Protein Kinase A Stimulates Binding of Multiple Proteins to a U-Rich Domain in the 3’-Untranslated Region of Lactate Dehydrogenase A mRNA That Is Required for the Regulation of mRNA Stability

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We have explored the molecular basis of the cAMP-induced stabilization of lactate dehydrogenase A (LDH-A) mRNA and identified four cytoplasmic proteins of 96, 67, 52, and 50 kDa that specifically bind to a 30-nucleotide uridine-rich sequence in the LDH 3’-untranslated region with a predicted stem-loop structure. Mutational analysis revealed that specific protein binding is dependent upon an intact primary nucleotide sequence in the loop as well as integrity of the adjoining double-stranded stem structure, thus indicating a high degree of primary and secondary structure specificity. The critical stem-loop region is located between nucleotides 1473 and 1502 relative to the mRNA cap site and contains a previously identified cAMP-stabilizing region (CSR) required for LDH-A mRNA stability regulation by the protein kinase A pathway. The 3’-untranslated region binding activity of the proteins is up-regulated after protein kinase A activation, whereas protein dephosphorylation is associated with a loss of binding activity. These results imply a cause and effect relationship between LDH-A mRNA stabilization and CSR-phosphoprotein binding activity. We propose that the U-rich CSR is a recognition signal for CSR-binding proteins and for an mRNA processing pathway that specifically stabilizes LDH mRNA in response to activation of the protein kinase A signal transduction pathway.

There is increasing evidence demonstrating that the rate of mRNA turnover and its regulation by effector agents play significant roles in controlling mRNA steady-state levels (1, 2). We have previously demonstrated, for example, that not only does the relatively high steady-state level of LDH-A mRNA after β-adrenergic agonist or phorbol ester stimulation of rat C6 glioma cells reflect an increased transcriptional rate (3, 4) but that cells also have the ability to enhance the intracellular mRNA level by a mechanism that regulates the rate of degradation and half-life of mRNA through activation of protein kinases A and C (5). Selective mRNA stabilization in eukaryotic cells by stimulants of second messenger pathways, al-

though not as widely and thoroughly studied as transcriptional control, is a regulated property that can determine the level of expression of a gene product. It is known that individual mRNAs within eukaryotic cells display a wide range of stabilities, with half-lives ranging from a few minutes for highly regulated gene products, such as oncogenes, to over 24 h for very stable species, such as β-globin mRNA (1, 6–8).

Lactate dehydrogenase A subunit mRNA is characterized by a relatively short half-life of about 45–55 min (4, 5). Agents that activate protein kinases A or C cause a 9-fold or 4-fold increase, respectively, in the half-life of LDH-A mRNA (4, 5). Recent analysis of the factors regulating stability of LDH-A mRNA has led to the recognition of a cAMP-stabilizing region (CSR), the presence of which in the 3’-UTR is absolutely necessary for cAMP regulation of LDH mRNA stability to occur (9). In the present report, we describe the extension of our studies toward elucidation of the molecular determinants of protein kinase A-regulated mRNA stability with focus on putative regulatory RNA-binding proteins that interact with the CSR. We identified at least four cytoplasmic proteins of about 96, 67, 52, and 50 kDa that show selective binding affinity for the CSR. Their CSR binding activity is significantly enhanced in rat C6 glioma cells stimulated with activators of protein kinase A and after phosphatase inhibition and functionally correlates with increased LDH-A mRNA stabilization.

EXPERIMENTAL PROCEDURES

Materials—Materials used, synthesis of oligonucleotides, and cell culture methodology were previously described (9).

Synthesis of Uniformly Labeled Truncated LDH-A 3’-UTR Transcripts—DNA oligonucleotides were annealed, ligated into pGEM-3Zf(+) and used as templates for in vitro transcription of 32P-labeled RNA probes according to standard procedures (10). Synthetic RNA oligonucleotides were purchased from OligoTherapeutics (Wilsonville, OR). All 32P-labeled RNA probes were purified by denaturing polyacrylamide gel electrophoresis. Shorter RNA oligonucleotide fragments were synthesized in vitro using a T7 RNA polymerase kit (Promega). Oligonucleotide probes were 5’-end-labeled using [γ-32P]ATP and T4 polynucleotide kinase.

Preparation of Protein Extracts for RNA-Protein Binding Assay—Cells were collected, and the ribosome salt wash (RSW) was prepared as described by Ross (11). Proteinase inhibitors phenylmethylsulfonyl fluoride (0.1 mM), benzamidine (20 mM), leupeptin (50 mM), and pepstatin (1 μM) were added to all cell extracts. The extracts were stored in small aliquots at −80 °C. The RSW fraction contained 95% of the RNA-protein binding activity and was used for all further RNA-protein binding studies.

Gel Mobility Shift Assays—Cell extracts (5–10 μg of RSW protein) were incubated in RNA binding buffer (10 mM Hepes, 40 mM KCl, 5 mM MgCl2, 1 mM dithiothreitol, 0.5 mM EDTA, 5% glycerol, 1 μg/ml heparin, and 0.5 μg/ml yeast RNA) with 32P-labeled RNA probe (50,000 cpm) on ice for 1 h in a total volume of 25 μl. In the competition experiments, competitor RNA was added simultaneously with the 32P-labeled RNA probe. Electrophoresis was carried out on 6% nondenaturing polyacrylamide gels (molar ratio of acrylamide:bisacrylamide, 60:1) in 45 mM
Glioma cells with the protein kinase A catalytic subunit expression ultraviolet products, Inc.). After irradiation, RNase A (0.2
were placed on ice and exposed to 600 mJ of ultraviolet light (5-cm
Gels were dried and exposed to Hyperfilm-MP (Amersham Pharmacia
used in RNA-protein binding studies.

exposed to film for 1–2 days.

2-min washes with binding buffer. The membrane was air-dried and
binding buffer for 1 h. Unbound RNA probe was removed using three

Carried out as described by us (5).

32P-labeled 3

50% methanol, followed by 2% 10% acetic acid, and for 1% 5%
Tris, 45 mM boric acid, and 1.2 mM EDTA buffer, pH 7.4, for
4 h at 4 °C.

Experiments attempting to identify the region of LDH-A 3'-UTR
able to binding cellular factor(s), we prepared truncated

Identification of LDH-A 3'-UTR-Protein Complexes—In experiments attempting to identify the region of LDH-A 3'-UTR capable of binding cellular factor(s), we prepared truncated

RESULTS

Identification of LDH-A 3'-UTR-Protein Complexes—In experiments attempting to identify the region of LDH-A 3'-UTR capable of binding cellular factor(s), we prepared truncated

We were unable to identify binding of protein to fragment Δ1.

To identify the protein binding region(s) of Δ2 more precisely,
we prepared three slightly overlapping truncated fragments of

2, namely Δ3, Δ4, and Δ5, (see Fig. 1) and tested them for
protein binding. Of the three fragments, only fragment Δ4,
consisting of bases 1453–1527, gave a positive shift, the intensity
of which was more pronounced in extracts from Sp-cAMPS-
treated cells (Fig. 2, Δ4, compare lanes C and CA). Screening of
further truncated fragments derived from Δ4 (fragments Δ6, Δ7, and Δ8) produced two shifted RNA-protein complexes that localized binding of protein to fragment Δ7; that is, to the 30-base region consisting of bases 1472–1501. It is important to note that the band density of the Δ7 complex with proteins from Sp-cAMPS-stimulated glioma cells was markedly increased. A concentration of 5 × 10
was needed to elicit maximum stimulatory response as measured by band density. When binding of protein from Sp-cAMPS-stimulated cells to Δ7 was assayed as a function of increasing protein concentration, a linear increase of band density was observed (not shown).

Binding Specificity of Cytoplasmic Protein Factors for the Δ7 Fragment—Competition experiments were carried out to define the specificity of RNA-protein complex formation. We allowed complexes to form in the presence of RNA probe and unlabeled competitor RNA. Under these conditions, Δ7-protein complex formation was competitively eliminated by increasing molar concentrations of unlabeled fragment Δ7 but not by the nonspecific Δ8 fragment (see Fig. 3A), indicating the specificity of Δ7-protein complex formation. We used an additional specificity test by adding increasing concentrations of four different ribonucleotides to the binding reaction. Fig. 3B shows that poly(U) but not poly(A), poly(G), or poly(C) competitively inhibited the formation of the Δ7-protein complex. The ability of polynucleotides to compete suggests that one of the features recognized by the protein binding activity is the presence of abundant uridine residues and that complex formation is at least partially dependent upon the presence of uridine bases. Indeed, the Δ7 fragment contains a relatively high percentage of uridine (about 50%, as compared with 28% of the entire LDH 3'-UTR).

Identification of LDH-A 3'-UTR-binding Proteins by Northwestern Protein Blotting—Whereas the above studies allowed us to identify the presence of 3'-UTR-binding protein(s) in rat C6 glioma cells and to define the binding region, more detailed characterization and determination of the number of putative protein factors usually requires the application of difficult and extensive protein purification procedures. A further disadvantage may be the inadvertent selection of only one particular protein form and not of others or even proteolytically modified proteins. In consideration of these potential problems, we have applied Northwestern blotting to identify glioma cell proteins that selectively bind to the 30-nucleotide Δ7 fragment. Cytoplasmic proteins from Sp-cAMPS-stimulated glioma cells were separated by SDS-polyacrylamide gel electrophoresis, blotted onto membranes, and probed with a 32P-labeled Δ7 fragment. Despite the great number of cytoplasmic proteins present in the gel seen after Coomassie Brilliant Blue staining to verify equal loading of protein and then dried. Gels were exposed to film for autoradiography or to a phosphorimager plate (Fuji imaging plate, type BAS-III) for quantitation.

UV RNA-Protein Cross-linking—RNA-protein binding reactions were carried out in 96-well tissue culture dishes. The binding conditions were identical to those described for the gel mobility shift. Samples were placed on ice and exposed to 600 mJ of ultraviolet light (5-cm distance for 15 min using a 254 nm ultraviolet lamp, model R-260; Ultraviolet Products, Inc.). After irradiation, RNase A (0.2 μg/μl) and RNase T1 (40 units/μl) were added, and samples were incubated at 37 °C for 15 min. The irradiated samples were adjusted to 62.5 mM Tris, pH 6.8, 2% SDS, 5% β-mercaptoethanol and heated to 90 °C for 2 min. An aliquot was subjected to electrophoresis on an 8% SDS-polyacrylamide gel along with prestained protein molecular size markers (rainbow marker, United States Biochemicals). The gels were fixed for 2 h in 50% methanol, followed by 2 h in 10% acetic acid, and for 1 h in 5% methanol/7% acetic acid, after which the gels were dried. In competition experiments, protein bands were visualized with Coomassie Brilliant Blue staining to verify equal loading of protein and then dried. Gels were exposed to film for autoradiography or to a phosphorimager plate for quantitation as described above.

Northwestern Protein Blotting Assay—Twenty micrograms of protein extract were subjected to electrophoresis on 0.1% SDS-10% polyacrylamide gels. Electrophoresis was carried out for about 5 h at a constant current of 100 mA. Proteins were subsequently blotted onto nitrocellulose membranes for 2 h at 1 mA using a Hoefer electrophoresis apparatus. To repress the proteins, nitrocellulose membranes were washed three times for 30 min at room temperature in binding buffer (10 mM Tris, pH 7.5, 1 mM EDTA, 50 mM NaCl, 0.02% bovine serum albumin, 0.02% Ficoll 400, and 0.02% polyvinylpyrrolidone). The blotted proteins were then probed with 32P-labeled RNA (104 cpm/ml) in binding buffer for 1 h. Unbound RNA probe was removed using three 2-min washes with binding buffer. The membrane was air-dried and exposed to film for 1–2 days.

Protein Kinase A Catalytic Subunit Inhibition—Stable transfection of glioma cells with the protein kinase A catalytic subunit expression vector pM7-CaEVneo (12) and expression of catalytic subunit were carried out as described by us (5).

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was needed to elicit maximum stimulatory response as measured by band density. When binding of protein from Sp-cAMPS-stimulated cells to Δ7 was assayed as a function of increasing protein concentration, a linear increase of band density was observed (not shown).

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these data, the 96-, 70-, 67-, 61-, 52-, and 50-kDa proteins can be identified as possessing high specific binding affinity for the Δ7 fragment. The 42- and 44-kDa bands are competed only at a relatively high concentration (100-fold) of Δ7.

**UV-mediated Δ7-Protein Cross-linking Analysis**—RNA-protein complexes can be formed by irradiation of cells or cell extracts with UV light (13, 14). UV-mediated RNA-protein complex formation was carried out as an experimental approach complementary to Northwestern blotting to ascertain the specificity of interaction of the proteins with the Δ7 fragment. UV irradiation of proteins in the RSW from unstimulated and Sp-cAMPS-stimulated glioma cells with different 32P-labeled LDH 3'-UTR probes resulted in a covalent linkage of Δ4 and Δ7 with several cytoplasmic proteins of apparent molecular masses of 96, 67, 52, and 50 kDa (Fig. 5). When 3'-UTR fragments Δ3, Δ5, Δ6, and Δ8 were used as probes, no labeled RNA-protein complexes were detected. Complex formation of Δ4 and Δ7 with proteins from Sp-cAMPS-stimulated glioma cells was significantly more prominent than with proteins from unstimulated cells. We were unable by this method to identify the 70-, 61-, 44-, and 42-kDa bands seen by Northwestern blot analysis (see Fig. 4).

**Competition Reveals Specificity of Δ7-Protein Interactions**—We next addressed the question of RNA-protein binding specificity by analyzing the ability of homologous and nonhomologous RNAs to compete with 32P-labeled Δ7 for protein binding in UV cross-linking assays. Radiolabeled Δ7 fragment was mixed with increasing molar amounts of unlabeled competitor, combined with protein extract from Sp-cAMPS-stimulated glioma cells, irradiated, and analyzed as described above. The results are shown in Fig. 6. Addition of a 5–50-fold molar excess of competitors Δ5 and Δ6 had no effect on Δ7-protein binding, and the appearance of the 96, 67-, 52-, and 50-kDa protein
bands was not affected. In contrast, addition of as little as a 5-fold molar excess of fragments Δ4 and Δ7 reduced band density and a 50-fold molar excess efficiently eliminated all four protein bands. These results indicate that labeling of the four protein bands is the result of formation of specific complexes between proteins in the RSW and the 30-base Δ7 fragment.

The Secondary Structure of 3′-UTR Is Critical for Protein Binding — To examine the putative role of the secondary structure on Δ7-protein binding, we used two different experimental approaches. First, the Δ7 probe was heat-denatured at 95 °C for 10 min and either rapidly cooled in ice to retain a denatured structure or slowly cooled to room temperature (over a 40-min period) to allow reformation of the stable secondary conformation. If secondary structure of the 3′-UTR is vital for Δ7-protein complex formation, its disruption should abolish binding of proteins. UV cross-linking analysis shows that this was indeed the case (Fig. 7). Whereas no heating (lane 3) or heating followed by slow cooling (lane 2) allowed binding of proteins to Δ7, rapid cooling of the Δ7 probe in ice prevented protein binding to the denatured probe (lane 1).

In the second experimental approach, in which we also aimed to identify the base sequence of the binding site in more detail, Δ7 was modified by mutation in two different ways. First, we mutated bases 1486–1492 within the single-stranded loop structure of Δ7, resulting in mutation m1. The second mutation of Δ7 (m2) altered bases 1479–1483, which participate in forming the double-stranded stem of wild-type Δ7 (see predicted secondary structure in Fig. 13). Whereas mutation of bases 1486–1492 in m1 has no effect on secondary structure, mutation of bases 1479–1483 in m2 destroys the double-stranded stem and leads to a change of secondary structure. We then tested wild-type Δ7 and mutated constructs for protein binding. Fig. 8 shows that whereas wild-type Δ7 produced a positive gel shift, mutated fragment m1, as well as fragment m2, failed to form shifted RNA-protein complexes. This data indicates that Δ7-protein complex formation not only requires a correct primary single-stranded base sequence within the loop but also critically depends on an intact double-stranded stem-loop structure of Δ7. Mutational changes of other bases (for instance, bases 1498–1502) had no effect on Δ7-protein complex formation (not shown). The functional significance of these data becomes evident when considered together with our previous findings that similar mutational changes in either the primary sequence of the single-stranded loop or in the double-stranded stem of Δ7, e.g. conditions that abrogate Δ7-protein complex formation, abolish the effect of cAMP on mRNA stabilization (9). This strongly suggests a functional relationship between RNA-protein binding and mRNA stability.

Mechanism Underlying Up-regulation of Δ7-Protein Binding Activity — In view of the above data suggesting the involvement of protein kinase A and thus protein phosphorylation in LDH-A mRNA stabilization, we investigated the effect of modulators of protein kinase A in more detail. Activation of protein kinase A with either forskolin (an activator of adenylyl cyclase (15)) or Sp-cAMPS clearly led to a marked increase of binding activity (Fig. 9, lanes 3 and 4) as analyzed by electrophoretic mobility shift assay. Furthermore, exposure of glioma cells to okadaic acid (20 nM) mimicked the effects of protein kinase A activators and resulted in a shifted Δ7-protein complex of similar density as the complexes obtained with extracts from Sp-cAMPS and forskolin-stimulated cells (Fig. 9, lane 5). Okadaic acid is one of the tumor promoters that does not activate protein kinase C (16). Rather, it is a powerful inhibitor of protein phosphatases-1 and -2A in vitro as well as in vivo (17). Treatment of Sp-cAMPS-stimulated glioma cell extracts with calf intestinal phosphatase markedly reduced Δ7-protein complex formation.
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**Fig. 8.** Electrophoretic band-shift analysis of \(^{32}P\)-labeled fragment \(\Delta 7\) and mutated \(\Delta 7\) fragments (m1 and m2) with RSW protein from Sp-cAMPS-stimulated cells. The base sequence of the fragments is listed, and mutated bases are underlined. Gel shift was carried out without protein (−) and with 5 µg of protein (\(\Delta 7, m1,\) and \(m2\)). Two open arrows indicate RNA-protein complexes of higher electrophoretic mobility, which appear only inconsistently in repetitive experiments. Two filled arrows indicate consistently appearing complexes.

**Fig. 9.** Effect of modulators of protein phosphorylation on RNA gel shift. Electrophoretic band-shift analysis was carried out using \(^{32}P\)-labeled \(\Delta 7\)-RNA as probe with RSW protein prepared from untreated glioma cells (lane 2) and cells treated with Sp-cAMPS (lane 3), forskolin (lane 4), okadaic acid (lane 5), and calf intestinal phosphatase (lane 6). Lane 1 contains RNA probe without protein. For experimental details, see under “Experimental Procedures.”

These results strongly suggest that regulation of RNA-protein binding activity occurs through reversible phosphorylatable modification of binding protein(s) involving protein kinase A in combination with okadaic acid-sensitive protein phosphatase.

The effect of modulators of protein kinase A activity on \(\Delta 7\)-protein binding was additionally analyzed by UV-mediated cross-linking analysis. The results are shown in Fig. 10. Treatment of glioma cells with Sp-cAMPS, forskolin, or okadaic acid led to a marked increase of binding of the 96-, 67-, 52-, and 50-kDa proteins to the \(\Delta 7\) fragment (Fig. 10, lanes 2–5). Treatment of cell extracts from Sp-cAMPS-stimulated cells with calf intestinal phosphatase significantly reduced the affinity of all four binding proteins to \(\Delta 7\) (lane 6).

**Fig. 10.** UV-catalyzed RNA-protein cross-linking assay. UV cross-linking assays of \(\Delta 7\)-protein binding in RSW protein extracts prepared from untreated glioma cells (lane 2) and cells treated with Sp-cAMPS (lane 3), forskolin (lane 4), okadaic acid (lane 5), and calf intestinal phosphatase (lane 6). Lane 1 contains RNA probe without protein.

Overexpression of the Catalytic Subunit of Protein Kinase A Leads to Up-regulation of \(\Delta 7\)-Protein Binding—In order to investigate the contribution of the catalytic subunit of protein kinase A on \(\Delta 7\)-protein binding, the functional effect of overexpression of the catalytic subunit of protein kinase A was analyzed by electrophoretic gel mobility shift and UV cross-linking. We used an expression vector, pMT-CoEVneo (12), designed to express the catalytic subunit of protein kinase A directed by the metallothionein promoter, the activity of which can be induced with metal ions (18). Our previous studies have shown that following Zn\(^{2+}\) treatment of a rat C6 glioma cell line stably transfected with pMT-CoEVneo, catalytic subunit activity levels increased about 7–8-fold as compared with untreated cells (5). Evaluation of the \(\Delta 7\)-protein binding activity under these experimental conditions showed a marked increase of shifted \(\Delta 7\)-protein complex (see Fig. 11; compare lanes 4 and 5) with protein from transfected and Zn\(^{2+}\)-induced cells. Protein from untransfected control cells showed little or no complex formation and shift with \(\Delta 7\) under these conditions (Fig. 11, lanes 2 and 3).

The effect of overexpression of the catalytic subunit on the binding of the \(\Delta 7\) fragment to protein was also evaluated by UV cross-linking assay. As shown in Fig. 12, binding activity of \(\Delta 7\) to the 96-, 67-, 52-, and 50-kDa proteins was markedly increased (lane 5) in pMT-CoEVneo-transfected and Zn\(^{2+}\)-treated cells. Little or no binding of the proteins was observed in nontransfected cells (lanes 2 and 3) and in transfected cells that had not been treated with Zn\(^{2+}\) (lane 4).

**DISCUSSION**

In the present study, we have continued our investigation into mechanisms that are involved with and responsible for the regulation of LDH-A mRNA stability by the protein kinase A pathway. In our previous report (9) we have identified a CSR in the 3′-UTR of LDH-A mRNA that is responsible for the stability regulation of LDH-A mRNA by the protein kinase A pathway. Here, we provide information concerning the presence, molecular size, and cAMP regulation of proteins that exhibit marked binding affinity for the CSR. The evidence was obtained by three independent experimental methods, i.e., electrophoretic gel shift analysis, UV-mediated cross-linking of RNA and protein, and Northwestern protein blotting assay. Specificity of the CSR-protein associations was demonstrated by competition assays using homologous and nonhomologous RNAs. Furthermore, competition experiments with different ribohomopolymers revealed that the four cytoplasmic CSR-binding proteins (CSR-binding proteins 1–4) require a uridine-rich binding region and depend upon a specific primary nucleotide sequence, as well as an unaltered secondary structure of CSR, to display selective binding activity.

A key finding and novel feature of our data is the dependence
of active CSR-protein binding upon phosphorylatory modification of the CSR-binding proteins by protein kinase A and that their binding functionally correlates with LDH-A mRNA stabilization. Based on the experimental evidence, we propose that four CSR-binding proteins are the target of protein kinase A and that their interaction with the CSR plays a major role in regulating intracellular LDH-A mRNA levels via the protein kinase A signal pathway. In a previous paper (9) we have shown that the interaction of either adenosine-uridine binding factor (AUBF) or SG-uridine-rich binding protein (SG-URBP), with their AU-rich recognition sequences, may be involved in the regulation of RNA-protein binding a concurrent stabilization of mRNA occurs. There are several examples demonstrating that mRNA-protein binding affinity varies inversely with a stabilizing effect on mRNA. For instance, Tholakanunnel et al. (29) reported the β-adrenergic agonist-mediated induction of the M, 35,000 β-adrenergic receptor mRNA-binding protein, which binds to β2-adrenergic receptor mRNA, concurrently undergoing agonist-mediated destabilization. Likewise, adrenergic stimulation up-regulates binding of AUF1 to β-adrenergic receptor mRNA but simultaneously destabilizes the receptor mRNA. Whereas the above cited data are examples of up-regulation of mRNA-protein binding by cAMP (26), it is also of interest that Nachaliel et al. (30) reported the identification of a 100-kDa protein, the affinity of which changes with the state of activity of protein kinase A.

An increasing number of proteins, other than RNases, are being identified that are believed to act as trans-regulatory factors. These factors, in concert with cis-elements in the mRNA sequence, may be key proteins involved in the regulation of mRNA stability (for reviews, see Refs. 2 and 7). For instance, the binding affinity of a family of proteins that bind to AU- and U-rich sequences can be modulated through reversible phosphorylation by protein kinases other than protein kinase A (19, 20). In those instances in which mRNA stability is regulated by cAMP (4, 5, 21–26), the binding of several proteins to AU-rich regions is up-regulated by cAMP (22, 23, 26–28). Both Stephens et al. (27) and Peng and Lever (28) have suggested that the interaction of either adenosine-uridine binding factor (AUBF) or SG-uridine-rich binding protein (SG-URBP), with their AU-rich recognition sequences, may be involved in the regulation of RNA-protein binding a concurrent stabilization of mRNA occurs. There are several examples demonstrating that mRNA-protein binding affinity varies inversely with a stabilizing effect on mRNA. For instance, Tholakanunnel et al. (29) reported the β-adrenergic agonist-mediated induction of the M, 35,000 β-adrenergic receptor mRNA-binding protein, which binds to β2-adrenergic receptor mRNA, concurrently undergoing agonist-mediated destabilization. Likewise, adrenergic stimulation up-regulates binding of AUF1 to β-adrenergic receptor mRNA but simultaneously destabilizes the receptor mRNA. Whereas the above cited data are examples of up-regulation of RNA-protein binding by cAMP (26), it is also of interest that Nachaliel et al. (30) reported the identification of a 100-kDa protein, the affinity of which changes with the state of activity of protein kinase A.

All of these data point to the complexities of establishing a unified mechanism of action of the protein kinase A signal transduction pathway in regulating mRNA stability. Considering the similarities of the response of the LDH-A, PEPCK, renin, α2A adrenergic receptor, SGLT1, and GLUT1 mRNAs to cAMP, namely mRNA stabilization (4, 5, 21, 23, 24, 27, 31), some common features in the mechanism of mRNA stabiliza-
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tion and RNA-protein binding might be expected. However, despite some overall structural similarities (e.g., presence of U-rich regions), there appear to be no strict homologies between 3′-UTR sequences that are major determinants of mRNA stability (32, 33). Comparison of 3′-UTR nucleotide sequences that are implicated in cAMP-mediated protein binding shows little sequence similarity other than the presence of seemingly randomly distributed tracts of polypyrimidine and AUUU or AUUUU motifs (34), which do not seem sufficient to destabilize mRNA or regulate stability by trans-factors. However, comparison of the 3′-UTR of rat PEPCK mRNA, a messenger RNA that is stabilized by cAMP (31), with LDH-A 3′-UTR reveals a 15-base PEPCK 3′-UTR fragment (bases 1119–1135) (34), which shows a high degree of base sequence similarity with LDH-A CSR, as follows: LDH CSR, 5′-CUGUAUAUUGUGU-3′; PEPCK, 5′-CUGUAU-AUAUGUGU-3′.

In preliminary studies, we have tested the PEPCK fragment for protein binding using electrophoretic mobility shift assay with glioma cell protein extract. The autoradiographs showed two shifted bands that were specifically competed with the PEPCK fragment and LDH CSR (data not shown). However, because so far few data are available concerning the role of PEPCK 3′-UTR base sequences on stability of its mRNA other than the report by Nachaal et al. (30), the functional role, if any, of the 15-base PEPCK 3′-UTR in PEPCK mRNA stabilization remains to be elucidated.

Our own studies have provided several lines of evidence indicating that the CSR-binding proteins possess specific affinity for the primary U-rich CSR sequence and that binding affinity is sensitive to changes in 3′-UTR secondary structure. First, CSR-protein complex formation is protein concentration-dependent and is prevented by excess specific but not by non-specific competitor RNA. Second, mutation of primary structure (see Figs. 8 and 13) and elimination of the secondary structure by appropriate mutational changes of the CSR nucleotide sequence (at nucleotides 1478–1492) and by heat denaturation completely abolished binding of proteins. Fig. 13 shows a computer-generated predicted secondary structure of a LDH-A 3′-UTR fragment (nucleotides 1450–1523) containing the CSR. The position of the CSR was predicted to be within a 12-nucleotide single-stranded loop of a larger stem-loop structure. Mutations of the single-stranded primary sequence in the loop (nucleotides 1486–1492), which should not alter RNA secondary structure, resulted in decreased protein binding (see Fig. 8). Furthermore, when secondary structure was destroyed by heat denaturation or mutation of bases 1479–1483 in the double-stranded stem structure, protein binding was severely impaired. This finding may have significant functional consequences because we have shown that identical base mutations to the CSR, as well as a functional CSR, depends on stringent nucleotide sequence requirements in the loop, as well as proper conformation of the stem-loop structure.

The LDH-A CSR primary base sequence may not per se be a general consensus motif for cAMP regulation of mRNA stability, but it is conceivable that the overall secondary/tertiary structure allowed by an element such as CSR, together with specific binding proteins, possesses properties that are critical in determining mRNA stability. For instance, sequences of differing primary nucleotide structure in cAMP-stabilized mRNAs may allow the formation of similar or identical conformations that have the ability to recognize and specifically interact with trans-regulatory phosphoproteins. However, until the various 3′-UTR-binding proteins are identified and characterized, their specific functional roles in mRNA stabilization and the significance of their phosphorlyative modification by protein kinase A remain to be elucidated.

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