A bulged *lin-4/lin-14* RNA duplex is sufficient for *Caenorhabditis elegans* *lin-14* temporal gradient formation

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The *Caenorhabditis elegans* heterochronic gene *lin-14* generates a temporal gradient of the LIN-14 proteins to control stage-specific patterns of cell lineage during development. Down-regulation of LIN-14 is mediated by the *lin-14* 3' untranslated region (UTR), which bears seven sites that are complementary to the regulatory *lin-4* RNA. Here we report molecular and genetic evidence that RNA duplexes between the *lin-4* and *lin-14* RNAs form in vivo and are necessary for LIN-14 temporal gradient generation. *lin-4* RNA binds in vitro to a *lin-14* mRNA bearing the seven *lin-4* complementary sites but not to a *lin-14* mRNA bearing point mutations in these sites. In vivo, the *lin-4* complementary regions are necessary for *lin-14* 3' UTR-mediated temporal gradient formation. Based on *lin-14* 3' UTR sequence comparisons between *C. elegans* and *C. briggsae*, four of the seven *lin-4/lin-14* RNA duplexes are predicted to bulge a *lin-4* C residue, and three sites are predicted to form nonbulged RNA duplexes. Reporter genes bearing multimerized bulged C *lin-4* binding sites show almost wild-type temporal gradient formation, whereas those bearing multimerized nonbulged *lin-4* binding sites do not form a temporal gradient. Paradoxically, *lin-4* RNA binds in vitro to nonbulged *lin-14* RNA more avidly than to the bulged *lin-14* RNA. This suggests that a specific secondary structure of *lin-4/lin-14* RNA duplex that may be recognized by an accessory protein, rather than an RNA duplex per se, is required in vivo for the generation of the LIN-14 temporal gradient.

[Key Words: *C. elegans*; development; heterochronic; bulged RNA; translation; *lin-14*, *lin-4*; 3' UTR]

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Genetic and molecular analyses in *Drosophila* and *Caenorhabditis elegans* have shown that gene pathways specific to one axis of the developing animal—for example, anterior/posterior [Nüsslein-Volhard 1991], dorsal/ventral [Rushlow et al. 1988; Serano and Cohen 1995], or temporal [Ruvkun and Giusto 1989]—generate control gene activities that form a gradient over that developmental axis to specify patterned arrays of cells. In many cases, these pattern formation genes have been shown to be regulated post-transcriptionally [Wang and Lehmann 1991; Wharton and Struhl 1991]. The *C. elegans* heterochronic genes regulate the temporal sequence of the cell lineage by such a mechanism. These genes post-transcriptionally generate a temporal gradient of a key regulator, the LIN-14 protein (LIN-14) [Ambros 1989; Ruvkun and Giusto 1989; Wightman et al. 1993].

During wild-type *C. elegans* development, blast cells execute stage-specific patterns of cell lineage at each of the four larval stages and terminally differentiate during the adult stage [Ambros and Horvitz 1984]. Mutations in

the heterochronic genes *lin-4*, *lin-14*, *lin-28*, and *lin-29* cause temporal transformations in these stage-specific patterns of cell lineage in many tissues and cell types [Chalfie et al. 1981; Ambros and Horvitz 1984]. Depending on the heterochronic mutant, stage-specific developmental events occur at either earlier or later stages than they would normally.

An instructive role for *lin-14* in temporal pattern formation was established by the observation that *lin-14* gain-of-function and loss-of-function mutations have the opposite phenotype [Ambros and Horvitz 1984]. Loss-of-function (lf) *lin-14* alleles cause precocious execution at early larval stages of cell fates appropriate for later larval stages [Ambros and Horvitz 1984]. Gain-of-function (gf) *lin-14* alleles cause the opposite transformation in temporal cell fate—reiterations of early cell fates at later stages.

lin-14 controls stage-specific cell lineages by generating a temporal gradient of the LIN-14 nuclear proteins [Ruvkun and Giusto 1989]. The high level of LIN-14 at early stages specifies an early cell lineage, and the falling level of the LIN-14 causes a switch to late cell lineages. The normal temporal regulation of LIN-14 abundance is required for the wild-type sequence of temporal cell

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fates. Heterochronic mutations that elevate LIN-14 levels at late stages cause reiterations of early cell lineages, whereas mutations that decrease LIN-14 levels at early stages cause precocious expression of late cell lineages (Ruvkun and Giusto 1989; Arasu et al. 1991; Wightman et al. 1991).

Temporal regulation of LIN-14 abundance during wild-type development occurs at a post-transcriptional step (Wightman et al. 1993). This post-transcriptional regulation is mediated by the *lin-14* 3' untranslated region (UTR) that is sufficient to confer such regulation on an unrelated reporter gene (Wightman et al. 1993). *lin-4* gene activity is required for the post-transcriptional temporal regulation of *lin-14* or reporter genes bearing the *lin-14* 3' UTR. In the region of the *lin-14* 3' UTR that is deleted in *lin-14* (gf) mutations are seven copies of a 14- to 19-nucleotide sequence that is complementary to a portion of the regulatory *lin-4* RNA (Lee et al. 1993). This suggests that *lin-4* down-regulates *lin-14* translation by forming multiple RNA duplexes with the *lin-14* mRNA (Wightman et al. 1993).

Here we show that multiple elements in the *lin-14* 3' UTR are conserved between *C. briggsae* and *C. elegans*, but that the *lin-4* complementary elements account for the *lin-14* temporal gradient forming activity. A *lin-4*/*lin-14* RNA duplex forms in vitro. In vivo, particular *lin-4*/*lin-14* RNA duplexes that bulge a nucleotide are active in temporal gradient formation, whereas non-bulged RNA duplexes are not. We propose that a specific RNA secondary structure is required for post-transcriptional generation of the LIN-14 temporal gradient.

Results

Conserved elements in the *lin-14* 3' UTR

Comparison of nucleic acid sequences between *C. elegans* and the related nematode *C. briggsae* species has successfully identified regulatory elements by their DNA sequence conservation (Zucker-Aprison and Blumenthal 1989; Heschel and Baillie 1990; Xue et al. 1992; Lee et al. 1993). The *C. briggsae* *lin-14* 3' UTR mediates formation of the LIN-14 temporal gradient in *C. briggsae*, and a *lacZ* reporter gene fused to the *C. elegans* *lin-14* 3' UTR forms a temporal gradient in *C. briggsae* (Wightman et al. 1993). Therefore, those sequences that are required for *lin-14* temporal regulation should be conserved between the two species. Comparison of the *C. briggsae* and *C. elegans* *lin-14* 3' UTR sequences reveals multiple blocks of conserved sequence, separated by stretches with no conservation (Fig. 1A). The longest stretch of exactly conserved sequence is 30 nucleotides (24250–24280). The region that is deleted by both *lin-14*(gf) mutations (24168–25516) contains conserved blocks of various lengths scattered throughout. Additional conserved blocks of sequence are found between the deletion breakpoint of the weaker gf mutation (*n536*) and the polyadenylation signal (24819–25515). The position and stem complementarity of the most stable stem-

loop structure is also conserved (Fig. 1A). In contrast, almost no sequence conservation was found in the 600 bases immediately 3' to the *C. elegans* stop codon (position 23912), in the region 3' to the polyadenylation signal, and in introns, suggesting that these regions may be dispensable for *lin-14* function.

Seven repeated 9- to 15-nucleotide sequences that are complementary to a portion of the *lin-4* RNAs are completely conserved between the two species (Fig. 1B). Because the portion of the *lin-4* RNAs that is complementary to these repeated elements is also completely conserved between *C. elegans* and *C. briggsae* (Lee et al. 1993), this result is consistent with the model that multiple *lin-4* RNAs hybridize to the 3' UTR of the *lin-14* mRNA.

A *lin-4*/*lin-14* RNA duplex forms in vitro

To observe RNA duplex formation in vitro, a ³²P-labeled chemically synthesized 24-nucleotide *lin-4* RNA that corresponds to the major *lin-4* transcript (Lee et al. 1993) was incubated with in vitro transcribed *lin-14* 3' UTR RNA (nucleotides 23845–25637) bearing the seven putative *lin-4* binding sites, and complexes were resolved by native gel electrophoresis (Fig. 2). The wild type *lin-4* RNA binds to wild-type *lin-14* 3' UTR RNA but not to a *lin-14* (7Xmut) 3' UTR RNA that bears helix-disrupting point mutations (UCA to AGU; Fig. 1B) in each of the seven *lin-4* complementary regions (Fig. 2). Mutant *lin-4*(*ma161*) RNA carrying the C to U *lin-4*(*ma161*) base substitution in the proposed *lin-14* complementary region (at *lin-4* nucleotide number 5 in Fig. 1B) shows lower affinity than the *lin-4*(+) RNA to the wild-type *lin-14* 3' UTR RNA, and no binding to the *lin-14*(7Xmut) 3' UTR RNA (Fig. 2). This in vitro data shows that the postulated RNA duplex between the *lin-4* and *lin-14* RNAs in fact forms. In addition, it suggests that the strong *lin-4*(*ma161*) allele affects the stability of the *lin-4*/*lin-14* RNA duplex, consistent with thermodynamic calculations of the effects of the C to U transition on duplex formation (Lee et al. 1993; Wightman et al. 1993). Because these same mutations in the *lin-14* 3' UTR affect its function in vivo (see below), this in vitro assay may reflect the in vivo function of these RNAs.

Mutations that disrupt in vitro formation of *lin-4*/*lin-14* RNA duplex disrupt temporal gradient formation in vivo

To further test the *lin-4*/*lin-14* RNA duplex model, we compared in vivo temporal gradient formation by the *lin-14*(7Xmut) 3' UTR, bearing helix-disrupting mutations in each of the seven proposed *lin-4* binding sites, to that of the wild-type *lin-14* 3' UTR. Temporal gradient formation was compared in animals expressing wild-type *lin-4* RNA as well as in the *lin-4*(*e912*) strain that deletes the entire *lin-4* region (Lee et al. 1993). To suppress any indirect effects on temporal gradient formation resulting from the heterochronic effects of the *lin-4* mutant from direct effects on the *lin-14* 3' UTR, hetero-

Figure 1. (A) A comparison of the *lin-14* 3' UTR sequences from *C. elegans* and *C. briggsae*. The top line shows the *C. briggsae* sequence (GenBank accession no. U67400); the bottom line shows the *C. elegans* sequence (Wightman et al. 1991). Sequences that are conserved are shown with dashed lines between the *C. briggsae* and *C. elegans* sequences. Potential *lin-4* binding sites are designated by boxes that are numbered to correspond to those shown in detail in Fig. 1B. The *lin-14(gf)* mutations and polyadenylation site for the *C. elegans* sequence are labeled below the line. Positions in this figure are numbered according to genomic sequences of cosmid T25C12, which contains the *lin-14* gene. Dots indicate gaps introduced into both sequences to allow alignment. The most stable stem and loop structure are shown as double-headed arrows (stem and stem', loop). In both *C. elegans* and *C. briggsae*, this stem-loop has the lowest predicted free energy of any predicted stem-loop in the 3' UTR (-38.3 and -27.3 kcal/mole, respectively) (Jaeger et al. 1989). In the region of the stems the sequence is conserved between the two species. At most positions where the sequence differs between the two species, compensatory changes in the other half of the stem would maintain base-pairing. This observation suggests that a stem-loop structure may be required at this position, but that a particular sequence may not be required. This *C. elegans* stem-loop structure is not deleted in either *lin-14(gf)* mutation, indicating that the structure is not sufficient for temporal regulation, and the analysis below suggests that it is not necessary for temporal gradient formation. (B) Locations of the proposed *lin-14* 3' UTR RNA/*lin-4* RNA duplexes and other conserved regions in the *lin-14* 3' UTR. Four out of seven *lin-4* binding sites (1, 2, 4, and 6) bulge a *lin-4* C from the duplex (arrow). Examples of a bulged C *lin-4/lin-14* RNA duplex (#2) and a nonbulged RNA duplex (#5) and the alterations of these structures predicted from *lin-14(7Xmut)* 3' UTR (underlined outline AGU) and the *lin-4(ma161)* RNA sequence (outline U) are shown.

Figure 2. In vitro binding analysis of *lin-4* RNA and *lin-14* 3' UTR RNA. *lin-14* RNAs were prepared by in vitro transcription from a T3 promoter, and *lin-4* RNAs were synthesized chemically. 32 P-labeled *lin-4* RNA was incubated with *lin-14* 3' UTR RNA at room temperature and analyzed by agarose gel (1%) electrophoresis. Unbound labeled *lin-4* RNA migrates to the bottom of the gel, whereas *lin-4/lin-14* RNA complexes migrate more slowly. The *lin-4(+)* RNA binds to a *lin-14(+)* RNA but not to the *lin-14(7Xmut)* RNA carrying mutations in each of the seven *lin-4* binding sites. *lin-4(ma161)* RNA binds *lin-14(+)* RNA more weakly. The amount of *lin-14* RNAs for each lane is equivalent as measured by binding to a DNA oligonucleotide outside of the *lin-4* complementary region (data not shown).

chronic defects were suppressed by a *lin-14(n179ts)* point mutation in the protein coding region (B. Reinhart and G. Ruvkun, pers. comm.), which is benign in this assay (Fig. 3). Whereas transgenic animals carrying a *lacZ* reporter gene fused to a wild-type *lin-14* 3' UTR show *lin-4*-dependent down-regulation from the L1 to L4 stage (130-fold), animals bearing the reporter gene fused to the *lin-14(7Xmut)* 3' UTR bearing helix-breaking mutations in each of the seven *lin-4* complementary regions show only 8-fold down-regulation that is not *lin-4*-dependent (Fig. 3). The 8-fold down-regulation of the *lin-14(7Xmut)* 3' UTR is equivalent to the level of *lin-4*-independent down-regulation shown by the control *unc-54* 3' UTR (Wightman et al. 1993).

Thus a *lin-14(7Xmut)* 3' UTR that is specifically disrupted in the seven *lin-4* complementary sites is 16-fold less active in temporal gradient formation than the wild-type *lin-14* 3' UTR (Fig. 3). Similarly, temporal gradient-forming activity of the wild-type *lin-14* 3' UTR in a *lin-4* mutant background is decreased 5- to 30-fold relative to wild type, whereas lack or presence of *lin-4* gene activity has no effect on the *lin-14* 3' UTR lacking *lin-4* binding sites (Fig. 3). Thus all of the *lin-14* 3' UTR temporal gradient forming activity detectable by this fusion gene assay is mediated by the *lin-4* complementary region of the *lin-14* 3' UTR. This data supports the model that the

Figure 3. Down-regulation of β -galactosidase activity in transgenic wild-type or *lin-4* mutant animals carrying reporter genes fused to the *lin-14(+)*, *lin-14(7Xmut)*, or control *unc-54* 3' UTRs. The y-axis represents the ratio of β -galactosidase activity in L1 stage animals relative to L4 stage animals. The same transgenic arrays that are analyzed in wild type were transferred by mating to the *lin-4* mutant background, so that differences between temporal gradient formation in *lin-4(+)* and *lin-4* mutant backgrounds are not a result of differences in the transgenic arrays. (*) The integrated line [pC10UT; Wightman et al. 1993] was analyzed in the *lin-4(ma161)* genetic background. To uncouple reporter gene activities from indirect effects of *lin-4* on temporal cell fates mediated by heterochronic *lin-14* activity, fusion gene expression was monitored in a *lin-4(e912);lin-14(n179ts)* strain at 20°C. At this temperature, the temperature-sensitive LIN-14(*n179ts*) protein suppresses the lack of *lin-4* mediated LIN-14 down-regulation and vice versa, so that animals show no heterochronic Lin-4 or Lin-14 phenotype. Temporal regulation of reporter gene expression in *lin-4(e912);lin-14(n179ts)* at 20°C was very similar to that observed in *lin-4(e912)* background (Wightman et al. 1993). The disruption of down-regulation in *lin-4(e912);lin-14(n179ts)* is not a result of the *lin-14(n179ts)* mutation; the same *lin-14(+)* 3' UTR reporter gene in *lin-14(n179ts)* shows 105-fold temporal down-regulation, which is similar to down-regulation of this transgene in wild type. Animals carrying the *lin-14(7Xmut)* 3' UTR reporter gene show negligible temporal gradient generation activity in either *lin-4(e912);lin-14(n179ts)* (9-fold) or wild type (8-fold). The residual 3- to 8-fold down-regulation of reporter genes bearing the *unc-54* 3' UTR or the *lin-14(7Xmut)* 3' UTR may reflect the temporal regulation of the collagen promoter or the surface area-to-volume ratio dilution effect of the larger L4 animals. The specific β -galactosidase activities [Δ optical density (O.D.) 574/min./mg protein] of the transgene with wild-type *lin-14* 3' UTR are 2.61 ± 0.30 (L1) and 0.018 ± 0.002 (L4) [*lin-4(+);lin-14(+)*], 11.18 ± 0.84 and 0.57 ± 0.074 [*lin-4(e912);lin-14(n179ts)*], 0.99 ± 0.37 and 0.23 ± 0.02 [*lin-4(ma161);lin-14(+)*], 6.85 ± 0.15 and 0.066 ± 0.006 [*lin-4(+);lin-14(n179ts)*]. For transgenes of *lin-14(7Xmut)* 3' UTR they are 2.0 ± 0.67 and 0.24 ± 0.055 [*lin-4(+);lin-14(+)*] and 4.5 ± 0.93 and 0.48 ± 0.058 [*lin-4(e912);lin-14(n179ts)*]. For transgenes with *unc-54* 3' UTR they are 9.0 ± 4.4 [*lin-4(+);lin-14(+)*] and 1.4 ± 0.7 [*lin-4(e912);lin-14(n179ts)*] (Wightman et al. 1993).

duplex formation between *lin-4* RNA and *lin-14* 3' UTR is essential for formation of the LIN-14 temporal gradient.

Bulged lin-4/lin-14 RNA duplexes are active in temporal gradient formation

In RNA-protein interactions such as ribosomal assembly and translational regulation, loops or bulges in RNA secondary structures have been shown to be important for protein interactions and regulatory events (Dingwall et al. 1990; Klausner et al. 1993; Hjalte and Wagner 1995). Four of the seven proposed *lin-4/lin-14* RNA duplexes bulge a *lin-4* C residue and three form thermodynamically more stable nonbulged duplexes in this region (arrow in Fig. 1B). The same set of *lin-4/lin-14* RNA duplex structures in the same 5' to 3' order are also predicted from the *C. briggsae* *lin-4* and *lin-14* RNA sequences (Fig. 1B; Lee et al. 1993).

In addition, the most likely *lin-4* bulged C nucleotide in the four putative bulged RNA duplexes is changed to a U in the strong *lin-4(ma161)* mutant (Fig. 1B; Lee et al. 1993). The *lin-4(ma161)* mutation disrupts LIN-14 temporal gradient formation in vivo and by the *col-10/lacZ/lin-14(+)* 3' UTR reporter gene (Fig. 3) as potently as the *lin-4(e912)* mutation that deletes *lin-4* entirely (Lee et al. 1993). These genetic data suggest that the bulged C RNA duplexes as well as the nonbulged RNA duplexes play an essential role in LIN-14 temporal gradient formation. However, thermodynamic calculations as well as in vitro binding studies (Fig. 2; see below) suggest that if an RNA duplex per se is the essential feature for temporal gradient formation, the three more stable *lin-4/lin-14* RNA duplexes would also contribute to *lin-14* 3' UTR mediated down-regulation.

To distinguish the role of the two types of *lin-4* binding sites in temporal gradient formation, we assayed reporter genes bearing multimerized (six copies) single bulged C or nonbulged *lin-4* binding sites (Fig. 4A). These multimerized *lin-4* binding sites were assayed for temporal down-regulation in the context of the *unc-54* 3' UTR in both wild type and the *lin-4(e912);lin-14(n179ts)* background (Fig. 4B). Reporter genes with six copies of the bulged C *lin-4/lin-14* RNA duplex generate a *lin-4* dependent temporal gradient (120-fold temporal down-regulation) that is similar to that generated by the *lin-14(+)* 3' UTR (Fig. 4B). Reporter genes bearing multimerized *lin-4* binding sites predicted to form nonbulged *lin-4/lin-14* RNA duplexes show much less down-regulation that is not *lin-4*-dependent (Fig. 4B).

These data suggest that the bulged C *lin-4/lin-14* RNA duplexes alone can confer the temporal gradient generating activity of the *lin-14* 3' UTR. In combination with our previous observation that a reporter gene carrying two nonbulged and one bulged C *lin-4* binding site shows intermediate temporal gradient generating activity (Wightman et al. 1993), these data suggest that multiple *lin-4* bulged C binding sites are essential for temporal gradient forming activity.

In vitro binding studies are consistent with the thermodynamic calculations of RNA duplex stability (Jaeger et al. 1989). RNAs bearing six copies of the bulged C or nonbulged *lin-4* binding sites were incubated with ³²P-labeled *lin-4* RNA and analyzed as above (Fig. 4C). *lin-*

4(+) RNA binds strongly in vitro to a *lin-14* RNA bearing multimerized nonbulged *lin-4* binding sites (calculated $\Delta G = -23.1$ kcal/mole per RNA duplex), although it does not bind in vitro to a *lin-14* RNA bearing multimerized bulged C *lin-4* binding sites (calculated $\Delta G = -17.0$ kcal/mole) (Fig. 4C). The *lin-4(ma161)* RNA also binds to the nonbulged *lin-14* RNA, consistent with thermodynamic predictions based on the G::U base pairing (calculated $\Delta G = -20.5$ kcal/mole). These results are paradoxical to the lack of temporal gradient formation by a 3' UTR bearing nonbulged *lin-4* binding sites and the generation of a temporal gradient by the 3' UTR bearing bulged C *lin-4* binding sites. The results suggest that formation of RNA duplexes is not sufficient for temporal gradient formation. Rather, the bulged C structural feature of the *lin-4/lin-14* RNA duplex is essential for temporal gradient formation. The lack of in vitro stability of the *lin-4/lin-14* RNA duplex bearing the bulged *lin-4* C suggests that a protein factor may recognize this bulged C to stabilize the *lin-14/lin-4* duplex in vivo.

Discussion

The lin-4/lin-14 RNA duplex is necessary and sufficient for temporal gradient formation

The *lin-14* 3' UTR mediates the generation of a LIN-14 temporal gradient by post-transcriptional down-regulation of LIN-14 translation from the nontemporally regulated *lin-14* mRNA (Wightman et al. 1993). The *lin-4* regulatory RNA (Lee et al. 1993) also acts post-transcriptionally (Wightman et al. 1993). This *lin-4*-dependent post-transcriptional regulation via a *lin-14* 3' UTR can be conferred to an unrelated reporter gene showing that *lin-4* acts via the *lin-14* 3' UTR (Wightman et al. 1993).

The experiments we report here show that the *lin-4* RNA binds to conserved elements in the *lin-14* 3' UTR and that the *lin-4* complementary sites of the *lin-14* 3' UTR are necessary for *lin-14* 3' UTR-mediated *lin-4*-dependent temporal gradient formation. In addition we show that particular *lin-4/lin-14* RNA duplexes that bulge a C residue are sufficient to confer such temporal gradient generation activity.

A bulged C lin-4/lin-14 RNA duplex generates a temporal gradient

We find that a multimerized bulged C *lin-4* binding site is sufficient to confer the same *lin-4*-dependent temporal down-regulation as the *lin-14* 3' UTR bearing all of these multiple conserved blocks. Other experiments have shown that a 3' UTR bearing three *lin-4* binding sites generates only partial temporal gradient activity (Wightman et al. 1993), suggesting that more than three such sites are essential for full temporal gradient-generating activity. Because six bulged C sites are sufficient for full temporal gradient formation, it is likely that only the *lin-4* complementary regions of the *lin-14* 3' UTR are essential for temporal gradient formation.

Figure 4. (A) Structure of transgenes bearing multimerized bulged or nonbulged *lin-4/lin-14* RNA duplexes. Multimerized bulged C or nonbulged *lin-4/lin-14* RNA duplex sites were placed downstream of *lacZ* in the context of *unc-54* 3' UTR. (B) Ratio of β -galactosidase activity of the bulged and nonbulged transgenes. *lin-4*-dependent temporal down-regulation of β -galactosidase activity is shown for *col-10/lacZ/3'* UTR reporter genes bearing six copies of the bulged C *lin-4* complementary site but not in the reporter genes bearing six copies of the nonbulged site. (C) In vitro binding analysis of *lin-4* RNA and *lin-14* RNA that has six copies of the bulged C or nonbulged *lin-4* RNA binding sites. *lin-14* RNAs were synthesized by T3 RNA polymerase in vitro and *lin-4* RNAs were synthesized chemically. 32 P-labeled *lin-4* RNA was incubated with *lin-14* RNA at room temperature and analyzed by a 6% native polyacrylamide gel electrophoresis. Unbound *lin-4* RNA migrates to the bottom of the gel, and complexes migrate more slowly. *lin-4(ma161)* RNAs bind to the nonbulged *lin-14* RNAs but do not bind to the bulged C *lin-4* sites. The amount of RNA for each lane was normalized (data not shown).

It is surprising that only the bulged C *lin-4* complementary site is sufficient for temporal gradient generation. Both the *C. briggsae* and *C. elegans* *lin-14* 3' UTRs bear four bulged C *lin-4*-complementary sites and three perfect duplex *lin-4* complementary sites (Fig. 1B; Wightman et al. 1993), suggesting that the perfect duplex sites must also have some essential function as well. They might be necessary for down-regulation in cooperation with bulged *lin-4* binding sites, although they have no activity by themselves. For example, because the nonbulged sites are predicted to have the highest affinity, they could be the first RNA duplexes to form as *lin-4* RNA levels increase (Hjalt and Wagner 1995). Such an RNA duplex may melt other *lin-14* mRNA structures to expose the next most affine site, to foster cooperative binding of *lin-4* RNA molecules to sites showing lower affinity, such as the bulged C sites. Or it is possible that more than four bulged C sites may generate too steep a LIN-14 temporal gradient, which would disrupt temporal patterning.

The essential function of the bulged C sites is supported by the strong *lin-4(ma161)* mutant that is predicted to bulge a U rather than a C at these RNA duplex sites (Fig. 1B). This *lin-4* mutation strongly disrupts formation of a temporal gradient by the *lin-14(+)* 3' UTR (Fig. 3). *lin-4(ma161)* is also predicted to substitute a slightly destabilizing G::U for a G::C base pair in the three predicted nonbulged *lin-14/lin-4* RNA duplex regions (calculated $\Delta\Delta G = 2.6$ kcal/mole) (Figs. 2 and

4C). The slight effect of this mutation on the perfect duplex stability, coupled with our demonstration that the bulged C elements are the active temporal gradient generating elements, strongly suggests that the *lin-4* bulged C nucleotide is essential for down-regulation of *lin-14* mRNA translation (Dingwall et al. 1990; Hjalt and Wagner 1995). It is possible that such bulged U sites in *lin-4(ma161)* cannot be recognized by protein factors that normally couple the formation of these *lin-4/lin-14* RNA structures to down-regulation of *lin-14* mRNA translation (Fig. 5).

In vitro binding experiments support the model that *lin-4* regulates *lin-14* by binding to the *lin-4* complementary sites (Fig. 2). However, some of the results, although thermodynamically predicted, are paradoxical to the in vivo results. For example, the proposed bulged C RNA duplexes do not form under these in vitro conditions that allow formation of the predicted perfect duplexes (Fig. 4C), but the bulged C *lin-14* sites form a temporal gradient in vivo (Fig. 4B). This suggests that in vivo transiently formed bulged C RNA duplexes may be stabilized by protein factors that specifically recognize this structure. If the bulged C residue was essential for recognition of this RNA duplex, the bulged U *lin-4(ma161)/lin-14* RNA duplexes may fail to be stabilized by the association to such a duplex binding protein. Such an unbound *lin-4(ma161)* RNA might be degraded; in fact the level of the *lin-4(ma161)* RNA is lower than in wild type (R. Feinbaum and V. Ambros, pers. comm.). However, the

Figure 5. Model for the down-regulation of LIN-14 by formation of bulged and non-bulged *lin-4/lin-14* RNA duplexes. A transacting factor recognizes the four bulged *lin-4/lin-14* RNA duplexes, and these protein–RNA complexes inhibit translation of the *lin-14* mRNA. However, it does not recognize the three nonbulged RNA duplexes. The factor might recognize the specific bulged C of the duplex (lower right) and stabilize the *lin-4/lin-14* RNA duplex. However, the factor may fail to recognize a bulged U in the transient *lin-4*(*ma161*)/*lin-14* RNA duplex (lower left).

lin-4(*ma161*) mutation may destabilize the *lin-4* RNA by a distinct mechanism, or may affect *lin-4* promoter activity (R. Feinbaum and V. Ambros, pers. comm.).

RNA duplexes are A-form double helices that have equally sized major and minor grooves, neither of which is wide enough to allow entry of an alpha-helical protein domain, unlike B-form DNA duplexes, which have a much larger major groove and correspondingly smaller minor groove (Weeks and Crothers 1993; Chen and Frankel 1995). Bulges and loops in RNA structures have been shown to form recognition elements for RNA-binding proteins (Predki et al. 1995; Scripture and Huber 1995). For example, the specific bulged structure in the stem region of Tar RNA is recognized by Tat protein (Puglish et al. 1992). Similarly, the *lin-4* bulged C might provide a scaffold for RNA-binding proteins.

Our conclusion that all of the temporal gradient-generating activity of the *lin-14* 3' UTR is resident only in the *lin-4* complementary regions was not predicted from the analysis of the LIN-14 gradient in *lin-4* null mutants and *lin-14*(*gf*) mutants. This analysis shows more LIN-14 down-regulation in the *lin-4* null mutant than in the strong *lin-14*(*n355gf*) mutant (Wightman et al. 1993). This result has been confirmed qualitatively by immunofluorescence studies of LIN-14 levels (G. Ruvkun, unpubl.). One possible explanation of this difference is that *lin-4* may also regulate the expression of other factors that destabilize LIN-14 (e.g., a *lin-4*-regulated LIN-14 co-factor or protease; Arasu et al. 1991); in a *lin-4* mutant such destabilizing factors may be up-regulated, whereas in a *lin-14*(*gf*) mutant, they may be down-regulated by wild-type *lin-4* RNA.

While the in vitro and in vivo experiments strongly

suggest that the *lin-4* RNA binds directly to the complementary regions of the *lin-14* 3' UTR, this in vivo interaction has not been demonstrated by compensatory *lin-4* and *lin-14* mutations (Datta and Weiner 1991; Reich et al. 1992). A compensatory *lin-4* mutation (AGU to UCA; Fig. 1B) is not expected to down-regulate a wild-type *lin-14* 3' UTR but should down-regulate the *lin-14*(*7Xmut*) 3' UTR that bears the complementary mutations in the seven *lin-4* binding sites. However, such an engineered *lin-4* mutant RNA is unstable or not expressed (P. Olsen and V. Ambros, pers. comm.), precluding such a formal in vivo proof. Our proposed *lin-4/lin-14* RNA structure is supported by the conservation between *C. elegans* and *C. briggsae* of both *lin-4* and *lin-14* sequences in the complementary regions. The locations and the detailed features of the proposed bulged C and nonbulged regions are also conserved.

Multiple blocks of conserved *lin-14* 3' UTR sequence between *C. elegans* and *C. briggsae* highlight elements that may be required for LIN-14 protein temporal gradient formation or other functions such as polyadenylation (Fig. 1A). The conserved blocks that are complementary to the *lin-4* RNA are essential for in vitro binding of the *lin-4* RNA to the *lin-14* 3' UTR (Fig. 2) and for in vivo temporal gradient formation (Fig. 3). The other conserved features of the *lin-14* 3' UTR may be binding sites for *trans*-acting factors that act in addition to or cooperatively with *lin-4*. Alternatively, they may participate in other regulatory activities of the *lin-14* 3' UTR (e.g., regulation in particular cell types or finer scale temporal regulation). Phylogenetically conserved elements in 3' UTRs of other *Drosophila* and *C. elegans* pattern formation genes post-transcriptionally regulate gene expres-

sion, especially during germ-line and early maternal development (Irish et al. 1989; MacDonald 1990; Ahringer and Kimble 1991; Wharton and Struhl 1991; Goodwin et al. 1993; Evans et al. 1994; Curtis et al. 1995; Murata and Wharton 1995). Proteins that bind these elements to mediate translational regulation have been identified (Kim-Ha and Macdonald 1995; Murata and Wharton 1995; Dubnau and Struhl 1996). It is probable that the conserved elements in the *lin-14* 3' UTR are also recognized by regulatory proteins.

Regulatory RNAs that generate RNA duplexes have been shown to control prokaryotic plasmid replication, transposition, and gene expression (Persson et al. 1988; Wagner and Simons 1994) as well as eukaryotic viral and host gene expression (Mellits et al. 1990; Klausner et al. 1993). Given the involvement of RNA duplexes in gene regulation from bacteria to metazoans, we expect that the mechanism by which the bulged C *lin-4*/*lin-14* RNA duplex is recognized to down-regulate translation may be general.

Materials and methods

Molecular biology

A *C. briggsae* genomic library in λ Charon 4 (David Baillie, Simon Fraser University, Burnaby, British Columbia, Canada) was screened with 32 P-labeled 3.8 *Eco*RI fragment (Wightman et al. 1991; Fig. 1) and washed at low stringency (55°C, 2 $\times$ SSC) (Ausubel et al. 1993). Insert fragments were subcloned and sequenced.

For construction of a *lacZ* fusion gene bearing the *lin-14* 3' UTR with a set of three point mutations in each of the seven *lin-4* complementary sites (7*Xmut*), two restriction sites (*Stu*I and *Hind*III) were created in the *lin-14* 3' UTR at positions 24567 and 25062 (Fig. 1A) by site-directed mutagenesis. Four long DNA oligonucleotide primers (nucleotides 24562–24692, 24693–24830, 24831–24956, and 24955–25066; they are numbered according to genomic sequences of cosmid T25C12 that contains the *lin-14* gene, GenBank accession no. Z66566) that substitute AGU for UCA at each of the seven *lin-14*/*lin-4* RNA duplex regions (see Fig. 1B) were chemically synthesized and purified on a denaturing 12% polyacrylamide gel. Eight short DNA oligonucleotide primers that bridge the long DNA primers were used to join the entire set together by sequential PCR. The 3' end of the short bridging DNA primers was designed to be adjacent to a 3' A to render inconsequential nonspecific addition of extra A nucleotides by Taq polymerase. The final PCR product was cloned into the *Stu*I and *Hind*III sites of the *lin-14* 3' UTR and these two restriction sites were changed back to wild-type sequence by site-directed mutagenesis. The *lin-14*(7*Xmut*) 3' UTR replaced the wild-type *lin-14* 3' UTR in the *col-10/lacZ* reporter gene pC10UT (Wightman et al. 1993). The sequence was confirmed.

Fusion genes with multimerized bulged and nonbulged *lin-4* binding sites were constructed by iterative cloning of DNA fragments generated by two pairs of oligonucleotide primers (5'-CTAGTACACTCACACAACTCAGGAACCTTT-3' and its complementary oligomer for the bulged sites and 5'-CTAGTACACTCACACAACTCAGGAACCTTT-3' and its complementary oligomer for the non-bulged sites). Six copies of these *lin-4* binding sites were obtained through six cycles of subcloning and subsequently placed into the *col-10/lacZ/unc-54* 3' UTR reporter gene.

In vitro RNA binding assay

To generate the various *lin-14* RNAs for in vitro binding, subclones of wild-type *lin-14* 3' UTR, *lin-14*(7*Xmut*) 3' UTR, 6 $\times$ bulged C *lin-4* binding site from the *lin-14* 3' UTR, and 6 $\times$ nonbulged *lin-4* binding site from the *lin-14* 3' UTR were transcribed using T3 RNA polymerase and purified by precipitation, and their concentration was estimated by ethidium bromide staining after agarose gel electrophoresis. Wild-type and mutant *lin-4* RNAs (5'-GUUCCCUGAGACCUCUGUGAG-3' for wild type and 5'-GUUCCUUGAGACCUCUGUGAG-3' for *ma161*) were chemically synthesized (Biotechnology Resource Laboratory, Yale Medical School). The in vitro transcribed *lin-14* RNAs (0.2 μ g) were incubated with 32 P-labeled *lin-4* RNA at room temperature for 2 hr in 20 mM HEPES at pH 8.0, 5 mM MgCl₂, 5% glycerol, 10 mg/ml tRNA of *Escherichia coli*, 1 unit RNase inhibitor. This mixture was resolved on a native 1% agarose or 6% polyacrylamide gel ($\frac{1}{2} \times$ TBE buffer) at 100 V for 1.5 hr. The gel was dried and exposed to film.

Temporal gradient assays

col-10/lacZ/lin-14 3' UTR fusion genes were coinjected with the marker plasmid pRF4 as described previously (Mello et al. 1991; Wightman et al. 1993). Between 1 and 3 independent lines were obtained for each construct. The same fusion gene arrays assayed for β -galactosidase activity in wild type were crossed into *lin-14*(n179ts) or *lin-4*(e912);*lin-14*(n179ts) genetic backgrounds (Ambros 1989) for those assays. Thus the same arrays were tested in *lin-4*(e912) and wild type.

Staged preparations from transformed strains bearing the various *lacZ* constructs were obtained by treating each strain with alkaline hypochlorite to isolate a population of eggs (Wood 1988). Eggs were allowed to hatch overnight in M9 buffer to yield a pure population of L1 stage animals. Some of this preparation was frozen and the rest was fed *E. coli* strain XL1-blue (which does not produce β -galactosidase activity) until the L4 stage. Bacteria in the gut were removed by multiple rinses with sterile distilled water.

β -galactosidase activity was assayed using a chlorophenol red- β -D-galactopyranoside substrate (CPRG, Boehringer Mannheim) (Simon and Lis 1987). Approximately 20 μ l of staged animals were sonicated for 10 sec using an Ultrasonics W-375 Sonicator in 500 μ l of assay buffer (50 mM potassium phosphate at pH 7.5, 1 mM MgCl₂, 1 mM Pefabloc SC, Boehringer Mannheim) and spun for 5 min in a microfuge at 4°C. Supernatant was transferred to a disposable cuvette, additional assay buffer added to a total of 200 μ l, and 300 μ l of 1 mg/ml CPRG in assay buffer added to each. Samples were mixed and incubated at room temperature. A_{574} was measured on a Beckman spectrophotometer every 30 min for 2 hr. Activity was calculated by dividing the change in A_{574} over time by the amount of total protein of transgenic animals in each extract (determined by Bradford Method, Bio-Rad). Multiple dilutions of test extracts showed that this assay yielded results that increased linearly with the amount of extract over the range of O.D. used in these experiments. Extracts made from *E. coli* strain XL1-Blue, on which worms were fed, or wild-type *C. elegans* fed this strain yielded no detectable activity in this assay.

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A bulged lin-4/lin-14 RNA duplex is sufficient for *Caenorhabditis elegans* lin-14 temporal gradient formation.

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