Specificity of microRNA target selection in translational repression

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MicroRNAs (miRNAs) are a class of noncoding RNAs found in organisms as evolutionarily distant as plants and mammals, yet most of the mRNAs they regulate are unknown. Here we show that the ability of an miRNA to translationally repress a target mRNA is largely dictated by the free energy of binding of the first eight nucleotides in the 5′ region of the miRNA. However, G:U wobble base-pairing in this region interferes with activity beyond that predicted on the basis of thermodynamic stability. Furthermore, an mRNA can be simultaneously repressed by more than one miRNA species. The level of repression achieved is dependent on both the amount of mRNA and the amount of available miRNA complexes. Thus, predicted miRNA:mRNA interactions must be viewed in the context of other potential interactions and cellular conditions.

[*Keywords*: microRNA; translational repression; 3′ UTR]

Received January 6, 2004; revised version accepted January 29, 2004.

The canonical RNA interference (RNAi) pathway begins with the cleavage of long, double-stranded RNA into an intermediate RNA species of ~21 nt known as short, interfering RNA (siRNA; for review, see Zamore 2002; Dykxhoorn et al. 2003). These siRNAs are double-stranded, with 5′ phosphates and 2-nt 3′ overhangs, indicators of RNase III cleavage, and, indeed, the enzyme Dicer was identified as responsible for their generation (Bernstein et al. 2001). One of the two strands of the siRNA is incorporated into the RNA induced silencing complex (RISC; Hammond et al. 2000; Martinez et al. 2002; Khvorova et al. 2003; Schwarz et al. 2003). This strand then guides RISC to perfectly complementary mRNAs and cleaves them, resulting in their degradation. Several labs cloned short RNA species to find endogenous siRNAs, and these efforts led to the discovery of miRNAs as a large class of noncoding RNAs (Lagos-Quintana et al. 2001; Lau et al. 2001; Lee and Ambros 2001).

MicroRNAs are ~22-nt single-stranded RNA species found in a wide variety of organisms, ranging from plants to worms to humans (for review, see Lai 2003; Bartel 2004). The founding member of the miRNA class, the *Caenorhabditis elegans* gene *lin-4*, as well as its target, the nuclear protein lin-14, were first identified in a screen for worms with defects in cell lineage progression (Horvitz and Sulston 1980; Chalfie et al. 1981). After more than a decade of research, it was determined that *lin-4* did not code for a protein, but rather a small RNA species with imperfect complementarity to several sites in the 3′ untranslated region (UTR) of *lin-14* (Lee et al. 1993). Because expression of *lin-4* led to a decrease in lin-14 protein level without a decrease in mRNA level, this phenomenon was dubbed translational repression (Wightman et al. 1991, 1993). Biochemical analysis revealed that the repressed mRNAs remain in polysomes, suggesting that the block in expression occurs after translation initiation, although little is known about the mechanism (Olsen and Ambros 1999; Seggerson et al. 2002).

Although the mechanism of miRNA action remains elusive, their biogenesis is rapidly becoming clear. Primary miRNA transcripts are first processed in the nucleus by the RNase III enzyme Drosha to produce a hairpin RNA of ~70 nt (Lee et al. 2003). In a pathway dependent on Exportin-5, this pre-miRNA is then exported into the cytoplasm (Yi et al. 2003; Lund et al. 2004), where Dicer then cuts the hairpin (Grishok et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001; Knight and Bass 2001; Lee et al. 2002). Correlative evidence suggests that the same rules governing siRNA strand choice also hold for determining which side of the hairpin becomes the mature strand of the miRNA (Schwarz et al. 2003). The complex containing active miRNAs and the RISC involved in RNAi are similar if not identical, as endogenous miRNAs can cleave mRNAs with perfect complementarity (Hutvagner and Zamore 2002), and exogenously introduced siRNAs can translationally repress mRNAs bearing imperfectly complementary binding sites (Doench et al. 2003; Saxena et al. 2003; Zeng et al. 2003).

In addition to *lin-4* regulation of *lin-14*, there are now several other miRNAs with known targets. In *C. el-*

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Article published online ahead of print. Article and publication date are at http://www.genesdev.org/cgi/doi/10.1101/gad.1184404.

egans, *let-7* regulates both *lin-41* (Reinhart et al. 2000; Slack et al. 2000) and *hbl-1* (Abrahante et al. 2003; Lin et al. 2003), and *lin-4* also regulates *lin-28* (Moss et al. 1997). In *Drosophila*, the *bantam* gene was found to encode an miRNA that regulates the proapoptotic gene *hid* (Brennecke et al. 2003). *miR-2* and *miR-13* were predicted to regulate genes containing the K-box motif (Lai 2002), and recent experimental work has validated this prediction (Boutla et al. 2003). MicroRNAs have also been implicated in fat metabolism (Xu et al. 2003) and hematopoietic lineage differentiation (Chen et al. 2004), although no targets were confirmed in these studies. Of note, these mRNAs tend to contain several binding sites for the miRNA, emphasizing the potential importance of synergistic binding of the miRNA to the target. This synergism has been directly demonstrated, as addition of multiple binding sites into a 3′ UTR resulted in more efficient inhibition of translation than that expected from the sum of the effect of each binding site individually (Doench et al. 2003).

Computational approaches have recently been used to identify potential miRNA targets (Enright et al. 2003; Lewis et al. 2003; Stark et al. 2003). The methods used by Lewis et al. (2003) and Stark et al. (2003) incorporated conservation of the mRNA target site in related organisms to separate signal from noise. Additionally, the studies by Enright et al. (2003) and Stark et al. (2003) relied on inferences from known miRNA:mRNA interactions, a relatively small data set. There are hundreds of identified miRNAs, with the vast majority of their potential targets unknown, and we thus decided to experimentally investigate the miRNA:mRNA pairing rules.

Results

As we and others have previously demonstrated, an siRNA can translationally repress a target mRNA with imperfectly complementary binding sites in its 3′ UTR, and thus the siRNA functions as an miRNA (Doench et al. 2003; Saxena et al. 2003; Zeng et al. 2003). To determine if any region of the miRNA:mRNA interaction was of primary importance, 3′-UTR constructs were designed to contain two base mismatches to the miRNA, tiled across the length of the binding site (Fig. 1A). Two identical mutant binding sites, separated by 4 nt, were flanked by two of the original binding sites, each 11 nt away, and cloned in the 3′ UTR of the *Renilla* luciferase gene. This arrangement mimics known miRNA target mRNAs, which tend to have several binding sites, and potentially allows synergetic interactions for translational repression (Ha et al. 1996; Doench et al. 2003; Lewis et al. 2003). These constructs were cotransfected into HeLa cells with a control plasmid encoding firefly luciferase, either with or without the CXCR4 siRNA. Luciferase assays revealed that mutations creating mismatches with the 5′ region of the miRNA inactivated the repression, whereas the other mutations had no effect (Fig. 1B). For example, mutant H, mismatched at positions 3 and 4, and mutant G, mismatched at positions 5 and 6, do not silence reporter expression beyond the threshold of approximately fivefold repression, which is contributed by the two flanking, original sites. The other mutants silence expression ~12-fold, which is equivalent to that observed with four original sites. As determined by ribonuclease protection assay (RPA), the CXCR4 siRNA did not have a significant effect on steady-state luciferase mRNA levels (Fig. 1C).

To test if positions 3–6 of the miRNA were uniquely important for repression, additional 3′-UTR mutants were constructed, creating individual mismatches between the miRNA and mRNA or bulges in the miRNA or mRNA (Fig. 1D). For all these constructs, interactions in the 3′ region of the miRNA were held constant, and two mutant sites were flanked by two original sites, as in Figure 1A. Luciferase assays revealed that some mutations hindered repression more than others, and that most mutations were neither fully active nor fully inactive for repression. As a means of quantifying the potential interaction, the free energy of the first 8 nt of the miRNA binding to the various UTR constructs was calculated, using the mFold server (Zuker 2003). Plotting the calculated ΔG against the fold repression revealed a strong correlation (Fig. 1E). Interactions with a free energy less than approximately –5 kcal/mole were not active in repression beyond the fivefold repression contributed by the two flanking sites, whereas those greater than –6 kcal/mole were optimally active, yielding 12-fold repression. Under these conditions, there appears to be a critical free energy required for effective repression.

The importance of interactions with the 3′ region of the miRNA was investigated in constructs in which the binding site for the 5′ region of the miRNA was held constant. Three additional mutant binding sites were made, mismatching 4 nt at a time, and a fourth mutant mismatching the entire 3′ region of the miRNA (Fig. 2A). ΔG was then calculated, again using mFold and introducing a small loop to simulate the binding of the 5′ region of the miRNA (see Materials and Methods), and plotted against fold repression (Fig. 2B). Unlike the 5′ region of the miRNA, interactions in the 3′ region were of minimal importance, as all mutants generated ~12-fold repression, with a single exception; this construct was repressed only 6.7-fold. In this case, the introduced mutations probably allowed the mRNA to form a stable hairpin, as revealed by mFold, potentially leading to decreased accessibility for the miRNA.

In the above examples, in which interactions in the 3′ region were not important, the stability of the miRNA:mRNA interaction in the 5′ region was high (–9.1 kcal/mole). If this interaction was energetically weaker but still fully effective, mutations in the 3′ region might become more important. Thus, two 5′-region mutants were combined with a 3′-region mutant, again flanked by two original CXCR4-binding sites (Fig. 2C). Whereas the 5′-region mutants each give full repression with a perfectly complementary 3′ region (11.2- and 12.1-fold repression), they yielded no repression above baseline (4.1- and 4.1-fold repression) when base-pairing in the 3′ region was very weak. We conclude that the 5′ region of

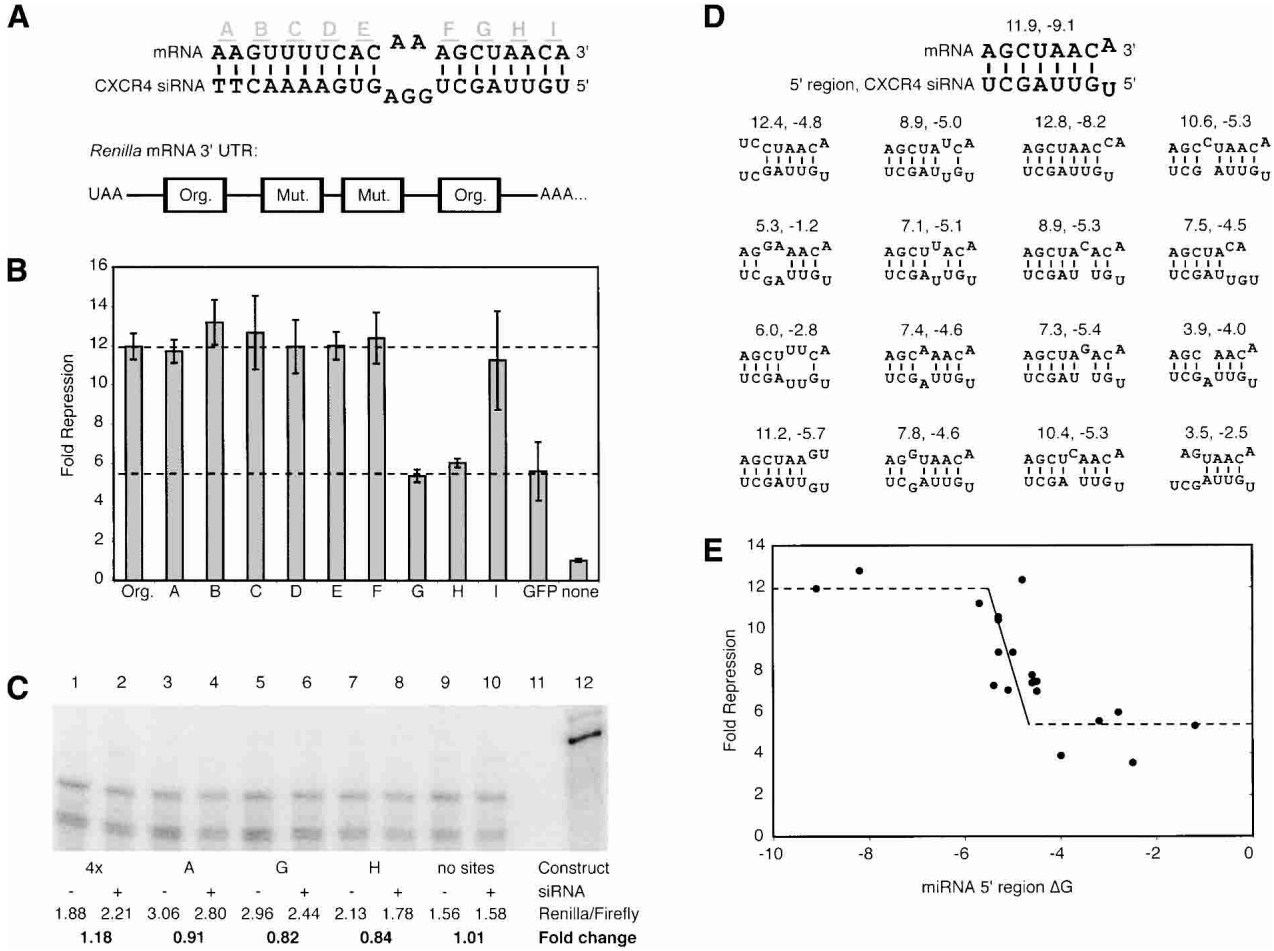

Figure 1. 5′ region of the miRNA determines translational repression. (*A*) Schematic of the CXCR4 siRNA, antisense strand, base-pairing to a designed 3′-UTR-binding site. The two 3′-most nucleotides are deoxythymidines. Mutations were made in the mRNA to form mismatches with the siRNA. In each case, the 2-nt sequence of the mRNA was mutated to that of the siRNA. For example, mutant B contains a GU-to-CA mutation. (*B*) Luciferase assay of mutant constructs. Constructs were transfected ±siRNA, and fold repression was determined. The *upper* broken line corresponds to repression with four original sites, and the *lower* broken line corresponds to repression with two original sites flanking two binding sites for an unrelated siRNA (targeting GFP), and thus serves as the lower bound for repression. The experiment was performed three times, and averages are presented ±standard deviation. (*C*) Ribonuclease protection assay of steady-state mRNA levels. The *upper* band corresponds to firefly luciferase mRNA (control), and the *lower* band to *Renilla* luciferase mRNA (targeted). Lane *12* is 5% of input probe, and lane *11* shows that no species are protected in untransfected HeLa cells. 4x is the construct with four original CXCR4 sites, and mutants A, G, and H are described in *A*. The *Renilla* mRNA level was normalized to the firefly, and then the fold change was calculated for each construct, dividing the +siRNA value into the –siRNA value; a value <1 indicates a decrease in relative *Renilla* mRNA levels. (*D*) Twelve additional mutants with alterations in the binding site for the first 8 nt of the miRNA along with mutants F, G, H, and I from *A*. The structure predicted by mFold is shown, and the original binding site is shown for comparison. The two numbers *above* each binding site correspond to the fold repression achieved and the calculated ΔG value. (*E*) ΔG for the first 8 nt of the miRNA binding to the mRNA, plotted against fold repression, for the mutants in *D* as well as mutants F–I from *A*. The broken lines correspond to the same bounds as in *B*.

the miRNA is the more important determinant of repression, but that the 3′ region can also modulate this effect.

The role of G:U wobble base pairs, which are thermodynamically favorable and are common in RNA secondary structure, was investigated in the context of miRNA:mRNA interactions. Three mutant UTRs were constructed with single G:U wobbles, and one mutant was constructed with G:U wobble at three positions. Surprisingly, a single G:U wobble was detrimental to translational repression despite having a favorable ΔG value, and three G:U wobble pairings eliminated activity entirely (Fig. 3). A G:U wobble at position 3 in the 5′ region reduced repression from 12- to 6-fold in spite of the fact that this pairing was not predicted to reduce the stability of the miRNA:mRNA interaction. Similarly, the mutant with three G:U base-pairings had a theoretical stability of –6.3 kcal/mole in the 5′ region, a value consistent with full repression with previous mutants, but was inactive in this assay.

To confirm that many of the above observations were

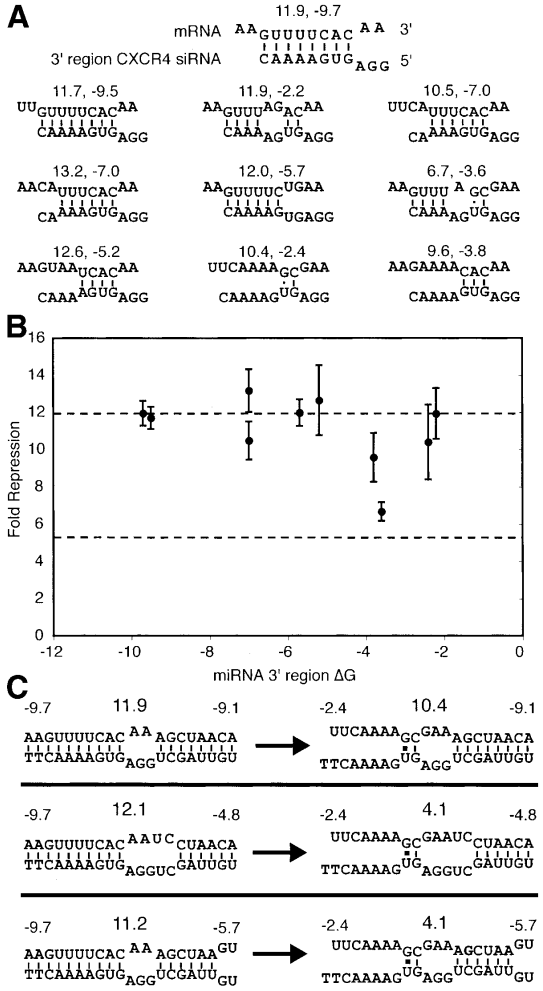

Figure 2. 3′ region of the miRNA is rarely critical for repression. (*A*) Nine mutants with alterations in the binding site for the 3′ region of the miRNA. The structure predicted by mFold is shown, and the original binding site is shown for comparison. The nine sites shown are mutants A–E from Figure 1A, and four additional mutant constructs. The two numbers *above* each binding site correspond to the fold repression achieved and the calculated ΔG value. (*B*) ΔG of the 3′ region of the miRNA binding to the mRNA was calculated, and plotted against fold repression (±standard deviation from three independent experiments). The horizontal broken lines are the same as in Figure 1. (*C*) Effect of combined 5′- and 3′-binding site mutations. The *left* column shows the original binding site and two 5′-binding site mutant constructs. The number *centered above* the binding site is the fold repression achieved, and the smaller numbers are the ΔG values for the binding of the 5′ and 3′regions of the miRNA. Each construct on the *left* was then mutated in the 3′-region-binding site.

also true for an endogenous miRNA, nine 3′ UTRs were constructed, containing two binding sites each, that are predicted to base-pair to endogenous *let-7a* miRNA with varying ΔG values in the 5′ region (Fig. 4A). We note that, unlike in previous experiments, these constructs do not contain flanking binding sites. *let-7a* was chosen because it is known to be highly expressed in HeLa cells, and paralogs expressed in HeLa cells share the same 8 nt in the 5′ region (Lagos-Quintana et al. 2001; Lim et al. 2003). Again, the degree of repression correlated with the ΔG values (Fig. 4B, gray bars). However, under conditions of pairing with endogenous *let-7a*, construct D, with a free energy value of –6.3 kcal/mole, was essentially inactive for repression. This contrasts with previous results with transfected siRNAs in which values of –5 to –6 kcal/mole were active. To determine if this difference could be due to the concentration of miRNA, the experiment was repeated with additional *let-7a* introduced as an siRNA (Fig. 4B, white bars). As expected, additional *let-7a* did not lead to any repression of constructs with weak ΔG values (constructs B, C, and E). Interestingly, only a modest increase in repression (38%) was observed for construct A, with the strongest ΔG value (–11.0 kcal/mole), yet for construct D, with a near-threshold ΔG value of –6.3 kcal/mole, additional *let-7a* miRNA greatly increased repression (189%). Thus, miRNAs likely exist in a concentration-dependent association with their binding sites, and the presence of more miRNAs increases these interactions, resulting in more repression. This model predicts that increasing the amount of mRNA would have the opposite effect. Indeed, exchanging the weak herpes virus thymidine kinase promoter for the strong CMV promoter in the construct with four original CXCR4 sites led to a dramatic decrease in repression, from 12-fold to <4-fold (data not shown).

The activity of the *let-7a* constructs also confirmed the detrimental effect of G:U wobble pairing (Fig. 4, constructs G, H, and I). A construct with a strong ΔG value, but with a G:U wobble at position 5, was not repressed with endogenous *let-7a* (construct G). Only upon addition of more *let-7a* could this construct be repressed.

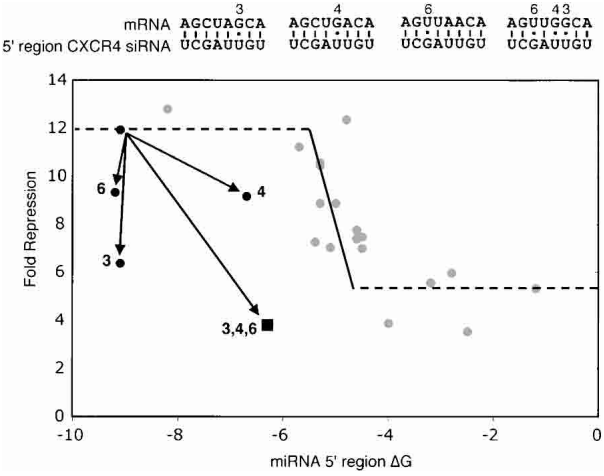

Figure 3. G:U wobble in the 5′ region of the miRNA hinders repression. The 5′ region of the CXCR4 siRNA binding to the mRNA is shown, as well as four mutant constructs that create G:U wobble pairing. These constructs were assayed and plotted on top of the data presented in Figure 1E. Arrows point from the original binding site to the four mutant constructs, and are labeled with the position of the G:U wobble. Data points indicate the average of three independent experiments.

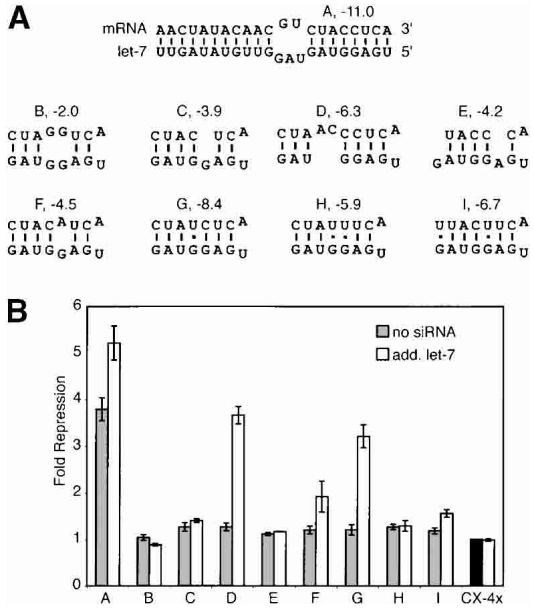

Figure 4. Endogenous *let-7a* confirms importance of miRNA 5′ region. (*A*) Schematic of a 3′-UTR-binding site, and its predicted interaction with endogenous *let-7a*, along with eight mutant binding sites for the 5′ region of endogenous *let-7a*, together with the ΔG value. Constructs G, H, and I contain G:U wobble base pairs. (*B*) Fold repression for the various constructs shown in *A*. The fold repression achieved by endogenous *let-7a* is in gray. Expression values were first normalized internally to firefly luciferase expression, then across samples to the control construct, with four CXCR4 sites, shown in black. The constructs were then transfected with additional *let-7a*, and the fold repression is shown in white, again normalized to the expression of the control CXCR4–4x construct. Values are averages from three independent experiments, ±standard deviation.

Furthermore, constructs with two G:U wobbles (constructs H and I) were not repressed by endogenous *let-7a*, nor did they significantly respond to additional *let-7a*.

We next examined the spacing requirements on the mRNA for miRNA interaction. Constructs with four original CXCR4 sites were used, and the distance between the two internal sites was varied. 3′ UTRs with the two internal CXCR4 sites spaced by 4 or 0 nt showed similar repression (Fig. 5, constructs A and B). To investigate possible steric hindrance between binding sites, constructs were designed such that the binding site for the 3′ region of one CXCR4 siRNA would overlap with the binding site for the first four 5′ nucleotides of another CXCR4 siRNA. To ensure that each internal site had a similar affinity for the miRNA, the binding site for the 3′ region was disrupted in both sites. Perhaps surprisingly, this construct showed no decrease in repression (Fig. 5, construct C). However, if this overlap between the two sites was increased to 9 nt, the construct gave the same amount of repression as only one internal site (Fig. 5, cf. constructs D and E). Because a binding site can prevent access to a sufficiently close binding site, these results suggest that a factor stably associates with the mRNA. Indeed, miRNAs are thought to act by binding to their target mRNAs rather than by a catalytic mechanism requiring only a transient association between the miRNA and mRNA.

Combinatorial regulation, in which two factors simultaneously regulate a single gene, is a common feature of eukaryotic cells. To test if a single mRNA could be repressed by more than one miRNA, two 3′-UTR constructs were made, each of which contained two sites for the CXCR4 siRNA and two sites for a GFP siRNA (Fig. 6A). To avoid possible competition between the two siRNAs for access to protein assembly factors, the siRNAs were transfected at a less than saturating concentration (1 nM). The results indicate that two miRNAs can indeed simultaneously translationally repress a single mRNA (Fig. 6B). When either construct, GFP-CXCR4-CXCR4-GFP or CXCR4-GFP-GFP-CXCR4, was transfected with either siRNA alone, the degree of repression was approximately threefold. In contrast, cotransfection with both siRNAs resulted in approximately eightfold repression. Clearly, these reporters are being regulated by both siRNAs.

Figure 5. Distance requirements for miRNA accessibility. The binding sites inserted between two original CXCR4 sites are shown; for clarity, one of the CXCR4 siRNAs is shown in gray. The distance between the two sites was progressively reduced, until the 5′ region of one site moved into the 3′ region of the adjacent site. The fold repression achieved is indicated to the *right* of each schematic, the average of three independent experiments.

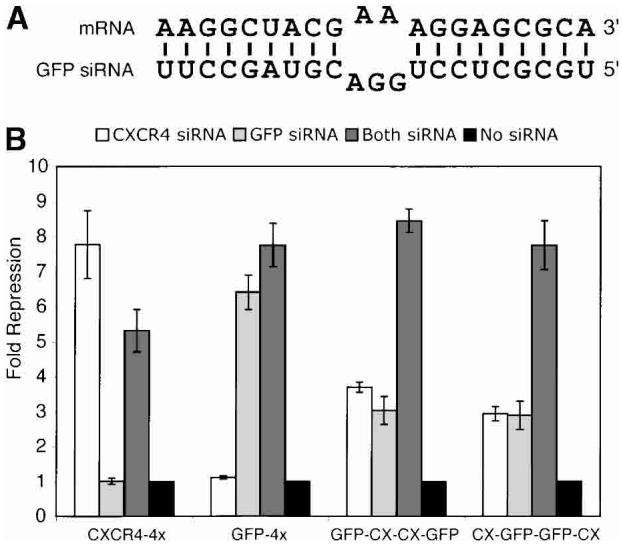

Figure 6. Two miRNAs can simultaneously repress an mRNA. (*A*) Schematic of a binding site for an siRNA originally used to target GFP. (*B*) Four constructs were transfected with either the GFP siRNA, the CXCR4 siRNA, both siRNAs, or no siRNA. One construct had four CXCR4 sites, one had four GFP sites, and two constructs had two of each, in the arrangement indicated. Fold repression was determined, normalized to the no siRNA transfection. The average of three independent experiments is shown, ±standard deviation.

Discussion

We can draw several conclusions about miRNA:mRNA interactions from this study. First, the pairing of the miRNA 5′ region to the mRNA is sufficient to cause repression, and the ΔG value of this interaction is an important determinant of activity. The 3′ region of the miRNA is less critical, but can modulate activity in certain circumstances. Interestingly, G:U wobble pairing is highly detrimental to miRNA function despite its favorable contribution to RNA:RNA duplexes. These results support conclusions of recent computational investigations into miRNA target selection (Enright et al. 2003; Lewis et al. 2003; Stark et al. 2003), but also point toward potential improvements on the various methods. For example, the study by Lewis et al. (2003) required exact complementarity between seven of the first eight nucleotides of an miRNA and its target. However, our results suggest that a model based on a free energy of interactions is likely to better capture the possible targets of an miRNA. Stark et al. (2003) used thermodynamic parameters to generate their list of targets, but ranked their targets by the overall stability of the miRNA:mRNA interaction; our data show that the 5′ region contributes more to specificity and activity. The study by Enright et al. (2003) allowed for G:U wobble pairing, but our results indicate that these interactions are strongly selected against in translational repression, perhaps as a means of preserving target specificity. Furthermore, the computational predictions allowed the possibility that a given mRNA can be regulated by more than one miRNA species, and our experiments validate this assumption.

Our studies on an endogenous miRNA, *let-7a*, indicate that a potential target must be evaluated in its cellular context. We demonstrate that a binding site that is not repressed by endogenous levels of miRNA becomes repressed upon addition of exogenous miRNA. Thus, the level of expression of both the mRNA and the miRNA, as well as potential competing binding sites on other mRNAs, need to be taken into account to determine whether the mRNA is endogenously regulated by the miRNA. Validation of predicted miRNA:mRNA interactions by ectopic expression of either the mRNA target at artificially low levels, or the miRNA at artificially high levels, may "confirm" an interaction that does not exist in vivo. It is well-established that many miRNAs are limited in their expression to certain stages in development or to certain tissues and cell types (Bartel 2004). Computational prediction would be aided by taking into consideration expression profiling of both miRNA and mRNA levels, and biochemical methods or genetic analysis may be needed for definitive proof of an miRNA:mRNA interaction.

This study brings into focus the question of miRNA specificity. Indeed, miRNAs are an abundant species of RNA both in terms of the sheer number of miRNAs in the genome, currently estimated at 200–255 for the human genome (Lai 2003), and in terms of their expression levels, as some miRNAs are expressed at >1000 copies per cell (Lim et al. 2003). Additional factors may also be important for determining in vivo targets of miRNAs, such as the FMRP protein, a known regulator of mRNA translation that has been implicated in RNA silencing complexes (Caudy et al. 2002; Ishizuka et al. 2002). Alternatively, specificity may be entirely dictated by the sequence of the miRNA itself. That the thermodynamic stability of a region spanning only 8 nt, a surprisingly low information content, is sufficient for miRNA activity may indicate a broad role for miRNAs in the regulation of gene expression.

Materials and methods

Plasmid construction

Two original CXCR4 sites, with XhoI and SpeI restriction sites between them, were inserted into the XbaI site in the 3′ UTR of the pRL-TK plasmid (Promega). The mutant binding sites were then inserted by ligating annealed oligonucleotides into the XhoI and SpeI sites. Oligonucleotides were purchased from QIAGEN, and all constructs were confirmed by sequencing. The *let-7a* and GFP constructs were made with the same strategy.

Cell culture and transfections

HeLa cells were maintained in DMEM with 5% calf serum and 5% inactivated fetal bovine serum, supplemented with glutamine and penicillin/streptomycin. The day before transfection, cells were seeded at 10^5 cells/well in a 24-well plate in antibiotic-free media, such that they would be 95% confluent at the

time of transfection. Transfections were done with Lipofectamine 2000 according to the manufacturer's protocol (Invitrogen). Here, 0.7 μg of pRL-TK plasmid and 0.1 μg of pGL3-Control plasmid (Promega) were used per well, and each sample was transfected in duplicate or triplicate. Transfections were done in a final volume of 0.5 mL, using siRNA at a final concentration of 5 nM (~0.03 μg). siRNAs were purchased from Dharmacon and prepared according to the manufacturer's instructions. Luciferase assays were performed 24 h after transfection using the Dual-Glo luciferase kit (Promega).

Ribonuclease protection assay

HeLa cells were transfected in six-well plates by scaling up the 24-well plate protocol by a factor of 5. Total RNA was collected with the RNAeasy kit, including an on-column DNase treatment (QIAGEN), 24 h after transfection. RNA probes were constructed by cloning PCR products into TOPO vectors (Invitrogen). The pGL3 probe corresponds to nucleotides 1142–1429 and was cloned into pCRII-TOPO, and the pRL-TK probe corresponds to nucleotides 1068–1297 and was cloned into pCR2.1-TOPO (position 1 of the plasmid as defined by the manufacturer). Transcription templates were linearized by SpeI restriction digestion (New England Biolabs) and transcribed in the presence of radiolabeled CTP (Perkin Elmer) using the T7 MAXIscript kit (Ambion). To allow for equivalent signals from the two mRNAs, the firefly luciferase probe was made with a fivefold lower specific activity. Ribonuclease protection assays were then performed with the RPA III kit, using 10 μg of RNA (Ambion). Gels were visualized on a Molecular Dynamics Storm 860 PhosphorImager, and quantitated with ImageQuant software version 1.2.

mFold analysis

To determine ΔG values for the binding of the 5′ region of the miRNA, the various mRNA-binding sites were entered followed by "LLL" and then the first 8 nt of the miRNA. The LLL tells mFold to treat the sequence as two separate RNA strands, and thus the initiation free energy, ΔI, is properly incorporated into the ΔG value (Zuker 2003). To determine ΔG values for the 3′ region, the mRNA-binding sites were entered followed by a loop of sequence nnnGGGnnnnCCCnnn and then the 3′ region of the miRNA. The ΔG value of the loop alone is –1 kcal/mole, and this is included in the data shown. Because the siRNA used had two deoxythymidines at the 3′ end, these were omitted from the free energy calculations, as indicated in the figures.

Acknowledgments

The authors thank C. Petersen for consistently helpful discussions and R. Bodner and D. Bartel for a critical reading of the manuscript. We also thank the Burge and Bartel labs for sharing data prior to its publication. J.G.D. is a Howard Hughes Medical Institute Predoctoral Fellow. This work was supported by United States Public Health Service MERIT Award R37-GM34277 from the National Institutes of Health and PO1-CA42063 from the National Cancer Institute to P.A.S. and partially by a Cancer Center Support (core) grant P30-CA14051 from the National Cancer Institute.

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