Cooperative enhancement of translation by two adjacent microRNA-122/Argonaute 2 complexes binding to the 5' untranslated region of hepatitis C virus RNA

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Abstract

The liver-specific microRNA-122 (miR-122) binds to two conserved binding sites in the 5' UTR of hepatitis C virus (HCV) RNA. This binding was reported to enhance HCV RNA replication, translation and stability. We have analysed binding of miR-122/Argonaute 2 (Ago2) complexes to these sites using anti-Ago2 co-immunoprecipitation of radioactively labelled HCV RNAs along with ectopic miR-122 in HeLa cells. Our results show that the miR-122 target sites can be addressed separately. When both target sites were addressed simultaneously, we observed a synergistic binding of both miR/Ago2 complexes. Consistently, simultaneous binding of both miR-122/Ago2 complexes results in cooperative translation stimulation. In the binding assays as well as in the translation assays, binding site 1 has a stronger effect than binding site 2. We also analysed the overall RNA stability as well as the 5' end integrity of these HCV RNAs in the presence of miR-122. Surprisingly, using short HCV reporter RNAs, we did not find effects of miR-122 binding on overall RNA stability or 5' end integrity over up to 36 h. In contrast, using full-length HCV genomes that are incapable of replication, we found a positive influence of miR-122 on RNA stability, indicating that features of the full-length HCV genome that do not reside in the 5' and 3' UTRs may render HCV RNA genome stability miR-122 dependent.

INTRODUCTION

Hepatitis C virus (HCV) is a member of the genus Hepacivirus in the family Flaviviridae and has a single-strand RNA genome of positive orientation that replicates in the cytoplasm of hepatocytes [1, 2]. The single polyprotein ORF of the viral genome encodes the structural and non-structural (NS) proteins [1], and is flanked by the 5' UTR and the 3' UTR (see Fig. 1). The ORF is translated under the control of an internal ribosome entry site (IRES) element that is located mainly in the highly structured 5' UTR but spans about 20 nucleotides into the core protein coding region [3]. The IRES recruits the translation initiation machinery to an internal position on the viral RNA, thereby avoiding the need for a 5' terminal cap nucleotide. In turn, this allows the replication signals to reside mainly in the 5' and 3' UTRs at the genome ends, while some additional cis-elements involved in replication control are located in the 3' terminal non-structural protein 5B (NS5B) coding region [1]. The 3' UTR of the HCV RNA genome is not only involved in replication but also stimulates HCV translation [4, 5].

While binding of the virus to the low density lipoprotein receptor and other cell surface proteins facilitates entry of the virus into hepatocytes [6], one important intracellular determinant of hepatotropism is the liver-specific microRNA-122 (miR-122). This microRNA (miRNA) constitutes about 60–70 % of all miRNA in hepatocytes [7] and is nearly not present in other tissues, rendering it largely liver specific. The HCV genome contains five or six (depending on genotype) target sites for miR-122 (see Fig. 1a): two highly conserved sites (S1 and S2) are located in the 5' UTR, one in the otherwise variable region of the 3' UTR and two highly conserved sites in the NS5B coding region, while another site in the NS5B region is conserved only in some isolates [8].

miR-122 has been proposed to support several functions in the HCV replication cycle. While the conserved miR-122 target sites in the NS5B region and the 3' UTR appear to have functions in replication [9, 10], the target sites in the 5' UTR have been associated with multiple functions in the viral life cycle. Binding of miR-122 to the 5' UTR target sites was reported to be involved in the stimulation of genome

The two miR-122 target sites S1 and S2 are located in the very 5′-region of the 5′ UTR [28]. The seed target sequences (i.e. the nucleotides opposite to miRNA nucleotides 2–5) of sites S1 and S2 are located in a region that is conserved to be single-stranded [8] between the stem and loops (SLs) I and II in the 5′ UTR close to the 5′ end of the genome (Fig. 1b) [11, 13, 17]. In addition, the miR-122 molecule binding to the most 5′ site S1 binds with its so-called supplementary region near the miRNA’s 3′ end [29] to three or four nucleotides (depending on the HCV isolate) at the very 5′ end of the HCV RNA, thereby bridging the SL I of the HCV RNA. The second miR-122 molecule that addresses site S2 binds with its supplementary region to target nucleotides between both seed target sites (Fig. 1b). This arrangement of two miR-122 molecules that bind close to each other and cover the HCV genome’s very 5′ end was proposed to be directly involved in protecting the HCV genome 5′ end from exonucleolytic degradation [30]. For the effect of miR-122 on the stimulation of translation, the HCV IRES but not heterologous IRES elements must be downstream of the miR-122 binding sites for full regulation of translation by miR-122 [21], suggesting a specific interaction of the miR-122–protein complexes with the translation machinery that depends on IRES sequence and/or structural features.

The binding of miR-122 to these sites is conferred by protein complexes that contain an Argonaute (Ago) protein [14, 21, 23, 24]. In cellular control of gene expression, such miRNA/Ago complexes are known to mediate translation repression and/or RNA degradation when binding to the 3′ UTRs or coding regions of normal mRNAs [31]. Moreover, adjacent miRNA target sites in a 3′ UTR have been shown to act synergistically on translation regulation [32, 33]. In the HCV 5′ UTR, the two adjacent sites are conserved to have virtually the same distance in all isolates [8]. This raises the question if both miR-122/Ago complexes in the HCV 5′ UTR exert their different functions on RNA replication and genome accumulation, translation stimulation and RNA stability either separately or in combination. For example, it could be speculated that site S1, which is close to the HCV 5′ end, is involved in RNA stability, while
RESULTS

Separate binding of miR-122/Ago2 complexes to the HCV 5' UTR binding sites

The binding of miR-122/Ago2 complexes to the HCV 5' UTR was tested with an RNA that contains the HCV 5' UTR plus the first 23 nucleotides of the HCV core coding region (see Fig. 1b). This allows correct folding of the IRES stem–loop IV [3] and binding of the small ribosomal 40S subunit and of eIF3 [34], mimicking a situation as it exists when the HCV 5' UTR RNA is initially exposed to cytoplasmic cellular components. The miR-122 binding sites in this HCV RNA were present either in wild-type (wt) sequence or mutated: in the variant 'S1mS2m', both miR-122 target sequences had been mutated differentially to allow independent binding of compensatory mutated miR-122m1 to site S1m, binding of miR-122m2 to site S2m, or both in combination (Fig. 2a). This HCV RNA was 32P-labelled during in vitro transcription and transfected into HeLa cells along with ectopic duplex miR-122 or its variants. HeLa cells do not contain endogenous miR-122 but allow HCV replication when supplemented with ectopic miR-122 [35, 36]. Six hours after transfection, cells were lysed, and Ago2-miRNA/target RNA complexes were recovered from the cell lysate by co-immunoprecipitation (co-IP) using an anti-Ago2 antibody [23]. From these complexes, RNA was extracted and resolved on gels, and the 32P-labelled HCV RNA was visualized by autoradiography.

In this assay, the wt miR-122 binds strongly to the wt HCV RNA (Fig. 2a, lane 4), whereas the mutated miR-122m1 and -m2 variants bind only very weakly (lanes 6 and 7). In turn, when the mutated HCVS1mS2m RNA was used, binding of the wt miR-122 was abolished (Fig. 2b, lane 4), whereas the mutated miR-122m1 and miR-122m2 bind strongly to the HCVS1mS2m RNA (Fig. 2b, lanes 6 and 7). In order to check if each of the miR-122 variants does indeed bind to its corresponding binding site, we specifically blocked either site S1m or S2m using locked nucleic acid (LNA) blocker oligonucleotides that specifically bind to either site S1m or S2m and by that interfere with miR-122m1 or -m2 binding to these sites. When site S1m was addressed using miR-122m1, the blocker LNAm1 could not interfere with miR-122m1 binding, whereas LNAm1 completely abolished miR-122m1 binding (Fig. 2c, lanes 3 and 4). In turn, binding of miR-122m2 was not affected by LNAm1 but completely abolished by LNAm2 (Fig. 2d, lanes 5 and 6). Thus, the miR-122 variants S1m and S2m specifically address their corresponding target sites in the HCV 5' UTR as shown in Fig. 1(b).

The two miR-122/Ago2 complexes bind cooperatively to the HCV 5' UTR

In order to find out if both miR-122/Ago2 complexes bind cooperatively to the HCV 5' UTR, we addressed both binding sites separately or in combination and compared binding efficiencies (Fig. 3). Binding of miR-122m1 to site S1m was slightly stronger than binding of miR-122m2 to site S2m (Fig. 3a, compare lanes 2 and 4). When both sites were addressed in combination (lane 3), binding was much stronger than binding to each single target site only. Binding was quantified from three independent experiments (Fig. 3b), showing that site 1 binds the miRNA substantially stronger than site 2. Most importantly, binding of both sites in combination ('m1 m2') was more than 1.7-fold stronger than the sum of binding strength when sites were addressed separately (backgrounds subtracted). This shows that binding of both miRNAs to the two closely spaced sites 1 and 2 in the HCV 5' UTR occurs in a cooperative way.

Cooperative stimulation of translation

In order to check if this cooperative binding of miR-122/Ago2 complexes also has implications for a possible cooperativity in translation stimulation, we tested this hypothesis using translation reporter RNAs (Fig. 4a). These constructs contained the HCV 5' UTR as shown in Fig. 1(b), but fused to a Renilla luciferase reporter gene and the HCV 3' UTR, which is required for efficient translation [5]. The miR-122 target site in the variable region of the HCV 3' UTR was present but not addressed by wild-type miR-122 in our experiments using HeLa cells.

When the HCV 5' UTR contained wt miR-122 binding sites (Fig. 4b), addition of miR-122 resulted in stimulation of translation by the HCV IRES as previously shown [17]. When an anti-miR-122 LNA oligonucleotide was used to sequester the ectopically added wt miR-122, translation stimulation was abolished (Fig. 4b). In contrast, when wt miR-122 was used along with the mutated HCVS1mS2m 5' UTR (Fig. 4c), wt miR-122 could not stimulate translation (reaction 2). However, miR-122m1 stimulated translation directed by the HCV IRES (reaction 3), while we could not detect significant stimulation of translation when miR-122m2 was used alone (reaction 4), most likely due to the weaker binding to site S2m observed in the co-IP assays shown above (compare Fig. 3b).

When both miR-122m1 and -m2 were used in combination, we observed a much stronger stimulation of translation (Fig. 4c, reaction 5). This translation stimulation was more than fourfold stronger than the mere sum of translation efficiencies that were obtained by using miR-122m1 or -m2
separately (after the background was subtracted). Binding of miR-122m1 and -m2 could be significantly inhibited using a blocker oligonucleotide that competes with binding to both target sites (reaction 6). These results indicate that miR-122/Ago2 complexes bind to the HCV 5’ UTR and stimulate translation cooperatively.

**RNA stability is not affected by miR-122/Ago2 binding to the HCV 5’ UTR in short reporter constructs**

In previous studies [24–26], an effect of miR-122 binding to the HCV 5’ UTR on RNA genome stability was shown. However, in these studies, the authors used full-length genomes which contain all or nearly all HCV sequences. To exclude possible effects of other cis-elements located elsewhere in the HCV genome or functions encoded in the genome on HCV RNA stability, we investigated the effect of miR-122 on RNA stability using a reduced HCV reporter system (Fig. 5a).

This HCVS1mS2m reporter RNA was transfected into HeLa cells along with miR-122 variants by electroporation. By electroporation, all RNA that enters the cell is provided to the cytosol of the cell immediately. Thereby, it is avoided that a fraction of the reporter RNA may be contained in liposomes or endosomes for longer time like in liposome-mediated transfection, which could lead to false-positive results in RNA stability assays since the RNA to be re-extracted and analysed may then have been protected from biological degradation. Then, 6, 12 or 36 h after electroporation, total RNA was extracted from the cells, and the HCV reporter RNA was detected using an RPA (Fig. 5d, e). The 32P-labelled RNA probe spans the very 5’ end of the HCV 5’ UTR, thereby allowing not only to quantify the amounts of the transfected RNA but also to detect its 5’ end integrity (see below). The amount of probe was titrated to be not limiting for the detection of recovered HCV RNA in the RPA (Fig. 5b, c). When 0.1 ng of radioactive probe was used, reduction of the amount of sample RNA resulted in a sharp

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**Fig. 2.** Both miR-122 target sites in the HCV 5’ UTR can be addressed separately. (a) *In vitro* synthesized 32P-labelled wild-type (wt) HCV 5’ UTR-IREs RNA (see Fig. 1b) was transfected into HeLa cells along with miRNA duplexes as indicated, using either wt miR-122 (122) or mutated variants m1 or m2 (also see Fig. 1b). miR-124 duplexes were used as negative control. Six hours after transfection, cells were lysed. Total RNA was isolated from 10% of the lysate to serve as control for RNA integrity after lysis (lysate RNA, middle panel). The rest of the lysate was used for co-IP with anti-Ago2 antibody (co-IP RNA, upper panel). Immunoprecipitated material (10%) was used for an anti-Ago2 Western blot (WB, lower panel) to check for successful Ago2 immunoprecipitation. An anti-eIF3 antibody was used as positive control and an anti-Flag antibody as negative control. RNAs were isolated from the beads after co-IP, resolved on denaturing 6% polyacrylamide (PAA) gels and visualized by autoradiography. The input RNA (lane 1) shows a small aliquot of the *in vitro* transcript used for transfections as a size marker for comparison with the RNAs recovered from cells. (b) co-IP experiment as in (a), but using mutated HCV S1mS2m RNA (see Fig. 1). (c) co-IP experiment as in (b) using mutated HCV S1mS2m RNA, largely addressing mutated miR-122 target site S1m. In addition, blocker locked nucleic acid (LNA) oligonucleotides were used to block either site S1m or S2m as indicated. (d) co-IP experiment as in (c) using mutated HCV S1mS2m RNA, largely addressing site S2m.
drop of signal intensity (Fig. 5b, lanes 4 and 5; see also longer exposure in lanes 4' and 5'), demonstrating that the probe was not limiting. The same effect was also observed when the 10-fold amount of radioactive probe was used (Fig. 5c), and signal strengths were similar with both amounts of probe, confirming that the amount of probe was not limiting in the RPAs.

When we analysed the stability of the HCV reporter RNA transfected along with either no miRNA, miR-122m1, miR-122m2 or both miR-122m1 and -m2 in combination, we observed that the HCV reporter RNA was quite stable for 6 and 12 h, while RNA amounts were significantly lower after 36 h (Fig. 5d, and also see quantitative evaluation shown in Fig. 6). From the quantitative data (Fig. 6), we estimate that the half-life of the HCV reporter RNA is in the range of about 25 to 30 h.

However, we could not observe substantial differences in the amount of the recovered HCV reporter RNA, irrespective of if miR-122m1 or -m2 were provided alone or in combination, or if no miRNA was present at all. This was the case 6 h after electroporation (Fig. 5d, lanes 5–8) as well as 12 h (lanes 13–16). While the experiment shown in Fig. 5(d) shows a slight preference for stronger degradation in the absence of miRNAs binding to the 5' UTR after 36 h (lanes 21–24), this was not the case in other experiments (compare Fig. 5e, right panel). The quantitative evaluation of all experiments (Fig. 6) shows that this effect is not significant even 36 h after electroporation. Essentially the same results were also obtained when the experiment was performed in HuH-7.5 hepatoma cells (Fig. 5f), indicating that no cellular factors other than miR-122 influence the result.

Since this result was surprising to us in the light of previously published studies [24–26], we also resolved the radioactive probes protected by the recovered HCV reporter RNAs on high-resolution 12% polyacrylamide gels (Fig. 5e). In these gels, the HCV reporter RNAs recovered (lanes 5–8, 13–16 and 21–24) yielded exactly the same band (together with some additional minor bands) as a control experiment in which the in vitro-transcribed HCV reporter RNA was directly hybridized with the radiolabelled probe without electroporation into cells (compare lanes 3, 11 and 19). Thus, HCV 5' end integrity was not affected by binding of miR-122/Ago2 complexes to the two miR-122 binding sites at the HCV RNA 5' end.

**Comparison of HCV reporter constructs and HCV full-length genomes for RNA stability**

Previous studies in which an effect of miR-122 on HCV RNA stability had been shown [24–26] usually used full-length HCV genomes, which differ from our HCV reporter constructs in that they contain not only the HCV 5' UTR and 3' UTR but also all the HCV structural and non-structural protein coding regions. Therefore, we electroporated our HCV 5' UTR–Rluc–3' UTR reporter RNA (Fig. 7a) side by side with full-length HCV genome RNA (Fig. 7b) in which the NS5B replicase was either wild-type (wt) or contained a GND active site mutant to monitor only RNA degradation in the absence of genome replication. For these experiments, we used HuH-7.5 hepatoma cells without addition of exogenous miR-122 duplexes to provide miR-122 under conditions as natural as possible. Consequently, miR-122 target site mutants in the RNAs were used to monitor possible differences in RNA stability. RNA abundance in the cells was then monitored as before after 6, 12 and
36 h using RT-qPCR (in the Rluc region for the reporter RNA and in the NS3 region for the full-length RNA). The miR-122 binding sites in the HCV 5′ UTR in these constructs were either wt or contained a mutation of the miR-122 binding site 1 (S1m) or a mutation of binding site 2 (S2m) in the HCV 5′ UTR, relating these experiments to the single site mutants used before like in Fig. 5(d-f). The results are shown in Fig. 7(c, d).

With the short HCV Rluc reporter RNA, we observed a quite small decline of RNA abundancy from 6 to 12 h and a further slight decline at 36 h (Fig. 7c). This essentially corresponds to the decline in RNA abundancy observed in the RPAs above (Figs 5 and 6) even though we consider that the qPCR may be more sensitive to changes in template abundancy. Both detection methods may have different dynamics in the hybridization step (long RPA probes and long hybridization time in the RPA versus short primers and short hybridization times in the RT-qPCR), and the dynamic range of the phosphorimager screens may limit the detection range in the RPAs. Interestingly, also in this RT-qPCR assay, we observed no substantial difference in RNA abundancy irrespective of if the wt or mutated miR-122 sites were present in the HCV 5′ UTR, confirming the results obtained in the RPA experiments (Figs 5 and 6).

Then, we electroporated full-length HCV genome RNA in which the NS5B replicase active site was mutated to GND. This HCV genome RNA is unable to replicate and does not produce progeny genome RNAs, allowing to focus on stability of the transfected RNAs only. In contrast to the results with the short HCV Rluc reporter RNA, we found a more pronounced decline in RNA abundancy after 36 h, and the HCV full-length RNA with the wild-type miR-122 binding sites in the 5′ UTR was degraded slightly faster already within 6 h (Fig. 7d, left panel). These results indicate that the presence of the full-length HCV genome has a substantial influence on HCV RNA genome stability. In contrast, with HCV genomes that have a wild-type NS5B replicase, abundancy of the genome with wild-type miR-122 binding sites caught up to higher amounts due to active genome replication, whereas full-length genomes with miR-122 target site mutants were more substantially degraded (Fig. 7d, right panel), confirming the results of previous studies using full-length HCV genomes [24–26].

**DISCUSSION**

miRNA/Ago2 complexes were shown to act cooperatively when they are recruited to adjacent sites on target miRNAs by bulged miRNAs [37]. Accordingly, genome-wide analysis of miRNA target sites revealed that cooperativity of miRNA–target interactions is a widespread phenomenon that may play an important role in miRNA-mediated gene regulation [32, 33]. Molecular modelling of two Ago2 molecules that are recruited to a target miRNA by bulged miRNAs in close proximity suggests that amino acid contacts between the two Ago2 proteins confer cooperativity [33]. The distance between two cooperatively acting miRNA target sites in miRNAs was found to be in the range of 0 to about 35 nucleotides, with a preference for both sites being directly adjacent [32, 33]. In the HCV 5′ UTR, both miR-122 target sites perfectly meet this requirement by being directly adjacent: the last nucleotide of the first miR-122 binding site S1 (ACACUCC) is always very close to the first nucleotide of the supplemental binding site of binding site S2 (see Fig. 1b). Moreover, in both miR-122 binding sites S1 and S2 in the HCV 5′ UTR, there are always nucleotides that do not match to miR-122 to avoid cleavage of
Fig. 5. Effects of miR-122 on the stability of transfected HCV reporter RNAs with the HCV 5' UTR. (a) The HCV reporter RNA as in Fig. 4(a) but with mutated miR-122 binding sites S1m and S2m. The 32P-labelled antisense probe used for the RNase protection assay (RPA) is shown below. From 5' to 3', this probe contains 1 non-hybridizing nucleotide, 159 hybridizing nucleotides (grey box) reverse complementary to the 5' terminal 159 nucleotides of the HCV S1mS2m 5' UTR sequence, plus 114 non-hybridizing nucleotides. (b) Titration of the RPA. HeLa cells were transfected with the HCV reporter RNA shown in (a). Total RNA was re-extracted from the cells, and different amounts (as indicated) of the total RNA were hybridized with 0.1 ng of the 32P-labelled antisense probe shown in (a) (lanes 4–8). Lanes 4'–8' are a longer exposure of lanes 4–8. Purified in vitro transcripts were used as controls and length markers as indicated (lanes 1–3). (c) Titration as in (b), but with 1 ng of radiolabelled probe. (d–f) RNA stability assays with the HCV reporter RNA shown in (a) using RPAs. HeLa cells were electroporated with the HCV reporter RNA shown in (a), either without miR-122 variant or along with duplex miR-122m1, miR-122m2 or both as indicated. As negative control, no RNA was transfected. Six, 12 or 36 h after electroporation, total RNA was extracted from the cells. One microgram of total RNA was hybridized to 0.1 ng of the 32P-labelled antisense probe shown in (a) and subjected to RPA analysis. Samples (50 %) with the resulting protected fragments were resolved on denaturing 6 % polyacrylamide gels (d) and visualized by autoradiography. Gels shown in the different panels have been exposed for the same time to phosphorimager screens. Lane 1, 0.0033 ng of undigested probe. Lane 2, 0.0033 ng of probe digested with RNase. Lane 3, 0.05 ng probe hybridized to 2.5 ng HCV S1mS2m reporter RNA in vitro transcript and digested by RNase. Lane 4, RPA with total RNA extracted from mock-transfected cells. Lanes 5–8, RPA with total RNA extracted from cells transfected with HCV S1mS2m reporter RNA and duplex miRNAs as indicated. Total RNAs from cells lysed after 12 and 36 h were processed accordingly. (e) Analysis of 25 % of the samples on denaturing 12 % polyacrylamide gels, visualized by autoradiography. 'Fragm.' labels the 159 nucleotide probe fragment protected by HCV RNA in the RPA. Gels shown in the different panels have been exposed for the same time. (f) The same experiment as in (d) but in HuH-7.5 hepatoma cells. Gels shown in the different panels have been exposed for the same time. VR, variable region.
the HCV RNA genome by Ago2 protein. For binding site S1, this is the conserved stem-loop I. For site S2, in all HCV isolates, there are some nucleotides conserved not to match to miR-122 and by that to form the bulge between seed and supplemental site of S2, while the number and exact sequence of these nucleotides may slightly vary [8]. Taken together, the 5' end of the HCV genome is conserved to recruit two miR-
122/Ago complexes in a cooperative way but avoid cleavage by Ago2. This concept is confirmed by our finding that binding of miR-122/Ago2 complexes to the HCV 5' UTR and stimulation of translation occur cooperatively.

The aspect of cooperative binding of miR-122/Ago complexes comes into play when analysing the individual contribution of both binding sites in all aspects of miR-122 action on the HCV genome, i.e. HCV genome accumulation, RNA stability and translation stimulation. Genome accumulation is only rescued if both binding sites are

Fig. 6. Summary of the RNA stability assays with the HCV reporter RNA in HeLa cells like shown in Fig. 5(d) from four independent experiments (for quantification details, see Methods). The asterisk indicates significance (P<0.05) according to a one-tailed Student's t-test for corresponding reactions (e.g. m1 vs m1) at 12 versus 36 h.

HCV-Rluc

Fig. 7. RNA stability of HCV full-length genomes compared with short HCV translation reporter constructs. The HCV translation reporter RNA (a) is the same as used in Figs 4 and 5. The Jc1 full-length HCV genome (b) was used either as wt sequence or with the GND mutation in the NS5B polymerase active centre to inactivate HCV RNA replication. (c, d) RNA stability assays. (c) HuH-7.5 cells were electroporated with the HCV translation reporter RNA shown in (a). (d) HuH-7.5 cells were electroporated with the HCV Jc1 GND genome or the Jc1 wt genome shown in (b). Total RNA was extracted from the cells after 6, 12 or 36 h, and RNA abundancy was quantified by Reverse Transcription and quantitative PCR (RT-qPCR). Values were normalized to the highest value obtained with either the Rluc, NS5B GND or NS5B wt construct at 6 h after transfection, normalized to GAPDH mRNA abundance, and means and standard deviations were calculated from three (Rluc RNAs) or four (full-length RNAs) independent experiments. VR, variable region.
addressed [13, 27, 30]. Both binding sites are also involved in RNA stability [22, 25]. As well, both miR-122 binding sites are involved in translation stimulation [17, 21, 22]. Taken together, we conclude that both miR-122 target sites in the HCV 5’ end are bound cooperatively, while this binding event then results in various downstream effector functions. Protection of the HCV genome 5’ end against 5’ exconuclease degradation can be imagined to be conferred by miR-122/Ago complexes acting as a mechanical barrier, and enhanced HCV RNA genome accumulation can be an indirect consequence of enhanced RNA stability, whereas the mechanisms underlying translation stimulation are not yet clear.

We find that miR-122 binding site S1 in the HCV 5’ UTR binds miR-122/Ago2 complexes stronger and also acts more efficiently in translation stimulation than binding site S2. In contrast to our results, Mortimer and Doudna showed in in vitro assays that miR-122 binds stronger to binding site S2 than to site S1 [38]. However, these in vitro assays were performed in the absence of Ago protein that first presents the so-called nucleation sequence within the seed sequence (miRNA nucleotides 2–5) to the target [39, 40], and only after conformational changes in the Ago protein additional nucleotides of the small RNA are presented to match to the target [29, 40]. Thus, the binding preferences of miRNAs may largely differ between in vitro hybridization and Ago-mediated miRNA binding in vivo. Binding site S1 in the HCV 5’ UTR has 7 seed nucleotides, but in the supplemental site only 4 or in some isolates even only 3 nucleotides support binding (10 or 11 nucleotides in total). Binding site S2 contains a seed sequence of only 6 nucleotides (CACUCC), but in vitro up to 6 nucleotides of the supplemental site can additionally bind to the HCV RNA (12 nucleotides in total). These differences may explain why miR-122 binds better to site S2 than to site S1 in vitro [38].

In contrast, in the in vivo situation, Ago protein mediates miR-122 binding. As a consequence, the supplemental site is less relevant in vivo since only nucleotides 13–16 of miR-122 have been shown to bind to the supplemental site of site S2 [25, 30]. The fact that site S1 has seven seed nucleotides instead of six nucleotides in site S2 may then be key for the higher affinity of miR-122/Ago complexes for site S1 in vivo. In our mutants, the stability of the miR-122-target duplexes was largely unchanged for site 1 since the G+C content in the combined seed plus supplemental sequences changed from 6 out of 10 nucleotides to 5 out of 11 nucleotides, leaving the overall melting temperature of both sites combined at the same value. For site 2, the G+C content in the combined seed plus supplemental sequences changed from 6 out of 11 nucleotides to 5 out of 11 nucleotides, resulting in a slight decrease of the melting temperature of the combined seed plus supplemental sites by 2°C. However, this change could not be avoided since all seed and supplemental sites needed to be mutated differently while maintaining the predicted secondary structure of the HCV target sequence. In accordance with our results, also other studies [22, 27] found in vivo that binding of miR-122 to site S1 has a stronger effect on replication than binding to S2. These findings are also well consistent with the idea that miR-122/Ago complexes protect the HCV 5’ end against exconucleolytic degradation [30], a function in which site S1 is of particular importance since the loss of miR-122 binding to that supplemental site may lead to the loss of protection of the four very 5’ terminal nucleotides of the HCV genome [25]. We can only speculate if the binding of the small ribosomal 40S subunit to the SL II may cause weaker binding of miR-122 to site S2. In turn, weaker binding of miR-122 to site S2 could be required to allow a more dynamic ‘on/off’ behaviour when miR-122/Ago complexes binding to site S2 are interacting with the directly adjacent translation initiation machinery. This idea is consistent with the fact that the seed sequence of site S2 is conserved to be only six nucleotides long and is not followed by a stabilizing adenosine residue [40] in all HCV isolates [8].

Binding of miR-122 to the HCV 5’ UTR was reported to reduce the sensitivity of HCV RNA for degradation [24–27]. In particular, miR-122 binding reduces degradation of the HCV RNA by the 5’ exonucleases Xrn1 [26, 27] and Xrn2 [41]. In contrast, our results with the short HCV reporter RNAs show that miR-122 has virtually no influence on the stability of a reporter RNA that contains the HCV 5’ UTR but nearly no HCV coding sequences. In accordance with our results with the short reporter RNAs, in one other study, HCV reporter RNAs similar to those in our study were used, and the authors did not find differences in RNA stability due to miR-122 binding, whereas miR-122 stimulated translation from these RNAs [42]. In contrast, we found stronger degradation in full-length genomes (Fig. 7d). Although our mutations in the full-length genomes were limited only to single miR-122 target site mutants, the full-length genomes in which the miR-122 target sites are mutated are subject to more extensive degradation than the wild-type genome, confirming the results of previous studies with full-length genomes [24–26].

As a consequence, we must assume that additional sequences within the large coding region have an influence on HCV RNA stability, either on the level of RNA cis-signals or by expressed proteins. On the one hand, several cis-elements were recently described mainly in the NS5B coding region in addition to those in the 5’ and 3’ UTR [43, 44]. Such signals could be involved in long-range interactions with cis-elements in the 5’ and 3’ UTR as well as in the core and NS5B coding regions [45] and may somehow contribute to miR-122-mediated stabilization of HCV genomes. On the other hand, it is important to note that P-body components like PatL1, LSM1, DDX3 and DDX6 are involved in HCV translation and replication [46–48]. Together with other P-body components, Xrn1 is also recruited to lipid droplets upon HCV infection and co-localizes with the HCV core protein [49]. Thus, it may be possible that Xrn1 is just accidentally co-recruited to the site of HCV replication along with other P-body components. Hence, the

protection of the HCV 5’ end by miR-122 may be a countermeasure of the virus to cope with a problem that is accidentally caused by other changes in cellular metabolism, which are induced by the virus to promote its replication. Thus, degradation of the HCV genome 5’ end by 5’ exonucleases may not be a principal feature of the HCV RNA genome 5’ end but may be context dependent, i.e. degradation can occur preferentially when 5’ exonucleases are artificially co-recruited to the sites of replication. This idea would be consistent with the discrepancies between former studies that recruited to the sites of replication. This idea would be consistent with the discrepancies between former studies that use (near) full-length constructs and show substantial sensitivity to degradation [24–27], and those studies that use shorter constructs not including the HCV protein coding region that do not show substantial degradation ([17, 42] and this study).

Importantly, these considerations imply that those previous studies in which short HCV reporter constructs were used ([17, 19–21, 23, 42] and this study) indeed identified the stimulation of HCV IRES-dependent translation by miR-122 as being one of the molecular mechanisms of miR-122 function in the HCV life cycle, independent of reporter RNA stability.

METHODS

Transcription templates and RNA synthesis

The templates for in vitro-transcribed HCV reporter RNAs were generated by PCR from plasmid pUC18-J6/JFH1 IRES-hRLuc-3’-UTR and its derivatives. This plasmid contains a T7 promoter, followed by the 340 nucleotide HCV genotype 2a 5’ UTR and 33 nucleotides of core coding sequence from plasmid pFL-J6/JFH1 [50], a 9 nucleotide linker sequence, the coding sequence for humanized Renilla luciferase (hRLuc; Promega), 12 linker nucleotides, the 3’ terminal 21 nucleotides of the HCV NS5B coding region including the stop codon and the HCV 3’ UTR. Full-length HCV genomic RNAs were transcribed from plasmid pJc1 [51].

For in vitro transcriptions using T7 RNA polymerase, PCR fragment templates were generated from the HCV reporter constructs and gel purified. Transcriptions of unlabelled RNAs were performed in the presence of 500 μM ATP, GTP, CTP and UTP. RNA integrity and concentrations were determined by gel images and photometric analyses. Transcription of the radiolabelled HCV 5’-UTR RNAs to be transfected was performed using T7 RNA polymerase, in vitro transcriptions using T7 RNA polymerase, PCR

The radioactively labelled probe RNA for the RPA was transcribed from a PCR fragment generated from a plasmid in which a SP6 promoter was inserted in reverse orientation. The resulting radioactive probe RNA (see Fig. 5a) contains one linker nucleotide, followed by the reverse complement of the most 5’-terminal 159 nucleotides of the genotype 2a HCV sequence [50] with the miR-122 target sites mutated (S1m, S2m) as described in Fig. 1, plus 114 unrelated nucleotides. After transcription in the presence of [32P]UTP (see above), the radiolabelled probe RNA was purified on a 7 M urea/6% polyacrylamide gel and checked by autoradiography.

The concentrations of the labelled RNAs were calculated from the amount of the limiting radiolabelled nucleotide used in the in vitro transcription and considering the estimated efficiency of label incorporation (% nucleotides incorporated into the RNA versus % non-incorporated nucleotides) after analytic gel electrophoresis and autoradiography.

RNA oligonucleotides and RT-qPCR primers

RNA and DNA oligonucleotides were supplied by www.biomers.net. miRNA duplexes were formed between the guide (mat) and its complementary passenger strand (*). The RNA sequences were as follows:

miR-122 mat, 5’-(phos)UGGAGUGUGACAAUGGGUG UUGG-3’; miR-122*, 5’-(phos)AACGCCAUAUCACACUAAA UA-3’; miR-122m1 mat, 5’-(phos)UGUAGUCUGACAAAG UCGUUUG-3’; miR-122m1*, 5’-(phos)AACGACUUUAUACAGAC UCAUA-3’; miR-122m2 mat, 5’-(phos)UGGAAUGUGACACUG UGUUGG-3’; miR-122m2*, 5’-(phos)AACGCAGUUAUCACUA UAAAUA-3’; miR-124mat, 5’-(phos)UAAGGACGACGGUGAA UGCCA-3’; miR-124*, 5’-(phos)GUGUUCAGCGGACCUUGA UU-3’.

miRNA duplexes were generated by heating the single strands (100 μM) to 90 °C and cooling down at 1 °C s⁻¹ to 4 °C.

2’-O-methylated blocker DNA oligonucleotide ‘a’ (used in Fig. 4c):

5’-(phos)AACGCAGUUAUCACUAAAAUA.

Locked nucleic acid (LNA)/DNA mixmer blocker oligonucleotides (used in Fig. 2c, d) (with: +G, +A, +T, +C, phosphorothioate DNA base: G*, A*, T*, C*):


LNA oligonucleotide for sequestering miR-122 ‘α-miR-122-LNA’ (used in Fig. 4b):

+C*C*A*+T*T*G*+T*C*A*+C*A*C*+T*C*+C.

qPCR primer for hRLuc reporter RNA:

Forward: hRLuc1-for, 5’-AATCGAGCCGCTTGGAG TTG-3’;
Reverse and RT: hRLuc1-rev, 5’-TAGCTCCCTCAGA CATTGCCG-3’.

qPCR primer for HCV NS3 region (plus strand):

RT: NS3-RT, 5’-GTATGCCAGGGCATTCAAG-3’.
Transfections and co-IPs

For transfections using Lipofectamine 2000, $2.5 \times 10^6$ HeLa cells were seeded in 9 cm plates and transfected on the next day (at approximately 90 % confluency) with 3 µg HCV 5' UTR or reporter RNA and 1–3 µg of active miRNA (i.e. up to 6 µg of miR duplex=800 pmol duplex). The RNA amounts per cell and the HCV reporter RNA/miRNA ratios correspond to the conditions found previously to be optimal for the co-IP were purified by proteinase K digestion, phenol/chloroform extraction and ethanol precipitation in the presence of GlycoBlue. RNAs were separated by gel electrophoresis on 7 M urea/6 % polyacrylamide gels and analysed using a PerkinElmer Cyclone plus phosphorimager.

To calculate the miRNA binding strength from the phosphorimager scans, first the background binding intensity was subtracted, values normalized to the value obtained after 6 h without miRNAs, and means and standard deviations calculated.

RT-qPCR

For quantification of RNA abundancy by RT-qPCR, cells were electroporated, washed thoroughly with PBS and lysed by TRIzol reagent (Ambion). After collection of lysates, total RNA was extracted with chloroform and precipitated with isopropanol in the presence of GlycoBlue reagent. Next, total RNA was resuspended in RNase-free water, and Dnased treatment of one-third of each sample was conducted with 2 U of DNaseI, followed by enzyme removal by RNA Clean-up kit (Life Technologies). Finally, total RNA samples were eluted in equivalent amounts of RNase-free water. cDNA was produced using the qScript Flex cDNA kit (Quanta Biosciences) according to the manufacturer’s instructions. For the quantitative PCR, the prepared cDNA was used with the PerfeCTa SYBR Green FastMix (Quanta Biosciences) according to the manufacturer’s instructions; all measurements were conducted using Eppendorf Mastercycler ep realplex2. Mock-transfected cells were used as a control of primers’ specificity. For HCV-Rluc reporter RNA detection, RT and qPCR primers in the Rluc region were used. HCV full-length sequences were detected in the NS3 region. Values were normalized for GAPDH mRNA expression.

RNase protection assay

The RNase protection assay (RPA) was essentially performed as described previously [5, 52], with the following modifications. The PCR templates for the unlabelled HCV reporter RNAs (as used for luciferase expression experiments) to be electroporated were gel purified before transcription. After transcription, template DNA was digested by DNase, and RNAs were purified using the GeneJET RNA Cleanup and Concentration Micro kit (Thermo Scientific). These purified RNAs were then used for electroporation. For electroporations for RNA stability tests, $4 \times 10^6$ HeLa cells, 4 µg of unlabelled HCV reporter RNA and 2 µg of miRNA duplexes were used. After electroporation of cells, total RNA was re-extracted from the cells using TRIzol. The $^{32}$P-labelled probe RNA was gel purified after transcription; the PCR fragment for in vitro transcription of the probe was 1340 nucleotides long to ensure complete removal of residual template DNA from the 274 nucleotide probe RNA by gel purification. For the RPA, 1 µg of total RNA re-extracted from (transfected) cells was mixed with 0.1 ng of $^{32}$P-labelled probe RNA (unless stated otherwise) in a volume of 30 µl. The amount of probe was titrated to be not limiting (see Fig. 5b, d). For quantification of bands, phosphorimager screens were exposed for the same time for all assays within a given experiment. Band intensity was quantified with OptiQuant software (PerkinElmer), backgronds subtracted, values normalized to the value obtained after 6 h without miRNAs, and means and standard deviations calculated.

Acknowledgements

We thank Charles M. Rice (New York, USA) for plasmid pFL-J6/JFH-1 and Thomas Pietschmann (Hannover, Germany) and Ralf Bartenschlager (Heidelberg, Germany) for plasmid pJc1.

Conflicts of interest

The authors declare that there are no conflicts of interest.


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