

# Identification of specific *let-7* microRNA binding complexes in *Caenorhabditis elegans*

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## ABSTRACT

Little is known about the protein complexes required for microRNA formation and function. Here we used native gel electrophoresis to identify miRNA ribonucleoprotein complexes (miRNPs) in *Caenorhabditis elegans*. Our data reveal multiple distinct miRNPs that assemble on the *let-7* miRNA *in vitro*. The formation of these complexes is affected but not abolished by *alg-1* or *alg-2* null mutations. The largest complex (M\*) with an estimated molecular mass of >669 kDa cofractionates with the known RISC factors ALG-1, VIG-1, and TSN-1. The M\* complex and two complexes, M3 and M4, with similar molecular weights of ~500 kDa, also assemble on all other miRNAs used in our experiments. Two smaller complexes, M1 (~160 kDa) and M2 (~250 kDa), assemble on the members of the *let-7* miRNAs family but not *lin-4* or *mir-234*, and their formation is highly dependent on specific sequences in the 5' seed region of *let-7*. Moreover, an unidentified protein, p40, which only appears in the M1 and M2 complexes, was detected by UV triggered cross-linking to *let-7* but not to *lin-4*. The cross-linking of p40 to *let-7* is also dependent on the *let-7* sequence. Another unidentified protein, p13, is detected in all *let-7* binding complexes and *lin-4* cross-linked products. Our data suggest that besides being present in certain large miRNPs with sizes similar to reported RISC, the *let-7* miRNA also assembles with specific binding proteins and forms distinct small complexes.

**Keywords:** miRNA; siRNA; *let-7*; RISC

## INTRODUCTION

MicroRNAs (miRNAs) belong to a class of noncoding small RNAs and have been shown to regulate gene expression through repressing translation and/or cleaving mRNAs (Bartel 2004; Valencia-Sanchez et al. 2006). *let-7* is one of the founding members of the miRNA family and was identified through genetic mutation as a temporal patterning regulator in *Caenorhabditis elegans* (Reinhart et al. 2000; Ambros 2001; Banerjee and Slack 2002; Grosshans and Slack 2002; Pasquinelli and Ruvkun 2002). Previous studies indicated that in *C. elegans*, *let-7* regulates *lin-41* and *let-60* by binding to sequences in the 3'-untranslated regions (3'-UTRs) of these mRNAs, which are partially complementary to *let-7* (Lee et al. 1993; Wightman et al. 1993; Slack et al.

2000; Vella et al. 2004). However, the mechanism of action of miRNAs, including *let-7*, in regulating gene expression is still not clear. *let-7* is a useful model for understanding miRNA-mediated regulation because it has been extensively studied in *C. elegans* development and is conserved in many organisms. Moreover, because human *let-7* regulates the *let-60* homolog, the *RAS* oncogene in lung tissue (Calin et al. 2004a,b; Takamizawa et al. 2004; Johnson et al. 2005), studies on *let-7* are also important for human cancer research.

In *C. elegans*, systematic genetic analysis has indicated that a number of proteins function in the miRNA pathway (Ketting et al. 1999; Tabara et al. 1999; Grishok et al. 2001). For example, DCR-1, an RNase III-like enzyme, is involved in the processing of hairpin-like pre-miRNAs to generate mature miRNAs (Bernstein et al. 2001; Billy et al. 2001; Grishok et al. 2001; Hutvagner et al. 2001; Ketting et al. 2001). ALG-1 and ALG-2, which belong to the Argonaute (Ago) family, play crucial roles in *let-7* and *lin-4* miRNA-mediated regulation (Grishok et al. 2001) and associate with *let-7* miRNP complexes (Hutvagner et al. 2004). TSN-1 (Tudor staphylococcal nuclease homolog-1) and VIG-1 (vasa intronic gene homolog-1), which were found associated with RNA interference activity in *Drosophila*, have

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been shown to also be required for proper *let-7*-mediated regulation in *C. elegans* (Caudy et al. 2003). Two *let-7*-containing ribonucleoprotein complexes with sizes of 500 kDa and 250 kDa, similar to the sizes of siRNA-induced silencing complexes (siRISC) found in *Drosophila* (Pham et al. 2004), have been identified by gel filtration of *C. elegans* extracts (Caudy et al. 2003). Among them, TSN-1 and VIG-1 comigrate with *let-7* in the 250-kDa protein complex (Caudy et al. 2003). Further studies on miRNA-containing ribonucleoprotein complexes and discoveries of additional unknown cofactors in these complexes would be helpful to understand miRNA related mechanisms of action.

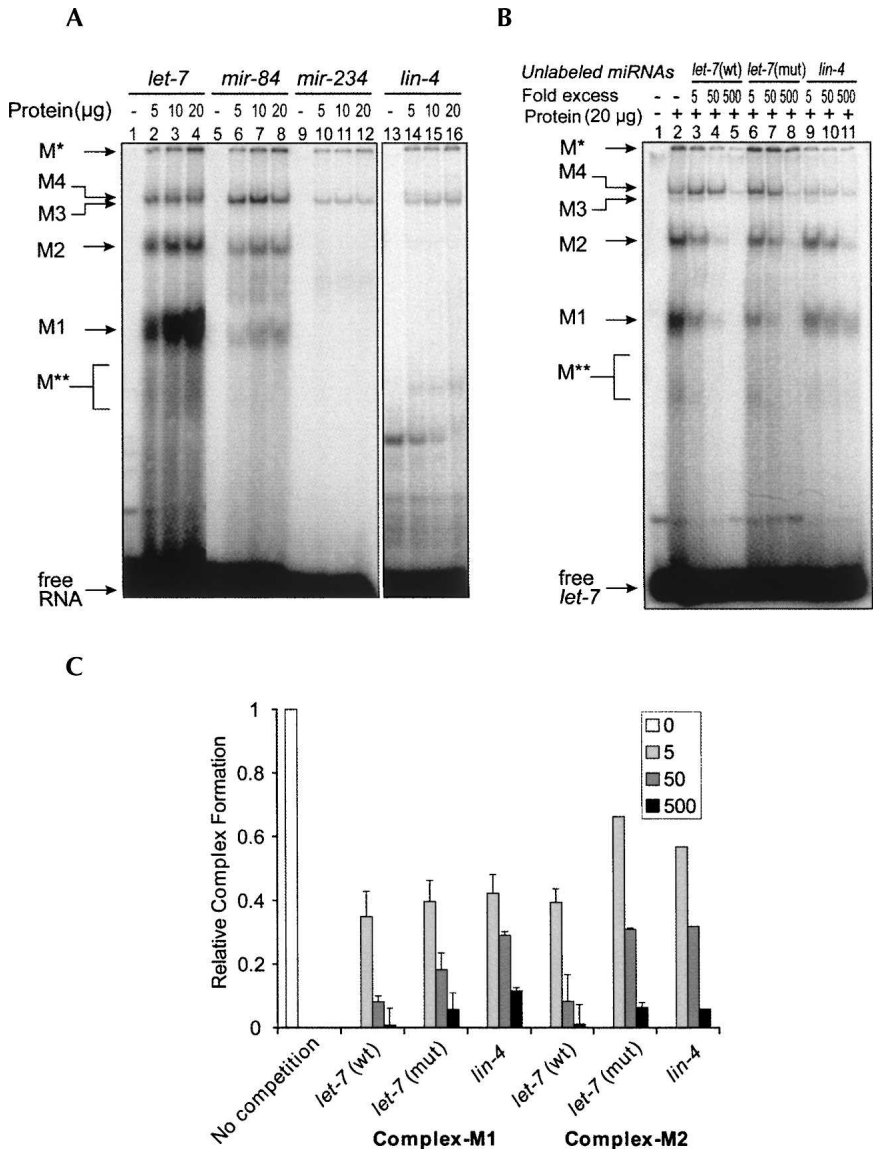
In this study, we used native gel electrophoresis to characterize *let-7* miRNP complexes in *C. elegans*. Our data indicate the presence of multiple and distinct ribonucleoprotein complexes assembling with *let-7* in vitro. These complexes are affected by *alg-1* and *alg-2* null mutations, suggesting that they are relevant to miRNA function. In addition to large complexes with estimated molecular mass ranges from ~500 kDa to >669 kDa, which also assemble with other miRNAs, two small complexes of ~250 kDa and ~160 kDa seem specific to the *let-7* miRNA family, and their formation depends on the sequence in the 5' seed region. By UV cross-linking, we detected two unidentified proteins, p40 and p13, which may directly associate with *let-7*. p13 appears in all *let-7* binding complexes and is also cross-linked to *lin-4*. p40 appears only in the small complexes, and its appearance is dependent on the *let-7* sequence.

**RESULTS**

**The *let-7* miRNA forms multiple ribonucleoprotein complexes in *C. elegans* extracts**

To elucidate the molecular mechanism of the *let-7* miRNA in its function, we have identified and characterized multiple *let-7* miRNA ribonucleoprotein (miRNP) complexes in *C. elegans*. We first prepared cytosolic extracts from mixed-stage *C. elegans* animals and examined whether these extracts supported the formation of miRNPs.

Results from gel retardation experiments using end-labeled mature *let-7* miRNA indicated that *let-7* could form at least four distinct miRNP complexes. In this study, we refer to these complexes as M1, M2, M3, and M4 (Fig. 1A). In addition to the M1–M4 complexes, we found another large complex (M\*), which was not capable of entering the gel, and a small and less distinct complex (M\*\*). These complexes were sensitive to SDS and proteinase K (data not shown), suggesting that they contain protein components. The reaction mixture for gel retardation



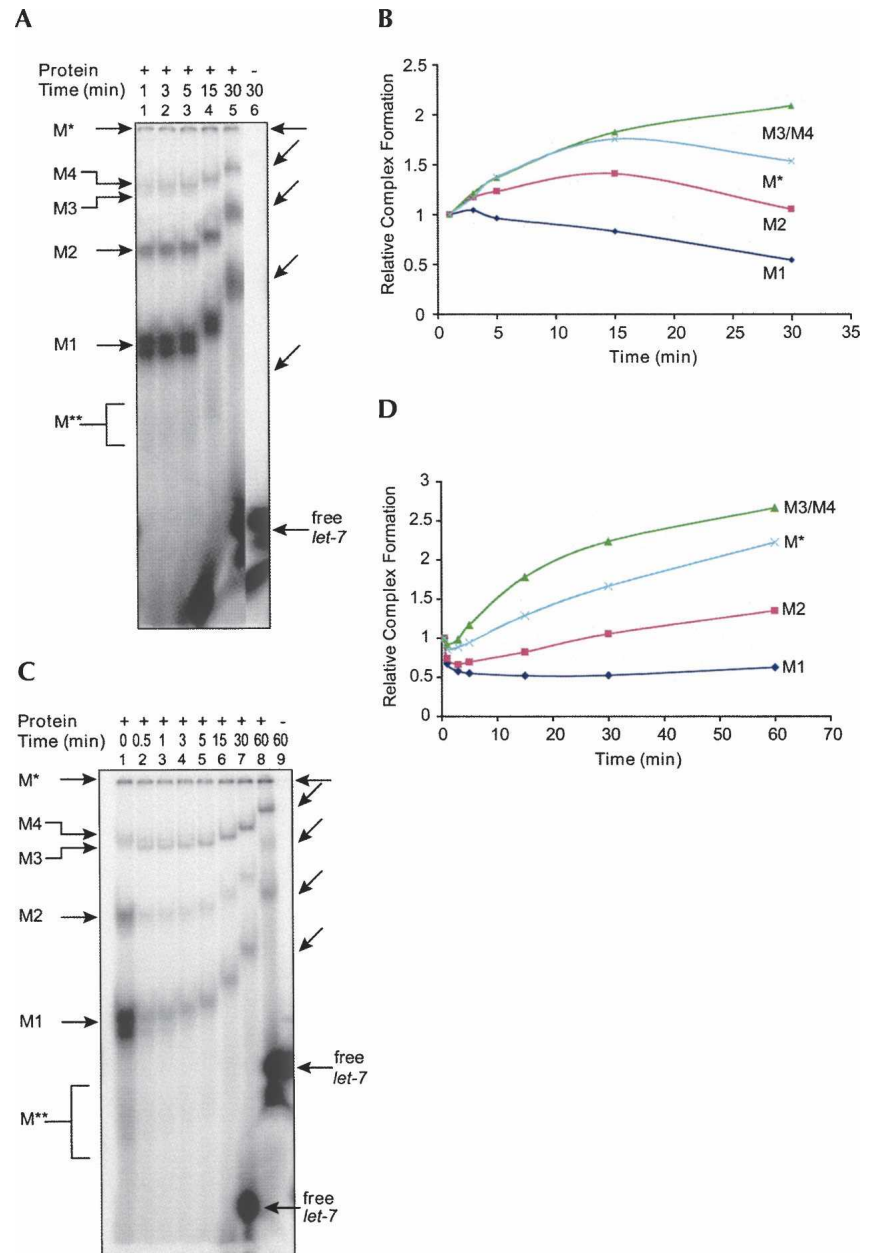
**FIGURE 1.** Multiple ribonucleoprotein complexes assemble with *let-7* in vitro. (A) Radio-labeled *let-7*, *mir-84*, *mir-234*, or *lin-4* was incubated with different amounts of *C. elegans* cell extracts and analyzed by native gel electrophoresis. The ribonucleoprotein complexes named in this study are indicated on the left. (B) Up to 500-fold excess of unlabeled *let-7*(wt), *let-7*(mut), and *lin-4* RNAs were used to compete for the formation of *let-7* binding complexes. (C) Quantification of fold decrease in the complexes M1 and M2 in B. The reaction without any unlabeled RNA served as control. The bars represent the error from two independent experiments.

contained a 250-fold excess of tRNA over labeled *let-7* to reduce the background caused by nonspecific RNA binding proteins.

We tested for complex formation with *lin-4*, *mir-234*, and a member of the *let-7* miRNA family, *mir-84* (Lim et al. 2003; Abbott et al. 2005). *mir-84* formed all the same complexes as *let-7* did albeit with reduced efficacy for M1 and M2 (Fig. 1A). Interestingly, *lin-4* and *mir-234* formed low amounts of M\* and M3/M4 complexes but no M1 and M2 complexes. This indicated that M1 and M2 complexes preferentially assemble with *let-7* or members of the *let-7* miRNA family. In contrast, the M\* and M3/M4 complexes seem to lack this selectivity. We also compared the ability of unlabeled *lin-4*, wild-type *let-7*, or mutant *let-7(n2853)* RNA (which contains a G-to-A base substitution at position 5 of *let-7*) (Reinhart et al. 2000) to compete for complex formation. We found that *lin-4* did not compete for formation of the M1 and M2 complexes as efficiently as *let-7* did, which is consistent with the inability of *lin-4* to form these complexes (Fig. 1B,C). The competition by mutant *let-7* was also weaker than that by wild-type *let-7*, although the difference was less pronounced. All of the above observations point to the possibility that formation of the M1 and M2 complexes is specific to *let-7* and is dependent on the *let-7* sequence (see below for further studies).

We performed time-course experiments to observe the formation of these complexes. Since it was not possible to arrest the formation of these complexes, we followed the protocols used in previous studies on RISC formation in *Drosophila* and loaded the binding reaction mixture directly into a running native gel at each time point (Pham et al. 2004). In the first set of experiments, we performed the binding reaction without a competitor miRNA. We found that the M3/M4 complexes increased in abundance over the time course of the experiment. The M\* complex at first accumulated at a rate similar to that of M3/4, then the abundance slightly decreased (Fig. 2A,B).

The M1 complex formed quickly and abundantly, then decreased over the time course of the experiment. The abundance of M2 increased for a short period and then decreased to its original amount. In the second set of



**FIGURE 2.** *let-7* miRNPs form in different ways. (A) Time-course analysis of miRNP assembly. Gel retardation samples were withdrawn at different durations of time and loaded into a running native gel. The differential mobility of the complexes is due to time differences between each loading. Lane 6 corresponds to the free label loaded at the same time as lane 5. The shifted positions of complexes are also shown by slanted arrows on the right. In lanes 1–3, the free probe ran off the gel because of earlier loading. (B) Amounts of complexes in A at different time intervals compared to the beginning of the reaction (1 min). (C) A gel retardation reaction was chased with an excess (20-fold) of unlabeled *let-7* miRNA after the first minute of incubation. At indicated time intervals, the samples were withdrawn and analyzed as described in A. (D) Amounts of complexes in C at different time intervals compared to the beginning of the pulse chase (0.5 min after addition of unlabeled *let-7* miRNA).

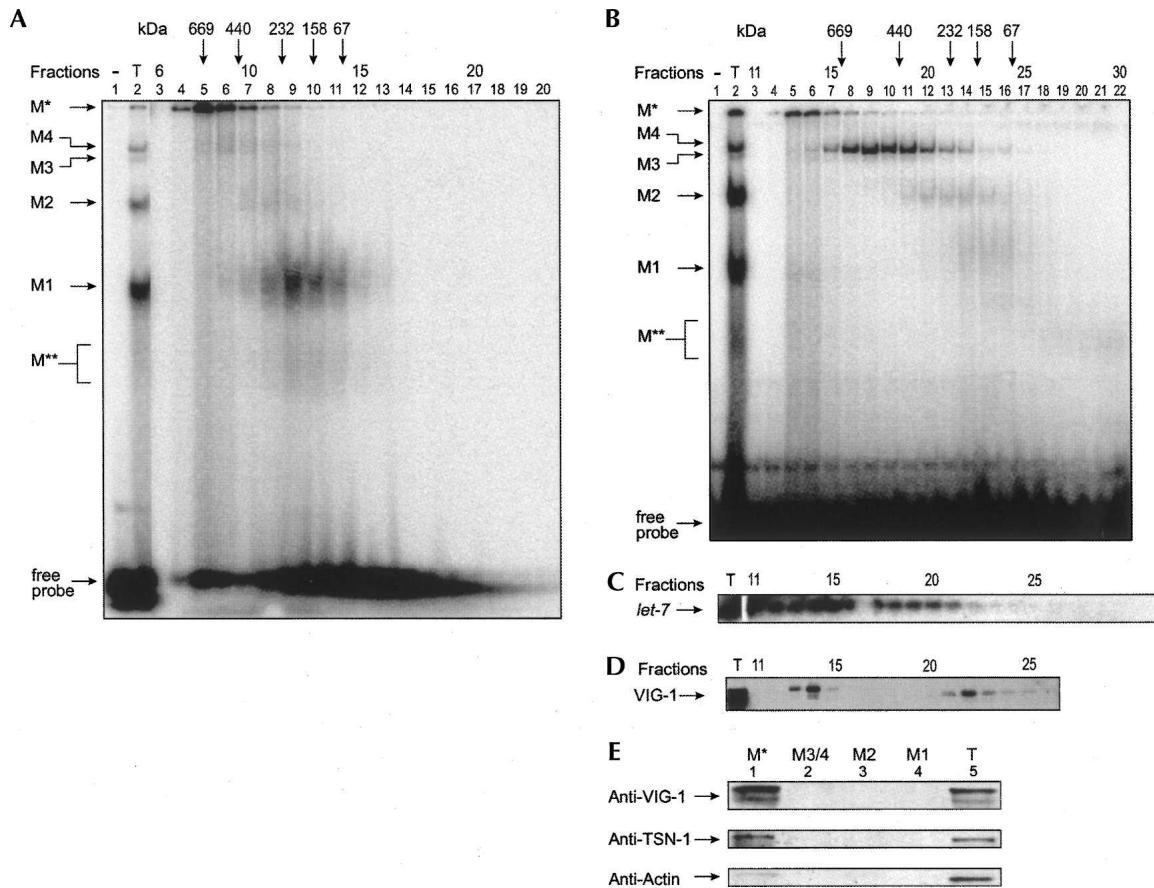
experiments, we started the binding reaction with radiolabeled *let-7* miRNA, and after 1 min of incubation, complex formation was competed by a 20-fold excess of unlabeled *let-7* miRNA. The M\* and M3/M4 complexes were almost not affected in formation and still increased their binding to labeled *let-7* over time (Fig. 2C,D). The smaller complexes, especially M1, were very susceptible to the competition by unlabeled *let-7*, resulting in low amounts of the complexes carrying labeled *let-7*. After that, the formation of M1 complex was barely changed over time, while the M2 complex slightly increased its binding to labeled *let-7* (Fig. 2C,D). A possible explanation is that the formation of smaller complexes M1 and M2 rapidly reaches the equilibrium state and these complexes could become less stable, and/or bound RNAs in the M1 and M2 complexes are quickly able to exchange and so was rapidly replaced by excess unlabeled *let-7* (Fig. 2C). The larger complexes M\* and M3/M4 may form more slowly and could be more stable, or the incorporated *let-7* is less exchangeable. This may also explain why certain concentrations of unlabeled *let-7* did not compete for the formation of M3/M4 complexes (Fig. 1B). Studies with *Drosophila* extracts indicate that siRNAs enter into multiple complexes and that the larger complexes arise from smaller ones (Pham et al. 2004; Tomari et al. 2004). It is also possible that the accumulation of the larger complexes M3/M4 is due to the transition from smaller complexes M1 or M2, and also a consequence of higher stability.

### Fractionation studies of *let-7* binding complexes

Gel filtration experiments with *C. elegans* extracts have shown that *let-7* miRNA cofractionates in protein complexes of ~500 and ~250 kDa in size (Caudy et al. 2003). We performed similar experiments and fractionated radiolabeled *let-7* miRNP complexes on a superdex-200 gel exclusion gravity column. We found radiolabeled M\*, M3/M4, and M2 complexes migrating at >669 kDa, ~500 kDa, and ~250 kDa, respectively. M1 and M\* complexes migrated at ~160 kDa (Fig. 3A). Interestingly, we found slightly different results when we first fractionated the native extracts and then assayed for *let-7* miRNP formation via gel shift (Fig. 3B). While the sizes of the complexes remained approximately the same, the relative abundance of the label in each complex was different. M1 and M2 complexes, the abundant forms in the nonfractionated extract (Fig. 3B, lane 2), formed poorly upon addition of radiolabeled *let-7* miRNA in fractionated extracts (Fig. 3B, lanes 10–18). One explanation is that fractionation may segregate some necessary associating factors for formation of the M1 and M2 complexes and causes a reduction in new *let-7* incorporation or the instability of these complexes. Pre-formed complexes containing *let-7* miRNA (Fig. 3A) may be less affected or more stable in the fractionation process.

The complex M\*, which was unable to enter the 5% native gel, was also observed in fractions that correspond to the void volume, which contain very-high-molecular-weight protein complexes of >669 kDa. Previous fractionation studies by Caudy et al. (2003) showed that endogenous *let-7* comigrates with complexes of ~500 kDa and ~250 kDa. Since we have identified a >669-kDa complex (M\*), in addition to the ~500-kDa and ~250-kDa complexes, assembling with exogenous *let-7*, we investigated how endogenous *let-7* miRNA fractionates in our extracts. We isolated RNA from each fraction from the experiment shown in Figure 3B and probed for the presence of endogenous *let-7* miRNA by Northern blot. We found that the majority of the *let-7* miRNA fractionated in the void volume corresponding to M\* and significant amounts of *let-7* also cofractionated with the ~500-kDa (M3/M4) complexes (Fig. 3C). Less *let-7* miRNA cofractionated with the ~250-kDa (M2) complex, and negligible amounts of *let-7* miRNA corresponded to the ~160-kDa (M1) complex. Considering the rapid formation and the abundance of M1 and M2 complexes when incubated with exogenous *let-7*, lower amounts of endogenous *let-7* cofractionated with the smaller complexes M1 and M2 also point to their higher susceptibility or exchange rate for the substrate. This observation reveals the existence of a major *let-7*-containing complex of >669 kDa in addition to the previously found ~500-kDa and ~250-kDa complexes (Caudy et al. 2003). The difference between these two sets of results could be due to the extracts used for fractionation. Caudy and colleagues used ribosome-associated extracts for gel filtration, whereas we have used a 16,100g supernatant, which corresponds to the total cytosolic extract.

VIG-1 and TSN-1 have previously been shown to cofractionate with an ~250-kDa complex, but not with an ~500-kDa miRNP complex (Caudy et al. 2003). We examined our fractions for the presence of VIG-1 protein. We found VIG-1 cofractionating with a complex that peaks at ~200 kDa overlapping partially with M1 and M2 (~160 kDa and ~250 kDa) complexes but not with M3/M4 (~500 kDa) complexes (Fig. 3D). To our surprise, we found VIG-1 also cofractionated with the larger complex M\* (>669 kDa). We isolated the proteins from these complexes from native gels and analyzed them by Western blot for the presence of VIG-1 and TSN-1. We found VIG-1 and TSN-1 proteins only in the M\* complex but not in the M1, M2, or M3/M4 complexes (Fig. 3E). A control protein, actin, was barely present in the proteins isolated from the M\* complex compared to the total proteins, indicating preferential enrichment of VIG-1 and TSN-1 proteins in M\*, but not in other complexes. Although earlier fractionation studies (Caudy et al. 2003) suggested the presence of VIG-1 in an ~250-kDa complex, Western blot analysis of proteins from these complexes suggests that VIG-1 is not present in our M1 (~160 kDa) or M2 (~250 kDa) complexes, but instead is present only in the M\* complex. Therefore, in addition to



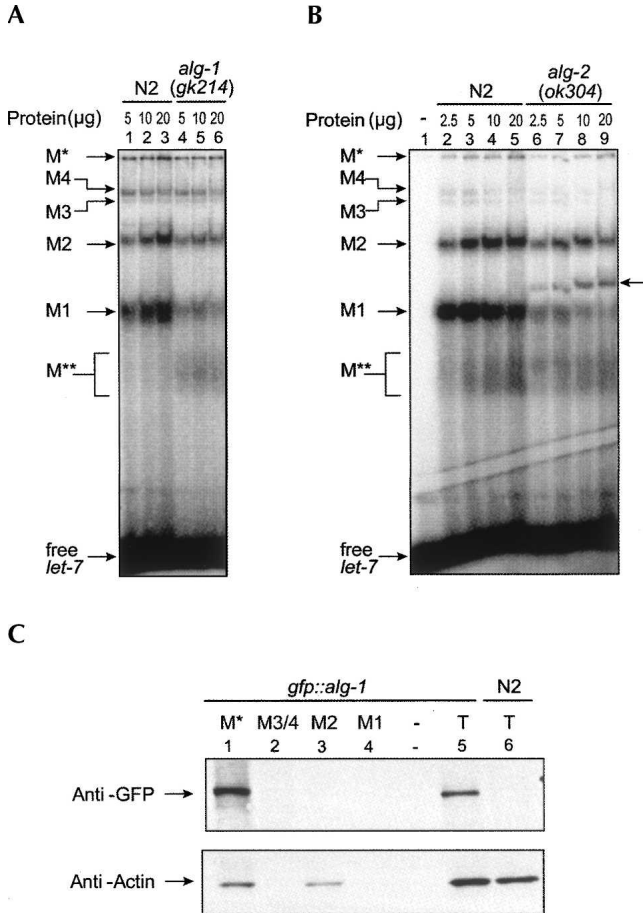
**FIGURE 3.** Fractionation assay of *let-7* miRNPs. (A) Fractionation of pre-formed complexes with radiolabeled *let-7* miRNA by gel filtration. The positions of eluted molecular weight markers are represented by arrows. (B) *C. elegans* cytosolic extracts were fractionated, and then each fraction was assayed for miRNP formation with radiolabeled *let-7* miRNA. (T) Complex formed with 20  $\mu$ g of protein of total extracts. (C) RNAs in fractionated samples from B were analyzed by Northern blot for the presence of endogenous *let-7* miRNA. (T) RNA isolated from total extract. (D) Indicated fractions were assayed for the presence of VIG-1 protein by Western blot. (E) Proteins isolated from indicated *let-7* binding complexes were separated by SDS-PAGE and analyzed for the presence of VIG-1, TSN-1, or actin proteins by Western blot. (T) Total extract.

the  $\sim$ 250-kDa complex identified in the earlier study from *C. elegans* (Caudy et al. 2003), we have found that a larger *let-7*-containing complex M\* ( $>$ 669 kDa) also contains VIG-1 and TSN-1. However, since our Western blot analysis did not reveal that VIG-1 or TSN-1 associates with M1 ( $\sim$ 160 kDa) or M2 ( $\sim$ 250 kDa) complexes (Fig. 3E), cofractionation of VIG-1 with M1 and M2 complexes could be coincidental.

### Formation of *let-7* miRNP complexes is affected by *alg-1* or *alg-2* mutations

In *C. elegans*, genetic studies have shown that two closely related Argonaute proteins, *alg-1* and *alg-2* (referred to here as *alg-1/2*), are necessary for maturation and/or activity of *lin-4* and *let-7* miRNAs (Grishok et al. 2001). Coimmunoprecipitation experiments from *C. elegans* extracts have shown that ALG1/2 proteins interact with the *let-7* miRNA (Hutvagner et al. 2004). ALG1/2 are

homologous to RDE-1, which is essential for RNAi in *C. elegans* (Tabara et al. 1999), and to the Argonaute-like protein Ago2, which is known to be present in siRISC of human and *Drosophila* extracts (Hammond et al. 2001; Hutvagner and Zamore 2002; Martinez et al. 2002; Mourelatos et al. 2002) and responsible for the cleavage activity (Liu et al. 2004; Meister et al. 2004). Therefore, we asked whether Argonaute-like genes *alg-1* and *alg-2* are necessary for the formation of these complexes. We prepared extracts from *alg-1(gk214)* and *alg-2(ok304)* knockout mutants and assayed for the formation of *let-7* binding complexes. We observed a substantial decrease in the amount of M1 and M2 complexes and a slight decrease in the amount of other complexes in both *alg-1* and *alg-2* deletion mutants (Fig. 4A,B). Interestingly, the absence of ALG-2 also leads to the formation of a distinct complex intermediate in size between complexes M1 and M2 (Fig. 4B). Since *alg-1* and *alg-2* have been shown necessary for maturation and activity of the *let-7* miRNA (Grishok et al. 2001),



**FIGURE 4.** Formation of the *let-7* complexes is affected by *alg-1* or *alg-2* mutations. (A) Gel retardation of labeled *let-7* miRNA by extracts prepared from wild-type (N2) or *alg-1(gk214)* animals. (B) Gel retardation of labeled *let-7* miRNA by extracts prepared from wild-type (N2) or *alg-2(ok304)* animals. Arrow corresponding to lanes 5–9 indicate the appearance of an extra complex in *alg-2(ok304)* mutants. (C) Proteins isolated from indicated *let-7* binding complexes formed in the cell extract prepared from a transgenic line of *C. elegans* that expresses GFP-tagged ALG-1 were separated by SDS-PAGE. The presence of GFP-tagged ALG-1 or actin proteins was analyzed by Western blot. (T) Total extract.

and hence to mediate proper developmental timing, the susceptibility to *alg-1* or *alg-2* mutations of the *let-7* miRNP complexes discovered here implies the relevance of these complexes to *let-7* function. Extracts prepared from other developmental timing (heterochronic) defective mutants *lin-28(n719)* and *lin-41(ma104)* did not affect formation of *let-7* miRNP complexes (data not shown), suggesting that these effects are specific to *alg-1* and *alg-2* and not associated with the heterochronic pathway per se. Since extracts prepared from the *alg-1(gk214)* and *alg-2(ok304)* null mutants (Grishok et al. 2001) did not completely abolish the formation of complexes, we speculate that ALG-1 or ALG-2 may be partially substituting for one another. Such redundancy between *alg-1* and *alg-2* has already been observed for their functions in temporal patterning,

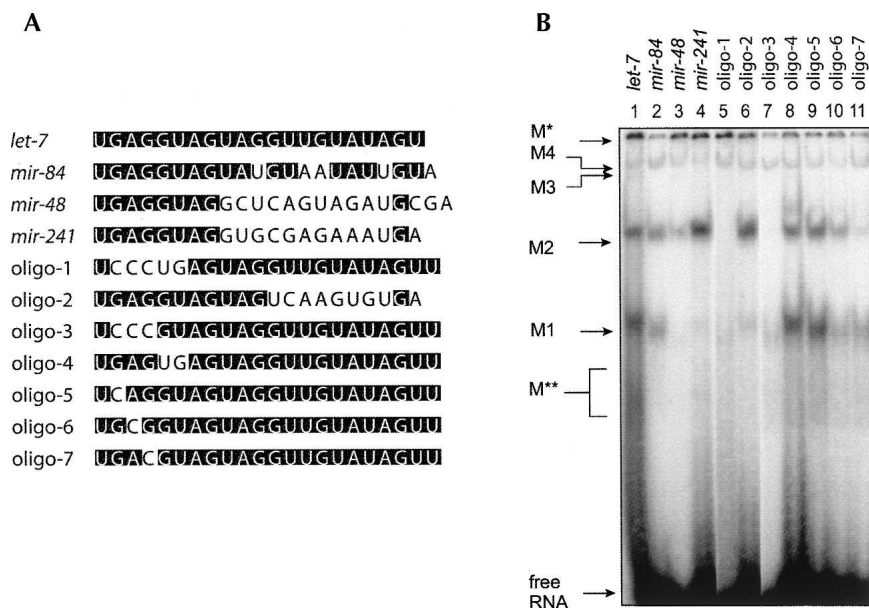
miRNA biogenesis, and miRNA function (Grishok et al. 2001). However, we are unable to directly test this hypothesis because the *alg-1; alg-2* double mutant is lethal.

We have assayed the presence of ALG-1 protein in different *let-7* binding complexes in cell extract prepared from a transgenic line of *C. elegans* that expresses GFP-tagged ALG-1. Similar to VIG-1 and TSN-1, ALG-1 was detected only in the M\* complex (Fig. 4C). This is consistent with previous results showing that ALG-1 cofractionated only with a single complex larger than 650 kD in size (Tops et al. 2006) and suggests that only the M\* complex is a candidate for functional miRISC. The reason that the smaller complexes M1 and M2, which do not contain ALG-1 protein, are more susceptible to *alg-1* or *alg-2* mutations is not clear. One speculation is that these complexes may play roles after the formation or functioning of Ago-containing *let-7* miRISC or prior to this in a feedback loop, and their formation is affected by the absence of ALG-1 or ALG-2 proteins. Another explanation is that expression of some factors required for these complexes is regulated by *alg-1* and *alg-2*.

#### Sequence requirements for *let-7* miRNP formation

The M1 and M2 complexes assemble with *let-7* and *mir-84*, a member of the *let-7* family, but not *lin-4* or *mir-234* (Fig. 1). In contrast, the M\* and M3/4 complexes are able to assemble with all miRNAs we tested so far, although in varying amounts. Thus, the M1 and M2 complexes appear specific to the *let-7* family, while the others are not. To test this hypothesis, we applied the same assay to two other members of the *let-7* family, *mir-48* and *mir-241*, and also to artificial chimeric miRNAs designed by mixing the sequences of *let-7* and *lin-4*. All short RNAs were heated before incubation with cell extracts to reduce the possibility of secondary structure formation. *mir-48* and *mir-241* are identical to *let-7* in the 5' seed region but less conserved in the sequences 3' to the seed. *mir-48* and *mir-241* formed the M2 complex but only very small amounts of M1 complexes (Fig. 5B, lanes 3,4). Oligo-1, which contains the *lin-4* seed and the *let-7* sequence outside the seed, formed a very small amount of M1 complex but did not form M2 (Fig. 5B, lane 5). Oligo-2, which contains nucleotides 1 to 11 of *let-7*, covering the seed region, and nucleotides 12 to 21 of *lin-4*, formed a significant amount of M2 complex in addition to the small amount of M1 complex (Fig. 5B, lane 6). This suggests that the formation of the M2 complex is responding to the sequence of the *let-7* seed region. On the other hand, all the four RNAs formed much smaller amounts of the M1 complex, compared to *let-7* and *mir-84*, implying that intense formation of M1 complex may require the entire sequence to be more like *let-7*.

We further investigated the requirement of the *let-7* seed sequence for complex formation. We modified the *let-7*



**FIGURE 5.** The *let-7* sequence is required for formation of the M1 and M2 complexes. (A) Sequence alignment of RNA oligonucleotides used in the gel retardation analysis in *C. elegans* extracts. (Solid boxes) Nucleotides identical to the *let-7* sequence at the same positions. (B) RNA oligonucleotides listed in A were analyzed for ribonucleoprotein complex formation by native gel electrophoresis.

miRNA by replacing nucleotides in the seed region with the corresponding *lin-4* sequences (Fig. 5A, oligo-3 to oligo-7). Interestingly, replacing the nucleotides 2~4 completely abolished the M2 complex formation and also reduced the amount of M1 complex significantly (Fig. 5B, oligo-3, lane 7), whereas replacing nucleotides 5 and 6 did not show obvious negative effects (Fig. 5B, oligo-4, lane 8). This suggests that the nucleotides 2~4 in the *let-7* seed are required for M2 complex formation and are also important for M1. Then we modified each single nucleotide from the nucleotides 2 to 4 and found that single nucleotide substitutions at nucleotides 3 or 4 showed negative effects on formation of both the M1 and M2 complexes (Fig. 5B, lanes 9–11). In contrast, the existence of M3/M4 complexes was not affected by these sequence modifications. These results argue again that the M1 and M2 complexes are specific to *let-7* and also reveal a putative *cis*-element on *let-7* required for the formation of these complexes.

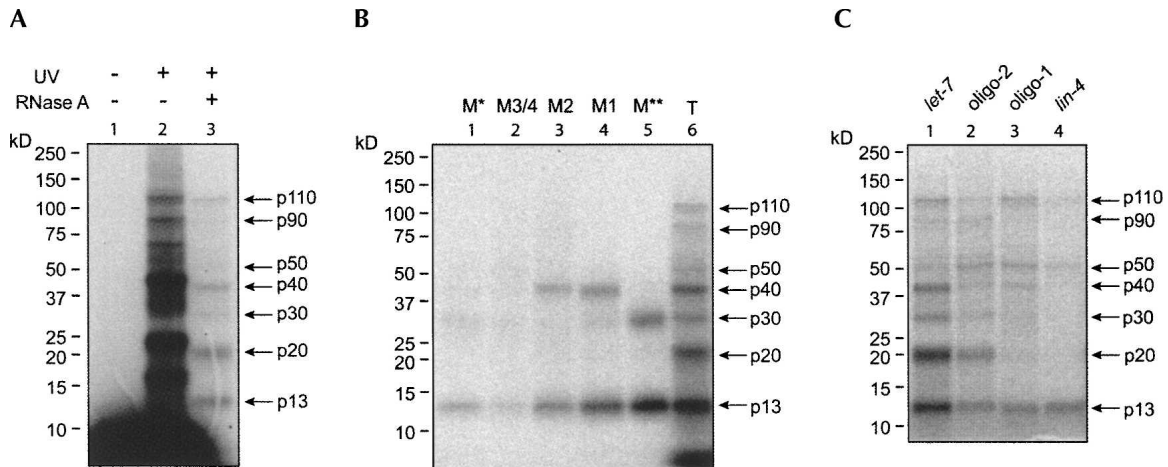
### Sequence-dependent binding of proteins to *let-7*

Although we did not find miRISC components like ALG-1, TSN-1, or VIG-1 in the M1 and M2 complexes, their susceptibility to *alg-1* and *alg-2* mutations implied a relevance to miRNA function. In addition, their formation depends on the *let-7* sequence, suggesting that they may play roles in *let-7*-related functions. We sought to identify proteins present in these *let-7* miRNP complexes and to determine whether those associating proteins are specific to

*let-7*. To detect *let-7*-associating proteins, we used ultraviolet light (254 nm wavelength) to cross-link RNA to protein in a reaction mixture containing *C. elegans* extract and 5'-radiolabeled *let-7* miRNA. Proteins cross-linked to *let-7* were assayed by SDS-PAGE with or without RNase A treatment. After RNase A digestion, only proteins that were cross-linked to the 5'-end of *let-7* could be detected and would be predicted to migrate close to positions corresponding to their molecular weights. At least seven cross-linked proteins, in sizes of ~110, ~90, ~50, ~40, ~30, ~20, and ~13 kDa, were detected with medium to strong signals (Fig. 6A), indicating their close spatial relationships to *let-7* or even a direct association with *let-7*.

We performed an “in-gel” UV cross-linking to see whether these proteins are present in the *let-7* binding complexes we discovered. After the *let-7* binding complexes were separated on a native gel, we sliced the gel into pieces containing each *let-7* binding complex and exposed them to UV light. Proteins eluted from each gel piece were assayed by SDS-PAGE. Only p13, p30, and p40 were observed after electrophoresis, indicating that other proteins may lose their associations with *let-7* during electrophoresis. p13 was present in all complexes, suggesting its relevance in protein components among the *let-7* binding complexes. Interestingly, p40 appeared to be present only in M1 and M2 complexes, and p30 was only present in the less distinct M\*\* complex (Fig. 6B). These results also reveal that these *let-7* binding complexes may share common components but could still contain specific factors.

To determine the requirements of these *let-7* binding proteins for target sequences, we performed UV cross-linking with reaction mixtures in which other miRNAs, including *lin-4* and artificial ones containing parts of *let-7* sequences, were radiolabeled at the 5'-end and incubated with cell extract, respectively. Oligo-2, which contains the seed region of *let-7* and is capable of forming abundant M2 complex in addition to a low amount of M1 complex (Fig. 5B), was cross-linked to all proteins that were cross-linked to *let-7*, but the signals seemed to be weakened (except for p50) (Fig. 6C, lane 2). Oligo-1, which is modified in the seed region and only formed a low amount of M1 complex and no M2 complex (Fig. 5B), was not cross-linked to p20 and showed less association with p30 and p40 (Fig. 6C, lane 3). Only p110, p50, and p13 were detected in the cross-linking reaction using *lin-4*, in which only the M\* and M3/M4 complexes were detected. The presence of proteins specifically cross-linked to the *let-7* miRNA seems to



**FIGURE 6.** *let-7* specific binding proteins were detected by UV cross-linking. (A) Radiolabeled *let-7* was incubated with cell extract, and proteins were cross-linked to the probe with UV light. After RNase A treatment, cross-linked proteins were analyzed by SDS-PAGE and autoradiography. (B) Radiolabeled *let-7* was incubated with cell extract, and *let-7* binding complexes were separated on a native gel. Gel pieces containing each complex were sliced out and exposed to UV light. Cross-linked proteins were eluted and treated by RNase A and then analyzed by SDS-PAGE. (C) Radiolabeled *let-7*, *lin-4*, *oligo-1*, and *oligo-2* RNAs were incubated with cell extract and exposed to UV light. Cross-linked proteins were treated by RNase A and T1 and analyzed by SDS-PAGE.

correspond to formation of the M1 and M2 complexes, which was highly dependent on the *let-7* sequence (Fig. 5). These results suggest sequence-specific association of proteins to *let-7*, like the binding of p40, and indicate that *let-7* associates with a set of specific proteins to assemble distinct complexes. However, some associating proteins discovered here, for example, p13, are not specific to *let-7* since they were also cross-linked to *lin-4* and may represent common factors in miRNPs.

## DISCUSSION

We have observed the formation of multiple *let-7* miRNP complexes in *C. elegans*, ranging in size from 160 to >669 kDa, similar to several RISC complexes described in previous studies (Hammond et al. 2000; Nykanen et al. 2001; Martinez et al. 2002; Pham et al. 2004). Our results also indicate that these *let-7* miRNP complexes form in different ways and with different stabilities. A trend we observed is that the larger complexes of >669 kDa and ~500 kDa have lower exchange rates for the substrate and higher stabilities. This is supported by observations that these larger complexes are more resistant to competitive unlabeled *let-7* and to the fractionating process. Although the smaller M1 and M2 complexes form rapidly when exogenous *let-7* is added into a binding reaction, the bound substrates seem to exchange actively. Moreover, we found that the major fractions of endogenous *let-7* were cofractionated with the larger complexes but not the M1 and M2 complexes; hence, it is possible that the M1 and M2 complexes are intermediate complexes with less stability and the stable large complexes M\* and M3/M4 are more likely the major complexes that maintain bound *let-7* in vivo. The M\*

complex might represent a functional miRISC since it was cofractionated with abundant endogenous *let-7* and contains ALG-1 and two known factors, VIG-1 and TSN-1, which have been shown to associate with RISC and *let-7* (Caudy et al. 2002, 2003). It is worth noting that in *Drosophila* extracts, RISC activity has been observed in the R\* complex, similar to M\*, which does not enter the gel and fractionates in the void volume, as well as in an 80S complex that is likely to be a part of the R\* complex (Pham et al. 2004).

The dynamic nature of RISC complexes described in results from Pham et al. (2004) and Tomari et al. (2004) suggests that smaller siRNA-associated complexes convert into larger, active complexes. It is possible that miRNA-associated complexes also perform a similar assembly pathway. *let-7* could be transferred from the M1 and M2 complexes to the larger complexes, or a portion of the smaller complexes with incorporated substrate further assembles to stable larger complexes. However, our results show that free *let-7* is able to enter into larger complexes in fractionated extracts in which M1 and M2 are absent. Thus the formation of larger complexes M\* and M3/M4 can be separated from the formation of M1 and M2. In addition, some other miRNAs we have tested, including *lin-4*, *mir-234*, and several artificially designed short RNAs containing parts of *let-7* or *lin-4* sequences, are all able to form the larger complexes M\* and M3/M4 but not the M1 and M2 complexes. It is possible that most miRNAs can enter large miRNP complexes of ~500 kDa or >669 kDa to accomplish their function, but some miRNAs, including *let-7*, also enter multiple complexes with smaller sizes. These smaller miRNP complexes may play an aforementioned role in dynamic formation of the larger and active complexes or



have unknown functions. For now in our experiments, we found that formation of the smaller M1 and M2 complexes is susceptible to *alg-1* or *alg-2* null mutations although ALG-1 is not detected in these complexes. We speculate that the M1 and M2 complexes may function at the steps prior to or following the formation of Argonaute-containing complexes.

While there seems to be no specificity for the formation of the M\* and M3/M4 complexes when different miRNAs were used, interestingly, the M1 and M2 complexes seem specific to the *let-7* miRNA family. We investigated the sequence specificity for complex formation by using a few artificially designed miRNAs containing parts of *let-7* or *lin-4* sequences. Changing or replacing different parts of the *let-7* sequence can dramatically affect the formation of M1 and M2 complexes. We found that nucleotides 2 to 4 of *let-7* are required for the formation of M2 and are also important for M1. Here we cannot eliminate the possibility that changes in the sequence alter secondary structure and hence affect complex formation. However, the most plausible short intramolecular base pairs predicted by mfold (Zuker 2003) in the sequences of the *let-7* family members, which all form some amount of the M2 complex, are different from each other (data not shown), suggesting that a special secondary structure is not required for complex formation. Also, there is no clear correlation between the predicted structures of the short RNAs tested in this study and their abilities to form the M1 and M2 complexes. In addition, the free energy changes ( $\Delta G$ ) estimated by mfold for all chimeric short RNAs to form secondary structure are similar to those for *let-7* and *lin-4* (data not shown), which are presumed to associate with RISC component proteins in physiological conditions and to serve as single-stranded RNA fragments for base-pairing with their targets. Thus we propose that our results are more likely due to the changes in the primary sequences.

By UV cross-linking, we detected a number of proteins associating with *let-7* in a binding reaction in which labeled *let-7* was incubated with cell extract. Among them, p40 was specifically detected in the M1 and M2 complexes, and its cross-linking to the miRNA is highly sensitive to changes in the *let-7* sequence. p40 is hence a strong candidate for a *let-7*-specific associating factor. p13 was detected in all *let-7* binding complexes, including M\* and M3/M4 that were also formed with other miRNAs we tested. p13 was also cross-linked to *lin-4*. One speculation is that p13 is not specific to *let-7* and could generally exist in many miRNPs formed with different miRNAs. However, in our experiments, more abundant p13 was cross-linked to *let-7*, perhaps due to the formation of M1 and/or M2 complexes. This implies that the formation of smaller *let-7* complexes, which may contain some *let-7*-specific binding proteins, also contribute to recruitment of other binding proteins, which may be miRISC components and lack a preference of selectivity to miRNAs. Recent studies have shown that mouse Lin28 is

able to bind pri-*let-7* and pre-*let-7* miRNAs and specifically inhibits the pri-*let-7* processing in embryonic cells (Viswanathan et al. 2008). This has revealed a possibility that some RNA-binding proteins selectively associate with the pri-miRNAs or pre-miRNAs and manipulate their biogenesis or function. Our results indicate that the mature *let-7* miRNA could associate with specific proteins and be incorporated into distinct complexes as well. Since our complexes formed in the *lin-28*-null mutant, we have likely identified novel *let-7* binding proteins in addition to LIN-28. It remains to be seen what the functions of these proteins are.

## MATERIALS AND METHODS

### Preparation of *C. elegans* extracts

Wild-type (N2) animals were grown at 20°C in 50 mL of S medium (Stiernagle 1999), supplemented with *Escherichia coli* HB101. After 48–60 h of growth, the mixed-staged animals were collected by sedimentation on ice. The animals were washed with 0.1 M NaCl three times and frozen at –80°C. Packed animals (1 mL) were thawed on ice and washed twice with 4 volumes of wash buffer (50 mM Tris-HCl at pH 7.5 and 10 mM potassium acetate) followed by centrifugation at 3000 rpm for 2 min. The animals were resuspended in 4.0 mL of homogenization buffer (50 mM Tris-HCl at pH 7.5, 10 mM potassium acetate, 5 mM DTT, 10 U/mL SUPERase-In [Ambion], and 1× Complete protease inhibitor [Roche]) and incubated for 20 min on ice with intermittent agitation. The animals were transferred to a 7-mL glass dounce homogenizer for 30 strokes on ice using pestle B. The homogenate was transferred to a 15-mL tube, 2 mM magnesium acetate was added, and the concentration of potassium acetate was adjusted to 100 mM. The homogenate was incubated for 20 min on ice with intermittent agitation and then centrifuged at 16,100g for 30 min at 4°C. The supernatant was stored at –80°C and used as cell extracts in this study.

In the experiments using *alg-1* and *alg-2* mutants (Fig. 4), wild-type (N2) or mutant animals were grown on NGM plates with *E. coli* OP50 as bacterial food. Mixed-staged animals were collected from 10 plates (~100  $\mu$ L of packed animals) and washed three times by M9 buffer (22 mM KH<sub>2</sub>PO<sub>4</sub>, 42 mM Na<sub>2</sub>HPO<sub>4</sub>, 85 mM NaCl, and 1 mM MgSO<sub>4</sub>). These animals were frozen at –80°C until use. The animals were washed and homogenized using a pellet pestle (Kimble-Knotes) and the lysis buffers described above.

### RNA oligonucleotides

Synthetic RNAs were obtained from Dharmacon Research Inc. or Integrated DNA Technologies, Inc. as follows:

*let-7*(wt) (5'-UGAGGUAGUAGGUUGUAUAGU-3');  
*let-7*(mut) (5'-UGAGAUAGUAGGUUGUAUAGU-3');  
*mir-84* (5'-UGAGGUAGUAUGUAAUUAUUGUA-3');  
*mir-48* (5'-UGAGGUAGGCUCAGUAGAUUGCGA-3');  
*mir-241* (5'-UGAGGUAGGUGCGAGAAAUGA-3');  
*mir-234* (5'-UUAUUGCUCGAGAAUACCCUU-3');  
*lin-4* (5'-UCCCUGAGACCUCAAGUGUGA-3');

oligo-1 (5'-UCCUGAGUAGGUUGUAUAGUU-3');  
 oligo-2 (5'-UGAGGUAGUAGUCAAGUGUGA-3');  
 oligo-3 (5'-UCCCGUAGUAGGUUGUAUAGUU-3');  
 oligo-4 (5'-UGAGUGAGUAGGUUGUAUAGUU-3');  
 oligo-5 (5'-UCAGGUAGUAGGUUGUAUAGUU-3');  
 oligo-6 (5'-UGCGGUAGUAGGUUGUAUAGUU-3'); and  
 oligo-7 (5'-UGACGUAGUAGGUUGUAUAGUU-3').

To monitor ribonucleoprotein complex formation in vitro, oligonucleotides were labeled at the 5'-end using T4 polynucleotide kinase and [ $\gamma$ - $^{32}$ P]ATP. Reaction mixtures (25  $\mu$ L) contained 1 $\times$  kinase buffer (NEB, 50 mM Tris at pH 7.5, 5 mM MgCl<sub>2</sub>), 2.0  $\mu$ M RNA, 50  $\mu$ Ci of [ $\gamma$ - $^{32}$ P]ATP (3000 Ci/mmol, NEN), and 25 units of T4 polynucleotide kinase. Reactions were performed for 30 min at 37°C. Free [ $\gamma$ - $^{32}$ P]ATP nucleotides were removed by passing the mixture through a Spandex G-25 column (Roche). The specific activities of the RNAs were typically 1  $\times$  10<sup>6</sup> c.p.m./pmol.

### Native gel electrophoresis

RNA binding reaction mixtures (10  $\mu$ L) containing 20 mM Tris (pH 7.5), 1 mM magnesium acetate, 1 mM calcium chloride, 0.01% NP-40, 2 mM ATP, 250 ng/ $\mu$ L *E. coli* tRNA, 4  $\times$  10<sup>5</sup> c.p.m. end-labeled RNA (~40 nM), and different amounts of extracts were incubated for 60 min at room temperature and then loaded onto a 5% native polyacrylamide gel. The native gel was subjected to electrophoresis in 1 $\times$  TBE at 100 V for 120 min, dried on 3 MM Whatman paper, and exposed to a PhosphorImager plate. The PhosphorImager signals were quantified using ImageQuant 5.1 software (Molecular Dynamics).

### Gel filtration

For fractionation of pre-formed *let-7* binding complexes, a gravity column was prepared using an Econo-Column (Bio-Rad) column and 2 mL of packed Superdex-200 beads (Amersham-Pharmacia), which were equilibrated with gel filtration buffer (20 mM Tris-HCl at pH 7.5, 50 mM potassium acetate, 1 mM magnesium acetate, 1 mM calcium chloride, and 5% glycerol). One hundred microliters of RNA-binding reaction containing 200  $\mu$ g of protein was carried out for 60 min and separated on the column by gravity. Around 30 fractions (~100  $\mu$ L each) were collected assayed by native gel electrophoresis as described above.

For gel filtration of crude *C. elegans* extracts, 400- $\mu$ L extracts with 6 mg/mL total protein were loaded onto a Superdex-200 HR 10/30 column that was equilibrated with gel filtration buffer. The proteins were separated at 4°C at the rate of 0.35 mL/min using AKTA FPLC (Pharmacia), and 60 fractions (0.5 mL each) were collected. To monitor the ability to form *let-7* binding complexes, samples of each fraction were mixed with radiolabeled *let-7* and assayed for miRNP formation by native gel electrophoresis as described above.

To detect endogenous *let-7*, indicated fractions (300  $\mu$ L) were mixed with 100  $\mu$ L of 4 $\times$  proteinase K buffer (400 mM Tris-HCl at pH 7.5 and 4% SDS). Proteinase K was added to a final concentration of 450  $\mu$ g/mL, and the reaction was carried out for 20 min at 65°C. Then the samples were extracted by phenol/chloroform/isoamyl alcohol (25:24:1), and the RNAs were precipitated in the presence of 20  $\mu$ g of glycogen. The RNAs were separated on a 10% TBE urea gel and transferred to a nylon membrane. The *let-7* miRNA was probed by end-labeled DNA oligonucleotide complementary to *let-7* (Johnson et al. 2003).

### UV cross-linking

RNA binding reaction mixtures (20  $\mu$ L) containing 1 mg/mL protein from cell extract and  $^{32}$ P-labeled miRNA were placed onto a pre-chilled 96-well plate on ice and exposed to 254-nm light for 15 min in a Stratalinker 2400 (Stratagene). The reactions were treated by 5 U/mL RNase A and/or 50 U/mL RNase T1 for 30 min at room temperature and then analyzed by denaturing gel electrophoresis using a Criterion XT Bis-Tris 4%–12% gradient gel (Bio-Rad) and autoradiography.

For the “in-gel” UV cross-linking experiment, a 150- $\mu$ L RNA-binding reaction containing 1 mg/mL protein from cell extract and  $^{32}$ P-labeled miRNA was subjected to electrophoresis on a 5% native preparative gel. Without drying, the gel was exposed to a BioMax MS film (Kodak) for 1 h. The film was aligned with the gel, and the gel slices containing each complex were individually excised. For samples subjected to UV cross-linking, gel slices were placed on a clean glass plate on ice and exposed to 254-nm light for 15 min. The gel slices were minced into small pieces. For the RNase A treatment, the gel pieces were mixed with 150  $\mu$ L of diluted RNase A (250 U/mL in 50 mM Tris-HCl at pH 7.5 buffer) and incubated for 30 min at room temperature. Then 50  $\mu$ L of 4 $\times$  sample buffer (Bio-Rad) were added, and the samples were agitated vigorously on a vortex mixer for 3 h. To extract protein efficiently, the gel slices were frozen and thawed twice intermittently. Eluted proteins were analyzed by denaturing gel electrophoresis as described above.

### Immunoblotting

Proteins in gel filtration fractions or eluted from native gel slices were separated on SDS denaturing gels and transferred to nitrocellulose membranes (Schleicher and Schuell). Polyclonal anti-VIG-1, anti-TSN-1, and anti-GFP (A11122; Molecular Probes) antibodies, and monoclonal anti-Actin antibody (clone C4; ICN Pharmaceuticals) were used as primary antibodies. Western blots were developed by ECL (Amersham Biosciences).

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