Identification of HuR as a protein implicated in AUUUA-mediated mRNA decay

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Expression of many proto-oncogenes, cytokines and lymphokines is regulated by targeting their messenger RNAs for rapid degradation. Essential signals for this control are AU-rich elements (AREs) in the 3' untranslated region (UTR) of these messages. The ARE is loosely defined as the five-nucleotide sequence AUUUA embedded in a uracil-rich region. A trans-acting factor, presumably a protein, binds the ARE and initiates recognition by the destabilization machinery. Numerous candidate ARE-binding proteins have been proposed. We show that a 32 kDa protein in HeLa nuclear extracts characterized previously has RNA-binding specificity that correlates with the activity of an ARE in directing mRNA decay. Purification and subsequent analyses demonstrate that this 32 kDa protein is identical to a recently identified member of the Elav-like gene family (ELG) called HuR. The *in vitro* binding selectivity of HuR is indicative of an ARE sequence's ability to destabilize a mRNA *in vivo*, suggesting a critical role for HuR in the regulation of mRNA degradation.

Keywords: AU-rich element/HuR/mRNA decay/UTR

Introduction

Post-transcriptional regulation of messenger RNA (mRNA) levels is an important control point in gene expression. Significant attention has focused on a class of early response genes (ERGs), which code for cytokines, lymphokines or proto-oncogenes. Their short-lived mRNAs are generally transcribed for a limited time in response to extracellular stimuli and then rapidly removed from the cytoplasm by targeted degradation. This instability is regulated by a *cis*-acting adenylate-uridylate-rich element (ARE) found in the 3' untranslated region (UTR) of the mRNA (Caput *et al.*, 1986; Shaw and Kamen, 1988; for reviews, see Belasco and Brawerman, 1993; Chen and Shyu, 1995). The initial experimental observation was that a 51-nucleotide sequence transplanted from the 3' UTR of an unstable mRNA (granulocyte-macrophage colony-stimulating factor, GM-CSF) to the 3' UTR of a stable message (β -globin) was sufficient to target the stable mRNA for rapid degradation (Shaw and

Kamen, 1988). Conversely, removal of an ARE can stabilize a labile message and confer a transforming phenotype on a proto-oncogene (Miller *et al.*, 1984; Meijlink *et al.*, 1985; Lee, W.M. *et al.*, 1988).

Not surprisingly, there appear to be multiple classes of AREs, since many ERG messages are regulated independently in different cell types. Growth factor mRNAs in certain cell lines can be selectively stabilized over proto-oncogene mRNAs in response to external stimuli such as phorbol esters, cell surface antibodies and calcium ionophores (Schuler and Cole, 1988; Lindsten *et al.*, 1989). Whether this results from differential destabilization or selective stabilization is not clear, since non-ARE-associated mRNA stabilizing sequences also exist in the GM-CSF 3' UTR (Iwai *et al.*, 1991). Other explanations for differential regulation stem from the discovery that the *c-fos* and *c-myc* proto-oncogene mRNAs contain additional destabilizing elements found throughout their length (Shyu *et al.*, 1989, 1991; Chen *et al.*, 1994).

Another complex issue is whether ongoing translation is required for destabilization. Early work showed that translational inhibitors stabilized ERG mRNAs, suggesting a requirement for either a labile protein factor or translation of the mRNA body itself (Greenberg *et al.*, 1986). Several studies support the latter explanation: inhibition of translation by introducing either a strong stem-loop structure into the 5' UTR (Aharon and Schneider, 1993) or a premature stop codon into the open reading frame (ORF) (Savant-Bhonsale and Cleveland, 1992) inhibited translation and prevented destabilization. Re-introduction of an internal ribosome entry site downstream of the stem-loop re-established translation and destabilized the mRNA (Aharon and Schneider, 1993). Additional reports both confirmed and directly opposed these findings. One group found that insertion of strong stem-loops could stabilize a message regardless of location, as long as they were placed 5' to the ARE (Curatola *et al.*, 1995). An iron response element (IRE) in the 5' UTR of a mRNA was used to control intracellular localization and translation; with either the *c-fos* ARE or GM-CSF ARE in the 3' UTR, the message was destabilized only when loaded on polysomes (Winstall *et al.*, 1995). Conversely, inhibition of translation by introduction of an IRE was reported not to stabilize an mRNA carrying the *c-fos* ARE in its 3' UTR (Koeller *et al.*, 1991). Similarly, translation has been uncoupled from destabilization for both the *c-fos* and GM-CSF AREs using stem-loop structures in 5' UTR (Chen *et al.*, 1995).

In an effort to explain these apparent contradictions, Shyu and co-workers (Chen and Shyu, 1994; Chen *et al.*, 1995) defined three different classes of destabilizing elements: AUUUA-lacking elements and AUUUA-containing elements subdivided into those with scattered AUUUA motifs (typified by proto-oncogene mRNAs) and

those with multiple, overlapping iterations of the AUUUA pentanucleotide (typified by growth factors). Division into these three classes was supported by careful examination of the decay kinetics of growth factor and proto-oncogene AUUUA-containing ARE mRNAs, including differences in poly-A tail removal (Chen *et al.*, 1995) and characterization of a non-AUUUA-containing ARE in the *c-jun* proto-oncogene (Peng *et al.*, 1996). Specific sequences which can serve as destabilizing elements *in vivo* have recently been more precisely defined by deletion mutagenesis and construction of artificial AREs. Zubiaga and co-workers (1995) pared down the *c-fos* ARE to a minimal destabilizing element and used this information to construct artificial AREs. Lagnado *et al.* (1994) adopted a similar strategy starting directly with artificial AREs. Both groups concluded that a single AUUUA motif is insufficient for destabilization, whereas one copy of the nine-nucleotide sequence UUAUUUAUU is weakly destabilizing and two copies provide strong destabilization.

Numerous proteins have been implicated in mRNA destabilization by virtue of their ability to bind the ARE. Most of these are in the 30–45 kDa size range. First identified was a three-subunit complex of 44 kDa termed AUBF (AU-rich sequence binding factor; Malter, 1989). This phosphorylated protein is up-regulated in peripheral blood cells and a T-cell leukemia line upon treatment with phorbol esters or calcium ionophores, consistent with a role in cytokine and lymphokine regulation (Malter and Hong, 1991); it also recognizes proto-oncogene AREs (Gillis and Malter, 1991). Development of an *in vitro* destabilization system allowed identification and characterization of a fraction which accelerates the decay of ARE-containing messages. It contains two polypeptides of 37 kDa and 40 kDa called AUF1 (Brewer, 1991); a cDNA encoding the 37 kDa protein has been cloned and contains two RNA recognition motifs (RRMs) as well as a glutamine-rich C-terminal domain (Zhang *et al.*, 1993; Ehrenman *et al.*, 1994). Three activities termed AU-A, AU-B and AU-C likewise bind to ARE sequences (Bohjanen *et al.*, 1992). AU-B and AU-C appear to be structurally related and have specificity for lymphokine and cytokine AREs (Bohjanen *et al.*, 1991), while AU-A (34 kDa) is not selective and appears to shuttle between the nucleus and cytoplasm (Katz *et al.*, 1994). Two studies using UV-crosslinking have identified proteins of 82, 71, 66 and 37 kDa (You *et al.*, 1992) as well as 70, 45, 40, 38 and 32.5 kDa (Nakamaki *et al.*, 1995) which crosslink to AU-rich sequences from the *c-fos* and GM-CSF 3' UTR, respectively. In the latter study, actinomycin-D treatment depleted the 32.5 kDa protein from the nuclear fraction, yet none of the binding activities was affected by chemicals known to stabilize growth factor mRNAs (Nakamaki *et al.*, 1995). Immunoprecipitation experiments showed that while the 45, 40 and 38 kDa proteins were crossreactive with anti-AUF1 antibodies, the 40 and 38 kDa proteins were also crossreactive with anti-hnRNP C antibodies (Nakamaki *et al.*, 1995). The hnRNP A1 and C proteins had previously been shown to associate with AU-rich sequences (Hamilton *et al.*, 1993). Affinity chromatography using AUUUA multimers led to the isolation of a protein with hydratase activity (Nakagawa *et al.*, 1995). Ma and co-workers (1996) cloned HuR, a 36 kDa member of the *elav* family of RRM-containing

proteins and showed that it binds specifically *in vitro* to ARE sequences, similar to other ELAV proteins reported previously (Levine *et al.*, 1993; Gao *et al.*, 1994).

Of particular interest to us has been an apparent 32 kDa protein identified by Vakalopoulou *et al.* (1991) in nuclear extracts from HeLa cells. A correlation between UV-crosslinking of the protein *in vitro* and ability to destabilize *in vivo* was initially established for a small number of ARE sequences. We subsequently demonstrated that this protein also binds three virus-encoded small nuclear RNAs (snRNAs) which are expressed in marmoset T-cells transformed by *Herpesvirus saimiri* (Myer *et al.*, 1992). This suggested that the viral snRNAs may contribute to transformation by sequestering the 32 kDa protein in a stable, nuclear particle and thereby modulating mRNA stability in the cell.

Here, we have further characterized the HeLa 32 kDa protein with respect to its ARE-binding specificity. Using sequences previously tested *in vivo*, we find that the 32 kDa protein in nuclear extract interacts *in vitro* only with those that serve as effective destabilizing elements *in vivo*. Even similar sequences which function quite differently *in vivo* are faithfully distinguished by protein binding *in vitro*. We have purified the 32 kDa protein and identified it as being identical to HuR (Ma *et al.*, 1996). We show that binding of HuR *in vitro* to previously untested RNAs can predict the ability of these sequences to serve as functional AREs *in vivo*. Our results argue that HuR is a component of the mRNA destabilization machinery.

Results

RNA binding specificity of the 32 kDa protein

The recent *in vivo* dissection of ARE sequences (Lagnado *et al.*, 1994; Zubiaga *et al.*, 1995) provides a benchmark to which the binding specificity of any protein suspected to be involved in mRNA decay can be compared. We used two different assay systems to analyze the performance of the 32 kDa protein (Vakalopoulou *et al.*, 1991; Myer *et al.*, 1992) in binding competition reactions in HeLa nuclear extract. UV-crosslinking allows direct visualization of proteins bound to a radiolabeled RNA: short-wave (254 nm) light forges a covalent polypeptide–RNA bond, thereby tagging the protein with a radioactive moiety which allows detection by autoradiography. Gel retardation experiments, also using radiolabeled RNA, require fewer manipulations and probably more closely mimic *in vivo* conditions as a protein must remain stably bound to the substrate throughout the assay procedure.

To assess the binding of various sequences to the 32 kDa protein, each was tested for its ability to compete with a readily bound substrate in HeLa cell nuclear extract. The (AUUU)₄A RNA probe contains four tandemly repeated copies of the AUUUA motif, which is the core of the ARE sequence (Caput *et al.*, 1986; Shaw and Kamen, 1988) and has been shown to be an effective destabilizing element *in vivo* (Zubiaga *et al.*, 1995). The competitor RNAs (Table I) were transcribed in the presence of a trace amount of radioactive nucleotide to facilitate gel purification and quantitation. They were then combined at 10-, 20- and 50-fold molar excess with the (AUUU)₄A RNA, which was labeled to very high specific activity

Table I. Summary of binding to the 32 kDa protein *in vitro* and ability to destabilize a mRNA *in vivo*

Construct	Sequence	Binding ^a	Destabilization
(AUUU) ₄ A	AUUUAUUUAUUUAUUUA	++	+ + c,d
(AUUU) ₂ A	AUUUAUUUA	—	— b, c
N1	UU AUUUAUU	— ^e	+ c,d,f
N2	UU AUUUAUU GACCU AUUUAUU	++	+ + c, d
7S7	AUUUAUGACCUAUUUUAU	—	— c,d
(AU ₃ AGC) ₄ A	AUUUAGCAUUUAGCAUUUAGCAUUUAGC	—	— c
(AGGU) ₄ A	AGGUAGGUAGGUAGGU	—	— d
(AUGU) ₄ A	AUGUAGUAGUAGUAGU	++	+ d
(CUUU) ₄ C	CUUUCUUUCUUUCUUUC	—	— d
(AU) ₈ A	AUAUAUAUAUAUAUAUA	— ^f	n.d.
(AUU) ₅ A	AUUUAUUUAUUUAUUUA	— ^f	— b
(AUUUU) ₃ A	AUUUUUUUUUUUUUUUA	+ ^f	n.d.

Column 1 gives the names of all constructs. The (AUUU)₄A construct was previously called AUUUA-3Z (Myer and Steitz, 1995). Column 2 shows the variable portion of the sequence of the RNAs transcribed from the constructs in column 1. AUUUA pentanucleotides are in bold. All transcripts contain GGGCGAAUUC at their 5' ends and GUC at their 3' ends, as described in Materials and methods. Column 3 gives a qualitative assessment of each RNA's ability to bind the 32 kDa protein (HuR) in competition with the (AUUU)₄A RNA as assayed by both UV-crosslinking and mobility shift (see Figure 1). Column 4 is a qualitative assessment of each sequence's ability to function as an ARE *in vivo* when tested by transient transfection (see Figure 5; Lagnado *et al.*, 1994; Zubiaga *et al.*, 1995). n.d. indicates not done.

^aSee Figure 1.

^bLagnado *et al.* (1994).

^cZubiaga *et al.* (1995).

^dSee Figure 5.

^eBinding detected only by direct UV-crosslinking, see text.

^fData not shown.

with [α -³²P]UTP. If the competitor RNA binds the 32 kDa protein in HeLa nuclear extract, it should reduce both the crosslinking and mobility shift efficiency of the (AUUU)₄A RNA probe.

The results of such competitions are shown in Figure 1 [(A), UV-crosslinking; (B) gel retardation]. Lane 1 shows binding of (AUUU)₄A in the absence of any competitor RNA. Lanes 2–4 show competition with increasing amounts of the (AUUU)₄A sequence itself. Effective competition demonstrates that the 32 kDa protein is limiting under the assay conditions. Each of the competitor RNAs was then tested (Figure 1) and the results summarized in Table I.

For those sequences examined by Zubiaga *et al.* (1995) we found a positive correlation between ability to destabilize *in vivo* and bind the 32 kDa protein *in vitro*. Especially interesting are the N2 and 7S7 substrates. The N2 RNA contains two copies of the N1 minimal destabilizing sequence (UUAUUUAUU) separated by five nucleotides, while 7S7 is shorter by only four nucleotides, having the outside two uracils in each repeat unit deleted. *In vivo*, these substrates showed striking differences in their behavior, with N2 being a very active destabilizing element and 7S7 being inert (see Zubiaga *et al.*, 1995 and Figure 5). *In vitro*, the two RNAs show the same striking difference: N2 binds the 32 kDa protein very well (lanes 14–16), while 7S7 binding is not detectable (lanes 11–13).

The only one of the six previously tested substrates for which the *in vitro* binding and *in vivo* stabilizing results do not correlate is the N1 RNA (see Table I). This RNA appears not to bind at all in either competition assay (Figure 1). N1 was found to be a weak destabilizing element *in vivo* (Lagnado *et al.*, 1994; Zubiaga *et al.*, 1995); its apparent lack of binding *in vitro* may therefore reflect a very low affinity for the protein that is easily competed by lesser amounts of the (AUUU)₄A RNA. This speculation was confirmed by labeling the N1 RNA to

high specific activity and performing UV-crosslinking in the absence of any other RNA. The 32 kDa protein did crosslink to the N1 RNA, albeit much more weakly (~50-fold less) than to N2, (AUUU)₄A or (AUGU)₄A (data not shown).

In addition to examining sequences previously tested *in vivo*, we designed novel substrates to ask whether *in vitro* binding of an RNA to the 32 kDa protein would serve as a reliable predictor of that sequence's ability to destabilize *in vivo* (see below). The three mutants we made were modifications of the parent (AUUU)₄A substrate, since very few mutant sequences targeting the core AUUUA have been investigated. The middle uracil was changed to a guanosine in the (AUGU)₄A competitor (Figure 1, lanes 26–28), while in (AGGU)₄A both of the first two Us were converted to Gs (lanes 17–19). In (CUUU)₄C, all of the adenosines were changed to cytosines (lanes 20–22). Of these three mutants, only the (AUGU)₄A RNA competed effectively for binding to the 32 kDa protein. The other two mutants do not bind and would be predicted to be non-destabilizing.

Underexposure of the native gel (Figure 1B) revealed that the shifted complex formed on the (AUUU)₄A RNA consists of two bands with similar mobility. We explored the possibility that two different shifted complexes happened to co-migrate by directly examining the bound proteins. We separated nuclear extract that had been UV-crosslinked to radiolabeled (AUUU)₄A RNA (as in the competition reactions) on a native gel and isolated the shifted complex. After RNase treatment to remove excess RNA and fractionation on a denaturing SDS gel, we observed two crosslinked proteins: the 32 kDa protein, as well as a 46 kDa protein (data not shown). The larger protein is seen as the second most prominent band in the direct crosslinking assay (Figure 1A). Further experiments confirmed that the two crosslinking activities and their corresponding mobility shifts are separable chromato-

graphically (data not shown). Therefore, we argue that each protein independently imparts a similar mobility shift to the (AUUU)₄A RNA, rather than the shifted complex being a result of both proteins binding simultaneously to the same RNA.

Purification of the 32 kDa protein

To characterize the 32 kDa protein further, we purified it using UV-crosslinking to the (AUUU)₄A RNA as the

assay. HeLa cell nuclear extract (15 ml, equivalent to 7.5×10^9 cells) was precipitated with saturated ammonium sulfate (final concentration 50%) and the pellet was loaded onto a gel filtration column (Superdex 200, Pharmacia). The 32 kDa protein was found in fraction 13 (Figure 2A, lane 8), which is well included in the column and is approximately where a 32 kDa monomeric protein would be expected to elute. It was important to run the column at relatively high salt concentration (400 mM NaCl) because at lower salt levels (150 mM) the protein was completely excluded, suggesting its association with large complexes in nuclear extract (data not shown). Fractionation on other sizing columns indicated that such complexes are entirely excluded from Sephacryl S-300 (exclusion limit of 1.5 MDa) and partially in the void volume of an S-500 column (exclusion limit >8 MDa; data not shown).

Heparin is often useful for the purification of nucleic acid-binding proteins since its polyanionic structure mimics the sugar-phosphate backbone. The sample from the Superdex 200 column was diluted and loaded onto a heparin-Sepharose column. The 32 kDa protein bound and was eluted by a linear salt gradient at ~ 280 mM NaCl (Figure 2B). The slightly larger crosslinked species in Figure 2B, fractions 20 and 21, was identified by Western blotting as the hnRNP A1 protein (data not shown). The bulk of all protein, as well as hnRNP A1, eluted at higher salt (320 mM NaCl). A characteristic smaller doublet co-eluting with the 32 kDa protein (seen in fractions 14–19) often appeared during purification and is diagnostic for the 32 kDa protein. We believe these peptides to be stable degradation products of the 32 kDa protein.

Fractions containing peak activity from the heparin column (Figure 2B, fractions 15–18) were collected, dialyzed to lower salt concentration (100 mM NaCl) and pH (6.3), and loaded onto a Mono S column (Pharmacia). Notably, despite the anionic nature of both the heparin and the Mono S resins, the 32 kDa protein would not bind to Mono S at pH 7.9, thereby necessitating prior dialysis. When bound proteins were eluted with a linear salt gradient, crosslinking activity peaked in fractions 24–26 (Figure 2C). Western blot analysis identified peaks of the hnRNP A1 and A2 proteins in fractions 19–20 and 15–16, respectively. All fractions were concentrated to <50 μ l and fractionated on a denaturing SDS gel. After the gel was stained with Coomassie blue and destained, the 32 kDa protein was excised (Figure 2D, lanes 9–11) and subjected to tryptic digestion and amino acid sequencing.

Fig. 1. Binding specificity of the 32 kDa protein. The ability of the 32 kDa protein to bind the [α -³²P]UTP-labeled (AUUU)₄A RNA in the presence of competitor RNAs (see Table I) was assayed by both UV-crosslinking (A) and mobility shift (B). In (A) and (B), lane 1 shows binding in the absence of added specific competitor; all lanes contain 3 μ g of yeast RNA as non-specific competitor. Each unlabeled, specific competitor is presented in three lane sets in both panels with the first lane (2, 5, 8, 11, 14, 17, 20, 23 and 26) being a 10-fold molar excess of the indicated competitor, the second lane (3, 6, 9, 12, 15, 18, 21, 24 and 27) being a 20-fold molar excess, and the third lane (lanes 4, 7, 10, 13, 16, 19, 22, 24 and 28) a 50-fold excess. In (A), the mobility of the crosslinked 32 kDa protein in the 12.5% denaturing SDS gel is indicated with an arrow. In (B), the mobilities of the free probe (FP) and shifted complex (C) on the 4% non-denaturing gel are noted.

Purification was started with 150 mg of protein and resulted in ~2 µg product. Recovery is estimated at 20% based on UV-crosslinking activity (data not shown), resulting in an ~15 000-fold purification.

To confirm that the material in the final purified fraction was the same as the 32 kDa protein which crosslinks to the (AUUU)₄A RNA in HeLa nuclear extract, we performed

partial proteolytic digestion analysis. Comparing digestion profiles of similarly sized crosslinked proteins is a very sensitive method for determining their relationship. As shown in Figure 3, the crosslinked material in the final purified fraction (lanes 5–8) has a digestion profile identical to that of the 32 kDa protein from crude HeLa extract (lanes 1–4). Thus, we have purified the 32 kDa protein implicated in mRNA destabilization from HeLa cells.

The 32 kDa protein is HuR

Two peptide sequences obtained from the purified 32 kDa protein (TNLIVNYLPQNMTQDELRL and VLVDQTTGLSR) were exact matches to tryptic fragments predicted for a protein sequence found in the database. HuR is a recently described (Ma *et al.*, 1996) member of the *elr*-A class of the elav-like gene (ELG) family (Good, 1995). It is expressed ubiquitously and is highly conserved with 92% identity between *Xenopus laevis* and humans (Ma *et al.*, 1996).

To confirm that HuR is identical to the 32 kDa protein we had purified, we again relied on comparison of the partial proteolytic digestion profiles of the 32 kDa protein from HeLa cells and recombinant HuR. The ORF encoding HuR was amplified from a human placental cDNA library by PCR and cloned into a bacterial expression vector (pet 11b, Novagen). As shown in the Coomassie-stained gel of total bacterial protein (Figure 4A), only a single band with a molecular weight of ~32 kDa is strongly and specifically produced upon induction with IPTG. In bacterial extracts prepared by sonication, much of the protein is insoluble, but significant quantities of soluble HuR remain (data not shown).

The binding activity of recombinant HuR in bacterial extracts was tested by UV-crosslinking to the (AUUU)₄A RNA. As shown in Figure 4B, extracts made from bacteria expressing HuR give a strong crosslinking signal (lane 2) of identical mobility to the 32 kDa protein crosslinked in HeLa cell nuclear extract (lane 1). When extracts made from bacteria carrying the parent plasmid with no HuR insert were used, no significant crosslinks were observed (data not shown).

Fig. 2. Purification of the 32 kDa protein from HeLa cell nuclear extract. HeLa cell nuclear extract was fractionated and assayed by UV-crosslinking to the [α -³²P]UTP-labeled (AUUU)₄A RNA. Panels (A–D) are all 12.5% denaturing SDS protein gels. (A) The 0–50% ammonium sulfate cut of crude nuclear extract was fractionated on a Superdex 200 gel filtration column at 400 mM NaCl. Crosslinking of the starting material (NE) and of selected column fractions are shown. The 32 kDa protein is indicated by a line. Fraction 3 is the column void volume and fraction 13 was processed further. (B) The sample from the sizing column was diluted 1:2 with running buffer to a final salt concentration of 133 mM NaCl and loaded on a heparin–Sepharose column. After washing, the proteins were eluted with a linear salt gradient. The load (L), column flow-through (FT), and selected gradient fractions were assayed. Fractions 15–18 were processed further. The migration of the 32 kDa protein is denoted with a line. (C) After dialysis to a lower pH and salt concentration, the sample was applied to a Mono S column and proteins eluted with a linear 100–500 mM NaCl gradient. The material loaded onto the column (L) and fractions 14–27 were assayed. (D) After concentration, the Mono S fractions were separated on a preparative gel and stained with Coomassie blue. The identities of the 32 kDa protein, hnRNP A1 and hnRNP A2 (as determined by Western blotting) are noted. The most prominent band in fractions 24–26 was excised and submitted for protein sequencing.

Fig. 3. Comparative protease mapping of the purified 32 kDa protein and the protein which crosslinks in crude HeLa nuclear extract. Either crude extract (lanes 1–4) or Mono S-purified material (lanes 5–8) was crosslinked to the [α - 32 P]UTP-labeled (AUUU) $_4$ A RNA and excised from a preparative gel. These gel slices were loaded onto a second, 15% acrylamide/0.1% SDS gel and subjected to increasing amounts of V8 protease before separation.

The definitive comparative proteolysis map is shown in Figure 4C. Crosslinked protein from HeLa cell extract (lanes 1–4) produced a digestion profile identical to that of crosslinked protein from HuR-expressing bacterial extract (lanes 5–8). Additionally, *in vitro* binding experiments (data not shown) confirmed that the RNA binding specificity of recombinant HuR is identical to that of the human 32 kDa protein originally identified by Vakalopoulou *et al.* (1991).

HuR binding correlates with *in vivo* destabilization activity

To confirm that *in vitro* binding of HuR to an RNA sequence reflects that RNA's ability to destabilize *in vivo*, we undertook a series of transient transfection assays (Figure 5). Constructs for these experiments were based on those previously reported (Zubiaga *et al.*, 1995) and all control plasmids were gifts of the authors. Untested sequences were introduced into the same parent plasmid pB–N1 (see Materials and methods and Zubiaga *et al.*, 1995) to insure that any observed stability differences were due exclusively to the new sequences being tested.

NIH 3T3 cells were co-transfected with the plasmid encoding the β -globin mRNA with the ARE, and a control plasmid pEF–BOS–CAT (Zubiaga *et al.*, 1995), which constitutively expresses the CAT mRNA. After serum starvation, expression of the β -globin mRNA cloned downstream of the *c-fos* promoter was initiated by addition of serum. Cells were collected every 90 min, and the RNA isolated and analyzed by RNase protection (Figure 5A). The level of the CAT mRNA was determined at each point and all numbers standardized to this internal control. The data are shown in Figure 5B and summarized in Table I. The maximum signal in each case was obtained between 90 min and 3 h; this level was considered as 100%. The peak expression of mRNA at earlier times (1–1.5 h) in previous studies (Lagnado *et al.*, 1994; Zubiaga *et al.*, 1995) can probably be attributed to the cellular response to the different transfection system (lipofectamine) used here. Another difference is that the stable mRNAs are more stable in our system than in previous reports (Lagnado *et al.*, 1994; Zubiaga *et al.*, 1995).

As is evident in Figure 5, control mRNAs which contain

Fig. 4. The 32 kDa protein is identical to HuR. (A) A Coomassie blue-stained gel showing the total protein from *E. coli* cells carrying an expression plasmid containing the HuR open reading frame. Lane 1 shows the profile before induction and lane 2 at 2 h after induction with 1 mM IPTG. A 32 kDa band (noted with a line) is produced. (B) Extracts of HeLa cell nuclei (lane 1) or bacteria (lane 2) induced as in (A) were subjected to UV-crosslinking using the [α - 32 P]UTP-labeled (AUUU) $_4$ A RNA. No prominent bands were seen in extracts made from bacteria carrying the parent plasmid (data not shown). The migration of the 32 kDa protein and recombinant HuR are denoted with a line. (C) Crosslinked protein was generated in HeLa and bacterial extracts [as in (B)] were analyzed as described in Figure 3. Lanes 1–4 show the digestion pattern of crosslinked protein from HeLa cell nuclear extract while lanes 5–8 show that from HuR-expressing bacterial extracts.

AREs previously shown to be functional [the *c-fos* ARE, (AUUU) $_4$ A and N2] are unstable in our transfection system with levels below 35% at 7 h post-induction. Likewise, mRNAs shown to be stable (the parent β -globin pBBB and 7S7) exhibit RNA levels >80% after 7.5 h. Additionally, we observe that the activity of N1 is cell type-specific in our hands: it destabilizes in NIH 3T3 cells (see Figure 5), but not in mouse L-cells (data not shown). Another explanation for the discrepancy between N1's behavior in the binding (Figure 1) and destabilization (Figure 5) assays is that N1 is simply too short for stable binding.

The instability conferred by the previously untested sequences correlates with their *in vitro* binding specificity. Both the (AGGU) $_4$ A and (CUUU) $_4$ C RNAs are ineffective as AREs, with 95% and 91% RNA remaining at the 7.5 h time point respectively (Figure 5A and B). In contrast, the (AUGU) $_4$ A sequence clearly destabilizes, with <61% RNA remaining. Thus, *in vitro* binding of a RNA to HuR successfully predicts a sequence's ability to function as an ARE *in vivo*.

Fig. 5. Transient transfection analysis of ARE sequences. (A) Plasmids containing either previously tested or untested AREs were transiently transfected into NIH 3T3 cells using lipofectamine. After serum starvation, expression was stimulated by serum addition, cells harvested, RNA isolated and analyzed by RNase protection. 90-min time points are shown, beginning when the serum burst was delivered (Time = 0) to 7.5 h post-induction (Time = 7.5). (B) The signals obtained from RNase protection were quantitated on a Molecular Dynamics PhosphorImager and plotted. All values were scaled to the level obtained from constitutively expressed CAT mRNA which was co-transfected and also analyzed by RNase protection. 100% RNA is arbitrarily assigned to the time point which gave the highest signal. For pB-(CUUU)₄C, the 3.0-h rather than the 1.5-h time point was used because of the aberrantly low CAT signal for the 1.5-h point. The data obtained in these experiments have been repeated several times.

Discussion

Despite the identification of numerous proteins with affinity for ARE elements, none has been definitively linked to ARE-mediated destabilization. Without a reliable *in vitro* assay for mRNA decay, identification of participating proteins must rest on the correlation of *in vitro* binding with *in vivo* activity. Here, we have shown that one protein, the 32 kDa protein identified by Vakalopoulou *et al.* (1991), has binding specificity for RNAs that correlates directly with a sequence's documented ability to serve as a functional ARE *in vivo*. Furthermore, binding specificity is a reliable predictor of a previously untested RNA's destabilizing activity. By purifying the 32 kDa protein, we have shown that it is identical to HuR (Ma *et al.*, 1996).

HuR is a human homologue of the *Drosophila elav* (embryonic lethal, abnormal vision) gene. Elav was originally identified in a screen designed to identify nervous system defects and is essential for proper neural development (Campos *et al.*, 1985; Robinow *et al.*, 1988). Subsequent work has shown several distinct elav-like genes (ELGs) in all higher eukaryotes; they have been classified into four groups (*elr* A–D) based on their sequence similarity and tissue specificity of mRNA expression (Good, 1995). The *elr* D and C genes (human genes HuD and HuC/ple21, respectively) are neuron-specific and serve as very early markers for commitment to development into neuronal cells (Marusich and Weston, 1992; Good, 1995). The *elr* B protein (human gene Hel-N1/Hel-N2) is expressed in nervous tissue, as well as in testis, ovaries and certain tumor cell lines (Gao *et al.*, 1994; Good, 1995). The *elr* A gene (human gene HuR) is the most widely expressed family member, but may be

limited to vertebrates since it has not been identified in *Drosophila*, despite efforts to do so (Good, 1995). All ELGs contain three RRM with a characteristic tethering domain between the second and third RRM (Robinow *et al.*, 1988; Levine *et al.*, 1993; Good, 1995; Ma *et al.*, 1996). The human gene products are targets for antibodies produced by individuals with autoimmune disorders such as paraneoplastic encephalomyelitis and sensory neuropathy (Graus *et al.*, 1985; Dalmau *et al.*, 1990). The human gene products Hel-N1/Hel-N2 have been implicated in mRNA destabilization and translation based on its *in vitro* ARE-binding specificity (Levine *et al.*, 1993). In an effort to identify a member of the ELG family which might be a participant in destabilization in all tissues, the human *elr* A family member HuR was cloned using degenerate PCR (Ma *et al.*, 1996). Gel retardation assays with HuR–GST fusion proteins confirmed that HuR has a binding specificity consistent with its involvement in ARE-mediated mRNA destabilization (Ma *et al.*, 1996).

The binding of HuR in HeLa cell extracts was originally shown to be inversely correlated with mRNA accumulation for a total of eight sequences (Vakalopoulou *et al.*, 1991). In this report we extend this correlation to nine additional sequences. Moreover, we have assayed the binding of each mutant by competition against a single sequence [(AUUU)₄A] instead of direct crosslinking to the mutant sequence. This eliminates the possibility that changing the sequence affects only crosslinking (but not binding) by removal or alteration of a nucleotide that is chemically essential for formation of the crosslink.

The sequence discrimination of HuR binding is quite striking. The N2 sequence, which contains two non-overlapping copies of UUAUUUAUU, binds well and

destabilizes with near wild-type ARE efficiency (see Figures 1 and 5; also Zubiaga *et al.*, 1995). Removing the four outside U-residues from the two copies of UUAUUUAUU (7S7) completely eliminates binding and ARE function. In reciprocal experiments, the *in vitro* binding of three previously untested sequences successfully predicted their *in vivo* ARE-function. We also determined by competition that HuR does not bind to (AU)₈A or (AUU)₅A but does bind (AUUUU)₃A (data not shown). Although none of these sequences has been tested *in vivo*, a sequence similar to the (AUU)₅A RNA that contains six AUUA repeats was found to be non-functional *in vivo*, consistent with our binding data (Lagnado *et al.*, 1994). Given the correlation between HuR binding and destabilization, we predict that the (AUUUU)₃A sequence should serve as a functional ARE, while the (AU)₈A should not.

The identification of the 32 kDa protein as HuR should help resolve the role of ongoing mRNA translation in destabilization. Originally, an inverse correlation between binding of the 32 kDa protein and RNA accumulation was observed for RNA in nuclear, as well as cytoplasmic fractions (Vakalopoulou *et al.*, 1991). This suggested that translation may not be essential for RNA destabilization and/or that there may be an independent, but related, nuclear destabilization pathway. We originally became interested in ARE-containing mRNAs because a group of viral small nuclear RNAs (snRNAs) contain ARE-like sequences at their 5' ends. We showed that the stable snRNAs encoded by *Herpesvirus saimiri* (the HSURs; *Herpesvirus saimiri* U-RNAs), upon transformation of host T lymphocytes, are packaged into snRNPs which contain the 32 kDa protein (Myer *et al.*, 1992). Recent work designed to test the hypothesis that HSURs bind and sequester HuR in stable nuclear particles, thereby preventing it from recognizing ARE-containing mRNAs, has produced surprising results. snRNAs containing ARE-like sequences are less stable than snRNAs in which the ARE has been mutated to eliminate HuR binding (X.Fan, V.Myer and J.Steitz, manuscript in preparation). This suggests that HuR may be involved in numerous destabilization pathways, at least some of which clearly do not require translation.

It is possible that HuR is identical to the AU-A activity of Bohjanen and co-workers (Bohjanen *et al.*, 1992; Katz *et al.*, 1994). AU-A has a molecular weight of ~34 kDa, predominates in nuclear fractions and binds indiscriminately to ARE sequences regardless of their origin (Bohjanen *et al.*, 1992). It is not subject to the up-regulation seen for AU-B and AU-C (Bohjanen *et al.*, 1992) and shuttles between the nucleus and cytoplasm (Katz *et al.*, 1994), reminiscent of the hnRNP A proteins (Pinol-Roma and Dreyfuss, 1991). HuR likewise binds to AREs from both GM-CSF and *c-fos* (Vakalopoulou *et al.*, 1991). We speculate that HuR, although having no detectable nuclease activity itself (data not shown), may be a general targeting factor which specifies any RNA for rapid removal by directing assembly of a degradation complex. Selective class-specific regulation could then be imposed either by removal of HuR from the mRNA or by directly masking HuR's function. The use of comparative protease mapping should help distinguish the interrelationship and identities of the numerous ARE-binding proteins identified so far.

One possible role for HuR which cannot be ruled out by the present data is that HuR is, in fact, a stabilizing factor. In such a model, HuR would bind to the ARE and restrict access of the degradation machinery to the mRNA in a regulated fashion. This protection would then be antagonized by specific proteins to initiate decay. Thus, HuR would bind well to functional AREs and protect them until degradation of such ARE-containing mRNAs was deemed necessary by the cell. HuR would not bind to non-ARE-like sequences, as they would not be recognized by the degradation machinery and would thus require no protection. Experiments to distinguish between HuR as a destabilizing or stabilizing factor are now possible using transfection to modulate the levels of the protein within the cell. The demonstration that the 32 kDa protein is identical to HuR, and that this protein is central to mRNA destabilization, will facilitate subsequent development of immunological tools and other reagents necessary for molecular dissection of this important aspect of gene regulation.

Materials and methods

DNA oligonucleotide synthesis, cloning and transcription

All plasmids for *in vitro* transcription of RNAs used in competition or binding experiments were created identically to the AUUUA-3Z plasmid (Myer and Steitz, 1995). Complementary oligonucleotides containing an internal *HincII* site were cloned into the *EcoRI* and *HindIII* sites of the vector pGEM-3Z (Promega). After digestion with *HincII* and transcription by T7 RNA polymerase, the resulting RNA sequence is 5'-GGGCG-AAAUC-variable-GUC-3'. The variable sequences are listed in Table I. Transcription was performed in the presence of [α -³²P]UTP at a specific activity of either 30 000 c.p.m./ng for the highly labeled (AUUU)₄A probe (AUUUA-3Z plasmid) or 80 c.p.m./ng for competitor RNAs. Transcripts were purified on a 10% polyacrylamide gel and eluted by overnight incubation in RNA elution buffer (10 mM Tris, pH 7.5, 400 mM NaCl, 0.1% SDS). After ethanol precipitation, RNAs were resuspended in water and counted in a scintillation counter. The yields and concentrations were then calculated based on the specific activities of each transcript.

Plasmids for transient transfection assays contained a genomic copy of the rabbit β -globin gene driven by the serum-inducible human *c-fos* promoter. A 51-base pair sequence from the *c-fos* 3' UTR (bp 3099 to bp 3149 as measured from the translation start site) followed by various degradation sequences was inserted directly after the β -globin stop codon. The plasmids pBBB-ARE, pB-N1, pB-N2, pB-7S7, as well as the internal control plasmid pEF-BOS-CAT, have been described previously (Zubiaga *et al.*, 1995) and were the generous gifts of Dr J.G.Belasco. The plasmid pBBB was a gift from Dr A.-B.Shyu (Shyu *et al.*, 1989). Plasmids pB-(AUUU)₄A, pB-(CUUU)₄C, pB-(AGGU)₄A and pB-(AUGU)₄A were generated in such a way that the N1 (UUAU-UUAUU) sequence in pB-N1 was replaced by each of these four different 17 base pair sequences.

The pGEM-3Z plasmid (Promega) for transcribing the antisense β -globin RNA contained the globin genomic sequence from nucleotides 217 to 730 (exon 2 flanked by two introns), while that for antisense CAT contained the sequence from nucleotides 238 to 385 as measured from the translation start site. After linearization by restriction enzyme *EcoRI* or *HindIII* (for globin or CAT, respectively) near the 5' end of the sense sequence, the plasmids were transcribed *in vitro* by SP6 or T7 RNA polymerase (for globin or CAT, respectively) in the presence of [α -³²P]UTP. RNAs were gel-purified as above.

DNA oligonucleotides were synthesized by John Flory at the Keck Foundation Biotechnology Resource Laboratory at Yale University on an Applied Biosystems DNA synthesizer. All cloning was performed according to standard procedures (Maniatis *et al.*, 1982).

Cell culture and nuclear extracts

For preparation of nuclear extracts, frozen HeLa cells were purchased from the National Cell Culture Center (Minneapolis, Minnesota). Extracts were prepared essentially as described by Dignam *et al.* (1983) and modified as in Myer and Steitz (1995).

Murine NIH 3T3 fibroblasts were passed in Dulbecco's modified Eagle's medium (DMEM; Gibco) with 10% fetal bovine serum (FBS; Gibco).

Binding and competition experiments

Binding reactions containing 10 µl of HeLa cell nuclear extract, 3 µg yeast cRNA (Sigma), 0.5 µl 100 mM ATP (Pharmacia), 400 000 c.p.m. (AUUU)₄A probe labeled to high specific activity and competitor RNA (labeled to low specific activity) at 10-, 20- or 50-fold molar excess in a total of 20 µl were incubated for 15 min at 30°C. From each reaction, 3 µl were removed and loaded directly onto a 4% polyacrylamide/1× TBE non-denaturing gel and electrophoresed at room temperature; gels were dried and exposed to film at room temperature. The remainder of the reaction mixture was crosslinked with 254 nm light for 15 min at 4°C, treated with RNase A for 15 min at 37°C, and fractionated on a 12.5% polyacrylamide/1% SDS gel as described (Myer *et al.*, 1992).

Purification of the 32 kDa protein

Saturated ammonium sulfate (pH 8.0) was added to 15 ml of HeLa cell nuclear extract (equivalent to 7.5×10^9 cells) to a final concentration of 50% and rocked for 30 min at 4°C. Precipitated proteins were removed by centrifugation at 12 000 r.p.m. for 15 min in an SS-34 rotor (Sorvall). The pellet was resuspended to 4 ml total volume with buffer D [20 mM HEPES, pH 7.9, 10% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 0.05% Thesit (Boehringer-Mannheim)] supplemented with 400 mM NaCl and loaded onto a 320-ml Superdex 200 column (Pharmacia) equilibrated with buffer D + 400 mM NaCl. Fractions (10 ml each) were collected starting at 90 ml with the exception of fraction 13, which was 15 ml. 10 µl of each fraction were removed for assay by UV-crosslinking. Reaction mixtures consisted of 10 µl column fraction, 300 000 c.p.m. of [α -³²P]UTP (AUUU)₄A probe (30 000 c.p.m./ng), 0.5 µl 100 mM ATP, 5 µg *E. coli* tRNA and H₂O to a total volume of 20 µl. Subsequent crosslinking and processing of reactions was performed exactly as for the competition experiments.

Fraction 13 was diluted 1:2 with buffer D to a final volume of 45 ml (NaCl concentration 133 mM) and loaded onto a 1 ml heparin-Sepharose hi-trap column (Pharmacia). After washing with 10 column volumes (CVs) of buffer D + 133 mM NaCl, a 15-CV linear gradient to 400 mM NaCl was applied and 1 ml fractions collected. Crosslinking assays were performed as above.

Fractions containing peak activity were pooled (4 ml total) and dialyzed against 1 l of buffer E (50 mM MES, pH 6.2, 10% glycerol, 0.2 mM EDTA, 0.5 mM DTT, 1 mM PMSF, 0.05% Thesit) + 100 mM NaCl for 2 h, with one buffer change. The sample was then loaded onto a 1 ml Mono S FPLC column (Pharmacia) and washed with 5 CVs of buffer E + 100 mM NaCl. A 20-CV linear gradient to 500 mM NaCl was then applied and 1 ml fractions collected. From each fraction, 150 µl were removed, 10 µl of which assayed as above. The remaining 850 µl were concentrated to ~50 µl final volume in a Centricon-10 (Amicon). These fractions were separated on a preparative 12.5% acrylamide/0.1% SDS gel, which was stained with Coomassie brilliant blue R-250 (Bio-Rad) and destained; the appropriate bands were excised and submitted to the Keck Foundation Protein Sequencing Facility at Yale University for tryptic digestion and amino acid sequencing.

Protease mapping

Partial proteolytic digestion was performed as described (Cleveland *et al.*, 1977; Myer *et al.*, 1992) using protein crosslinked to the (AUUU)₄A RNA labeled to high specific activity.

Recombinant HuR expression

To express HuR in bacteria, 200 ng of the PCR primers HuR-N (5'-GCC GCG CTC GAG CAT ATG TCT AAT GGT TAT GAA GAC CAC ATG GCC GAA GAC) and HuR-C (5'-GCC CCG CTC GAG CAT ATG TCA TTA TTT GTG GGA CTT GTT GGT TTT GAA GG) were used to amplify the HuR ORF (Ma *et al.*, 1996) from a human placental cDNA library. The mixture, containing 1 µg library DNA, 1× amplification buffer (Maniatis *et al.*, 1982), 200 µM dNTPs, 1.5 mM MgCl₂ and 1 µl *Taq* polymerase (Perkin-Elmer), was cycled 40 times (1 min at 94°C, 1 min at 65°C, 1 min at 72°C) and the product was PCA (phenol:chloroform:isoamyl alcohol, 25:25:1) extracted, chloroform extracted, ethanol precipitated, digested with *Nde*I and fractionated on a 1% agarose gel. The DNA band of ~1 kbp was excised from the gel and ligated into the *Nde*I site of the pet-11b vector (Novagen). The resulting clone generated a protein with the exact N- and C-termini predicted by the ORF of the HuR cDNA (Ma *et al.*, 1996). Log phase BL21 *E. coli* (250 ml) carrying this plasmid were

induced for 2 h at 37°C in the presence of 1 mM IPTG. Total protein was examined by removing 1 ml samples before induction and after 2 h, pelleting each bacterial sample in a microcentrifuge, resuspending the pellet in 100 µl of SDS loading buffer, boiling and fractionating 10 µl on a 12.5% acrylamide/0.1% SDS gel. Proteins were visualized by Coomassie blue staining. Bacterial extract was made by pelleting 250 ml of induced bacteria, resuspending and sonicating in 20 ml buffer D. After sonication, the bacteria were subjected to four freeze-thaw cycles and debris was removed by centrifugation at 12 000 g for 10 min. Extracts were frozen at -80°C. UV-crosslinking was performed as in the purification assays.

Transfections and RNase protection assays

Murine NIH 3T3 cells were grown in 100 mm dishes to between 50 and 70% confluency and switched to OPTI-MEM I reduced serum medium (Gibco) for lipofectamine (Gibco) transfection. DNA plasmids (12 µg of the reporter plasmid plus 4 µg of the control pEF-BOS-CAT) were mixed with 48 µl of lipofectamine reagent in OPTI-MEM I reduced serum medium for 45 min and added to cells for overnight incubation. After recovery in 10% FBS-DMEM for 3–6 h, the transfected cells were serum-starved in 0.5% FBS-DMEM for 24 h and then stimulated with 20% FBS-DMEM as described (Zubiaga *et al.*, 1995). Following stimulation, total RNA was collected at various time points using TRIzol reagent (Gibco) and then treated with RQ1 DNase (Promega). The degradation of β -globin mRNA was analyzed by T1 RNase protection assays as described (Lee, S.I. *et al.*, 1988), with the following modifications: for each time point, equal amounts of DNase-treated RNA (20–30 µg) were combined with equal counts of ³²P-labeled anti- β -globin and anti-CAT probes ($2\text{--}4 \times 10^5$ c.p.m.). The RNA-probe mixture was heated at 85°C for 5 min, incubated at 45°C overnight to allow annealing and then digested with T1 RNase (1 unit/10 µg RNA; Calbiochem) at 30°C for 1 h. The samples were electrophoresed in a 6% polyacrylamide/TBE gel, dried and exposed to film. Results were quantitated with a Molecular Dynamics PhosphorImager.

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