

Identification of Cellular Factors Associated with the 3'-Nontranslated Region of the Hepatitis C Virus Genome*

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Chronic infection by hepatitis C virus (HCV) is the leading cause of severe hepatitis that often develops into liver cirrhosis and hepatocellular carcinoma. The molecular mechanisms underlying HCV replication and pathogenesis are poorly understood. Similarly, the role(s) of host factors in the replication of HCV remains largely undefined. Based on our knowledge of other RNA viruses, it is likely that a number of cellular factors may be involved in facilitating HCV replication. It has been demonstrated that elements within the 3'-nontranslated region (3'-NTR) of the (+) strand HCV genome are essential for initiation of (-) strand synthesis. The RNA signals within the highly conserved 3'-NTR may be the site for recruiting cellular factors that mediate virus replication/pathogenesis. However, the identities of putative cellular factors interacting with these RNA signals remain unknown. In this report, we demonstrate that an RNA affinity capture system developed in our laboratory used in conjunction with LC/MS/MS allowed us to positively identify more than 70 cellular proteins that interact with the 3'-NTR (+) of HCV. Binding of these cellular proteins was not competed out by a 10-fold excess of nonspecific competitor RNA. With few exceptions, all of the identified cellular proteins are RNA-binding proteins whose reported cellular functions provide unique insights into host cell-virus interactions and possible mechanisms influencing HCV replication and HCV-associated pathogenesis. Small interfering RNA-mediated silencing of selected 3'-NTR-binding proteins in an HCV replicon cell line reduced replicon RNA to undetectable levels, suggesting important roles for these cellular factors in HCV replication. *Molecular & Cellular Proteomics* 5:1006–1018, 2006.

More than a decade ago, hepatitis C virus (HCV)¹ was discovered as the major causative agent of parenteral non-A

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¹ The abbreviations used are: HCV, hepatitis C virus; 3'-NTR, 3'-nontranslated region; 3'-UTR, 3'-untranslated region; ARE, A/U-rich element; siRNA, small interfering RNA; PTB, polypyrimidine tract-binding protein; RNP, ribonucleoprotein; hnRNP, heterogeneous nu-

non-B hepatitis (1, 2), and the number of HCV-infected individuals worldwide is currently estimated at 170 million. Although some infected individuals are able to clear the virus without treatment, most infections persist leading in about 50% of all cases to chronic hepatitis, which may develop into chronic active hepatitis, liver cirrhosis, and hepatocellular carcinoma. The main therapeutic regimen currently in clinical use is a combination treatment consisting of high doses of interferon- α and the nucleoside analog ribavirin, and a large percentage of patients receiving this regimen are not responsive. These stark facts underscore the importance of expediently developing new strategies to combat this viral infection.

The hepatitis C virus is a positive strand RNA virus of the flaviviridae family having a genome roughly 9.6 kb in length (3). The RNA genome contains a single ORF that encodes a polyprotein of ~3000 amino acids that is processed by a combination of host- and virus-encoded proteases (4) into four structural proteins (core, E1, E2, and p7) and six non-structural (NS2, NS3, NS4A, NS4B, NS5A, and NS5B) proteins. Replication of HCV is initiated at the 3'-NTR of the RNA genome, although little is known about the mechanism of initiation or the factors required for this process. The NS5B protein is the RNA-dependent RNA polymerase, or HCV replicase, responsible for replication of the HCV genome whose structure and enzymatic activities have been well characterized (5, 6). Although it has been established that NS5B can initiate primer-independent RNA synthesis, the mechanistic details of this process remain the source of some controversy (7–9). Hong *et al.* (10) have proposed that a structural motif within NS5B positions the terminal nucleotides of the genome in such a way that *de novo* synthesis is initiated from the 3'-end of the genome. The initiation of HCV virus replication may very well involve the aid of cellular factors in this process. As discussed below, cellular factors that bind the 3'-NTR of HCV may also regulate viral RNA stability, transport, and localization.

Until recently, there was no system for studying HCV replication under *in vivo* conditions other than in the chimpanzee.

clear ribonucleoprotein; MP, mobile phase; KH, K homology; RRM, RNA recognition motif; HIV, human immunodeficiency virus; HIV-1, human immunodeficiency virus, type 1; PSF, PTB-associated splicing factor; FUSE, far upstream element; RHA, RNA helicase; FBP, far upstream element-binding protein.

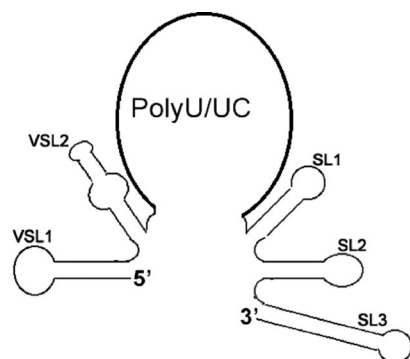


FIG. 1. The predicted secondary structure of HCV 3'-NTR (+). The 3'-NTR contains three structurally distinct domains: (i) an upstream variable region folded into two stem-loop structures designated as *VSL1* and *VSL2*, (ii) a large poly(U)/UC tract, and (iii) a highly conserved 98-nucleotide 3'-terminal segment that forms three stem-loop structures designated as *SL1*, *SL2*, and *SL3*. Both poly(U)/UC tract and 98-nucleotide conserved segments are required for infectivity (15, 32) and viral replication (16, 17).

Therefore, examining the precise role(s) of either RNA signals or proteins involved in replication presented a formidable challenge. Lohmann *et al.* (11) were the first to report development of a subgenomic HCV dicistronic RNA that replicated efficiently in cell culture. Subsequently other groups also reported that subgenomic replicons of HCV were maintained in cell culture systems, some of which revealed adaptive mutations in NS3, NS5A, and NS5B that further enhanced replication capacity (12–14). Therefore, it is now possible to study various aspects of HCV replication and pathogenesis in cell culture, although the absence of the HCV structural genes in the subgenomic replicons prevents a comprehensive view of HCV biology and virus-host interactions. The replicon system has been used to identify conserved regions in the 3'-NTR of HCV required for viral replication (15–17), although the exact function(s) of these RNA signals remains unclear.

The conserved nontranslated 5'- and 3'-terminal regions of the viral genome have multiple regulatory elements that are essential for viral replication and expression of viral genes. Although the 5'-NTR of HCV contains the internal ribosomal entry site required for cap-independent translation of HCV RNA (18), the 3'-NTR is also predicted to be highly structured (Fig. 1) and is the site for initiation of viral replication (7). In addition to proteins associated with the translational machinery, several proteins have been reported to interact with the 5'-NTR, including polypyrimidine tract-binding protein (PTB) (19–21), La autoantigen (22), nucleolin (23), and EIF2B γ (24). Previous investigations have also reported some cellular proteins associated with the 3'-NTR of HCV, namely PTB, (25–27) heterogeneous nuclear ribonucleoprotein C (hnRNP C) (28), La autoantigen (29, 30), and HuR protein (31). In efforts to elucidate the structure and composition of the nucleoprotein complex at the 3'-NTR of HCV, we developed a novel RNA affinity capture system in which a biotinylated oligonucleotide is annealed to one end of a runoff transcript corresponding to

the (+) strand 3'-NTR of HCV. Subsequent immobilization of this partial duplex on paramagnetic streptavidin-coated beads and incubation with hepatocyte extracts allowed us to isolate cellular proteins bound to the 3'-NTR RNA. One-dimensional SDS-PAGE followed by tryptic digest of selected bands and LC/MS/MS analysis positively identified more than 70 cellular proteins that bind with high affinity to the 3'-NTR of HCV. Although some of the proteins we identified may represent irrelevant interactions with the HCV RNA, some have already been implicated in the life cycles of other viruses, and yet others surely represent novel insights into the protein machinery utilized by HCV in its replication strategy.

EXPERIMENTAL PROCEDURES

Plasmids and Oligonucleotides

A 243-bp fragment of the 3'-terminal region of the HCV genome (32) was cloned in the pET3b vector (Novagen, Inc.) 65 nucleotides downstream of the T7 promoter in between *Nde*I and *Bam*HI sites to generate pVP506. The transcript prepared after linearizing pVP506 with *Bam*HI contains an additional 65-nucleotide flanking sequence derived from the plasmid upstream of the 3'-NTR. For hybridization to the 5'-flanking sequences of the 3'-NTR runoff transcript, we synthesized a 39-mer oligonucleotide with the sequence 5'-GTA TAT CTC CTT CTT AAA GTT AAA CAA AAT TAT TTC TAG-3' having biotin conjugated to the 3'-terminal G nucleotide.

Preparation of Transcripts

The 308-base 3'-NTR runoff transcripts were generated following linearization of the pVP506 plasmid with *Bam*HI using the T7 transcription kit from Roche Applied Science. The 311-base transcripts encoding the 3'-untranslated region of the human collagen gene *COL1A2* were prepared using SP6 RNA polymerase (Invitrogen) following linearization of the plasmid (33) with *Pst*I. Following treatment with RNase-free DNase I to remove template DNA, the RNA was precipitated with lithium chloride and resuspended and stored in RNase-free water containing 40 units/ml SUPERaseIN RNase inhibitor (Ambion) at -80°C .

Cell Culture and Preparation of Hepatocyte Cell Extracts

Huh7 and En5-3 cells, a clonal cell line derived from Huh7 (34), were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum, 2 $\mu\text{g}/\text{ml}$ blasticidin (Invitrogen), penicillin, and streptomycin. Cell extracts from hepatocytes (Huh7 or En5-3) were prepared using the procedure of Dignam *et al.* (35) and stored under liquid nitrogen. Briefly cells were homogenized in 250 mM sucrose, 10 mM HEPES (pH 8.0), 2 mM PMSF, 50 mg/ml antipain, 0.5 mg/ml leupeptin, 0.7 mg/ml pepstatin. For subfractionation of cell homogenate, the homogenate was centrifuged successively at $3000 \times g$, $10,000 \times g$, and $50,000 \times g$, and the $10,000 \times g$ and $50,000 \times g$ pellets were suspended in buffer containing 0.5% Nonidet P-40 for 1 h at 4°C and then centrifuged at $10,000 \times g$ and $50,000 \times g$, respectively. The supernatants were dialyzed against 20 mM HEPES, pH 8.0, 0.5 mM DTT, 0.5 mM PMSF, 10% glycerol at 4°C for 3–5 h. The $10,000 \times g$ extract was stored as mitochondrial extract, whereas the $50,000 \times g$ extract was stored as endoplasmic reticulum extract and stored under liquid nitrogen. Supernatant from the post- $50,000 \times g$ fraction (soluble cytosolic fraction) was used in the binding experiments. Protein concentrations in each extract were determined by Bradford assay (Bio-Rad).

RNA Affinity Capture Experiments

For small scale experiments we used 20 μ l of En5-3 cell extract, 75 μ l of the streptavidin-coated paramagnetic bead suspension (Dyna, Inc.), and 100 pmol of RNA:39-mer duplex. The experiments scaled up for proteomic analysis were 5 \times for each of these constituents. Briefly the 3'-NTR runoff transcript was annealed to the 5'-biotin-labeled 39-mer DNA oligonucleotide. The streptavidin bead preservative solution was removed by magnetic separation, beads were washed two times with binding buffer containing 1 \times protease inhibitor mixture (mini-EDTA-free, Roche Applied Science), 1 mM DTT, 100 mM NaCl, 20 mM HEPES, pH 7.5, 20 units/ml SUPERaseIN (Ambion) and were mixed with the RNA:39-mer duplex. This mixture was incubated on ice in binding buffer for 30 min to immobilize the RNA:39-mer duplex on the beads. The buffer solution was removed, and En5-3 cell extracts were then mixed with the beads to capture proteins binding the 3'-NTR RNA. Beads were incubated with the cell extracts on ice for a total of 1 h with occasional gentle vortexing. For competition experiments, a 10-fold molar excess of MS2 RNA (Roche Applied Science) was added to the cell extracts prior to incubation with the beads, and for poly(rU) competition, a 2–16-fold molar excess of polyuridylic acid (Amersham Biosciences, average length = 250 nucleotides) was added. Competition experiments with 3'-UTR RNA of human collagen gene were also performed by addition of the competitor (20–100 pmol) to the cell extracts prior to incubation with NTR-bound beads. After the incubation, beads were washed three times with the binding buffer. Elution was done by adding 30 μ l of binding buffer and 30 μ l of 2 \times SDS gel loading dye to washed beads and heating at 95 $^{\circ}$ C for 5 min prior to magnetic separation of beads from eluted proteins. Samples were resolved by 8–16% gradient SDS-PAGE, and the gel was stained with SYPRO Ruby dye (Molecular Probes, Inc.) for visualization of protein bands, which were excised for tryptic digest.

Tryptic Digest and Mass Spectrometry

Circular cores of gel bands were excised and placed in a 96-well TecPro plate (Tecan) containing 100 μ l of Milli-Q water for processing in an automated protocol on the Tecan GENESIS instrument. Gel pieces were washed two times with 50 mM ammonium bicarbonate in 30% acetonitrile. Reduction was done by incubating gel pieces for 30 min at 37 $^{\circ}$ C in solution containing 10 mM DTT, 50 mM ammonium bicarbonate, 30% acetonitrile. Subsequently alkylation was done by incubating gel pieces for 30 min at 37 $^{\circ}$ C in solution containing 45 mM iodoacetamide, 50 mM ammonium bicarbonate, 30% acetonitrile. Gel pieces were then dehydrated by washing two times with 80% acetonitrile and drying at 60 $^{\circ}$ C for 10 min. Dried gel pieces were subjected to trypsin digest by adding solution containing 50 mM ammonium bicarbonate, 10 ng/ml trypsin (Trypsin Promega Gold MS grade). Following a 2-h incubation at 37 $^{\circ}$ C for digestion, reactions were adjusted to 1% trifluoroacetic acid for extraction of peptides from the gel, and the liquid phase was eluted through the capillaries in the bottom of the TecPro plate. A Micromass CapLC system was coupled with a Waters UH104 Q-TOF instrument to perform the LC/MS/MS analysis. A 300- μ m C₁₈ precolumn and 15-cm, 75- μ m-inner diameter C₁₈ nano-LC column were purchased from LC Packings. 15- μ l in-gel digested samples were concentrated by spinning in an Eppendorf Vacufuge for 15 min to reduce the volume to \sim 7 μ l of which 6.4 μ l was injected onto a precolumn without further purification at a flow rate of 20 μ l/min. Peptides held on the precolumn were eluted onto the nano-LC column at a flow rate of 4 μ l/min. The composition of MP A and MP B were 0.1% formic acid in 98:2 H₂O:ACN and 5:95 H₂O:ACN solvent, respectively. A 36-min gradient with MP B increasing from 3 to 45% was used to separate the peptides. The scan times for MS and MS/MS experiments were 1.5

and 2.0 s, respectively. Multiply charged ions with MS intensity higher than 15 counts were automatically chosen for MS/MS. In the case that there were many ions with intensity higher than 15 counts, the number of ions chosen for MS/MS simultaneously was set to 3. For a certain ion, when either total ion current of MS/MS was over 2500 counts or total MS/MS scan time was over 6.0 s, the Q-TOF instrument automatically switched from MS/MS mode to MS mode. Following data acquisition, ProteinLynx 2.1 from Waters was used to process and generate the peaklist file, and both ProteinLynx 2.1 and Mascot were used for database searching. Only proteins with a protein score >100 and at least two matching peptides were considered positively identified proteins.

siRNA Experiments

Sequences of siRNAs Used—Sequences of siRNAs were as follows: Ddx5: sense, 5'-GGG UUC UAA AUG AAU UCA Att-3'; anti-sense, 5'-UUG AAU UCA UUU AGA ACC Cag-3' (Qiagen); HuR: siRNA used was HiPerformance Validated from Qiagen catalog number SI00300139; FBP: sense, 5'-GGG ACA UCA CUG A AU UCA Att-3'; anti-sense, 5'-UUG AAU UCA GUG AUG UCC Ctg-3' (Ambion); control siRNA: sense, 5'-UUC UCC GAA C GU GUC ACG Utt-3'; anti-sense, 5'-ACG UGA CAC GUU CGG AGA Att-3' (Qiagen).

Primer Sequences Used for RT-PCR—Primer sequences used were as follows: HCV 5'-NTR: forward, 5'-CGG GAG AGC CAT AGT GG-3'; reverse, 5'-AGT ACC ACA AGG CCT TTC G-3'; actin: forward, 5'-CAG GCA CCA GGG CGT GAT GG-3'; reverse, 5'-AGG CGT ACA GGG ATA GCA CA-3'.

HCV Subgenomic Replicon and Cell Line—MH 14 cells (a kind gift from Dr. Makoto Hijikata) carrying replicating HCV replicons were grown in Dulbecco's modified Eagle's medium (Cellgrow) supplemented with 10% fetal calf serum, 100 μ g/ml penicillin, 100 μ g/ml streptomycin, 300 μ g/ml G418. The cells (2 \times 10⁵/well) were grown in a 6-well plate for 24 h and then transfected with 20 nM siRNAs targeted against Ddx5, FBP, and HuR following the manufacturer's protocol using siPort amine as the transfection reagent (Ambion). The transfected cells were further grown for 72 h. In one set, the cells were washed, lysed, and analyzed for total protein (BCA protein assay, Pierce). An equal quantity of protein from each set was used for Western blot analysis. Another set of cells were processed for the isolation of total mRNA and subsequent RT-PCR analysis for HCV RNA and actin mRNA.

RESULTS

RNA Affinity Capture Is a Robust Tool for Characterizing 3'-NTR-Protein Interactions—Traditional methods for characterizing RNA-protein interactions, such as UV cross-linking and RNA gel mobility shift assays (36), can provide interesting information regarding size and other biophysical properties of RNA-interacting proteins but cannot provide identity information without additional analysis. Therefore, these techniques represent unnecessary intermediate steps when the ultimate objective is the identification of RNA-associated proteins. Although DNA affinity capture has been used to isolate cellular proteins associated with specific sequences (37) and a strategy similar to ours has been used to isolate RNP complexes from cells harboring subgenomic HCV replicons (38), this technique has not been successfully applied to comprehensive protein profiling of complexes formed with specific viral RNA species. Prior methods for biotinylation of RNA involve either cumbersome chemical modification and purification

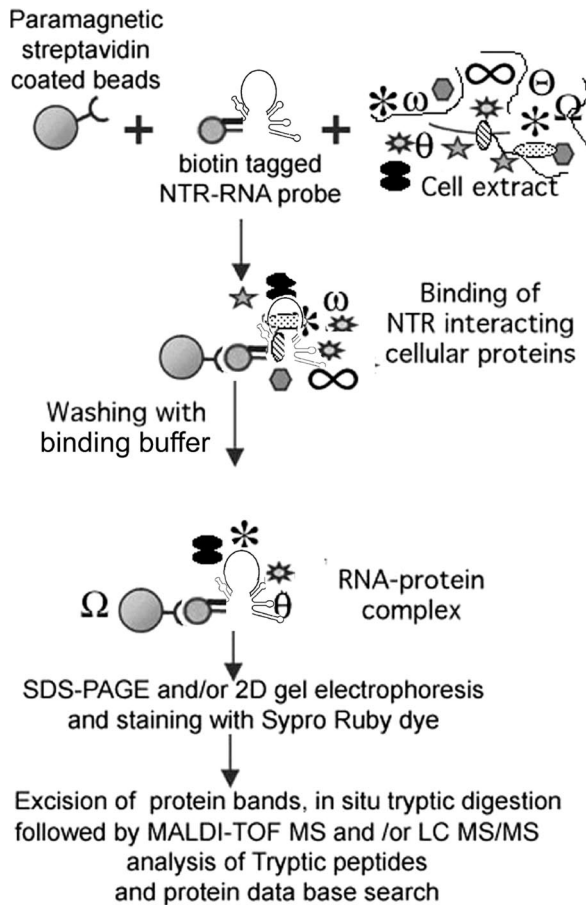


FIG. 2. Scheme for RNA affinity capture of cellular proteins interacting with HCV 3'-NTR.

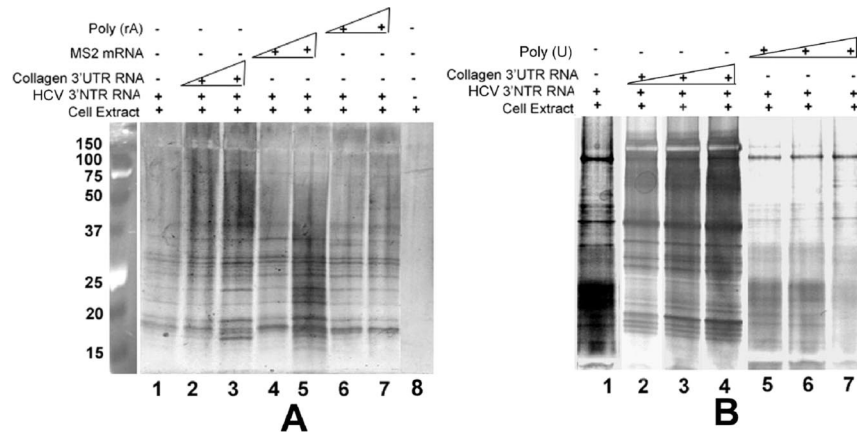


FIG. 3. RNA affinity capture in the presence and absence of competitor RNA. A, the RNA transcripts of the 3'-UTR of the human collagen gene, MS2 bacteriophage mRNA, and poly(rA) were used as competitor RNAs in the binding reaction. The HCV 3'-NTR RNA annealed with biotinylated 39-mer DNA oligonucleotide was incubated with cell extract on ice in the presence of individual competitor RNAs. After 1 h of incubation, the biotinylated 3'-NTR RNA:39-mer DNA duplex was immobilized on paramagnetic streptavidin beads, washed extensively with the binding buffer, and subjected to SDS-PAGE analysis. The gel was stained with SYPRO Ruby dye. Lane 1 shows cellular proteins captured on 3'-NTR RNA in the absence of competitor RNA; lanes 2 and 3, in the presence of 1- and 5-fold excess of collagen 3'-UTR RNA; lanes 4 and 5, in the presence of 1- and 5-fold excess of MS2 mRNA; and lanes 6 and 7, in the presence of 1- and 5-fold excess of poly(rA), respectively. Lane 8 represent incubation of streptavidin beads alone with the cell extract. The molecular markers are shown on the left in A. B, all the components of the competition experiment with collagen 3'-UTR of A were scaled up to 2-fold, and the concentration of the competitor 3'-UTR as well as poly(U) was in excess of 1–10-fold over HCV 3'-NTR RNA. Lane 1, cellular proteins captured on 3'-NTR RNA in the absence of competitor; lanes 2–4, in the presence of 1-, 5-, and 10-fold excess of collagen 3'-UTR RNA; lanes 5 and 6, in the presence of 1-, 5-, and 10-fold excess of poly(U).

steps (39, 40) or a random internal labeling of transcripts (41) that results in a heterogeneous population with regard to location(s) of the biotin label and that may disrupt important secondary structure within the RNA and diminish the availability of the RNA for specific association with proteins. By annealing a biotinylated oligonucleotide complementary to flanking sequences at the 5'-end of the HCV 3'-NTR RNA and then immobilizing on paramagnetic streptavidin beads (see "Experimental Procedures"), we overcame the need for labeling of the RNA, maintained the native structure of the target RNA trap, and facilitated its association with interacting cellular proteins. This strategy is outlined in Fig. 2.

Binding of Cellular Protein to HCV 3'-NTR in the Presence of Competitor RNAs—Because the 3'-NTR of HCV may serve a function similar to that of the 3'-untranslated regions (3'-UTRs) of some cellular mRNAs, such as regulating RNA stability, we reasoned that binding of proteins to the 3'-NTR of HCV might be competed out by addition of a cellular mRNA 3'-UTR to the binding reactions. We performed an experiment in which NTR RNA was incubated with the cytosolic fraction of the cell extract in the presence of 3'-UTR RNA of the human collagen gene (33) as a competitor at the ratio of 1:1 and 1:5 of 3'-NTR RNA:competitor RNA (Fig. 3A, lanes 2 and 3). In another set of binding reactions, we also included MS2 bacteriophage mRNA (Fig. 3A, lanes 4 and 5) as well as poly(rA) (Fig. 3A, lanes 6 and 7) as competitors. The 3'-NTR RNA annealed with biotinylated 39-mer oligonucleotide DNA along with individual competitor RNA(s) was incubated with the soluble cytosolic extract for 1 h on ice followed by selective trapping of the NTR RNA:39-mer DNA duplex on strepta-

vidin beads. After extensive washing, beads were subjected to SDS-PAGE followed by staining with SYPRO Ruby dye. The intensity of proteins bound to HCV 3'-NTR RNA was actually increased in the presence of collagen 3'-UTR RNA, and this increase in intensity was correlated with the concentration of the competitor (Fig. 3A, lanes 2 and 3). Similar results were also obtained with MS2 bacteriophage mRNA and poly(rA) competitors.

In the next experiment, we repeated the competition experiment with 3'-UTR by scaling up the reaction components 2-fold. The ratio of HCV 3'-NTR RNA to collagen 3'-UTR RNA competitor was kept at 1:1, 1:5, and 1:10 (Fig. 3B, lanes 2–4). We also included poly(rU) as the competitor at a similar ratio of the 3'-NTR RNA *versus* the competitor RNA (Fig. 3B, lanes 5–7). As shown in the figure, the binding intensity of NTR binders was significantly increased with the increase in the concentrations of collagen 3'-UTR as the competitor RNA. Some additional new bands were also seen in the presence of collagen 3'-UTR. In contrast, a strong competition by poly(U) was observed as most of the 3'-NTR binders are competed out in the presence of this competitor, suggesting that the poly(U)-U/C-rich region of 3'-NTR is a hot spot for binding of most of the cellular proteins. The enhanced binding of specific cellular proteins to HCV 3'-NTR in the presence of competitor RNAs (except in the presence of poly(U)) was unexpected but is not difficult to explain. In the absence of competitor RNA, nonspecific cellular proteins, which show appreciable binding to the 3'-NTR in the absence of competitor RNA, may interfere with binding of factors that bind specifically to the structure and/or sequence elements of the NTR RNA. These nonspecific proteins loosely associated with the 3'-NTR may be susceptible to removal during subsequent washing of the beads, whereas specific NTR binders may withstand these rigorous wash steps. This masking effect is reduced in the presence of competitor RNAs, which likely sequester the bulk of these nonspecific proteins and thus result in enhanced binding of the relevant NTR-associated factors that remain bound following washing and are visualized in the gel.

Identification of Cellular Proteins Interacting with HCV 3'-NTR—For identification of specific cellular protein binding to the 3'-NTR, the experiment was scaled up 5-fold. The HCV 3'-NTR transcript immobilized on the beads was incubated with cytosolic cellular fraction from hepatic (Huh7) cells in the absence or presence of 10-fold excess of nonspecific MS2 bacteriophage as the competitor RNA. Following extensive washings, the cellular proteins bound to immobilized HCV 3'-NTR were solubilized and subjected to SDS-PAGE. As shown in Fig. 4 (lane 1), most of the cellular proteins captured on the 3'-NTR were resistant to competition by a nonspecific RNA from bacteriophage MS2 (lane 2). Our results demonstrate that this system can provide great specificity in the capture of RNA-binding proteins and adjusted to the appropriate scale yields sufficient protein mass for subsequent LC/MS/MS analysis. Alternative methods for RNA affinity capture are being developed in our laboratory

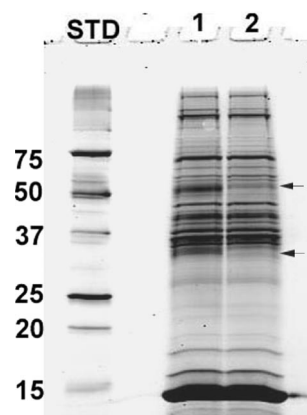


FIG. 4. Preparative scale affinity capture and SDS-polyacrylamide gel electrophoresis of cellular proteins interacting with HCV 3'-NTR (+). Paramagnetic streptavidin beads bound with biotinylated 39-mer oligonucleotide hybridized with the 3'-NTR transcript were incubated with cell extract in the presence and absence of 10-fold excess of total mRNA from bacteriophage MS2 as the competitor RNA for nonspecific proteins. The bound RNA-protein complex was trapped on paramagnetic streptavidin beads and extensively washed with binding buffer, and the bound RNA-protein complexes were subjected to SDS-PAGE on 8–16% gradient polyacrylamide gels and stained with SYPRO Ruby dye. Lanes 1 and 2 represent cellular proteins bound to 3'-NTR in the absence and presence of the competitor RNA, respectively. Molecular mass (kDa) of the marker proteins are shown on the left. The upper arrow indicates the position of a smaller isoform of PTB, and the lower arrow indicates the position of a smaller isoform of hnRNP A1, both of which were competed out in the presence of the competitor RNA. A major band seen at the 13-kDa position corresponds to the streptavidin component of the beads. STD, standard.

for isolation of RNA-associated proteins under native conditions so that these proteins may be used in functional assays following affinity purification.

The individual protein bands (Fig. 4, lane 2) were excised from the gel and processed for LC/MS/MS analysis to achieve the highest level of confidence in our identification. We used LC/MS/MS tandem mass spectrometric detection, which has the advantages of (i) separating tryptic peptides prior to mass spectrometric analysis, (ii) providing sequence information for fragmented peptides, and (iii) identifying proteins in protein mixtures. As shown in Fig. 5, LC/MS/MS analysis of individual protein bands revealed many different proteins bound to the 3'-NTR RNA. These proteins are listed in Table I with their accession number obtained from the protein database (NCBI). Many of these proteins belong to the hnRNP family of proteins, whereas others, such as Ku70, NF90, Y-box transcription factor, and RNA helicase A, represent quite a diverse set of cellular factors. We also confirmed earlier findings that reported the association of both PTB and hnRNP C with the 3'-NTR of HCV (25, 28) as well as HuR protein (31) and glyceraldehyde-3-phosphate dehydrogenase (42). A recent report describing the binding of NF90 to the 3'-NTR of bovine viral diarrhea virus (43) suggested a

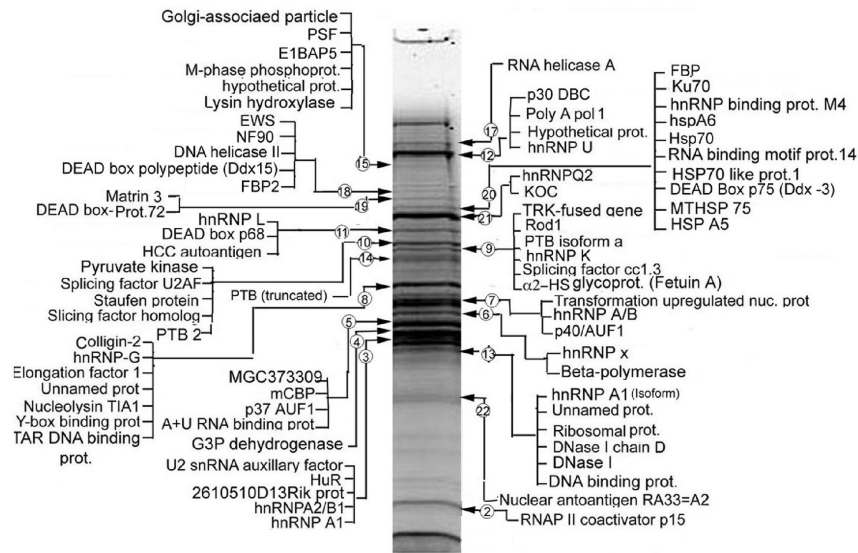


FIG. 5. **Proteomic analysis of cellular proteins captured on the 3'-NTR of HCV.** The individual protein bands from *lane 1* or *lane 2* of Fig. 3 were excised and trypsinized *in situ*, and the resulting tryptic peptides were subjected to LC/MS/MS as described under "Experimental Procedures." Multiply charged ions with MS intensity higher than 15 counts were automatically chosen for MS/MS. Following data acquisition, ProteinLynx 2.1 from Waters was used to process and generate the peaklist (PKL) file, and both ProteinLynx 2.1 and Mascot were used for NCBI database searching. *RNAP*, RNA polymerase; *HCC*, hepatocellular carcinoma; *G3P*, glyceraldehyde-3-phosphate; *mCBP*, murine poly(C)-binding protein.

role for this protein in replication of positive strand RNA viruses, and we also identified this protein as binding to the 3'-NTR of HCV.

Interestingly we detected multiple isoforms of some proteins bound to the 3'-NTR RNA, and only certain isoforms were displaced from the NTR by nonspecific mRNA. For example, the 38-kDa form of hnRNP A1, the major component of band 3 (Fig. 5), was not dissociated from the NTR RNA in the presence of excess competitor RNA. However, a smaller isoform of hnRNP A1, found to be the major component of band 13 (Fig. 5), was efficiently displaced by the competitor RNA (Fig. 4, *lower arrow*). This observation, although initially perplexing, can be explained in the light of previous findings that demonstrate that the larger isoform has higher affinity for single-stranded DNA than the smaller isoform due to the presence of an additional nucleic acid binding domain contained within the C-terminal region (44) absent in the smaller isoform. Similarly, we observed that a smaller isoform of PTB (45, 46), the major component of band 14 (Fig. 5), was efficiently competed out by the competitor RNA, whereas the larger isoforms (bands 9 and 10) were not displaced from the 3'-NTR RNA (Fig. 4, *upper arrow*).

Silencing of RNA Helicase Expression Inhibits HCV Replication—RNA helicases are abundant in cells and serve diverse functions. There is recent evidence that some cellular RNA helicases influence replication of RNA viruses (47, 48). We identified several RNA helicases associated with the 3'-NTR of HCV and selected two of these for further investigation into their possible influence on the replication of HCV repli-

cons in a cell culture system. Using siRNA, we silenced expression of the RNA helicase p68 (Ddx5) and FBP, an ARE-binding protein also shown to possess RNA helicase activity. Additionally we silenced expression of HuR, an ARE-binding protein shown to stabilize cellular RNAs by binding to U-rich regions, (49–51) and that has been shown previously to bind to the 3'-NTR of HCV (31). The siRNA was delivered into MH 14 cells, a cell line carrying actively replicating HCV replicons. Fig. 6 shows that expression of Ddx5 and FBP was reduced by 75 and 80%, respectively, whereas expression of HuR was reduced by ~50% as demonstrated by Western blotting analysis (*lane 4*) by the siRNA specifically targeting these three proteins. Neither control siRNA (*lane 3*) nor RNA transfection reagent alone (*lane 2*) had any influence on expression of these proteins. RT-PCR examining the presence of HCV replicon RNA (5'-NTR) revealed a direct correlation between the reduction in expression of the selected RNA helicase proteins and the reduction in HCV replicon RNA. In the case of HuR, the observed ~50% reduction in protein expression also correlated well with the reduction in HCV replication. Actin RNA controls demonstrated that the loss of HCV RNA following the silencing of these two proteins is not a global effect or a result of cell death. These data suggest that each of these cellular proteins found to be associated with the 3'-NTR of HCV in our affinity capture assay are important for HCV replication. Although down-regulation of HuR expression resulted in a marginal inhibition of HCV replication as judged by RT-PCR, the magnitude of this inhibition was quite dramatic when expression of either FBP or p68 was silenced.

TABLE I

Cellular proteins bound to the 3'-NTR RNA transcript

RNAP, RNA polymerase; HCC, hepatocellular carcinoma; G3P, glyceraldehyde-3-phosphate; mCBP, murine poly(C)-binding protein; snRNA, small nuclear RNA. EWS, Ewing sarcoma; DBC, deleted in breast cancer; KOC, KH domain containing protein overexpressed in cancer.

No.	Cellular protein identified	Molecular mass	Accession no.
		<i>kDa</i>	
1	Similar to hnRNP A1	12.3	gi-34879290
2	RNAP II co-activator p15	14.3	gi-1709514
3	Unnamed protein	15.9	gi-12845960
4	U2 snRNA auxiliary factor	27.8	gi-5803207
5	DNase I	28.9	gi-118922
6	Ribosomal protein	29.9	gi-306553
7	hnRNP A/B-related protein	29.8	gi-5052976
8	DNase I, chain D	29.0	gi-229691
9	A + U RNA binding factor	30.1	gi-2547076
10	p37 AUF1	31.4	gi-433344
11	mCBP	34.9	gi-495128
12	Y-box-binding protein 1	35.7	gi-112410
13	DNA-binding protein	35.8	gi-181914
14	HuR	36.0	gi-38201714
15	G3P dehydrogenase	36.0	gi-7669492
16	Similar to MGC37309	36.3	gi-34857163
17	2610510D13Rik protein	37.0	gi-23274114
18	hnRNP X	37.5	gi-5453854
19	hnRNP A1	38.8	gi-133254
20	β -Polymerase	38.2	gi-190156
21	Elongation factor 1- α	39.6	gi-4530096
22	α_2 -HS glycoprotein	38.3	gi-27806751
23	p40/AUF1	42.0	gi-2773158
24	Nucleolysin TIA1	42.9	gi-6094480
25	TRK-fused gene	43.4	gi-21361320
26	hnRNP K	50.9	gi-473912
27	hnRNP G	47.4	gi-542850
28	Colligin-2	46.5	gi-2118393
29	Unnamed protein	50.1	gi-31092
30	TAR DNA-binding protein	44.7	gi-6678271
31	PTB 2	57.4	gi-10863997
32	Splicing factor U2AF	53.4	gi-107723
33	Splicing factor homolog	54.2	gi-543010
34	Staufen protein	55.2	gi-4572588
35	Rod 1	56.4	gi-4514554
36	Pyruvate kinase	57.8	gi-125598
37	Splicing factor cc1.3	58.8	gi-4757926
38	PTB isoform a	59.6	gi-4506243
39	hnRNP L	60.1	gi4557645
40	HCC autoantigen	61.8	gi-4883681
41	RNA-binding protein KOC	63.6	gi-2105469
42	p68 RNA helicase (Ddx5)	66.8	gi-226021
43	M phase phosphoprotein	66.7	gi-1770458
44	hnRNP Q2	66.6	gi-15809588
45	FBP	67.4	gi-1082624
46	EWS	68.4	gi-4885225
47	FUSE-binding protein 2	68.3	gi-1575607
48	Ku70	69.8	gi-4503841

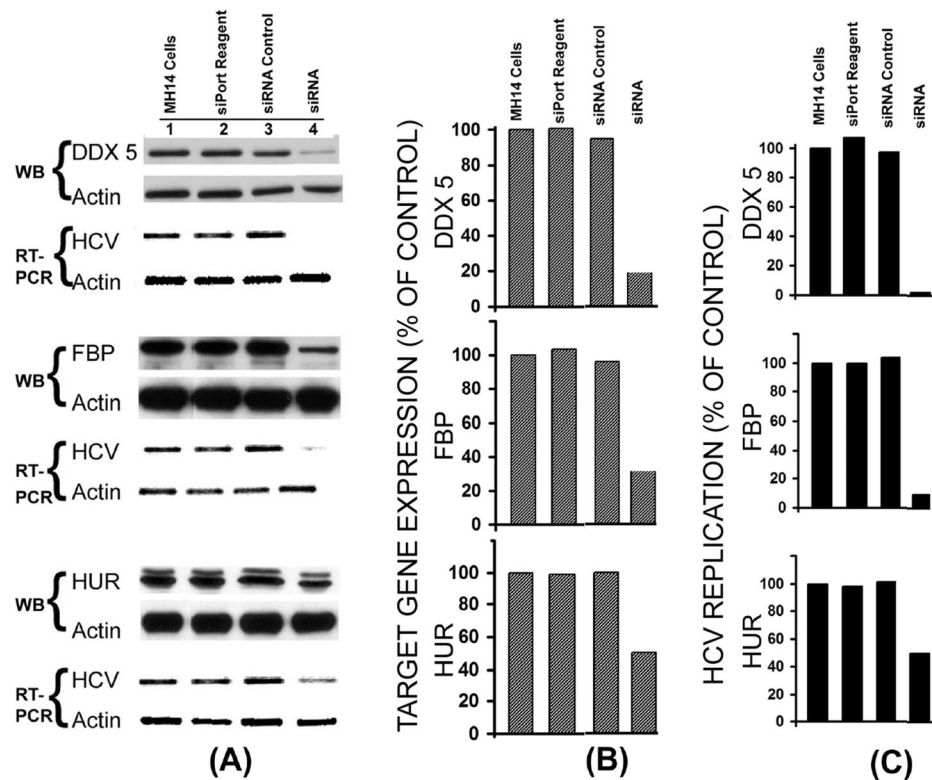
TABLE I—continued

No.	Cellular protein identified	Molecular mass	Accession no.
		<i>kDa</i>	
49	RNA binding motif protein 14	69.4	gi-5454064
50	Heat shock 70-kDa-like protein 1	70.6	gi-1346319
51	HspA6	70.8	gi-87626
52	Hsp70	70.8	gi-5729877
53	DEAD box protein 72 (Ddx17)	72.3	gi-3122595
54	Hsp A5	72.0	gi-87528
55	DEAD box protein 3 (Ddx3)	73.2	gi-3023628
56	MTHSP75	73.7	gi-292059
57	PSF	76.1	gi-4826998
58	hnRNA-binding protein M4	77.5	gi-479852
59	hnRNP U	79.6	gi-16041796
60	NF90	82.7	gi-5006602
61	DNA helicase II	82.8	gi-10863945
62	Lysine hydroxylase	84.6	gi-4505889
63	hnRNP U	88.9	gi-284156
64	DEAH box polypeptide (Ddx15)	92.7	gi-4557517
65	Matrin 3	94.5	gi-21626466
66	E1B-AP5	95.7	gi-7512403
67	RNA polymerase	98.6	gi-30387455
68	Golgi-associated particle	102.4	gi-486784
69	p30 DBC	102.7	gi-24432106
70	Poly(A) polymerase-1	113.0	gi-130781
71	RNA helicase A (Dhx9)	140	gi-3915658

DISCUSSION

Modern tools for the comprehensive compositional analysis of ribonucleoprotein complexes have been in dire scarcity. Traditional techniques for characterizing RNA-protein interactions, such as UV cross-linking or RNA electrophoretic mobility shift assays, yield limited information with regard to protein identity and function. SDS-PAGE gels of cross-linked species can inform on the size of proteins and can even confirm protein identity if antibodies to suspected proteins are available for subsequent Western blotting. RNA electrophoretic mobility shift assays can be useful for detecting RNA binding activities in cell extracts, quantifying binding affinity for particular proteins, and also detecting protein-protein interactions in the appearance of supershifted species. However, the only modern technique capable of positively identifying numerous proteins simultaneously within a complex mixture is mass spectrometry. The most powerful system for performing this feat is LC/MS/MS, which couples the high resolution of the HPLC instrument with tandem mass spectrometry, providing both mass information and sequence information for all of the tryptic peptides. We harnessed the power of this technology to address major questions about virus-host interactions in the life cycle of hepatitis C virus. A recent report describing the identification of protein species present in the human spliceosome (52) undertook the large scale cross-linking and subsequent purification of various cross-linked species for mass spectrometric analysis, which

FIG. 6. Inhibition of HCV replication by siRNA-mediated down-regulation of Ddx5, FBP, and HuR. A derivative of Huh7 cell lines (MH 14) carrying replicating HCV replicon were grown for 24 h and transfected with 20 nM anti-Ddx5, -FBP, and -HuR siRNA duplexes or with control siRNA duplexes. A mock transfection was also performed using only transfection reagent. Cells were grown for 72 h after transfection, and total protein and RNA were isolated. Lane 4 in A shows siRNA-mediated down-regulation of targeted host cell proteins as assessed by Western blotting and inhibition of HCV replication by RT-PCR (5'-NTR of the HCV genome). The down-regulated protein bands of Ddx5, FBP, and HuR and corresponding RT-PCR of HCV RNA quantified by Quantity One software (Bio-Rad) are shown in B and C, respectively. WB, Western blot.



represents an excellent development toward combining affinity capture and mass spectrometry. Also HCV ribonucleoprotein complexes have been purified using an affinity approach in which the captured complexes were subjected to Western analysis for viral proteins only (38). We used runoff RNA transcripts to capture and examine the entire protein binding profile of the 3'-NTR of the HCV genome to gain insight into the host components of the HCV replication machinery.

A recent report describing the comprehensive proteomic analysis of a large ribonucleoprotein complex reveals the presence of many cellular proteins (53). Consistent with these observations, we found >70 human proteins associated with the 3'-nontranslated region of the hepatitis C virus RNA genome in our affinity capture assay. Given the size and predicted structure of the 3'-NTR, it may not seem feasible that all of these proteins would bind to the NTR RNA simultaneously. However, it is quite possible that some of the identified proteins compete for binding. As recently demonstrated by Lal *et al.* (50), both HuR and AUF1 bind to common labile target mRNAs, and this coordinated binding likely determines the stability of these RNAs. With a vast array of RNA binding and ARE-binding proteins in the cell, it seems logical that more than two proteins might compete for binding to target mRNAs at any given time. Depending upon the phase of cell growth, differentiation, or responses to stress, the expression of specific ARE-binding proteins would likely be modulated, influencing the downstream regulation of stability of target mRNAs. Alternatively as in the case of interleukin-2 mRNA

stabilization by NF90, increased export of ARE-binding proteins from the nucleus to the cytoplasm (following T cell activation) could be another general mechanism utilized by cells to regulate mRNA turnover.

One group of proteins we identified that may have significant implications in their possible role(s) as regulators of viral RNA stability comprises several AU-rich element-binding proteins. The AU-rich element-binding proteins we confirmed as HCV 3'-NTR binders include AUF1/hnRNP D, HuR, FBP, FBP2 (KH-type splicing regulatory protein), hnRNP C, YB-1, and NF90. Interestingly although some of these proteins have been shown to be involved in targeting mRNAs for degradation by binding to AREs within the 3'-UTRs of cellular mRNAs, such as AUF1 (54), hnRNP L (55), and KH-type splicing regulatory protein (56). Others such as HuR (49, 51), hnRNP C (57), and NF90 (58), have been shown to increase the stability and longevity of certain mRNAs. Our observation that HuR expression is correlated with HCV replicon RNA abundance in MH 14 cells supports the contention that HuR serves to stabilize the viral RNA message as it has been observed to stabilize cellular RNAs. It is also possible that HuR facilitates or stimulates HCV replication at the level of initiation of RNA synthesis, thus increasing HCV RNA abundance. The binding of both positive and negative regulators of RNA stability to the 3'-NTR of HCV suggests a complex set of interactions governing the fate of HCV viral RNA. The possibility that the 3'-NTR of HCV may serve a function similar to that of the 3'-UTRs of cellular mRNA with regard to regulating RNA

stability certainly presents an expanded view of virally encoded regulatory mechanisms utilizing host factors.

Although the hnRNP family members are frequently related in primary sequence as well as structure (59), these proteins can perform rather diverse functions in the cell. The hnRNP K protein, strongly expressed in hepatoma cells across species (60), has both RNA and DNA binding properties (61, 62) and shuttles back and forth between the nucleus and cytoplasm, thus being implicated in mRNA processing and transport. Like FBP, a highly homologous ARE-binding protein, hnRNP K can also stimulate transcription from the *c-myc* promoter, (61, 63, 64) The K homology (KH) domain, now recognized as an RNA binding motif common to many proteins, was originally identified in hnRNP K (65), and structural information for these domains has recently become available (66–68). Interestingly hnRNP K has been shown to interact with the core protein of HCV (69), and given the multifunctional character of hnRNP K this interaction may contribute to HCV pathogenesis. hnRNP C contains a different RNA binding motif from that of K, namely the RNP motif, also called RNA recognition motif (RRM). However, hnRNP C and hnRNP K both bind U-rich regions with very high affinity (70) and were both captured in our NTR binding assay.

One of the most intensively studied hnRNPs, A1, contains RRM motifs and is implicated in several functions including splicing and export of mRNAs and telomere biogenesis (71). Additionally hnRNP A1 has been shown to bind the 3'-untranslated region of mouse hepatitis virus and may help bridge the 3'- and 5'-ends of the mouse hepatitis virus genome (72). Besides these functions, A1 has been shown to play a role in alternative splicing of HIV-1 RNA transcripts by influencing splice site utilization (73).

PTB is another hnRNP family member that has been extensively characterized. PTB is a basic protein of ~59 kDa but also exists as other isoforms as mentioned above. PTB is involved in pre-mRNA splicing (74), has been shown to modulate the stability of CD154 mRNA through binding to the 3'-UTR (75), and also has been shown to stimulate binding of RNA polymerase II to HIV-1 TAR RNA (76). We also identified PTB-associated splicing factor (PSF), a protein containing two RRM motifs responsible for RNA binding, that was initially identified as an interacting partner of PTB (77). PSF has been shown recently to play an important role in posttranscriptional regulation of HIV-1 gene expression by binding to instability elements within the *env* gene (78). Other hnRNPs identified in our RNA affinity assay include hnRNPs L, U, G, A0, X, and Q2.

Although the vast majority of proteins we identified associated with the 3'-NTR are RNA-binding proteins, they serve many varied functions. Ku70 exists as part of a heterodimer with Ku80 (79) and is involved in the nonhomologous end joining pathway in V(D)J recombination. An interaction between Ku80 and poly(A) polymerase has been shown to be important for suppressing chromosomal aberrations in liver cancer formation (80). Additionally the Ku70/Ku80 het-

erodimer has been shown to associate with human telomeres through interaction with human telomerase (81). This protein has also been shown to play an important role in the life cycle of HIV-1 (82). Rod 1 was discovered in yeast by virtue of its ability to confer resistance phenotypes (83), and the mammalian homolog was cloned soon thereafter (84) and found to be an RNA-binding protein that blocks differentiation and preferentially binds poly(U) stretches. Staufen protein, originally identified in *Drosophila* as being critical for the localization of specific mRNAs in early fly development, is reported to bind double-stranded RNA (85, 86). The human homolog hStaufen interacts with NS1 protein of influenza virus (87) and also binds the 3'-UTR of bicoid mRNA (88). Recently hStaufen has been shown to be incorporated into HIV-1 virions and to play a role in the generation of infectious virus particles (89).

NF90, initially identified as a transcription factor whose expression is up-regulated in activated T cells (90), also interacts with the DNA-dependent protein kinase complex, which includes Ku70/Ku80 (91), and can act as both a positive and negative regulator of gene expression (92). Although a predominantly nuclear protein, the cytoplasmic abundance of NF90 increases upon T cell activation.

FBP, one of the ARE-binding proteins we identified that appears to be important for HCV replication (Fig. 6), activates transcription of the *c-myc* gene by binding to an element called the far upstream element (FUSE). FBP binds the 3'-UTR of the GAP-43 mRNA (93) along with PTB, and these two may jointly regulate stability of the mRNA. FBP was shown to be identical to the DNA helicase V protein and contains a powerful ATP-dependent DNA helicase activity (94). FBP has RGG motifs common to many RNA and DNA helicases, is highly conserved (95), and has been compared with hnRNP K in its ability to target cognate sequences in negatively supercoiled DNA. FBP shares some homology with hnRNP K, which has also been shown to bind the "CT" element in the *c-myc* promoter (61) and is present in undifferentiated but not differentiated cells (96). The structure of FBP (KH3 and KH4) bound to a 29-bp fragment from FUSE has been solved by NMR (97).

Human Hsp70 protein, captured in our binding assay, has been shown recently to facilitate nuclear import of HIV-1 preintegration complexes (98) and was also found to be incorporated into virion particles (99), suggesting a significant role for this host protein in the life cycle of another positive-stranded RNA virus. RNA helicase (RHA), abundant in our capture assay, contains two copies of a double-stranded RNA binding domain at its N terminus and an RGG box at its C terminus that binds single-stranded nucleic acids (100). RHA unwinds both duplex RNA and DNA in an ATP-dependent fashion (101, 102). Hence it is not surprising that RHA displays high affinity for the 3'-NTR RNA, which contains both double-stranded and single-stranded regions. In addition to RHA, we identified other DEAD box family members including p68 (Ddx5) and Ddx3, shown to be overexpressed in hepatocellular carcinoma tissue (103). The p68 helicase is also appar-

ently important for HCV replication as demonstrated by the decrease in replicon RNA upon silencing of p68 expression (Fig. 6). Although HCV encodes its own RNA helicase activity in the NS3 protein, cellular RNA helicases such as FBP and Ddx5 may greatly enhance viral (+) RNA synthesis by facilitating the unwinding of the viral RNA template (-) strand. Both the binding of cellular RNA helicases to the 3'-NTR of HCV, confirmed in our MS analysis, and the decrease in HCV replicon RNA observed upon silencing of FBP and Ddx5 strongly support this hypothesis.

Because we identified several proteins associated with the 3'-NTR that are known to modulate mRNA stability by means of binding to the 3'-untranslated regions of cellular genes as discussed above, it is quite feasible that HCV may utilize *cis*-acting signals similar to those found in the 3'-UTRs of cellular genes to regulate the stability of its own RNA. Although AU-rich elements have been identified most predominantly in the 3'-UTRs of short lived cytokine and proto-oncogene mRNAs and seem to be responsible for rapid decay of these mRNAs (104, 105), it is not logical that HCV would evolve to encode a mechanism for rapid degradation of its own genetic material. AREs are divided into three separate classes (104), which surely interact with ARE-binding proteins differentially. The U-rich region in the 3'-NTR of HCV would clearly fall into the third class, which does not contain the repeated consensus AUUUA sequence present in the class I and class II AREs. As already noted, we identified some ARE-binding proteins that serve to stabilize mRNAs and others that promote mRNA decay. Because the poly(U)/UC tract within the 3'-NTR has been shown to be essential for HCV replication, the indisposability of this region may be related to the binding of mRNA-stabilizing proteins. In other words, HCV viral RNA may be rapidly degraded in the absence of this U-rich tract, leading to severely reduced viral replication.

Recent studies have elegantly described the sequences within the 3'-NTR of HCV that are essential for replication of subgenomic replicons in cell culture (16, 17). The current challenge is the identification of cellular and/or viral proteins interacting with these HCV-encoded signals, which then mediate downstream events in infected cells. These downstream events may include, in addition to viral RNA synthesis, the up-regulation or down-regulation of cellular genes, influences on posttranscriptional regulation of mRNA, and changes in cellular metabolism. Indeed our findings indicate that some of the proteins bound to the 3'-NTR are involved in regulating mRNA stability and pre-mRNA splicing as well as other activities in the cell. Although our RNA affinity capture system yielded much new information, the exact sequences and/or structures within the HCV 3'-NTR required for binding each of these individual proteins remain unknown.

The KH domains of FBP also bind to single-stranded nucleic acid rich in U (RNA) or T (DNA) (97), suggesting that FBP may also bind the 3'-NTR in the U-rich region. *Drosophila* Staufen protein has been shown to bind to the 3'-UTR of

bicoid mRNA, which is predicted to form three stem-loop structures (88), much like the 98-nucleotide 3'-terminal region of the HCV NTR. Future studies examining the altered protein binding profiles of NTR RNA carrying mutations or deletions will more precisely define the structural and/or sequence elements responsible for binding specific proteins or classes of proteins we identified. It is intriguing, although perhaps not surprising, that a number of NTR-binding proteins including Hsp70, Ku70, hnRNP A1, hStaufen, and PSF have been shown to play important roles in the life cycle of HIV-1, another positive-stranded RNA virus. Although HCV and HIV have quite different replication strategies with HCV replicating in the cytoplasm of infected cells and HIV utilizing a DNA intermediate that is integrated into the host genome, our findings suggest that host factors may be recruited by these viruses for common purposes that are beneficial to the virus such as RNA stabilization, splicing regulation, and influencing host gene expression.

We detected both positive and negative regulators of mRNA stability in our affinity capture assay. Additionally we found several RNA helicases associated with the 3'-NTR of the HCV genomic RNA. Our gene silencing studies demonstrated that Ddx5 and FBP may play critical roles in HCV replication. HuR, a well characterized ARE-binding protein shown to stabilize cellular mRNAs, also appears to stabilize HCV RNA. Studies currently underway in our laboratory are aimed at elucidating the mechanism by which the helicases FBP and Ddx5 facilitate replication of the HCV replicons. Additional studies will reveal how HCV replicons influence the expression of these and other potential HCV regulators (3'-NTR binders) in cell culture. Potential influences of viral non-structural proteins at the level of transcription, translation, and proteolysis are being examined. Also additional gene silencing experiments may provide clues to the identities of other host proteins involved in HCV replication.

Just as the polymerases of many pathogens including HIV, herpes simplex virus, and others, the NS5B polymerase of HCV was identified as a target for the development of anti-HCV drugs early on in the investigation of hepatitis C virus. Other potential targets for antiviral intervention include the protease and helicase activities found in the nonstructural proteins of HCV. Due to a prior lack of information regarding the signals within the nontranslated regions of the HCV genome that are essential for both translation and replication and a scarcity of modern tools for either characterizing or disrupting RNA-protein interactions, these regions did not appear to be attractive targets for intervention. In light of our findings, the wealth of RNA-protein interactions observed within the 3'-NTR may, in fact, represent excellent new targets for inhibition of viral replication. Possible strategies for disrupting essential RNA-protein interactions include not only the use of genome-targeted antisense inhibitors for blocking viral replication but also the regulation or inhibition of host proteins involved in viral replication.

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