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The hepatitis C viral nonstructural protein 5A stabilizes growth-regulatory human transcripts

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ABSTRACT

Numerous mammalian proto-oncogene and other growth-regulatory transcripts are upregulated in malignancy due to abnormal mRNA stabilization. In hepatoma cells expressing a hepatitis C virus (HCV) subgenomic replicon, we found that the viral nonstructural protein 5A (NS5A), a protein known to bind to viral RNA, also bound specifically to human cellular transcripts that encode regulators of cell growth and apoptosis, and this binding correlated with transcript stabilization. An important subset of human NS5A-target transcripts contained GU-rich elements, sequences known to destabilize mRNA. We found that NS5A bound to GU-rich elements *in vitro* and in cells. Mutation of the NS5A zinc finger abrogated its GU-rich element-binding and mRNA stabilizing activities. Overall, we identified a molecular mechanism whereby HCV manipulates host gene expression by stabilizing host transcripts in a manner that would promote growth and prevent death of virus-infected cells, allowing the virus to establish chronic infection and lead to the development of hepatocellular carcinoma.

INTRODUCTION

For viruses to survive and replicate, they often control the host cellular environment by manipulating host gene expression. It is becoming increasingly clear that viral manipulation of host posttranscriptional regulatory mechanisms plays critical roles in viral pathogenesis. For example, Kaposi's sarcoma herpes virus globally down-regulates expression of host cellular transcripts by expressing the shut-off alkaline exonuclease (SOX), which mediates transcript degradation (1). Herpes simplex virus selectively degrades certain host transcripts through a viral endonuclease (2) but stabilizes and up-regulates a specific subset of host cellular transcripts through the viral ICP27 protein (3). By utilizing posttranscriptional mechanisms, viruses are able to selectively manipulate the expression of host transcripts to create a cellular environment that inhibits antiviral host defense mechanisms and allows the establishment of viral infection.

Hepatitis C virus (HCV), which belongs to the family *Flaviviridae* family of viruses (4), is a RNA virus with a 9.6 kb RNA genome of positive polarity that encodes structural and nonstructural proteins required for HCV replication (5). HCV infects approximately 184 million people worldwide (6) and causes hepatitis, liver cirrhosis and hepatocellular carcinoma (7). The mechanisms leading to liver cirrhosis and cancer in the HCV-infected individual are not understood. In the current era, cure of HCV infection with new antivirals is possible, but even patients with virological cure have an increased risk of developing hepatocellular carcinoma (8,9). Therefore, understanding the molecular progression of HCV infection to hepatocellular carcinoma is a priority.

Due to its small genome, HCV must use the host protein synthesis machinery to produce viral proteins required for viral replication. One of these viral proteins, non-structural protein 5A (NS5A), is multifunctional and influences many viral and cellular processes. The entire protein-protein interaction network of the HCV proteome was mapped by

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high throughput yeast two hybrid screening, and NS5A was found to interact with proteins functioning in focal adhesions, gap-junctions and host cellular signaling pathways (10). NS5A also influences cell cycle control by interacting with p53 and modulation of p21 (11–14). During viral genome replication, NS5A interacts with the RNA-dependent RNA-polymerase NS5B (15,16), an interaction that is essential to maintain the HCV subgenomic replicon in Huh 7 cells (15,17). NS5A also binds to G- and U-rich sequences in HCV genomic RNA (18). The amino terminal domain 1 and the adjacent unstructured region of NS5A interacts with HCV genomic RNA (16) and modulates its template selection (19). Crystal structures of domain 1 show a RNA-binding zinc finger which coordinates a Zn^{2+} ion (20,21). We hypothesized that NS5A functions to regulate the expression of host genes at posttranscriptional levels through its ability to bind to G and U rich RNA sequences.

We previously described a GU-rich element (GRE) that is enriched in the 3′ untranslated region (UTR) of rapidly degraded cellular transcripts expressed in primary human T cells (22). GRE-containing transcripts encode numerous proto-oncogene proteins and other proteins involved in cell growth regulation or apoptosis (23). Insertion of the GREs from JUN, JUNB or TNFRSF1B mRNAs into the 3′ UTR of a beta-globin reporter transcript conferred instability onto the otherwise stable beta-globin transcript. Further investigation showed that the CUGBP1 and ETR-3—Like Factor 1 (CELF1) protein functions as a GRE-binding protein and mediates the decay of GRE-containing transcripts (22), perhaps by recruiting other enzymatically active proteins to the transcript. The Xenopus homologue of CELF1, EDEN-BP, also binds to GREs and is involved in deadenylation of mRNA during oocyte maturation (24). In mammalian cell extracts, CELF1 interacts with poly A ribonuclease and mediates transcript deadenylation (25). The GRE and CELF1 define a posttranscriptional mechanism for coordinately regulating the expression of multiple transcripts involved in cellular growth and apoptosis, and we hypothesized that HCV manipulates this mechanism through its NS5A protein to create a cellular environment that prevents cell death and promotes growth of virus-infected cells.

In this report, we demonstrate that the HCV NS5A protein does indeed bind to host transcripts, including a large set of GRE-containing transcripts and mediates their stabilization. Expression of a subgenomic HCV replicon in the Huh 7.5 human hepatoma cell line led to binding by NS5A to host GRE-containing transcripts, which correlated with the stabilization of these transcripts. NS5A expressed exogenously in HeLa cells bound to reporter transcripts in a GRE-dependent manner, and this binding led to transcript stabilization, demonstrating that NS5A has RNA-stabilizing activity in cells. A purified recombinant NS5A polypeptide bound to GRE sequences in a zinc finger-dependent manner. Mutation of the zinc finger abolished the RNA-binding and the mRNA stabilizing activity of NS5A. Together, these data suggest that HCV manipulates host cellular mRNA decay through NS5A-mediated stabilization of host transcripts. Because GRE-containing NS5A target transcripts encode proto-oncogenes and other important regulators of cell growth and apoptosis, HCV-induced stabilization of these transcripts would prevent cell death and promote growth of virus-infected cells, allowing the virus to establish a chronic infection and thereby promote the development of hepatocellular carcinoma.

MATERIALS AND METHODS

Cell culture

The human hepatoma cell line Huh 7.5 (Huh) is a derivative of the Huh7 human hepatoma cell line attenuated in the RIG-I/interferon regulatory factor 3 (IRF-3) pathway (26). The Huh 7.5 SI cell line (Huh-HCV) stably expresses the HCV-Con1 replicon with an adaptive mutation in NS5A-coding sequence producing the S.2204.I variant (18). This cell line was generated by transfecting 1.6×10^6 Huh 7.5 cells with 2 μg of *in vitro* transcribed replicon RNA using TransMessenger transfection system (Qiagen). After transfection, 1×10^5 cells were seeded in 100-mm diameter dishes and 12–14 h later the cells were placed under G418 selection (500 μg/mL) for 3 weeks and colonies were further expanded. Huh and Huh-HCV cells were propagated in Dulbecco's modified eagle's medium (DMEM, Gibco) supplemented with 10% FBS (Atlanta biologicals), 0.1 mM nonessential amino acids (Gibco), 1% L-Glutamine (Gibco) and 100 units/ml of penicillin/streptomycin (Gibco). For Huh-HCV cells, 500 μg/ml of G418 (Calbiochem) was added to the medium. HeLa Tet-off cells (Clontech) were cultured in minimal essential medium alpha (Gibco) containing 10% tetracycline-free FBS (Clontech), 1% L-glutamine (Gibco) and 100 units/ml of penicillin/streptomycin (Gibco).

Plasmids

The pTracerC green fluorescence protein (GFP) expression plasmid and the pcDNA3 plasmid were purchased from Invitrogen. The tet-responsive beta-globin expression plasmid pTetBBB (BBB) (27) was a gift from Dr Ann-Bin Shyu (University of Texas-Houston). We previously described insertion of the *JUNB* GRE, mutated *JUNB* GRE or *IL2* ARE sequences into the 3′ UTR of the beta-globin sequence of the BBB plasmid to create the BBB-GRE, BBB-mGRE and BBB-ARE plasmids, respectively (22,28). To generate pCDNA3.1–5A and pCDNA3.1-del32–5a NS5A was PCR-amplified from the plasmid pHCVbart.rep1b/Ava-II (29), a gift from Dr Charles Rice (Rockefeller University) with primers FW-5a, 5′-GCGTCTAGAATGGGCTCCGGCTCGTGG CTA-3′ or FW-del32-5a 5′-GCGTCTAGAATGGGCGG AGTCCCCTTCTTC-3′ and RV-5a, 5′-GCGCAAGCTTC TATTAGCAGCAGACGACATC-3′ and inserted into the XbaI and HindIII sites of pCDNA3.1 (Invitrogen). The NS5A expression plasmid pCDNA3.1–5A was used to express NS5A in HeLa cells and pCDNA3.1-del32–5a was used to express the NS5A ΔN mutation. The QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used to introduce four cysteine to serine mutations (C39S, C57S, C59S, and C80S) into these plasmids to create pCDNA3.1–5A-4C-4S and pCDNA3.1-del32–5a-4C-4S which express the NS5A 4C-4S and NS5A ΔN-4C-4S mutations, respectively.

Plasmids for expression of recombinant NS5A polypeptides in *Escherichia coli* were created by cloning PCR amplified sequences from NS5A into the pSUMO plasmid (LifeSensors Inc). The following PCR primers were used to amplify the NS5A-domain 1+ coding sequence from the plasmid pHCVbart.rep1b/Ava-II (29): 5′-GCG GGT CTC AAG GTG GAG TCC CCT TC-3′ and 5′-GCG CGC AAG CTT CTA TTA GGA GTC ATG CCT GGT AGT GCA TGT TGC-3′. The amplified product was subcloned into the pSUMO plasmid using the BsaI and HindIII sites to generate pSUMO-NS5A-domain1+. The QuikChange Site-Directed Mutagenesis Kit (Stratagene) was used to mutate four cysteines to serines (C39S, C57S, C59S and C80S) in pSUMO-NS5A-domain1+ to create pSUMO-5A-domain1+ 4C-4S.

Purification of NS5A-domain 1+ WT and 4C-4S proteins

The NS5A polypeptides expressed from the pSUMO-based plasmids were expressed as fusion proteins with SUMO at the amino terminus. Overexpression of protein in this system was performed in the Rosetta (DE3) strain of *E.* coli and purified as described previously (30).

RNA sequencing and Actinomycin D mRNA decay assays

To measure the decay of endogenous cellular transcripts in Huh or Huh-HCV cells, actinomycin D (5 μg/ml, Sigma) was added to the media, and total cellular RNA was collected after 0, 3 and 6 h using the RNeasy kit (Qiagen). Genome-wide RNA sequencing was performed on technical duplicate samples to assess mRNA expression levels and mRNA decay rates as described previously (31). The zero time point was used to determine mRNA expression levels. Sequencing reads were mapped to the human genome (hg19) using Bowtie 2.0 with default settings. Tophat (v2.0.13) and Cufflinks (v2.2.1) were used subsequently to generate Fragments Per Kilobase of transcript per Million (FPKM) mapped reads that were quantified using custom R scripts. Transcript decay rates were determined following addition of actinomycin D based on a model of first order decay.

To measure the decay of beta-globin reporter transcripts in Huh and Huh-HCV cells, the cells were transfected in a 15 cm dish using 150 μl Lipofectamine 2000 (Invitrogen) and 15 μg of the beta-globin expression plasmids BBB, BBB-GRE or BBB-ARE as well as 8 μg of the pTracerC GFP expression plasmid. After transfection, each 15 cm dish of cells was split into three 10 cm dishes. Cells were treated 48 h later with 5 μg/ml of actinomycin D (Sigma), and total RNA was isolated after 0, 3 or 6 h using the RNeasy kit (Qiagen), following manufacturer's recommendations. Residual genomic or plasmid DNA was removed by digesting 1 μg of each sample with 1 unit of DNAse I (NEB) for 30 min at 37°C. cDNA was prepared with Superscript II enzyme (Invitrogen) and oligo dT_{15} primer from 1 μg of total RNA for each time point. Controls without RT were also prepared from 1 μg of total RNA. Quantitative real time PCR was performed in triplicate for each sample, and relative concentrations were calculated based on standard curves for each primer set. The PCR reaction primers were:

Beta-globin: forward 5′-GAGGGTCTGAATCACCT GGA-3′ and reverse 5′-GCCAAAATGATGAGACAGC A-3′.

GFP: forward 5′-TGGAAACATTCTCGGACACA-3′ and reverse 5′-CTTTTCGTTGGGATCTTTCG-3′. These primers were mixed with the template and the IQ™ SYBR® Green Supermix (Biorad) and amplified SYBR-green amounts were measured in an iCycler (Biorad) over time. Beta-globin levels were normalized to GFP levels.

RNA-immunoprecipitation followed by RNA sequencing or RT-PCR

To identify mRNA targets of NS5A, RNA-IP was performed as described previously (32) on Huh or Huh-HCV cells using a rabbit polyclonal antibody for NS5A which was produced at Covance Research Products (Denver, PA) using the purified recombinant protein NS5A-His as the antigen (16,18), RNA was purified from the immunoprecipitated material using the RNeasy kit (Qiagen) following manufacturer's instructions. Genome-wide RNA sequencing was performed as described previously (31). For each transcript, we calculated a NS5A-binding parameter called the fold change in enrichment (FCE) defined as:

$$\mathrm{FCE} = \frac{(\mathrm{NS5A\ IP/input})\ \mathrm{Huh-HCV}}{(\mathrm{NS5A\ IP/input})\ \mathrm{Huh}}$$

where the ratio of the RNA-Seq expression level for each transcript from the NS5A IP to the level from input RNA based on duplicate samples for Huh-HCV cells is divided by the same ratio for Huh cells.

To assess NS5A binding to reporter transcripts, Huh-HCV (15 cm dish) were transfected with 15 μg of BBB or BBB-GRE reporter plasmids and 8 μg of pTracerC plasmid in 100–150 μl of Lipofectamine 2000. RNA-IP was performed as described previously (32) using an anti-His antibody (Santa Cruz Biotech Inc.), anti-NS5A antibody (16), or anti-PABP antibody (Immuquest). RNA was purified from the input and immunoprecipitated material using the RNeasy kit (Qiagen) following manufacturer's recommendations. cDNA was prepared using Superscript II enzyme (Invitrogen), and PCR was performed with the beta-globin and GFP PCR primers described above.

In vitro RNA–protein binding assay

The fluorescence polarization assay was performed using a Beacon fluorescence polarization system (Amersham Biosciences) as described previously (18). Recombinant NS5A domain 1+ protein (0–1500 nM) and the 3′-fluorescein-labeled GRE RNA oligonucleotides or mutant RNA oligonucleotides shown in Table 1 were gently mixed in binding reaction buffer (20 mM HEPES, pH 7.5, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 100 μM $ZnCl_2$ and 100 mM NaCl) and incubated briefly at 25°C. Binding of NS5A domain 1+ was measured by the change in polarization. All steps were performed in reduced light. Data were fit to a hyperbola by using KaleidaGraph software (Synergy Software).

Table 1. NS5A affinity (K_d) for binding to RNA oligonucleotides

RNA	Sequence	$K_{d\,(nM)}$
1	GGCUGAGGCAGG	10 ± 1.5
2	GGGUGGGGGUGG	20 ± 2.7
3	UGUUUGUUUGUCCC	100 ± 10
4	UCUUUCUUUCUCCC	700 ± 200
5	UAUUUAUUUAUCCC	1000 ± 400
6	AAAAAAAAAAAAAA	1000 ± 150

Tet-off mRNA decay assay

HeLa Tet-off cells (15 cm dish) were transfected with 15 μg of the parental BBB reporter plasmid or BBB plasmids containing 3′ UTR inserts along with 15 μg of the NS5A expression plasmid, mutated NS5A expression plasmids or mock expression plasmid, and 8 μg of the pTracerC GFP expression plasmid in 100–150 μl of Lipofectamine 2000. After transfection each 15 cm dish was split into five 10 cm dishes and 48 hours later cells were treated with 300 ng/ml of doxycycline. After 0, 1.5, 3, 4.5 or 6 h, total RNA was extracted using the RNeasy kit (Qiagen) following manufacturer's recommendations, and for each sample, 10 μg of RNA was analyzed by northern blot using beta-globin and GFP probes as described previously (33). To ensure expression of NS5A or mutated NS5A, protein was extracted from cells in duplicate plates 48 hours after transfection and analyzed by western blotting as described previously (34) using an anti-NS5A antibody (16) and an anti-ERK 1/2 antibody (Cell signaling).

RESULTS

Host cellular transcripts are stabilized in HCV-expressing cells

We measured mRNA expression levels and mRNA decay rates on a genome-wide basis using Actinomycin D mRNA decay assay in the human hepatoma cell line, Huh 7.5 (Huh), and the same cell line expressing an HCV subgenomic replicon (Huh-HCV). Actinomycin D was added to Huh and Huh-HCV cells to block transcription and total cellular RNA was collected at 0, 3, and 6 h. This RNA was analyzed by genome-wide RNA sequencing (RNA-Seq) to calculate levels of transcript expression and transcript half-life based on a model of first order decay. We found a total of 16 714 transcripts were expressed in either Huh or Huh-HCV cells, 14 894 transcripts were expressed in Huh cells, 15 542 transcripts were expressed in Huh-HCV cells, and 14 359 transcripts were expressed in both. Of these, 6012 transcripts (36%) were up-regulated by >20% and 3903 transcripts (23%) were down-regulated by >20% in Huh-HCV cells compared to Huh cells. We calculated the half-life of each transcripts in both Huh and Huh-HCV cells and found 4945 transcripts (30%) were stabilized (the log of the decay slope increased by > 50%) and 5131 transcripts (31%) were destabilized (the log of the decay slope decreased by > 50%) in Huh-HCV cells compared to Huh cells. A master file containing all the primary gene expression and mRNA decay data is found in Supplementary Table S1. Overall, these data suggest that expression of the HCV subgenomic replicon had a dramatic impact on host gene expression and mRNA decay.

NS5A binding to host cellular transcripts correlates with transcript stabilization

Since the HCV NS5A protein is an RNA-binding protein known to bind to HCV genomic RNA, we hypothesized that NS5A might also bind to host cellular mRNAs. To test this hypothesis, we used an anti-NS5A antibody to immunoprecipitate (IP) NS5A from cytoplasmic extracts prepared from Huh-HCV and Huh cells, and we used RNA-Seq to identify and quantify co-purified host cellular mRNA transcripts. The anti-NS5A antibody used in this study was previously shown to be specific for NS5A in Western blot and to specifically immunoprecipitate NS5A from Huh cells expressing HCV replicon (16,18). For each transcript, we calculated a NS5A-binding parameter called the fold change in enrichment (FCE) as defined in the Materials and Methods. We defined NS5A targets as host transcripts with a FCE > 3 or transcripts present in the NS5A IP from Huh-HCV cells but absent in the NS5A IP from Huh cells and identified 960 NS5A target transcripts. A pathway analysis of these NS5A target transcripts revealed that 701 transcripts were related to cancer, 294 transcripts were related to cell growth and proliferation, and 290 transcripts were related to cell death (Ingenuity Pathway Assistant software). Supplementary Table S2 shows examples of NS5A target transcripts, and a complete listing of target transcripts is found in Supplementary Table S3. Figure 1 shows examples of functional pathways that contain NS5A target transcripts involved in apoptosis (Figure 1A) and cell growth/proliferation (Figure 1B). Thus, we found that NS5A target transcripts were highly enriched for transcripts encoding regulators of cell growth, cell death and cancer.

We hypothesized that cytoplasmic binding by NS5A to host cellular transcripts could alter their mRNA half-life. To evaluate this, we assessed changes in the mRNA decay rates of NS5A target transcripts in Huh-HCV cells compared to Huh cells. Of the 960 NS5A target transcripts, 556 (58%) were stabilized in Huh-HCV cells. In contrast, among 8731 transcripts that were not NS5A targets (FCE < or = 1), only 2116 (24%) were found to be stabilized (Figure 2A, blue bars). This difference was highly significant ($P < 2.2 \times 10^{-16}$). In addition to enrichment in transcript stabilization, we also found that NS5A target transcripts were highly enriched for up-regulation and stabilization (Figure 2A, red bars; $P < 2.2 \times 10^{-16}$). For a subset of NS5A target transcripts, we used quantitative RT-PCR to verify that they were stabilized and upregulated in Huh-HCV cells (Supplementary Table S4). Overall, our data suggest NS5A targets are highly enriched for transcript stabilization and up-regulation in Huh-HCV cells.

GRE-containing reporter transcripts are stabilized in Huh-HCV cells

The HCV NS5A protein functions as an RNA-binding protein with preference for G- and U-rich sequences (18). We showed that GU-rich sequences, known as GREs, present

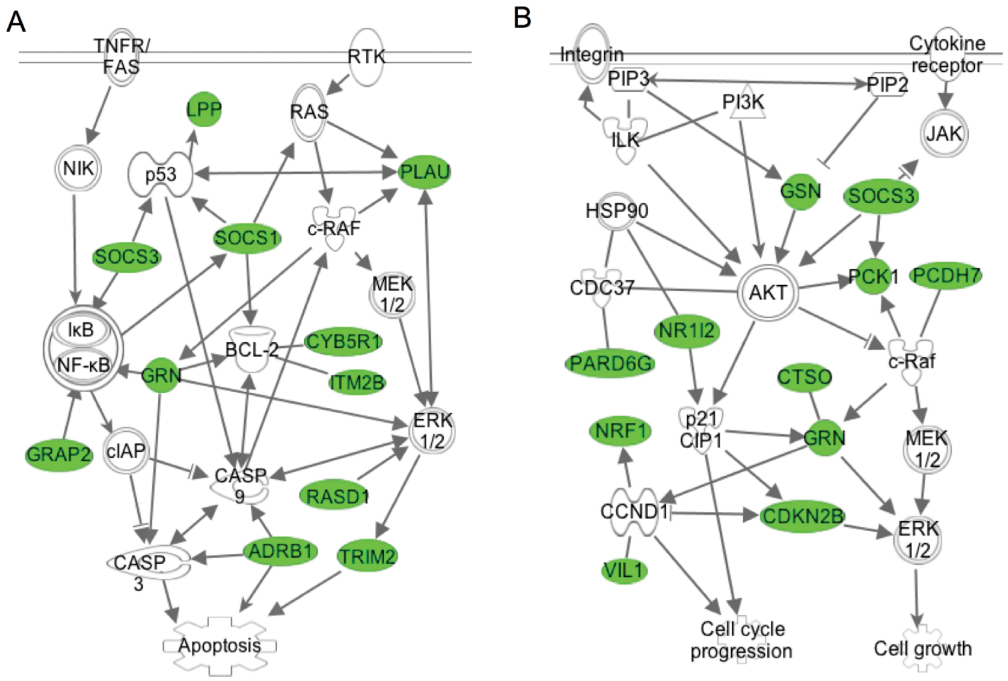

Figure 1. GRE-containing NS5A target transcripts encode regulators of apoptosis (**A**) and cell growth/proliferation (**B**). Transcripts depicted in green are NS5A target transcripts that contain GREs based on a FCE > 3 as defined in the Materials and Methods. These pathway figures were created using Ingenuity Pathway Assist software (Qiagen Inc).

in the 3′UTR of certain human transcripts target them for rapid mRNA degradation (22). We found that 118 NS5A target transcripts contain GRE sequences in their 3′ UTRs, as defined previously (22,33). We derived a cumulative distribution of the FCE for NS5A target transcripts that contain a GRE or do not contain a GRE and found greater binding by NS5A to targets that contained a GRE ($P = 0.003$, two sample Kolmogorov-Smirnov test, Supplementary Figure S1). Many other NS5A targets contain 3′ UTR sequences that are similar to the GRE and are rich in G or U residues. A *de novo* motif search was performed using Partek software to look for conserved sequences in the 3′ UTRs of NS5A targets based on the RNA sequencing data. The top 12-mer motif, shown in Figure 2B (top sequence), is highly G rich and resembles a consensus sequence that was previously found in CELF1 target transcripts (bottom sequence) (33). Also, a motif resembling a polyU sequence was among the top 11-mer motifs identified as a consensus sequence present in NS5A target transcripts (Supplementary Figure S2). These data are consistent with previous findings that NS5A could bind to polyU or polyG sequences (18). Since G and U rich sequences, similar to known NS5A binding sites, were conserved in the 3′ UTRs of NS5A target transcripts, we hypothesized that NS5A could impact the decay of transcripts containing G and U rich sequences. Our finding that the consensus NS5A target sequences shown in Figure 2B (top) has similarity to previously published GRE sequences (33) suggested a possible relationship between NS5A binding sites and GREs.

To determine if GU-rich sequences regulate host mRNA decay in HCV-expressing cells, we transfected Huh or Huh-HCV cells with beta-globin reporter constructs in which the *JUNB* GRE (BBB-GRE) and the *IL2* ARE (BBB-ARE) were inserted into the 3′ UTR. The *JUNB* GRE and the *IL2* ARE have been previously shown to function as mediators of rapid mRNA decay (22,35). The cells were also co-transfected with a GFP reporter construct to control for transfection efficiency. Transcription was inhibited by the addition of actinomycin D, and total RNA was isolated after 0, 3 and 6 h. Expression of the reporter transcripts was measured by quantitative RT-PCR and was normalized to the expression of the GFP transcripts. In this set of experiments, the BBB-GRE transcript decayed rapidly in Huh cells with a half-life of 80 ± 3 min and was stabilized ($P = 0.005$) in Huh-HCV cells with a half-life of 392 ± 18 min. In contrast, the BBB-ARE transcript decayed rapidly in Huh cells with a half-life of 133 ± 3 min but exhibited only minor and insignificant stabilization in Huh-HCV cells with a half-life of 158 ± 4 min (Figure 3). This finding that the GRE-containing reporter transcript exhibited specific stabilization in replicon-containing cells suggests that a mechanism exists for selective recognition of the GRE in HCV replicon-containing cells.

Binding by the HCV NS5A protein to host cellular transcripts correlates with transcript stabilization

Because the HCV NS5A protein binds preferentially to RNA containing GU-rich sequences, we speculated that NS5A could be responsible for the stabilization of GRE-containing transcripts in Huh-HCV cells. We performed RNA-IP assays to determine if we could identify a physical interaction between NS5A and the GRE in cells that contained the subgenomic HCV replicon. We transfected Huh-HCV cells with the beta-globin reporter construct (BBB)

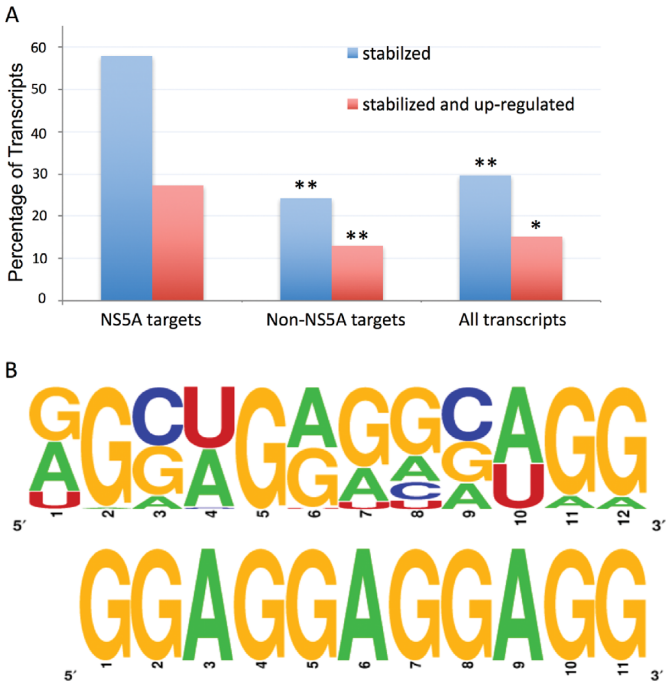

Figure 2. NS5A target transcripts are highly enriched for transcript stabilization and up-regulation. (**A**) The percentage of NS5A target transcripts, non-NS5A target transcripts and all transcripts that were stabilized (blue bars) or stabilized and up-regulated (red bars) is shown. ** represents statistically significant differences (P-value $< 10^{-16}$, Fisher's exact test with R) and *represents statistically significant differences (P-value $< 10^{-11}$, Fisher's extact test, R) in the percentages comparing NS5A target transcripts to Non-NS5A target transcripts or all transcripts. (**B**) Top: A motif search was performed to look for conserved consensus sequences in the 3′ UTRs of NS5A target transcripts. The top 12-mer motif is shown. The position in the signal (bases) is depicted on the horizontal axis. The height of each stack of letters on the vertical axis is proportional to the residue frequency in the given position. Bottom: The motif previously found in CELF1 target transcripts that resembles the top 12-mer motif shown above.

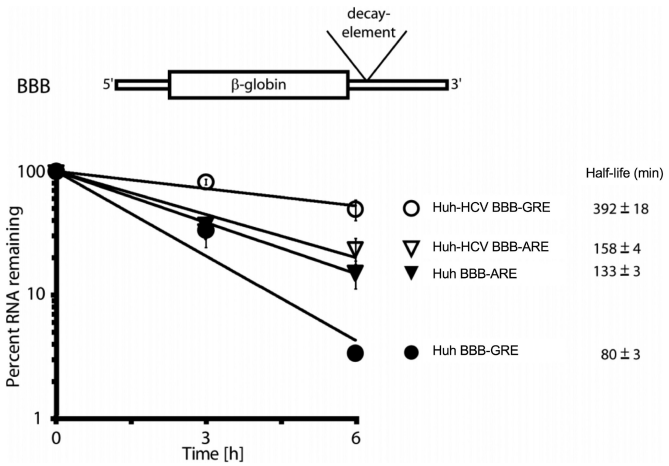

Figure 3. GRE-containing host mRNA transcripts are stabilized in Huh cells stably expressing an HCV subgenomic replicon (Huh-HCV). Huh or Huh-HCV cells were transfected with the BBB-GRE or BBB-ARE beta-globin reporter constructs. Actinomycin D was added to stop transcription and total cellular RNA was isolated after 0, 3 or 6 h. Specific mRNA levels were determined by quantitative real time RT-PCR. Beta-globin transcript levels at each time point were normalized to the transcript levels from a co-transfected GFP reporter. Transcript levels at the 0 time point were set to 100%, and the percent mRNA remaining was plotted as a function of time. The error bars indicate the standard error of the mean (SEM) from three experiments. Transcript half-life and SEM are shown to the right of each graph.

or the same reporter carrying the *JUNB* GRE in its 3′UTR (BBB-GRE). Two days after transfection, cell lysates were immunoprecipitated with an anti-His antibody (negative control), anti-PABP antibody (positive control) or anti-NS5A antibody, and RNA was isolated from the input material (I) and the immunoprecipitation pellet (P). Reverse transcription PCR was performed on this RNA to evaluate levels of beta-globin transcript and Hypoxanthine Phosphoribosyltransferase (HPRT) transcript, which is used as a housekeeping gene control (Figure 4). In cells transfected with the BBB-GRE construct, the beta-globin transcript was present in the NS5A pellet but the HPRT transcript was not (lane 10), whereas in cells transfected with the BBB construct, neither the beta-globin nor the HPRT transcripts were present in the NS5A pellet (lane 4). This result suggests that NS5A bound specifically in cells only to beta-globin reporter transcript that contained a GRE. Thus, NS5A was capable of binding to the GRE in HCV replicon-containing cells.

We performed *in vitro* RNA-binding assays to measure the binding affinity (Kd) of NS5A for the GRE and other sequences, including RNA sequences derived from the consensus sequence shown in Figure 2B, top (Table 1). For these assays, we used a polypeptide that contained the amino terminal domain (domain 1) of NS5A. The structure of domain 1 of NS5A has been determined by X-ray crystallography (20,21). The protein crystallized as a homodimer, and the structural integrity of the dimer was dependent on the tetra-cysteine-coordinated Zn^{2+} ion (Figure 5A). We purified a recombinant NS5A polypeptide that contained domain 1 plus 36 additional carboxy terminal amino acids; we refer to this protein as NS5A domain 1+. We also purified a NS5A derivative that was identical except the 4 cysteines in the zinc finger were changed to serines (referred to as 4C-4S). We used a fluorescence polarization assay (18) to measure binding of these NS5A polypeptides to fluorescein-labeled RNA oligonucleotides that contained a minimal GRE sequence (RNA #3 in Table 1). NS5A domain 1+ was titrated into a binding mixture containing a 3′-fluorescein labeled RNA substrate. The median polarization (mP) was plotted as a function of NS5A domain 1+ concentration, and the equilibrium dissociation constant was determined by fitting the data to a hyperbola. NS5A domain 1+ bound with high affinity to the GRE and this binding depended on the presence of zinc (Figure 5B). The mutated NS5A polypeptide (4C-4S), incapable of binding Zn^{2+} because of complete disruption of the Zn^{2+}-binding site, failed to bind to the GRE (Figure 5C). Converting the GRE sequence into CU- or AU-rich sequences resulted in a 7- to 10-fold reduction in the observed affinity of NS5A domain 1+ for these RNAs (Table 1; compare RNA #4 and #5 to #3). Two RNA sequences derived from the consensus sequence in Figure 2B bound to NS5A domain 1+ with high affinities (Table 1, RNA #1 and #2). Overall, our data support a direct and

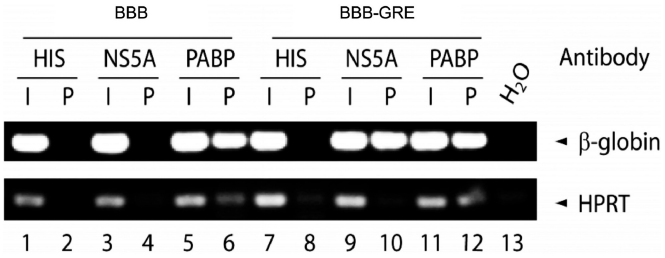

Figure 4. NS5A binds to GRE-containing transcripts in cells. Huh7-HCV cells were transfected with the BBB, or BBB-GRE reporter plasmids. Cell lysates were immunoprecipitated using specific antibodies against the His-tag (HIS), NS5A or the poly A binding protein (PABP). RNA isolated from the input (I) or the pellet fraction (P) was reverse transcribed and amplified by PCR using beta-globin and HPRT specific primers, and the RNA was separated by electrophoresis. Water (H_2O) was used as a contamination control for the PCR.

specific interaction between NS5A and the GRE in cells and *in vitro*.

NS5A directly stabilizes GRE-containing transcripts

Based on our findings that GRE-containing transcripts were stabilized in Huh-HCV cells and that the HCV NS5A protein was found to bind to GRE sequences, we hypothesized that binding by NS5A to GRE-containing transcripts mediates transcript stabilization. To test our hypothesis, we transfected HeLa cells with the BBB, BBB-GRE, BBB-mGRE or BBB-ARE beta-globin reporter constructs and co-transfected them with constructs that expressed wild-type NS5A (NS5A) or the following NS5A derivatives: 4C-4S, ΔN or ΔN-4C-4S. A GFP expression construct was also co-transfected to control for transfection efficiency. Transcription from the tet-responsive promoter was blocked by the addition of doxycycline, and total RNA was extracted after 0, 1.5, 3, 4.5 or 6 h. Transcript degradation was measured over time by northern blot (Figure 6) and the expression of NS5A or derivatives were monitored by western blot (Supplementary Figure S3). As expected, the BBB-reporter alone and the BBB-mGRE reporter were very stable, whereas the BBB-GRE reporter decayed more rapidly (half-life = 337 ± 42 minutes). When the NS5A construct was co-transfected, however, the BBB-GRE transcript was stabilized (1091 ± 117 min; $P = 0.0005$). This stabilizing effect was not observed when the four cysteines that form the NS5A zinc finger were mutated to serine (4C-4S). This indicated that a zinc finger-mediated NS5A-GRE interaction was required for stabilization. Published reports showed that the N-terminal helix of NS5A is required for tethering the NS5A protein to the viral replication compartment of the endoplasmatic reticulum (36–38). Deletion of the N-terminal helix releases this protein into the cytoplasm and decreases viral replication (39). We found that deletion of the N-terminal 36 amino acids (ΔN) had no effect on the ability of NS5A to stabilize the GRE-containing reporter transcript. Deletion of the N-terminal 36 amino acids and perturbation of the NS5A zinc finger at the same time (ΔN-4C-4S), however, abrogated the stabilizing activity of NS5A. In contrast to the GRE-reporter, the ARE reporter transcript (BBB-ARE) displayed equal decay in the presence or absence of NS5A with half-life of 240 ± 15 minutes and 228 ± 25 minutes, respectively. This result indicates that NS5A specifically and selectively mediated the stabilization of GRE-containing, but not ARE-containing transcripts.

DISCUSSION

Introduction of a HCV subgenomic replicon into Huh cells led to the selective stabilization of host cellular transcripts through a mechanism that involved binding of NS5A directly to GU-rich sequences. Not all stabilized transcripts showed increased abundance. This could be due to other mechanisms that may affect transcript abundance to maintain homeostasis. For NS5A target transcripts we observed a significantly higher number of transcripts with increased abundance compared to non-NS5A target transcripts (Figure 2A). We showed that NS5A binds directly with high affinity to GRE sequences, and may bind to other G or U-rich sequences that were conserved in the 3′ UTRs of NS5A target transcripts. Our finding in HeLa cells that exogenous expression of NS5A specifically stabilizes GRE-containing transcripts clearly demonstrates that NS5A possesses mRNA-stabilizing activity (Figure 6). This function of NS5A enables HCV to control host cellular gene expression.

The results presented here support previous work suggesting that NS5A functions as an RNA-binding protein. NS5A interacts with HCV genomic RNA (18,19) and was shown to bind to polyU or polyG sequences (18). Our results indicate that a G-rich consensus sequence was present in the 3′ UTRs of NS5A target transcripts (Figure 2B). This sequence resembles the G-rich consensus sequence previously found in CELF1 target transcripts (33). In addition, NS5A is also reported to bind to poly(U/UC) sequence in the 3′UTR of HCV RNA and downregulates viral RNA translation (40). We found similar poly(U/UC)-rich sequences were present within the 3′UTR of NS5A target transcripts (Supplementary Figure S2). It is reported that GRE sequences are very common in the genome. Although GREs were found in NS5A target transcripts, they were not enriched. Our data shows that the GRE can function as a site for NS5A, but not all GRE-containing transcripts are NS5A targets. This may be due to secondary structures, genomic context, or availability for binding sites.

Our results confirm the RNA-binding activity of NS5A and suggest that the GRE sequence, UGUUUGUUUGU (22), is a binding target for NS5A. We showed by RNA immunoprecipitation that an anti-NS5A antibody specifically co-immunoprecipitated GRE-containing reporter transcripts, but not reporter transcripts that lacked a GRE (Figure 4). These results suggest that the GRE functions as a target of NS5A within cells. Our *in vitro* binding experiments showed that NS5A binding to the GRE was dependent upon the C_4 zinc finger motif in domain 1 since no GRE-binding activity was observed when the four cysteines were mutated to serines (Figure 5C). Also, no binding presented in the absence of zinc (Figure 5B), suggesting that coordination of zinc is critical for binding. Crystal structures of domain 1 dimers revealed the presence of a cleft near the zinc-coordinating sites that may facilitate RNA binding

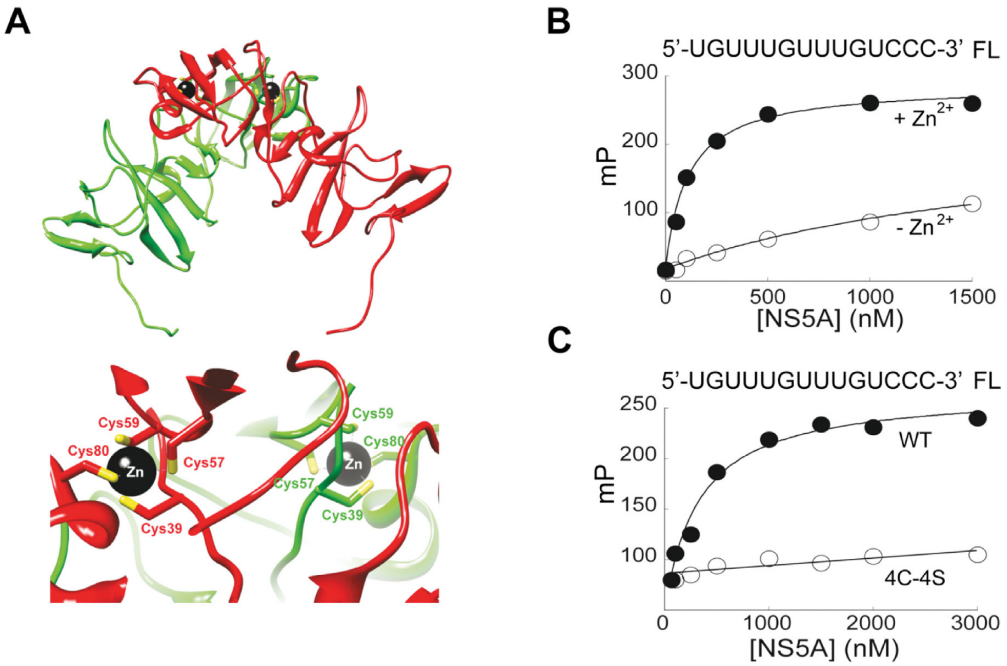

Figure 5. Recombinant NS5A binds to GRE RNA in a manner that is dependent on an intact zinc-binding site and the presence of zinc. (**A**) The upper panel shows a ribbon diagram of dimeric NS5A domain 1 that was prepared by using reference number 1ZH1 from the Protein Data Bank. One subunit is colored red and the other is colored green. The lower panel zooms in on the zinc-binding site of each subunit. Four conserved cysteines are required for zinc binding. (**B**) The NS5A domain 1+ polypeptide was titrated into a binding reaction buffer (20 mM HEPES pH 7.5, 5 mM $MgCl_2$, 10 mM 2-mercaptoethanol, 100 mM NaCl) in the absence (-Zn^{2+}) or presence (+Zn^{2+}) of 100 μM $ZnCl_2$ and incubated briefly at 25°C in a final volume of 100 μl. Binding of NS5A was measured by the change in polarization (mP). The change in fluorescence polarization was plotted as a function of NS5A domain 1+ concentration and fit to a hyperbola by using KaleidaGraph (Synergy Software). (**C**) Experiments were performed as described in panel B in the presence of zinc using the NS5A domain 1+ polypeptide (WT) or the derivative whose zinc-binding site was inactivated by converting the four cysteine residues to serine residues (4C-4S).

(20,21) (Figure 5A). Collectively, our findings suggest that the zinc finger motif contributes to the functional form of NS5A that enables specific interaction of NS5A with GRE RNA.

We previously showed that the GRE mediates rapid mRNA decay by binding to CELF1 (22), which appears to recruit components of the cellular mRNA decay machinery such as poly A ribonuclease (25). In the presence of NS5A, the rapid decay of the GRE-containing reporter was completely abolished (Figure 6), suggesting that NS5A might potentially antagonize the activity of CELF1. CELF1 and NS5A appear to have overlapping binding specificities since they both bind directly to G-rich sequences, including the GRE, but a subset of NS5A target transcripts might not be targets of CELF1. These proteins have different mechanisms for RNA binding. NS5A binding involves zinc finger domains, whereas CELF1 binding involves RNA recognition motifs, yet their binding target sites appear to overlap, at least in the case of GRE sequences. Further work is needed to better define the sites for binding by these proteins to host transcripts.

Viruses have developed mechanisms to manipulate host gene expression at posttranscriptional levels in order to subvert antiviral defense mechanisms and to create an environment in the virus-infected cell that will prevent cell death and allow viral replication. Kaposi's sarcoma virus (1) and Herpes simplex virus (2) produce nucleases that mediate host mRNA decay. Herpes simplex virus also produces the ICP27 protein which mediates the stabilization of AU-rich element-containing host transcript (3) and Epstein-Barr virus infection leads to the stabilization of mRNAs through the activation of the stress activated protein kinase p38 (41). Several studies found differential host gene expression in cell cultures infected with HCV using high-throughput approaches and screens (42–46), but the mechanism of how the host gene expression is regulated by the virus is not well understood. Luna et al. proposed that microRNA-122 sequestration by HCV RNA may lead to stabilization of miR-122 targets, facilitating the oncogenesis of HCV (47). Moon et al. demonstrated another mechanism that HCV utilizes to affect host mRNA decay. They found the 5′UTR of HCV genomic RNA can stall and suppress the cellular 5′-3′ exoribonuclease Xrn1, leading to global stabilization of cellular transcripts (48). The mechanism by which HCV stabilizes GRE-containing transcripts, described in this report, depends on the direct interaction between the NS5A protein and GU-rich sequences within host cellular mRNA. Through this mechanism, HCV blocks the host mRNA degradation machinery and selectively stabilizes a set of GU-rich transcripts that may be necessary for establishment or maintenance of chronic HCV infection.

Many of the short-lived GRE-containing transcripts in mammalian cells encode proto-oncogenes and other important regulators of cell growth and apoptosis, constituting a network of coordinately regulated transcripts (23). These GRE-containing transcripts include proto-oncogene tran-

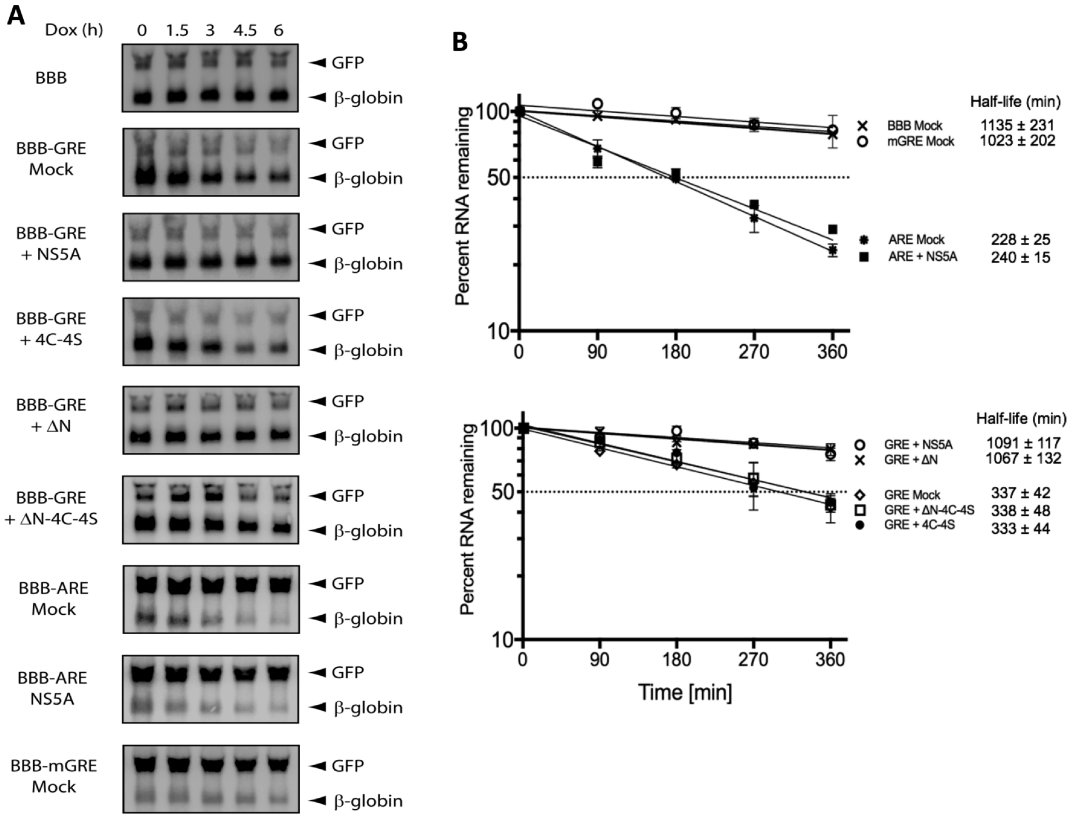

Figure 6. Exogenously expressed NS5A stabilizes GRE-containing reporter genes. (**A**) HeLa tet-off cells were transfected with BBB, BBB-GRE, BBB-ARE or BBB-mGRE reporter plasmids as well as a plasmid that express NS5A, NS5A-4C-4S, ΔN-NS5A, ΔN-NS5A-4C-4S or a mock control plasmid. A GFP expression plasmid was included in each to control for transfection efficiency. Transcription from the tet-responsive promoter was stopped with 300 ng of doxycycline and RNA harvested after 0, 1.5, 3, 4.5 or 6 h was analyzed by northern blotting using GFP and beta-globin probes. (**B**) The experiment shown in (A) was performed three times, and the northern blot signals were quantified by a Storm 820 phosphorimager (Amersham Biosciences). For each time point, the intensity of the beta-globin reporter was normalized to the intensity of the GFP band, and the band intensity at the 0 time point was set to 100%. The percent of mRNA remaining was plotted over time. The error bars indicate the standard error of the mean (SEM) from three experiments. The calculated transcript half-life and SEM are shown to the right of each graph.

scripts such as *JUN*, *JUNB*, *JUND*, and *ETS2*, transcripts encoding regulators of apoptosis such as *BCL10*, *BAG*, *MAP3K5*, and *TNFRSF1*, and transcripts encoding other regulators of cell growth including *EIF4EBP2*, *EIF4G3*, *SMAD7* and *HOXC10*. After infection with HCV, NS5A-mediated stabilization and overexpression of these GRE-containing transcripts would be predicted to promote cell growth and prevent cell death in order to allow a chronic infection to be established. It is possible that new drugs that block the NS5A interaction with the GRE could be developed that would prevent establishment of chronic HCV infections and inhibit the maintenance or propagation of infection. Indeed, drugs like ledipasvir that target NS5A but whose mechanism of action remain unclear (49) might function by perturbing the ability of NS5A to bind GREs or similar G or U rich sequences and modulate host gene expression.

Infection with HCV increases the risk for hepatocellular carcinoma development as well as certain lymphomas (50,51). The onset of the tumors, however, occurs many years after HCV infection, suggesting that multiple events are required to transform hepatic cells to become malignant (52). Progression of liver fibrosis to cirrhosis in HCV-infected patients will generate a local milieu that predisposes to liver cancer. In this environment, changes occur to the hepatic parenchyma, with hepatocyte injury, which contributes to sequential genetic hits that culminate in malignant transformation (53). However, the mechanisms involved in this process are largely unknown. Moreover, multiple studies have showed a direct role for HCV in hepatic carcinogenesis and transgenic mice expressing the HCV polyprotein can develop liver cancer in the absence of inflammation, hepatic cirrhosis or immune recognition of the transgene (13,54,55). The stabilization and overexpression of GRE-containing transcripts including proto-oncogene transcripts and transcripts encoding regulators of apoptosis may contribute to the development of cancer by promoting cell growth and preventing the death of genetically damaged cells. Later events that finally lead to uncontrolled growth of these cells might destroy the fail-safe mechanisms that are initially able to cope with the increased stability of these important messages.

In conclusion, our findings describe a novel role of NS5A in stabilizing host GRE-containing transcripts, providing new insights into HCV pathogenesis and carcinogenesis that may lead to new treatments to prevent HCV infection and understand the progression to liver cancer.

DATA AVAILABILITY

Gene expression omnibus (GEO) accession number GSE102910.

SUPPLEMENTARY DATA

Supplementary Data are available at NAR online.

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