Structural Determinants in AUF1 Required for High Affinity Binding to A + U-rich Elements*

(Received for publication, July 21, 1997, and in revised form, August 27, 1997)

Christine T. DeMaria†, Yue Sun, Laura Long‡, Belinda J. Wagner¶, and Gary Brewer**

From the Department of Microbiology and Immunology and the #Department of Plastic and Reconstructive Surgery, Bowman Gray School of Medicine, Wake Forest University, Winston-Salem, North Carolina 27157-1064

AUF1 is an RNA-binding protein that contains two nonidentical RNA recognition motifs (RRMs). AUF1 binds to A + U-rich elements (AREs) with high affinity. The binding of AUF1 to AREs is believed to serve as a signal to an mRNA-processing pathway that degrades mRNAs encoding many cytokines, oncoproteins, and G protein-coupled receptors. Because the ARE binding activity of AUF1 appears central to the regulation of many important genes, we analyzed the domains of the protein that are important for this activity. Examination of the RNA binding affinity of various AUF1 mutants suggests that both RRMs may be required for binding to the human c-fos ARE. However, the two RRMs together are not sufficient. Highest affinity binding of AUF1 to an ARE requires an alanine-rich region of the N terminus and a short glutamine-rich region in the C terminus. In addition, the N terminus is required for dimerization of AUF1. However, AUF1 binds an ARE as a hexameric protein. Thus, protein-protein interactions are important for high affinity ARE binding activity of AUF1.

RNA processing is an important component of regulated gene expression in eukaryotic cells. Together, the rates of transcription, pre-mRNA splicing, mRNA transport, translation, and mRNA degradation determine the steady-state amount of mRNA, and hence protein, that will be present in a cell at a given time. Each of these processes of RNA metabolism involves RNA-binding proteins that exhibit specific protein-RNA interactions (reviewed in Ref. 1). Thus, defining how such proteins interact with their RNA substrates is integral to understanding the complex control of RNA processing.

A variety of conserved protein motifs mediate specific protein-RNA interactions (2). Perhaps the most common and well characterized of these motifs is the RNA recognition motif (RRM), also called a consensus sequence RNA-binding domain. This motif consists of 80–90 amino acids containing two conserved sequences: a highly conserved octamer motif, RNP-1, and a less conserved hexamer motif, RNP-2 (2, 3). The general structure of an RRM consists of a $\beta$-$\alpha$-$\beta$-$\alpha$-$\beta$-$\alpha$ folding pattern in a four-stranded $\beta$-sheet with the two $\alpha$-heliccs packed against one face of the sheet. The RNP-1 and RNP-2 motifs lie on the two central strands at the center of the $\beta$-sheet. These motifs probably provide general RNA binding activity. The mechanisms by which RRMs provide sequence-specific or structure-specific RNA recognition are unknown. However, recognition of specific targets is thought to be provided by unique amino acids located in intradomain loops and tails. Nonetheless, this seemingly simple view is complicated by two observations. (i) Multiple RRMs within some proteins are required for high affinity RNA binding (see Ref. 3 and references therein); and (ii) complex communication can occur between amino acids in the intradomain loops and tails (4). Thus, the efforts of many laboratories are being directed toward dissecting the molecular mechanisms of specific RNA recognition by RNA-binding proteins.

We previously molecularly cloned the RNA-binding protein AUF1. AUF1 binds with high affinity to a variety of AREs present in the 3′-UTRs of mRNAs encoding many cytokines, oncoproteins, and G protein-coupled receptors (5–8). Binding of AUF1 to an ARE is thought to act as a signal to target the mRNA for rapid degradation. Because the ARE binding activity of AUF1 appears central to the regulation of many important genes, we analyzed the domains of the protein that are important for this activity. Binding assays with various mutants suggest that both RRMs may be required for ARE binding, but they are not sufficient. Amino acids flanking the RRMs seem to contribute to ARE binding, but their contribution is not simply to maintain or allow proper folding of the protein. Instead, the flanking amino acids appear to allow protein-protein interactions that may be crucial for high affinity ARE binding activity. In particular the alanine-rich N terminus is important for homodimerization of AUF1. However, AUF1 binds an ARE as a hexameric protein. Thus, additional protein-protein interactions are involved in the high affinity ARE binding activity of AUF1.

EXPERIMENTAL PROCEDURES

All enzymes and plasmid vectors were obtained from Promega Corp. (Madison, WI) unless otherwise noted. All plasmid constructs were confirmed by both restriction analyses and either dyeoxy sequencing with Sequenase (version 2.0, U.S. Biochemical Corp.) or by cycle sequencing with Taq Polymerase (Perkin-Elmer).

Construction of Prokaryotic Expression Vectors—Wild-type and mu-
tandent AUF1 constructs were cloned into pTrcHis vectors (Invitrogen) to create fusion proteins with an N-terminal histidinyl (His6) tag followed by an epitope tag consisting of the 11-amino acid, T7 gene 10 leader peptide. Reading frames of His6-AUF1 expression constructs were confirmed by DNA sequencing at the junctions of the pTrcHis vectors and the inserts.

The entire coding region of p37+AUF1 resides on a 910-base pair BsmAI (New England Biolabs) fragment spanning nucleotides 236–1146 of the pBS8 cDNA sequence. This fragment was blunted and inserted into the Smal site of the pGEM-7Z+ vector to yield plasmid pGEM7ZP37CR (7). This construct served as the parent for various restriction fragment additions into pTrcHisB at the Asp718 (BamH I)-HindIII sites to create vectors encoding AUF1-(1–286), AUF1-(1–194), AUF1-(1–291–286), AUF1-(1–291–294), and AUF1-(1–257). Plasmid pTrcHisCAUF1-(92–286) was constructed by inserting the BglII-HindIII fragment from pGEM7ZP37CR into BglII-HindIII-digested pTrcHisAUF1-(69–229) and AUF1-(69–229) were created by PCR amplification of pGEM7ZP37CR, digesting the BglII fragment of pGEM7ZP37CR into BglII-HindIII-digested pTrcHisAUF1-(69–229) and pTrcHisCAUF1-(69–229), respectively. AUF1-(29–286) was created by PCR amplification of pGEM7ZP37CR, digesting the PCR product with KpnI and HindIII, and ligating it to KpnI-HindIII-cut pTrcHisB. Plasmid pTrcHisBAUF1-(1–229) was constructed by a three-way ligation of the Asp718-BglII fragment from AUF1-(1–229) with the BglII-HindIII fragment of pGEM7ZP37CR (202–286), and AUF1-(1–239/248–286) was created by polymerase chain reaction-directed deletion of the cDNA sequences in pTrcHisBp37CR encoding the glutamine-rich region of the protein.

### Purification of His6-AUF1 Proteins, Radiolabeling of RNA Substrates, and Determination of Apparent Ks Values—Wild-type and mutant His6-AUF1 fusion proteins were expressed and purified as described by Pende et al. (7). The concentration of each His6-AUF1 protein was estimated by comparison of the intensities of the full-length protein bands on an SDS-polyacrylamide gel. RNA containing the c-fos ARE was synthesized by transcription of BglII-digested plasmid pG919β-ARE (5), using T3 RNA polymerase and [32P]UTP (800 Ci/mmol). Apparent Ks values were determined by electrophoretic mobility shift assays and PhosphoImager analyses as described by DeMaria and Brewer (8). Free probe concentration was plotted versus His6-AUF1 concentration, and apparent Ks values were determined as the protein concentration at which 50% of the RNA was bound (9).

### Circular Dichroism Measurements—Purified wild-type His6-AUF1-(1–286) and His6-AUF1-(1–194) protein samples were diluted into a buffer containing 10 mM Tris-HCl (pH 7.0), 5 mM magnesium acetate, and 100 mM potassium acetate to a final protein concentration of 5 μM. CD spectra were recorded from 190–230 nm at room temperature on a Jasco 720 spectropolarimeter using a cuvette with a 0.05-cm path length. The ellipticity values (mdeg) were converted to mean residue ellipticity (deg cm² dmol⁻¹) according to the following formula (10): [θ] = 100 × N × α /

\[
[\text{Distance traveled from meniscus by His6-AUF1}] = \frac{s_{\text{obs}}}{s_{\text{stand}}} = \frac{[\text{Distance traveled from meniscus by standard}] - [\text{Distance traveled from meniscus by His6-AUF1}]}{[\text{Distance traveled from meniscus by standard}]} \quad (\text{Eq. 1})
\]

For each protein, the sedimentation coefficient was determined as the average of the values obtained from comparison of each of the standards in two separate experiments.

### Molecular Mass Determinations of RNA-bound and -unbound His6-AUF1 Proteins—The monomeric molecular mass of each protein was determined from the amino acid composition. The native molecular mass of each protein was calculated from the equation (14),

\[
M = 6\pi n R_N u/(1 - \nu^2) \quad (\text{Eq. 2})
\]

where \(n\) is the viscosity (g/cm•s), \(R_N\) is the Stokes radius (cm), \(N\) is Avogadro’s number, and \(s\) is the sedimentation coefficient. The partial specific volumes, \(\overset{\circ}{s}\), of His6-AUF1-(1–286), His6-AUF1-(1–194), His6-AUF1-(1–239/248–286), and His6-AUF1-(29–194) were calculated as 0.7217 ml/g, 0.7217 ml/g, 0.7301 ml/g, and 0.7270 ml/g, respectively, from amino acid composition by the method of Laue et al. (15). The solvent density, \(\rho\), was measured as 1.0059 g/ml. The frictional ratio, \(f_{fr}\), of each protein was calculated from the equation (14),

\[
f_{fr} = R_s/[(36/5)ANs^{1/2}] \quad (\text{Eq. 3})
\]

### RESULTS

Identification of the Domains of AUF1 Critical for Its High Affinity ARE Binding Activity—Using quantitative electrophoretic mobility shift assays, we showed previously that purified, recombinant His6-p37+AUF1 fusion protein binds the human c-fos ARE with an apparent \(K_s\) of 7.8 ± 0.4 nM. (The wild-type fusion protein will hereafter be referred to as His6-AUF1-(1–286).) As shown in Fig. 1, the predicted AUF1 polypeptide contains two tandem, nonidentical RRMs. Adja-
cent to RRM1 is an alanine-rich, N-terminal region of 68 amino acids. C-terminal to RRM2 is an 8-amino acid region containing six glutamine residues. This is followed by a short C-terminal region. To examine the involvement of the RRs and other regions of AUF1 in ARE binding, we constructed several N- and C-terminal truncation mutants of His6-AUF1 that were purified by Ni2+-chelate chromatography. Each purified recombinant fusion protein was tested for binding to the wild-type, 32P-labeled c-fos ARE by gel mobility shift assays. The dissociation constant, or apparent $K_d$, was calculated for each protein by determining the protein concentration at which 50% of the RNA probe was bound (8).

His6-AUF1(92–286) and His6-AUF1-(1–194) lack a portion of either RRM1 or RRM2, respectively, and these mutant proteins bound the c-fos ARE with respective 100- and 70-fold lower affinities than wild-type His6-AUF1 (Fig. 1). These results suggest that both RRs are important for high affinity ARE binding. In support of this suggestion, His6-AUF1(1–257), which retains both RRs but lacks the C-terminal 29 amino acids, bound the c-fos ARE with an apparent $K_d$ of 5.3 nM, similar to the 7.8 nM apparent $K_d$ for wild-type His6-AUF1 (Fig. 1; Ref. 8). However, both RRM1 and RRM2 together are not sufficient for ARE binding, since His6-AUF1(69–229) bound the c-fos ARE with 280-fold lower affinity than the wild-type protein. Nonetheless, the necessity of the RRs is demonstrated by a mutant, His6-AUF1(1–29/195–286) (depicted in Fig. 4), in which most of the amino acids comprising the RRs were deleted. Comparison of this mutant with the wild-type protein using UV-cross-linking analysis with 32P-labeled c-fos ARE revealed undetectable binding by His6-AUF1(1–29/195–286) (data not shown). Together, these experiments indicate that the RRs of AUF1 are necessary for ARE binding, but they are not sufficient.

Since the C-terminal 29 amino acids of AUF1 are dispensable for high affinity ARE binding, the importance of both the N-terminal and glutamine-rich regions for ARE binding was examined. His6-AUF1(69–257) bound the c-fos ARE with 180-fold lower affinity than the wild-type protein (Fig. 1). His6-AUF1(69–257) and His6-AUF1(1–257) differ only by the presence of the N-terminal region in the latter; therefore, the N-terminal region of AUF1 is required for high affinity ARE binding activity. His6-AUF1(1–229) was then created to assess the importance of amino acids C-terminal to RRM2 for ARE binding. His6-AUF1(1–229) bound the c-fos ARE with an affinity of 207 nM, 27-fold lower than the affinity displayed by the wild-type protein (Fig. 1). Thus, amino acids C-terminal to RRM2 are important for high affinity ARE binding. To determine if the glutamine-rich region contributes to high affinity binding, this 8-amino acid region (amino acids 240–247) was deleted from the full-length protein. This mutant, His6-AUF1(1–239/248–286), bound the c-fos ARE with an apparent $K_d$ of 36 nM, a 4.5-fold lower affinity than that of the wild-type protein (Fig. 1). This result suggests that the glutamine residues located in this domain do contribute to the ARE binding activity of AUF1. However, based upon the low binding affinity of His6-AUF1(1–229), amino acids in the C terminus flanking the glutamine residues also contribute to the RNA binding activity.

As a control for the possibility that one or more of the mutant proteins with low affinity binding had lost RNA binding activity during purification, ARE binding activity was analyzed in aliquots of the same bacterial extracts used for purification of the wild-type and mutant polypeptides. By UV-cross-linking analysis using these bacterial lysates and 32P-labeled c-fos ARE (8), ARE binding was either undetectable or occurred with at least 100-fold lower affinity than wild-type His6-AUF1 for the mutant proteins that bound the c-fos ARE with low affinity in the mobility shift assays shown in Fig. 1 (data not shown). Therefore, putative loss of RNA binding activity during purification of the mutant proteins is unlikely. The results can be summarized as follows. (i) While the RRs appear important for high affinity ARE binding activity, both RRM1 and RRM2 together are not sufficient for ARE binding. (ii) The N-terminal region of AUF1 is required for high affinity ARE binding activity. (iii) Amino acids C-terminal to RRM2, particularly the glutamines and amino acids flanking the glutamines, are also important for high affinity ARE binding. However, the C-terminal 29 amino acids appear not to contribute to RNA binding, since their truncation has no effect on ARE binding affinity.

Circular Dichroism Analyses—For many RR-containing proteins, either one RR or a combination of the RRs is usually sufficient for high affinity RNA binding (see “Discussion”). Thus, the (2200 nM) ARE binding affinity of the His6-AUF1(69–229) mutant (containing RRM1 plus RRM2 alone) is unusual among RR-containing proteins. To examine the possibility that the low ARE binding affinity of this mutant might be simply due to its misfolding, the thermodynamic stabilities of the wild-type protein and the AUF1(69–229) mutant were measured. Both proteins have CD spectra similar to other RR-containing proteins (3, 16, 17), suggesting that the RRs are folded in a structure similar to that observed for this class of proteins (Fig. 2, upper panels). A combination of chemical denaturation with various concentrations of GdnHCl and ellipticity measurements at 222 nm was used to reveal changes in α-helical content as a function of GdnHCl concentration (Fig. 2, bottom panels). Application of the denaturant binding model of Pace (12) with these denaturation curves revealed that the thermodynamic stabilities (ΔG°f unfold) of the wild-type protein and the AUF1(69–229) mutant are similar (1.941 ± 0.008 kcal/mol versus 1.9 ± 0.1 kcal/mol, respectively). Therefore, it is unlikely that the low affinity of the AUF(69–229) mutant for the ARE is due to misfolding of the polypeptide. We conclude from the ARE-binding studies that amino acids flanking the RRs are necessary for the high affinity ARE binding activity of AUF1. However, the analyses of the thermodynamic stabilities suggest that they do not simply serve to permit proper folding of the RRs. Nonetheless, the amino acids flanking the RRs must play some role in the RNA binding function of AUF1. Since some RNA-binding proteins self-associate (e.g. see Ref. 18), it is possible that protein domains flanking the RRs of AUF1 are involved in AUF1 self-association. To address this question, the hydrodynamic properties of wild-type and mutant His6-AUF1 proteins were examined to determine their native molecular mass in solution.

Determination of Molecular Masses of His6-AUF1 Proteins—Gel filtration analysis of His6-AUF1(1–286) was used to determine the Stokes radius of the native protein. Four protein standards were used to calibrate the Sephacryl S-300 column (Fig. 3, upper panel). His6-AUF1(1–286) at a concentration of 6 μM was loaded onto the column, and fractions were collected. The amount of His6-AUF1(1–286) present in each fraction was determined spectrophotometrically, and relative amounts were plotted versus fraction number (Fig. 3, upper panel). Analysis of the gel filtration data by the method and equations of Ackers (19) yielded a Stokes radius of 3.6 nm (Table 1). Gel filtration using 500 nM His6-AUF1(1–286) produced a protein elution profile identical to that shown in Fig. 3 (data not shown), demonstrating that the protein did not form detectable aggregates at the higher (6 μM) concentration. In addition, both purified cellular AUF1 and recombinant AUF1 (without a His6 tag) synthesized by translation in vitro displayed the same elution profile as that in Fig. 3 (data not shown). This control
**FIG. 1.** Analysis of ARE binding activity of mutant His6-AUF1 proteins. AUF1 contains two nonidentical RNA recognition motifs (labeled as RRM1 or RRM2, respectively), each of which contains two conserved RNP boxes depicted as solid black bars. AUF1 also contains an 8-amino acid glutamine-rich region C-terminal to RRM2 (labeled Q). Binding of each protein to the c-fos ARE was analyzed by electrophoretic mobility shift assays in which increasing concentrations of purified fusion protein were incubated with 100 pmol 32P-labeled RNA. Apparent \( K_d \) values were

\[ \begin{align*}
\text{RRM1} & \quad \text{RRM2} \\
K_d = 7.8 \text{ nM} & \quad K_d = 5.3 \text{ nM} \\
K_d = 36 \text{ nM} & \quad K_d = 207 \text{ nM} \\
K_d = 530 \text{ nM} & \quad K_d > 800 \text{ nM} \\
K_d = 1390 \text{ nM} & \quad K_d = 2200 \text{ nM}
\end{align*} \]
demonstrates that the gel filtration profile shown in Fig. 3 is not unique to the bacterially expressed protein. Gel filtration analyses were also performed for His6-AUF1-(1–194) and His6-AUF1-(92–286), since these mutant proteins do not bind an ARE with high affinity and together span the length of the wild-type protein. For each, 6 μM protein was loaded onto, and eluted from, the column, and the amount of protein present in each fraction was determined spectrophotometrically (Fig. 3, upper panel). Analysis of these data yielded Stokes radii of 3.5 and 2.6 nm for His6-AUF1-(1–194) and His6-AUF1-(92–286), respectively (Table I). The larger Stokes radii of His6-AUF1-(1–286) and His6-AUF1-(1–194) suggested that either each of these proteins contains more than one polypeptide or that each is a highly anisotropic monomer. To distinguish between these two possibilities, velocity sedimentation analyses were performed to determine sedimentation coefficients, which were then used in combination with the Stokes radii to calculate molecular mass values that are not dependent upon the shape of the molecule (14, 20).

**Fig. 2. Circular dichroism spectra of wild-type and mutant His6-AUF1 proteins.** The upper panels show a comparison of the CD spectra for the wild-type His6-AUF1-(1–286) (left panel) and mutant His6-AUF1-(69–229) (right panel) fusion proteins. The lower panels show GdnHCl denaturation of His6-AUF1-(1–286) (left panel) and His6-AUF1-(69–229) (right panel) monitored by CD at 222 nm as described under “Experimental Procedures.”

determined by quantitation of free RNA bands, plotting concentration of RNAfree versus concentration of His6-AUF1, and determining the protein concentration at which 50% of the RNA was bound. Representative plots of RNAfree concentration versus fusion protein concentration are shown, where superscript numbers refer to the amino acids included in the protein. In each case, the apparent $K_d$ value shown is the average of three experiments.
AUFI-(1–194), and His6-AUFI-(92–286) were determined by centrifugation through 5–20% sucrose density gradients by the procedures of Martin and Ames (21) using $\beta$-amylace (8.9 S), BSA (4.3 S), and trypsin inhibitor (2.3 S) as standards. Fig. 3 (lower panel) shows a sucrose gradient elution profile from one experiment, in which relative protein abundance was plotted versus fraction number. For each His6-AUFI protein, the sedimentation coefficient, $s$, was obtained by comparison to each of the standards in two separate experiments. The resulting averaged values are 4.6 S, 3.9 S, and 2.6 S for His6-AUFI-(1–286), His6-AUFI-(1–194), and His6-AUFI-(92–286), respectively (Table I). These values, along with the Stokes radii and partial specific volumes for each protein were used to calculate native molecular masses and frictional ratios (see “Experimental Procedures”).

As presented in Table I, the molecular masses calculated for...
The His<sub>6</sub>-AUF1 proteins analyzed are listed across the top, with numbers in parentheses corresponding to the amino acids of AUF1 contained in the polypeptide. Stokes radii and sedimentation coefficients were determined from gel filtration and velocity sedimentation centrifugation, respectively, as described under “Experimental Procedures.” For each protein, the sedimentation coefficient was determined as the average of the values obtained in two separate experiments. The native molecular mass values, M, were determined from R<sub>s</sub> and s values, and frictional ratios were calculated from M and R<sub>s</sub> values as described under “Experimental Procedures.”

| His<sub>6</sub>-AUF1 | AUF1-(1–286) | AUF1-(1–194) | AUF1-(29–194) | AUF1-(92–286) |
|-------------------|-------------|-------------|-------------|-------------|
| Stokes radius, R<sub>s</sub> (nm) | 3.6 | 3.5 | 3.6 | 2.6 |
| Sedimentation coefficient, s | 4.6S | 3.9S | 1.4S | 2.6S |
| Monomer mass (Da) | 35,922 | 25,110 | 22,360 | 26,614 |
| Native mass (Da) | 68,294 | 56,294 | 21,450 | 28,717 |
| Frictional ratio, f/f<sub>0</sub> | 1.3 | 1.4 | 2.0 | 1.3 |

**TABLE I**

Physical properties of wild-type and mutant His<sub>6</sub>-AUF1 proteins

The native His<sub>6</sub>-AUF1 proteins analyzed are listed across the top, with numbers in parentheses corresponding to the amino acids of AUF1 contained in the polypeptide. Stokes radii and sedimentation coefficients were determined from gel filtration and velocity sedimentation centrifugation, respectively, as described under “Experimental Procedures.” For each protein, the sedimentation coefficient was determined as the average of the values obtained in two separate experiments. The native molecular mass values, M, were determined from R<sub>s</sub> and s values, and frictional ratios were calculated from M and R<sub>s</sub> values as described under “Experimental Procedures.”

| His<sub>6</sub>-AUF1 | AUF1-(1–286) | AUF1-(1–194) | AUF1-(29–194) | AUF1-(92–286) |
|-------------------|-------------|-------------|-------------|-------------|
| Stokes radius, R<sub>s</sub> (nm) | 3.6 | 3.5 | 3.6 | 2.6 |
| Sedimentation coefficient, s | 4.6S | 3.9S | 1.4S | 2.6S |
| Monomer mass (Da) | 35,922 | 25,110 | 22,360 | 26,614 |
| Native mass (Da) | 68,294 | 56,294 | 21,450 | 28,717 |
| Frictional ratio, f/f<sub>0</sub> | 1.3 | 1.4 | 2.0 | 1.3 |

**TABLE II**

Physical properties of wild-type His<sub>6</sub>-AUF1 protein bound to the c-fos ARE

The His<sub>6</sub>-AUF1-(29–194) suggests a prolate ellipsoid with an axial ratio of 20 or an oblate ellipsoid with an axial ratio of 30. While this analysis does not allow one to predict the actual shape of the molecule, these proteins are clearly not spherical structures.

Since the native form of the AUF1 protein in solution is a dimer, the form of AUF1 protein bound to its RNA target sequence was next determined. A binding reaction was performed with the wild-type fusion protein and 32P-labeled c-fos ARE. The protein concentration used was 20 nM, which is in the range of the apparent K<sub>d</sub> for binding to the c-myc and c-fos AREs (8). The protein was cross-linked to RNA using ultraviolet light to prevent dissociation of the protein from RNA during sample processing (22). Unbound RNA was digested with RNase A. Reactions were fractionated by gel filtration or by sucrose gradient centrifugation. RNA-bound His<sub>6</sub>-AUF1-(29–286) protein was detected by Cerenkov counting due to label transfer. The Stokes radius of the 32P-labeled His<sub>6</sub>-AUF1-(29–286) protein is 6.4 nm, and its sedimentation coefficient is 8.6 S (Table II). From these values, the calculated molecular mass of the 32P-labeled His<sub>6</sub>-AUF1-(29–286) protein is 227,700 Da. Since its monomer mass is 35,922 Da, the mass of the labeled protein is consistent with a hexameric structure for AUF1 when bound to the c-fos ARE. Three controls were performed.

(i) A binding reaction with His<sub>6</sub>-AUF1 protein and 32P-labeled rabbit β-globin 3′-UTR was analyzed by gel filtration and sucrose gradient centrifugation. No 32P-labeled protein was recovered from the gel filtration column or the sucrose gradient. RNA-bound His<sub>6</sub>-AUF1-(1–286) protein yielded only background counts in the fractions expected of a protein complex with a Stokes radius of 6.4 nm and a sedimentation coefficient of 8.6 S. (ii) A UV-cross-linked binding reac-
tion containing radiolabeled c-fos ARE and His<sub>6</sub>-AUF1(1–286) was also analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography. The radiolabeled His<sub>6</sub>-AUF1(1–286) polypeptide exhibited the same mobility as the protein alone (i.e. in the absence of RNA) (data not shown; e.g. see Fig. 1 in Ref. 8); no higher molecular weight forms were observed. This suggests that the large mass of the 32P-labeled protein is not due to RNA bridging. Thus, we conclude that the hydrodynamic properties of the RNA-bound form of AUF1 are consistent with it being a hexamer.

The frictional coefficients of His<sub>6</sub>-AUF1(1–286) and the ARE-bound form of the protein were also compared (Table II). A frictional ratio of 1.3 for the unbound form corresponds to either a prolate or an oblate ellipsoid with an axial ratio of 6 (see Ref. 20). By contrast, a frictional ratio of 1.6 for the ARE-bound form of His<sub>6</sub>-AUF1 corresponds to either a prolate or an oblate ellipsoid with an axial ratio of 13. Again, while this analysis does not allow us to predict the actual shape of the molecule, the His<sub>6</sub>-AUF1 oligomer bound to RNA is clearly not spherical in structure.

**DISCUSSION**

We have shown previously that AUF1 binds AREs with high affinity and that the magnitude of this binding affinity is comparable with the affinities exhibited by several other RNA-binding proteins that recognize specific sequences or structures (8). In this study, our objective was to elucidate the structural features of AUF1 that mediate its ARE binding activity. Our results (summarized in Fig. 4) suggest that while both RRMs may be required for high affinity ARE binding, they are not sufficient. Additionally, our results suggest that amino acids adjacent to the RRMs participate in AUF1 binding to its cognate RNA target.

The prevailing paradigm for RNA-binding proteins that contain more than one RRM is that RRMs are essentially modular. The number of RRMs typically found within this class of RNA-binding proteins ranges between two and four (23). RRMs can be tandemly arranged or separated by intervening polypeptide sequence. In some RNA-binding proteins with two or more RRMs, a subset of one or more RRM-containing regions is generally sufficient for RNA binding (24–29). Some other RNA-binding proteins do require all of their RRMs for RNA binding activity (30, 31), but as a general rule, other regions of the protein are dispensable (31–33).

Our experiments suggest that AUF1 does not follow this paradigm of RRM modularity. In this respect, AUF1 exhibits a greater similarity to RNA-binding proteins that contain only a single RRM, which often do require amino acids adjacent to the RRM for high affinity target binding (2, 23, 34). For instance, the *Drosophila* Tra2 protein contains one RRM that is necessary but not sufficient for RNA binding (34). Like AUF1, Tra2 requires at least part of the region C-terminal to the RRM for RNA binding activity. Deleting a 90-amino acid region N-terminal to the Tra2 RRM has no effect on RNA binding. By contrast, AUF1 requires regions N-terminal to RRM1 and C-terminal to RRM2 for high affinity ARE binding activity.

Analyses of the hydrodynamic properties of various mutant proteins in the absence of target RNA indicate that the alanine-rich, N-terminal 28 amino acids of AUF1 are necessary for dimerization, while the glutamine-rich region and the C-terminal domain appear not to be important for dimerization. Truncation of the N-terminal 28 amino acids from the full-length protein abolishes dimerization (Table I) and lowers its ARE binding affinity almost 10-fold to 62 nM (data not shown; Fig. 4). This result suggests that self-association may be crucial for high affinity ARE binding activity.

Ala-rich regions of proteins are known to mediate pro-
tion, also bind RNA as tetramers. These package 700-nucleotide increments of heterogenous nuclear RNA into triangular complexes (22). The hnRNP (A1)B/B2 tetramers and (A2)B1 tetramers also package heterogenous nuclear RNA in a fashion similar to the hnRNP C proteins (40). Alternatively, since AUF1 forms complexes with a number of intracellular proteins (5, 41), AUF1 bound to an ARE as a hexameric protein complex might provide a large surface for interacting, effector proteins to bind. This function, however, need not be exclusive of a putative packaging role for AUF1.

In summary, our experiments indicate that the RRMs in AUF1 are not modular in function and that the ability of AUF1 to dimerize via its alanine-rich N-terminal domain is important for high affinity ARE binding. Furthermore, they suggest that AUF1 binds to its RNA target as a hexameric complex. Future experiments will examine how these protein-protein interactions work in concert with the RRMs to permit ARE recognition by AUF1.

Acknowledgments—Oligodeoxynucleotide synthesis and cycle sequencing were performed by the core laboratories of the Comprehensive Cancer Center of Wake Forest University. We thank Gerald Wilson and Doug Lyles for comments on the manuscript and John Parks for assistance with the spectropolarimeter.

REFERENCES
1. McCarthy, J. E. G., and Kollmus, H. (1995) Trends Biochem. Sci. 20, 191–197
2. Burd, C. G., and Dreyfuss, G. (1994) Science 265, 615–621
3. Serin, G., Joseph, G., Ghisolfi, L., Bauzan, M., Erard, M., Amalric, F., and Bouvet, P. (1997) J. Biol. Chem. 272, 13109–13116
4. Zeng, Q., and Hall, K. (1997) RNA 3, 303–314
5. Zhang, W., Wagner, B. J., Ehrenman, K., Schaefer, A. W., DeMaria, C. T., Crater, D., DeHaven, K., Long, L., and Brewer, G. (1995) Mol. Cell. Biol. 15, 7652–7665
6. Ehrenman, K., Long, L., Wagner, B. J., and Brewer, G. (1994) Gene (Amst.) 148, 315–319
7. Peña, A., Tremmel, K. D., DeMaria, C. T., Blaxall, B. C., Minobe, W. A., Sherman, J. S., Bisognano, J. D., Bristow, M. R., Brewer, G., and Port, J. D. (1996) J. Biol. Chem. 271, 8495–8501
8. DeMaria, C. T., and Brewer, G. (1996) J. Biol. Chem. 271, 12179–12184
9. Carey, J. (1991) Methods Enzymol. 208, 103–117
10. Parks, J. S., and Gebre, A. K. (1997) J. Lipid Res. 38, 266–275
11. Sparks, D. L., Lund-Katz, S., and Phillips, M. C. (1992) J. Biol. Chem. 267, 25839–25847
12. Pace, C. N. (1986) Methods Enzymol. 131, 266–280
13. Carey, J., Cameron, V., de Haseth, P. L., and Uhlenbeck, O. C. (1983) Biochemistry 22, 2601–2609
14. Barnett, S. F., Friedman, D. L., and LeStourgeon, W. M. (1989) Mol. Cell. Biol. 9, 492–498
15. Lave, T. M., Shah, B. D., Ridgeway, T. M., and Pelletier, S. L. (1992) in Analytical Ultracentrifugation in Biochemistry and Polymer Science (Harding, S. E., Rowe, A. J., and Horton, J. C., eds) pp. 90–125, Royal Society of Chemistry, Cambridge, United Kingdom
16. Lu, J., and Hall, K. B. (1995) J. Mol. Biol. 247, 739–752
17. Shamooy, A., Abdul-Manan, N., Patten, A. M., Crawford, J. K., Pelliigrini, M. C., and Williams, K. R. (1994) Biochemistry 33, 8272–8281
18. Zapp, M. L., Hope, T. J., Parslow, T. G., and Green, M. R. (1991) Proc. Natl. Acad. Sci. U. S. A. 88, 7734–7738
19. Ackers, G. K. (1964) Biochemistry 3, 723–730
20. Freifelder, D. (1982) Physical Biochemistry: Applications to Biochemistry and Molecular Biology, pp 460–467, W. H. Freeman & Co., New York
21. Martin, R. G., and Ames, B. N. (1961) J. Biol. Chem. 236, 1372–1379
22. Huang, M., Rech, J. E., Northington, S. J., Flicker, P. F., Mayeda, A., Krainer, A. R., and LeStourgeon, W. M. (1994) Mol. Cell. Biol. 14, 518–533
23. Nagai, K., Oubridge, C., Ito, N., Avis, J., and Evans, P. (1995) Trends Biochem. Sci. 20, 235–240
24. Burd, C. G., Matunis, E. L., and Dreyfuss, G. (1991) Mol. Cell. Biol. 11, 3419–3424
25. Chung, S., Jiang, L. Cheng, S., and Furneaux, H. (1996) J. Biol. Chem. 271, 11518–11524
26. Dember, L. M., Kim, N. D., Liu, K.-Q., and Anderson, P. (1996) J. Biol. Chem. 271, 2783–2788
27. Kuhn, U., and Pieler, T. (1996) J. Mol. Biol. 256, 20–30
28. Levine, T. D., Gao, F., King, P. H., Andrews, L. G., and Keene, J. D. (1993) Mol. Cell. Biol. 13, 3494–3504
29. Sachs, A. B., Davis, R. W., and Kornberg, R. D. (1987) Mol. Cell. Biol. 7, 3208–3216
30. Green, S. R., Manche, L., and Mathews, M. B. (1995) Mol. Cell. Biol. 15, 358–364
31. Zamore, P. D., Patton, J. G., and Green, M. R. (1992) Nature 355, 609–614
32. Scherly, D., Dathan, N. A., Boelens, W., van Venrooij, W. J., and Mattaj, I. W. (1990) EMBO J. 9, 3675–3681
33. Green, S. R., and Mathews, M. B. (1992) Genes Dev. 6, 2478–2490
34. Amrein, H., Hedley, M. L., and Maniatis, T. (1994) Cell 76, 735–746
35. Yeung, K., Kim, S., and Reinberg, D. (1997) Mol. Cell. Biol. 17, 36–45
36. King, K. M., Lesson-Wood, L. A., Weintraub, B. D., and Chung, J. H. (1996) Mol. Cell. Biol. 16, 4366–4377
37. Wang, J., and Bell, L. R. (1994) Genes Dev. 8, 2072–2085
38. Chase, J. W., and Williams, K. R. (1986) Annu. Rev. Biochem. 55, 103–136
39. Chen, C. Y., and Shyu, A.-B. (1995) Trends Biochem. Sci. 20, 465–470
40. Barnett, S. F., Theiry, T. A., and LeStourgeon, W. M. (1991) Mol. Cell. Biol. 11, 864–871
41. Kileldjian, M., DeMaria, C. T., Brewer, G., and Novick, K. (1997) Mol. Cell. Biol. 17, 4870–4876