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Timing in *Caenorhabditis elegans* by Blocking LIN-14 Protein Synthesis after the Initiation of Translation

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lin-4 **encodes a small RNA that is complementary to sequences in the 3′ untranslated region (UTR) of** ***lin-14*** **mRNA and that acts to developmentally repress the accumulation of LIN-14 protein. This repression is essential for the proper timing of numerous events of** ***Caenorhabditis elegans*** **larval development. We have investigated the mechanism of** ***lin-4*** **RNA action by examining the fate of** ***lin-14*** **mRNA** ***in vivo*** **during the time that** ***lin-4*** **RNA is expressed. Our results indicate that the rate of synthesis of** ***lin-14*** **mRNA, its state of polyadenylation, its abundance in the cytoplasmic fraction, and its polysomal sedimentation profile do not change in response to the accumulation of** ***lin-4*** **RNA. Our results indicate that association of** ***lin-4*** **RNA with the 3′ UTR of** ***lin-14*** **mRNA permits normal biogenesis of** ***lin-14*** **mRNA, and normal translational initiation, but inhibits step(s) thereafter, such as translational elongation and/or the release of stable LIN-14 protein.** **© 1999 Academic Press**

Key Words: **heterochronic genes; translation;** ***C. elegans;*** ***lin-4*** **RNA; polyribosomes.**

INTRODUCTION

In the nematode *Caenorhabditis elegans,* the heterochronic gene pathway controls the coordinated timing of larval developmental events, including cell division, differentiation, cell migration, and death in a variety of tissues (Ambros, 1989). Mutations in heterochronic genes result in temporal transformations of cell fates such that cells adopt fates normally specific to cells at earlier or later times in development. For example, loss-of-function mutations in *lin-14* cause precocious expression of cell fates that normally occur at later larval stages. *lin-14* gain-of-function mutations result in retarded phenotypes, in which cell fates specific to early larval stages are reiterated (Ambros and Horvitz, 1987). These and other genetic properties of *lin-14* indicated that *lin-14* must be active in the early L1, to specify the early cell fates, and then must be down-regulated late in the L1 and during the L2 to permit the expression of later fates (Ambros and Horvitz, 1987; Moss and Ambros, 1997). LIN-14 protein level decreases between the L1 and the L2 stages, while *lin-14* mRNA level is relatively constant, suggesting that LIN-14 is regulated posttranscriptionally (Wightman *et al.,* 1993).

The down-regulation of LIN-14 is specified by the action of *lin-4. lin-4* encodes a 22-nucleotide regulatory RNA that is thought to act by antisense base-pairing with sequences in the 3′ untranslated region (3′ UTR) of *lin-14* mRNA (Lee *et al.,* 1993). *lin-4* RNA is expressed beginning late in the L1 stage, consistent with its playing a critical role in the down-regulation of LIN-14 protein at that time (Feinbaum and Ambros, 1999). *lin-4* also represses translation of *lin-28* mRNA, and *lin-28* is completely unrelated to *lin-14* in sequence, except for the presence of *lin-4*-complementary sequences in the 3′ UTRs of both genes (Moss *et al.,* 1997).

Translational control mechanisms involving *cis*-acting 3′ or 5′ UTR regulatory sequences that bind *trans*-acting regulatory factors are frequently found to play significant roles in the regulation of gene activity, particularly during development (Wickens *et al.,* 1996). An example of regulation via a 5′ UTR element is the negative control of ferritin biosynthesis under low-iron conditions, in which a *trans*-acting regulatory protein interacts with a stem–loop structure in the 5′ UTR of ferritin mRNA and prevents the formation of a preinitiation complex (Aziz and Munro, 1987). Examples of mRNAs translationally regulated via 3′ UTR elements include numerous mRNAs in the *Drosophila* embryo that specify body pattern through localized translation (Wickens *et al.,* 1996). For example, *hunchback*

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mRNA is translationally repressed in the posterior of the fly embryo by the combined action of the Nanos and Pumilio proteins. A protein related to Pumilio represses translation of the *fem-3* mRNA in *C. elegans* and mediates a developmental switch from spermatogenesis to oogenesis in the hermaphrodite (Zhang *et al.*, 1997). Similarly, translational control elements in the 3′ UTR of the *C. elegans tra-2* mRNA mediate the developmental control of TRA-2 expression and hence regulate sexual phenotype (Goodwin *et al.*, 1993). A second worm developmental timing gene, *let-7*, has been shown to encode a small RNA like *lin-4* (but completely different in sequence) and candidate regulatory targets of *let-7* have been identified by their *let-7*-complementary 3′ UTR sequences (F. Slack, B. Reinhart, and G. Ruvkun, personal communication). In many cases of translational regulation, *trans*-acting factors that bind UTR elements to effect translational control have been identified; however, to date, *C. elegans lin-4* and *let-7* are the only cases in which a *trans*-acting regulator has been demonstrated to be a small antisense RNA.

The mechanisms by which 3′ UTR elements mediate translational regulation are not clear. Models that suggest the involvement of a closed loop between 5′ and 3′ UTR sequences (Jacobson, 1996) imply that repressors that bound in 3′ UTR sequences could affect 3′–5′ interactions required for initiation of translation. For example, *oskar* mRNA in *Drosophila* is translationally repressed by the binding of Bruno protein to elements in the *oskar* 3′ UTR, and this repression is overcome by the action of derepressor activity acting through a 5′ UTR element (Gunkel *et al.*, 1998; Lie and Macdonald, 1999), suggesting that an interaction between 3′ and 5′ sequences is critical for regulation of *oskar* mRNA translation. In certain cases, the binding of *trans*-acting factors to 3′ UTR regulatory elements can lead to deadenylation of the target mRNA. Poly(A) length can influence rates of translational initiation, presumably via interactions between factors bound to the mRNA 5′ and 3′ ends of mRNAs (Wickens, 1992; Salles *et al.*, 1994; Tarun and Sachs, 1995, 1996; Lieberfarb *et al.*, 1996). However, poly(A) length can also affect other facets of mRNA processing and function, including the rate of mRNA degradation, so changes in poly(A) length do not necessarily signify a direct regulation of initiation (Jacobson, 1996). For example, binding of the *Drosophila* translational repressor Pumilio to hunchback mRNA results in deadenylation, but Pumilio can act on an mRNA containing an internal ribosome entry site (IRES) (Wharton *et al.*, 1998), suggesting that the translational repression by Pumilio occurs at a step downstream of the 5′ cap site. Although Pumilio could act by blocking a translation step after initiation, it is also possible that Pumilio could be affecting an initiation step common to cap-dependent and IRES-dependent translation, such as assembly of 80S ribosomes (Wharton *et al.*, 1998). Since some mRNAs seem to be translationally repressed despite apparently normal translational initiation (Kaspar and Gherke, 1994; Berry *et al.*, 1988; Ch'ng *et al.*, 1990; I. Clark and E. Gavis, personal communication), it is apparent that translational repressors associated with 3′ UTR sequences can affect protein synthesis from an mRNA at steps subsequent to initiation, such as elongation or the discharge of functional protein.

In this study, we describe the results of experiments directed at understanding the mechanism by which the *lin-4* regulatory RNA down-regulates LIN-14 protein during *C. elegans* larval development. Our results indicate that translational repression of *lin-14* mRNA by the 22-nucleotide *lin-4*S RNA does not appear to involve changes in the polyadenylation status or intracellular partitioning of *lin-14* mRNA. Furthermore, *lin-4*S RNA and the translationally repressed *lin-14* mRNA are both associated with polyribosomes, suggesting that *lin-4*S, complexed with its target mRNA, blocks protein synthesis at a point after the initiation of translation.

MATERIALS AND METHODS

Nematode Methods

C. elegans strain N2 was maintained and cultured in liquid or on NGM plates according to established methods (Wood, 1988). In order to obtain populations of nematodes at various larval stages, animals were synchronized by hatching embryos in the absence of food. Food (bacterial strain HB101) was added to the suspension of synchronized larvae and the worms were grown to the desired larval stage and then harvested. Nomarski microscopy was used to confirm the synchrony of staged animals; this method provided populations in which greater than 90% of the larvae were in the desired larval stage.

Western Blots

Western analysis was performed according to standard methods (Ausubel *et al.*, 1989). Protein extracts were prepared by boiling nematodes in 0.05 M Tris–HCl, pH 7.5, 4% SDS, 0.1 M β-mercaptoethanol. After appropriate dilution, protein extract concentrations were determined by Bradford assay and by SDS–PAGE followed by Coomassie blue staining. Ponceau S staining was used to assess the quantitative transfer of protein from the polyacrylamide gel to nitrocellulose membrane. LIN-14 protein was detected with a LIN-14 specific antisera (Ruvkun and Giusto, 1989) using the ECL chemiluminescence kit (Amersham).

Nuclear Run-on Analysis

Nuclei from early L1 and late L2/L3 stage *C. elegans* larvae were isolated as described, except that larvae were frozen in liquid nitrogen and ground using a mortar and pestle (Candido *et al.*, 1989). Nuclei were stored in aliquots at −80°C in 40% glycerol, 50 mM Tris–Cl, pH 8.0 (25°C), 5 mM $MgCl_2$, and 0.1 mM EDTA. Nucleus concentration was estimated by measuring DNA concentration using Hoechst dye 33258 (Labarca and Paigen, 1980). For run-on analysis, nuclei were diluted into 50 mM Tris–Cl, pH 8.0, 2.0 M salt to disrupt protein-bound DNA. Nuclear run-on assays were performed as described, but with some modification (Schauer and Wood, 1990). Briefly, a standard reaction consisted of 10^8 nuclei in a final volume of 0.150 ml containing 100 mM Tris–Cl, pH 8.0; 100 mM KCl; 50 mM NaCl; 5 mM $MgCl_2$; 1 mM DTT; 0.2 mM

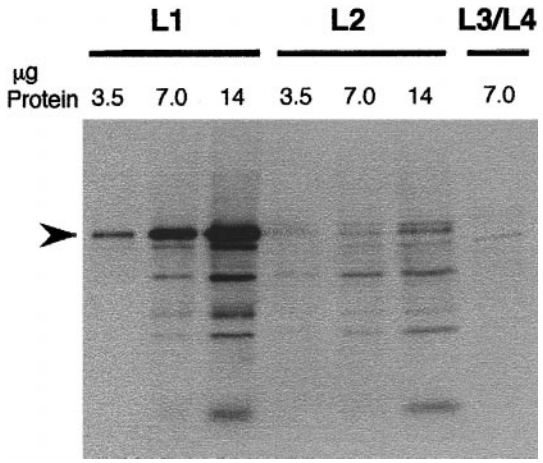


FIG. 1. Down-regulation of LIN-14 protein after the L1 stage. A Western blot of protein extracts from L1, L2, and L3/L4 larvae shows the dramatic decrease in the steady-state level of LIN-14 protein (arrowhead) from the L1 to the L2. The additional smaller bands detected on the Western blot do not seem to be related to LIN-14 protein; they are not immunoprecipitated with the LIN-14 antiserum and are not routinely detected on Western blots (data not shown).

PMSF; 30% glycerol; 3 mM each ATP, CTP, and GTP; 0.01 mM UTP; and 0.5 mCi [^{32}P]UTP (800 Ci/mmol). The reaction was incubated for 50 min at 25°C, digested with RQ1 DNase (Promega), and subsequently digested with proteinase K. The reaction was extracted once with 1:1 phenol:chloroform and unincorporated nucleotide was removed using a G-50 spin column. The standard reaction was scaled up as necessary to obtain greater quantities of labeled run-on transcripts. For the experiment shown in this paper, a fragment of the 3′ UTR of the actin gene (*act-1*) (Krause *et al.*, 1989), the eIF-4A cDNA (Roussell and Bennett, 1992), and a genomic fragment of *lin-14* from about 200 bp upstream of exon 4 to just downstream of the polyadenylation signal (Wightman *et al.*, 1991) were subcloned into pBluescript (Stratagene). Prior to being run on an agarose gel, these constructs were linearized at either one restriction enzyme site within the vector (for actin and eIF-4A) or two flanking and one internal restriction enzyme site to separate pBluescript vector DNA from the *lin-14*-specific DNA. One microgram of each digested DNA sample was run on a 1.0% agarose gel and transferred to nitrocellulose membrane (MSI). The filter was baked for 2 h at 80°C and cut in half to separate two identical sets of lanes. Run-on transcripts from L1 or L2/L3 stage nuclei were hybridized to the nitrocellulose filters as described, except that a phosphorimager was used to detect and quantify hybridized transcripts (Molecular Dynamics) (Schauer and Wood, 1990).

Poly(A) Tail Length and Sequencing

The poly(A) tail length of *lin-14* mRNA was determined using the RT/PCR poly(A) test essentially as described (Salles and Strickland, 1995). RNA was extracted from *C. elegans* at three developmental stages using a TRIzol (Gibco BRL) method. One hundred nanograms of each of these RNA samples was used in the reverse transcription reaction and 1/12 of each of the synthesized cDNA pools was used in the PCR with a *lin-14* sense-specific primer, 5′-GTTCTCTCTATCCA CCCCAACTTCCTCTC-3′. This primer is located approximately 360 bp upstream of the predicted polyadenylation signal (Wightman *et al.*, 1991). PCRs for autoradiography contained 10 μCi of [α-^{32}P]dATP. Fifty percent of each reaction was digested with *Hin*fI to confirm the specificity of the PCR. Sequence analysis confirmed that the 3′ UTR of *lin-14* mRNA was specifically reverse transcribed and amplified and confirmed the location of the *lin-14* cleavage and polyadenylation sites as previously determined by cDNA sequencing (Wightman *et al.*, 1991). The *Hin*fI site is approximately 95 bp from the poly(A) site, and so poly(A) length was estimated by *Hin*fI digestion of the *lin-14* RT-PCR products followed by fractionation on 10% nondenaturing polyacrylamide gels. ϕX174 *Hin*fI-digested, ^{32}P-end-labeled DNA fragments were used as molecular weight markers. PCRs for sequencing were unlabeled and were run on 2% agarose gels and stained with ethidium bromide and the products were purified. Sequence was obtained from an ABI 373 sequencer operated by the Dartmouth Medical School Molecular Biology Core Facility (Hanover, NH) and analyzed using the computer program Sequencher (GeneCodes). This method of poly(A) tail analysis was successfully tested on *fem-3* mRNA, a *C. elegans* mRNA known to undergo large polyadenylation length changes from the embryo to the larval stages (Ahringer *et al.*, 1992; data not shown).

Polyribosome Sedimentation

Total polyribosomes from *C. elegans* lysates were centrifuged on sucrose gradients essentially as previously described (Davies and Abe, 1995). Larvae were frozen in liquid nitrogen and ground using a mortar and pestle. The frozen powder was thawed on ice in 5 vol of buffer A (200 mM Tris–HCl, pH 8.5, 50 mM KCl, 25 mM $MgCl_2$, 2 mM EGTA, 0.1 mg/ml heparin, 2% polyoxyethylene 10-tridecyl ether, and 0.75% sodium deoxycholate). The lysate was centrifuged in an SW55Ti rotor for 10 min at 27,000*g* at 4°C, and the postmitochondrial supernatant (PMS) was loaded directly onto one or more 12-ml 20–60% (w/v) sucrose gradients made in buffer B (50 mM Tris–HCl, pH 8.5, 25 mM KCl, and 10 mM $MgCl_2$). The tubes

TABLE 1

Normalized *lin-14* RNA Levels in Various Cell Fractions of L1 and L2 Larvae

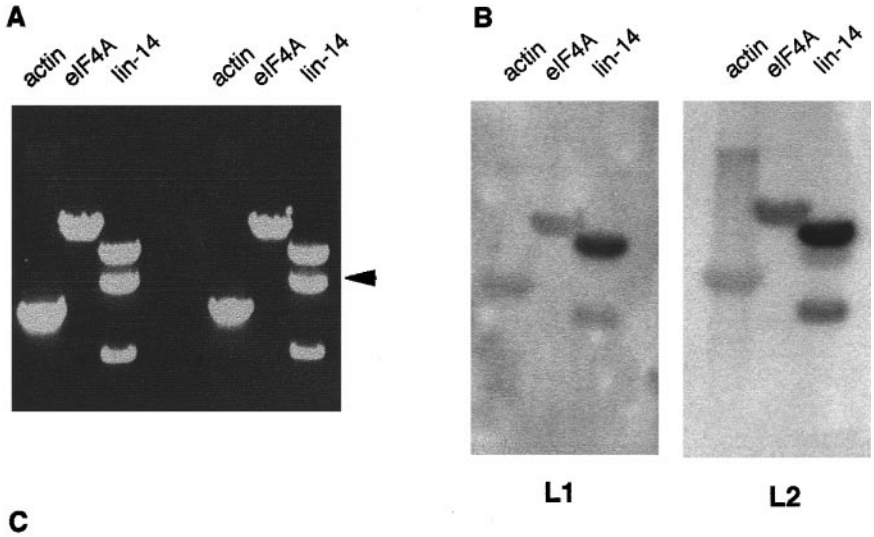
L1/L2[a]		PMS[b]/Total[c]	
Total RNA[c]	PMS RNA[b]	L1	L2
2.3 (±0.96)[d]	1.5(±1.0)[d]	1.1(±0.46)[d]	1.9(±0.91)[d]

[a] The ratios of *lin-14* RNA levels in fractions of extracts from L1 and L2 larvae were determined from phosphorimager images of polyacrylamide gels after RNase protection analysis (see Material and Methods). *lin-14* RNA levels in each sample were normalized to eIF-4A RNA levels before ratios were calculated.

[b] Amount of *lin-14* RNA (normalized to eIF-4A RNA) in the postmitochondrial supernatant (PMS) fraction of L1 and L2 extracts.

[c] Total amount of *lin-14* RNA (normalized to eIF-4A RNA) in L1 and L2 extracts prior to centrifugation to produce the PMS fractions.

[d] Average and standard deviation of three experiments.



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Gene	L1	L2
lin-14	3.8	6.1
eIF4A	1.0	1.9
actin	0.5	0.6

FIG. 2. *lin-14* is transcribed at comparable levels in the L1 and L2 stages. (A) Ethidium bromide-stained agarose gel of fractionated DNA. Two sets of identical lanes containing 1 mg each of the indicated DNAs were run on 1.2% agarose gel. Location of the pBluescript vector is indicated by the arrowhead. (B) DNA from gel shown in A was denatured and transferred to nitrocellulose, and the two sets of lanes were separated and probed with labeled nuclear run-on transcripts prepared from either L1 or L2 nuclei. (C) Signal intensities were quantified using a phosphorimager and normalized to the eIF-4A signal detected in the L1.

were centrifuged in an SW41Ti rotor for 90 min at 40,000 rpm at 4°C. Gradients were fractionated using an ISCO gradient collector while continuously monitoring absorbance at 254 nm. Individual fractions were extracted once with phenol:chloroform (1:1) and precipitated with ethanol. In some cases, as noted in the figure legends, the PMS was loaded onto 1 ml of 60% (w/v) sucrose in buffer B and centrifuged in an SW55Ti rotor for 2 h at 50,000 rpm at 4°C to collect polyribosomes. Following this, the polyribosome pellet was resuspended in buffer A and loaded onto one or more 12-ml 20–60% (w/v) sucrose gradients made in buffer B. Fractions were analyzed by RNase protection.

Metrizamide Gradient Analysis

Postmitochondrial supernatants were prepared from L1 and L2 stage nematodes, and polyribosomes were purified as described above. The polyribosome pellets were resuspended in a total volume of 4.0 ml of a solution of 50% metrizamide, 10 mM Hepes–KOH, pH 7.5, 10 mM KCl, 3 mM $MgCl_2$, 1 mM DTT (Dissous *et al.*, 1976). Resuspended material was layered onto 1.0 ml of the same solution (but with 70% metrizamide) in a 5-ml centrifuge tube. Centrifugation runs were for 90 h at 45,000 rpm, 4°C in a SW55Ti rotor. Twelve fractions from each gradient were collected and the refractive index of the fractions was measured with an Abbe refractometer. Fractions were extracted with phenol: chloroform and ethanol precipitated. RNase protection was used to identify those fractions containing *lin-14* RNA.

RNase Protection Assays

RNase assays were done with the RPA II kit (Ambion). ^{32}P-labeled antisense probes were synthesized using T7 RNA polymerase according to standard methods (Ausubel *et al.*, 1989). The *lin-4* probe plasmid template, pMspI, was previously described (Lee *et al.*, 1993).The *lin-14* probe was prepared from a PCR template and is protected by about 280 nucleotides of the 3′ UTR immediately following the stop codon (Wightman *et al.*, 1991). The eIF-4A probe was prepared from a PCR template and is protected by 110 nucleotides of eIF-4A sequence starting approximately 90 nucleotides downstream of the start codon (Roussell and Bennett, 1992). RNase protection samples were run on either 5 or 20% denaturing polyacrylamide gels to fractionate *lin-14* and eIF-4A or *lin-4* protected probes, respectively.

RESULTS

Posttranscriptional Down-regulation of LIN-14

To explore the mechanism by which *lin-4*RNA down-regulates the level of LIN-14 protein during *C. elegans* development, we examined the fate of *lin-14* mRNA in the presence and absence of *lin-4* RNA. From previous studies, it was apparent that *lin-4* activity brought about a decrease in LIN-14 protein between the first and the second larval stages, while the level of *lin-14* mRNA remained constant (Wightman *et*

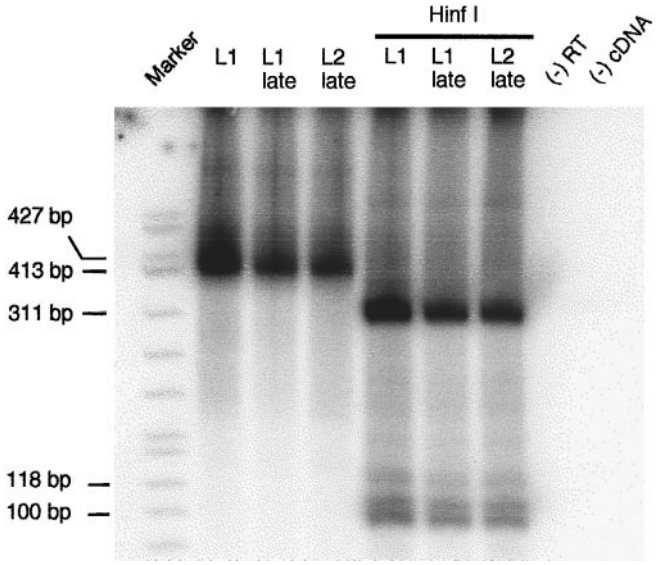


FIG. 3. A nondenaturing 5% polyacrylamide gel of 3′ UTR RT-PCR products was exposed to a phosphorimager screen and shows that *lin-14* mRNA poly(A) tail length is unchanged from the L1 to the L2 and extends to about 30 nucleotides. The minimum expected product length is 360 bp of 3′ UTR + 30 bp of oligo$(dT)_{12}$ anchor primer = 390 bp. The observed length is approximately 400–420 bp, which indicates a poly(A) length of about 10–30 nt. *Hin*fI digestion of the PCR product produces a result consistent with these estimates, as the *Hin*fI site is about 95 bp from the poly(A) addition site, and the length of the *Hin*fI digestion product is about 110 bp less than the undigested product. This suggests a poly(A) length of about 15 nt. No differences are apparent among the products from the three larval stages.

al., 1993). We also found that LIN-14 protein decreases at least 10-fold from the L1 to the L2 relative to total protein (Fig. 1). In contrast, the normalized steady-state level of *lin-14* mRNA changed by no more than a factor of approximately 2 from the L1 to the L2 (Table 1), consistent with previous findings (Wightman *et al.*, 1993). Nuclear run-on experiments were performed to examine the transcriptional state of *lin-14* in the L1 and the L2. Very similar rates of *lin-14* RNA synthesis were observed in nuclei from L1 and L2 larvae, assuming that the eIF-4A transcription levels are equivalent in the L1 and the L2 (Fig. 2). These data are consistent with previous data indicating that *lin-14* activity is developmentally regulated posttranscriptionally.

lin-14 Poly(A) Tail Length Is Constant between the L1 and the L2

To determine whether the regulation of LIN-14 protein synthesis by *lin-4* involves effects on *lin-14* polyadenylation, the poly(A) tail length of *lin-14* mRNA was determined from RNA isolated in the early L1 (5 h on food), late L1 (16 h on food), and late L2 (Fig. 3). RT/PCR analysis was performed using oligo(dT) and a primer within the *lin-14* 3′ UTR. The resulting PCR product was analyzed by restriction digestion and gel electrophoresis. The length of the poly(A) was deduced based on the length of the observed product relative to the position of the poly(A) addition site in the *lin-14* sequence. From early L1 through the L2, the average poly(A) length of *lin-14* mRNA appeared essentially unchanged (Fig. 3). There was some heterogeneity in the apparent length of the poly(A) of *lin-14* mRNA within a particular larval stage, varying from 12 nucleotides (the lower limit of our measurement owing to the 12 thymidines in the oligo(dT) anchor primer) to approximately 30 nucleotides. Some of the observed variation could be a consequence of the method, so the actual poly(A) length of *lin-14* mRNA may be less heterogeneous than the results suggest.

Polyribosome Association of lin-14 mRNA Is Unchanged between the L1 and the L2

To determine whether the decrease in LIN-14 protein between the L1 and the L2 is a consequence of an inhibition of translational initiation, we examined the sedimentation profile of polyribosomes containing *lin-14* mRNA. Extracts were prepared from synchronized populations of L1 and L2 larvae as described under Materials and Methods, and portions of each extract were analyzed in parallel by sucrose gradient sedimentation to fractionate polyribosomes (Fig. 4) and by Western blotting to confirm down-regulation of LIN-14 protein (Fig. 5). In three independent experiments, the fraction of total *lin-14* mRNA in each size class of polyribosomes was essentially unchanged between the L1 and the L2 stages. A careful examination of the distribution of the *lin-14* mRNA indicates that there may be slightly more *lin-14* mRNA associated with approximately seven or more ribosomes in the L1 compared to the L2, but this difference is within experimental variation of the measurements (Figs. 5B and 5C). Measurement of LIN-14 protein levels relative to total protein in the same worm samples used for polyribosome analysis suggests an approximately 12- to 15-fold down-regulation of LIN-14 between the L1 and the L2 in these experiments (Fig. 5A).

The reduction in LIN-14 protein between the L1 and the L2 did not seem to result from an overall reduction in the level of *lin-14* RNA or a sequestering of *lin-14* RNA in a subcellular fraction away from polyribosomes. First, the total amount of *lin-14* mRNA in the L1 is only about twice the amount in the L2 (Table 1). Second, the amount of *lin-14* RNA contained in the postmitochondrial supernatant (the fraction loaded on sucrose gradients to separate polyribosomes) is virtually unchanged between the L1 and the L2 (Table 1), indicating that no significant portion of *lin-14* mRNA becomes sequestered away from the translational apparatus in the L2.

To test the possibility that *lin-14* mRNA shifts from polyribosomes in the L1 to another (unidentified) complex of similar sedimentation profile in the L2, we tested

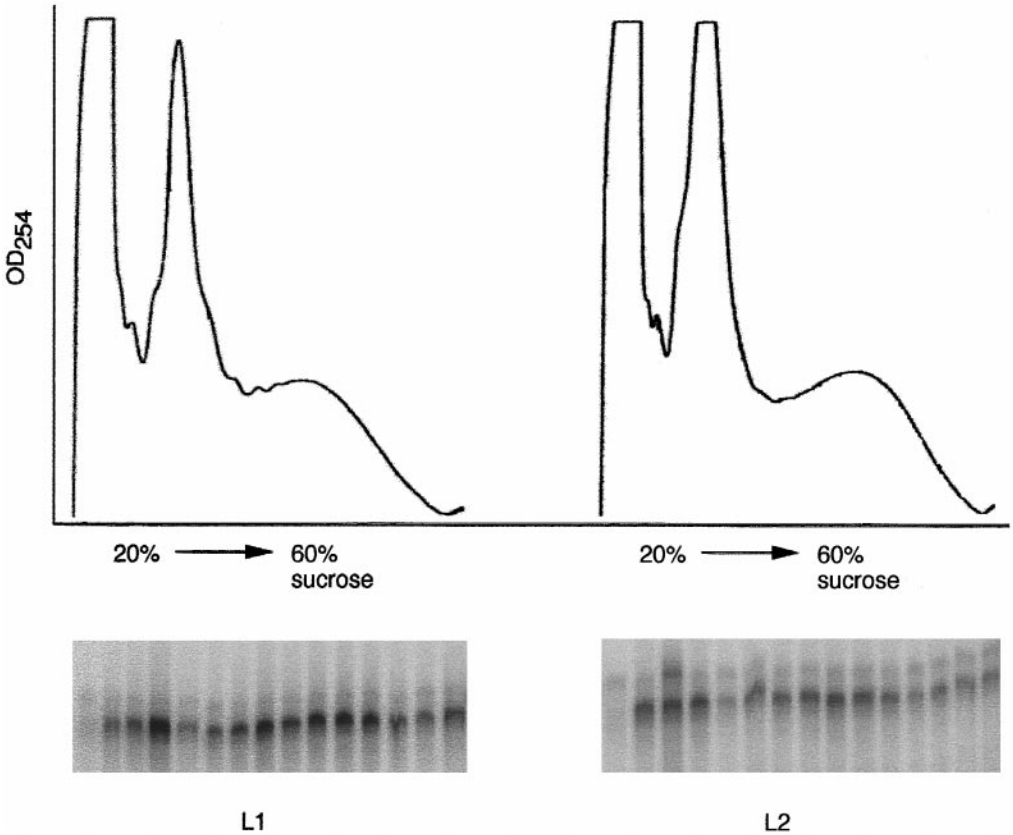
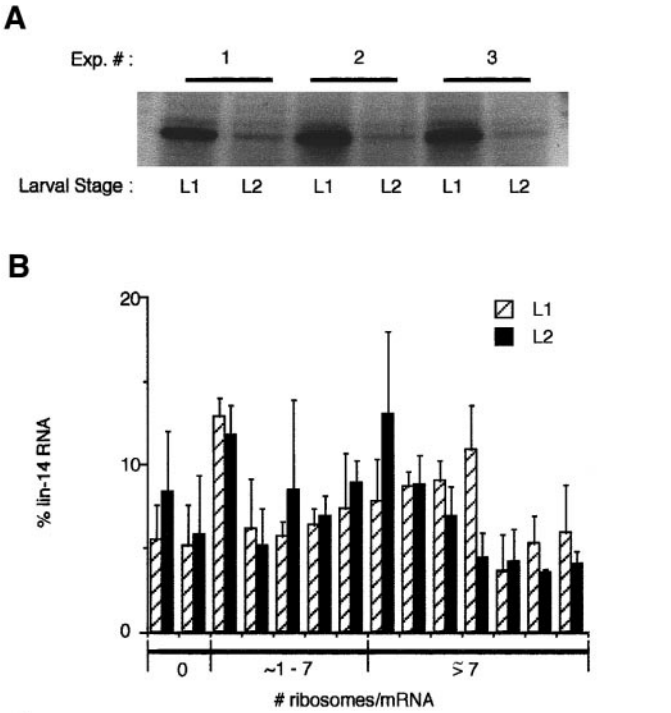


FIG. 4. The distribution of ribosomes associated with *lin-14* mRNA in the L1 is unchanged from the distribution observed in the L2. Polyribosomes were fractionated on sucrose gradients. RNase protection assays, using a *lin-14* antisense probe, on each of 16 fractions are shown below for LI and L2 larvae. The protected fragments were run on the same gel, but the autoradiogram has been split in two for clarity.

whether the *lin-14* mRNA-associated complexes in the L2 exhibit other properties of polyribosomes, specifically, EDTA sensitivity and characteristic buoyant density. In the absence of Mg^{2+}, mRNA is released from polyribosomes as the ribosomal subunits dissociate (Henshaw, 1968). In the presence of the chelating agent EDTA, *lin-14* mRNA from L2 animals no longer cosediments with polyribosomes in the fast-sedimenting gradient fractions, but sediments in the fractions at the top of the sucrose gradient, consistent with most of *lin-14* RNA being released from polyribosomes by EDTA (Fig. 6). (We have not examined further the EDTA-treated *lin-14* mRNA to determine if after release from polyribosomes it is associated with a smaller RNP). Also consistent with the identity of L2 *lin-14* mRNA complexes as polyribosomes, the complexes from L1 and L2 larvae exhibited identical buoyant density in metrizamide density gradient analysis (Dissous *et al.*, 1976; Fig. 7). We conclude that *lin-14* mRNA is associated with a polyribosome complex in the L2 and that its overall composition is similar to that of the actively translating polyribosome in the L1. These results indicate that the control of *lin-14* translation occurs at a point downstream of translation initiation.

Polyribosomes Contain lin-14 and lin-4 RNAs

The sequence complementarity between the *lin-14* 3′ UTR and *lin-4* RNA suggests base-pairing of *lin-4* and *lin-14* RNAs (Lee *et al.*, 1993; Wightman *et al.*, 1993). In sucrose gradients of extracts from L2 larvae, we found that about 10% of the total *lin-4* RNA cosedimented with polyribosomes (Fig. 8). *lin-4* RNA was also detected, though at slightly lower levels, in polyribosome fractions from *lin-14(n355n679)* animals (data not shown). The *lin-14(n355n679)* allele contains a deletion of all the potential *lin-4* RNA binding sites (Wightman *et al.*, 1991) and so this residual polyribosome association of *lin-4* RNA in *lin-14(n355n679)* animals could reflect association with other mRNAs, including *lin-28* mRNA (Moss *et al.*, 1997), and/or with components of the translation machinery.



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% lin-14 mRNA associated with:	L1	L2
1 or more ribosomes	89 +/- 1.0 %	86 +/- 6.7 %
1 to 7 ribosomes	51 +/- 4.8 %	45 +/- 9.0 %
8 or more ribosomes	38 +/- 5.5 %	41 +/- 5.9 %

FIG. 5. This represents the results of three independent experiments of the type shown in Fig. 4. (A) A Western blot for LIN-14 protein from *C. elegans* populations used for polyribosome analysis. (B) Polyribosome gradient fractions were analyzed with RNase protection, and *lin-14* RNA levels normalized to absorbance at 254 nm are plotted as the percentage of total *lin-14* mRNA in the gradient against fraction number (the top two and bottom two fractions of the sucrose gradient were each pooled). Error bars indicate ± standard deviation of the three experiments. (C) This table was derived from the data shown in B.

DISCUSSION

The down-regulation of LIN-14 protein between the L1 and the L2 larval stages is critical for the proper timing of postembryonic development in *C. elegans*. The evidence presented in this paper helps to discriminate among possible mechanisms for the down-regulation of LIN-14. First, we found that the levels of *lin-14* mRNA in the L1 and L2 differ by a factor of less than 3 and that the rate of transcriptional initiation for *lin-14* RNA is constant. This suggests that LIN-14 protein down-regulation does not involve changes in the half-life or synthesis of *lin-14* mRNA and is consistent with a mechanism occurring at the level of translation. Second, the poly(A) tail length of *lin-14* mRNA is unchanged between the L1 and the L2 stages, suggesting that mechanisms in which translation efficiency is related to poly(A) tail length are not applicable to LIN-14 down-regulation. Finally, since we can account for all the *lin-14* mRNA in the L1 and the L2 as being associated with ribosomes, it is unlikely that the *lin-14* mRNA in the L2 is somehow inaccessible to the translational apparatus.

We found that the sucrose gradient sedimentation profile of *lin-14* mRNA in extracts of L2 larvae is essentially identical to that in extracts from L1 animals. EDTA sensitivity and metrizamide gradient analysis of *lin-14* mRNA complexes obtained from L2 extracts are consistent with the identification of these complexes as polyribosomes (Henshaw, 1968; Dissous *et al.*, 1976). The simplest interpretation of this observation is that *lin-14* mRNA translation is regulated by *lin-4* at a step after initiation. A significant fraction of *lin-4* RNA is also associated with polyribosomes, suggesting that inhibition of *lin-14* mRNA translation by *lin-4* involves the formation of a ribosome-associated *lin-4::lin-14* RNA complex.

Although some well-studied translational control mechanisms have been shown to act at the level of translation initiation (Mathews *et al.*, 1996), in certain other cases, mRNAs have been reported to associate with polyribosomes without producing detectable protein, similar to the situation for *lin-14* mRNA under *lin-4* repression. For example, IL-1β mRNA in human monocytes and ribulose-1,5-bisphosphate carboxylase mRNAs in amaranth seedlings are found associated with polyribosomes though no protein synthesis is detectable (Kaspar and Gherke, 1994; Berry *et al.*, 1988). In a subline of U937 cells, no creatine kinase B (CKB) activity was detected, though the CKB mRNA was associated with polyribosomes; this inhibition of CKB level is mediated by the CKB 3′ UTR and hence signifies an apparent translational repression of CKB mRNA on polysomes (Ch'ng *et al.*, 1990). CKB translation also appears to be similarly regulated in developing rat brain, where CKB protein levels increase dramatically during postnatal development of the cerebrum, while CKB mRNA abundance and polyribosome distribution do not change over the same period (G. Molloy, personal communication). Similarly, the translational repression of unlocalized *nanos* mRNA in *Drosophila* embryos, which is mediated by *nanos* 3′ UTR sequences (Gavis and Lehmann, 1994), does not result in an altered polyribosome profile of *nanos* mRNA, indicating a block after initiation of translation (I. Clark and E. Gavis, personal communication). Although all these examples seem to involve translational control after initiation, the precise point(s) of regulation has not yet been identified.

Translational repression by *lin-4* RNA could occur at one or more postinitiation steps, including elongation, termination, or the release of functional protein. Our data do not critically test these various possibilities. We could not employ tests for puromycin sensitivity to examine the

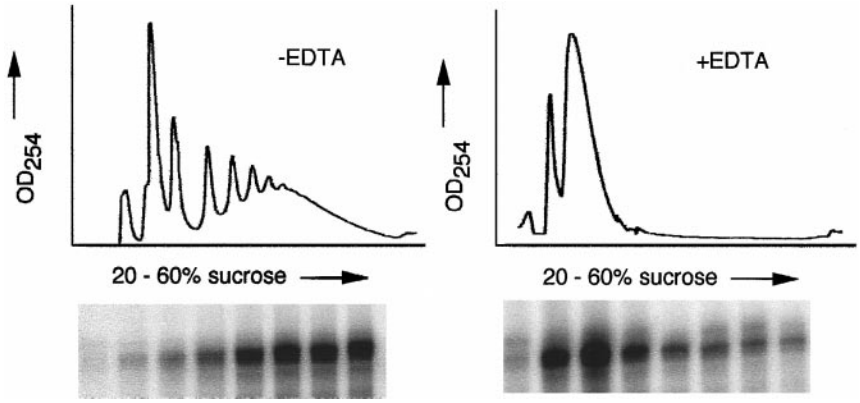

FIG. 6. *lin-14* mRNA polyribosomes from L2 larvae are EDTA sensitive. Polyribosomes were purified from L2 larvae and split into two aliquots and EDTA was added to one sample (to 0.15 M) prior to fractionation on sucrose gradients (see Materials and Methods). The left shows the general polyribosome distribution and the corresponding distribution of *lin-14* mRNA determined by RNase protection analysis in the absence of EDTA, while the right shows the results of polyribosomes treated with EDTA.

elongation competence of the *lin-14*-containing polyribosomes, as was possible, for example, for translationally arrested IL-2β mRNA (Kaspar and Gehrke, 1994). Under the conditions we used, live worms seemed to be impermeable to puromycin, and larva extracts were not translationally active (data not shown).

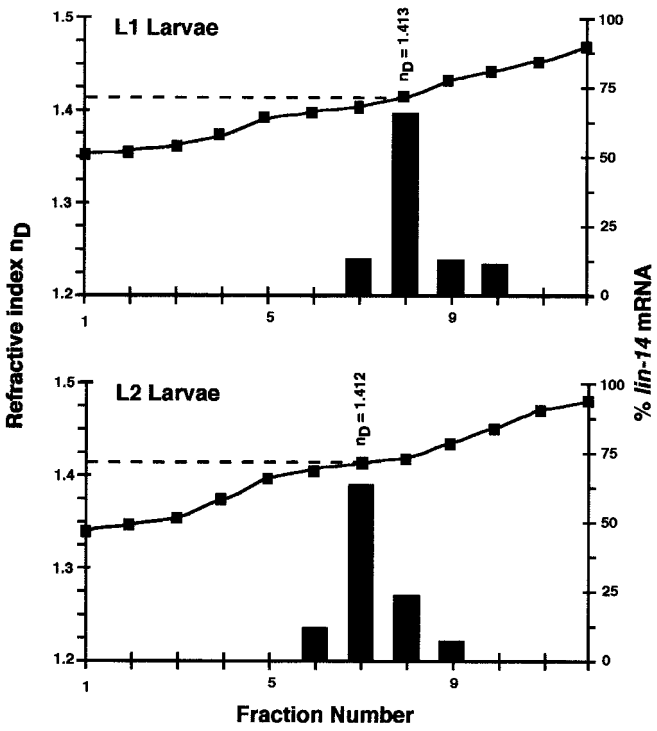

FIG. 7. Metrizamide gradient analysis (Dissous *et al.*, 1976) of polyribosomes purified from L1 and L2 larvae shows that the distributions of *lin-14* mRNA are identical, with the peak fractions in each larval stage sharing the same density. RNase protection was used to quantify the position of *lin-14* mRNA in the gradient. Curves marked by filled squares show the shape of the gradient.

Nevertheless, certain considerations stemming from our data are illuminating with regard to possible postinitiation mechanisms of *lin-4* action. First, reduction in the rate of *lin-14* mRNA elongation would be expected to cause a change in the size of *lin-14* polyribosomes. Since transcription of *lin-14* mRNA persists unabated after the L1 stage, a very strong block of *lin-14* translational elongation would be expected to eventually shift *lin-14* mRNA to smaller polyribosomes as new *lin-14* transcripts would accumulate a small number of translationally arrested 5′-proximal ribosomes. A moderate effect on *lin-14* elongation that resulted in elongation becoming limiting but not completely blocked would lead to an increase in the size of *lin-14* polyribosomes (Mathews *et al.*, 1996). Similarly, a specific inhibition of *lin-14* translational termination would be predicted to result in an increase in the number of ribosomes per *lin-14* mRNA. Thus, our observation that the *lin-14* mRNA polyribosome profile is developmentally constant implies that the mechanism of *lin-4* action does not involve simply an inhibition of elongation or termination. It is possible that the constant *lin-14* polyribosome profile reflects a fortuitous combination of inhibitory effects, such as reduction of both elongation and initiation, that happen to cancel out with respect to polyribosome size. Alternatively, *lin-4* RNA may leave *lin-14* mRNA translation unaffected, but somehow target the nascent LIN-14 protein for rapid degradation. Further experiments are required to distinguish among these various possible mechanisms.

The fact that *let-7* of *C. elegans* also encodes a small antisense RNA (F. Slack, B. Reinhart, and G. Ruvkun, personal communication), and the fact that *lin-4* regulates not only LIN-14 synthesis via the *lin-14* 3′ UTR, but also controls LIN-28 protein synthesis via the *lin-28* 3′ UTR (Moss *et al.*,

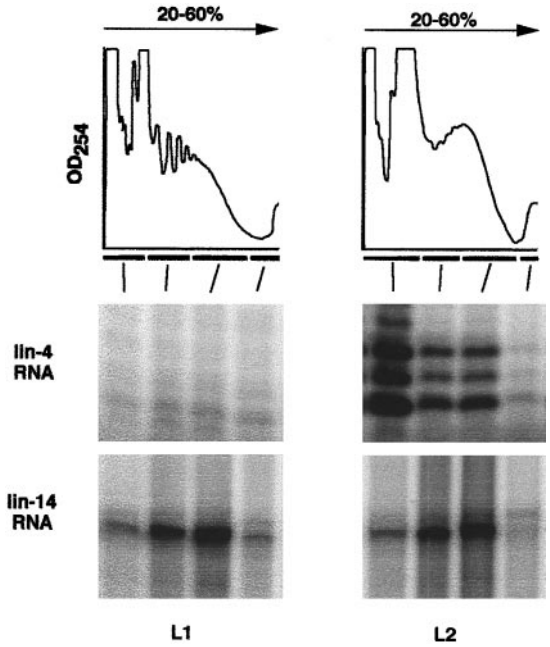

FIG. 8. *lin-4* S RNA cosediments with polyribosomes. Postmitochondrial supernatants from L1 larvae (left) and L2 larvae (right) were fractionated on sucrose gradients and fractions were analyzed for the presence of *lin-4* RNA and *lin-14* mRNA, shown in the middle and at the bottom, respectively. The 22-nt *lin-4* RNA protects from nuclease digestion probe fragments of 20, 21, and 22 nt, probably as a result of breathing of the *lin-4* RNA::probe hybrid during digestion (Lee *et al.*, 1993).

1997), suggests that translational control by small antisense RNAs could be more common than previously anticipated. It is not yet clear whether the mechanism of *lin-28* repression by *lin-4* is identical, in detail, to that of *lin-14* repression, but preliminary analysis indicates that polyribosome profiles of *lin-28* RNA are similar between early and late stages of development (K. Seggerson and E. Moss, personal communication). It remains to be determined how common are cases of translational control by small RNAs like *lin-4* and whether the mechanism of *lin-4* RNA action shares features with the mechanisms involving other RNA and/or protein translational repressors.

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