A Hairpin-like Structure within an AU-rich mRNA-destabilizing Element Regulates trans-Factor Binding Selectivity and mRNA Decay Kinetics*

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In mammals, rapid mRNA turnover directed by AU-rich elements (AREs) is mediated by selective association of cellular ARE-binding proteins. These trans-acting factors display overlapping RNA substrate specificities and may act to either stabilize or destabilize targeted transcripts; however, the mechanistic features of AREs that promote preferential binding of one trans-factor over another are not well understood. Here, we describe a hairpin-like structure adopted by the ARE from tumor necrosis factor α (TNFα) mRNA that modulates its affinity for selected ARE-binding proteins. In particular, association of the mRNA-destabilizing factor p37AUF1 was strongly inhibited by adoption of the higher order ARE structure, whereas binding of the inducible heat shock protein Hsp70 was less severely compromised. By contrast, association of the mRNA-stabilizing protein HuR was only marginally affected by changes in ARE folding. Consistent with the inverse relationship between p37AUF1 binding affinity and the stability of ARE folding, mutations that stabilized the ARE hairpin also inhibited its ability to direct rapid mRNA turnover in transfected cells. Finally, phylogenetic analyses and structural modeling indicate that TNFα mRNA sequences flanking the ARE are highly conserved and may stabilize the hairpin fold in vivo. Taken together, these data suggest that local higher order structures involving AREs may function as potent regulators of mRNA turnover in mammalian cells by modulating trans-factor binding selectivity.

The rate of mRNA turnover is highly variable among the cytoplasmic mRNA population and thus plays a significant role in regulating the steady-state concentrations of individual mRNA species available to program protein synthesis. In mammalian cells, different transcripts exhibit a range of decay kinetics spanning over 2 orders of magnitude, largely due to the presence of discrete cis-acting elements contained within each mRNA (1, 2). Most mRNAs encoding cytokines, inflammatory mediators, and proto-oncogenes are inherently unstable, often exhibiting cytoplasmic half-lives of 1 h or less. Rapid turnover of these transcripts is principally due to the activity of AU-rich elements (AREs), a broad family of mRNA-destabilizing sequences localized to the 3′-untranslated regions (3′-UTRs) of many labile mRNAs (3). The intrinsic lability of ARE-containing mRNAs enables their cytoplasmic concentrations to be rapidly modulated following acute changes in their synthetic rates (4, 5). Additionally, modulation of ARE-directed mRNA decay pathways by selected signal transduction systems can regulate the cytoplasmic levels of some mRNAs independent of, or in concert with, changes in the synthetic rate (6–8).

The ability of AREs to direct mRNA turnover is mediated by the activity of selected ARE-binding proteins (9–11). To date, over 25 such factors have been identified, although the regulatory significance of most remains unknown. Some proteins, including AUF1 (12–14), tristetraproline (TTP) (15, 16), and KSRP (17, 18), appear to promote ARE-directed mRNA turnover, whereas others, including members of the Hu family of RNA-binding proteins, function to prevent the degradation of some ARE-containing transcripts (14, 19, 20). Recent studies, however, have indicated that different AREs or subdomains thereof may be targeted by distinct populations of ARE-binding proteins. For example, whereas Hu proteins bind relatively promiscuously to RNA substrates in vitro, with little selectivity beyond a general preference for sequences rich in uridylate residues (21–23), some AU-rich sequences functioning as potent mRNA destabilizers are insensitive to HuR activity (24, 25). By contrast, TTP binding to RNA is highly discriminatory, requiring an AUUUA or AUUUUA motif within a U-rich tract for high affinity interactions (26, 27).

The mRNA-destabilizing factor AU1 exists as a family of four protein isoforms generated by alternative pre-mRNA splicing and are named according to their apparent molecular weights (28). The p45AUF1 and p42AUF1 isoforms are principally nuclear, whereas p40AUF1 and p37AUF1 are generally present in both nuclei and cytoplasm (29–31). To date, the p40AUF1 and p37AUF1 isoforms have been most closely associated with the regulation of ARE-directed mRNA turnover (12–14, 31). In particular, extensive studies have documented p37AUF1 interactions with a broad spectrum of AREs and related RNA sequences (12, 32–36); however, the specific features of RNA substrates that discriminate high from low affinity AU1 targets remain unclear. p37AUF1 binds U-rich RNA substrates by sequential association of protein dimers (37) and induces local changes in RNA conformation upon binding (38). However,

The abbreviations used are: ARE, AU-rich element; Cy3, cyanine-3; Dox, doxycycline; Fl, fluorescein; FRET, fluorescence resonance energy transfer; GST, glutathione S-transferase; TNFα, tumor necrosis factor α; TTP, tristetraproline; UTR, untranslated region.

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p37AUF1 binding to the ARE from tumor necrosis factor α (TNFα) mRNA is inhibited by the presence of Mg2+ in vitro, concomitant with the adoption of a condensed but undefined RNA structure (33, 38). Both inhibition of AUF1 binding and RNA structural condensation resulting from Mg2+ treatment were specific for the ARE sequence, since the cation did not significantly alter the affinity of p37AUF1 binding to polyuridylic substrates, nor did Mg2+ induce substantial conformational changes in these RNA ligands (33).

Together, these data suggested that one or more higher order RNA structures involving the TNFα ARE could inhibit association of AUF1 and, by extension, that local higher order RNA structures involving AREs may serve as significant determinants directing their binding preferences for different trans-acting factors. In this study, we have used structural modeling, nuclease mapping, and fluorescence-based approaches to define a candidate folded structure for the TNFα ARE in solution. Quantitative assessment of the thermodynamic consequences of selected site-directed mutations within the TNFα ARE further supports the proposed folded model. Stabilization of the folded ARE structure potently inhibited binding of p37AUF1 to this element in vitro. However, trans-factor binding was not universally responsive to the folded state of the TNFα ARE. The 70-kDa inducible heat shock protein, Hsp70, also associates with U-rich RNA substrates (39), but its ARE-binding activity was significantly less sensitive to the stability of RNA structures involving the TNFα ARE. The 70-kDa inducible heat shock protein, Hsp70, also associates with U-rich RNA substrates (39), but its ARE-binding activity was significantly less sensitive to the stability of RNA structures involving the TNFα ARE (33, 38). Both inhibition of AUF1 binding and the ARE constitute important determinants of RNA-binding activity for selected ARE-binding proteins, in some cases possibly as significant in magnitude and scope as RNA primary structural information.

**EXPERIMENTAL PROCEDURES**

**RNA Substrates—**Synthesis, deprotection of 2’-hydroxyl groups, and purification of RNA substrates were performed by Dharmaseq Research or Integrated DNA Technologies. The oligoribonucleotide ARE (38) includes the core ARE sequence from TNFα mRNA (5′-GUAG UAUUAUUUAUAAUAUUAUUAUUAUUAUUGAC-3′). Selected site-directed mutants are identified where applicable. 5′-linked cytidine-3 (Cy3) and 3′-linked fluorescein (Fl) groups are identified by relevant prefixes and suffixes, respectively, where applicable. Lyophilized RNA pellets were reconstituted in 10 mM Tris-HCl (pH 8). RNA yields and fluorophore labeling efficiencies were quantified by absorbance spectroscopy, incorporating fractional contributions of fluorescent dyes to A260 for RNA substrates containing Fl and/or Cy3 moieties as previously described (38, 40). For fluorescence experiments, RNA substrate concentrations were based on the abundance of Fl-conjugated RNA, which typically exceeded 90% of the total synthesis. For experiments requiring 5′-hydroxyl RNA, 5′-hydroxyl RNA substrates were radiolabeled using T4 polynucleotide kinase and [γ-32P]ATP to specific activities of 3–5 × 10⁶ cpm/μmol as described (37).

**Preparation of Recombinant Proteins—**Standard DNA subcloning techniques were used to modify the bacterial expression plasmid pB/HT-p37AUF1. All constructs were verified by restriction digests and automated DNA sequencing. The pB/HT-p37AUF1 plasmid was transformed into Escherichia coli TOP10 cells, and expression of the encoded His6-p37AUF1 recombinant protein was induced by arabinose. The His6-p37AUF1 protein was purified from lysed cells using Ni2+-affinity chromatography as described (33). The His6-Hsp70 protein was similarly expressed from plasmid pBAD/HisC-hsp70 and purified using Ni2+-affinity chromatography as described (39).

A cDNA encoding the open reading frame of HuR was amplified by reverse transcription-PCR using mRNA purified from the human monocytic leukemia cell line THP-1 and then subcloned into pGEX-3X (Amersham Biosciences). Following sequence verification, a glutathione S-transferase (GST)-HuR chimeric protein was expressed in E. coli TOP10 cells by induction with isopropyl-1-thio-β-D-galactopyranoside and purified by glutathione affinity chromatography using a GST of 30 kDa column (Amersham Biosciences) recommended by the manufacturer. GST lacking the HuR moiety was similarly expressed from unmodified pGEX-3X and purified. All recombinant proteins were quantified using Coomassie Blue-stained SDS-PAGE gels against a titration of bovine serum albumin.

**Bioinformatics, RNA Structural Modeling, and Nuclease Mapping—**Sequence alignments were prepared using the ClustalW program at the San Diego Supercomputer Center Biology Workbench (available on the World Wide Web at www.workbench.sdsc.edu). Candidate local RNA secondary structures were identified using mFold version 2.3 (available on the World Wide Web at www.bioinfo.rpi.edu/~zuker/mfold) (41, 42).

Nuclease mapping experiments were performed using a modification of the RNase V1 endonuclease protocol provided by Ambion. Briefly, 5′-32P-labeled RNA substrates (25 fmol) were incubated in a final volume containing 25 mM HEPES-HCl (pH 7.4), 50 mM KCl, and 4 μg of yeast tRNA. Where indicated, MgCl2 was included to 5 mM. In reactions lacking Mg2+, 0.5 mM EDTA was added to scavenge residual divalent cations. After equilibration at 25 °C, RNase A (0.045 Kunitz U; Sigma) was added, and incubation continued for 5 min. Reactions were stopped, and RNA fragments were precipitated using the inactivation/precipitation buffer (Ambion). Reaction products were fractionated on a 12% denaturing acrylamide gel, which was then dried and visualized using a PhosphorImager (Amersham Biosciences). Single base RNA ladders were prepared by hydroxide ion-mediated cleavage following protocols provided by Ambion.

**FRET-based Assays of RNA Folding—**Folding events involving RNA substrates were monitored by changes in the distance between 3′-linked Fl donor and 5′-linked Cy3 acceptor moieties using fluorescence resonance energy transfer (FRET). The efficiency of FRET (EFRET) is inversely related to the sixth power of the scalar distance (r) between a fluorescent donor and acceptor (Equation 1). Rf is the Förster distance, defined as the distance between a donor-acceptor pair where FRET efficiency is 50% (43, 44).

\[
EFRET = R_F^6 (R_f^6 + r^6)
\]

(1)

Rf for the Fl-Cy3 donor-acceptor pair linked to single-stranded nucleic acids has been calculated as 55.7 Å (45).

\[
EFRET = 1 - (F_D/F_0)
\]

(2)

However, without quantitative acceptor labeling, EFRET will be underestimated due to the presence of RNA molecules labeled only with Fl within the (Cy3 + Fl)-labeled RNA substrate population. To correct for this, donor emission from (Cy3 + Fl)-labeled RNA substrates (F_D>Cy3) was interpreted by Equation 3, where F_0 is the efficiency of acceptor labeling, in this case Cy3 (typically 75–90%).

\[
F_D>Cy3 = F_D + F_f + F_f \cdot (1 - f_0)
\]

(3)

Incorporating this function into Equation 2 and substituting \( F_D = F_f \) yields Equation 4.

\[
EFRET = 1 - \left[ \frac{F_D>Cy3}{F_f + F_f (1 - f_0)} \right]
\]

(4)

The ability of Mg2+ to stabilize folding of RNA substrates was quantified by measuring EFRET as a function of Mg2+ concentration at constituting immediately downstream of this sequence tag to yield plasmid pB/HT-p37AUF1. All constructs were verified by restriction digests and automated DNA sequencing. The pB/HT-p37AUF1 plasmid was transformed into E. coli TOP10 cells, and expression of the encoded His6-p37AUF1 recombinant protein was induced by arabinose. The His6-p37AUF1 protein was purified from lysed cells using Ni2+-affinity chromatography as described (33). The His6-Hsp70 protein was similarly expressed from plasmid pBAD/HisC-hsp70 and purified using Ni2+-affinity chromatography as described (39).
stant temperature essentially as described (38), with \( E_{\text{FRET}} \) calculated using Equation 4. Considering the conformation of the RNA substrate to be in equilibrium between an open, cation-free state and a folded, cation-associated state across a titration of Mg\(^{2+}\) concentrations allows these data to be resolved by Equation 5, derived from the Hill model (47).

\[
E_{\text{FRET}} = E_o + (E_i - E_o) \times \left( \frac{[\text{Mg}^{2+}]^n}{[\text{Mg}^{2+}]^{n+1}} \right)
\]

(Eq. 5)

Here, \( E_o \) and \( E_i \) represent the intrinsic \( E_{\text{FRET}} \) values for the open and folded RNA states, respectively, \( [\text{Mg}^{2+}] \) is the concentration of cation that yields 50% folded RNA, and \( h \) is the Hill coefficient.

The thermodynamic stability of RNA folding was evaluated by thermal denaturation in paired experiments containing either (Cy3 + Fl) double-labeled (for \( F_{\text{Cy3Fl}} \) or Fl single-labeled (for \( F_{\text{Fl}} \)) oligonucleotides. Samples contained 20 nM RNA in 10 mM Tris-HCl (pH 8), 50 mM KCl, 1 \( \mu \)g/\( \mu \)l heparin, and 0.1 \( \mu \)g/\( \mu \)l acetylated bovine serum albumin. Where indicated, Mg\(^{2+}\) was added as MgCl\(_2\), whereas samples lacking Mg\(^{2+}\) contained 0.5 mM EDTA. Samples were equilibrated in solution for 5 min at 10 °C, and then fluorescence was measured (\( \lambda_{\text{em}} = 490 \text{ nm}, \lambda_{\text{em}} = 518 \text{ nm}, 5-\text{nm band pass} \) in 0.5 °C intervals as the temperature increased from 10 to 70 °C at 1 °C/min using a Cary Eclipse fluorescence spectrophotometer (Varian Instruments) with a Peltier temperature controller and in-cell temperature probe. Thermodynamic parameters describing the folding of RNA substrates were extracted from the partial derivative of \( E_{\text{FRET}} \) (\( \Delta T = 4 °C \)) as a function of temperature. For a given structural transition, the reaction temperature yielding a local maximum in the derivative plot was defined as the apparent melting temperature (\( T_m \)), whereas the temperature above \( T_m \) yielding half-maximal derivative \( \Delta T \) was defined as \( T_d \). Using these parameters, the change in the van’t Hoff enthalpy (\( \Delta H_{\text{VH}} \)) for a unimolecular RNA structural transition is given by Equation 6 (48).

\[
\Delta H_{\text{VH}} = \frac{-3.5}{(1/T_m) - (1/T_d)}
\]

Taking \( T_m \) together with the experimentally derived \( T_m \), thus allows the transition free energy (\( \Delta G_{\text{calc}} \)) to be calculated as a function of reaction temperature (\( T \)) using Equation 7 (48).

\[
\Delta G_{\text{calc}} = \Delta H_{\text{VH}} \left( \frac{1}{T} - \frac{1}{T_m} \right)
\]

\( \Delta G_{\text{calc}} \) is the transition free energy (\( \Delta G_{\text{calc}} \)) for a unimolecular RNA structural transition.

Protein-RNA Binding Assays—Qualitative assessment of binding events between \( ^{32}\text{P}\)-labeled RNA substrates and recombinant proteins was performed using gel mobility shift assays essentially as described (49), except that Mg\(^{2+}\) was not included in binding reactions. Protein-RNA binding affinity was quantitatively measured by monitoring the changes in the fluorescence anisotropy of Fl-conjugated RNA substrates across the range of protein essentially as described (33, 37). Concomitant measurement of total fluorescence emission for each reaction demonstrated that association of either His\(_6\)-p37\( ^{\text{AF1}} \), His\(_6\)-Hsp70, or GST-HuR with Fl-labeled RNA substrates did not significantly affect their fluorescence quantum yield (data not shown).

AUF1 proteins interact with the TNFa ARE by sequential association of protein dimers yielding a tetrameric protein complex on the RNA substrate (P,R). Assembly of this complex as a function of protein dimer concentration (\( P_R \)) is thus resolved by tandem association equilibrium constants (33, 37); \( K_d \) describes the initial interaction between the RNA substrate and a protein dimer, whereas \( K_c \) describes the interaction between the RNA-protein dimer complex (P,R) and a subsequent protein dimer. Under conditions of constant quantum yield and limiting RNA substrate (i.e., [RNA] vs [P,R] < 1/k\(_d\)), \( K_d \) and \( K_c \) are derived from the total measured anisotropy of the Fl-conjugated RNA substrate (A\(_r\)) as a function of protein concentration by Equation 8,

\[
A_r = \frac{A_{\text{Fl}} + A_{\text{P2R}}K_d[P,R] + A_{\text{AP2}}K_cK_d[P,R]^2}{1 + K_d[P,R] + K_cK_d[P,R]^2}
\]

(Eq. 8)

where \( A_r \), \( A_{\text{Fl}} \), and \( A_{\text{P2R}} \) represent the intrinsic anisotropy values of the free RNA, the RNA-protein dimer complex, and the RNA-protein tetramer complex, respectively. \( A_{\text{Fl}} \) is measured directly from binding reactions lacking protein (\( n \approx 4 \), whereas all other constants are resolved by nonlinear regression using PRISM version 3.03 software (GraphPad). The formation of equimolar complexes between His\(_6\)-Hsp70 and Fl-labeled TNFa ARE substrates was resolved using a binary association function (Equation 9) as described previously (39).

Quantitative assessment of binding between GST-HuR and Fl-labeled RNA substrates was similarly prefomed using fluorescence anisotropy, but with two significant variations. First, preliminary on-rate analyses indicated significantly slower association kinetics for GST-HuR versus His\(_6\)-p37\( ^{\text{AF1}} \) or His\(_6\)-Hsp70 binding to ARE substrates (data not shown). Accordingly, RNA-binding reactions containing GST-HuR were incubated for a minimum of 1 h prior to measurement of fluorescence anisotropy. Second, the cooperative nature of GST-HuR binding to ARE substrates (detailed under "Results") necessitated analysis by a variant of the Hill model given in Equation 10, where \( A_{\text{P2R}} \) is the intrinsic anisotropy of the saturated Fl-RNA-GST-HuR\(_c\) complex, and \( P_{1/2} \) is the concentration of protein giving half-maximal binding.

\[
\Delta H_{\text{VH}} = \frac{T}{T_m} - \frac{T}{T_m}
\]

(Eq. 10)

Measurement of mRNA Decay Kinetics—The cellular decay rates of \( \beta\)-globin-ARE reporter mRNAs were measured using doxycycline (Dox) time course assays, slightly modified from previous descriptions (50). Briefly, the plasmid pTRE-R\(_{\beta}\)-wt and derivative plasmids was transfected using SuperFect (Qiagen) into HeLa Tet-Off cells (BD Biosciences) along with the control plasmid pSV1-c, encoding \( \alpha\)-globin. 48 h post-transfection, transcription from the pTRE-\( \beta\)-derived plasmids was arrested by the addition of Dox (2 \( \mu \)M). At selected time points thereafter, RNA was harvested and analyzed for \( \alpha\)-globin and \( \beta\)-globin mRNA levels using the Direct Protect RNase Protection Assay (Ambion). RNase protection assay reactions were programmed with 1 fmol each of \( ^{32}\text{P}\)-labeled antisense \( \beta\)-globin (complementary to portions of intron 2 and exon 3) and \( \alpha\)-globin (complementary to exon 2) riboprobes, prepared by in vitro transcription from plasmid DNA templates to specific activities of 2–4 \( \times 10^6 \) cpm/fmol. Following normalization to \( \alpha\)-globin mRNA, turnover rates of \( \beta\)-globin and \( \beta\)-ARE mRNAs were calculated by nonlinear regression of the percentage of \( \beta\)-globin or \( \beta\)-ARE mRNA remaining as a function of time following Dox treatment, yielding first-order decay constants (k).

RESULTS

A Candidate Stem-Loop Folded Structure for the TNFa ARE—Previous data indicated that an RNA substrate containing the core TNFa ARE sequence adopts a unimolecular, condensed structure in a cation-dependent manner (33, 38). Since folding of this substrate was preferentially facilitated by highly charged, spatially compact cations, we concluded that multivalent cations probably stabilize the condensed ARE structure by targeted counterion neutralization at regions of high negative charge density. To identify potential local RNA structures involving the core TNFa ARE, we first analyzed a 38-nucleotide RNA sequence containing this element (ARE(38)) using the mFold algorithm (41, 42). At 25 °C, mFold returned a hairpin-like structure punctuated by symmetrical U:U mismatches as the thermodynamically preferred RNA conformation (Fig. 1A). Suboptimal predicted structures were principally shift-shift variants of this candidate fold.

This candidate ARE structure was first tested by nuclease mapping. RNase A, which preferentially cleaves 3' of single-stranded pyrimidine residues, completely digested the ARE(38) substrate in the absence of Mg\(^{2+}\) under the conditions tested (Fig. 1B). By contrast, the addition of 5 mM Mg\(^{2+}\) conferred significant nuclease resistance, consistent with protection of single-stranded regions. Control experiments using an unstructured RNA substrate verified that the catalytic activity of RNase A did not significantly differ in the presence or absence of Mg\(^{2+}\) (data not shown). Whereas inclusion of Mg\(^{2+}\) strongly decreased the sensitivity of the ARE(38) substrate to RNase A, specific cleavage sites observed in the presence of the cation localized to unpaired uridylate residues predicted by mFold,
with the exception of U31 (Fig. 1A, arrows). These data support Mg\(^{2+}\)-dependent adoption of the folded ARE(38) structure predicted by mFold based on presentation of unpaired uridylate residues at predicted sites and general loss of accessibility for the single strand-specific nuclease, consistent with adoption of intramolecular base pairs in the presence of the cation.

**Site-directed Mutations within the TNFα ARE Modulate the Stability of RNA Folding**—Whereas the nuclease mapping data are largely consistent with the stem-loop ARE structure predicted by mFold, this model was further tested by creating single point mutations within the ARE(38) sequence that were predicted by mFold based on presentation of unpaired uridylate residues at predicted sites and general loss of accessibility for the single strand-specific nuclease, consistent with adoption of intramolecular base pairs in the presence of the cation.

![Diagram](http://www.jbc.org/)

Fig. 1. A hairpin model for TNFα ARE folding. A, the thermodynamically optimal hairpin fold of the ARE(38) RNA substrate predicted by mFold under conditions of 1 M Na\(^+\) and 25 °C. The arrows represent sites retaining sensitivity to RNase A after Mg\(^{2+}\)-induced RNA folding, determined in B, B, RNase A footprinting assays. The 5'\,-32P-labeled ARE(38) substrate was digested with RNase A in the presence or absence of 5 mM MgCl\(_2\), as described under “Experimental Procedures.” A 13-nucleotide 32P-labeled RNA substrate was included as a marker (Mkr) along with a single nucleotide ladder generated by partial alkaline hydrolysis of the ARE(38) substrate (′OH). C, nucleotide substitutions near the termini of the ARE(38) substrate used to test the proposed ARE structure.

Subsequent to the transitory adoption of the A4-U31 base pair, the U32-C32 pair.

Characterizing the Mg\(^{2+}\)-stabilized structural condensation of ARE substrates in the absence of monovalent cations has provided useful information regarding the physical basis for local RNA remodeling by Mg\(^{2+}\) (38) and the contributions of predicted base pairs to stabilization of the folded ARE structure (above). However, several factors necessitated the inclu-
sion of monovalent cations in subsequent experiments designed
to elucidate the biochemical significance of ARE folding. First,
nucleic acid interactions with mono- versus multivalent cations
occur through diverse modes with varying affinities, which can
promote distinct structural consequences (51). Second, the in-
tracellular environment includes a broad spectrum of cations of
differing valence and charge distribution. Finally, interactions
between RNA substrates and cellular proteins are often sensi-
tive to ionic strength (52, 53). In particular, association of
AUF1 with AU-rich RNA substrates is almost completely in-
hibited under hypotonic ([K+] = 25 mM) conditions (38), thus
precluding omission of monovalent cations from any study of
AUF1-RNA binding affinity. Based on these concerns, assess-
ing the role of ARE folding in the regulation of
trans-factor binding affinity required the establishment of parameters de-
scribing the stability of folded ARE structures in mixed cationic
environments. Analysis of the Mg2+ dependence of Cy3-
ARE(38)-Fl folding in the presence of 50 mM KCl yielded three
principal observations (Fig. 2).

FIG. 2. Cation dependence of ARE folded stability evaluated using FRET-based assays. A, schematic of a fluorescently labeled RNA substrate with 5'-Cy3 and 3'-Fl labels. The efficiency of FRET (E_FRET) between the Fl and Cy3 fluorophores is dictated by the scalar distance between them, described by Equation 1. As such, the transition of a population of RNA molecules from an unfolded (left) to a hairpin-like folded state (right) may
be monitored by an increase in E_FRET, reflecting the decreased distance between the RNA 5' and 3' termini upon folding. B and C, emission spectra of the Cy3-ARE(38)-Fl (B) and ARE(38)-Fl (C) RNA substrates with increasing concentrations of Mg2+ (0, 30, 70, 200, and 1000 μM) excited at 490 nm. D, E_FRET of (Cy3 + Fl)-labeled ARE(38) (closed circles), ARE(A3→C) (open circles), ARE(U32→C) (open triangles), and ARE(G3→U; U32→C) (closed triangles) substrates as a function of Mg2+ concentration in the absence of monovalent cation. All points represent the mean ± σ_n-1 of triplicate experiments. Nonlinear regression of each data set (solid lines) was performed using Equation 5. E, E_FRET of the (Cy3 + Fl)-labeled ARE(38) substrate as a function of Mg2+ concentration in the absence (dotted line) or presence of 5 mM Mg2+ (solid line). F, thermal denaturation analyses for the Cy3-ARE(38)-Fl substrate in the absence (dotted line) or presence of 5 mM Mg2+ (solid line). The local maximum indicates the characteristic melting temperature for the RNA structural transition (T_m), whereas the temperature above T_m yielding half-maximal
E_FRET/T is defined as T_s for calculation of thermodynamic parameters as described under “Experimental Procedures.”

TABLE I

| RNA substrate       | [Mg2+]_1/2 μM | h^a |
|---------------------|---------------|-----|
| ARE(38)             | 73 ± 3        | 1.53 ± 0.09 |
| ARE(A3→C)           | 114 ± 3       | 1.54 ± 0.05 |
| ARE(U32→C)          | 26 ± 2        | 1.01 ± 0.06 |
| ARE(G3→U; U32→C)    | 124 ± 3       | 1.37 ± 0.04 |

^a The concentration of cation required for half-maximal RNA sub-
strate folding ([Mg2+]_1/2) and the Hill coefficient (h) were calculated from
E_FRET versus [Mg2+] data sets (Fig. 2D) using Equation 5. All values are
based on triplicate independent experiments and represented as the
mean ± σ_n-1.
by Equation 5 gives $E_{d} = 0.35$ ± 0.02, indicating that this RNA substrate is partially folded in the monovalent salt background, independent of Mg$^{2+}$. Second, the concentration of Mg$^{2+}$ required to stabilize ARE folding is dramatically increased in the presence of KCl ([Mg$^{2+}$])$_{0.5}$ = 700 ± 30 μM, consistent with a competitive relationship between K$^+$ and Mg$^{2+}$ in their association with structured RNA molecules (51).

Finally, as the concentration of Mg$^{2+}$ increases, $E_{FRET}$ of the Cy3-ARE(38)-Fl RNA substrate approaches a limiting value ($E_d$) of greater than 0.9. By Equation 1, this indicates that the average distance between the Fl and Cy3 moieties in the folded state is no more than 39 Å. Given that the average diameter of an A-form double helix is ~26 Å (54) and that the donor and acceptor dyes are tethered to the Cy3-ARE(38)-Fl substrate by 6- and 3-atom linkers, respectively, this calculated interfluorophore distance is consistent with the mFold model of the folded ARE(38) structure (Fig. 1A), where the 5'- and 3'-termini are closely spaced in solution.

Thermodynamic parameters describing the stability of Cy3-ARE(38)-Fl substrate folding in a background of 50 mM KCl were estimated by thermal denaturation. In the absence of Mg$^{2+}$, the Cy3-ARE(38)-Fl substrate adopted a condensed conformation at low temperature denoted by high $E_{FRET}$ (Fig. 2F, dotted line). However, a decrease in $E_{FRET}$ as temperature increased indicated release of the folded structure. The change in van’t Hoff enthalpy accompanying this transition was calculated from the partial derivative of $E_{FRET}$ as a function of temperature (Fig. 2G) as described under “Experimental Procedures,” which in turn permitted solution of the apparent free energy of folding ($\Delta G_{\text{fold}}^{0}$) at 25 °C using Equation 7. In the absence of Mg$^{2+}$, the Cy3-ARE(38)-Fl substrate is unfavorable at 25 °C ($\Delta G_{\text{fold}}^{0} > 0$), but the addition of Mg$^{2+}$ significantly shifts the equilibrium in favor of the folded state in a dose-dependent manner, based on both increases in the apparent melting temperature ($T_m$) and concomitant decreases in $\Delta G_{\text{fold}}^{0}$ (Table II). For example, inclusion of 5 mM Mg$^{2+}$ (Fig. 2, P and G, solid line) increased $T_m$ of the Cy3-ARE(38)-Fl substrate by 11.5 °C, yielding $\Delta G_{\text{fold}}^{0} = -2.44$ kcal/mol at 25 °C relative to substrate folding in the absence of Mg$^{2+}$. Thermal denaturation analyses of fluorescent RNA substrates containing the ARE(A$^{1}\rightarrow$C) and ARE(U$^{32}\rightarrow$C) sequences further validated the predicted roles of the mutated bases in stabilization of the ARE hairpin structure. For each concentration of Mg$^{2+}$ tested, values of $\Delta G_{\text{fold}}^{0}$ indicated that the folded structure of the Cy3-ARE(A$^{1}\rightarrow$C)-Fl substrate was significantly less stable than that formed with the Cy3-ARE(38)-Fl substrate, whereas folding of the Cy3-ARE(U$^{32}\rightarrow$C)-Fl substrate was significantly more stable (Table II). Taken together, the increased concentration of Mg$^{2+}$ required to stabilize the folded Cy3-ARE(A$^{1}\rightarrow$C)-Fl substrate relative to the Cy3-ARE(38)-Fl substrate (Table I), coupled with the decreased value of $\Delta G_{\text{fold}}^{0}$ for the A$^{1}\rightarrow$C mutant (Table II), is consistent with participation of A$^{4}$ in an A-U base pair contact in the wild type ARE structure. Similarly, the improved stability and diminished Mg$^{2+}$ requirement of the folded Cy3-ARE(U$^{32}\rightarrow$C)-Fl substrate relative to the wild type ARE supports formation of a G-C base pair in the mutant RNA structure. Since the U$^{32}\rightarrow$C substitution would weaken an RNA structure containing a U-A base pair involving U$^{32}$, and only two guanosine residues are present upstream of U$^{32}$ in the ARE(38) sequence, these findings are consistent with a base pair interaction between U$^{32}$ and either G$^{1}$ or G$^{3}$ in cation-stabilized folding of the wild type TNFα ARE.

A Compensatory Double Mutation Highlights an Interaction between G$^{3}$ and U$^{32}$ during ARE Folding—In order to confirm G$^{3}$ as the interacting partner of U$^{32}$ in the folded ARE structure, a compensatory double mutation was made by changing G$^{3}\rightarrow$U and U$^{32}\rightarrow$C (Fig. 1C). Cation titration and thermal denaturation experiments of RNA substrates containing these substitutions yielded three principal observations. First, significantly higher concentrations of Mg$^{2+}$ were required to stabilize the folded state of the Cy3-ARE(G$^{3}\rightarrow$U; U$^{32}\rightarrow$C)-Fl substrate relative to the Cy3-ARE(U$^{32}\rightarrow$C)-Fl substrate (Fig. 2D, Table I). Second, the cooperativity of ARE substrate folding with respect to Mg$^{2+}$ that was lost by the U$^{32}\rightarrow$C substitution was largely restored in the double mutant (Table I). Finally, thermal denaturation experiments demonstrated that the improvement in folding energy conferred by the U$^{32}\rightarrow$C substitution relative to the wild type ARE was completely lost by concurrent mutation of G$^{3}\rightarrow$U at all Mg$^{2+}$ concentrations tested (Table II). Taken together, these data indicate that the G$^{3}\rightarrow$U substitution abrogates the stabilizing influence of the U$^{32}\rightarrow$C mutation on ARE folding, consistent with the formation of a noncanonical G$^{3}$-U$^{32}$ base pair in the folded structure of the wild type ARE substrate.

**Table II**

| RNA substrate | [Mg$^{2+}$] | $T_m$ | $T_m^{0}$ | $\Delta G_{\text{fold}}^{0}$ at 25 °C |
|---------------|-------------|------|----------|----------------------------------|
| ARE(38)       | 0           | 20.5 | 26.0     | $0.86 + (0.09 - 0.07)$            |
|               | 1           | 27.0 | 32.0     | $-0.43 + (0.05 - 0.04)$           |
|               | 5           | 32.0 | 37.0     | $-1.52 + (0.17 - 0.14)$           |
| ARE(A$^{1}\rightarrow$C) | 0            | 18.5 | 23.0     | $1.92 + (0.32 - 0.24)$            |
|               | 1           | 25.0 | 30.5     | $0.00 + (0.11 - 0.09)$            |
|               | 5           | 30.5 | 35.5     | $-0.85 + (0.07 - 0.06)$           |
| ARE(U$^{32}\rightarrow$C) | 0           | 26.5 | 31.5     | $-0.32 + (0.04 - 0.03)$           |
|               | 1           | 34.0 | 39.0     | $-2.45 + (0.35 - 0.27)$           |
|               | 5           | 39.5 | 44.0     | $-3.58 + (0.45 - 0.36)$           |
| ARE(G$^{3}\rightarrow$U; U$^{32}\rightarrow$C) | 0           | 19.0 | 24.5     | $1.15 + (0.12 - 0.10)$            |
|               | 1           | 24.0 | 30.5     | $0.16 + (0.01 - 0.01)$            |
|               | 5           | 30.0 | 37.5     | $-0.73 + (0.05 - 0.05)$           |

a. Solved from thermal denaturation analyses of (Cy3 + Fl)-labeled RNA substrates as described under “Experimental Procedures” and in Fig. 2G, with triplicate experiments yielding indistinguishable results.

b. Solved using Equations 6 and 7, with error estimates based on ±0.25 °C uncertainties in the values of $T_m$ and $T_m^{0}$.
To determine whether the sensitivity of trans-factor binding to the folded stability of ARE substrates was limited to p37AUF1, similar experiments were performed using recombinant forms of the ARE-binding proteins Hsp70 and HuR. Association of His6-Hsp70 with the ARE(38)-Fl substrate was decreased by 6-fold by the mRNA-stabilizing factor, p37AUF1, and the mRNA stabilizing protein, HuR, for the unfolded TNFα ARE (Tables III and IV; 0 mM Mg^{2+}) indicate that both proteins may effectively compete for binding to this RNA substrate. However, inhibition of p37AUF1 binding to the folded TNFα ARE substrate without significant changes in HuR binding affinity (Tables III and IV; 5 mM Mg^{2+}) suggests that stabilization of the TNFα ARE hairpin structure perturbs this equilibrium in favor of the mRNA-stabilizing factor. It follows, therefore, that the ability of the TNFα ARE to direct rapid mRNA turnover in cis may be closely linked to the stability of ARE folding. To test this hypothesis, sequences encoding the ARE(38), ARE(A^4→C), ARE(U^32→C), and ARE(G^3→U; U^52→C) elements were sub-

| Protein | K_{a}^{\alpha} | K_{a}^{\beta} | K_{d}^{\alpha} | K_{d}^{\beta} | n |
|---------|----------------|---------------|---------------|---------------|----|
| His6-p37AUF1 | 0.10 ± 0.01 | 0.98 ± 0.10 | 1.5 ± 0.10 | 68 ± 3 |
| 1 | 0.62 ± 0.09 | 1.6 ± 0.09 | 0.65 ± 0.07 | 150 ± 3 |
| 5 | 0.07 ± 0.01 | 15 | 0.3 ± 0.07 | 300 ± 2 |
| His6-Hsp70 | 0.09 ± 0.01 | 0.09 ± 0.01 | 10 | 2 |
| 0 | 0.08 ± 0.01 | 13 | 3 |
| 5 | 0.017 ± 0.002 | 58 | 4 |

a Association binding constants were solved from A_{t} versus [protein] data sets (Fig. 3) using Equation 8 (for His6-p37AUF1) or Equation 9 (for His6-Hsp70) and are expressed as mean ± s_{n-1} for n = 3 or mean ± spread for n = 2.

b Dissociation constants solved as K_{d} = 1/K.

### TABLE III

| Protein | K_{a}^{\alpha} | K_{a}^{\beta} | K_{d}^{\alpha} | K_{d}^{\beta} | n |
|---------|----------------|---------------|---------------|---------------|----|
| His6-p37AUF1 | 0.10 ± 0.01 | 0.98 ± 0.10 | 1.5 ± 0.10 | 68 ± 3 |
| 1 | 0.62 ± 0.09 | 1.6 ± 0.09 | 0.65 ± 0.07 | 150 ± 3 |
| 5 | 0.07 ± 0.01 | 15 | 0.3 ± 0.07 | 300 ± 2 |
| His6-Hsp70 | 0.09 ± 0.01 | 0.09 ± 0.01 | 10 | 2 |
| 0 | 0.08 ± 0.01 | 13 | 3 |
| 5 | 0.017 ± 0.002 | 58 | 4 |
cloned into the 3′-UTR of a β-globin reporter gene under the control of a tetracycline-responsive promoter (Fig. 5A). Following transfection into HeLa/Tet-Off cells, the cellular decay rates of these transcripts were assessed using Dox time course assays (Fig. 5B), followed by a solution of first order decay constants (Fig. 5C). Introduction of the ARE(38) sequence into the 3′-UTR of β-globin mRNA decreased the mRNA half-life by 2.7-fold (Table V; cf. β-G-ARE(U32→C) versus β-G-ARE(38)). Conceivably, inhibition of ARE-directed mRNA turnover by the U32→C substitution may result either from stabilization of ARE folding or by loss of a primary RNA structural determinant for trans-factor binding. However, the U32→C substitution was unable to slow ARE-directed mRNA decay kinetics in the presence of the compensatory G3→U mutation (Table V; cf. β-G-ARE(U32→C) versus β-G-ARE(G3→U; U32→C)), indicating that mutation of U32→C inhibits mRNA decay through stabilization of ARE folding via interaction with G3 (Fig. 1) and not simply by abrogation of trans-factor binding contacts through U32. Taken together, these data show that a mutation that stabilizes the folded structure of the TNFα ARE (Tables I and II) also decreases the ability of this ARE to direct rapid mRNA turnover in cells, consistent with a model whereby the stability of ARE folding may influence the equilibrium between various trans-acting factors competing for this cis-acting element.

**Fig. 4. Evaluation of GST-HuR binding to the ARE(38)-Fl RNA substrate in vitro.** A, gel mobility shift assays were performed using 5′-32P-labeled ARE(38)-Fl RNA substrate and a titration of recombinant GST-HuR or GST protein in the absence of Mg2+. Lanes indicated NP contained no recombinant protein. Bands corresponding to complexes containing the 32P-RNA substrate and one or more GST-HuR molecules are indicated by the arrowheads. B, association of GST-HuR with the ARE(38)-Fl RNA substrate was analyzed by fluorescence anisotropy as described for Fig. 3 (solid circles, 0.5 mM EDTA; open circles, 5 mM Mg2+). Additional binding reactions contained recombinant GST protein in place of GST-HuR (triangles). GST-HuR binding data were resolved by a cooperative association model given by Equation 10.

**Table IV**

| [Mg2+] | [GST-HuR] | n | m | h |
|--------|-----------|---|---|---|
| mm     | nm        |
| 0      | 0.54 ± 0.06 | 1.5 ± 0.1 | 4 |
| 1      | 0.74 ± 0.02 | 1.5 ± 0.2 | 2 |
| 5      | 0.8 ± 0.1   | 1.62 ± 0.09 | 2 |

*The concentration of GST-HuR required for half-maximal RNA binding ([GST-HuR])0 and the Hill coefficient (h) were calculated from Ak versus [GST-HuR] data sets (Fig. 4B) using Equation 10 and are expressed as the mean ± s.e. for n = 4 or mean ± spread for n = 2.

**Fig. 5.** Influence of Mg2+ stabilized RNA folding on equilibrium binding of GST-HuR to the ARE(38)-Fl RNA substrate.
cases, base pair contacts involving sequences flanking the core ARE were invariant; rather, slight differences in predicted folding energy were the result of alternative base pairing in the core ARE region.

The high degree of sequence conservation flanking the core ARE sequence from mammalian TNFα mRNAs suggests one or more important regulatory roles for these domains. Phylogenetic comparisons of structural models indicate that one such role may be to direct the conformational presentation of the core ARE. Given that even modest stabilization of ARE folding significantly inhibits the binding activity of p37AUF1 and, to a lesser extent, Hsp70 (Fig. 3 and Table III) while only minimally influencing HuR binding (Fig. 4B and Table IV), these data support the hypothesis that local RNA folding potential may constitute an important determinant of ARE function or regulation in vivo, by presenting a novel mechanism for discrimination of trans-factor binding events.

**DISCUSSION**

From this work, several lines of evidence indicate that the TNFα ARE may adopt a stem-loop formation in solution analogous to the model predicted by mFold (Fig. 1A). First, the ARE(38) substrate is partially protected from digestion by RNase A in the presence of Mg2+ , consistent with occlusion of single-stranded regions in the presence of the cation (Fig. 1B). Second, nucleotides remaining sensitive to RNase A in the presence of Mg2+ map almost exclusively to unpaired uridylate residues in the folded model. Third, FRET analyses indicate a limiting distance of 39 Å between fluorophores linked to the 5′ and 3′ termini of the ARE(38) substrate in the folded state (Fig. 2E). Fourth, single nucleotide substitutions predicted to stabilize (U; U32→C) or destabilize (A; C) the ARE stem-loop structure, as well as a double mutant ARE (G; C→U; U32→C) predicted to abrogate stabilization by U32→C, all yielded these anticipated effects, demonstrated by (i) changes in the Mg2+ concentrations required to stabilize RNA substrate folding (Table I) and (ii) changes in the thermodynamic stability of each RNA substrate (Table II). Finally, sequences spanning and flanking the TNFα ARE are highly conserved among mammals, with substitutions and covariance between species consistent with maintenance of the TNFα core ARE near the terminal loop of an extended hairpin structure (Fig. 6).

Additional details, however, raise the likelihood that the stem-loop model presented in Fig. 1A may be representative of a limited population of similar structures involving the TNFα ARE. For example, mFold also predicted a small number of suboptimal structures retaining overall stem-loop character but slip-shifted relative to the most stable candidate (Fig. 6D and data not shown). Shifting base pair contacts by only 2 or 3 nucleotides within a subset of ARE hairpin structures would exert fairly minor influences on the average distance between the termini of the ARE(38) substrate in solution (and hence minimally influence $E_{FRET}$) but may
account for occasional exposure of U31 to single-stranded nucleases (Fig. 1B). Furthermore, the mFold models do not account for potential energetic contributions from base stacking within symmetrical bulges or formation of non-Watson-Crick U-U base pairs (56, 57). Finally, with the specificity of ARE folding driven by small numbers of contiguous A-U or G-U base pairs, it is likely that ARE stem-loop structures would exhibit rapid conformational dynamics in solution (58).

In this study, stabilizing the folded state of RNA substrates containing the core TNFα ARE by addition of Mg2+ inhibited p37AUF1 binding and oligomerization activities (Table III) concomitant with occlusion of single-stranded RNA sequence determinants contributing to protein recognition. In this manner, accessibility of ARE target sites for trans-acting factors may be considered in terms of a partition function, where the opportunity for associative RNA-protein contacts is influenced by both the length of RNA required for a given RNA-protein interface and the stability and/or dynamics of folded RNA structures involving this site. By this model, the binding activity of p37AUF1 was suppressed by formation of a stable hybrid (Fig. 1A).

**Fig. 6. Conservation of sequences and predicted structures of the TNFα mRNA spanning the ARE.** A, the human TNFα 3′-UTR sequence spanning and flanking the core ARE was aligned using ClustalW with corresponding sequences from goat, sheep, mouse, dolphin, and whale. Nucleotide numbers are relative to the TNFα mRNA translational initiation codon from each species, and the core ARE sequence is capitalized. Bases conserved with the human sequence are given by dots, dashes indicate bases deleted in individual species, and base substitutions between species are listed where applicable. Data base sequences were extracted from the following accession numbers: human, Homo sapiens, NM_000594; goat, Capra hircus, X145282; sheep, Ovis aries, X56756; mouse, Mus musculus, X02611; dolphin, Tursiops truncates, AB049358; beluga whale, Delphinapterus leucas, AF320323. B, the optimal mFold predicted structure for the human TNFα 3′-UTR sequence flanking and spanning the ARE. The core ARE sequence is boxed. Base substitutions are indicated for goat (1), sheep (2), dolphin (3), and whale (4). C, the optimal mFold predicted structure for the mouse TNFα 3′-UTR domain homologous to the human sequence in B. Base substitutions in the human sequence are given. del denotes a base that is deleted in the human sequence, whereas > indicates the location of a GUG trinucleotide found in the human TNFα mRNA. D, a suboptimal mFold prediction for the murine TNFα ARE hairpin structure. Base pairs involving sequences further 5′ and 3′ are identical to those shown in C.
p37AUF1 is likely to be highly sensitive to the RNA folding potential of ARE substrates, since p37AUF1 requires relatively large (>20-nucleotide) AU-rich sequences for high affinity binding (32). By contrast, ARE-binding proteins capable of forming stable complexes with significantly smaller RNA substrates may be less sensitive to the folded stability of ARE substrates. For example, the neuronal HuR-related protein, HuD, binds a 13-nucleotide, AU-rich RNA substrate with $K_d < 10 \text{ nM}$ (22), whereas the zinc finger domain of TTP forms complexes of similar affinity with RNA substrates as small as 9 nucleotides (26). The strong influence of ARE folding on the binding activity of His$_6$-p37AUF1, coupled with the very modest inhibition of GST-HuR binding to the folded ARE substrate (this work) are supportive of this model. Furthermore, the inhibition of cellular ARE-directed mRNA turnover resulting from a modest increase in the stability of ARE folding (Fig. 5 and Table V) suggests that some ARE-binding proteins also show sensitivity to local RNA structure in the cellular environment. Given the growing number of ARE-binding proteins identified to date, local RNA structures involving these elements may thus constitute an important determinant of trans-factor selectivity.

Current models of ARE-directed mRNA decay indicate that association of some ARE-binding factors serves to target nucleases (17, 18) or other ancillary proteins (59) to the RNA substrate. However, given that AREs from some mRNAs extend more than 120 nucleotides in length (3) and that flanking sequences may be conserved to a much greater degree than required solely for maintenance of local secondary structure (e.g. Fig. 6A), it is likely that assembly of multisubunit, trans-acting complexes may include multiple RNA-protein interactions near the ARE. Accordingly, alterations in local RNA topology could potentially influence ARE-directed mRNA turnover kinetics by promoting or restricting any number of RNA-protein binding events. A recent report showing that ARE-directed mRNA decay kinetics can be modulated by hybridization of antisense sequences adjacent to the ARE lends further support for this model (60). The relationship between local ARE structure and regulation of mRNA decay is complicated, however, by observations that ARE-binding proteins themselves are capable of remodeling local RNA structure. For example, the zinc finger domains of TTP and the related protein TIS11d both retain bound RNA substrates in elongated conformations (26, 27). In contrast, the two N-terminal RNA recognition motifs of HuD induce a bend in associated ARE substrates (21). AUF1 proteins present a more complex case, in that both p37AUF1 and p40AUF1 structurally condense RNA substrates upon binding (38, 61). However, in THP-1 monocytic leukemia cells, phorbol ester-induced stabilization of ARE-directed mRNA decay activity not only by selective recruitment of ARE-binding proteins but also by the ability of such factors to remodel flanking RNA structures. As a result, we speculate that gene-specific control of ARE-directed mRNA decay may ultimately be possible by modulating the stability or dynamics of local ARE folding events.

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REFERENCES

1. Guhaniyogi, J., and Brewer, G. (2001) Gene (Amst.) 265, 11–23
2. Blackshear, P. J. (2002) Biochem. Soc. Trans. 30, 945–952
3. Chen, C.-Y. A., and Shyu, A.-B. (1999) Trends Biochem. Sci. 20, 465–470
4. Ross, J. (1995) Microbiol. Rev. 59, 423–450
5. Hargrove, J. L., and Schmidt, F. H. (1989) PASE II 3, 2360–2370
6. Prevel, M. A. E., Bakheer, T., Silva, A. M., Hissong, J. G., Khabar, K. S. A., and Williams, B. R. G. (2003) Mol. Cell. Biol. 23, 425–436
7. Dean, J. L. E., Sarsfield, S. J., Tsoumakakos, E., and Saklatvala, J. (2003) J. Biol. Chem. 278, 39470–39476
8. Briata, P., Hengo, C., Corte, G., Moroni, C., Rosenfeld, M. G., Chen, C.-Y., and Gherzi, R. (2003) Mol. Cell. 12, 1201–1211
9. Wilson, G. M., and Brewer, G. (1999) Prog. Nucleic Acids Res. Mol. Biol. 62, 157–291
10. Zhang, T., Krays, V., Huez, G., and Gueydan, C. (2002) Biochem. Soc. Trans. 30, 952–958
11. Chen, C.-Y., Acton, S. J., Sexton, A. E., and Morrison, A. R. (2004) J. Biol. Chem. 279, 8196–8205
12. Lapucci, A., Donnini, M., Papucci, L., Witort, E., Tempestini, A., Bevilacqua, A., Nolvin, A., Brewer, G., Schiavone, N., and Capaccioli, S. (2002) J. Biol. Chem. 277, 16158–16161
13. Sarkar, B., Xi, Q., He, C., and Schneider, R. J. (2003) Mol. Cell. 23, 6685–6693
14. Raineri, I., Wegmueller, D., Gross, B., Certo, U., and Moroni, C. (2004) Nucleic Acids Res. 32, 1279–1288
15. Lai, W. S., Carballo, E., Thom, J. M., Ennas, A. C., and Blackshear, P. J. (2000) J. Biol. Chem. 275, 18727–18737
16. Lai, W. S., Carballo, E., Strum, J. R., and Blackshear, A. E., Phillips, R. S., and Blackshear, P. J. (1999) Mol. Cell. Biol. 19, 4311–4323
17. Chen, C.-Y., Gherzi, R., Ong, S.-E., Chan, E. L., Raijmakers, R., Pruijn, G. J. M., Stoelcklin, G., Moroni, C., Mann, M., and Karin, M. (2001) Cell 107, 451–464
18. Gherzi, R., Lee, K.-Y., Briata, P., Wegmüller, D., Moroni, C., Karin, M., and Chen, C.-Y. (2004) Mol. Cell 14, 571–583
19. Feng, S. S. Y., Chen, C.-Y. A., Xu, N., and Shyu, A.-B. (1998) EMBO J. 17, 3461–3470
20. Fan, X. C., and Steitz, J. A. (1998) EMBO J. 17, 3448–3460
21. Wang, X., and Hall, T. M. T. (2001) Nat. Struct. Biol. 8, 141–145
22. Park-Lee, S., Kim, S., and Laird-Offringa, I. A. (2003) J. Biol. Chem. 278, 39801–39808
23. Lopez de Silanes, I., Zhan, M., Lal, A., Yang, X., and Gorospe, M. (2004) Proc. Natl. Acad. Sci. U. S. A. 101, 2987–2992
24. Winzen, R., Grewishanker, G., Bollig, F., Redich, N., Resch, K., and Holtmann, H. (2004) Mol. Cell. Biol. 24, 4835–4847
25. Chen, C.-Y. A., Xu, N., and Shyu, A.-B. (2002) Mol. Cell. Biol. 22, 7268–7278
26. Brewer, B. Y., Malicka, J., Blackshear, P. J., and Wilson, G. M. (2004) J. Biol. Chem. 279, 27870–27877
27. Persson, B. D., Martinez-Vilalta, M. A., Dyson, J. H., and Wright, P. E. (2004) Nat. Struct. Mol. Biol. 11, 257–264
28. Wagner, B. J., DeMaría, C. T., Sun, Y., Wilson, G. M., and Brewer, G. (1998) Genomics 48, 195–202
29. Zhang, W., Wagner, B. J., Ehrenkern, K., Schuefer, A. W., DeMaría, C. T., Crater, D., DeHaven, K. Long, L., and Brewer, G. (1993) Mol. Cell. Biol. 13, 7652–7665
30. Aron, Y., Kuriyama, R., Kayama, F., and Kato, S. (2000) Arch. Biochem. Biophys. 386, 228–236
31. Wilson, G. M., Sun, Y., Huyhn, Y., and Brewer, G. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7302–7307
32. Wilson, G. M., Sun, Y., Huyhn, Y., and Brewer, G. (2000) Proc. Natl. Acad. Sci. U. S. A. 97, 7302–7307
Folding of an AU-rich RNA Element Regulates mRNA Decay

43. Clegg, R. M. (1992) Methods Enzymol. 211, 353–388
44. Lakowicz, J. R. (1999) Principles of Fluorescence Spectroscopy, 2nd Ed., pp. 367–394, Kluwer Academic/Plenum, New York
45. Norman, D. G., Granger, R. J., Uhrin, D., and Lilley, D. M. J. (2000) Biochemistry 39, 6317–6324
46. Klostermeier, D., and Mullar, D. P. (2001) Methods 23, 240–254
47. Heilman-Miller, S. L., Thirumalai, D., and Woodson, S. A. (2001) J. Mol. Biol. 306, 1157–1166
48. Breslauer, K. J. (1995) Methods Enzymol. 259, 221–242
49. Wilson, G. M., and Brewer, G. (1999) Methods 17, 74–83
50. Lasa, M., Mahtani, K. R., Finch, A., Brewer, G., Saklatvala, J., and Clark, A. R. (2000) Mol. Cell. Biol. 20, 4265–4274
51. Draper, D. E. (2004) RNA 10, 335–343
52. Draper, D. E. (1995) Annu. Rev. Biochem. 64, 593–620
53. Fisher, B. M., Ha, J.-H., and Raines, R. T. (1998) Biochemistry 37, 12121–12132
54. Nelson, D. L., and Cox, M. M. (2005) Lehninger Principles of Biochemistry, 4th Ed., p. 284, W.H. Freeman and Co., New York
55. Xu, N., Chen, C.-Y. A., and Shyu, A.-B. (1997) Mol. Cell. Biol. 17, 4611–4621
56. Santa Lucia, J. Jr., Kierzek, R., and Turner, D. H. (1991) Biochemistry 30, 8242–8251
57. Leontis, N. B., Stombaugh, J., and Westhof, E. (2002) Nucleic Acids Res. 30, 3497–3531
58. Herschlag, D. (1995) J. Biol. Chem. 270, 20871–20874
59. Larocia, G., Cuesta, R., Brewer, G., and Schneider, R. J. (1999) Science 284, 499–502
60. Meisner, N. C., Hackermuller, J., Uhl, V., Aszodi, A., Jaritz, M., and Auer, M. (2004) ChemBioChem 5, 1432–1447
61. Wilson, G. M., Lu, J., Sutphen, K., Suarez, Y., Sinha, S., Brewer, B., Villanueva-Feliciano, E. C., Ylsa, R. M., Charles, S., and Brewer, G. (2003) J. Biol. Chem. 278, 33039–33048
A Hairpin-like Structure within an AU-rich mRNA-destabilizing Element Regulates trans-Factor Binding Selectivity and mRNA Decay Kinetics

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