Structural Remodeling of an A + U-rich RNA Element by Cation or AUF1 Binding*

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Association of AUF1 with A + U-rich elements (AREs) induces rapid cytoplasmic degradation of mRNAs containing these sequences, involving the recruitment or assembly of multisubunit trans-acting complexes on the mRNA. Recently, we reported that Mg2⁺-induced conformational changes in the ARF from tumor necrosis factor α mRNA inhibited AUF1 binding and oligomerization activities on this substrate (Wilson, G. M., Sutphen, K., Chuang, K., and Brewer, G. (2001) J. Biol. Chem. 276, 8695–8704). In this study, resonance energy transfer was employed to characterize structural changes in RNA substrates in response to cation- and AUF1-binding events. An RNA substrate containing the tumor necrosis factor α ARE displayed a weak conformational transition in the absence of added cations but was cooperatively stabilized by Mg2⁺. Additional assays demonstrated a strong preference for small, multivalent cations, suggesting that the folded RNA structure was stabilized by counterion neutralization at discrete regions of high negative charge density. Association of AUF1 with cognate RNA substrates also induced formation of condensed RNA structures, although distinct from the folded structure stabilized by multivalent cations. Taken together, these experiments indicate that association of AUF1 with an ARE may function to remodel local RNA structures, which may be a prerequisite for subsequent recruitment of additional trans-acting factors.

The synthetic rates of many eukaryotic gene products are regulated by the cytoplasmic stabilities of mRNAs encoding them (reviewed in Refs. 1 and 2). A + U-rich elements (AREs) are sequences located in the 3'-untranslated regions of many labile mammalian mRNAs, including some encoding oncoproteins, inflammatory mediators, cytokines, and G protein-coupled receptors, that target these transcripts for rapid cytoplasmic turnover (3). AREs are variable in length but often consist of a number of overlapping AUUUA pentamers embedded within or adjacent to a U-rich region. Messenger RNA turnover directed by AREs is typically characterized by rapid deadenylation of the mRNA, followed by degradation of the mRNA body (3–5).

The ability of an ARE to promote rapid mRNA turnover in cis is dependent on interactions between this element and any of a number of cytoplasmic ARE-binding factors. One such factor, HuR, is associated with stabilization of the ARE-containing transcript (6, 7), while others, including AUF1 and tristetraprolin, are associated with acceleration of mRNA decay (8–12). While these and several other proteins are known to associate with AREs (reviewed in Ref. 13), few details are available regarding either their mechanisms of action or the conditions influencing ARE binding of one trans-acting factor over another. Characterization of the interactions between these proteins and their ARE substrates is an essential step toward a broader understanding of the mechanisms linking ARE recognition with the initiation or inhibition of mRNA decay processes.

The ARE-binding factor AUF1 has been extensively studied as a mediator of rapid, ARE-directed mRNA turnover both in vivo (10, 11, 14, 15) and in cell-free mRNA decay systems (9, 16). AUF1 exists as a family of four protein isoforms generated from a common pre-mRNA, and all associate with RNA substrates via two tandemly arranged RNA recognition motifs (RRMs) (17). Individual isoforms are designated by their apparent molecular weights as p37 AUF1, p40 AUF1, p42 AUF1, and p45 AUF1. The principal cytoplasmic isoforms, p37 AUF1 and p40 AUF1, associate with several other cellular proteins (18), including eIF4G, poly(A)-binding protein, and the heat shock proteins Hsp70 and Hsc70 (19). In vitro, recombinant p37 AUF1 associates directly with AREs and forms protein oligomers on these RNA substrates by sequential binding of protein dimers (20–22). The formation of multimeric AUF1 complexes on AREs, coupled with the ability of this protein to associate with other cellular factors involved in the regulation of translation and mRNA stability, indicates that AUF1 may function as an ARE-targeting system, assembling or recruiting selected additional trans-acting factors to the mRNA, which ultimately lead to the induction of RNA catabolism. Accordingly, mechanisms that modulate the ability of AUF1 to interact with AREs are likely to be important regulators of mRNA stability.

Previously, we demonstrated that association of recombinant p37 AUF1 to the ARE from tumor necrosis factor α (TNFα) mRNA was inhibited by the presence of magnesium ions, concomitant with the adoption of a spatially condensed, unimolecular RNA structure exhibiting restricted backbone flexibility (22). Inhibition of AUF1 binding by Mg2⁺ was sequence-specific with respect to the ARE substrate, since AUF1 binding to a

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The abbreviations used are: ARE, A + U-rich element; Cy3, cyanine 3; DTT, dithiothreitol; Fl, fluorescein; RET, resonance energy transfer; RRM, RNA recognition motif; TNFα, tumor necrosis factor α.
comparably sized polyuridylate sequence (U₃₂) was not significantly affected by the cation. In this study, we have further characterized the structural consequences of both cation- and AUF1-binding events to these RNA substrates. Using resonance energy transfer (RET) to monitor global changes in the solution structure of the TNFα ARE, we observed that this RNA substrate adopts a weak, higher order RNA structure that is cooperatively stabilized by Mg²⁺ in a sequence-specific manner. This structure was optimally stabilized by compact, multivalent cations, indicating that ion-mediated stabilization probably occurs by counterion neutralization at localized regions of high negative charge density, resulting from the juxtaposition of negatively charged groups in the folded RNA structure. AUF1 binding also induced conformational changes in both the TNFα ARE and U₃₂ substrates. Based on calculation of intramolecular distances between the 5' and 3'-ends of the RNA substrates, however, these protein-folded structures are shown to be distinct from those stabilized by cations and appear to be a direct consequence of AUF1 binding and oligomerization. Together, these experiments define a mechanism for structural condensation of AREs that regulates their AUF1 binding activity and suggest that AUF1-induced, localized remodeling of these RNA structures may contribute to the recruitment of additional trans-acting factors to the AUF1-ARE complex.

**EXPERIMENTAL PROCEDURES**

**RNA Substrates**—RNA substrates used in this study are listed in Table I. Each RNA oligonucleotide (2'-hydroxyl) was synthesized, deprotected, and purified by ion exchange high performance liquid chromatography by Integrated DNA Technologies (Coralville, IA). The purity and integrity of each RNA substrate were verified by matrix-assisted laser desorption-ionization mass spectrometry. Dried RNA pellets were dissolved in 10 mM Tris-HCl (pH 8.0) and quantified by absorbance spectroscopy. Estimates of RNA extinction coefficients at 260 nm were calculated as described (23). For fluorescein (Fl)-conjugated RNA probes, absorbance due to the fluorophore at 260 nm was subtracted from total measured absorbance at 260 nm by quantitation of the Fl moiety at 493 nm using a Fluorolog-3 (Jobin Yvon, Edison, NJ) with fluorescein excitation (480 nm) and emission (520 nm) filters. Ten anisotropy measurements were taken for each binding reaction. Since association of Fl-RNA with RNA substrates (22), the total measured anisotropy (Aₜ) was interpreted as a function of the intrinsic anisotropy (Aᵢ) and fractional concentration (fᵢ) of each fluorescent species as described in Equation 1 (26–28).

$$Aₜ = Aᵢ + AᵢKᵢ[Pᵢ] + AᵢKᵢ[Pᵢ]²$$

(Eq. 1)

where $Aᵢ$, $AᵢKᵢ$, and $AᵢKᵢ²$ represent the intrinsic anisotropy values of the free RNA (R), AUF1 dimer-bound RNA (PᵢR), and AUF1 tetramer-bound RNA (PᵢR), respectively. All equation parameters were solved by nonlinear regression of $Aₜ$ versus $[Pᵢ]$ data sets using PRISM version 2.0 (GraphPad, San Diego, CA) as described (20).

**Analysis of RNA Folding by RET**—Conformational changes in RNA substrates were evaluated by changes in the intramolecular distance between their 5' and 3' termini. This distance was monitored by RET through a fluorescent species as described in Equation 4 (22). Association of RNA substrates by measuring the decrease in fluorescence emission of the RET donor (λₑ = 490 nm, λₑm = 518 nm, 5-nm bandwidth) in the presence of the acceptor (29, 30). The Förster distance for the Fl-Cy3 fluorophore pair conjugated to DNA has been calculated as 55.7 Å (31).

$$R₀ = 9780(n²)/4Qₜλᵢλₑ$$

(Eq. 4)

where $n$ is the orientation factor for dipole-dipole coupling (typically 5%), $Qₜ$ is the quantum yield of the donor in the absence of the acceptor, and $λᵢλₑ$ is the overlap integral between donor emission and acceptor excitation (29, 30). The Förster distance for the Fl-Cy3 fluorophore pair conjugated to DNA has been calculated as 55.7 Å (31).

In this study, $R₀$ was determined for double-labeled RNA substrates by measuring the decrease in fluorescence emission of the RET donor ($λₑ = 490 nm, λₑm = 518 nm, 5-$nm bandwidth) in the presence of the acceptor (29, 32). Accordingly, all experiments required the assembly of three parallel samples: (i) a blank containing no fluorophore, (ii) the donor in the absence of the acceptor (TNF-FI or U₃₂-FI), and (iii) the donor in the presence of the acceptor (Cy-TNF-FI or Cy-U₃₂-FI).

**Preparation of Recombinant AUF1 and Assessment of RNA Binding Activity**—Recombinant His₆-p37AUF1 was purified by Ni²⁺ affinity chromatography from lysates of E. coli Top10 cells transformed with pBAD/HisB-p37AUF1 following arabinose induction as described (22). Desalted protein preparations were quantified by comparison of Coomasie Blue-stained SDS-polyacrylamide gels containing His₆-p37AUF1 and a titration of bovine serum albumin as described (25).

The RNA binding activity of His₆-p37AUF1 preparations was evaluated by measuring changes in the fluorescence anisotropy of 5'-Fl-labeled RNA substrates essentially as described (20, 22). Binding reactions contained varying amounts of His₆-p37AUF1 and 0.2 μM Fl-RNA in a total volume of 100 μl containing 10 mM Tris-HCl (pH 8.0), 50 mM KCl, 2 mM dithiothreitol (DTT), 0.5 mM EDTA, and 0.1 μg/μl acetylated bovine serum albumin. Heparin (1 μg/μl) was required to inhibit non-specific association of AUF1 with RNA substrates (22). Fluorescence anisotropy was measured using a Beacon 2000 Variable Temperature Fluorescence Polarization System (Panvera, Madison, WI) with fluorescein excitation (490 nm) and emission (535 nm) filters. Ten anisotropy measurements were taken for each binding reaction. Since association of His₆-p37AUF1 with Fl-RNA and Fl-U₃₂ does not significantly alter the fluorescence quantum yields of these RNA substrates (22), the total measured anisotropy (Aₜ) was interpreted as a function of the intrinsic anisotropy (Aᵢ) and fractional concentration (fᵢ) of each fluorescent species as described in Equation 1 (26–28).

$$Aₜ = Aᵢ + AᵢKᵢ[Pᵢ] + AᵢKᵢ²[Pᵢ]$$

(Eq. 2)
Cation- and AUF1-dependent RNA Remodeling

Subtraction of blank sample fluorescence from donor samples lacking or containing the RET acceptor yielded \( F_D \) and \( F_{DA} \), respectively. \( E_{RET} \) was then calculated using Equation 5 (30, 33).

\[
E_{RET} = 1 - \left( \frac{F_{DA}}{F_D} \right)
\]

All fluorescence readings were taken using a Cary Eclipse fluorescence spectrophotometer (Varian Instruments, Walnut Creek, CA), with accessories indicated where applicable.

For assays of ion-dependent RNA folding, samples (0.2-m final volume) were assembled exactly as described for ion-dependent RNA folding experiments in this work has been limited to the number (\( R \)). Preliminary kinetic analyses measuring ion-induced changes in degree of denaturation experiments in this work has been limited to the number (\( R \)).

Thermal melting experiments were performed using the Peltier multiscill holder and temperature controller (Varian). Samples (2 ml) were assembled exactly as described for ion-dependent RNA folding experiments, except that preliminary incubations were performed at 5-10°C for 10 min. Temperature was then ramped to 80°C at 1°C/min using Eclipse Thermal Application software (Varian), with fluorescence readings taken every 0.5°C. A limitation of evaluating thermal denaturation of RNA by RET is that the value of \( R \) may be strongly influenced by temperature. Significant changes in the values of both \( Q_0 \) and \( J(\alpha) \) (Equation 4) were observed for the FI-Cy3 donor-acceptor pair at elevated temperatures (>40°C, data not shown), thus precluding the assignment of interferocrophore distances (\( r \)) or RNA folding enthalpy (\( \Delta H \)) in these experiments. As such, the interpretation of thermal denaturation experiments in this work has been limited to the number of RNA structural transitions under different reaction conditions and an approximation of the melting temperatures (\( T_m \)) of each transition.

For protein-induced RNA folding experiments, samples were assembled as described for assessment of RNA binding activity by fluorescence anisotropy (see above), except that the full volume was made to 2 ml and the concentration of fluorescent RNA substrate was increased to 2 nm. Samples lacking Mg\(^{2+}\) contained 6 mM EDTA. The increase in RNA concentration was necessary to improve signal-to-noise in the assay but required the employment of additional algorithms to determine the fractional concentration of each complex in RET binding assays containing titrations of recombinant AUF1 (for details, see below).

Data Analysis—Previously, we characterized ion-dependent folding of the TNF\(_\alpha\) ARE in terms of a two-component system, based on changes in the anisotropy of fluorophores conjugated to the 5'- or 3'-ends of the RNA (22). In this system, the RNA substrate was considered to be in equilibrium between a flexible, ion-free state and a structurally constrained, ion-associated state. Application of this model to changes in \( E_{RET} \) values for double-labeled RNA substrates across a titration of cation concentrations (\( [\text{Ion}] \)) allowed these data to be resolved by a variant of the Hill model (34) given in Equation 7.

\[
E_{RET} = E_a + (E_i - E_a) \times \left( \frac{[[\text{Ion}] / [\text{Ion}]_{1/2}]}{1 + [\text{Ion}] / [\text{Ion}]_{1/2}} \right)
\]

Here, \( E_a \) and \( E_i \) represent the intrinsic values of \( E_{RET} \) for the unfolded and folded RNA conformations, respectively, while \( \alpha \) represents the Hill coefficient, and \([\text{Ion}]_{1/2}\) represents the concentration of ion yielding 50% folded RNA. Values for all constants were derived by nonlinear regression of \( E_{RET} \) versus [\text{Ion}] plots using PRISM version 2.0 (GraphPad).

Using the sequential dimer binding model for UAF1 oligomerization on an RNA substrate, \( K_1 \) and \( K_2 \) were solved by nonlinear regression of anisotropy versus [\text{P}] plots using Equation 2 as described above. However, to estimate the fractional concentrations of AUF1-RNA complexes in RET experiments, a higher concentration of RNA (2 nm) was necessary to improve the signal/noise ratio. As such, \([\text{P}]_{\text{total}} \sim [\text{P}]_{\text{free}} \) would be invalid. Accordingly, an equation set lacking this approximation was used to determine the concentration of each fluorescent species during RET experiments involving titrations of AUF1, when \( K_1 \) and \( K_2 \) are known. By conservation of mass, the concentrations of all RNA and protein components of these binding reactions are related by Equations 8 and 9. The equilibrium relationships describing \([\text{P}]_1 \) and \([\text{P}]_2 \) in terms of \([\text{P}]_{\text{free}} \) are given by Equations 10 and 11.

\[
[P]_{\text{total}} = [P]_{\text{free}} + [P]_1 + [P]_2
\]

\[
[R]_{\text{total}} = [R]_{\text{free}} + [R]_1 + [R]_2
\]

\[
[P]_1 = K_1[R]_{\text{total}}[P]_{\text{free}}
\]

\[
[P]_2 = K_2[R]_{\text{total}}[P]_{\text{free}}^{1/2}
\]

All real solutions of \((P\_2_{\text{free}}, R\_1_{\text{free}}) \sim (0,0)\) for this system of equations were determined for given values of \([P]_{\text{total}}, [R]_{\text{total}}, K_1, \) and \( K_2 \) using Mathematica version 4.1 (Wolfram Research, Champaign, IL). Calculated values of \([P]_1 \) and \([R]_2 \) were then used to derive \([P]_1 \) and \([P]_2 \) using Equations 10 and 11, which were then expressed as fractional concentrations of \([R]_{\text{total}}\).

RESULTS

Utility of RET in Characterization of Mg\(^{2+}\)-induced RNA Folding Events—Initial experiments were performed to validate the use of RET to monitor folding of RNA substrates. Following excitation at 490 nm, the F1 moiety may release energy by quantum emission (\( \lambda_{\text{max}} = 518 \) nm) or by nonquanti-
gen events, including molecular contact with a quenching compound (Fig. 1A). However, if an appropriate acceptor (Cy3) is present in suitable proximity to the excited FI group, energy may be transferred from FI to Cy3 by RET, a long range dipole-dipole interaction that effects acceptor excitation without the transfer of a photon (29, 30). This nonradiative transfer of energy to Cy3 results in a decrease in fluorescence emission from FI, allowing the efficiency of this process to be calculated using Equation 5.

Previously, we demonstrated that Mg\(^{2+}\)-inhibited association of AUF1 with the TNF\(_\alpha\) ARE (22). Inhibition of protein binding occurred concomitantly with a cation-dependent conformational change in the RNA substrate that decreased its flexibility. To verify that Mg\(^{2+}\)-induced changes in ARE conformation could be resolved by RET, the emission spectra (\( \lambda_{\text{max}} = 490 \) nm) of RNA substrates containing the TNF\(_\alpha\) ARE linked to either the donor alone (TNF-FI), the acceptor alone (Cy-TNF), or both fluorophores (Cy-TNF-FI) were evaluated in the presence or absence of Mg\(^{2+}\) (Fig. 1B). Three observations confirmed the utility of RET for monitoring these conformational changes in the TNF\(_\alpha\) ARE. First, FI emission (\( \lambda_{\text{max}} = 518 \) nm) of Cy-TNF-FI is dramatically decreased in the presence of 1 mM Mg\(^{2+}\), indicating that energy from this fluorophore is being lost to nonquantum events in the presence of the cation. Second, the Mg\(^{2+}\)-dependent diminution of FI emission is not observed for RNA substrates lacking the acceptor moiety (TNF-FI). This demonstrates that the loss of FI emission in the double-labeled substrate is not due to quenching interactions with the RNA sequence, Mg\(^{2+}\), or other components of the reaction mixture. Third, the loss of FI emission from Cy-TNF-FI in the presence of Mg\(^{2+}\) is accompanied by an increase in emission from Cy3, detectable at 564 nm. Based on the panel displayed in Fig. 1B, total fluorescence emission of Cy-TNF-FI at this wavelength is clearly not increased in the presence of Mg\(^{2+}\). However, the enhancement of Cy3 emission is obvious when total fluorescence intensity is corrected for emission from FI (Table II). In the absence of Mg\(^{2+}\), the emission of Cy-TNF-FI at 564 nm is attributable solely to the sum of FI and Cy3 emission at this wavelength resulting from direct excitation of each fluorophore at 490 nm (Table II, -Mg\(^{2+}\) column). In the presence of Mg\(^{2+}\), however, the contribution of FI emission from Cy-TNF-FI at 564 nm is very small, based on a
proportional decrease in Fl emission at 518 nm. Since the emission of Cy3 resulting from direct excitation at 490 nm is not enhanced by the presence of Mg\(^{2+}\), the Mg\(^{2+}\)-induced increase in Cy3 emission from Cy-TNF-Fl at 564 nm was calculated based on the intensity of Cy-TNF-Fl fluorescence at 518 nm and the ratio of fluorescence intensities of the Fl moiety at 564 nm versus 518 nm, calculated using the single-labeled TNF-Fl substrate. The \( F_{564}/F_{518} \) ratio for this fluorophore (0.239) is not significantly affected by the presence of 1 mM Mg\(^{2+}\) (data not shown). There is no significant emission from Cy3 at 518 nm (Fig. 1B).

Additional experiments were performed to ensure that changes in RET efficiency reflected global changes in RNA conformation, rather than modulation of the dipole-dipole interaction by inhibition of fluorophore mobility (29, 30). First, the anisotropy of the Fl moiety of TNF-Fl was compared in the absence and presence of 1 mM Mg\(^{2+}\) (cf. 0.038 ± 0.001 in 0.5 mM EDTA versus 0.057 ± 0.001 in 1 mM MgCl\(_2\), \( n = 3 \)). In both cases, the anisotropy of the Fl moiety is very low, indicating that Fl retains most of its rotational freedom in the presence of Mg\(^{2+}\), allowing its orientation to be effectively randomized relative to the Cy3 acceptor. The small increase in Fl anisotropy observed in the presence of Mg\(^{2+}\) is probably due to conformational restraint within the RNA itself in the presence of the cation (22). In a second experiment, the fluorescence emission spectrum of each RNA substrate reported in Fig. 1B was reevaluated, but with the addition of excitation and emission polarizers set at 54.7° relative to each other. This “magic angle” polarizer arrangement is effective for minimizing the contribution of linear polarization of fluorophores linked to highly structured nucleic acids, such as the double helical domains of the hairpin (35) and hammerhead (36) ribozymes. In this case, however, the inclusion of polarizers resulted in a large decrease in sensitivity but did not detectably alter the spectral characteristics of any of the fluorescent TNFs ARE RNA substrates tested either in the absence or presence of Mg\(^{2+}\) (data not shown). Taken together, these experiments indicate that the Fl moiety exhibits sufficient rotational freedom in this context that dipole-dipole interactions with Cy3 resulting from linear polarization of the RNA molecule are likely to be minimal. Accordingly, we have interpreted the loss of Fl emission in the presence of Cy3 and Mg\(^{2+}\) in this system as RET between a fluorescent donor-acceptor pair as a consequence of ion-dependent changes in RNA structure. Based on Equation 3, this implies the adoption of a condensed RNA conformation in the presence of the cation.

**Folding of the TNFs ARE Is Consistent with a Single Conformational Transition That Is Cooperatively Stabilized by Mg\(^{2+}\)**—Following validation of the RET system for monitoring ion-induced conformational changes in the TNFs ARE, thermal denaturation experiments were performed to determine whether multiple RNA structural transitions might be contributing to the ion-folded state (37, 38). Samples containing the Cy-TNF-Fl substrate with or without Mg\(^{2+}\) were slowly heated, and \( E_{\text{RET}} \) was monitored as a function of temperature. Unfolding of the condensed ARE structure was detected by a temperature-dependent decrease in \( E_{\text{RET}} \), resulting from an increase

**TABLE II**

| Emission source | \(-\text{Mg}^{2+}\) | \(+\text{Mg}^{2+}\) (1 mM) |
|-----------------|-----------------|-----------------|
| \( F_{\text{total}} \) | 18.2 | 17.7 |
| \( F_{\text{Cy3-direct}} \) | 9.1 | 8.6 |
| \( F_{\text{Fl-direct}} \) | 9.5 | 1.5 |
| Difference (\( F_{\text{Cy3-RET}} \)) | -0.4 | 7.6 |

\( ^a \) \( F_{\text{total}} \) was measured at 564 nm using the Cy-TNF-Fl substrate.

\( ^b \) \( F_{\text{Cy3-direct}} \) was measured at 564 nm using the Cy-TNF substrate.

\( ^c \) In the absence of Mg\(^{2+}\), \( F_{\text{Fl-direct}} \) was measured at 564 nm using the TNF-Fl substrate. In the presence of Mg\(^{2+}\), the intensity of Fl emission from Cy-TNF-Fl at 564 nm was calculated based on the intensity of Cy-TNF-Fl fluorescence at 518 nm and the ratio of fluorescence intensities of the Fl moiety at 564 nm versus 518 nm, calculated using the single-labeled TNF-Fl substrate. The \( F_{564}/F_{518} \) ratio for this fluorophore (0.239) is not significantly affected by the presence of 1 mM Mg\(^{2+}\) (data not shown). There is no significant emission from Cy3 at 518 nm (Fig. 1B).

**FIG. 1**. Evaluation of RNA folding by RET. A, a schematic of an RNA substrate labeled at its 3′-end with Fl (RET donor) and at its 5′-end with Cy3 (acceptor). Quantum and nonquantum events resulting from excitation of the fluorophores at 490 nm are depicted by arrows and are described under “Results.” B, emission spectra of fluorescent RNA substrates excited at 490 nm. Samples (2 ml) contained 20 nM RNA in 10 mM Tris-HCl (pH 8.0) and 2 mM DTT in the presence of 0.5 mM EDTA (solid lines) or 1 mM MgCl\(_2\) (dotted lines). Spectra were collected at 22 °C across \( \lambda_{\text{exc}} = 500–600 \) nm in 1-nm intervals using a 5-nm bandwidth. Eight fluorescence readings were taken at each interval. Each tracing represents the average of five scans corrected for solvent background fluorescence.
in the distance between the 5’ and 3’ termini of the RNA. Thus, if RNA unfolding requires multiple structural transitions, increasing temperature would be expected to effect stepwise changes in $E_{\text{RET}}$ with each step corresponding to a distinct change in RNA conformation. This analysis is simplified by plotting the partial derivative of $E_{\text{RET}}$ as a function of temperature (Fig. 2). Here, each structural transition may be identified as an individual peak, positioned about its characteristic melting temperature ($T_m$). For this study, we have approximated the $T_m$ of each RNA structure as the temperature at which $E_{\text{RET}}$ varies maximally. By this definition, a local maximum in the derivative plot corresponds to the $T_m$ of an individual structural transition.

In the absence of Mg$^{2+}$, the Cy-TNF-Fl substrate exhibited a single transition between a condensed RNA conformation (high RET) and an extended conformation (low RET) (Fig. 2, solid circles). The condensed structure was very weak, with $T_m = 10^\circ$C. The appearance of this folded RNA structure at low temperatures in the absence of added Mg$^{2+}$ suggests that the TNFα ARE may exist in equilibrium between an unfolded state and an unstable, folded state, independent of divalent cations. In the presence of increasing concentrations of Mg$^{2+}$, however, the condensed RNA conformation was stabilized, presenting $T_m$ values of 26°C at 0.1 mM Mg$^{2+}$ and 34°C at 1 mM Mg$^{2+}$ (Fig. 2, open circles and triangles, respectively). The appearance of a single derivative peak in all thermal denaturation experiments indicates that one conformational transition probably accounts for the temperature-dependent change in RET observed for this RNA substrate. Given the minimal degree of self-complementarity in the TNFα ARE, this conformational change is unlikely to include significant contributions from Watson-Crick base pairs and hence probably reflects a tertiary RNA structural event.

To further characterize the dependence of Mg$^{2+}$ in stabilization of the folded TNFα ARE, $E_{\text{RET}}$ of Cy-TNF-Fl was measured at 22°C across a range of Mg$^{2+}$ concentrations (Fig. 3A). Assuming a two-state model for this RNA substrate (unfolded versus folded), the sensitivity of the condensed Cy-TNF-Fl structure for [Mg$^{2+}$] was well described by the Hill model, with half-maximal folding of RNA observed with 75 ± 2 μM Mg$^{2+}$ (mean ± 95% confidence interval) at this temperature. The Hill coefficient resolved to 1.7 ± 0.1, demonstrating that stabilization of the folded Cy-TNF-Fl substrate involved multiple magnesium ions associating in a cooperative manner. In the absence of Mg$^{2+}$, $E_{\text{RET}}$ resolved to <0.1 for this RNA substrate, indicating an average interfluorophore distance of >80 Å by Equation 3. Based on the regression solution for Equation 7, $E_{\text{RET}}$ for the folded RNA ($E_f$) resolved to 0.902 ± 0.005, giving an average distance of 38 ± 1 Å between the fluorescent donor and acceptor in this structure. Unlike the Cy-TNF-Fl substrate, however, $E_{\text{RET}}$ of Cy-U32-Fl displayed only modest changes with increasing [Mg$^{2+}$], consistent with previous findings that the flexibility of an RNA substrate containing U32 is only minimally affected by this cation (22). This dramatic difference in the structural sensitivity of Cy-TNF-Fl versus Cy-U32-Fl to Mg$^{2+}$ indicates that adoption of a folded structure in the TNFα ARE is dependent upon the ARE sequence. When coupled with the observation that Mg$^{2+}$ does not inhibit association of AUF1 to U32 substrates (22), these data further support the hypothesis that it is the Mg$^{2+}$-dependent change in the structure of the TNFα ARE that is largely responsible for inhibition of AUF1 binding activity to this substrate in the presence of the cation.

**Ion Dependence of ARE Folding**—In addition to Mg$^{2+}$, many other cations can induce or stabilize higher order RNA structures. The cation preference of an RNA structure is strongly dependent on the nature of the interactions between the ions and the RNA substrate (39–43). Accordingly, we measured the ability of several different ions varying in both valence and charge density to stabilize the folded state of Cy-TNF-Fl. For each cation, both the Hill coefficient and the ion concentration...
Corresponding to half-maximal RNA folding were calculated using Equation 7 (Table III). Stabilization of the folded TNF-ARE structure was well described by the Hill model for all ions tested (Fig. 3B). The Hill coefficient for each ion titration resolved to >1, indicating that cooperative association of multiple ions was responsible for stabilization of the folded RNA substrate. The upper limit of E_{RET} (given by E_{Hill} in Equation 7) was also similar for all ions tested, consistent with an average distance of 38 ± 2 Å between the folded RNA donor and acceptor fluorophores. The appearance of similar scalar distances between the termini of all ion-stabilized Cy-TNF-Fl structures suggests that a common or closely related RNA conformation is stabilized by each ion.

Among small inorganic cations, a strong correlation was observed between the charge of the ion and the efficiency with which it stabilized the folded Cy-TNF-Fl substrate. The most efficient ARE folding was observed with Co(III)H_{6}^{3+}, which produced half-maximal RNA folding at 77 ± 2 mim. Co(III)H_{6}^{3+} is a trivalent analog of hydrated magnesium ion (Mg(H_{2}O)_{6}^{2+}) and effectively induces or stabilizes a variety of RNA structures (42–45). Comparing all inorganic ions tested, a +1 change in the valence of the cation decreased the ion concentration producing half-maximal RNA folding by ~1000-fold (Table III; cf. Co(III)H_{6}^{3+} versus Mg^{2+}, Ca^{2+}, Mn^{2+} versus Na^{+}, K^{+}). In addition to valence, the distribution of positive charges within the cation also influenced its ability to stabilize the folded ARE substrate. Substitution of spermine^{3+} for Co(III)H_{6}^{3+} resulted in a 17-fold increase in the concentration of cation required for the transition midpoint. Like other di- and trivalent metal ions, Co(III)H_{6}^{3+} presents a high concentration of positive charge, while the positive charges of spermine^{3+} are distributed across a span of ~13 Å (42). Taken together, the ion titration experiments demonstrated that folding of the TNF-ARE into its spatially condensed conformation is most effectively stabilized by ions possessing multiple, densely packed positive charges. The implications of these ion preferences are detailed further under “Discussion.”

### RNA Conformational Changes in Response to AUF1 Binding and Oligomerization

—While divalent cation-stabilized folding of the TF20 ARE inhibits the association of AUF1 to this RNA substrate (22), the RNA structural consequences of AUF1 ARE binding events were unknown. A series of experiments was designed to investigate the potential of AUF1 to modulate the conformation of RNA substrates, with particular emphasis on distinguishing RNA conformational changes resulting from the initial AUF1 binding event versus subsequent oligomerization of the protein on the RNA. To achieve this objective, it was first necessary to establish fractional concentration profiles of AUF1-RNA complexes across a titration of protein concentrations, based on the solutions of equilibrium binding constants describing their assembly. Subsequently, E_{RET} measurements of double-labeled RNA substrates were taken across the same AUF1 titrations. Correlations between changes in E_{RET} and the fractional concentrations of individual AUF1-RNA complexes would thus indicate which, if any, stage of complex assembly coincided with conformational changes in RNA substrates.

The ability of recombinant His_{6}-p37AUF1 to bind and oligomerize on RNA substrates containing the TNF-ARE or U_{32} was determined by measurements of fluorescence anisotropy of 5'-Fl-labeled RNA substrates across a titration of protein concentrations (Fig. 4A). In the absence of divalent cations, association of AUF1 with these substrates is well described by sequential binding of protein dimers to form a tetrameric AUF1 complex on the RNA, with distinct equilibrium binding constants (K_{1} and K_{2}) describing each phase of tetramer assembly (22). This model allowed values of K_{1} and K_{2} for each RNA substrate to be determined by nonlinear regression of measured anisotropy versus the concentration of His_{6}-p37AUF1 dimer using Equation 2 as described under “Experimental Procedures.” In binding reactions containing the Fl-TNF-ARE substrate, triplicate AUF1 titrations yielded equilibrium binding constants of 1.06 ± 0.09 × 10^{-5} M^{-1} and 4.0 ± 0.8 × 10^{-5} M^{-1} for K_{1} and K_{2}, respectively. Similar experiments with the Fl-U_{32} substrate gave binding constants of 2.4 ± 0.4 × 10^{-4} M^{-1} and 1.3 ± 0.4 × 10^{-3} M^{-1} for K_{1} and K_{2}, respectively. Based on the solutions of these equilibrium binding constants, distribution profiles of RNA-protein complexes were constructed for binding reactions containing either Fl-TNF-ARE or Fl-U_{32} substrates and selected concentrations of AUF1 (Fig. 4B), as described under “Experimental Procedures.”

#### Tables

**Table III**

| Ion          | Hill coefficient | [I_{on}]_{1/2} |
|--------------|------------------|----------------|
| Co(H_{2}O)_{6}^{3+} | 2.2 ± 0.1        | 7.7 ± 0.2 × 10^{-8} |
| Spermidine^{3+}   | 1.8 ± 0.1        | 1.31 ± 0.06 × 10^{-6} |
| Mg^{2+}       | 1.7 ± 0.1        | 7.5 ± 0.2 × 10^{-5} |
| Ca^{2+}      | 1.9 ± 0.1        | 4.3 ± 0.2 × 10^{-5} |
| Mn^{2+}      | 1.32 ± 0.06     | 3.8 ± 0.2 × 10^{-5} |
| Na^{+}       | 1.8 ± 0.2       | 6.0 ± 0.3 × 10^{-5} |
| K^{+}        | 1.6 ± 0.1       | 6.1 ± 0.3 × 10^{-2} |

*The Hill coefficient (h) and concentration of cation associated with half-maximal RNA folding ([I_{on}]_{1/2}) were calculated by application of Equation 7 to the E_{RET} versus [cation] data sets presented in Fig. 3B. Each constant is expressed as the mean ± 95% confidence interval of triplicate data sets solved by simultaneous nonlinear regression.
ARE substrate. By contrast, $E_{RET}$ of Cy-U32-Fl (Fig. 4C, right panel) increased only slightly with the formation of P2R ([AUF1] = 10 nM dimer) and rather appeared to increase concomitantly with the concentration of P4R (Fig. 4B, right panel). While these differences in RNA folding may reflect altered binding characteristics of AUF1 to ARE versus poly(U) substrates, limited RNA folding in the Cy-U32-Fl P2R complex may alternatively result from the homogeneous nature of the poly(U) sequence. AUF1 binding to Cy-U32-Fl probably generates an extensive population of binding complexes, due to the multiplicity of potential AUF1-binding sites on the U32 substrate. As such, it is possible that the influence of AUF1 binding on overall $E_{RET}$ in the P4R state is minimized by the presence of subpopulations where AUF1 associates close to the ends of the RNA substrate, where localized folding might have little effect on the overall scalar distance between fluorophores linked to the 5′ and 3′ termini of the RNA. In any case, these experiments demonstrate that binding and/or oligomerization of AUF1 with an RNA substrate induces conformational changes in the RNA that are condensed relative to an unfolded RNA.

An additional control experiment was prompted by the observation that AUF1 binding to Fl-labeled TNFα ARE or U32 substrates significantly increases the anisotropy of the Fl moiety (Fig. 4A). To verify that linear polarization artifacts resulting from AUF1 binding were not contributing to RET between the fluorophores of the double-labeled RNA substrates, $E_{RET}$ of binding reactions containing the highest concentration of AUF1 (250 nM protein dimer) and either Cy-TNF-Fl or Cy-U32-Fl was measured with or without “magic angle” polarizers. In both cases, the observed value of $E_{RET}$ was not significantly affected by the inclusion of polarizers (data not shown). These observations demonstrated that any contributions of linear polarization of these RNA substrates to RET are likely to be minimal (29, 30). Accordingly, increases in $E_{RET}$ of Cy-TNF-Fl and Cy-U32-Fl with increasing AUF1 concentrations are consistent with protein-induced changes in the conformation of the RNA substrates.

Since AUF1 binding induced the formation of a spatially condensed RNA structure on an otherwise largely unfolded RNA substrate, an experiment was performed to determine how association of AUF1 might modulate the structure of a cation-stabilized, folded RNA. We have described the association of AUF1 with the TNFα ARE in the presence of Mg2+ by a convergent binding model (22), which accounts for the ion-dependent inhibition of AUF1 binding in terms of a rapid equilibrium between unfolded and ion-stabilized RNA folded states and decreased affinity of the initial AUF1-binding event for the
folded RNA substrate (Fig. 5A). Furthermore, this model predicted the ejection of Mg\(^{2+}\) prior to formation of the AUF1 tetramer-ARE complex. In the absence of AUF1, the Cy-TNF-FI substrate exhibited high \(E_{\text{RET}}\) in the presence of 2 mM Mg\(^{2+}\). Maintenance of the Cy3 and Fl groups in close proximity is thermodynamically unfavorable event that also contributes to inhibition of AUF1 oligomerization in the presence of this cation. The model also predicts that, prior to association with AUF1 in the presence or absence of Mg\(^{2+}\), there are two principal observations. First, thermal denaturation experiments suggest that the RNA most effectively because they can neutralize a greater number of negative charges within a smaller space. Average distances between the fluorescent donor-acceptor pairs were calculated using Equation 3 from figures of two independent experiments.

DISCUSSION

For decades, studies have addressed the ability of cations to electrostatically induce or stabilize specific higher order RNA structures (46–48). Laing et al. (40) have characterized three distinct classes of metal binding sites contributing to the maintenance of RNA structures. First, cations may associate non-specifically to RNA, exemplified by the stabilization of hairpin structures as ionic strength increases. Second, a cation may coordinate directly to a specific binding site on the RNA, generally distinguished by a significant preference for one particular ion over others of comparable size or valence. Ion-specific binding of both monovalent (41) and divalent cations (40,49) to RNA substrates has been documented. Such specific ion-RNA contacts may also exist within a subset of catalytic RNAs, for which tertiary structures may be stabilized by a spectrum of ions, but where catalysis is largely Mg\(^{2+}\)-dependent (42, 45). In a third class of metal ion-RNA interaction, several different divalent ions may effectively associate with relatively high affinity to distinct regions of high negative charge density in the folded RNA structure. RNA folding generates high local concentrations of negative charge from the juxtaposition of backbone phosphates, which can neutralize multivalent cations, but does not require specific RNA contacts with the ion. This mode of ion-RNA interaction has been characterized in the structural stabilization of several RNA tertiary structures (42–45).

Cation-stabilized folding of the TNF\(\alpha\) ARE is most consistent with the third class of ion-RNA interaction (Fig. 6), based on two principal observations. First, thermal denaturation experi-

**Fig. 5.** Modulation of the Mg\(^{2+}\)-folded TNF\(\alpha\) ARE structure by association of AUF1. A, the convergent binding model for AUF1 binding and oligomerization on the TNF\(\alpha\) ARE was derived from differences in the anisotropy profiles of fluorescent TNF\(\alpha\) ARE substrates associating with AUF1 in the presence or absence of Mg\(^{2+}\) (22). In the presence of Mg\(^{2+}\) the TNF\(\alpha\) ARE is in equilibrium between an ion-free, unfolded state (R) and a folded state (R’), stabilized by the association of multiple \((x)\) magnesium ions. Both of these forms may associate with an AUF1 dimer to generate P\(_R\) and P\(_{R’}\), respectively, but R’ exhibits 6-fold lower affinity for the protein. This model also predicts that, prior to association of a second AUF1 dimer to form the tetramer-associated RNA (P\(_R\), P\(_{R’}\) must unfold and eject its associated Mg\(^{2+}\) ions, a thermodynamically unfavorable event that also contributes to inhibition of AUF1 oligomerization in the presence of this cation. B, \(E_{\text{RET}}\) was measured for Cy-TNF-FI in binding reactions containing various concentrations of AUF1 in the presence of 2 mM Mg\(^{2+}\). Each point represents the mean ± spread of two independent experiments.

**Fig. 6.** Folding of the TNF\(\alpha\) ARE stabilized by counterion neutralization. In this two-dimensional representation, the TNF\(\alpha\) ARE exists in a rapid equilibrium between a disordered, unfolded state (left) and a folded state resulting from sequence-specific, intramolecular contacts within the RNA (center). This folded state is unstable, due to the formation of regions of high negative charge density (boxed). Cations stabilize the folded structure by neutralizing the juxtaposed negative charges (right). Condensed, multivalent cations stabilize the folded RNA most effectively because they can neutralize a greater number of negative charges within a smaller space. Average distances between the fluorescent donor-acceptor pairs were calculated using Equation 3 from figures.
ments revealed that the Cy-TNF-F1 substrate folded, albeit weakly, in the absence of multivalent cations, demonstrating that direct cation contact was not obligate for adoption of the RNA fold. Second, several cations were capable of stabilizing the folded TNFα ARE structure; however, lower concentrations were required for trivalent versus divalent or monovalent cations (Fig. 3B, cf. Co\((\text{NH}_3)_6\)\(\text{Co}^{3+}\) versus Mg\(^{2+}\), Ca\(^{2+}\), Mn\(^{2+}\) versus Na\(^{+}\), K\(^{+}\)), and compact versus elongated cations (Fig. 3B, cf. Co\((\text{NH}_3)_6\)\(\text{Co}^{3+}\) versus spermidine\(^{3+}\)). This demonstration that the efficiency of ARE structural stabilization increases with both cation valence and charge density strongly indicates that localized regions of high negative charge density serve as cation targets in the folded ARE substrate.

Some interesting regulatory possibilities are raised by the selectivity of these ion-stabilized RNA folding events for a subset of AUF1-binding sequences. Dramatic differences between the conformations of the TNFα ARE versus the U\(_{32}\) substrate indicated that base-specific, intramolecular contacts are probably involved in formation of the folded ARE structure, possibly involving the interspersed adenosine residues within the ARE substrate. Since cation-stabilized folding of the TNFα ARE correlates with inhibition of AUF1 binding to this substrate (22), such RNA folding events may serve to modulate the accessibility of individual AREs for ARE-binding proteins. Constitutively, this mechanism may engender preferences for one ARE-binding protein over another, with resultant ARE-specific changes in mRNA decay rates or in susceptibility to regulatory signaling. Alternatively, other RNA-binding proteins associating with or near AREs may function as modulators of mRNA turnover by promoting or restricting ion-stabilized ARE structures or by altering the equilibrium between competing local RNA folding events.

Association of AUF1 with its RNA targets also correlated with the adoption of a relatively condensed RNA structure. Unlike ion-stabilized RNA folding, AUF1-induced RNA conformational changes were observed with both the TNFα ARE and U\(_{32}\) substrates, suggesting that protein binding promotes the formation of an RNA conformation distinct from that stabilized by cations. This was further indicated by the significant difference in the distance between the termini of the TNFα ARE substrate when folded by AUF1 versus cations. The relatively high affinity of AUF1 for ARE substrates (20, 22), together with the strong correlation between ARE length and AUF1 binding affinity (12), indicates that several RNA contact sites spread across a significant distance are probably utilized upon AUF1 binding. In addition, truncation of AUF1 protein domains flanking the RRMs severely inhibits binding to ARE substrates, suggesting that contacts between an ARE and AUF1 also involve dispersed regions within the protein (21). Taken together, the number and scale distribution of contact points between AUF1 and its cognate RNA substrates may be responsible for the ability of AUF1 to alter the local conformation of its RNA targets.

In addition to AUF1, other proteins containing RRMs may also modulate the local conformation of RNA targets. Two RRMs of nucleolin serve to stabilize a weak RNA stem-loop containing the nucleolin recognition element (50), while heterogeneous nuclear ribonucleoprotein A1 has also been proposed to fold RNA targets into stem-loop structures (51). In both cases, stabilization of unique RNA folded structures may reflect RNA chaperone activities for these proteins (50, 52, 53) by preventing incorrect local RNA structures that may be kinetically disadvantageous for subsequent tertiary folding events (54). By contrast, protein fragments containing either the two RRMs of sex-5/1 or the two N-terminal RRMs from poly(A)-binding protein (56) promote extended RNA conformational states when bound to cognate RNA targets. The significance of these elongated, protein-induced RNA conformations is currently unknown.

While no specific function has yet been ascribed to RNA remodeling by AUF1, additional studies of ARE-directed mRNA decay in vitro and of the cytoplasmic environment of AUF1 present some possibilities for speculation. First, AUF1 functions within the context of a multisubunit complex to promote rapid mRNA decay. In extracts from K562 cells, the mRNA destabilizing activity containing AUF1 is recovered as a large (7 S) complex (16). Recombinant or cellular AUF1 purified to homogeneity are ineffective in accelerating mRNA turnover in cell-free mRNA decay systems, indicating that ancillary factors are essential for induction of RNA catabolism. Second, co-immunoprecipitation experiments have identified other cellular factors involved in the regulation of translation and/or mRNA turnover associating with cytoplasmic AUF1 (19). Finally, recombinant AUF1 forms multimers on ARE substrates (20–22), generating an RNA-protein complex whose hydrodynamic properties indicate maximization of surface area (21). Taken together, these observations suggest that the AUF1-ARE complex serves to nucleate subsequent trans-acting factor recruitment and that AUF1-induced remodeling of local RNA structures may participate in this process by exposing critical binding determinants for association of these factors.

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