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Overexpression of HuR, a nuclear–cytoplasmic shuttling protein, increases the *in vivo* stability of ARE-containing mRNAs

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The messenger RNAs of many proto-oncogenes, cytokines and lymphokines are targeted for rapid degradation through AU-rich elements (AREs) located in their 3′ untranslated regions (UTRs). HuR, a ubiquitously expressed member of the Elav family of RNA binding proteins, exhibits specific affinities for ARE-containing RNA sequences *in vitro* which correlate with their *in vivo* decay rates, thereby implicating HuR in the ARE-mediated degradation pathway. We have transiently transfected HuR into mouse L929 cells and observed that overexpression of HuR enhances the stability of β-globin reporter mRNAs containing either class I or class II AREs. The increase in mRNA stability parallels the level of HuR overexpression, establishing an *in vivo* role for HuR in mRNA decay. Furthermore, overexpression of HuR deletion mutants lacking RNA recognition motif 3 (RRM 3) does not exert a stabilizing effect, indicating that RRM 3 is important for HuR function. We have also developed polyclonal anti-HuR antibodies. Immunofluorescent staining of HeLa and L929 cells using affinity-purified anti-HuR antibody shows that both endogenous and overexpressed HuR proteins are localized in the nucleus. By forming HeLa–L929 cell heterokaryons, we demonstrate that HuR shuttles between the nucleus and cytoplasm. Thus, HuR may initially bind to ARE-containing mRNAs in the nucleus and provide protection during and after their export to the cytoplasmic compartment.

Keywords: ARE/Elav proteins/HuR/mRNA decay/ nuclear–cytoplasmic shuttling protein

Introduction

Selective mRNA turnover is an important mechanism of eukaryotic gene regulation. The expression of proto-oncogenes, lymphokines and cytokines is usually transient, requiring rapid mRNA removal through destabilization following the cessation of transcription. AU-rich elements (AREs) located in their 3′ untranslated regions (3′ UTRs) comprise a major class of *cis*-elements that target these mRNAs for rapid degradation (Caput *et al.*, 1986; Shaw and Kamen, 1986; for reviews, see Belasco and Brawerman, 1993; Chen and Shyu, 1995). Loss of this negative regulatory control conferred by AREs has been shown to be associated with transforming phenotypes (Miller *et al.*, 1984; Meijlink *et al.*, 1985; Lee,W. *et al.*, 1988). Besides mRNAs, small nuclear RNAs (snRNAs) can also be targeted by AREs for rapid degradation, presumably through similar decay pathway(s) (Fan *et al.*, 1997). ARE-mediated decay may also be differentially regulated. For instance, in a monocyte tumor cell line, c-*fos* mRNA is degraded constitutively, whereas the GM-CSF mRNA is stable (Schuler and Cole, 1988); in a human T cell line upon co-stimulation with anti-CD28 antibody, GM-CSF and interleukin-3 (IL-3) mRNAs have been reported to be selectively stabilized (Lindsten *et al.*, 1989). It is not known, however, whether the decay pathway(s) are somehow inhibited or stabilizing pathway(s) are activated to override the decay pathways under such circumstances.

Most AREs contain multiple copies of the sequence AUUUA (Caput *et al.*, 1986; Shaw and Kamen, 1986; for reviews, see Belasco and Brawerman, 1993; Chen and Shyu, 1995). During ARE-mediated decay, shortening of the poly(A) tail precedes the degradation of the mRNA body (Shyu *et al.*, 1991; Chen *et al.*, 1995). Based on their sequence features, deadenylation and degradation kinetics, AUUUA-containing AREs have been classified into two groups (Shyu *et al.*, 1991; Chen *et al.*, 1995; Xu *et al.*, 1997). Class I AREs, found mainly in proto-oncogene mRNAs (such as c-*fos*), contain one to three copies of dispersed AUUUA motifs in a U-rich region and mediate distributive synchronous poly(A) shortening followed by rapid degradation of the mRNA body. In contrast, class II AREs, found mostly in cytokine mRNAs (such as GM-CSF), contain multiple copies of clustered AUUUA motifs and direct asynchronous deadenylation, suggesting a processive nucleolytic digestion of the poly(A) tail followed by mRNA decay (Shyu *et al.*, 1991; Chen *et al.*, 1995; Xu *et al.*, 1997).

To define the cellular degradation machinery responsive to ARE signals, much effort has been devoted to identifying ARE-binding proteins (Malter, 1989; Bohjanen *et al.*, 1991, 1992; Brewer, 1991; Malter and Hong, 1991; Vakalopoulou *et al.*, 1991; Myer *et al.*, 1992, 1997; Hamilton *et al.*, 1993, 1997; Zhang *et al.*, 1993; Katz *et al.*, 1994; Nakagawa *et al.*, 1995; Wennborg *et al.*, 1995). We have focused on a protein of 32 kDa apparent molecular weight, first identified by UV-crosslinking to the c-*fos* ARE in HeLa cell extracts (Vakalopoulou *et al.*, 1991). This protein also binds to the AU-rich regions of Herpesvirus saimiri U snRNAs (HSURs) 1, 2 and 5, which are highly expressed in virally transformed marmoset T cells (Lee,S. *et al.*, 1988; Lee and Steitz, 1990; Myer *et al.*, 1992). Repeated copies of AUUUA at the 5′ end of HSUR 1 have been shown to target this and other snRNAs for rapid degradation upon transient transfection into several mammalian cell lines (Fan *et al.*, 1997).

Characterization of the purified 32 kDa protein demonstrated that it is identical to HuR (also called HuA; Myer *et al.*, 1997), a ubiquitously expressed member of the Hu

family of proteins (Ma *et al.*, 1996). Three other Hu proteins, namely Hel-N1 (or HuB), HuC and HuD, have been previously identified as target antigens in paraneoplastic encephalomyelitis sensory neuronopathy (the Hu syndrome), which is characterized by diverse neuronal degeneration associated with small cell lung cancer (SCLC) (Dalmau *et al.*, 1991; Szabo *et al.*, 1991; Levine *et al.*, 1993). It is believed that patients develop autoantibodies against Hu antigens abnormally expressed in SCLC, which then attack the central nervous system, leading to neuronal dysfunction and finally death (for reviews, see Posner, 1995; Darnell, 1996). All four Hu proteins contain three highly conserved RNA binding domains belonging to the RRM (RNA recognition motif; also called RBD) superfamily (Burd and Dreyfuss, 1994), but their auxiliary regions (the sequence N-terminal to RRM 1 and the hinge region between RRMs 2 and 3) differ. All Hu proteins exhibit high affinity for AU-rich RNA sequences (Levine *et al.*, 1993; Gao *et al.*, 1994; Abe *et al.*, 1996; Ma *et al.*, 1996, 1997; Jain *et al.*, 1997; Myer *et al.*, 1997). Deletion studies conducted on HuC, HuD and HuR have suggested that RRMs 1 and 2 are responsible for ARE-binding, whereas RRM 3 binds simultaneously to the poly(A) tail of an mRNA (Abe *et al.*, 1996; Ma *et al.*, 1997).

Hu proteins are highly conserved. Their RRMs exhibit strong homology to those of the *Drosophila* RNA binding proteins Elav (embryonic lethal, abnormal vision) and sxl (sex-lethal). Elav was originally cloned in a screen designed to identify *Drosophila* nervous system defects and was found to be essential for normal neural development (Campos *et al.*, 1985; Robinow *et al.*, 1988). Homologs of each of the four human Hu proteins have been identified in other vertebrates, such as *Xenopus* (Good, 1995), zebrafish (Good, 1995), chicken (Wakamatsu and Weston, 1997) and mouse (Okano and Darnell, 1997). They have been classified into four groups called Elav-like ribonucleoproteins A–D (elr A–D, or Hu A–D, respectively), based on both sequence similarities in the auxiliary regions and the tissue specificity of their mRNA expression (Good, 1995; Okano and Darnell, 1997). Elr B, C and D are neuron-specific, while elr A is expressed ubiquitously at the mRNA level (Good, 1995; Okano and Darnell, 1997). Human HuR (Hu A) protein is the most closely related to *Drosophila* Elav and sxl among the four human Hu proteins and has been considered ancestral to the other three (Okano and Darnell, 1997). It is over 99% identical to mouse HuA at the amino acid level (Ma *et al.*, 1996), 98.2% to chicken (Wakamatsu and Weston, 1997) and over 90% to the *Xenopus* protein (Good, 1995; Ma *et al.*, 1996).

HuR has been implicated in ARE-mediated rapid mRNA destabilization because of a direct correlation between its *in vitro* affinity for a variety of ARE sequences and the ability of these sequences to direct *in vivo* degradation of a reporter mRNA (Myer *et al.*, 1997). To ascertain HuR's involvement in mRNA decay *in vivo*, we have assayed the decay rates of ARE-containing mRNAs in mouse L929 cells overexpressing HuR and have observed stabilization of mRNAs containing AREs of both class I and II (c-*fos* and GM-CSF). We have also established the subcellular localization of HuR in HeLa and mouse L cells as nuclear. Yet, both the endogenous and epitope-tagged HuR actively shuttle between nucleus and cytoplasm. We discuss models for HuR function in mammalian cells.

Results

Expression of HuR in cultured mammalian cell lines

We raised rabbit polyclonal antibodies against His-tagged HuR recombinant protein (kindly prepared by Dr V.Myer) and affinity-purified them through a Ni^{2+}-agarose column coupled to His-tagged recombinant mouse HuR (mHuA) protein (see Materials and methods; the pET-mHuA plasmid was kindly provided by Dr R.Darnell). The rabbit antiserum and the affinity-purified antibody were analyzed by Western blot using HeLa whole-cell extract (Figure 1A, lanes 1 and 2). To verify that the single band detected in Figure 1A, lane 2 (indicated by an arrow) is indeed HuR, the purified antibody was depleted by passing the solution through an affinity column that was pre-loaded with His-mHuA at a 5-fold excess relative to the antibody. Use of this depleted antibody preparation resulted in a complete loss of signal (Figure 1A, lane 3) in a strip loaded with the same amount of HeLa cell lysate as in lanes 1 and 2 and run on the same gel.

Since HuR expression has only been reported to occur at the RNA level in non-neuronal mammalian tissues (Ma *et al.*, 1996; Okano and Darnell, 1997), we examined HuR protein expression in various cell lines through Western analysis. Total cell lysates from 1670 (marmoset T cells), HEK 293 (human embryonic kidney cells), NIH 3T3 (mouse fibroblasts), L929 (mouse connective tissue cells), HeLa (human epithelial cells) and COS (monkey kidney fibroblasts) were prepared by sonication and immunoblotted using the affinity-purified anti-HuR antibody. As shown in Figure 1B, HuR protein levels are high in 1670, HEK 293, HeLa and COS cells (Figure 1B, lanes 1, 2, 5 and 6, respectively) and lower, but still detectable in NIH 3T3 and L929 cells (lane 3 for 3T3 cells; lanes 4 and 7 for L cells). The blots were stripped and re-probed with anti-tubulin monoclonal antibody as an internal loading control. We conclude that HuR protein is expressed in a variety of mammalian cell lines.

We next investigated whether transient transfection of a human HuR cDNA [pCDNA3-HuR, driven by the human cytomegalovirus (CMV) immediate early gene promoter] into mouse L929 cells could provide overexpression of HuR. As analyzed by Western blot, a protein of the same size as endogenous HuR (~36 kDa) is detected in transfected cell lysates (Figure 1B, lane 8); the level of the HuR band is increased ~3-fold compared with non-transfected cells (Figure 1B, lane 7). Since the transient transfection efficiency of mouse L cells using DEAE–dextran is ~30% (Fan *et al.*, 1997), the overexpression of HuR is therefore estimated to be ~8-fold relative to the endogenous level in those L cells that have been transfected, similar to the level of HuR protein expression in HEK 293 cells (Figure 1B, lane 2). A C-terminal Flag-tagged HuR, expressed from the same CMV promoter (pCDNA3-HuR-C-Flag), was also transiently transfected into L cells and immunoblotted with anti-HuR. A protein of ~37 kDa, consistent with the addition of the Flag epitope, is detected in these transfected cells (Figure 1B, lane 9) at approximately the same level as in the non-

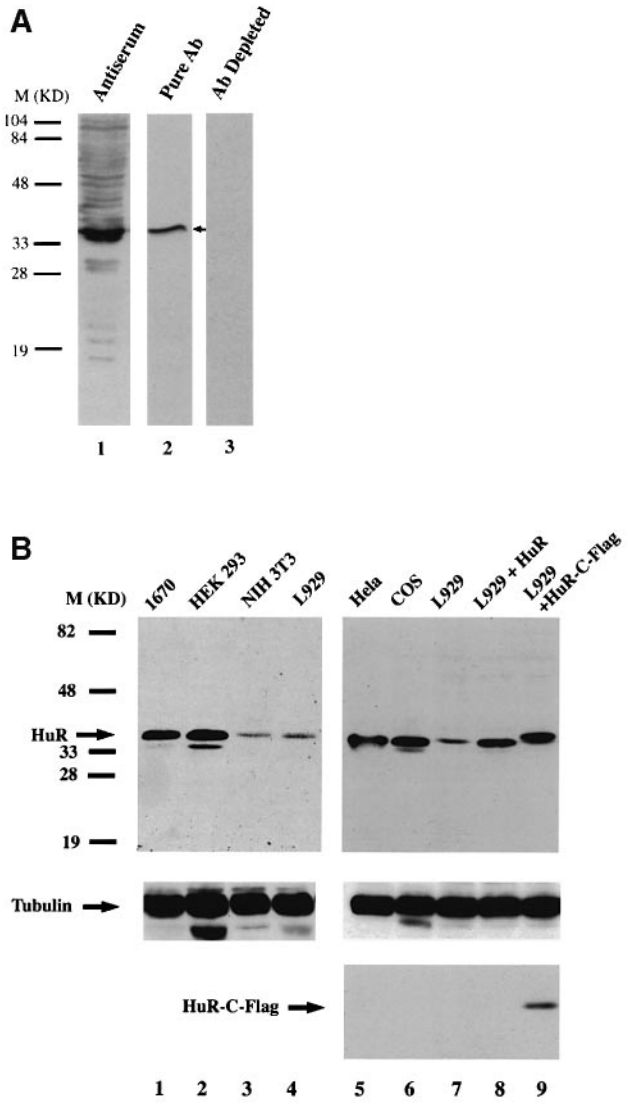

Fig. 1. Western blot analysis of HuR expression. (**A**) Specificity of the anti-HuR polyclonal antibody. Rabbit polyclonal antibody was raised against His-tagged recombinant HuR protein, affinity-purified through a Ni^{2+}-agarose column coupled to recombinant His-mHuA protein. HeLa total cell extract was fractionated (50 μg protein/lane) on 12.5% SDS–PAGE and blotted with crude anti-HuR rabbit antiserum at 1:2000 dilution (lane 1), or with affinity-purified anti-HuR polyclonal antibody at 0.7 μg/ml (also 1:2000, lane 2). The affinity-purified anti-HuR was depleted by passage through an antigen affinity column pre-loaded with a 5-fold excess (molar ratio) mHuA relative to the antibody, and blotted at the same (1:2000) dilution (lane 3). The HuR signal is indicated by an arrow in lane 2. (**B**) Expression of HuR in cultured cell lines. Total cell lysates from 1670, HEK 293, NIH 3T3, L929, HeLa and COS were prepared by sonication, fractionated at 200 μg/lane on 12.5% SDS–PAGE and immunoblotted using the affinity-purified anti-HuR antibody at 0.7 μg/ml (lanes 1–7, respectively). The blots were stripped and re-probed with anti-tubulin monoclonal antibody (Calbiochem) at 1 μg/ml as an internal control. L929 cells transiently transfected with pCDNA3-HuR and pCDNA3-HuR-C-Flag were analyzed in the same way (lanes 8 and 9, respectively). This blot was also stripped and re-blotted with anti-Flag (Sigma) at 1 μg/ml dilution (lanes 5–9). The signals were analyzed using the NIH Image Program. HuR levels in 1670, HEK 293, NIH 3T3, HeLa, COS and L929+HuR were found to be ~6-, 7-, 0.6-, 5-, 5- and 3-fold, respectively, relative to that in L929 cells. The overexpressed HuR-C-Flag signal (lane 9) was ~2-fold relative to the endogenous HuR. The secondary bands in lanes 2 and 6 are likely to be gel artifacts, rather than degradation products, as they do not appear in repeated Western blots of the same extracts.

tagged HuR transfectants (Figure 1B, lane 8). The blot was stripped and probed with anti-Flag monoclonal antibody, yielding a band at 37 kDa only in extracts from cells transfected with HuR-C-Flag (Figure 1B, lane 9). These results further verify the specificity of the affinity-purified anti-HuR polyclonal antibody.

Nuclear localization of HuR

While HuR was originally isolated from HeLa cell nuclear extract (Myer *et al.*, 1997), a fraction of the protein had also been reported to be cytoplasmic (Vakalopoulou *et al.*, 1991). We investigated the subcellular localization of HuR by indirect immunofluorescence. HeLa cells grown on coverslips were fixed with 3% paraformaldehyde for 20 min, permeabilized with 0.5% Triton for 15 min, and stained using the affinity-purified anti-HuR polyclonal antibody. The cells were co-stained with either the Y12 monoclonal antibody that recognizes Sm snRNPs (which exhibit nuclear localization with nucleolar exclusion) (Lerner *et al.*, 1981) or with the Y10B monoclonal antibody, which is immunoreactive with ribosomal RNA (rRNA; showing cytoplasmic and nucleolar staining) (Lerner *et al.*, 1981). The secondary antibody for anti-HuR, goat-anti-rabbit IgG, was coupled to Texas red, and that for the monoclonal antibodies, goat-anti-mouse IgG, to FITC. Normal rabbit IgG and anti-HuR pre-cleared by exposure to the mHuA antigen affinity column were also applied at the same dilution as negative controls.

As shown in Figure 2, staining of HuR in HeLa cells with the affinity-purified anti-HuR antibody reveals a nearly exclusive nuclear localization, with nucleolar exclusion (Figure 2B and F). Normal rabbit IgG and the antigen-depleted anti-HuR antibody yield no immunofluorescence signals (Figure 2Q and N, respectively), while Y12 (Figure 2C, J, O and R) and Y10B (Figure 2G) stain their respective antigens as expected. HuR's nucleoplasmic distribution is confirmed by the superimposed images (Figure 2D and H): it displays an overlapping pattern with Sm snRNPs, as shown by the orange color (Figure 2D), and is complementary to the rRNA staining (Figure 2H). A second fixation/permeabilization method was also used; the cells were fixed with 2% formaldehyde for 30 min followed by 3 min at –20°C in acetone. HuR exhibited the same nucleoplasmic staining (data not shown) as in Figure 2, suggesting that the fixation procedures do not alter the intracellular distribution of HuR.

Mouse L929 cells untransfected or transfected with pCDNA3-HuR-C-Flag were also subjected to immunofluorescent staining. As shown in Figure 2, endogenous HuR is localized in the L cell nucleus (Figure 2I), but the signal is weaker than in HeLa cells, consistent with the results of Western analysis (Figure 1B). Overexpressed HuR-C-Flag in transfected L cells (indicated by arrows in Figure 2K and L) is likewise predominantly nucleoplasmic (Figure 2K). Double staining with both affinity-purified anti-HuR polyclonal (Figure 2K) and anti-Flag monoclonal antibodies (Figure 2L) confirms the co-localization of the endogenous and the Flag-tagged HuR proteins.

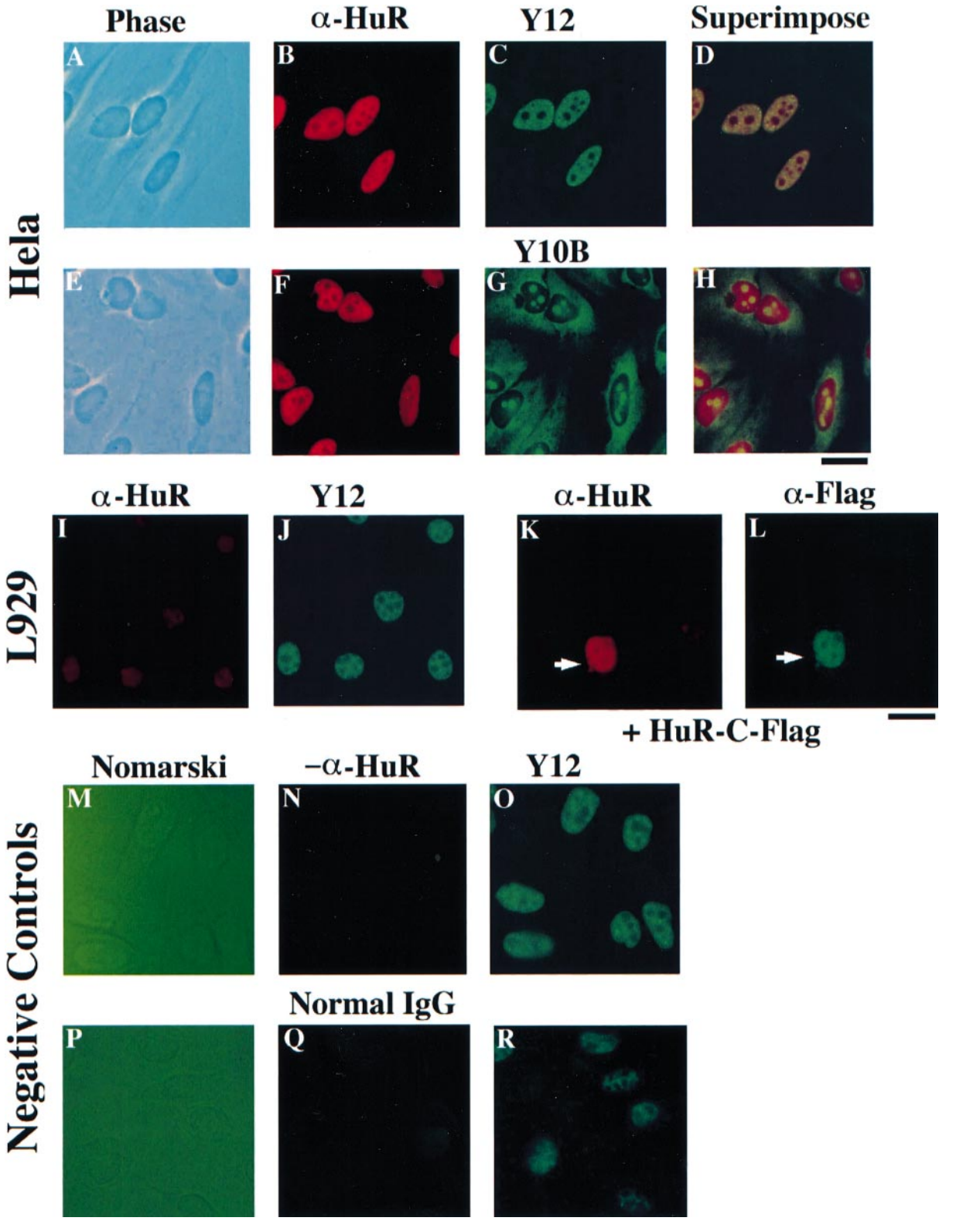

Fig. 2. Nucleoplasmic localization of endogenous and transfected HuR in HeLa and L929 cells. HeLa (**A**–**H**, **M**–**O**), mouse L929 cells (**I** and **J**, **P**–**R**) and L929 cells transfected with pCDNA3-HuR-C-Flag (**K** and **L**) were fixed with 3% paraformaldehyde for 20 min, permeabilized with 0.5% Triton X-100 and stained with either the affinity-purified anti-HuR polyclonal antibody (B, F, I and K), affinity-purified anti-HuR depleted by exposure to mHuA antigen (N), or normal rabbit IgG (Q). Cells were co-stained with either the Y12 monoclonal antibody that recognizes Sm snRNPs in the nucleoplasm (C, J, O and R), Y10B monoclonal antibody against rRNA in the nucleoli and cytoplasm (G), or anti-Flag monoclonal antibody (Sigma) (L). The transfected L929 cell overexpressing HuR-C-Flag is indicated by an arrow (K and L). The secondary antibody for anti-HuR, goat-anti-rabbit IgG (Jackson ImmunoResearch Laboratory), was coupled to Texas red, and that for the monoclonal antibodies, goat-anti-mouse IgG, to FITC. Superimposed red and green images are shown in (D) (for B and C) and (H) (for F and G). (A) and (E) show HeLa cells in phase-contrast while (M) and (P) are Nomarski images. Bars, 10 μm.

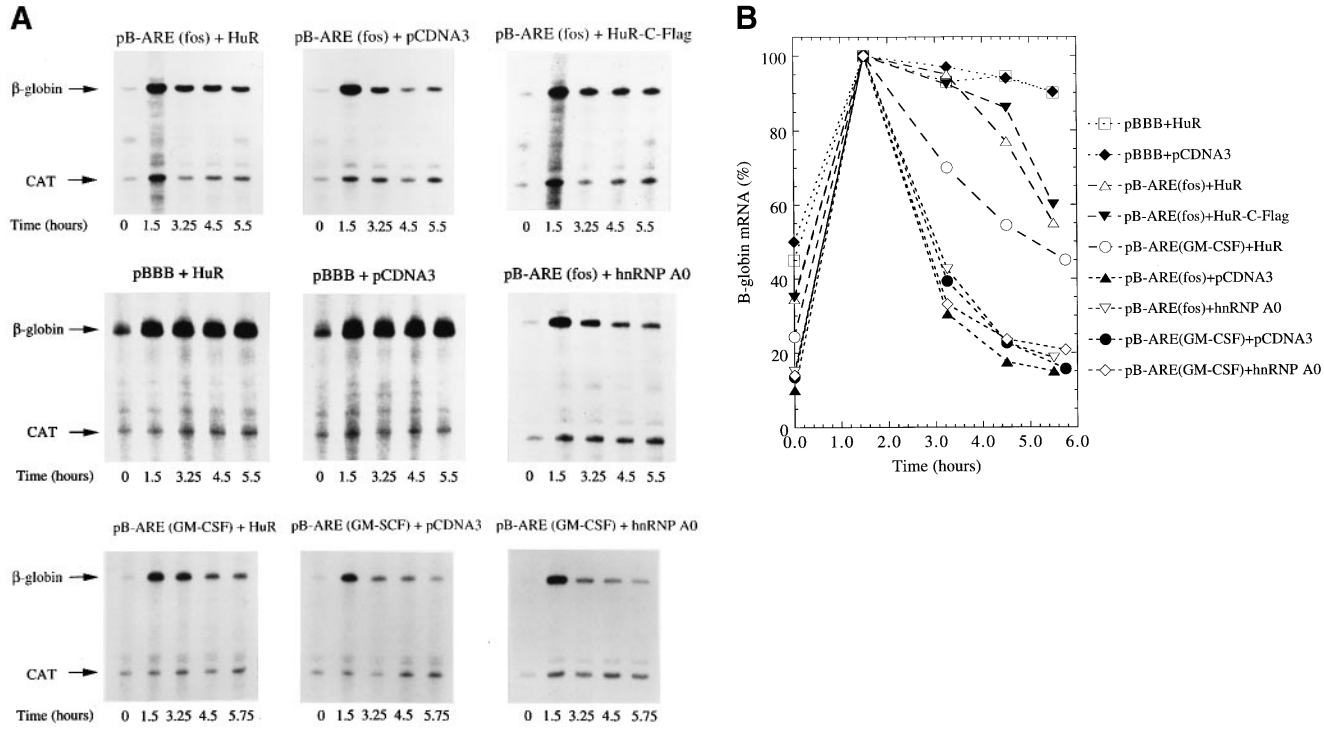

Fig. 3. Overexpression of HuR stabilizes β-globin reporter mRNAs containing both class I (c-*fos*) and II (GM-CSF) AREs. (**A**) Transient transfection analyses. The β-globin reporter [pB-ARE(fos), pB-ARE(GM-CSF) or pBBB] and CAT (pEF-BOS-CAT) plasmids were transiently co-transfected into mouse L929 cells along with pCDNA3 plasmids overexpressing HuR, HuR-C-Flag or hnRNP A0, as indicated. After serum starvation, β-globin transcription was stimulated by serum addition and cells were harvested at the time intervals indicated. Total RNA was isolated for T1 RNase protection analyses using both a β-globin and a CAT antisense probe. (**B**) Time-course of β-globin mRNA decay. The β-globin signals in the RNase protection assays (as in Figure 3A) were quantitated on a Molecular Dynamics PhosphorImager, standardized to the CAT internal control (the lower band of the doublet was quantitated as the major T1 digestion product) and plotted. 100% was arbitrarily assigned to the time point with the highest signal. The results are the average of duplicate experiments (except for pCDNA3-HuR, which is the average of three repeats); the variability at each time point was within 5% relative to the maximal signal.

Overexpression of HuR stabilizes ARE-containing mRNAs

The correlative data that previously implicated HuR in the ARE-mediated mRNA decay pathway (Myer *et al.*, 1997) could not distinguish whether HuR functions as a stabilizer or a destabilizer. Overexpression and correct nuclear localization in transfected cells provided an opportunity to dissect HuR's role further by examining the *in vivo* decay of ARE-containing reporter mRNAs co-transfected with HuR. We chose the β-globin reporter gene with a serum inducible c-*fos* promoter and a c-*fos* ARE [pB-ARE(fos)], utilized in many transient transfection studies of mRNA stability (Shyu *et al.*, 1989; Schiavi *et al.*, 1994; Zubiaga *et al.*, 1995; Fan *et al.*, 1997; Myer *et al.*, 1997). The 51-nucleotide class I c-*fos* ARE (Shyu *et al.*, 1989) was shown previously to have a high affinity for HuR (Myer *et al.*, 1992).

Plasmids pCDNA3-HuR and pB-ARE(fos) (Myer *et al.*, 1997) were co-transfected into mouse L cells along with a control plasmid pEF-BOS-CAT, which constitutively expresses the CAT mRNA (Zubiaga *et al.*, 1995; Myer *et al.*, 1997). After 24 h of serum starvation, cells were collected at 0, 1.5, 3.25, 4.5 and 5.5 h following serum induction; total RNA was isolated and analyzed by RNase T1 protection (Figure 3A). The data, averaged from several experiments and standardized to the CAT mRNA internal control, are plotted in Figure 3B. The maximum signal in each case was considered 100%. Plasmid pCDNA3 alone, without the HuR insert, was assayed as a negative control.

As is evident from Figure 3A and B, in the negative control cells (which are co-transfected with the pCDNA3 vector alone), the pB-ARE(fos) mRNA level decays to $<$15% 5.5 h post-induction (half-life = 1.2 h), as observed previously (Fan *et al.*, 1997). In contrast, in cells overexpressing HuR, pB-ARE(fos) mRNA decay is slowed, exhibiting an RNA level of 55% at the 5.5-h time point (half-life = 3.5 h). Also note that the pB-ARE(fos) mRNA level at the 0 time point, which represents its steady-state level, is higher in cells overexpressing HuR (~35%) than in the negative control cells (~10%). Flag-tagged HuR (pCDNA3-HuR-C-Flag) was also co-transfected into L cells and likewise yielded increased pB-ARE(fos) mRNA levels (half-life = 3.7 h; Figure 3A and B), showing that the epitope tag does not interfere with the stabilizing ability of HuR.

A possible explanation for the higher pB-ARE(fos) mRNA level in cells overexpressing HuR could be up-regulation of transcription from the c-*fos* serum-inducible promoter. The β-globin mRNA alone (pBBB mRNA) exhibits basal level decay once transcription from the c-*fos* promoter is turned off 0.5–1.5 h after serum induction (Fan *et al.*, 1997). If HuR were to up-regulate transcription, we would expect to see a relative increase of pBBB mRNA levels in cells overexpressing HuR. Both pCDNA3-HuR and pCDNA3 were therefore separately co-transfected with the pBBB plasmid. The pBBB mRNA exhibited no difference in its pattern of expression (Figure 3A and B, □ versus ◆), arguing that the high level of pB-

ARE mRNA in cells overexpressing HuR results from RNA stabilization, rather than increased transcription.

Since HuR is an RNA-binding protein of the RRM superfamily, we considered the possibility that overexpression of any RRM-containing protein might non-specifically produce the same stabilizing effect as HuR. We therefore tested another RRM protein, hnRNP A0, chosen for two reasons. First, A0 also has high (but not specific) affinity for AUUUA-repeats (Myer and Steitz, 1995), and second, we estimate that the cellular levels of HuR and A0 proteins are within the same order of magnitude (Myer *et al.*, 1997). HnRNP A0 was cloned under the same CMV promoter in the pCDNA3 plasmid, shown to produce the same level of overexpression, and assayed in the same way as HuR transfectants. As shown in Figure 3A and B, pB-ARE(fos) mRNA stability is not affected by overexpression of hnRNP A0 (▽ versus ▲; half-lives of 1.4 versus 1.2 h). We also examined a third, more abundant RRM-containing protein, hnRNP D (Pinol-Roma *et al.*, 1988; Zhang *et al.*, 1993; Ehrenman *et al.*, 1994; Kajita *et al.*, 1995), which exists in several alternatively spliced isoforms (Dempsey *et al.*, 1998). We overexpressed three hnRNP D cDNAs from the CMV promoter (one full-length, one with the second exon alternatively spliced and one with the seventh exon spliced out; all kind gifts of Dr N.Maizels), but observed no effect on the stability of pB-ARE(fos) mRNA (data not shown). We therefore conclude that the mRNA stabilization conferred by HuR is not a general property of RRM-containing RNA-binding proteins.

We also asked whether HuR's stabilizing effect is specific for ARE-containing mRNAs only, or acts on labile mRNAs containing another type of destabilizing element. A human β-globin reporter construct β-39, which contains a nonsense mutation at codon 39, is subject to nonsense-mediated rapid decay (Baserga and Benz, 1988; a kind gift from Dr S.Baserga). Overexpression of HuR did not increase its level when examined in transfected L cells (data not shown), confirming HuR's specificity for stabilizing ARE-containing mRNAs.

Finally, we asked whether overexpression of HuR would stabilize an mRNA with a class II ARE. The 62-nucleotide GM-CSF ARE (Shaw and Kamen, 1986) was cloned into the β-globin 3′ UTR in the pB-ARE plasmid at the same position as the c-*fos* ARE and assayed in the same way. As shown in Figure 3A and B, the pB-ARE(GM-CSF) mRNA was also stabilized to >40% at 5.75 h (half-life = 2.5 h) after induction in cells overexpressing HuR. Conversely, in cells transfected with vector alone or with hnRNP A0, pB-ARE(GM-CSF) mRNA levels decayed to <20% (half-lives = 1.3 or 1.2 h, respectively). We conclude that overexpression of HuR can stabilize mRNAs containing AREs of both class I and II.

One explanation for the observed stabilization is that excess HuR traps ARE-containing mRNAs in the nucleus, keeping them away from the degradation machinery. We eliminated this possibility by cloning the AU-rich sequence into the 3′ UTR of a luciferase reporter gene, and carrying out luciferase activity assays. In eight independent transient transfection experiments, we found the luciferase activity to be 2.0 ± 0.4-fold higher in cells overexpressing HuR than in control cells transfected with vector alone. Enhanced translation of the ARE-containing reporter mRNA indicates that the major effect of overexpressing HuR cannot be to sequester bound mRNAs in the nucleus.

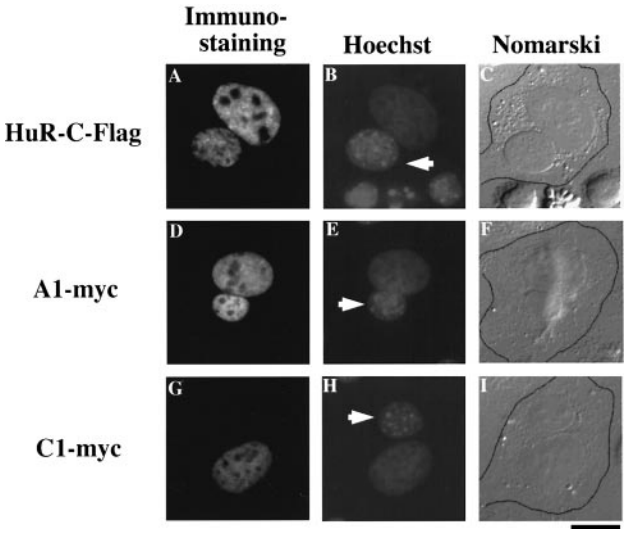

Fig. 4. HuR shuttles between the nucleus and cytoplasm. HeLa cells were transiently transfected with plasmid pCDNA3-HuR-C-Flag, pCDNA3-hnRNP A1-myc (Pinol-Roma and Dreyfuss, 1992), or pCDNA3-hnRNP C1-myc (Pinol-Roma and Dreyfuss, 1992), fused with mouse L929 cells to form heterokaryons using 50% PEG, and incubated for a further 4 h in the presence of the protein synthesis inhibitor cycloheximide. The co-culture was then fixed and stained using either the anti-Flag monoclonal antibody (Sigma) for HuR-C-Flag (**A**), or the 9E10 anti-myc-tag monoclonal antibody for hnRNP A1-myc (**D**) or for hnRNP C1-myc (**G**). The heterokaryons were also stained with Hoechst dye 33258 to distinguish the human and mouse nuclei: mouse nuclei show bright dots, indicated by arrows in (**B**), (**E**) and (**H**). Nomarski images of the heterokaryons are outlined (**C**, **F** and **I**). Bar, 10 μm.

HuR shuttles between nucleus and cytoplasm

We have shown that HuR is a nuclear protein and that its overexpression interferes with mRNA decay, which is believed to occur in the cytoplasm. This raised the possibility that HuR may shuttle between nucleus and cytoplasm, binding initially to an ARE-containing mRNA in the nuclear compartment, but then providing protection during its cytoplasmic life. We utilized the ability of HeLa and mouse L929 cells to form heterokaryons (Pinol-Roma and Dreyfuss, 1992) to test whether HuR in fact shuttles between nucleus and cytoplasm. Since the C-terminal Flag-tagged HuR exhibits the same subcellular localization (Figure 2) and mRNA stabilization activity (Figure 3) as the non-tagged HuR, we transfected HeLa cells with HuR-C-Flag and then fused them with mouse L cells. If HuR shuttles, the tagged protein visualized by immunostaining using anti-Flag antibodies should be transported to the mouse nuclei, even in the absence of protein synthesis. A myc-epitope-tagged hnRNP A1, which had been shown previously to shuttle (Pinol-Roma and Dreyfuss, 1992; Michael *et al.*, 1995; Siomi and Dreyfuss, 1995), was transfected in parallel as a positive control. The non-shuttling hnRNP C1 (also myc-tagged) provided a negative control (Pinol-Roma and Dreyfuss, 1992; Nakielny and Dreyfuss, 1996).

As shown in Figure 4, at 4 h after fusion in the presence of the protein synthesis inhibitor cycloheximide, HuR-C-Flag appears in both the HeLa and L cell nuclei of the heterokaryons (Figure 4A). [The HeLa and mouse nuclei

can be distinguished by co-staining with Hoechst dye (Moser *et al.*, 1975), which produces bright dots in mouse cell nuclei, indicated by arrows in Figure 4B, E and H)]. HuR's behavior therefore mimics that of the known shuttling protein hnRNP A1-myc, which is likewise detected in both HeLa and L nuclei (Figure 4D). Conversely, the non-shuttling protein hnRNP C1-myc is confined to the HeLa nucleus in the heterokaryon (panel G), confirming that the amount of cycloheximide used is sufficient to shut down protein synthesis.

We have also investigated the ability of HuR to shuttle by a different approach. Since the endogenous HuR level is much lower in L than in HeLa cells, we made heterokaryons from non-transfected HeLa and L cells, and stained them with affinity-purified anti-HuR antibody (data not shown). We observed that the HuR signal in L cell nuclei increased several fold upon heterokaryon formation, as compared with the much weaker staining of the non-fused L cell nuclei. We conclude that the endogenous HuR protein, like the tagged one, can be transported from one nucleus to another in heterokaryons, confirming that HuR is a shuttling protein.

RRM 3 is important for HuR function

To confirm that the mRNA stabilizing function can be attributed directly to HuR overexpression, we examined HuR deletion mutants. Previous *in vitro* studies using HuR mutants suggested that the first two RRMs are responsible for ARE binding, while RRM 3 simultaneously associates with the poly(A) tail (Abe *et al.*, 1996; Ma *et al.*, 1997). We therefore prepared constructs containing deletions of RRM 3 and tested their mRNA stabilizing activity in transfected cells.

HuR M1, which contains only the first two RRMs (aa 1–185), and HuR M2, which also lacks RRM 3 but retains the hinge region between RRMs 2 and 3 (aa 1–244), were cloned into the pCDNA3 vector and transiently transfected into mouse L cells. In Western blots using the affinity-purified anti-HuR antibody, protein bands of the sizes expected for M1 and M2 (~20 and 27 kDa, respectively) appeared in transfected L cells (Figure 5A, lanes 2 and 3, respectively); their levels are 1.1- and 0.6-fold relative to the endogenous HuR band. Assuming that antigenic epitopes are distributed evenly along the HuR polypeptide chain and that the transfection efficiency is comparable with that determined previously (30%, Fan *et al.*, 1997), we estimate M1 and M2 levels to be ~4.5- and 2.4-fold, respectively, that of the endogenous HuR in those cells that are transfected. Plasmids pCDNA3-HuR-M1 and -M2 were then co-transfected with pB-ARE(fos) and pEF-BOS-CAT. As shown in Figure 5C and D, the pB-ARE(fos) mRNA level fell to $<$20% by 5.5 h post-stimulation in cells overexpressing HuR M1 or HuR M2 (half-lives = 1.0 or 1.1 h, respectively), thereby exhibiting the same decay pattern as in cells transfected with the plasmid pCDNA3 alone (Figure 3A and B).

As a positive control for these experiments, it was necessary to lower the expression of full-length HuR in transfected cells. We therefore used pCDNA3-HuR at one-third the amount (Figure 5B, lane 3) of previous transfections (Figure 5B, lane 2) and confirmed by Western blot that the expression level was ~2-fold above the endogenous HuR (Figure 5B, lane 1) in transfected cells, similar to the levels obtained for M1 and M2. Yet, this 3-fold lower overexpression of HuR still exhibited a stabilizing effect, elevating the labile pB-ARE(fos) mRNA level to $>$35% at 5.5 h post stimulation (Figure 5C and D, □; half-life = 2.1 h). We conclude that only a 2-fold overexpression of full-length HuR can stabilize an ARE-containing mRNA, whereas deletion mutants M1 and M2 are indeed non-functional in this assay.

Discussion

HuR has been implicated in the ARE-containing mRNA decay pathway because its binding to various AU-rich sequences correlates with their abilities to confer rapid degradation on host mRNAs (Myer *et al.*, 1997). We show here that overexpression of HuR, but not of truncated HuRs lacking RRM 3, in mouse L929 cells stabilizes ARE-containing mRNAs, providing the first *in vivo* evidence that HuR can influence this degradation pathway. Similar conclusions are presented by Peng *et al.* (1998), and Levy *et al.* (1998) have recently reported that HuR stabilizes the labile vascular endothelial growth factor (VEGF) mRNA specifically under hypoxic conditions. Immunofluorescent staining of human HeLa and mouse L929 cells reveals that both the endogenous and overexpressed HuR are localized in the nucleoplasm. The formation of heterokaryons between mouse L929 and HeLa cells demonstrates that HuR shuttles between nucleus and cytoplasm.

How does HuR function in ARE-mediated mRNA decay?

The observation that HuR overexpression increases the half-life of ARE-containing mRNAs can be interpreted in at least two ways. One possibility is that HuR acts as part of a large complex (Savant-Bhonsdale and Cleveland, 1992) whose assembly is required for ARE-targeted mRNA decay. If another factor(s) in this complex is (are) limiting, then overexpression of HuR could sequester such component(s), thereby precluding assembly of the active degradation complex (a squelching effect; Ptashne and Gann, 1990). According to this model, RRM 3 of HuR would be critical (either directly or indirectly) for the squelching interaction, since the overexpression of HuR mutants (M1 and M2) lacking this domain does not stabilize the reporter mRNA.

Alternatively, HuR may function *in vivo* to slow the decay of ARE-containing mRNAs, suggesting that the rate of mRNA degradation is determined by the relative balance of stabilizing and destabilizing factors. Such an activity has been proposed previously for another Hu family member, the neuronal-specific protein Hel-N1. When expressed ectopically in mouse adipocytes, it retards the decay rate of the ARE-containing glucose transporter gene (GLUT 1) mRNA (Jain *et al.*, 1997).

While investigating HuR expression in various cell lines, we noticed that HuR protein levels are higher in transformed lines, including HeLa, HEK 293, COS and 1670, and lower in immortalized lines such as L929 and NIH 3T3 (Figure 1B). Since proto-oncogene, cytokine and lymphokine mRNAs characteristically contain ARE sequences, finding higher HuR protein levels in faster-growing cells is consistent with HuR having a stabilizing effect on these mRNAs, some of which may contribute

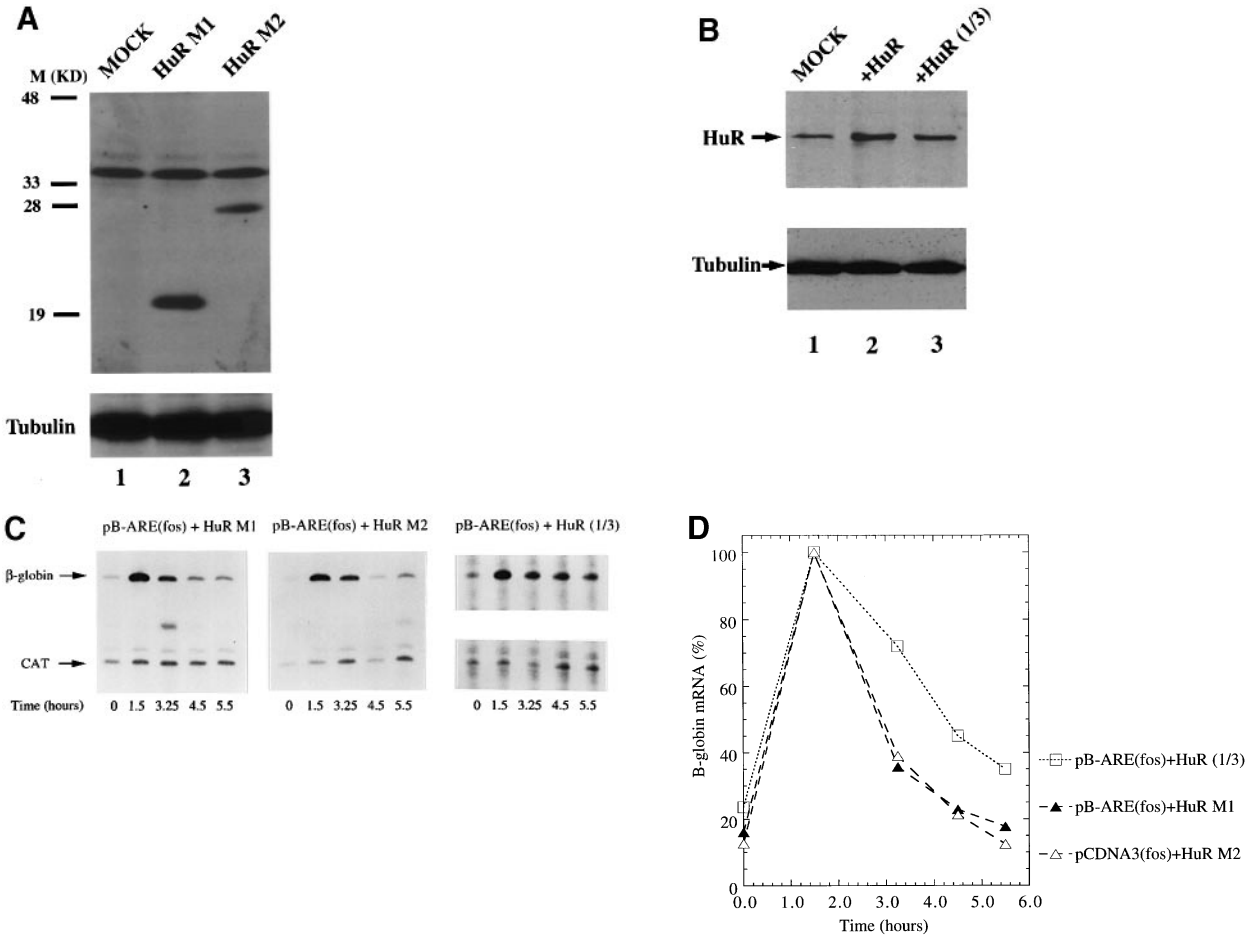

Fig. 5. RRM 3 is required for the stabilization activity of HuR. (**A**) Expression of HuR deletion mutants M1 and M2 in mouse L929 cells. L929 cells were transiently transfected with plasmids pCDNA3-HuR M1 (containing aa 1–185, lane 2), pCDNA3 M2 (containing aa 1–244, lane 3), or pCDNA3 alone (lane 1) as a control. Total cell extracts were prepared by sonication, fractionated (200 μg protein/lane) on 12.5% SDS–PAGE, and immunoblotted using the affinity-purified anti-HuR polyclonal antibody at 0.7 μg/ml. The blot was then stripped and re-probed with anti-tubulin monoclonal antibody (Calbiochem) at 1 μg/ml as an internal control. The Western blots were quantitated using the NIH image program: the bands corresponding to HuR M1 and M2 (lanes 2 and 3, respectively) were found to be 1.1- and 0.6-fold of the endogenous HuR (after normalization to the endogenous tubulin signal). (**B**) Controlled overexpression of HuR. Plasmid pCDNA3-HuR, either at the same level as in previous transfections (3 μg; lane 2) or one-third of that (1 μg supplemented with 2 μg pCDNA3 plasmid; lane 3) was transiently transfected into L929 cells. Three μg of plasmid pCDNA3 was also transfected as a control (lane 1). Total cell extracts were prepared by sonication and 100 μg of each was fractionated on 12.5% SDS–PAGE and Western blotted using the affinity-purified anti-HuR polyclonal antibody at a concentration of 0.7 μg/ml. The blot was then stripped and re-blotted with anti-tubulin monoclonal antibody (Calbiochem) at 1 μg/ml as an internal control. The Western blots were quantitated using the NIH Image program. When normalized to the tubulin signals, the HuR band in lanes 2 and 3 was found to be 3.0- and 1.7-fold, respectively, of the that of the endogenous HuR in lane 1. (**C**) Effect of HuR deletion mutants on pB-ARE(fos) mRNA stability. The pB-ARE(fos) and CAT (pEF-BOS-CAT) plasmids were transiently co-transfected into mouse L929 cells along with pCDNA3 plasmids expressing M1 (3 μg), M2 (3 μg) or HuR (1 μg plus 2 μg of pCDNA3). After serum starvation, β-globin transcription was stimulated by serum addition and cells were harvested at the time intervals indicated. Total RNA was isolated for T1 RNase protection analyses using both a β-globin and a CAT antisense probe. (**D**) Time-course of reporter β-globin mRNA decay. The β-globin mRNA signals in RNase protection assays (as in Figure 5C) were quantitated on a Molecular Dynamics PhosphorImager, standardized to the CAT internal control (the lower band of the doublet was quantitated as the major T1 digestion product) and plotted. 100% was arbitrarily assigned to the time point with the highest signal. These results are the average of duplicate experiments, with variability at each time point being within 5% relative to the maximal signal.

to the transformed phenotype. In a recent report concerning Hu protein expression during chicken neurogenesis, HuR was observed to exhibit higher expression levels in the ventricular zone of the spinal cord and was implicated in maintaining proliferation of neuronal precursor cells (Wakamatsu and Weston, 1997). However, when HuR alone was transiently transfected into either neuronal cells (Wakamatsu and Weston, 1997) or mouse fibroblasts (data not shown), no significant change in growth phenotype was observed. It will be interesting to learn whether HuR levels are actively up-regulated in cells treated in ways known to increase the stability of ARE-containing mRNAs (Lindsten *et al.*, 1989; Bohjanen *et al.*, 1991).

Considering the stabilization model discussed above, our observation that overexpression of HuR mutants M1 and M2 lacking RRM 3 does not slow the degradation of the ARE-containing reporter mRNA is particularly intriguing. This RRM has been assigned a role in associating with the poly(A) tail, while RRMs 1 and 2 suffice for ARE-sequence binding (Abe *et al.*, 1996; Ma *et al.*, 1997). Our results therefore suggest that interactions with both the ARE and poly(A) are essential for effective mRNA stabilization by HuR. We have further probed this idea by determining the effect of HuR overexpression on a labile, ARE-containing snRNA HSUR 1, which also binds HuR (Fan *et al.*, 1997). After transient transfection of mouse

L cells, wild-type HSUR 1 levels were found not to be altered by overexpression of HuR compared with vector alone with respect to a co-transfected stable snRNA control (data not shown). Since HSUR 1 contains no poly(A) tail, this result is in accord with the notion that binding of RRM 3 to poly(A) is required for HuR to stabilize a RNA. Deadenylation has been shown to be the first step of degradation (Shyu *et al.*, 1991; Chen *et al.*, 1995), suggesting that HuR binding may serve initially to protect the mRNA from deadenylation. In this regard, it is interesting that the *Xenopus* homolog of HuR, elrA, has been implicated in polyadenylation of maternal mRNAs in fertilized eggs (Wu *et al.*, 1997). Conversely, detailed Northern analyses of ARE-containing reporter mRNAs have revealed no effect of overexpression of HuR on deadenylation kinetics (Peng *et al.*, 1998). Gene knockout experiments are therefore required in order to establish more definitively whether HuR acts mechanistically as a stabilizing or destabilizing protein.

HuR, a ubiquitous nuclear protein that shuttles between nucleus and cytoplasm

If HuR plays a pivotal role in mRNA stability, HuR protein should be expressed ubiquitously in all vertebrate tissues. Accordingly, we have detected HuR protein expression in a wide variety of mouse tissues (data not shown), as well as in many mammalian tissue culture cell lines (Figure 1B) through Western blot analysis with our polyclonal antibody. Previously, HuR mRNA was reported to be ubiquitously expressed in human (Ma *et al.*, 1996; Okano and Darnell, 1997) and *Xenopus* cells (Good, 1995), even though HuR protein in non-neural mouse tissues was not detectable using patient anti-Hu autoantibodies (Okano and Darnell, 1997). In contrast, widespread expression of HuR throughout the chick embryo has been observed at both the mRNA and protein levels (Wakamatsu and Weston, 1997).

It was initially a surprise to find that the immunofluorescent staining of both endogenous and transfected HuR proteins is predominantly nucleoplasmic. This is the case for a variety of mammalian cell lines tested, including HeLa (Figure 2), L929 (Figure 2), NIH 3T3 (data not shown) and CHO (Chinese hamster ovary; data not shown). On the other hand, ARE-mediated rapid decay of mRNA is generally believed to occur in the cytoplasm, even though translation is not absolutely required (Koeller *et al.*, 1991; Chen *et al.*, 1995; Fan *et al.*, 1997). We therefore investigated whether HuR shuttles actively between the nucleus and cytoplasm. This expectation was confirmed for both the tagged transfected protein (Figure 4) and for endogenous HuR (data not shown). As has been suggested for those hnRNP proteins that shuttle (for review, see Nakielny and Dreyfuss, 1997), HuR may initially bind to ARE-containing mRNAs in the nucleus, accompany them to the cytoplasm and then return rapidly to the nucleus after release from the mRNA. Release could occur upon mRNA decay (if HuR is a destabilizing protein) or upon encountering some active signal (if HuR is stabilizing) that displaces HuR and exposes the mRNA to nuclease attack.

The identification of HuR as a shuttling protein also rationalizes the possibility that HuR may have different subcellular localizations during different stages of development. In contrast to our results with mammalian somatic cells, the *Xenopus* homolog of HuR, elrA, is localized in the cytoplasm of oocytes and fertilized eggs (Wu *et al.*, 1997). Such a dynamic intracellular distribution could be a reflection of HuR's diverged but overlapping target sequence specificity and functional activity: *Xenopus* elrA binds to eCPE, U_5AU_{12-27}, and has been implicated in cytoplasmic polyadenylation in fertilized eggs (Wu *et al.*, 1997), where ARE-mediated mRNA decay does not occur (Kruys *et al.*, 1987; Marinx *et al.*, 1994); conversely, HuR binds to AUUUA repeats (Myer *et al.*, 1997) and stabilizes ARE-containing mRNAs in mammalian tissue culture cell lines (Figure 3), possibly through secondary interaction with their poly(A) tails (Abe *et al.*, 1996; Ma *et al.*, 1997).

HuR may contain a novel shuttling motif

Nuclear proteins are transported into or out of the nucleus via specific sequences (for reviews, see Dingwall and Laskey, 1991; Gorlich and Mattaj, 1996; Nakielny and Dreyfuss, 1997). The well-characterized nuclear localization signal (NLS) either contains a cluster of basic amino acids (as in the SV40 large T antigen) or is bipartite with two basic amino acids in the first cluster, separated by a 10-aa spacer from a second cluster of three or more basic amino acids (as in nucleoplasmin or HIV-1 Rev). The NLS is recognized by the importin α/β complex, docked to the nuclear pore complex (NPC) and translocated to the nucleoplasm through the Ran GTPase cycle in a temperature- and transcription-independent manner. The classical nuclear export sequence (NES) comprises a stretch of 10 amino acids rich in leucine [as in Rev or the protein kinase inhibitor (PKI)] (for reviews, see Izaurralde and Mattaj, 1995; Nakielny and Dreyfuss, 1997). The CRM 1 (Chromosomal Region Maintenance) protein, which exhibits homology to importin β, has recently been shown to be responsible for intracellular transport mediated by the NES; Ran GTPase is also involved (Fornerod *et al.*, 1997; Fukuda *et al.*, 1997; Stade *et al.*, 1997).

In addition to the NLS and NES motifs, two shuttling sequences, namely the 38-aa M9 sequence of hnRNP A1 and A2 (Siomi and Dreyfuss, 1995) and the 24-aa KNS (hnRNP K shuttling domain) of hnRNP K (Michael *et al.*, 1997), have been identified. They possess both NLS and NES activities, with their NLS activities being dependent on the transcriptional activity of RNA polymerase II (Pinol-Roma and Dreyfuss, 1992; Michael *et al.*, 1997). In the case of M9, Ran GTPase and transportin, which is related to importin β, have been shown to be involved in protein translocation through the NPC (Pollard *et al.*, 1996; Fridell *et al.*, 1997; Izaurralde *et al.*, 1997).

The hinge region between RRMs 2 and 3 of HuR has a basic sequence, $_{204}$RRFGGPVHHQAQRFRF, similar but not identical to the consensus bipartite NLS, since it has only two basic amino acids in the second cluster. HuR does not, however, have any M9-, KNS- or NES-like sequences, suggesting that it may contain a novel shuttling/NES motif. It is conceivable that removal of RRM 3 (in HuR mutant M2) or of the hinge region in addition (in the case of mutant M1) may have disturbed the NLS, NES or shuttling motifs of HuR. Therefore, we cannot exclude the possibility that mutants M1 and M2 do not function to stabilize the ARE-containing mRNA (Figure 5) because they have a different subcellular location or because they lack the ability to shuttle. More mutants are

required to pinpoint the sequences within HuR that are responsible for its stabilizing function, shuttling activity and its simultaneous binding to both the ARE and poly(A) tail.

Diverse functions for Elav-like proteins

Despite the fact that they exhibit high sequence homology and bind to similar target RNA sequences, Elav-like proteins appear to engage in quite different cellular functions. *Drosophila* Elav has been implicated in regulating alternative splicing essential for neuronal development (Robinow *et al.*, 1988; Koushika *et al.*, 1996) and sxl is a splicing and translation regulator involved in the *Drosophila* sex determination pathway (Valcarcel *et al.*, 1993). The vertebrate proteins elr B, C and D, on the other hand, are predicted to be involved in neuron-specific functions because they are expressed primarily in neuronal tissues. In 3T3-L1 adipocytes, ectopically expressed Hel-N1 protein increases the stability and translatability of glucose transporter 1 (GLUT 1) mRNA, apparently by binding to the AU-rich sequence in its 3′ UTR (Jain *et al.*, 1997). In cells of neuronal origin, Hel-N1 binds to a subset of poly(A)$^+$ mRNPs that associate with polysomes and the microfilament cytoskeletal network (Antic and Keene, 1998), suggesting an involvement in mRNA translation, localization and neuronal differentiation (Antic and Keene, 1997). The *Xenopus* homolog of HuR, elrA, has been linked to polyadenylation of certain maternal mRNAs that contain an embryonic-type cytoplasmic polyadenylation element (eCPE), $U_5AU_{12–27}$, in fertilized eggs (Wu *et al.*, 1997). Our data, as well as that of Peng *et al.* (1998) and Levy *et al.* (1998), implicate the mammalian elrA, HuR, in mRNA stabilization. It will be interesting to learn whether all these other Elav-like proteins shuttle from nucleus to cytoplasm and whether shuttling is an obligatory aspect of their functioning in cellular RNA metabolism.

Materials and methods

Cell culture and transient transfections

Murine L929 cells were passed in MEM medium (Gibco-BRL) supplemented with 10% horse serum (HS, Gibco-BRL). HeLa and 1670 cells were maintained in RPMI 1640 medium (Gibco-BRL) with 10% fetal bovine serum (FBS, Gibco-BRL). NIH 3T3, COS and HEK 293 cells were grown in 10% FBS-DMEM medium (Gibco-BRL). Three weeks prior to heterokaryon experiments, L929 cells were adapted in 10% FBS-DMEM.

L929 cells were grown in 100 mm dishes to 50–70% confluence and then switched to MEM medium supplemented with 10% NuSerum (Gibco) for DEAE–dextran transfection. Plasmids were resuspended in 40 μl TBS, mixed with 80 μl 10 mg/ml DEAE–dextran (Sigma)/TBS and then overlaid on cells. After incubation for 4 h, cells were shocked with 10% DMSO/PBS for 1 min and allowed to recover in 10% HS/MEM overnight. For mRNA degradation analyses, transfected L929 cells were serum-starved in 0.5% HS/MEM for 24 h and then serum-stimulated by addition of 20% HS/MEM. Cells were collected and RNA was isolated at 0, 1.5, 3.0, 4.5 and 5.5 h. A total of 6 μg DNA was applied for transfection of a 100 mm plate: to overexpress RNA binding proteins cloned in pCDNA3 (Invitrogen), 3 μg of plasmid was used, supplemented with 3 μg of salmon sperm DNA; for mRNA degradation analyses, 3 μg of pCDNA3-HuR plasmid was co-transfected with 2 μg of reporter construct and 1 μg of pEF-BOS-CAT control construct.

HeLa cells were transfected using the Calcium Phosphate Transfection System (Gibco-BRL) according to the manufacturer's directions. Luciferase assays were carried out using the Luciferase Assay System (Promega) according to the manufacturer's protocols.

Plasmid constructions

To generate the mammalian expression plasmid pCDNA3-HuR, human HuR cDNA was PCR-amplified from a human fetal brain cDNA library using the following pair of oligonucleotides: 5′-CGGGAATTCATACAATGTCTAATGGTTATGAAGACC and 5′-CGGTCTAGAGAGCGTTATTTGTGGGACTTG; the PCR fragment was then digested with restriction enzymes *Eco*RI and *Xba*I, and cloned into the eukaryotic expression vector pCDNA3 (Invitrogen). Plasmid pCDNA3-HuR-C-Flag was generated by PCR from pCDNA3-HuR by using the 5′ overhang oligonucleotide 5′-TATCTAGATTACTTGTCATCGTCGTCCTTGTAGTCTTTGTGGGACTTGTTGGTTTTG and an oligonucleotide that contains the T7 RNA polymerase promoter sequence (T7 primer; Invitrogen), and then cloning the product into the *Eco*RI and *Xba*I sites in the pCDNA3 vector. Plasmids pCDNA3-HuR M1 and M2, which contain amino acids 1–185 (RRMs 1 and 2) and 1–244 (RRMs 1, 2 plus the hinge between RRMs 2 and 3), respectively, were PCR-amplified from pCDNA3-HuR using the T7 primer and the oligonucleotide 5′-CAATCTAGACTATTAGGCTGCAAACTTCACTGCGATGG (for M1), or 5′-CAATCTAGACTATTACCAGCCGGAGGAGGCGTTTCCTG (for M2), and then cloned into the *Eco*RI and *Xba*I sites of pCDNA3 vector. A 1.65 kb luciferase gene was PCR-amplified using the pGL-Basic vector as template (Promega) and subcloned into the *Hin*dIII and *Xho*I sites in pCDNA3; an AT-rich sequence containing five repeated copies of ATTTA was inserted into its 3′ UTR to generate the luciferase reporter construct. All clones generated by PCR were sequenced to confirm the fidelity of amplification. The reporter plasmids pB-ARE(fos), pBBB and pEF-BOS-CAT have been described previously (Shyu *et al.*, 1989; Zubiaga *et al.*, 1995; Myer *et al.*, 1997). The plasmid pB-ARE(GM-CSF) was generated by replacing the 51-nucleotide c-*fos* ARE (Shyu *et al.*, 1989) in pB-ARE(fos) with the 62-nucleotide GM-CSF ARE (Shaw and Kamen, 1986). The pGEM-3Z plasmids (Promega) that were transcribed to produce the antisense β-globin and CAT RNAs used in the RNase protection assays have been described previously (Myer *et al.*, 1997). Plasmids pCDNA3-hnRNP A1-myc and pCDNA3-hnRNP C1-myc were kindly provided by Dr G.Dreyfuss and have been described previously (Michael *et al.*, 1995; Nakielny and Dreyfuss, 1996). Plasmid pET-mHuA for overexpression of the recombinant His-tagged mouse HuA (HuR) was generously provided by Dr R.Darnell (Okano and Darnell, 1997). Plasmid β-39 was provided by Dr S.Baserga (Baserga and Benz, 1988).

Antibody production, purification and Western blot analysis

Purified His-tagged recombinant HuR protein (Myer *et al.*, 1997) was injected into rabbits with Freund's adjuvant three times at 3-week intervals by the Yale University Animal Care Facility. The rabbit antibodies were precipitated from serum with $(NH_4)_2SO_4$ (40% final concentration) at 4°C overnight, centrifuged at 3000 *g* for 30 min and dialyzed into PBS. A 4 ml antigen affinity column was prepared with 6 mg of His-tagged mHuA protein bound to Ni^{2+}-agarose beads (Qiagen). The antibodies were then passed through the affinity column three times, washed with 5 vols of PBS, 5 vols of 2 M NaCl/20 mM Na_2HPO_4 pH 7.5, and eluted with 3 vols of 4 M $MgCl_2$. The purified antibody was dialyzed into PBS and concentrated to the starting serum volume using Centricon-30 (Bio-Rad). To verify its antigen specificity, the purified anti-HuR antibody was passed through an affinity column previously loaded with His-tagged mHuA at 5-fold molar excess than the antibody. The flow-through was then subjected to Western analysis.

For Western blot analysis, protein samples fractionated on 12.5% polyacrylamide/1% SDS gels were transferred to a nitrocellulose membrane and probed with primary antibodies as described (Ausubel *et al.*, 1994). Secondary antibodies were either horseradish peroxidase (HRP)-conjugated donkey anti-rabbit antibody for polyclonal antibodies or HRP-conjugated donkey anti-mouse antibody for monoclonal antibodies; blots were developed using the ECL system (Amersham) according to the manufacturer's directions. Signals were quantitated using the NIH Image program version 1.6 (NIH).

Immunofluorescence microscopy

HeLa cells and mouse L929 cells were grown under the conditions described above on coverslips for over 36 h. Cells were fixed with 3% paraformaldehyde in PBS for 20 min at room temperature, washed with PBS twice for 5 min, permeabilized with 0.5% Triton X-100/1% normal goat serum (Jackson ImmunoResearch Laboratory) in PBS for 15 min, and washed with 1% normal goat serum/PBS three times for 10 min each. The coverslips were then incubated with primary antibodies in 1% normal goat serum/PBS at room temperature for 1 h. The purified anti-HuR antibody was diluted to 5 μg/ml; the anti-Flag tag monoclonal

antibody (Sigma) was diluted to 10 μg/ml; mouse ascites fluids containing Y12 (anti-Sm; Lerner *et al.*, 1981), Y10B (anti-rRNA; Lerner *et al.*, 1981) or 9E10 (anti-myc tag, kindly provided by Dr G.Dreyfuss) monoclonal antibodies were applied at 1:1000 dilution. The coverslips were washed twice for 10 min and incubated with the secondary antibodies at 5.6 μg/ml in 1% normal goat serum/PBS at room temperature for 1 h. Secondary antibodies were goat anti-rabbit IgG coupled to Texas red or goat anti-mouse IgG coupled to FITC (Jackson Immuno-Research Laboratory). For analyses of HeLa-L929 heterokaryons, the Hoechst dye 33258 (Sigma) was included at 1 μg/ml with the secondary antibody incubation. The samples were then washed three times for 10 min each and mounted. Figure 2A–H were photographed through an AX-70 microscope (Olympus) with a 40× UPlan Fl objective. Figure 2I–T were photographed through an Axiophot microscope (Zeiss, Germany) with a 40× Plan-Apochromat oil objective. Figure 4A–I were photographed through an Axioplan 2 microscope (Zeiss) with a 63× Plan-Apochromat oil objective.

A second fixation/permeabilization method was used to verify the protein subcellular localization. Cells were fixed with 2% formaldehyde in PBS for 30 min at room temperature followed by 3 min in acetone at –20°C (Siomi and Dreyfuss, 1995), and then stained as described above.

RNase protection assays

Total RNA was collected from transfected cells using Trizol reagent (Gibco-BRL) and treated with RQ1 DNase (Promega). RNase T1protection assays were performed as described (Lee,S. *et al.*, 1988; Fan *et al.*, 1997), with the following modifications: DNase-treated RNA (20–30 μg for mRNA) was combined with $2–4\times10^5$ c.p.m. of the appropriate [α-^{32}P]UTP-labeled antisense probe, heated at 85°C for 5 min, incubated at 45°C overnight to allow annealing and then digested with T1 RNase (1.2 unit/10 μg RNA, Calbiochem) at 30°C for 1 h. Samples were electrophoresed on a 6% polyacrylamide–TBE gel. Results were quantitated with a Molecular Dynamics PhosphorImager.

Heterokaryon formation

Heterokaryons of HeLa and mouse L929 were formed as described (Michael *et al*., 1995) with the following modifications: HeLa cells were seeded onto glass coverslips at 2×10^5 cells/coverslip at 24 h post-transfection. Following overnight incubation, L929 cells, which had been incubated for 30 min in the presence of 75 μg/ml cycloheximide (Sigma), were seeded onto the same coverslip at 3×10^5 cells/coverslip. The co-cultures were then incubated for 3 h in 75 μg/ml cycloheximide, fused with 50% PEG 3350 (Sigma) for 2 min, washed in PBS and returned to medium containing 100 μg/ml cycloheximide for another 4 h incubation, followed by paraformaldehyde fixation for immunostaining as described above.

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