

Base Pairing between Hepatitis C Virus RNA and MicroRNA 122 3' of Its Seed Sequence Is Essential for Genome Stabilization and Production of Infectious Virus

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MicroRNA 122 (miR-122) facilitates hepatitis C virus (HCV) replication by recruiting an RNA-induced silencing complex (RISC)-like complex containing argonaute 2 (Ago2) to the 5' end of the HCV genome, thereby stabilizing the viral RNA. This requires base pairing between the miR-122 "seed sequence" (nucleotides [nt] 2 to 8) and two sequences near the 5' end of the HCV RNA: S1 (nt 22 to 28) and S2 (nt 38 to 43). However, recent reports suggest that additional base pair interactions occur between HCV RNA and miR-122. We searched 606 sequences from a public database (genotypes 1 to 6) and identified two conserved, putatively single-stranded RNA segments, upstream of S1 (nt 2 and 3) and S2 (nt 30 to 34), with potential for base pairing to miR-122 (nt 15 and 16 and nt 13 to 16, respectively). Mutagenesis and genetic complementation experiments confirmed that HCV nt 2 and 3 pair with nt 15 and 16 of miR-122 bound to S1, while HCV nt 30 to 33 pair with nt 13 to 16 of miR-122 at S2. In genotype 1 and 6 HCV, nt 4 also base pairs with nt 14 of miR-122. These 3' supplementary base pair interactions of miR-122 are functionally important and are required for Ago2 recruitment to HCV RNA by miR-122, miR-122-mediated stabilization of HCV RNA, and production of infectious virus. However, while complementary mutations at HCV nt 30 and 31 efficiently rescued the activity of a 15C,16C miR-122 mutant targeting S2, similar mutations at nt 2 and 3 failed to rescue Ago2 recruitment at S1. These data add to the current understanding of miR-122 interactions with HCV RNA but indicate that base pairing between miR-122 and the 5' 43 nt of the HCV genome is more complex than suggested by existing models.

Hepatitis C virus (HCV) is a positive-strand RNA virus classified in the genus *Hepacivirus* of the family *Flaviviridae*. It is strongly hepatotropic and has an unparalleled ability among RNA viruses to establish lifelong persistent infections in the majority of persons it infects (for a review, see reference 18). About 4 million Americans are persistently infected with HCV, resulting in approximately 12,000 deaths annually due to cirrhosis and liver cancer, with particularly high rates of disease among those coinfecting with human immunodeficiency virus (HIV) (6). Worldwide, more than 130 million people are infected, with an estimated 350,000 HCV-related deaths each year (23). HCV is thus important from a medical and public health perspective. It is also important as an example of an RNA virus that has achieved a uniquely close evolutionary relationship with its human host, upon which its replication depends. This relationship is exemplified by the novel role played by a particular, liver-specific microRNA (miRNA), miR-122, in the HCV life cycle.

miRNAs are ~22-nucleotide (nt) RNAs that originate from lengthier transcripts and that regulate gene expression, typically posttranscriptionally, by mediating mRNA deadenylation and degradation and/or translational repression (reviewed in reference 8). This requires loading of the "guide strand" of a miRNA duplex into an argonaute (Ago) protein-containing miRNA-induced silencing complex (miRISC) and, typically, the binding of its "seed sequence" (nt 2 to 8 of the miRNA) to the 3'-untranslated region (3'UTR) of target mRNAs. miRNAs are evolutionarily conserved, and as such, they play a controlling role in cellular metabolism by regulating a majority of all genes. At present, miRBASE (<http://www.mirbase.org/>) lists over 1,400 distinct human miRNAs, each of which targets a number of mRNAs. Of these, miR-122 (hsa-mir-122 or MI000442) is uniquely expressed

at high abundance in the adult liver, accounting for over 50% of the mature miRNAs in hepatocytes (2). miR-122 is also expressed in Huh-7 human hepatoma cells, which are widely used to propagate laboratory strains of HCV (2).

Jopling et al. (17) demonstrated that the ability of synthetic HCV RNA to replicate in transfected Huh-7 cells is dependent upon a direct interaction of miR-122 with the 5'UTR segment of the positive-strand RNA. miR-122 binds to two sites, near the 5' end of the HCV genome (S1 and S2), at which conserved viral sequences are complementary to the seed sequence of miR-122 (nt 2 to 7) (16) (Fig. 1A). Binding of the miR-122 seed sequence to S1 and S2 promotes protein expression from transfected synthetic viral RNA and has been suggested to enhance the efficiency of translation initiated by the HCV internal ribosome entry site (IRES) (14, 15). However, a comparison of the yields of infectious virus produced by cells transfected with HCV RNA with mutations in the miR-122 binding sites versus mutations in the IRES led us to conclude that promotion of translation is insufficient to

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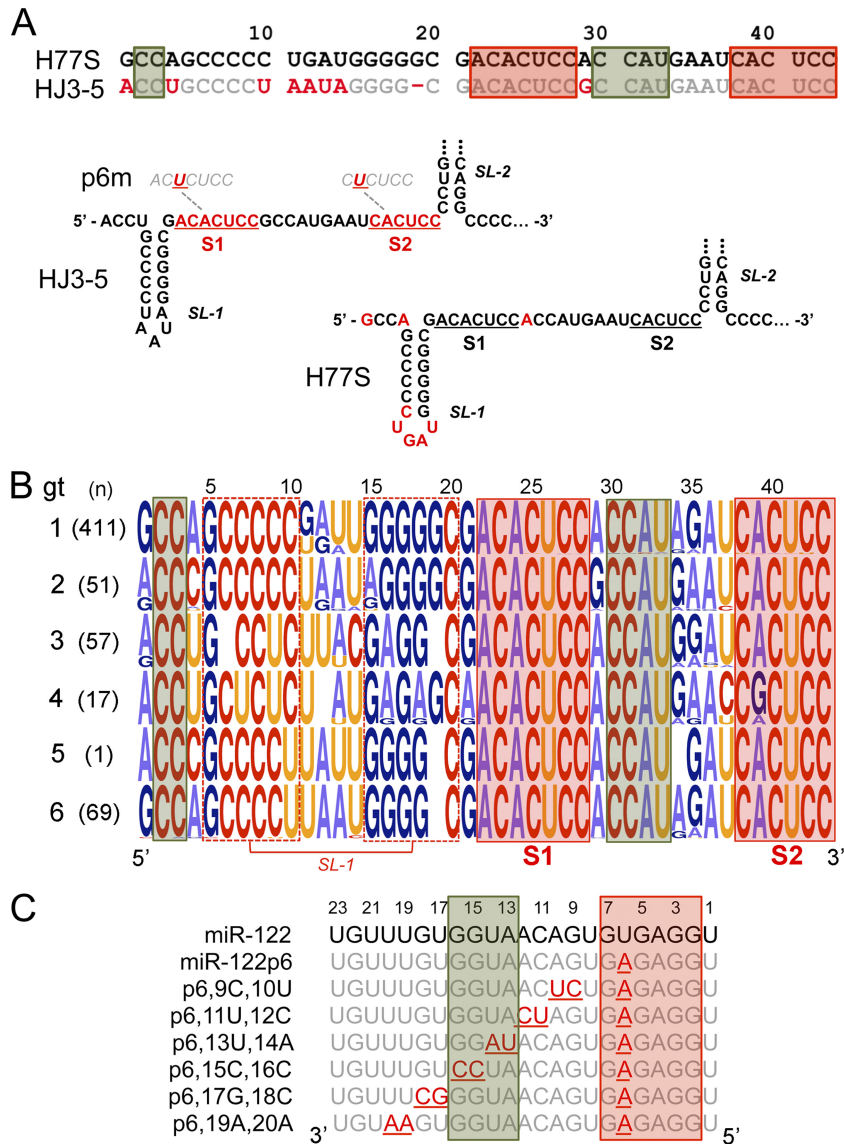


FIG 1 Conserved complementary sequences near the 5' end of the HCV genome suggest supplementary binding of miR-122 to HCV RNA 3' of the miR-122 seed sequences at both S1 and S2. (A) (Top) Alignment of the terminal 5'UTR sequences in H77S (genotype 1a) and HJ3-5 (genotype 2a; derived from JFH-1) viruses. Conserved sequences known to base pair with the seed sequence of miR-122 are highlighted in red boxes. Additional conserved sequences complementary to miR-122 are highlighted in green. Nucleotides in HJ3-5 that differ from those in H77S are shown in red font. (Bottom) Putative secondary structure of the RNA segments, showing stem-loop 1 (SL-1) and the base of stem-loop 2 (SL-2). The S1 and S2 seed match sequences are highlighted in red in the HJ3-5 sequence, with the single base changes in the p6m mutants of S1 and S2 shown above. Bases that differ between the genotype 1a and 2a sequences are highlighted in red in the H77S structure. (B) Logo depiction of sequence conservation in the 5'UTRs of different HCV genotypes. The number and height of each character reflect the diversity in bases and conservation of sequence at each position. Highly conserved sequences are highlighted in red and green as in panel A. Base-paired segments in stem-loop SL-1 are shown in open boxes. The number of sequences of each genotype studied is shown to the left. (C) Guide-strand sequences of miR-122 mutants and related mutants. Point mutations are shown in red. Passenger-strand sequences (Table 1) were modified to maintain the melting temperature of each duplex between 54.3 and 54.9°C. Bases are numbered in a 5'-to-3' fashion at the top but shown in a 3'-to-5' manner to facilitate visualization of base pairing with positive-strand HCV RNA. Nucleotides involved in base pair interactions are shaded in red and green as in panels A and B.

fully explain the requirement for miR-122 (15). These data suggest that miR-122 promotes HCV replication by a mechanism other than translation, stabilizing the viral RNA or possibly promoting the initiation of new viral RNA synthesis (15, 28). Consistent with this prediction, we recently confirmed that binding of miR-122 results in the recruitment of an Ago2-containing RISC-like complex that physically stabilizes the viral RNA genome (26). This miR-122–Ago2 complex slows the decay of positive-strand HCV

RNA both in RNA-transfected cells and in cells persistently infected with HCV, most likely by protecting the RNA from 5'-exonuclease attack (26).

In addition to base pairing involving the seed sequence, the recognition of cellular mRNA targets by miRNAs may also entail supplementary or accessory interactions of bases 3' of the seed sequence (1, 12). Such additional base pairing optimally involves nt 13 to 16 and spares nt 9 to 12 of the miRNA, presumably be-

cause such base pairing does not require helix formation throughout the length of the guide strand (as occurs with perfectly complementary small interfering RNA [siRNA]) and thus may not perturb its association with Ago in the RISC (1, 12). Several recent reports provide evidence that the interaction of miR-122 with HCV RNA similarly involves such 3' supplementary interactions (20, 22, 24). Although details differ between these studies, these interactions appear to contribute to the ability of miR-122 to promote the amplification of HCV RNA in transfected cells or protein expression from reporter RNAs containing the HCV IRES in their 5'UTR. Here we confirm and extend these observations, using a genetic approach to demonstrate 3' supplementary base pair interactions between miR-122 and the 5' termini of both genotype 1a and genotype 2a viral RNAs. We show that 3' supplementary base pairing contributes to the recruitment of Ago2 and the stabilization of HCV RNA by miR-122 and that it is also required for optimal production of infectious virus in cell culture.

MATERIALS AND METHODS

Cells. Huh-7, Huh-7.5, and FT3-7 cells (a clonal derivative of Huh-7 cells) were maintained as described previously (15, 25). Murine embryo fibroblasts (MEFs) were the kind gift of Alexander Tarakhovskiy, Rockefeller University, and were maintained in cell culture as described previously (21).

Plasmids. pHJ3-5 (19), the related S1, S2, and S1-S2 p6m mutants (15), and pH77S/GLuc2A-AAG (25, 30) have been described previously. The *Gussia princeps* luciferase (GLuc) coding sequence, followed by the foot-and-mouth disease virus 2A sequence, was inserted between p7 and NS2 in pHJ3-5 or pH77S.3 and the related S1, S2, and S1-S2 p6m, S1p3m, and S1p23m miR-122 binding site mutants, using the strategy adopted previously for H77S (25). Single- and multiple-base substitutions were created within pHJ3-5 and pHJ3-5/GLuc2A by site-directed mutagenesis of the segment spanning the EcoRI and AgeI sites. Base substitutions were similarly introduced into pH77S.3/GLuc2A and pH77S/GLuc2A-AAG by site-directed mutagenesis of the sequence spanning the NotI and AgeI sites. Base changes and the integrity of the surrounding sequences were confirmed by DNA sequencing.

RNA transcription. RNA transcripts were synthesized *in vitro* as described previously (25).

RNA oligonucleotides. RNA oligonucleotides have been described previously (15) and were synthesized by Dharmacon. Additional mutant miRNAs used in this study are shown in Table 1. All miRNAs were transfected as duplexes (15).

miRNA supplementation and HCV replication. FT3-7 cells seeded previously into a 6-well culture plate were transfected with a miRNA duplex (50 nM) by use of Lipofectamine 2000 (Invitrogen) as recommended by the manufacturer. At 24 h, the cells were retransfected with replication-competent *in vitro*-transcribed HCV RNA (1.25 μ g/well) for 6 h, using the TransIT mRNA transfection reagent (Mirus Bio) according to the manufacturer's suggested protocol. The cells were retransfected with miRNA 24 h later and refed fresh medium every 24 h thereafter. Supernatant fluid samples were collected for virus titration or GLuc assay at 24-h intervals, unless noted otherwise. Total cellular RNA was extracted for Northern blots.

miRNA supplementation and HCV RNA stability and translation. Synthetic HCV RNA containing a replication-lethal mutation in the NS5B coding region (20 μ g) and duplex miRNA (1 μ M) were mixed with 1×10^7 wild-type (wt) MEF cells in a 4-mm cuvette and pulsed once at 400 V, 250 μ F, and infinite Ω in a Gene Pulser Xcell Total system (Bio-Rad). Synthetic capped and polyadenylated RNA encoding *Cypridina* luciferase (CLuc) was cotransfected to monitor transfection efficiency. The cells were plated into three wells of a six-well culture plate, and cell culture supernatant fluid was collected for GLuc assay and total cellular RNA extracted for Northern blotting at 3-h intervals.

TABLE 1 miR-122 mutants used in this study

miR-122 mutant	Guide (5'-3') and passenger-strand (3'-5') RNA sequences ^a
miR-122	UGGAGUGUGACAAUGGUGUUUGU AUAUUACACACUAAUACCGCAA
miR-122p6	UGGAGAGUGACAAUGGUGUUUGU AUAUUACACACUAAUACCGCAA
miR-122p6,9C,10U	UGGAGAGUCUCAUUGGUGUUUGU AUAUUACACAGAAUACCGCAA
miR-122p6,11U,12C	UGGAGAGUGAUCAUUGGUGUUUGU AUAUUACACACUAAUACCGCAA
miR-122p6,13U,14A	UGGAGAGUGACAAUAGGUGUUUGU AUAUUACACACUAAUACCGCAA
miR-122p6,15C,16C	UGGAGAGUGACAAUCCUGUUUGU AUAUUACACACUAAUAGGGCAA
miR-122p6,17G,18C	UGGAGAGUGACAAUGGGCUUUGU AUAUUACACACUAAUACCGUAAA
miR-122p6,19A,20A	UGGAGAGUGACAAUAGGUGAAUGU AUAUUACACACUAAUACCGCUUA
miR-122m15C,16C	UGGAGAGUGACAAUCCUGUUUGU AUAUUACACACUAAUAGGGCAA

^a The top sequence for each mutant represents the guide strand, and the lower sequence represents the passenger strand. Underlined bases have been mutated from the wild-type sequence.

Infectious virus titration. Huh-7.5 cells were transfected with duplex miR122p6 (50 nM) and then seeded into 48-well plates at a density of 5×10^4 cells/well and maintained in a 37°C 5% CO₂ environment. At 24 h, the cells were inoculated with serial dilutions of virus-containing medium (100 μ l). Cells were fed 300 μ l fresh medium 24 h later. Following 48 h of additional incubation, cells were fixed, labeled with HCV core-specific antibody, and examined under an inverted UV fluorescence microscope as described previously (25, 30). Clusters of infected cells identified by specific staining for core antigen were considered to constitute a single infectious focus-forming unit (FFU); virus titers are reported as FFU/ml.

Ago2-RNA immunoprecipitation (IP). MEFs were electroporated with 10 μ g HCV RNA and 1 μ M duplex miRNAs. Six hours later, cells were harvested in lysis buffer (150 mM KCl, 25 mM Tris-HCl, pH 7.4, 5 mM EDTA, 1% Triton X-100, 5 mM dithiothreitol [DTT], Complete protease inhibitor cocktail [Roche], 100 U/ml RNaseOUT [Invitrogen]). Lysates were centrifuged for 30 min at $17,000 \times g$ at 4°C, filtered through a 0.45- μ m syringe filter, and incubated with anti-Ago2 monoclonal antibody (MAB) (2D4; Wako Chemicals) or isotype control IgG at 4°C for 2 h, followed by the addition of 30 μ l of protein G Sepharose (GE Healthcare) for 1 h. The Sepharose beads were washed 3 times in lysis buffer, and RNA was extracted using an RNeasy minikit (Qiagen). HCV RNA associated with Ago2 protein was detected by reverse transcription-PCR (RT-PCR), using a SuperScript One-Step RT-PCR kit with Platinum *Taq* DNA polymerase (Invitrogen) and primers specific for the HCV core sequence as described previously (25).

Luciferase assay. Cell culture supernatant fluids were collected at 24-h intervals following RNA transfection and replaced completely with fresh medium. Secreted GLuc and CLuc activities were measured as described previously (25).

Northern blotting. Northern blotting for HCV RNA and actin mRNA (loading control) and quantitation of these results were carried out as described previously (15, 25).

Genotype-specific HCV 5'UTR sequence logos. HCV 5'UTR sequences of at least 300 bases were retrieved from the European HCV database (euHCVdb [<http://euHCVdb.ibcp.fr/euHCVdb/>]) (4). Genotype-specific (27) sequence alignments were computed using ClustalW (3) and MUSCLE (7), with minor manual modifications in the SeaView alignment editor (11). Sequence logos were computed using WebLogo (<http://weblogo.berkeley.edu/logo.cgi>) (5).

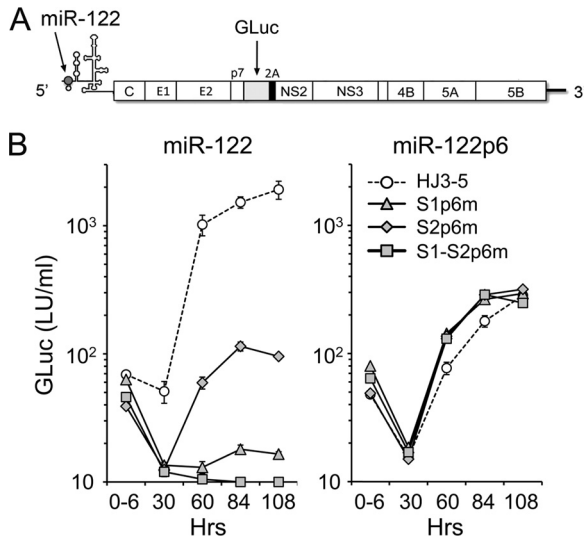


FIG 2 Replication of HJ3-5 RNAs with or without point mutations in the S1 and S2 seed match sites. (A) Organization of synthetic GLuc-expressing HCV genomic RNAs. The GLuc sequence was inserted downstream of p7, followed by the 2A sequence of foot-and-mouth disease virus. (B) GLuc activities in supernatant fluids of cultures transfected with HJ3-5 or related S1 and S2 mutant RNAs and either wt miR-122 (left) or the miR122p6 mutant (right). FT3-7 cells were transfected with the oligoribonucleotides 24 h before and after transfection of the viral RNAs. Supernatant fluids were collected to assay GLuc secreted into the medium between 0 and 6 h following HCV RNA transfection and over successive 24-h periods ending at 30, 60, 84, and 108 h posttransfection. Results shown represent the mean ratios of GLuc activities in replicate cultures \pm ranges and are representative of multiple independent experiments. LU, light units.

RESULTS

miR-122 bases involved in productive interactions with HCV RNA. A conserved CCAU sequence (HCV nt 30 to 33) upstream of the S2 site in HCV RNA (Fig. 1A, top panel) is complementary to nt 13 to 16 of miR-122 and is thus available for base pairing with the miRNA 3' of its seed sequence (miR-122 nt 2 to 7) (12). In addition, a conserved CC sequence (HCV nt 2 and 3) upstream of stem-loop 1 represents a similar potential site of 3' supplementary base pairing at S1. An analysis of over 600 individual viral sequences deposited in the European HCV database (euHCVdb) indicated that these miR-122-complementary bases are highly conserved across all 6 HCV genotypes (Fig. 1B). As indicated above, Machlin et al. (20) recently showed that 3' supplementary interactions involving nt 15 and 16 of miR-122 and nt 2 and 3 and nt 30 and 31 of the viral RNA contribute to the ability of miR-122 to promote the accumulation of viral RNA following its transfection into permissive cells (20). To further characterize supplementary base pairing of miR-122 outside its seed sequence to HCV RNA, we synthesized a panel of miR-122 mutants in which successive pairs of bases, starting at nt 9 and 10 and going through nt 19 and 20, were replaced with their Watson-Crick complement (Fig. 1C) (the analysis of miR-122 mutants with base substitutions at nt 21 to 23 will be reported elsewhere). Each of these mutated miR-122s had a U-to-A substitution at nt 6 ("p6" mutation). We tested the ability of the mutants, transfected as duplex miRNAs into HCV-permissive FT3-7 cells, to promote the replication of HCV RNA carrying complementary substitutions ("p6m") (Fig. 1A, bottom panel) within the S1, S2, or both S1 and S2 seed se-

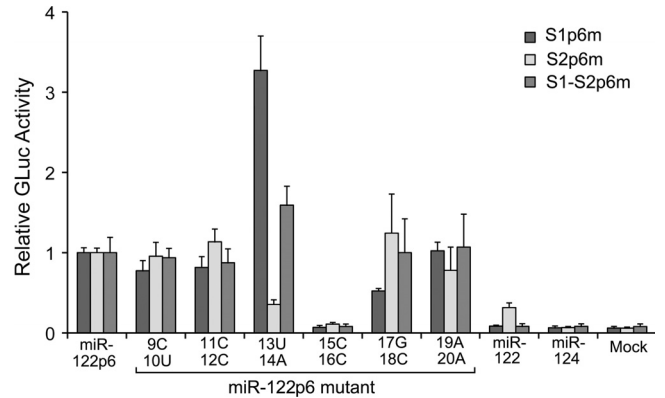


FIG 3 Capacity of various miR-122 mutants to support HCV RNA replication. HJ3-5 RNAs bearing either single or double S1 and S2 p6m mutations, as indicated, were cotransfected into cells with the indicated wt or mutant duplex miR-122s, using the same protocol as that described in the legend to Fig. 2B. Results shown represent the mean ratios of GLuc activities in triplicate cultures at 84 h \pm standard deviations (SD), normalized for each viral RNA to that obtained with miR-122p6. The data shown are representative of multiple independent experiments. Mock, no oligonucleotide.

quence binding sites (15). HCV RNAs with a p6m base substitution in either S1 or S2 are severely handicapped in replication, especially when the mutation is at the S1 site, but can be rescued by transfection of the complementary miR-122 p6 mutant (15). This strategy functionally isolates the S1 and S2 miR-122 binding sites, allowing requirements for miR-122 sequence to be determined individually at each seed match site, and is similar to the strategy we used previously to dissect the contributions of the two sites to miR-122-mediated increases in viral translation versus replication (15). Where necessary, the passenger-strand RNA in the duplex was modified to maintain a melting temperature for each duplex that was similar to that of the parental miR-122p6 duplex (Table 1).

The genome-length HCV RNA used in this series of experiments was HJ3-5 (19), a chimera that contains the genotype 2a 5'UTR sequence rather than the genotype 1a H77 5'UTR sequence studied by Machlin et al. (20) (see Fig. 1A for a comparison of the relevant sequences). It was modified by the insertion of the GLuc (*Gussia princeps* luciferase) sequence within the polyprotein, which allows replication to be monitored by measuring GLuc activity secreted into supernatant fluids of transfected cell cultures (Fig. 2A). As expected (15), viral RNAs with p6m mutations in S1 or S2 produced much less GLuc than RNAs containing the wt seed match sequences when cotransfected into cells with wt miR-122 (Fig. 2B, left panel). The GLuc expression pattern suggested a lack of replication of the double S1-S2p6m mutant. In contrast, the GLuc activity produced by each of these RNAs was substantially increased when the RNAs were cotransfected with miR-122p6 (Fig. 2B, right panel), with the S1-S2p6m mutant replicating with an efficiency similar to that of the other RNAs.

Each of the modified miR-122p6 duplexes (Fig. 1C) was cotransfected into cells with either the S1p6m, S2p6m, or S1-S2p6m HJ3-5 HCV RNA, and GLuc activity secreted into the culture supernatant fluids was monitored over the ensuing 84 h as a measure of genome amplification. The results are presented in Fig. 3, which shows the GLuc activity at 84 h posttransfection, normalized for each of the viral RNAs to that produced by cotransfection with miR-122p6 (which lacks base substitutions outside the seed

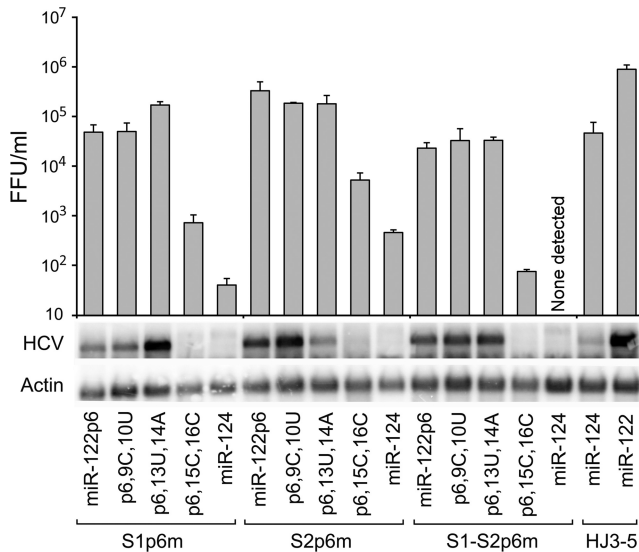


FIG 4 miR-122 nt 13 to 16 are essential for support of HCV RNA replication and production of infectious virus in cell culture. (Top) FT3-7 cells were transfected with HJ3-5 RNA and related p6m mutants 24 h after transfection with the indicated duplex oligoribonucleotides and then retransfected 24 h later with the same oligoribonucleotides. After an additional 48 h of incubation, supernatant fluids were harvested for titration of HCV infectivity in a focus-forming assay using Huh-7.5 cells transfected with miR-122p6 24 h prior to inoculation. (Bottom) RNA extracted from the HCV RNA-transfected cells was subjected to Northern analysis for HCV RNA. β -Actin mRNA served as a loading control.

sequence). As additional controls, the HCV RNA-transfected cells were also supplemented with wt miR-122 or miR-124, an unrelated brain-specific miRNA. miR-122p6 mutants with base substitutions in nt 9 to 12 and 17 to 20 functioned as well as miR-122p6 in promoting genome amplification, indicating that these bases are not important for the interaction with HCV RNA. However, very different results (either decreased or increased GLuc expression) were obtained with miR-122p6 mutants with base substitutions at nt 15 and 16 or nt 13 and 14 (Fig. 3). miR-122p6,15C,16C was markedly impaired in the ability to rescue the amplification of HCV RNAs with p6m mutations at either the S1 or S2 site (Fig. 3). In contrast, while miR-122p6,13U,14A had a significantly reduced capacity to rescue amplification of the S2p6m mutant, it demonstrated 3-fold greater activity than miR-122p6 in promoting replication of the S1p6m RNA and was able to enhance replication of the double S1-S2p6m mutant (Fig. 3). Northern blotting of RNAs extracted from cells transfected with HJ3-5 virus RNAs containing only the miR-122 binding site mutations (no GLuc2A insertion) confirmed these results, as well as the absence of any effect of substitutions at miR-122 nt 9 and 10 (Fig. 4, bottom panels).

The marked decrease we observed in the ability of the miR-122p6,15C,16C mutant to promote amplification of HCV RNA with a genotype 2a 5'UTR confirms the loss of activity of a similar miR-122 mutant against genotype 1a RNA reported by Machlin et al. (20) and is consistent with 3' supplementary base pair interactions between miR-122 and bases upstream of both the S1 (nt 2 and 3) and S2 (nt 30 and 31) sites in the viral RNA (as suggested in Fig. 1). However, the results we obtained with miR-122 mutated at nt 13 and 14 were quite different from those described previously

for a genotype 1 virus. Machlin et al. (20) reported an ~70% decrease in the ability of a similar miR-122 mutant to promote HCV RNA accumulation when bound at the S1 seed match site, while we observed a >3-fold increase in activity (Fig. 3). We show below that these differences, which are large and very significant for miRNA activities, reflect genotype-specific variation in the 5'UTR sequences used in the two studies.

Base substitutions in miR-122 and promotion of infectious virus yield. Since prior studies have not determined the relevance of 3' supplementary miR-122 mutations to the production of infectious virus, we measured the yields of infectious virus produced from cells cotransfected with the HJ3-5 S1p6m, S2p6m, or S1-S2p6m RNA and key miR-122p6 mutants. Although the dynamic range of the FFU assay used for titration of infectious virus is far greater (extending over >4 orders of magnitude) than that of Northern blotting for HCV RNA, the results generally mirrored each other (Fig. 4). There was no detectable virus produced by the double S1-S2 mutant RNA in the absence of cotransfected miR-122p6. Virus yields from the single S1 and S2 mutants were also very low, but production of infectious virus from each of these HCV RNAs was boosted 100- to 1,000-fold by cotransfection with miR-122p6. In contrast, miR-122p6,15C,16C was severely impaired in the ability to support production of infectious virus by each of the three HJ3-5 mutants. Nonetheless, it did cause an approximately 10-fold increase in infectious virus yield compared with miR-124, indicating that 3' supplementary base substitutions involving nt 15 and 16 of miR-122 are not absolutely essential for the interaction with HCV RNA and for miR-122 boosting of infectious virus production. The base substitutions at nt 13 and 14 had less impact on infectious virus yield. However, cotransfection of miR122p6,13U,14A with the S1 mutant RNA resulted in a significant increase in virus yield compared with that with miR122p6 and in a slight reduction of yield compared to that with the S2 mutant. These results are consistent with the GLuc results obtained in the transient-transfection experiments shown in Fig. 3. Although disruption of miR-122 binding at either the S1 or S2 binding site has a strong negative effect on viral RNA amplification and infectious virus yields, the results shown in Fig. 4 confirm a greater dependency on the S1 versus S2 seed match site for virus replication, as we suggested previously (15).

Complementary mutations at HCV nt 2 and 3 and nt 30 to 33 rescue miR-122 mutants with substitutions at nt 13 to 16. The strong negative impact of cytosine-for-guanine substitutions at nt 15 and 16 of miR-122 in the experiments described above is consistent with miR-122 base pairing to the conserved cytosines at positions 2 and 3 and positions 30 and 31 in HCV RNA (Fig. 1). This was confirmed by Machlin et al. (20), who demonstrated rescue of miR-122 promotion of viral RNA accumulation when mutations complementing base substitutions at nt 15 and 16 of miR-122 were constructed within the viral RNA. However, the negative impact of the 13U and 14A substitutions observed with miR-122 interactions at the S2 site (Fig. 3) suggested that the supplementary base pairing upstream of S2 extends in a 3' direction on the viral RNA, to include nt 32 and 33. To confirm this, we constructed HJ3-5/GLuc2A-S2p6m RNAs with base substitutions at nt 32 and 33 that were complementary to those in miR-122p6,13U,14A. We also constructed viral RNAs with guanine substitutions at nt 2 and 3 and nt 31 and 32 to complement the base changes in the miR-122p6,15C,16C mutant. The replication of each of these viral RNA mutants was impaired compared with

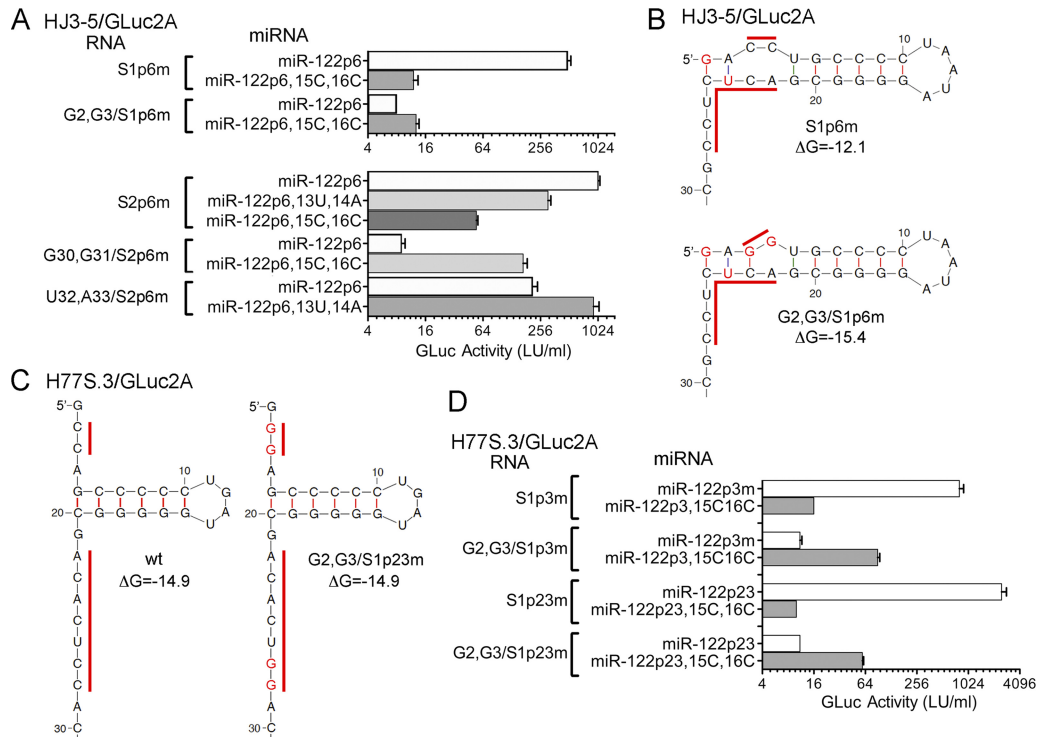


FIG 5 Functional rescue of miR-122 mutants by complementary substitutions in HCV RNA. (A) Complementary substitutions at nt 2 and 3 and nt 30 to 33 of HCV RNA rescue promotion of its amplification by miR-122p6 mutants with substitutions at nt 13 and 14 and/or nt 15 and 16. Replication-competent HJ3-5/GLuc2A RNAs with p6m mutations in S1 (top) or S2 (bottom) and additional base substitutions at nt 2 and 3, 30 and 31, or 32 and 33 were transfected into FT3-7 cells with the indicated oligonucleotides, as described in the legend to Fig. 2. Results shown represent mean GLuc activities in supernatant fluids 96 h following transfection \pm ranges. Minimal but statistically significant rescue was observed with S1p6m RNA, while rescue was relatively efficient with S2p6m RNA. (B) mfold prediction of secondary structure at the 5' end of the HJ3-5/GLuc2A-S1p6m transcripts with and without complementary G2 and G3 substitutions. Note that the first guanine base is generated from the T7 promoter sequence in the plasmid, whereas the first nucleotide of H77S is naturally guanine (see Fig. 1). (C) mfold predictions of secondary RNA structures of H77S.3/GLuc2A and H77S.3/GLuc2A-G2,G3-S1p23m. (D) Ability of complementary mutations in H77S.3/GLuc2A-S1p3m and -S1-p23m RNAs to rescue activity of cognate miR-122 mutants with 15C and 16C substitutions.

that of the cognate HJ3-5/GLuc2A-S1p6m or -S2p6m parent (as determined by GLuc expression in transient-transfection assays) (Fig. 5A), with the G2,G3 and G30,G31 mutants affected more than the U32,A33 mutant. With each mutant, the reduction in replication capacity was rescued at least partially by cotransfection with miR-122p6,15C,16C (G2,G3 and G30,G31 mutants) or miR-122p6,13U,14A (U32,A33 mutant). These results provide genetic evidence for the suspected supplementary base pairing, including its extension to nt 32 and 33 as suggested in Fig. 1B.

While we observed a reproducible, ~ 2 -fold enhancement in GLuc expression from HJ3-5/GLuc2A-S1p6m,G2,G3 when it was cotransfected with its cognate miR-122p6,15C,16C mutant, its replication remained very inefficient. One possible explanation for this is an aberrant RNA secondary structure when guanines are inserted in lieu of the cytosines at positions 2 and 3 in the genotype 2a 5'UTR. mfold predictions (<http://mfold.rna.albany.edu>) of secondary structure in the HJ3-5/GLuc2a-S1p6m,G2,G3 mutant RNA suggest that it forms a stable stem-loop ($\Delta G = -15.4$ kcal) at its extreme 5' end (Fig. 5B). We considered this a likely explanation for the lack of complete rescue of replication with the complementary miR-122 mutant, since Machlin et al. (20) did not report any difficulty in rescuing a similar mutant genotype 1a RNA. To confirm this, we constructed two G2,G3 mutants constructed in the background of H77S.3/GLuc2A, in which the viral sequence is entirely genotype 1a. In addition to the G2,G3 substi-

tutions, we made base changes at position 3 or both positions 2 and 3 in the S1 seed match site (p3m and p23m mutants). None of these mutations alter the predicted secondary structure at the 5' end of the genotype 1a genome (Fig. 5C). Despite this, cotransfection of the complementary miR-122p3,15C,16C and miR-122p23,15C,16C mutants with these HCV mutants only partially rescued the large defect in replication capacity imposed by the G2,G3 substitutions (Fig. 5D). We concluded that while these bases are engaged in supplementary 3' base pairing with miR-122, there is an additional requirement for cytosines at positions 2 and 3. One possibility is that these bases are involved in recognition of the 5' end of the genome by the HCV replicase (9).

3' supplementary base pair interactions are required for miR-122 stabilization of HCV RNA. We have shown recently that binding of a miR-122-Ago2 complex stabilizes HCV RNA and results in decreased rates of decay of both synthetic viral RNA transfected into cells and replicating viral RNA in infected cells (26). To determine whether 3' supplementary base pair interactions of miR-122 contribute to this stabilizing action, we monitored RNA decay in cells cotransfected with a replication-incompetent HCV RNA and various miR-122 mutants. We used MEFs for these experiments because they do not express miR-122. This ensures that results are not confounded by expression of endogenous miR-122. Cells were transfected with genotype 1a H77S RNA modified to express GLuc and containing a replication-lethal mu-

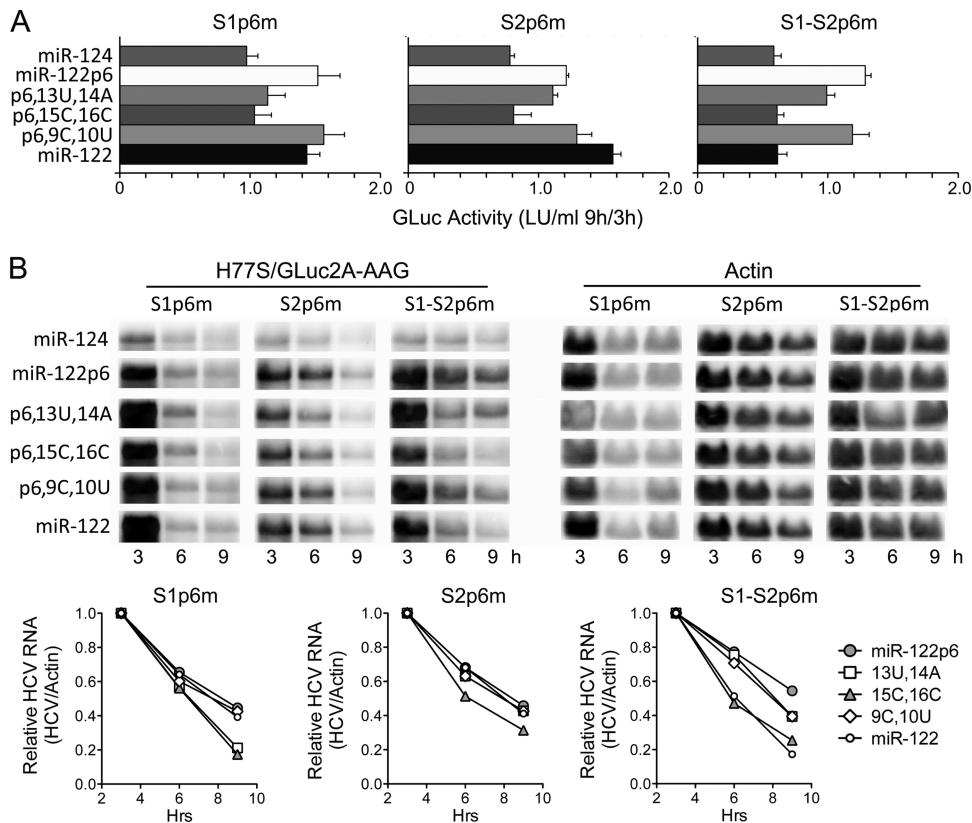


FIG 6 3' supplementary interactions involving nt 13 to 16 contribute to stabilization of HCV RNA by miR-122. (A) GLuc expression from replication-incompetent, genotype 1a H77S/GLuc2A-AAG and related p6m S1 and S2 mutant RNAs that were coelectroporated into MEFs with the indicated miR-122 duplexes. Results shown represent the means \pm ranges of the ratios of GLuc activities in supernatant fluids from duplicate transfected cultures at 9 h versus 3 h and are representative of replicate experiments. (B) Northern analysis of HCV RNA and β -actin mRNA (loading control) in RNAs extracted from cells 3, 6, and 9 h after electroporation of the indicated viral RNAs and duplex miRNAs. Quantitation of HCV RNA (HCV/actin mRNA) in representative Northern blots is shown below, normalized to that present at 3 h (arbitrarily set to 1.0), to compare the stability of the RNA cotransfected with different miR-122 mutants.

tation within the NS5B coding sequence (H77S/GLuc2A-AAG). As we have shown previously (25), GLuc expression from this RNA results exclusively from translation of the input RNA, peaking at around 3 h posttransfection. Thus, the ratio of GLuc activity in culture supernatant fluids at 9 h versus 3 h posttransfection reflects the rate of decay of the transfected RNA (25). p6m mutations were placed within the S1 or S2 seed match sites, as described above (Fig. 1A). As expected, the ratio of GLuc activity expressed by cells transfected with the H77S/GLuc2A-AAG/S1-S2p6m double mutant at 9 h versus 3 h was enhanced when the mutant was cotransfected with miR-122p6 but not miR-122 or the control, brain-specific miR-124 (Fig. 6A, right panel). miR-122p6,9C,10U was as capable as miR-122p6 at enhancing the level of GLuc expression at 9 h versus 3 h, but miR-122p6,15C,16C was no more active than miR-124 or miR-122. These results are consistent with our earlier observations that base pairing involving nt 15 and 16 of miR-122 is required for efficient replication of HJ3-5 RNA (Fig. 3 to 5). Stabilization of the HCV RNA was confirmed by Northern blots of total cellular RNA extracted 3, 6, and 9 h after transfection. These results showed greater abundance of the viral RNA at 9 h when it was cotransfected with miR-122p6 or miR-122p6,9C,10U than when it was cotransfected with miR-122p6,15C,16C, miR-122, or miR-124 (Fig. 6B).

In contrast to miR-122p6,15C,16C, miR-122p6,13U,14A

demonstrated only a modest reduction in the ability to promote GLuc expression at 9 h versus 3 h (compared to that of miR-122p6) (Fig. 6A, right) or in the ability to stabilize the S1-S2p6m RNA in Northern blots (Fig. 6B). Thus, while base pairing at nt 13 and 14 of miR-122 contributes to the stabilization of viral RNA, it appears to be less important than the interactions involving nt 15 and 16.

We also examined the ability of the mutated miRNAs to stabilize H77S/GLuc2A-AAG RNA with mutations within either the S1 or S2 seed match sites. Unlike the case for the double S1-S2p6m mutant, GLuc assays (Fig. 6A, left and center panels) and Northern blots (Fig. 6B) revealed that these mutants were both stabilized by wild-type miR-122. This likely reflects interactions with the alternative, nonmutated binding site (e.g., the S2 site in the S1p6m mutant) and is consistent with both miR-122 binding sites contributing to stabilization of the RNA. As expected, the miR-122p6,15C,16C mutant had a smaller effect than that of the control (miR-122p6) on the stability of either the S1p6m or S2p6m mutant, based on GLuc expression (Fig. 6A) and HCV RNA abundance at 9 h (Fig. 6B). Base pairing of nt 15 and 16 at both binding sites is thus important for stabilization. However, in contrast to the substantial enhancement we observed with miR-122p6,13U,14A relative to miR-122p6 in promoting replication of the HJ3-5/GLuc2A-S1p6m mutant (Fig. 2 and 3), miR-122p6,13U,14A was less active

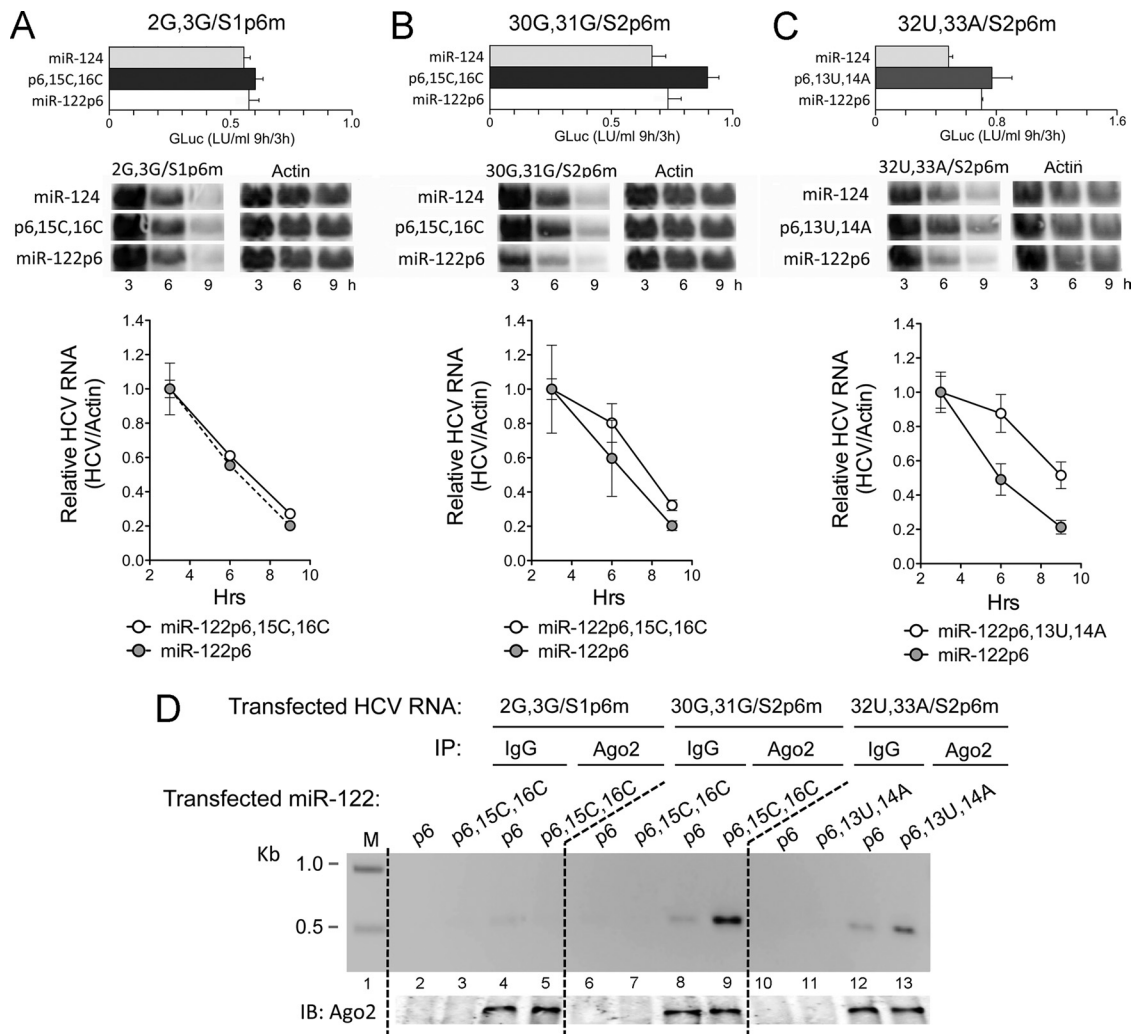


FIG 7 Complementary mutations at HCV nt 30 and 31 restore a stabilization phenotype to miR-122p6 mutants with base substitutions at positions 15 and 16. H77S/GLuc2A-AAG RNAs carrying an S1 or S2 p6m mutation and additional nucleotide substitutions at nt 2 and 3 (A), nt 30 and 31 (B), or nt 32 and 33 (C) were transfected into MEFs with the indicated miRNA duplexes. The top panel of each of these figures shows the ratio of GLuc activity at 9 h versus 3 h. The middle panels show Northern blots of the HCV RNA at 3, 6, and 9 h, with actin mRNA included as a loading control. At the bottom of each panel is shown the quantitation of Northern blotting results from duplicate transfections, comparing HCV RNA to actin mRNA at 3, 6, and 9 h (means \pm ranges). Results were normalized to the HCV RNA abundance at 3 h (arbitrarily set to 1.0) in order to compare the stability of the viral RNA cotransfected with miR-122p6 versus the complementary mutant. (D) Ago2-RNA immunoprecipitation results obtained with lysates from cells cotransfected with the mutant HCV RNAs in panels A to C and either miR-122p6 or related mutants.

than miR-122p6 in stabilizing the H77S/GLuc2A-S1p6m mutant (Fig. 6A and B). Subsequent experiments (see below) demonstrated that this difference in the activity of the miR-122p6,13U,14A mutant with the H77S versus HJ3-5 RNA was due to differences in the nucleotide sequences of the two viral RNAs at nt 4.

Additional experiments were carried out to demonstrate whether the mutated miRNAs were capable of stabilizing H77S/GLuc2A-AAG RNAs carrying complementary mutations. These studies revealed that supplementation with miR-122p6,15C,16C resulted in no significant increase in the expression of GLuc 9 h versus 3 h following transfection of 2G,3G/S1p6m RNA compared with that with miR-122p6 (Fig. 7A, top panel). This was confirmed in quantitative Northern blots showing that miR-122p6,15C,16C did not enhance the stability of the 2G,3G/S1p6m RNA (Fig. 7A, middle and bottom

panels). This is consistent with the minimal rescue of replication of the 2G,3G/S1p6m mutant by miR122p6,15C,16C (Fig. 5, top panel) and suggests that there may be other factors controlling miR-122 interactions at S1. In contrast, miR-122p6,15C,16C was capable of stabilizing the 30G,31G/S2p6m mutant, as evidenced by GLuc assays and in Northern blots (Fig. 7B), while miR-122p6,13U,14A similarly stabilized 32U,33A/S2p6m RNA (Fig. 7C). These last results provide strong support for the supplemental 3' base pairing between nt 13 to 16 of miR-122 and the HCV sequence upstream of S2 proposed in Fig. 1, and they indicate its importance in stabilization of the viral genome.

3' supplementary interactions of miR-122 with HCV RNA contribute to the recruitment of Ago2. miR-122-mediated stabilization of HCV RNA is dependent upon recruitment of Ago2 to the viral 5'UTR (26). To ascertain the involvement of the 3' sup-

plementary base pair interactions in this process, we used RT-PCR to determine whether HCV RNA was physically associated with Ago2 immunoprecipitated from lysates of cells cotransfected with mutated viral RNAs and their cognate miR-122 mutants, as in the experiments shown in Fig. 7A to C. The technical details of these Ago2-RNA co-IP experiments are provided in Materials and Methods and have been published previously (26). The results of these experiments confirmed that base pairing of nt 13 and 14 and nt 15 and 16 of miR-122 with nt 32 and 33 and nt 30 and 31 in HCV, upstream of the S2 seed match site, is important for Ago2 recruitment (Fig. 7D). HCV RNA was enriched in Ago2 precipitates from cells transfected with the 32U,33A/S2p6m and 30G,31G/S2p6m HCV RNAs and the complementary p6,13U,14A and p6,15C,16C miR-122 mutants, respectively, versus miR-122p6, which lacks the relevant complementary base changes (Fig. 7D, lane 13 versus lane 12 and lane 9 versus lane 8). In contrast, very little HCV RNA was coimmunoprecipitated with Ago2 from lysates of cells transfected with the 2G,3G/S1p6m viral RNA, and there was no enrichment of the RNA in cells cotransfected with the complementary p6,15C,16C miR-122 mutant (Fig. 7D, compare lanes 4 and 5). These results are consistent with the inability of complementary miR-122 mutants with base changes at positions 15 and 16 to fully rescue replication and/or stabilization of either genotype 1a or 2a HCV RNA mutants with cytosine-to-guanine mutations at nt 2 and 3 (Fig. 5A and D). Again, these results suggest that the interaction of miR-122 with HCV RNA upstream of the S1 seed match site may be more complex than simple supplementary base pairing between nt 15 and 16 of miR-122 and nt 2 and 3 of HCV RNA.

Nucleotide 4 of genotype 1 HCV RNA base pairs with nt 14 of miR-122. The results described above demonstrate a surprising difference in the impact of the miR-122p6,13U,14A mutant on replication of HJ3-5 RNA when the miRNA is bound at the S1 seed match site versus its effect on protein expression and stability of the replication-incompetent H77S RNA. While it was significantly more potent than miR-122p6 in promoting the replication of HJ3-5/GLuc2A-S1p6m RNA (Fig. 3), it had little ability to stimulate translation or to stabilize H77S/GLuc2A-S1p6m RNA (Fig. 6A and B). A comparison of the nucleotide sequences of the H77S (genotype 1a) and HJ3-5 (genotype 2a) 5'UTRs (Fig. 1A) suggested a possible explanation for these contrasting effects. Among other differences, base position 4 is adenine in H77S (and most other genotype 1 viruses) but uracil in HJ3-5 (Fig. 1A). (Note, however, that this base is cytosine in most genotype 2 viruses [Fig. 1B].) The presence of A4 in H77S would allow an extension of base pairing at C2 and C3 to include A4, as it could base pair with U14 of miR-122 (Fig. 8A, top panel). Since this pairing is not possible with the miR-122p6,13U,14A mutant, the loss of this pairing could explain the reduced ability of the latter to promote translation or stabilization of the H77S RNA, as shown in Fig. 6A and B. On the other hand, while the genotype 2a HJ3-5 sequence does not allow base pairing with wild-type miR-122 at this position, it does allow it with the miR-122p6,13U,14A mutant (Fig. 8A, bottom panel). Thus, functionally important base pairing between nt 4 of the HCV genome and nt 14 of miR-122 could explain the increase in replication of the HJ3-5/S1p6m RNA cotransfected with miR-122p6,13U,14A, as shown in Fig. 3.

To determine whether loss of base pairing at nt 4 of the H77S RNA is responsible for the inability of miR-122p6,13U,14A to stabilize this RNA, we replaced the adenine at position 4 of the

H77S RNA with a uracil. This mutation restores the potential for base pairing with nt 14 in miR-122p6,13U,14A. As anticipated, it conferred upon miR-122p6,13U,14A the ability to enhance GLuc expression and to stabilize the H77S RNA to a greater extent than that with miR-122p6 (Fig. 8B). These results are supportive of base pairing between nt 14 of miR-122 and nt 4 of the genotype 1 HCV RNA.

To further assess how miR-122 base pairs with the first 4 nucleotides of the genotype 2 5'UTR, we examined the impact of G2,G3 and G2,G3,A4 substitutions in HJ3-5/GLuc2A RNA cotransfected with either wt miR-122 or miR-122m15C,16C. There were no mutations in either seed match sequence in these experiments. The G2 and G3 substitutions resulted in a sharp reduction in replication of the RNA, as determined by GLuc expression 72 h after transfection, and in the absence of any stimulation when the RNA was cotransfected with wt miR-122 (Fig. 8C). However, GLuc expression was approximately doubled when the G2,G3 RNA was cotransfected with miR-122m15C,16C yet still remained much less than that observed with wt HJ3-5 (Fig. 8C). This provides additional support for base pair interactions between nt 2 and 3 of HCV and nt 15 and 16 of the miRNA but again shows that complementary mutations in miR-122 only partially rescue guanine substitutions at nt 2 and 3 of the genome (Fig. 5, top panel). The additional introduction of an adenine substitution at nt 4 resulted in a marked boost in replication when the RNA was cotransfected with miR-122m15C,16C but had no apparent impact when the RNA was cotransfected with wt miR-122 (Fig. 8C). There was also no increase in replication after cotransfection of miR-122m15C,16C when only the A4 substitution was introduced into HJ3-5 RNA, although this led to a 4-fold increase in GLuc expression over that in wt HJ3-5 in the absence of miR-122 supplementation (Fig. 8C). This likely reflects formation of the additional base pair at nt 4 with nt 14 of endogenous miR-122 (Fig. 8D). Consistent with this, replication was further increased when the A4 RNA was cotransfected with wt miR-122. Collectively, these data provide strong support for functionally important base pair interactions between nt 4 of HCV and nt 14 of miR-122. However, this interaction is possible only when the base composition of HCV includes an adenine at nt 4 and is permissive for it (i.e., in genotype 1 and genotype 6 viruses, as shown in Fig. 1B).

DISCUSSION

The data we present here both confirm and extend previous studies suggesting that bases 3' of the seed sequence in miR-122 form accessory Watson-Crick interactions with HCV RNA at base positions located upstream of both the S1 and S2 seed match sequences. Previous studies by Machlin et al. (20) demonstrated that the introduction of complementary mutations at HCV nt 2 and 3 and nt 30 and 31 rescued the ability of miR-122 with base substitutions at nt 15 and 16 to promote amplification of a subgenomic genotype 1a HCV RNA in transfected cells. We confirmed this in studies of genome-length RNA possessing a genotype 2a 5'UTR (Fig. 5). However, we provide new genetic complementation data suggesting that functionally important base pairing extends to nt 14 of miR-122 and HCV nt 4 (Fig. 8), upstream of the S1 seed match site, and to nt 13 and 14 of miR-122 and HCV nt 32 and 33, upstream of S2 (Fig. 5). We also extend the understanding of the functional significance of these 3' supplementary base pair interactions of miR-122 by showing that they are important for the

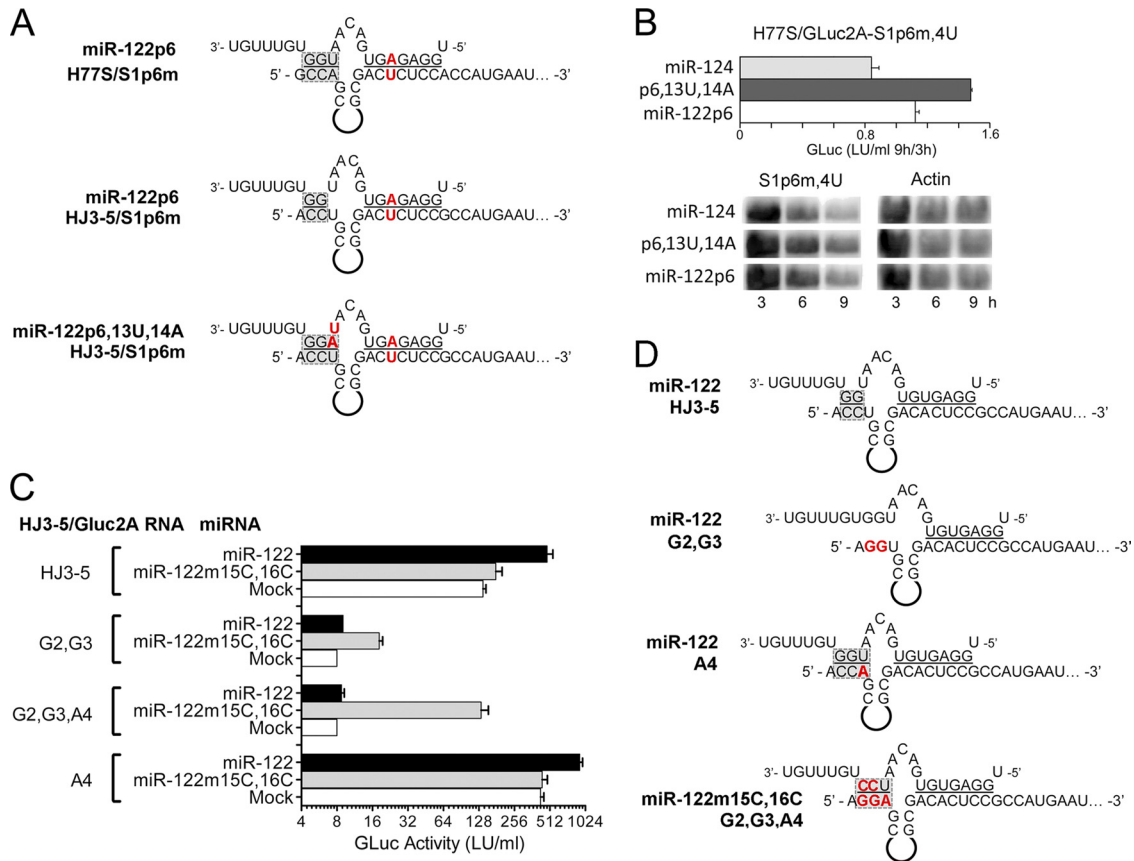


FIG 8 Influence of HCV nt 4 on the interaction with miR-122. (A) The adenine present at nt 4 of H77S (genotype 1a) (top) RNA provides an opportunity for an additional base pair to form with U14 of miR-122. This is not possible with HJ3-5 (genotype 2a) (middle) RNA, in which nt 4 is uracil. However, U4 of HJ3-5 can pair with A14 of miR-122p6,13U,14A (middle), potentially explaining why this mutant has more activity than wt miR-122 at the S1 site in HJ3-5 (see Fig. 3). (B) Like the case with HJ3-5, miR-122p6,13U,14A has greater activity than wt miR-122 in stabilizing the H77S/GLuc2A-S1p6m,4U mutant. Data shown represent the ratios of GLuc activities at 9 h versus 3 h (top) and Northern blots of the RNA at 3, 6, and 9 h posttransfection. (C) GLuc expressed by FT3-7 cells cotransfected with HJ3-5/GLuc2A or related mutant RNAs with wt miR-122 versus miR-122m15C,16C (no p6m mutation in either). “Mock” indicates no oligoribonucleotide. See the legend to Fig. 6 for additional details. (D) Putative base pair interactions involving nt 2 to 4 of HCV and wt miR-122 or miR-122m15C,16C.

production of infectious virus progeny from RNA-transfected cells (Fig. 4) and that they contribute significantly to the ability of miR-122 to both recruit Ago2 to the viral genome (Fig. 7D) and physically stabilize the viral RNA and lower its rate of decay in cells (26) (Fig. 6 and 7A to C).

For at least a subset of miRNAs, the specificity with which cellular mRNAs are targeted depends partly upon sequences 3' of the seed sequence (nt 2 to 8). The base pairing that occurs between these miRNAs and their target mRNAs outside the seed sequence typically spares nt 9 to 12 of the miRNA and involves primarily nt 13 to 16 (1, 12). This is likely driven by the architecture of the miRISC and, more specifically, by the need to maintain proper orientation of the miRNA guide strand loaded into an argonaute protein (1). The seed sequence (~nt 2 to 8) is positioned to the exterior of this complex, where it is preoriented in a manner facilitating its base pairing with the mRNA target. The extension of contiguous base pairs beyond nt 8 of the miRNA would result in a helical structure that is likely to disrupt its association with Ago (1). Thus, the lack of involvement of nt 9 to 12 of miR-122 in base pair interactions with the HCV genome, as indicated by our studies, is likely to facilitate continued association of miR-122 with

Ago2 yet permit additional base pair interactions, involving nt 13 to 16, that enhance the affinity and specificity of the target interaction. In contrast to our findings, recent studies using selective 2'-hydroxyl acylation analyzed by primer extension (SHAPE) to analyze the impact of miR-122 on the structure of the 5'UTR concluded that base pairing outside the seed sequence at S2 involves nt 9 to 12 of miR-122 (22). This likely reflects the fact that these studies were done *in vitro*, in the absence of Ago2. Supplementary base pairing of miRNAs with their targets 3' of the seed sequence may not be common (13). However, the pattern of accessory base pair interactions revealed here for miR-122 and HCV RNA at S2 is consistent with what is known of the interaction of miRNAs with their mRNA targets in the context of a loaded miRISC, and thus consistent with miR-122 recruiting Ago2 to the HCV 5'UTR (26).

In addition to genome-length HCV RNAs such as those used in this study, short reporter RNAs containing the authentic 5'- and 3'UTR sequences of HCV have been used to demonstrate a role for miR-122 in promoting translation directed by the HCV IRES, as well as potential 3' supplementary base pair interactions of miR-122 (14, 24). However, it remains uncertain how well these

reporter RNAs model the interactions of miR-122 with replication-competent viral RNAs. While the potential for base pairing between nt 2 to 4 of a reporter RNA and 3' miR-122 sequences was demonstrated in a recent study by the loss of miR-122 regulation of reporter activity when substitutions were made at nt 3 and 4 of the reporter, no effect on miR-122 regulation was apparent with substitutions at nt 30 to 34, upstream of the S2 seed match site (24). These results contrast sharply with those shown in Fig. 5A, where the regulation of viral RNA replication by miR-122 was significantly ablated by G30,G31 and U32,A33 substitutions in HJ3-5/GLuc2A RNA yet rescued by complementary mutations in miR-122. These differences demonstrate that studies with short reporter RNAs need to be interpreted with caution and, where possible, confirmed with replication-competent RNAs.

Although our finding that miR-122 nt 15 and 16 are important for replication of genotype 1 HCV RNA (Fig. 3) is in agreement with the work of Machlin et al. (20), we observed substantial differences in the impact of other nucleotide substitutions, particularly at the most 5' S1 binding site. We found that miR-122p6,13U,14A was >3-fold more potent than miR-122p6 in promoting the replication of HJ3-5/GLuc2A-S1p6m RNA (Fig. 3 and 4), while Machlin et al. (20) reported that a very similar miR-122 mutant had a reduced ability to support HCV RNA replication. This difference is related to variation in the 5'UTR sequences studied. In contrast to its increased ability to promote replication of HJ3-5 RNA, which contains a genotype 2a 5'UTR, the 13U,14A miR-122 mutant was unable to stimulate translation of or stabilize H77S/GLuc2A-S1p6m RNA (Fig. 6A and B), which, like the subgenomic HCV RNA studied by Machlin et al. (20), contains a genotype 1a 5'UTR. This was reversed by replacing the adenine normally present at position 4 of the genotype 1a 5'UTR with the uracil present in genotype 2a viruses (Fig. 8B). These results thus demonstrate that there are genotype-specific differences in base pairing of HCV RNA with miR-122. They also show that there is a potential for productive base pair interactions at nt 4 of genotype 1 and 6 HCVs that does not exist in other HCV genotypes (Fig. 1B).

There is also a potential for base pair formation between nt 1 of HCV (either guanine or adenine) and nt 17 of miR-122 (uracil) (Fig. 1B). Although we did not investigate this in further detail, the decreased ability of miR-122p6,17G,18C to promote HJ3-5/S1p6m replication (Fig. 3) suggests that it may be important. It is striking, however, that the potential loss of base pairing at HCV nt 1 has less impact than the loss of pairing at nt 4 (compare Fig. 3 and 8C). A model of the seed sequence and 3' supplementary base pair interactions of miR-122 with the genotype 1a H77S virus, including the recruitment of two miR-122 molecules to the 5' end of the RNA, is shown in Fig. 9. Although the base pair interactions upstream of the S1 seed match site shown in this model are supported by data presented in this communication, it is important that we were able to achieve only partial rescue of the negative effects of guanine substitutions at nt 2 and 3 of the HCV genome by cotransfection of miR-122 mutants with potentially complementary substitutions at nt 15 and 16 (Fig. 5A and D and 8C). We have no explanation for why this was not observed by Machlin et al. (20), as we encountered this issue with HCV RNAs containing either the genotype 1a or genotype 2a 5'UTR (Fig. 5A and D). *in silico* predictions suggest that the lack of complete rescue was not due to aberrant folding of the 5'-terminal HCV sequence (Fig. 5C), although this cannot be ruled out entirely.

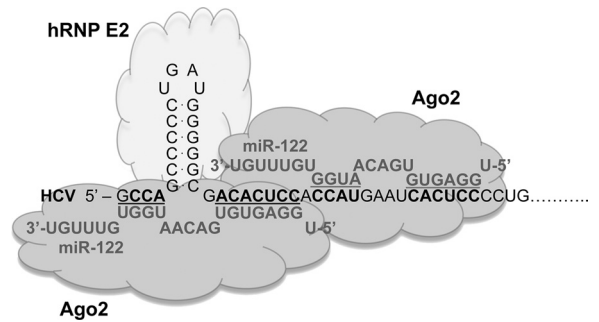


FIG 9 Base pair interactions between miR-122 and the 5'UTR terminal sequences of genotype 1a H77S RNA demonstrated by mutational studies. The model shown includes two distinct Ago2 molecules recruited by miR-122 molecules bound to the S1 and S2 seed match sequences. PCBP2 (hRNP E2) is shown bound to stem-loop 1 as described by Fukushi et al. (10). Interactions of miR-122 with the 5' 30 nt of the HCV sequence are likely to be more complex than that shown here, since complementary substitutions at nt 2 and 3 of the HCV genome fail to rescue Ago2 recruitment by miR-122p6,15C,16C, targeting the S1 seed match site, and only partially rescue the ability of miR-122p6,15C,16C to promote HCV RNA accumulation in transfected cells.

One possibility, mentioned above, is that the native cytosines are required for recognition by the replicase complex. However, the residual defect in replication of the HJ3-5/GLuc2A-G2,G3 mutant cotransfected with miR-122 containing complementary 15C and 16C substitutions was overcome to a significant extent by an adenine substitution at nt 4 of HCV that is predicted to extend the interaction of miR-122 with nt 2 and 3 of the HCV RNA by an additional base pair (Fig. 8C and D). This suggests that the lack of complete rescue of the 2G,3G mutants may be due to a failure to recreate the native interactions of miR-122 with 5' HCV RNA upstream of the S1 seed match site rather than a defect in replicase recognition of HCV RNA. This notion is reinforced by the failure of the complementary mutants to stabilize the viral RNA, as shown in Fig. 7A, or to efficiently recruit Ago2, as indicated in the Ago2-RNA co-IP experiment shown in Fig. 7D (left panel). Thus, the interactions of miR-122 with HCV RNA upstream of the S1 seed match site are likely to be more complex than those shown in Fig. 9. It is notable that SHAPE was not able to map the effect of miR-122 on RNA structure upstream of S1 (22), and it is possible that there are several alternative structures, only one of which is shown in Fig. 9. Several conserved cytosines in nt 6 to 10 are predicted to contribute to a stable stem-loop structure in the 5'UTR (SL-1 in Fig. 1B) but, alternatively, could pair with G15 and G16 of miR-122. However, this stem-loop has been suggested to bind poly(rC) binding protein 2 (PCBP2; also known as hRNP E2) (Fig. 9) and to be required for HCV replication (10, 29). Thus, although the data we present here provide a more detailed view of the interaction of miR-122 with HCV RNA than that available previously, it is clear that additional studies will be needed to resolve the structure(s) of the complex formed by miR-122 at the extreme 5' end of the viral RNA.

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