Negative regulatory sequences in the *lin-14* 3'-untranslated region are necessary to generate a temporal switch during *Caenorhabditis elegans* development

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The heterochronic gene *lin-14* controls the temporal sequence of developmental events in the *Caenorhabditis elegans* postembryonic cell lineage. It encodes a nuclear protein that normally is present in most somatic cells of late embryos and L1 larvae but is absent at later stages. Two *lin-14* gain-of-function mutations delete 3'-untranslated sequences causing an inappropriately high level of the *lin-14* nuclear protein late in development. These mutations identify a negative regulatory element that controls the formation of the *lin-14* protein temporal gradient. The 21-kb *lin-14* gene is differentially spliced to generate three *lin-14* transcripts that encode protein products with variable amino-terminal regions and a constant carboxy-terminal region. The sequence of the gene revealed no protein sequence similarity to any proteins in various data bases.

[Key Words: *C. elegans*; *lin-14*; heterochronic gene; gain-of-function mutation; 3' UTR]

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Regulatory genes have been shown to control the spatial and temporal configuration of the many cell types generated during development in *Caenorhabditis elegans*, *Drosophila*, *Xenopus*, and presumably all metazoans. Many of these genes are expressed in striking spatial or temporal patterns that are consistent with the developmental defects caused by mutations in the corresponding genes (Costa et al. 1988; Ingham 1988; Ruiz i Altaba and Melton 1989; Ruvkun and Giusto 1989). A major step in the dissection of the molecular basis of pattern formation is to discern how these genes become activated in spatial and temporal patterns.

Temporal pattern formation genes have been identified by heterochronic mutations in *C. elegans*, which perturb the time at which many cell lineages and cell types are generated during postembryonic development (Ambros and Horvitz 1984, 1987; Ambros 1989). The *lin-14* heterochronic gene plays a central role in this temporal regulation of the cell lineage. Loss-of-function (*lf*) *lin-14* alleles cause the precocious execution of cell lineages normally observed in descendent cells one or two larval stages later. Gain-of-function (*gf*) *lin-14* alleles affect the same cell lineages but cause the opposite trans-

formations in cell fate: Early cell lineages are normal, but later cells reiterate the early cell lineages normally associated with their ancestor cells. For example, in the development of the lateral hypodermis of *lin-14(lf)* mutants, the blast cell T skips its characteristic larval stage 1 (L1) sublineage and, instead, expresses a sublineage normally associated with its granddaughter blast cell T.ap. In *lin-14(gf)* mutants, the blast cell T expresses its normal L1-specific sublineage, but during L2 its granddaughter T.ap reiterates this L1-specific sublineage normally associated with blast cell T (Ambros and Horvitz 1984). The *lin-14(gf)* mutations have been shown by genetic criteria to cause more *lin-14* gene activity than normal, whereas the *lin-14(lf)* mutations are due to loss of *lin-14* gene activity (Ambros and Horvitz 1987). These data suggest that during normal development a relatively high *lin-14* gene activity during early larval stages, for example, in cell T, is reduced later in development, for example, in cell T.ap, to form a temporal developmental switch.

The product of the *lin-14* gene is a nuclear protein, the abundance of which forms a steep temporal gradient during *C. elegans* development: It is present at high levels in most but not all nuclei of embryos and L1 animals but is undetectable at later stages (Ruvkun and Giusto 1989). For example, in the case of the T lineage, during normal development the *lin-14* protein is present at high levels

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in the T-cell nucleus but is not observable in the T.ap-cell nucleus. In *lin-14(gf)* mutants, this temporal gradient is disrupted: The *lin-14* protein is observed at all stages of development (Ruvkun and Giusto 1989), for example, in both cells T and T.ap (as well as T.apap, etc.), and these cells reiterate L1-specific cell lineages. In some *lin-14* null mutants, no *lin-14* protein is detectable, and cells express L2-specific lineages during the L1 stage (Ambros and Horvitz 1987; Ruvkun and Giusto 1989). Thus, the normally sharp decrease in *lin-14* protein levels during the L1 stage causes cells to switch from L1-specific cell lineages to L2-specific cell lineages; and in *lin-14(gf)* mutants, the inappropriate presence of the *lin-14* protein at L2 and later stages prevents this temporal switch in cell fate.

Here we report that the normal down-regulation in *lin-14* protein level during *C. elegans* development depends on sequences in the 3'-untranslated region (UTR) of the *lin-14* mRNA. *lin-14(gf)* mutation disrupt this negative regulatory element, thereby disabling the down-regulation of *lin-14* protein levels. We also report that the *lin-14* gene encodes multiple proteins with no sequence similarity to any known genes or proteins.

Results

lin-14 gene structure and DNA sequence

To determine the wild-type *lin-14* DNA sequence and changes in selected *lin-14* mutants, we isolated cDNA clones from mixed stage and embryonic wild-type (N2) *C. elegans* and from a mutant strain [MT1842 = *lin-14(n536n838)*], which contains a *lin-14(gf)* mutation (*n536*) and a *cis*-acting *lin-14(lf)* mutation (*n838*). In addition to the cDNAs, the corresponding genomic regions from wild type were sequenced to determine the locations of introns and exons (Figs. 1 and 2). The sequences of these cDNAs and the corresponding genomic regions

revealed that the *lin-14* gene contains 13 exons with intron sizes varying from 44 bases to 12 kb. Differential splicing of exons 1–3 or exon 4 to the common exons 5–13 was observed in the cDNAs (Figs. 1 and 2). Exon 4 is located within the 12-kb intron (between exons 3 and 5) of the other transcripts and is separated by a 101-bp intron from exon 5. Polymerase chain reaction (PCR) with primers specific for exons 1 and 5 revealed a third transcript, which contains exons 1 and 3 spliced to exon 5 but lacks exon 2 (data not shown). The presence of all three transcripts was verified by PCR and RNase protection by using primers and probes from exons 1–5 (see Materials and methods). We designate the exon 4-containing transcript *lin-14A*, the exon 1,2,3-containing transcript *lin-14B1*, and the exon 1- and 3-containing transcript *lin-14B2*. The total lengths of the *lin-14B1*, *lin-14B2*, and *lin-14A* transcripts were inferred from cDNAs and genomic DNA sequence to be 3272, 3197, and 3338 bases, respectively, without the poly(A) tail, all of which are similar to the 3.5-kb *lin-14* mRNA species detected with probes specific to either transcript (Ruvkun et al. 1989). PCR with primers specific for exons 1, 4, and 5 showed that no *lin-14* transcripts containing exons 1 and 4 are produced, whereas, as also shown by the existing cDNA clones, *lin-14* transcripts containing exons 1 and 5 and exons 4 and 5 are produced (data not shown). In addition, no consensus splice acceptor sequences are located within 140 bases of the 5' end of exon 4. Although the 5' ends of these transcripts have not been determined, these data suggest that the *lin-14A* transcript and the *lin-14B1/B2* transcripts may have distinct 5' ends and may use distinct promoters.

lin-14 proteins

The longest open reading frame in the *lin-14B1* transcript starts with an ATG in exon 2 at position 3193 of

Figure 1. A molecular map of the *lin-14* region, showing the organization of the gene with the three *lin-14* transcripts inferred from cDNA and PCR clones isolated from both wild-type (N2) and *lin-14* mutant strains. The cDNAs were isolated by using either the 3.8-kb *EcoRI* fragment, the 7.6-kb *BglII* fragment, or the 5.0-kb *BglII* fragment as probe [(B) *BglII* and (E) *EcoRI*]. Exons containing open reading frames are shown in black, whereas those containing untranslated regions are shown in white. Exons are numbered below. Those genomic regions that have been sequenced are shown under the genomic restriction map and generally flank the exons detected in the cDNA clones. This analysis could have missed exons that are incorporated rarely into *lin-14* transcripts. The locations of the two *lin-14(gf)* mutations in the 3' UTR are shown above and below the genomic restriction map.

Figure 2. (See following page for legend.)

the genomic DNA sequence and ends at position 19,919, yielding a protein of 539 amino acids (Fig. 3) (59,091 daltons). Two codons upstream of the first AUG is a stop codon, which confines the reading frame. Splicing of exon 1–3 in the *lin-14B2* transcript generates a stop codon in-frame at the splice junction. The first AUG downstream of this junction yields a smaller protein of 515 amino acids (56,718 daltons), which could also be produced by the *lin-14B1* transcript. In the *lin-14A* transcript, exon 4 contains an open reading frame that splices to the common reading frame in exon 5 (Fig. 3). The reading frame is open from the 5' end of exon 4 and can be extended >236 bases farther upstream on the genomic DNA sequence to a stop codon at position 15,788. The first methionine codon downstream of the stop codon at 15,788 is found at position 16,148, suggesting that this may be the amino terminus of a 537-amino-acid *lin-14A* protein (59,305 daltons). This protein has a 63-amino-acid amino-terminal domain that is distinct from the 65-amino-acid amino-terminal domain of the putative *lin-14B1* protein, and the 38-amino-acid amino-terminal domain of *lin-14B2*. No sequence similarity between the different amino termini of the *lin-14* proteins could be detected. All of the putative initiation codons compare poorly with the *C. elegans* consensus start codons (C. Fields, pers. comm.). The predicted *lin-14A* and *lin-14B1* proteins are so similar in molecular mass that they should not be resolvable on SDS gels. In fact, only one migrating species at ~67 kD is detected on immunoblots with anti-*lin-14* antibodies (Fig. 4). Because the apparent molecular mass of this species is so different from those predicted by the DNA sequence, we could not assess which transcripts encode the proteins detected.

Data bank searches with the amino acid sequences of both *lin-14* proteins revealed no significant sequence similarity to any proteins. Because the *lin-14* protein is nuclear, we also examined the sequence closely for motifs identified in other nuclear proteins (Ginsberg et al. 1984; Bell et al. 1988; Evans and Hollenberg 1988; Murre et al. 1989; Scott et al. 1989) and found no similarity. A 20-amino-acid region (positions 417–436) common to both *lin-14* proteins could potentially form an amphi-

pathic α -helix with two basic regions separated by an acidic region on the hydrophilic face (Fig. 3).

The *lin-14* proteins also contain a high proportion of proline, glutamate, serine, and threonine, or PEST residues, as has been observed in various unstable proteins (Rogers et al. 1986; Nash et al. 1988). Given that the protein appears to be quickly degraded late in L1 (Ruvkun and Giusto 1989), these sequences could mediate that instability.

lin-14(n355) and *lin-14(n536)* gain-of-function mutations are located in the 3' UTR

Both *lin-14(gf)* mutations were shown previously to map within the 3.8-kb *EcoRI* fragment at the 3' end of the gene (Ruvkun et al. 1989). We isolated genomic DNA segments from this region from both *lin-14(gf)* mutants *n355* and *n536* to pinpoint the location of each mutation by DNA sequencing. The *n355* mutation is an insertion or inversion of at least 10 kb of unknown DNA sequences at position 20,172, 256 bp 3' to the termination codon of the *lin-14* protein-coding region common to all *lin-14* transcripts (Figs. 1 and 2). No other change was found between this breakpoint and the end of the *lin-14* protein-coding region. This breakpoint separates the distal 1.35 kb of the 3' UTR and the normal polyadenylation signal site from the proximal part of the *lin-14* transcripts.

The other *lin-14(gf)* mutation, *n536*, is a 607-bp deletion starting at position 20,216, 300 bp downstream from the 3' end of the *lin-14* open reading frame (Fig. 2). In addition, a C → T transition at position 20,167, 7 bases upstream of the *n355* breakpoint, was detected. We did not determine which of these two changes causes the *lin-14* mutant phenotype. The region deleted by the *n536* mutation overlaps the region that is rearranged in the *n355* mutation.

To verify that these mutations affected the transcript as well, we examined the *lin-14* message detected on Northern blots of RNA from each mutant. As shown previously, instead of the normal 3.5-kb *lin-14* RNA, *n355* mutants produce two species of 3.0 and 2.2 kb, and

Figure 2. (Continued)

in italics. A gap of 95 bp separates the end of the e31 cDNA from the next cDNAs farther downstream (549 and 505; both isolated from wild type). These cDNAs also were generated from the *lin-14* transcript; the 549 cDNA hybridizes on Northern blots to the same size mRNAs as the e31 cDNA: 3.5 kb in wild-type and 3.0 kb in *lin-14(n536)* mutants. Because no good splice acceptor or donor sites are present in the 95-base interval, we can assume that this region is transcribed without interruption. The 570 cDNA also hybridizes to the 3.5-kb *lin-14* mRNA and extends farther downstream, ending in a 27-base-long poly(A) tail. A good polyadenylation signal site (AATAAA) is present 11 bp upstream from the poly(A) tail. The *lin-14B1* transcript that we assemble from these cDNA clones is 3272 bases long without the poly(A) tail. The sequence of the *lin-14B2* transcript was determined by the sequencing of a clone derived from PCR of N2 cDNA. The *lin-14A* transcript was identified by the 557 cDNA clone. This transcript is 3338 bases long. The locations of the *lin-14(n536)* and *lin-14(n355)* mutations are shown above the wild-type sequence. The location of the *n838* mutation was ascertained from three cDNA clones isolated from this mutant and compared to the wild-type DNA sequence in this region from one cDNA and genomic clones, as well as the sequence of this region from the genomic clone from the *n536* mutant. DNA sequence analysis of the fragment inserted into the *lin-14* 3' UTR in the *lin-14(n355)* mutant revealed a consensus *C. elegans* splice donor site 7 bases into the rearrangement. This donor site may be spliced to an acceptor site located ~800 bases 3' in the inserted DNA to generate the 2.2-kb transcript, whereas the 3.0-kb transcript may be not spliced at this point. The inverted repeat in the 3' UTR is shown below the sequence with arrows for each stem-loop. Within the main loop are two smaller stem-loops, also shown with inverted arrows. Restriction sites for *EcoRI* and *BglII* are shown in bold. Italicized boldface type is used to demark the polyadenylation site. The sequence has been entered into GenBank/EMBL; accession numbers are x60230, x60231, x60232, and x60233.

Figure 3. Protein sequence of *lin-14*. The three possible *lin-14* proteins predicted from the *lin-14* DNA sequence. The *lin-14A*-specific amino-terminal region is shown above the *lin-14B1*- and *lin-14B2*-specific protein. The crossover from the carboxyl terminus of the *lin-14A*-specific region to the amino terminus of the common region shows how the *lin-14A* protein sequence continues in the *lin-14* common carboxy-terminal region. The star above position 25 designates the first methionine present in the predicted *lin-14B2* protein, as splicing of exon 1–3 generates an in-frame stop codon just 5' to this methionine codon. Both *lin-14B1* and *lin-14B2* products could use this methionine as a translation initiator. Basic residues are shown in boldface type. Regions containing a high proportion of PEST residues are underlined; the region containing the amphipathic helix is boxed. The *n838* mutation is shown above the wild-type sequence.

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lin-14A
1 MDLPGTSSNDFVQPFSTFYPSPTSPPMHQAALLPALTALYPQLLQLPHLKSDYLIRSDMSAF
lin-14B1/B2
1 MGKGQPKPEKRIEQSVVDLCKRTVAMNLLQCYPTTTVDENMCEEWNGTSTQSVAAQCQGIELRKEVTDL 70
71 RQAVNLILPLMLPLYPTIGNGFNATGLAAQPTLQHVIIQQSLLRKRPAQTPTVPQPECPGQIRPVLSSPAA 140
141 ALQNVIMLNPWIMGSSSLKPAASPTLFPNGQIPTTIGETSLQGTDDQTVKWIQPSVSDSNGQKTSSAASAGD 210
211 NQNIDVIGDGSESPTSSNHSQAQIALMTSQQTFLNALKDSSFLFTNPVPTVETAPPLRVAPPINGTTNGT 280
      T (n838)
281 AKAGGPERKPRKPVNDIVKIVRNQDLSEENISMFEIPVPKATASDPTFRPVSEQQIIQQIIQKKYEEM 350
351 EVGECMIQLCKLAEKRVFGPRLMSQTTVAGLNHSNYANLPKIGICYIQHVCRKVLYDKFENEEDFWDKFF 420
421 REAMRKLAAACRRVRHAKKTKHNREEAQAEMLSKRYGEDMPFNLNAGLIRPKVETVSPEANILNSDQIK 490
491 SQLESLEFAHIPKTESETPLIEIIQQNISLFTHLIRTKVESQSPPLQGGFQ 539

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n536 mutants produce a single species of 3.0 kb (Ruvkun et al. 1989). Both the 2.2-kb and the less abundant 3.0-kb *lin-14(n355)* transcripts were detected with probes that detect exons 5' to the breakpoint but not with probes that detect sequences 3' to the breakpoint (Fig. 5). These data suggest that the two *lin-14* transcripts seen in this mutant are generated by differential splicing or polyadenylation of a transcript(s) containing the normal exons 5' to the breakpoint and foreign sequences 3' to the breakpoint.

The 607-bp deletion present in genomic clones of *n536* is consistent with the shorter 3.0-kb transcript detected in this mutant. The *lin-14(n536)* 3.0-kb transcript is detected by probes to the left and right of the deletion (Fig. 5). Therefore, transcript from this mutant contains sequences flanking the *n536* deletion and presumably utilizes the normal polyadenylation signal.

The location of both *lin-14(gf)* mutations in the 3' UTR suggests that they do not affect the *lin-14* protein. Immunoblot analyses of the *lin-14* proteins from wild-type and both the gain-of-function mutants support this prediction: No difference in the size of the *lin-14* proteins was observed in these mutants (Fig. 4).

In the case of the *n355* mutation, either the deleted 1.35 kb of *lin-14* 3' UTR or the additional foreign sequences fused to the 3' end of the *lin-14* message could cause the inappropriate production of *lin-14* protein after the L1 stage. However, given that the *n536* deletion causes a similar gain-of-function phenotype, these data suggest that the 3' UTR of *lin-14* contains a negative regulatory element that mediates the down-regulation of *lin-14* protein levels after mid-L1. Both *lin-14(gf)* mutations delete this element, so that in these mutants the level of the *lin-14* protein is higher than normal after L1.

Because an element in the 3' UTR might be expected to act on the message, the sequence of the *lin-14* 3' UTR was examined for structures of possible significance in the RNAs. Computer analysis of the *lin-14* RNA sequence identified one significant secondary structure in the 3' UTR. A stem-loop structure with 21 of 22 matching pairs in the main stem and two side stems of 7 bp is

located from 19,980 to 20,084, ~59 bases downstream from the end of the *lin-14* protein-coding region and ~69 bases upstream of the *n355* breakpoint (Fig. 6). This structure would be expected to have a free energy of -38.3 kcal/mole. Randomization of the 107-base region that comprises this stem-loop generated structures of a mean free energy of -15 kcal/mole ± 2.5 ($n = 53$). Thus, the *lin-14* stem-loop structure is ~9 s.d. lower energy than those that arise randomly in a sequence of the same base composition. By an independent statistical calculation, the probability of randomly generating just the 21-base inverted repeat of the stem-loop is 10^{-7} for a 3.5-kb sequence.

lin-14 loss-of-function mutations cause a decrease in *lin-14* protein levels

Animals bearing the mutation *lin-14(n536n838)*, a rever-

Figure 4. Immunoblot with anti-*lin-14* and anti-myosin antibodies. Immunoblots with antibodies raised against the *lin-14A* protein (Ruvkun and Giusto 1989) revealed proteins of 67 kD in L1 animals from both wild-type and the two gain-of-function mutants *n536* and *n355*. These proteins are not detected in the *lin-14(n536n540lf)* mutant (Fig. 7e; Ruvkun and Giusto 1989), showing that the bands detected are the *lin-14* proteins and not cross-reacting antigens. Pharyngeal myosin is shown as a control.

Figure 5. Northern blots of wild-type and gain-of-function mutants. Poly(A)⁺ RNA from N2 (lane 1), *lin-14(n355)* (lane 2), and *lin-14(n536); lin-4(e912)* (lane 3) animals, probed with cDNA 549 (A), a wild-type clone that spans the location of the *n355* and *n536* breakpoints, and with cDNA 570 (B), which maps to the right of the breakpoints (see Fig. 2). The 2.2-kb *lin-14(n355)* mRNA hybridizes to the probe to the left of the breakpoint but not to the probe to the right of the breakpoint. The 3.0-kb band in *n355* is only faintly apparent in this blot. The 3.0-kb *lin-14(n536)* mRNA hybridizes to both probes. The *lin-4(e912)* mutation has no effect on *lin-14* mRNA size or level (data not shown). The lanes shown are all from one Northern blot probed sequentially with the two probes.

tant of the *lin-14(n536gf)* mutant, display a lower level of *lin-14* protein than the parent strain *n536* or wild type during the L1 and L2 stages in all cell lineages (Fig. 7). This revertant was sequenced to pinpoint the location of the *n838* mutation. Three cDNAs were isolated from a *lin-14(n536n838)* double mutant. A comparison of the cDNA sequences from the *n536n838* mutant to the wild-type cDNA and genomic DNA sequences from wild type and *n536* revealed one point mutation in the coding region. Although we have not shown that this mutation is the actual cause of the Lin-14 phenotype (e.g., it could be caused by a second, as yet undetected, mutation), it was the only change in the entire *lin-14* protein-coding region of the mutant. As expected for ethylmethane sulfonate (EMS)-induced mutations (Moerman et al. 1986), this mutation is a G → A transition; it changes an alanine residue at position 322 into a threonine residue, thus identifying this position as necessary for *lin-14* protein stability or function (Fig. 2).

The *lin-14(n355n534)* mutant, a revertant of *lin-14(n355gf)*, similarly makes detectable but reduced amounts of *lin-14* protein. L1 animals display very weak staining in all cells, whereas later larval stages show faint staining only in nerve ring neurons (Fig. 7d). Therefore, in this mutant also, reversion of the gain-of-function mutant is associated with a decreased *lin-14* protein level.

These mutants contrast with *lin-14(n536n540)*, a putative null mutant, which displays no *lin-14* protein staining at any stage (Fig. 7e).

Discussion

A negative regulatory element in the lin-14 3' UTR is necessary to generate the lin-14 temporal switch

We have demonstrated that the normal down-regulation of the level of *lin-14* protein, which occurs during late L1 and causes cells to switch from L1-specific fates to L2-specific fates, depends on a regulatory element in the 3' UTR of the *lin-14* gene. Two *lin-14(gf)* mutations inappropriately accumulate *lin-14* protein in nuclei at the L2–L4 and adult stages (Ruvkun and Giusto 1989). Analysis of these mutations revealed that both are lesions in the 3' UTR of the *lin-14* gene, 270 and 300 bases downstream from the 3' end of the open reading frame (Fig. 2). These mutations delete sequences from the 3' UTR of the *lin-14* mRNA and, in turn, cause an inappropriately high level of *lin-14* protein at post-L1 stages (Figs. 5 and 7). There is no change in the *lin-14* protein-coding region in these mutants, and Western blots showed no change in the size of the *lin-14* proteins (Fig. 4). Because the *lin-14(gf)* mutations are not in protein-coding regions, they do not act by altering the activity or stability of the *lin-14* protein. The location of the regulatory element in the 3' UTR suggests that the element controls a post-transcriptional process such as transcript stability, nuclear export, or translation.

Post-transcriptional regulation has been demonstrated in the iron-regulated transferrin receptor and ferritin genes in mammals (Casey et al. 1988). In these cases, related stem-loop structures in the 3' UTR of the transferrin receptor mRNA regulate transcript stability; and in the 5' UTR of the ferritin gene mRNA, control translation (Casey et al. 1988). Mutations in these stem-loop structures disrupt the regulation (Rouault et al. 1988). In the case of the ferritin 5' UTR stem-loop structure, proteins have been identified that bind to those elements (Leibold and Munro 1988). In the mouse protamine gene, 3' UTR sequences have been shown to mediate negative translational control (Braun et al. 1989). In yeast, genetic evidence has been obtained that proteins bound to the 3' end of mRNAs can influence their translation: Suppressors of mutations in poly(A)-binding protein have been isolated in a ribosomal protein gene (Sachs and Davis 1989). In the human immunodeficiency virus (HIV), the *rev* trans-activator protein has been shown to interact

Figure 6. Stem-loop predicted in the 3' UTR. Location and computer-generated structure (see Materials and methods) of the stem-loop predicted in the 3' UTR of exon 13 between the 3' end of the open reading frame and the breakpoints of the *n536gf* and *n355gf* mutations. Numbers refer to nucleotide positions in Fig. 2.

Figure 7. In situ immunofluorescent detection of *lin-14* nuclear protein in wild-type, *lin-14(gf)*, and *lin-14(lf)* mutant animals. Specimens were fixed and incubated with affinity-purified anti-*lin-14* antibodies followed by a secondary fluorescein isothiocyanate-labeled (FITC) goat anti-rabbit antibody and 4,6-diamido-2-phenylindole (DAPI)-stained to visualize all nuclei. Immunofluorescent photographs were taken with FITC (F) filters to show *lin-14* protein staining or DAPI (D) filters to show all nuclei. Representative nuclei of a cell type are identified by white lines: (G) Gonad; (H) hypoderm; (I) intestine; (M) body wall muscle; (N) nerve ring; (V) ventral cord neurons. Photomicrographs are generally lateral views, in one of two planes. If a hypodermal cell is pointed out, the plane of focus is lateral and large nuclei are all hypodermal (H) and small nuclei are neuronal (N). If intestinal (I) and ventral cord neurons (V) are labeled, the plane of focus is medial and all large nuclei are intestinal and the line of small nuclei ventrally are the ventral nerve cord and small dorsal nuclei are muscle. The head of the animal is identified in most photomicrographs by the intensely staining nerve ring nuclei (N). (a) (Top left) FITC; wild-type early L1 stage, lateral plane, showing bright *lin-14* protein staining in all hypodermal nuclei; (top middle) FITC of same L1 animal with plane of focus at the midline showing intense *lin-14* staining in the large intestinal nuclei and the smaller nuclei of the ventral cord neurons toward the interior (ventral) and the dorsally located body wall muscle nuclei on the outer section of this animal. The nerve ring also shows intense *lin-14* nuclear staining. (Top right) DAPI of same L1 animal with plane of focus at the midline as in the top middle, (Bottom left) FITC; wild-type L2 stage animal focused at the midline showing no detectable *lin-14* staining; (bottom right) DAPI of same animal showing ventral cord neuronal nuclei interspersed with Pn.a-derived neural nuclei and Pn.p ventral hypodermal nuclei that do not accumulate *lin-14* protein. (b) (Top left) FITC; lateral view of *lin-14(n536gf)* mutant, early L1 stage, showing intense staining of hypodermal, ventral nerve cord, and nerve ring nuclei. (Top right) FITC; ventral view of *lin-14(n536)* L2, stage showing *lin-14* protein in the ventral cord neurons and in muscle nuclei. Both gain-of-function mutants show inappropriate accumulation of *lin-14* protein in L2 and later stages, although the *n536* allele is weaker than the *n355* allele and *lin-14* protein staining is consistently about half as intense in *lin-14(n536)* mutant animals after the L1 stage. (c) (Top) FITC; *lin-14(n536n838lf)* mutant L1 and L2 stages (L1 is the smaller animal above), showing weak *lin-14* protein staining in neuronal and hypodermal nuclei of the L1 stage animal and no *lin-14* protein in the L2 stage animal, unlike the *lin-14(n536gf)* mutants. (Bottom) DAPI. (d) (Top left) FITC; *lin-14(n355n534lf)* mutant L1 stage, which is a partial revertant of the *n355(gf)* mutant, showing little or no *lin-14* protein in most nuclei in the two animals shown. (Bottom) DAPI. (Top right) FITC; *lin-14(n355n534)* L3 stage, displaying faint *lin-14* nuclear protein staining in some neurons of the anterior nerve ring. All other cell lineages of the later developmental stages show no detectable *lin-14* protein (data not shown). (e) (Top left) FITC; lateral view of *lin-14(n536n540lf)* mutant L1 stage, showing no *lin-14* protein in any nuclei. (Bottom) DAPI. (Top right) FITC; *lin-14(n536n540)* L1 immunostained with anti-myosin antibodies, showing body wall myosin (Miller et al. 1986) to prove that these animals were fixed and permeabilized properly.

with an RNA structure in the viral transcript (Zapp and Green 1989). This system may mediate export of the mRNA from the nucleus because mutations in the *rev* gene disrupt export of the HIV unspliced transcript from the nucleus (Emerman et al. 1988). Sequences in the 3' UTR of *fem-3* have been shown to mediate its polyadenylation (Ahringer and Kimble 1991). These experiments reveal that sequences in various regions of the transcript can regulate polyadenylation, export of a transcript from the nucleus, the half-life of the transcript, or translation of that transcript.

These precedents suggest that during late L1, the negative regulatory element in the *lin-14* 3' UTR may normally down-regulate the stability of the *lin-14* mRNA, its polyadenylation, its export from the nucleus, or its translation. The secondary structure predicted in the *lin-14* 3' UTR (Fig. 6) could mediate this regulation, and the *lin-14(gf)* mutations might delete sequences that are necessary for the proper function or formation of this RNA structure. However, it is not clear that this structure is actually involved in the process of down-regulation because neither gain-of-function mutant actually deletes the stem-loop itself.

We suggest that at the mid-L1 stage, a newly activated or synthesized regulatory factor binds to the 3' UTR of the *lin-14* mRNA and down-regulates its processing or translation to protein. A candidate for binding to the 3' UTR of the *lin-14* mRNA is the *lin-4* gene product. The heterochronic lineage defects of a *lin-4* mutant are the same as those of *lin-14(gf)* mutants (Chalfie et al. 1981; Ambros and Horvitz 1984), and *lin-14(lf)* mutants are epistatic to *lin-4* mutants (Ambros 1989), suggesting that *lin-4* negatively regulates *lin-14*. As in *lin-14(gf)* mutants, the level of the *lin-14* protein is inappropriately high in late larval stages in a *lin-4* mutant, showing that *lin-4* negatively regulates the expression of *lin-14* (Arasu et al., this issue). Although this *lin-4*-mediated regulation of *lin-14* need not be direct, analysis of whether the *lin-4* product binds to the *lin-14* 3' UTR will test whether *lin-4* regulates *lin-14* in this manner.

The lin-14 gene encodes multiple protein products

The *lin-14* DNA sequence revealed 13 exons in the *lin-14* gene, with introns ranging in size from ~12 kb to 44 bp. The *lin-14B1* and *lin-14B2* unspliced transcripts are ~15 kb larger than the *lin-14A* unspliced transcript. The transcripts encode proteins with distinct amino-terminal domains and a common carboxy-terminal region. This production of multiple related protein products is similar to that observed for the *Ultrabithorax* (*Ubx*), *E74*, and *E75* genes from *Drosophila* (Beachy et al. 1985; Burtis et al. 1990; Segraves and Hogness 1990). Two of the *Ubx* proteins are expressed in distinct but overlapping cell types and specify distinct cell fates (Mann and Hogness 1990). Similarly, the multiple *lin-14* protein products could be expressed in particular sets of cells or at particular developmental times to specify distinct cell types.

A combination of genetic and molecular evidence sug-

gests that the *lin-14B1* and *lin-14B2* proteins could encode a gene activity distinct from the *lin-14A* protein. On the basis of distinct heterochronic defects associated with some *lin-14* alleles and intragenic complementation between two such alleles, Ambros and Horvitz (1987) proposed two separately mutable *lin-14* functions: the *lin-14b* function that specifies L2 and later cell fates, but not L1 cell fates, and the *lin-14a* function that specifies L1 cell fates, but not later cell fates (Ambros and Horvitz 1987). Most *lin-14(lf)* alleles are *lin-14a⁻b⁻* and are therefore defective in both early and late cell fates. One of these *lin-14a⁻b⁻* mutants, *n536n540*, makes no detectable *lin-14* protein (Fig. 7e). *lin-14b* gene function is specifically affected by *lin-14(n360)* rearrangement in the 2.3-kb *EcoRI* fragment that contains exons 2 and 3 of the *lin-14B1* transcript (Fig. 1; Ruvkun et al. 1989). In addition, we have shown that no transcripts containing exon 3 (i.e., *lin-14B1* and *lin-14B2*) are present in this mutant (B. Wightman, P. Arasu, and G. Ruvkun, unpubl.). Thus, *lin-14b* gene activity could be encoded by the *lin-14B1* and *lin-14B2* transcripts. If this is the case, because the *lin-14b* gene function has a late L1 temperature-sensitive period compared to mid-L1 for the *lin-14a* gene function (Ambros and Horvitz 1987), the *lin-14B1* and *lin-14B2* proteins may be synthesized or activated later than the *lin-14A* protein.

The DNA sequence of the only existing *lin-14a⁻b⁽⁺⁾* allele, *n536n838*, revealed an A → T mutation in exon 8, which is common to all *lin-14* proteins (Figs. 2 and 3). Therefore, this mutation is not specific to the *lin-14A* transcript, leaving open the possibility that *lin-14a* activity is due to the combined action of *lin-14A* and *lin-14B1/B2* proteins. The mutation adds an additional threonine to a region of the protein rich in prolines, glutamates, serines, and threonines; these amino acids in other systems have been shown to be correlated with unstable proteins (Rogers et al. 1986). The *n838* mutation causes the level of *lin-14* protein to decrease much more steeply during L1 than either wild-type or the parent *n536* strain (Fig. 7). Therefore, the *n838* mutation may destabilize the *lin-14* proteins to mask the effects of the *n536* regulatory mutation. Because this mutant expresses L2 fates precociously during L1 but is normal at later stages, the falling level of *lin-14* protein must be insufficient to specify early fates yet sufficient to specify later fates. This suggests that *lin-14a* gene activity may have a higher threshold requirement for *lin-14* protein than *lin-14b* gene activity. Whether this requirement is for *lin-14A* protein alone or the other *lin-14* proteins as well has not yet been determined.

lin-14 nuclear proteins are not similar to any known class of regulatory protein

Although the nuclear localization of *lin-14* proteins suggested that we might find sequence similarities to other known nuclear proteins, we have found no homology to the classes of transcription factors and RNA-splicing proteins sequenced so far. Analysis of the *lin-14* protein sequences did, however, reveal provocative potential

structures. The common region of the *lin-14* proteins contains a potential amphipathic helix with two basic patches and an acidic patch of amino acid residues on the helical face opposite the hydrophobic face (Fig. 3). A computer search with just this region revealed no homologs in the data base. Amphipathic helices with basic patches have been observed in the *myc* family of transcription factors (Murre et al. 1989). The predicted amphipathic helix is located within the protein sequence element CX₃HXCX₂₈CX₄HX₅H, which is slightly reminiscent of zinc finger proteins but distinct from those observed so far in this gene family (Evans and Hollenberg 1988).

The absence of known proteins showing sequence similarity to the *lin-14* proteins suggests that they represent the first example of this regulatory gene class. Because the *lin-14* proteins are localized to the nucleus, they may function to regulate gene expression. The potential basic amphipathic helix hints that the protein could bind to DNA or RNA. The *lin-14* proteins could activate L1 stage lineage-specific genes and/or repress L2 stage and later lineage-specific genes. Genetic epistasis experiments suggest that the downstream gene *lin-29* may be negatively regulated by the *lin-14* proteins (Am bros 1989).

Generation of the *lin-14* temporal gradient

During wild-type development, the *lin-14* protein levels decrease by a factor of >25 from mid-L1 to late L1, a period of 6 hr (B. Wightman and G. Ruvkun, unpubl.). The *lin-14* protein levels decrease at the same rate in both dividing and nondividing cells (Ruvkun and Giusto 1989), suggesting that, at least in nondividing cells, breakdown of the nuclear membrane is not necessary for the degradation of the *lin-14* protein and that nuclear proteases must control this process. In addition, these observations suggest that in the nondividing cells, cell-cycle-regulated proteases, like those that control cyclin protein levels (Murray and Kirschner 1989), probably do not regulate *lin-14* protein levels. Protease(s) that degrade the *lin-14* protein could be induced late in the L1 stage or could be present constitutively, with their effects only visible after the synthesis of new *lin-14* protein is abolished in late L1. The presence of PEST sequences in the *lin-14* protein and our observation that addition of a PEST amino acid by the *n838* mutation decreases the level of *lin-14* protein supports the notion that the rate of *lin-14* protein degradation is relevant to the formation of the *lin-14* protein gradient.

Other heterochronic genes could control developmental timing of the *C. elegans* cell lineage by participating in the generation of the *lin-14* temporal gradient at any of these levels, by participating in the reception of this signal, or by interacting with the *lin-14* protein to specify distinct cellular responses to this general temporal control signal. In Arasu et al. (this issue), we show that the two other heterochronic genes, *lin-28* and *lin-4*, act antagonistically to set the level of the *lin-14* protein during postembryonic development. We also show that the het-

erochronic gene *lin-29* does not affect the level of the *lin-14* protein and thus is a downstream gene. Therefore, the *lin-28* and *lin-4* products are candidates to interact directly with the *lin-14* mRNA or protein, and the *lin-29* gene is a potential downstream gene to which the *lin-14* nuclear protein could bind to regulate its expression.

Materials and methods

Cloning procedures

cDNA was synthesized from poly(A)⁺ RNA according to Ausubel et al. (1987) and cloned into λ gt10 with either *Xba*I–*Eco*RI linkers or *Not*I polylinkers. Some libraries were obtained from Stuart Kim (Stanford University, Stanford, CA). λ gt10 libraries were screened according to standard methods by using either the ³²P-labeled 5-kb *Bgl*II restriction fragment or the ³²P-labeled 3.8-kb *Eco*RI restriction fragment as probe. cDNAs and genomic *Eco*RI and *Bgl*II restriction fragments from cosmid EEG4 (see Ruvkun et al. 1989) were subcloned into Bluescribe + (pBS+). Genomic DNA from the *lin-14(n355)* mutant strain was prepared as described in Ruvkun et al. (1989), partially digested with *Sau*3A, and cloned into pJB8 cosmid vector. Cosmid-bearing colonies were screened by using a ³²P-labeled 1.9-kb *Eco*RI restriction fragment. Genomic DNA from the *lin-14(n536)* mutant strain was prepared the same way, completely digested with *Hind*III, and size-selected on an agarose gel; fragments of ~6 kb were cloned into pBS+. The resulting library was screened with a ³²P-labeled 3.8-kb *Eco*RI fragment.

PCR studies

cDNA was prepared from N2 (wild-type) poly(A)⁺ RNA and used as a template for PCR. Paired oligonucleotides corresponding to sequences in exons 1 and 2, exons 1 and 4, exons 1 and 5, and exons 4 and 5 were used in separate reactions. The *lin-14B2* clone was obtained by blunt-end ligation of the products of the exon 1–5 reaction into BS+.

Northern and RNase protection analysis

Northern blots were standard 1.2% agarose–formaldehyde gels transferred to nitrocellulose and probed with ³²P-labeled probes as indicated (Ausubel et al. 1987). For RNase protection assays, total RNA obtained from N2 was hybridized to antisense RNA probes produced in vitro by standard methods (Ausubel et al. 1987). Probes were produced from a genomic subclone spanning exons 4 and 5 and a cDNA clone that included exons 1–3 and 5, both in BS+. Hybridizations were performed at 42°C and digested with RNase A and RNase T1, and the resulting fragments were analyzed on urea–polyacrylamide gels.

Sequencing

Sequencing was performed on either single-stranded or double-stranded templates according to the chain-termination method with ³⁵S-labeled dATP (Amersham and New England Nuclear) and Sequenase (U.S. Biochemical). In brief, cDNAs in pBS+ were sequenced from both ends with forward and reverse primers. Sequence was then extended by using synthesized oligonucleotides (Applied Biosystems) as primers until all sequence was double stranded. Genomic subclones were sequenced from the ends by using forward and reverse primers. Sequence was extended with newly synthesized primers or previously synthesized primers derived from the cDNAs. Genomic sequence of

parts of some introns is only single stranded. The *n355* genomic clone was directly sequenced on cosmid DNA, from just before the end of the open reading frame to beyond the breakpoint, by using primers derived from the cDNA sequence. cDNA clones obtained from mutants containing the *n355* mutation were sequenced, demonstrating no other divergence from wild-type sequence from exon 10 to the 3' end of the coding sequence (data not shown). The *n536* genomic clone was sequenced in the region flanking the *n838* point mutation and from 50 bp 5' of the *n355* breakpoint to 120 bp 3' of the *n536* breakpoint.

Sequences were entered on Apple Macintosh systems with a talking sequence editor written by T.R. Bürglin. Sequences were aligned and assembled with a multiple sequence editor generously provided by Will Gilbert, and sequences were analyzed with the GCG programs (Devereux et al. 1984).

Protein similarity searches were performed by using FASTA with k-tuple of 2. No similarity score greater than that of the sequence randomized was detected. RNA secondary structure and free energy calculations were determined with the GCG Fold program.

Immunofluorescence

C. elegans wild-type (N2) and mutant animals were fixed and immunostained with anti-*lin-14* antibodies as described by Ruvkun and Giusto (1989), and animals were staged as described by Ambros and Horvitz (1987). Anti-myosin antibodies against the body wall muscle and pharyngeal muscle were kindly provided by David Miller (Duke) and used at a dilution of 1 : 200. Fluorescent nuclei were visualized with a Zeiss Universal microscope as reported previously (Ruvkun and Giusto 1989).

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