Regulation of hepatitis C virus by microRNA-122

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Abstract

Most metazoan miRNAs (microRNAs) bind to sites in the 3′-UTRs (untranslated regions) of mRNA targets and negatively regulate protein synthesis. The liver-specific *miR-122*, however, exerts a positive effect on HCV (hepatitis C virus) RNA levels by binding directly to a site in the 5′-UTR of the viral RNA. HCV translation and RNA stability are unaffected, and therefore *miR-122* is likely to act at the level of viral replication. The *miR-122*-binding site in HCV RNA was examined to determine whether the nature of the site is responsible for the unusual mode of action for a miRNA. When the site was placed in the 3′-UTR of a reporter mRNA, *miR-122* repressed translation, and therefore the location of the *miR-122*-binding site dictates its effect on gene expression. Additionally, a second binding site for *miR-122* was identified in the HCV 5′-UTR, and *miR-122* binding to both sites in the same viral RNA was found to be necessary for viral replication. The two sites are adjacent and are separated by a short spacer, which is largely conserved between HCV genotypes. The binding site requirements for *miR-122* to positively regulate HCV replication provide an insight into this unusual mode of miRNA action.

Introduction

miRNAs (microRNAs) are non-coding RNAs expressed by a wide range of eukaryotic organisms that are important in the control of gene expression [1]. Several hundred miRNAs are expressed in mammals, initially as part of large precursor transcripts, which subsequently undergo nuclear and cytoplasmic processing to yield 21–23 nt single-stranded mature miRNA molecules. miRNAs can mediate cleavage of exactly complementary RNA targets, but in metazoan systems most miRNAs have been shown to function by binding with imperfect complementarity to 3′-UTR (untranslated region) sites as part of a complex of proteins known as the miRNP. This binding resulted in inhibition of gene expression, which occurred at the level of translation initiation or by a post-initiation block in different studies. Localization of mRNA to processing (P) bodies and subsequent degradation has also been observed, and it is likely that all three mechanisms of repression may function in different circumstances [2].

In higher organisms miRNAs show a high degree of specificity of expression, both according to tissue type and developmental stage. *miR-122* was identified as a highly liver-specific miRNA and was found to account for approx. 70% of total liver miRNA content, with approx. 66000 copies per cell [3].

HCV (hepatitis C virus) is a hepatotropic positive-sense RNA virus that establishes persistent infections in liver tissue [4]. Infection can eventually lead to cirrhosis of the liver and hepatocellular carcinoma. Current antiviral therapies are frequently ineffective, and a greater understanding of the virus is important for the development of new treatments. We examined liver cell lines and found that *miR-122* is expressed in Huh7 cells, which are supportive of HCV replication, but not in a different human liver cell line, HepG2 [5]. Huh7 cells stably expressing a dicistronic HCV replicon, in which the EMCV (encephalomyocarditis virus) IRES (internal ribosome entry site) directs translation of the complete HCV polyprotein, whereas HCV 5′- and 3′-UTRs allow replication to occur [6], were also examined, and *miR-122* was expressed in these cells. We were interested to see what impact the presence of high levels of *miR-122* had on HCV replication, and used a 2′-O-methylated antisense oligonucleotide to sequester *miR-122* in Huh7 cells. Both in Huh7 cells stably containing the dicistronic genotype 1b HCV replicon described above, and in cells transiently expressing a full-length type 1a HCV replicating RNA [7], sequestration of *miR-122* resulted in a dramatic reduction in HCV RNA levels [5].

Mutagenesis indicated that *miR-122* binds to a site in the 5′-UTR of HCV RNA and that its effect on HCV is due to this binding. The *miR-122*–binding site we identified is in an unstructured region of the 5′-UTR, upstream of the HCV IRES, and is conserved across all six genotypes of the virus (Figure 1A, seed match 1). We observed no effect of *miR-122* binding on HCV translation or RNA stability, and concluded that *miR-122* positively regulates HCV at the level of viral replication [5].

The *miR-122*-binding site from HCV mediates repression of gene expression when placed in a heterologous 3′-UTR

The discovery of this novel mode of action for a miRNA suggested that particular features of *miR-122* or its binding site in HCV might cause this unusual mode of regulation. A major determinant of productive miRNA binding has been found to be exact Watson–Crick complementarity to nt 2–7 of the miRNA, known as the ‘seed’. This seed match

Key words: hepatitis C virus (HCV), internal ribosome entry site (IRES), microRNA, *miR-122*, viral replication.

Abbreviations used: HCV, hepatitis C virus; IFN, interferon; IRES, internal ribosome entry site; miRNA, microRNA; TOP, terminal oligopyrimidine tract; UTR, untranslated region.

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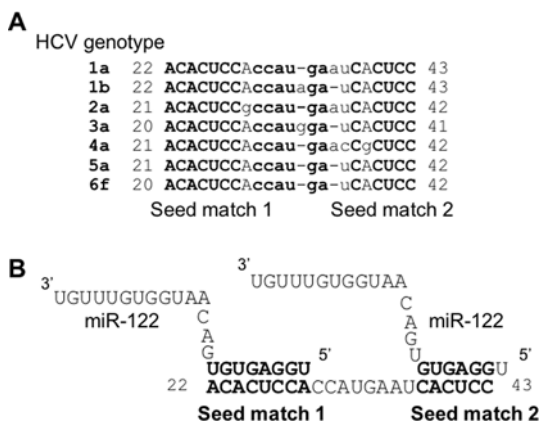
Figure 1 | ***miR-122*** **binds to two adjacent sites in the 5′-UTR of HCV RNA**

(**A**) Sequence conservation of the region spanning the two *miR-122* seed matches across genotypes of HCV. Nucleotides forming the seed matches are shown in capitals, and conserved nucleotides in bold. The numbers indicate the position of this sequence relative to the 5′-end of each HCV isolate. The isolates shown are H77c (type 1a), HCV-N (1b), JFH-1 (2a), NZL1 (3a), HEMA51 (4a), FRT41 (5a) and TH271 (6f). (**B**) Model for concurrent binding of two *miR-122* molecules to the adjacent binding sites in the HCV 5′-UTR.



should ideally be flanked by A residues pairing to nt 1 and 8 of the miRNA [8]. Further pairing to the 3′ region of the miRNA contributes to binding affinity, but is less crucial. The *miR-122*-binding site in HCV fits this model (Figure 1B, seed match 1), despite the fact that *miR-122* binding to this site does not lead to repression of HCV translation [5].

The region spanning the *miR-122*-binding site was amplified from the HCV 5′-UTR by PCR, and inserted in the 3′-UTR of the firefly luciferase gene. Transfection of this firefly luciferase plasmid into Huh7 cells and sequestration of *miR-122* showed that *miR-122* mediates repression of luciferase expression from this mRNA, and quantitative RT (reverse transcriptase)–PCR indicated that luciferase mRNA levels are unaffected, so repression occurs at the level of translation [9]. Mutagenesis of the *miR-122*-binding site, and introduction of mutant *miR-122* that can bind to the mutant site, showed that the *miR-122* response is partly mediated by binding to this site. However, some *miR-122*-dependent down-regulation of luciferase expression persisted when the site was mutated. A second *miR-122* seed match exists adjacent to, and downstream of, the first one. This seed match (seed match 2) extends from nt 2 to 7 of *miR-122* and lacks the A residues pairing to positions 1 and 8 that are seen in seed match 1 (Figure 1B). However, this second seed match is conserved across HCV genotypes (Figure 1A, seed match 2), suggesting a possible functional role. Mutagenesis of both seed matches in the 3′-UTR of luciferase abolished *miR-122*-dependent repression of luciferase expression, which was restored by introduction of mutant *miR-122* that binds to the mutant site [9]. This indicated that *miR-122* mediates repression of gene expression via both these adjacent sites in the context of a 3′-UTR.

miR-122 regulates HCV replication via two adjacent binding sites

These findings raised the question as to whether *miR-122* binds to both adjacent seed matches in the context of HCV RNA. Point mutations were introduced into the *miR-122* seed match 2 in a plasmid encoding an HCV type 1a (H77) genomic RNA, containing five adaptive mutations that allow efficient replication in Huh7 cells [7]. Following the introduction of the mutant RNA into cells and a five-day culture period to allow replication to occur, no HCV RNA was detectable. Moreover, the introduction of a *miR-122* mutant that can bind to this mutant site led to the restoration of HCV RNA levels, indicating that *miR-122* binding to this second site is necessary for HCV replication to occur. Levels of HCV RNA containing mutations in both *miR-122*-binding sites could also be restored by transfection of mutant *miR-122* molecules that can bind to the sites. Importantly, when different mutations were introduced into each of the two sites, individual *miR-122* mutants that could bind to both sites had to be introduced into cells in combination for HCV replication to occur. This indicates that *miR-122* must bind to both sites within the same HCV molecule [9]. However, it is not yet known whether this is due to a requirement for overlapping binding to the two sites (Figure 1B), or for a sequential binding process.

Multiple binding sites for the same or different miRNAs are frequently observed in 3′-UTRs, and some co-operativity occurs when miRNAs bind to adjacent sites, with an optimal distance of 13–35 nt between the start of two seed matches [10]. However, the essential requirement for binding to the two adjacent *miR-122* seed matches that we find in HCV RNA has not been observed in other systems. An additive, but inessential, effect of multiple copies of the *miR-122*-binding site from HCV was observed in the context of the 3′-UTR of luciferase mRNA, indicating that the absolute requirement for the double binding site is specific to HCV replication. The sites are separated by an 8 nt spacer that is largely conserved across HCV genotypes (Figure 1A), and preliminary data indicate that the length and sequence of the spacer are important for HCV replication [9]. It is possible that *miR-122* binding to the adjacent sites allows the recruitment of an oligomeric protein complex to the 5′-UTR of HCV and that this is required for viral replication.

Implications for miRNA function

The finding that a miRNA can bind to the same site in different contexts and mediate different events suggests that miRNA activity is more flexible than was previously thought. Most efforts, both experimental and computational, to detect miRNA-binding sites have been directed towards the 3′-UTR. Several recent studies, however, suggest that miRNA activity may extend beyond repression of gene expression via 3′-UTR sites. It was shown that *miR-369-3* binds to AU-rich

elements in the 3′-UTR of the TNFα (tumour necrosis factor α) mRNA and mediates a switch from repression to activation of mRNA translation upon serum starvation. Similar effects were observed with a *let-7* target, and recruitment of the FXR1 (fragile X mental retardation-related protein 1) protein as part of the miRNP was responsible for this change in activity [11]. Further study is needed to indicate how general a mechanism this is, but it provides an indication that the cellular environment and the composition of the miRNP may have a considerable effect on miRNA activity.

Very recently, *miR-10a* was shown to interact with sites in the 5′-UTR of 5′-TOP (5′-terminal oligopyrimidine tract) mRNAs [12]. These mRNAs encode components of the ribosome and translational machinery. The TOP sequence at the 5′-end of these mRNAs governs a rapid increase in translation in response to growth signals. *miR-10a* was shown to bind downstream of, and adjacent to, the TOP sequence, and this binding was required for activation of translation. Interestingly, the binding sites for *miR-10a* in TOP mRNAs did not have extensive seed matches, although the overall thermal stability of the miRNA–mRNA duplex was good. Other target sites that are similar to this, and may not be present in 3′-UTRs, are likely to have been missed by current bioinformatic site identification tools. As with *miR-122*, *miR-10a* can mediate different processes by binding to sites in different locations, as 3′-UTR-binding sites for this miRNA were also identified, and *miR-10a* binding to these sites results in repression of gene expression. The mechanism by which *miR-10a* positively regulates translation of TOP mRNAs is not yet known.

The miRNA pathway and viruses

The role for *miR-122* in HCV replication fits in with increasing evidence of interplay between viral infection and the miRNA pathway. Several DNA viruses express miRNAs, and these can regulate both viral and cellular transcripts and play a role in viral infection or latency. Other studies have suggested that cellular miRNAs directly target and repress PFV-1 (primate foamy virus 1) and VSV (vesicular-stomatitis virus), but the role of these miRNAs in viral infection has not been established, and it is likely that viruses would evolve to escape deleterious host miRNA interactions [13]. As yet, a positive effect on viral replication by direct binding of a cellular miRNA has only been observed in HCV, but it is possible that this mechanism may extend to different viruses.

A recent paper showed that IFN (interferon) β treatment of cells results in up-regulation of a number of miRNAs [14]. Five of these have predicted targets in the HCV RNA genome, and their overexpression exerts a negative effect on HCV. Mutagenesis indicated that direct binding to their sites in HCV RNA mediates this effect for two of these miRNAs. Interestingly, *miR-122* expression decreased upon IFN treatment, and overexpression of *miR-122* partially alleviated the antiviral effects of IFN [14].

Further research has indicated that *miR-122* is required for HCV replication in the genotype 2a JFH-1 system, which permits production of infectious virus in Huh7 cells. Therefore, it appears that the conservation of the *miR-122*-binding sites across genotypes of HCV is functionally important and that the *miR-122* requirement for HCV replication extends across genotypes. Moreover, RNAi (RNA interference)-mediated knockdown of components of the miRNA and RNAi pathways resulted in inhibition of HCV replication [15]. This indicates that the positive effect of *miR-122* on HCV replication overrides the effects of any negatively acting miRNAs or siRNAs.

The finding that a miRNA is required for HCV infection has great potential for the development of novel antiviral therapies. Excitingly, intravenous injection of LNA (locked nucleic acid)-modified oligonucleotides complementary to *miR-122* into African green monkeys led to effective, reversible inhibition of *miR-122* activity in the liver [16]. The relief of *miR-122* inhibition of cellular targets, most of which are involved in cholesterol metabolism, resulted in a decrease in total plasma cholesterol levels. No toxicity was observed over the course of the study, suggesting that targeting *miR-122* is a promising avenue to pursue for the treatment of HCV infection. A greater understanding of the role for *miR-122* binding to the two adjacent sites we have identified in HCV is important for the future development of novel therapies.

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