How Do MicroRNAs Regulate Gene Expression?

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Several thousand human genes, amounting to about one-third of the whole genome, are potential targets for regulation by the several hundred microRNAs (miRNAs) encoded in the genome. The regulation occurs posttranscriptionally and involves the ~21-nucleotide miRNA interacting with a target site in the mRNA that generally has imperfect complementarity to the miRNA. The target sites are almost invariably in the 3′-untranslated region of the messenger RNA (mRNA), often in multiple copies. Metazoan miRNAs were previously thought to down-regulate protein expression by inhibiting target mRNA translation at some stage after the translation initiation step, without much effect on mRNA abundance. However, recent studies have questioned these suppositions. With some targets, an increase in the rate of mRNA degradation by the normal decay pathway contributes to the decrease in protein expression. miRNAs can also inhibit translation initiation, specifically the function of the cap-binding initiation factor, eIF4E. Repressed target mRNAs as well as miRNAs themselves accumulate in cytoplasmic foci known as P-bodies, where many enzymes involved in mRNA degradation are concentrated. However, P-bodies may also serve as repositories for the temporary and reversible storage of untranslated mRNA, and reducing the expression (knockdown) of several distinct P-body protein components can alleviate miRNA-mediated repression of gene expression.

Introduction

A PubMed search of "microRNA" or "miRNA" gives just over 1200 citations, all of them published since the turn of the millennium. These numerous publications have been concerned primarily with using experimental and in silico approaches to identify miRNAs and the target genes that they regulate in various organisms, elucidating the biogenesis of miRNAs, and investigating how the spectrum of miRNAs differs in different tissues and disease states. Thus, by mid-2005, it was known that the human genome encodes several hundred different microRNAs (*1*) and that the pattern of miRNA expression is often perturbed in disease states (*2*–*4*). Bioinformatic approaches (*1*) further suggested that the mammalian miRNA repertoire might collectively regulate several thousand genes, even though only a handful of these predicted targets have been validated to date. By contrast, very few of these studies addressed the mechanism of miRNA-mediated repression. Primary publications and reviews written before that date generally proposed that miRNAs (i) do not promote degradation of their target mRNAs, but (ii) they do down-regulate target mRNA translation at some stage after the initiation step.

Over the past 18 months, there has been a steady flow of provocative reports on mechanisms of miRNA-mediated repression, and, far from consolidating and building upon these earlier ideas, many of them directly contradict one or another of these original assertions. The aim of this Review is to illuminate and explain the controversies generated by these recent publications. However, we have been unable to resolve many of the apparent contradictions, and our advice to the reader is to keep an open mind to the possibility that there may be more than one mechanism by which miRNAs effect posttranscriptional regulation of gene expression.

This Review will therefore focus specifically on mechanisms, with particular reference to metazoans. Lower eukaryotes are mentioned only to the extent that they have contributed to our understanding of mechanisms likely to operate in higher organisms, and plants are ignored altogether, as the mechanisms of plant miRNA biogenesis and action seem distinctly different. Mechanisms of RNA interference (RNAi) by short interfering RNAs (siRNAs) are also beyond the scope of this Review except insofar as they relate directly to miRNA-mediated repression.

siRNAs Versus miRNAs: Definitions, Similarities, and Differences

The generally accepted distinction between "miRNAs" and "siRNAs" is that the former are ~21-residue RNAs derived from longer RNAs that include a ~70-nucleotide (nt) imperfectly base-paired hairpin segment that is the precursor of the mature miRNA, whereas siRNAs are of similar length but are derived from longer, perfectly complementary double-stranded RNA precursors of endogenous or exogenous origin (*5*). In both cases, biogenesis proceeds through a staggered duplex intermediate, usually with ~19 base pairs (bp), and always with unpaired 3′ extensions of 2 nt and a 5′-phosphate on each strand. Especially in the case of mammalian cells, these ~21-nt RNAs are often introduced by transfection of chemically synthesized versions of this staggered duplex RNA, or alternatively as DNA constructs that will give rise to such a duplex after transcription and processing. One of the two strands, the one with the lower-stability base-pairing at its 5′ end, is then assimilated into a complex with several proteins, while the other, the so-called passenger strand, is degraded [reviewed in (*6*)]. The resulting RNA-protein complex is generally known as RISC (RNA-induced silencing complex), or for miRNAs, it is sometimes known as an miRNP (miRNA-protein complex).

For RNAi, the 21-residue siRNA, or its longer double-stranded RNA precursor, is designed to be perfectly complementary to part of the target mRNA sequence, which leads to endonucleolytic cleavage of the mRNA at the site of complementarity (*6*),

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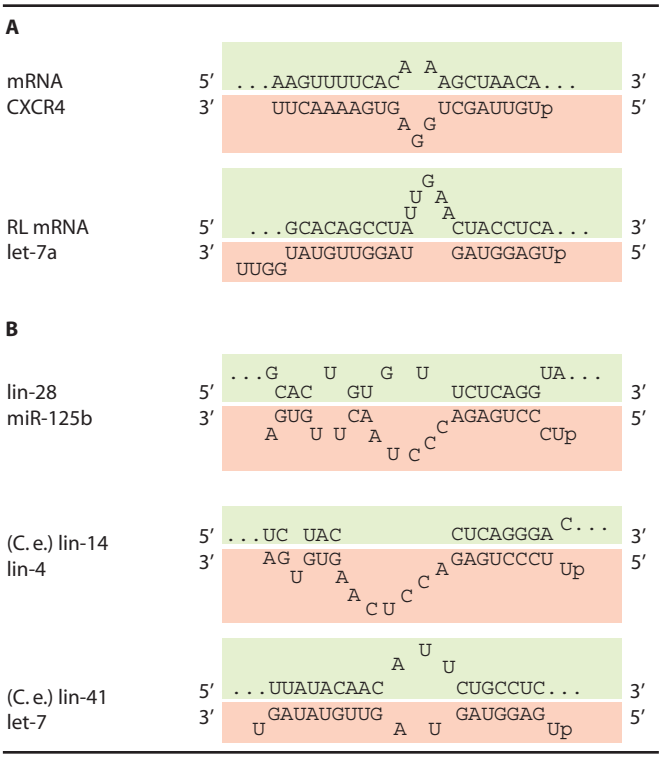

Fig. 1. Examples of the imperfect complementarity between various miRNAs and their bulged target sites. Micro-RNA sequences are in red, mRNA target sites in green. The two miRNA/mRNA pairs in (**A**) have been widely used in studies of the mechanisms of miRNA-mediated repression discussed here. The first is an artificial system based on an siRNA designed for RNAi of CXCR4 mRNA, but adapted to serve as a miRNA mimic through the design of bulged target sites in the reporter mRNA 3′-UTR. The second is the interaction of (endogenous) let-7a miRNA with a reporter target site based on the predicted interaction of let-7a with human *lin-28* mRNA. The three examples in (**B**) are endogenous miRNAs and their predicted (miR-125b/human *lin-28* mRNA) or validated (*C. elegans lin-4* and *let-7* miRNAs) targets sites. Relevant references cited in this work are as follows (note that these are not necessarily the publications that first identified the miRNA/target site interaction): mRNA/CXCR4 (*8*, *11*, *12*, *33*, *79*); RL mRNA/let-7a (*29*); *lin-28*/miR-125b (*47*); (C.e.) *lin-14/lin-4* (*22*–*25*, *49*); (C.e.) *lin-41/let-7* (*49*).

followed by degradation of the two fragments by enzymes of the normal mRNA decay pathways. Cleavage may not be entirely abrogated by a very limited number of mismatches, depending on their position, and so miRNAs that have a similar near-perfect complementarity to their target sites can also promote degradation by this route (*7*). Generally, however, the interaction of an miRNA with its predicted or validated target involves quite extensive mismatched bulges, especially in the central region (Fig. 1), and to a lesser extent in the miRNA 3′ end. This type of interaction does not generally lead to mRNA degradation via endonucleolytic cleavage, but results in a decrease in protein expression that is usually (but not always) greater than the decrease in mRNA abundance, suggesting that the main reason for down-regulation is reduced efficiency of translation rather than increased mRNA degradation. Validated and predicted miRNA target sites are almost invariably located in the 3′-untranslated region (3′-UTR) of a gene, often in multiple copies.

It is important to note that the formal distinction between an siRNA and an miRNA is based solely on their different origins and biogenesis, irrespective of the degree of complementarity to the target site and irrespective of whether the ~21-nt RNA promotes endonucleolytic cleavage of the target mRNA, or repression of the target by some other mechanism. According to these definitions, a ~21-nt RNA introduced into cells by transfecting a chemically synthesized staggered duplex intermediate should be called an "siRNA," even if its sequence has been designed to be precisely the same as that of a natural miRNA. This is a potential source of confusion to those outside the immediate field, and in order to avoid such difficulties, it might be preferable to refer to such an RNA as a si(mi)RNA, to highlight the fact that although it is technically an siRNA, in fact it is one that is designed to exactly mimic a bona fide miRNA.

Because the different outcomes depend on the degree of complementarity between the short RNA and its target, it follows that a ~21-nt RNA designed to promote endonucleolytic cleavage of a particular mRNA with a perfectly complementary target site could also act as a translational repressor of another mRNA if this has appropriate mismatched bulged target sites. The validity of this argument has been demonstrated for an siRNA designed for RNAi of mRNA encoding the CXCR4 chemokine receptor (*8*). This siRNA has subsequently been widely used to study how interaction of a ~21-nt RNA with imperfectly complementary 3′-UTR sites results in repression of mRNA translation. For the reasons explained above and because the sequence of this small RNA shows no resemblance to any known natural miRNA, we will refer to it as CXCR4 siRNA, but, except where otherwise stated, it should be understood that it is always being used in conjunction with mismatched target sites and is therefore serving as a surrogate for a typical microRNA.

The mismatch between an miRNA and its target site can take on different configurations, with a central unpaired bulge in either the miRNA or mRNA strand, or both. Figure 1 shows both natural miRNAs paired to their natural target, and the pairing of the artificial CXCR4 siRNA with its laboratory-designed target site. In the latter case, the exact configuration of the unpaired bulge(s) affects the degree of repression, but this may not be applicable for other miRNA-mRNA pairs (*9*). Two extensive studies of how target site sequence and complementarity influence repression by either natural *Drosophila* miRNAs overexpressed in the wing imaginal disc or by the CXCR4 siRNA transfected into HeLa cells are in very close agreement as to what is important (*10*, *11*). First and foremost, residues in the 5′ portion of the miRNA (residues 2 to 8, the so-called seed) upstream of the discontinuity should be (almost) perfectly complementary to the mRNA. The degree of repression is dependent on the stability of the pairing in this region, with a rather sharp cut-off, and in addition, A-form geometry seems important because G-U pairs in this region decrease repression to a greater extent than can be explained purely on the grounds of mRNA stability. If the 5′ portion of the miRNA is optimally base-paired, pairing between the 3′ portion and the mRNA target is not critical, but a high degree of complementarity here can rescue repression when the pairing of the miRNA "seed" is marginally suboptimal. In the CXCR4 system, the degree of repression is related to the number of target sites, at least up to six such sites, the maximum tested so far (*8*, *12*). In addition, different miRNAs can act in a combinatorial

way, in the sense that cotransfection of two different siRNAs with a reporter construct that had two 3′-UTR bulged target sites for each of them resulted in a similar degree of repression, as when there were four identical target sites for just one of the siRNAs (*11*). Many endogenous mRNAs have just one or two predicted sites for interaction with endogenous miRNAs, yet seem to be quite efficiently repressed, which raises questions as to whether the artificial systems such as CXCR4 may be intrinsically less potent repressors in some way. This idea is given some support by recent indications that the sequences flanking miRNA target sites, or the context of such sites, can influence the biological outcome of miRNA-mRNA interactions (*13*).

The contrasting outcomes that are dependent on whether interaction of the ~21-nt small RNA with the mRNA target site is perfectly complementary or has substantial bulged mismatches are presumably due to different proteins being present in the RISC and miRNP or, if the protein composition is really identical, then to the degree of complementarity influencing how these proteins act. The protein compositions of RISC assembled on perfectly complementary siRNAs of exogenous origin, and the miRNPs formed with endogenous miRNAs, are often described as similar or overlapping rather than absolutely identical. However, as the functional distinction between between a perfectly complementary interaction and one with mismatches is only apparent after the ~21-nt RNA has interacted with the target site, any differences are likely to arise concomitantly with, or shortly after, the establishment of this interaction.

Both types of complex include members of the Argonaute family of proteins. Of the four mammalian Argonaute proteins, only Ago2 is capable of siRNA-mediated endonucleolytic cleavage, with its ribonuclease H (RNase H)–like domain cutting the phosphodiester bond in the target mRNA opposite the 10th and 11th residue in the siRNA (*14*, *15*). Cocrystal structures show that the 5′-phosphate on the siRNA is essential to place this phosphodiester bond in the active site (*16*, *17*). Such endonucleolytic cleavage would not be expected with most miRNA-mRNA interactions because the A-form geometry would be severely disrupted by the unpaired bulges in this region (Fig. 1).

Knockdown (via RNAi) of each Ago protein individually in human embryonic kidney (HEK) 293 cells showed that Ago2 was the major contributor to miRNA-mediated repression of target mRNA translation (*18*). Nevertheless, there is evidence that Ago1, Ago3, and Ago4 are each capable of promoting this repression to some extent (*14*, *19*), even though they have no known enzymatic activity. In contrast, the two Argonaute proteins in *Drosophila* appear to have largely nonoverlapping functions, with Ago1 involved mainly or exclusively in miRNA-mediated repression, and Ago2 restricted to siRNA-mediated endonucleolytic cleavage (*20*, *21*).

Evidence That miRNAs Can Inhibit Translation at Some Stage After Initiation

The pre-2005 hypothesis that miRNA-mediated repression of target gene expression is due to inhibition of mRNA translation at some stage after the initiation step was based on just two studies of a single *Caenorhabditis elegans* miRNA, *lin-4* miRNA (the first microRNA to be discovered, long before the designation "microRNA" had been invented), and two larval mRNA targets, *lin-14* mRNA, which has up to seven potential 3′-UTR sites for bulged interaction with *lin-4* miRNA (*22*, *23*), and *lin-28* mRNA. Expression of *lin-4* miRNA commences late in the L1 larval stage, and by the late L2 or early L3 stage, the amounts of LIN-14 and LIN-28 proteins are less than 10% of that seen in mid-L1, but the abundance of *lin-28* mRNA is essentially unchanged, whereas that of *lin-14* mRNA is about half of that in the L1 stage (*24*, *25*), with no appreciable difference in poly(A) tail length (15 to 30 A residues).

In sucrose gradient analyses of polysomes at the late L2 or early L3 stage, the repressed *lin-14* and *lin-28* mRNAs were found in the same fractions as in a polysome distribution analysis of mid-L1 larvae, where both would be efficiently translated (*24*, *25*). When these polysomes from late L2 larvae were analyzed on gradients containing EDTA, both mRNAs were found near the top of the gradient, and on metrizamide gradients the *lin-14* mRNA was found in fractions of the same buoyant density as those containing polysomes from L1 larvae (*24*, *25*). Thus, by these two commonly used criteria, *lin-14* and *lin-28* mRNAs appeared to be genuinely in polysomes in late L2 and early L3 larvae, even though their translation was repressed at these stages. These tests do not, however, prove that the polysomes are dynamic and capable of elongation, an issue that can be examined by seeing whether the repressed mRNA moves into smaller polysomes on incubation with puromycin, or with a specific inhibitor of initiation (polysome runoff assays). These functional tests are best done in intact cells, in case the translational repression might be relieved by the mere act of preparing a cell-free extract, and should be done over a relatively short time period (~5 min) so that differences in the rate of reaction with puromycin or the rate of runoff can be detected. However, puromycin and specific initiation inhibitors do not penetrate *C. elegans* larvae, and so the best that could be done was to isolate polysomes from early L3 larvae and add them to reticulocyte lysate for polysome runoff, which showed that in a rather long incubation period (45 min), the *lin-28* mRNA moved out of polysomes as effectively as did an mRNA that was not repressed in the L3 larvae (*25*). The question of whether this runoff produced any full-length LIN-28 protein was not examined.

The results described above are not a peculiarity of *C. elegans* larval development. Similar results were obtained when DNA transfections of 293T cells were used to study the repression of a luciferase reporter with six bulged sites for interaction with CXCR4 siRNA. Introduction of the CXCR4 siRNA reduced overall luciferase expression by more than 95%, with relatively little effect on target mRNA abundance (*12*). Likewise, the presence or absence of the CXCR4 siRNA had no influence on the polysomal distribution of the target mRNA, nor did it affect the movement of this mRNA into smaller polysomes on a brief (3 min) incubation of the cells with puromycin. On a 5-min incubation with a specific inhibitor of initiation, the target mRNA moved into smaller polysomes, as expected, but the shift was distinctly greater under conditions of repression by CXCR4 siRNA. This was interpreted as premature ribosome drop-off, or a failure of processivity (*12*). However, if such drop-off was occurring continuously, it is hard to see why the steady-state polysomal distribution of the target mRNA under repressed conditions would be so similar to the unrepressed distribution, unless there was a fortuitous increase in initiation frequency that precisely compensated for the decrease in ribosome transit time. A plausible alternative explanation is that the target mRNA is slightly more sensitive to the initiation inhibitor when it is repressed by the CXCR4 siRNA than it is under unrepressed conditions.

In this system, both cistrons of a dicistronic reporter with either the hepatitis C virus (HCV) or cricket paralysis virus

(CrPV) IRES (internal ribosome entry site) driving downstream cistron translation were susceptible to repression by CXCR4 siRNA, with the downstream cistron possibly even more sensitive than the upstream one (*12*). Because initiation driven by the CrPV IRES occurs by a highly unusual mechanism not requiring any of the canonical translation initiation factors (*26*), this result is consistent with the view that the repression mechanism does not operate at the stage of initiation, but at some later step.

Cosedimentation of miRNAs with polysomes has been taken as further evidence that repression occurs on target mRNAs when they are actually in polysomes, and this is a valid argument in cases where most of the cellular complement of a particular miRNA is polysome-associated. However, if there is only a minor fraction cosedimenting with polysomes, this could possibly represent miRNAs associated with those mRNA molecules that are not fully repressed. In late L2 stage *C. elegans* larvae, some 10% of the total *lin-4* miRNA cosedimented with polysomes (*24*). Similarly, a small fraction of the miR-124a present in a human neuronal cell line cosedimented with polysomes in an EDTA-sensitive manner (*27*), and a more substantial fraction of this and seven other miRNAs examined was polysome-associated in rat forebrain neurons (*28*).

It is difficult to produce an explanation for an mRNA having almost exactly the same polysomal distribution under conditions of both strong miRNA-mediated repression and no repression. As Olsen and Ambros pointed out when this was first discovered for *lin-14* mRNA, inhibition of termination or an inhibition of elongation that is less than 100% complete would cause polysomes to increase in size (*24*). Conversely, an inhibition of elongation that is literally 100% complete will freeze preexisting mRNA in polysomes, but any newly synthesized mRNA will become associated with only a monomeric 80*S* ribosome. In the context of miRNA regulation of translation, neither of these scenarios has, to our knowledge, ever been observed. On the basis of polysome dynamics alone, an miRNA-mediated 90% decrease in protein output with no change in the polysome distribution of the target mRNA would require a 90% reduction in initiation frequency coupled with a balancing 90% reduction in elongation rate, a somewhat implausible scenario.

These considerations led to the suggestion, likewise made by Olsen and Ambros, that initiation and elongation rates on the repressed mRNA may be normal, and the strong reduction in protein product yield could be the result of a specific cotranslational degradation of the nascent protein chain (*24*). For this reason, the question of whether any full-length protein product can be detected following polysome runoff, or in pulse-labeling experiments, is important. In the Petersen *et al*. experiments, pulse-labeling for 3 min failed to detect any product (*12*), even though the reporter had an N-terminal epitope tag to facilitate product detection, an observation that at face value is consistent with the cotranslational degradation hypothesis.

However, there is an ingenious experiment that argues otherwise. An mRNA encoding a protein with an N-terminal signal sequence for targeting the product to the endoplasmic reticulum (ER), and with three bulged sites for let-7a miRNA, was just as susceptible to repression by endogenous HeLa cell let-7a as was a luciferase reporter that encoded a cytoplasmic product (*29*). Because the ER-targeted nascent protein chain is unlikely to be accessible to any cytoplasmic proteolytic system, this result argues against the cotranslational degradation hypothesis. It should be noted, however, that in other experiments these authors found a polysome distribution of the repressed mRNA suggestive of inhibited initiation (see below), and thus it remains a possibility that cotranslational degradation of the nascent protein might occur specifically in those experimental conditions where the repressed mRNA cosediments with large polysomes.

Evidence That miRNAs Can Inhibit Initiation Dependent on eIF4E

The idea that miRNAs regulate mRNA translation at some stage after the initiation step was challenged by the publication of two papers about a year ago that point toward initiation as the regulated step. The first, by Pillai *et al*., studied repression by endogenous let-7a miRNA in HeLa cells, using luciferase reporters with three bulged target sites in the 3′-UTR, with sequestration of let-7a using an antisense 2′-*O*-methyl oligonucleotide serving as a control (*29*). In DNA transfections, luciferase expression was reduced by 80 to 90% at 48 hours after transfection, but reporter mRNA abundance was reduced by 20%. Polysome profiles showed a distribution of the repressed mRNA that was skewed toward the top of the gradient, peaking in the 40*S* to disome region, strongly indicative of inhibited initiation (*29*). A similar sucrose gradient distribution of endogenous cationic amino acid transporter–1 mRNA was seen under conditions where it was repressed by endogenous miR-122 in Huh7 cells, which shows that this distribution is not a peculiarity of overexpressed luciferase reporter targets (*30*).

In direct RNA transfections assayed at 6 hours after transfection, 7-methylguanosine (m^7GpppG)–capped polyadenylated mRNAs (where ppp represents a triphosphate moiety) that would be translated by the scanning mechanism were susceptible to repression by let-7a, and were only slightly less sensitive to this repression if the poly(A) tail was absent. By contrast, a m^7GpppG-capped polyadenylated reporter driven by the HCV IRES was completely resistant, as were uncapped or adenosine (ApppG)–capped nonpolyadenylated mRNAs dependent on the encephalomyocarditis virus (EMCV) IRES (*29*). Because initiation driven by the EMCV IRES requires all the canonical initiation factors except eIF4E, the cap-binding factor (*31*), and the HCV IRES functions independently of eIFs 4A, 4B, 4E, and 4G (*32*), this result indicates that the repression mechanism affects the function of eIF4E rather than any other factor.

These results are markedly similar to those in the second paper, by Humphreys *et al*., who cotransfected HeLa cells with CXCR4 siRNA and various luciferase reporter mRNAs, each with four bulged target sites for this siRNA (*33*), the very same reporters as used by the Sharp group (*8*, *11*). At 16 hours after transfection, the degree of CXCR4 siRNA-dependent repression of a m^7GpppG-capped polyadenylated mRNA was greater than 80% or about 50% if the poly(A) tail was absent. In addition, 50% repression was seen with an ApppG-capped polyadenylated mRNA that would be translated by the scanning ribosome mechanism, and an ApppG-capped polyadenylated mRNA with an EMCV IRES, whereas no repression was detectable with nonpolyadenylated versions of these RNAs or an ApppG-capped nonpolyadenylated mRNA with the CrPV IRES (*33*).

The sensitivity of the m^7GpppG-capped mRNA to repression, contrasted with the lack of response of the nonpolyadenylated mRNAs with the viral IRESs, is in complete agreement with Pillai *et al*. and points to the same conclusion—an inhibition of eIF4E function (*29*, *33*). However, although the nonpolyadenylated EMCV IRES construct was not repressed, adding a poly(A) tail

increased its translation efficiency (in the absence of the CXCR4 siRNA) by a factor of ~2.5, but rendered it susceptible to a 50% repression by this siRNA (*33*). In other words, the siRNA (almost) completely negated the stimulatory effect of the poly(A) tail. This suggests that apart from any effect on eIF4E function, the CXCR4 siRNA may also interfere with the "closed loop," in which poly(A) binding protein (PABP) bound to the poly(A) tail interacts with the eIF4G component of the eIF4F complex (Fig. 2) bound either to the 5′ end of the mRNA (strongly in the case of m^7GpppG-capped RNAs and more weakly with an ApppG-cap) or to an internal site in the EMCV IRES (*34*, *35*). This poly(A)-PABP–dependent "closed loop" enhances the efficiency of initiation of both scanning-dependent mRNAs and mRNAs dependent on the EMCV IRES, but the HCV IRES is unaffected by the presence or absence of a poly(A) tail (*36*–*39*).

One can envisage two alternative ways by which the CXCR4 siRNA could disrupt the "closed loop," either via a disruption of the protein-protein and protein-RNA interactions necessary to maintain the loop, or by simply promoting deadenylation of the mRNA. Although no notable effect of the siRNA on overall reporter mRNA stability was detected in these experiments, the methods used would not have detected changes in poly(A) tail length (*33*).

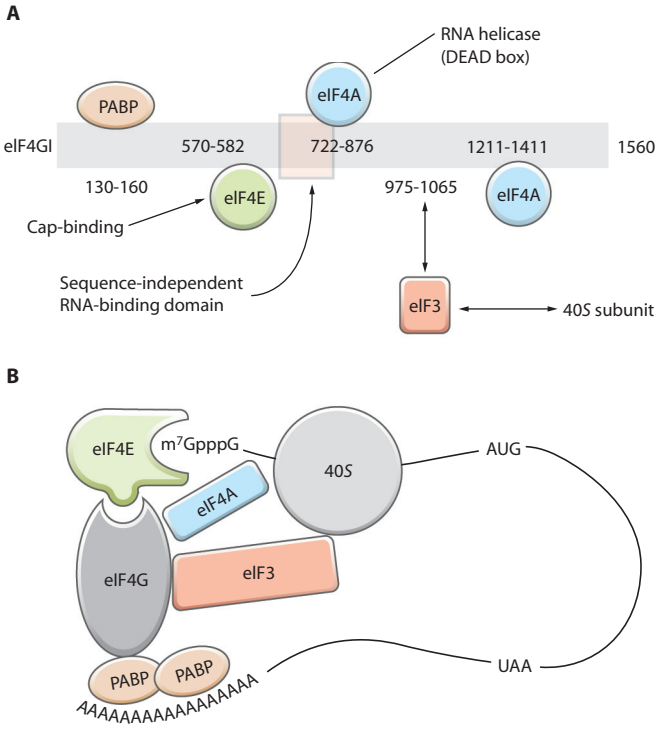

Fig. 2. Schematic diagram of (**A**) the eIF4F complex and (**B**) the "closed loop" formed by the interaction of PABP bound to the poly(A) with the eIF4G component of the eIF4F complex bound to the 5′-cap. Mammalian cells have two eIF4Gs encoded by different genes. Various isoforms of the more abundant species, eIF4GI, arise from initiation at different AUG codons by leaking scanning. The isoform shown, which is generally the most abundant, is the second largest and lacks the 40 N-terminal amino acids of the longest form. Apart from the direct interactions with eIF4A, eIF4E, and PABP, eIF4G also interacts directly with eIF3, which itself binds tightly to the 40*S* ribosomal subunit. Thus, there is an indirect eIF4G/40*S* subunit interaction bridged by eIF3, and this is thought to be instrumental in loading the 40*S* subunit on the mRNA and in facilitating ribosome scanning.

Can These Apparently Conflicting Results Be Reconciled?

There are two, apparently unrelated, discrepancies between the data described above and the results of Petersen *et al.*: (i) the difference in polysome distribution of the repressed mRNA, and (ii) the issue of whether the viral IRESs are insensitive to repression (*12*, *29*, *33*). Although technical details of the polysome analyses are not identical, there is no obvious difference that might explain the disagreement. Both Pillai *et al.* and Petersen *et al.* used DNA transfections for these experiments, albeit over different time periods (48 and 20 hours, respectively) (*12*, *29*). One clear difference is that Pillai *et al.* relied on endogenous let-7a to effect the repression (*29*), whereas Petersen *et al.* used exogenous CXCR4 siRNA (*12*), which might well have resulted in a higher intracellular concentration of the short RNA. This, together with the fact that Petersen *et al.* had six bulged target sites in their reporter as compared to just three in the reporter used in the other paper, may account for the higher repression in their experiments, more than 95% as compared to 80 to 90% in Pillai *et al.* One possibility is that at lower miRNA/mRNA target ratios and lower repression ratios, inhibition is exclusively or largely at the initiation step, whereas with increasing severity of repression a secondary effect on elongation rate or processivity sets in.

As for the issue of whether IRES-dependent mRNAs are insensitive to repression, in this case there are differences in procedure that deserve consideration as a possible explanation for the discrepancy: DNA transfections of constructs that would produce a capped dicistronic mRNA (*12*), as opposed to RNA transfections of monocistronic IRES-containing RNAs (*29*, *33*). The capped dicistronic mRNA generated in the Petersen *et al.* procedure would certainly bind the eIF4F complex (Fig. 2), and the expected repression of the upstream cistron was indeed observed (*12*). Is it possible that this repression of the upstream cistron, however it may occur, could spread so as to also affect the downstream cistron? Unfortunately, this conjecture is undermined by the results obtained by Pillai *et al.* with another type of dicistronic mRNA (*29*), in which downstream cistron expression was driven, not by a natural viral IRES, but by tandem phage λ Box B sites in the intercistronic spacer, and coexpression of an eIF4G-λ N peptide fusion protein, which would bind specifically to the Box B sites (Fig. 3C). In DNA transfection assays, the presence of three bulged target sites for let-7a in the 3′-UTR of this dicistronic construct resulted in 65% repression of upstream cistron expression with apparently no effect on the downstream cistron (*29*).

Different cell types and different miRNAs are unlikely to explain the conflicting evidence implicating different mechanisms of miRNA action. Petersen *et al.* and Humphreys *et al.* used precisely the same siRNA (CXCR4) and reporters with bulged target sites of precisely the same sequence (though the number of sites differed), yet came to completely different conclusions (*12*, *33*). Likewise, there appears to have been sufficient overlap of different cell types used by each group to be able to eliminate this as the explanation. Thus, there is no clear evidence pointing to one of the two radically different outcomes being the right answer and the other wrong, and the collective published data show no sizable majority vote in favor of either explanation. If this and other

recent reviews (*40*) appear to have a bias in favor of initiation being the regulated step, this is probably because there are numerous precedents for regulation of eukaryotic initiation by protein-mRNA interactions at 3′-UTR sites, but no precedents for cotranslational degradation of the nascent protein, or for high-frequency premature ribosome drop-off. However, a more detached and unbiased position would take the view that miRNAs may be able to mediate repression by either mechanism, but as these two outcomes are so radically different, this would also imply that there must be at least one unrecognized or uncontrolled variable that determines which of the two putative alternative mechanisms is predominant in particular experimental situations.

There are actually some precedents, dating from over 20 years ago, for unexpected polysome association of near-silent mRNAs. Heat shock of *Drosophila* cells generally leads to a complete disappearance of polysomes (due to inhibition of initiation) followed by the appearance of polysomes specifically translating heat-shock mRNAs. However, in one report, some 30 to 50% of the housekeeping mRNAs were still polysome- associated 1 hour after heat shock, even though the expression of the encoded proteins was reduced by at least 90% (*41*). Similarly, prolonged incubation of HeLa cells with amino acid analogs induces the synthesis of stress proteins and shuts off synthesis of housekeeping proteins, yet the mRNAs encoding these proteins remain largely polysome-associated (*42*). Inhibition of initiation was thought to be the immediate response to such stress, but it was suggested that an additional elongation defect might set in somewhat later. More recent confocal microscopy work reveals an interesting parallel with miRNA-mediated repression in that these near-silent housekeeping mRNAs would be largely localized in cytoplasmic bodies known as stress granules (*43*, *44*), whereas mRNAs subject to miRNA-mediated repression localize to another type of cytoplasmic foci known as P-bodies (see below).

Of course, the big difference between these situations and miRNA-mediated repression is that the latter is selective for (one or a few) particular mRNA species, whereas the stress conditions affect the behavior of bulk mRNA. There are, however, some examples in which a specific mRNA that appears to be inactive is nevertheless still found in polysomes: thymidine kinase mRNA in differentiating myoblasts (*45*), and *nanos* mRNA in *Drosophila* embryos (*46*). The polysomes with repressed *nanos* mRNA were sensitive to EDTA, they decreased in size on prolonged incubation of the embryo extract with puromycin, and polysome runoff resulted in release of most of the *nanos* mRNA, yet no Nanos protein product could be detected (*46*). These results are similar to the original observations on the status of repressed *lin-14* and *lin-28* mRNAs in *C. elegans* L2/L3 larvae (*24*, *25*), and to the results of Petersen *et al*. (*12*).

miRNAs Can Also Promote Target mRNA Degradation

In almost all published studies, there is at least some miRNA-dependent decrease in mRNA abundance, which has usually been ignored, as it is often relatively small compared with the inhibition of expression of the encoded protein. For example, Petersen *et al*. observed a greater-than-95% decrease in reporter expression, but only a 50% decrease in mRNA abundance (*12*). Nevertheless, an explanation for this 50% decrease in mRNA abundance is called for.

Some recent publications report larger miRNA-dependent decreases in mRNA abundance, and emphasize mRNA degradation as an important aspect of miRNA-mediated repression of gene expression. Microarray analyses of HeLa cell mRNAs showed that expression of the brain-specific miR-124 or the muscle-specific miR-1 decreased the abundance of different subsets of 100 to 200 mRNAs in each case, specifically mRNAs that are of low abundance in brain and muscle, respectively (*47*).

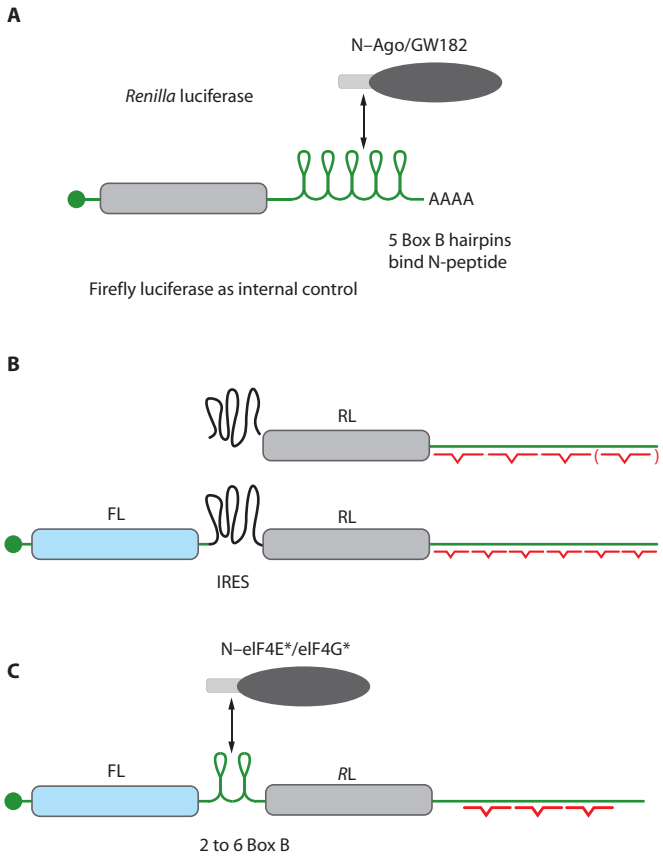

Fig. 3. Examples of the IRES and tethered-function constructs described here. (**A**) A reporter construct with five phage λ Box B hairpins in its 3′-UTR is cotransfected with a construct designed to express a fusion protein consisting of phage λ N-peptide fused to a human or *Drosophila* Ago protein, or GW182 (and often with an epitope tag spacer, which is not shown). The high-affinity interaction between the N-peptide moiety and Box B hairpins tethers the Ago or GW182 to the target mRNA. (**B**) Schematic diagram illustrating the type of monocistronic and dicistronic mRNAs with viral IRESs discussed here. (**C**) A dicistronic mRNA in which the downstream cistron expression is dependent on an artificial IRES system consisting of two or more phage λ Box B hairpins. The DNA construct is cotransfected with constructs designed to express a fusion protein consisting of phage λ N-peptide fused to mammalian eIF4E or eIF4G mutants (eIF4E*, eIF4G*) unable to function in cap-dependent initiation. The eIF4G mutant is unable to interact with eIF4E but retains the functionally important interactions with eIF3 and eIF4A (Fig. 2). The eIF4E mutant cannot bind to 5′-caps, but retains the potential for interaction with wild-type endogenous eIF4G, and thus the binding of the N-peptide/eIF4E* fusion protein to the Box B hairpins recruits endogenous eIF4G to the vicinity of the downstream cistron initiation site. Relevant references cited in this work are as follows: (A) (*19*, *20*, *29*); (B) (*12*, *26*, *29*); (C) (*29*).

In addition, miRNA-dependent degradation of mRNAs that have 3′-UTR AU-rich destabilizing elements has been reported, even though it is not the "seed" sequence of this miRNA (miR-16) that is complementary to the AU-rich sequence (*48*). Even more surprising is a recent report that in *C. elegans* larvae, *lin-4* and *let-7* miRNAs promoted extensive degradation of *lin-14*, *lin-28*, and *lin-41* target mRNAs (*49*), notwithstanding the previous evidence for repression of translation of these mRNAs (*24*, *25*). However, in none of these cases was there evidence that degradation occurred by the endonucleolytic mechanism characteristic of RNAi by perfectly complementary siRNAs (*40*).

Three recent papers, two concerned with mammalian systems and the other with *Drosophila* cells, also demonstrate miRNA-mediated acceleration of target mRNA degradation, not by an siRNA-like mechanism of endonucleolytic cleavage, but rather, through the normal pathway of deadenylation, followed by decapping and subsequent degradation of the body of the mRNA by 5′→3′ exonuclease activity (*18*, *20*, *50*). The only difference from the normal pathway is that the miRNP bound to its 3′-UTR target sites can apparently act as a barrier to the 5′→3′ exonuclease, leading to accumulation of deadenylated decay intermediates with 3′-end sequences (*18*, *49*).

Thus, in *Drosophila* cells, target mRNA concentrations could be restored by RNAi knockdown not only of Ago1 but also of the deadenylating (Not1 and Ccr4) or decapping enzymes (Dcp1/2), and in the last case deadenylated capped mRNAs accumulated (*20*). In mammalian cells with a target gene under control of the c-*fos* promoter (to allow a transient transcriptional pulse), an acceleration of deadenylation (and subsequent decay of the mRNA body) dependent on miR-125b expression was seen even with a reporter that had just a single bulged 3′-UTR target site for this miRNA (*50*). The accelerated deadenylation was not dependent on translation of the mRNA, because it was not substantially affected by insertion of a stable hairpin in the 5′-UTR. Nor did premature decapping occur on the mRNAs that had not yet been completely deadenylated (*50*).

Similarly, in zebrafish embryos, the expression of the miR-430 family of microRNAs, which starts at ~5 hours after fertilization, led to deadenylation and subsequent degradation of several hundred maternal mRNAs with predicted bulged target sites (and injected reporter mRNAs with such sites) at the stage when zygotic transcription is activated. This serves to clear the maternal mRNAs rapidly from the embryo and thus makes the translation machinery available for the new transcripts. In mutant embryos that do not express the miR-430 microRNAs, deadenylation and degradation of the maternal transcripts still occurred but were delayed, which led to abnormalities in brain development (*51*).

Thus, mRNA degradation and inhibition of translation apparently both contribute to the overall repression of gene expression by miRNAs, but the relative importance of each mechanism may vary according to the cell type and the stability or configuration of the particular miRNA-mRNA pair (*18*, *20*, *50*). This dual explanation for decreased expression is well illustrated by the data for reporters with the 3′-UTRs of three different *Drosophila* genes: With the nerfin 3′-UTR, overall reporter protein output was reduced 90% by miR-9b expression, but mRNA levels were reduced by only 20%, indicative of an effect predominantly on translation efficiency; with the CG10011 3′-UTR, protein output and mRNA abundance were both reduced by the same extent (80%) by miR-12 expression, suggesting almost no effect on translation efficiency per se; and the Vha68-1 3′-UTR/miR-9b pair gave results intermediate between these two extremes (*20*).

A consequence of deadenylation is disruption of the "closed loop" (Fig. 2), resulting in a severe reduction in translation efficiency, which will fall effectively to zero after the subsequent step of decapping. How much the low translation efficiency of this capped deadenylated species contributes to the true repression of translation that is observed will depend on the lifetime of this transient decay intermediate and its steady-state concentration relative to that of the polyadenylated species. These considerations are well illustrated by the three *Drosophila* mRNAs mentioned above, where RNAi knockdown of decapping enzymes caused the target mRNA abundance to increase (by trapping the decay intermediates as capped deadenylated transcripts), but the increase in reporter protein output was smaller, so that the degree of true translational repression actually increased, in the sense that the ratio of protein output to the amount of mRNA was reduced (*20*). However, because siRNA-mediated depletion of deadenylating enzymes also increased target mRNA concentrations (to a lesser extent), but this time with very little change in the degree of actual translational repression (the ratio of protein output to mRNA abundance), it seems likely that under normal conditions, translational repression is occurring mainly on polyadenylated mRNAs, with comparatively little contribution from the low translation efficiency of the capped deadenylated mRNA decay intermediates. (The situation may be somewhat different in RNA transfection assays, where there is no ongoing production of fully polyadenylated mRNAs.)

In mammalian cells expressing (from a CMV promoter) a polyadenylated luciferase reporter mRNA with six bulged target sites for miR-125b, coexpression of this miRNA reduced mRNA abundance by about 70%, and luciferase protein expression by more than 90%, indicative of a 65% decrease in the actual translation efficiency (*50*). When the poly(A) tail was replaced by the 3′-terminal stem-loop of histone H1.3, miR-125b coexpression had little effect on mRNA abundance (just a 10% decrease), but reduced translation efficiency by about 70%. The close correspondence between the reduction in true translation efficiency in these two experiments likewise suggests that the poor translatability of capped deadenylated decay intermediates, which would be present only in the former case, contributes little to the observed translational repression. The issue of whether the inhibition was exerted at initiation of translation or at some subsequent step in protein synthesis was not addressed in this study, but if future work indicates inhibition of initiation, it is worth bearing in mind that, despite the absence of a poly(A) tail, there is evidence that a "closed loop" (Fig. 2) can form with histone mRNAs and that this stimulates their translation (*52*, *53*).

miRNAs and Their mRNA Targets Accumulate in P-Bodies

Another important development has been the discovery that Argonaute proteins, miRNAs, and their target mRNAs accumulate in cytoplasmic foci usually known as P-bodies, or processing bodies, which are detected by confocal microscopy and immunostaining or the use of fusions between candidate P-body proteins and a fluorescent protein (*29*, *54*, *55*). The protein composition of these foci always includes enzymes that are important in the normal pathway of mRNA degradation, but is

more complex in higher eukaryotes than in yeast, where it includes the Dcp1/Dcp2 decapping complex, Xrn1 5′-3′ exonuclease, most of the Lsm proteins (Lsms 1 through 7, a family of related RNA-binding proteins necessary for mRNA decay), the Dhh1p putative RNA helicase (known as RCK or p54 in vertebrates), and Pat1p, but not ribosomes, nor any of the eight translation initiation factors examined, nor components of the exosome complex of 3′→5′ exonucleases (*56*–*58*). Although Pat1p homologs have not been tested, P-bodies in higher eukaryotes seem to have the same proteins plus many additional components, most of them absent from the yeast proteome, and because budding yeast neither expresses siRNAs or miRNAs nor shows any response to ~21-residue RNAs, at least some of these additional components in higher eukaryotes could play a role in the mechanisms of siRNA- and miRNA-mediated mRNA silencing. Interesting potential candidates include the following proteins (*59*–*62*): GW182, a putative RNA-binding protein with glycine-tryptophan repeats; eIF4E (but no other translation initiation factors nor PABP nor ribosomes); and the eIF4E-binding protein, 4E-T (but not 4E-BP1, another eIF4E binding protein). The *Drosophila* 4E-T homolog, known as Cup, has been implicated in the repression of specific maternal mRNAs (*63*–*65*), whereas 4E-BP1 interaction with eIF4E results in global inhibition of initiation (*66*).

P-bodies are highly motile within the cell cytoplasm (*44*) and fluctuate in size and number. In situations where P-bodies seem to disappear as visible foci, it is not clear whether their components become completely dispersed in the cytoplasm, or whether "mini" P-bodies persist that are undetectable by confocal microscopy. P-bodies not only contain (untranslated) mRNA and especially mRNA decay intermediates, but they require this RNA for their integrity (*57*, *60*). Thus, the foci disappear in yeast mutants with defective deadenylation, which would reduce the level of decay intermediates, and increase in size and number in mutants defective in downstream steps of the decay process, such as mutants in the Dcp1/Dcp2 decapping complex or the Xrn1 5′→3′ exonuclease (*56*, *57*). Similar results are seen in mammalian cells following RNAi knockdown of mRNA degradation enzymes (*60*, *61*), and in addition, P-bodies decrease following depletion or knockdown of GW182, 4E-T, Lsm1, and the RCK/p54 helicase (*61*, *67*). Inhibition of translation elongation (e.g., by cycloheximide) causes the foci to disappear, whereas inhibition of initiation results in an increase in size and number (*56*–*58*, *60*, *61*), suggesting that an mRNA has to be cleared of ribosomes before it can localize to P-bodies. Thus, the P-body localization of the mRNA targets of miRNA-mediated repression can be taken as additional, though circumstantial, evidence that it is initiation that is the inhibited step.

Irrespective of whether repression was effected by endogenous let-7a miRNA or exogenous CXCR4 siRNA, a fraction of the target mRNA was localized to foci resembling P-bodies (*29*, *54*). However, Pillai *et al*. emphasize that the foci in which the repressed mRNA and let-7 miRNA were localized did not always coincide precisely with the Dcp-containing foci (*29*): 67% were perfectly overlapping with Dcp-1 foci, and 37% were described as being adjacent. Furthermore, there were a few Dcp foci apparently lacking the target mRNA, and vice versa, as though there might be two subtypes of foci that might fuse occasionally or exchange mRNA. In a similar vein, adjacent location and possible fusion between P-bodies and stress granules has also been noted (*43*, *44*, *68*), leading to the suggestion that after storage and sorting in stress granules, some mRNAs may pass to P-bodies for degradation.

Entry of an mRNA into Dcp1/Dcp2 foci does not inevitably lead to its degradation, as shown by the fact that glucose starvation of yeast, which results in a global inhibition of initiation, drives mRNAs into P-bodies, but on refeeding they exit the foci and become (re)assimilated into active polysomes (*58*, *69*). Reversibility of miRNA-dependent repression and P-body localization has been demonstrated for the cationic amino acid transporter (CAT-1) mRNA, which has three or four [depending on which poly(A) addition site was used] bulged 3′-UTR sites for interaction with miR-122 (*30*). In Huh7 cells, the endogenous miR-122 reduced CAT-1 protein levels by about 65% under fed conditions (as judged by the results of introducing an antisense oligonucleotide to this miRNA), and both the CAT-1 mRNA and the miR-122 could be detected in P-bodies. On amino acid starvation there was a rapid increase in the amount of CAT-1 protein, with no immediate change in mRNA abundance; rather, the preexisting CAT-1 mRNA left the P-bodies and was mobilized into polysomes. An unexpected additional finding was that both the activation of translation and the mobilization from P-bodies required a particular AU-rich element (ARE) in the 3′-UTR and the presence of the ARE-binding protein, HuR (*30*). Together, these observations confirm that miRNA-mediated repression and P-body localization of the target mRNA are potentially reversible processes. P-bodies can be regarded as a repository for untranslated mRNA, some of which may subsequently be degraded, whereas some may be returned to translation. An unresolved question is whether the translational activation of CAT-1 mRNA is accompanied by complete dissociation of the miRNP from the target mRNA, or whether it is just a case of the repressive action being overridden in some way without dissociation.

It was estimated that some 20% of let-7a miRNA and 20% of its target mRNA in HeLa cells were localized in foci, under conditions in which the target was repressed by at least 80% (*29*). The results of biochemical fractionation of cell extracts prepared by lysis with digitonin suggest that the repression of the rest of the target mRNA occurred in "mini" P-bodies smaller than the threshold size detectable by confocal microscopy, rather than uniformly and completely dispersed throughout the cytoplasm. Centrifugation of these extracts at 14,000*g* pelleted almost all the target mRNA (but not the tubulin mRNA examined as an unrepressed control), most of the let-7a miRNA (in the case of HeLa cells), and all of the endogenous miR-122 (Huh7 cells), together with all of the cellular complement of the P-body marker proteins, Xrn1 and Dcp1 (*29*, *30*).

It seems intuitive that it is the inhibition of translation rather than the mere binding of the miRNP to the target mRNA that drives the repressed mRNA into P-bodies. Thus, it would be reasonable to assume that in the experiments where mRNAs with the EMCV, HCV, or CrPV IRESs were resistant to repression (*29*, *33*), an miRNP-mRNA complex still forms on these mRNAs, yet presumably they do not get localized to P-bodies. It would be intriguing to know what happens as regards P-body accumulation with a dicistronic mRNA (Fig. 3C), in which the upstream cistron is sensitive to miRNA-mediated repression but the downstream cistron is insensitive (*29*).

Another case where it seems most unlikely that the target RNA enters P-bodies is the interaction of miR-122 with HCV RNA, which occurred at a 5′-proximal site upstream of the

IRES and was necessary for the replication of the viral RNA, without having any direct effect on its translation (*70*). Here again, there is no reason to suppose that an miRNP complex with Ago proteins does not form on the viral RNA, yet it seems most unlikely that this results in the miRNP/viral RNA complex accumulating in P-bodies.

siRNA-Mediated Knockdown and Tethered Function Approaches to Identify the Essential Mediators of miRNA-Dependent Repression of Translation

Approaches toward elucidating the mechanism of repression include (i) identifying which proteins interact with Argonaute proteins, (ii) testing whether miRNA-mediated repression is relieved by siRNA-mediated knockdown or depletion of these and other likely candidate proteins, and (iii) tethered function assays (Fig. 3).

Proteins that interact with mammalian Ago1 or Ago2 proteins in an RNase-insensitive manner, and colocalize in P-bodies, include the Dcp1-Dcp2 decapping complex, GW182, and RCK/p54 helicase, which is the vertebrate homolog of yeast Dhh1p (*54*, *67*, *71*). The interactions with Dcp1/2 and GW182 still occurred with Ago mutants deficient in RNA binding that did not localize to P-bodies (*54*, *67*), and the interaction with p54 still occurred in cells depleted of Lsm1 (a protein found in P-bodies and implicated in RNA processing), which dispersed the foci (*71*). Again, it is not clear whether these interacting proteins were completely dispersed in these situations. The *Drosophila* GW182 homolog binds to and localizes with Ago1, but not Ago2 (*20*), which functions mainly or exclusively in the siRNA-mediated endonucleolytic pathway.

When *Drosophila* cells were depleted of these various proteins, relief of miRNA-mediated repression was seen with knockdown of Ago1, GW182, and Dcp1/Dcp2, in descending order of efficacy (*20*, *72*). Depletion of Xrn1 had no effect, nor did Ago2 knockdown, though this did relieve siRNA-mediated endonucleolytic cleavage of mRNA with a perfectly complementary target site (*72*). In mammalian cells, repression of reporters with either four or six bulged target sites for CXCR4 siRNA was likewise relieved by GW182 depletion and, to a slightly lesser extent, by knockdown of p54 helicase or Dcp2, but not by Xrn1 knockdown (*67*, *71*). Depletion of Lsm1, which caused dispersal of P-bodies, had no effect on the degree of repression, implying that the usual localization in visible foci is a consequence of repression rather than a prerequisite for it (*71*). In the case of a reporter with a single site perfectly complementary to the introduced siRNA, depletion of GW182 again gave some relief, though not as much as Ago2 knockdown, and Dcp1/2 or Xrn1 depletion had no effect (*67*, *71*).

In view of the indications that miRNA-mediated repression in some way affects eIF4E function (*29*, *33*), it would be interesting to test the outcome of 4E-T knockdown. Another candidate worth testing would be the higher-eukaryote homolog of yeast Pat1p, which has been shown to act as a global translational repressor together with Dhh1p (the yeast p54 homolog), in an additive rather than overlapping way (*69*). In strains deleted for both, the normal response to glucose starvation, which includes the breakdown of polysomes, cessation of translation, and mobilization of all mRNAs into P-bodies, no longer occurred (*69*).

Finally, tethered function assays have shown that coexpression of Ago-λ N-peptide fusions and a reporter with tandem phage λ Box B sequences in its 3′-UTR (Fig. 3A) caused the reporter to behave as if it were repressed by microRNAs. Its translation was specifically repressed at the initiation step by up to 80%, dependent on the number of Box B sites, but independent of their position in the 3′-UTR, with no appreciable decrease in mRNA abundance (*19*, *29*). Curiously, although repression promoted by tethering Ago bypasses the requirement for an miRNA, repression and P-body localization did not occur with tethered Ago PAZ domain mutants that are defective in binding the miRNA/mRNA complex (*67*).

Tethering GW182 to an mRNA in the same way mimicked miRNA-mediated repression in *Drosophila* cells, causing a 75% decrease in the abundance of the reporter mRNA and a greater than 90% overall reduction of luciferase output, implying a 75% inhibition of translation per se (*20*). The same effects of tethered GW182 were seen even in cells depleted of Ago1. Thus, tethering the relevant Argonaute protein can bypass the miRNA requirement for repression, and tethering GW182 bypasses the Ago requirement, at least in *Drosophila* cells, suggesting that GW182 acts downstream of Ago proteins.

As for p54, tethering it to the 3′-UTR of a reporter mRNA specifically repressed reporter translation in *Xenopus* oocytes, implying that p54 plays an important role in the represssion of maternal mRNA in these oocytes (*73*, *74*). The *Drosophila* p54 homolog, Me31B, has also been implicated in the repression of certain maternal mRNAs in early embryos, and in this capacity it appears to act as a corepressor working in conjunction with Cup, the *Drosophila* homolog of mammalian 4E-T (*75*, *76*). It is not yet known if tethering 4E-T/Cup to the 3′-UTR also represses translation, and, if so, whether it or p54 acts downstream of GW182 or vice versa.

Recapitulation of miRNA-Mediated Repression in Vitro

Many of the uncertainties about the exact mechanism of translational repression might be resolved if repression could be recapitulated in an in vitro system, as has been achieved for siRNA-mediated endonucleolytic cleavage (*77*, *78*). Until recently, no such in vitro recapitulation of miRNA-mediated repression had been reported, although we presumed that it had been attempted in many laboratories without success. However, success has recently been achieved with the same siRNA (CXCR4) and a luciferase mRNA with the same bulged target sites as used by Petersen *et al.* (*12*) and Humphreys *et al.* (*33*). The essential trick was to pre-anneal the miRNA to the target mRNA before addition to the rabbit reticulocyte lysate (RRL) system (*79*). Although this requirement for pre-annealing means that the miRNP-mRNA complex was not assembled by the normal route that is thought to occur in intact cells, the outcome had all the hallmarks of miRNA-mediated regulation established in vivo: increased repression with increasing number of target sites; a strict requirement for a 5′-phosphate on the miRNA; requirement for perfectly complementary contiguous base-pairing of residues 2 to 8 of the miRNA, coupled with a much less stringent requirement for contiguous base-pairing of the 3′ end; no repression if the 3′-end of the 21-nt miRNA was extended by 10 residues perfectly complementary to the target mRNA; and no repression with a 2′-*O*-methyl derivative of the 21-nt mRNA. Although the commercial RRL system used is one that appears to have been optimized for translation of uncapped mRNA, repression was only seen if the mRNA was both capped and had a standard-length poly(A) tail (200 A residues), but uncapped mRNAs were repressed if the tail was extended to unphysiological lengths (2000 residues).

Maximal repression (70% reduction) of luciferase output was seen at short incubation times (10 min), and the time-course data implied that the CXCR4 siRNA increased the lag time before the luciferase activity first appeared, after which the actual rate of increase was not very different. Although these kinetics could imply a delay in the first initiation events, or alternatively a slower rate of elongation, it could also be that the repression mechanism is rather labile over time, a suggestion which would be consistent with the finding that repression by CXCR4 siRNA was more sensitive to freeze-thawing of the RRL than was the basal translation activity (*79*). Although the data do not allow an unambiguous identification of which step of translation is being inhibited, the requirement for a cap and a poly(A) tail strongly suggests that it is initiation rather than elongation that is affected.

If the reporter mRNA had a single 3′-UTR site perfectly complementary to the CXCR4 siRNA, it appeared to be extensively degraded in this system, implying that despite the abnormal pathway, a competent RISC with an active Ago2 can assemble in RRL, although confirmation of this requires a demonstration that the reporter was cleaved in the expected site.

A Possible Model?

We propose a speculative model to account for the available findings (Fig. 4). Figure 4A depicts all the different ways by which miRNAs have been proposed to regulate their target mRNAs, other than the rare cases where metazoan miRNAs have near-perfect complementarity with an mRNA target site and therefore promote endonucleolytic cleavage (*7*). There are two reasons why we are unable to enlarge upon the possible mechanisms of perturbation of elongation that result in the repressed mRNA being found in polysomes of approximately the same size as when it is unrepressed. First, it is not yet at all clear whether this is due to cotranslational cleavage of the nascent protein chain, or to premature ribosome drop-off (coupled with a compensating increase in initiation frequency), or to a reduced elongation rate coupled with a similar decrease in the rate of initiation. Second, none of the components of miRNPs, and none of the proteins that have been found to interact with Ago proteins, is thought to interact with, or have any impact upon an elongating ribosome..

However, we are able to elaborate the model in respect of miRNA-mediated regulation of translation initiation and miRNA-dependent acceleration of mRNA degradation (Fig. 4B). In this scheme, binding of an Ago protein at the site of miRNA interaction with its bulged target sequence leads to recruitment of GW182, RCK/p54, the Dcp1/Dcp2 decapping complex, 4E-T, and the higher eukaryote homolog of yeast Pat1p (although there is as yet no evidence for or against recruitment of these last two components). With the assistance of p54, 4E-T interacts with eIF4E bound to the 5′-cap, to form an inhibitory "closed loop" similar to the postulated mechanism for repression of maternal mRNAs in *Drosophila* embryos by the combined action of Cup and Me31B, the *Drosophila* homologs of 4E-T and p54, respectively (*63*–*65*, *75*). An additional assumption is that the bound Ago protein recruits, either directly or indirectly, the Not1 and Ccr4 deadenylation factors, which results in accelerated shortening of the poly(A) tail and consequently accelerated mRNA degradation. This model cannot explain why repressed mRNAs and miRNAs sometimes end up in polysomes (*12*, *24*, *25*), nor

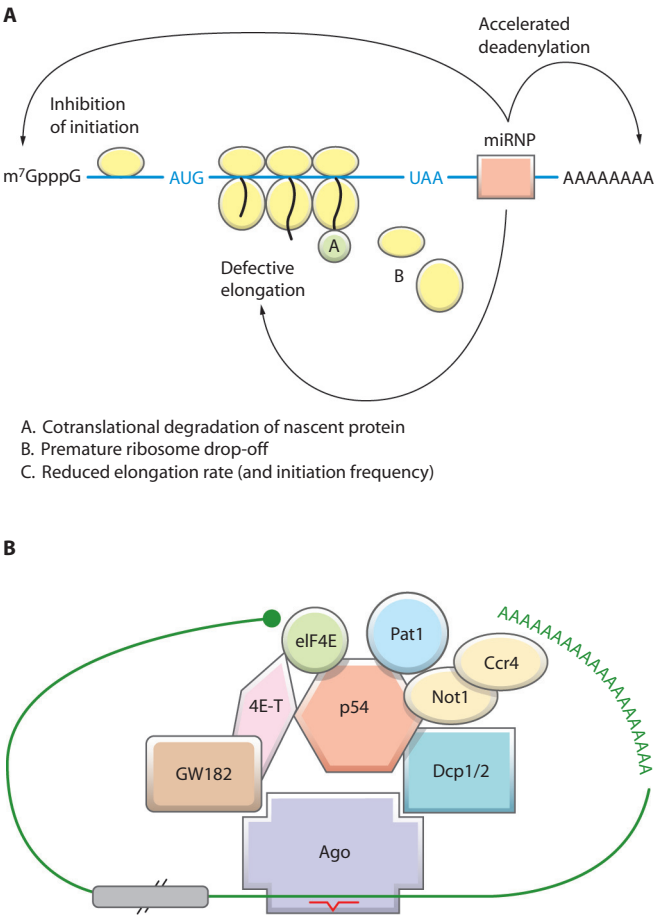

Fig. 4. Ultraspeculative model for how miRNAs may be able to regulate gene expression at the posttranscriptional level. (**A**) A schematic depiction of the different mechanisms proposed for the regulation of target mRNA function by miRNAs. Three alternative ways in which miRNA-mediated repression might perturb translation elongation are listed, with the green sphere representing an unknown ribosome-associated protease degrading the nascent protein chain cotranslationally. (**B**) Diagram showing how the binding of an Ago protein to the site of miRNA/mRNA interaction might lead to inhibition of translation initiation and an enhanced rate of deadenylation. (See text for an explanation.) For convenience, the diagram depicts an mRNA with only a single target site for the miRNA. With multiple sites there is the possibility that protein interactions at one site could be directed mainly toward inhibition of initiation, and those at another site toward deadenylation, or even toward aberrant elongation.

can it explain how and why translation dependent on the HCV and CrPV IRESs is susceptible to repression in some experiments (*12*). But apart from these problems, the model is consistent with the other results discussed here, subject to the minor caveat that the weak repression seen with ApppG-capped polyadenylated mRNAs (with or without an EMCV IRES) in the RNA transfection assays of Humphreys *et al.* would have to be explained as a secondary consequence of deadenylation rather than a direct result of any inhibition of initiation (*33*).

Concluding Remarks

A comment found with increasing frequency in the literature is that there may well be more than one mechanism by which miRNAs repress expression of their target genes. There is clear evidence for this in respect to deadenylation-dependent degradation and true translational repression, and it does not stretch credulity too far to imagine that the miRNA/mRNA interaction could lead to both these outcomes. What does defy the imagination, on the other hand, is how the same miRNA-mRNA target pairs can show inhibition of translation initiation in one system, but inhibition at some step after initiation in another. We embarked on this Review with the hope of finding an explanation for this apparent contradiction. We have failed in this aim, but some pointers to future directions have emerged on the route to this failure.

First, more attention should be paid than hitherto to the poly(A) tail length of the target mRNA. Moreover, target mRNA abundance should in future be assessed by methods (e.g., Northern blotting) that, unlike RNase protection or reverse transcription–polymerase chain reaction amplification (RT-PCR) of a short internal segment, can distinguish full-length mRNA from degradation intermediates. Second, it would be interesting to know whether depletion of critical P-body components such as GW182 and RCK/p54 also relieves the repression in those systems that lead to polysomal accumulation of the repressed mRNA. Third, it would be useful if those who find that target mRNAs with an HCV, CrPV, or EMCV IRES are insensitive to repression in RNA transfection assays were to confirm this result with dicistronic mRNAs, and also in DNA transfections of mono- and dicistronic constructs. For this purpose, it would also be worthwhile replacing the HCV IRES with the phylogenetically related, but much more potent, IRES from classical swine fever virus (*80*) as a counter to the argument (*40*) that translation dependent on some viral IRESs may be unresponsive to miRNAs simply because these IRESs are inefficient. Fourth, because initiation on all the viral IRESs used to date is not only independent of eIF4E but also does not involve any ribosome scanning, it seems important to extend the range of IRESs studied to those that require eIF4E and those where there is ribosomal scanning, so as to confirm that it is eIF4E function rather than scanning that is inhibited. Fifth, further use of depletions can be expected to identify more candidate proteins required for repression, and tethered function assays coupled with knockdowns may reveal the hierarchy in which these function. Sixth, further development of the cell-free systems (*79*) can be expected to illuminate the proximal mechanism of repression, though it would be somewhat surprising if such systems were able to shed light on the mechanisms of deadenylation and target mRNA accumulation in P-bodies. Finally, in order to facilitate reconciliation of the biochemical fractionation data with the confocal microscopy results, it would be extremely useful to know what happens to P-bodies, and where they and their constituent proteins partition, in the procedures used by different laboratories to prepare cytoplasmic extracts for fractionation by sucrose density gradient centrifugation.

Note added in proof. There have been two very recent publications on these controversial issues. Both agree that repression occurs while the target mRNA is polysome-associated, implying that the repression must be exerted at some stage after initiation. In sucrose gradient analyses of HeLa cell polysomes, virtually the whole cellular complement of three representative miRNAs (let-7a, miR-16, and miR-21) cosedimented with polysomes in an EDTA-sensitive manner (*81*). This polysomal association was dependent on base-pairing between the miRNA and mRNA, because the let-7a miRNA was found at the top of the gradient if the cells had been transfected with a 2′-*O*-methyl oligonucleotide antisense to this miRNA. Moreover, the polysomes were dynamic by the criteria that the miRNAs were found nearer the top of the gradient if the cells had been incubated with puromycin or with initiation inhibitors before lysis (*81*).

In the other study, HeLa cells were transfected with a DNA construct with a thymidine kinase promoter that had the Renilla luciferase coding region upstream of the *C. elegans lin-41* 3′-UTR, which has two validated target sites for let-7a miRNA (*82*). As compared with a control lacking these two sites, luciferase expression was reduced by 90%, with a decrease in target mRNA abundance of 30%, implying an ~80% reduction in true translation efficiency. About half of the target mRNA, as well as half the endogenous let-7a miRNA and AGO proteins, cosedimented with large polysomes (with the other half found at the top of the gradients), but they sedimented nearer the top of the gradient following incubation with puromycin or under conditions of inhibited initiation (*82*). These results were taken as evidence that repression was occurring at some stage after initiation, and the authors specifically favor the cotranslational nascent protein degradation hypothesis, because, like Petersen *et al.* (*12*), they were unable to detect any nascent peptide product under repressed conditions, even though the reporter had an N-terminal epitope tag to facilitate detection of such peptide products.

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