Developmental Biology **210,** 87–95 (1999)
Article ID dbio.1999.9272, available online at http://www.idealibrary.com on IDEAL®

The Timing of *lin-4* RNA Accumulation Controls the Timing of Postembryonic Developmental Events in *Caenorhabditis elegans*

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The *lin-4* gene encodes a small RNA that is required to translationally repress *lin-14* toward the end of the first larval stage of *Caenorhabditis elegans* development. To determine if the timing of LIN-14 protein down-regulation depends on the temporal profile of *lin-4* RNA level, we analyzed the stage-specificity of *lin-4* RNA expression during wild-type development and examined the phenotypes of transgenic worms that overexpress *lin-4* RNA during the first larval stage. We found that *lin-4* RNA first becomes detectable at approximately 12 h of wild-type larval development and rapidly accumulates to nearly maximum levels by 16 h. This profile of *lin-4* RNA accumulation corresponded to the timing of LIN-14 protein down-regulation. Transgenic strains that express elevated levels of *lin-4* RNA prior to 12 h of development display reduced levels of LIN-14 protein and precocious phenotypes consistent with abnormally early loss of *lin-14* activity. These results indicate that the temporal profile of *lin-4* RNA accumulation specifies the timing of LIN-14 down-regulation and thereby controls the timing of postembryonic developmental events. © 1999 Academic Press

Key Words: **heterochronic genes; *lin-4* RNA; LIN-14, developmental timing; *C. elegans*.**

INTRODUCTION

The development of a multicellular organism from a single-celled egg requires the temporal and spatial coordination of cell division, cell death, differentiation, and morphogenesis. The placing of the various parts of an animal in their correct positions, and the generation of those parts at the right times, reflects underlying temporal and spatial patterns of gene activity. Broadly speaking, the temporal and spatial control of gene expression during development derives from the temporal or spatial distribution of key regulatory molecules. For example, gradients of bicoid and nanos in the *Drosophila* egg specify the positions at which other regulatory genes, such as *hunchback*, are expressed and thereby govern the number and types of body segments (St. Johnston and Nusslein-Volhard, 1992). Similarly, the secreted proteins DPP and Wingless of *Drosophila* form graded extracellular signals that can elicit different target gene responses in cells at different distances from the source of the signal (Nellenn *et al.*, 1996; Zecca *et al.*, 1996). By analogy to spatial determination of cell fates, the temporal control of developmental events can be governed by temporal changes in the level of regulatory molecules; for example, stage-specific levels of hormonal signals specify the timing of larval developmental events in *Drosophila* (Richards, 1981), and in the nematode *Caenorhabditis elegans*, a temporal gradient of LIN-14 protein defines the timing and sequence of larval developmental events (Ruvkun and Guisto, 1989).

C. elegans genes that act to control temporal patterns of development have been identified by the isolation of mutants defective in the expression of stage-specific larval events (Ambros and Horvitz, 1984; reviewed by Ambros and Moss, 1994; Slack and Ruvkun, 1997). Mutations in these heterochronic genes disrupt the temporal sequence of development, causing particular developmental events to occur either earlier or later than normal (Ambros and Horvitz, 1984). A regulatory network of three heterochronic genes, *lin-4, lin-14,* and *lin-28,* plays a critical role in controlling the progression of events accompanying the first three larval stages (Ambros, 1989). LIN-14 activity is required for the expression of L1-specific cell fates; in *lin-14(0)* mutants, L1-specific developmental events are deleted from diverse cell lineages and instead L2-specific events occur precociously (Ambros and

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0012-1606/99 $30.00

Horvitz, 1984). Both *lin-14* and *lin-28* activity are required for the proper timing of L2 stage-specific developmental events. In a subset of *lin-14* loss-of-function (*lf*) mutants and in *lin-28(0)* mutants, L2-specific cell fates are skipped and L3 stage-specific events occur precociously. The functions of *lin-14* and *lin-28* are required for expression of the early programs of *C. elegans* larval development, and the progressive decrease in their activities, owing to repression by *lin-4*, is essential for the expression of later cell fates (Ambros and Horvitz, 1987; Moss *et al.*, 1997).

lin-4 encodes a 22-nt RNA repressor of *lin-14* and *lin-28* translation (Lee *et al.*, 1993). Although the precise mechanism of this translational repression is not understood, the presence in the 3′ untranslated regions of *lin-14* and *lin-28* mRNA of elements complementary to *lin-4* (Wightman *et al.*, 1993; Moss *et al.*, 1997) suggests that *lin-4* RNA base-pairs with its target mRNAs and the formation of that base-paired complex inhibits translation *in cis*. In *lin-4(0)* mutants, in which the repressor is absent, or in *lin-14* gain-of-function mutants, in which the *lin-14* mRNA is missing elements critical for repression of translation by *lin-4* (Wightman *et al.*, 1991, 1993), L1-specific developmental events are reiterated at later larval stages (Ambros and Horvitz, 1984). Likewise, when the *lin-4* binding site is removed from a *lin-28:GFP* transgene, a dominant retarded phenotype results due to the reiteration of L2-specific events (Moss *et al.*, 1997). In addition to being subject to translational repression by *lin-4* RNA, the levels of LIN-14 and LIN-28 are also coupled by mutual feedback circuits (Arasu *et al.*, 1991; Moss *et al.*, 1997).

A full understanding of the temporal signals and regulatory mechanisms governing the timing of developmental events in *C. elegans* requires a precise characterization of the role of *lin-4*. One key question is whether *lin-4* RNA acts as a master regulatory molecule that is expressed in a temporal profile that critically determines the temporal profile of *lin-14* and *lin-28* translation or whether *lin-4* is uniformly expressed and simply plays a permissive role in the down-regulation of its targets. Since LIN-14 and LIN-28 are both down-regulated by *lin-4*, either LIN-14 or LIN-28 can be monitored to assay *lin-4*-dependent translational repression *in vivo*. Here we monitor LIN-14 protein levels and LIN-14-dependent phenotypes and show that the temporal profile of *lin-4* RNA level defines the timing of LIN-14 down-regulation and thereby controls the timing of stage-specific events throughout postembryonic development.

MATERIALS AND METHODS

General C. elegans Methods

Methods for growth and culture of *C. elegans* were as described in Wood *et al.* (1988) unless otherwise noted. The wild-type strain used here was N2, *C. elegans* var Bristol.

Transgenic Worms

The 693-bp *lin-4*-rescuing fragment (Lee *et al.*, 1993) was amplified with PCR primers RFMGH25 and RFMGH46. The *lin-4* DNA PCR product (200 ng/μl) and approximately 10 ng/μl of cosmid DE9 [*daf-7*(+)] were injected into *daf-7(e1372ts)* animals raised at 15°C using standard microinjection procedures (Mello *et al.*, 1991). Transgenic animals rescued for *daf-7* defects bypassed the growth-arrested dauer stage at 25°C. *daf-7* was used as a co-injection marker because it provides a growth selection for transgenic animals at 25°C, facilitating the recovery of transgenic animals that are otherwise severely abnormal owing to heterochronic phenotypes. Injected animals were placed at 25°C. Eight of fourteen transgenic lines obtained exhibited gross phenotypes similar to *lin-14(lf)* mutants, including egg-laying defects; a short, kinky tail; precocious adult lateral alae; and abnormal vulval morphogenesis. One transgenic extrachromosomal array that conferred a high penetrance of these precocious phenotypes was integrated by gamma-ray irradiation (Epstein and Shakes, 1995). The two resulting independent integrated transgenic lines were VT731 *daf-7(e1372ts); maIs101[daf-7$^+$;lin-4$^+$]* and VT780 *daf-7(e1372ts); maIs100[daf-7$^+$;lin-4$^+$]*. VT779*maIs100[daf-7$^+$;lin-4$^+$]; maIs105[pVT301 col-19::gfp]* was derived from VT780 by crossing VT780 hermaphrodites with *maIS105* males and recovering F2 animals that were homozygous for both transgenes (as evidenced by their developmental and fluorescence phenotypes).

Oligonucleotides

The oligonucleotides used in this study were RFMGH10, 5′ GTACCCGGAGAGCCCAGGTGTGAAGCATCAATAGTACACTCACACTTGAGGTCTCAGGGAACCTATAGTGAGTCGTATTA 3′; RFMGH25, 5′ GTCGACGAGACGCCGAGTCT 3′; and RFMGH46, 5′ CGACTTCTGAAAATAATCGT 3′.

Phenotypic Analysis

Animals at defined developmental stages were examined using Nomarski differential interference contrast microscopy and were scored, using previously described techniques, for precocious L2-specific cell lineage patterns (Ambros and Horvitz, 1984), precocious dauer larva formation (Liu and Ambros, 1989), precocious vulva development (Euling and Ambros, 1996), precocious adult-specific cuticle formation (Ambros, 1989), and precocious expression of *col-19::gfp* (Liu *et al.*, 1995; Abrahante and Rougvie, 1998).

Northern Blot Analysis

Animals were synchronized by hatching for 16 h in S medium (Wood *et al.*, 1988) in the absence of food. Subsequently, animals were fed and grown on NGM agarose plates for specified times. Animals were washed off the plates in S Basal, collected by centrifugation, and washed four or five times until all visible traces of bacteria were removed. Samples were divided and prepared for RNA analysis and antibody staining.

RNA was prepared by standard one-step guanidinium method (Ausubel *et al.*, 1994). Total RNA, 30 μg per lane, was resuspended in RNA formamide loading buffer (80% formamide, 1 mM EDTA, 0.1% bromphenol blue, 0.1% xylene cyanol), denatured at 80°C for 3 min, and then loaded on a (0.75 mm × 16 cm × 16 cm) 11% urea-acrylamide gel (Sequagel). The RNA was separated by electro-

phoresis for 3 h at 250 V in 1× TBE. RNA was electrophoretically transferred to Zetaprobe membrane in 0.5× TBE at 200 mA overnight at 4°C. RNA was crosslinked to the membrane by UV irradiation (1200 μJ; Stratagene UV Stratalinker) and the membrane was subsequently baked at 80°C for 30 min. The probe for the Northern blots was the antisense *lin-4* oligonucleotide (RFMGH10) 5′-end labeled with T4 polynucleotide kinase and [γ-^{32}P]ATP under standard conditions. Hybridization and washing conditions with the antisense *lin-4* oligonucleotide probe were according to ZetaProbe manufacturer specifications for hybridization with oligonucleotide probes. After hybridization and washing, the membrane was exposed to XAR X-ray film overnight with an intensifying screen. Northern blots were stripped and reprobed with an anti-U6 oligonucleotide probe as previously described (Lee *et al.*, 1993). Subsequent to autoradiography, the amount of *lin-4* RNA or U6 RNA was quantified using a PhosphorImager (Molecular Dynamics).

Immunohistochemistry

Worms (30–40 μl) were fixed for immunostaining as described for whole-mount fixation of larvae and adults (Ruvkun and Finney method in Epstein and Shakes, 1995) with the following modifications: After permeabilization by freezing and thawing, worms were incubated for 30 min on ice with gentle agitation, washed twice in TTB, and then incubated in TTB, 1% β-mercaptoethanol for 2 h at 37°C with gentle shaking. BO_3 Buffer (1×) was modified slightly, the final concentration being 0.03 M H_3BO_3 and 0.017 M NaOH. After the reduction and oxidization steps, the worms were washed in Antibody Buffer A for 2 h at room temperature.

Immunostaining was modified from the Ruvkun and Finney method (Epstein and Shakes). Worms were doubly immunostained with anti-LIN-14 antiserum (IIIc fraction 1) and MH27. One hundred microliters of anti-LIN-14 antiserum (diluted 1:77) and MH27 (diluted 1:1400) in Antibody Buffer A was added to 10 μl of fixed worms and incubated overnight at 4°C. After incubation with the primary antibodies, worms were washed four times in quick succession with 1.0 ml of Antibody Buffer B and then incubated with 100 μl of secondary antibodies in Antibody Buffer A overnight at 4°C. Secondary antibodies were as follows: FITC goat anti-rabbit (anti-LIN-14) and rhodamine goat anti-mouse (MH27). Secondary antibodies were preadsorbed at a dilution of 1:100 in Antibody Buffer A against 30 μl of fixed N2 worms overnight at 4°C. Stained worms were stored at 4°C in the dark and then mounted using 0.5% *n*-propylgallate, 70% glycerol, 40 mM Tris, pH 9.0, 1 μg/ml DAPI (modified from Epstein and Shakes, 1995).

Photomicrography

Animals were photographed using color slide film, or images were captured directly from the microscope using an Optronics DEI750 three-chip color camera and Scion LG7 video capture board and a Macintosh 8500 AV computer. Color slides were scanned to produce a digital image. Images were processed using Adobe PhotoShop. Sets of images that are compared (Figs. 2A and 2B) were grouped together as raw images and were then processed identically.

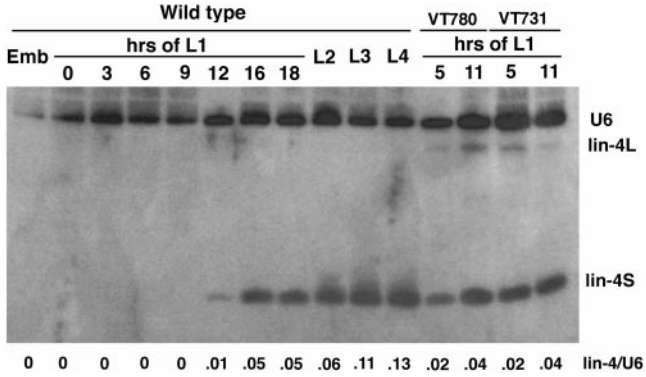

FIG. 1. Developmental Northern blot of *lin-4* RNA levels in larvae. Larvae were synchronized by starvation at hatching (Shakes and Epstein, 1995), fed with *Escherichia coli*, and allowed to develop for defined periods of time at 20°C. Populations of wild-type worms or transgenic animals overexpressing *lin-4* RNA (VT780 or VT731) were harvested at defined stages of development and RNA was prepared and analyzed by Northern blotting with *lin-4* or U6 probe, as described under Materials and Methods. RNA samples were from embryos (Emb), L1 larvae of various times after feeding of starvation-synchronized newly hatched larvae (hrs of L1), and L2, L3, or L4 larvae. Development was monitored through the L1 stage by Nomarski microscopy and by immunofluorescence staining with MH27 antibody. Developmental hallmarks in the L1 were V cells dividing at 6 h, P cells dividing at 12 h, and completion of the molt at 18 h. The filter was hybridized with a probe that detects *lin-4* RNAs, exposed to X-ray film, and analyzed with a PhosphorImager to quantify *lin-4*S signals. A fainter *lin-4*L signal is also visible in some samples, but was not quantified. The filter was then reprobed with U6 probe, exposed to a second X-ray film, and analyzed with a PhosphorImager to quantify the level of U6 signal, which serves as a gel-loading standard. The two X-ray film images were scanned to create PhotoShop images and were combined digitally. (Some scratches were removed using PhotoShop touchup utilities.) The amount of *lin-4* RNA in each sample is measured as ratio of *lin-4*S to U6 (lin-4/U6).

RESULTS

Expression of lin-4 RNA Is Temporally Regulated

To determine whether *lin-4* RNA levels are temporally regulated, we examined the expression of *lin-4* RNA during development by Northern blot analysis. *lin-4* encodes two transcripts, the relatively abundant 22-nt *lin-4*S transcript and the 61-nt minor transcript, *lin-4*L (Lee *et al.*, 1993). *lin-4*S RNA was not detected in embryos, or early L1 larvae, and was first detected during the latter half of the L1 larval stage, 12 h after larval development began (Fig. 1). *lin-4*S levels continued to increase through the L1 and during subsequent stages of development (*lin-4*S RNA is also present in high levels in adult worms; data not shown). *lin-4*L is expressed at very low levels and was difficult to detect by these Northern blots. The temporal profile of *lin-4*S RNA in the wild type parallels the timing of LIN-14

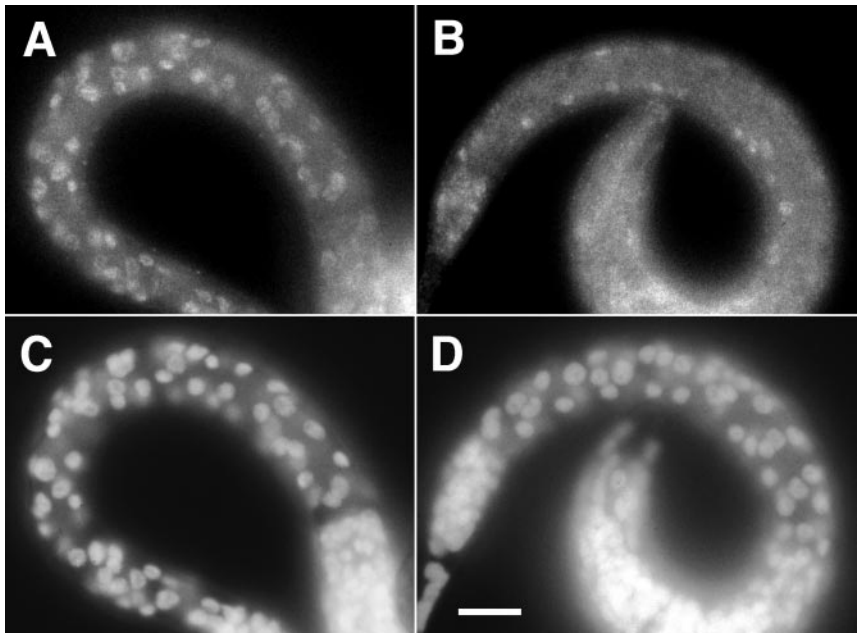

FIG. 2. Immunohistochemistry of LIN-14 protein. Animals were synchronized by starvation at hatching, fed with *E. coli*, allowed to develop for 5.5 h at 20°C, and fixed and stained with fluorescent LIN-14 antisera and DAPI as described under Materials and Methods. (A and B) FITC anti-Lin-14; (C and D) DAPI. Wild-type 5.5-h larvae (A) display uniform background fluorescence and brighter fluorescence concentrated in nuclei of neurons and hypodermal cells. VT780 5.5-h larvae (B) display essentially only background staining, with some weak fluorescence detected in certain nuclei. In A and B, the plane of focus is the same and includes the lateral hypodermal nuclei (compare DAPI fluorescence of C and D). Original negatives were scanned and processed identically using Adobe PhotoShop. Bar, 5 μm.

down-regulation, which begins in the L1 stage and operates throughout larval development (Ruvkun and Guisto, 1989).

Premature Expression of lin-4 RNA Causes a Premature Decrease in LIN-14 Protein

We generated transgenic lines that overexpress *lin-4* RNA by microinjection of the 693-bp *lin-4* rescuing region at high DNA concentration (200 ng/μl). The expression of *lin-4* RNA was examined by Northern blot analysis for two integrated lines, VT780 and VT731, that were derived from these high-copy injections. VT780 and VT731 express *lin-4*S and *lin-4*L early in the L1 at a time when no *lin-4* RNA is detectable in wild-type animals. The level of *lin-4*S in the transgenic animals at 5.5 h postfeeding was greater than that in N2 animals at 12 h postfeeding (Fig. 1). The level of *lin-4* RNA continues to increase in the transgenic animals during the L1. By 11 h postfeeding, a time when *lin-4*S is barely detectable in N2, the *lin-4*S levels in VT780 and VT731 are similar to those found in N2 animals at 16 h postfeeding (Fig. 1). In both transgenic overexpressing lines at 5.5 and 11 h postfeeding, the level of *lin4*L was greater than that seen in wild type at any stage.

To determine whether precocious accumulation of high levels of *lin-4* RNA could result in premature down-regulation of LIN-14 protein, we examined LIN-14 protein levels by antibody staining in the *lin-4*-overexpressing lines and compared them to N2. At hatching, animals of the *lin-4*-overexpressing lines (strains VT780 and VT731) express LIN-14 protein at approximately normal levels in the nuclei of blast cells and neurons (data not shown). However, early in the L1 (at 5 h of development), the number and intensity of anti-LIN-14 fluorescent nuclei were greatly reduced in VT780 or VT731 animals compared to similarly staged wild-type animals (Fig. 2). Some nuclei still had detectable levels of expression at this stage (Fig. 2B), suggesting that LIN-14 expression in some cells may be less sensitive to *lin-4* overexpression than in other cells, but we did not note any consistent anatomical pattern of differential sensitivity to *lin-4*. It is notable that this precocious reduction in LIN-14 protein levels in hypodermal nuclei occurs at a time when *lin-14* protein is required for L1 stage-specific hypodermal developmental events. By 11 h of development, when LIN-14 protein is still easily detectable in wild-type hypodermal nuclei, there was no detectable LIN-14 staining in hypodermal cells of VT780 and VT731 animals (data not shown).

Precocious Expression of lin-4 RNA Results in Precocious Phenotypes

Overexpression of *lin-4* RNA early in development results in reduced levels of LIN-14 protein, which suggested that animals would exhibit precocious phenotypes like

TABLE 1

Precocious Phenotypes of *lin-4*-overexpressing Animals[a]

Developmental stage	% precocious phenotype[b]	No. of cells	No. of animals
L1	15	84	13
L2 molt	83	72	12
Dauer larva	84	na	239
L3 molt	96	230	24

[a] VT779 *maIs100; maIs105* and VT780 *maIs100; daf-7(e1372ts)* animals were used interchangeably in these experiments, with no apparent difference in phenotype.

[b] Phenotypes scored depend on developmental stage: L1, % V cell lineages that expressed the normally L2-specific cell division patterns; L2 molt, % animals that expressed the normally L3-specific vulva precursor cell divisions; dauer larva, % total dauer larvae that were arrested at the end of the L1 stage (instead of the end of the L2 stage, as in the wild type); L3 molt, % seam cells that expressed adult-specific lateral alae. For the most part, different sets of animals were scored for different phenotypes.

those of *lin-14(lf)* mutants. VT780 and VT731 animals exhibited gross phenotypes, including egg-laying defects, a slight Dumpy shape, a deformed tail, and a malformed vulva, all of which are characteristic of *lin-14(lf)* mutants. Furthermore, examination of the timing of several stage-specific cell fates in the *lin-4*-overexpressing lines confirmed that they exhibited precocious cell lineage defects like those of *lin-14(lf)* mutants.

The precocious defects of *lin-4*-overexpressing lines included precocious expression of L2-specific cell division patterns in the L1, a phenotype like that of *lin-14(lf)* mutants. In the wild type, young L1 animals contain 6 hypodermal stem cells (V1–V6) positioned along the lateral line or "seam" on each side of the animal (Sulston and Horvitz, 1977). These 6 stem cells ("seam cells") each divide once in the L1, generating a new seam cell and a nondividing hypodermal cell. Thus, at the end of the wild-type L1, the number of seam cells from the V1–V6 cell lineages on each side of the larva remains at 6. Five of these 6 seam cells then generate, in the L2, 2 seam cell progeny each, increasing the complement of V lineage seam cells to 11 per side. In *lin-14(lf)* mutants, the lateral hypodermal seam cells do not exhibit their normal LI behavior, but instead precociously express in the L1 stage the duplicative division that is normally specific to the L2 (Ambros and Horvitz, 1984). This precocious cell lineage defect results in the production of more than (as much as a doubling of) the normal complement of L1 seam cells. Although not 100% penetrant, this precocious L2 hypodermal lineage defect was clearly in evidence in *lin-4*-overexpressing lines; 15% of the 84 hypodermal V cell lineages examined exhibited L2-specific lineages in the L1 (Table 1), as determined by the presence of extra seam cells in late L1 animals (Fig. 3).

Overexpression of *lin-4* RNA affects the timing of dauer larval formation. The dauer larva is a developmentally arrested and morphologically distinct alternative L3 adapted to prolonged survival under adverse conditions (Cassada and Russell, 1975). Under conditions of overcrowding, starvation, and elevated temperature, *C. elegans* larvae will arrest development and differentiate into the dauer larva form at the L2 molt. The capacity to express the dauer larvae program is restricted to the L2 molt in the wild type. *lin-14* is required for this tight temporal regulation of dauer formation, and *lin-14(lf)* mutants can become dauer larvae one stage early, at the L1 molt (Liu and Ambros, 1989). Like *lin-14(lf)* mutants, *lin-4*-overexpressing animals execute dauer larva formation at the L1 molt (Table 1).

Transgenic lines that overexpress *lin-4* also precociously express certain L3-specific cell lineage patterns in the L2. In wild-type animals, the vulva is formed from the descendants of 3 vulval precursor cells (VPCs) P5.p, P6.p, and P7.p (Sulston and Horvitz, 1977). The VPCs are born in the L1 and do not begin to divide until the mid L3, and they execute two rounds of division during the late L3. Subsequently, 10 of the 12 progeny generated from P5.p, P6.p, and P7.p divide again at or slightly after the L3 molt to generate the 22 cells that form the vulva. In *lin-14(lf)* and *lin-28(lf)* mutants, the VPCs begin their division during mid-L2, about 5–6 h earlier than in wild type, and all of the vulval cells are precociously generated by the early L3 (Ambros and Horvitz, 1984; Euling and Ambros, 1996). When we examined *lin-4*-overexpressing animals at the L2 molt, we found that 83% of the VPCs examined had already divided (Table 1). In these animals the VPCs divide an estimated 6–8 h earlier than normal, and typically two rounds of division were completed by the L2 molt (Fig. 4).

lin-4-overexpressing animals exhibited precocious expression of the larval-to-adult (L/A) switch in the lateral hypodermal seam cells, producing adult cuticle structures one stage early (Fig. 5). In wild-type animals, the lateral seam cells divide at each of the first three molts. The seam cells cease dividing in the L4 and they produce adult cuticle at the L4 molt. The production of adult cuticle can be distinguished from larval cuticle by the presence of cuticular ridges, called alae, that run along the seam of the animal and by the expression of adult-specific collagen genes such as *col-19* (Singe and Sulston, 1978; Liu and Ambros, 1995). In *lin-14(lf)* mutants, the L/A switch occurs one stage too early and animals produce adult cuticle at the L3 molt (Ambros and Horvitz, 1984; Ambros, 1989; Liu *et al.*, 1985). *lin-28(lf)* mutants also form adult cuticle at the L3 molt but, unlike *lin-14(lf)* mutants, some seam cells in *lin-28* animals produce adult cuticle two stages early, at the L2 molt (Ambros and Horvitz, 1984). To determine whether *lin-4*-overexpressing animals similarly executed precocious adult lateral hypodermal programs, VT780 and VT731 animals in the L3 molt were collected and examined for the presence of adult lateral alae and for the expression of the adult-specific reporter gene *col-19::GFP* (Liu *et al.*, 1985; Abrahante and Rougvie, 1998). Adult lateral alae, characterized by three cuticular ridges, was present on over 96%

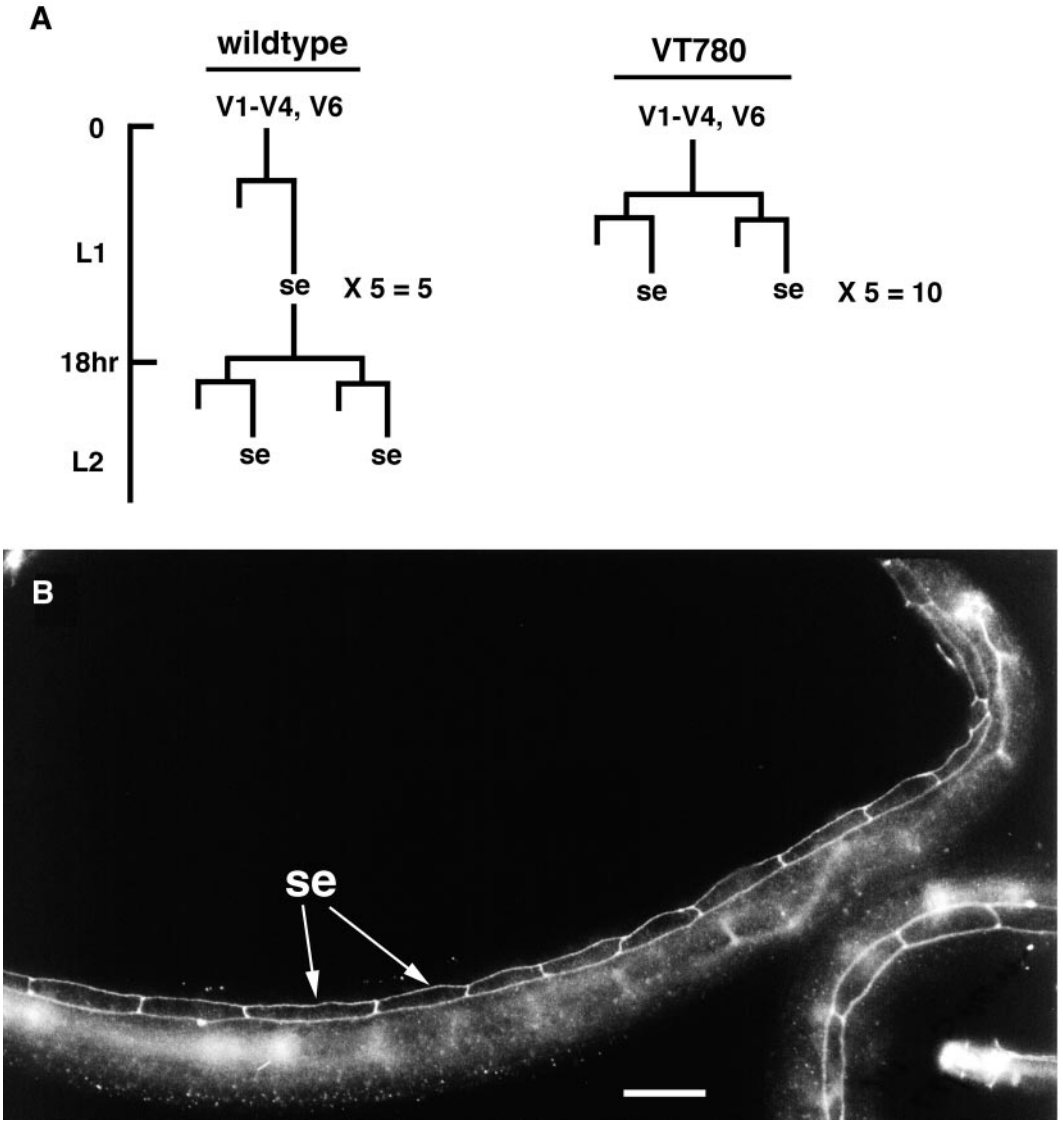

FIG. 3. Extra seam cells of VT780 animals. Animals with reduced LIN-14 owing to elevated levels of *lin-4* RNA would be expected to display a *lin-14(lf)* phenotype in the L1 stage. This cell lineage defect (A) involves the deletion of an L1-specific single cell division in the lateral hypodermis and the substitution of a normally L2-specific double cell division. The result of this defect is extra seam cells (se) between the head and the tail by the late L1 stage—as many as 5 extra seam cells from the V1–V4 and V6 lineages. (B) An 11.5-h VT780 larva stained with rhodamine-labeled MH27 monoclonal antibody, which detects cellular junctions and outlines seam cells. This phenotype is highly variable, and the animal shown here represents the extreme, with an estimated 5 extra seam cells easily discernible. Staining with MH27 antibody reveals seam cells derived from the H and T lineages as well as the V lineages and therefore the number of seam cells in the pictured animal is greater than the 11 predicted to be produced by the precocious division of the V cells. The original negative was scanned and processed using Adobe PhotoShop. Bar, 5 μm.

of the seam cells in these animals at the L3 molt (Table 1; Fig. 5), and seam cells efficiently expressed *col-19::GFP* at this stage (Fig. 5). We did not observe evidence of adult lateral alae or *col-19::GFP* expression earlier than the L3 molt in *lin-4*-overexpressing animals (of more than 20 animals examined at earlier stages).

DISCUSSION

To determine whether *lin-4* RNA plays a critical regulatory role in the timing of *C. elegans* larval developmental events, we examined the temporal profile of *lin-4* RNA accumulation during wild-type development and analyzed

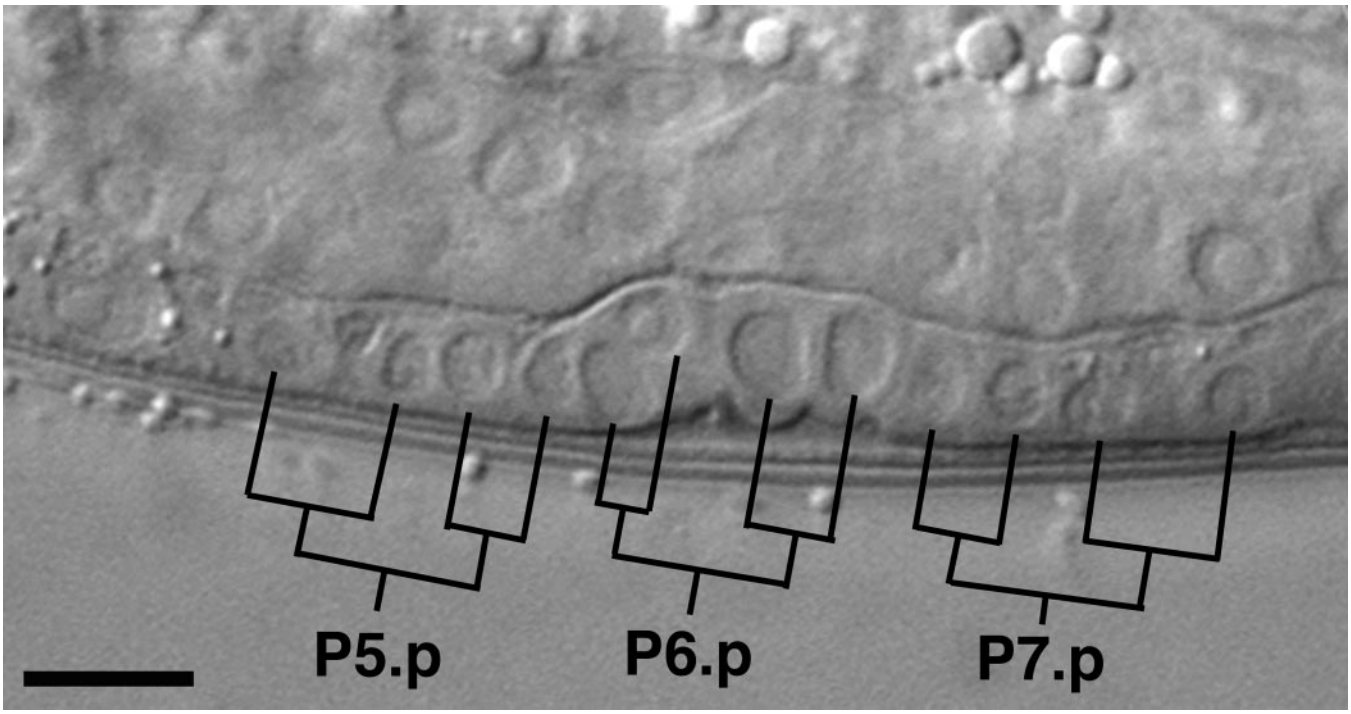

FIG. 4. DIC image of precocious vulval development in a VT779 hermaphrodite at the L2 molt. Vulval precursor cells P5.p, P6.p, and P7.p have divided through two rounds at the L2 molt, approximately 6–8 h earlier than normal. At this stage, P5.p, P6.p, and P7.p would normally be undivided. Bar, 3 μm.

the effects of expressing *lin-4* RNA earlier than normal. The results of these experiments suggest that the timing of *lin-4*S RNA accumulation defines the timing of LIN-14 down-regulation during the L1 stage and thus specifies the timing and sequence of larval developmental programs. This conclusion is derived from two findings. First, we found that the onset of *lin-4* RNA accumulation in the wild-type L1 stage corresponds to the time that the level of LIN-14 protein normally begins to decrease, as determined previously by immunohistochemistry and Western blotting of staged larvae (Ruvkun and Giusto, 1989). Second, we observed that overexpression of *lin-4* RNA in the L1 stage results in precocious down-regulation of LIN-14 protein and precocious expression of larval phenotypes.

The *lin-4* gene encodes two small RNAs; the 22-nt *lin-4*S is the major transcript, and the 61-nt *lin-4*L is a very minor transcript (Lee *et al.*, 1993). How these two molecules are generated, whether *lin-4*S is processed from *lin-4*L or *lin-4*L represents readthrough of *lin-4*S termination signals, is unclear. If *lin-4*L is the precursor of *lin-4*S, then the relatively high levels of *lin-4*L observed in the overexpressing lines may be due to a bottleneck in processing the overexpressed *lin-4*L early in the L1 or might represent a population of molecules incapable of being processed. For various reasons, including the relative abundance of *lin-4*S, and the fact that *lin-4*L is predicted to have secondary structure that would impede its base-pairing with target mRNAs, *lin-4*S is considered to be the *lin-4* gene product that acts as a translational repressor (Lee *et al.*, 1993). *lin-4*S is the major *lin-4* transcript in the overexpressing lines as well, suggesting that *lin-4*S is responsible for the premature down-regulation of LIN-14 and the precocious phenotypes exhibited by the overexpressing lines.

Most of the precocious phenotypes in *lin-4*-overexpressing lines were strong, consistent with a potent premature repression of LIN-14 in these animals. However, the L1 defects (precocious expression of L2 cell lineage patterns in the L1 stage) were relatively weak. Perhaps *lin-4* RNA is not expressed at sufficiently high levels for full repression of LIN-14 synthesis until later in the L1 in these transgenic animals. Another possible explanation for the relatively weak L1 defects in these lines could be that *lin-14* mRNA for some reason becomes more sensitive to repression by *lin-4*S as L1 development proceeds. It is also noteworthy that we did not observe the strong precocious adult cuticle defect associated with mutations in another *lin-4* target gene, *lin-28*. Specifically, *lin-28(lf)* animals frequently display adult cuticle formation at the L2 molt, one stage earlier than in *lin-14(lf)* animals (and two stages earlier than in the wild type), but in *lin-4*-overexpressing lines, adult cuticle formation was not observed earlier than the L3 molt. We did not monitor LIN-28 levels directly, and so it is possible that LIN-28 is less sensitive than is LIN-14 to repression by *lin-4* in the early L1 stage.

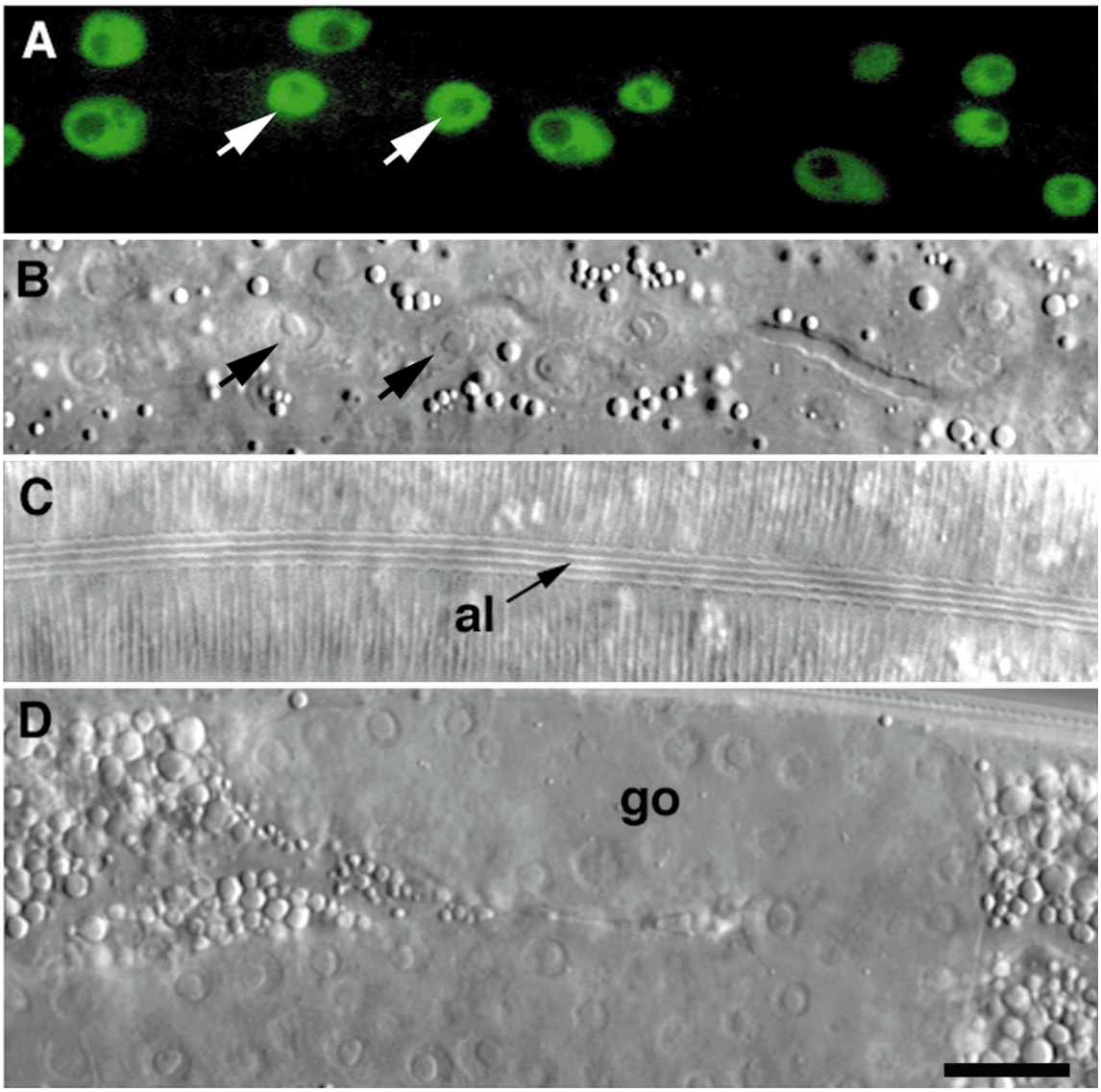

FIG. 5. Precocious expression of adult-specific hypodermal characteristics in the L4 in a strain overexpressing *lin-4*. A VT779 animal at the L3 molt was immobilized with levamisole and examined by fluorescence (A) and DIC (B–D) microscopy. A–D are of the same animal. A and B are fluorescent and DIC images, respectively, in the same plane of focus. C and D are DIC images of the same animal as in A and B, but showing different planes of focus. The strain contains *maIs105[col-19::gfp]*, which normally expresses GFP from the adult-specific *col-19* promoter beginning at the L4 molt. This strain expresses *col-19::gfp* (A) precociously at the L3 molt in hypodermal nuclei (two of which are indicated by arrows in A and B). C shows precocious adult-specific lateral alae (al). D shows one gonadal arm at a state of reflex typical of the L3 molt. Images were captured and processed digitally as described under Materials and Methods. Bar, 5 μm.

Does *lin-4* RNA level function as a gradient or a switch? The fact that precocious elevation of *lin-4* RNA in the L1 stage causes a premature reduction in LIN-14 protein and a precocious phenotype indicates that the amount of *lin-4* RNA in the early L1 affects the temporal fates of cells, probably via its effects on LIN-14 level. However, these results do not necessarily mean that the precise developmental profile of *lin-4* RNA abundance in the wild type directly dictates the developmental profile of LIN-14 protein. There may be time-dependent factors other than *lin-4* (for example, regulators of LIN-14 protein stability) that also govern the kinetics of LIN-14 down-regulation. For example, although *lin-4* RNA is expressed continuously after the L1 stage, we do not know if *lin-4* RNA is required to maintain repression of LIN-14 protein synthesis at the later larval stages. So it is possible that *lin-4* is required only to trigger the onset of *lin-14* translational repression, and other factors suffice to complete and maintain the down-

regulation of LIN-14. According to this latter view, overexpression of *lin-4* RNA would bring about precocious development simply by advancing the time that LIN-14 begins to decrease in quantity. Further work is required to determine whether *lin-4* RNA is continuously required after the L1 for repression of LIN-14 translation.

We do not know what developmental signals temporally regulate *lin-4* RNA levels. Presumably a chain of regulatory events originating with a signal that requires feeding leads to the accumulation of *lin-4* RNA in the mid-to-late L1. In principle, the level of *lin-4* RNA could be developmentally regulated by transcriptional and/or posttranscriptional mechanisms. The overexpression of *lin-4* RNA in the *lin-4* transgenic lines employed here could result from the summation of basal transcription from multiple transgenes or from titration of negative regulatory factors that repress *lin-4* RNA accumulation. A full understanding of how *lin-4* is regulated during wild-type development will require the characterization of the relative roles of transcriptional and posttranscriptional mechanisms in the control of *lin-4* RNA levels, the identification of the RNA polymerase that transcribes *lin-4,* the identification of upstream temporal regulatory genes and their products, and the characterization of *cis*-regulatory elements of the *lin-4* gene that mediate the action of those regulators. method in Epstein and Shakes, 1995) with the following modifications: After permeabilization by freezing and thawing, worms were incubated for 30 min on ice with gentle agitation, washed twice in TTB, and then incubated in TTB, 1% β-mercaptoethanol for 2 h at 37°C with gentle shaking. BO_3 Buffer (1×) was modified slightly,

ACKNOWLEDGMENTS

We thank Gary Ruvkun for providing research space for R.F. and we thank the Massachusetts General Hospital Department of Genetics and Hoechst Aktiengesellschaft for financial support for R.F. We also thank members of the Ambros and Ruvkun labs for helpful discussions and advice. This work was supported by PHS Grant GM34028 to V.A. and by postdoctoral fellowships to R.F. from the Daymon Runyon-Walter Winchell Foundation and the United States Public Health Service (GM15396).

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Received for publication January 21, 1999
Accepted February 9, 1999