop of PCK-7, respectively.

Fig. 9. Binding of recombinant AUF1 to [32P]-labeled PCK-6 and Mut-6 RNAs. The [32P]-labeled PCK-6 and Mut-6 RNAs were incubated in the absence and presence of AUF1. The samples were treated with RNase T1 to digest the unbound probe, resolved on a native polyacrylamide gel, and imaged on a PhosphorImager.

Fig. 10. Binding of recombinant AUF1 to various deleted and mutated forms of PCK-7 RNA. (Panel A) [32P]-labeled PCK-7 RNA, the deleted segments of PCK-7 RNA (PCK-8, PCK-9, and PCK-10) and an unrelated RNA transcribed from pBlueScript-SK(-) (pBS) were incubated in the absence or presence of recombinant AUF1. (Panel B) [32P]-labeled PCK-7 RNA and the mutated segments of PCK-7 RNA (Mut-1, Mut-2, Mut-3 and Mut-4) were incubated with recombinant AUF1. All of the samples were treated with RNase T1 to digest the unbound probe and the remaining complexes were resolved on a native polyacrylamide gel and imaged on a PhosphorImager.
Fig. 1
A

| Hours Dox Treatment | 0 | 0.5 | 1 | 2 | 3 | 4 |
|---------------------|---|-----|---|---|---|---|
| βG-PCK-1 RNA       |   |     |   |   |   |   |
| 18S RNA             |   |     |   |   |   |   |

\[ \text{βG-PCK-1} \quad t_{1/2} = 1.2 \text{ h} \]

B

| βG coding region | PCK 3' UTR | bGH | AAAA(A~200)...
|-----------------|-----------|-----|----------------
|                  |           |     |                |

\[ \text{RNase H oligo} \quad \uparrow \quad \uparrow \quad \text{TTTTT(A~30) oligo dT} \quad 315 \text{ nt} \]

| Time (min) |
|------------|
| 0  | 30 | 60 | 120 | 180 | 240 |

Poly(A)

|   | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
|---|---|---|---|---|---|---|---|
|   |   |   |   |   |   |   |   |

315 nt

**Fig. 2**

βG-PCK-1
Fig. 3
Fig. 4
A.

| PCK-6/7 | - | + | + | + |
|---------|---|---|---|---|
| 1       |   |   |   |   |
| 2       |   |   |   |   |
| 3       |   |   |   |   |

102 kDa
55 kDa
45 kDa
38 kDa

B.

| Antibodies: | - | - | CP1 | CP2 | HuR | AUF1 |
|-------------|---|---|-----|-----|-----|------|
| [32P]-PCK-7: | + | + | +   | +   | +   | +    |
| RRC extract: | - | + | +   | +   | +   | +    |

RNA–Protein complexes

RNase T1
Digested PCK-7

Fig. 5
Fig. 6
A.

| Competitor: | PCK-1 | PCK-7 | pBS |
|-------------|-------|-------|-----|
| -           | -     | 30    | 200 | 500 |
| PCK-1:      | +     | +     | +   | +   |
| p40 AUF1:   | -     | +     | +   | +   |

![Image of gel blot with bands labeled 1 to 10]

B.

| Competitor: | PCK-6/7 | pBS |
|-------------|---------|-----|
| -           | -       | 30  | 200 | 500 |
| PCK-6/7:    | +       | +   | +   |
| p40 AUF1:   | -       | +   | +   |

![Image of gel blot with bands labeled 1 to 7]

C.

| Competitor: | PCK-2 | pBS |
|-------------|-------|-----|
| -           | -     | 30  | 200 |
| PCK-2:      | +     | +   | +   |
| p40 AUF1:   | -     | +   | +   |

![Image of gel blot with bands labeled 1 to 5]

Fig. 7
A.  
\[ \text{PCK-6} \quad 5'\text{guauguuaaauuauuuuauac}\ 3' \]
\[ \text{PCK-Mut-6} \quad 5'\text{guauguuaaacGCAAGCAGGuac}\ 3' \]

B.  
\[ \text{PCK-7} \quad 5'\text{acugcCCUUCUUuCaauauugaaauagguauccugacca}\ 3' \]
\[ \text{PCK-8} \quad 5'\text{acugcCCUUUCBUuCCUUCUUuCaauauugaaauagguauccugacca}\ 3' \]
\[ \text{PCK-9} \quad 5'\text{acugcCCUUCUuaCCUUCUUuCaauauugaaauagguauccugacca}\ 3' \]
\[ \text{PCK-10} \quad 5'\text{acugcCCUUCUUuCaauauugaaauagguauccugacca}\ 3' \]

C.  
\[ \text{MUT-1} \quad 5'\text{acugAGAGGGCAAAccuuucuuuaauaauuaugaaauagguauccugacca}\ 3' \]
\[ \text{MUT-2} \quad 5'\text{acugccuuucuuuaGAGAGCCAGacGaGaGCgaauaagguauccugacca}\ 3' \]
\[ \text{MUT-3} \quad 5'\text{acugAGAGGGCAAAgAGAGCCAGacGaGaGCgaauaagguauccugacca}\ 3' \]
\[ \text{MUT-4} \quad 5'\text{acugccuurucuuaccuuucAAAaGaCaCAGaaauagguauccugacca}\ 3' \]

**Fig. 8**
| Probes used: | PCK-6 | PCK-Mut-6 |
|-------------|-------|-----------|
| p40 AUFI:   | -     | +         |
|             | 1     | 2         |
|             | -     | +         |
|             | 3     | 4         |

RNA–Protein complex

RNase T1 Digested Probes

Fig. 9
A.

|                | $[^{32}P]^{-}$PCK-7 | $[^{32}P]^{-}$PCK-8 | $[^{32}P]^{-}$PCK-9 | $[^{32}P]^{-}$PCK-10 | $[^{32}P]^{-}$pBS |
|----------------|---------------------|---------------------|---------------------|---------------------|------------------|
| AUF1 (p40)    | -                   | +                   | -                   | +                   | -                |
| 1              | 2                   | 3                   | 4                   | 5                   | 6                |
| 7              | 8                   | 9                   | 10                  |                     |                  |

RNA–Protein complexes

RNase T1 Digested Probes

Fig. 10
B.

Probes used: Mut-1 Mut-2 Mut-3 Mut-4 PCK-7

p40 AUF1:

|    1 |    2 |    3 |    4 |    5 |
|-----|-----|-----|-----|-----|
| +   | +   | +   | +   | +   |

RNA–Protein complex

Fig. 10
3'-Nontranslated region of phosphoenolpyruvate carboxykinase mRNA contains multiple instability elements that bind AUF1
Sachin Hajarnis, Jill M. Schroeder and Norman P. Curthoys

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