RESEARCH COMMUNICATION

The *C. elegans* microRNA *let-7* binds to imperfect *let-7* complementary sites from the *lin-41* 3′UTR

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***Caenorhabditis elegans let-7*, a founding member of the microRNA family, is predicted to bind to six sites in the 3′UTR of the mRNA of its target gene, *lin-41*, to down-regulate LIN-41. Here, we demonstrate that wild-type *let*-7 microRNA binds in vitro to RNA from the *lin-41* 3′UTR. This interaction is dependent on two conserved *let*-7 complementary sites (LCSs). A 27-nucleotide sequence between the LCSs is also necessary for down-regulation in vivo. LCS mutations compensatory to the lesion in *let-7(n2853)* can partially restore *lin-41* 3′UTR function in a *let-7(n2853)* background, providing the first experimental evidence for an animal miRNA binding directly to its validated target in vivo.**

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Genes that regulate the timing of *Caenorhabditis elegans* post-embryonic development are called heterochronic genes. Several heterochronic genes are regulated post-transcriptionally through elements in their 3′UTR. This regulation is mediated by small temporal RNAs (stRNAs), like *lin-4* and *let-7*, expressed at the first larval stage (L_1) and L_3 stage, respectively (Wightman et al. 1993; Feinbaum and Ambros 1999; Reinhart et al. 2000; Johnson et al. 2003). stRNAs are predicted to interact with partially complementary sequences in the 3′UTRs of their target genes and down-regulate gene expression through an unknown mechanism; however, the direct binding of an stRNA to its validated target has not been demonstrated experimentally in vivo.

lin-4 is thought to act by antisense base pairing to seven complementary sites in the *lin-14* 3′UTR to down-regulate *lin-14* post-transcriptionally (Lee et al. 1993; Wightman et al. 1993). The 3′UTR of *lin-14* is sufficient for temporal down-regulation; experiments using a *lacZ* reporter gene fused to the *lin-14* 3′UTR show stage-specific down-regulation (Wightman et al. 1993). The seven complementary sites in the *lin-14* 3′UTR are also conserved in *Caenorhabditis briggsae*, a closely related *Caenorhabditis* species, and are deleted in *lin-14* gain-of-function mutants, demonstrating that these sites are crucial to normal *lin-14* regulation (Wightman et al. 1993; Ha et al. 1996). Binding of *lin-4* to *lin-14* is thought to block protein synthesis or accumulation after the initiation of translation (Olsen and Ambros 1999).

let-7 controls the larval-to-adult (L/A) transition and *let-7* mutants display retarded terminal differentiation of seam cells (Reinhart et al. 2000). *lin-41* mutants display precocious terminal differentiation of these cells at the L_4 stage (Slack et al. 2000). As judged by LIN-41/GFP fusion, LIN-41 appears to be down-regulated in the seam cells during the L_4 stage, coinciding with the up-regulation of *let-7* RNA (Slack et al. 2000). As in the case with *lin-14*, the *lin-41* 3′UTR placed behind a heterologous promoter is sufficient to down-regulate a reporter gene. This down-regulation requires *let-7* and a region in the *lin-41* 3′UTR with sites complementary to *let-7*.

let-7 and *lin-41* are conserved from *C. elegans* to mammals, and LCSs are found in *Drosophila* and zebrafish *lin-41* 3′UTRs (Pasquinelli et al. 2000; Slack et al. 2000). This conservation suggests that the mechanism of gene regulation is also present in higher eukaryotes. *let-7* has targets in addition to *lin-41*, namely, *hbl-1*, which has eight predicted LCSs in its 3′UTR (Lin et al. 2003).

lin-4 and *let-7* are the founding members of a larger family of microRNAs (miRNAs). MiRNAs are genomically encoded, untranslated RNA molecules of ~20–25 nucleotides (nt), transcribed as precursors that can form a hairpin loop (Ambros et al. 2003). For example, the *let-7* miRNA precursor RNA is ~70 nt and is processed into a mature 22-nt molecule by the RNAse III nuclease Dicer (*dcr-1* in *C. elegans*; Bernstein et al. 2001; Grishok et al. 2001).

Numerous groups have reported the cloning of >100 miRNAs using HeLa cell culture, *Drosophila* (Lagos-Quintana et al. 2001; Mourelatos et al. 2002), *C. elegans* (Lau et al. 2001; Lee and Ambros 2001; Lim et al. 2003), plants (Reinhart et al. 2002), and embryonic stem cells (Houbaviy et al. 2003). Like *let-7*, (Pasquinelli et al. 2000), some of the recently discovered miRNAs have been shown to be developmentally regulated and are highly conserved in *Drosophila* and humans (Lau et al. 2001; Mourelatos et al. 2002). The function of some of these novel miRNAs has now been deciphered. In *Drosophila*, *bantam* encodes a miRNA involved in cell proliferation and cell death (Brennecke et al. 2003), and *mir-14* plays a role in cell death and fat metabolism (Xu et al. 2003). Loss of *mir-15/16* leads to chronic lymphocytic leukemia (CLL) in humans (Calin et al. 2002). It is now apparent that a novel mechanism of gene regulation involving untranslated RNA molecules, first described with *lin-4* and *let-7*, may be a general and ancient one.

To investigate the mechanism(s) by which animal miRNAs regulate their targets, we have dissected the interaction between the *let-7* miRNA and one of its target genes, *lin-41*. We have determined that the sequences both necessary and sufficient for *lin-41* down-regulation

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map to three elements in the *lin-41* 3′UTR, two of which are complementary to *let-7*. We also show that *let-7* binds both in vitro and in vivo to RNA from the *lin-41* 3′UTR containing these two conserved LCSs, presenting the first evidence for a direct interaction between an animal miRNA and imperfect complementary sites in a validated miRNA target.

Results and Discussion

Multiple conserved LCSs in the lin-41 *3′UTR*

The 1.1-kb *lin-41* 3′UTR is sufficient to down-regulate a *lacZ* reporter gene driven by the *col-10* promoter in a *let-7*-dependent manner, during late larval development in *C. elegans* (Reinhart et al. 2000). Additionally, an 85-bp deletion of the *lin-41* 3′UTR compromises temporal down-regulation of the reporter (Reinhart et al. 2000). Within this 85-bp region, are at least two sequences complementary to the *let-7* miRNA, except for small bulged regions within the predicted duplexes. We found four additional putative LCSs, sites 3–6, in the *lin-41* 3′UTR with a lower predicted binding affinity to *let-7* than have the previously reported sites (LCS 1–LCS 2, Supplemental Fig. S1a,b). Of the six predicted LCSs in the 3′UTR of *lin-41* (Supplemental Fig. S1b), three are removed (LCS 1–LCS 3) in the 85-bp deletion (pFS1031, Fig. 1A).

LCS 1 and LCS 2 are conserved in C. briggsae

At the protein level, *C. elegans lin-41* is well conserved in *C. briggsae* (Stein et al. 2003). The NHL domain (Slack and Ruvkun 1998; Slack et al. 2000) of LIN-41 is 95% conserved between species as determined by BLAST analysis. In addition, sequences in the 3′UTR of *C. briggsae lin-41* are also conserved, and the *C. briggsae lin-41* 3′UTR was able to partially down-regulate a reporter gene during *C. elegans* development (see below).

To determine which of the six 3′UTR LCS sequences may be important for *lin-41* function, we performed a sequence alignment between the *C. elegans* and *C. briggsae lin-41* 3′UTRs (Supplemental Fig. S2). LCS 1 and LCS 2 are 100% conserved, suggesting they are important for LIN-41 down-regulation. LCS 3–LCS 6, in contrast, are

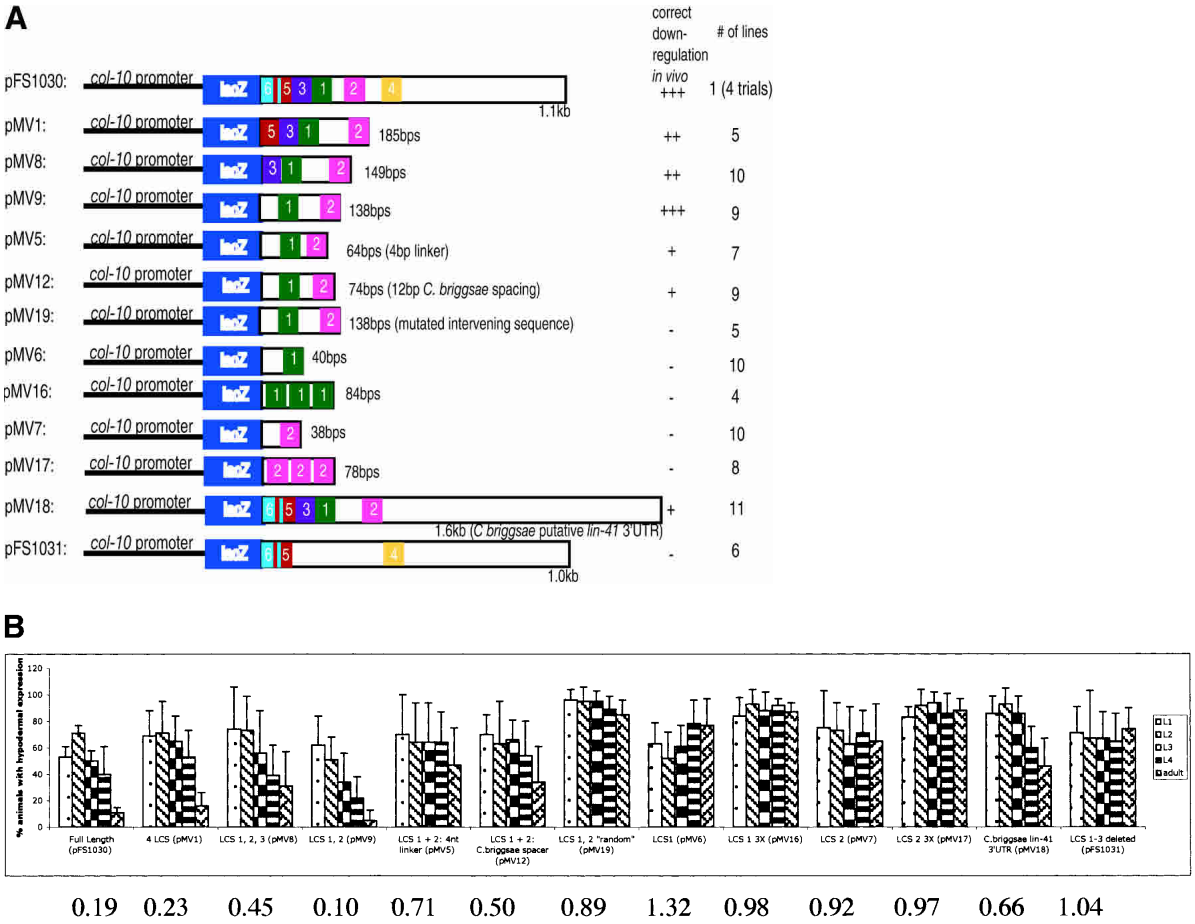

Figure 1. LCSs are necessary and sufficient for proper down-regulation in vivo. (*A*) Schematic of in vivo LCS constructs. All share a common *col-10* promoter fused to the *Escherichia coli lacZ* reporter gene. Each construct contains various LCSs from the *Caenorhabditis elegans lin-41* 3′UTR inserted into the *unc-54* 3′UTR. pMV18 contains *Caenorhabditis briggsae lin-41* DNA. (+++) 0%–12% of animals with hypodermal expression at the adult stage); (++) 13%–32%); (+) 33%–60%); (–) >60%. (*B*) In vivo *lacZ* expression analysis. We analyzed multiple lines for all constructs and averaged the data for each construct. Error bars indicate standard deviation between lines/trials where indicated. See Supplemental materials for number of lines/animals scored. Numbers below graph refer to ratio of hypodermal *lacZ* expression between adult vs. L_1–L_3 animals.

poorly conserved, suggesting that they are less relevant for *lin-41* function.

We investigated the ability of the *C. briggsae lin-41* 3′UTR to down-regulate a reporter gene in *C. elegans* by fusing 1.5 kb of sequence downstream of the *C. briggsae lin-41* stop codon to our *lacZ* reporter (Fig. 1A, pMV18). *lacZ* expression was down-regulated over time in vivo; 46% of adult animals expressed the reporter gene as compared with ~90% of early larval animals. However, this down-regulation was less effective than the *C. elegans lin-41* 3′UTR (Fig. 1B, pFS1030), in which only 11% of adult animals showed expression compared with ~80% of early larval animals. This demonstrates that sequences in the *C. briggsae lin-41* 3′UTR are capable of providing partial *lin-41* down-regulation.

Two conserved LCSs are sufficient for LIN-41 *down-regulation in vivo*

We determined the minimal sequence in the *lin-41* 3′UTR that is necessary and sufficient for proper *lin-41* down-regulation. Like the entire 3′UTR (pFS1030; Reinhart et al. 2000), LCS 1, LCS 2, LCS 3, and LCS 5 together (pMV1) were found to be sufficient for proper reporter gene down-regulation (Fig. 1B). Further, only LCS 1 and LCS 2 together (pMV9) could direct proper down-regulation of the reporter; 5% of adult animals expressed the reporter gene. Interestingly, neither LCS 1 (pMV6) nor LCS 2 (pMV7) alone was sufficient to effect down-regulation of the reporter gene. Increasing the number of bulged siRNA/miRNA-binding sites in the 3′UTR of a reporter gene increases the degree of repression (Doench et al. 2003). To test whether we could achieve reporter gene down-regulation with multiple copies of either LCS 1 or LCS 2, we concatemerized either LCS 1 (pMV16) or LCS 2 (pMV17) three times with a 4-nt linker between each LCS. This did not result in *lin-41* down-regulation in vivo (Fig. 1B). These experiments indicate either that only both copies of LCS 1 and LCS 2 together are sufficient for *lin-41* down-regulation, and/or that the intervening 27-nt sequence between LCS 1 and LCS 2 is also required. We distinguished between these below.

The intervening sequence between LCS 1 and LCS 2 is required for full down-regulation

To determine the significance of the 27-nt spacer sequence between LCS 1 and LCS 2, we investigated the contribution of the spacer sequence length and the specific spacer sequence itself. We found that LCS 1 and LCS 2 with an artificial 4-nt linker was insufficient for reporter down-regulation (pMV5, Fig. 1B). Similarly, replacing the 27-nt native sequence with the *C. briggsae* 12-nt spacer sequence (pMV12) also did not restore complete reporter down-regulation. These experiments demonstrate that the spacer sequence is important for 3′UTR down-regulation activity. A shortened sequence replacing the native 27-nt *C. elegans* spacer sequence may cause the *let-7* molecules to encounter steric hindrance and interfere with their ability to effectively bind *lin-41* in vivo and/or function properly. Alternatively, the intervening sequence may provide a binding site for additional necessary factors, either protein or RNA.

To test the importance of the specific sequence of the 27-nt spacer, we altered the sequence, but not length of the spacer between LCS 1 and LCS 2 (pMV9 vs. pMV19, Supplemental Materials). We found that 85% of adult animals carrying the altered 27-nt sequence in pMV19 showed *lacZ* expression compared with 5% of pMV9 containing adult animals with the native 27 nt (Fig. 1B). This demonstrates that the specific sequence between LCS 1 and LCS 2 is required for *lin-41* down-regulation. We speculate that this sequence binds additional necessary factors not yet identified, but we cannot rule out that by mutating the 27-nt sequence we may have introduced alternative secondary structure that may inhibit the binding of *let-7*. *Mfold* predictions do not, however, suggest secondary structure in this region of the 3′UTR (data not shown; Mathews et al. 1999; Zuker et al. 1999).

Point mutations in the LCS compromise reporter gene regulation in wild-type animals in vivo

To understand better the in vivo interaction of the *let-7* miRNA with its target sequences in the *lin-41* 3′UTR, we engineered point mutations in the LCSs. The *let-7(n2853)* lesion is a G to A point mutation at position 5 on the *let-7* RNA (Reinhart et al. 2000), that is predicted to disrupt the base pairing between LCS and *let-7* (Supplemental Fig. S1b; Fig. 2A). Consistent with this, *let-7(n2853)* animals carrying *col-10-lacZ*- LCS 1, LCS 2, LCS 3, and LCS 5 (pMV1) failed to down-regulate the reporter gene at the adult stage, demonstrating that wild-type *let-7* RNA is required for proper down-regulation of this reporter in vivo (Fig. 2B).

We engineered nucleotide substitutions compensatory to the *let-7(n2853)* mutation in reporter constructs carrying the LCSs, changing the corresponding C to U in LCS 1 or LCS 2, or both LCS 1 and LCS 2 (Fig. 2A) of pMV1. The presence of a point mutation (designated *) in all three constructs in a wild-type background compromised down-regulation of the reporter gene at the adult stage in vivo, with between 40% and 50% of adults expressing the reporter (pMV10, pMV1011, and pMV1013, Fig. 2C). This compares with the <20% expression at the adult stage seen with the control wild-type plasmid, pMV1.

Thus, single point mutations in LCS 1 or LCS 2 that potentially disrupt *let-7/lin-41* duplex formation resulted in decreased reporter down-regulation in wild-type animals (Fig. 2A,C). Although these data provide evidence that LCS 1 and LCS 2 are necessary for full LIN-41 down-regulation, surprisingly, we did not observe an additive effect when LCS 1 and LCS 2 were mutated concurrently; the expression pattern of pMV13 at the adult stage was comparable with pMV10 and pMV1011 at the same stage (Fig. 2C). There may be some other sequence(s) responsible for target down-regulation in the LCS 1* (pMV10) and LCS 2* (pMV11) constructs, a role perhaps played by the 27-nt spacer sequence between LCS 1 and LCS 2. These data, along with the deletion study (pFS1031, ΔLCS 1–LCS 3, Fig. 1B) indicate that both LCS 1 and LCS 2 and the specific 27-nt spacer sequence are necessary and sufficient for the regulation of our reporter, and by inference, *lin-41* itself.

let-7 *binds to specific complementary sequences from the* lin-41 *3′UTR in vivo*

To establish in vivo experimental evidence for the direct binding of the *let-7* RNA to the *lin-41* 3′UTR, we tested whether *let-7(n2853)* animals with reporter constructs

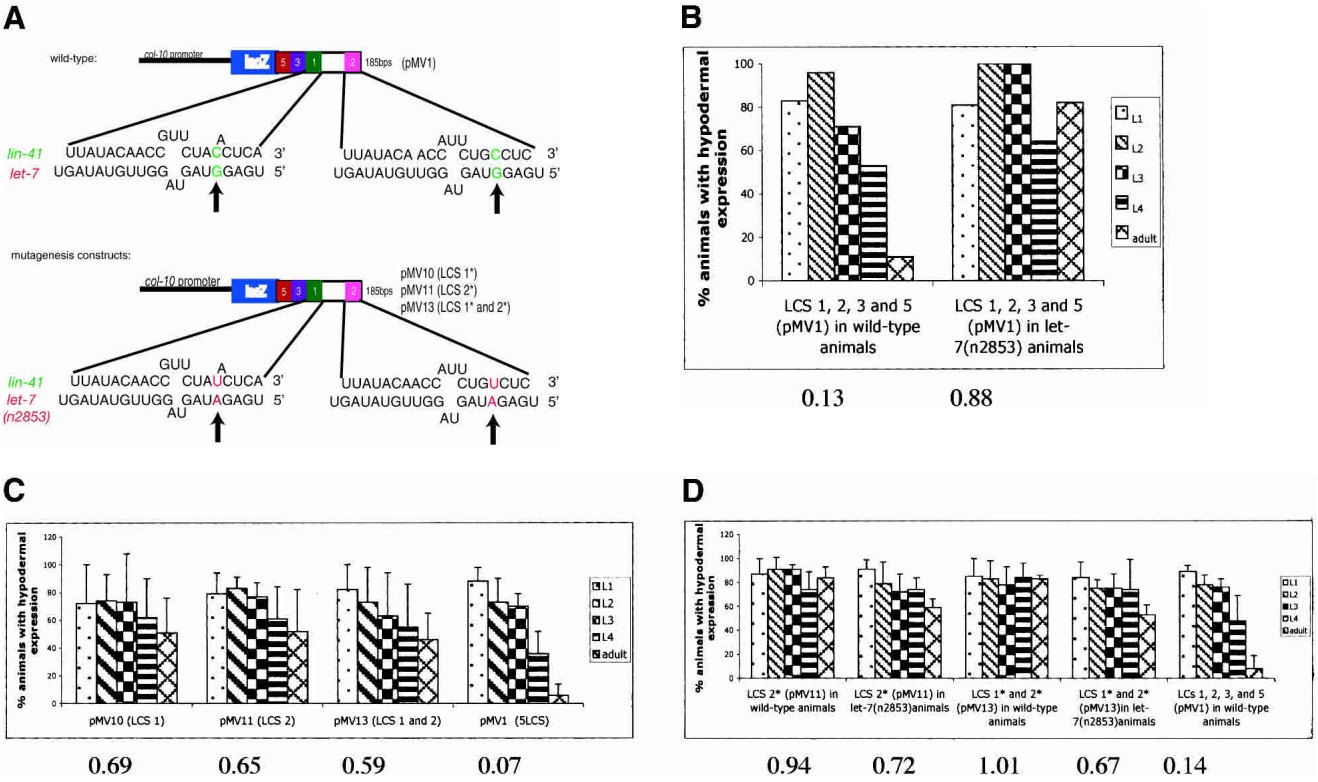

Figure 2. Effect of point mutations in LCS 1 and LCS 2. (*A*) The sequence of the wild-type LCS 1 and LCS 2, the predicted base pairing with the *let-7* RNA, and mutations compensatory to *let-7(n2853)* in the LCSs of the *lin-41* 3′UTR. Arrows indicate base nucleotide substitutions (C to U) made in LCS 1, LCS 2, or both, and predicted base pairing with the *let-7(n2853)* RNA. (*B–D*) See Supplemental materials for number of lines/animals scored. All data were analyzed as in Fig. 1B. Numbers below graph refer to ratio of hypodermal expression between adult vs. L_1–L_3 animals. (*B*) Down-regulation of *col-10-lacZ*-LCS 1, LCS 2, LCS 3, and LCS 5 (pMV1) is abrogated in *let-7(n2853)* animals. (*C*) Effect of LCS 1* and LCS 2* mutations in a wild-type background. (*D*) A *let-7(n2853)* compensatory mutation in LCS 2 and LCS 1 partially restores down-regulation in a *let-7(n2853)* background. Lines that showed the most severe lack of down-regulation at the adult stage in a wild-type background were assayed.

with compensatory LCS* mutations could show reporter gene down-regulation at the adult stage. This was accomplished by crossing animals carrying mutant transgenic arrays carrying the LCS 2* (pMV11) or both LCS 1* and LCS 2* (pMV13) mutations into *let-7(n2853)* animals (Fig. 2A). Comparing *lacZ* expression patterns of LCS 2* (pMV11) in *let-7(n2853)* and wild-type adult animals, we saw 25% greater adult reporter gene down-regulation in *let-7(n2853)* animals; *lacZ* expression was seen in 84% of wild-type animals versus 59% of *let-7(n2853)* animals (Fig. 2D). These data indicate that enabling at least one mutant *let-7* molecule to bind to one LCS in the *lin-41* 3′UTR and form a *let-7* RNA (*n2853*)/*lin-41** RNA duplex (at LCS 2) partially rescues down-regulation at the adult stage in vivo. We hypothesized that increasing the potential binding sites for mutated *let-7* (LCS 1* and LCS 2*, pMV13) would increase the repression of *lacZ* expression at the adult stage. However, we observed only 30% greater (slightly higher than that of LCS 2* alone) adult reporter gene down-regulation in *let-7(n2853)* animals; *lacZ* expression was seen in 83% of wild-type animals (pMV13) versus 53% of *let-7(n2853)* animals carrying LCS 1* and LCS 2* (Fig. 2D). The demonstration that compensatory mutations in LCS 1 and LCS 2 partially restore reporter down-regulation in a *let-7(n2853)* mutant strongly suggests that *let-7* binds directly to at least LCS 2.

A likely explanation as to why the compensatory mutations in LCS 1 and LCS 2 did not restore full down-regulation of *lin-41* at the adult stage in a *let-7(n2853)* background is that *let-7* RNA levels are reduced in *let-7(n2853)* animals (Reinhart et al. 2000). If *let-7* levels are below a threshold for activity in a *let-7(n2853)* mutant, no amount of complementarity would completely rescue. Another possibility is that another LCS (LCS 3 and/or LCS 5, LCS 6, for example) may need to be mutated (*) to allow for better down-regulation of *lin-41* at the adult stage in a *let-7(n2853)* background.

let-7 *binds to RNA from the* lin-41 *3′UTR in vitro*

We tested whether the *let-7* RNA can bind to RNA from the *lin-41* 3′UTR in vitro. ^{32}P-labeled in vitro-transcribed wild-type *let-7* RNA and mutant *let-7(n2853)* RNA were incubated separately with three in vitro-transcribed versions of *lin-41* 3′UTR RNA, respectively, and the resulting complexes were resolved by native gel electrophoresis (Fig. 3B). The wild type *let-7* binds to and retards the mobility of the full-length *lin-41* 3′UTR (pEYC1; Fig. 3A,B), as well as RNA with LCS 1, LCS 2, LCS 3, and LCS 5 (pEYC3; Fig. 3A,B, lane 4), but not to a *lin-41* 3′UTR deleted for LCS 1–LCS 3 (pEYC2; Fig. 3A,B, lanes 2,3). *let-7* also bound to the RNA sequence containing LCS 1, LCS 2, and LCS 3 (Fig. 3C, lane 6) but not to in vitro-transcribed RNA containing either LCS 1 or LCS 2 alone (Fig. 3c, lanes 2,3). However, we could detect

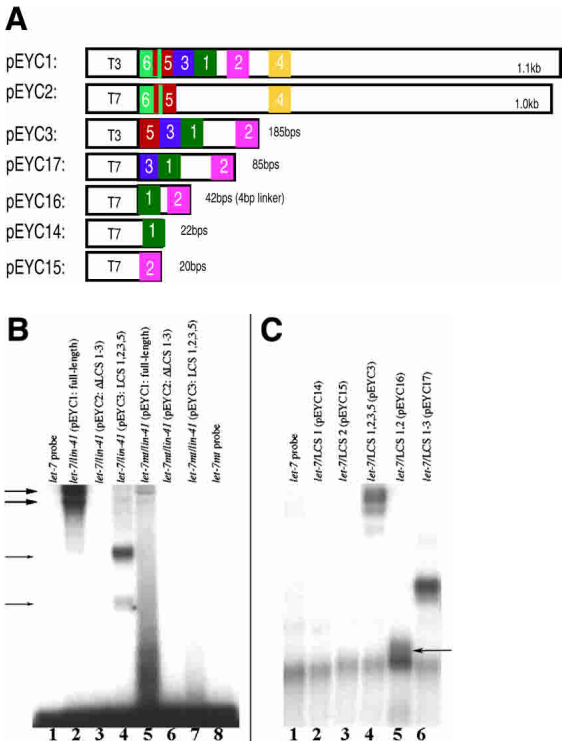

Figure 3. *let-7* can bind to sequences from the *lin-41* 3′UTR in vitro. (*A*) Schematic of in vitro LCS constructs used to generate RNAs. (*B*) Wild-type *let-7* RNA, but not *let-7(n2853)* mutant RNA can bind the full-length *lin-41* 3′UTR RNA. Lanes as indicated; retarded products indicated by two large arrows (lane *1*), and two small arrows (lane *4*). (*C*) LCS 1 and LCS 2 are sufficient to bind *let-7* RNA in vitro. Lanes as indicated. The *let-7* free probe has run off the gel and is not shown.

binding of *let-7* to a synthetic RNA that contained both LCS 1 and LCS 2 linked to each other by a 4-nt linker. These data showed that *let-7* RNA can bind to *lin-41* 3′UTR RNA in vitro, and that a sequence containing LCS 1, LCS 2, and LCS 3 within a 185-nt region is necessary and sufficient for binding. The minimal sequence required for *let-7* binding was LCS 1 and LCS 2 (Fig. 3C, lane 5), supporting the finding that the minimal sequence determined to be sufficient for *lin-41* down-regulation in vivo contains LCS 1 and LCS 2 (see above). We observe multiple retarded complexes (small arrows, Fig. 3B, lane 4) that may represent multiple loading of *let-7* molecules at various LCSs.

The mutant variant of *let-7* RNA, carrying a single point mutation corresponding to *let-7(n2853)* shows lower in vitro-binding affinity than the wild-type *let-7* RNA to the full-length *lin-41* 3′UTR RNA (Fig. 3B, lane 5) and to the RNA with LCS 1, LCS 2, LCS 3, and LCS 5 (Fig. 3B, lane 7). This suggests that the *let-7(n2853)* mutation destabilizes the *let-7/lin-41* RNA duplex, consistent with the thermodynamic calculations of the effects of the G to A transition on duplex formation (data not shown; Supplemental Fig. S1b).

Conclusion

Our data define the minimal region responsible for *lin-41* 3′UTR down-regulation activity in vivo. This region contains two sequences complementary to *let-7*, LCS 1, and LCS 2, as well as a specific 27-nt spacer sequence. This work reveals that more than one miRNA complementary site is required for down-regulation activity. Perhaps *let-7* miRNAs act in a cooperative fashion, stabilizing each other's interaction with the 3′UTR of their target gene, thereby increasing the degree of regulation imparted on their target gene. It has been shown in HeLa cell culture that increasing the number of miRNA/siRNA-binding sites increases the degree of repression of a target gene (Doench et al. 2003). By using in vivo transgenic arrays of a reporter gene and endogenously expressed *let-7*, we find that it is not enough to merely multimerize miRNA complementary sites. We predict that there are probably other factors, RNA or protein, that need to be present along with wild-type *let-7* to ensure proper *lin-41* repression at the adult stage. In the case of *lin-41*, this activity may reside in the LCS spacer sequence.

We altered the sequence of the 27-nt spacer and found that these changes severely affect *lin-41* down-regulation activity. The spacer sequence may be important for secondary structure of the *lin-41* 3′UTR and/or for binding of additional required factors such as a specific RNA-binding protein and/or complex, or another miRNA. Conserved sequences flanking other miRNA-binding sites have been found in the *hbl-1* 3′UTR that are similar to NRE elements known to bind regulatory proteins such as *Pumillio* (Lin et al. 2003).

In this study, we demonstrate that our *let-7* and *lin-41* RNAs directly bind each other in vitro, and in vivo strongly suggesting that native *let-7* and *lin-41* bind each other. We have demonstrated for the first time that an animal miRNA binds to its target RNA in vivo through imperfect complementary sequences.

Materials and methods

Plasmid constructions, injections, and lacZ *expression analysis*

All plasmids with designation pMV#:DNA fragments were digested with *Sac*II and *Nco*I and ligated into the *unc-54* 3′UTR of the B29 vector (Reinhart et al. 2000) cut with *Sac*II and *Nco*I behind the *Escherichia coli lacZ* reporter gene. B29 contains the *col-10* promoter fused to *lacZ*, and the *unc-54* 3′UTR, a 3′UTR thought to be absent of temporal regulatory elements (Wightman et al. 1993). Refer to published methods for pFS1030 and pFS1031 (Reinhart et al. 2000). For primer sequences and insert data, see Supplemental Table 1. Resulting plasmid sequences were verified by sequencing, injected into wild-type animals (5 ng/μL) with injection marker pRF4 *(rol-6)* (80 ng/μL) to create extrachromosomal array lines. Refer to Supplemental materials for β-galactosidase assays. Control animals contained extrachromosomal array *mgEx540* (pFS1030, 5 ng/μL; Slack et al. 2000).

Site-directed mutagenesis and in vitro RNA binding

To make mutations in the *lin-41* 3′UTR LCS 1 and LCS 2 (designated 1* and 2*) plasmids pMV10, pMV11, and pMV13 were constructed as described in Supplemental materials. In vitro *let-7/lin-41* duplex formation is described in Supplemental materials.

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The *C. elegans* microRNA *let-7* binds to imperfect *let-7* complementary sites from the *lin-41* 3′UTR

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