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RNA-binding protein HuR autoregulates its expression by promoting alternative polyadenylation site usage

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ABSTRACT
RNA-binding protein HuR modulates the stability and translational efficiency of messenger RNAs (mRNAs) encoding essential components of the cellular proliferation, growth and survival pathways. Consistent with these functions, HuR levels are often elevated in cancer cells and reduced in senescent and quiescent cells. However, the molecular mechanisms that control HuR expression are poorly understood. Here we show that HuR protein autoregulates its abundance through a negative feedback loop that involves interaction of the nuclear HuR protein with a GU-rich element (GRE) overlapping with the HuR major polyadenylation signal (PAS2). An increase in the cellular HuR protein levels stimulates the expression of long HuR mRNA species containing an AU-rich element (ARE) that destabilizes the mRNAs and thus reduces the protein production output. The PAS2 read-through occurs due to a reduced recruitment of the CstF-64 subunit of the pre-mRNA cleavage stimulation factor in the presence of the GRE-bound HuR. We propose that this mechanism maintains HuR homeostasis in proliferating cells. Since only the nuclear HuR is expected to contribute to the auto-regulation, our model may explain the longstanding observation that the increase in the total HuR expression in cancer cells often correlates with the accumulation of its substantial fraction in the cytoplasm.

INTRODUCTION
Eukaryotes employ multiple posttranscriptional mechanisms to adjust their gene expression programs in response to external cues and changes in the physiological status of the cell (1,2). A major part in the posttranscriptional hierarchy is played by RNA-binding proteins (RBPs) that regulate virtually every aspect of the messenger RNA (mRNA) metabolism, from nuclear processing reactions and export to the cytoplasm to translation and stability (3,4). The extent of this regulatory paradigm is illustrated by the fact that hundreds of distinct RBPs are encoded in mammalian genomes and many of these proteins still await their detailed functional characterization (5).

Most RBPs interact with fairly short or degenerate RNA elements that occur in many RNA species (6,7). Interestingly, mRNAs encoding components of a specific biological pathway often contain binding sites for a particular RBP or a group of related RBPs, which may facilitate coordinated regulation of these mRNA species (8,9). A striking example of such regulatory network is provided by a cohort of RBPs involved in mRNA turnover and/or translation regulation (TTR-RBPs) (10). Examples of TTR-RBPs include AUF1/hnRNP D, BRF1, KSRP, tristetraprolin (TTP), TIAl and TIAR proteins, as well as four mammalian homologs of the Drosophila ELAV protein (embryonic lethal abnormal vision): the ubiquitously expressed HuR/HuA/ELAVL1 and the nervous system-specific HuB/ELAVL2, HuC/ELAVL3 and HuD/ELAVL4 proteins (10–13).

Similar to other TTR-RBPs, Hu proteins normally function by interacting with AU- or U-rich sequence elements (ARE) frequently found within 3'-untranslated regions (3'-UTRs) and sometimes other parts of mRNAs encoding proto-oncogenes, cytokines, lymphokines and other functionally coherent protein subsets (12,14–17). Hu proteins tend to stimulate mRNA stability and translational efficiency, although in several cases they have been shown to promote mRNA decay or repress its translation (10,12,14–16,18–31).

HuR protein and its nervous system-specific paralogs contain three RNA-recognition motif (RRM) domains. Of these, the two N-terminal domains bind U-rich RNA sequences whereas the C-terminal RRM has been...
proposed to stabilize Hu-RNA complexes, bind poly(A) sequences and be involved in protein-protein interactions [15] and references therein. The second and the third RRM s are connected by a basic hinge peptide containing the HuR nucleocytoplasmic shuttling sequence (HNS) (32). HuR protein localizes predominantly to the nucleus; however, its cytoplasmic fraction has been reported to increase transiently at specific stages of the cell cycle or in response to stress and growth factors (16,27,28,30,33–35).

In addition to their involvement in the mRNA stability and translation pathways, Hu proteins have been implicated in regulation of multiple alternative pre-mRNA splicing and cleavage/polyadenylation events in the nucleus (15,17,36–38), evocative of the similar activities of the Drosophila ELAV protein (39–41). Pre-mRNA cleavage and polyadenylation in metazoan organisms rely on the activity of several protein factors (42–44). Of these, cleavage/polyadenylation specificity factor (CPSF) binds to the polyadenylation signal (PAS) and catalyzes the pre-mRNA cleavage. The 3′-end of the cleaved pre-mRNAs is then polyadenylated by the nuclear polyadenylate polymerase (PAP). Other important components of the cleavage/polyadenylation machinery are cleavage factors I and II (CFI and CFII) and the cleavage-stimulation factor (CstF). The CstF-64 subunit of the CstF interacts with GU-rich sequence elements that normally occur downstream of the future cleavage site [downstream sequence elements (DSEs)]. Notably, Hu proteins may compete with CstF-64 for binding to U-rich sequences adjacent to the PASs of some cellular and viral pre-mRNAs (37).

Despite the advanced state of our knowledge of the HuR functions, the mechanisms regulating its expression are not understood completely. Cellular HuR protein abundance may undergo major changes in specific physiological situations. For example, it is dramatically reduced in HeLa cells during heat shock as a result of proteosome-dependent degradation (45). HuR protein levels are also diminished in senescent and quiescent cells (33,46). In senescent cells, this effect appears to be mediated by a positive feedback auto-regulatory loop, in which interaction of HuR protein with the 3′-UTR of its own mRNA is required for the mRNA export from the nucleus to the cytoplasm (46). Since the HuR protein localization in senescent cells is exclusively nuclear, the export of the HuR mRNA is arrested leading to its reduced translation (46).

Several alternatively polyadenylated isoforms have been described for both human and mouse HuR mRNAs (47,48). Although the mechanism regulating the PAS choice is currently unknown, the longer mRNA isoforms encoding human HuR have been shown to be destabilized by a functional ARE sequence (48). Interestingly, overexpressed HuR protein partially rescued the stability of these mRNA species (48), which might potentially provide another positive feedback mechanism contributing to the reduced HuR expression in senescent cells or under some other circumstances.

On the other hand, the HuR levels are often increased in tumors as compared to the normal cells of the respective lineages (22,49,50). This is often accompanied by the HuR accumulation in the cytoplasm, which may increase the expression of proto-oncogenes, anti-apoptotic and pro-metastatic proteins, angiogenic growth factors and immunosuppressive cytokines, and decrease the expression of tumor suppressors (14,16,19,21,22,50–52). The cellular HuR levels and its cytoplasmic localization may therefore be used as prognostic markers for tumor severity (51–63). The elevated HuR expression in cancer cells may in part be due to the reduced levels of microRNAs miR-16, miR-125 or/and miR-519 that have been shown to target the HuR mRNA (64–67). However, it is not clear whether the lack of repression by these three miRNAs is sufficient to account for the elevated HuR expression in all cancer types. Moreover, the possible causative link between the cytoplasmic accumulation and the total HuR expression has not been studied.

Notwithstanding the above examples, HuR levels remain relatively constant throughout the cell cycle and during several forms of stress other than heat shock (27,35,45). This may suggest the existence of an unknown homeostatic mechanism controlling the HuR expression in proliferating cells. Interestingly, the expression of the ELAV protein in Drosophila is autoregulated through a negative feedback loop, which involves ELAV protein-regulated switch in the alternative splicing pattern of the ELAV pre-mRNA (41,68). In this study, we investigated the mechanisms controlling the HuR abundance in proliferating mammalian cells.

**MATERIALS AND METHODS**

**Plasmids**

Full-length mouse HuR 3′-UTR was PCR-amplified from BAC RP24-147J6 (BACPAC Resources Center at Children’s Hospital Oakland Research Institute, Oakland, CA, USA) using KAPA HiFi DNA polymerase (KAPA Biosystems) and EMO224/EMO225 primers (Supplementary Table S1) and cloned into the psiCHECK-1 vector (Promega) to generate pEM429 plasmid (also referred to as RLuc-3HuR-wt). To construct pEM430 plasmid (RLuc-3′HuR-PAS2mut), the PAS2 sequence in pEM429 was mutated with EMO128/EMO129 primers (Supplementary Table S1; mutated nucleotides underlined) using a modified Quikchange site-directed mutagenesis protocol (Stratagene), in which PfuTurbo was substituted with KAPA HiFi DNA polymerase. ARE sequence was deleted from pEM429 and pEM430 using mutagenic primers EMO1117/EMO1118 (Supplementary Table S1) giving rise to pEM801 (RLuc-3′HuR-ΔARE) and pEM657 (RLuc-3′HuR-PAS2mut/ΔARE), respectively. Reporter plasmid containing mouse HuR ARE (pEM806) was created by cloning the corresponding PCR product (EMO1646/EMO1647; Supplementary Table S1) into the psiCHECK-1 vector. To construct a lentiviral plasmid encoding a HuR-specific shRNA (pEM505), annealed EMO835/EMO836 oligonucleotides (Supplementary Table S1) were cloned into the pTRIPZ vector (Open Biosystems, Huntsville, AL, USA) at the XhoI and
EcoRI sites. pSV3-neo was obtained from the ATCC. To generate the pEM821 plasmid, the fragment encoding the SV40 large T antigen (LTA) was removed from pSV3-neo using AvaI and HpaI (NEB). pBOS-T7-HuR and pEF-BOS have been described previously (69,70). Vector pEM705 encoding a bicistronic dTomato-IRES-EGFP cassette flanked with Lox2272 and LoxP sites has been described (71). To prepare the pEM725 plasmid for HILO-RMCE knock-ins of the T7-HuR gene, pBOS-T7-HuR-derived PCR fragment amplified with EMO1088/EMO1089 (Supplementary Table S1) was cloned at the XhoI–NotI sites of the pEM705 vector in place of the dTomato-IRES-EGFP cassette. To generate the pEM707 and pEM708 plasmids, the wild-type or the PAS2-mutated GU-rich element (GRE)/PAS2 fragments were amplified from pEM429 or pEM430, respectively, using EMO703/EMO704 (Supplementary Table S1). The PCR fragments were digested with PstI–EcoRI (underlined) and cloned into pEM705 at the NsiI–MfeI sites. Plasmids pEM849 and pEM850 encoding the GRE and the ARE-containing fragments used in the PAS2-mutated GRE/PAS2 fragments are cloned into pEM705 at the NsiI–MfeI sites. Plasmids pEM849 and pEM850 encoding the GRE and the ARE-containing fragments used in the biotinylated RNA pull-down assays were generated by cloning the corresponding PCR fragments amplified with EMO703/EMO704 and EMO1518/EMO1519 primer pairs (Supplementary Table S1) into the pCR4Blunt-TOPO vector (Invitrogen). Maps of all pEM plasmids are available upon request.

Cell cultures

Cell lines were obtained from ATCC and propagated at 37°C in the presence of 5% CO₂. HEK293T, HeLa, HeLa S3, L929, and NIH 3T3 were cultured in DMEM/High glucose medium (Hyclone) supplemented with 10% Fetal Bovine Serum (FBS, Characterized grade; Hyclone), 1 mM sodium pyruvate (Invitrogen) and 1x penicillin-streptomycin (100 U/ml penicillin, 100 µg/ml streptomycin; Invitrogen). P19 cells were cultured in αMEM medium containing 10% FBS and 1x penicillin–streptomycin. When required, media were supplemented with 5 µg/ml blasticidin S, 2–5 µg/ml puromycin or 2 µg/ml doxycycline. Lipofectamine 2000 (Invitrogen) was used to transfect cells with plasmid DNA as recommended.

Transgene integration using recombination-mediated cassette exchange

The high efficiency/low background (HILO) recombination-mediated cassette exchange (RMCE) procedure has been described elsewhere (71). Briefly, P19 and NIH 3T3 cells were transduced with a lentiviral vector encoding a Cre RMCE acceptor site comprised of a blasticidin S resistance gene (Bsd) flanked by the mutually incompatible Lox2272 and LoxP sites. The blasticidin-resistant clones containing a single genomic copy of the proviral acceptor site were co-transfected with a modified version of the Cre recombinase-expressing plasmid pCAGGS-Cre (72) and an RMCE donor plasmid containing a desired cassette flanked by the Lox2272 and LoxP sites. The donor plasmids additionally contained a puromycin resistance marker, which allowed the selection of the correctly recombined cell populations with puromycin.

RNA interference

ON-TARGETplus SMARTpool siRNAs against mouse HuR or TTP (Dharmacon/Thermo Scientific) were delivered into cells using Lipofectamine 2000 (Invitrogen) as recommended. Cells transfected with siRNAs were harvested 78 h post-transfection. Lentivirus vector encoding a HuR-specific shRNA was prepared by co-transfecting HEK293T cells with the pEM505 plasmid and the Lenti-X HT packaging mix using Lentiphos HT transfection reagent (Clontech) as recommended. Medium was changed 12 h after transfection and the plate was incubated for another 48 h before collecting the supernatant containing the lentivirus vector particles. The supernatant was cleared from the cellular debris by centrifugation at 1000 g for 5 min and frozen at −80°C in small, single use aliquots. Virus titers were determined using HEK293T cells as recommended (Open Biosystems). Stable cell lines were obtained from individual lentivirus-transduced puromycin-resistant colonies. shRNA expression was induced with doxycycline for 48–72 h.

Reverse transcription–quantitative PCR

Reverse transcription–quantitative PCR (RT–qPCR) was carried out as described (73). Briefly, total RNA was purified from cell cultures using Trizol (Invitrogen) as recommended. RNA samples were treated with 50 units/ml of RNase-free DNase (Promega) at 37°C for 30 min to remove genomic DNA contamination. Reverse transcription (RT) was carried out using SuperScript III (Invitrogen) and random decamer (N10) primers at 50°C for 1 h. cDNA samples were analyzed by quantitative PCR using Fast SYBR Green Master Mix and a StepOnePlus Real-Time PCR System (Applied Biosystems) as recommended. Primers used for qPCR are listed in Supplementary Table S1. The expression of targets of interest was normalized to the expression level of the Hprt gene (primers EMOS92/593; Supplementary Table S1). Reactions were performed in triplicate.

In vitro pre-mRNA cleavage assays

Radiolabeled RNA substrates for the cleavage assays were prepared using T7 RNA polymerase transcription as recommended (Promega). Reactions (20 µl) contained 50 U of [α-32P]UTP (Perkin Elmer), 0.8 mM Ribo mG Cap analog (Promega) and 1 µg of either pEM810 plasmid linearized with PmeI (NEB) or pEM811 plasmid linearized with EcoRI (NEB). Following 1.5 h incubation at 37°C, the labeled RNAs were treated with RNase-free DNase (Promega; 1 unit/µg of template DNA) for 15 min at 37°C, extracted with a 1:1 mixture of phenol and chloroform, precipitated with ethanol and rehydrated in DEPC-treated water (Invitrogen). Pre-mRNA cleavage assays were carried out in principle as described (74). Reaction mixtures (25 µl) contained 40% (v/v) HeLa S3 nuclear extract (dialyzed against buffer D containing 20 mM HEPES, pH 7.9, 1.5 mM MgCl₂, 100 mM KCl, 20% Glycerol, 0.5 mM DTT and 0.05 mM EDTA), 0.08 mM ATP (Promega), 1 mM cordycepin (Sigma),
20 mM phosphocreatine (Sigma), 1.5% PEG8000 (Sigma), 0.01% NP-40, 2 mM DTT, 10 U rRNAsin (Promega), 0.1 mg/ml yeast tRNA and 60 fmol of 32P-labeled substrate RNA. Reactions were incubated at 30°C for 2.5 h followed by digestion with 0.8 mg/ml proteinase K (Fermentas) at 37°C for 15 min. The reactions were extracted with phenol-chloroform, precipitated with ethanol and analyzed by 5% polyacrylamide-7 M urea gel electrophoresis. The radioactive signals were visualized using a Typhoon Trio imager (GE Healthcare).

**Biotinylated RNA pull-down assays**

Biotinylated RNA probes were prepared using in vitro transcription of appropriate linearized plasmid templates with T7 or T3 RNA polymerases (Promega) in the presence of the biotin-UTP labeling NTP mixture (Roche) as recommended. The reactions were incubated for 2 h at 37°C, followed by incubation with RQ1 DNase (Promega; 1 U/µg of template DNA) for 15 min at 37°C. The biotinylated RNAs were then extracted with phenol-chloroform (1:1) mixture, precipitated with ethanol and rehydrated in DEPC-treated water. HuR/RNA complexes were allowed to form at room temperature for 30 min in 20 µl mixtures containing 2 µg biotinylated RNA probe, 40% (v/v) nuclear extract (~100 µg in total), 4 µg yeast tRNA, 50 µg heparin and 40 units of rRNasin (Promega), 20 mM Hepes–KOH, pH 7.9, 20% glycerol, 100 mM KCl, 0.2 mM EDTA, 0.5 mM DTT (in addition to the concentrations of these components in the nuclear extract). CstF-64/RNA complexes were assembled at 30°C for 20 min in 50 µl reactions containing 1 µg biotinylated RNA probe, contained 40% (v/v) nuclear extract diluted with buffer D to 1 mg/ml (i.e. 20 µg in total), 2 µg yeast tRNA, 0.8 mM ATP. The complexes were further stabilized by UV cross-linking at 254 nm, 1.8 J/cm², 0.01% NP-40, 2 mM DTT, 20 mM phosphocreatine, 10 U of rRNAsin (Promega). Both HuR/RNA and the UV-cross-linked CstF-64/RNA complexes were then incubated with 10 µl Streptavidin Sepharose (Sigma) for 1 h at 4°C followed by three washes with 8 mM HEPES–KOH, pH 7.9, 0.6 mM MgCl2, 40 mM KCl, 8% Glycerol, 0.2 mM DTT and 0.02 mM EDTA. The bead-associated proteins were eluted by treating the beads with 1 mg/ml RNase A for 15 min at 37°C (this step was omitted for the HuR/RNA complexes) followed by boiling with SDS–PAGE loading buffer for 5 min. The eluted proteins were analyzed by immunoblotting.

**Estimating mRNA half-lives**

HEK293T cells were transfected with pEM429 (RLuc-3HuR-wt) or pEM430 (RLuc-3HuR-PAS2mut) plasmids overnight to allow the expression of the corresponding RLuc mRNAs. On the next day, the RNA polymerase II inhibitor actinomycin D (Sigma) was added to cultures to the final concentration of 5 µg/ml and the total cellular RNA was extracted with Trizol at 0–8-h time points. RT–qPCR with RLuc-specific primers (EMO1264/EMO1265; Supplementary Table S1) was used to follow the mRNA degradation time course of the data normalized to the RPLP0 mRNA RT–qPCR signals (primers EMO2185/EMO2186; Supplementary Table S1) were plotted as a function of the actinomycin D treatment time and fitted to a single exponential decay function to determine the apparent mRNA half-lives.

**RNA immunoprecipitation**

Ten million NIH 3T3 cells UV cross-linked at 254 nm, 0.4 J/cm² were either directly resuspended in the lysis buffer [50 mM HEPES (pH 7.0), 60 mM KCl, 5 mM MgCl2 and 0.5% NP-40, 1 mM DTT, 0.1 U/µl rRNAsin] or used to prepare nuclear extracts with the NE-PER kit (Pierce) as recommended. The extracts pre-cleared by incubation with protein A or G Sepharose beads (GE) at 4°C for 4 h were incubated with rabbit anti-HuR antibody (Millipore), anti-CstF4 mouse monoclonal antibody (clone 3A7; a gift from Clinton MacDonald, Texas Tech University) or corresponding preimmune IgG controls at 4°C overnight. The antigen-antibody complexes were mixed with protein A or G beads, as appropriate, and incubated at 4°C for 4 h with constant agitation. The beads were washed with the lysis buffer followed by incubation with the lysis buffer supplemented with 0.1% SDS and 0.5 mg/ml proteinase K (Fermentas) at 50°C for 30 min. RNAs were extracted with phenol-chloroform and precipitated with ethanol. Traces of genomic DNA were removed by incubating the samples with RQ1 DNase (Promega) for 15 min at 37°C, which was followed by RT–PCR analyses with either mouse HuR-specific primers designed to amplify a cDNA fragment spanning the 3’-terminal exon–exon junction (EMO2294/2295; Supplementary Table S1) or similarly designed Hprt-specific primers (EMO592/593; Supplementary Table S1).

**RESULTS**

**Mouse HuR expression is autoregulated by a negative feedback mechanism**

To understand the regulation of HuR expression in proliferating mammalian cells, we integrated a single copy of a constitutively expressed T7 epitope-tagged mouse HuR transgene into the P19 mouse embryonic carcinoma cell genome using the high-efficiency and low-background recombination-mediated cassette exchange (HILO-RMCE) technique recently developed in our laboratory (Figure 1A). Using immunostaining with T7 tag-specific antibodies we confirmed that virtually all cells in the transgenic populations expressed T7-HuR with T7 tag-specific antibodies we confirmed that virtually all cells in the transgenic populations expressed T7-HuR in the parental P19 cells (Figure 1C).
To ensure that this effect was not cell line-specific, we integrated the T7-HuR expression cassette into the genome of mouse NIH 3T3 cells using HILO-RMCE and analyzed the expression of the transgenic and endogenous HuR proteins by immunoblot (Figure 1D). The transgenic T7-HuR appeared to be expressed in NIH 3T3 more efficiently than in P19, potentially due to a higher CAG activity in the former line. The T7-HuR overexpression diminished the expression of the endogenous HuR to a dramatically low level as compared to the control transgenic cells lacking the T7-HuR gene (Figure 1D). We concluded that the expression of the endogenous HuR protein is reduced in the presence of the transgenic T7-HuR. (D) Immunoblot analysis of the HuR expression in the NIH 3T3 cell population expressing the T7-HuR transgene (Tg) and control transgenic cells lacking the T7-HuR (control). Similar to (C), the expression of the endogenous HuR protein is reduced in the presence of T7-HuR. (C and D) βTub, β-tubulin loading control.

HuR 3'-UTR contains cis-elements responsible for the negative autoregulation

To test if, similar to the Drosophila ELAV protein, the HuR protein may repress its expression by interacting with the 3'-terminal part of its pre-mRNA, we generated a Renilla reniformis luciferase reporter gene fused with a ~5 kb fragment comprising the 3' untranslated region (3'-UTR) of the mouse HuR gene (RLuc-3'HuR). The 3'-UTR of the mouse HuR gene contains several alternative PASs including PAS1, PAS2, PAS3 and five closely positioned signals that we refer to as PAS4a-e (Figure 2A). Of these, PAS2 appears to be used most frequently since it is supported by the largest number of sequenced cDNA clones (Figure 2B). When human embryonic kidney (HEK) 293T cells were co-transfected with the RLuc-3'HuR construct and either a T7-HuR expression plasmid (pBOS-T7-HuR) or the corresponding empty vector (pEF-BOS), the RLuc expression was reduced 4.7-fold ($P = 2.7 \times 10^{-4}$; t-test) in the presence of T7-HuR as compared to the pEF-BOS control (Figure 2C). In contrast, T7-HuR did not affect the expression of the control RLuc construct containing a 3'-UTR sequence from the SV40 virus (Figure 2C). Thus, the mouse HuR 3'-UTR contains sequence(s) mediating the negative feedback regulation.

HuR protein differentially regulates the abundance of different alternatively polyadenylated forms of its mRNA

To examine whether the excess of the HuR protein repressed the RLuc-3' HuR expression by reducing the mRNA abundance, we repeated the co-transfection experiment and analyzed the RNA samples by RT–qPCR. Four primer pairs were designed to account for the possibility of alternative PAS usage (F1/R1, F2/R2, F3/R3 and F4/R4; Figure 2A). Quantitative amplification of fragments upstream of the PAS2 indicated that the RLuc-3' HuR mRNA expression indeed decreased 2.5–2.6-fold ($P < 0.001$; t-test) in the T7-HuR sample as compared to the pEF-BOS control (Figure 2D).
Surprisingly, however, the RT-qPCR signals corresponding to the sequences downstream of the PAS2 increased 1.4–1.5-fold (P < 0.0001; t-test) (Figure 2D). This suggested that, in addition to reducing the total HuR mRNA abundance, T7-HuR overexpression simultaneously caused a ≥3.5-fold (1.4 × 2.5) increase in the expression of the mRNA forms terminated at a downstream PAS (Figure 2D).

We wondered if the endogenous HuR mRNA was regulated in the similar manner. As judged by the northern blot analysis, the predominant HuR mRNA form terminated at the PAS2 sequence in all three mouse cell lines used in this study (P19, NIH 3T3 and L929) express predominantly PAS2-terminated HuR mRNA species. Expected positions of the four HuR mRNA forms are marked on the left. (F and G) RT-qPCR analyses showing that the expression of the transgenic T7-HuR protein in P19 and NIH 3T3 cells leads to a downregulation of the total HuR mRNA levels and a simultaneous increase in the PAS4-terminated mRNA fraction. Parental P19 cells are used as a control in (F), whereas NIH 3T3 cells containing a transgenic vector sequence are used as a control in (G). Data in (C and D) and (F and G) are averaged from three experiments ± SD.
mouse cell lines used in this study (P19, NIH 3T3 and L929; Figure 2E). To examine if elevated HuR expression changed the relative abundance of the alternatively polyadenylated HuR mRNA forms, we analyzed the parental P19 cells and the P19 cells expressing the T7-HuR transgene by RT–qPCR with primer pairs shown in Figure 2A. Similar to the effect on RLuc-3’HuR transcripts, the transgenic T7-HuR expression diminished the total levels of the endogenous HuR mRNA 1.2–1.6-fold (P < 0.05, t-test), while simultaneously elevating the expression of the PAS4-terminated mRNA species 1.4–1.5-fold (P < 0.05, t-test). More dramatic changes were detected in the T7-HuR expressing NIH 3T3 cells, where the total HuR mRNA levels decreased 3.0-fold (P = 3.0 × 10⁻³, t-test) and the abundance of the PAS4 species rose 2.1-fold (P = 0.02, t-test) (Figure 2G). Taken together, these data suggest that the HuR auto-regulation involves a change in the ratio between its long and short mRNA species.

Two alternative models for HuR homeostasis

The longer form of the human HuR mRNA has previously been shown to contain a functional ARE sequence that destabilized a reporter mRNA in a HuR protein-repressible manner (48). Inspection of the mouse and other mammalian HuR 3’-UTRs revealed similar ARE sequences downstream of the PAS2 site (Figure 2A and Supplementary Figure S1A). On the other hand, HuR and its mammalian paralogs have previously been shown to repress cleavage and polyadenylation at PAS sites occurring within U-rich sequence contexts (37). Interestingly, the mouse PAS2 is embedded within a GRE, which is conserved across mammalian species (Figure 2A and Supplementary Figure S1B). We therefore considered two alternative models for the HuR autoregulation. (i) An excess of the HuR protein can stabilize the ARE-containing mRNA forms but reduce the total RLuc-3’HuR mRNA abundance through an independent mechanism. (ii) Alternatively, HuR can inhibit the pre-mRNA processing at the PAS2 site while failing to provide sufficient protection to the ARE-containing mRNA species terminated at the downstream PAS sequences. The following experiments were carried out to distinguish between these two possibilities.

The long forms of mouse HuR mRNA are destabilized through an ARE-dependent mechanism

To estimate the stability of the longer forms of mouse HuR mRNA, we inactivated the PAS2 in the RLuc-3’HuR-wt reporter construct by mutating the AU UAAA hexamer (Supplementary Figure S1B) to AUGGA A and analyzed the mRNA species produced from the wild-type and the mutant genes in HEK293T cells by northern blotting (Figure 3A and B). Four distinct species of the RLuc-3’HuR-wt transcripts were detected that matched the predicted lengths of the corresponding alternatively polyadenylated forms (Figure 3B). As expected, the PAS2-terminated form was the most abundant one. Mutation of the PAS2 site completely blocked the pre-mRNA processing at this position and, surprisingly, at the downstream PAS3 sequence (Figure 3B). The functional inactivation of PAS2 and PAS3 did not result in a proportional increase in the abundance of the longer mRNA species, consistent with the model that the PAS4-terminated transcripts are less stable than those terminated at the upstream PASs. Indeed, the apparent half-life of the RLuc-3’HuR-PAS2mut mRNA was noticeably shorter than that of the RLuc-3’HuR-wt mRNA (128 min vs. 346 min; Figure 3C). Moreover, the RLuc-3’HuR-PAS2mut construct generated 8.7 times less RLuc protein than the wild-type reporter gene (Figure 3D; P = 7.0 × 10⁻⁶; t-test).

To determine whether the ARE is responsible for the instability of the PAS4-terminated transcripts, we deleted the ARE sequence in the wt and the PAS2mut RLuc-3’HuR reporters (Figure 3A) and repeated the luciferase assay. The expression of the genes lacking the ARE was significantly higher than their ARE-containing counterparts, which was especially evident in the PAS2mut background (Figure 3E; 4.3-fold increase; P = 4.1 × 10⁻⁸; t-test).

TTP has been shown to promote the ARE-dependent decay of the long forms of the human HuR mRNA (48). We therefore knocked down TTP by RNA interference (RNAi) (Figure 3F) and analyzed the expression levels of the PAS2mut and PAS2mut/ΔARE versions of the RLuc-3’HuR genes using the luciferase assay. Down-regulation of TTP increased the expression of PAS2mut reporter gene containing the functional ARE sequence 2.2-fold (P = 0.011; t-test), whereas the expression of the PAS2mut gene lacking the ARE did not change significantly (Figure 3G). We concluded that, similar to the human HuR gene, the long forms of the mouse HuR mRNA are destabilized by the ARE sequences and that TTP is an essential trans-factor in this pathway.

ARE protection by the HuR protein fails to account for the increased abundance of the long HuR mRNA form

To test if an excess of the HuR protein can rescue the ARE-dependent instability of the PAS4-terminated mouse HuR mRNA species, we co-transfected HEK293T cells with the RLuc-3’HuR-PAS2mut construct and either the T7-HuR expression plasmid or the pEF-BOS vector and assayed the RLuc activity. The HuR overexpression had no significant effect on the RLuc expression (Supplementary Figure S2). However, when we repeated the assay with a RLuc reporter containing a 244 bp fragment of the mouse HuR 3’-UTR spanning the ARE sequence we detected a modest but statistically significant stimulation of the RLuc expression by T7-HuR (1.4-fold; P = 5.6 × 10⁻⁷; Supplementary Figure S2), in line with the previously published results (48).

Since the ARE-dependent mRNA decay is thought to be a predominantly cytoplasmic process, we analyzed the intracellular localization of the T7-HuR protein transiently over-expressed in the HEK293T cells by immunofluorescence (Supplementary Figure S3). Notably, most of the T7-specific signal was enriched in the nucleus, similar to
the behavior of the endogenous HuR (Supplementary Figure S3). Taken together, these data suggest that although the HuR protein might potentially counter the ARE-dependent decay of its mRNA under some circumstances, this activity does not explain the increase in the abundance of the long HuR RNA species upon HuR overexpression.

HuR inhibits PAS2 utilization in vivo

Given its nuclear enrichment, we wondered if the HuR protein can regulate the use of the PAS2 site in vivo. For this purpose, we assembled a bicistronic cassette that consisted of dTomato and EGFP genes separated by a 433 bp fragment containing the mouse HuR PAS2 within its natural context (Figure 4A). A single copy of this cassette (Bic-PAS2wt) placed downstream of a strong constitutive promoter (CAG) was stably integrated into the P19 cell genome using HILO-RMCE. We reasoned that the pre-mRNA processing at the PAS2 site should produce a monocistronic dTomato mRNA whereas skipping of the PAS2 site would generate a bicistronic mRNA containing coding both dTomato and EGFP coding sequences. Therefore, we knocked down HuR in the transgenic P19 cells with HuR-specific siRNAs (Figure 4B) and analyzed the ratio between the EGFP and dTomato expression levels by RT-qPCR (Figure 4C). Down-regulation of HuR led to a 1.9-fold decrease in the EGFP/dTomato ratio \( (P = 2.8 \times 10^{-3}) \) as compared to the control samples treated with a non-targeting control siRNA (siControl) and the expression levels of the TTP mRNA were analyzed 48 h post-transfection using RT–qPCR. (G) HEK293T cells pre-treated with siRNAs as described in (F) were transfected with RLuc-3 HuR-PAS2mut reporters with (PAS2mut) or without the ARE sequence (PAS2mut/ARE) and the RLuc activity measured 24 h after transfection.
HuR inhibits the PAS2-specific pre-mRNA cleavage

To examine whether the HuR protein can directly interfere with its pre-mRNA processing, we carried out an in vitro pre-mRNA cleavage assay using HeLa nuclear extract and radioactively labeled RNA substrates. We initially tested a substrate containing the HuR PAS2 sequence in its natural context but this RNA failed to be cleaved under a range of conditions (data not shown). We therefore designed a composite SV40-PAS2 substrate containing the late PAS of the SV40 virus preceded by the SV40-specific sequence and followed by the HuR-derived GU-rich DSE (Figure 5A). This recombinant RNA was readily cleaved in vitro with the efficiency comparable to that of the control substrate containing the SV40 late polyadenylation site within its natural viral context (compare the first lanes in Figure 5B and C).

Notably, the addition of increasing amounts of purified HuR protein led to a potent inhibition of the SV40-PAS2 cleavage (Figure 5C and D). The SV40 substrate cleavage was also inhibited at high HuR concentrations as previously described (37) but this effect was substantially less pronounced than in the case of SV40-PAS2 (Figure 5B and D). These results indicate that the HuR protein inhibits the PAS2 utilization by blocking the pre-mRNA cleavage reaction and that this activity depends on the GU-rich sequences adjacent to the PAS2.

HuR interacts with the GRE sequences adjacent to the PAS2

Pre-mRNA cleavage normally requires the presence of a U- or GU-rich DSE, which is recognized by the CstF-64 subunit of the cleavage stimulation factor (CstF) (42–44). Since the HuR PAS2 occurs within GU-rich context (Supplementary Figure S1B), we hypothesized that the high-affinity HuR binding to this sequence may interfere with the CstF recruitment.

To test if HuR could interact with the GRE sequence, we carried out pull-down assays using biotinylated RNA probes (Figure 6A). HuR interaction with the GRE sequence was readily detected by this assay (Figure 6B). Comparably strong interaction was observed when we used a probe containing the mouse HuR ARE sequence and no interaction was detected with a probe containing the ARE antisense sequence. Similar results were obtained when we repeated this experiment with HeLa and HEK293T cell nuclear extracts (Supplementary Figure S5 and data not shown). Thus, HuR protein physically interacts with the PAS2-associated GRE sequence.
HuR inhibits the recruitment of the cleavage stimulation factor CstF-64

To test if the interaction of HuR with the GRE may hinder the CstF-64 recruitment, we introduced a doxycycline-inducible HuR-specific shRNA into the HEK293T cell genome using lentiviral transduction and prepared nuclear extracts from the cells that were either preincubated for 72 h with 2 μg/ml doxycycline or left untreated. Immunoblot analysis confirmed that HuR was virtually completely depleted from the nuclear extract upon doxycycline treatment (Figure 6C). We then analyzed the CstF-64 association with the GRE-specific RNA probe using the biotinylated RNA pull-down assay comprising an additional UV cross-linking step to stabilize the CstF-RNA complex (Figure 6A and D). The analysis of the streptavidin-retained fraction with a CstF-64-specific antibody showed that the CstF-64 interaction with the RNA probe was considerably more efficient in the HuR-depleted extract than in the extract containing the normal HuR levels (Figure 6D).

To confirm that both HuR and CstF-64 can interact with the endogenous HuR mRNA, we immunoprecipitated RNA–protein complexes from the NIH 3T3...
cells using antibodies against HuR or CstF-64 and analyzed the samples by RT–PCR with HuR cDNA-specific primers annealing upstream of the PAS2. An RT–PCR product of an expected size was detected in both immunoprecipitations and no signal was detected in the control sample precipitated with a pre-immune mouse IgG protein (Figure 6E). Notably, the HuR-specific antibodies failed to efficiently precipitate the Hprt mRNA, consistent with the absence of extensive GU-rich sequences within this transcript (Figure 6F). On the other hand, the anti-CstF-64 immunoprecipitation sample contained readily detectable Hprt mRNA amounts (Figure 6E).

We then repeated the RNA immunoprecipitation analysis using the anti-CstF-64 antibody and nuclear extracts from transgenic NIH 3T3 cells expressing either a control sequence or T7-HuR (Figure 6F). Strikingly, the T7-HuR expression virtually abolished the CstF-64 interaction with the HuR mRNA while having no effect on the CstF-64 interaction with the Hprt mRNA (Figure 6F). We concluded that HuR inhibits the PAS2 utilization by reducing the CstF-64 recruitment.

Cytoplasmic accumulation of the HuR protein in the cytoplasm of cells expressing SV40 virus LTA correlates with an overall increase in the HuR expression

Our results so far support the model in which HuR controls its expression through regulating the alternative polyadenylation of its own pre-mRNA in the nucleus. Such mechanism could maintain the cellular HuR homeostasis assuming that changes in the total HuR abundance would translate into proportional changes in its nuclear levels. Interestingly, cancer cells have been reported to accumulate a larger fraction of the total HuR protein in the cytoplasm than normal non-malignant cells (49,51–63). Since, according to our model, only nuclear HuR can contribute to its homeostatic auto-regulation, an increase in the cytoplasmic HuR fraction should result in elevated total HuR protein levels.

To test this prediction, we generated two transgenic populations of NIH 3T3 cells, one carrying the SV40 virus LTA and a G418 resistance gene (NIH 3T3-SV3neo) and the other only the G418 resistance gene (NIH 3T3-neo). The LTA-transformed NIH 3T3-SV3neo cells proliferated considerably faster than the NIH 3T3-neo cells (Supplementary Figure S6A), in line with earlier reports (75,76). Compared to the control cells, the cytoplasmic concentration of the HuR protein increased in the SV3neo cells whereas its nuclear concentration was somewhat diminished (Supplementary Figure S6B). Consistent with our model, the increase in the cytoplasmic HuR levels in the NIH 3T3-SV3neo cells was accompanied by an increase in the overall HuR protein expression (Supplementary Figure S6C).

To test if the slightly reduced nuclear HuR protein concentration in the NIH 3T3-SV3neo had any effect on the total HuR mRNA expression and the relative abundance of the alternatively polyadenylated mRNA species, we carried out RT–qPCR using primers specific to either all mRNA forms (F1/R1, see diagram in Figure 2A) or just the long species terminated at the PAS4 sites (F4/R4). This analysis showed that the expression of the SV3neo cassette indeed correlated with a 1.6-fold increase in the total HuR mRNA levels ($P = 4.3 \times 10^{-3}$) and a 2-fold decrease in the ratio between the PAS4-terminated and the total mRNA levels (Supplementary Figure S6D and E). These data suggest that the overall increase in the HuR protein levels in SV40 LTA-transformed NIH 3T3 cells might be due to both its accumulation in the cytoplasm where it fails to contribute to the negative feedback regulation and a decrease in its nuclear presence leading to an elevated production of the PAS2-terminated mRNA species.

DISCUSSION

Here we show that mammalian HuR protein auto-regulates its expression through a negative feedback loop, whereby it binds to its own pre-mRNA and increases the expression of long mRNA species containing a destabilizing ARE sequence (Figure 7). This mechanism may ensure HuR homeostasis when the nucleocytoplasmic distribution of this protein remains relatively constant or undergoes programmed reversible fluctuations as it happens during the cell cycle (16,27). On the other hand, our model may provide at least a partial explanation to the earlier reports that the increase in the total HuR expression levels in cancer cells is often accompanied by sequestration of a large fraction of HuR in the cytoplasm (49,51–63). Indeed, since only the nuclear HuR is expected to contribute to the negative feedback auto-regulation, a sustained HuR accumulation in the cytoplasm would result in the overall increase in the intracellular HuR protein levels (Supplementary Figure S6).

The newly identified homeostatic mechanism shares common features with the auto-regulatory loop described for the Drosophila ELAV protein (41,68). In both cases, Hu/ELAV proteins interact with 3′ segments of their corresponding pre-mRNAs. However, an important distinction between the two systems is that the ELAV protein functions by changing the pattern of alternative splicing, which leads to the appearance of a non-functional ELAV mRNA (41). On the other hand, HuR stimulates the expression of unstable alternatively polyadenylated HuR mRNAs.

Mammalian HuR and its nervous system-specific paralogs have previously been shown to regulate cleavage and polyadenylation of CT/CGRP and an SV40 virus pre-mRNA by interacting with U-rich sequences adjacent to the PAS hexanucleotides (37). Similar to our results, HuR reduces CstF-64 binding to these pre-mRNAs. Interestingly, the Drosophila ELAV protein also represses the 3′-end pre-mRNA processing of some pre-mRNA species but the CstF-64 binding is not affected in this case (39) Thus, despite the apparent functional similarity between ELAV and the Hu proteins, distinct molecular mechanisms may underlie the ELAV/Hu regulation in different metazoan organisms.
Based on our data, HuR represses the PAS2 utilization by interacting with the adjacent GRE (Supplementary Figure S1B). Since the GRE contains multiple copies of the optimal HuR binding motifs (7), we predict that binding of several HuR protein molecules upstream and/or downstream of the PAS2 may be required to interfere with the recruitment of CstF-64 and potentially other pre-mRNA cleavage/polyadenylation factors. Since HuR protein is capable of dimerization through the RRM3 domain (69,77), it is conceivable that its interaction with the GRE sequence occurs in a cooperative manner. This would ensure that the PAS2 is promptly inactivated when the nuclear HuR concentration exceeds a threshold level. Further studies will address this possibility.

Notably, the HuR mRNA species terminated at the PAS2 contain the 5′-terminal half of the GRE sequence, which may theoretically interact with the HuR protein on its own. Wang and colleagues (46) have shown that HuR indeed binds the 3′-terminal segment of the PAS2-terminated HuR mRNA, although the exact location of the binding site(s) has not been determined. The authors propose that this interaction may be required for the export of HuR mRNA, which reduces the HuR expression in senescent cells as a result of nuclear retention of the HuR protein–mRNA complex (46). Thus, the PAS2-adjacent GU-rich sequences may potentially play opposite roles in senescent and proliferating cells being a part of a positive feedback in the former and a negative feedback in the latter.

Khabar and colleagues (48) have previously demonstrated that the ARE sequence from the human HuR destabilizes a reporter mRNA in a TTP-dependent manner and that overexpressed HuR protein rescues the mRNA stability. Consistent with this data, we find that mouse HuR also stimulates the expression of reporters constructs containing a short fragment of the mouse HuR 3′-UTR that spans the ARE sequence. However, mouse HuR failed to significantly stimulate the expression of a reporter construct containing the ARE within the full-length HuR 3′-UTR context. It is possible that the HuR protection of the ARE sequence is hindered in the full-length 3′-UTR by the RNA secondary structure or unknown trans-factors. Interestingly, human HuR has been shown to bind its mRNA at a site located downstream of the PAS2 but distinct from the ARE (10). Although the biological significance of this interaction has not been studied, it is possible that the mouse counterpart(s) of this sequence modulate the HuR ARE-protective effect.

RBPs play an essential part in the posttranscriptional control of gene expression, which requires precise regulation of their cellular levels (1–4). A growing body of work suggest that this goal is often achieved through a variety of auto-regulatory homeostatic mechanisms where RBP proteins interact with their own (pre-)mRNAs in a manner that triggers a reduced mRNAs stability or/and translational efficiency. (2,78–80). It appears that HuR, a master regulator of the mRNA metabolism in proliferating mammalian cells, provides a compelling example of this regulatory trend.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR Online: Supplementary Materials and Methods, Supplementary Table S1, Supplementary Figures S1–6 and Supplementary References [42,73,82].

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