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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fates. Heterochronic mutations that elevate LIN-14 levels at late stages cause repetitions 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 around 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 in 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-
RNA duplex regulates *C. elegans* temporal gradient

**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* 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 cosmids 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* 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* 3' UTR (underlined outline AGU) and the *lin-4* (ma161) RNA sequence (outline U) are 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 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).

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 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; Klauser et al. 1993; Hjalt and Wagner 1995). Four of the seven proposed lin-14/lin-4 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(mal61) mutant (Fig. 1B; Lee et al. 1993). The lin-4(mal61) 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-14/lin-4 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-

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. 32P-labeled fin-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(mal61) 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(mal61) 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(mal61) 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 ΔΔG = 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(mal61) 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 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(mal61)/lin-14 RNA duplexes may fail to be stabilized by the association to such a duplex binding protein. Such an unbound lin-4(mal61) RNA might be degraded; in fact the level of the lin-4(mal61) RNA is lower than in wild type (R. Feinbaum and V. Ambros, pers. comm.). However, the
RNA duplex regulates *C. elegans* temporal gradient

**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(mal61)/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 cofactor 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(Xmut)* 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 32P-labeled 3.8 EcoRI fragment (Wightman et al. 1991; Fig. 1) and washed at low stringency (55°C, 2x 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 (7Xmut), two restriction sites (Stul and HindIII) 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, 24692–24830, 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.

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(7Xmut) 3' UTR, 6' bulged C lin-4 binding site from the lin-14 3' UTR, and 6' 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'-GUUCCUGAGACCCUGUGAG-3' for wild type and 5'-GUUCCUGAGACCCUGUGAG-3' for ma161) were chemically synthesized [Biotechnology Resource Laboratory, Yale Medical School]. The in vitro transcribed lin-14 RNAs (0.2 µg) were incubated with 32P-labeled 3.8-4 RNA at room temperature for 2 hr in 20 mM HEPES at pH 8.0, 0.5 mM MgCl2, 5% glycerol, 10 mg/ml RNA of Escherichia coli, 1 unit RNase inhibitor. This mixture was resolved on a native 1% agarose or 6% polyacrylamide gel (1/3 X 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 co-injected 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(179ts) or lin-14(e912);lin-14(179ts) genetic backgrounds [Ambros 1989] for those assays. Thus the same arrays were tested in lin-14(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 µl of staged animals were sonicated for 10 sec using an Ultrasonics W-375 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 µl of staged animals were sonicated for 10 sec using an Ultrasonics W-375 Sonicator in 500 µl 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 µl, and 300 µl of 1 mg/ml CPRG in assay buffer added to each. Samples were mixed and incubated at room temperature. A540 was measured on a Beckman spectrophotometer every 30 min for 2 hr. Activity was calculated by dividing the change in A540 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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