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HuR Displaces Polypyrimidine Tract Binding Protein To Facilitate La Binding to the 3′ Untranslated Region and Enhances Hepatitis C Virus Replication

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ABSTRACT

HuR is a ubiquitous, RNA binding protein that influences the stability and translation of several cellular mRNAs. Here, we report a novel role for HuR, as a regulator of proteins assembling at the 3′ untranslated region (UTR) of viral RNA in the context of hepatitis C virus (HCV) infection. HuR relocalizes from the nucleus to the cytoplasm upon HCV infection, interacts with the viral polymerase (NS5B), and gets redistributed into compartments of viral RNA synthesis. Depletion in HuR levels leads to a significant reduction in viral RNA synthesis. We further demonstrate that the interaction of HuR with the 3′ UTR of the viral RNA affects the interaction of two host proteins, La and polypyrimidine tract binding protein (PTB), at this site. HuR interacts with La and facilitates La binding to the 3′ UTR, enhancing La-mediated circularization of the HCV genome and thus viral replication. In addition, it competes with PTB for association with the 3′ UTR, which might stimulate viral replication. Results suggest that HuR influences the formation of a cellular/viral ribonucleoprotein complex, which is important for efficient initiation of viral RNA replication. Our study unravels a novel strategy of regulation of HCV replication through an interplay of host and viral proteins, orchestrated by HuR.

IMPORTANCE

Hepatitis C virus (HCV) is highly dependent on various host factors for efficient replication of the viral RNA. Here, we have shown how a host factor (HuR) migrates from the nucleus to the cytoplasm and gets recruited in the protein complex assembling at the 3′ untranslated region (UTR) of HCV RNA. At the 3′ UTR, it facilitates circularization of the viral genome through interaction with another host factor, La, which is critical for replication. Also, it competes with the host protein PTB, which is a negative regulator of viral replication. Results demonstrate a unique strategy of regulation of HCV replication by a host protein through alteration of its subcellular localization and interacting partners. The study has advanced our knowledge of the molecular mechanism of HCV replication and unraveled the complex interplay between the host factors and viral RNA that could be targeted for therapeutic interventions.

Hepatitis C virus (HCV) was discovered in 1989 as a major cause of chronic non-A non-B hepatitis (1). HCV is an enveloped positive-strand RNA virus classified in the *Hepacivirus* genus within the *Flaviviridae* family. The single-stranded uncapped 9.6-kb RNA codes for a long polyprotein of ~3,000 amino acids which is then processed to structural and nonstructural proteins. The RNA genome is flanked by the 5′ and 3′ untranslated regions (UTRs), which are essential for translation and replication of the viral RNA. The viral proteins are synthesized by internal ribosome entry site (IRES)-mediated translation. These proteins initiate extensive remodeling of the intracellular membranes to create a detergent-resistant scaffold for viral replication, termed as the "membranous web" (2). The progeny viral genomes are then assembled on the surface of cytoplasmic lipid droplets through interaction of the core and envelope proteins with the viral replicase (3).

HCV, being an obligate intracellular parasite, is entirely dependent on host factors for its propagation. This is highlighted by the necessity of ectopic expression of a number of human proteins and microRNAs (miRNAs) for reconstitution of the viral life cycle in mouse-derived cell lines (4). The cellular proteins that are necessary for HCV IRES-mediated translation and replication have been identified through small interfering RNA (siRNA) knockdown screens and other independent studies. Some of these proteins, such as the human La autoantigen (La) (5, 6), polypyrimidine tract binding protein (PTB) (5), heterogeneous nuclear ribonucleoprotein Q (hnRNPQ) (7), hnRNPL (8), poly(C)-binding protein 2 (PCBP2) (9), and ribosomal protein S5 (RPS5) (10), have been shown to modulate HCV translation by binding to the HCV IRES. Also, the competition between La protein and the viral NS3 protease for binding to the IRES has been shown to regulate the translation-replication switch in HCV (11). Apart from proteins, miRNAs are also involved in regulation of HCV translation. The most well-studied miRNA in the context of HCV is miR-122, a microRNA preferentially expressed in the liver cells that stimulates HCV translation and replication and alters its stability (12, 13). Likewise, viral replication is also dependent on several host

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proteins, including cyclophilins (14, 15), human vesicle-associated membrane protein-associated protein of 33 kDa (hVAP-33) (16), phosphatidylinositol-4-kinase III alpha (PI4KIII-α) (17), oxysterol-binding protein 1 (OSBP1) (18), NF90 and hnRNPL (19), etc. The importance of studying the role of these host proteins in the viral life cycle is underlined by the fact that many pharmaceutical agents directed against them, such as cyclophilin antagonists (20) and miR-122 inhibitors (21), are in clinical trials.

Several host proteins that regulate viral replication are also known to directly interact with the 5′ UTR, 3′UTR, or the coding region of the viral RNA. In a recent study, RNA binding proteins (RBPs) interacting with the HCV 5′ UTR were identified through affinity pulldown experiments. Among them, hnRNPL and NF90 were found to affect HCV replication (19). La and PCBP2 proteins have been shown to interact with both of the UTRs and link the 5′ and 3′ ends of the viral genome, facilitating replication (22, 23). Other independent studies on RNA binding proteins like hnRNPD, hnRNPA1, HuR, Unr, hnRNPU, etc., have indicated that they interact with the viral RNA (24–27). However, the molecular basis of regulation of viral replication by these RBPs remains a mystery.

In the present study, we have explored the role of one such RNA binding protein, HuR (human antigen R), in HCV replication. HuR, a ubiquitously expressed member of the Hu family which shuttles between the nucleus and cytoplasm in response to stress, has also been shown to bind to the HCV 3′ UTR and help in HCV translation as well as replication (27–30). However, the molecular mechanism of its participation in these processes is not clear. Since the HCV 3′ UTR is the site of assembly of the viral replication complex, we hypothesized that HuR, as a part of this complex, could be involved in cross talk with other cellular cofactors interacting with the HCV 3′ UTR to regulate replication. We have exploited the nuclear-cytoplasmic shuttling and RNA binding properties of HuR to address this question and to delineate the molecular mechanism by which HuR affects HCV replication.

MATERIALS AND METHODS

Plasmids and constructs. pcD-HCV-3′UTR was generated by amplifying nucleotides (nt) 9374 to 9605 of the HCV 3′ UTR and cloning them in HindIII-EcoRI sites of the pcDNA3 vector to generate pcD-HCV-3′UTR. pET28a-HuR-full-length, pET28a-HuR-RRM1 (nt 1 to 294; RRM1 is RNA recognition motif 1), pET28a-HuR-RRM(1+2) (nt 1 to 555), and pET28a-HuR-RRM(1+2+H) (nt 1 to 732; H indicates the hinge region) were cloned by amplifying the HuR coding sequence from pGEX-5X-2-HuR (a generous gift by Imed Gallouzi, McGill University, Canada) by PCR using the following primers: a forward primer containing an EcoRI site, 5′-GGAATTCATGTCTAATGGTTATGAAGACCACATGGCCG-3′, and a reverse primer containing an XhoI site, 5′-CCGCTCGAGTTATTTGTGGGACTTGTT-3′. The reverse primers used for amplification of nt 1 to 294 and nt 1 to 555 of the coding sequence were 5′-CCGCTCGAGCTACGGGCGAGCATACGACACC-3′ for pET28a-HuR-RRM1, 5′-CCGCTCGAGCTAGGCTGCAAACTTCACTGTG-3′ for pET28a-HuR-RRM(1+2), and 5′-CCGCTCGAGCTACCAGCCGGAGGAGGCGTTT-3′. The amplicons were cloned in EcoRI-XhoI sites of pET28a for expression of N-terminal His-tagged proteins. pRSETA-La (a generous gift from Jack Keene, Duke University, Durham, NC), pET28a-PTB (a generous gift from J. G. Patton, Vanderbilt University Medical Center, USA), and pThNS5BCΔ21 (a generous gift from Neerja Kaushik Basu, New Jersey Medical School, USA) were used for purification of the corresponding His-tagged proteins. pcDNA3-myc-HuR was obtained by cloning the HuR coding sequence in pcDNA3-myc vector. The HCV monocistronic replicon construct pFKi383hygubiNS3-3′5.1 (31), the bicistronic replicon constructs SGR-JFH1/Luc (where Luc is luciferase) and SGR-JFH1/Luc-GND (32), and the full-length HCV-JFH1 construct (33) (generous gifts from Ralf Bartenschlager and Takaji Wakita) were used in the present study.

Cell lines and transfections. Huh7 and Huh7.5 cells were provided by the laboratory of Charles M. Rice, Rockefeller University, and Apath, LLC (New York, NY, USA), and maintained in Dulbecco's modified Eagle's medium (DMEM; Sigma) with 10% fetal bovine serum (Gibco, Invitrogen). HCV monocistronic replicon-containing cells (34) were additionally cultured in the presence of 25 μg/ml hygromycin (Sigma). Lipofectamine 2000 (Invitrogen) was used for siRNA transfections according to the manufacturer's protocol. For generation of the infectious virus, HCV-JFH1 RNA was *in vitro* transcribed and transfected into Huh7.5 cells. Supernatant from the transfected cells was concentrated and used for viral infection as described previously (19). Uninfected Huh7.5 cells were used as a mock control.

siRNA transfection. Silencing by RNA interference (RNAi) was performed as described (23). siRNA targeting HuR, PTB, or La and a nontargeting siRNA (Dharmacon) were used in the study. Briefly, 14 h after seeding of replicon cells, 50 nM or 100 nM siRNA was transfected using Lipofectamine 2000 transfection reagent in Opti-MEM (Invitrogen). At 96 h posttransfection the cells were harvested, and the extracts were used for Western blot analysis as described below. In case of transient transfections, siRNA was transfected first, and HCV RNA was transfected 24 h later. Cells were harvested at the time points after HCV RNA transfection indicated on the figures.

Semiquantitative reverse transcription-PCR (RT-PCR). Total RNA from replicon cells was isolated with TRI reagent (Sigma) as per the manufacturer's protocol. Additionally, the isolated RNA was treated with 10 units of DNase I (Promega), extracted with acidic phenol-chloroform, and precipitated with 3 M sodium acetate (pH 5.2) and 2 volumes of absolute alcohol. In the first step, cDNA was synthesized using specific reverse primers for the HCV 5′ UTR and glyceraldehyde-3-phosphate dehydrogenase (GAPDH). This was used as the template for PCR amplification in the second step using specific forward and reverse primers for the HCV 5′ UTR and GAPDH. Amplification products were viewed in a 2% agarose–Tris-acetate EDTA (TAE) gel.

qRT-PCR analysis. Total RNA was isolated from cells using TRI reagent. HCV negative-strand RNA was quantified by two-step quantitative RT-PCR (qRT-PCR) using a DyNAmo HS SYBR green qPCR kit (Finnzymes). cDNA was synthesized with Moloney murine leukemia virus (M-MLV) reverse transcriptase at 42°C for 1 h (Promega) using 1 μg of total RNA by adding primers targeting HCV negative-strand RNA and glyceraldehyde 3-phosphate dehydrogenase (GAPDH) mRNA in same reaction mixture. qRT-PCR was done using 100 ng of cDNA in a 10-μl reaction mixture according to the manufacturer's instructions for 40 cycles. The comparative threshold cycle (C_T) method was used to calculate fold change in HCV RNA levels ($2^{-\Delta\Delta CT}$) and normalized to GAPDH.

Western blot analysis. Protein concentrations of the extracts were determined by Bradford assay (Bio-Rad), and equal amounts of cell extracts were separated by SDS–12% PAGE and transferred onto a nitrocellulose membrane (Pall Corporation). Samples were then analyzed by Western blotting using the desired antibodies, which included anti-HuR antibody (3A2; Santa Cruz), anti-NS5B antibody (ab35586; Abcam), anti-NS3 antibody (ab65407; Abcam), anti-calnexin antibody (ab92573; Abcam), anti-La antibody (ab75927; Abcam), anti-caveolin-2 antibody (ab97476; Abcam), or anti-PTB antibody (Calbiochem), followed by the respective secondary antibody (horseradish peroxidase-conjugated anti-mouse or anti-rabbit IgG [Sigma]). Mouse monoclonal anti-β-actin peroxidase-conjugated antibody (A3854; Sigma) was used as a control for equal loading of total cell extracts. Antibody complexes were detected using an Immobilon Western system (Millipore).

MTT assay. Cell viability after treatment with siRNA targeting either a nonspecific control or HuR (siNSP or siHuR, respectively) was checked

by an MTT [3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide] assay as described earlier (35).

Immunofluorescence staining. For immunofluorescence staining, ~0.2×10^6 Huh7.5 cells were seeded on coverslips in a 12-well plate for 14 h, followed by transfection of either a nonspecific RNA or HCV-JFH1 RNA (1 μg). At 48 h posttransfection, cells were washed twice with 1× phosphate-buffered saline (PBS) and fixed using 4% paraformaldehyde at room temperature (RT) for 20 min. After permeabilization by 0.1% Triton X-100 for 2 min at room temperature, cells were incubated with 3% bovine serum albumin (BSA) at 37°C for 1 h, followed by incubation with the antibodies indicated on the figure for 2 h at 4°C, and then detected by Alexa-633-conjugated anti-mouse or Alexa-488 conjugated anti-rabbit secondary antibody for 30 min (Invitrogen). Images were taken using a Zeiss microscope, and image analysis was done using the Zeiss LSM or ZEN software tool. Colocalization was quantified using the overlap coefficient, where a coefficient of 0 indicates no colocalization and a coefficient of 1 indicates complete colocalization. More than 20 cells were analyzed per field in six different fields for each experiment.

GST pulldown assay. A glutathione *S*-transferase (GST) pulldown assay was performed according to a protocol described earlier (36). Briefly, GST and GST fusion proteins were first immobilized on glutathione-Sepharose beads. The coated beads were then incubated with 1 mg of precleared cell extract in GST lysis buffer (20 mM Tris-Cl, pH 8.0, 200 mM NaCl, 1 mM EDTA, 0.5% NP-40, protease inhibitors) for 2 h at 4°C. After four washes with the lysis buffer, the proteins bound to the beads were eluted in Laemmli buffer and analyzed by Western blotting.

***In vitro* transcription.** RNAs were transcribed *in vitro* from different linearized plasmid constructs under T7 promoters in runoff transcription reactions. pcDNA3 vectors containing the HCV 5′ UTR or HCV 3′ UTR were linearized with EcoRI. The linear DNA samples were electrophoresed on agarose gels, extracted using a gel elution kit (Qiagen), and used as templates for synthesis of unlabeled or ^{32}P-labeled RNA using T7 RNA polymerase (Fermentas) and [^{32}P]UTP (PerkinElmer Life Sciences). The transcription reaction was carried out under standard conditions (Fermentas protocol) using 2.5 μg of linear template DNA at 37°C for 1.5 h. After alcohol precipitation, the RNA was resuspended in 20 μl of nuclease-free water. One microliter of the radiolabeled RNA sample was spotted onto DE81 filter paper, washed with phosphate buffer, and dried, and the incorporated radioactivity was measured using a liquid scintillation counter.

Protein purification. Recombinant HuR or HuR deletion mutant proteins were prepared in *Escherichia coli* BL21(DE3) cells transformed with the appropriate pET28a vectors. The expression of recombinant HuR was induced with 0.5 mM isopropyl-1-thio-β-D-galactopyranoside (IPTG) at an optical density of 0.6 at 660 nm and grown for another 5 h. The cells were pelleted, resuspended in lysis buffer (50 mM Tris, pH 7.5, 300 mM NaCl, 0.1 mM phenylmethylsulfonyl fluoride [PMSF]), and disrupted on ice by sonication. All subsequent steps were carried out at 4°C. The lysates were cleared by centrifugation at 10,000 rpm for 30 min and incubated with a Ni-nitrilotriacetic acid (NTA)-agarose slurry (Qiagen) with rocking for 6 h. The lysate was loaded onto a column, and the flow-through was discarded. The column was washed with 20 ml of wash buffer (50 mM Tris, pH 7.5, 300 mM NaCl, and 40 mM imidazole). The bound protein was eluted with 500 μl of elution buffer containing 500 mM imidazole. The eluted proteins were dialyzed at 4°C for 4 to 6 h in 10 times the volume of dialysis buffer (50 mM Tris, pH 7.4, 100 mM KCl, 7 mM β-mercaptoethanol [β-ME], 20% glycerol), aliquoted, and stored in a −70°C freezer. His-PTB and His-La proteins were purified as described earlier (11, 33).

Preparation of S10 extracts. S10 extracts were prepared as described by Ray and Das (37). Briefly, Huh7 or replicon cells were grown in DMEM (Sigma), supplemented with 10% FBS (Gibco, Invitrogen). A monolayer of cells was harvested, pelleted down, and washed three times with cold isotonic buffer (35 mM HEPES, pH 7.4, 146 mM NaCl, 11 mM glucose), resuspended in 1.5× packed cell volume of hypotonic buffer (10 mM HEPES, pH 7.4, 15 mM KCl, 1.5 mM Mg-acetate, and 6 mM β-ME), and then incubated on ice for 10 min for swelling. Cells were then transferred to a Down's homogenizer and disrupted by 50 strokes on ice. The lysate was incubated in 1× incubation buffer (10× buffer is 200 mM HEPES, 1.2 M KCl, 50 mM Mg-acetate, and 60 mM β-mercaptoethanol) for 10 min. Cytoplasmic extract (S10 supernatant) was isolated by centrifuging the lysate at 10,000 × *g* for 30 min at 4°C. The supernatant was dialyzed for 2 to 4 h against 100 volumes of dialysis buffer (10 mM HEPES, 90 mM KCl, 1.5 mM Mg-acetate, 7 mM β-ME, 20% glycerol).

UV-induced cross-linking of proteins with RNA and immunoprecipitation (IP) assays. UV-induced cross-linking was carried out as described by Ray and Das (37). Briefly, α-^{32}P-labeled RNA probes were allowed to form complexes with S10 extracts or with recombinant proteins in 1× RNA binding buffer (5 mM HEPES, pH 7.6, 25 mM KCl, 2 mM $MgCl_2$, 3.8% glycerol, 2 mM dithiothreitol [DTT], and 0.1 mM EDTA) and then UV irradiated for 20 min. The mixture was treated with 30 μg of RNase A (Sigma), separated on an SDS–10% polyacrylamide gel (SDS-PAGE), and analyzed by phosphorimaging.

For immunoprecipitation (IP), the RNase A-treated reaction mixtures (30 μg of total protein) were made up to 500 μl with polysome lysis buffer (100 mM KCl, 5 mM $MgCl_2$, 10 mM HEPES, pH 7.0, 0.5% NP-40, 1 mM DTT, 100 U/ml RNasin) and precleared with protein G-Sepharose beads for 1 h at 4°C. The samples were spun at 1,000 × *g* for 2 min to pellet the beads, and the supernatant was removed. Protein G-Sepharose beads were incubated with 2 μg of anti-HuR antibody (Santa Cruz) or anti-PTB antibody (Calbiochem) overnight at 4°C in a total volume of 200 μl of polysome lysis buffer and added to the precleared lysates, followed by incubation for 3 h with continuous mixing on a rotator device at 4°C. The beads were washed four times with polysome lysis buffer. SDS sample buffer was then added to the beads and boiled to release the immunoprecipitated protein, and the supernatant was electrophoresed on an SDS–10% PAGE gel. The gel was dried, exposed, and developed by autoradiography.

Coimmunoprecipitation assay. Coimmunoprecipitation assays to detect protein-protein interactions were performed in 1× radioimmunoprecipitation assay buffer (RIPA) as described before (23). Briefly, cells were lysed in 1× RIPA buffer and incubated with antibody-saturated protein G-Sepharose beads for 4 h. The beads were washed four times using 1× RIPA buffer. SDS sample buffer was then added to the beads, boiled to release the immunoprecipitated protein, and resolved by SDS-PAGE.

Filter binding assay. α-^{32}P-labeled HCV 3′ UTR RNA was incubated with increasing concentrations of recombinant HuR, PTB, or La protein at 30°C for 15 min in RNA binding buffer (5 mM HEPES [pH 7.6] 25 mM KCl, 2 mM $MgCl_2$, 3.8% glycerol, 2 mM DTT, and 0.1 mM EDTA). The reaction mixtures were loaded onto nitrocellulose filters equilibrated with RNA binding buffer and allowed to dry. The filters were then washed with RNA binding buffer twice and once with 100% ethanol and dried. The counts retained on the filters were measured using a scintillation counter. A graph of protein concentration versus the percentage of RNA retention was plotted, and the apparent dissociation constant (K_d) was derived from it.

A 5′–3′ coprecipitation assay. A 5′-to-3′ coprecipitation assay was performed as described by Kumar et al. (23). Briefly, α-^{32}P-labeled HCV 3′ UTR RNA was incubated with recombinant HuR/PTB/La proteins for 30 min at 37°C. Two micrograms of biotinylated HCV 5′ UTR RNA was allowed to bind 25 μl of streptavidin beads (Roche) for 30 min at room temperature in RNA binding buffer (14 mM HEPES [pH 7.5], 6 mM Tris-HCl [pH 7.5], 1 mM EDTA, 1 mM DTT, and 60 mM KCl). The sample was then incubated with the RNA protein complex for 20 min at 37°C. The beads were collected using a MagnaBind Magnet (Thermo Scientific). The coprecipitated RNA protein complexes were washed using wash buffer (50 mM Tris-HCl [pH 7.4], 300 mM KCl, 1 mM $MgCl_2$ and 0.05% NP-40), followed by proteinase K treatment for 30 min at 50°C. The RNA was treated with phenol-chloroform (pH 5.2) and precipitated

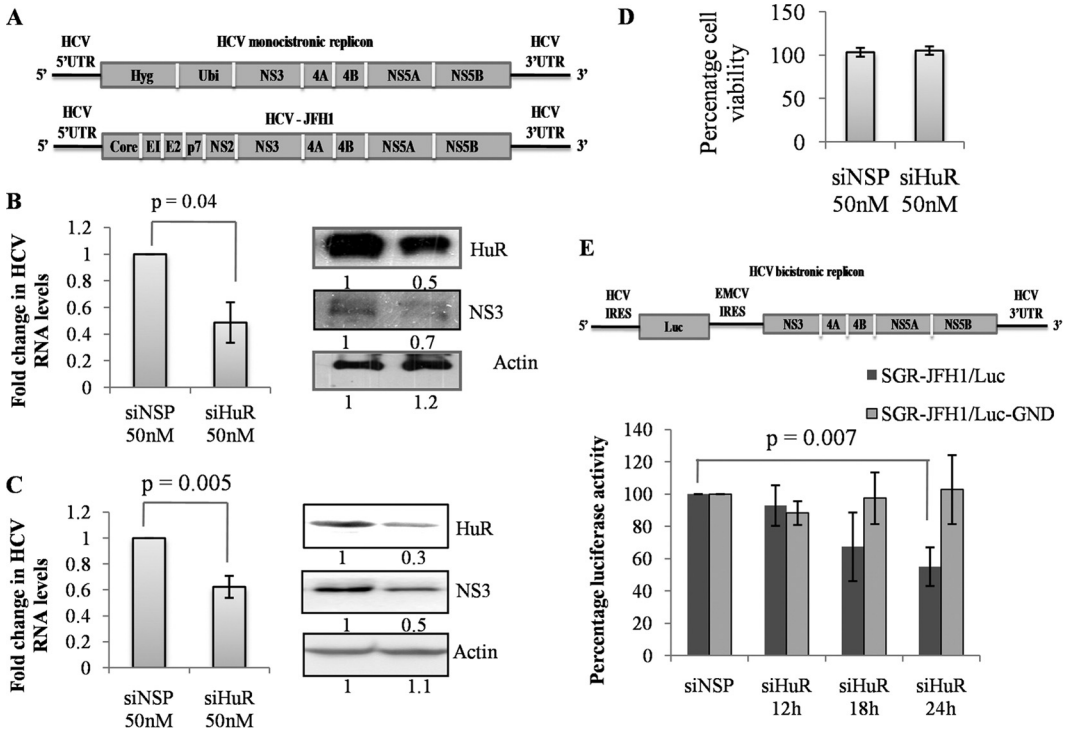


FIG 1 Effect of partial silencing of HuR. (A) Schematic of HCV monocistronic, subgenomic replicon pFKi383hygubiNS3-3′5.1, and HCV-JFH1 constructs. (B) HCV replicon-containing cells were transfected with either 50 nM siRNA against HuR (siHuR) or a nonspecific control siRNA (siNSP). After 96 h, HCV negative-sense RNA levels were analyzed by real-time PCR. Data from three biologically independent experiments are expressed relative to the control and presented as means ± SD. HuR and NS3 protein levels were analyzed by Western blotting, and the densitometry values after normalization with actin are indicated. (C) Huh7.5 cells were transfected with either 50 nM siHuR or siNSP, and 24 h later, transfection of HCV-JFH1 RNA was carried out. At 48 h posttransfection, the HCV negative-strand RNA levels were measured by qRT-PCR. Data from three biologically independent experiments are expressed relative to the control and presented as means ± SD. HCV NS3 protein levels were analyzed by Western blotting, and the densitometry values after normalization with actin are indicated. (D) Huh7.5 cells were transfected with either 50 nM siHuR or siNSP, and cell viability (as a percentage) was measured by MTT assay at 48 h posttransfection. (E) Huh7.5 cells were transfected with either 50 nM siHuR or siNSP, and 24 h later, either SGR-JFH1/Luc RNA or SGR-JFH1/Luc-GND RNA (shown in the schematic) was transfected into the cells. Luciferase activity normalized to the luciferase activity at the 6-h time point was determined. Data from three biologically independent experiments are expressed relative to the control and presented as means ± SD.

with an equal volume of ammonium acetate and twice the volume of 100% ethanol. The precipitated RNAs were resolved on a 7 M urea–6% acrylamide gel and autoradiographed.

RNP IP assay. To assess the association of HuR/La/PTB with HCV RNA, an ribonucleoprotein complex immunoprecipitation (RNP IP) assay was carried out as described earlier (38). Briefly, whole-cell lysates were prepared in polysome lysis buffer, and IP was performed using 2 μg of either IgG isotype control antibody (Imgenex) or anti-HuR, anti-PTB, or anti-La antibody. Protein G beads (Sigma) were preblocked with 0.4% BSA for 30 min at RT and then incubated with the respective antibodies in polysome lysis buffer for 16 h at 4°C. One milligram of cell lysate was used per reaction volume. Lysates were precleared with protein G beads for 1 h at 4°C and incubated with the desired antibody for 4 h at 4°C. Beads were washed with polysome lysis buffer four times and treated with 0.1% SDS and 30 μg of proteinase K at 50°C for 30 min, followed by RNA isolation and RT-PCR to detect the presence of HCV RNA.

Membrane flotation analysis. A membrane flotation assay was performed as described previously (2). Briefly, cells were lysed in hypotonic buffer by passage 20 times through a 25-gauge needle. Cell debris and nuclei were removed by centrifugation at 1,000 × *g* for 5 min. Cell lysates were mixed with 1.5 ml of 72% sucrose in low-salt buffer (LSB). The mixture was overlaid with 55% sucrose in LSB followed by 0.75 ml of 10% sucrose in LSB. The sucrose gradients were centrifuged at 38,000 rpm for 14 h at 4°C using a Beckman SW55 Ti rotor. Fractions of 500 μl were collected, and 100 μl from each fraction was resolved by SDS-PAGE followed by Western blotting for the desired proteins. In order to analyze the proportion of proteins in the detergent-resistant membrane (DRM) fractions, cell lysates were treated with 1% NP-40 for 30 min on ice before ultracentrifugation.

RESULTS

Partial knockdown of HuR by RNA interference leads to reduced HCV replication. To study the role of HuR in HCV replication, partial silencing of HuR was achieved using siRNAs targeting the protein. Initially, HCV replicon-containing cells (Fig. 1A) were used with which efficient HCV replication takes place, but infectious virions are not produced. These cells were transfected with siRNAs against HuR (siHuR) or a nontargeting control siRNA (siNSP), used as a negative control. The negative-sense HCV RNA levels were analyzed 96 h after siRNA transfection by quantitative reverse transcription-PCR (qRT-PCR). Results suggested that partial depletion of HuR leads to a significant reduction (~60%) in HCV RNA levels compared to the level in cells transfected with a nonspecific siRNA (Fig. 1B, siNSP). HCV-NS3 protein levels were also reduced considerably, indicating that there is a decrease in viral protein synthesis. Similar experiments were carried out in the HCV-JFH1 infectious RNA-transfected cells in which the complete life cycle of the virus is reproduced. Here also a modest decrease (40%) in HCV RNA levels and a significant reduction in NS3 protein levels were observed upon

siRNA-mediated depletion of HuR (Fig. 1C). These results suggest a possible role for HuR in the HCV life cycle, either at the translation or at the replication stage. There was no significant effect on cell viability upon HuR knockdown as measured using the MTT assay (Fig. 1D).

To determine whether HuR affects translation or replication of HCV RNA, Huh7.5 cells were transiently transfected with bicistronic RNA derived from either an SGR-JFH1/Luc or SGR-JFH1/Luc-GND (replication-defective) construct, in the presence of siNSP or siHuR. Luciferase activity in the cell lysates was measured at 12 h, 18 h, and 24 h posttransfection and was normalized to the activity value at 6 h posttransfection. The luciferase activity values from three biologically independent experiments were plotted as the percentage of the luciferase activity in lysates of siHuR-treated cells relative to that in siNSP-treated cells (Fig. 1E). There was a considerable decrease in the luciferase activity in SGR-JFH1/Luc RNA-transfected cells upon partial silencing of HuR. However, we observed no significant difference in the luciferase activity upon HuR silencing in SGR-JFH1/Luc-GND RNA (replication-deficient)-transfected cell lysates. Results indicate that HuR plays a more important role in HCV replication than in IRES-mediated translation. However, we do not rule out that the residual HuR protein in these cells was sufficient to support translation of the limited number of nonreplicating copies of the GND mutant RNA.

HuR relocalizes upon HCV infection and interacts with NS5B. HuR resides mostly in the nucleus and is known to relocalize to the cytoplasm under certain cellular stress conditions. Since HCV is a cytoplasmic virus, we wanted to investigate whether there is a change in the subcellular localization of HuR upon infection, making it more accessible to the viral RNA. For this purpose, Huh7.5 cells were transfected with HCV-JFH1 RNA, and at 48 h posttransfection, the cells were subjected to immunostaining with anti-HuR and anti-NS5B antibodies. While HuR was found only in the nucleus in uninfected cells, it was predominantly localized to the cytoplasm upon HCV-JFH1 RNA transfection (Fig. 2A). Interestingly, HuR was also found to colocalize with NS5B, indicating that HuR might interact with the viral polymerase. The overlap coefficient of HuR and NS5B was calculated, using the ZEN software program, from at least six different fields to be 0.57, thus confirming that the two proteins substantially colocalize in the cell. The intensity plot profiles derived using ImageJ also indicate colocalization. The same observations were made in Huh7.5 cells infected with the HCV-JFH1 virus (Fig. 2B). To further confirm this observation, GST pulldown assays were performed where GST-HuR was used as a bait protein and incubated with replicon cell lysates, followed by Western blotting with anti-NS5B antibody. HuR was found to interact with NS5B, and the interaction was independent of RNA since it did not decrease upon treatment with RNase A (Fig. 2C). Interestingly, we observed an increase in NS5B pulldown upon RNase A treatment. The binding of RNA to a protein often influences its interaction with other proteins by altering the exposure of its protein binding domains and vice versa. It is possible that when cellular RNA is degraded, HuR, which is an RNA binding protein, might be more available for interaction with NS5B and could account for the increase in NS5B coprecipitation. We also confirmed direct physical interaction between HuR and NS5B, using recombinant GST-HuR and recombinant NS5B proteins (Fig. 2D). A similar GST pulldown assay was performed with replicon cell lysates, followed by Western blotting with anti-NS3 antibody. Results indicated that HuR could also interact with NS3 protein. However, in contrast to the NS5B-HuR interaction, RNase A treatment led to a substantial decrease in NS3-HuR interaction, indicating the possible involvement of RNA in the interaction (Fig. 2E).

Since a major fraction of NS5B, NS3, and other proteins that participate in viral replication are localized to the detergent-resistant intracellular membrane fractions, where the replication complexes reside, we examined the possibility of association of HuR with these sites of viral replication. Cells transfected with HCV-JFH1 RNA were subjected to membrane flotation analysis in the presence or absence of 1% NP-40. The detergent-soluble membrane (DSM) and detergent-resistant membrane (DRM) fractions were segregated by ultracentrifugation and subjected to Western blot analysis to detect the proportion of HuR in the DRMs (Fig. 2F). In Huh7.5 cells, HuR was present mostly in the detergent-soluble cytosolic fractions. In HCV-JFH1 RNA-transfected cells, HuR was found to be associated with the membrane fractions as well as the cytosolic fractions. Even after NP-40 treatment, there was a significant proportion of HuR detectable in the detergent-resistant membrane fractions. The distributions of calnexin, a DSM marker, and caveolin-2, a DRM marker, were used to confirm the partitioning of DSM and DRM into distinct fractions upon NP-40 treatment. These observations indicate that HuR is indeed present at the sites of viral replication. To further understand how the presence of HuR at these sites can modulate viral replication, we turned to the basic RNA binding property of HuR for clues.

HuR interacts specifically with the HCV 3′ UTR, and RRM3 appears to be more important for the interaction. Competition UV cross-linking experiments suggested that GST-HuR interacts predominantly with the HCV 3′ UTR with a very low affinity for the 5′ UTR (Fig. 3A). We used UV cross-linking immunoprecipitation studies to establish that HuR interacts with the HCV 3′ UTR, which is consistent with earlier observations (22). In this experiment, cytosolic extract (S10) prepared from cells harboring the HCV replicon-containing cells was incubated with ^{32}P-labeled HCV 3′ UTR, followed by UV cross-linking, and the complexes were resolved by SDS-PAGE. A number of cellular proteins were found to interact with the 3′ UTR. The band corresponding to HuR was identified by immunoprecipitation. HuR was specifically precipitated from the cross-linked cytosolic extracts using anti-HuR antibody but not with the IgG isotype control antibody (Fig. 3B). Next, we sought to confirm the interaction of HuR with HCV RNA within the cells. For this purpose, Huh7 cells were transfected with replicon RNA for 48 h, followed by immunoprecipitation using anti-HuR antibody. RNA was isolated from the immunoprecipitated RNP complexes, and the associated HCV RNA was detected using semiquantitative RT-PCR (Fig. 3C).

HuR contains three RNA recognition motifs, RRM1, RRM2, and RRM3. In order to identify the specific RRM important for binding to HCV RNA, truncated forms of the full-length (FL) HuR protein were generated which contained either RRM1 alone (RRM1), RRM1 and RRM2 (RRM1+2) or RRM1, RRM2, and the hinge region (RRM1+2+H). Also, the individual RRMs were cloned and expressed to obtain proteins corresponding to RRM1, RRM2, and RRM3. The purified proteins were resolved by SDS–15% PAGE and visualized by silver staining. ^{32}P-labeled HCV 3′ UTR RNA was incubated with increasing molar concentrations of these recombinant proteins in a UV cross-linking assay (Fig. 3D).

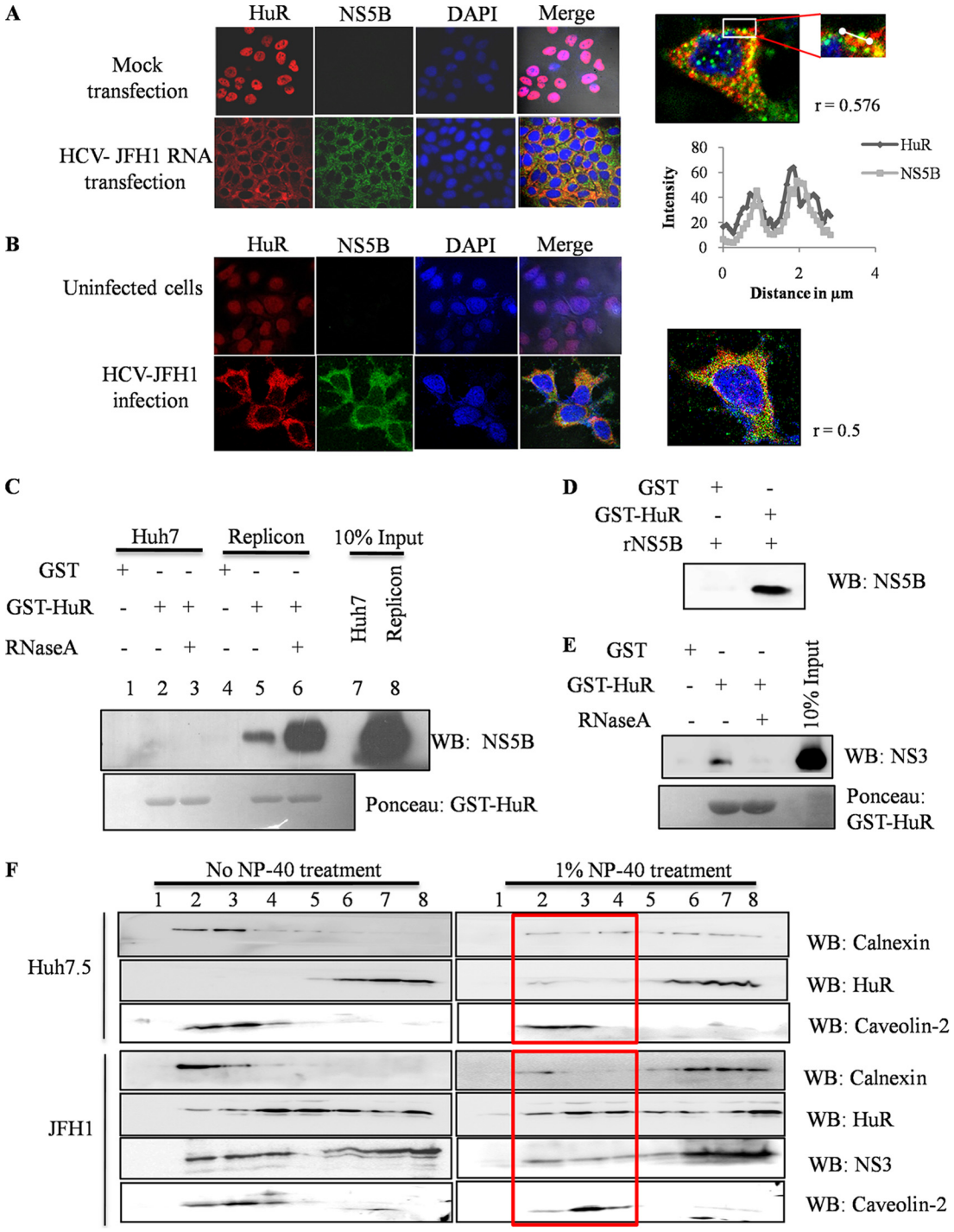


FIG 2 Interaction of HuR with NS5B. (A) Huh7.5 cells were mock transfected with luciferase-expressing RNA or with infectious HCV-JFH1 RNA, and immunofluorescence staining was carried out at 48 h posttransfection using Alexa Fluor-conjugated secondary antibody against HuR (red) or NS5B (green). The nucleus was counterstained with 4′,6′-diamidino-2-phenylindole (DAPI). Image analysis was carried out using ImageJ, and the line scans and plot profiles have been depicted. Pearson's colocalization coefficient for HuR and NS5B was calculated from at least six different fields using the ZEN software program. (B) The same experiment as described above was carried out in Huh7.5 cells infected with the HCV-JFH1 virus. (C) A GST pulldown assay was performed by incubating GST- or GST-HuR-coated beads with Huh7 or replicon cell lysates as indicated. The beads were either treated with RNase A or left untreated. The beads along with the associated proteins were resuspended in Laemmli buffer and resolved by SDS–10% PAGE. NS5B protein was detected by Western blotting (WB) using anti-NS5B antibody. Ponceau staining of the membrane indicates the amount of GST-HuR pulldown. (D) A GST pulldown assay was performed by incubating recombinant GST or GST-HuR with recombinant NS5B. (E) A GST pulldown assay was performed by incubating GST- or GST-HuR-coated beads with replicon cell lysates, followed by detection of NS3 protein by Western blot analysis. (F) Cell lysates were prepared from either mock-transfected or JFH1-transfected Huh7.5 cells, with or without NP-40 treatment. The membrane and cytoplasmic fractions were separated by ultracentrifugation and resolved on by SDS-PAGE followed by Western blot analysis. Calnexin and caveolin-2 were used as markers for detergent-soluble membrane (DSM) and detergent-resistant membrane (DRM), respectively. The proportions of NS3 and HuR proteins in each fraction were analyzed. Fractions containing the DRM are boxed in red.

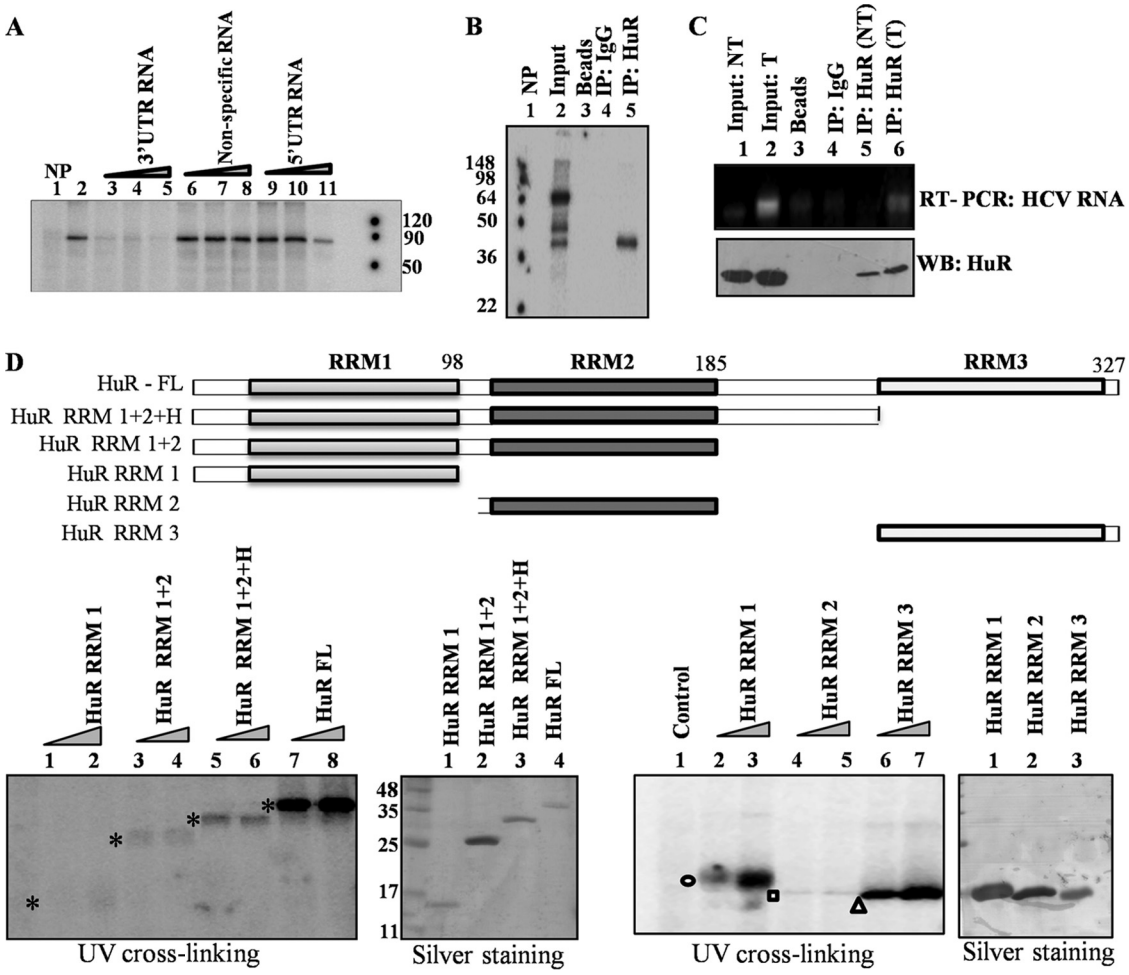
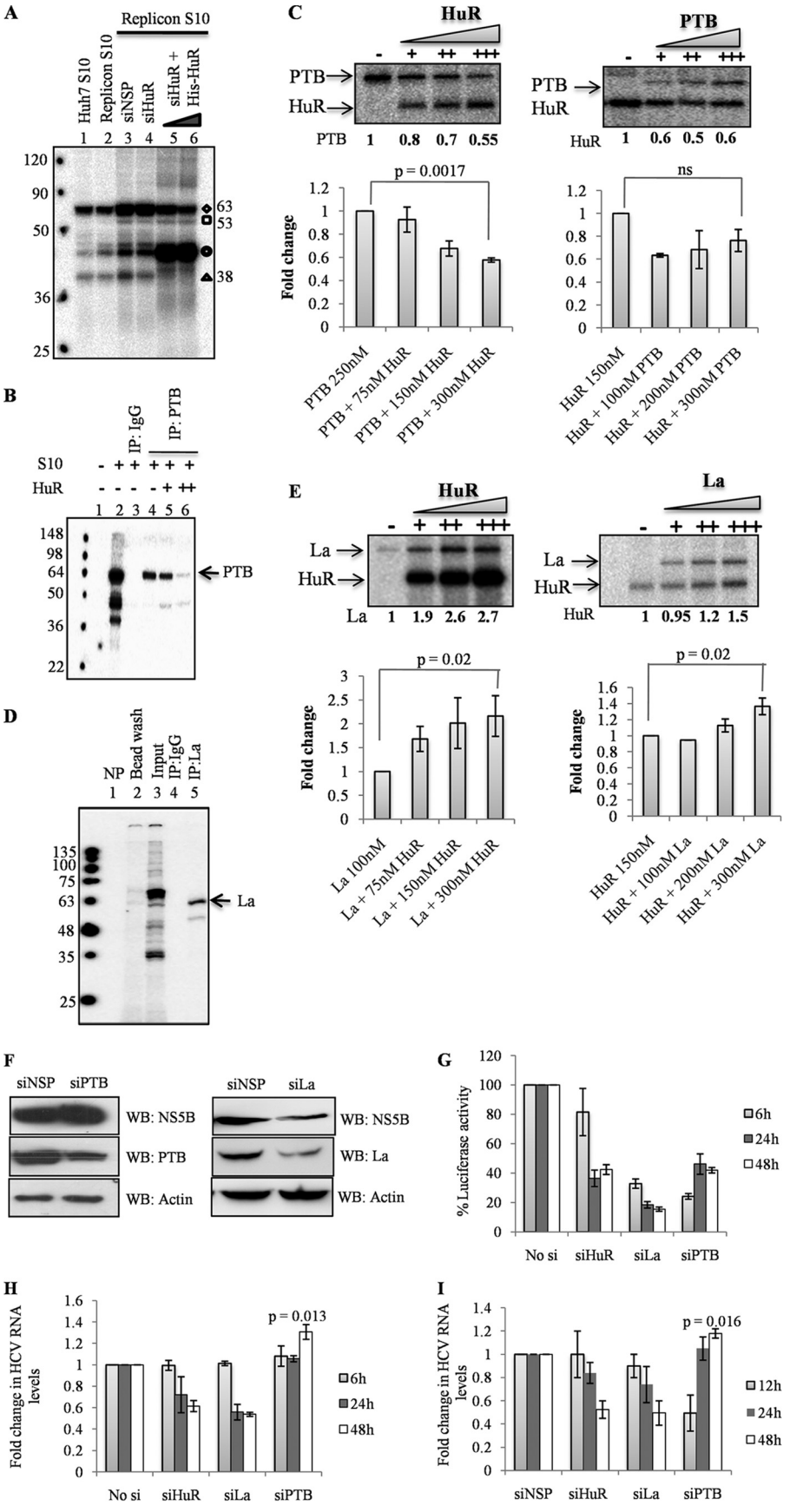


FIG 3 Interaction of HuR with the HCV 3′ UTR. (A) A competition UV cross-linking experiment was carried out with ^{32}P-labeled HCV 3′ UTR riboprobe and recombinant HuR in the presence of a 100-, 200-, or 400-fold molar excess of unlabeled 3′ UTR RNA or a nonspecific RNA or 5′ UTR RNA. The protein was separated on an SDS–10% polyacrylamide gel (SDS-PAGE) and analyzed by phosphorimaging. (B) After UV cross-linking of the ^{32}P-labeled HCV-3′ UTR RNA with replicon cell S10 extract, immunoprecipitation was carried out using the respective antibodies, followed by incubation with protein G-Sepharose beads. The beads were either used alone (lane 3) or after saturation with IgG isotype control antibody (lane 4) or anti-HuR antibody (lane 5). Lane 1, no protein (NP); lane 2, 25% of the UV-cross-linked S10 extract (input). Numbers to the left represent relative mobilities of the molecular mass markers. (C) Huh7.5 cells were transfected with HCV monocistronic replicon RNA followed by RNP IP using anti-HuR antibody. NT, no transfection; T, HCV replicon RNA transfection. (D) The schematic shows full-length (FL) HuR protein (amino acids 1 to 327) and the truncated forms which were cloned in pET28a for expression of the corresponding His-tagged proteins. In the panel shown on the left, the indicated proteins at 100 nM and 200 nM concentrations (corresponding to the first and second lanes, respectively, of each pair of lanes) were used in UV cross-linking with the radiolabeled HCV 3′ UTR. The asterisks indicate the bands corresponding to the respective proteins. The bands corresponding to the RRM1 (○), RRM2 (□), and RRM3 (△) proteins are indicated in the UV cross-linking panel shown on the right. The corresponding silver staining gels for the purified proteins are also shown.

The full-length protein containing all three RRMs showed maximum binding. When RRM3 was deleted, there was a drastic decrease in the binding. When both RRM3 and the hinge region were deleted, only a minimal level of binding was detectable. The use of individual RRMs in a direct UV cross-linking assay also indicated that RRM3 contributes maximally to the binding of HuR to the 3′ UTR followed by RRM1. These experiments suggest that RRM3, the hinge region, and RRM1 play an important role in the interaction of HuR with HCV RNA.

HuR competes with PTB but facilitates La binding to the HCV 3′ UTR. In order to understand the mechanism by which HuR affects HCV replication, UV cross-linking assays were carried out using cytoplasmic extracts to check whether silencing of HuR influences binding of other proteins to the HCV 3′ UTR. S10 extracts were prepared from replicon cells transfected with a 100 nM concentration of either siNSP or siHuR for 96 h. Also, in separate reactions, HuR-depleted S10 extracts were supplemented with increasing concentrations of recombinant HuR *in vitro* and then used in the UV cross-linking assay (Fig. 4A). First, we observed a dose-dependent decrease in the binding of one of the proteins in the ~63-kDa range upon addition of recombinant HuR. This protein has been identified previously as PTB (39). We confirmed the identity of this band by immunoprecipitation of PTB from S10 extract UV cross-linked with radiolabeled 3′ UTR RNA (Fig. 4B). We found that the amount of 3′ UTR radiolabeled RNA associated with PTB significantly decreases upon addition of increasing amounts of recombinant HuR to the S10 extract, indicating that HuR can displace PTB from the 3′ UTR. Also, direct



UV cross-linking assays were carried out with the recombinant proteins, His-HuR and His-PTB. In this assay, too, binding of recombinant PTB to the HCV 3′ UTR was found to decrease with increasing amounts of HuR (Fig. 4C). Upon addition of PTB, there was an initial decrease in HuR binding, which could be due to the two proteins sharing common binding sites on the HCV 3′ UTR. However, there was no dose-dependent change in HuR binding with the addition of increasing concentrations of PTB. Another protein in the ~53-kDa range showed a decrease in binding to the 3′ UTR when incubated with siHuR-treated S10 extract. However, its binding was restored upon supplementing recombinant HuR. Based on the apparent molecular weight and migration pattern of this protein (40), we suspected it to be the human La autoantigen which has been extensively studied in our lab. We confirmed the identity of this protein by immunoprecipitation of La from S10 extracts UV cross-linked with radiolabeled 3′ UTR RNA (Fig. 4D). Next, UV cross-linking assays were carried out with recombinant HuR and recombinant La proteins. The binding of La protein to the 3′ UTR increased by ~2.5-fold upon addition of increasing amounts of HuR. Interestingly, the addition of increasing amounts of recombinant La could also enhance HuR binding to the 3′ UTR to a small extent (Fig. 4E). Thus, it appears that HuR and La help each other in binding to the HCV 3′ UTR. The statistical significance for each of these experiments has been presented below the respective images in Fig. 4 as bar graphs depicting the means ± standard deviations (SD) from three biologically independent experiments.

Our observations based on the data shown in Fig. 4A to E indicate that HuR affects the binding of PTB and La to the HCV 3′ UTR. Earlier reports have shown that PTB displaces NS5B at the 3′ UTR and negatively regulates HCV replication (41) while La helps in circularization of the HCV genome and is a positive modulator (23). We confirmed the effects of these proteins on HCV replication by transfection of siRNAs against PTB and La in cell lines harboring the HCV monocistronic replicon. Indeed, we observed an increase in HCV NS5B levels upon siRNA-mediated silencing of PTB and a decrease in NS5B levels upon partial knockdown of La (Fig. 4F). In order to uncouple the effects on translation and replication, we used the SGR-JFH1/Luc bicistronic RNA. We observed that silencing any of the three proteins HuR, La, or PTB decreases the HCV IRES-driven firefly luciferase activity, indicating that all three proteins are essential either for IRES-mediated translation or the replication of the SGR-JFH1/Luc bicistronic RNA (Fig. 4G). In the experiment shown in Fig. 1E, using the SGR-JFH1/Luc-GND construct, we demonstrated that HuR predominantly affects HCV replication rather than translation. La protein has been shown to be essential for both IRES-mediated translation and replication (5, 6, 23), which is consistent with our observations (Fig. 4G and H). These results indicate that HuR and La are positive regulators of HCV replication. However, silencing of PTB led to a reduction in the luciferase activity and marginal but significant increase in the HCV negative-strand RNA levels, indicating that PTB helps in HCV IRES-mediated translation but could have a negative influence on HCV replication (Fig. 4H). The effects of these proteins on HCV replication was also studied in the HCV-JFH1 system by transfection of siRNAs against HuR, PTB, and La. HCV RNA levels were measured 12 h, 24 h, and 48 h posttransfection. As before, we observed that the depletion of HuR and La led to a considerable decrease in HCV RNA levels at 24 h and 48 h posttransfection. Knockdown of PTB caused a transient decrease in HCV RNA levels at 12 h, which could be a consequence of the decrease in translation, while a small but significant increase was observed in the HCV RNA levels at 48 h (Fig. 4I). The knockdown of HuR, La, and PTB has been confirmed in all the above-described experiments by Western blot analysis (data not shown). Taken together, these results indicate that HuR and La support HCV replication. PTB is required for HCV IRES-mediated translation but could have a negative influence on replication.

HuR, La, and PTB proteins interact with the HCV 3′ UTR with different affinities. The interplay observed between HuR, PTB, and La led us to determine the binding sites and binding affinities of these proteins for the HCV 3′ UTR. The 3′ UTR comprises three major regions: a variable region, a poly(U/UC) region,

FIG 4 Effect of HuR on cellular proteins binding to HCV 3′ UTR. (A) ^{32}P-labeled HCV 3′ UTR riboprobe was UV cross-linked to proteins in Huh7 S10 extract (lane 1), S10 extract from cells containing HCV monocistronic subgenomic replicon (lane 2), and S10 extract treated with a 100 nM concentration of either siNSP or siHuR (lane 3 or 4, respectively). S10 extract from siHuR-treated cells was supplemented with either 200 nM or 400 nM recombinant HuR (lane 5 or 6, respectively). □, the protein whose binding was affected by both silencing and supplementing HuR; ◇, protein displaced by HuR; △, band corresponding to endogenous HuR; ○, supplemented recombinant HuR. Numbers to the right represent approximate molecular masses of the indicated bands. (B) After UV cross-linking of ^{32}P-labeled HCV-3′ UTR RNA with replicon cell S10 extract, immunoprecipitation was carried out with protein G-Sepharose beads. The beads were either used alone (lane 3) or after saturation with anti-PTB antibody (lanes 4 to 6). In the experiment shown in lanes 5 and 6, UV cross-linking was performed in the presence of 100 nM (+) and 200 nM (++) recombinant HuR, respectively, followed by immunoprecipitation with anti-PTB-bound protein G-Sepharose beads. Lane 1, no protein; lane 2, 25% of the UV-cross-linked S10 extract used as input. (C) UV cross-linking of 150 nM PTB with the HCV 3′ UTR was carried out in the presence of 75 nM (+), 150 nM (++), and 300 nM (+++) recombinant HuR. Densitometry values are indicated for the band intensity corresponding to PTB. Also, UV cross-linking of 150 nM HuR was carried out in the presence of 100 nM (+), 200 nM (++), and 300 nM (+++) recombinant PTB. Densitometry values are indicated for the band corresponding to HuR. The densitometry values (means ± SD) from three biologically independent experiments are given below the respective images. (D) After UV cross-linking of ^{32}P-labeled HCV-3′ UTR RNA with replicon cell S10 extract, immunoprecipitation was carried out with protein G-Sepharose beads. The beads were saturated with either IgG isotype control (lane 4) or anti-La antibody (lane 5). Lane 1, no protein; lane 2, proteins that were present in the wash; lane 3, 25% of the UV-cross-linked S10 extract (input). (E) Experiments were performed with recombinant HuR and recombinant La as described for panel C for HuR and PTB, respectively. (F) HCV replicon-containing cells were treated with 50 nM siNSP or siPTB or siLa. Cells were harvested 96 h later, and the HCV NS5B protein levels were checked by Western blotting. Silencing of PTB and La was confirmed using the respective antibodies. Actin was used as a loading control. (G) Huh7.5 cells were transfected with 50 nM siNSP, siHuR, siLa, or siPTB. Cells were transfected 24 h later with SGR-JFH1/Luc RNA and harvested at the indicated time points. The luciferase activity in each lysate was measured and plotted as the percent change in activity. (H) In parallel to the luciferase activity measured above, RNA was isolated, and HCV negative-strand RNA levels were measured by qRT-PCR. Data for the fold change in the RNA levels are the averages of three biologically independent experiments. Knockdown of each of these proteins was confirmed by Western blotting (data not shown). (I) Huh7.5 cells were transfected with 50 nM siNSP, siHuR, siLa, or siPTB. The cells were transfected 24 h later with HCV-JFH1 RNA and harvested at the indicated time points. The RNA was isolated, and HCV negative-strand RNA levels were measured by qRT-PCR. Data for the fold change in the RNA levels are the averages of three biologically independent experiments. Western blotting was performed to confirm the knockdown of each of these proteins (data not shown).

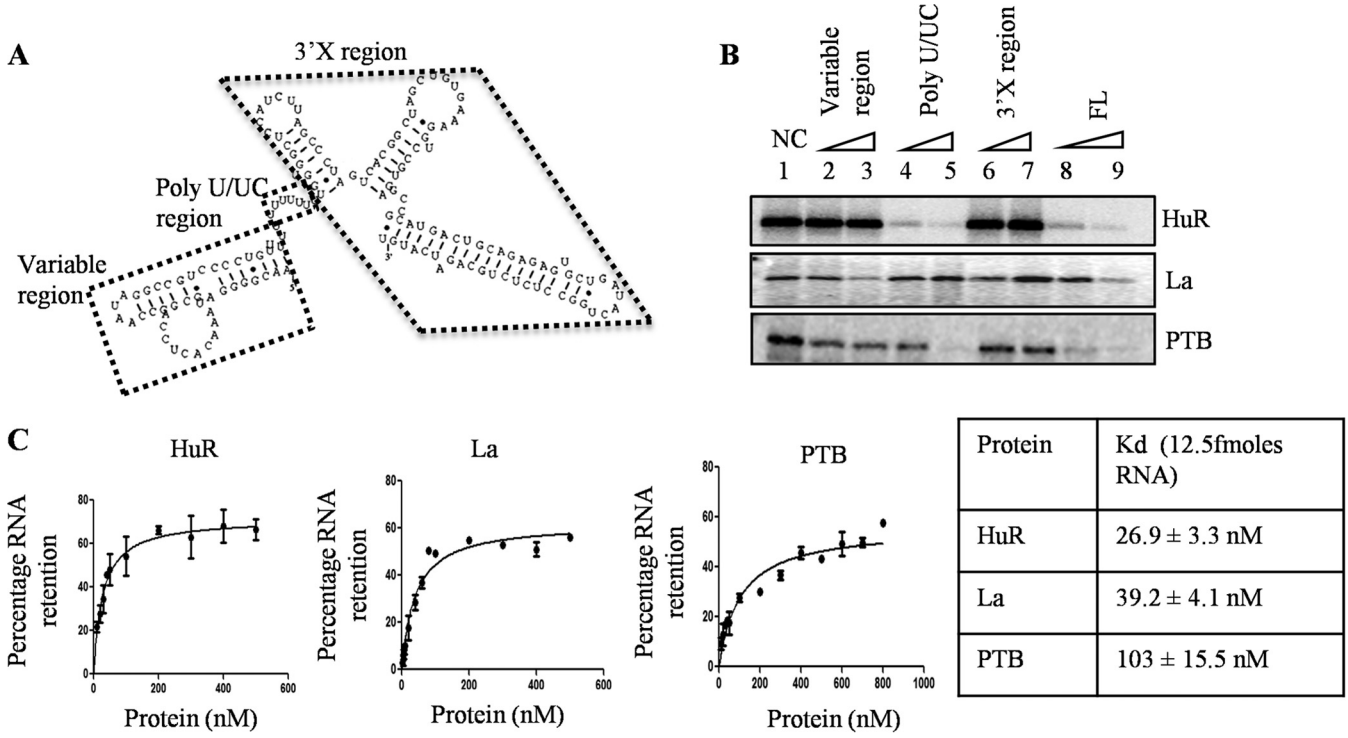


FIG 5 Characterization of HuR, PTB, and La binding to the 3′ UTR. (A) Schematic of the HCV 3′ UTR comprising the variable, poly(U/UC), and 3′ X regions. (Adapted from reference 39 with permission.) (B) ^{32}P-labeled HCV 3′ UTR riboprobe was used in UV cross-linking reactions with either recombinant HuR, PTB, or La proteins. Unlabeled RNA corresponding to the variable region, poly(U/UC) region, 3′ X region, and the full-length 3′ UTR were used in 50- and 100-fold excess concentrations (corresponding to the first and second lanes, respectively, of each pair of lanes) to identify the region which effectively competes with the radiolabeled RNA to bind to each of these proteins. (C) ^{32}P-labeled HCV 3′ UTR riboprobe was incubated with increasing concentrations of HuR, PTB, or La and loaded onto nitrocellulose filters. The filters were washed and dried, and the counts retained were measured using a scintillation counter. The percentage of RNA retention was plotted, and the apparent dissociation constant, which is a measure of the affinity of protein binding, was calculated.

and the 3′ X region (Fig. 5A). Competition UV cross-linking assays were used to identify the specific regions that are involved in the interaction with HuR, PTB, and La. The respective recombinant proteins were incubated with a radiolabeled full-length 3′ UTR. Unlabeled RNAs (50- and 100-fold excess) corresponding to each of the three regions as well as the full-length 3′ UTR were used for competition. We observed that the unlabeled RNA corresponding to the poly(U/UC) region was able to effectively compete out both HuR and PTB from the 3′ UTR while La was competed out mostly by unlabeled RNA corresponding to the variable region (Fig. 5B). These results indicate that HuR and PTB predominantly interact with the poly(U/UC) region of the 3′ UTR while La interacts with the variable region, providing a basis for competitive binding of HuR and PTB as well as for the cooperativity in binding of HuR and La. The relative binding affinities of these proteins were determined by filter binding assays in which the ^{32}P-labeled HCV 3′ UTR was incubated with increasing concentrations of each recombinant protein. The amount of RNA retained on the nitrocellulose filters was plotted to obtain the saturation curve. The apparent dissociation constant (K_d), that is, the protein concentration at which 50% of the RNA is bound, was calculated using GraphPad Prism (Fig. 5C). Results showed that recombinant HuR, PTB, and La bind to the 3′ UTR with apparent K_ds of 27 nM, 39 nM, and 103 nM, respectively, supporting our observations in which HuR binding could displace PTB but not vice versa (Fig. 4D). This could be attributed to the 4-fold difference in the binding affinities of HuR and PTB; however, we do not rule out that this could also be a result of the difference in relative abundances of the two proteins in the cytoplasm. Both HuR and La showed high affinity for binding to the HCV 3′ UTR.

HuR-La interplay facilitates HCV genome circularization. To examine whether HuR also directly interacts with La or PTB, whose binding it affects at the 3′ UTR, GST pulldown assays were performed using GST-HuR and Huh7 or HCV replicon-containing cell lysates, followed by Western blot analysis to detect La or PTB. There was no detectable coprecipitation of PTB with GST-HuR. However, we found that La protein present in the cell lysates coprecipitates with GST-HuR. Even upon treatment with RNase A, there was no significant decrease in the amount of coprecipitated La protein (after normalization with the amount of GST-HuR pulldown), suggesting that a direct protein-protein interaction could exist between HuR and La (Fig. 6A). This direct interaction was further confirmed by incubating GST-HuR with recombinant La. To rule out the possibility that RNA molecules could mediate the interaction, the purified proteins were treated with RNase A as described before (42). We observed significant coprecipitation of recombinant La with GST-HuR compared to that with GST alone. However, the possibility that RNA fragments protected by these proteins could influence the interaction cannot be ruled out (Fig. 6B). We performed coimmunoprecipitation assays to determine whether HuR interacts with La in cell culture. Huh7.5 cells were transfected with HCV-JFH1 RNA, followed by

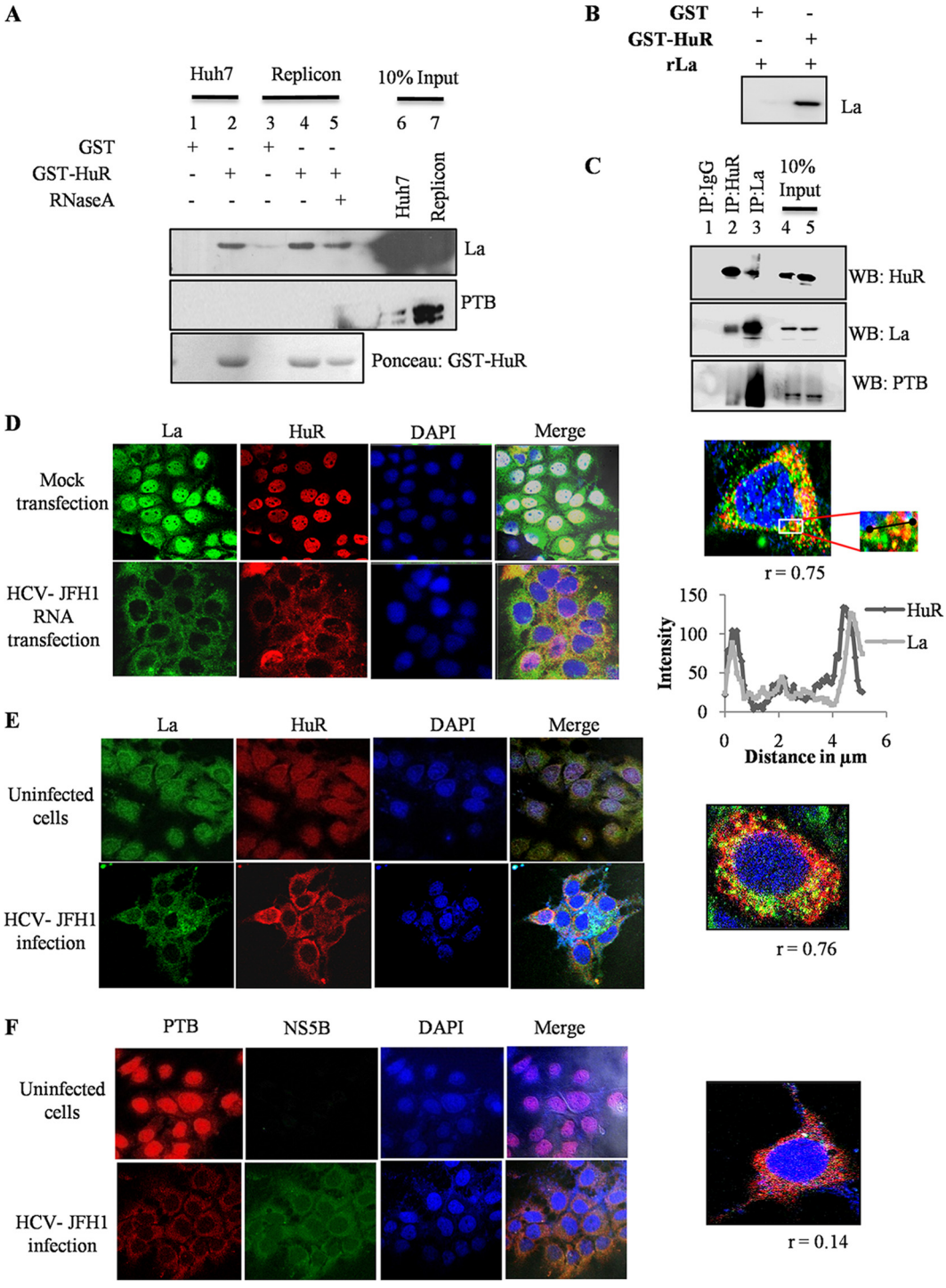


FIG 6 Interactions between HuR and La at the 3′ UTR. (A) A GST pulldown assay was performed by incubating GST- or GST-HuR-coated beads with Huh7 or replicon cell lysates. The beads were either treated with RNase A or left untreated. The beads along with the associated proteins were resuspended in Laemmli buffer and resolved on by SDS–10% PAGE. La and PTB proteins were detected by Western blotting using the respective antibodies. Ponceau staining of the membrane indicates the amount of GST-HuR pulldown. (B) A GST pulldown assay was performed by incubating recombinant GST- or GST-HuR-coated beads with recombinant La (rLa). (C) Coimmunoprecipitation assays were performed in Huh7.5 cells transfected with HCV-JFH1 RNA at 48 h posttransfection using protein G-Sepharose beads. The beads were saturated with an IgG isotype control (lane 1), anti-HuR (lane 2), or anti-La (lane 3) antibody. Lanes 4 and 5 are identical and represent 10% of the RIPA extract (input). (D) Huh7.5 cells were mock transfected or transfected with infectious HCV-JFH1 RNA, and immunofluorescence staining was carried out at 48 h posttransfection using Alexa Fluor-conjugated secondary antibody against HuR (red) or La (green). The nucleus was counterstained with 4′,6′-diamidino-2-phenylindole (DAPI). Image analysis was carried out using ImageJ, and the line scans and plot profiles have been depicted. Pearson's colocalization coefficient for HuR and La was calculated from at least six different fields using the ZEN software program. (E) The same experiment as described above was carried out in Huh7.5 cells infected with the HCV-JFH1 virus. (F) Immunofluorescence staining was carried out at 48 h post-HCV-JFH1 infection using Alexa Fluor-conjugated secondary antibody against PTB (red) or NS5B (green).

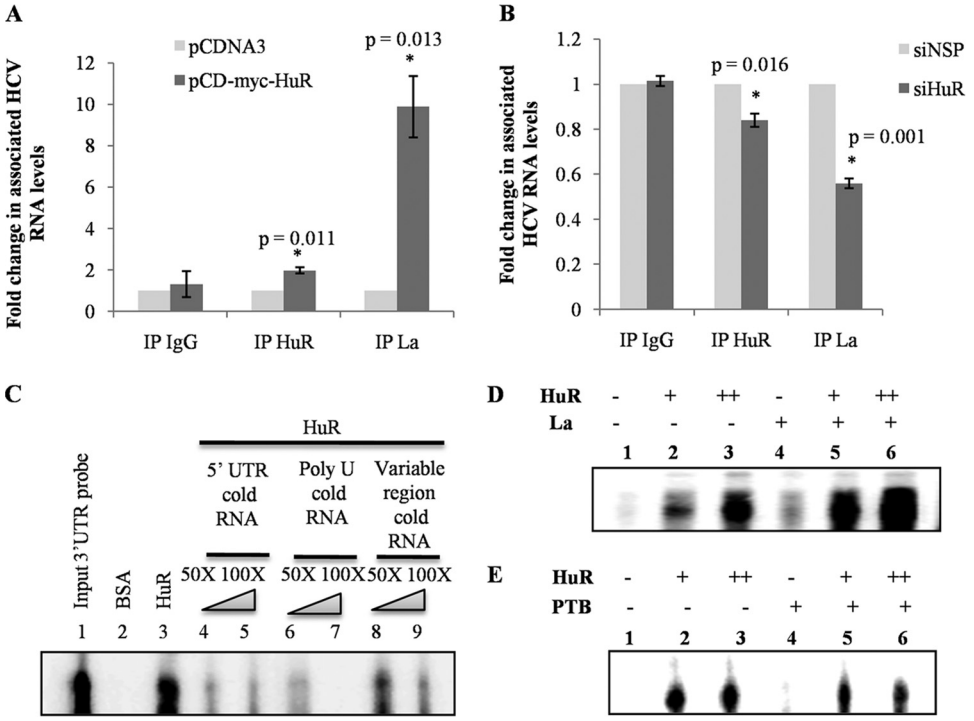


FIG 7 HuR-La interplay and its effects. (A) Huh7.5 cells were transfected with either pcDNA3 or pcDNA3-myc-HuR. Twenty-four hours later these cells were transfected with SGR-JFH1/Luc RNA and incubated further for 24 h. RNP IP was performed using either an IgG isotype control antibody or anti-HuR, anti-La, or anti-PTB antibody. The amount of HCV RNA associated with each of these proteins was measured by qRT-PCR. Data for the fold change in the associated HCV RNA in the presence of either pcDNA3 or pcDNA3-myc-HuR are means ± SD of three biologically independent experiments. (B) Huh7.5 cells were transfected with either siNSP or siHuR. Twenty-four hours later these cells were transfected with SGR-JFH1/Luc RNA, and RNP IPs were performed as described above. Data for the fold change in the associated HCV RNA in the presence or knockdown of HuR are means ± SD of three biologically independent experiments. (C) Biotin-labeled 5′ UTR RNA was incubated with HuR-bound 3′ UTR riboprobe followed by pulldown using streptavidin beads. Competition reactions were carried out using unlabeled 5′ UTR RNA, poly(U/UC) RNA, or variable-region RNA at 50- and 100-fold excess concentrations. The RNAs were resolved on a urea-polyacrylamide gel. The amount of radiolabeled RNA associated with the biotinylated RNA in each case was determined by autoradiography. A representative image from three independent experiments has been depicted. (D) ^{32}P-labeled HCV 3′ UTR riboprobe was incubated either with recombinant HuR or recombinant La or with both proteins as indicated. The complexes were then incubated with 5′ UTR biotinylated RNA conjugated to streptavidin beads to coprecipitate the protein-bound 3′ UTR. An autoradiograph of the coprecipitated 3′ UTR RNA is represented in the figure. (E) A similar experiment as described for panel D was also carried out with recombinant HuR and PTB proteins. + and ++ indicate 100 nM and 200 nM concentrations, respectively, of the proteins used in the assay.

immuno-pulldown using antibody against HuR or La. The antibody against endogenous HuR could immunoprecipitate La, and the antibody against endogenous La could immunoprecipitate HuR. However, under the conditions we used for the experiment, we could not detect an interaction with PTB (Fig. 6C). We attempted to identify the domain of HuR that is important for binding to La. It appeared that La binding sites are distributed throughout HuR since all the regions in HuR showed some interaction with La in both GST pulldown assays and coimmunoprecipitation assays (data not shown). Immunofluorescence staining indicated that HuR and La relocalize to the cytoplasm in HCV-JFH1-transfected Huh7.5 cells and in HCV-JFH1-infected cells. The determination of the colocalization coefficient from at least six different fields indicated significant colocalization of HuR with La with an overlap coefficient of ~0.75, as calculated using the ZEN software program (Fig. 6D and E). In the case of PTB also, we observed a relocalization, but its colocalization with the viral protein NS5B was not very evident in our study (overlap coefficient, 0.14) (Fig. 6F).

Next, we aimed at validating the interplay between HuR and La in HCV RNA-transfected cells through RNP IP analyses as a further confirmation for cooperative/competitive protein binding. For this purpose, myc-tagged HuR was ectopically expressed, and its effect on La and PTB binding to the HCV 3′ UTR was studied. Huh7 cells were transiently transfected with either the empty vector (pcDNA3) or a HuR expression vector (pcD-myc-HuR), followed by transfection with SGR-JFH1/Luc RNA. The RNA-protein complexes were immunoprecipitated with anti-HuR, anti-La, or anti-PTB antibody, and the amount of HCV RNA associated with each of these proteins was analyzed by qRT-PCR. The RNA associated with each protein upon HuR overexpression was normalized to that of the vector control (Fig. 7A). Upon HuR overexpression, we observed a ~2-fold increase in the HCV RNA associated with HuR, while there was an ~8-fold increase in HCV RNA associated with La, which supports our earlier conclusion (Fig. 4E) that HuR helps in La binding to the viral RNA in cell culture. Similar experiments were carried out under HuR knockdown conditions also. We observed that when HuR is silenced, there is a ~1.3-fold decrease in the HCV RNA associated with HuR, while there is a ~2-fold decrease in the RNA associated with La (Fig. 7B). However, the competitive effect on PTB was not evident in either case.

Given the evidence that HuR helps in La binding to the 3′ UTR, the next obvious question was how this might help in viral replication. Previous studies from our laboratory have established La as an important mediator of HCV genome circularization and thus HCV replication. Therefore, we investigated whether HuR promotes La-mediated 5′-to-3′ linkage of the HCV genome. We performed a 5′-to-3′ coprecipitation assay where a streptavidin-linked biotinylated 5′ UTR was incubated with radiolabeled a 3′ UTR in the presence of either recombinant HuR or recombinant La or both HuR and La proteins. The protein-RNA complex was isolated by pulldown of the biotinylated 5′ UTR using magnetic beads. The amount of associated radiolabeled 3′ UTR RNA was detected by resolving the complex on a urea-acrylamide gel, followed by autoradiography. Surprisingly, we observed that HuR alone could also mediate circularization in spite of its low-affinity binding with the HCV 5′ UTR and that the effect was specific to HuR (Fig. 7C). Also, we observed a dramatic increase in the 5′-to-3′ linkage when both HuR and La were present in the reaction mixture compared to the linkage with either HuR alone or La alone, indicating that the increase is due to more than just the additive effect of the two proteins (Fig. 7D). However, there was no such enhancement observed with PTB (Fig. 7E). These results suggest that HuR enhances La-mediated circularization of HCV RNA, which is crucial for efficient RNA replication.

DISCUSSION

The HCV-3′ UTR constitutes *cis*-acting elements that are critical for viral replication and infectivity (43). Viral and host proteins interacting with these regions could play an important role in regulating the viral life cycle. The expression and binding of these proteins may not be simultaneous but instead may be dependent on cell cycle phases and the stages of the virus life cycle and disease (27). HuR is an RNA binding protein that has previously been shown to bind to the HCV 3′ UTR and has also appeared in some whole-genome siRNA knockdown studies in the context of HCV (28, 29, 44, 45). Our study using both HCV replicon and JFH1 infectious cell culture systems clearly demonstrates that depletion of HuR leads to a significant reduction in the HCV RNA and protein levels. HuR has been shown to stimulate IRES-driven translation of HCV RNA (25). Interestingly, in our assay it appears that HuR plays a more dominant role in the replication step of the viral life cycle than in translation. It is possible that the residual HuR protein present in cells after partial depletion is sufficient to support translation of the limited number of nonreplicating copies of the GND mutant RNA used in the study.

The redistribution of HuR from the nucleus to the cytoplasm upon HCV infection also supported the role of this protein in viral replication. HuR relocalization has been observed upon infection with alphaviruses but not dengue virus which, like HCV, belongs to the *Flaviviridae* family (46). Hence, it may not be a general phenomenon observed in all members of the *Flaviviridae* family. The presence of high-affinity HuR binding sites in the 3′ UTR of these viruses might dictate the role played by HuR in their life cycles and thus its subcellular localization. However, in our study we do not rule out that the relocalization could also be a general response to cellular stress caused by HCV infection.

But protein localization alone does not indicate a possible mechanism of replication regulation. The mechanisms underlying the regulation of viral replication by host proteins can be varied depending on the cellular function of the protein and its domain organization. Some examples include fatty acid synthase, FASN, that interacts with and enhances the activity of the viral RNA polymerase, NS5B (47), and PI4KIIIα, which binds to NS5A and facilitates replication (48). With regard to RNA binding proteins, PCBP2 and La favor genome circularization and hence replication (22, 23). IGF2BP1 binds to the 5′/3′ UTR, helps in recruitment of eukaryotic translation initiation factor 3 (eIF3) to the 5′ UTR, and enhances IRES-mediated translation (49). DDX3X binds specifically to the HCV 3′ UTR and affects lipid droplet biogenesis, virus assembly, and secretion (50). Similarly, our study demonstrates that the RNA binding protein HuR directly interacts with the viral replicase NS5B and perhaps helps in its recruitment to the viral replication factories. In fact, we did observe significant colocalization of HuR with NS5B in infected cells and also RNA-independent protein-protein interactions between the two, which could facilitate the recruitment process. Since HuR is usually found in a complex with proteins like IGF2BP1, SYNCRIP, and DDX3X that are important in viral replication, its interaction with NS5B might help in bringing these proteins into close proximity with the viral replicase and tethering them to the 3′ UTR (51). The viral replicase is known to be localized in detergent-resistant membrane fractions, and the redistribution of HuR to these fractions in infected cells confirms its presence at the site of active RNA replication.

HuR is an RNA binding protein with multiple RRMs. It has two N-terminal RNA recognition motifs (RRMs) followed by a nucleocytoplasmic shuttling sequence and a C-terminal RRM. Among the three RNA binding motifs of HuR, RRM3 appeared to be most important for the interaction, followed by the hinge region and RRM1. Reports suggest that RRM3 is required for cooperative assembly of HuR oligomers on RNA molecules (52), indicating that HuR might be forming an oligomeric complex at the HCV 3′ UTR. Given the HCV RNA binding property of HuR, we hypothesized that it might indirectly modulate replication by influencing the binding of other proteins (such as La and PTB) regulating HCV replication at the 3′ UTR. In fact, the ability of HuR to participate in such regulatory networks has been demonstrated in a few contexts, such as promotion of HIF-1α mRNA translation through cooperative binding with PTB (53) and promotion of occludin mRNA translation by HuR through displacement of CUGBP1 (54). Our studies demonstrate cooperative binding of La and HuR to the HCV 3′ UTR as well as competitive binding of HuR and PTB.

PTB is a member of the hnRNP family of RNA binding proteins. It has four RNA recognition motifs that interact with polypyrimidine-rich regions and regulate RNA splicing, stability, and translation. In our study we observed that HuR displaced PTB from the HCV 3′ UTR, but PTB could not compete out HuR. Interestingly, HuR has been shown to physically interact with PTB under certain stress conditions, such as hypoxia (40). But in the context of HCV-JFH1 infection, we could not detect a significant interaction. Both of these proteins were found to interact predominantly with the poly(U/UC) region of the 3′ UTR. However, the binding affinity of HuR was higher than that of PTB, explaining why it could compete out PTB but not vice versa. The displacement of PTB by HuR could also be due to the difference in the relative abundances of the two proteins in the cell or because of the oligomerization property of HuR, and these aspects need to be further studied. Previously, it has been reported that PTB negatively regulates HCV replication by inhibiting NS5B binding to

the 3′ UTR (41). In support of this finding, we also noticed that partial depletion of PTB marginally increases NS5B levels in HCV replicon-containing cells. Similarly, we observed a marginal but significant increase in HCV replication in the SGR-JFH1/Luc bicistronic system as well as the HCV-JFH1 cell culture system. Taken together, the data led us to hypothesize that HuR relocalization to the cytoplasm upon HCV infection might displace PTB from the 3′ UTR, thus allowing NS5B to initiate HCV replication. The displacement of PTB by HuR in cell culture was not evident in our immunoprecipitation studies, probably because of additional associations of PTB with the HCV 5′ UTR. It is also possible that changes in PTB association occur in a different time frame; alternatively, PTB might behave differently *in vivo* in the presence of other cellular proteins and pathways than it does in its interactions *in vitro*.

The human La protein is an RNA binding phosphoprotein that has been implicated in facilitation of HCV IRES-mediated translation and replication (6). We confirmed the observation by siRNA-mediated knockdown of La, which reduced HCV RNA levels and protein expression. From our study, we also noticed that HuR facilitates La binding to the 3′ UTR. The data suggested another mechanism through which HuR could positively regulate HCV replication, that is, by stimulating binding of the positive regulator La to the HCV 3′ UTR. La and HuR interacted with distinct regions of the 3′ UTR: La interacts with the variable region, and HuR interacts with the poly(U/UC) region. It is possible that a change in RNA conformation induced by one of these proteins might expose the binding site for the other. Both proteins relocalized from the nucleus to the cytoplasm upon HCV infection and also colocalized in infected cells. GST pulldown and coimmunoprecipitation assays indicated a physical interaction between HuR and La. Further studies would be required to understand the significance of HuR-La interactions in HCV replication. In accordance with our *in vitro* UV cross-linking studies, we observed that HuR overexpression increased the association of La while knockdown of HuR decreased the association of La with HCV RNA in cell culture as quantified by RNP IP assays. Also, coprecipitation assays clearly showed that HuR could significantly enhance La-mediated circularization of HCV RNA, suggesting a possible mechanism by which cooperative binding of HuR and La could positively regulate HCV replication.

It is possible that the initial stoichiometry of the three proteins (La, PTB, and HuR) in the cytoplasm favors translation over replication. However, at later time points, the increased relocalization of La and HuR to the cytoplasm promotes HCV RNA circularization and HCV replication. The displacement of PTB from the 3′ UTR might contribute to the switch to the replication mode. Based on our observations, we hypothesize that there could be a triangular interplay between cellular proteins HuR, PTB, and La which, in turn, affects recruitment of NS5B to the 3′ UTR and positively regulates HCV replication. This study also highlights how the same protein can be exploited to exert different functions in the context of different viruses. In this case, it is HuR which can regulate the viral life cycle either by stabilizing the viral RNA (Sindbis virus) (55) or by regulating viral translation (HIV-1) (56) or by altering protein binding at the HCV 3′ UTR, as shown in the present study using HCV as a model system.

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S.S. and S.D. wrote the main manuscript text and prepared figures, and S.S., A.K, R.M, D.V, and N.M performed the experiments. S.S. and S.D. designed and analyzed the experiments and the results.

We confirm that there are no conflicts of interest.

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AUTHOR CORRECTION

Correction for Shwetha et al., "HuR Displaces Polypyrimidine Tract Binding Protein To Facilitate La Binding to the 3′ Untranslated Region and Enhances Hepatitis C Virus Replication"

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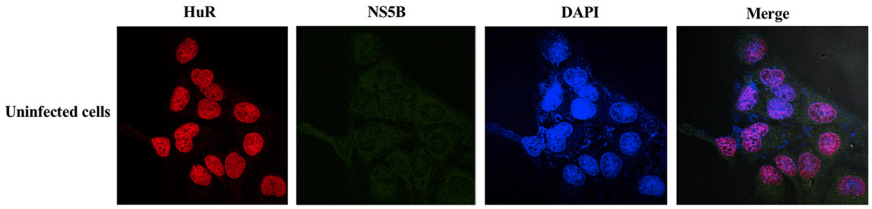
Volume 89, no. 22, p. 11356–11371, 2015, https://doi.org/10.1128/JVI.01714-15.
Page 11361, Fig. 2B: Due to an error in figure assembly, the top row in Fig. 2B is an inadvertent duplicate of the top row in Fig. 6F.

Figure 2B, top row, should appear as shown below.



Correction of this image does not change the interpretation of results or the conclusions of the paper.

Citation Shwetha S, Kumar A, Mullick R, Vasudevan D, Mukherjee N, Das S. 2019. Correction for Shwetha et al., "HuR displaces polypyrimidine tract binding protein to facilitate La binding to the 3′ untranslated region and enhances hepatitis C virus replication." J Virol 93:e00874-19. https://doi.org/10.1128/JVI.00874-19.

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