3564–3569 *Nucleic Acids Research, 1997, Vol. 25, No. 18*

The Elav-like proteins bind to AU-rich elements and to the poly(A) tail of mRNA

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Received June 25, 1997; Accepted July 15, 1997

ABSTRACT

The Elav-like proteins are specific mRNA binding proteins which are required for cellular differentiation. They contain three characteristic RNP2/RNP1-type RNA binding motifs. Previously we have shown that the first and second RNA binding domains bind to AU-rich elements in the 3′-UTR of mRNA. In this paper we show that the Elav-like proteins exhibit poly(A) binding activity. This activity is distinct from poly(A) binding activities that have been previously described. The Elav-like proteins specifically bind to long chain poly(A) tails. We have shown that the third RNA binding domain encompasses this poly(A) binding activity. Using poly(A)–Sepharose beads in a 'sandwich' assay we have shown that the Elav-like proteins can bind simultaneously to the AU-rich element and to the poly(A) tail.

INTRODUCTION

The Elav-like proteins are a group of closely related RNA binding proteins that were first described in *Drosophila* (1). Recently, Elav-like genes have been cloned from higher organisms (2–8). There are four members of the human family, HuD, HuC, Hel-N1 and HuR (3–5,7). HuR is expressed at the RNA level in all proliferating cells, whereas HuD, HuC and Hel-N1 are normally expressed on terminal differentiation of neurons (7,9,10). The human members of this family are of particular interest since they are tumor antigens associated with a wide variety of human tumors. (5,11,12). The Elav-like proteins contain three highly conserved RNA recognition motifs (RRM). Two of these RRMs are in tandem and are separated from the third by a basic segment (3,4,7,13). A significant insight into the mechanism of action of these proteins was provided by the observation that they specifically bind to AU-rich elements in the 3′-untranslated region (UTR) of mRNA. (7,13–17). These AU-rich elements were originally characterized by Shaw and Kamen, who were the first to show that the AU-rich element in the 3′-UTR of GM-CSF mRNA regulates its expression at the post-transcriptional level (18). The current model is that the AU-rich elements are recognized by a specific endonuclease, which cleaves the transcript and renders it acessible to an exonuclease (19). Thus, mRNAs that contain these elements have a very short half-life and are usually present at a very low steady-state level. The level of these mRNAs can be dramatically increased by factors that also bind to the AU-rich elements and inhibit the degradative activity. Recent evidence has indicated that the Elav-like proteins are such factors and selectively inhibit the decay of mRNAs that contain AU-rich elements (20–23). In previous studies we have shown that the first and second RRMs of HuD and HuR bind specifically and with high affinity to AU-rich elements (7,16). In this paper we show that these Elav-like proteins also bind to the poly(A) tail of mRNA. This is mediated by the third RRM and may promote an interaction between AU-rich elements and the poly(A) tails of mRNA.

MATERIALS AND METHODS

Materials

HuR, HuD and deletion mutants of HuD were purified as GST fusion proteins as previously described (7,16). Poly(A)–Sepharose 4B and Sepharose 4B were from Pharmacia. BA 85 nitrocellulose filters were from Schleicher & Schuell. RNase T1 was obtained from Calbiochem. Poly(A), poly(G), poly(U) and poly(C) were from Sigma.

Preparation of RNA transcripts

RNA transcripts were synthesized from plasmid DNA using [α-^{32}P]ATP or [^{32}P]GTP and were gel purified before use (16). The specific activity of these transcripts is expressed as c.p.m./pmol nucleotide. The 3′ myc and 3′ myc$(A)_{87}$ transcripts were derived from *Afl*II and *Hin*dIII digests of pMycSD$_3$, a gift of Dr Gary Brewer (24). The 3′ myc(I) transcript was derived from a *Ssp*I digest of pMycSD$_3$. The 3′ myc(II) transcript was derived from an *Afl*II digest of pMycSD$_3$/Δ5. pMycSD$_3$/Δ5 was created by deletion of an *Eco*RI–*Apo*I fragment from pMycSD$_3$. The $(A)_{87}$ transcript was derived from a *Hin*dIII digest of pSD$_3$, also a gift of Dr Gary Brewer. Poly$(A)_{600av}$ was hydrolyzed with alkali and end-labeled using T4 kinase and [γ-^{32}P]ATP to a specific activity of 1×10^6 c.p.m./pmol ends and gel purified.

RNase T1 selection assay

Reaction mixtures (0.02 ml) contained 50 mM Tris, pH 7.0, 150 mM NaCl, 0.25 mg/ml BSA, 0.25 mg/ml tRNA, radiolabeled

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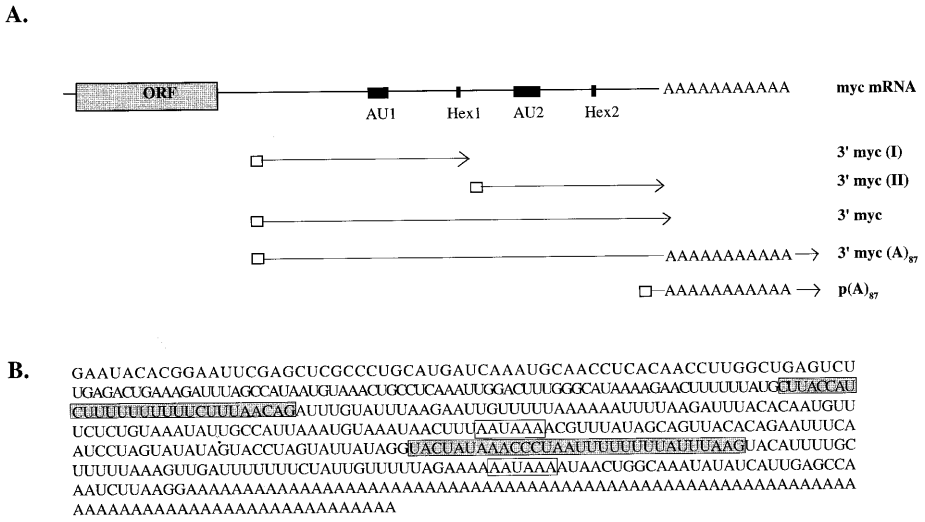

Figure 1. The structure of the c-myc 3′-UTR and of the RNA transcripts used in this study. (**A**) Hex1 and Hex2 indicate the hexanucleotide poly(A) addition signals for the minor and major transcripts respectively. The major transcript is shown. AU1 and AU2 denote the HuR and HuD binding sites. The open squares indicate the Sp6 promoter elements. (**B**) The sequence of 3′myc$(A)_{87}$. The shaded boxes indicate the binding sites for HuR and HuD. The Hex1 and Hex 2 poly(A) addition signals are indicated by the enclosed boxes of AAUAAA.

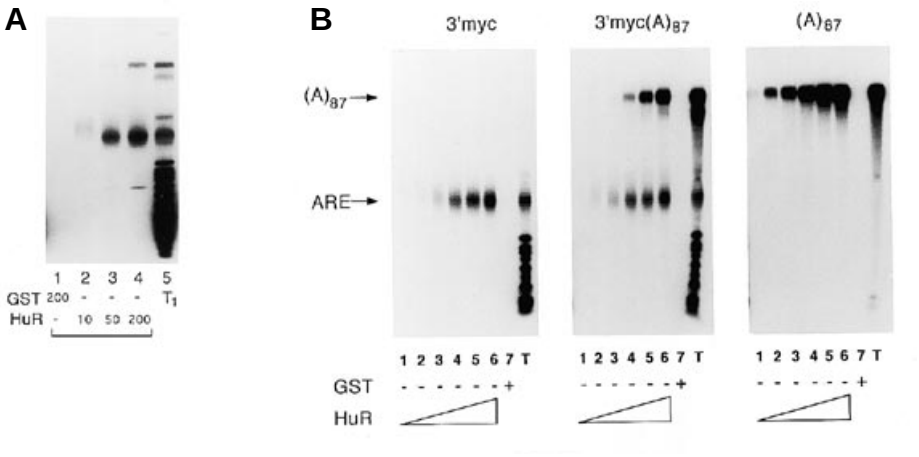

Figure 2. HuR binds to the AU-rich elements and poly(A) tail of 3′ myc$(A)_{87}$. (**A**) ^{32}P-Labeled 3′ myc$(A)_{87}$ RNA (14 fmol, 1×10^5 c.p.m./pmol GMP) was incubated with the indicated concentrations of HuR and GST at 37°C for 10 min. After treating the reaction mixture with RNase T1 the reaction mixtures were filtered through nitrocellulose. RNA fragments bound to the nitrocellulose were extracted and electrophoresed in a 12% acrylamide–8 M urea gel. Lane 5 shows the T1 digest of the transcript prior to selection. (**B**) The indicated ^{32}P-labeled RNAs (20 fmol, 1×10^4 c.p.m./pmol AMP) were incubated with HuR at 37°C for 10 min. In the first and second panels the concentration of HuR was 5, 10, 20, 50, 100 and 200 nM in lanes 1–6 respectively and the GST concentration was 200 nM. In the third panel the concentration of HuR was 20, 50, 100, 200, 500 and 1000 nM in lanes 1–6 respectively and the GST concentration was 1000 nM. The reaction mixtures were treated with RNase T1 and selected as described above. In each panel lane T shows the T1 digest of the transcript prior to selection. The amount loaded corresponds to 50% of the transcript used in the selection.

RNA and purified HuR as indicated. After 10 min incubation at 37°C, RNase T1 (5 U) was added and the reaction continued for a further 10 min. The mixtures were diluted 1:6 with buffer F (20 mM Tris, pH 7.0, 150 mM NaCl) and filtered through nitrocellulose (BA 85; Schleicher & Schuell). After washing the nitrocellulose twice with buffer F, the bound RNA was eluted by phenol/chloroform extraction. The resultant RNA was mixed with formamide buffer, denatured at 65°C for 3 min and analyzed by 12% polyacrylamide–urea gel electrophoresis. The gel was fixed with 1:1:8 acetic acid:methanol:water, dried on DE-81 paper with a backing of gel drying paper and exposed to the XAR5 film at –70°C overnight.

Nitrocellulose filter binding assay

Reaction mixtures (0.02 ml) contained 50 mM Tris, pH 7.0, 150 mM NaCl, 0.25 mg/ml BSA, 2.5 µg/ml tRNA, labeled RNA and purified protein as indicated. After 10 min incubation at 37°C the mixtures were diluted 1:6 with buffer F and filtered through nitrocellulose (BA85; Schleicher & Schuell). After washing the filter twice with buffer F, bound radioactivity was determined by Cerenkov counting. Each point is corrected for the amount of RNA bound in the absence of protein, which is usually <1% of the input.

RESULTS

The Elav-like proteins bind to AU-rich elements and to poly(A)

We have previously shown that the human Elav-like proteins bind specifically to the 3′-UTR of c-myc mRNA (7,16). Using RNase T1 selection analysis we discovered that the 3′ myc mRNA contains two independent HuR binding sites that we have labeled AU1 and AU2 (see Fig. 1). The two sites were confirmed by RNase T1 selection analysis of transcripts 3′ myc(I) and 3′ myc(II) (Fig. 1). In view of the relationship betweeen mRNA decay and polyadenylation (25), we decided to examine interaction of the Elav-like proteins with a polyadenylated transcript. Using pMycSD$_3$ and [^{32}P]GTP we synthesized a c-myc transcript that contained a poly(A) tail of 87 nt. The HuR binding sites on this transcript were assayed by the RNase T1 selection assay (7). As expected, HuR bound specifically to the AU1 and AU2 sites. (Fig. 2A, lanes 1–4). We were surprised, however, to observe that a larger band was also selected (Fig. 2A, lanes 3 and 4). This band was the same size as the (A)$_{87}$ fragment present in the total RNase T1 digest of the transcript [the (A)$_{87}$ fragment is labeled by virtue of a G residue in the restriction site at the end of the template DNA]. Thus we concluded that HuR exhibits a poly(A) binding activity. This activity was intrinsic to the HuR protein, as no fragments were selected by high concentrations of GST. We next determined whether HuR could directly bind to poly(A). Using the pSD$_3$ plasmid (see Fig. 1) we synthesized the (A)$_{87}$ tail itself. To increase the sensitivity of the assay we labeled the transcripts with [^{32}P]ATP. Figure 2B shows that HuR bound to (A)$_{87}$, even in the absence of the AU1 and AU2 sites. Thus HuR has an independent poly(A) binding activity. It is important to point out that HuR exhibits a significantly lower affinity with (A)$_{87}$ than with the AU1 and AU2 elements.

Characterization of the poly(A) binding activity

Next we investigated the properties of the poly(A) binding activity in more detail. We were surprised to observe that HuR did not bind to poly(A)$_{30}$. Thus we investigated whether HuR had a requirement for longer poly(A) tails. End-labeled poly(A) of uniform size distribution was prepared and incubated with HuR or GST. The bound poly(A) was selected by nitrocellulose filtration and analyzed by gel electrophoresis. Figure 3 shows that binding was first detectable with poly(A)$_{80}$, was half-maximal with poly(A)$_{180}$ and saturated at poly(A)$_{300}$. This result was confirmed by purification of poly(A) of defined size and determination of their binding affinity. As in previous studies, the interactions between the Elav-like proteins and RNA were quantitated using a nitrocellulose filter binding assay (7,26). A low concentration of labeled RNA was incubated with increasing concentrations of HuR. The reaction mixtures were filtered through nitrocellulose and the bound radioactivity determined. As predicted from the selection analysis, virtually no complex was formed with poly(A)$_{30}$ (Fig. 4A). Increasing reactivity was observed with poly(A)$_{100}$, poly(A)$_{150}$ and poly(A)$_{200}$, reaching saturation at poly(A)$_{300}$. A plot of log[complex/free poly(A)] versus log(HuR concentration) revealed a straight line in each case (Fig. 4A). This suggested a simple interaction with little cooperativity. The affinity of HuR for poly(A) is significantly less than that for the AU-rich elements. This is quantitatively shown in Figure 4B. The apparent K_d for 3′ myc is 4 nM, whereas the

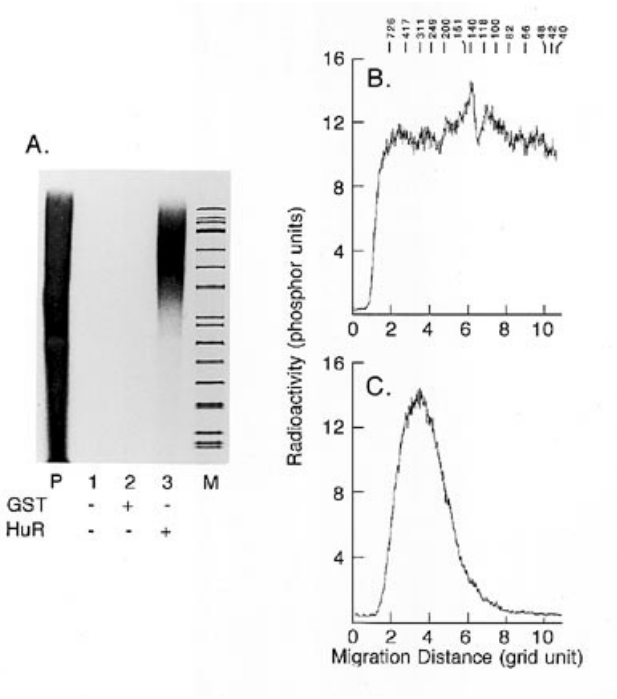

Figure 3. Size selection analysis of poly(A) binding activity. (**A**) Poly(A) of 600 nt (average size) was cleaved with dilute alkali and labeled to yield a uniform size distribution from 24 to 800 nt. This population of poly(A) was then labeled at the 5′-end using ATP and T4 kinase. The ^{32}P-labeled poly(A)$_{24–800}$ (50 fmol, specific activity 1.1×10^6 c.p.m./pmol) was then incubated with GST or HuR (200 nM) at 37°C. After 10 min the protein-bound poly(A) was selected by absorption to nitrocellulose, eluted and electrophoresed in a 6% acrylamide–8 M urea gel. The labeled poly(A)$_{24–800}$ is shown in lane P. The selected products after incubation with no protein, GST or HuR protein are shown in lanes 1–3 respectively. A marker digest of ΦX174 DNA is shown in lane M. A quantitative analysis (by scanning in a PhosphorImager) of lanes P and 3 is shown in (**B**) and (**C**) respectively.

apparent K_d for poly(A)$_{300}$ is 146 nM. As before, the intrinsic nature of these activities is indicated by the lack of reactivity with GST (Fig. 4B). There is also little difference in binding between 3′ myc and 3′myc(A)$_{87}$ (Fig. 4B). Thus the AU-rich element is the primary determinant of HuR binding. Next we examined the specificity of the poly(A) binding activity. We investigated whether the HuR–poly(A) complex could be displaced by other homopolymers. Figure 4C shows that the HuR–poly(A)$_{87}$ complex was displaced by poly(A)$_{600}$ (50% displacement at 0.2 molar excess) and to a lesser extent by poly(G)$_{600}$ (50% displacement at 0.8 molar excess). Neither poly(U)$_{600}$ (50% displacement at 32 molar excess) nor poly(C)$_{600}$ (no displacement at 1000-fold molar excess) significantly displaced the HuR–poly(A)$_{87}$ complex.

The poly(A) binding activity is resident in the third RNA binding domain

The most striking and unique structural feature of the Elav-like family of proteins is the presence and organization of the three putative RNA recognition motifs (Fig. 5). In each case the two tandemly arranged RNA recognition motifs are connected to the third RNA recognition motif by a highly basic segment that we

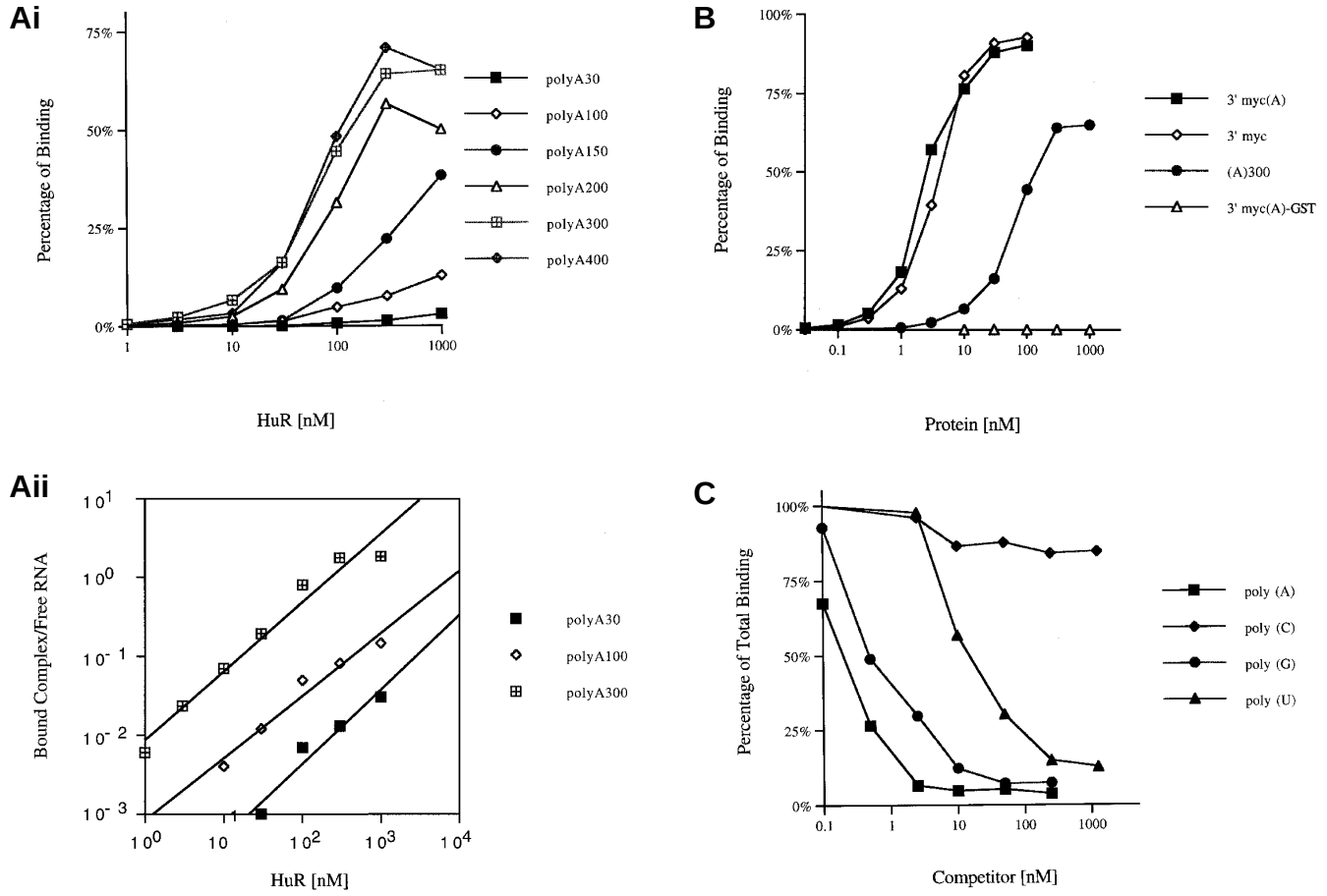

Figure 4. (**A**) The affinity of HuR for poly(A)$_{30}$, poly(A)$_{100}$, poly(A)$_{150}$, poly(A)$_{200}$, poly(A)$_{300}$ and poly(A)$_{400}$. RNA–protein complex formation was assayed by nitrocellulose filtration. An aliquot of 14 fmol each RNA (specific activity 1.1×10^6 c.p.m./pmol) was incubated with the indicated concentration of HuR for 10 min at 37°C. (i) Plot of percentage RNA bound versus log HuR concentration. (ii) Plot of log complex/free RNA versus log HuR concentration. (**B**) The affinity of HuR for 3′ myc, 3′ myc(A)$_{87}$ and (A)$_{300}$. RNA–protein complex formation was assayed by nitrocellulose filtration. ^{32}P-Labeled 3′ myc(A)$_{87}$, 3′ myc (9 and 13 fmol, specific activity 1×10^4 c.p.m./pmol AMP) and (A)$_{300}$ (14 fmol, specific activity 1.1×10^6 c.p.m./pmol) were incubated with the indicated concentration of HuR and GST for 10 min at 37°C. (**C**) Competition analysis of poly(A) binding activity. RNA binding was determined by nitrocellulose filtration. p(A)$_{87}$ (20 fmol, specific activity 1×10^4 c.p.m./pmol) was incubated with 200 nM recombinant HuR and the indicated molar excess of poly(A)$_{600}$, poly(G)$_{600}$, poly(U)$_{600}$ and poly(C)$_{600}$. Eight femtomoles of p(A)$_{87}$ were bound in the absence of competitor and set as 100%.

have termed the ‘basic segment’. We have previously shown that the first and second RRMs of HuD are necessary and sufficient for binding to AU-rich elements (16). We now sought to establish which domains are involved in poly(A) binding activity. We used the mutant constructs derived from HuD (16; Fig. 5). HuD bound to the ARE and poly(A) tail of 3′ c-myc(A)$_{87}$ (Fig. 5, lane 2). The first and second RNA binding domains (HuD I,II) did not bind to the poly(A) tail. Although HuD I,II does bind to AU-rich elements, it does so with significantly lower affinity (the apparent K_d for HuD is 16 nM, whereas the apparent K_d for HuD I,II is 125 nM) (16). Thus, at the concentration used here, little or no binding to the AU1 and AU2 elements was anticipated. In contrast, the third RNA binding domain (HuD III) bound avidly to the poly(A) tail (Fig. 5, lane 4). Thus we concluded that the first and second RNA binding domains interact with AU-rich elements whereas the third RNA binding domain interacts with the poly(A) tail.

The Elav-like proteins bind simultaneously to the AU-rich element and the poly(A) tail of mRNA

Next we investigated whether the Elav-like proteins can contact both sites simultaneously. To examine this, we bound HuR to poly(A)–Sepharose beads (HuR/pA-S4B beads), removed unbound HuR by washing and then examined the ability of the beads to bind the AU-rich element. Figure 6 shows that the labeled 3′ myc transcript bound to poly(A)–Sepharose beads preincubated with HuR (HuR/pA-S4B beads) but not to poly(A)–Sepharose beads preincubated with GST (GST/pA-S4B beads). The amount of transcript bound increased with increasing concentration of HuR. The labeled 3′ myc transcript did not bind to Sepharose beads that were preincubated with HuR (HuR/S4B beads). This is a difficult experiment to perform since the off-rate of the HuR–poly(A) complex is fast. Thus the demonstration that 10% of the myc transcript can be simultaneously bound at saturating

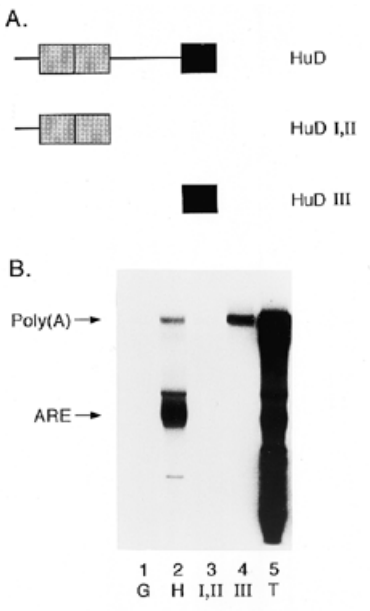

Figure 5. Analysis of RNA binding domains. (**A**) Structure of the mutant HuD derivatives. The residues of HuD contained in each construct are as follows: pGEX-HuD I,II, 2–216; pGEX-HuD III, 279–373. (**B**) The purified HuD derivatives (200 nM) were mixed with ^{32}P-labeled 3′ myc(A)$_{87}$ (100 fmol, specific activity 5×10^3 c.p.m./pmol AMP). Following incubation at 37°C for 10 min the reaction mixtures were analyzed by the RNase T1 selection assay.

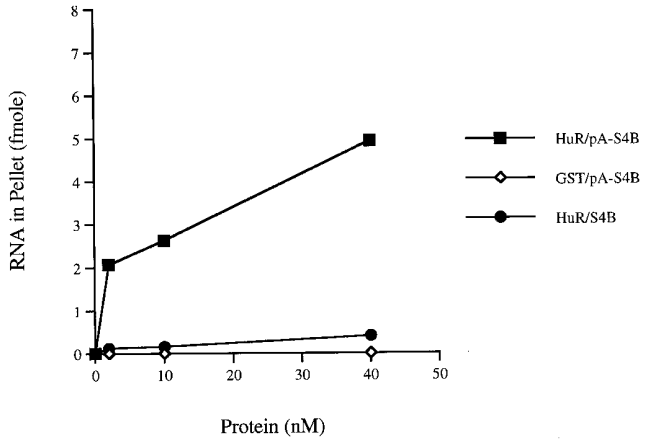

Figure 6. HuR binds to poly(A) and ARE simultaneously. Sepharose 4B and poly(A)–Sepharose 4B beads were preincubated with the indicated concentration of HuR in a 50 µl reaction containing 50 mM Tris–HCl, pH 7.0, 150 mM NaCl, 2.5 µg tRNA/ml, 250 µg BSA/ml and 0.01% NP-40. The mixture was shaken at room temperature for 1 h. The beads were spun down and the supernatant removed. The beads were washed with 200 µl wash buffer (50 mM Tris–HCl, pH 7.0, 150 mM NaCl, 0.01% NP-40). The washed beads were then resuspended in 50 µl containing 50 mM Tris–HCl, pH 7.0, 150 mM NaCl, 2.5 µg tRNA/ml, 250 µg BSA/ml and 0.01% NP-40 and 3′ c-myc RNA transcript (50 fmol, specific activity 5.0×10^3 c.p.m./pmol AMP). The mixture was incubated at room temperature for 15 min with shaking. The beads were washed with 3 ml wash buffer (500 µl each wash). Bound radioactivity was determined by Cerenkov counting.

HuR concentration is significant. We concluded that the Elav-like proteins can form a bridge between the poly(A) tail and the AU-rich element.

DISCUSSION

The Elav-like proteins, HuD, HuC, Hel-N1 and HuR, stabilize specific mRNAs via an interaction with AU-rich elements in their 3′-UTR (7,14–17,20–22). In this paper we have shown that HuD and HuR proteins have an additional property, namely a novel poly(A) binding activity. Recently (after this paper was submitted for publication) the HuC protein was also shown to have poly(A) binding activity (27). Thus this activity is probably a feature of all Elav-like proteins. This activity may not have been detected in previous studies of Hel-N1 since poly(A) was used as a non-specific competitor (17,28). The properties of the Elav-like poly(A) binding activity are quite different from other poly(A) binding proteins. The cytoplasmic poly(A) binding protein (PABI) binds to $(A)_{10}$ and the K_d does not significantly change with increased chain length (29). The nuclear poly(A) binding protein (PABII) binds to an oligo(A) tail >10–11 nt and remains associated [in a complex with poly(A) polymerase] until the tail is elongated to a length of 250 nt (30). Thus these activities have a minimal binding site of 10–15 nt, whereas the Elav-like proteins prefer polymers >70 nt. The affinity of the Elav-like proteins for poly(A) is relatively low [an apparent K_d of 146 nM for $(A)_{300}$] compared with a K_d of 5 nM for PABI (29,31) and a K_d of 2 nM for PABII (32). The Elav-like poly(A) binding activity is, however, similar to both PABI and PABII in that its activity is displaced by a molar excess of poly(A) and poly(G) but not efficiently by poly(U) or poly(C). The observation that the Elav-like proteins preferentially bind to long polymers of poly(A) is surprising. Typically, proteins exhibit increased binding to polymers as a result of protein–protein interactions. This does not appear to be the case with the Elav-like proteins. There is no obvious cooperativity in their interaction with either short or long chain poly(A). Thus the possibility remains that the third RNA binding domain recognizes a secondary or tertiary structure that is only evident in long chain poly(A).

The Elav-like proteins contain three RNA binding domains. Previous studies have shown that the third RNA binding domain is not required for AU-rich element binding (16). This domain is crucial, however, since mutation of Gly426 to glutamic acid in *Drosophila* Elav leads to a temperature-sensitive phenotype (33). We have shown here that the third RNA binding domain contains the poly(A) binding activity. The bifunctionality of the Elav-like proteins is not surprising. The first and second RNA binding domains are much more closely related in sequence to the corresponding domains among different organisms than to the third RNA binding domain (2,5–7,13). This supports the notion that they have different functions.

The demonstration of a poly(A) binding activity resident in the third RNA binding domain of the Elav-like proteins has important implications for understanding their mechanism of action. Our current model is that binding of the Elav-like protein inhibits the action of a specific endonuclease that recognizes the AU-rich element. It is possible that effective inhibition of the endonuclease may require association of the Elav-like proteins with both the AU-rich element and the poly(A) tail. This would ensure that deadenylated message would not be stabilized. Alternatively, the Elav-like proteins may sequester the poly(A) tail of a target mRNA, with a consequent inhibitory effect on a poly(A) exonuclease activity. The poly(A) mRNA–protein complex described here and the recent development of an *in vitro* system

that recapitulates the regulated turnover of mRNA (21) may provide a way to answer these questions.

ACKNOWLEDGEMENTS

We thank Barry Nevins for his patience in preparing the manuscript. This work was supported by the NIH (NS29682), The Byrne Fund and an NCI core grant (P30-CA08748).

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