

Protein Ligands to HuR Modulate Its Interaction with Target mRNAs In Vivo

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Abstract. AU-rich elements (AREs) present in the 3' untranslated regions of many protooncogene, cytokine, and lymphokine messages target them for rapid degradation. HuR, a ubiquitously expressed member of the ELAV (embryonic lethal abnormal vision) family of RNA binding proteins, selectively binds AREs and stabilizes ARE-containing mRNAs in transiently transfected cells. Here, we identify four mammalian proteins that bind regions of HuR known to be essential for its ability to shuttle between the nucleus and the cytoplasm and to stabilize mRNA: SET α , SET β , pp32, and acidic protein rich in leucine (APRIL). Three have been reported to be protein phosphatase 2A inhibitors. All four ligands contain long, acidic COOH-terminal tails, while pp32 and APRIL share a second motif: rev-like leucine-rich repeats in their NH₂-terminal regions. We show that pp32 and APRIL are nucleocytoplasmic shuttling proteins that interact with the nuclear export factor CRM1 (chromosomal region maintenance pro-

tein 1). The inhibition of CRM1 by leptomycin B leads to the nuclear retention of pp32 and APRIL, their increased association with HuR, and an increase in HuR's association with nuclear poly(A)⁺ RNA. Furthermore, transcripts from the ARE-containing *c-fos* gene are selectively retained in the nucleus, while the cytoplasmic distribution of total poly(A)⁺ RNA is not altered. These data provide evidence that interaction of its ligands with HuR modulate HuR's ability to bind its target mRNAs in vivo and suggest that CRM1 is instrumental in the export of at least some cellular mRNAs under certain conditions. We discuss the possible role of these ligands upstream of HuR in pathways that govern the stability of ARE-containing mRNAs.

Key words: AU-rich elements • RNA stability • nucleocytoplasmic shuttling • CRM1 • protein phosphatase inhibitors

Introduction

Messenger RNA degradation is a mechanism by which eukaryotic cells regulate gene expression and influence cell growth and differentiation (Sachs, 1993). It is dependent upon both cis-elements in the RNA and trans-acting factors. The best-characterized cis-element in mammalian messages is the AU-rich element (ARE)¹ (Chen and Shyu, 1995). AREs are present in the 3' untranslated regions of many mRNAs, including those of proto-oncogenes, cytokines, and lymphokines, and target these RNAs for rapid degradation (Caput et al., 1986; Shaw and Kamen, 1986). Direct or indirect interactions of these sequences with specific protein factors are believed to govern mRNA half-life.

The overexpression of HuR stabilizes messages containing AREs in transient transfection experiments (Fan and Steitz, 1998a; Peng et al., 1998). HuR (or HuA) is a ubiquitously expressed member of the ELAV (embryonic lethal abnormal vision) family of RNA binding proteins (Good, 1995; Ma et al., 1996), originally identified in *Drosophila* as essential for neural development (Campos et al., 1985). There are three neural-specific Hu family members in mammals: HuB (or HelN1/N2) (Akamatsu et al., 1999; Jain et al., 1997), HuC (Akamatsu et al., 1999), and HuD (Chung et al., 1997). All four Hu proteins contain three RNA recognition motifs (RRMs). Both gel shift (Myer et al., 1997) and UV-cross-linking (Fan et al., 1997) experiments have provided evidence that HuR binding parallels the in vivo ability of ARE sequences to direct mRNA degradation. ARE recognition appears to be mediated by the first two RRM of HuR; the third RRM has been suggested to bind the poly(A) tail (Ma et al., 1997). In transient transfection assays, deletion of RRM3 alone abolishes HuR's ability to stabilize ARE-containing reporter

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¹Abbreviations used in this paper: APRIL, acidic protein rich in leucine; ARE, AU-rich element; GST, glutathione S-transferase; LMB, leptomycin B; PP2A, protein phosphatase 2A; RRM, RNA recognition motif.

mRNAs (Fan and Steitz, 1998a). Although predominantly nuclear, HuR shuttles between the nucleus and the cytoplasm by virtue of a novel shuttling sequence, HNS, located in the hinge region between its second and third RRM (Fan and Steitz, 1998a,b). This has led to the suggestion that HuR may initially bind mRNAs in the nucleus and accompany them into the cytoplasm to provide ongoing protection from the degradation machinery. Recent *in vivo* cross-linking experiments and gradient analyses established that HuR can bind poly(A)⁺ RNA in both cellular compartments and that a substantial fraction of cytoplasmic HuR is found associated with polysomes (Gallouzi et al., 2000).

ARE-mediated mRNA stability is subject to regulation. Cell stress (Gorospe et al., 1998), stimulation (Lindsten et al., 1989; Ming et al., 1998), and transformation (Hirsch et al., 1995) have all been shown to stabilize ARE-containing mRNAs. Several lines of evidence suggest the involvement of signal transduction pathways. Stimulation of quiescent primary T cells with antibodies directed against CD3/CD28 receptors stabilizes several mRNAs containing AREs (Lindsten et al., 1989). The stabilization of ARE-containing mRNA has been associated with the activation of c-jun NH₂-terminal kinase, which is correlated with lower decay rates of IL-3 mRNA in mast cells (Ming et al., 1998). Stabilization has also been linked to the activation of MAP kinase-activated protein kinase 2 (Dean et al., 1999; Winzen et al., 1999) in HeLa cells. Phosphatases, like kinases, have been implicated in ARE-mediated stability. Cyclosporin A, an antagonist of calcineurin (protein phosphatase 2B), destabilizes IL-3 mRNA in autocrine tumor cell lines (Nair et al., 1994). However, the molecular details by which any of these pathways impact mRNA stability is not known. Considering the multiple players and their various cellular roles, the mechanisms are likely to be complex.

To begin to understand molecular interactions underlying the regulation of ARE-mediated mRNA stability, we looked for HuR binding partners. Here, we use affinity chromatography to identify four protein ligands to HuR in HeLa cell extracts. All of these proteins contain unusually long acidic stretches at their COOH termini. Although initially recognized in other contexts, three of the ligands have been reported to be inhibitors of protein phosphatase 2A (PP2A) (for review, see Millward et al., 1999). We have examined the subcellular location and trafficking of these ligands and have delineated the nature of their interactions with HuR. We provide evidence for the *in vivo* association of HuR with these ligands, as well as data suggesting that their association modulates HuR interactions with ARE-containing mRNAs.

Materials and Methods

Plasmid Constructions

Plasmids described in this manuscript were synthesized using oligonucleotides containing restriction sites adjacent to the coding regions. Amplified products were digested with the appropriate enzymes and cloned into their respective vector(s). Two glutathione-S-transferase (GST)-HuR constructs were used. That encoding the protein used in the initial purification (see Fig. 1 B) was created by amplifying human HuR cDNA from pcDNA3-HuR (Fan and Steitz, 1998a) and inserting it into the BglII and

EcoRI sites of pGEX-2TK (Amersham Pharmacia Biotech) to form an in-frame fusion with GST. The second GST-HuR construct and mutants 1–5, used in the *in vitro* binding assays, have been reported (Gallouzi et al., 2000). Like these constructs, the remaining HuR mutants were created by amplifying portions of HuR (see Fig. 4, legend) from pcDNA3-HuR (Fan and Steitz, 1998a) and inserting them into the EcoRI and NotI sites of pGEX-5X-2 (Amersham Pharmacia Biotech) to form in-frame fusions with GST. SET β , pp32, and acidic protein rich in leucine (APRIL) cDNAs were amplified from a human kidney library (kindly provided by R. Lifton, Howard Hughes Medical Institute, Yale University, New Haven, CT). The cDNAs encoding SET β and a truncated form of APRIL (amino acids 1–194) were inserted into the EcoRI and NotI sites of pGEX-5X-2. pp32 cDNA was inserted into the BamHI and NotI sites of the same vector. pp32-Flag and APRIL-Flag were created by amplifying their cDNAs with a downstream oligonucleotide containing the eight-amino acid Flag sequence and inserting them into the EcoRI and NotI, or BamHI and NotI sites of pcDNA3, respectively. Truncated portions of pp32 (see Fig. 4, legend) were created by PCR and inserted into the BamHI and NotI sites of pcDNA3. All oligonucleotide sequences can be obtained from the authors upon request.

Recombinant Protein Expression, Purification, and *In Vitro* Binding Assay

Plasmids for bacterial expression were transformed into BL21 cells. The protocol for the growth, induction, and lysis of these cells has been reported (Frangioni and Neel, 1993). The purification of the GST fusion proteins followed the protocol supplied by pGEX manufacturer (Amersham Pharmacia Biotech).

In vitro transcription and translation was performed using the TNT T7 Quick-Coupled Transcription/Translation System (Promega) in accordance with the protocol supplied by the manufacturer.

For the *in vitro* binding assays, affinity matrices were prepared by saturating 10 μ l of packed glutathione sepharose beads with one of the tagged versions of HuR. After purification, the matrix was reequilibrated with 20 mM Hepes, pH 7.9; 100 mM KCl, and incubated with either 200 μ l of HeLa nuclear extract (see Fig. 4, A and B) or equimolar amounts of the *in vitro* translated proteins (as judged by autoradiography) (see Fig. 4 C) for 1 h. The beads were then washed with two 1-ml volumes of the reequilibration buffer. Bound material was eluted with 20 mM Hepes, pH 7.9, and 1 M KCl, and TCA precipitated before loading on gels.

Purification and Identification of HuR Binding Proteins

After purifying GST-HuR in batch, \sim 2.5 mg of the protein (as judged by SDS-PAGE analysis with reference to standards), bound to the glutathione sepharose, was packed into a column and re-equilibrated with 50 column volumes of 20 mM Hepes, pH 7.9, 100 mM KCl. Afterwards, RNase-treated nuclear extract derived from 5.5×10^9 HeLa cells (Dignam et al., 1983) was slowly passed over the column (5 ml/h) and the column was washed with re-equilibration buffer. Interacting proteins were eluted with a 0.1–2 M KCl gradient. Protein identification was performed by the Keck Foundation Biotechnology Resource Laboratory at Yale University. The peptides recognized by mass spectrometry or Edman sequencing are as follows: SET α : 155–167; SET β : 11–26, 12–26, 27–44, 45–55, 60–70, 65–70, 71–77, 110–119, 124–137, 142–154, 169–176; pp32: 6–12, 7–12, 21–28, 68–75, 69–75, 100–110, 100–111, 102–111, 111–116, 138–150, 138–153, 238–249; APRIL: 6–12, 7–12, 68–75, 69–75, 76–87, 87–99, 100–110, 100–111, 102–110, 102–111, 117–132, 138–150. Isoelectric points were calculated using the pI/Mw program, part of the Swiss Institute of Bioinformatics' Expert Protein Analysis System.

The amount of RNase A used in treating the nuclear extract was determined by titration. Extract was incubated with varying amounts of RNase A at 25°C for 15 min. Subsequently, the RNA in each sample was pCp labeled and its degradation assessed by gel fractionation. 1 μ g of enzyme was sufficient to digest the RNA from 10^6 HeLa cells.

Antibodies, Immunoblotting, and Quantitation

Recombinant GST-SET β , GST-pp32, and a GST-tagged truncated form of APRIL (amino acids 1–194) were expressed and purified as described above, and dialyzed against PBS. Rabbits were injected with 500 μ g of the proteins at 3-wk intervals by Yale University's Immunization Services. Antibodies were purified by affinity chromatography: serum was passed through two columns sequentially, the first containing GST (Amersham Pharmacia Biotech) to remove anti-GST antibodies and the second con-

taining the antigen to obtain the purified antibody. Both columns were prepared by conjugating the proteins to cyanogen bromide-activated Sepharose (Amersham Pharmacia Biotech) following the manufacturer's protocol. The flow-through from the first column was applied to the second. The purified antibodies were obtained by eluting the second column with 100 mM Tris, pH 2.5 (Harlow and Lane, 1988).

For immunoblot analysis, proteins were fractionated on 12% denaturing polyacrylamide gels and transferred to nitrocellulose. Proteins derived from human tissues were obtained from CLONTECH Laboratories, Inc. The blots were probed as described (Gallagher et al., 1994). The anti-ligand antibodies were used at 1:5,000; the anti-CRM1 (chromosomal region maintenance protein 1; kindly provided by G. Grosveld, St. Jude Children's Research Hospital, Memphis, TN) (Fornerod et al., 1997a) was used at 1:1,000; the 4B10 anti-hnRNP A1 (kindly provided by S. Pinol-Roma, Mount Sinai School of Medicine; New York, NY) was used at 1:1,000; the 3A2 anti-HuR antibody (Gallouzi et al., 2000) was used at 1:30,000. The secondary antibody was either HRP-conjugated donkey anti-rabbit or HRP-conjugated donkey anti-mouse. The blots were developed using the ECL system (Amersham Pharmacia Biotech) according to the manufacturer's directions.

All quantitations were performed using the National Institutes of Health image 1.62 program.

Cell Lysate, Glycerol Gradient, and Coimmunoprecipitation Assays

HeLa whole-cell lysate known to preserve complexes was prepared at 1.5×10^8 cells/ml as described (Gu et al., 1997) and was clarified by centrifugation at 40,000 rpm for 40 min. The supernatant was loaded onto 4 ml 5–20% glycerol gradients made with 10 mM triethanolamine, pH 7.9, 100 mM KCl, 5 mM MgCl₂, and 1 mM DTT, and centrifuged at 40,000 rpm (SW50.1 rotor; Beckman Coulter) for 12 h. Immunoprecipitations were performed from gradient fractions as described (Gallouzi et al., 1998), except that each fraction was diluted threefold in lysis buffer (Gu et al., 1997) before incubation with the antibody.

Cell Culture and Transient Transfections

Suspension HeLa cells were obtained from the National Cell Culture Center. Adherent HeLa cells and murine L929 cells were maintained in MEM medium (GIBCO BRL) supplemented with 10% fetal bovine serum (GIBCO BRL).

Adherent HeLa cells were transfected by incubating them with a calcium phosphate precipitate of the plasmid of interest (Loflin et al., 1999).

Heterokaryon Formation, Immunofluorescence Microscopy, In Situ Hybridization, and In Vivo Cross-linking

The immunofluorescence protocol has been reported (Fan and Steitz, 1998a). The anti-Flag monoclonal antibody M2 (Sigma-Aldrich) was diluted to 10 µg/ml. The anti-Myc monoclonal antibody (Sigma-Aldrich) was used at a 1:1,000 dilution. The polyclonal antibodies were used at ~5 µg/ml. Two secondary antibodies were used, both at concentrations of 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 included with the secondary antibodies at 1 µg/ml.

HeLa and L929 fusions were formed as described (Fan and Steitz, 1998a). Leptomycin B (kindly supplied by M. Yoshida, The University of Tokyo, Tokyo, Japan) was used at a concentration of 10 ng/ml.

In situ localization of poly(A)⁺ RNA has been described (Gallouzi et al., 2000). For in situ localization of specific mRNAs, the cells were serum starved for 24 h and leptomycin B (LMB) was added (5 ng/µl) 8 h before serum stimulation. 30 min after serum stimulation, the cells were fixed and permeabilized (Gallouzi et al., 2000). 5 ng/µl of message-specific oligonucleotide probes [two for *c-fos* (Calbiochem and gift of J.-L. Veyrune, Institut de Genetique Moleculaire de Montpellier, CNRS, Montpellier, France) and one for GAPDH (Calbiochem)] were used to establish the *c-fos* and GAPDH mRNA cellular distribution. The probes had been previously 3'-end labeled with digoxigenin according to the protocol supplied by Boehringer. In situ hybridization was performed according to Gallouzi et al. (2000).

In vivo cross-linking was performed as described (Pinol-Roma et al., 1989).

Results

Affinity Purification of Four Previously Identified Proteins as HuR Ligands

Potential complexes containing HuR were examined by density gradient centrifugation. HeLa whole-cell extract was prepared in a manner known to preserve complexes (Gu et al., 1997), but treated with ribonuclease A (RNase A, see Materials and Methods) to prevent RNA from tethering HuR to other RNA binding proteins. After fractionation on a 5–20% glycerol gradient, immunoblots were probed with the 3A2 anti-HuR monoclonal antibody (Gallouzi et al., 2000). This analysis revealed that endogenous HuR (36 kD) is dispersed throughout the gradient (Fig. 1 A, top), in contrast to the discrete profile of His-tagged recombinant HuR (bottom). These data suggest that HuR in HeLa cells may engage in several complexes not mediated by RNA.

We used affinity chromatography to identify HuR binding partners. Human HuR cDNA was subcloned downstream of the glutathione-S-transferase coding region to

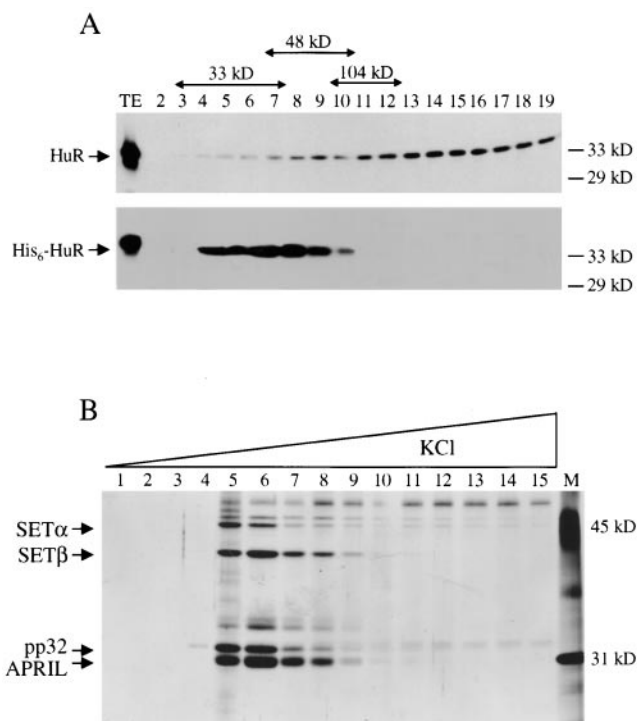


Figure 1. HuR exists in complexes and interacts with four protein ligands in vitro. (A) HeLa whole-cell extract (Gu et al., 1997) was fractionated on a 5–20% glycerol gradient. Fractions were run on a 12% gel and immunoblots probed with the 3A2 anti-HuR antibody (Gallouzi et al., 2000). As markers, phosphorylase B (104 kD), ovalbumin (48 kD), and carbonic anhydrase (33 kD) were run on a parallel gradient. (B) To identify HuR binding proteins, GST-HuR was immobilized on glutathione Sepharose (Amersham Pharmacia Biotech) and incubated with HeLa nuclear extract pretreated with RNase A. After washing, bound proteins were eluted from the column with a KCl gradient (0.1–2 M), fractionated on a 12% denaturing gel, and visualized by silver staining. Fractions were pooled, run on a second gel, and the indicated proteins were identified (see Materials and Methods).

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SET $\alpha$  : MALKRQSP LPPQKKKPRPPALGPFETSAAGL PPKK : 37
SET $\beta$  : -----MSAQAAKVSVKELNSNHGADETS : 24
pp32 : ----- : -
APRIL : ----- : -

          *          20          *
SET : EKEQQEAIHIDEVQNEIDRLNEQASEEILKVEQKYN : 74/61
pp32 : -----MEMGRRILHLELRNRTPSDVKELVLDNRSN : 30
APRIL : -----MDMKRRILHLELRNRTPAVRRELVLNDNCKSN : 30

          40          *          60          *
SET : KLRQPFQKRSFLIAKIPNFVITTFVNHPOVSALLGE : 111/98
pp32 : -----EGKLEGLTDEFFELFELSTINVGLTSIANLPKLNK : 67
APRIL : -----DGKLEGLTAEFFVNLFFLSEINVGLTSVSNLPKLPK : 67

          80          *          100          *
SET : EDDEALHLYLTRVEVTEFEDIKSGYRIDFYDENPYFE : 148/135
pp32 : -----KLELSDNRVSCGGLVLABKCPNLTHLNLSGNKIKDLS : 104
APRIL : -----KLELSDNRVSCGGLDMLABKLPNLTHLNLSGNKIKDLS : 104

          120          *          140
SET : NKVLSKRFHFLNEEGDPPSSKSTEIKWKSGKDLTKRSSQ : 185/172
pp32 : -----TEPLKKEMLKSLDLFNCVETNLNDYRENVFKLLPQ : 141
APRIL : -----TEPLKKEMLKSLDLFNCVETNLNDYRENVFKLLPQ : 141

          160          *          180
SET : TQNKASRRQHEEPESFFTFWTDHSDAGADELGEVVIK : 222/209
pp32 : -----LTYLDGYDRDDKEAPDSDAEGYVEG---LDDEBEDE : 175
APRIL : -----LTYLDGYDRDDQKAPDSDAEVDGVDEEEEDDEEGE : 178

          200          *          220
SET : DDIIWPNLYYLVPDMDDDEEGEGEEDDDDEEEEGLE : 259/246
pp32 : -----EPEYDEDAVVEDEDEDEEFGEEEDVSGEEEDDEE : 212
APRIL : -----DEDDDEGEDEEEDDEDEDEVEGDDEDDDEVSEEE : 215

          240          *          260
SET : DIDEDEDEDEDEDEDEDEDEDEEG--EEGE--EDEGFDD : 290/277
pp32 : -----GYNDGEVDDDEDEEELGEEERGQKRRKREPEDEGED : 249
APRIL : -----FGLDEBDEDEDEDEDEDEEESGKGEKRRKRETDDEGED : 251

          280          *
SET : ----- : 290/277
pp32 : ----- : 249
APRIL : ----- : 251

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Figure 2. Sequences of the HuR binding proteins. Sequences shaded in black indicate identities, while gray denotes similarities. pp32 contains two rev-like leucine rich repeats (residues 63–71, and 112–120), whereas APRIL contains three (residues 61–69, 85–93, and 110–118). The calculated molecular weights of SET α , SET β , pp32, and APRIL are: 33.5, 32.1, 28.6, and 28.8 kD, respectively. Note that APRIL migrates faster than pp32 in SDS-PAGE despite the fact that it is two amino acids longer. Residue three of our SET α cDNA clone is different from that reported (proline; Nagata et al., 1995).

form an in-frame fusion. This fusion protein, which mimics HuR binding to RNA in UV-cross-linking and competition experiments (data not shown), was expressed in bacteria and purified on glutathione sepharose. RNase A-treated HeLa nuclear extract (Dignam et al., 1983) from 5.5×10^9 cells was passed over the GST-HuR column (since HuR is predominantly nuclear; Fan and Steitz, 1998a) and, after washing, bound proteins were eluted with a 0.1–2 M KCl gradient. 1/10 of each fraction was analyzed by SDS-PAGE followed by silver staining (Fig. 1 B), showing several proteins that peak at ~ 250 mM KCl. None of these proteins bound to either the GST protein alone or the column matrix (data not shown). The remainder of fractions 5–10 were pooled, run on a second gel, and stained with Coomassie brilliant blue. The four proteins

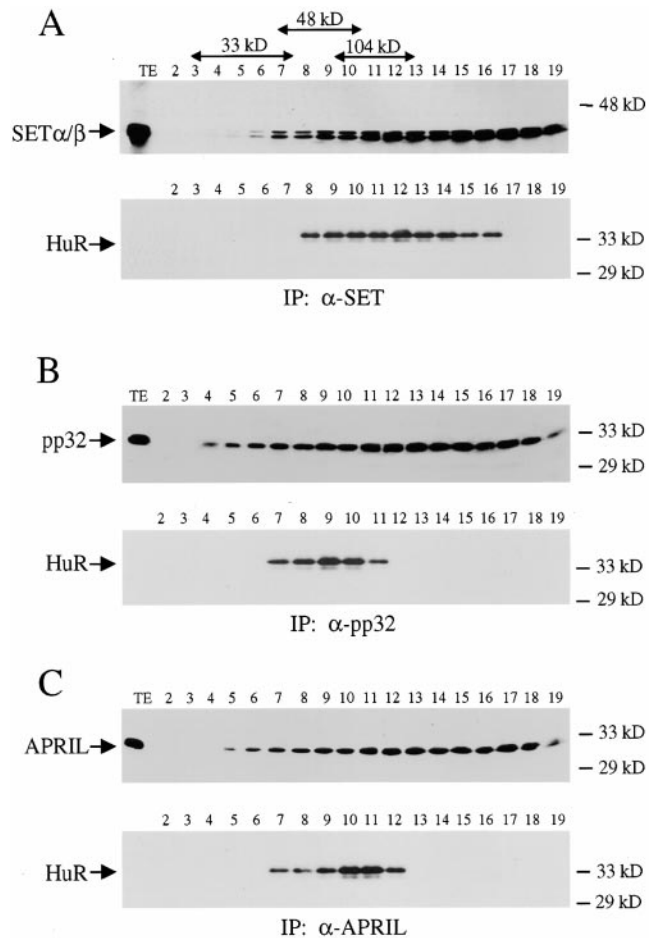


Figure 3. Coimmunoprecipitation of HuR from a glycerol gradient of HeLa whole-cell extract using antiligand antibodies. HeLa whole-cell extract was prepared and fractionated on a glycerol gradient as described in Fig. 1. A portion of each fraction was electrophoresed on a 12% denaturing gel, transferred to nitrocellulose, and probed with antiligand antibodies (see Materials and Methods). 10-fold larger portions were immunoprecipitated with anti-SET (A), anti-pp32 (B), or anti-APRIL (C). These precipitates were analyzed as above, but probed with the 3A2 anti-HuR monoclonal antibody. One gradient was used for all of the results in this figure. The molecular weight markers are described in Fig. 1.

denoted in Fig. 1, ranging from ~ 28 to ~ 45 kD, were excised from the gel, treated with trypsin, and analyzed by a combination of mass spectrometry and Edman degradation (see Materials and Methods).

Each of the four proteins (Fig. 2) had been previously described and three had been given several different names. We use either the protein's original name or that most commonly seen in the literature: SET α/β (von Lindern et al., 1992; Matsumoto et al., 1993), pp32 (Malek et al., 1990), and acidic protein rich in leucine (APRIL; Mencinger et al., 1998). Three of these proteins (SET α , SET β , and pp32) had been identified as inhibitors of PP2A (Li et al., 1996; Saito et al., 1999). Their other reported activities are detailed in the Discussion.

The four HuR binding proteins exhibit striking structural similarity (Fig. 2). All contain a long, acidic COOH-terminal tail: residues 242–290 of SET α , 222–277 of SET β , 164–249 of pp32, and 162–251 of APRIL. These tails prob-

ably contribute to the proteins' mobilities in SDS-polyacrylamide gels (Fig. 1 B), which are slower than predicted from their molecular weights (Fig. 2, legend) (Graceffa et al., 1992). The calculated isoelectric points for SET α , SET β , pp32, and APRIL are accordingly low: 4.23, 4.12, 3.99, and 3.94, respectively. Beyond their common acidic regions, the HuR binding proteins divide into two subsets: SET α and SET β , and pp32 and APRIL. SET α and SET β are identical over their 253 COOH-terminal amino acids, diverging only for the first 37 amino acids of SET α and the first 24 amino acids of SET β ; thus, these proteins are probably splice variants of one another, as previously noted (Matsumoto et al., 1993). In contrast, pp32 and APRIL exhibit 71% sequence identity (Fig. 2, black boxes) and 81% sequence similarity (gray boxes) and are clearly products of separate genes. They contain a second structural similarity: rev-like leucine-rich repeats in their NH₂-terminal regions (see Fig. 2, legend, and below).

We examined the tissue distribution of the four HuR binding proteins in various human tissues by immunoblotting (see Materials and Methods) and found, in agreement with reports on several mammalian species, that each is selectively expressed in certain tissues. The SET proteins are present in brain, heart, lung, spleen, and kidney (data not shown) (Nagata et al., 1998). pp32 is expressed in brain (Matsuoka et al., 1994) and kidney (data not shown), as well as in cells capable of self-renewal, such as those of the intestinal crypts and prostate (Kadkol et al., 1998; Malek et al., 1990). APRIL is present in brain, kidney, liver, skeletal muscle, and testis (data not shown) (Mencinger et al., 1998). It has been previously observed that HuR is several-fold more abundant in mammalian tissue culture cells that divide most rapidly (Fan and Steitz, 1998a). This is also true of all four HuR binding proteins (data not shown).

Detection of Complexes between HuR and Its Ligands in Cell Extract

Polyclonal antibodies were raised against GST-tagged versions of SET β , pp32, and a portion of APRIL (see Materials and Methods). These antibodies were affinity purified (Materials and Methods) and used to probe the gradient-fractionated, RNase-treated cell extract analyzed in Fig. 1 A. The four ligands are dispersed throughout the gradient (Fig. 3, A–C, top), suggesting that they, like HuR, are involved in multiple complexes. As anticipated, polyclonal antibodies directed against SET β cross-react with SET α .

The interaction of HuR with its ligands in these gradients was confirmed by immunoprecipitating each fraction with antisera to the various ligands, followed by probing immunoblots of the precipitates with the monoclonal 3A2 anti-HuR antibody (Gallouzi et al., 2000). Fig. 3, A–C (bottom), reveals that SET α and/or SET β interact with HuR in complexes with a range of sizes that might include additional proteins. In contrast, pp32- and APRIL-containing complexes are smaller and more discrete (peaking at 50–60 kD), suggestive of simple heterodimeric interactions with HuR.

Immunoprecipitations performed on unfractionated HeLa whole-cell extract revealed that ~5% of total cellular HuR binds to each of these four ligands (data not shown). Since no hnRNP A1, another RNA-binding pro-

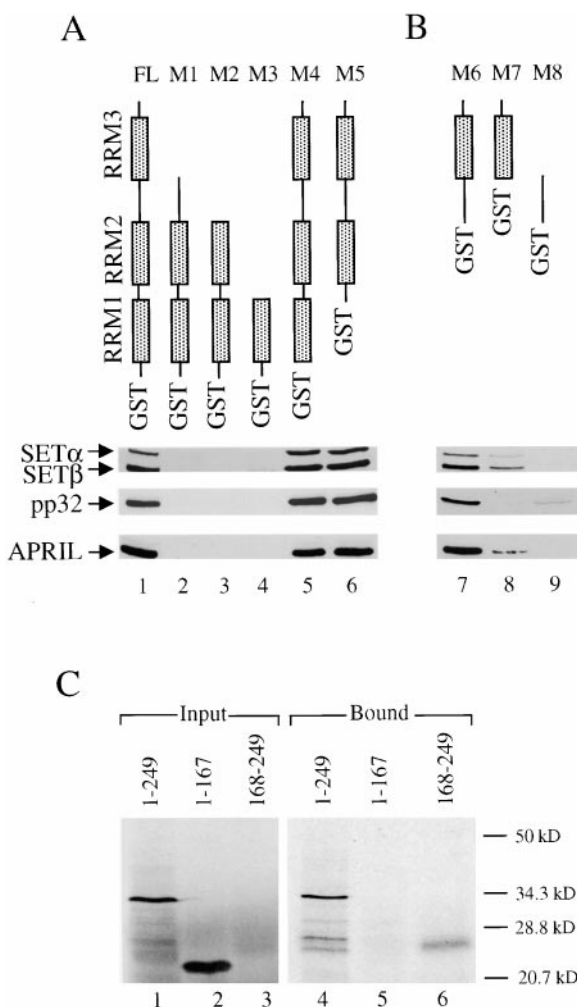


Figure 4. RRM3 and the hinge region of HuR are required for ligand binding, while the acidic tail of pp32 binds HuR. (A and B) Eight deletion mutants of GST-HuR were overexpressed in *Escherichia coli*, purified on glutathione sepharose, and incubated with HeLa nuclear extract treated with RNase A. The beads were then washed and the ligands were eluted, run on a 12% denaturing gel, transferred to nitrocellulose, and probed with affinity purified antibodies to SET α/β , pp32, or APRIL. FL denotes full length HuR (326 amino acids) fused to GST. Those amino acids of HuR included in the mutants are as follows: M1, 2–242; M2, 2–189; M3, 2–100; M4, 19–326; M5, 101–326; M6, 190–326; M7, 243–326; M8, 190–242. (C) Plasmids encoding full-length pp32 (249 amino acids, lane 1), its NH₂-terminal region (amino acids 1–167, lane 2), and its acidic tail (amino acids 168–249, lane 3) were transcribed and translated in vitro in the presence of ³⁵S-methionine and incubated with GST-HuR on glutathione sepharose. Bound polypeptides were run on a denaturing gel and detected by autoradiography (lanes 4–6). While the migration of all pp32 polypeptides is retarded (presumably because of their acidic nature), that of the acidic tail (amino acids 168–249, lanes 3 and 6) is most retarded.

tein that is at least 100-fold more abundant than HuR (Kiledjian et al., 1994), was detected in these same precipitates (data not shown), we conclude that HuR's interactions with SET α/β , pp32, and APRIL are highly specific. The converse immunoprecipitation experiments (in which HuR was quantitatively immunoprecipitated and the pre-

capitates probed for the four binding proteins) indicated that SET α , SET β , pp32, and APRIL are at least 10-fold more abundant than HuR in HeLa cells (data not shown); thus, only a small fraction of each ligand is associated with HuR.

RRM3 and the Hinge Region of HuR Are Required for Ligand Binding

Previous studies have shown that RRM3 is crucial for HuR's ability to stabilize ARE-containing mRNAs (Fan and Steitz, 1998a) and that its hinge region contains a nucleocytoplasmic shuttling sequence (Fan and Steitz, 1998b). To ask whether interactions between HuR and SET α , SET β , pp32, and APRIL involve these important regions, the ligands' binding sites on HuR were mapped by deletion analysis (Fig. 4, A and B). Eight HuR mutants, designed to preserve conserved domains (as defined by Okano and Darnell, 1997), were subcloned downstream of GST. The resulting fusion proteins were immobilized on glutathione sepharose, incubated with RNase A–treated HeLa nuclear extract, and their ability to bind the HuR ligands examined by immunoblotting with affinity purified anti–ligand antibodies.

Fig. 4, A and B, shows that while all four HuR binding proteins interact robustly with GST-tagged full-length HuR (lane 1), removal of HuR's third RRM (lane 2), or longer sequences from the COOH terminus (lanes 3 and 4) completely abolishes ligand binding. This is not the case for deletions that remove the NH₂ terminus or the first RRM of HuR (lanes 5 and 6). More detailed studies revealed that RRM3 alone retains some ability to bind SET α/β and APRIL (lane 8), while the hinge region alone binds reduced amounts of pp32 (lane 9). However, both RRM3 and the hinge are required for efficient retention of all four ligands (lane 7).

We also investigated which regions of the ligands are necessary for interaction with HuR (Fig. 4 C). Here, *in vitro* translated (³⁵S-methionine labeled) full-length and NH₂- and COOH-terminal portions of pp32 (lanes 1–3) were incubated with immobilized GST–HuR, and the bound material was gel fractionated and detected by autoradiography (lanes 4–6). The pattern of retained polypeptides compared with the input reveals that the acidic tail of pp32 (amino acids 168–249) accounts for its interaction with GST–HuR (compare lanes 3 and 6), whereas the NH₂-terminal portion (amino acids 1–167) does not bind (lane 2 vs. 5). Although we were unable to produce comparable ³⁵S-labeled subfragments of SET or APRIL for study, it seems probable that these proteins likewise bind HuR via their acidic COOH-terminal tails.

pp32 and APRIL Appear Nuclear, but Shuttle between the Nucleus and the Cytoplasm

To determine where HuR interactions with its ligands might occur in the cell, immunofluorescence experiments were performed. Confocal microscopy using affinity-purified antibodies showed that the four HuR binding proteins differ in their subcellular location in HeLa cells. Antibodies directed against SET α/β produced nuclear as well as cytoplasmic staining (Fig. 5, panels 2 and 4), while pp32 and APRIL appeared strictly nuclear (panels 6 and 8, and 10 and 12, respectively). Two other laboratories have re-

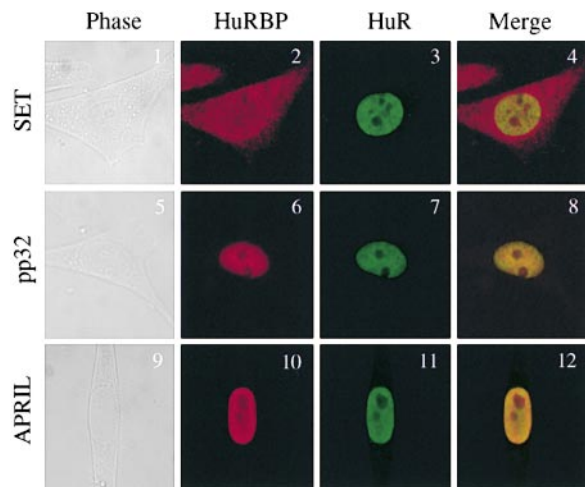


Figure 5. *In vivo* localization of HuR binding proteins. HeLa cells were fixed, permeabilized, incubated with affinity-purified polyclonal antiligand antibodies and the 3A2 monoclonal anti–HuR antibody, and visualized by confocal microscopy. The secondary antibodies were Texas red–conjugated anti–rabbit and Alexa 488 conjugated anti–mouse, respectively.

ported that pp32 is nuclear (Malek et al., 1990; Matilla et al., 1997), whereas two have observed cytoplasmic presence as well (Vaesen et al., 1994; Ulitzur et al., 1997b). HA-tagged versions of both SET α and SET β have previously been observed to be nuclear when overexpressed in HeLa cells (Nagata et al., 1998).

Because pp32 and APRIL are nuclear proteins, it was possible to employ the heterokaryon assay (Schmidt-Zachmann et al., 1993) to determine whether they, like HuR, shuttle between the nucleus and the cytoplasm. pp32 and APRIL were tagged with the eight amino-acid Flag epitopes at their COOH termini, expressed in HeLa cells, and shown to retain their nuclear location by immunofluorescence (Figs. 6 and 7 C, panels 6 and 9). To produce heterokaryons, transfected cells were incubated for 3 h with mouse L929 cells in the presence of cycloheximide (to block translation) and fused using polyethylene glycol. After an additional 3-h incubation period (also in the presence of cycloheximide), the coculture was fixed and immunofluorescence was performed (Fig. 6). To distinguish the human and mouse nuclei, the heterokaryons were treated with Hoechst 33258, which stains the human nucleus uniformly and the mouse nucleus in a spotted fashion (Moser et al., 1975).

Like HuR (Fan and Steitz, 1998a) (Fig. 6, panel 12), pp32 (panel 6) and APRIL (panel 9) exhibited shuttling behavior, appearing in both the human and mouse nuclei of heterokaryons. Myc-epitope–tagged hnRNP C1 (Nakielny and Dreyfuss, 1996), a nonshuttling hnRNP protein, provided a negative control (panel 3) (Pinol-Roma and Dreyfuss, 1992). Shuttling may explain the appearance of pp32 in the cytoplasm of some cell types (Vaesen et al., 1994; Ulitzur et al., 1997b).

pp32 and APRIL Bind CRM1 and Their Nuclear Export Is Inhibited by Leptomycin B

pp32 and APRIL contain 2 and 3 leucine-rich repeats in the NH₂-terminal portions of their sequences (Fig. 7 A),

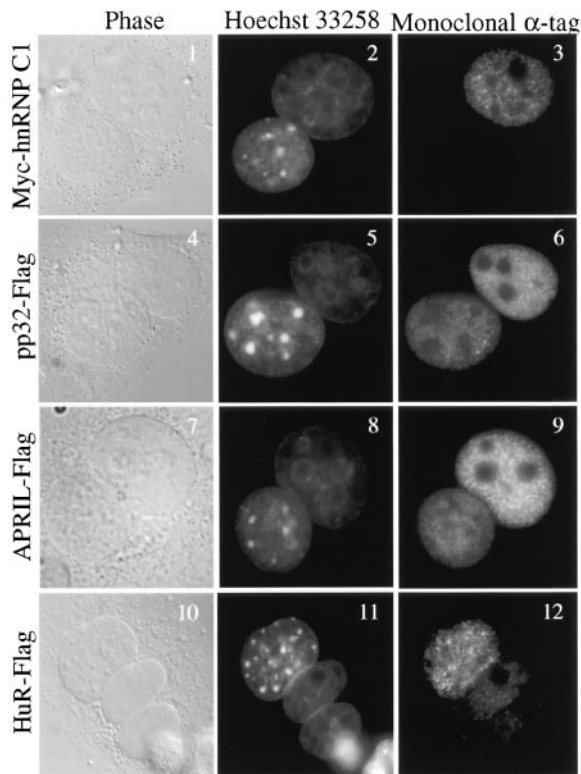


Figure 6. pp32- and APRIL-Flag shuttle. HeLa cells were transiently transfected with vectors encoding Myc-hnRNP C1 (Nakielny and Dreyfuss, 1996), pp32-Flag, APRIL-Flag, or HuR-Flag (Fan and Steitz, 1998a), cocultured with mouse L929 cells, and fused as described (Fan and Steitz, 1998a). Both L929 cells (before fusion) and heterokaryons were treated with cycloheximide. Fixed cells were probed with either the M2 anti-Flag monoclonal or the 9E10 anti-Myc monoclonal (Sigma-Aldrich); the secondary antibody was a goat anti-mouse antibody conjugated to Alexa 488. Cells were also stained with Hoescht 33258 (Sigma-Aldrich) to distinguish the human and mouse nuclei; the mouse (but not human) nuclei display a speckled pattern.

respectively. For rev and several other proteins, this motif constitutes a nuclear export signal (NES) that binds to CRM1, a nuclear export receptor (Fornerod et al., 1997b). CRM1, in turn, binds Ran GTP (Fornerod et al., 1997b; Stade et al., 1997) and several nuclear pore and nucleoporin-like proteins [e.g., RIP (Neville et al., 1997), the CAN/Nup88 complex (Fornerod et al., 1997a), NLP-1 (Farjot et al., 1999)]. LMB, an antifungal and antitumor agent (Hamamoto et al., 1983a,b; Komiyama et al., 1985), inhibits nuclear export by covalently modifying a critical cysteine residue in CRM1 (Kudo et al., 1999), thereby preventing the formation of the trimeric NES-CRM1-RanGTP complex (Fornerod et al., 1997b). Experiments performed both in *Saccharomyces cerevisiae* (Neville and Rosbash, 1999) and with HeLa extract (Kudo et al., 1998) have shown the reaction of leptomycin B with CRM1 to be extremely specific and efficient.

To determine whether their leucine-rich repeats enable pp32 and APRIL to interact with CRM1, coimmunoprecipitation experiments were performed (Fig. 7 B). HeLa whole-cell extract was precipitated with polyclonal anti-pp32 or -APRIL antiserum, and immunoblots of the pre-

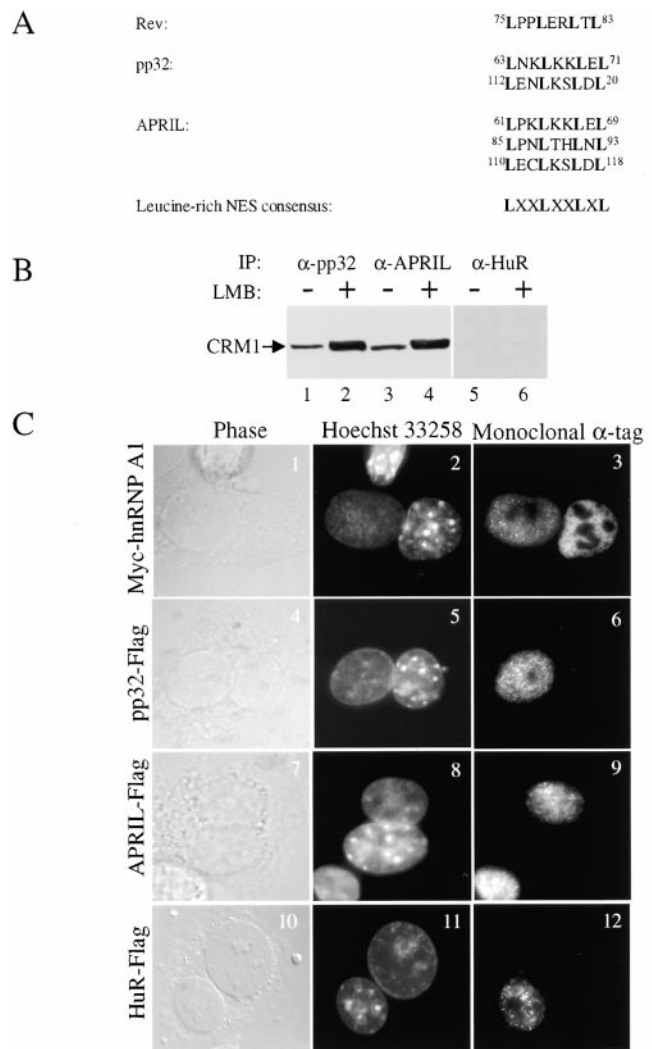


Figure 7. pp32 and APRIL complex with CRM1, and their nuclear export is inhibited by leptomycin B. (A) pp32 and APRIL contain several rev-like leucine-rich repeats. (B) HeLa whole-cell extract was prepared from cells grown in either the presence or absence of 10 ng/ml leptomycin B (12-h treatment). Anti-pp32 or -APRIL antiserum or 3A2 anti-HuR antibody (in amounts determined to quantitatively precipitate the target protein) was used for immunoprecipitation and the precipitates were probed with a polyclonal anti-CRM1 antibody (Fornerod et al., 1997a). (C) HeLa cells transfected with Myc-hnRNP A1 (Michael et al., 1995), pp32-Flag, APRIL-Flag, or HuR-Flag (Fan and Steitz, 1998a) were incubated with 10 ng/ml of leptomycin B for 12 h. They were then fused with L929 cells, permeabilized, and immunofluorescence was performed as reported (Fan and Steitz, 1998a). The antibodies and dye used in this experiment are identical to those described in Fig. 6.

cipitates were probed with a polyclonal anti-CRM1 antibody (Fornerod et al., 1997a). Indeed, CRM1 can be detected in the immunoprecipitates (Fig. 7 B, lanes 1 and 3). Moreover, complex formation was stimulated in cells treated with LMB (lanes 2 and 4). While only ~1% of CRM1 was immunoprecipitated by anti-pp32 or -APRIL serum when extract was prepared from cells grown in the absence LMB, ~5% of CRM1 was coimmunoprecipitated with pp32 and APRIL in extracts derived from cells

treated with LMB (see Materials and Methods). Immunoprecipitations performed using the 3A2 anti-HuR (Gallouzi et al., 2000) and the 4B10 anti-hnRNP A1 (Pinol-Roma et al., 1988) monoclonal antibodies provided negative controls. As suggested by their sequences, neither HuR (Fig. 7 B, lanes 5 and 6) nor hnRNP A1 (data not shown) detectably interacted with CRM1 in extracts prepared from cells grown in the absence or presence of LMB. The converse experiments, in which extracts were immunoprecipitated with an anti-CRM1 antibody and immunoblots of these precipitates were probed with the 3A2 anti-HuR and 4B10 anti-hnRNP A1 antibodies, confirm these results (data not shown).

We next asked whether leptomycin B inhibits the nuclear export of pp32 and APRIL, using HuR and hnRNP A1, which lack leucine-rich repeats, as controls. Heterokaryon fusion experiments were performed as above, except that for 12 h before incubation of the coculture, HeLa cells transfected with plasmids encoding pp32-Flag, APRIL-Flag, HuR-Flag (Fan and Steitz, 1998a), or Myc-hnRNP A1 (Michael et al., 1995) were incubated with leptomycin B (Fig. 7 C). LMB treatment continued during the coculture and after fusion. Finally, the cells were fixed and immunofluorescence was performed. As expected, the nuclear export of pp32- and APRIL-Flag is significantly inhibited by leptomycin B. While these proteins migrate from the human nucleus to the mouse nucleus in untreated cells (see Fig. 6, panels 6 and 9), they are restricted to the human nucleus in the presence of LMB (Fig. 7 C, panels 6 and 9). In contrast, Myc-hnRNP A1 and HuR-Flag are capable of shuttling between the nuclei under either condition (Fig. 7 C, panels 3 and 12, respectively), confirming that LMB is specific in targeting CRM1-mediated protein export. We obtained the same results when HeLa cells were incubated with LMB for just 3 h before coculturing (data not shown). These data strongly suggest that CRM1 governs the nuclear export of both pp32 and APRIL.

Leptomycin B Increases HuR's Interaction with pp32 and APRIL and Alters HuR's RNA-binding Activity

Although LMB does not interfere with the nuclear export of HuR, as judged by heterokaryon fusion assays (Fig. 7), we reasoned that the abnormal retention of pp32 and APRIL in the nucleus might nevertheless impact their interaction with HuR. To test this, we examined complex formation between HuR and its ligands after treatment of cells with LMB. HeLa cells were grown in the presence of leptomycin B, whole-cell extract was prepared, and immunoprecipitations were performed with anti-pp32, -APRIL, or -SET antisera. Subsequently, the precipitates were probed on immunoblots with the 3A2 monoclonal anti-HuR antibody (Gallouzi et al., 2000). Fig. 8 A shows that, after LMB treatment, the association of HuR with pp32 and with APRIL increases (compare Fig. 8, lanes 1 with 2, and 3 with 4). While only a small amount of HuR coimmunoprecipitated with pp32 and APRIL in extract prepared from cells not treated with LMB (~5%), six- and fivefold more HuR associated with these ligands in extract derived from LMB-treated cells, respectively (see Materials and Methods). This is not true of the interaction between SET α/β and HuR, as the same amount of HuR was coim-

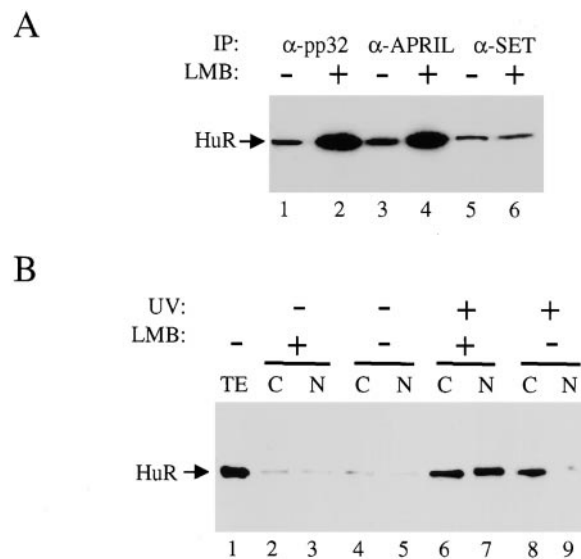


Figure 8. Leptomycin B treatment of HeLa cells results in increased ligand association with HuR, as well as a change in HuR's *in vivo* pattern of RNA binding. (A) HeLa whole-cell extract was prepared from cells grown either in the presence or absence of LMB, precipitated with anti-pp32, -APRIL, or -SET antiserum, and the precipitates were probed with the 3A2 anti-HuR monoclonal antibody. (B) HeLa cells grown in either the presence or absence of LMB were subjected to UV irradiation to induce HuR-RNA cross-links and fractionated into nucleus (N) and cytoplasm (C) (Pinol-Roma et al., 1989). The poly(A)+ RNA in each fraction was purified on oligo(dT)-sepharose and degraded with RNase (see Materials and Methods). Subsequently, cross-linked HuR was detected by immunoblotting with the 3A2 antibody. In lanes 2–5, the cells were treated exactly the same, but the UV treatment was omitted. Lane 1 shows the protein present in 1% the amount (compared with the other lanes) of UV-treated extract (total extract, TE) before fractionation.

munoprecipitated in the presence and absence of LMB (compare Fig. 8, lanes 5 and 6). The cellular levels of pp32, APRIL, and HuR do not change after LMB treatment (data not shown).

To ask whether increased interaction with its ligands might alter HuR binding to its target mRNAs, we performed *in vivo* UV cross-linking in HeLa cells in both the presence and absence of LMB. Subsequently, nuclear and cytoplasmic fractions were prepared and poly(A)+ RNA was purified from each fraction by oligo(dT)-cellulose chromatography using conditions that release noncovalently attached proteins from the RNA (Fig. 8 B, lanes 2–5) (Pinol-Roma et al., 1989). Cross-linked, poly(A)+ RNA/protein complexes were eluted, treated with RNase, and the presence of HuR was detected by probing immunoblots with the 3A2 monoclonal anti-HuR antibody. In the absence of leptomycin B, HuR cross-linked to only cytoplasmic poly(A)+ RNA (Fig. 8 B, lanes 8 and 9), as previously observed (Gallouzi et al., 2000). We have interpreted this result to mean that HuR binds nuclear mRNA shortly before its export. However, in cells treated with leptomycin B, HuR's RNA binding profile was altered: HuR cross-linked equally to poly(A)+ RNA in both the nucleus and the cytoplasm (lanes 6 and 7, respectively). This is not true of hnRNP A1, which cross-linked to nuclear

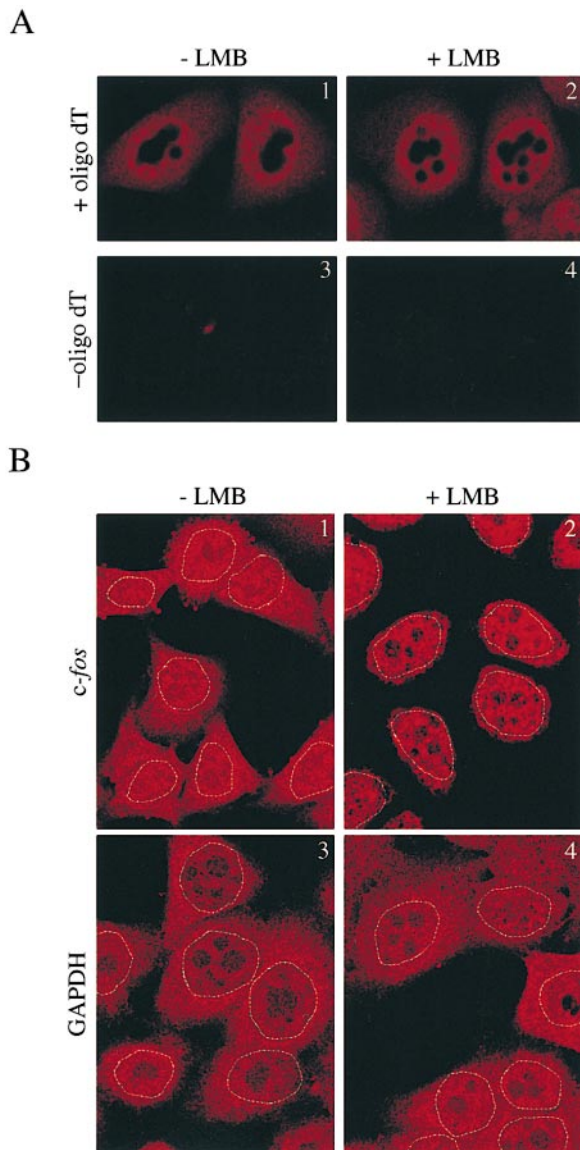


Figure 9. LMB causes *c-fos* mRNA nuclear retention. (A) HeLa cells grown with or without LMB (see Materials and Methods) were fixed, permeabilized, probed with a digoxigenin-labeled oligo(dT₃₅) probe, and visualized by confocal microscopy with comparable exposure times. A rhodamine-labeled antidigoxigenin antibody (Boehringer) was used for detection. (B) The distribution of *c-fos* and GAPDH mRNA was examined in HeLa cells with (2 and 4) and without (1 and 3) LMB treatment, as above. The 5' digoxigenin-labeled antisense oligonucleotide probe complementary to nucleotides 288–328 of *c-fos* mRNA (Calbiochem) (1 and 2) was used at 5 ng/μl with a 1:200 dilution of sheep antidigoxigenin Fab-rhodamine antibody (Boehringer). Identical results were obtained with a *c-fos* 3' untranslated region probe complementary to nucleotides 3363–3473 (a gift of J.-L. Veyrune). Cells in 3 and 4 were treated the same as those in 1 and 2, except that the hybridization was performed using a digoxigenin-labeled oligonucleotide complementary to nucleotides 46–85 of GAPDH mRNA (Calbiochem) as a probe. All antisense probes gave single bands on Northern blots (not shown). The dotted lines indicating the nuclear boundaries were constructed from the phase images of the same cells. Treatment of the fixed cells with RNase before hybridization with probes yielded no signal, as in A, 3 and 4. Likewise, no signal was detected with the *c-fos* probes without serum induction.

poly(A)⁺ RNA in cells grown in both the absence (Pinol-Roma et al., 1989) and presence (data not shown) of LMB.

Recently, several laboratories have suggested that CRM1 plays a role in mRNA export in *S. cerevisiae*, *Xenopus* oocytes, and rat 3Y1 cells (Pasquinelli et al., 1997; Stade et al., 1997; Watanabe et al., 1999). Therefore, it might be argued that the change in HuR's cross-linking profile after LMB treatment simply represents the interaction of HuR with higher levels of poly(A)⁺ RNA now in the nucleus. However, Fig. 9 A shows that the treatment of HeLa cells with leptomycin B, using the conditions described, does not significantly affect the cellular distribution of total poly(A)⁺ RNA, as judged by in situ hybridization using a digoxigenin-labeled oligo(dT) probe and a rhodamine-conjugated antidigoxigenin antibody (panels 1–4). Quantitation of these results revealed that nuclear retention of poly(A)⁺ RNA was only marginally increased (~3%) after LMB treatment (see Materials and Methods), similar to data reported by other laboratories for *S. cerevisiae* or the *Xenopus* oocyte system (Fischer et al., 1995; Segref et al., 1997; Neville and Rosbash, 1999). We conclude that the nuclear cross-linking of HuR to poly(A)⁺ RNA in cells treated with LMB represents an in vivo perturbation of HuR's activity, apparently through its interaction with pp32 and/or APRIL. Importantly, while LMB does not significantly alter the distribution of poly(A)⁺ RNA in HeLa cells, it does alter the localization of an ARE-containing, *c-fos* mRNA (Fig. 9 B). Cells were serum stimulated to increase the production of *c-fos* mRNA to detectable levels (Greenberg and Ziff, 1984) in the presence or absence of leptomycin B, fixed, and assayed by in situ hybridization using digoxigenin-labeled complementary oligonucleotides (see Materials and Methods). Unlike GAPDH mRNA (compare panel 3 with 4), *c-fos* mRNA is selectively retained in the nuclei of LMB-treated cells (compare panel 1 with 2). This result was obtained using two different probes to *c-fos* mRNA, one spanning the 5' untranslated region (UTR) and proximal coding sequence and one complementary to a region within the 3' UTR (see Fig. 9, legend). The somewhat spotted staining pattern of *c-fos* mRNA has been observed previously for another short-lived mRNA (Veyrune et al., 1996). These data reveal that CRM1 is instrumental in the nuclear export of particular mRNAs (perhaps all ARE-containing mRNAs) at least under certain conditions.

Collectively, these data argue that increased in vivo interactions with pp32 and APRIL are associated with a change in HuR's binding to ARE-containing mRNAs in cells treated with leptomycin B. Thus, the HuR ligands we have identified may also modulate HuR function in cells under normal conditions.

Discussion

We have isolated and characterized four abundant HeLa cell proteins that specifically associate with HuR: SETα, SETβ, pp32, and APRIL. Coimmunoprecipitation of binding protein/HuR complexes from glycerol gradient-fractionated, RNase-treated cell extracts suggests that the aggregates are of relatively low molecular weight, arguing that these proteins (particularly pp32 and APRIL) interact directly with HuR. All four ligands contain highly acidic

COOH-terminal tails. Since other human proteins possessing equally long acidic regions exist [e.g., nucleolin (Srivastava et al., 1990), nucleosome assembly protein (Simon et al., 1994), transcription factor UBF (Jantzen et al., 1990)], the selection of these proteins argues that their association with HuR reflects more than simply the affinity of an RNA binding protein for a negatively charged polymer. Only the extreme NH₂-terminal sequences vary between SET α and SET β , indicating that they are splice variants of one another. pp32 and APRIL exhibit a high degree of sequence identity (71%) and similarity (81%), and both contain several rev-like leucine-rich motifs.

Like HuR, pp32 and APRIL are nucleocytoplasmic shuttling proteins. The leucine-rich repeats present in both proteins hinted that their nuclear export might occur through binding of the nuclear export receptor, CRM1. Coimmunoprecipitation of CRM1 with pp32 and APRIL and the observation that leptomycin B inhibits their shuttling provides confirmation of this export pathway. Interestingly, LMB significantly increases the interaction of pp32 and APRIL not only with CRM1 (however, see previous *in vitro* studies with rev; Askjaer et al., 1998), but also with HuR. The concomitant increased cross-linking of HuR to nuclear poly(A)⁺ RNA suggests that the association of HuR with its ligands may modulate HuR's interaction with its target ARE-containing mRNAs *in vivo*. At least under these conditions, CRM1 appears to be instrumental in the export of an ARE-containing mRNA (*c-fos*).

The HuR binding proteins that we have identified appear in the literature in a remarkable diversity of contexts. However, a common finding is that SET α , SET β , and pp32 are inhibitors of protein phosphatase 2A (Li et al., 1996; Saito et al., 1999). Another shared property of the HuR ligands is their association with cell growth or differentiation. The SET proteins have long been implicated as players in leukemogenesis. The SET locus was first identified as a partner in chromosomal translocations with the CAN/Nup214 locus in patients with acute undifferentiated leukemia (von Lindern et al., 1992). Subsequently, SET β was observed in a complex with HRX leukemic fusion protein and PP2A in myeloid leukemic cell extracts (Adler et al., 1997). SET α and SET β have been shown to associate with histone/DNA complexes (as template activating factor 1, TAF1, α and β) and to remodel chromatin structure *in vitro* (Okuwaki and Nagata, 1998). Both SET β and pp32 were purified as HLA class II-associated proteins from human lymphoblastoid cells (Vaesen et al., 1994). pp32 is expressed at high levels in neoplastic cells, as well as in normal tissues competent for self-renewal (Walensky et al., 1993). The pattern of expression of pp32 (LANP) during postnatal development suggested a role in the differentiation of cerebellar neurons (Matsuoka et al., 1994). More recently, pp32 has been observed to inhibit the oncogene-mediated transformation of rat embryo fibroblasts (Chen et al., 1996) and to be present in intranuclear aggregates of mutant ataxin-1 that are associated with (Matilla et al., 1997), but not essential for (Klement et al., 1998), neural degeneration. Alternatively, pp32 has been observed to associate with microtubule-associated proteins, including MAP2, MAP4, and tau *in vitro* (mapmodulin; Ulitzur et al., 1997a). APRIL was only recently identified (Mencinger et al., 1998) and therefore has not been well studied.

Our localization of the protein domains responsible for intermolecular contacts with HuR suggests that the ligands' common acidic tails are critical (Fig. 4). In the case of pp32, both its acidic region (amino acids 168–249) and other portions of the molecule have been previously demarcated as functionally important. Amino acids 150–174 of pp32 are absolutely required for inhibiting the appearance of transformed foci elicited by oncogene pairs (Brody et al., 1999), whereas the first 147 amino acids of pp32 (as LANP) interact with ataxin-1 (Matilla et al., 1997). pp32's ability to modulate the interaction of microtubule-associated proteins with microtubules (as mapmodulin) has also been assigned to its acidic, COOH-terminal domain (Ulitzur et al., 1997a). In SET, it is the NH₂-terminal region that is required for PP2A inhibitor activity, whereas the acidic tail contributes to chromatin remodeling *in vitro* (Saito et al., 1999). The acidic tail of SET is included in the SET-CAN fusion implicated in leukemogenesis (von Lindern et al., 1992). Thus, some of these ligand interactions with other cellular molecules would be predicted to be compatible with, while others would be expected to compete with, their binding to HuR. This remains to be investigated.

In HuR, deletion of the third RRM alone abrogates recognition by SET α , SET β , pp32, and APRIL, whereas the hinge region and RRM3 comprise a minimal substrate for efficient interaction with these ligands (Fig. 4). Previously, these two domains have been characterized as being important for HuR's ability to shuttle between the nucleus and the cytoplasm and to bind and stabilize ARE-containing mRNAs (Ma et al., 1997; Fan and Steitz, 1998a), respectively. Interestingly, the third RRM has been shown to be important for the functioning of other Hu-family proteins. For instance, RRM3 is not only essential for the full activity of HuB and HuC in inducing a neuronal phenotype upon overexpression in PC12 cells, but it also acted as a dominant negative protein when cotransfected with wild-type HuB or HuC, and even when expressed *in vivo* in cells of the embryonic central nervous system (Akamatsu et al., 1999). Similarly, RRM3 and the hinge region of HuD were observed to be important for neurite-inducing activity in PC12 cells (Kasashima et al., 1999). While it remains to be shown that HuB, HuC, and HuD associate with the HuR ligands we have characterized, the high degree of conservation among the Hu proteins (Okano and Darnell, 1997) suggests that interaction is likely. Perhaps by binding to HuR and the other Hu proteins, the HuR ligands facilitate the stabilization of ARE-containing mRNAs and promote cellular differentiation.

It was surprising to find that leptomycin B produces greater cross-linking of HuR to nuclear poly(A)⁺ RNA (Fig. 8), even though CRM1 does not directly bind HuR, and LMB fails to inhibit HuR shuttling (Fig. 7). Specifically, increased ligand binding induced by LMB might have been expected to decrease HuR shuttling (perhaps by sequestering HNS), leading to greater retention of HuR in the nucleus and therefore greater cross-linking to nuclear RNA. However, it is not clear from our experiments whether interaction with its ligands does not in fact alter the nucleocytoplasmic trafficking of HuR: even though ~50% of HuR is bound by pp32 and APRIL in the presence of leptomycin B (Fig. 8), shuttling of the remaining

free HuR could generate the positive result obtained in the heterokaryon experiment of Fig. 7. Rather, our observation of HuR shuttling in the presence of LMB can only be interpreted to indicate that the HNS of HuR (which contains no leucine-rich repeats) is not recognized by CRM1 (Fig. 7), but instead by some other export receptor.

An alternative explanation for the LMB-induced increase in the cross-linking of HuR to nuclear poly(A)+ RNA is that ligand binding stabilizes HuR's interaction with ARE-containing mRNAs. Since *in vivo* cross-linking experiments like those in Fig. 8 B have demonstrated that SET α / β , pp32, and APRIL do not detectably bind RNA directly (data not shown), the change is likely to be propagated through RRM3 of HuR. Conceivably, the acidic tail of a ligand could occupy the RNA binding site of RRM3 and thereby displace the poly(A) tail of the mRNA. It is not clear whether this would decrease or increase HuR binding to ARE-containing mRNAs since the other two RRMs of HuR have been reported to have much higher affinity and to be specific for ARE sequences (Ma et al., 1997). Further analyses are required. It will also be important to establish whether interaction with its ligands under normal conditions enhances HuR's binding to ARE-containing mRNAs, as it appears to do in the presence of leptomycin B (Fig. 8 B).

Whatever the mechanism, LMB clearly causes the selective retention of *c-fos*, an ARE-containing mRNA, in the nucleus (Fig. 9 B). Such abnormal nuclear retention could also be the basis for numerous reports of ARE-containing mRNA stabilization in cells subjected to various stress conditions (Andrews et al., 1987; Lindsten et al., 1989; Hirsch et al., 1995; Gorospe et al., 1998; Levy et al., 1998; Ming et al., 1998). Clearly, it is most important to investigate in each case whether the mRNA has been transported to the cytoplasm and is undergoing translation. Although it is possible that leptomycin B inhibits other components of the nucleocytoplasmic trafficking machinery, the most straightforward interpretation of the results in Fig. 9 B is that CRM1 is involved in *c-fos* mRNA export via HuR interaction with its ligands. Thus, CRM1 should be considered a potential nuclear export receptor for certain cellular mRNAs (perhaps all ARE-containing messages). The possibility that the dominant export pathway for a particular mRNA may switch, subject to changes in cellular physiology, is also important to consider.

Our discovery of three HuR ligands (SET α , SET β , and pp32) that were previously identified as inhibitors of protein phosphatase 2A (Li et al., 1996; Saito et al., 1999) suggests that a regulatory cascade involving this enzyme influences ARE-mediated mRNA stability. PP2A is a highly conserved multimeric serine/threonine phosphatase, affecting a wide variety of cellular functions, including cell cycle progression, DNA replication, transcription, splicing, development, and morphogenesis (Millward et al., 1999). It is dynamic, capable of reversible interconversion between holoenzyme forms in response to stimulation (Zhu et al., 1997). PP2A's ability to dephosphorylate both the substrates of kinases and kinases themselves appears to be an important aspect of its function. This phosphatase regulates the activities of several major protein-kinase families, including those of the AGC subgroup [e.g., protein kinase B (Andjelkovic et al., 1996), protein kinase C (Hansra et

al., 1996), and p70 S6 kinase (Ballou et al., 1988)], the calmodulin-dependent kinases (Westphal et al., 1998), and members of the ERK MAP-kinase pathway (Anderson et al., 1990; Gomez and Cohen, 1991). The recent demonstration that expression of MAP kinase-activated protein kinase 2 leads to stabilization of ARE-containing mRNAs (Dean et al., 1999; Winzen et al., 1999) highlights the importance of particular kinases. Our data argue that in these signaling cascades, the HuR binding proteins act directly on HuR, with PP2A placed farther upstream in the pathway(s) that regulate ARE-mediated mRNA stability.

Since we and others have observed that pp32 and APRIL are phosphoproteins (data not shown) (Walensky et al., 1993; Ulitzur et al., 1997b), it is tempting to speculate that their shuttling, or their interactions with HuR, could be regulated by phosphorylation. This idea is particularly attractive since our attempts to identify a phosphorylated form of HuR have been unsuccessful (data not shown). Experiments are currently underway to determine whether particular phosphorylated forms of pp32 and APRIL selectively bind and influence the activity of HuR.

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