

Target mRNAs are repressed as efficiently by microRNA-binding sites in the 5' UTR as in the 3' UTR

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In animals, microRNAs (miRNAs) bind to the 3' UTRs of their target mRNAs and interfere with translation, although the exact mechanism of inhibition of protein synthesis remains unclear. Functional miRNA-binding sites in the coding regions or 5' UTRs of endogenous mRNAs have not been identified. We studied the effect of introducing miRNA target sites into the 5' UTR of luciferase reporter mRNAs containing internal ribosome entry sites (IRESs), so that potential steric hindrance by a microribonucleoprotein complex would not interfere with the initiation of translation. In human HeLa cells, which express endogenous let-7a miRNA, the translational efficiency of these IRES-containing reporters with 5' let-7 complementary sites from the *Caenorhabditis elegans* lin-41 3' UTR was repressed. Similarly, the IRES-containing reporters were translationally repressed when human Ago2 was tethered to either the 5' or 3' UTR. Interestingly, the method of DNA transfection affected our ability to observe miRNA-mediated repression. Our results suggest that association with any position on a target mRNA is mechanistically sufficient for a microribonucleoprotein to exert repression of translation at some step downstream of initiation.

internal ribosome entry sites | repression | method of transfection | let-7 miRNP | translation efficiency

MicroRNAs (miRNAs) are ≈ 21 -nt RNAs that, in animals, bind through imperfect base pairing to the 3' UTR of their target mRNAs and interfere with translational output. Even though miRNAs induce mRNA deadenylation or degradation in some cases (1–4), protein synthesis of these mRNAs appears to be repressed as well (2). However, the exact mechanism by which miRNAs diminish protein yield remains unclear. miRNAs have been proposed to interfere with translation at both the initiation and elongation stages, or translation may be unaffected, with nascent polypeptides being degraded instead (ref. 5; reviewed in refs. 6 and 7). Alternatively, target mRNAs may be translationally repressed because they are physically isolated, such as in P-bodies, from translating ribosomes (6, 7). Thus, miRNAs might effect repression by multiple means, or current analyses may be too limited to gain a thorough understanding of a single mechanism (7).

A large body of data suggests that mRNAs are translationally repressed by miRNAs at a step after translation initiation. A number of reports demonstrate the association of translationally repressed messages with polyribosomes (8–14). Moreover, miRNAs have been found to cosediment with polyribosomes, most likely through their association with mRNAs (13, 15, 16). Petersen *et al.* (14) observed that both cistrons of a dual-reporter mRNA were repressed by a synthetic miRNA, arguing for a postinitiation effect; they suggested that repression was not due to an interruption of translation initiation but instead to ribosome drop-off.

In contrast, other reports have concluded that translation initiation is affected by the presence of miRNA-binding sites in the mRNA 3' UTR. Both Pillai *et al.* (17) and Humphreys *et al.* (18) observed that the introduction of an internal ribosome entry

site (IRES) into an mRNA bypasses miRNA-mediated repression. Pillai *et al.* (17) found that an endogenous miRNA prevented the association of a target reporter mRNA with polyosomes. Finally, repressed mRNAs targeted by miRNAs have been seen to accumulate in P-bodies (2, 17, 19–23), suggesting that mRNA sequestration from the translation apparatus may also be critical.

Animal miRNAs have so far been reported to functionally target endogenous mRNAs only through sites in the 3' UTR. Although target sites for endogenous miRNAs can be identified in ORFs and 5' UTRs, they are less frequent and appear less effective than those in the 3' UTR (24–26). In zebrafish, exogenous reporter mRNAs containing target sites for let-7 miRNA in either the coding region or the 5' UTR were silenced after injection of exogenous let-7 (27). Similarly, endogenous geminin mRNA was translationally repressed when exogenous siRNAs with imperfect complementarity to the coding region were introduced into HeLa cells (28). In both of these cases, the physical presence of the microribonucleoprotein (miRNP) complex on the message may have interfered with ribosome scanning during initiation and/or with reading of the message. To circumvent possible effects of steric hindrance on ribosome access to the ORF, we inserted miRNA-binding sites into the 5' UTRs of messages containing IRESs.

In our studies we transiently transfected HeLa cells with plasmids encoding firefly (FF) luciferase reporters containing let-7 complementary sites (LCSs) from the *Caenorhabditis elegans* lin-41 3' UTR. RNA transfections were also performed with comparable G- or A-capped, polyadenylated *in vitro*-transcribed mRNAs. We chose these LCSs because the endogenous human let-7a expressed in HeLa cells is identical in sequence to *C. elegans* let-7 (29, 30). The hepatitis C virus (HCV) IRES (31, 32) was placed upstream of the luciferase ORF to bypass most of the steps necessary for translation initiation on capped mRNAs. Because ribosomes enter these IRES-containing reporter mRNAs at an internal start codon, we could examine whether the position of miRNA-binding sites, or a tethered miRNP component, relative to the ORF, effects repression.

Results

A Target Reporter mRNA Is Repressed by Endogenous let-7a miRNA in HeLa Cells. Previously, a region of the *C. elegans* lin-41 3' UTR containing two conserved LCSs separated by a 27-nt spacer was

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Abbreviations: HCV, hepatitis C virus; IRES, internal ribosome entry site; LCS, let-7 complementary site; miRNA, microRNA; miRNP, microribonucleoprotein; FF, firefly; R, *Renilla*.

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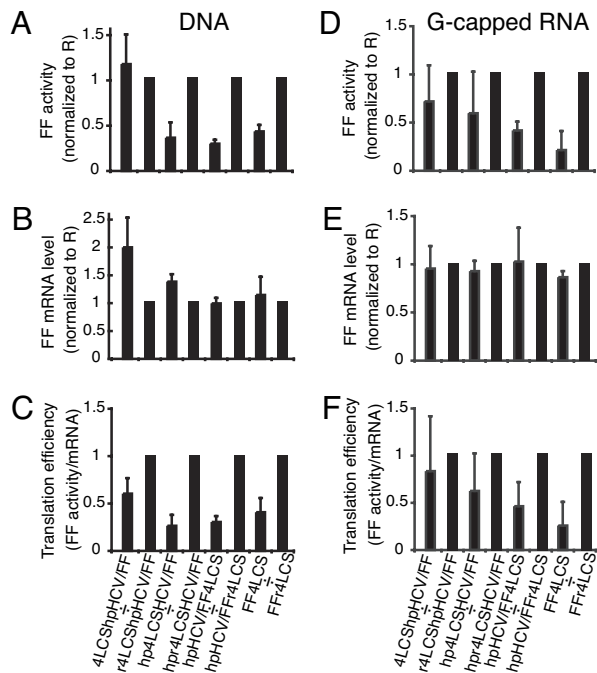


Fig. 4. Electroporation yields similar conclusions for DNA versus RNA transfections. (A–C) HeLa cells were transfected with the plasmid constructs depicted in Figs. 1B and 2A by electroporation. FF luciferase activity was assayed, and translation efficiency was calculated as in Fig. 1C–F. Error bars represent standard deviations from three experiments. (D–F) HeLa cells were transfected by electroporation with G-capped, polyadenylated mRNAs *in vitro*-transcribed from the plasmids described in A and assayed as above. Error bars represent standard deviations from three experiments.

mediated transfection reagent different from the TransIT-HeLaMONSTER we used for DNA transfections, the experiments in Fig. 2 were repeated with this new reagent (Fig. 3A–C). DNA transfections gave the same results regardless of which cationic lipid formulation was used (compare Fig. 2E with Fig. 3C). Specifically LCSs 5' to the ORF in our IRES-containing reporters promoted repression of translation, whereas LCSs in the 3' UTR did not exhibit miRNA-mediated repression.

miRNA-Mediated Translational Repression Is Affected by the Transfection Method. While we were completing these studies, Barreau *et al.* (41) reported that caution should be used with liposome-mediated transfections of RNA and advised that electroporation should be used instead. We therefore transfected HeLa cells by electroporation with both our DNA and RNA constructs (Fig. 4). Interestingly, our RNA transfection results were reproducible using either cationic lipid-mediated transfection or electroporation, with the LCSs effecting repression in all positions (Figs. 3F and 4F), but note the larger error bars for RNA electroporations). In contrast, the DNA electroporation results for LCSs in the 3' UTR showed a >2-fold repression (Fig. 4C), in direct contrast to the lack of repression observed by liposome-mediated transfection (Figs. 2E and 3C). These results highlight that caution should be taken when using different transfection methods and that conclusions should not be based on a single method.

Translational Repression of a Reporter mRNA Occurs When Human Ago2 Is Tethered to Either Its 5' or 3' UTR. Finally, we examined the effect of tethering the human Ago2 (hAgo2) protein, a core component of the miRNA-induced silencing complex, to the 5' UTR rather than the 3' UTR, where it is known to cause translation repression (42). IRES-containing mRNAs with the

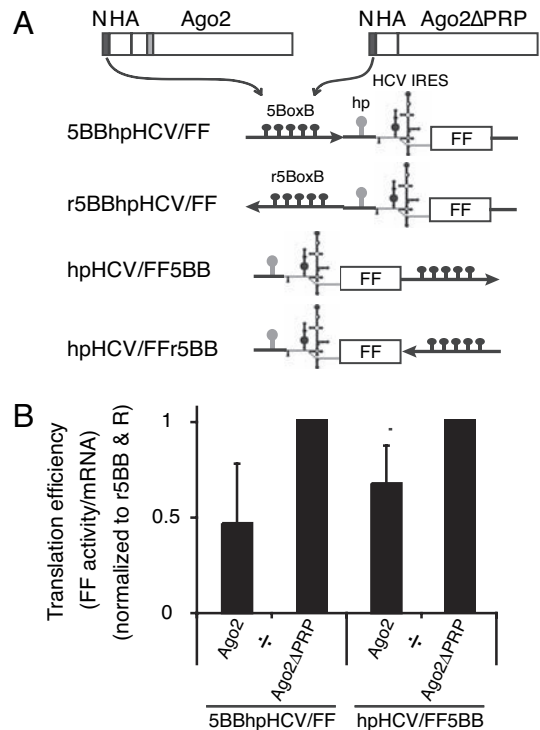


Fig. 5. Translation is repressed when human Ago2 is tethered to either the 5' or 3' UTR of IRES-containing reporters. (A) The hAgo2 protein, with the λ N and HA domains fused to its N terminus, or the same protein with the first 62 aa of Ago2 deleted (Δ PRP), was coexpressed with IRES-containing FF reporter RNAs. The reporters contained five boxB (5BB) N-protein-binding sites located 5' to the hairpin (hp) upstream of the HCV IRES in the 5' UTR or in the 3' UTR. Control constructs contained five reversed boxB sequences (r5BB). (B) HeLa cells were transfected by using TransIT-HeLaMONSTER (Mirus) with the plasmids described in A, and, after normalization of the luciferase activity to the mRNA levels as in Fig. 1F, the forward boxB constructs were normalized to the reversed boxB constructs and the tethered Ago2 results were normalized to the Ago2 Δ PRP results (value set to 1). Error bars represent standard deviations from three to four experiments. Similar results were obtained with normalization to values obtained with no coexpression of the N fusion protein.

LCSs substituted by five phage boxB hairpins in either the 5' or 3' UTR (Fig. 5A) were coexpressed with a N-HA-Ago2 fusion protein (42), both from DNA plasmids. Because ribosomes enter the IRES-containing message at an internal start codon, binding of the fusion protein should not interfere with ribosome entry into the FF ORF. A mutant N-HA-hAgo2 protein, in which the first 62 aa of Ago2 containing the PRP domain were deleted (Δ PRP) (Fig. 5A), was used as a control because it was previously shown that tethering this mutant protein does not induce repression (42). After normalization to the R reporter, a control reporter containing the five boxB hairpins in reverse orientation and mRNA levels, an \approx 2-fold level of repression was observed (Fig. 5B), comparable to that published previously (43). This repression occurred regardless of whether hAgo2 was tethered to the 5' or 3' UTR of the mRNA (Fig. 5B).

Discussion

The mechanism by which miRNAs block protein synthesis remains unclear. For our investigation, we used FF luciferase reporters containing let-7 miRNA complementary sites derived from the *C. elegans* lin-41 3' UTR and transiently transfected HeLa cells with either DNA plasmids or comparable *in vitro*-transcribed mRNAs. After normalization for mRNA levels, we observed that translation was repressed 2-fold by endogenous let-7a miRNPs (Figs. 1F, 3C, F, and I, and 4C and F). It is

possible that degradation of a target mRNA occurs after the cell has made the decision not to translate and that our approach of normalizing for mRNA levels thereby diminishes the extent of translational repression; however, although the degree of repression observed would change, our conclusions would not. We then used reporter constructs in which the HCV IRES was introduced into the 5' UTR, bypassing most of the steps necessary for translation initiation on capped mRNAs. This allowed us to examine the effect of the position relative to the ORF of the miRNA-binding sites and of a tethered miRNA-induced silencing complex component.

When IRES-containing mRNAs with miRNA-binding sites were introduced into HeLa cells by electroporation of *in vitro*-transcribed mRNAs (either G- or A-capped) or of DNA plasmids, translation repression occurred whether the target sites were 5' or 3' to the luciferase ORF (Figs. 3*F* and *I* and 4). It is not clear whether mRNA circularization brings sites located in the 3' UTR into proximity with the 5' end of the ORF; A-capped mRNAs would not be expected to undergo the usual cap-polyA interactions (40), but the HCV IRES may promote circularization by a noncanonical mechanism (44). Comparable repression was observed upon tethering the core miRNP protein hAgo2 to the 5' or 3' end of an IRES-containing mRNA (Fig. 5). We conclude that miRNP interaction with the mRNA is a minimal criterion for translational repression in mammalian cells but that the position of miRNA target site(s) relative to the ORF is not mechanistically important for the ability of an miRNP complex to repress translational output.

Conflicting reports have argued for and against the hypothesis that translation initiation is the step affected by the presence of miRNA-binding sites in an mRNA. Our RNA transfection data (Figs. 3*F* and *I* and 4*F*) indicate that miRNA-binding sites in the 5' or 3' UTR of an IRES-containing mRNA lead to repression, whether the 5' end carries a G-cap or A-cap. Use of A-capped mRNAs ruled out the possibility that stalled scanning ribosomes contributed artifactually to this result. Our conclusion that protein synthesis directed by an miRNA-targeted mRNA is affected at a step downstream of translation initiation agrees with the studies of Petersen *et al.* (14), which demonstrated repression using two other IRESs in a bicistronic construct containing miRNA-binding sites in the 3' UTR. In contrast, when we used cationic lipid to transfect DNA plasmids, we observed no repression of an IRES-containing message with miRNA-binding sites in the 3' UTR (Figs. 2*E* and 3*C*). This result agrees with the conclusions of both Pillai *et al.* (17) and Humphreys *et al.* (18), who likewise used cationic lipids but transfected *in vitro*-transcribed IRES-containing mRNAs. Because DNA transfections by electroporation exhibited repression in all cases, agreeing with our RNA transfection results by any means of transfection, we conclude that the let-7a miRNA exerts its effect after the initial steps of translation initiation.

An unsettling realization to emerge from our experiments is that the method of transfection affects whether miRNA repression is observed for a targeted mRNA. Although miRNA-binding sites in the 5' UTR of IRES-containing reporters led to repression when DNA or RNA was transfected using either cationic lipids or electroporation, the same was not the case for constructs with miRNA-binding sites in the 3' UTR (Figs. 2*E*, 3*C*, and 4*C*). One obvious difference between these transfection methods is that transfected RNAs would likely remain cytoplasmic, whereas an RNA transcribed from a DNA plasmid would assemble into a nuclear RNA-protein complex that is later exported to the cytoplasm. Also, cationic lipid transfections can be more efficient than electroporation (45), so the possibility exists that a large amount of DNA taken up by the cell might saturate the ability of the miRNA system to repress translation. However, we took care to use the lowest possible amount of DNA or RNA in each transfection that would allow detection of

the RNA in our RNase protection assays, needed for normalization purposes. Initial tests ensured that the same results were obtained when twice the amount of DNA was transfected using the cationic lipid method (data not shown). We must conclude that the method of transfection affects the ability of an mRNA to be repressed by endogenous miRNAs. Thus, a single transfection method may not be reliable for drawing conclusions concerning the efficacy of translational repression by miRNAs.

We deduce from our assays of HCV IRES-containing mRNAs that the early steps of translation initiation are not involved in miRNA-mediated repression. Thus, either a very late stage of initiation, elongation, or termination is affected or the protein product may be degraded. So far, most examples of translational control target translation initiation (reviewed in refs. 46 and 47). A late initiation step that could be targeted in our IRES constructs is joining of the 60S ribosomal subunit to the 48S mRNA complex, such as in the regulation of LOX mRNA translation (48). This mRNA contains motifs in its 3' UTR that bind hnRNP-K and hnRNP-E1 and prevent 60S subunit joining; it is repressed even when translation is driven by the encephalomyocarditis virus or classical swine fever virus IRES. Yet we favor models involving some step downstream of translation initiation because many miRNA-targeted mRNAs have been found associated with polysomes (5, 8–14). Another example of translational control where the data argue for inhibition at both initiation and postinitiation stages is the *nanos* mRNA. A site in its 3' UTR binds the Smaug protein, which recruits the Cup repressor protein, which, in turn, binds to eIF4E and inhibits cap-dependent initiation (49); nonetheless, Clark *et al.* (50) have demonstrated that the repressed *nanos* mRNA is associated with translating ribosomes. In the case of miRNA-mediated repression, it seems most likely that the presence of a large miRNP complex anywhere on the same mRNA enables an interaction with the translating ribosomes that somehow interferes with translocation or peptidyl transferase activity during elongation. The exact mechanism remains to be elucidated.

Materials and Methods

DNA Constructs. FF4LCS and its reverse, mutated control were created by subcloning the FF luciferase and 4LCS (containing a duplicated sequence from the *C. elegans* lin-41 3' UTR) into pcDNA3 (Invitrogen), with a CMV promoter, or into pRL-TK (Promega, Madison, WI), with a HSV-TK promoter and R luciferase removed. The mutated 4LCS maintained the same base composition (UUAUACAACCGUUCUACACUCA→UACCAUUAAGUUCUACACUCA).

pc4LCSHpHCV/FF, pphp4LCSHCV/FF, pCHCV/FF4LCS, and their reverse controls were made by subcloning into pcDNA3 4LCS, a hairpin, and an HCV/FF fusion containing the HCV IRES (383 bp) fused to FF luciferase, in the order indicated. The hp/HCV IRES/4LCS DNA constructs had 5' UTRs ranging from 521 to 723 bp and 3' UTRs ranging from 157 to 333 bp. pc5BBhpHCV/FF, pphpHCV/FF5BB, and their reverse controls were created by cloning the HCV/FF fusion, five boxB domains, and a hairpin into pcDNA3. These constructs had 5' UTRs of 521 or 836 bp and 3' UTRs of 157 or 470 bp. R luciferase was amplified from another vector and introduced into pcDNA3, to create pcR, or replaced R in pRL-TK to produce phR. phR generates an RNA transcript that has a 65-nt 5' UTR and a 231-nt 3' UTR.

For complete details of plasmids, see [supporting information \(SI\) Materials and Methods](#).

RNA Transcription. Cold, G-capped RNAs were *in vitro*-transcribed using the mMMESSAGE mMACHINE kit (Ambion, Austin, TX) from plasmids described above cleaved with DraIII. A-capped RNAs were transcribed using this same kit, but the 2× NTP/CAP solution was replaced with a 40 mM Tris buffer

containing 1.5 mM ATP, 7.5 mM other NTPs, 6 mM A cap-A(5')ppp(5')G (NEB, Ipswich, MA), and 15 mM MgCl₂. The RNAs were polyadenylated with the Poly(A) Tailing kit (Ambion), extracted with phenol/chloroform, and precipitated with ammonium acetate and isopropanol. The hp/HCV IRES/4LCS RNA constructs had 5' UTRs ranging from 481 to 683 nt and 3' UTRs ranging from 559 to 735 nt (before polyadenylation). The R RNA had a 5' UTR of 15 nt and a 3' UTR of 634 nt.

Transfection Protocols. For TransIT-HeLaMONSTER (Mirus) transfections, HeLa JW36 cells were transfected with DNA plasmids. One of six wells received 1.5 μg of pFF4LCS or its reversed, mutated control along with 25 ng of pcR or 200 ng of pcFF4LCS or its control along with 2 μg of phR. A total of 200 ng of pcHCV/FF construct was transfected with 2 μg of phR. The RNA from three wells and luciferase activity from one well were analyzed.

For Lipofectamine 2000 (Invitrogen) plasmid transfections, 200 ng of each pcFF construct per well with 2 μg of phR was transfected. Four wells were used per construct, with luciferase activity and RNA determined as described above. For RNA transfections, 1 μg of G-capped or A-capped FF RNA was transfected along with 1 μg of R RNA. Two wells of a six-well plate were transfected per construct; one well was harvested for luciferase activity, and one well was harvested for RNA.

For plasmid transfection by electroporation, 3 × 10⁶ cells were added to 7.5 μg of a single pcFF construct and 7.5 μg of phR and immediately transferred to two wells of a six-well plate. One well was harvested for luciferase activity, and one well was harvested for RNA. Electroporation with RNA transcripts was done with 9 μg of G-capped FF RNA and 9 μg of R RNA added to 6.625 × 10⁶ cells. After pulsing, the cells were transferred to three wells

of a six-well plate; one well was harvested for luciferase activity, and two wells were harvested for RNA.

Tethering hAgo2. A total of 100 ng of pc5BBhpHCV/FF, pchpHCV/FF5BB, or their reverse controls were transfected by TransIT-HeLaMONSTER (Mirus) along with 500 ng of pNHAgo2 or pNHAgo2ΔPRP and 2 μg of phR into one well of a six-well plate as described above.

Luciferase Assay. The contents of one well were isolated by using the Dual-Luciferase Reporter Assay according to the manufacturer's directions. A total of 50 μl of the 500 μl of 1× PLB were assayed by a 20/20n luminometer (Turner BioSystems, Savannah, GA) in a 0.5-ml Eppendorf tube using 100 μl of LARII and 100 μl of Stop & Glo Reagent. FF and R luciferase activities were measured.

RNase Protection Assay. The RNase protection protocol was adapted from one published earlier (45) and is described in detail in *SI Materials and Methods* and elsewhere (51).

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