RNA (2001), *7*:1348–1361. Cambridge University Press. Printed in the USA.

DOI: 10.1017.S1355838201016089

Protein ligands mediate the CRM1-dependent export of HuR in response to heat shock

IMED-EDDINE GALLOUZI,* CHRISTOPHER M. BRENNAN, and JOAN A. STEITZ

Department of Molecular Biophysics and Biochemistry, Howard Hughes Medical Institute,
Yale University School of Medicine, New Haven, Connecticut 06536, USA

ABSTRACT

AU-rich elements (AREs) located in the 3′ UTRs of the messenger RNAs (mRNAs) of many mammalian early response genes promote rapid mRNA turnover. HuR, an RRM-containing RNA-binding protein, specifically interacts with AREs, stabilizing these mRNAs. HuR is primarily nucleoplasmic, but shuttles between the nucleus and the cytoplasm via a domain called HNS located between RRM2 and RRM3. We recently showed that HuR interacts with two protein ligands, pp32 and APRIL, which are also shuttling proteins, but rely on NES domains recognized by CRM1 for export. Here we show that heat shock induces increased association of HuR with pp32 and APRIL through protein–protein interactions and that these ligands partially colocalize with HuR in cytoplasmic foci. HuR associations with the hnRNP complex also increase, but through RNA links. CRM1 coimmunoprecipitates with HuR only after heat shock, and nuclear export of HuR becomes sensitive to leptomycin B, an inhibitor of CRM1. Export after heat shock requires the same domains of HuR (HNS and RRM3) that are essential for binding pp32 and APRIL. In situ hybridization and coimmunoprecipitation experiments show that LMB treatment blocks both hsp70 mRNA nuclear export and its cytoplasmic interaction with HuR after heat shock. Together, our results argue that upon heat shock, HuR switches its export pathway to that of its ligands pp32 and APRIL, which involves the nuclear export factor CRM1. HuR and its ligands may be instrumental in the nuclear export of heat-shock mRNAs.

Keywords: APRIL; CRM1; ELAV; hsp70 mRNA; HuR; mRNA export; pp32

INTRODUCTION

It is well established that heat shock of mammalian cells both induces transcription and enhances the stability of hsp70 and other stress-related mRNAs (Banerji et al., 1986). In contrast, most other mRNAs are transcriptionally down-regulated and become sequestered within the nucleus after heat shock (Sadis et al., 1988; Gallouzi et al., 2000). This suggests the existence of dedicated pathways for the nuclear export and stabilization of stress-induced mRNAs upon heat shock.

The messenger RNAs of many mammalian early response genes, including cytokines, lymphokines, and protooncogenes, contain AU-rich elements (AREs) in their 3′ untranslated regions (3′ UTR; Chen & Shyu, 1995; Jacobson & Peltz, 1996). These, together with numerous *trans*-acting proteins, confer regulated instability on the message (Chen & Shyu, 1995; DeMaria & Brewer, 1996; Jacobson & Peltz, 1996; Antic & Keene, 1997; Brennan & Steitz, 2001). AREs have been divided into three classes based on sequence and decay properties (Chen & Shyu, 1995). Class I AREs, such as the c-*fos* ARE, contain one to three copies of the pentanucleotide AUUUA within U-rich regions. Class II AREs, like the GM-CSF ARE, also exist in a U-rich context and consist of at least two overlapping copies of a critical nonamer UUAUUUA(U/A)(U/A) (Lagnado et al., 1994). Class III AREs, for example, the c-*jun* ARE, contain a U-rich sequence lacking the typical AUUUA element but nevertheless signal mRNA degradation.

HuR, a member of the embryonic lethal abnormal vision (ELAV) family of RNA-binding proteins, is ubiquitously expressed in mammalian cells (Ma et al., 1996). It interacts with AREs of all three classes (Brennan & Steitz, 2001) and stabilizes ARE-containing messages upon overexpression in transfected mammalian cells (Fan & Steitz, 1998b; Peng et al., 1998). The ELAV family was originally identified in *Drosophila melanogaster* as essential for development and maintenance of the nervous system (Campos et al., 1985). Three closely related neural-specific Hu family members exist in mammals: HuB (or HelN1/N2), HuC, and HuD (Good, 1995; Okano & Darnell, 1997). Architecturally, Hu pro-

Reprint requests to: Joan A. Steitz, Department of Molecular Biophysics and Biochemistry, Howard Hughes Medical Institute, Yale University School of Medicine, 295 Congress Avenue, New Haven, Connecticut 06536, USA; e-mail: joan.steitz@yale.edu.

*Present address: McGill University, Biochemistry Department, McIntyre Boulevard, 3655 Promenade Sir William Osler, Montreal, QC, Canada, H3G 1Y6.

teins contain three well-conserved RNA recognition motifs (RRMs; Antic & Keene, 1997) and differ from one another in two regions, the N-terminus and the region located between RRM2 and RRM3, known as the hinge region (Fan & Steitz, 1998a). The first two RRMs of HuD participate in ARE recognition whereas the third RRM has been suggested to bind the poly(A) tail of a target mRNA (Ma et al., 1997). The hinge region is essential for HuR and HuD cellular localization and function (Fan & Steitz, 1998a; Akamatsu et al., 1999; Kasashima et al., 1999). Indeed, a systematic deletion analysis showed HuR to contain a shuttling domain, HNS (for HuR nucleocytoplasmic shuttling), that comprises 52 amino acids within the hinge region and does not appear to belong to any previously described class of shuttling signal (Fan & Steitz, 1998a).

We recently documented the interaction of HuR with acidic phosphoprotein ligands called pp32, SETα, and SETβ (Brennan et al., 2000), previously suggested to be inhibitors of protein phosphatase 2A (PP2A; Li et al., 1996; Saito et al., 1999). A deletion analysis of HuR demonstrated that both the hinge region and RRM3 are needed for strong interactions with these, as well as a fourth ligand, APRIL (acidic protein rich in leucine; Brennan et al., 2000). Like HuR, pp32 and APRIL are primarily nucleoplasmic, but shuttle between the nucleus and the cytoplasm. However, pp32 and APRIL contain leucine-rich domains (Brennan et al., 2000) homologous to nuclear export signals (NES) known to interact with CRM1 (chromosomal region maintenance protein 1), the nuclear export receptor for the HIV-1 Rev protein (Fornerod et al., 1997). Treatment of HeLa cells with the CRM1-specific inhibitor leptomycin B (LMB; Komiyama et al., 1985) led to a complete inhibition of pp32 and APRIL shuttling, but had no effect on HuR nucleocytoplasmic movement (Brennan et al., 2000). Yet, HuR's in vivo crosslinking to poly A^+ RNA was affected by LMB; it switched from being completely cytoplasmic to being detectable in both the nuclear and the cytoplasmic compartments (Brennan et al., 2000). Moreover, at least one ARE-containing mRNA (c-*fos*) accumulated in the nucleus after LMB treatment (Brennan et al., 2000).

These data suggested that HuR's export to the cytoplasm might occur by at least two different pathways, one being CRM1-dependent involving its protein ligands and the other being CRM1-independent and requiring its endogenous shuttling signal HNS. In vivo UV crosslinking revealed that in cells grown at 37 °C, where HuR appears nuclear, it interacts with poly A^+ RNA only in the cytoplasm (Gallouzi et al., 2000). However, upon heat shock, we observed that HuR becomes partially cytoplasmic, but interacts with poly A^+ RNA predominantly in the nucleus (Gallouzi et al., 2000). These contrasting observations prompted us to study HuR cellular distribution and associations with its ligands, as well as with mRNAs, under conditions of heat shock.

RESULTS

pp32 and APRIL colocalize with HuR in the cytoplasm after heat shock

To determine whether HuR ligands might contribute to the cytoplasmic localization of HuR upon heat shock, we first examined the cellular localization of pp32, APRIL, and SETα/β by indirect immunofluorescence. HeLa cells, before and after incubation for 1 h at 45 °C, were fixed, permeabilized, and doubly stained with affinity-purified polyclonal antibodies directed against the HuR ligands (Brennan et al., 2000) and the 3A2 anti-HuR monoclonal antibody (Gallouzi et al., 2000). Figure 1 shows that whereas HuR, pp32, and APRIL appear exclusively nucleoplasmic in cells grown at 37 °C (panels 1–3, 7–9), upon heat shock they migrate to the cytoplasm and partially colocalize in the cytoplasmic foci previously observed (Gallouzi et al., 2000; panels 4–6 and 10–12). Approximately 12–15% of the total HuR, pp32, and APRIL was detected in the cytoplasm (see Materials and Methods), and pp32 and HuR localize to a larger extent than APRIL and HuR. The fact that pp32 and APRIL are about 10 times more abundant in cells than HuR (Brennan et al., 2000) explains why their cytoplasmic colocalization with HuR is incomplete. In contrast, the RNA-binding proteins hnRNP A1 and La (Smith et al., 1985; Pinol-Roma & Dreyfuss, 1992) remained entirely nuclear (Fig. 1, panels 13–16). Similarly, heat shock induced no change in the cellular localization of HuR's other ligands, SETα and SETβ (data not shown), which are normally cytoplasmic as well as nuclear (Brennan et al., 2000). If cells are given 3 h incubation at 37 °C following heat treatment, HuR, pp32, and APRIL completely recolocalize to the nucleus (data not shown); more than 95% of the cells survive our heat shock conditions (Gallouzi et al., 2000).

To investigate whether the colocalization of pp32 and APRIL with HuR in the same cytoplasmic foci reflects an increase in their interaction upon heat shock, we performed coimmunoprecipitation experiments. HeLa cell total extracts, pretreated with RNase A, were exposed to polyclonal antibodies against the HuR ligands. Indeed, probing the immunoprecipitates with the monoclonal anti-HuR antibody (Fig. 2A) revealed that associations between pp32 and HuR, and APRIL and HuR, increased after heat shock (lanes 5 and 9). Whereas only 4% of HuR is complexed with pp32 or APRIL under normal conditions (Fig. 2A, lanes 3 and 7), approximately 15% of HuR associated with each of these proteins after heat shock. In contrast, the association of HuR with the SET proteins decreased fourfold upon heat treatment (Fig. 2A, lanes 12 and 14; see Materials and Methods).

We previously observed that HuR cosediments with its protein ligands in multiprotein complexes (Brennan et al., 2000). Because heat shock increases the inter-

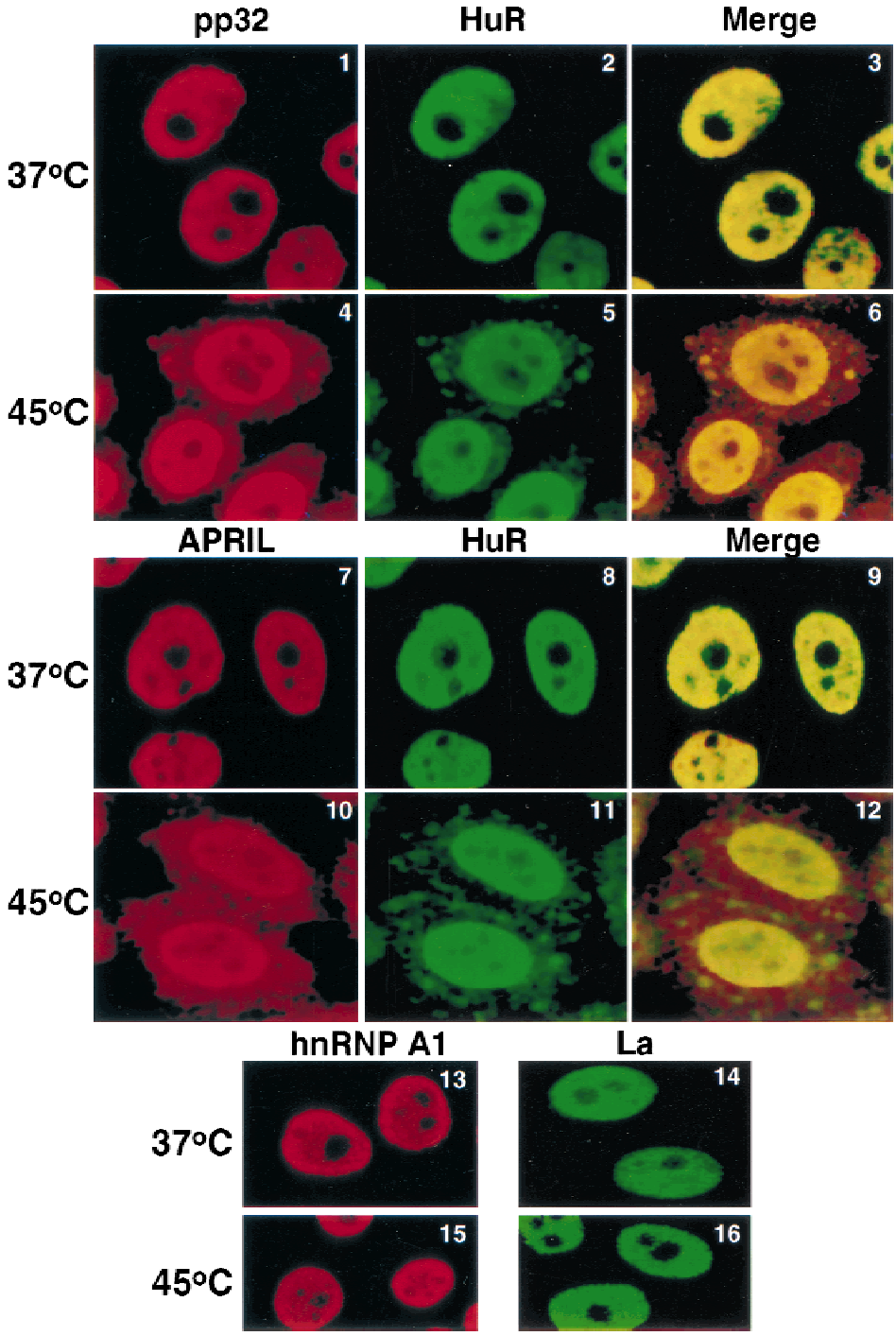


FIGURE 1. Cellular localization of HuR and its ligands upon heat shock. pp32 and APRIL colocalize with HuR in cytoplasmic foci after heat shock. HeLa cells without (panels 1–3, 7–9, 13, 14) or with (panels 4–6, 10–12, 15, 16) incubation for 1 h at 45 °C were fixed, permeabilized, and incubated with affinity-purified antiligand polyclonal antibodies and monoclonal anti-HuR (3A2), or with anti-hnRNP A1 (4B10) or anti-La monoclonal antibodies as indicated (see Materials and Methods). The secondary antibodies were Texas red-conjugated anti-rabbit for anti-pp32 and anti-APRIL, Texas red-conjugated anti-mouse for anti-hnRNP A1, or Alexia 488-conjugated anti-mouse for anti-HuR. All images were captured using a Zeiss confocal microscope.

action of HuR with pp32 and APRIL, we assessed the effect of stress on the sedimentation of these proteins in 5–20% glycerol gradients (Fig. 2B). Extracts were pretreated with RNase A so that only protein–protein interactions would be observed. By immunoprecipitating gradient fractions with antiligand antibodies and probing with anti-HuR, we observed that heat shock shifts pp32- and APRIL-associated HuR into faster sed-

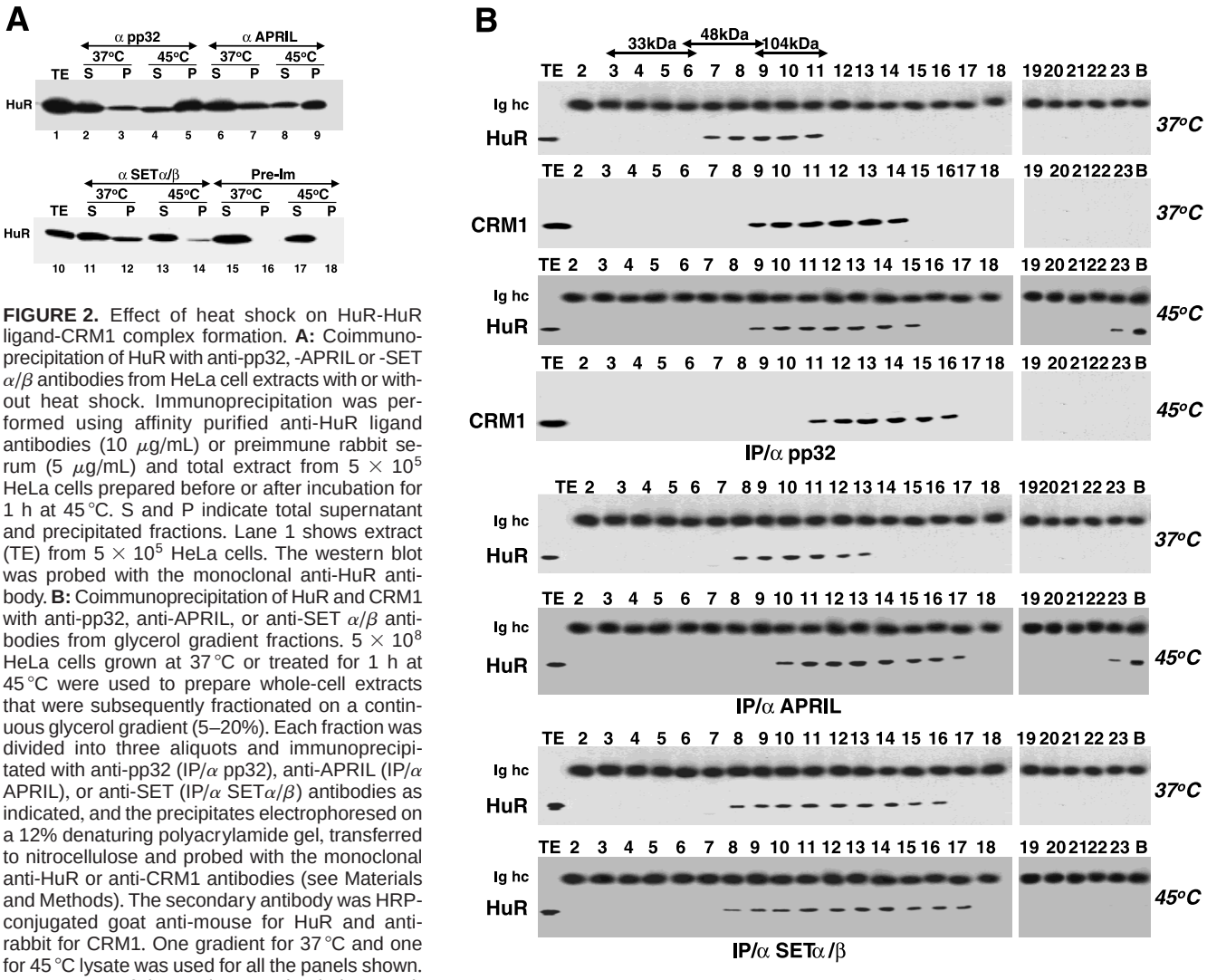


FIGURE 2. Effect of heat shock on HuR-HuR ligand-CRM1 complex formation. **A:** Coimmunoprecipitation of HuR with anti-pp32, -APRIL or -SET α/β antibodies from HeLa cell extracts with or without heat shock. Immunoprecipitation was performed using affinity purified anti-HuR ligand antibodies (10 μg/mL) or preimmune rabbit serum (5 μg/mL) and total extract from 5×10^5 HeLa cells prepared before or after incubation for 1 h at 45 °C. S and P indicate total supernatant and precipitated fractions. Lane 1 shows extract (TE) from 5×10^5 HeLa cells. The western blot was probed with the monoclonal anti-HuR antibody. **B:** Coimmunoprecipitation of HuR and CRM1 with anti-pp32, anti-APRIL, or anti-SET α/β antibodies from glycerol gradient fractions. 5×10^8 HeLa cells grown at 37 °C or treated for 1 h at 45 °C were used to prepare whole-cell extracts that were subsequently fractionated on a continuous glycerol gradient (5–20%). Each fraction was divided into three aliquots and immunoprecipitated with anti-pp32 (IP/α pp32), anti-APRIL (IP/α APRIL), or anti-SET (IP/α SETα/β) antibodies as indicated, and the precipitates electrophoresed on a 12% denaturing polyacrylamide gel, transferred to nitrocellulose and probed with the monoclonal anti-HuR or anti-CRM1 antibodies (see Materials and Methods). The secondary antibody was HRP-conjugated goat anti-mouse for HuR and anti-rabbit for CRM1. One gradient for 37 °C and one for 45 °C lysate was used for all the panels shown. One percent of the total extract loaded on each gradient is shown in the TE lane. The B lane shows material recovered from the bottom of the centrifuge tube after collection of the fractions. As markers, phosphorylase B (104 kD), ovalbumin (48 kD), and carbonic anhydrase (33 kD) were run on a parallel gradient. The band marked Ig hc is the immunoglobulin heavy chain. Only one band of the correct size for CRM1 was observed in the blots with anti-CRM1 antibody.

imenting complexes (Fig. 2B, compare lanes 8–10, panels 37 °C, with lanes 10–14, panels 45 °C), whereas the profile of HuR complexes with SETα/β remained unchanged (Fig. 2B, compare lanes 9–15, panel 37 °C, with lanes 8–15, panel 45 °C). These results suggest that HuR-pp32 and HuR-APRIL complexes recruit new factor(s) at 45 °C.

HuR increases its association with hnRNP complexes upon heat shock

Although the amount of HuR in the cytoplasm increases at least 12-fold after heat shock of HeLa cells, its interaction with cytoplasmic poly A^+ RNA is no longer detectable; instead, HuR crosslinks mostly to nuclear poly A^+ RNA (Gallouzi et al., 2000). Moreover, in contrast to other types of stress, heat shock does not alter hnRNP A1's nuclear localization nor its nuclear interaction with poly A^+ RNA (Gallouzi et al., 2000; van der Houven van Oordt et al., 2000). We therefore asked whether the shift of HuR into larger complexes after heat shock (Fig. 2) might involve hnRNP proteins.

Total-cell extracts prepared from HeLa cells maintained at 37 °C or exposed to 45 °C for 1 h, either untreated or treated with RNase A, were immunoprecipitated with anti-HuR, anti-hnRNP A1 (Pinol-Roma &

Dreyfuss, 1992), anti-hnRNP C (Choi & Dreyfuss, 1984), or anti-Sm (Lerner et al., 1981) monoclonal antibodies. Immunoblots of the precipitates were then probed with anti-HuR or anti-hnRNP A1 antibodies. Whereas less than 1% of total hnRNP A1 precipitated using the anti-HuR antibody (Fig. 3A, lane 1) from the 37 °C extract, the interaction increased fourfold upon heat shock (Fig. 3A, lane 3). The converse immunoprecipitation, using the anti-hnRNP A1 antibody and then probing with anti-HuR, yielded the same result (Fig. 3B). Likewise, about 3% of the HuR coprecipitated with anti-hnRNP C, but only after heat shock (Fig. 3D), whereas interaction between hnRNP C and A1 were seen at 37 °C as well (Fig. 3C). At both temperatures, all these interactions were completely abolished by RNase A treatment (Fig. 3A–D). Anti-Sm antibodies were unable to precipitate HuR from any extract tested, providing a negative control (Fig. 3E).

Together, these results indicate that HuR's association with the hnRNP complex is enhanced upon heat stress, but that this interaction is dependent upon RNA, presumably mRNAs and pre-mRNAs that tether together these nuclear RNA-binding proteins. Thus, these interactions cannot be the additional protein–protein contacts that contribute to the more rapid sedimentation of protein–protein complexes involving HuR and its ligands in gradients (Fig. 2B).

Heat-shock-induced nuclear export of HuR is LMB sensitive

We previously showed that the nucleocytoplasmic shuttling of pp32 and APRIL is inhibited by leptomycin B (LMB), which inhibits the nuclear export of NES-containing proteins by covalently modifying a critical cysteine residue in the nuclear export receptor CRM1

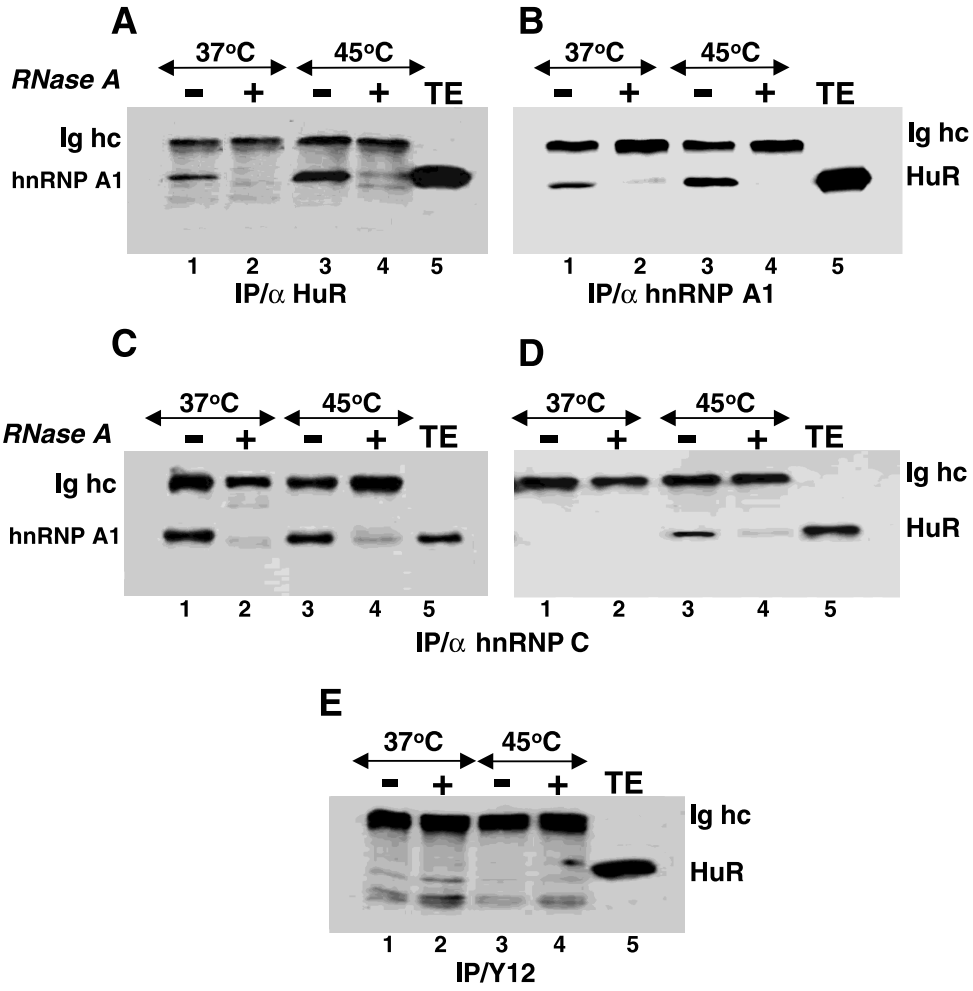
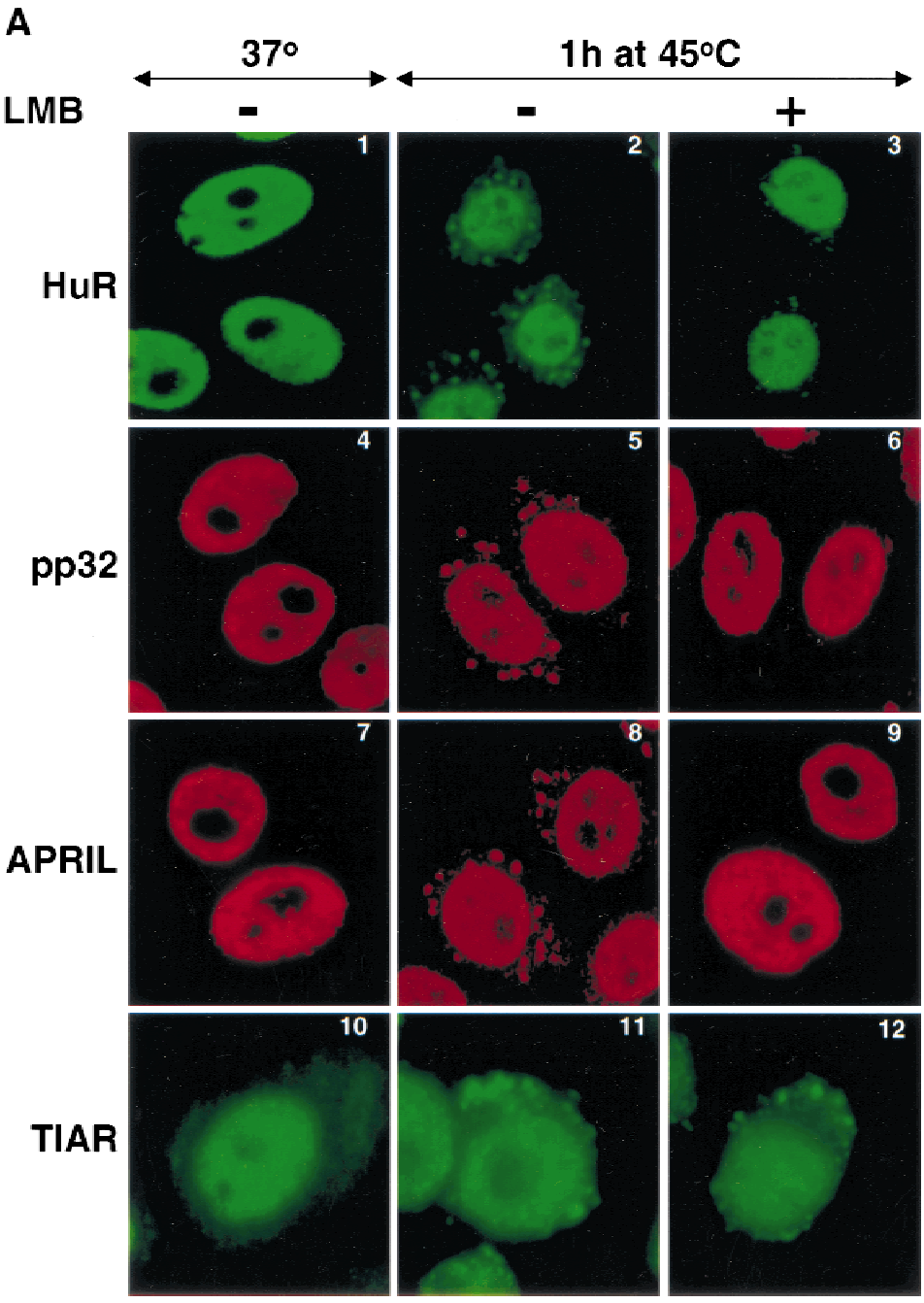


FIGURE 3. HuR's association with the hnRNP complex increases after heat shock. Total HeLa cell extracts were prepared before (lanes 1 and 2) or after (lanes 3 and 4) heat shock, and either pre-treated (+) (lanes 2 and 4) or not (−) (lanes 1 and 3) with 10 μg RNase A per 10^6 cells for 30 min at 37 °C. Immunoprecipitations were carried out on lysate from 5×10^6 cells using anti-HuR (3A2) (**A**), anti-hnRNP A1 (4B10) (**B**), anti-hnRNP C (4F4) (**C** and **D**), or anti-Sm (Y12) (**E**) monoclonal antibody. Western blots of the precipitates used the 3A2 or 4B10 antibody to identify HuR or hnRNP A1, respectively. Lanes 5, marked TE, show 1/10 the amount of extract used in the immunoprecipitations in lanes 1–4. The upper band (Ig hc) is the immunoglobulin heavy chain. Quantitations were performed by comparing lane 1 to lane 3 in each panel. These results were repeated three times.

(Kudo et al., 1999). Our observation that HuR colocalizes with pp32 and APRIL in cytoplasmic foci upon heat shock (Fig. 1) suggested that the stress-induced export of HuR might be CRM1-dependent. We therefore treated HeLa cells with LMB (see Materials and Methods) prior to heat shock and visualized pp32, APRIL, and HuR using their respective antibodies.

Figure 4A shows that LMB significantly reduces the colocalization of HuR with pp32 and APRIL in cytoplasmic foci (panels 3, 6, and 9 compared to 2, 5, and 8). Whereas approximately 12–15% of pp32, APRIL, and HuR were translocated to the cytoplasm after heat shock, LMB reduced this localization to less than 1% for each of these proteins. As a control, we examined the cellular localization of TIAR, an RNA-binding protein known to interact with the AU-rich sequence present in the 3′ UTR of the tumor necrosis factor alpha mRNA (Gueydan et al., 1999). TIAR was previously reported to accumulate in cytoplasmic stress granules upon heat shock (Kedersha et al., 1999). Addition of LMB did not alter this localization (Fig. 4A, compare panels 11 and 12).

HuR does not contain leucine-rich repeats and does not coimmunoprecipitate with CRM1 at 37 °C (Brennan



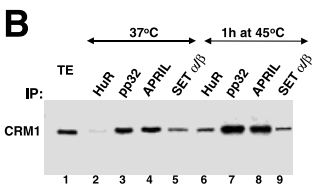


FIGURE 4. Heat shock induces HuR nuclear export through the CRM1 pathway and enhances HuR association with CRM1. **A:** Leptomycin B (LMB) blocks the heat shock induced cytoplasmic localization of HuR, pp32, and APRIL. HeLa cells were grown on coverslips with (+) or without (−) 5 ng/mL LMB for 8 h, including incubation for 1 h at 45 °C as indicated, fixed, and immunofluorescently stained as described in Materials and Methods. Anti-HuR (panels 1–3) or anti-TIAR (panels 10–12) monoclonal antibodies and anti-pp32 (panels 4–6) or anti-APRIL (panels 7–9) affinity-purified polyclonal antibodies were used. Detection was carried out using fluorescein isothiocyanate for HuR and TIAR, or rhodamine-labeled anti-rabbit secondary antibody for pp32 and APRIL. All images were captured using a Zeiss confocal microscope. **B:** Heat shock increases HuR association with CRM1. HeLa whole-cell extract was prepared from cells grown to subconfluency at 37 °C and either treated for 1 h at 45 °C (lanes 6–9) or maintained at 37 °C (lanes 2–5). Anti-pp32 (lanes 3 and 7), anti-APRIL (lanes 4 and 8), or anti-SET α/β (lanes 5 and 9) antiserum or 3A2 anti-HuR antibody was used for immunoprecipitation (IP) of lysate from 5×10^5 cells and the precipitates were probed with polyclonal anti-CRM1 antibody. This experiment was repeated and quantitated three times, with comparable results.

et al., 2000). However, because the interactions between HuR, pp32, and APRIL increase significantly upon heat shock, we asked whether incubation of HeLa cells for 1 h at 45 °C would result in detectable association between CRM1 and HuR. Whole-cell extracts were prepared before and after heat shock, and coimmunoprecipitates obtained using anti-pp32, anti-APRIL, anti-SET α/β, or anti-HuR antibodies were probed with anti-CRM1 polyclonal antibody (Fornerod et al., 1997). Coimmunoprecipitation of HuR and CRM1 was increased about 10-fold following heat treatment (Fig. 4B, lane 6 compared to lane 2), whereas the association of pp32 and APRIL with CRM1 increased 3-fold upon heat shock (Fig. 4B, lanes 7 and 8 compared to lanes 3 and 4). Indeed, when the gradients of Figure 2B were reprobed with anti-CRM1 antibodies, a shift to higher molecular weights was observed for pp32-CRM1 complexes (Fig. 2B, CRM1 panels, lanes 9–14 at 37 °C, versus lanes 11–16 at 45 °C) and for APRIL-CRM1 complexes (data not shown). These results are consistent with an association of CRM1 with HuR through its ligands pp32 and APRIL after heat shock.

HuR domains HNS and RRM3 together are necessary and sufficient for export through the CRM1 pathway

At 37 °C, HuR shuttles between the nucleus and the cytoplasm via a 52 amino acid domain, HNS, located in the linker separating RRM2 and RRM3 (Fan & Steitz, 1998a). Both HNS and RRM3 have been shown to be necessary for HuR interaction with pp32 and APRIL (Brennan et al., 2000). To define the domain(s) of HuR needed for localization in cytoplasmic foci after heat shock, we fused portions of HuR to C-terminal myc-tagged nucleoplasmin core (NPc), a well-studied carrier that does not contain the NPc NLS domain (Fan & Steitz, 1998a; Michael et al., 1995). These constructs (Fig. 5A) were transiently transfected to HeLa cells and after 24 h, the cells were fixed for immunofluorescence either before or after incubation for 1 h at 45 °C. Probing with anti-myc monoclonal antibody revealed that only the chimera containing both the HNS and RRM3 domains of HuR accumulated significantly in cytoplasmic foci upon heat shock (Fig. 5B, panel 8). This localization was completely inhibited by treatment with LMB (including the period of heat shock; Fig. 5B, panel 9). Only a weak cytoplasmic signal and many fewer foci were detected with the MYC-NPc-RRM3 construct (Fig. 5B, panel 5) and none with the MYC-NPc-HNS chimera upon heat stress (Fig. 5B, panel 2). The negative control, NPc-NLS, containing the classical NLS (nuclear localization signal) of SV40 T antigen, remained confined to the HeLa nucleus under all conditions (Fig. 5B, panels 10, 11, and 12). These data demonstrate that the HNS and the RRM3 domains of HuR together are necessary and sufficient for efficient protein localization in cytoplasmic foci under stress. Because these same two domains of HuR have been delineated as necessary for efficient interaction with pp32 and APRIL (Brennan et al., 2000), we conclude that these ligands most likely confer CRM1-dependent export on HuR after heat shock.

A role for HuR in nuclear export of mRNA after heat shock?

The hsp70 mRNA contains a class III ARE sequence in its 3′ UTR, homologous to that of the beta-adrenergic receptor (β-ADR) mRNA (Hunt & Morimoto, 1985; Wu et al., 1985; Blaxall et al., 2000), that was recently shown to interact with HuR (Blaxall et al., 2000). Although in vivo crosslinking suggested that HuR interaction with poly A^+ RNA shifted predominantly to the nucleus after heat shock, 30% of cytoplasmic HuR cosedimented with polysomes (Gallouzi et al., 2000). Thus, it seemed conceivable that heat shock transcripts representing a small percentage of cellular poly A^+ RNA might associate with HuR in the cytoplasm. We therefore asked whether hsp70 mRNA export (and stabilization) upon heat shock of HeLa cells might involve HuR.

We first examined whether hsp70 mRNA export, like that of HuR, occurs via the CRM1 pathway after heat shock. In situ hybridization experiments using an antisense probe corresponding to the 5′ end of hsp70 mRNA (see Material and Methods; Fig. 6A) were performed on HeLa cells either grown at 37 °C or treated for 1 h at 45 °C, in the absence and the presence of LMB. As expected, at 37 °C, the hsp70 mRNA signal was very low, in both the nucleus and the cytoplasm, with or without LMB treatment (Fig. 6A, panels 1 and 2). Heat shock increased hsp70 mRNA expression about 15-fold (see Materials and Methods), producing signal in both the nuclear and cytoplasmic compartments (Fig. 6A, panel 3). Significantly, LMB treatment resulted in nuclear sequestration of newly synthesized hsp70 mRNA (Fig. 6A, panel 4). The same cells probed with the anti-HuR antibody showed the expected heat-induced localization of HuR in cytoplasmic foci, but only in the absence of LMB (Fig. 6A, compare panels 5 and 6 to panels 7 and 8). LMB had no effect on GAPDH mRNA cellular distribution at either temperature (data not shown). These observations suggest that export of hsp70 mRNA, like HuR, occurs via the CRM1 pathway upon heat shock.

We then examined the interaction of HuR with hsp70 mRNA by immunoprecipitation followed by northern blot analysis. Nuclear and cytoplasmic fractions were prepared from HeLa cells grown in the absence or presence of LMB at 37°C or treated for 1 h at 45 °C, and immunoprecipitated RNAs (Fig. 6C) were compared with total RNA (Fig. 6B). At 37 °C, the hsp70 mRNA was undetectable in either cellular compartment in the presence or absence of LMB (Fig. 6B, hsp70 mRNA panel,

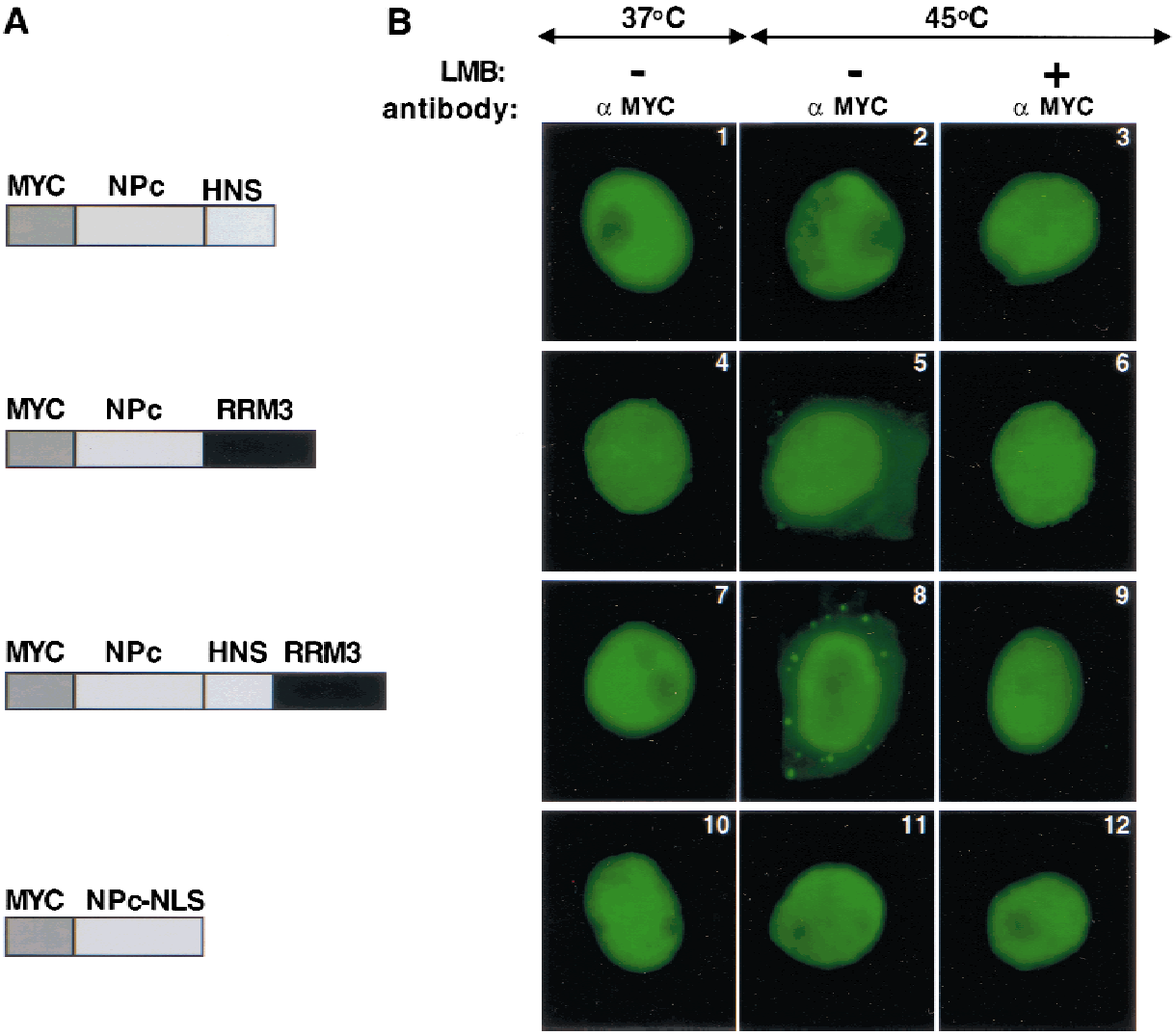


FIGURE 5. Delineation of HuR domains involved in cytoplasmic localization upon heat shock. **A:** Schematic diagram of the nucleoplasmin (NPc) fusion proteins containing HuR fragments HNS (amino acids 192–244), RRM3 (amino acids 245–326) or the SV 40 NLS (Fan & Steitz, 1998a). **B:** HeLa cells were transiently transfected with the myc-tagged nucleoplasmin expression vectors diagramed in **A**. Twenty-four hours later, cells maintained at 37 °C were either treated or not with 5 ng/mL LMB for 7 h and then heat shocked (as indicated) for 1 h at 45 °C in the presence or absence of LMB. After fixing with 3% paraformaldehyde for 20 min and permeabilization with 0.5% Triton X-100, the cells were stained with anti-myc antibody 9E10. The NPc-NLS vector provided a negative control.

lanes 1–4). Heat shock increased its level 12-fold (Fig. 6B, lanes 5 and 6). LMB treatment did not block hsp70 mRNA expression, but the RNA became restricted to the nuclear fraction (Fig. 6B, lane 8). In contrast, LMB affected neither the expression nor cellular distribution of GAPDH mRNA before and after heat shock (Fig. 6B, GAPDH mRNA panel, lanes 1–8). GAPDH mRNA was primarily cytoplasmic in normal cells (Fig. 6B, lanes 1–4) and accumulated in the nucleus in heat shocked cells (Fig. 6B, lanes 5–8), as reported previously for total poly A^+ RNA (Gallouzi et al., 2000). These results suggest that, in HeLa cells, hsp70 mRNA is selectively ushered to the cytoplasm via the CRM1 pathway.

After heat shock, anti-HuR antibody precipitated about 10% of hsp70 mRNA from both the nuclear and cytoplasmic compartments (Fig. 6C, lanes 5 and 6), whereas in the presence of LMB, hsp70 mRNA was coimmunoprecipitated only from the nuclear fraction (Fig. 6C, lanes 7 and 8). Although we previously observed that TIAR is relocated to the same cytoplasmic foci as HuR upon heat shock (Gallouzi et al., 2000), hsp70 mRNA was not immunoprecipitated by the anti-TIAR antibody (data not shown). Because HuR specifically interacts with the hsp70 mRNA upon heat shock in both the nuclear and the cytoplasmic compartments, and this association is inhibited by the same concentrations of LMB that block HuR export after stress, we conclude

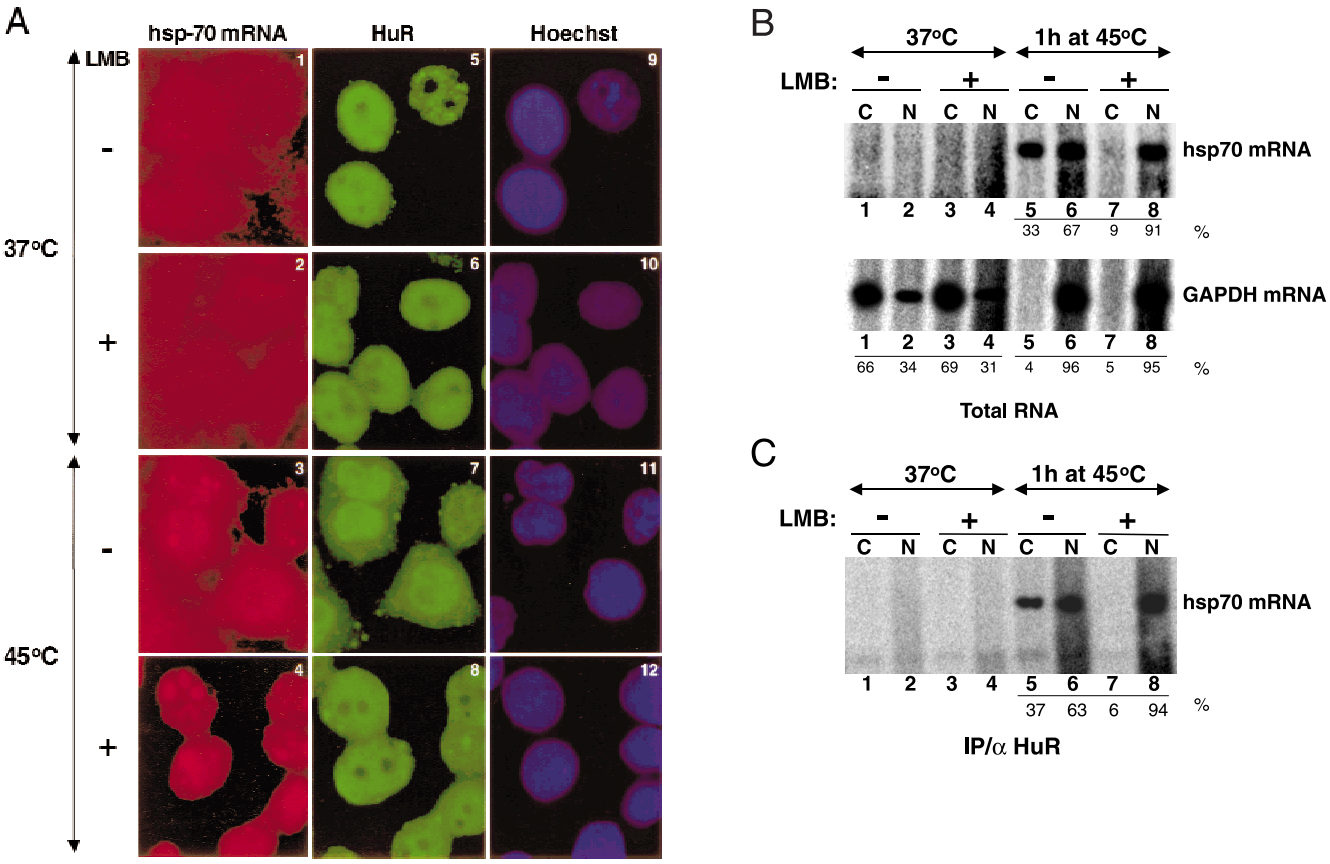


FIGURE 6. Inhibition of the CRM1 pathway abolishes hsp70 mRNA nuclear export and its cytoplasmic association with HuR upon heat shock. **A:** hsp70 mRNA and HuR cellular distribution with and without heat shock. HeLa cells were grown on coverslips, with (+) or without (−) 5 ng/mL LMB for 7 h prior to heat shock, and then incubated for 1 h at 45 °C (as indicated) in the presence or absence of LMB. The same fields of cells are shown for in situ hybridization (panels 1–4), immunofluorescence (panels 5–8), and Hoechst nuclear staining (panels 9–12). For in situ hybridization, a 3′ digoxigenin-labeled antisense oligonucleotide for hsp70 mRNA (Calbiochem) was used at 5 ng/μL with a 1:200 dilution of sheep antidigoxigenin Fab-rhodamine antibody. The monoclonal anti-HuR antibody (3A2) was detected using fluorescein isothiocyanate secondary anti-mouse antibody. In controls, treatment of the fixed cells with RNase before hybridization with digoxigenin-labeled probes or without the labeled probes yielded no signal (data not shown). The concentrated regions of hsp70 mRNA in the nuclei in panels 3 and 4 (45 °C) do not correspond to nucleoli; panels 7 and 8 show that HuR is present in those regions in the same cells. **B:** LMB produces hsp70 mRNA sequestration in the nuclear compartment. 10% of HeLa cytoplasmic or nuclear RNA from cells treated with LMB and heat shocked as described in the legend to Figure 6A were subjected to northern blot analysis. RNA was detected with ^{32}P-labeled cDNA probes encoding the hsp70 protein (a gift of N. Kedersha) and GAPDH protein (ATCC), as indicated. Band intensities were quantitated by PhosphorImager (Molecular Dynamics), with the percentages in each compartment given relative to the sum of the cytoplasmic (C) and nuclear (N) hsp70 mRNA or GAPDH mRNA band intensity. **C:** LMB treatment leads to hsp70 mRNA association with HuR only in the nuclear fraction. 90% of the cytoplasmic and nuclear extracts from 6B were incubated for 2 h at 4 °C with anti-HuR monoclonal antibody. Northern blotting and quantification of the mRNAs in the precipitates were carried out as described above for **B**.

that HuR could be involved in the export of hsp70 mRNA (and other stress-induced mRNAs) from the nucleus to the translational machinery in the cytoplasm.

DISCUSSION

Altered interactions of HuR with pp32 and APRIL and with the hnRNP complex after heat shock

The colocalization of HuR, pp32, and APRIL in cytoplasmic foci (Fig. 1) and their increased association (Fig. 2A) are consistent with the idea that pp32 and APRIL participate in HuR movement in heat shocked cells. The sedimentation shift upon heat stress (Fig. 2B) further suggests that HuR-pp32 and HuR-APRIL complexes engage additional proteins. Because the increased association of HuR with the hnRNP complex after heat shock is eliminated by RNase treatment, hnRNP proteins are unlikely to be these new partner(s). On the other hand, association of HuR with CRM1 via pp32 and APRIL (Fig. 4B) could explain the shifts seen in the gradients (Fig. 2B). Indeed, probing the pp32, APRIL, and SETα/β blots presented in Fig-

ure 2B with anti-CRM1 antibody revealed that CRM1 is present in the same fractions as HuR-pp32 and HuR-APRIL complexes after heat shock (Fig. 2B, CRM1 panels, and data not shown). Whereas the HuR/pp32 and HuR/APRIL complexes peak at about 80 kDa at 37 °C, they shift to about 170 kDa after heat stress, consistent with the addition of the 110-kDa CRM1 protein (Fukuda et al., 1997).

Heat shock also alters the interaction of HuR with the hnRNP complex (Fig. 3). The abundant hnRNP proteins interact with mRNA transcripts early during their biogenesis to regulate processing and nuclear export (Pinol-Roma & Dreyfuss, 1992; Nakielny & Dreyfuss, 1996, 1999). Dynamic remodeling of these complexes is evident from the observation that some hnRNP proteins, like hnRNP A1, shuttle between the nucleus and the cytoplasm, whereas others, like hnRNP C, are exclusively nuclear. Since in vivo crosslinking experiments showed that under normal conditions hnRNP A1 and HuR do not interact with poly A^+ mRNA in the same compartment (A1 in the nucleus, HuR in the cytoplasm), we previously hypothesized that HuR's interaction with its target mRNAs occurs at a late stage just before an RNA exits to the cytoplasm (Gallouzi et al., 2000). However, unlike hnRNP A1, HuR's interaction with poly A^+ RNA is significantly altered by heat shock: HuR crosslinks only to nuclear RNA after heat shock (Gallouzi et al., 2000). Because nuclear RNA molecules are assembled into hnRNP, it is not surprising that HuR's association with hnRNP A1 and C increases several-fold (dependent on RNA integrity; Fig. 3) after heat shock. This observation also suggests that the stabilization of some non-heat-shock messages under hyperthermia (Andrews et al., 1987) could be the consequence of their nuclear retention with HuR.

The CRM1 pathway becomes the major route for HuR nuclear export upon heat shock

Here we show that both the HNS and RRM3 domains of HuR [which are needed for its interaction with pp32 and APRIL (Brennan et al., 2000)] are necessary and sufficient to bring NPc to cytoplasmic foci upon stress (Fig. 5). LMB inhibits this activity, indicating CRM1 involvement and the participation of pp32 and APRIL in HuR export under these conditions. Although the effect of LMB could be indirect, perhaps because CRM1-mediated export is essential for maintaining the Ran-GTP gradient (Mattaj & Englmeier, 1998), we know that at least at 37 °C this gradients remains intact in mammalian cells; we previously observed that hnRNP A1 shuttling, which requires transportin 1 and Ran-GTP (Siomi et al., 1997), continues after 8 h of LMB treatment (Brennan et al., 2000). The fact that the HNS alone is unable to bring NPc to the cytoplasm after heat shock argues that HNS-dependent nucleocytoplasmic movement of HuR (Fan & Steitz, 1998a) is shut off. It may be that ligand binding simply competes for recognition of whatever export receptor normally couples with HNS. The observation that LMB treatment alone sequesters c-*fos* mRNA in the nucleus (Brennan et al., 2000) suggests that the ligand-dependent export pathway may operate to a limited extent even at 37 °C. The nature of the change allowing increased association of HuR with its protein ligands is unknown; we have not observed noticeable changes in the phosphorylation state of pp32 or APRIL upon heat shock (data not shown).

Recent evidence has shown that the CRM1 pathway for protein export remains active in heat shocked cells. Net, one of several transcription ternary complex factors (TCFs) and a transcriptional repressor, is mainly nuclear, but contains a leucine-rich NES as well as several NLS. After treatment with anisomycin, UV, or heat shock, Net accumulated in the cytoplasm, and this nuclear exclusion was inhibited by LMB (Ducret et al., 1999). In yeast, overexpressing the HIV-1 Rev protein, but not a Rev mutant, partially blocked the nuclear export of *SSA4* (the yeast homolog of mammalian hsp70) mRNA after heat shock (Saavedra et al., 1997). Thus, it is reasonable to propose that HuR may be exported by the CRM1 pathway in heat shocked cells.

Is HuR an export adapter protein for heat shock mRNAs?

The export of messenger RNA from the nucleus to the cytoplasm involves multiple pathways (Nakielny & Dreyfuss, 1999). CRM1 was first identified as an export receptor for the unspliced RNA of HIV-1 through its interaction with the viral-encoded adapter protein Rev (Neville et al., 1997). CRM1 is also involved in the export of the spliceosomal U snRNAs (Fornerod et al., 1997), where a phosphorylated isoform of PHAX (phosphorylated adapter for RNA export) serves as the specific adapter (Ohno et al., 2000). Recently, it was shown that the nuclear export of the 60S ribosomal subunit is mediated by yet another adapter protein, Nmd3p, in a CRM1-dependent fashion (Ho et al., 2000). In each of these cases, CRM1 recognizes the leucine-rich NES of the adapter protein, thereby escorting the bound RNA out of the nucleus through its interaction with at least one nucleoporin (NPC), CAN/Nup214 (Fornerod et al., 1997; Mattaj & Englmeier, 1998; Gorlich & Kutay, 1999; Ho et al., 2000). CRM1 itself is not known to interact directly with RNA.

The data presented in Figure 6A show that the same LMB concentration that prevents HuR appearance in cytoplasmic foci blocks the nuclear export of hsp70 mRNA in heat shocked cells. Moreover, use of a leucine-rich NES-containing peptide inhibitor after heat shock leads to complete nuclear sequestration of both hsp70

mRNA and HuR (data not shown). These observations suggest that HuR could be a part of a two-protein adapter complex for mRNA export during stress by associating with CRM1 through its ligands. pp32 and APRIL themselves have not been observed to interact directly with RNA (Brennan et al., 2000). The fact that the hsp70 mRNA in both the nucleus and cytoplasm after heat shock can be shown to be associated with HuR by coimmunoprecipitation (Fig. 6C) further supports an adapter role for HuR.

The presence of a class III type ARE in the 3′ UTR of hsp70 mRNA, in combination with the coimmunoprecipitation data in Figure 6C, indicates a direct interaction between hsp70 mRNA and HuR. This could also be true of the mRNAs for many other heat shock proteins. Of 18 stress-induced mammalian mRNAs surveyed, 15 have either a class I or class III ARE in their 3′ UTRs (data not shown). In our previous work, we were not able to detect an interaction between HuR and cytoplasmic poly A^+ RNA under stress conditions, even though some HuR could still be identified on polysomes (Gallouzi et al., 2000). This lack of crosslinking may simply be explained by the fact that the cytoplasmic abundance of heat shock mRNAs is low compared to the total mRNAs that exist in the cytoplasm of cells grown at 37 °C.

TIAR is an RNA-binding protein that, like HuR, contains three RRM domains and is mainly nuclear in cells grown at 37 °C (Tian et al., 1991; Kedersha et al., 1999). Upon heat shock, TIAR localizes in stress granules and acts as a general translation inhibitor (Kedersha et al., 1999; Piecyk et al., 2000), perhaps by sequestering mRNAs in stress granules away from the translation machinery (Kedersha et al., 1999, 2000). Our observations that TIAR export is LMB-independent (Fig. 4) and that it does not coimmunoprecipitate hsp70 mRNA (data not shown) argue that TIAR does not play a role in the nuclear export of heat shock mRNAs.

Two models for selective export of heat shock mRNAs after stress have been proposed for the yeast *Saccaromyces cerevisiae*. In yeast as in mammalian cells, heat shock leads to nuclear sequestration of the vast majority of poly A^+ RNA (Liu et al., 1996; Saavedra et al., 1996; Gallouzi et al., 2000); in contrast, heat shock mRNAs (e.g., *SSA4*, encoding yeast hsp70) transit to the cytoplasm for translation. The Cole laboratory has argued that Rip1p, a two hybrid interactor with HIV-1 Rev that is believed to associate with the NPC (Stutz et al., 1995), defines a pathway essential for export of *SSA4* and other heat shock mRNAs, even though Rip1p is dispensable under normal conditions (Saavedra et al., 1997). In contrast, the Rosbash laboratory has concluded that Rip1p participates in the export of both heat shock and non-heat-shock messages at high temperature and that CRM1 is not a major export receptor for heat shock mRNAs (Vainberg et al., 2000; Jensen et al., 2001). They suggest instead that competition explains the preferential export of heat shock mRNAs.

Our data for mammalian cells clearly support the idea of an alternative pathway and an active role for CRM1 in the export of heat shock mRNAs (Fig. 7). We propose that after heat shock, newly made hsp70 mRNA is escorted by hnRNP proteins from its site of transcription to the perinuclear area where it meets HuR (Gallouzi et al., 2000). HuR then binds, using RRM1 and -2 to recognize the class III ARE in the 3′ UTR of hsp70 mRNA, and brings in either pp32 or APRIL, which interacts with HuR's hinge region and RRM3 (Brennan et al., 2000). Addition of CRM1 to these assemblies through its ability to interact with the NES of the HuR ligands leads to the rapid export and subsequent trans-

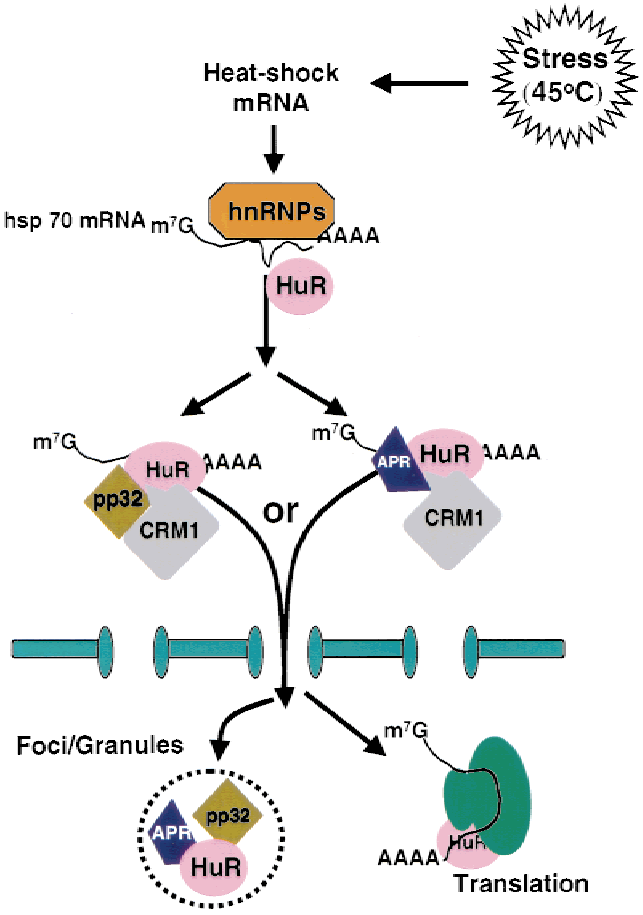


FIGURE 7. Model for HuR nucleocytoplasmic shuttling through the CRM1 pathway and its involvement in heat shock mRNA export. Heat shock induces hsp70 mRNA expression and the increased association of HuR with nuclear hnRNP complexes (Fig. 3), as well as increased localization of HuR and its ligands in the cytoplasm (Fig. 1). Under these stress conditions, HuR becomes associated with the CRM1 receptor through either pp32 or APRIL (APR; Fig. 4). Thus, the HuR-bound hsp70 mRNA (Fig. 6) is exported to the cytoplasm using the CRM1 pathway. Cytoplasmic HuR is localized in both polysomes and foci upon heat shock (Gallouzi et al., 2000), whereas HuR ligands are detected in foci (Fig. 1), but not in polysomes (data not shown). Hsp70 mRNA is not present in cytoplasmic foci (Fig. 6) and is highly translated.

lation of hsp70 mRNA. Thus, HuR could be the export adapter for hsp70 and perhaps other heat shock mRNAs.

pp32 and APRIL may establish a link between the cellular stress response, HuR, and protein phosphatase 2A

The half-lives of ARE-containing mRNAs can vary under conditions of stress, cell stimulation, and oncogenic transformation (Andrews et al., 1987; Lee et al., 1988; Hirsch et al., 1995). For example, levels of the cyclin kinase inhibitor, $p21^{waf1}$, are enhanced by UV treatment, involving stabilization of its mRNA by HuR (Gorospe et al., 1998; Wang et al., 2000). Similarly, the VEGF mRNA is stabilized in hypoxic cells, and in vitro experiments have implicated HuR in this stress response (Levy et al., 1998). Additionally, heat shock leads to c-*fos* mRNA stabilization in HeLa cells (Andrews et al., 1987).

It is perhaps not surprising that the HuR ligands pp32 and APRIL play a more prominent role in modulating HuR function in stressed than in normal cells. pp32 has been identified as a protein phosphatase 2A (PP2A) inhibitor (Saito et al., 1999); it is overexpressed in neoplastic cells and has been reported to inhibit oncogene-mediated transformation of rat embryo fibroblasts (Chen et al., 1996). APRIL is 71% identical to pp32 (Brennan et al., 2000) and is likely to have a similar function. PP2A is composed of a core heterodimer containing a protein called PR65 (A subunit) and a catalytic subunit (Janssens & Goris, 2001). Recently, it was reported that the heat shock transcription factor HSF2 interacts with PR65 to block its association with the catalytic subunit, thereby suggesting dual functions for HSF2: one regulating heat shock protein expression and the other regulating PP2A activity (Mencinger et al., 1998; Hong & Sarge, 1999). Thus, it is possible that pp32 and APRIL regulate PP2A activity as well as HuR cellular movement during heat shock. It will be of interest to investigate the link between these two activities.

MATERIALS AND METHODS

Plasmid construction

N-terminal myc-tagged NPc-HuR hinge region (MYC-NPc-HNS) and NPc-NLS (MYC-NPc-NLS) constructs were previously described (Michael et al., 1995; Fan & Steitz, 1998a). The NPc construct expresses the nucleoplasmin core, which does not contain an NLS domain (Michael et al., 1995; Fan & Steitz, 1998a). The MYC-NPc-HNS-RRM3 and the MYC-NPc-RRM3 chimeras were generated by PCR from the previously described expression construct pcDNA3-HuR (Fan & Steitz, 1998b) and cloned into the *Eco*RI and *Xho*I sites in the pcDNA3-NPc vector (Fan & Steitz, 1998b).

Cell culture, immunofluorescence, and in situ hybridization

Adherent HeLa cells were grown in DMEM (Gibco-BRL) supplemented with 10% fetal bovine serum (FBS, Gibco-BRL), penicillin, and streptomycin, following the manufacturer's directions (Gibco-BRL). HEPES (pH 7.9, 10 mM) was added before heat shock to maintain the pH. The cells were treated with 5 ng/mL LMB (a kind gift of Dr. M. Yoshida, University of Tokyo) for 7 h, including the 1 h incubation at 45 °C for heat shock experiments. Cells were heat shocked as described (Gallouzi et al., 2000) and either fixed for immunofluorescence or used to prepare extracts.

For immunofluorescence, HeLa cells were grown on coverslips overnight, treated as above, and processed as described (Fan & Steitz, 1998b) using the following antibody dilutions: anti-HuR monoclonal (3A2, 1:500; Gallouzi et al., 2000); anti-hnRNP A1 monoclonal (4B10, 1:1,000; kindly provided by S. Pinol-Roma, Mount Sinai School of Medicine, New York; Pinol-Roma and Dreyfuss, 1992); anti-La monoclonal, 1:1,000 (Smith et al., 1985); anti-TIAR monoclonal, 1:500 (kindly provided by N. Kedersha, Harvard Medical School, Boston, Massachusetts; Kedersha et al., 1999); and anti-pp32, anti-APRIL, and anti-SET rabbit polyclonal (5 μg/mL; Brennan et al., 2000). Secondary antibodies were used at 6–8 μg/mL: Alexa 488-conjugated anti-mouse (Molecular Probes) and Texas red-conjugated anti-rabbit (Molecular Probes). Hoechst dye 33258 (Sigma-Aldrich) was incubated with the secondary antibodies at 1 μg/mL.

In situ hybridization was performed as described (Gallouzi et al., 2000) with the following modifications. The cells were treated with 5 ng/mL LMB for 8 h including the 1-h heat treatment, then fixed and permeabilized. They were incubated with 5 ng/μL of message-specific oligonucleotide probe (corresponding to nucleotides 904–944 and 46–85 of the cDNAs for hsp70 and GAPDH, respectively; Calbiochem), 3′-end labeled with digoxigenin according to the protocol supplied by the manufacturer (Boehringer Mannheim) and detected with rhodamine-conjugated anti-digoxigenin antibody (Forrester et al., 1992).

Transient transfection

HeLa cells were grown as described above on coverslips and transiently transfected using Lipofectamin reagent (Gibco-BRL) according to the manufacturer s directions. For all experiments, 2 μg of cDNA were transfected into 10^6 cells and 24 h later, the cells were either incubated for 1 h at 45 °C or not in the presence or absence of 5 ng/mL of LMB (for 8 h, including 1 h of heat shock treatment), then used for immunofluorescence. The anti-MYC monoclonal antibody (Sigma-Aldrich) was used at 1:1,000 dilution. All quantitative analyses were performed using the National Institutes of Health (NIH) Image 1.62 program.

Cell lysate preparation, glycerol gradients, coimmunoprecipitation and immunoblotting

Lysates from HeLa cells grown at 37 °C or treated for 1 h at 45 °C were prepared and fractionated on 5–20% glycerol

gradients followed by immunoprecipitation as described (Brennan et al., 2000). Alternatively, lysates from 5×10^5 cells were incubated directly with the following antibodies preadsorbed to protein A-agarose (Pharmacia Fine Chemicals) for 12 h at 4 °C: 3A2 monoclonal anti-HuR (50 ng), 4B10 monoclonal anti-hnRNP A1 (50 ng), 4F4 anti-hnRNP C (50 ng; Choi & Dreyfuss, 1984), or affinity purified anti-HuR ligand antibodies (100 ng). After extensive washing, bound proteins were eluted by boiling in SDS-PAGE sample buffer (Brennan et al., 2000), analyzed by 12% SDS-PAGE, and transferred to nitrocellulose. The membrane was blocked with 10% nonfat milk in PBS and probed with 3A2 or 4B10 mouse monoclonal antibody as described (Gallouzi et al., 2000) or with a 1/1,000 dilution of rabbit anti-CRM1 (chromosomal region maintenance protein 1; kindly provided by G. Grosveld, St. Jude Children's Research Hospital, Memphis, Tennessee; Fornerod et al., 1997). The secondary antibody (1/5,000 dilution) was either HRP-conjugated donkey anti-rabbit or HRP-conjugated donkey anti-mouse. Blots were developed using ECL (Amersham Pharmacia Biotech) according to the manufacturer's protocol.

RNA extraction, northern blotting, hybridization, and probe preparation

Nuclear (N) and cytoplasmic (C) fractions from adherent HeLa cells were prepared as follows: 5×10^7 cells were washed with cold PBS, harvested in lysis buffer A (10 mM Tris, pH 8.5, 10 mM NaCl, 3 mM $MgCl_2$, 0.5% Nonidet P-40, 1 mM phenylmethyl sulfonyl fluoride [PMSF], 1 μM aprotinin and 1μM leupeptin) containing 20 mM of Vanadyl Ribonucleoside Complex (VRC; Gibco-BRL) and incubated for 5 min at 4 °C. After centrifugation at 15,000 rpm for 90 s, the supernatant was removed as the C fraction. The pellet was homogenized by five passages through a 25-gauge needle then incubated with 10 U/μL of DNase I (RQ 1-RNase Free; Promega) for 15 min at 37 °C to yield the N fraction. These fractions were either incubated for 3 h at 4 °C with 50 ng monoclonal anti-HuR or anti-TIAR antibody preadsorbed to protein A-agarose or used to prepare C and N total RNA. The immunoprecipitates from the N and C fractions were then incubated with 5 μg/mL of proteinase K (AMRESCO) for 1 h at 37 °C, phenol-chloroform extracted and ethanol precipitated. Northern blots were performed as described (Fujita et al., 1999). The hsp70 and GAPDH probes used for the hybridization were prepared by digesting plasmids containing hsp70 (kindly provided by N. Kedersha, Harvard Medical School, Boston, Massachusetts) or GAPDH (ATCC) cDNAs with *Eco*RI-*Bam*HI or *Eco*RI-*Not*I restriction enzymes, respectively. cDNA probes were radioactively labeled by random priming following the manufacturer's directions (Boehringer) to a specific activity of 3,000 Ci/mmol.

ACKNOWLEDGMENTS

We are most grateful to Drs. C.N. Cole, L.B. Weinstein, and E. Lund for comments on the manuscript and many helpful suggestions. We thank Dr. S. Pinol Roma and Dr. M.S. Swanson for providing the anti-hnRNP A1 antibody (4B10) and the anti-hnRNP C (4F4), respectively, and Dr. M. Yoshida for the LMB. This work was supported by Grant CA16038 from the National Institutes of Health. J. Steitz is an investigator of the Howard Hughes Medical Institute.

Received June 5, 2001; manuscript accepted without revision June 15, 2001

REFERENCES

Akamatsu W, Okano HJ, Osumi N, Inoue T, Nakamura S, Sakakibara S, Miura M, Matsuo N, Darnell RB, Okano H. 1999. Mammalian ELAV-like neuronal RNA-binding proteins HuB and HuC promote neuronal development in both the central and the peripheral nervous systems. *Proc Natl Acad Sci USA 96*:9885–9890.

Andrews GK, Harding MA, Calvet JP, Adamson ED. 1987. The heat shock response in HeLa cells is accompanied by elevated expression of the c-fos proto-oncogene. *Mol Cell Biol 7*:3452–3458.

Antic D, Keene JD. 1997. Embryonic lethal abnormal visual RNA-binding proteins involved in growth, differentiation, and posttranscriptional gene expression. *Am J Hum Genet 61*:273–278.

Banerji SS, Berg L, Morimoto RI. 1986. Transcription and post-transcriptional regulation of avian HSP70 gene expression. *J Biol Chem 261*:15740–15745.

Blaxall BC, Pellett AC, Wu SC, Pende A, Port JD. 2000. Purification and characterization of beta-adrenergic receptor mRNA-binding proteins. *J Biol Chem 275*:4290–4297.

Brennan CM, Gallouzi IE, Steitz JA. 2000. Protein ligands to HuR modulate its interaction with target mRNAs In vivo. *J Cell Biol 151*:1–14.

Brennan CM, Steitz JA. 2001. HuR and mRNA stability. *Cell Mol Life Sci 58*:266–277.

Campos AR, Grossman D, White K. 1985. Mutant alleles at the locus elav in *Drosophila melanogaster* lead to nervous system defects. A developmental-genetic analysis. *J Neurogenet 2*:197–218.

Chen CY, Shyu AB. 1995. AU-rich elements: Characterization and importance in mRNA degradation. *Trends Biochem Sci 20*:465–470.

Chen TH, Brody JR, Romantsev FE, Yu JG, Kayler AE, Voneiff E, Kuhajda FP, Pasternack GR. 1996. Structure of pp32, an acidic nuclear protein which inhibits oncogene-induced formation of transformed foci. *Mol Biol Cell 7*:2045–2056.

Choi YD, Dreyfuss G. 1984. Monoclonal antibody characterization of the C proteins of heterogeneous nuclear ribonucleoprotein complexes in vertebrate cells. *J Cell Biol 99*:1997–1204.

DeMaria CT, Brewer G. 1996. AUF1 binding affinity to A+U-rich elements correlates with rapid mRNA degradation. *J Biol Chem 271*:12179–12184.

Ducret C, Maira SM, Dierich A, Wasylyk B. 1999. The net repressor is regulated by nuclear export in response to anisomycin, UV, and heat shock. *Mol Cell Biol 19*:7076–7087.

Fan XC, Steitz JA. 1998a. HNS, a nuclear-cytoplasmic shuttling sequence in HuR. *Proc Natl Acad Sci USA 95*:15293–15298.

Fan XC, Steitz JA. 1998b. Overexpression of HuR, a nuclear-cytoplasmic shuttling protein, increases the in vivo stability of ARE-containing mRNAs. *EMBO J 17*:3448–3460.

Fornerod M, Ohno M, Yoshida M, Mattaj IW. 1997. CRM1 is an export receptor for leucine-rich nuclear export signals. *Cell 90*:1051–1060.

Forrester W, Stutz F, Rosbash M, Wickens M. 1992. Defects in mRNA 3′-end formation, transcription initiation, and mRNA transport associated with the yeast mutation prp20: Possible coupling of mRNA processing and chromatin structure. *Genes & Dev 6*:1914–1926.

Fujita M, Kawano T, Ohta A, Sakamoto H. 1999. Neuronal expression of a *Caenorhabditis elegans* elav-like gene and the effect of its ectopic expression. *Biochem Biophys Res Commun 260*:646–652.

Fukuda M, Asano S, Nakamura T, Adachi M, Yoshida M, Yanagida M, Nishida E. 1997. CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature 390*:308–311.

Gallouzi IE, Brennan CM, Stenberg MG, Swanson MS, Eversole A, Maizels N, Steitz JA. 2000. HuR binding to cytoplasmic mRNA is perturbed by heat shock. *Proc Natl Acad Sci USA 97*:3073–3078.

Good PJ. 1995. A conserved family of elav-like genes in vertebrates. *Proc Natl Acad Sci USA 92*:4557–4561.

Gorlich D, Kutay U. 1999. Transport between the cell nucleus and the cytoplasm. *Annu Rev Cell Dev Biol 15*:607–660.

Gorospe M, Wang X, Holbrook NJ. 1998. p53-dependent elevation of p21Waf1 expression by UV light is mediated through mRNA stabilization and involves a vanadate-sensitive regulatory system. *Mol Cell Biol 18*:1400–1407.

Gueydan C, Droogmans L, Chalon P, Huez G, Caput D, Kruys V. 1999. Identification of TIAR as a protein binding to the translational regulatory AU-rich element of tumor necrosis factor alpha mRNA. *J Biol Chem 274*:2322–2326.

Hirsch HH, Nair AP, Backenstoss V, Moroni C. 1995. Interleukin-3 mRNA stabilization by a *trans*-acting mechanism in autocrine tumors lacking interleukin-3 gene rearrangements. *J Biol Chem 270*:20629–20635.

Ho JH, Kallstrom G, Johnson AW. 2000. Nmd3p is a Crm1-dependent adapter protein for nuclear export of the large ribosomal subunit. *J Cell Biol 151*:1057–1066.

Hong Y, Sarge KD. 1999. Regulation of protein phosphatase 2A activity by heat shock transcription factor 2. *J Biol Chem 274*: 12967–12970.

Hunt C, Morimoto RI. 1985. Conserved features of eukaryotic hsp70 genes revealed by comparison with the nucleotide sequence of human hsp70. *Proc Natl Acad Sci USA 82*:6455–6459.

Jacobson A, Peltz SW. 1996. Interrelationships of the pathways of mRNA decay and translation in eukaryotic cells. *Annu Rev Biochem 65*:693–739.

Janssens V, Goris J. 2001. Protein phosphatase 2A: A highly regulated family of serine/threonine phosphatases implicated in cell growth and signalling. *Biochem J 353*:417–439.

Jensen TH, Patricio K, McCarthy T, Rosbash M. 2001. A block to mRNA nuclear export in *Saccharomyces cerevisiae* leads to hyperadenylation of transcripts that accumulate at the site of transcription. *Mol Cell 7*:887–898.

Kasashima K, Terashima K, Yamamoto K, Sakashita E, Sakamoto H. 1999. Cytoplasmic localization is required for the mammalian ELAV-like protein HuD to induce neuronal differentiation. *Genes Cells 4*:667–683.

Kedersha N, Cho MR, Li W, Yacono PW, Chen S, Gilks N, Golan DE, Anderson P. 2000. Dynamic shuttling of TIA-1 accompanies the recruitment of mRNA to mammalian stress granules. *J Cell Biol 151*:1257–1268.

Kedersha NL, Gupta M, Li W, Miller I, Anderson P. 1999. RNA-binding proteins TIA-1 and TIAR link the phosphorylation of eIF-2 alpha to the assembly of mammalian stress granules. *J Cell Biol 147*:1431–1442.

Komiyama K, Okada K, Tomisaka S, Umezawa I, Hamamoto T, Beppu T. 1985. Antitumor activity of leptomycin B. *J Antibiot (Tokyo) 38*:427–429.

Kudo N, Matsumori N, Taoka H, Fujiwara D, Schreiner EP, Wolff B, Yoshida M, Horinouchi S. 1999. Leptomycin B inactivates CRM1/exportin 1 by covalent modification at a cysteine residue in the central conserved region. *Proc Natl Acad Sci USA 96*:9112–9117.

Lagnado CA, Brown CY, Goodall GJ. 1994. AUUUA is not sufficient to promote poly(A) shortening and degradation of an mRNA: The functional sequence within AU-rich elements may be UUAUUUA (U/A)(U/A). *Mol Cell Biol 14*:7984–7995.

Lee WM, Lin C, Curran T. 1988. Activation of the transforming potential of the human *fos* proto-oncogene requires message stabilization and results in increased amounts of partially modified fos protein. *Mol Cell Biol 8*:5521–5527.

Lerner EA, Lerner MR, Janeway CA Jr, Steitz JA. 1981. Monoclonal antibodies to nucleic acid-containing cellular constituents: Probes for molecular biology and autoimmune disease. *Proc Natl Acad Sci USA 78*:2737–2741.

Levy NS, Chung S, Furneaux H, Levy AP. 1998. Hypoxic stabilization of vascular endothelial growth factor mRNA by the RNA-binding protein HuR. *J Biol Chem 273*:6417–6423.

Li M, Makkinje A, Damuni Z. 1996. Molecular identification of I1PP2A, a novel potent heat-stable inhibitor protein of protein phosphatase 2A. *Biochemistry 35*:6998–7002.

Liu Y, Liang S, Tartakoff AM. 1996. Heat shock disassembles the nucleolus and inhibits nuclear protein import and poly(A)+ RNA export. *EMBO J 15*:6750–6757.

Ma WJ, Cheng S, Campbell C, Wright A, Furneaux H. 1996. Cloning and characterization of HuR, a ubiquitously expressed Elav-like protein. *J Biol Chem 271*:8144–8151.

Ma WJ, Chung S, Furneaux H. 1997. The Elav-like proteins bind to AU-rich elements and to the poly(A) tail of mRNA. *Nucleic Acids Res 25*:3564–3569.

Mattaj IW, Englmeier L. 1998. Nucleocytoplasmic transport: The soluble phase. *Annu Rev Biochem 67*:265–306.

Mencinger M, Panagopoulos I, Contreras JA, Mitelman F, Aman P. 1998. Expression analysis and chromosomal mapping of a novel human gene, APRIL, encoding an acidic protein rich in leucines. *Biochim Biophys Acta 1395*:176–180.

Michael WM, Choi M, Dreyfuss G. 1995. A nuclear export signal in hnRNP A1: A signal-mediated, temperature-dependent nuclear protein export pathway. *Cell 83*:415–422.

Nakielny S, Dreyfuss G. 1996. The hnRNP C proteins contain a nuclear retention sequence that can override nuclear export signals. *J Cell Biol 134*:1365–1373.

Nakielny S, Dreyfuss G. 1999. Transport of proteins and RNAs in and out of the nucleus. *Cell 99*:677–690.

Neville M, Stutz F, Lee L, Davis LI, Rosbash M. 1997. The importin-beta family member Crm1p bridges the interaction between Rev and the nuclear pore complex during nuclear export. *Curr Biol 7*:767–775.

Ohno M, Segref A, Bachi A, Wilm M, Mattaj IW. 2000. PHAX, a mediator of U snRNA nuclear export whose activity is regulated by phosphorylation. *Cell 101*:187–198.

Okano HJ, Darnell RB. 1997. A hierarchy of Hu RNA binding proteins in developing and adult neurons. *J Neurosci 17*:3024–3037.

Peng SS, Chen CY, Xu N, Shyu AB. 1998. RNA stabilization by the AU-rich element binding protein, HuR, an ELAV protein. *EMBO J 17*:3461–3470.

Piecyk M, Wax S, Beck AR, Kedersha N, Gupta M, Maritim B, Chen S, Gueydan C, Kruys V, Streuli M, Anderson P. 2000. TIA-1 is a translational silencer that selectively regulates the expression of TNF-alpha. *EMBO J 19*:4154–4163.

Pinol-Roma S, Dreyfuss G. 1992. Shuttling of pre-mRNA binding proteins between nucleus and cytoplasm. *Nature 355*:730–732.

Saavedra C, Tung KS, Amberg DC, Hopper AK, Cole CN. 1996. Regulation of mRNA export in response to stress in *Saccharomyces cerevisiae. Genes & Dev 10*:1608–1620.

Saavedra CA, Hammell CM, Heath CV, Cole CN. 1997. Yeast heat shock mRNAs are exported through a distinct pathway defined by Rip1p. *Genes & Dev 11*:2845–2856.

Sadis S, Hickey E, Weber LA. 1988. Effect of heat shock on RNA metabolism in HeLa cells. *J Cell Physiol 135*:377–386.

Saito S, Miyaji-Yamaguchi M, Shimoyama T, Nagata K. 1999. Functional domains of template-activating factor-I as a protein phosphatase 2A inhibitor. *Biochem Biophys Res Commun 259*:471–475.

Siomi MC, Eder PS, Kataoka N, Wan L, Liu Q, Dreyfuss G. 1997. Transportin-mediated nuclear import of heterogeneous nuclear RNP proteins. *J Cell Biol 138*:1181–1192.

Smith PR, Williams DG, Venables PJ, Maini RN. 1985. Monoclonal antibodies to the Sjogren's syndrome associated antigen SS-B (La). *J Immunol Methods 77*:63–76.

Stutz F, Neville M, Rosbash M. 1995. Identification of a novel nuclear pore-associated protein as a functional target of the HIV-1 Rev protein in yeast. *Cell 82*:495–506.

Tian Q, Streuli M, Saito H, Schlossman SF, Anderson P. 1991. A polyadenylate binding protein localized to the granules of cytolytic lymphocytes induces DNA fragmentation in target cells. *Cell 67*:629–639.

Vainberg IE, Dower K, Rosbash M. 2000. Nuclear export of heat shock and non-heat-shock mRNA occurs via similar pathways. *Mol Cell Biol 20*:3996–4005.

van der Houven van Oordt W, Diaz-Meco MT, Lozano J, Krainer AR, Moscat J, Caceres JF. 2000. The MKK(3/6)-p38-signaling cascade alters the subcellular distribution of hnRNP A1 and modulates alternative splicing regulation. *J Cell Biol 149*:307–316.

Wang W, Furneaux H, Cheng H, Caldwell MC, Hutter D, Liu Y, Holbrook N, Gorospe M. 2000. HuR regulates p21 mRNA stabilization by UV light. *Mol Cell Biol 20*:760–769.

Wu B, Hunt C, Morimoto R. 1985. Structure and expression of the human gene encoding major heat shock protein HSP70. *Mol Cell Biol 5*:330–341.