The EMBO Journal Vol.17 No.12 pp.3461–3470, 1998

RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein

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An important paradigm for post-transcriptional regulation is the control of cytoplasmic mRNA stability mediated by AU-rich elements (AREs) in the 3′ untranslated region of transcripts encoding oncoproteins, cytokines and transcription factors. While many RNA-binding proteins have been shown to bind to AREs *in vitro*, neither the functional consequences nor the physiological significance of their interactions are known. Here we demonstrate a role for the embryonic lethal abnormal visual (ELAV) RNA-binding protein HuR in mRNA turnover *in vivo*. The ELAV family of RNA-binding proteins is highly conserved in vertebrates. In humans, there are four members; HuR is expressed in all proliferating cells, whereas Hel-N1, HuC and HuD are expressed in terminally differentiated neurons. We show that elevation of cytoplasmic HuR levels inhibits c-*fos* ARE-mediated RNA decay but has little effect on rapid decay directed by c-*jun* ARE. It appears that HuR has little effect on deadenylation but delays onset of decay of the RNA body and slows down its subsequent decay. We also show that HuR can be induced to redistribute from the nucleus to the cytoplasm and that this redistribution is associated with an altered function. Modulation of the ARE-mediated decay pathway through controlling distribution of the ELAV proteins between nucleus and cytoplasm may be a mechanism by which cell growth and differentiation is regulated.

Keywords: AU-rich element/ELAV protein/HuR/ nucleocytoplasmic shuttling/tetracycline-regulatory system

Introduction

Post-transcriptional regulation of the fate of mRNA in the cytoplasm, including mRNA localization, stability and translation, is now recognized as an important control point in mRNA metabolism (Dreyfuss *et al.*, 1996; Jacobson and Peltz, 1996; Wickens *et al.*, 1997). Control of selective mRNA degradation mediated by AU-rich elements (AREs) found in the 3′ untranslated region (UTR) of transcripts encoding oncoproteins, cytokines and transcription factors is a paradigm for post-transcriptional regulation (Chen and Shyu, 1995; Ross, 1995; Jacobson and Peltz, 1996). AREs are the most investigated RNA instability determinant among those characterized in mammalian cells. The widespread occurrence of AREs in mRNAs encoding proteins with diversified functions and synthesized under a variety of physiological conditions suggests that AREs are involved in the regulation of many biological processes (Chen and Shyu, 1995).

While a great deal has been learned concerning the key sequence features that specify the destabilizing function of the ARE (Chen and Shyu, 1995; Xu *et al.*, 1997), relatively little is known about the *trans*-acting factors that regulate or participate in the ARE-directed rapid mRNA decay. In the past few years, there have been many reports identifying ARE-binding activity in crude cell extracts. Among the proteins identified, at least 10 of them have been either cloned or found to be identical to known gene products. They include AUF1 (Zhang *et al.*, 1993), 3-oxoacyl-CoA thiolase (Nanbu *et al.*, 1993), glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Nagy and Rigby, 1995), hnRNP A1 (Hamilton *et al.*, 1993), hnRNP C (Hamilton *et al.*, 1993), AUH with enoyl-CoA hydratase activity (Nakagawa *et al.*, 1995) and the embryonic lethal abnormal visual (ELAV) family of RNA-binding proteins (reviewed in Antic and Keene, 1997). However, neither the functional consequences nor the physiological significance of the *in vitro* interactions have been directly demonstrated *in vivo*.

The ELAV family of proteins is of particular interest in that: (i) certain members of this family, e.g. mouse HuC and human HuR, have been shown to exhibit poly(A)-binding activity and appear to be able to bind simultaneously to the ARE and the poly(A) tail *in vitro* (Abe *et al.*, 1996; Ma *et al.*, 1997); (ii) overexpression of Hel-N1 leads to an enhancement of cytoplasmic expression of glucose transporter (GLUT1) mRNA which bears a U-rich region in its 3′ UTR (Jain *et al.*, 1997); (iii) their high affinity for AU- or U-rich sequences has been most extensively and rigorously addressed among other ARE-binding proteins (Levine *et al.*, 1993; Gao *et al.*, 1994; Good, 1995; Chung *et al.*, 1996, 1997; Gao and Keene, 1996; Ma *et al.*, 1996; Myer *et al.*, 1997); (iv) they bind to a subset of poly(A)$^+$ mRNA *in vivo*, forming the mRNP complexes that associate with ribosomes during translation (Gao and Keene, 1996). These observations support a role of ELAV proteins in modulating mRNA stability via the ARE. One other interesting feature of the ELAV proteins is their distribution both in the nucleus and the cytoplasm, consistent with being nucleocytoplasmic shuttling proteins (Antic and Keene, 1997). The observation that Hel-N1 is readily detected and mainly expressed in the cytoplasm of terminally differentiated neurons (Gao and Keene, 1996) is intriguing. It suggests that a change of distribution of ELAV proteins between the nuclear and cytoplasmic compartments may be used by ELAV proteins to fulfil their function in the cytoplasm.

In this report, we provide the first *in vivo* evidence that HuR, whose mRNA is ubiquitously expressed in all proliferating cells, participates in the regulation of the ARE-mediated mRNA turnover. We show that elevation of HuR in the cytoplasm through ectopic overexpression of exogenous HuR specifically inhibits the c-*fos* ARE-directed mRNA decay but has little effect on rapid decay mediated by the c-*jun* ARE. HuR appears to have little effect on the poly(A) shortening step. Instead, it delays the commencement of decay of the RNA body and slows down its subsequent decay. Moreover, the redistribution of HuR from nucleus to cytoplasm induced by nuclear transcription blockage is associated with the differential stabilization of mRNA bearing the c-*fos* ARE but not the c-*jun* ARE, suggesting a mechanism by which modulation of the ARE-mediated decay pathway is accomplished by controlling the nucleocytoplasmic distribution of ELAV proteins.

Results

Ectopic expression of human HuR in mouse NIH 3T3 cells specifically impedes c-fos ARE-mediated mRNA decay

To examine the potential participation of HuR in ARE-mediated mRNA turnover *in vivo*, we cloned a cDNA encoding HuR from a human placenta cDNA library by PCR (see Materials and methods). To obtain regulated overexpression of HuR in NIH 3T3 cells, we have utilized the tetracycline (tet)-regulatory system (Gossen and Bujard, 1992). The protein coding region of the HuR cDNA was subcloned into a mammalian expression vector containing the tet-promoter (see Materials and methods). In addition, we inserted an intron to enhance the RNA-processing efficiency in the nucleus. To avoid the normal translation stop codon in the cDNA being misrecognized as a nonsense codon, we introduced the intron in the 5′ UTR. It has been reported that if the stop codon is located upstream of the last intron, rapid mRNA decay may be triggered (Maquat, 1995). We also introduced a myc epitope-tag sequence (Cravchik and Matus, 1993) at the N-terminus of HuR to facilitate its detection. Western blot analysis (Figure 1A) shows that in the absence of tetracycline, expression of myc epitope-tagged HuR protein can be successfully detected in a NIH 3T3 stable cell line, termed B2A2, which expresses the tet-responsive *trans*-activator (tTA) (Xu *et al.*, 1998).

To examine the effect of ectopic expression of HuR on ARE-mediated decay in the cytoplasm, we transiently co-transfected the BBB+ARE plasmid and the plasmid bearing HuR cDNA into B2A2 cells. The decay of β-globin mRNA bearing the c-*fos* ARE (BBB+ARE$^{c\text{-}fos}$) intransiently transfected B2A2 cells constitutively expressing myc-tagged HuR in the absence of tetracycline was monitored. BBB+ARE$^{c\text{-}fos}$ mRNA was transiently transcribed from the c-*fos* promoter after serum induction of the growth-arrested B2A2 cells (Xu *et al.*, 1998). The results from time-course experiments show that the rapid decay of BBB+ARE$^{c\text{-}fos}$ mRNA in cytoplasm is not affected when the cloning vector without HuR cDNA is overexpressed (Figure 1C, top) but is significantly impeded when HuR protein is overexpressed (Figure 1C, bottom). The half-life of BBB+ARE$^{c\text{-}fos}$ mRNA increases from 35 to >240 min, a >7-fold effect. To demonstrate that the observed RNA stabilization effect exerted by HuR is specific for the ARE, we co-expressed BBB+ARE$^{c\text{-}fos}$ mRNA and a second mRNA, termed BFB mRNA, in B2A2 cells. BFB mRNA is a hybrid message made between β-globin and c-*fos* mRNAs and was shown previously to be targeted for rapid decay by the c-*fos* protein-coding determinant, which is distinct from the ARE in the 3′ UTR (Shyu *et al.*, 1989). Results in Figure 1D show that decay of BFB mRNA is not affected by HuR overexpression whereas BBB+ARE$^{c\text{-}fos}$ mRNA becomes stabilized, demonstrating that the RNA stabilization effect by HuR is specific for the ARE. The result also predicts that overexpression of HuR will have little effect on the rapid decay of c-*fos* proto-oncogene mRNA as it bears both the ARE and the protein coding determinant.

Previously, we have shown that in the ARE-mediated mRNA decay deadenylation precedes decay of the body of the mRNA (Chen and Shyu, 1994; Chen *et al.*, 1994, 1995). To address further which step in the c-*fos* ARE-mediated decay pathway is affected by HuR, we have performed additional time-course experiments with time points up to 480 min. Figure 2 shows that overexpression of HuR has little effect on the deadenylation rate. Instead, this leads to a delay in the onset of decay of the RNA body and also results in slowing down the rate of decay of the RNA body.

Overexpression of HuR leads to a dramatic increase of ARE-binding activity in the cytoplasm

We next used the gel mobility shift assay (You *et al.*, 1992) to determine if the increase in BBB+ARE$^{c\text{-}fos}$ mRNA stability is paralleled by a corresponding increase in the ARE-binding activity of HuR in the cytoplasm. Figure 3 shows that five RNA–protein complexes were detected by the c-*fos* ARE RNA substrate in cytoplasmic lysates from non-transfected cells (lane 1). In contrast, when cytoplasmic lysates prepared from B2A2 cells overexpressing myc-tagged HuR were used, a dramatic change in the complex formation occurred (Figure 3, lane 4). HuR overexpression led to the loss of the C1, C2, C4 and C5 bands and the formation of three new complexes (hC1, hC2 and hC3); it also enhanced the formation of C3 complex. The three new complexes and part of the C3 complex were supershifted to new positions both by the antibody to the myc-tag and by the antibody to HuR (Figure 3, lanes 5 and 6), demonstrating the presence of HuR in these complexes.

We further characterized the ARE-binding activity from endogenous HuR in non-transfected B2A2 cells. Figure 3 (lanes 1 and 3) shows that only modest HuR activity can be detected in the cytoplasm, as demonstrated by the supershift of C4 and C5 complexes using antibody against HuR. Together, these observations strongly suggest a direct binding of the HuR to c-*fos* ARE *in vivo*. The high level of binding of ectopically expressed HuR directly correlates with the stabilization of cytoplasmic BBB+ARE$^{c\text{-}fos}$ mRNA, and the very modest level of binding of the endogenous cytoplasmic HuR correlates with the rapid decay of BBB+ARE$^{c\text{-}fos}$ mRNA observed *in vivo* (Figure 1).

The relative amount of endogenous HuR and myc-tagged HuR was analyzed by Western blotting using

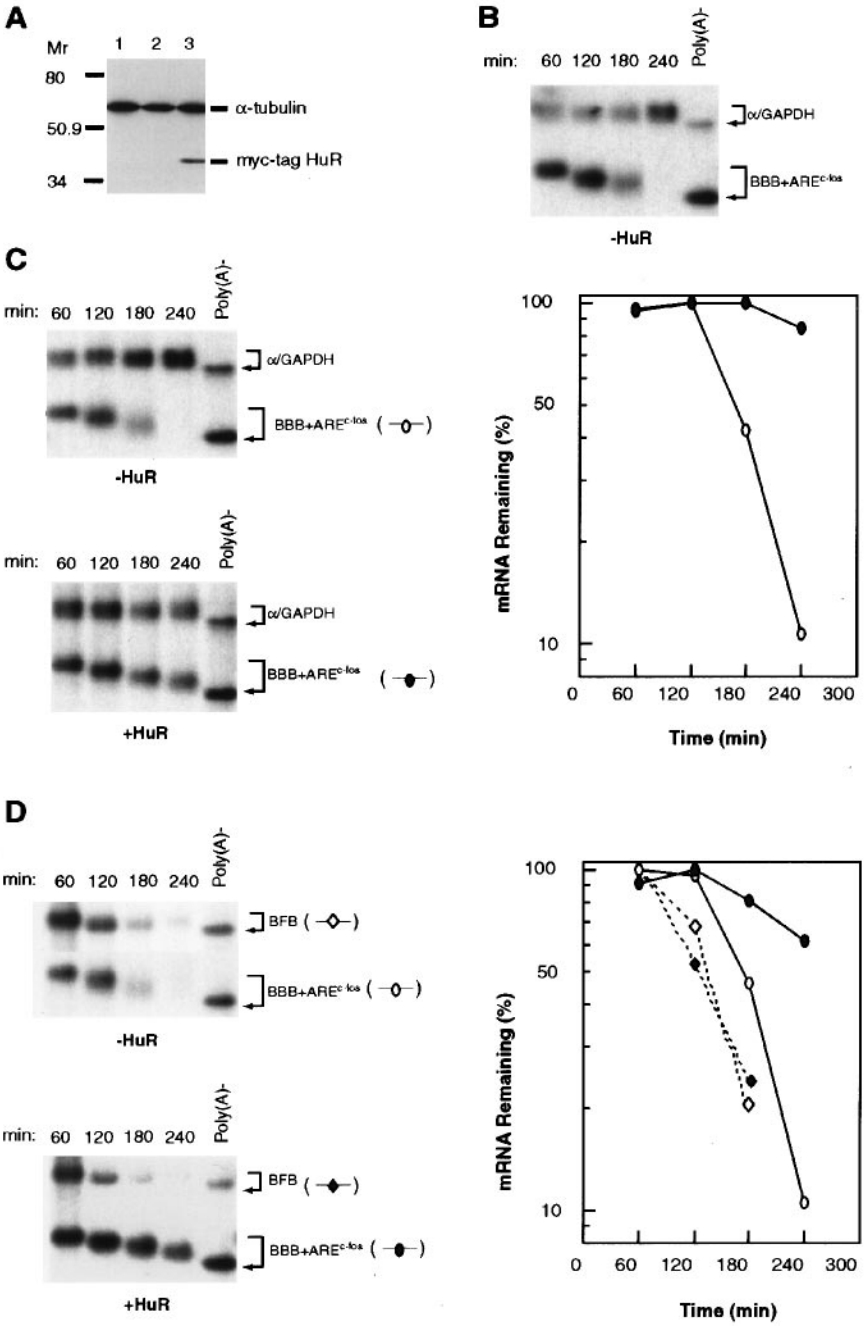

Fig. 1. Overexpression of HuR specifically inhibits the c-*fos* ARE-mediated mRNA decay in NIH 3T3 cells. (**A**) Western blot analysis of myc-tagged HuR expression in transfectants. Cytoplasmic lysates from non-transfected NIH 3T3 B2A2 cells (lane 1), cells transfected with cloning vector (lane 2), or cells transfected with a myc-tagged HuR expression vector (lane 3) were resolved on a 10% SDS–polyacrylamide gel and analyzed by Western blotting. The blot was probed with a control antibody against α-tubulin and an antibody against the myc-tag (Cravchik and Matus, 1993). (B–D) Northern blot analysis showing the specific inhibitory effect of HuR on the c-*fos* ARE-mediated mRNA turnover. B2A2 cells were transiently transfected with: pBBB+ARE$^{c\text{-}fos}$ (**B**); co-transfected with pBBB+ARE$^{c\text{-}fos}$ and pTet-Myc-over vector (**C**, top); co-transfected with pBBB+ARE$^{c\text{-}fos}$ and pTet-Myc-over-HuR (**C**, bottom); co-transfected with pBBB+ARE$^{c\text{-}fos}$ and pBFB (**D**, top); or co-transfected with pBBB+ARE$^{c\text{-}fos}$, pBFB and pTet-Myc-over-HuR (**D**, bottom). pSVα/GAPDH, which encodes a hybrid mRNA made between α-globin and GAPDH, was included in all the transfections. This hybrid mRNA was constitutively expressed and served as an internal standard. The times given at the top correspond to minutes after serum addition to growth-arrested B2A2 cells. Poly(A)$^-$ RNA was prepared *in vitro* by treating RNA samples from the 60 min time-point with oligo(dT) and RNase H. Also included in (C) and (D) are composite semi-log plots of the time-course experiments: (C, right panel, decay curves of BBB+ ARE$^{c\text{-}fos}$ in the absence (○) or presence (●) of ectopically expressed HuR; (D, right panel, decay curves of BBB+ ARE$^{c\text{-}fos}$ (solid line) in the absence (○) or presence (●) of ectopically expressed HuR, and decay curves of BFB control mRNA (dashed line) in the absence (◇) or presence (◆) of ectopically expressed HuR. The quantitation of data was obtained by scanning the radioactive blots with an imager (Packard).

antiserum to HuR (data not shown). Signal from myc-tagged HuR, which is expressed from less than ~25% cells in a typical transient transfection using 2 μg of myc-tagged HuR expressing plasmid, is at least 5-fold stronger than the signal from endogenous mouse HuR, which is likely to be expressed in all cells. As a result, the myc-

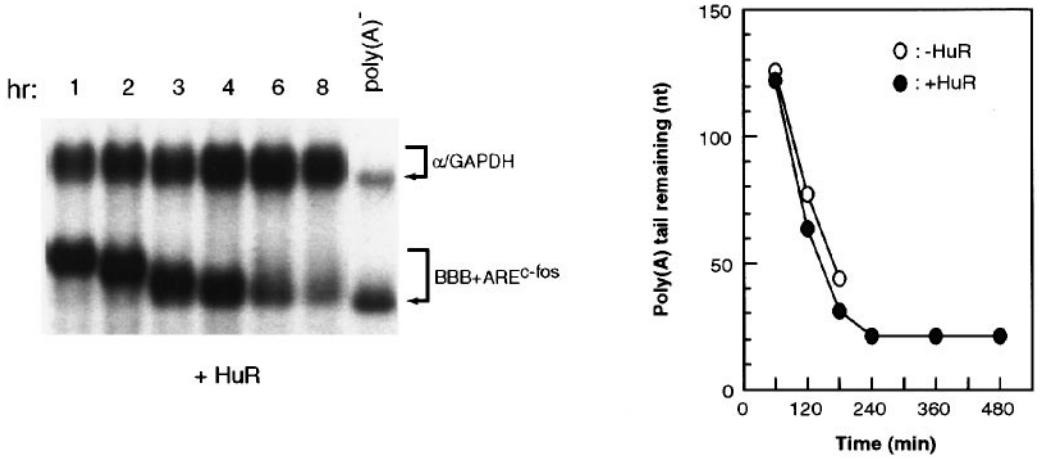

Fig. 2. Overexpression of HuR has no effect on the deadenylation step but delays the onset of decay of RNA body and slows down its subsequent decay. Left panel: Northern blot showing deadenylation and decay of BBB+ARE$^{c\text{-}fos}$ in the presence of ectopically expressed HuR. Right panel: the deadenylation rate for the BBB+ARE$^{c\text{-}fos}$ in the absence (○) or presence (●) of ectopically expressed HuR was calculated and plotted as described previously (Shyu *et al.*, 1991; Chen *et al.*, 1994). Transient transfection, RNA isolation, RNase H treatment and time-course experiments are as described in the legend to Figure 1.

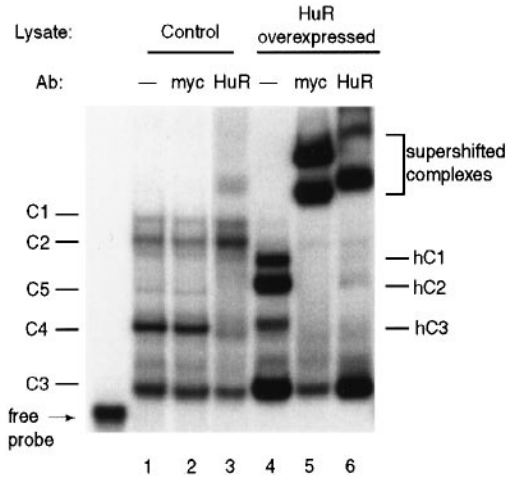

Fig. 3. The increase in BBB+ARE$^{c\text{-}fos}$ mRNA stability is paralleled by a corresponding increase in the ARE-binding activity of HuR in the cytoplasm. ^{32}P-labeled RNA transcribed *in vitro* from human c-*fos* ARE was incubated with cytoplasmic lysates from non-transfected NIH 3T3 B2A2 cells (lanes 1–3) or from cells transfected with myc-tagged HuR expressing vector (lanes 4–6). The RNA substrate was incubated with cytoplasmic lysate alone or in the presence of antibody against the myc-tag or HuR as indicated on the top of each lane. After RNase T1 digestion, the binding mixtures were analyzed by electrophoresis on a 6% non-denaturing polyacrylamide gel.

tagged HuR is at least 20-fold higher than the endogenous HuR in the cytoplasm if one takes into account the transient transfection efficiency using a calcium phosphate technique. This estimation is consistent with the significant difference seen in the mobility supershift assays shown in Figure 3, lanes 3 and 6.

Transcription blockage induces redistribution of HuR from nucleus to cytoplasm

The inhibition of the c-*fos* ARE-mediated destabilization by HuR is reminiscent of the specific inhibition of the c-*fos* ARE-directed destabilization observed when nuclear transcription is blocked (Chen *et al.*, 1995; Peng *et al.*, 1996). Because several hnRNP proteins that shuttle between nucleus and cytoplasm accumulate in the cytoplasm when transcription is blocked (Pinol-Roma and Dreyfuss, 1991; Dreyfuss *et al.*, 1996), we asked if HuR, also found in the nucleus and cytoplasm (Myer *et al.*, 1997; Okano and Darnell, 1997), leaves the nucleus and accumulates in the cytoplasm upon transcription inhibition by actinomycin D. We first analyzed by gel mobility shift assay whether there is any change of the ARE-binding activity of endogenous HuR both in the nucleus and in the cytoplasm when transcription is blocked. Figure 4A shows that the c-*fos* ARE supports the formation of two major complexes, C3 and C5, in nuclear extracts. When transcription is inhibited, the level of C5 complex is greatly reduced. Conversely, the formation of C5 is greatly enhanced when the cytoplasmic lysate is prepared after transcription is blocked (Figure 4B, lanes 1 and 2). Moreover, the C5 complex is specifically supershifted by the antibody to HuR but not by preimmune serum (Figure 4B, lanes 3–5), demonstrating an increase of HuR ARE-binding activity in cytoplasm when transcription is inhibited. These results support the conclusion that elevation of HuR in the cytoplasm leads to the specific inhibition of ARE function.

To determine whether the changes of HuR activity between the nuclear and cytoplasmic compartments result from protein redistribution, we carried out Western blot analysis. Figure 4C shows that upon transcription blockage, the nuclear HuR is reduced whereas the cytoplasmic HuR increases. The weak signal detected by the HuR antibody indicates that only a very limited amount of endogenous HuR is present in NIH 3T3 cells, which is consistent with the observations of Okano and Darnell (1997) that HuR can hardly be detected in NIH 3T3 cells and also explains the rather modest ARE-binding activity from endogenous HuR detected by gel mobility shift assay using ^{32}P-labeled ARE probe (comparing Figure 3, lanes 1–3 and Figure 4A and B with Figure 3, lanes 4–6). Moreover, preimmune serum also detected a background band that co-migrates with endogenous HuR both in nuclear and in cytoplasmic extracts. To demonstrate that the changes of HuR distribution between nucleus and cytoplasm are bona fide, we probed the same blot with

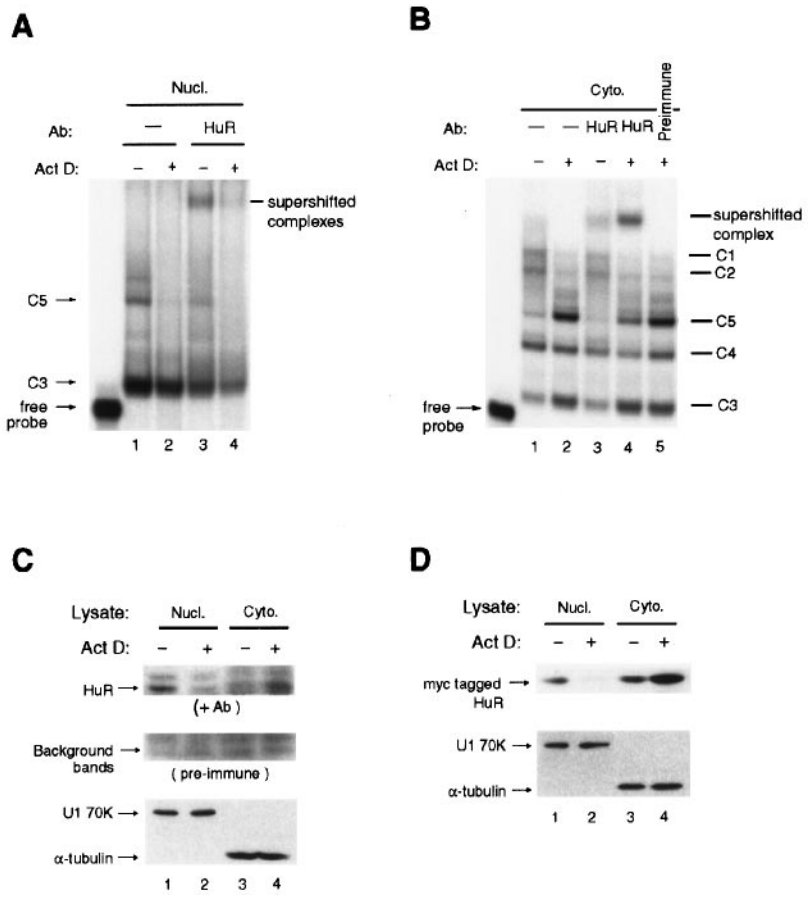

Fig. 4. Redistribution of endogenous HuR and exogenous myc-tagged HuR proteins between nucleus and cytoplasm. Gel mobility shift assays were carried out as described in the legend to Figure 3 with nuclear (**A**) or cytoplasmic (**B**) lysates from non-transfected NIH 3T3 B2A2 cells with (+) or without (–) actinomycin D treatment (5 μg/ml) as indicated. Western blot analysis of endogenous HuR (**C**) or myc-tagged HuR (**D**) expression in the nucleus or cytoplasm of NIH 3T3 B2A2 cells with or without actinomycin D treatment was also carried out. The blots were probed with an antibody against HuR (C) or myc-tag (D) plus a control antibody against U1 70K (for nuclear lysate) or against α-tubulin (for cytoplasmic lysate). Note that nuclear and cytoplasmic lysates were made from the same batch of cells and an aliquot of lysate equivalent to ~2×10^5 cells was used in each lane.

two control antibodies, one against the nuclear splicing factor U1 70K and the other against cytoplasmic α-tubulin. Neither protein changes their distribution after transcription is blocked (Figure 4C). The ability of HuR to redistribute from nucleus to cytoplasm was further investigated by examining the redistribution of ectopically expressed HuR by Western blot analysis. The results show that the same change can be seen in the case of overexpressed myc-tagged HuR which is readily detected both by an antibody to the myc-tag (Figure 4D) as well as by an antibody to the human HuR (data not shown). In contrast, we detected no change of distribution of either U1 70K or α-tubulin.

To substantiate the above observations of HuR redistribution, NIH 3T3 B2A2 cells transiently transfected with myc-tagged HuR overexpressing plasmid were also examined by indirect immunofluorescence microscopy. The subcellular location of myc-tagged HuR protein was determined using monoclonal antibody against myc-tag. As shown in Figure 5A and B, myc-tagged HuR can be detected mostly in the nucleus and partly in the cytoplasm. The lack of fluorescence in non-transfected cells demonstrates that the fluorescence is specific for myc-tagged HuR. In the meantime, our data show that upon transcription blockage by actinomycin D, HuR redistributes from the nucleus to the cytoplasm as indicated by a significant increase of fluorescence in the cytoplasm and a reciprocal diminution of fluorescence in the nucleus (Figure 5C and D). Taken together, our gel mobility supershift assays (Figure 4A and B), Western blot analyses (Figure 4C and D) and immunofluorescence microscopy study (Figure 5) support the notion that the enhancement of the ARE-binding activity of HuR in the cytoplasm upon nuclear transcription blockage results from the redistribution of HuR from nucleus to cytoplasm, which directly correlates with the inhibition of ARE-mediated mRNA destabilization.

HuR differentially regulates the mRNA decay mediated by the c-fos and c-jun AREs

Previously, we have observed that when nuclear transcription is blocked, the RNA destabilizing function of c-*fos* ARE is significantly impeded whereas that of the c-*jun* ARE is only moderately affected (Peng *et al.*, 1996). This suggests the hypothesis that HuR has different effects on these two AREs. We therefore performed time-course experiments to monitor the decay of β-globin mRNA bearing the c-*fos* ARE or the c-*jun* ARE in B2A2 NIH 3T3 cells transfected with increasing amounts of myc-tagged HuR cDNA. Figure 6A shows that while both AREs display HuR dose-dependent changes in their destabilizing function, the RNA destabilizing function of the c-*jun* ARE

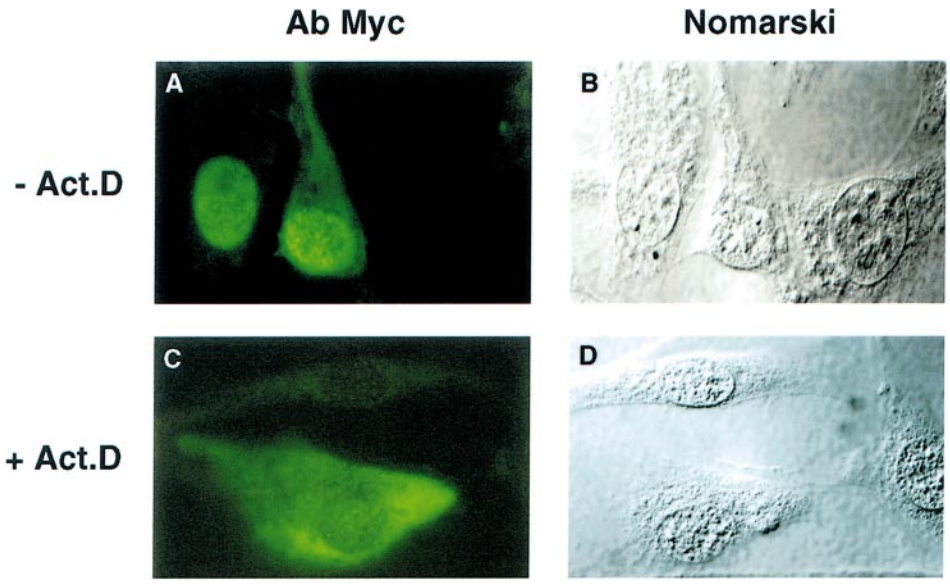

Fig. 5. Indirect immunofluorescence microscopy study of the HuR redistribution between the nucleus and the cytoplasm. NIH 3T3 cells transfected with pTet-Myc-over-HuR were subjected to either no (**A** and **B**) or transcription blockage (**C** and **D**) by actinomycin D (5 μg/ml) treatment for 2 h. After fixation with 100% methanol and permeabilization with 0.5% Triton X-100, cells were stained using anti-myc-tag antibody as primary antibody. The secondary antibody for anti-mouse-IgG (Sigma) was coupled to FITC. (B) and (D) show cells in Nomarski images. All images were captured and generated using an Olympus BX60 Microscope and a Spot-Digital camera (Diagnostics).

is much less sensitive to HuR overexpression compared with the significant stabilization of β-globin mRNA bearing the c-*fos* ARE. In the case of the c-*jun* ARE there is essentially no change of the rates for decay of the RNA body under different levels of HuR overexpression. It appears that the onset of decay of the RNA body is slightly delayed.

To determine whether the ability of HuR to exert its stabilizing effect may be related to its ability to bind an ARE, we performed *in vitro* competition experiments using the gel mobility shift assay. The results (Figure 6B) show that the addition of increasing amounts of homologous unlabeled c-*fos* ARE to the binding reaction mixtures results in a concentration-dependent reduction in the formation of the ^{32}P-labeled RNA–protein complexes containing HuR, whereas the addition of the unlabeled c-*jun* ARE has little effect on the formation of RNA–protein complexes containing HuR. Thus, these results demonstrate a correlation between the ability of HuR to exert its stabilizing effect and its ability to bind an ARE.

Discussion

In this study, we provide *in vivo* evidence that HuR can play an RNA-stabilizing role in the ARE-directed mRNA decay in mammalian cells. In an accompanying paper, Fan and Steitz (1998), using a different approach, also report a similar finding. Our data (Figure 2) further show that elevation of HuR in the cytoplasm does not affect the accelerated deadenylation step. Instead, it results in a delay of the onset of decay of the mRNA body following the deadenylation step and also prolongs the rate of decay of the mRNA body. We and others have shown previously that the ARE-mediated decay is a two-step mechanism, namely, deadenylation precedes decay of the body of the mRNA (Chen and Shyu, 1995). Therefore, overexpression of HuR appears to interfere with the second step in the ARE-mediated decay pathway. It is interesting to note that two recent reports showed that, *in vitro*, HuR can bind simultaneously to both the ARE and the poly(A) tail and that it prefers to bind a poly(A) tail of at least 70 nt in size (Ma *et al.*, 1997), suggesting a potential involvement of ELAV proteins in the deadenylation step. One possibility to reconcile these seemingly conflicting observations is that HuR might participate in the deadenylation step through its ARE- and poly(A)-binding activities and its subsequent return to the nucleus might then allow decay of the RNA body to occur. Thus, preventing a timely return of HuR to the nucleus might block the decay of the RNA body (also see below for further discussion).

The observation that HuR is found both in the nucleus and the cytoplasm suggests that HuR shuttles between the two. Using the heterokaryon approach with HeLa and mouse L929 cells, Fan and Steitz (1998) demonstrated that HuR can shuttle between nucleus and cytoplasm. In our laboratory, a systematic truncation of HuR has led to the identification of domains within HuR that are necessary for its nuclear localization (unpublished data), further corroborating its nucleocytoplasmic shuttling in NIH 3T3 cells. At least three lines of evidence support that the nucleocytoplasmic shuttling of HuR may be associated with an alteration in its function. First, elevation of HuR concentration in the cytoplasm specifically inhibits the c-*fos* ARE-mediated mRNA decay, which is paralleled by a corresponding increase of HuR activity in the cytoplasm (Figures 1–3). Secondly, the redistribution of HuR from nucleus to cytoplasm induced by transcription blockage results in a dramatic enhancement of the formation of an HuR-containing RNP complex on the c-*fos* ARE (Figure 4). This correlates well with the inhibition of the c-*fos* ARE-mediated decay when transcription is blocked. Thirdly, the differential stabilizing effect on the c-*fos* and c-*jun* AREs by elevating HuR in the cytoplasm correlates with the differential inhibition of the decay mediated by

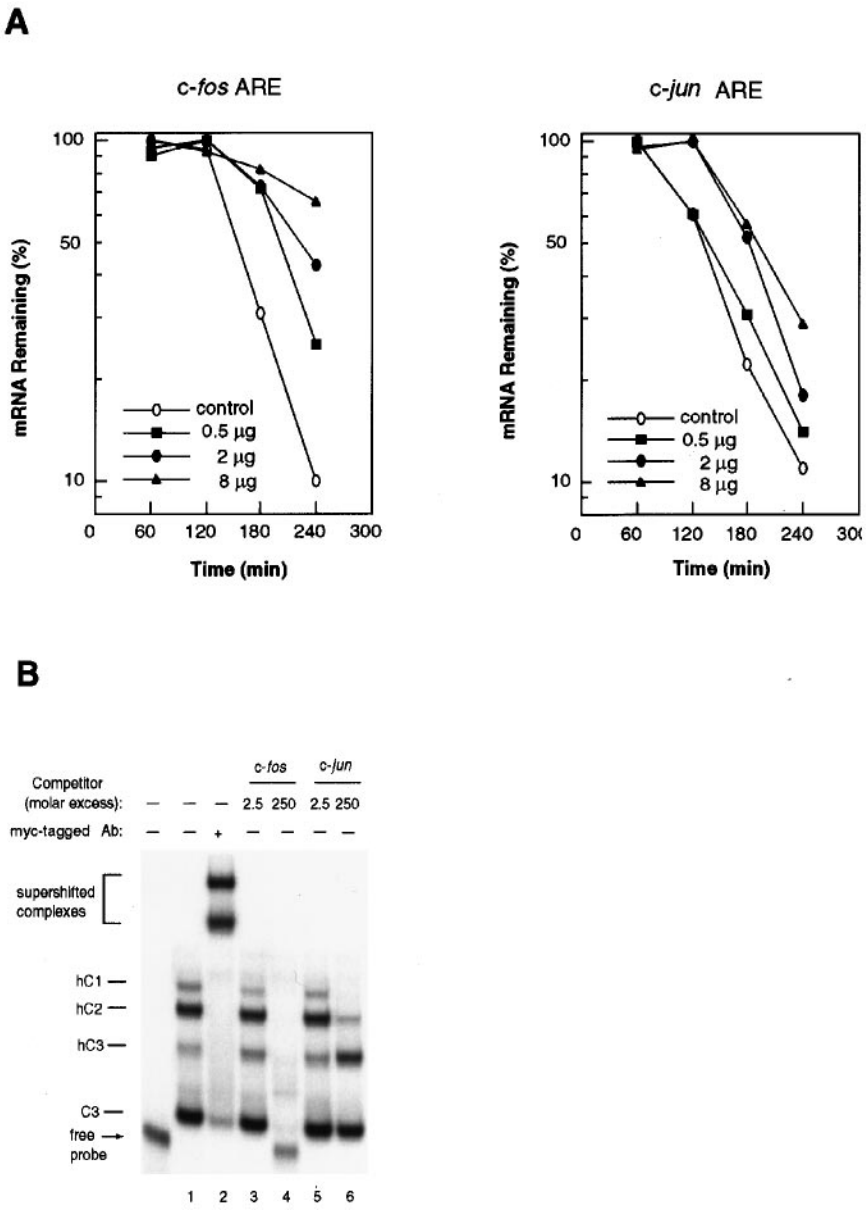

Fig. 6. Differential inhibition of the destabilizing function of the c-*fos* and c-*jun* AREs by HuR correlates with the ARE-binding ability of HuR. (**A**) Semi-log plots showing the decay of β-globin mRNA containing the c-*fos* (left) or c-*jun* (right) ARE in NIH 3T3 B2A2 cells co-transfected with carrier plasmid (○) or with 0.5 µg (■), 2 µg (●), or 8 µg (▲) of pTet-Myc-over-HuR. Transient transfections of NIH 3T3 B2A2 cells and Northern blot analysis were carried out as described previously (Shyu *et al.*, 1989). The quantitation of data was obtained by scanning the radioactive blots with an imager (Packard). (**B**) ^{32}P-labeled RNA transcribed *in vitro* from human c-*fos* ARE was incubated with cytoplasmic lysates from cells overexpressing myc-tagged HuR in the presence of increasing amounts of either unlabeled c-*fos* ARE or unlabeled c-*jun* ARE transcript as competitor. After RNase T1 digestion, the binding mixtures were analyzed by electrophoresis on a 6% non-denaturing low ionic strength polyacrylamide gel.

the two AREs induced by nuclear transcription blockage (Figure 6 and Peng *et al.*, 1996).

The shuttling of HuR between nucleus and cytoplasm is reminiscent of the nucleocytoplasmic shuttling of hnRNP proteins (Nakielny and Dreyfuss, 1997; Nigg, 1997). It is tempting to speculate that ELAV proteins may be bound to nuclear mRNAs bearing an ARE and remain associated during transit through the nuclear envelope. One possibility is that they are involved in proper transport of ARE-containing mRNA to the cytoplasm. As a result, in the nucleus ELAV proteins may play a passive role in protecting the mRNA from being degraded by binding to the ARE before the mRNA transport to the cytoplasm. Once the ELAV protein–mRNA (mRNP) complex arrives in the cytoplasm, the ELAV proteins might participate in the subsequent localization, translation and turnover of the mRNA before they return to the nucleus, depending on the physiological situation. The return of ELAV protein might then allow the subsequent assembly of a proper protein complex that would trigger the rapid decay of the RNA body. Therefore, preventing timely return of cytoplasmic ELAV proteins to the nucleus may interfere with the assembly of a functional ARE–protein complex, resulting in the inhibition of ARE-mediated mRNA decay.

What is the underlying mechanism by which ELAV proteins regulate cell growth and differentiation?

Depending on where the ELAV-containing mRNPs are localized in the cytoplasm, the nuclear localization signal (NLS) of an ELAV protein may be shielded from being recognized through its interaction with other *trans*-acting factors or cytoskeleton. This could in turn result in stabilization of an ARE-containing mRNA in the cytoplasm, thereby affecting cellular programs that trigger or maintain cell growth and differentiation. Alternatively, the attachment of ELAV-containing mRNP to the cytoskeleton may also prevent the ELAV protein from returning to the nucleus. Several recent studies concerning human ELAV proteins are consistent with this possibility. First, Morello and co-workers have found a correlation that HuR expression directly parallels c-*myc* mRNA expression in quiescent tissues and during liver development (Lafon *et al.*, 1998). When expression of the ARE-containing c-*myc* mRNA is up-regulated, there is a corresponding increase of HuR levels in these tissues. Secondly, Keene and co-workers have observed that ELAV-containing mRNPs localize along microtubule tracks both in cell bodies and in the processes of cortical neurons and that this association with the cytoskeleton is important for their translation (Antic and Keene, 1997). Moreover, neuron-specific Hel-N1 is readily detected and mainly expressed in the cytoplasm of terminally differentiated neurons (Gao and Keene, 1996). Thirdly, Ginzburg and co-workers have found that upon nerve growth factor stimulation in PC12 cells, an ARE-containing mRNA termed tau, which encodes a microtubule-associated protein, is stabilized (Sadot *et al.*, 1995). This stabilization is linked both to the binding of HuD to the ARE in the 3′ UTR of tau mRNA and to the ability of tau mRNA to associate with microtubule (I.Ginzburg, personal communication). Fourthly, a recent report (Wakamatsu and Weston, 1997) showed that mis-expression of chicken HuD in cultured neural crest cells results in an increase in the proportion of cells exhibiting neuronal morphology, molecular markers for neurons and neurotrophin dependence. It is plausible that in neuronal cells the neuron-specific expression of HuC, HuD and Hel-N1 in the cytoplasm may lead to differential up-regulation of ARE-bearing mRNAs encoding protein products necessary for proper development and function of neurons.

What is the molecular basis for selective stabilization of particular ARE-containing transcripts in a given biological scenario? Previously, we have shown that AREs consist of three distinct classes (Chen and Shyu, 1995). We hypothesize that modulating HuR distribution between the nucleus and cytoplasm could differentially alter the destabilizing function of distinct classes of ARE. This could form the basis by which a selective stabilization of a subset of ARE-bearing mRNAs is achieved, for example, cytokine mRNA stabilization during T-cell (Lindsten *et al.*, 1989) and mast-cell activation (Nair *et al.*, 1994), up-regulation of differentiation-specific neurofilament M mRNA expression in terminally differentiated neurons (Antic and Keene, 1997), as well as glucose transporter mRNA stabilization in adipocytes (Jain *et al.*, 1997). *In vivo* biochemical dissection of HuR and other ELAV proteins will provide further insight into the functional role of this family of RNA-binding proteins in RNA metabolism and developmental decisions.

Materials and methods

Plasmid constructions

When necessary, DNA with 5′- or 3′- protruding ends was treated with Klenow fragment or T4 DNA polymerase to make ends blunt. The HuR cDNA spanning the protein coding region was amplified from a human placental cDNA library (Clontech) by PCR with the following primers: 5′-ATCATGTCTAATGGTTATGAAGACC-3′ and 5′-GCTGATATCGCATGAGCGAGTTATTTGTGGG-3′. To construct the Tet-regulated over-expression vector pTet-Myc-over, a pair of oligomers encoding myc epitope-tag were synthesized (5′-CATGGAGCAAAAGCTGATTTCTGAGGAGGATCTGGTCGACGATATCT-3′, 5′-CTAGAGATATCGTCGACCAGATCCTCCTCAGAAATCAGCTTTTGCTC-3′). After annealing, Klenow polymerization reaction was performed to make a double-stranded DNA fragment flanked by a *Nco*I site at the 5′ end and a *Xba*I site at the 3′ end. The DNA fragment was digested with these enzymes and used to replace the CAT coding sequence excised from the plasmid pCAT3-control vector (Promega) by *Nco*I and *Xba*I digestions. A 363 bp *Sfi*I–*Xba*I fragment bearing the intron-containing 5′ UTR and the myc-tag sequence was excised from the resulting plasmid, blunt-ended and used to replace the *Eco*RI–*Bgl*II (blunt-ended) fragment in the plasmid pTet-BBB (Xu *et al.*, 1998) to generate the plasmid, pTet-Myc-over. To create the plasmid pTet-Myc-over-HuR, the 1013 bp PCR product spanning the HuR protein coding region was blunt-ended and subsequently subcloned into pTet-Myc-over at its unique *Eco*RV site so that it is in-frame with the myc-tag immediately upstream of it. The identity of the PCR-amplified HuR cDNA was confirmed by DNA sequencing. The construction of plasmids pBBB+ARE$^{c\text{-}fos}$, pBBB+ARE$^{c\text{-}jun*}$ (3′ 1), pBFB, pSVα/GAPDH and pT3 ARE$^{c\text{-}fos}$ has been described previously (Shyu *et al.*, 1989; Chen *et al.*, 1995; Peng *et al.*, 1996). To generate the plasmid pT3ARE$^{c\text{-}jun*}$, a 146 bp *Bgl*II fragment from plasmid pBBB+ARE$^{c\text{-}jun*}$ (3′ 1) was subcloned into the unique *Bam*HI site of plasmid pT3/T7a18 (Gibco-BRL).

Analysis of mRNA decay and deadenylation

Cell culture, DNA transfection, isolation of total cytoplasmic RNA and Northern blot analysis were conducted as described previously (Shyu *et al.*, 1996). Briefly, 15 cm-diameter dishes were seeded at a density of 2.0×10^6 cells per dish and incubated under 5% CO_2 for 16–20 h before transfection. Cells were transfected for ~16 h with a total of 20 μg of DNA which includes 3 μg of pBBB+ARE, 2 μg of pTet-Myc-over-HuR (note that in Figure 4 different amounts were used as specified in the figure legend), 2 μg of pSVa/GAPDH and enough carrier plasmid pT3/T7a18 (Gibco-BRL) to make a final amount of 20 μg DNA. The cells were then washed with phosphate-buffered saline (PBS), serum starved and stimulated with 20% bovine serum albumin (BSA) (Gibco-BRL). Gene-specific DNA probes were prepared by the method of random oligonucleotide priming for Northern blot analysis. The ^{32}P-labeled probes were produced by inclusion of [α-^{32}P]dCTP (>6000 Ci/mmol; Dupont). Note that since the mRNA encoding myc-tagged HuR bears the β-globin 3′ UTR, specifically to detect BBB+ARE mRNA but not the mRNA encoding myc-tagged HuR in overexpressing cells, a β-globin cDNA fragment lacking the 3′ UTR was used as probe template. All experiments described in this manuscript have been performed in duplicate or triplicate.

Preparation of NIH 3T3 cytoplasmic and nuclear extracts

Cytoplasmic lysates were prepared as described previously (You *et al.*, 1992). Briefly, cytoplasmic lysates were prepared from NIH 3T3 cells by lysis at 4°C in a lysis/extraction buffer containing 10 mM HEPES pH 7.6, 3 mM $MgCl_2$, 40 mM KCl, 2 mM DTT, 5% glycerol, 0.5% Nonidet P-40, 10 μg/ml of aprotinin, 10 μg of leupeptin per ml and 100 μg/ml PMSF. Nuclei were removed by centrifugation (1250 *g*, 4°C for 5 min). Nuclei were then resuspended and sedimented twice in the lysis/extraction buffer to avoid contamination by cytoplasmic proteins. Nuclear extracts were prepared from the pelleted nuclei in the nuclear extraction buffer containing 10 mM HEPES pH 7.9, 0.1 mM EGTA, 1.5 mM $MgCl_2$, 420 mM NaCl, 0.5 mM DTT, 0.5 mM PMSF and 25% glycerol. After incubation on ice for 20 min, cellular debris was removed by centrifugation (1250 *g*, 4°C for 5 min). Protein concentration was analyzed by the BCA protein assay reagent (Pierce).

Gel mobility shift assay and supershift analysis

Preparation of RNA probes by *in vitro* transcription and analysis of RNA–protein interactions were described previously (You *et al.*, 1992). Transcription reactions were performed according to Promega instruc-

tions, using T3 or T7 RNA polymerase. Labeled RNA transcripts were produced by inclusion of [α-^{32}P]UTP (Amersham or Dupont, 800 Ci/mmol) in the reactions. The specific activity of transcripts performed in this way is ~3–15×10^8 c.p.m./μg. The c-*fos* ARE probe is a sense transcript synthesized from *Hin*dIII-linearized pT3ARE (You *et al.*, 1992). Cytoplasmic lysate (6 μg of protein; equivalent to ~1.4–1.7×10^4 cells per μg protein) and ^{32}P-labeled RNA (1–4 ng) were incubated at room temperature for 15 min in a buffer containing 10 mM HEPES pH 7.6, 3 mM $MgCl_2$, 40 mM KCl, 2 mM DTT, 5% glycerol and 0.5% Nonidet P-40. Heparin (5 μg/ml, final concentration) and yeast total RNA (200 ng/ml, final concentration) were added to reduce non-specific binding. The volume of each reaction was 10 μl. Subsequently, unbound RNA was digested for 20 min by 0.6 U of RNase T1 (Calbiochem) at room temperature. RNA–protein complexes were resolved in 6% non-denaturing polyacrylamide gels. Gel mobility supershift analysis was performed by addition of 0.5 μl of antibody into binding reaction (final volume = 10 μl; 1:20 dilution) which has been incubated for 15 min at room temperature to allow RNA–protein interactions to occur, and the antibody–RNA–protein mixture was then incubated at room temperature for another 15 min. The RNA–protein–antibody complexes were resolved in 6% non-denaturing polyacrylamide gels. The purified monoclonal antibody against the myc-tag (0.1 μg/μl IgG) was purchased from Calbiochem. The polyclonal antibody to human HuR raised in rabbit (a gift from H.Furneaux) was raised against the peptide sequence spanning the first 13 amino acids of human HuR.

Western blot analysis

Cytoplasmic and nuclear lysates were resolved on a 10% SDS–polyacrylamide gel and analyzed by Western blotting using an ECL Western blotting kit (Amersham). The blots were probed with specific antibodies as described in the legends to figures. The purified monoclonal antibody against α-tubulin (DM1A) was purchased from Sigma and used at 1:20 000 dilution. The antibody against the U1 70K (mouse IgG) was kindly provided by S.Berget and used at 1:100 dilution. The antibodies to myc-tag and human HuR were as described above.

Immunofluorescence microscopy

NIH 3T3 cells were grown in DMEM with 10% CS on coverslips and transfected with pTet-Myc-over-HuR. After 48 h, one group of cells was subjected to 5 μg/ml of actinomycin D treatment for 2 h. Cells were fixed with 100% methanol for ~20 min at room temperature, washed three times with PBS (5 min each), permeabilized with 0.5% Triton X-100 in PBS for 10 min, and washed three times with PBS (15 min each). The coverslips were incubated with the anti-myc-tag monoclonal antibody in PBS with 1% BSA at 37°C for 1 h. The culture medium containing anti-myc-tag monoclonal antibody was collected and applied directly at 1:20 dilution (Cravchik and Matus, 1993). The coverslips were washed three times (15 min each) and further incubated with the goat anti-mouse IgG coupled to FITC (Sigma) at 1:100 dilution for 1 h at 37°C. The coverslips were then washed three times (15 min each) and mounted. Images were all viewed using an Olympus BX60 Microscope with a 100× oil objective. All images were subsequently captured by a Spot-Digital camera (Diagnostics) and processed for publication at 300 d.p.i. using Adobe Photoshop (4.0) software.

Acknowledgements

We thank J.A.DeMoss, J.Lever and M.Wilkinson for critical reading of the manuscript and their thoughtful comments, S.Berget for the antibody against U1 70K, H.Furneaux for the antibody to human HuR protein, and Mike Blackburn for helping us with indirect immunofluorescence microscopy study. This work was supported by a grant from National Institutes of Health and in part by a grant from the Council of Tobacco Research-USA, Inc. A.-B.S. is the recipient of an American Heart Association Established Investigator Award.

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Received February 3, 1998; revised March 17, 1998; accepted April 7, 1998