Cell, Vol. 88, 637–646, March 7, 1997, Copyright ©1997 by Cell Press

The Cold Shock Domain Protein LIN-28 Controls Developmental Timing in C. elegans and Is Regulated by the *lin-4* RNA

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Summary

Mutations in the heterochronic gene *lin-28* of C. elegans cause precocious development where diverse events specific to the second larval stage are skipped. *lin-28* encodes a cytoplasmic protein with a cold shock domain and retroviral-type (CCHC) zinc finger motifs, consistent with a role for LIN-28 in posttranscriptional regulation. The 3′UTR of *lin-28* contains a conserved element that is complementary to the 22 nt regulatory RNA product of *lin-4* and that resembles seven such elements in the 3′UTR of the heterochronic gene *lin-14*. Both *lin-4* activity and the *lin-4*-complementary element (LCE) are necessary for stage-specific regulation of *lin-28*. Deleting the LCE produces a dominant gain-of-function allele that causes a retarded phenotype, indicating that *lin-28* activity is a switch that controls choices of stage-specific fates.

Introduction

Animal development consists of a complex schedule of stage-specific developmental events. The proper succession of events within tissues and cell lineages and the coordination of events throughout the animal require the accurate control of the temporal components of cell fates, in conjunction with other fate components, such as position and sex. The heterochronic genes compose a genetic regulatory hierarchy that controls developmental timing in C. elegans larvae by specifying the temporal components of fates of cells in diverse tissues throughout the animal (Ambros and Horvitz, 1984; Ambros, 1989; reviewed in Ambros and Moss, 1994). Mutations in these genes cause either of two types of fate transformations, precocious and retarded, where cells adopt fates that normally occur either later or earlier in the same cell lineage. A variety of developmental events are affected by heterochronic mutations, including patterns of cell division, the lengths of specific cell cycles, and stage-specific terminal differentiation events (Ambros and Horvitz, 1984; Ambros, 1989; Euling and Ambros, 1996). A notable feature of the heterochronic genes as developmental regulators is the stage specificity of their action: in general, mutations in these genes cause the omission or reiteration of developmental events specific to a particular postembryonic developmental stage, suggesting that the heterochronic gene products are expressed and act stage-specifically.

How do the products of the heterochronic genes regulate diverse events, and how are the activities of the heterochronic genes themselves regulated temporally? To address these questions, we have undertaken the molecular characterization of the heterochronic gene *lin-28*. Mutations in *lin-28* cause precocious development, where many developmental events specific to the second larval stage (L2) are skipped and later events occur one stage earlier than normal (Ambros and Horvitz, 1984). For example, in *lin-28* mutant animals the pattern of division of the lateral hypodermal seam cells characteristic of the third larval stage (L3) occurs in the L2, and all later events in this lineage, including the synthesis of adult-specific cuticle, occur precociously (Ambros and Horvitz, 1984). The molting cycle ceases after the third stage so that the animal develops through only three larval stages, instead of the normal four. Other developmental events, such as the cell division and differentiation events that form the hermaphrodite vulva, are similarly affected in *lin-28* mutants (Euling and Ambros, 1996). Thus *lin-28* mutants are deformed and unable to lay eggs due to the precocious development of several cell lineages with respect to the gonad, which develops normally. One model for *lin-28* activity is that it is necessary to prevent the expression of L3-specific fates in the L2; restriction of *lin-28* activity to the L2 would thus allow the succession of L2- to L3-specific cell fates, and subsequent events would follow without direct influence by *lin-28*. However, the mechanism of *lin-28* action and its time of expression have not been known. Elucidating the actual role of this gene in the control of developmental timing requires the molecular characterization of its activity and regulation.

lin-28 shares its effect on the succession of L2- to L3-specific developmental events with another heterochronic gene, *lin-14*, mutations in which also cause precocious cell fate transformations (Ambros and Horvitz, 1984; Ambros, 1989; Euling and Ambros, 1996). The genetic analysis of *lin-14* and *lin-28* has not yet distinguished whether they act in series or in parallel to control the succession of stage-specific events (Ambros 1989; Euling and Ambros, 1996). *lin-14* and *lin-28* may act as cofactors and be simultaneously required to regulate shared downstream targets, or one may control the expression or activity of the other, the second being the more direct regulator of the targets. *lin-14* encodes a broadly expressed nuclear protein that has no discernible similarity to other known proteins (Ruvkun and Giusto, 1989; Wightman et al., 1991). Sequence analysis indicates that *lin-14* encodes a protein with an amphipathic helix near its C terminus, and it has been suggested that the LIN-14 protein may bind nucleic acids, although its biochemical function remains to be elucidated (Ruvkun et al., 1991).

Critical to normal developmental timing in C. elegans is the temporally regulated decrease in *lin-14* activity and protein level mediated by the heterochronic gene *lin-4* (Ambros and Horvitz, 1987; Wightman et al., 1991). *lin-4* encodes a 22 nt RNA that is believed to act through complementary elements in the 3′UTR of the *lin-14* mRNA, and through an unknown mechanism, to negatively regulate *lin-14* protein accumulation (Lee et al., 1993; Wightman et al., 1993). If the down-regulation of

lin-14 by *lin-4* does not occur properly, as in *lin-4 loss-of-function (lf)* mutants or *lin-14 gain-of-function (gf)* mutants that lack the complementary elements, a retarded phenotype results, where cells reiterate early stage-specific fates within a lineage and later events are delayed or prevented (Chalfie et al., 1981; Ambros and Horvitz, 1984; Ambros and Horvitz, 1987; Wightman et al., 1991). *lin-28(lf)* mutations are epistatic to *lin-4(lf)* and *lin-14(gf)* mutations, indicating that *lin-28* activity is required for the retarded phenotype (Ambros, 1989).

What is the relationship between *lin-14* and *lin-28*, how is *lin-28* regulated in development, and how might *lin-28* act on its targets? In this report, we show that *lin-28* encodes a cytoplasmic protein with a cold shock domain and retroviral-type zinc finger motifs and therefore is likely to be an RNA-binding protein. LIN-28 may interact with mRNA of downstream genes to affect their expression posttranscriptionally. We also have found that *lin-28* expression is temporally regulated by the *lin-4* RNA through a single *lin-4*-complementary element in the *lin-28* 3′UTR. The action of *lin-4* RNA is therefore not restricted to the *lin-14* gene and can act through a single complementary element in a different RNA context to control a distinct gene product. We demonstrate that *lin-28* lacking the *lin-4*-complementary element causes a retarded phenotype analogous to the retarded phenotype caused by *lin-14(gf)*, indicating that *lin-28* acts as a switch controlling the succession of L2- to L3-specific fates and that the down-regulation of both *lin-28* and *lin-14* by *lin-4* is essential to normal developmental timing. Furthermore, we have found that *lin-14* positively regulates *lin-28*, consistent with *lin-14* acting at least in part through *lin-28*.

Results

Location and Sequence of *lin-28*

lin-28 was previously mapped to the center of linkage group I (Ambros and Horvitz, 1984) and was further mapped to the 0.25 map units between the genes *mei-1* and *ceh-6* (Figure 1A; Clark and Mains, 1994; data not shown). This region is represented on the C. elegans physical map by overlapping clones that span approximately 200 kb. *lin-28* mutant animals were transformed with the yeast artificial chromosome Y37F9 that contains 180 kb of the DNA from this region, and one of 20 transformed lines was rescued for the *lin-28* precocious phenotype. Cosmids that contain some of the same sequences as Y37F9 were then tested, and one of these, ZC550, also rescued *lin-28* mutant animals. Deletions and subclones of ZC550 were tested, and the smallest of these, containing 4.6 kb of C. elegans genomic DNA, efficiently rescued the *lin-28* mutant phenotype (Figure 1A).

We sequenced the 4.6 kb rescuing genomic fragment and found it to contain a single predicted gene using Genefinder (see Experimental Procedures). A C. elegans cDNA library was screened, and one clone was identified that has 10 nt of the 22 nt sequence of the trans-spliced leader SL1 at one end and poly(A) at the other, indicating that the cDNA is full length. The intron–exon boundaries defined by the cDNA sequence match those predicted by Genefinder. The gene is composed of three exons, one nucleotide between the trans-spliced SL1 leader and the first AUG codon, a 227 codon open reading frame, and a 529 nt 3′UTR.

We sequenced DNA from several *lin-28* mutants and identified the molecular lesions associated with the corresponding *lin-28* alleles (Figure 1B). At least two of these alleles are likely to be null mutations: *lin-28(ga54)* is a mutation to a nonsense codon near the 5′ end of the second exon, and *lin-28(n719)* is a mutation of the universally conserved G residue in the 5′ splice donor of the second exon. A frameshift mutation introduced into the second exon by filling in an AgeI restriction site to create a 4 nt insertion renders the 4.6 kb genomic fragment unable to rescue *lin-28* mutant animals (data not shown). We conclude that the 4.6 kb genomic fragment and the corresponding cDNA define the *lin-28* gene. However, one of the rescuing genomic fragments begins only midway through the first intron, and thus lacks 5′ sequences including the first exon (Figure 1A, PstI fragment). Though we do not know the nature of the transcript produced from the 5′-truncated fragment, rescue by this fragment suggests that a LIN-28 protein missing the 48 amino acids encoded by exon 1 retains its necessary structure and biochemical activity.

We sequenced *lin-28* cDNAs from two other species of Caenorhabditis and found that the predicted amino acid sequence of C. elegans *lin-28* is overall 85% and 77% identical with homologs from C. remanei and C. vulgaris, respectively (Figure 1B). Most of the differences among the homologs are in two regions: in the middle of exon 1 and at the end of exon 3. The conservation of predicted amino acid coding at the beginning of exon 1 indicates that the first AUG codon encodes the initiator methionine. Exon 2 is 97% identical among the three species.

The 3′UTRs of the three homologs range in length from 487 nt to 590 nt and contain regions of contiguous or nearly contiguous nucleic acid identity within otherwise largely unconserved sequence (Figure 2A). The conserved sequences may be important for mRNA metabolism or regulation (see below). The most 3′ conserved region includes the presumed poly(A) addition signal.

lin-28 Encodes a Protein with a Cold Shock Domain and Retroviral-Type Zinc Finger Motifs

Analysis of the coding potential of *lin-28* by BLAST revealed two motifs found in RNA-binding proteins. Exon 2 encodes a region homologous to bacterial cold shock proteins and members of the eukaryotic Y-box family of proteins (Figure 1B). This protein family shares a cold shock domain (CSD) that contains the RNA-binding motifs RNP-1 and RNP-2 (Wistow, 1990; Landsman, 1992; Graumann and Marahiel, 1996). The CSD of *lin-28* is as much as 45% identical to those of other members of the CSD family and is more similar to those of the bacterial and plant proteins than the Y-box factors of vertebrates. Two mutant alleles of *lin-28*, *n1119*, and *ga74* have changes in conserved residues of the CSD, indicating the importance of the CSD for *lin-28*'s role in developmental timing (Figure 1B). Exon 3 encodes two zinc

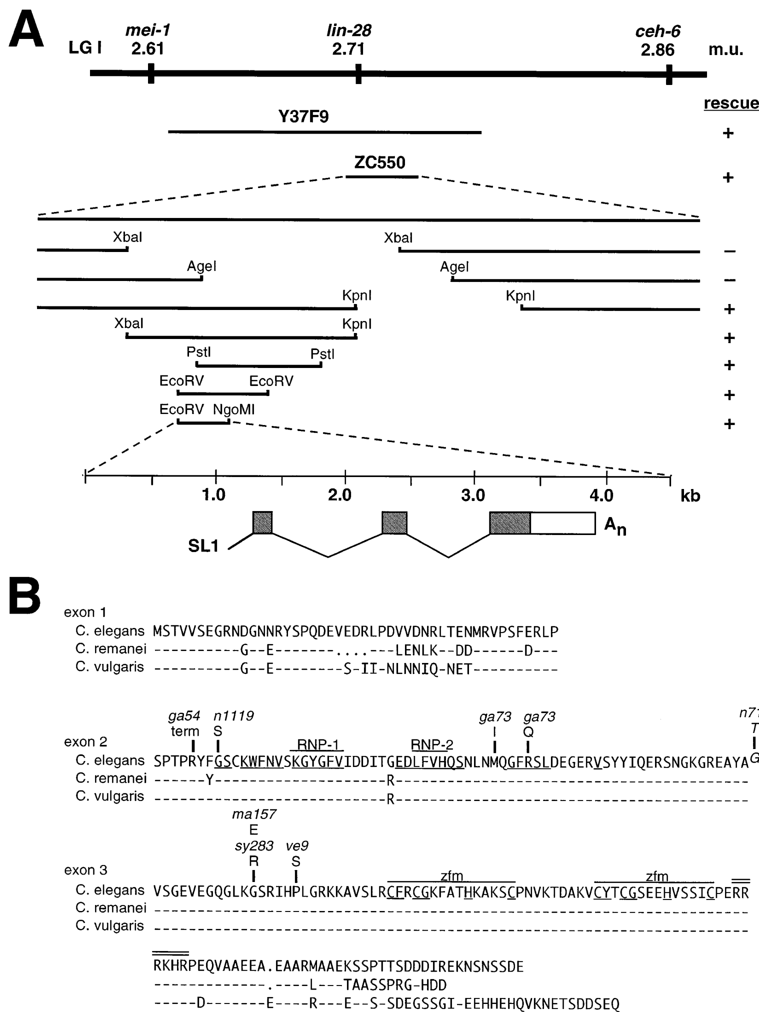

Figure 1. *lin-28* Encodes a Protein with a Cold Shock Domain and Retroviral-Type (CCHC) Zinc Fingers

(A) The genetic map positions of *lin-28* and other loci used in the mapping of *lin-28* are shown above the relative positions of the rescuing clones, yeast artificial chromosome Y37F9, and the cosmid ZC550, from the corresponding physical map of linkage group I (LGI). The genetic and physical maps are from ACEDB. Deletions and subclones derived from ZC550 were tested for rescuing activity, and the results are indicated to the right. At the bottom is the structure of the *lin-28* gene as determined by genomic and cDNA sequencing. The presence of the trans-spliced leader SL1 and poly(A) tail are indicated, and the coding region is shaded. The orientation of ZC550 and *lin-28* relative to LGI is reversed.

(B) The deduced amino acid sequence of LIN-28 from C. elegans is aligned with those of homologs from C. remanei and C. vulgaris. The sequences are arranged according to the three exons of the C. elegans gene. Mutations found in C. elegans *lin-28* mutant alleles are indicated above the line ([term], termination codon). *n719* contains a G-to-T change at the first nucleotide of the splice donor sequence at the 5′ end of the second intron. Important conserved amino acids of the cold shock domain (exon 2) and the retroviral-type zinc finger motifs (exon 3) are underlined. RNP-1 and RNP-2 motifs of the cold shock domain and the zinc finger motifs (zfm) are indicated. Basic residues C-terminal to the zinc finger motifs are double-overlined. (–), identity between sequences; (.), gap in alignment.

finger motifs that resemble those of retroviral nucleocapsid proteins that are involved in the packaging the viral genomic RNA into virions (Gorelick et al., 1988; Meric and Goff, 1989). Each zinc finger motif contains characteristically spaced cysteine and histidine residues in the order CCHC, as well as other amino acids that are conserved with retroviral and cellular proteins, and the two are spaced with respect to each other like those of retroviruses. Immediately C-terminal to the zinc finger motifs is a cluster of basic residues (Figure 1B); such clusters in RNA-binding proteins are important for interaction with RNA (Lazinski et al., 1989; de Rocquigny et al., 1993). These sequence homologies suggest that *lin-28* encodes an RNA-binding protein.

lin-28:GFP Is Expressed in the Cytoplasm of Diverse Cell Types

To determine the expression pattern of *lin-28*, we generated transgenic lines expressing a *lin-28*-green fluorescent protein (GFP) fusion and examined transformed animals by fluorescence microscopy. The fusion was made by inserting the GFP coding region at the 3′ end of the *lin-28* ORF in the rescuing 4.6 kb genomic fragment so that the *lin-28* coding and regulatory regions were preserved (see Experimental Procedures). The *lin-28:GFP* fusion rescues the mutant phenotype of *lin-28(n719)* animals (data not shown). In transgenic L1 larvae, fluorescence can be seen in diverse cell types, including hypodermis, muscle, and neurons, but not in the germline (Figure 3A; data not shown). Lineages that are abnormal in *lin-28* mutants, such as lateral and ventral hypodermis (Ambros and Horvitz, 1984; Euling and Ambros, 1996), express the *lin-28:GFP* fusion, suggesting that the gene acts cell autonomously. Other cell types, such as head neurons and body muscle, that do not show apparent defects in *lin-28* mutants also express *lin-28:GFP*. The fluorescence is in the cytoplasm in all of these cells, though occasionally fluorescence can be detected in some nuclei or nucleoli. The fluorescence is most intense in neurons and occasionally in hypodermal blast cells, and is often weak in or absent from the intestine (Figure 3; data not shown).

lin-28:GFP Expression Decreases from Early to Late Postembryonic Development

Expression of *lin-28:GFP* is greatest in late embryos and L1 larvae, detectable but diminished in the L2, and greatly diminished by the L3 and L4 stages (Figure 3). To

A

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.................................AATCATCTAGACACTGAGAATATTGATAGAGAAATAATGGAATATA
...............................................................................
CGAAGATTAAGAAGAAATTCGAAAGGAATCAGATGAAGATTTAGATGAAGATCTTTGAAAAAAACGTTTTTATTTGATTT

TGGTCTCAAATAGATTTTGTCTCGAAATTCTTGAACATCCAAATTTAAATGTGAAAATTTTAGGAAATAATTTTGAGCTT
........................AGATAGGAGAAAACTTCTTAGAATGAAAGCAGTGAAACTGAATAGCTGAAAGAAT
GTTTTCTTTCGGAAATTTTCTTTTGCATGCTTAAGTATTATATTTTCTGAAAAATTGTCAAACTTCAGACCACCCCCCTT

TCTGATAGTTTTTCAGCTTTTGAAATGTATTTTATTTTGAAACCAATATTTATTTCATTTTCTGTCTCATCTAATTCTCA
ATTTTTAGTTT........TTATTGTTTTCTATCTCTTCTTCATTGCTTCTTGTCTCGAAAAAAATCTTTTGCATGCTTA
TTTTGTTTTCTCTAAAAACCTAGGATCTGGTTTGAAGAGAGAAGAAAGTTTTTTTCTGAAAATATGATATACTTTTTTT

GTTTGCATGCGTTACTTTAACTTACACACTAACTCTTTTTT.TATTCAAATTTTGATGTTTTTTTTCAATTGTTTTACTC
CTTCTAACTAACAACATTTTTCTCGATAATATTAACTTTTC.TAATCAAATTTCCTTTTTTATcGTCCCCACTTACCCAG
TAAACAGTAATCGTTTTTTTTTACATTTAAATTTCATTTTATTATTCAAATTTCCTCAA.TTTCTTTGCCTCTTTCCCCC

AAA..TTTCCCCTCTAAACCATACTAC.CACCTACCTCCTCAAATTGCACTCTCAGGGATTCTTT...............
AAATGTTTCTTTTCACCACCATACTACTCATCTACCTCCTCAAATTGCACTCTCAGGGATTC...CAATTTTGAAACGTC
A...TTTTCTTTTCAATT...TACTAC.CATCTACCTCCTCAAATTGCACTCTCAGGGATTTTTTAAATGTTTCGGATAC

TTTTTTTCAAATAGAACTGATTGCACCTGTTTTCAGGATCTATAAA.............TCTTATAATATATTTAACTTTC
TATTCTAAAAAAGAAACTTACCGTCTTTATTTTTTATATATTGTAACTCAGGAAATTTGCGTCGATTCTGTCACTCTATG
TGTTTGTTTCCGAAACCTTATTATGTCCATTTTTATTATAATTTAACTCAGGAAATTTGCGTCGAAT...ACTACCTTAC

GTGTCTTTTCATGTTCATGTATTT...CT.T.GCTGAAATCCATCATTTTCTCTGAAAATCTCTACTCGTAAGTTCCGCT
CTAAACTTTTCTATT...GTATTTTTTCT.TTGCTGAAATC.ATTTTTGTCTCTGAAAATCTCCATCCCTCGACCCCCGT
TACCGTTTTGTTTTTTT.GTATTT...CTATTGCTGAAATC.AT..TT.TCTCTGAAAATCTCCACACCCTCACACCGTT

ATTGCGGGCGAA............CGCTGAAATAAATCGAGAGTTTTGTTTT  C.elegans
TTTTTAATTGAACGATACGAGCGGCGCTGAAATAAATCGAGAGTTT.GTT..  C.remanei
GCTTAATAAAACAACGAAGAGAGGCGCTGAAATAAATCGAGAGTTT.GTT..  C.vulgaris
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B

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lin-28      5'-UUG.CAC......UCUCAGGGA-
lin-14 -1     -UCA-UGCU......CUCAG.GAA-
lin-14 -2     -UCA.........-.CUCAG.GAA-
lin-14 -3     -UCG-CAUUU...-.CUCAGGGAA-
lin-14 -4     -UCA.U.......-.CUCAG.GAA-
lin-14 -5     -UC..UAC.......CUCAGGGAA-
lin-14 -6     -UU-.UGU.....-..UCAG.GAA-
lin-14 -7     -UCA.........-.CUCAGGG.A-
lin-4S      3' AGU.GUGAACUCCAGAGUCCCUU
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C

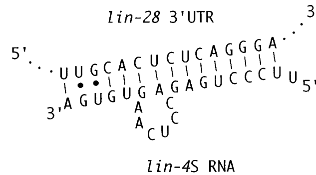

Figure 2. Conserved *lin-4* Complementary Element in the 3′UTR of *lin-28*

(A) An alignment of cDNA sequences of the *lin-28* 3′UTRs of C. elegans, C. remanei, and C. vulgaris. Aligned identical residues are highlighted. The 15 nt constituting the *lin-4*-complementary element are underlined; these nucleotides were deleted to generate the *lin-28(gf)* mutant (see Experimental Procedures). The 3′-most identical region contains the presumed poly(A) addition signal; poly(A) tails were found after the last bases shown. Alignments were generated using the GCG software and optimized manually.

(B) Alignment based on predicted base pairing between the 22 nt *lin-4* gene product (*lin-4*S) and the *lin-4*-complementary elements in *lin-28* and *lin-14* 3′UTRs. Only those sequences of the UTRs predicted to base-pair with *lin-4*S are shown. (−), one or more noncomplementary nucleotides; (.), gap in alignment.

(C) A proposed secondary structure formed between *lin-4*S and *lin-28* 3′UTR. The predicted base pairing and loop are characteristic of the base-paired structures predicted to form between *lin-4*S and the 3′UTR of *lin-14*.

determine whether fluorescence intensity in *lin-28:GFP* transgenic animals decreases with development, we followed development of individual larvae and found that none of the animals that were fluorescing at the L1 did so at the L4 stage (Table 1). Some neuron cell bodies, particularly those in the head, can occasionally be seen fluorescing weakly in late stages, and fluorescence in hypodermis and muscle is generally undetectable in L3-stage or older animals (Figure 3; data not shown). The decrease in expression is likely to reflect a posttranscriptional developmental regulation of *lin-28*, because Northern and RNase protection analysis reveals that

Table 1. Regulation of *lin-28* Expression by *lin-4* and *lin-14*

Strain	Genotype[a]	Percentage of Fluorescent L4 Animals[b]	Percentage of Adult Animals with Alae[c]
VT800	*lin-28:GFP*	0 (n = 105)	100[d] (n = 16)
VT802	*lin-4*(−); *lin-28:GFP*	100 (n = 45)	ND[e]
VT803	*lin-28(gf):GFP*[f]	98 (n = 44)	0 (n = 21)
VT805	*lin-4*(−); *lin-14(ts); lin-28:GFP*	4[g] (n = 52)	30[g] (n = 10)

[a] VT800, VT802, and VT805 carry the same transgenic array crossed into different genetic backgrounds. Full genotypes are listed under Experimental Procedures.

[b] An epifluorescence dissecting microscope was used to identify transgenic animals that were fluorescent in early larval development (L1 or L2 stage) and to examine the same animals later as L4 larvae.

[c] Transgenic animals were examined by DIC optics at the L4 molt or as young adults for presence of adult alae. An animal with no alae along at least one side was scored as negative. Somatic gonad and germline development served to indicate the age of the animal. Animals were determined to be transgenic by virtue of their previous fluorescence as L1 larvae, the presence of the Rol marker, or their fluorescence when they were scored for the alae phenotype.

[d] Some animals were observed to have gaps in adult alae; this phenotype is not observed in nontransgenic animals, and so is due to the multicopy *lin-28* transgene.

[e] The lack of alae was confirmed for some animals, consistent with the *lin-4* retarded phenotype (Ambros, 1989).

[f] Both *nDp4*$^{+}$ and *nDp4*$^{-}$ animals were scored, therefore some animals lacked a functional chromosomal copy of *lin-28*.

[g] Animals were examined at the restrictive temperature of 25°C. The retarded phenotype displayed by some VT805 animals is due to the multicopy transgene because nontransgenic animals do not display this phenotype (see note above). This strain is likely to be more sensitive to the level of *lin-28* activity than is the wild type.

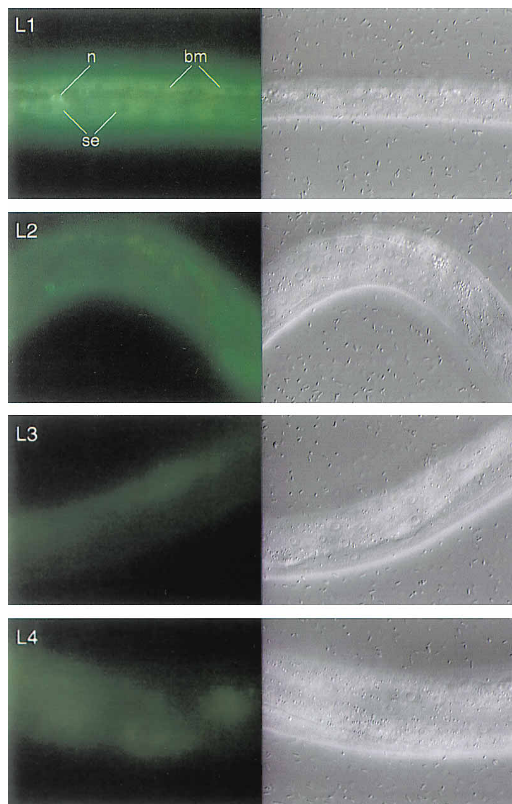

Figure 3. Decreasing Expression of *lin-28:GFP* after the L1 Stage

Fluorescence micrographs of animals carrying the *lin-28:GFP* transgene (VT800) are shown with a DIC image of the same field at each of the four larval stages. For each stage, a different animal is shown; each is representative of VT800 at the indicated stage. Fluorescence is greatest in the L1, is detectable but diminished in the L2, and is only autofluorescence later. In the L1 animal, cells of various types express *lin-28:GFP*, including hypodermal seam cells (se), body muscle (bm), and neurons (n).

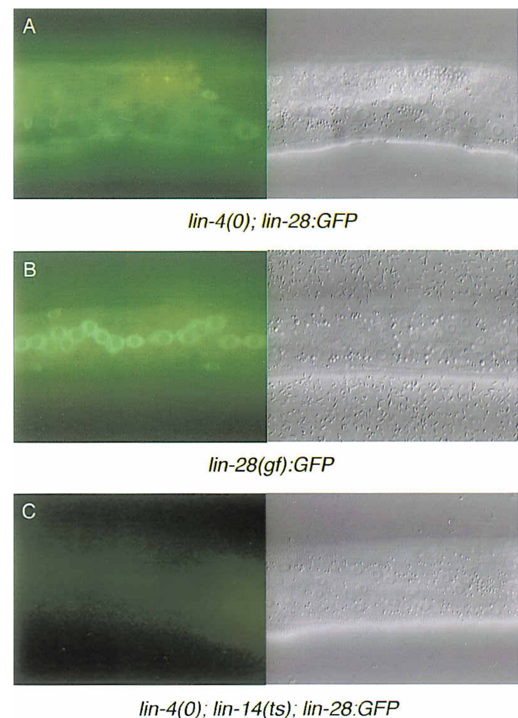

Figure 4. Regulation of *lin-28:GFP* Expression by *lin-4* and *lin-14*

Fluorescence micrographs of late stage animals showing the degree of fluorescence of LIN-28:GFP. All animals were identified as L4 or older based on the extent of somatic gonad and germline development. (A), *lin-4(e912); lin-28:GFP* (VT802); (B), *lin-28(gf):GFP* (VT803); and (C), *lin-4(e912); lin-14(n179ts); lin-28:GFP* (VT805) at 25°C.

the *lin-28* mRNA is present throughout postembryonic development (data not shown).

lin-28:GFP Is Regulated by *lin-4*

The 3′UTR of *lin-28* contains a 15 nt sequence that is similar to each of seven elements in the 3′UTR of the heterochronic gene *lin-14* (Figure 2). The seven elements in the *lin-14* mRNA are complementary to the *lin-4* RNA and are necessary for posttranscriptional regulation of *lin-14* (Lee et al. 1993; Wightman et al. 1993). Figure 2B shows an alignment of the *lin-4*-complementary element (LCE) of *lin-28* with the seven elements of *lin-14* based on how they are predicted to base pair with *lin-4* RNA. Figure 2C shows a model for base pairing between *lin-4* RNA and the LCE of *lin-28*; the predicted structure is similar to those predicted to form between *lin-4* RNA and the *lin-14* mRNA (Lee et al., 1993; Wightman et al., 1993).

The presence of an LCE suggested that *lin-28* expression is regulated by *lin-4*. To test this possibility, we crossed the *lin-28:GFP* transgenic array into a *lin-4(e912)* mutant background to examine the expression of the fusion in the absence of *lin-4* activity. Unlike in a wild-type background, *lin-28:GFP* was expressed in late larval stages and adults of *lin-4(e912)* animals (Figure 4A; Table 1). L1 larvae of *lin-4(e912)* transgenic animals fluoresce no more intensely than L1s of *lin-4(+)* transgenic animals (data not shown) and older *lin-4(−); lin-28:GFP* larvae display relatively undiminished fluorescence compared to the L1 (Table 1; compare Figure 3 [L4] to Figure 4A).

The LCE Is Required for Regulation of *lin-28:GFP* Expression

To determine whether the LCE is necessary for the regulation of *lin-28*, we constructed a *lin-28:GFP* fusion lacking the 15 nt LCE (see Figure 2). In contrast to the animals carrying the wild-type *lin-28:GFP* transgene, animals containing the transgene without the LCE fluoresce at late developmental stages (Figure 4B; Table 1). The LCE-deleted transgene caused a dominant retarded phenotype characterized by proliferation of lateral hypodermal cells and prevention of adult lateral alae formation (Figure 4B; Table 1). We refer to the LCE-deleted transgene as *lin-28(gf):GFP* in reference to the apparent *lin-28* gain-of-function phenotype that it causes.

Overproliferation of lateral hypodermal cells was characteristic of every *lin-28(gf):GFP* animal we examined, although the degree of proliferation varied. An example of extensive hypodermal proliferation is shown in Figure 4B, where the animal had 66 lateral hypodermal seam cells on one side in a region where wild-type C. elegans at the same stage has twelve (Sulston and Horvitz, 1977). Supernumerary nuclei were also commonly observed

in the hypodermal syncytium in *lin-28(gf):GFP* animals (data not shown). We interpret this proliferation as reflecting the reiteration of the L2-specific lateral hypodermal cell division pattern at each stage after the L2. The L2-specific division pattern is characterized by two rounds of cell division, in contrast to the single cell division in each lateral hypodermal lineage that normally occurs in the L3, and it is this L2-specific pattern that fails to occur in *lin-28(lf)* animals (Sulston and Horvitz, 1977; Ambros and Horvitz, 1984). This finding is consistent with *lin-28(gf):GFP* inhibiting or delaying the succession of L2- to L3-specific cell fates in lateral hypodermal lineages. Similarly, vulva development in *lin-28(gf):GFP* animals is often delayed or completely prevented (data not shown). Thus, the lateral and ventral hypodermal retarded defects of *lin-28(gf)* resemble those of *lin-4* and *lin-14(gf)* mutants (Chalfie et al., 1981; Ambros and Horvitz, 1984; see Discussion).

lin-4 Regulates *lin-28* Activity Independently of *lin-14*

As a further test of the regulation of *lin-28* by *lin-4*, we determined whether *lin-4* affects developmental timing through a downstream regulator other than *lin-14*. We employed a *lin-14(gf,ts)* allele that permits an animal to develop essentially normally, but where *lin-14* level is insensitive to *lin-4* owing to deletion of 3′UTR sequences (Ambros and Horvitz, 1987; Wightman et al., 1991). This allele contains two mutations: *n355*, which is a deletion of the *lin-14* 3′UTR including the seven LCEs, and *n679ts*, which reduces the level of the deregulated *lin-14* activity to near normal levels at 20°C and to a reduced level at 25°C (Ambros and Horvitz, 1987). In this genetic background, we asked whether the developmental timing phenotype is affected by the presence or absence of *lin-4* activity. If *lin-4* acts solely through *lin-14*, then in a genetic background where *lin-14* activity is essentially normal and is insensitive to *lin-4* activity, removing *lin-4* activity should have no effect. However, we observed a significant enhancement of the retarded phenotype under these conditions, indicating that *lin-4* has at least one target other than *lin-14* that affects developmental timing (Table 2). The enhancement of the retarded phenotype does not occur in the presence of a *loss-of-function* allele of *lin-28*, consistent with *lin-28* being an additional target of *lin-4* (Table 2). In the context of our finding that *lin-28* is directly regulated by *lin-4*, we interpret these observations to indicate that the overexpression of *lin-28*, independently of *lin-14*, accounts for part of the retarded phenotype of a *lin-4* mutant.

lin-14 Regulates *lin-28:GFP* Expression

To determine whether *lin-14* activity affects *lin-28:GFP* expression, we examined the fluorescence of late stage larvae of *lin-4(−); lin-14(ts); lin-28:GFP* animals. The genotype of this strain is essentially identical to the *lin-4(−); lin-28:GFP* animals that fluoresce late in development (pictured in Figure 4A and listed in Table 1), except that a temperature-sensitive allele of *lin-14* was introduced by mating. Although the L1 fluorescence is unaffected in this strain, late stage larvae display diminished fluorescence at the restrictive temperature (Figure 4C; Table 1). *lin-14* activity therefore positively regulates *lin-28:GFP* expression in late stages in the *lin-4* mutant. This result indicates that *lin-14* activity becomes necessary for the maintainance of *lin-28* expression some time during or after the L1.

Discussion

We have found that *lin-28* encodes a predominantly cytoplasmic protein with a cold shock domain and retroviral-type zinc finger motifs, suggesting a mechanism of action of LIN-28 involving RNA binding and posttranscriptional regulation of target genes. We have also discovered a conserved *lin-4*-complementary element in the 3′UTR of *lin-28* and have determined that both the *lin-4* RNA and the LCE are necessary for the temporal regulation of a *lin-28:GFP* transgene. Deletion of the LCE of *lin-28* causes a retarded phenotype that resembles those of *lin-4(lf)* and *lin-14(gf)* mutants. This *lin-28(gf)* retarded phenotype demonstrates that the regulation of *lin-28* activity is critical to normal developmental timing.

Table 2. Regulation of Developmental Timing by *lin-4* Independent of *lin-14*

Strain	Temperature	Genotype[a]	Percentage of Adult Alae[b]	
			L3 Molt	L4 Molt
MT1388	20°C	*lin-28*(+) *lin-4*(+) *lin-14(gf,ts)*	0 (n = 93)	100 (n = 62)
VT785	20°C	*lin-28*(+) *lin-4*(−) *lin-14(gf,ts)*	0 (n = 270)	10 (n = 279)
VT799	20°C	*lin-28*(−) *lin-4*(+) *lin-14(gf,ts)*	100 (n = 81)	NA[c]
VT798	20°C	*lin-28*(−) *lin-4*(−) *lin-14(gf,ts)*	100 (n = 97)	NA[c]
MT1388	25°C	*lin-28*(+) *lin-4*(+) *lin-14(gf,ts)*	100 (n = 134)	
VT785	25°C	*lin-28*(+) *lin-4*(−) *lin-14(gf,ts)*	6 (n = 200)	
			L2/L3 Molts[d]	
VT799	25°C	*lin-28*(−) *lin-4*(+) *lin-14(gf,ts)*	97 (n = 67)	
VT798	25°C	*lin-28*(−) *lin-4*(−) *lin-14(gf,ts)*	100 (n = 140)	

[a] Full genotypes are listed in Experimental Procedures.

[b] Percent seam cells expressing adult lateral alae was determined by DIC optics as described (Ambros and Horvitz, 1987; Ambros, 1989).

[c] Animals of these strains do not undergo a fourth molt due to their precocious development.

[d] VT799 and VT798 animals were not observed to execute the L3 molt at 25°C, as is expected for an extreme *lin-28; lin-14* phenotype (Ambros, 1989). They were assayed at either the L2 molt, or at a stage equivalent to the L3 or L4 based on extent of gonad development. However, since the L3 molt was not monitored in all animals, these data could include some alae formed at an L3 molt in some of the animals.

Thus, the *lin-4* RNA coordinately regulates the expression of two developmental timing regulators, and the *lin-28* activity level is at least as important as that of *lin-14* in determining the succession of L2- to L3-specific cell fates. We have also found that *lin-14* activity affects the expression of *lin-28*, indicating that *lin-14* controls the timing of developmental events at least in part by controlling the level of *lin-28* activity. It remains to be demonstrated whether or not *lin-14* acts exclusively through *lin-28* to control the succession of L2- to L3-specific fates. The direct targets of *lin-28* may be few, such as one or more downstream developmental timing regulators, or may be many, such as cell cycle regulators, differentiation factors, and other effectors of the diverse developmental events under heterochronic gene control.

Structure and Function of LIN-28 Protein

Because LIN-28 is primarily localized to the cytoplasm and consists of two domains that contain putative RNA-binding motifs, it is likely to function in posttranscriptional regulation. Cold shock domains are so-called because bacterial cold shock proteins share the sequence motifs characteristic of this family; however, eukaryotic CSD proteins are not known to be involved in the cold shock response (Wistow, 1990; reviewed in Wolffe et al., 1992; Wolffe, 1994; Sommerville and Ladomery, 1996). The CSD includes RNP-1 and RNP-2 motifs, which are characteristic of the large RRM family of RNA-binding proteins, and structural analysis suggests that the CSD and RRM proteins may interact with nucleic acids in similar ways (Landsman, 1992; Schindelin et al., 1993; Oubridge et al., 1994; Schroder et al., 1995; Graumann and Marahiel, 1996). The CSD protein FRGY2 is a major component of messenger ribonucleoprotein particles (mRNPs) responsible for the masking of maternal mRNAs from translation in Xenopus oocytes and has been shown to be a sequence-specific RNA-binding protein (Tafuri and Wolffe, 1990; Deschamps et al., 1992; Murray et al., 1992; Ranjan et al., 1993; Bouvet et al., 1995) The CSD protein p50 is the major core protein of cytoplasmic mRNPs in rabbit reticulocytes (Evdokimova et al., 1995). These proteins are postulated to form mRNPs by binding mRNAs with a high protein-to-RNA ratio and render the mRNA inaccessible to translation machinery (Spirin, 1996).

LIN-28 resembles other eukaryotic CSD proteins in that its N-terminal CSD is coupled to a C-terminal domain that is also implicated in RNA binding (Ladomery and Sommerville, 1994; Murray, 1994). The C-terminal region of LIN-28 has two zinc finger motifs like those of retroviral nucleocapsid proteins that are involved in the specific binding and encapsidation of viral genomic RNA (Gorelick et al., 1988; Meric and Goff, 1989; Dannull et al., 1994; reviewed in Darlix et al., 1995). Retroviral-type zinc finger motifs are found also in diverse cellular proteins that act by binding single-stranded nucleic acids (e.g., Rajavashisth et al., 1989; Sato and Sargent, 1991). The only other proteins known that have both a CSD and retroviral-type zinc fingers, although they otherwise differ substantially from LIN-28, are the GRP2 proteins of plants, whose functions are not known (de Oliveira et al., 1990; Obokata et al. 1991; Kingsley and Palis, 1994).

Regulation of *lin-28* Expression by *lin-4* RNA

Based on the following criteria, we believe that *lin-28* is directly regulated by the *lin-4* RNA. First, *lin-4* affects developmental timing through downstream targets other than *lin-14*. Second, *lin-28* contains a 15 nt LCE in its 3′UTR that is predicted to be complementary to the *lin-4* RNA, and this element is completely conserved among *lin-28* homologs from three Caenorhabditis species. Third, the *lin-28* LCE resembles each of the seven *lin-4*-complementary elements in the *lin-14* 3′UTR that are necessary for the translational regulation of that gene by *lin-4*. Fourth, both *lin-4* and the single LCE of *lin-28* are necessary for the stage-specific down-regulation of *lin-28*. Finally, deletion of the LCE causes a retarded phenotype.

Our findings indicate that an LCE is capable of working in different mRNA contexts and that a single LCE is sufficient to put a gene under the control of *lin-4*. Other than the LCEs, we note no significant similarities between the 3′UTRs of *lin-28* and *lin-14*. The *lin-14* 3′UTR is 1600 nt, whereas that of *lin-28* is 529 nt, and each contains sequences that are conserved among homologs but are not similar between the two genes (Figure 2A; Ha et al., 1996). The fact that two different genes are regulated by *lin-4* suggests that the action of this unusual regulatory molecule is not restricted to the *lin-14* mRNA or protein. It is possible that genes other than *lin-14* and *lin-28* may be under *lin-4* control. More generally, there may be other *lin-4*-like RNAs with specificity different from that of *lin-4* that regulate target genes with shared roles in cell fate.

The *lin-28(gf)* Retarded Phenotype

By deleting the LCE from the 3′UTR of *lin-28*, we have discovered that a *gain-of-function* allele of *lin-28* can cause a retarded phenotype. Because we observe proliferation of lateral hypodermal seam cells, we interpret this retarded phenotype as the result of the reiteration of the L2-specific cell lineage pattern—the same pattern that fails to occur in a *lin-28(lf)* mutant. In the wild type, the seam cells divide at each larval stage, and in general, the anterior daughter joins the hypodermal syncytium and the posterior daughter remains a blast cell. Therefore, in those stages when the seam cells each divide only once, the number of seam cells stays constant as the number of syncytial nuclei increases. It is only in the L2 of the wild type that certain seam cells (descendants of V1–V4, V6) undergo two rounds of cell division, thus increasing the number of midbody seam cells (descendants of H2, V1–V6) from 7 to 12. It is this double cell division pattern characteristic of the L2 that is absent in *lin-28(lf)* mutants and is apparently reiterated in *lin-28(gf)* transgenic animals. This proliferative aspect of the phenotype of *lin-28(gf)* differs from that of *lin-14(gf)* alleles because *lin-14* controls the L1/L2 fate decision and therefore reiterates L1-specific fates (one cell division in the lateral hypodermal lineages) when deregulated. *lin-28* must regulate, directly or indirectly, specific

targets in the lateral hypodermal blast cells that control the pattern of cell divisions.

The retarded phenotype is not restricted to the lateral hypodermal cells because we have also noted the delay or prevention of vulva precursor cell (VPC) division and vulva development in *lin-28(gf)* animals. *lin-28* has been shown to control the entry of VPCs into S phase after a long G1 and their acquisition of competence to respond to inductive patterning signals (Euling and Ambros, 1996). A delay in vulva development is consistent with *lin-28* actively preventing VPC S phase and competence. The targets of *lin-28* in the vulva precursor cells may be different from those in the lateral hypodermis, or alternatively, *lin-28* may act through a common downstream regulator in both cell types.

The Heterochronic Gene Hierarchy

Our finding that the loss-of-function and gain-of-function phenotypes of *lin-28* are opposite (precocious and retarded, respectively) indicates that *lin-28* functions as a developmental timing switch affecting the L2/L3 cell fate decision. The control of *lin-28* activity is at least as important as that of *lin-14*, which is involved in the L1/L2 and the L2/L3 decisions (Ambros and Horvitz, 1987). Loss-of-function mutations and gain-of-function mutations of these genes result in precocious and retarded development, respectively, and each gene must be on early and off later for normal larval development to unfold (Figure 5). *lin-4* down-regulates both *lin-14* and *lin-28* and thereby controls two developmental timing switches in parallel. We have not yet resolved whether *lin-14* or *lin-28* is the more direct regulator of downstream targets controlling the L2/L3 fate decision, or whether the two gene products independently regulate the same targets.

We have found that *lin-14* is required for *lin-28:GFP* expression in a *lin-4* mutant background after the L1. Similarly, Arasu et al. have shown that *lin-28* is required for the expression of *lin-14* in the absence of *lin-4* regulation after the L1 (Arasu et al. 1991). Thus *lin-14* and *lin-28* become mutually dependent for their expression at some time during or after the L1. *lin-4* is off in the early L1 and abundant by the L2 (R. Feinbaum et al., unpublished data). In both cases, the requirement for the positive regulation is independent of *lin-4* activity, so neither gene is likely to oppose *lin-4* RNA directly. We propose a model whereby at least two kinds of changes in *lin-14* and *lin-28* regulation occur from the L1 to the L2: *lin-4* activity begins to down-regulate both genes, and *lin-14* and *lin-28* become mutually dependent on each other (Figure 5). This mutual positive regulation between *lin-14* and *lin-28* may serve to coordinate the decrease of both of these critical gene activities and allow the proper succession of L1- to L2- to L3-specific cell fates.

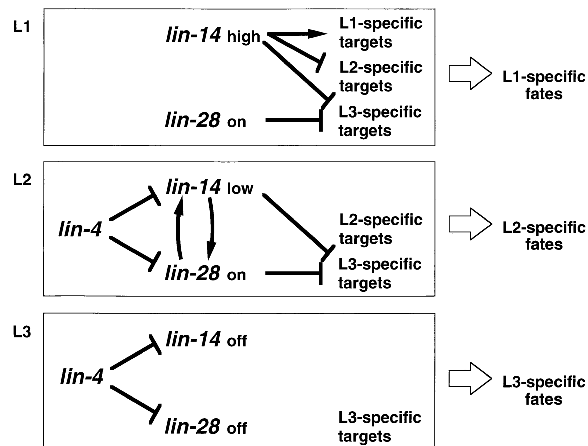

Figure 5. The Heterochronic Gene Hierarchy

A model for the succession of events in the first three larval stages of C. elegans development based on genetic epistasis and expression analysis of heterochronic genes. The stage-specific targets are unknown genes or gene products that cause cells to adopt stage-specific fates. A high level of *lin-14* is necessary for L1-specific fates, and this level is independent of *lin-28*. *lin-14*'s positive regulation of L1-specific targets and negative regulation of L2-specific targets may result from the same activity of LIN-14 protein. Both *lin-14* and *lin-28* activities are critical regulators of the succession of L2- to L3-specific cell fates. *lin-4* is a direct regulator of both *lin-14* and *lin-28*. When *lin-4* is on, both *lin-14* and *lin-28* are down-regulated, but each must retain a certain level of activity to allow L2-specific fates to occur. A mutual positive interaction between *lin-14* and *lin-28* contributes to the L2-specific activity of both genes. The regulation of *lin-14* and *lin-28* by each other could be indirect through unknown regulators. Although both are shown to negatively regulate L3-specific fates in parallel, we have not determined whether *lin-14* acts through *lin-28* to control the L2/L3 fate decision or *lin-14* regulates L3-specific targets independently. L3-specific fates occur later when both *lin-14* and *lin-28* activities become fully reduced.

Experimental Procedures

Nematode Methods

Nematode strains were grown and maintained as described (Wood et al., 1988). All animals were grown at 20°C unless otherwise indicated. The following strains were used: MT1388 *lin-14(n355n679ts)*, VT284 *lin-14(ma135)/szT1*, VT616 *lin-28(n719) unc-29(n1072); nDp4*, VT689 *lin-4(e912) unc-4(e120)/mnC1; him-5(n1440)*, VT785 *lin-4(e912) unc-4(e120); lin-14(n355n679ts)*, VT798 *lin-28(n947); lin-4(e912) unc-4(e120); lin-14(n355n679ts)*, VT799 *lin-28(n947); lin-14(n355n679ts)*, VT800 *maEx149*, VT801 *lin-14(n179ts); maEx149*, VT802 *lin-4(e912); maEx149*, VT803 *lin-28(n719) unc29(n1072); nDp4; maEx150*, and VT805 *lin-4(e912) unc-4(e120); lin-14(n179ts); maEx149*. *maEx149* was generated by coinjecting *lin-28:GFP* (pVT#218; 20 μg/mL), *rol-6(su1006)* (pRF4; 100 μg/mL), and *unc-36* (RlpI; 50 μg/mL). *maEx150* was generated by coinjecting *lin-28(gf):GFP* (pVT#221; 20 μg/mL), *rol-6(su1006)* (pRF4; 100μg/mL), and *unc-36* (RlpI; 50 μg/mL). *maEx151* was generated by coinjecting *lin-28(gf):GFP* (pVT#221; 20 μg/mL), *unc-36* (RlpI; 50 μg/mL), and *ncl-1* (C33C3; 100 μg/mL). RlpI and C33C3 were used to improve transformation efficiency and expression consistency. VT800, VT802, and VT805 were made by crossing VT689 males with VT801. VT803 was made by transforming VT616.

Transformation Rescue

Clones were tested for rescue of the *lin-28* mutant phenotype by transformation of VT616 using a clone of *rol-6(su1006)* as a coinjection marker (Mello et al., 1991). *lin-28* mutant animals are unable to lay eggs and develop adult lateral alae precociously in the L4 stage (Lin); rescued animals are able to lay eggs and have no precocious alae (nonLin). A clone was scored as positive for rescue if Unc nonLin animals were identified among the F1 or F2 progeny. A clone was negative for rescue if all Rol Unc animals examined were Lin. The yeast artificial chromosome Y37F9 was purified from a CHEF gel using low-gelling temperature agarose, agarase digestion, and a spin microconcentrator. Cosmids were prepared using a kit by Qiagen.

Cloning and Sequence Analysis

Cosmids and YACs were obtained from the C. elegans genome consortium, via The Sanger Centre, Cambridge, UK. Subclones were made in Bluescript SK(+). C. elegans *lin-28* cDNA was isolated from a library provided by Robert Barstead. cDNA libraries of C. vulgaris and C. remanei were constructed by Philip Olsen and R. C. L. (unpublished data) from strains provided by Scott Baird. The relationships among C. elegans, C. remanei, and C. vulgaris are discussed in Fitch et al. (1995). Libraries were probed with the 6 kb PstI genomic fragment containing *lin-28* (Figure 1A; Ausubel et al., 1994). Sequence was obtained on an ABI 373 automated sequencer and analyzed by Sequencher (Gene Codes), BLAST (Altschul et al., 1990) via the National Center for Biotechnology Information, Genefinder (L. Hillier and P. Green, unpublished data) via ACEDB (R. Durbin and J. Theirry-Meig, unpublished data; available at http://www.sanger.ac.uk), the GCG Sequence Analysis Software Package by Genetics Computer Group, Incorporated (Devereux et al. 1984), and MulFold (Jaeger et al., 1989).

Plasmid Constructions

The 4.6 kb *lin-28*-rescuing genomic fragment (EcoRV–NgoMI) was cloned into pBluescript SK(+) (EcoRV–XmaI). The GFP coding region was amplified from pPD95.02 (Chalfie et al., 1994; A. Fire et al., personal communication) and inserted at the 3′ end of the *lin-28* ORF in the 4.6 kb rescuing genomic fragment, using an XmaI site introduced into the last codon and an XbaI site 9 nt away in the 3′UTR, to generate pVT#218. The 15 nt LCE deletion in pVT#221(*lin-28[gf]:GFP*) was made by generating an altered SphI–EagI fragment by PCR amplification and replacing the corresponding fragment in pVT#218, and was confirmed by sequencing.

Microscopy and Photography

Images of living animals anesthetized with sodium azide were captured with an Optronics integrating CCD camera and the built-in video board of a Power Macintosh. Fluorescence images were taken with a 2 s exposure, and DIC images were taken with an automatic exposure. Figures were assembled using Adobe Photoshop. The color of the DIC images was removed and their contrast increased to help visualize nuclei; otherwise, the images are unaltered.

Acknowledgments

We are grateful to Susan Euling, Rhonda Feinbaum, and Deborah Marvel for their work toward locating *lin-28*; Paul Maines for strains and mapping data; Thomas Burglin for *ceh-6* mutant; David Eisenmann, Susan Euling, Ann Rougvie, and Paul Sternberg for *lin-28* alleles; Alan Coulson and the C. elegans genome consortium for cosmids and YACs; Diane Church for advice on purifying YACs; Mark Metzstein and Mark McPeek for assistance with sequence analysis; Philip Olsen for cDNA libraries and the epifluorescence dissecting microscope; Richard Roy for RNA; and Yang Hong, Eric Lambie, Philip Olsen, and Richard Roy for discussions. This work was supported by a US Public Health Service National Research Service postdoctoral fellowship (E. G. M.) and a US Public Health Service research grant GM34028 (V. A.). Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the NIH National Center for Research Resources.

Received November 6, 1996; revised January 30, 1997.

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Genbank Accession Numbers

The accession numbers for the sequences reported in this paper are U75912 (C. elegans cDNA), U75913 (C. remanei cDNA), U75914 (C. vulgaris cDNA), and U75915 (C. elegans genomic DNA).