Heterogeneous ribonucleoprotein K (hnRNP K) binds miR-122, a mature liver-specific microRNA required for hepatitis C virus replication*

Baochang Fan‡¶, F.X. Reymond Sutandy§¶, Guan-Da Syu§, Stefani Middleton‡, Guanghui Yi‡, Kuan-Yi Lu§, Chien-Sheng Chen§, and C. Cheng Kao‡¶

Heterogeneous ribonucleoprotein K (hnRNP K) binds to the 5’ untranslated region of the hepatitis C virus (HCV) and is required for HCV RNA replication. The hnRNP K binding site on HCV RNA overlaps with the sequence recognized by the liver-specific microRNA, miR-122. A proteome chip containing ~17,000 unique human proteins probed with miR-122 identified hnRNP K as one of the strong binding proteins. In vitro kinetic study showed hnRNP K binds miR-122 with a nanomolar dissociation constant, in which the short pyrimidine-rich residues in the central and 3’ portion of the miR-122 were required for hnRNP K binding. In liver hepatocytes, miR-122 formed a coprecipitable complex with hnRNP K. High throughput Illumina DNA sequencing of the RNAs precipitated with hnRNP K was enriched for mature miR-122. SiRNA knockdown of hnRNP K in human hepatocytes reduced the levels of miR-122. These results show that hnRNP K is a cellular protein that binds and affects the accumulation of miR-122. Its ability to also bind HCV RNA near the miR-122 binding site suggests a role for miR-122 recognition of HCV RNA.

EXPERIMENTAL PROCEDURES

MicroRNAs (miRNAs) are a class of noncoding RNA of ~22-nucleotides in length that can regulate gene expression by either targeting RNA for degradation or suppressing their translation through base pairing to the RNAs (1). Since their discovery in 1993 in Caenorhabditis elegans, miRNAs have been found in many species and are involved in the regulation of proliferation, differentiation, apoptosis, and development (1, 2). Moreover, miRNAs are also critical factors in the development of cancers, neurodegenerative diseases, and infectious diseases (3).

MiR-122 is a highly abundant RNA in hepatocytes that regulates lipid metabolism, regeneration, and neoplastic transformation (4–6). In addition, miR-122 is required for the replication of the hepatitis C virus (HCV), a positive-strand RNA virus that infects over 170 million people worldwide (7–9). MiR-122 binds to a conserved sequence in the 5’ untranslated region (UTR) of the HCV RNA to increase the stability of the HCV RNA (10). Silencing of miR-122 can abolish HCV RNA accumulation in non-human primates (11). The expression of human miR-122 in non-hepatic cells can confer the ability to replicate HCV RNA (12). MiR-122 is one of the most critical host factors for HCV replication.

We previously reported that the HCV RNA sequence that anneals to miR-122 is recognized by the heterogeneous ribonucleoprotein K (hnRNP K), a multifunctional RNA-binding protein known to be involved in RNA processing, translation, and the replication of several RNA viruses (13–15). In an unbiased screen for proteins from human proteome chips containing over 17,000 proteins, we identified 40 proteins that bind mature miR-122, including hnRNP K. Recombinant hnRNP K recognizes short pyrimidine sequences in miR-122 in vitro and a similar sequence in the HCV 5’ UTR. In hepatocytes endogenous hnRNP K can form a coprecipitable complex with miR-122, whether or not the cells contain replicating HCV. HnRNP K is thus a protein that binds a mature microRNA.

From the ‡Department of Molecular & Cellular Biochemistry, Indiana University, Bloomington, IN 47405, USA; §Graduate Institute of Systems Biology and Bioinformatics, National Central University, Jhongli 32001, Taiwan

Received April 2, 2015, and in revised form, August 7, 2015
Published September 1, 2015, MCP Papers in Press, DOI 10.1074/mcp.M115.050344

Author contributions: B.F., C.C., and C.K. designed the research; B.F., F.S., G.S., S.M., and G.Y. performed the research; C.K. contributed new reagents or analytic tools; B.F., F.S., G.S., S.M., G.Y., K.L., C.C., and C.K. analyzed the data; and F.S., K.L., and C.K. wrote the paper.

The abbreviations used are: hnRNP K, heterogeneous ribonucleoprotein K; miR-122, microRNA 122; HCV, hepatitis C virus; UTR, untranslated region; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; QRT-PCR, quantitative reverse-transcription, polymerase chain reaction; CLIP-Seq, Crosslinking-immunoprecipitation, DNA sequencing.
Human Proteome Chip Assay—The human protein chip was screened as described previously (15). The protein chip was pre-blocked with a solution of 3% BSA and 0.1 mg/ml of salmon sperm DNA under a slide coverslip and then incubated with the probe RNA for 1 h at 37 °C and 8 rpm using a BioMixer™ II (CapitalBio, Beijing, China). The coverslip was then removed and the chip was washed with 500 ml of phosphate buffered saline (PBS) amended with diethylpyrocarbonate and 0.05% Triton X-100 for 10 min. To quantify the relative amount of each protein spot on the human proteome chip, the chip was further probed with 80 μl of 1 μg/ml diluted DyLight™ 549-conjugated anti-GST monoclonal antibody (Rockland) in diethylpyrocarbonate-PBS and incubated for 45 min at 37 °C, 8 rpm. After two washes, the chip was dried and scanned using LuxScan™ 10K Microarray Scanner (CapitalBio). The detected binding signals were analyzed using the GenePix Pro 6.0 software.

Affinity Measurements by Interferometry—HnRNP K binding to miR-122 or its variants were quantified using an Octet RED96 System (ForteBio, Menlo Park, CA). GST-tagged hnrNP K (Abnova, Taipei Taiwan) was immobilized on anti-GST biosensors (ForteBio). The capture level of anti-GST biosensors was 2.6 ± 0.1 nm. Association and dissociation measurements were carried out in the presence or absence of miR-122 or its mutants serially diluted in PBS. A reference biosensor was included to determine the background signal. During the measurement, the analytes were subject to rotation at 1,000 rpm at 30 °C. Kinetic constants (k_on and k_off) were calculated from a 1:1 model fitting by using Octet software. Equilibrium dissociation constants (K_d) were calculated according to the Langmuir adsorption model for the steady-state response. Each reported value represents the results of a minimum of three independent experiments.

SL1-hnRNPK Crosslinking Assay—HnRNPK binding to SL1 or variants was determined as previously described in Fan et al. (15). Briefly, 100 ng of purified protein was mixed with 1 pmole of the RNA that was radiolabeled at its 5' terminus using polynucleotide kinase and [γ-32P]ATP. The reactions were irradiated in a CL-1000 UV cross-linker (Stratagene, Santa Clara, CA) at 1,200 mJ/cm² (254 nm) for 3 min. The protein–RNA mixtures were subjected to SDS-PAGE, and the protein–RNA complex was quantified by autoradiography using a PhosphorImager (GE Healthcare Biosciences, Pittsburgh, PA). The proteins in the gel were visualized by Coomassie brilliant blue staining.

Crosslinking-Immunoprecipitation Assay—A crosslinking and immunoprecipitation (CLIP) assay was performed to examine the interaction between hnRNP K and miR-122 in Huh7.5 cells (16). Briefly, 5 × 10^6 cells grown in 10 cm plates were washed twice with ice-cold PBS and irradiated on ice with 150 mJ/cm² at 254 nm (Stratalinker, Agilent, Santa Clara CA). The cells were harvested by digestion with trypsin, pelleted by centrifugation at 5,000 g for 5 min, and lysed with Nonidet P-40 lysis buffer (150 mM NaCl, 50 mM TrisCl, pH 8.0, 1.0% Nonidet P-40) on ice for 1 h with occasional mixing. The lysate was centrifuged at 16,000 g for 30 min, and the supernatant was collected and incubated at 4 °C for 3 h with protein A/G beads conjugated with mouse anti-human hnRNP K antibody (Abcam, Thermo Fisher Scientific, catalogue number Ab39975) equilibrated with Nonidet P-40 lysis buffer or a goat anti-mouse IgG control antibody treated in the same way (Santa Cruz). Materials bound to Protein A/G beads were washed three times with 50 mM Tris saline solution amended with 0.1% Triton X-100 (TBST) followed by addition of 200 μl of Laemmli sample buffer. The mixture was heated at 95 °C for 2 min and loaded on a 4–12% Bis-Tris gel. Gel electrophoresis was for 1 h at 150 V in 1x MES SDS running buffer (ThermoFisher Scientific, Fredricks, MD). Protein–RNA complexes were transferred from the gel to a nitrocellulose membrane using a wet transfer apparatus (BioRad, Hercules, CA) at 100 V for 2.5 h. Proteins on the membrane were stained with 0.1% Ponceau-S for 10 min, and the portion of the membrane immediately above the 100 kDa hnRNP K band was excised using a new razor blade. RNAs from the membrane were released by digestion with proteinase K, extracted with Trizol reagent (Invitrogen), and precipitated with three volumes of ethanol, 0.5 M ammonium acetate, and centrifugation at 16,000 × g for 30 min. The pellet was washed 2X with ice-cold 70% ethanol and dried. The eluted RNA was dissolved in water, polyadenylated with poly(A) polymerase (New England Biolab [NEB], Ipswich MA) and reverse transcribed using M-MulV reverse transcriptase (NEB) and anchored oligo-dT primer. Sequences for the primers used to amplify cDNAs will be made available upon request. High-throughput DNA sequencing was performed using the Illumina MiSeq system.

CLIP-Seq was performed as described previously (17, 18). Analyses of MiSeq reads were performed using the Galaxy User Interface. The reads were processed with the FASTX-tookit by trimming the 3' bases with a quality score lower than 20 using the FASTQ Quality Trimmer and removing the 3' adapter and discarding any reads shorter than 15-nt using Clip. Reads mapping to the specific RNA sequence were selected using Grep1.0.1, and the final read counts for each RNA were obtained after further analysis with the Compare two Datasets tool from the SharpLabTools Suite available through the Galaxy Tool Shed.

siRNA Knockdown of hnrNP K and Quantitative Reverse Transcription-PCR (qRT-PCR)—Huh7.5 cells (5 × 10^5 cells) or Huh7.5 cells harboring the replicating 1b/Con1 replicon (5 × 10^5 cells) were transfected with siRNA using Lipofectamine™ 2000 according to the manufacturer’s instructions (Invitrogen). Aliquots of the transfected cells were assessed for effects on cell proliferation and cytotoxicity using the Wst-1 reagent (Clontech). The total RNA was extracted from the cells with Trizol Reagent and quantified by spectrometric. The RNA (1 μg per sample) was reverse transcribed using M-MulV reverse transcriptase (NEB) and 4 μM of a randomized 9-nt primer mix. The levels of mRNA for hnrNP K were determined by qRT-PCR using the iQ™ SYBR Green kit (BioRad). Amplifications were performed with an initial incubation at 10 min at 95 °C, followed by up to 45 cycles of 15 s at 95 °C, 20 s at 60 °C, and 60 s at 72 °C. The mRNA levels were normalized to GAPDH mRNA level, and the changes were compared with the mock-transfected control as previously reported (18). MiR-122 levels were quantified using the protocol of Livak and Schmittgen (19).

RESULTS

Identification of Human Mature mir-122-Binding Proteins—The human proteome chip has allowed the global screening and rapid identification of ligands that can bind proteins (20). A version of the chip that contains human proteins has previously been used to screen for binding to specific RNAs (15). We used the chip to perform an unbiased screen of the human proteins that can bind to miR-122. A 22-nt miR-122 modified with a Cyanine 5 (Cy5) dye at its 5’ terminus was used as a probe (Fig. 1A). As a specificity control, a Cy5-labeled 22-nt RNA that contained a scrambled miR-122 sequence named Sc-22 was used to independently probe a chip and identify nonspecific RNA-
hnRNPK Binds miR-122

Fig. 1. **Identification of miR-122 binding proteins by using human proteome chips.** (A) The human proteome chip was first probed with Cy5-labeled miR-122. After washing with TBST, it was then probed with DyLight™549-labeled anti-GST to allow signal normalization (Fig. 1C). In all, 40 proteins were found to bind miR-122 with a mean of 1 S.D. above that of Sc-22. Bioinformatics analysis revealed that 13 of the 40 proteins contain nucleic-acid-binding motifs, and 10 are expressed in the liver (Fig. 1C and Table I). HnRNP K is one of the 10 proteins expressed in the liver that preferentially binds miR-122 (Table I).

We focused on hnRNP K and a possible interaction with miR-122 because hnRNP K was previously determined to bind to a stem-loop structure, SL1, within the HCV 5’ UTR. Intriguingly, SL1 contains the Seed 1 sequence that anneals to miR-122 (15).

HnRNP K Binds to Both miR-122 In Vitro through Short Pyrimidine Sequences—A consensus motif for the RNAs bound by hnRNP K was previously identified in *Xenopus laevis* by B. Szaro and colleagues (21). The consensus motif contains three discontinuous stretches of enriched for pyrimidines separated by spacer sequences (Fig. 2A). HnRNP K contains three KH domains that individually can bind to single-stranded RNA and DNAs of ca. 4-nt that are enriched for pyrimidines (22). MiR-122 possesses a short pyrimidine-rich sequence at its 3’ region, perhaps providing a sequence for hnRNP K binding through the KH domain (Fig. 2A). Interestingly, we note that the Seed 1 sequence within SL1 also contained a short pyrimidine-rich sequence and that Seed 2 contains a direct repeat of the Seed 1 sequence (Fig. 2A).

An interferometry assay was used to determine the region within miR-122 that contributes to the binding of hnRNP K. GST-tagged hnRNP K was immobilized onto a biosensor that can bind GST. Wild-type miR-122 or the scrambled RNA sequence, Sc-22, was added in increasing concentrations to the immobilized hnRNP K. The wild-type miR-122 rapidly associated with hnRNP K while Sc-22 did not (Fig. 2B). GST attached to the chip did not bind miR-122, and Sc-22 had very poor binding (Fig. 2C and data not shown). The binding isotherm for miR-122 was fitted to a 1:1 binding model, and the results suggest that binding had a dissociation constant of 14.9 nM. MiR-122 binding was characterized by rapid on and off rates (Fig. 2C). The binding of Sc-22 could not be modeled based on the concentrations tested.

Four variants of miR-122 that had substitutions along the length of miR-122 were tested for binding to hnRNP K. Mut1, which has three 5’ terminal guanylates replaced with adenylates, was minimally affected for binding to hnRNP K, with a $K_D$ of $\sim 26 \text{ nm}$ (Fig. 2C). Mut2, which had two uridylates within the purine-rich sequence changed to adenylates, was severely reduced for hnRNP K binding. Mut3 and mut4, which had a concentration of pyrimidines in the central and the 3’ region of miR-122 substituted with adenylates, were also reduced for hnRNP K binding (Fig. 2C). These results demonstrate that pyrimidines within the central and 3’ portion of miR-122 both contribute to effective hnRNP K binding.

Changes in the sequence of miR-122 could have impacted hnRNP K binding by altering the structures of the RNAs. To
determine whether a correlation to RNA structure exists, we first predicted the structure of miR-122 using the program mfold (23). MiR-122 formed a weakly stable hairpin structure (ΔG = -0.1 kcal/mol) with four base pairs in the stem (Fig. 3A). Mut1, which had only slightly weaker binding to hnRNP K, also formed a weakly stable hairpin. Interestingly, Mut2, which was predicted to form a more stable hairpin with six base pairs (ΔG = -2.4 kcal/mol), was defective for hnRNP K binding. These results suggest that hnRNP K binding to miR-122 is likely through a more flexible pyrimidine-rich sequence in miR-122. Mut3 and Mut4, which had substitutions in the pyrimidines and also lacked a predicted stable structure, were reduced for hnRNP K binding. These results suggest that hnRNP K preferentially binds the short pyrimidine-enriched sequences in the flexible portions of miR-122.

HnRNP K Binds to the Seed 1 Sequence in the HCV SL1—
HnRNP K binding to the pyrimidine-enriched sequences within miR-122 led us to further examine the motifs in the HCV SL1 required for hnRNP K binding. Similar to miR-122, SL1 has two pyrimidine-rich sequences. One of these is the Seed 1 sequence that binds miR-122 and is predicted by mfold to be unstructured in SL1 (10, Fig. 3B). The second is a stretch of homocytidylate that is predicted by mfold to base pair to a stretch of homoguanylates (15). To independently confirm that this is the case, we tested the hnRNP K binding of both SL1 and SL1 lacking the Seed 1 residues in an RNA named 1. The RNAs were radiolabeled at the 5' terminus and comparable amounts of the two RNAs were incubated with hnRNP K and subjected to UV crosslinking. HnRNP K and the RNAs were resolved by denaturing PAGE (Fig. 3C). SL1 formed

| Gene    | Uniprot ID | Description                        | Binds nucleic acid | Expressed in liver |
|---------|------------|------------------------------------|--------------------|-------------------|
| SLC10A6 | Q3KNW5     | Solute carrier family 10 member 6 | X                  |                   |
| WBSCR28 | Q6UE05     | Williams-Beuren syndrome chromosomal region 28 protein | X                  |                   |
| SLC35F5 | Q8WV85     | Solute carrier family 35 member 5  |                   |                   |
| POLR3E  | Q8NVJ0     | DNA-directed RNA polymerase III subunit RPC5 |                   | X                 |
| NF2     | P35240     | Merlin                             | X                  |                   |
| PARP11  | Q9NR21     | Poly [ADP-ribose] polymerase 11    | X                  |                   |
| CPA5    | Q8WXXQ8    | Carboxypeptidase A5                | X                  |                   |
| IFT140  | Q96RY7     | Intraflagellar transport protein 140 homolog | X                |                   |
| ELOVL3  | Q8HB3      | Elongation of very long chain fatty acids protein 3 | X                |                   |
| RNF148  | Q8N7C7     | RING finger protein 148            | X                  |                   |
| TLE6    | Q9HB08     | Transducin-like enhancer protein 6  | X                  |                   |
| PRLR    | P16471     | Prolactin receptor                 | X                  |                   |
| ACO2    | Q99798     | Aconitate hydratase, mitochondrial | X                  |                   |
| ACO11   | Q8WV83     | Acid-sensing ion channel 2         | X                  |                   |
| PCDHA7  | Q9J7T2     | Protocadherin alpha-7              | X                  |                   |
| SLC30A5 | Q8TAD4     | Zinc transporter 5                 | X                  |                   |
| GOLPH2  | Q8NB4J     | Golgi membrane protein 1           | X                  |                   |
| FAM118A | Q9NWS6     | Protein FAM118A                    | X                  |                   |
| HLA-DPB1| P04440     | HLA class II histocompatibility antigen, DP beta 1 chain | X                |                   |
| C3orf54 | Q96EL1     | Protein FAM212A                    | X                  |                   |
| PCDH21  | Q9J6P9     | Cadherin-related family member 1   | X                  |                   |
| ICOS    | Q9Y6W8     | Inducible T-cell costimulator      | X                  |                   |
| ERH     | P84090     | Enhancer of rudimentary homolog    | X                  |                   |
| CDC51   | Q96ER9     | Coiled-coil domain-containing protein 51 | X                |                   |
| BSCL2   | Q96G97     | Serpin                             | X                  |                   |
| ELL3    | Q9HB65     | RNA polymerase II elongation factor ELL3 | X                |                   |
| TCF12   | Q99081     | Transcription factor 12            | X                  |                   |
| NPM2    | Q86SE8     | Nucleoplasmid-2                    | X                  |                   |
| GFM1    | Q96RP9     | Mitochondrial Elongation factor G  | X                  | X                 |
| PCTK2   | Q00537     | Cyclin-dependent kinase 17         | X                  | X                 |
| TGOLN2  | O43493     | Trans-Golgi network integral membrane protein 2 | X                | X                 |
| PRKY    | O43930     | Putative serine/threonine-protein kinase PRKY | X                | X                 |
| MDH1B   | Q50G3      | Putative malate dehydrogenase 1B   | X                  | X                 |
| ACCL4   | O60488     | Long chain fatty acid CoA ligase 4  | X                  | X                 |
| ZBED1   | O96006     | Zinc finger BED domain-containing protein 1 | X                | X                 |
| HNRPK   | P61978     | Heterogeneous nuclear ribonucleoprotein K | X                | X                 |
| ZSCAN20 | P17040     | Zinc finger and SCAN domain-containing protein 20 | X                | X                 |
| DDX18   | Q9NPV1     | ATP-dependent RNA helicase DDX18    | X                  | X                 |

**TABLE I**

Candidate miR-122-binding proteins identified using the human protein chip

Molecular & Cellular Proteomics 14.11

2881
complex with hnRNP K, the majority of which corresponded to a monomeric mass of hnRNP K. A lower abundance complex migrated at the position of a dimeric hnRNP K (Fig. 3).

RNA/H9004, which lacks the Seed 1 sequence, was greatly reduced for interaction with hnRNP K. These results confirm that hnRNP K recognizes pyrimidine-rich Seed1 sequence in RNA.

HnRNP K Can Bind miR-122 in Cells—We seek to determine whether endogenous hnRNP K binds miR-122 in cultured hepatocytes. A protocol that was previously used to examine the interactions between viral proteins with RNA in cells was adapted (17, 18) (Fig. 4A). Briefly, Huh7.5 cells were first UV irradiated to generate covalent RNA-protein complexes. The cells were then lysed and immunoprecipitated with antibody specific to hnRNP K. Western blot analysis showed that the hnRNP K was quite specifically precipitated, as a control antibody did not result in detectable hnRNP K (Fig. 4B). The precipitated materials or regions in the membrane corresponding to the location of hnRNP K were digested with protease and the released RNAs were processed for quantitative RT-PCR. RNAs from immunoprecipitations performed with a control antibody did not detect miR-122 signal (data not shown). PCR performed with primers that do not recognize miR-122 and the RNAs did not result in detectable signal (data not shown). In addition, reactions performed without UV crosslinking resulted in only a low amount of detected miR-122. With UV crosslinking, a fourfold increase in miR-122 was observed (Fig. 4C). These results suggest that endogenous hnRNP K can associate with miR-122 in hepatocytes.

We also examined whether hnRNP K could interact with miR-122 in Huh7.5 cells harboring the replicating 1b/Con1 HCV replicon. When compared with the Huh7.5 cells, a higher amount of miR-122 was recovered in HCV+ Huh 7.5 cells in the absence of UV crosslinking. There was also a modest

![Fig. 2](image-url)
reduction in the total number of copies of miR-122 recovered by immunoprecipitation after UV crosslinking (Fig. 4C). However, similar to Huh7.5 cells, the level of miR-122 that selectively precipitated with hnRNP K increased in HCV/H11001 cells subjected to UV crosslinking. In these HCV/H11001 cells, we were also able to detect an enrichment of the HCV RNA using RT-PCR (Fig. 4D).

HnRNP K Preferentially Binds Mature miR-122—cDNAs synthesized from the RNAs that coprecipitated with hnRNP K were subjected to high-throughput Illumina DNA sequencing to analyze the form of miR-122 that binds to hnRNP K. Over 20,000 sequences that were readily identified to contain the miR-122 sequence in both the RNAs from Huh7.5 and from HCV+Huh7.5 cells (Fig. 4E). We also identified a low level of the sequence for RNase P, an abundant RNA in cells important for tRNA processing, and two additional miRNAs, miR-378 and miR-221. RNase P, miR378, and miR221 were not enriched with the immunoprecipitation for hnRNP K to the level observed for miR-122, suggesting that hnRNP K preferentially recognized miR-122 (Fig. 4E). However, similar levels of miR-122 were found to interact with hnRNP K in Huh7.5 cells harboring the HCV replicon (Fig. 4E), suggesting that HCV RNA replication is not required for hnRNP K interaction with miR-122.

Analysis of the reads that contained miR-122 sequence revealed that the vast majority (> 94%) contained mature miR-122 sequences of 22-nts in both cells that harbored the HCV replicon and those that did not. Less than 6% of the sequences had one to four nucleotides missing from the two termini of miR-122. Interestingly, the number of reads decreased when the uridylate-rich sequence at the 3' region of miR-122 was increasingly truncated (Fig. 4F). This is consistent with our observation that substitutions in the pyrimidine-enriched sequence negatively affected hnRNP K binding (Fig. 2C).

HnRNP K Level Affects miR-122 Accumulation In Vivo—To further define the effects of hnRNP K binding to miR-122, we used siRNAs to selectively knockdown hnRNP K in Huh7.5 cells. siRNA specific to hnRNP K reduced the hnRNP K

---

**Fig. 3. Potential secondary structures of miR-122 and SL1 and the role of the Seed 1 sequence in hnRNP K binding.** (A) Predicted secondary structures for miR-122 and two mutants that were affected for binding of hnRNP K. The RNA secondary structures were predicted using the computer program mfold (23) and the change in entropy (ΔG) is from the mfold calculations. Pyrimidine residues in the RNAs are highlighted in bold letters. The residues in Mut1 and Mut2 that were changed from the WT miR-122 are underlined. (B) The predicted secondary structure of SL1. The sequence for Seed 1 is boxed. RNA1 lacks the Seed 1 sequence. (C) The Seed 1 sequence in SL1 is required for optimal binding to hnRNP K. The gel images were from an assay where purified hnRNP K was crosslinked to RNAs radiolabeled at the 5' terminus using γ-32P-ATP and polynucleotide kinase. The quantities of the probes used are shown. The complexes were then separated by SDS-PAGE, and the locations of the radiolabeled RNAs were identified using a phosphorimager. The bottom images show the recombinant hnRNP K present in the SDS-PAGE and stained with Coomassie brilliant blue. The relative amounts of the monomeric hnRNP K-RNA complexes from three independent experiments are shown below the gel images.
mRNA relative to the GAPDH internal control to less than half of the mock-treated control (Fig. 5A). At this level of reduction, the cells exhibited only a model defect in the rate of proliferation, as determined by the Wst-1 assay (Fig. 5A). MiR-122 levels in these cells quantified using qRT-PCR were significantly reduced (Fig. 5B), suggesting that hnRNP K is required for the normal accumulation of miR-122 in Huh7.5 cells. Huh7.5 cells harboring genotype 1b/Con1 replicons had an overall lower level of miR-122 in the absence of anti-hnRNP K siRNA, but the knockdown of hnRNP K did not result in an additional decrease in miR-122 levels (Fig. 5B). It is possible that the presence of the HCV replicon stabilizes miR-122 even when hnRNP K levels are reduced.

**DISCUSSION**

This study was motivated by our previous observation that hnRNP K could bind the SL1 sequence in the 5'UTR of the HCV RNA that overlapped with the miR-122 binding site. A human proteome chip screened with miR-122 identified that hnRNP K as a miR-122 binding protein. Recombinant hnRNP K was able to bind to miR-122 in vitro through short pyrimidine sequences in miR-122. DNA sequencing revealed that hnRNP K preferentially binds mature miR-122.

hnRNP K contains three KH domains, each of which can accommodate RNAs and single-stranded DNAs of four nucleotides (22). Within the nucleic acid binding cleft of the KH domain is a hydrophobic pocket that interacts with the bases of single-stranded nucleic acids. The 3' terminal 4-nt of miR-122 (5'UUUG3') matches sequences preferentially bound by KH domains. However, a typical KH domain binds RNAs with low micromolar dissociation constants (22) and the \( K_D \) for miR-122 binding to hnRNP K was \( 15 \) nM. It is possible that multiple KH domains within hnRNP K contact pyrimidine-rich sequences.
sequences within miR-122 (24). Within the HCV sequence that binds miR-122, we have demonstrated that the Seed 1 se-
quence contributes to hnRNP K recognition. Notably, the Seed 2 sequence also contains a sequence rich in pyrimidines (5'HACUCC3'H) (10). It is likely that hnRNP K molecule(s) bind(s) both the Seed 1 and Seed 2 sequences.

Our data suggest that hnRNP K binding to miR-122 was not dependent on HCV RNA replication. However, miR-122 may be partially stabilized by HCV RNA infection when the level of hnRNPK was reduced. The increase in miR-122 stability could be due to sequestration of miR-122 by the HCV RNA (25). Since hnRNPK can bind both the HCV RNA and miR-122, it may have a role in bringing miRNA-122 to the HCV RNA. HnRNPK has been known to mediate the interaction of multiple molecules, including the HCV Core (26), and we have observed that the UV crosslinked hnRNPK-RNA complex appears to form a dimer when it binds RNA (Fig. 3C). We have attempted to determine whether recombinant hnRNPK could mediate the annealing of miR-122 and a target sequence in vitro, but the results were inconclusive. It is possible that factor(s) not present in our assay is/are needed to induce miR-122 to leave hnRNPK and bind to the target sequence. The phosphorylation of hnRNPK has been shown to decrease its binding to homopolymeric cytidylates (27).

MicroRNA biogenesis involves sequential interaction between the precursor and/or mature miRNA with regulatory proteins (28, 29). Argonaute 2, which processes miRNAs, has also been reported to increase miRNA stability. Additional factors are necessary for additional processing and stability of the miRNA. GLD-2 is known to selectively increase the stability of mature miRNAs through 3' monoadenylation during biogenesis (30). Our study shows that hnRNPK increases the stability of mature miR-122, possibly by protecting a non-base-paired portion of miRNA. The interaction between hnRNPK and miR122 will likely modulate miR-122 stability and the replication of HCV RNA.

Acknowledgments—We thank Dr. B. Szaro for helpful discussion on hnRNPK. We thank L. Kao for editing of the manuscript.

* This work was partially supported by the National Institute of Allergy and Infectious Diseases (Grants AI075015A; AI073335) to C.C. Kao; Taiwan National Health Research Institutes Career Development Grant (NHRI-EX103-10233SC) to C.-S. Chen; Taiwan National Science Council to C.-S. Chen (NSC101-2320-B-008-004-MY3; MOST103-2627-M-008-001); and Taiwan Aim for the Top University Project.
hnRNP K Binds mir-122

REFERENCES

1. Cai, Y., Yu, X., Hu, S., and Yu, J. (2009) A brief review on the mechanisms of miRNA regulation. Genomics Proteomics Bioinf. 7, 147–154

2. Brown, R.H. (2009) Reinnervating microRNA. Science 326, 1494–1495

3. Newman M.A., and Hammond, S.M. (2010) Emerging paradigms of regulation and transcription termination at the early growth response 1 (EGR1) gene wide binding survey reveals its role in regulating 3′-end RNA processing

4. Cai, Y., Yu, X., Hu, S., and Yu, J. (2009) A brief review on the mechanisms of miRNA regulation. Genomics Proteomics Bioinf. 7, 147–154

5. Haussecker, D., and Kay, M.A. (2010) miR-122 continues to blaze the trail for microRNA therapeutics. Mol. Ther. 18, 240–242

6. Li, C., Wang, Y., Wang, S., Wu, B., Hao, J., Fan, H., Ju, Y., Ding, Y., Chen, L., Chu, X., Liu, W., Ye, X., and Meng, S. (2013) Hepatitis B virus miRNA-mediated miR-122 inhibition upregulates PTG1-binding protein, which promotes hepatocellular carcinoma tumor growth and cell invasion. J. Virol. 87, 2193–2205

7. Jopling, C.L., Yi, M., Lancaster A.M., Lemon, S.M., and Sarnow, P. (2005) Modulation of Hepatitis C Virus RNA abundance by a liver-specific microRNA. Science 309, 1577–1581

8. Garcia-Sastre, A., and Evans, M.J. (2013) miR-122 is more than a shield for the hepatitis C virus genome. Prog. Natl. Acad. Sci. U.S.A. 110, 1571–1572

9. Gower, E., Estes, C., Blach, S., Razavi-Shearer, K., and Razavi, H. (2014) Global epidemiology and genotype distribution of the hepatitis C virus infection. J. Hepatol. 61, S45–S57

10. Jopling, C.L., Schütz, S., and Sarnow, P. (2008) Position-dependent function for a tandem microRNA miR-122-binding site located in the hepatitis C virus RNA genome. Cell Host Microbe 4, 77–85

11. Elmén, J., Lindow, M., Schütz, S., Lawrence, M., Petri, A., Obad, S., Lindholm, M., Hedtjärn, M., Hansen, H.F., Berger, U., Gullans, S., Kearney, P., Sarnow, P., Straarup, E.M., and Kauppinen, S. (2008) LNA-mediated miRNA silencing in non-human primates. Nature 452, 896–899

12. Fukuhara, T., Kambara, H., Shiokawa, M., Ono, C., Katoh, H., Morita, E., Okuzaki, D., Maehara, Y., Koike, K., and Matsuura, Y. (2012) Expression and characterization of monospecific monoclonal antibodies using a human proteome microarray. Mol. Cell. Proteomics 11, O111.016255

13. Dinh, P.X., Das, A., Franco, R., and Pattanakul, A.K. (2013) Heterogeneous nuclear ribonucleoprotein K supports vesicular stomatitis virus RNA replication by regulating cell survival and cellular gene expression. J. Virol. 87, 10059–10069

14. Mikula, M., Bomsztyk, K., Goryca, K., Chojnowski, K., and Ostrowski, J. (2013) Heterogeneous nuclear ribonucleoprotein (HnRNP K) genome-wide binding survey reveals its role in regulating 3′-end RNA processing and transcription termination at the early growth response 1 (EGR1) gene through XRN2 exonuclease. J. Biol. Chem. 288, 24788–24798

15. Fan, B., Lu, K.-Y., Sutandy, F.X.R., Chen, Y.-W., Konan, K., Zhu H., Kao, C.C., and Chen, C.-S. (2014) A human proteome microarray identifies that the heterogeneous nuclear Ribonucleoprotein K (hnRNP K) recognizes the 5′ terminal sequence of the hepatitis C virus RNA. Mol. Cell. Proteomics 13, 84–92

16. Ule, J., Jensen, K., Mele, A., and Darnell, R.B. (2005) CLIP: A method for identifying protein–RNA interaction sites in living cells. Methods 37, 376–386

17. Ni, P., Vaughan, R.C., Trageser, B., Hoover, H., and Kao, C.C. (2014) The plant host can affect the encapsidation of brome mosaic virus (BMV) RNA: BMV virions are surprisingly heterogeneous. J. Mol. Biol. 426, 1061–1076

18. Subba-Reddy, C.V., Yunus, M.A., Goodfellow, I.G., and Kao, C.C. (2012) Norovirus RNA synthesis is modulated by an interaction between the viral RNA-dependent RNA polymerase and the major capsid protein, VP1. J. Virol. 86, 10138–10149

19. Livak, K.J., and Schmittgen, T.D. (2001) Analysis of relative gene expression data using real-time quantitative PCR and the 2(-delta delta C(T)) method. Methods 25, 402–408

20. Jeong, J.S., Jiang, L., Abino, E., Marrero, J., Rho, H.S., Hu, J., Hu, S., Vera C., Bayron-Poueymiroy, D., Rivera-Pacheco, Z.A. (2012) Rapid identification of monospecific monoclonal antibodies using a human proteome microarray. Mol. Cell. Proteomics 11, O111.016255

21. Liu, Y., and Szaro, B.G. (2011) hnRNP K post transcriptionally co-regulates multiple cytoskeletal genes needed for axonogenesis. Development 138, 3079–3090

22. Valverde, R., Edwards, L., and Regan, L. (2008) Structure and function of KH domains. FEBS J. 275, 2712–2728

23. Züker, M. (2003) Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 31, 3406–3415

24. Nicastro, G., Taylor, I.A., Ramos, A. (2015) KH-RNA interactions: Back in the groove. Curr. Opin Struct. Biol. 30, 63–70

25. Luna, J.M., Scheel, T.K., Danino, T., Shaw, K.S., Mele, A., Fak, J.J., Nichiuchi, E., Takacs, C.N., Catanese, M.T., de Jong, Y.P., Jacobson, I.M., Rice, C.M., and Damell, R.B. (2015) Hepatitis C virus RNA functionality sequesters miR-122. Cell 160, 1099–1110

26. Li, Z., and Nagy, P.D. (2011) Diverse roles of host RNA-binding proteins in RNA virus replication. RNA Biol. 8, 305–315

27. Deijgaard, K., Leffers, H., Rasmussen, H.H., Madsen, P., Kruse, T.A., Gesser, B., Nielsen, H., and Celis, J.E. (1994) Identification, molecular cloning, expression and chromosome mapping of a family of transformati on upregulated hnrnp-k proteins derived by alternative splicing. J. Mol. Biol. 236, 33–48

28. Rüegger, S., and Grojáhns, H. (2012) MicroRNA turnover: when, how, and why. Trends Biochem. Sci. 37, 436–446

29. Zhang, Z., Qiu, Y.-W., Brewer, G., and Jing, Q. (2012) MicroRNA degradation and turnover: Regulating the regulators. Wiley Interdiscip. Rev. RNA 3, 593–600

30. Katoh, T., Sakaguchi, Y., Miyauchi, K., Suzuki, T., Kashiwabara, S., Baba, T., and Suzuki, T. (2009) Selective stabilization of mammalian microRNAs by 3′ adenylation mediated by the cytoplasmic poly(A) polymerase GLD-2. Genes Dev. 23, 433–438

31. Wen Y, Lin X, Fan, B., Ranjith-Kumar, C.T., and Kao, C.C. (2015) The juxtamembrane sequence of the hepatitis C virus polymerase regulates RNA synthesis and affects sensitivity to active site allosteric inhibitors. Virus Genes 51, 1–11