A cAMP-regulated RNA-binding Protein That Interacts with Phosphoenolpyruvate Carboxykinase (GTP) mRNA*

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Cyclic-AMP stabilizes phosphoenolpyruvate carboxykinase (GTP) (PEPCK) mRNA against degradation. To investigate the mechanism of this effect, RNA mobility shift assays were used to determine the interaction of cellular proteins with specific domains from the mRNA. We report here the identification of a protein with an affinity for sequences of PEPCK mRNA with a predicted stem-loop structure. RNA-protein complex formation was significantly reduced if the double-stranded RNA probe was preheated to 90 °C. The RNA-binding protein did not bind to the hairpin structure of poly(rI)-poly(rC), indicating some degree of sequence specificity and that the RNA-binding protein is not the interferon-induced double-stranded RNA-activated protein kinase. The binding activity was contained in the cytosolic fraction (100,000 × g) of rat hepatoma FTO-2B cells and was significantly enhanced by high concentrations of KCl. Chromatography on an anion exchanger separated the binding activity from a factor which, upon reconstitution, inhibited the interaction with the RNA probe. Incubation of cells with cAMP resulted in a 3-4-fold decrease in the activity of the RNA-binding protein. An inhibition in complex formation was observed with extracts as early as 60 min after exposure of cells to cAMP. Liver extracts from rats starved for 72 h also had reduced binding activity compared to extracts from fed animals. Cellular extracts treated with alkaline phosphatase exhibited an elevated level of complex formation. An analysis by SDS-polyacrylamide gel electrophoresis of the RNA-protein complex after ultraviolet light cross-linking demonstrated that the RNA-binding protein had a molecular mass of approximately 100 kDa. On the basis of these results, we suggest that liver cells contain a protein whose interaction with PEPCK mRNA is regulated by cAMP-dependent phosphorylation and which may be responsible for the cAMP-mediated control of PEPCK mRNA half-life.

Phosphoenolpyruvate carboxykinase (GTP) (EC 4.1.1.32) (PEPCK) is a key regulatory enzyme in hepatic gluconeogenesis. The steady-state level of the enzyme mRNA is regulated by a number of hormones, including glucagon (acting via cAMP), glucocorticoids, and insulin (reviewed by Granner and Pilkis, 1990). It is now well established that these hormones exert their activity by modulating the transcription rate of the PEPCK gene: both glucagon (or cAMP) and glucocorticoids are stimulatory whereas insulin is inhibitory (Liu and Hanson, 1991; O'Brien and Granner, 1991). In addition to its effect on the transcription rate of the gene (Lamers et al., 1982), cAMP has been shown to stabilize PEPCK mRNA against degradation (Hod and Hanson, 1988). Time course analysis of the cAMP effect in rat livers (Lamers et al., 1982) and FTO-2B rat hepatoma cells (Hod and Hanson, 1988) showed a transient increase in the transcription rate of the gene following a stimulus of cAMP, whereas the level of the mRNA remained elevated for several hours. An earlier study showed that the translation of the mRNA may be also stimulated by the cyclic nucleotide (Wicks and Mckibbin, 1972), although cAMP enhanced the stability of a polysome-free PEPCK mRNA (Hua and Hod, 1992). These studies demonstrate that the metabolism of PEPCK mRNA in the cytoplasm may play an important role in the overall control of PEPCK gene expression.

Several reports have demonstrated that RNA-protein interaction is critical in mediating the post-transcriptional control of gene expression. The coordinate reciprocal changes in the translation and mRNA stability of ferritin and transferrin receptor mRNAs, respectively, is one example (Klausner et al., 1993). These alterations are mediated by a single protein (iron response binding protein) capable of binding to a specific iron response element located in the 5′-UTR of ferritin and the 3′-UTR of transferrin receptor mRNAs.

Using RNA mobility shift assays and a label transfer analysis, we have identified an RNA-binding protein with a molecular mass of approximately 100 kDa. Complex formation with RNA is not sequence specific but rather seems to require a hairpin-loop structure. Evidence is presented for the acute regulation of the RNA-binding protein by cAMP.

EXPERIMENTAL PROCEDURES

Cell Culture—FTO-2B rat hepatoma cells (Killary et al., 1984) were maintained as monolayers in a 1:1 mixture of Dulbecco’s modified Eagle’s minimal essential medium and Ham’s F-12 medium supplemented with 10% bovine calf serum. All experiments were carried out 16–20 h prior to the experiment.

Preparation of Cell Extract from FTO-2B Cells—FTO-2B cells (0.5–1 × 10^6 cells/100-mm plate) were scraped into ice-cold phosphate-buffered saline containing 2 mM EDTA. Following centrifugation at 450 × g for 5 min, the cells were suspended in lysis buffer (1 ml/cells recovered from four plates) containing 10 mM Tris·Cl, pH 7.4, 40 mM KCl, 0.5 mM spermidine, 0.15 mM spermine, 2 mM EDTA, 1 mM EGTA, and 5 mM dithiothreitol. Cells were homogenized in a Dounce homogenizer (Kimble) using 10 strokes with a B pestle.

Nuclei and cell debris were removed by centrifugation at 13,000 × g for 10 min. The supernatant was centrifuged at 100,000 × g to obtain a post-polysomal extract and a polysomal pellet. To prepare a polysomal wash, the polysomal pellet was suspended for 1 h in a lysis buffer containing 0.5 mM KCl and then centrifuged at 100,000 × g to obtain a post-polysomal extract and a polysomal pellet.

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‡ The abbreviations used are: PEPCK, phosphoenolpyruvate carboxykinase; UTR, untranslated.
g. In experiments designed to determine the effect of cAMP, the lysis buffer was supplemented with 2 mM sodium orthovanadate, 50 mM β-glycerophosphate, and 50 mM NaF. Cellular extracts were stored at −70 °C. Protein concentrations were determined by the bicinchoninic acid protein assay (BCA) (Pierce Chemical Co.).

Animals and Preparation of Liver Cell Extract—Male Sprague-Dawley rats (Charles River Breeding Laboratories) at about 200 g were used for the experiments. Standard chow (when applicable) and water were available ad libitum. Rats were killed by decapitation, and the livers were freeze-clamped in liquid nitrogen and stored at −70 °C. Cellular extracts were prepared as described above for FTO-2B cells, except that 1 g of frozen tissue was homogenized with 9 volumes of ice-cold lysis buffer.

Preparation of RNA Transcripts—The RNA transcripts were made from single-stranded oligodeoxyribonucleotide templates by using T7-RNA polymerase as described (Milligan et al., 1987). Template oligodeoxyribonucleotides were synthesized on an Applied Biosystems 381A DNA synthesizer.

The RNA transcripts were as follows: Lp8, a 30-nucleotide probe encompassing nucleotides 2339-2368 from the 3'-UTR of the rat PEPCK mRNA (Beale et al., 1985); Lp7, a 30-nucleotide probe encompassing nucleotides 2027-2056 from the 3'-UTR of the rat PEPCK mRNA (Beale et al., 1985); Lp2, a 77-nucleotide probe encompassing nucleotides 570-646 from the coding region of the rat PEPCK mRNA (Beale et al., 1985); Lp3c, a 60-nucleotide probe encompassing nucleotides 2356-2415 from the 3'-UTR of the chicken PEPCK mRNA (Cook et al., 1986). Other RNA transcripts are described in the legend to Fig. 2.

Radioabeled RNA were synthesized using Transcription Pac-Kits (Epicentre Technologies). To prepare 32P-labeled RNA probes, [α-32P]CTP (Du Pont-New England Nuclear, 800 Ci/mmol) was included in the reaction mixture at about 100 Ci/mmole final specific radioactivity. 32S-Radiolabeled RNA transcripts were synthesized as described above except that cytidine 5'-[α-32S]thiotriphosphate (Du Pont-New England Nuclear, >1200 Ci/mmol) was used at a final specific radioactivity of about 15 Ci/mmole.

RNA Mobility Shift Assays—Binding reactions were carried out with cytoplasmic extract (0.15–1 mg/ml) and ~1 pmol of 32P-radio-labeled RNA probe in binding buffer containing 10 mM Tris-HCl, pH 7.6, 4 mM MgCl2, 100 μg/ml poly(rA) (Sigma), 1 mM dithiothreitol, and irradiated for 15 min at an intensity of 3000 microwatts/cm2. To determine whether cellular extracts contain proteins which bind to the alternating pyrimidine-purine base sequence, a 32P-labeled 30-nucleotide transcript from the center of the 102-base region (designated as Lp8) was incubated with cellular extracts from FTO-2B rat hepatoma cells as detailed under "Experimental Procedures." After digestion of the free RNA probe with RNase T1, the RNA-protein complexes were separated on a native polyacrylamide gel. Fig. 1 (lanes 2 and 3) shows one major complex which migrated with a KCl gradient between 0.4 and 0.45 M KCl (lane 2) and 0.5 M KCl (lane 3). The gel shift analysis was carried out at 70 °C. Complex formation was specific because neither a 0.5 M KCl polysomal wash at a protein concentration of 0.4 mg/ml (lane 2), or 1.5 mg/ml (lane 3); a 0.5 M KCl polysomal wash at a protein concentration of 0.4 mg/ml (lane 4) or 1.5 mg/ml (lane 5). The gel shift analysis was carried out as detailed under "Experimental Procedures."

RESULTS

Specificity of the RNA-Protein Interaction—Studies with several mRNAs have shown that double-stranded regions are binding sites for proteins and that such interactions may play an important regulatory role (Pandey et al., 1991; Manzella and Blackshear, 1992; Klausner et al., 1993). As a primary target for the analysis of protein interaction with PEPCK mRNA, the 102-nucleotide region of alternating purine-pyrimidine bases was selected. This sequence is contained in the 3'-UTR of the PEPCK mRNA (positions 2310–2405) and includes numerous repeats and palindromes (Beale et al., 1985). Secondary structure analysis revealed that this sequence has a potential to form a stable hairpin structure (Cook et al., 1986). The 3'-UTRs of the mRNA from the chicken (Cook et al., 1986) and the human (Stoffel et al., 1993) have similar predicted hairpin structures, despite only a marginal degree of homology in the nucleotide sequence forming the stem.

To determine whether cellular extracts contain proteins which bind to the alternating pyrimidine-purine base sequence, a 32P-labeled 30-nucleotide transcript from the center of the 102-base region (designated as Lp8) was incubated with cellular extracts from FTO-2B rat hepatoma cells as detailed under "Experimental Procedures." After digestion of the free RNA probe with RNase T1, the RNA-protein complexes were separated on a native polyacrylamide gel as described (Konarska and Sharp, 1986). Dried gels were exposed to X-Omat AR films (Eastman Kodak Company) at −70 °C, typically for 4–6 h.

Partial Purification of the RNA-binding Protein—An extract of FTO-2B cells was incubated with a Q-Sepharose (Pharmacia LKB Biotechnologies Inc.) column (at about 10 mg of protein/1 ml bed volume), pre-equilibrated with lysis buffer containing 40 mM KCl. After the column was washed with 2 bed volumes of lysis buffer, the RNA-binding protein was eluted by a KCl gradient between 0.4 and 0.75 M KCl. Fractions containing the RNA binding activity (eluted at 0.4–0.5 M KCl) were combined and concentrated by precipitation with polyethylene glycol (20%). The protein was suspended in a lysis buffer containing 150 mM KCl and applied to a heparin-Sepharose CL-6B (Pharmacia) column pre-equilibrated with the same buffer. The RNA-binding protein was eluted by a KCl gradient between 0.15 and 0.75 M KCl.

UV Light Cross-linking of the RNA-Protein Complex—RNA-protein binding reactions were carried out as described above. Following the incubation with RNase T1, binding reactions were placed on ice and irradiated for 15 min at an intensity of 3000 microwatts/cm2, using a Stratalinker UV light box (Stratagene, model 1800). The RNA-protein complexes were denatured by heating at 95 °C for 5 min in the presence of SDS (1%) and subjected to SDS-polyacrylamide gel electrophoresis on a 10% gel.

Several observations suggested that the RNA binding activity is a protein. For example, prior treatment of the cellular extract at 56 °C for 10 min, or inclusion of SDS (0.1% w/v) in the reaction assay, completely inhibited binding activity (data not shown). Additionally, the RNA-binding protein was partially purified by precipitation by ammonium-sulfate (40–50% saturation), and fractionation on Q-Sepharose and heparin-Sepharose as detailed under "Experimental Procedures." Whereas the binding reactions were routinely carried out at 4 °C, similar results were obtained at 37 °C (data not shown), suggesting that no enzymatic activity was involved in the RNA-protein interaction.

Fig. 1. An RNA-binding protein in the cytosol fraction of FTO-2B cells. Complex formation was performed with Lp8 as an RNA probe in the presence of bovine serum albumin (1.5 mg/ml) (lane 1); a post-polysomal 100,000 × g fraction from FTO-2B cells at a protein concentration of 0.4 mg/ml (lane 2), or 1.5 mg/ml (lane 3); a 0.5 M KCl polysomal wash at a protein concentration of 0.4 mg/ml (lane 4) or 1.5 mg/ml (lane 5). The gel shift analysis was carried out as detailed under "Experimental Procedures."
To determine the sequence specificity of the RNA-protein interaction, competition studies with different RNA probes were performed. Fig. 2 summarizes these studies. Panels A and B show that stable complexes were formed with Lp8 as well as with a sequence from a region between positions +2010 and +2073 in the 3'-UTR of PEPCK mRNA (Lp7). This sequence has been also predicted to form a stable stem-loop structure (Cook et al., 1986). 20–60-fold excess molar amounts of 35S-labeled Lp8 or Lp7 were sufficient to prevent complex formation with either their homologous 32P-labeled probes or with their counterparts. Other RNA probes with a predicted secondary stable structure, such as the second loop in the 5'-UTR of the rat mRNA (Lp2) or a loop structure from the 3'-UTR of the chicken PEPCK mRNA (Lp3c) (Cook et al., 1986), also competed out the protein interaction with either Lp8 or Lp7 (data not shown). In contrast, neither the double-stranded sequence of poly(rI)-(rC) (Fig. 2, panel C), nor sequences with no stable hairpin structures (Fig. 2, panel D), prevented complex formation.

To determine whether the double-stranded structure of the RNA is indeed critical in the interaction with the protein, the RNA probe was first heat denatured at 90 °C for 5 min and then either cooled down rapidly to 4 °C, to maintain the denatured structure, or gradually cooled down, to allow the secondary stable structure of the RNA to reform. Fig. 3 shows the results of this study. It is clear that disruption of the secondary structure of the RNA prevented complex formation (Fig. 3, lane 3). In contrast, binding was slightly enhanced if the probe was allowed to renature slowly to its stable structure (Fig. 3, lane 2). The results of these studies strongly suggest that the RNA-binding protein has a preferential affinity toward RNA structures containing double-stranded sequence. Since the hairpin structure of poly(rI)-(rC) did not form a complex with the RNA-binding protein, the protein may have some limited degree of sequence specificity, and that it is not the interferon-induced dsRNA-activated protein kinase (Hovanessian, 1989). Due to the similarity in the RNA-protein interactions with Lp8 and Lp7, both transcripts have been used exchangeably in the studies reported here.

**Effect of Ionic Strength on the RNA-Protein Interaction**—Fig. 4 shows an analysis of the RNA-protein interaction in the presence of various concentrations of KCl. It is evident from this experiment that complex formation was significantly enhanced when the binding reaction was carried out at a KCl concentration of 0.3 M or above. Fig. 4 also shows that at these KCl concentrations, binding was stimulated proportionally with the increase in the protein concentration in the binding reaction (lanes 4–9), whereas at low KCl concentrations, binding was inhibited as the concentration of the extract in the binding assay was raised (lanes 1–3; cf. Fig. 6).

A possible explanation of the results is that the cellular extract contains an inhibitory activity which is dissociable from the RNA-binding protein at high salt concentrations. To test this possibility, a cellular extract was fractionated on...
an anion-exchanger (Q-Sepharose), and the RNA-binding protein was eluted by a stepwise increase in KCl concentration from 0.04 to 0.4 M (see legend of Fig. 5). After chromatography on Sephadex G-25 to adjust the KCl concentration to 0.04 M, RNA-protein complex formation was measured. The activity recovered in the 0.4 M KCl fraction was significantly enhanced relative to that in the original cellular extract (compare lanes 1 and 3), even though complex formation was performed at low ionic strength (0.04 M KCl) (Fig. 5). While the wash-through fraction did not contain an RNA binding activity (lane 2), reconstitution of the wash-through fraction with the proteins eluted at 0.4 M KCl resulted in an inhibition of complex formation (Fig. 5, lanes 4-7). These results show that the cellular extract contained an inhibitory activity which may regulate the activity of the RNA-binding protein. We found that the inhibitory activity was sensitive to moderate heat treatment (56 °C for 10 min) or to repeated freezings and thawings (data not shown), indicating that this activity may be a protein.

Reduced Level of the RNA Binding Activity in cAMP-treated FTO-2B Cells—To determine whether the RNA-binding protein may be involved in the cAMP control of PEPPCK gene expression, we examined the RNA binding activity in cells treated with cAMP. Since most known effects of cAMP are mediated through phosphorylation events, cellular extracts were prepared in the presence of inhibitors of phosphatases, as described under "Experimental Procedures." Due to the profound effect of high salt concentrations on binding activity (Fig. 4), the control of the RNA binding activity was analyzed at low ionic strength conditions (0.04 M KCl). Fig. 6 shows complex formation analysis with extracts from FTO-2B cells incubated with different analogues of cAMP for 12 h. It is evident that cells treated with either CPT-cAMP, Bt2cAMP, or 8-Br-cAMP contained lower levels of the RNA binding activity. Densitometric scanning of the autoradiogram showed that complex formation was inhibited by 70-75%, in average, in extracts from cAMP-treated cells. Time course analysis (Fig. 7) demonstrated that the decay of the RNA binding activity was relatively rapid, declining to basal level within 3 h after exposure of cells to the cyclic nucleotide. The reason why the activity did not decline beyond 25-30% of the initial level is not yet known.

Reduced Level of the RNA Binding Activity in Livers of Starved Rats—The activity of the RNA-binding protein was also measured in liver of starved rats. The metabolic adaptation of the liver to starvation is associated with an elevated level of cAMP (Claus and Pilkis, 1981). Fig. 8 shows that complex formation was inhibited in liver extracts from 72-h starved animals, when the binding assay was carried out at 0.04 M KCl (lanes 1-4). Performing the assay at high ionic strength (lanes 5-8), revealed that extracts from livers of starved rats contained substantial amount of the RNA-binding activity, albeit, at a lower level than that in livers of of
Complex formation was analyzed with Lp7 and cytoplasmic extracts from livers of fed (left panel) or 72-h starved (right panel) rats. In lanes 1-8 the extracts were from rat livers and in lane F from FTO-2B cells. Assays mixtures contained either 0.04 (lanes 1-4) or 0.5 M KC1 (lanes 5-8 and lane F). The following protein concentrations were used: 0.2 (lanes 1 and 5); 0.4 (lanes 2 and 6); 0.6 (lanes 3 and 7); and 0.8 mg/ml (lanes 4, 8, and F).

Phosphatase Treatment Stimulates the RNA Binding Activity—To test whether phosphorylation is involved in the control the RNA binding activity, the effect of phosphatase on complex formation was examined. In this experiment, a cellular extract was incubated first at 37 °C for 30 min. Control samples (lanes 1, 3, 5, and 7) were incubated in the presence of the corresponding volumes of vehicle. Complex formation was carried out with Lp7 at a final KC1 concentration of 0.04 M (lanes 1-5). Lane 9, complex formation was carried out as above with non-phosphatase-treated extract and at a KC1 concentration of 0.5 M (lane 6). Lane 10, complex formation was carried out under these assay conditions. Further- more, UV cross-linked complexes of phosphatase-treated extract (Fig. 10, lanes 4 and 5) demonstrated that only the 100–110-kDa protein complex was significantly enhanced by the phosphatase treatment. Because the changes in the level of the 100–110-kDa protein complex correlated with the results of a gel-shift analysis of the same samples on a native gel, we concluded that the RNA-protein complex characterized in the present study has an apparent molecular mass of 100–110 kDa. Assuming that such a complex migrates on an SDS-polyacrylamide gel as a typical globular protein and considering the maximal size of the RNA probe (30 nucleotides), the estimated size of the RNA-binding protein identified in the present study is about 100 kDa.

DISCUSSION

Because the RNA-binding protein interacted with a variety of RNA probes with no apparent sequence similarities, it is likely that the RNA-binding protein recognizes structural domain(s) within the RNAs. This is strongly supported by the demonstration that complex formation was inhibited if the RNA probe was first heat denatured (Fig. 3). We tested in the course of the present study 10 different RNA probes (not all are shown), seven of which formed complexes with the RNA-binding protein. The most common feature among these short RNAs is their potential to fold into a stem-loop structure. The only exception was the poly(rI)-(rC) (Fig. 2). However, computer modeling suggests that this sequence is likely to form a double-stranded stem structure with a limited size loop. If indeed this is the case, it may suggest that binding is dependent on the presence of a loop and that the secondary structure of the RNA is critical in the recognition of the RNA by the RNA-binding protein. A similar dependence on a loop structure has been previously reported for the Tat protein of HIV-1 in its interaction with TAR, a cis-acting sequence in the virus RNA. The likelihood that TAR functions as a target for RNA-protein interaction was first suggested by the observation that a stable stem-loop structure is formed by intramolecular base pairing (Muesing et al., 1987). Mutations in the stem that altered the primary nucleotide sequence, but maintained its secondary structure, fully supported the func-
tion of Tat. However, alteration of nucleotides in the loop structure as well as in an adjacent bulge, abolished Tat response (Peng and Holland, 1988; Roy et al., 1990).

Complex formation with double-stranded sequences was highly specific and occurred even in the presence of a 10,000-fold molar excess of poly(rA). However, at the present time we cannot rule out the possibility that other sequences with a stem-loop structure are the true target for the RNA-binding protein for exertion of its biological activity. This possibility is exemplified by the HIV-1 trans-activator Rev protein which is highly specific in its ability to bind RNA containing stem-loop structure. Its affinity for the same structure which was composed of the complementary sequence was reduced some 20-fold (Heaphy et al., 1990). Alternatively, binding specificity may be determined by an interaction of the stem-loop structure with other sequences and/or by the presence of other cellular factors. Studies to define the exact binding specificity of the RNA-binding protein are in progress.

Christ et al. (1991) showed complex formation between the complete 3'-UTR (818 nucleotides) of PEPCK mRNA and a cytosolic factor from cultured rat hepatocytes. Since protein binding was noted with the sense and anti-sense sequences, as well as with a transcript from a non-linearized plasmid DNA, these authors concluded that a secondary structure due to RNA folding is responsible for the RNA-protein interaction at the 3'-UTR of PEPCK mRNA. The RNA binding activity detected by Christ et al. (1991) is probably different than the one identified in the present paper because incubation of hepatocytes with glucagon induced a temporary small increase in complex formation, while cAMP down-regulated the binding activity reported here (Figs. 6-8).

The lability (half-life <30 min) of the mRNA transcripts of a variety of proto-oncogenes, transcription factors, and lymphokine genes has been reported to be mediated by the association of several proteins (AU-specific binding proteins, AUBP) with reiterations of the sequence AUUUA located in the 3'-UTR of these mRNAs (Shaw and Kamen, 1986; Caput et al., 1986). Stephens et al. (1992) showed an increase in the activity of AUBP in 3T3-L1 preadipocytes treated with 8-bromo-cAMP. These investigators suggested that the interaction of AUBP with its AU-rich recognition sequence may be involved in the cAMP-control of GLUT1 mRNA stability. Even though PEPCK mRNA is short lived (t1/2 ~ 30 min) and its half-life is regulated by cAMP (Hod and Hanson, 1988), the mRNA contains only a single AUUUA motif (Beale et al., 1985). Because several copies of the AU-rich motif are required to mediate the interaction with AUBP (Bohjanen et al., 1991; Gillis and Malter, 1991; Vakalopoulou et al., 1991), it is unlikely that the single motif within PEPCK mRNA plays a regulatory role. This is supported by our finding that an RNA probe containing the PEPCK AUUUA sequence did not form a complex with a cellular extract from PTO-2B cells (not shown). It suggests that other RNA-protein interactions are important in the control of PEPCK mRNA turnover.

The interaction between protein factors and RNAs which fold into a stem-loop structure has been previously reported. This includes the interaction of the iron-response element-binding protein with its cognate elements in transferrin-receptor and ferritin mRNAs (Klausner et al., 1993), or the interaction of nuclear and polyribosomal proteins with a stem-loop at the 3' end of histone mRNA (Pandey et al., 1991). This hairpin structure has been shown to be responsible for the cell-cycle-mediated regulation of the half-life of histone mRNA (Pandey and Marzluff, 1987; Graves et al., 1987).

The studies reported here are consistent with a model in which the RNA-binding protein consists of a binding subunit and a regulatory component. We propose that the RNA-binding subunit has the molecular mass of about 100 kDa and that the association of the regulatory component with the binding subunit inhibits RNA-protein complex formation. We further suggest that the interaction between the binding subunit and the regulatory component is regulated by phosphorylation. Dephosphorylation of one or more sites results in the dissociation of the regulatory component and stimulation of the RNA-binding activity. In turn, phosphorylation of the RNA-binding protein by a cAMP-dependent protein-kinase inactivates the RNA-protein interaction, perhaps by inducing the association between the regulatory component and the binding subunit.

The functional consequences of the interaction between the RNA-binding protein and PEPCK mRNA sequences identified in the present study, are yet to be established. However, because of its affinity to double-stranded regions, an attractive hypothesis is that the RNA-binding protein has an RNA unwinding activity. Several structure-specific binding proteins which are RNA helicases have been described (Schmid and Linder, 1992). This family of proteins is best characterized by the translation initiation factor, eIF-4A. This factor, which binds to the 5' end of mRNA in conjunction with the eIF-4F complex, is involved in an ATP-dependent unwinding of secondary structure of the mRNA to allow the preinitiation complex to bind and scan for the initiator codon (Thach, 1992). Another putative member of this family of RNA helicases (Koonin, 1992) is the yeast protein UPF1, which was found to be an activator of mRNA turnover (Leeds et al., 1992). Our observation that the activity of the RNA-binding protein is reduced in liver cells treated with cAMP, condition which also stabilizes PEPCK mRNA against degradation (Hod and Hanson, 1988), is consistent with the possibility that the RNA-binding protein plays a destabilizing role by unwinding double-stranded sequences within the mRNA. Purification of the cAMP-dependent PEPCK mRNA-binding protein should assist in defining its functional role and mode of action.

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