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Stage-specific accumulation of the terminal differentiation factor LIN-29 during *Caenorhabditis elegans* development

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SUMMARY

The *Caenorhabditis elegans* gene *lin-29* is required for the terminal differentiation of the lateral hypodermal seam cells during the larval-to-adult molt. We find that *lin-29* protein accumulates in the nuclei of these cells, consistent with its predicted role as a zinc finger transcription factor. The earliest detectable LIN-29 accumulation in seam cell nuclei is during the last larval stage (L4), following the final seam cell division, which occurs during the L3-to-L4 molt. LIN-29 accumulates in all hypodermal nuclei during the L4 stage. The time of LIN-29 appearance in the hypodermis is controlled by the heterochronic gene pathway: LIN-29 accumulates in the hypodermis abnormally early, during the third larval stage, in loss-of-function *lin-14*, *lin-28* and *lin-42* mutants, and fails to accumulate in hypodermis of *lin-4* mutants. LIN-29 also accumulates stage-specifically in the nuclei of a variety of non-hypodermal cells during development. Its accumulation is dependent upon the upstream heterochronic genes in some, but not all, of these non-hypodermal cells.

Key words: *lin-29*, heterochronic, *Caenorhabditis elegans*, terminal differentiation

INTRODUCTION

The nematode *C. elegans* provides a unique opportunity to investigate the timing mechanisms that govern specific cell fate decisions in a developing organism. The ability to observe and track every cell during development (Sulston and Horvitz, 1977) has allowed the identification and isolation of animals with mutations in genes that provide temporal information, the heterochronic genes (Ambros and Horvitz, 1984).

The heterochronic genes control the relative timing of stage-specific events that occur as the worm develops through four post-embryonic larval stages (L1-L4) into an adult (Ambros and Horvitz, 1984). Our present work investigates one of these events: the terminal differentiation of the lateral hypodermal seam cells. The lateral seam cells divide in a stem cell-like pattern during the first three larval molts (Fig. 1A). During the final molt they terminally differentiate; they exit the cell cycle and fuse together to form the lateral seam syncytia. Each seam syncytium contributes to the synthesis of the cuticle, and secretes a set of adult-specific cuticular ridges, adult alae, that extend the length of the cuticle on each side (Singh and Sulston, 1978). In addition to the morphological distinction between the larval and adult cuticles, there are also biochemical differences, owing to the stage-specific expression of certain cuticle collagen genes (Cox and Hirsh, 1985; Liu et al., 1995).

Mutations in the heterochronic genes *lin-4*, *lin-14*, *lin-28*, *lin-29* and *lin-42* alter the timing of seam cell terminal differentiation (Ambros and Horvitz, 1984; Liu, 1990). Loss-of-function *lin-14*, *lin-28* and *lin-42* mutations cause precocious terminal differentiation, resulting in 'larvae' with characteristically adult cuticle. Conversely, in *lin-4*, *lin-29* and gain-of-function *lin-14* mutants, hypodermal seam cell terminal differentiation fails to occur and sexually mature 'adults' develop with larval-type cuticle. The larval program of seam cell division and larval-type cuticle synthesis is repeated in these animals during supernumerary molts.

Epistasis analysis has been used to generate a model for the action of the heterochronic genes in controlling seam cell terminal differentiation (Ambros, 1989; see Fig. 1B). According to this model, *lin-4* negatively regulates *lin-14* and *lin-28*, and these genes, in turn, negatively regulate *lin-29*. *lin-42* also acts as a negative regulator of *lin-29*, although its position with respect to the other heterochronic genes is not precisely understood (Liu, 1990). Among these five genes, *lin-29* is the most direct regulator of seam cell terminal differentiation (Fig. 1B). In order to understand how seam cell terminal differentiation is timed, we must determine how *lin-29* activity is restricted to the final larval stage by the action of the upstream heterochronic genes.

The cloning and molecular analysis of *lin-29* has revealed that it can encode two proteins, each containing the same five $(Cys)_2$-$(His)_2$ zinc finger domains (Rougvie and Ambros, 1995). Thus, LIN-29 probably acts by controlling the transcription of genes involved in seam cell terminal differentiation. Among the target genes for LIN-29 regulation are collagen genes that are expressed in a stage-specific fashion (Liu et al., 1995; Rougvie and Ambros, 1995). Certain collagen genes normally expressed only during larval stages exhibit continued expression in *lin-29* mutant adults, whereas collagen genes activated in wild-type adults are not activated in *lin-29* mutants (Liu et al., 1995). In vitro binding studies show that

LIN-29 binds to promoter sequences necessary for correct in vivo expression of at least some of these collagen genes (Rougvie and Ambros, 1995). Not all of the observed collagen gene mis-expression in *lin-29* mutants is restricted to the lateral seam (Liu et al., 1995), suggesting that *lin-29* also functions in other hypodermal cells. For example, normal expression of *col-19* begins during the L4-to-adult molt in the lateral seam, the main body hypodermis, and the hypodermal syncytia of the head and tail (Liu et al., 1995). In *lin-29* mutants, expression of *col-19* is not detected in any of these hypodermal cells. Studies of the adult-stage cuticle ultrastructure also suggest that the defects in *lin-29* mutants are not restricted to the lateral seam cells (Ambros and Horvitz, 1984). Taken together, these results suggest that there is a focus of *lin-29* activity in the hypodermis during the late L4 stage.

In contrast to the predicted time and place of LIN-29 action in the L4-stage hypodermis, RNA blot experiments detect *lin-29* expression at much earlier developmental times. *lin-29* transcripts are first detected during the L1 stage (Rougvie and Ambros, 1995), long before *lin-29* activity is required to trigger hypodermal seam cell terminal differentiation. In order to understand how *lin-29* activity is controlled, we must reconcile the early expression of *lin-29* mRNA with the late hypodermal phenotype observed in *lin-29* null mutants. Specifically, is *lin-29* protein accumulation restricted to the L4-stage hypodermis or is it present at earlier developmental stages or in other cell types?

Here we show that hypodermal cell nuclei accumulate *lin-29* protein in a temporally restricted fashion, beginning during the L4 stage, and we describe the effects of mutations in the upstream heterochronic genes on the time of LIN-29 accumulation. We also show that LIN-29 accumulation is not limited to hypodermal lineages; other cell types, including specific muscle and neuronal cells, accumulate LIN-29. LIN-29 accumulates prior to the L4 stage in many of these cells. Intriguingly, we find that expression of *lin-29* in a subset of these non-hypodermal cells is independent of the heterochronic gene pathway.

MATERIALS AND METHODS

Nematode strains and culture conditions

C. elegans were cultured as described by Brenner (1974). Mutant alleles are loss-of-function unless otherwise noted. Alleles used were: wild-type N2 var. Bristol (Brenner, 1974), *lin-14(n355*gf*)*, *lin-14(n536*gf*)*, *lin-28(n947)*, *lin-29(n546)*, *lin-29(n333)*, *lin-29(n836)* (Ambros and Horvitz, 1984), *lin-14(ma135)* (Ambros, 1989), *lin-42(n1089)* (Liu, 1990), *lin-29(n1368)*, *lin-29(n1440)* (Papp et al., 1991). *lin-29(ga93)* (a gift from D. Eisenmann and S. Kim) and *lin-29(ve5)* were isolated following ethyl methanesulfonate screens and behave as null alleles.

Preparation of antibody to LIN-29 protein

lin-29 sequences from +1362 to +2338 (Rougvie and Ambros, 1995) were cloned into the pGEX-2T expression vector (Smith and Johnson, 1988). This construct produces an in-frame fusion protein in which the C-terminal 86 amino acids of LIN-29 are fused to the C terminus of glutathione S-transferase (GST). The fusion protein was soluble when produced in *E. coli* DH5α, and was purified for use as an immunogen by binding to glutathione-coupled agarose beads (Sigma), eluting with free glutathione and electrophoresing on an SDS-polyacrylamide gel. The protein band was visualized by staining in 4 M sodium acetate (Higgins and Dahmus, 1979) and excised. The gel slices were macerated in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na_2HPO_4, 1.4 mM KH_2PO_4), mixed with RAS adjuvant (RIBI ImmunoChem Research, Inc.) and injected into two New Zealand White rabbits. The rabbits were boosted at 3, 6 and 9 weeks and bled at 11 weeks. The serum from each rabbit had a high titre against the fusion protein. LIN-29-specific antibodies were affinity-purified twice against a maltose binding protein::LIN-29 (MBP::LIN-29) fusion protein that had been electrophoresed and blotted onto nitrocellulose (Olmsted, 1981).

Preparation of worms for indirect immunofluorescence

Worms were prepared using a modification of a protocol developed by Finney and Ruvkun, (1990; Miller and Shakes, 1995). Briefly, staged populations of worms were washed several times with M9 (Brenner, 1974) over 30 minutes, then washed once in distilled water. Ruvkun Fixation Buffer (2×: 160 mM KCl, 40 mM NaCl, 20 mM ethylene glycol-bis (β-aminoethyl ether) N, N, N′, N′-tetraacetic acid (EGTA), 10 mM spermidine HCl, 30 mM sodium piperazine-N, N′-bis [2-ethanesulfonic acid] (pH 7.4) (PIPES), 50% (v/v) methanol)

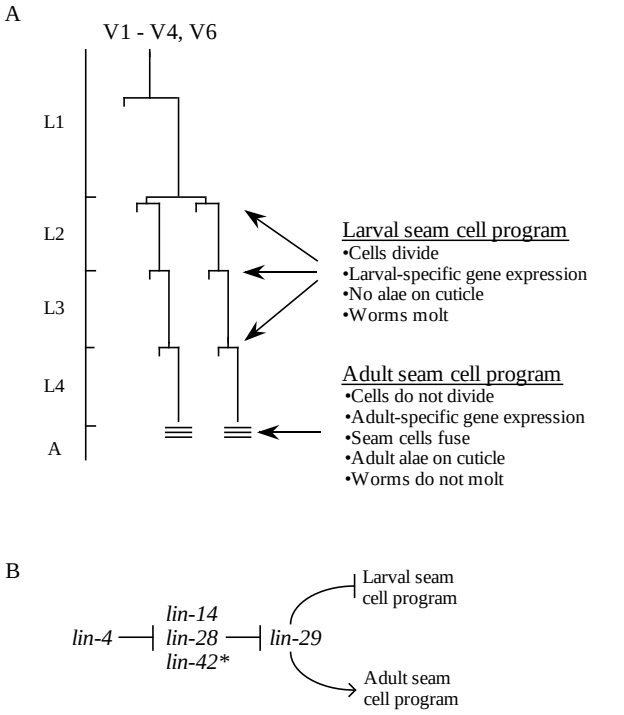

Fig. 1. Lateral hypodermal seam cell terminal differentiation and its control by the heterochronic genes. (A) The cell lineage pattern is shown for the hypodermal seam cells V1-V4 and V6. The hypodermal blast cells H1, H2, V5 and T also give rise to cells that contribute to the lateral seam (Sulston and Horvitz, 1977), but they undergo slightly different cell division patterns and have been omitted for simplicity. The triple horizontal bars indicate that the cells have terminally differentiated: they have fused and synthesized adult alae. (B) The heterochronic gene hierarchy for control of lateral hypodermal seam cell terminal differentiation. *lin-29* is the most direct known regulator of the switch from the larval to the adult seam cell program. *lin-14*, *lin-28* and *lin-42* are negative regulators of *lin-29* activity (Ambros, 1989; Liu, 1990). The asterisk indicates that the precise position of *lin-42* in this pathway is unknown. Although *lin-29* is completely epistatic to *lin-42(n1089)*, *lin-42(n1089)* is only partially epistatic to *lin-4* (Liu, 1990). This result may indicate that *lin-42(n1089)* is not a null allele.

was added to a final concentration of 1×, and formaldehyde was added to a final concentration of 2%. The mixture was frozen in a dry ice/ethanol bath, thawed and incubated on ice for 3.5 hours with occasional inversion. The worms were washed with Tris/Triton buffer [100 mM Tris-Cl (pH 7.4), 1% (v/v) Triton X-100, 1 mM ethylenediaminetetraacetic acid (EDTA)], and incubated in Tris/Triton/1% β-mercaptoethanol at 37°C with gentle agitation for 4 hours. The worms were washed with BO_3 buffer [0.01 M H_3BO_3 (pH 9.2), 0.01 M NaOH], and incubated in BO_3/10 mM dithiothreitol at 37°C with gentle agitation for 15 minutes. The worms were washed with BO_3 buffer, and incubated in BO_3/0.3% (v/v) H_2O_2 at room temperature with gentle agitation for 15 minutes, and then washed in BO_3 buffer and incubated in Buffer B [PBS, 0.1% (w/v) bovine serum albumin (BSA), 0.5% Triton X-100, 0.05% sodium azide, 1 mM EDTA] at room temperature for 30 minutes with gentle agitation. The worms were stored in Buffer A [Buffer A = Buffer B with 1% (w/v) BSA] at 4°C. These preparations are stable for several months.

Indirect immunofluorescence

Samples of fixed worms were incubated with twice-affinity-purified anti-LIN-29 antibody at a final dilution of 1:7.5 in Buffer A at room temperature for 8-12 hours with gentle agitation. The worms were then washed seven times in Buffer B over a 1-hour period and incubated in FITC-conjugated goat anti-rabbit antibody (Cappel) diluted 1:400 in Buffer A for 3.5-4 hours. Excess secondary antibody was removed by eight washes in Buffer B over a minimum of 2 hours. Hoechst dye was added to the penultimate wash at a concentration of 12.5 ng/ml to allow visualization of nuclei. The monoclonal antibody MH27 (kindly provided by M. Hresko and R. Waterston, Washington University) was used at a final concentration of 1:1500. When MH27 was used, FITC-conjugated goat anti-mouse (Cappel) secondary antibodies were used at a 1:600 dilution. Worms were mounted in 1 mg/ml ρ-phenylene diamine (in 1×PBS and 90% glycerol) and visualized by epifluorescence microscopy using a Nikon Microphot-FXA. Developmental stages of individual worms were assessed by examining the extent of gonad development, which is largely unaffected by the heterochronic mutants we have studied (Ambros and Horvitz, 1984). Cell identification and nomenclature is based on the classification by Sulston and Horvitz (1977) and Sulston et al. (1988).

Immunofluorescence controls

The specificity of the affinity-purified LIN-29 antibody and its resultant staining pattern were examined in several control experiments. First, samples stained with secondary antibodies alone showed only a uniform, low level of background fluorescence relative to samples incubated with both primary and secondary antibodies. Second, the whole rabbit serum affinity-purified against a $(His)_6$::LIN-29 fusion protein produced the same staining pattern as the serum purified against the MBP::LIN-29 fusion protein. Third, serum immuno-depleted against the MBP::LIN-29 fusion protein showed little or no staining when used at the same dilution. Similarly, preincubation of LIN-29 antibody, affinity purified against the MBP::LIN-29, with the GST::LIN-29 fusion protein at approximately 200 μg/ml, showed little or no staining when used at the same dilution. Preincubation of LIN-29 antibody with GST alone had no effect. Fourth, single label experiments with LIN-29 antibody or MH27 antibody alone demonstrated that their staining patterns are spatially non-overlapping.

The affinity-purified LIN-29 antibody detected the GST::LIN-29, $(His)_6$::LIN-29 and MBP::LIN-29 fusion proteins on western blots, but failed to detect proteins in whole worm or nuclear extracts using a variety of immuno-transfer and signal detection methods (data not shown).

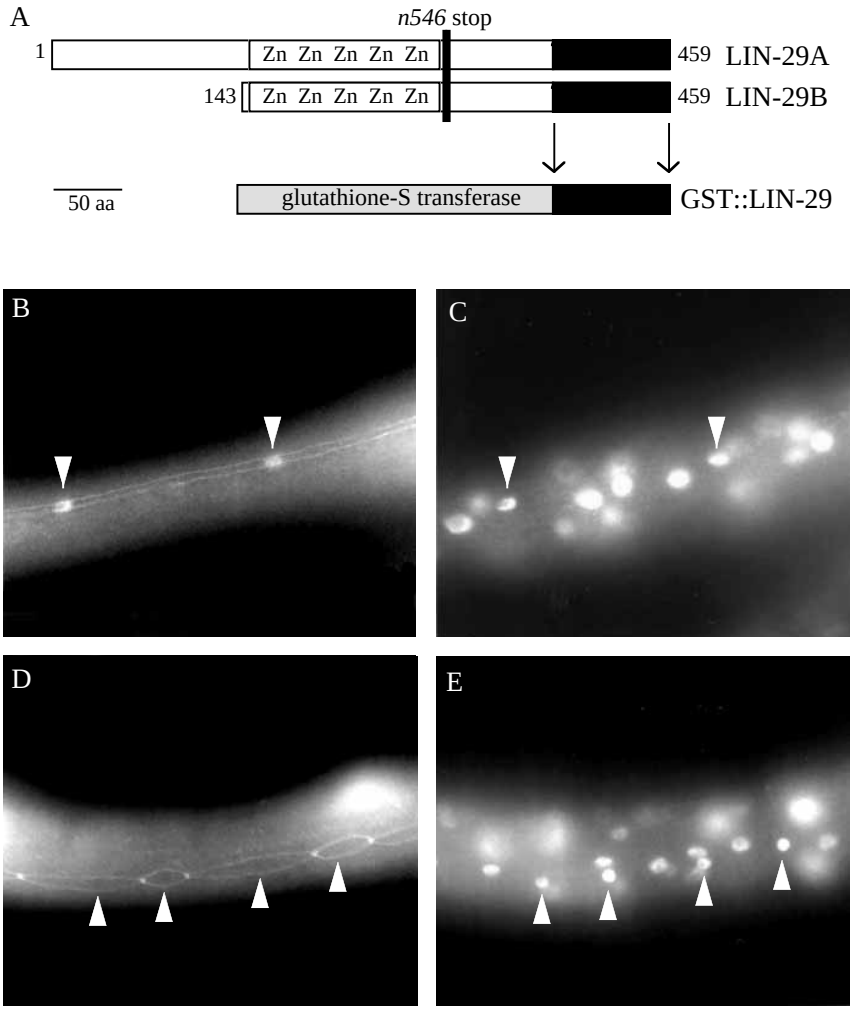

Fig. 2. LIN-29 is a nuclear protein. (A) Diagrams of the two *lin-29* predicted proteins are shown. LIN-29A is a 459-amino acid protein. LIN-29B is identical except for the lack of the N-terminal 142 amino acids. Zn indicates the position of each zinc finger. The 86-amino acid C-terminal domain included in the GST::LIN-29 fusion protein shown below is indicated by a filled box. The position of the premature stop in *lin-29(n546)* is indicated by a vertical line. (B-E) Arrowheads indicate the positions of lateral hypodermal seam cell nuclei. Unless otherwise noted, anterior is to the left in all figures and left lateral views are shown. (B) Wild-type adult stained with anti-LIN-29 and MH27 antibodies and visualized by indirect immunofluorescence. LIN-29 has accumulated in the seam cell nuclei. MH27 outlines the fused hypodermal seam, which is seen as two parallel lines. (C) Hoechst staining of worm in B to show nuclei. (D) *lin-29(n546)* mutant adult stained with anti-LIN-29 and MH27 antibodies. LIN-29 is not detected. Even though this is an adult, based upon gonadal development, the MH27 staining indicates that the seam cells have failed to fuse. (E) Hoechst staining of worm shown in D.

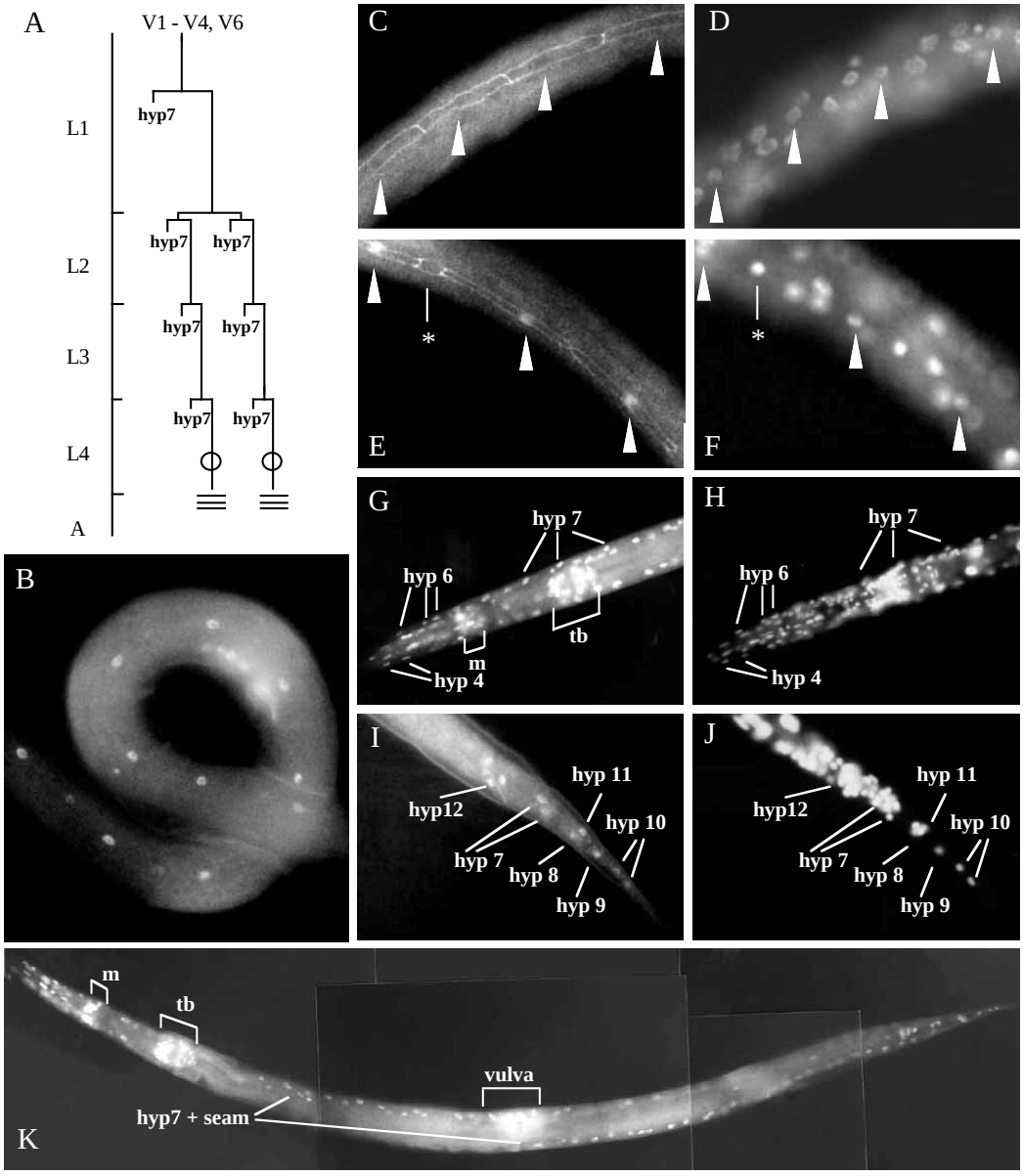

Fig. 3. Accumulation of LIN-29 during development. (A) The wild-type cell lineage pattern is shown for V1-V4 and V6 (Sulston and Horvitz, 1977). The seam cells that accumulate LIN-29 are indicated by open circles. (B) Wild-type L4-stage hermaphrodite stained with anti-LIN-29 antibodies. LIN-29 accumulates in seam cell nuclei positioned along the lateral seam (open circles in A). The worm is curled, with anterior to the left. The row of tightly spaced nuclei that have accumulated LIN-29 are the progeny of the sex myoblasts (see Fig. 6E). (C) Wild-type L3-stage hermaphrodite co-stained with anti-LIN-29 antibodies and MH27. MH27, in conjunction with the Hoechst staining (D), unambiguously identifies the lateral seam cells and their nuclei (arrowheads). LIN-29 is not detected in the L3 seam. (E) Wild-type L4-stage hermaphrodite co-stained with anti-LIN-29 antibodies and MH27. LIN-29 accumulates in L4-stage seam cell nuclei (arrowheads). The asterisk indicates the nucleus of a cell that is about to fuse to hyp7, and has not yet accumulated LIN-29. (F) Hoechst staining in the same focal plane. (G) The head of a wild-type animal during the L4-molt stained with anti-LIN-29 antibodies. Many nuclei in the head accumulate LIN-29 during the L4 stage. This includes all of the hypodermal nuclei, a subset of which are indicated. All of the nuclei that fuse into hyp7 (marked hyp7 in A) accumulate LIN-29 during the L4 stage. A subset are indicated in this figure. Nuclei of the pharynx, particularly the metacorpus (m) and terminal bulb (tb), accumulate LIN-29 (see also Fig. 6A). (H) Hoechst staining in the same focal plane. (I) The tail of a wild-type animal during the L4 molt stained with anti-LIN-29 antibodies. All nuclei of the tail hypodermis [hyp8-hyp12 (hyp12=P12.pa; Sulston et al., 1988); K.a; see Fig. 6C] accumulate LIN-29. Two nuclei of hyp7 are also present in this focal plane. (J) Hoechst staining of the same focal plane. (K) A wild-type animal during the L4-molt stained with anti-LIN-29 antibodies. Two rows of nuclei from hyp7 and the seam are visible in this focal plane and are indicated. m and tb, metacorpus and terminal bulb of the pharynx, respectively. LIN-29 accumulation in the vulval region (out of the plane of focus) is also indicated.

RNA analysis

RNA analysis was performed on synchronous populations of L4-stage worms as previously described (Liu et al., 1995; Rougvie and Ambros, 1995). Densitometry was performed with a Personal Densitometer SI (Molecular Dynamics) and the IPLab Gel Analysis System (Signal Analytics Corp.).

lin-29::lacZ fusions and analysis of β-galactosidase accumulation

We constructed *lin-29A* and *lin-29B promoter::lacZ* fusions using a modification of vectors pPD21.28 and pPD22.11, respectively (Fire et al., 1990), in which the *unc-54* 3′ end was replaced with the *lin-29* 3′ end. In *lin-29A::lacZ* a 4.0 kb genomic DNA fragment containing 162 bp of Exon 1 of lin-29A and extending 3.9 kb upstream was fused to *lacZ*. The lin-29B promoter appears to reside within the large fourth intron of lin-29A (Rougvie and Ambros, 1995). *lin-29B::lacZ* contains a 5.6 kb genomic fragment from the 3′ end of this intron and includes 2 kb upstream of the 37 bp non-coding lin-29B-specific exon 1b (Rougvie and Ambros, 1995) through the first 38 bp of exon 6b (see Fig. 9A for exon numbering). For each construct we established five independent transgenic lines, each carrying an extrachromosomal array of the reporter gene and pRF4

(*rol-6(su1006)*; Mello et al., 1991) as a transformation marker. We generated two independent integrated lines from a chosen extrachromosomal line. For each reporter fusion, the extrachromosomal and integrated arrays showed similar expression patterns. Expression of the *lin-29::lacZ* transgenes was monitored by indirect immunofluorescence as described above, using a monoclonal antibody against β-galactosidase (Promega).

RESULTS

To determine the complete spatial and temporal distribution of *lin-29* protein during development, we prepared an antibody that recognizes both predicted *lin-29* protein products, LIN-29A and LIN-29B. LIN-29A and LIN-29B contain the same five zinc fingers and differ only at their N termini, with LIN-29A containing an additional 142 amino acids (Rougvie and Ambros, 1995; Fig. 2A). We raised polyclonal antibodies against a glutathione S-transferase::LIN-29 fusion protein (GST::LIN-29) containing the C-terminal 86 amino acids of LIN-29 and affinity-purified them as described in Materials and Methods. The LIN-29 peptide fragment excludes the zinc finger domains to minimize possible cross-reactivity with other zinc finger proteins. Antibodies against this shared domain should reveal the complete pattern of LIN-29 accumulation when used in whole-mount immunolocalization studies.

LIN-29 protein accumulates stage-specifically in hypodermal nuclei

The anti-LIN-29 antisera recognized a nuclear antigen in lateral hypodermal seam cells in wild-type *C. elegans* (Fig. 2B), consistent with the predicted role of LIN-29 as a transcription factor. The specificity of the antibody preparation was demonstrated by the lack of signal in three out of seven independent *lin-29* mutants that are apparent null alleles by genetic criteria (Ambros and Horvitz, 1984; A. Rougvie, unpublished observations). One allele, *lin-29(n546)*, contains a premature stop codon that terminates the open reading frame prior to the domain used to generate the fusion protein (Rougvie and Ambros, 1995) (Fig. 2A), and thus the antisera should not react specifically with the *lin-29(n546)* protein product. We have used the monoclonal antibody MH27 to assay seam cell terminal differentiation. Because this antibody recognizes an antigen in hypodermal cell junctions (Waterston, 1988), it effectively outlines hypodermal cells and allows us to visualize seam cell fusion. Double-staining of fixed *lin-29(n546)* animals with anti-LIN-29 antibodies and MH27 demonstrates that the seam in these mutant adults is unfused (Fig. 2D). Furthermore, although LIN-29 was not detected, the MH27 staining shows that the preparation was permeable to antibodies.

The anti-LIN-29 antibodies revealed a differential pattern of *lin-29* protein accumulation during development. LIN-29 was not detected in hermaphrodite hypodermal nuclei prior to the L4 stage (Fig. 3C). Although we cannot rule out the possibility that LIN-29 is distributed diffusely throughout the hypodermal cytoplasm during the L3 and younger stages, we detected no difference in hypodermal cell staining when these animals were incubated with secondary antibody alone, relative to animals incubated with both primary and secondary antibodies. The earliest LIN-29 accumulation in lateral seam cell nuclei that we detected was shortly after their final division, during the L3- to L4-molt (Fig. 3B,E). LIN-29 accumulated in these hypodermal nuclei during the L4 stage, and remained detectable in the adult animal. At approximately the same time, LIN-29 was detected in the hypodermal nuclei of the head (hyp1-hyp6), tail (hyp8-hyp12, K.a) (Figs 3G,I, 6C), and the large hypodermal syncytium covering most of the animal (hyp7) (Fig. 3G,I,K). The accumulation of LIN-29 in hyp7 was typically observed following accumulation in the

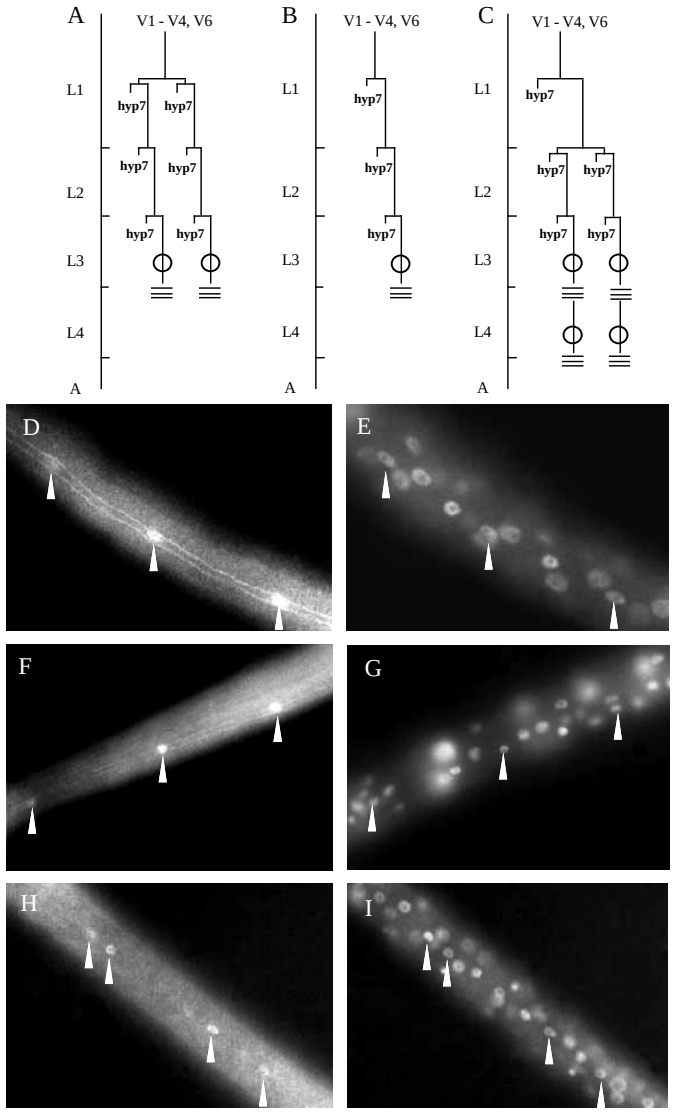

Fig. 4. LIN-29 accumulation in precocious heterochronic mutants. Animals were staged by examining the extent of gonad development, which is essentially unaffected by mutations in *lin-14*, *lin-28* and *lin-42*. Arrowheads indicate seam cell nuclei that have accumulated LIN-29. (A-C) The cell lineages of typical V1-V4 and V6 cells are shown for loss-of-function *lin-14*, *lin-28* (Ambros and Horvitz, 1984) and *lin-42* (Liu, 1990) mutants, respectively. (See Fig. 1 for details). (D) A third larval stage *lin-14(ma135)* animal co-stained with anti-LIN-29 antibodies and MH27. LIN-29 is present in the precociously fused seam. (E) Hoechst staining of same focal plane. (F) A third larval stage *lin-28(n947)* animal co-stained with anti-LIN-29 antibodies and MH27. LIN-29 is present in the precociously fused seam. (G) Hoechst staining. (H) A third larval stage *lin-42(n1089)* animal stained with anti-LIN-29 antibodies. LIN-29 is present precociously in the seam. (I) Hoechst staining.

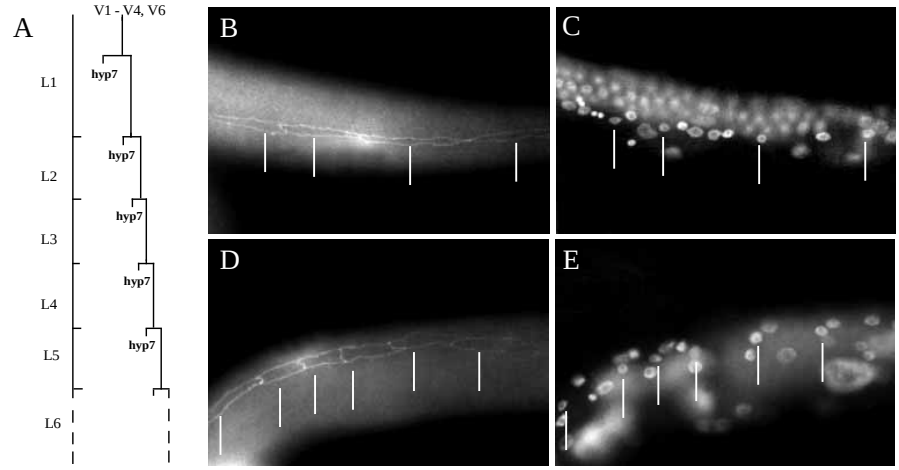

Fig. 5. Effects of retarded heterochronic mutants on *lin-29* protein accumulation. (A) Cell lineage of a representative V1-V4 and V6 cell is shown for loss-of-function *lin-4* and gain-of-function *lin-14* alleles (Ambros and Horvitz, 1984). The seam cells fail to terminally differentiate during the L4-molt and instead reiterate the larval pattern of cell division during supernumerary molts (L5, etc.). (B,D) 'L5-stage' worms co-stained with anti-LIN-29 antibodies and MH27. (C,E) Corresponding focal planes showing Hoechst-staining nuclei. Vertical lines indicate positions of seam cell nuclei in the unfused lateral seam. LIN-29 accumulation is not observed. (B,C) A *lin-4(e912)* animal. (D,E) A *lin-14(n355*gf) animal.

seam, and the signal was usually less intense. In summary, LIN-29 accumulates stage-specifically, beginning during the L4 stage and persisting into the adult stage, in all hypodermal cell nuclei of the worm. LIN-29 was also detectable in late-stage gravid adults, at a time when *lin-29* mRNAs are greatly reduced in abundance (Rougvie and Ambros, 1995).

LIN-29 protein accumulation is temporally controlled by the upstream heterochronic genes

In worms carrying loss-of-function alleles of *lin-14*, *lin-28* or *lin-42*, seam cell terminal differentiation occurs during the third molt, which is one stage earlier than in wild type (Fig. 4A-C). Instead of completing a final round of cell division during the third molt, the seam cells in these mutants fuse, and synthesize an adult-type cuticle. In *lin-14* and *lin-28* mutants, this third molt is the final molt (Ambros, 1989); however, *lin-42(n1089)* mutants can complete the third molt and proceed to the fourth (Liu, 1990).

We investigated whether the temporal changes in hypodermal cell terminal differentiation observed in these mutants correlate with changes in LIN-29 accumulation. The precocious mutants *lin-14(ma135)*, *lin-28(n719)* and *lin-42(n1089)* accumulate LIN-29 in seam cell nuclei prematurely, following the second molt (Fig. 4D,F,H). Precocious accumulation of LIN-29 occurs throughout the hypodermis in these mutants (not shown). In addition, we occasionally observed LIN-29 in L2-stage hypodermal nuclei of *lin-28* mutant worms, consistent with the occasional terminal differentiation of these cells during the L2 molt (Ambros, 1989).

Lateral seam cell terminal differentiation is delayed or eliminated in the retarded heterochronic mutants *lin-4* and *lin-14* gain-of-function (gf). In these mutants, *lin-14* escapes negative regulation by *lin-4*, and consequently *lin-14* protein remains present at abnormally late times (Arasu et al., 1991), preventing seam cell terminal differentiation (Ambros and Horvitz, 1984, 1987). The seam cells reiterate the larval program of cell division and larval cuticle synthesis during the L4 and supernumerary molts that occur in these animals (Fig. 5A).

In *lin-4(e912)* mutant animals, which carry a deletion of the *lin-4* gene (Lee et al., 1993), LIN-29 protein was undetectable in lateral hypodermal seam nuclei (Fig. 5B) and in other hypodermal nuclei at any stage. A similar result was obtained with *lin-14(gf)* alleles *n355* and *n536*, which decrease or eliminate the response of *lin-14* to *lin-4* translational repression (Ambros and Horvitz, 1987; Wightman et al., 1991, 1993; Lee et al., 1993). In the *lin-14(gf)* backgrounds, we found that LIN-29 did not accumulate in hypodermal nuclei during the L4 stage (Fig. 5D). We have, however, observed infrequent examples of weak accumulation of LIN-29 in old, gravid *lin-14(gf)* mutants that have undergone more than five molts (data not shown). We note that occasional seam cells have been observed to differentiate during the supernumerary fifth or sixth molts in *lin-14(n536*gf*)* mutants (Ambros and Horvitz, 1984).

Accumulation of LIN-29 is not restricted to hypodermal cells or to the L4 stage and can occur in a *lin-4*-independent fashion

LIN-29 was detected in many non-hypodermal cells in the head, tail and vulval region of the developing hermaphrodite (Fig. 6). In the head, LIN-29 accumulates in cells of the pharynx and in a subset of neurons (Fig. 6A). In the tail, LIN-29 accumulates in the rectal cells B, F and U (Fig. 6C). LIN-29 also accumulates in the sex myoblasts and their progeny (Fig. 6E), in the distal tip cells, the anchor cell, and in many vulval cells (Fig. 6G). The signal detected in these other cell types is due to LIN-29 accumulation as it not observed in *lin-29* null animals (not shown), and it is eliminated, or greatly reduced, by preincubation of the antibody with a LIN-29 fusion protein (see Materials and Methods). A detailed analysis of LIN-29 accumulation in the vulva and the somatic gonad will be presented elsewhere (Rougvie et al., unpublished data).

Although the accumulation of LIN-29 in the hypodermis is restricted to the L4 stage, accumulation in several of these other cell types is not. For example, the accumulation of LIN-29 in the anchor cell and the distal tip cells occurs during the L3 stage. In addition, many, if not all, of the cells that make up the pharynx contain low levels of LIN-29, beginning in the L1 stage and extending to the adult stage (Fig. 6I).

LIN-29 accumulation in some of the non-hypodermal cells is independent of *lin-4* activity. For example, LIN-29 accumulates in approximately 14 head neurons, in the anchor cell and in the distal tip cells in *lin-4(e912)* mutants (Fig. 7A,C). In contrast, most of the tail staining observed in wild-type animals is dependent on *lin-4* (not shown). Accumulation of

LIN-29 in the pharynx is noticeably reduced in *lin-4* mutants (Fig. 7A).

Temporal regulation of *lin-29*

lin-29A and lin-29B transcripts are both detectable prior to the L4 stage in wild-type animals (Rougvie and Ambros, 1995). The steady state abundance of these messages is similar in L3 versus L4-stage animals, despite the dramatic increase in the number of cells, largely hypodermal, that accumulate LIN-29 during the L4 stage (Fig. 3). One interpretation of these observations is that hypodermal transcription of *lin-29* occurs prior to the L4 stage and that this transcription is not affected by the upstream heterochronic genes. The observed temporal regulation of *lin-29* would then occur at the level of protein accumulation. Alternatively, the *lin-29* mRNAs detected prior to the L4 stage by northern analysis could solely reflect *lin-29* expression in non-hypodermal cells. We have taken two approaches towards addressing the level at which *lin-29* is regulated. We have asked if *lin-29* mRNA accumulation is dramatically decreased in retarded heterochronic mutants and whether *lin-29::lacZ* reporter gene fusions are expressed in the hypodermis prior to the L4 stage.

To test if the large difference in *lin-29* protein accumulation in retarded heterochronic mutants (Fig. 5B,D) relative to wild type reflects a corresponding reduction in steady state *lin-29* mRNA levels, we compared *lin-29* transcript levels in these animals. Both the lin-29A and lin-29B transcripts were detected in L4-stage mutant animals (Fig. 8). Densitometric

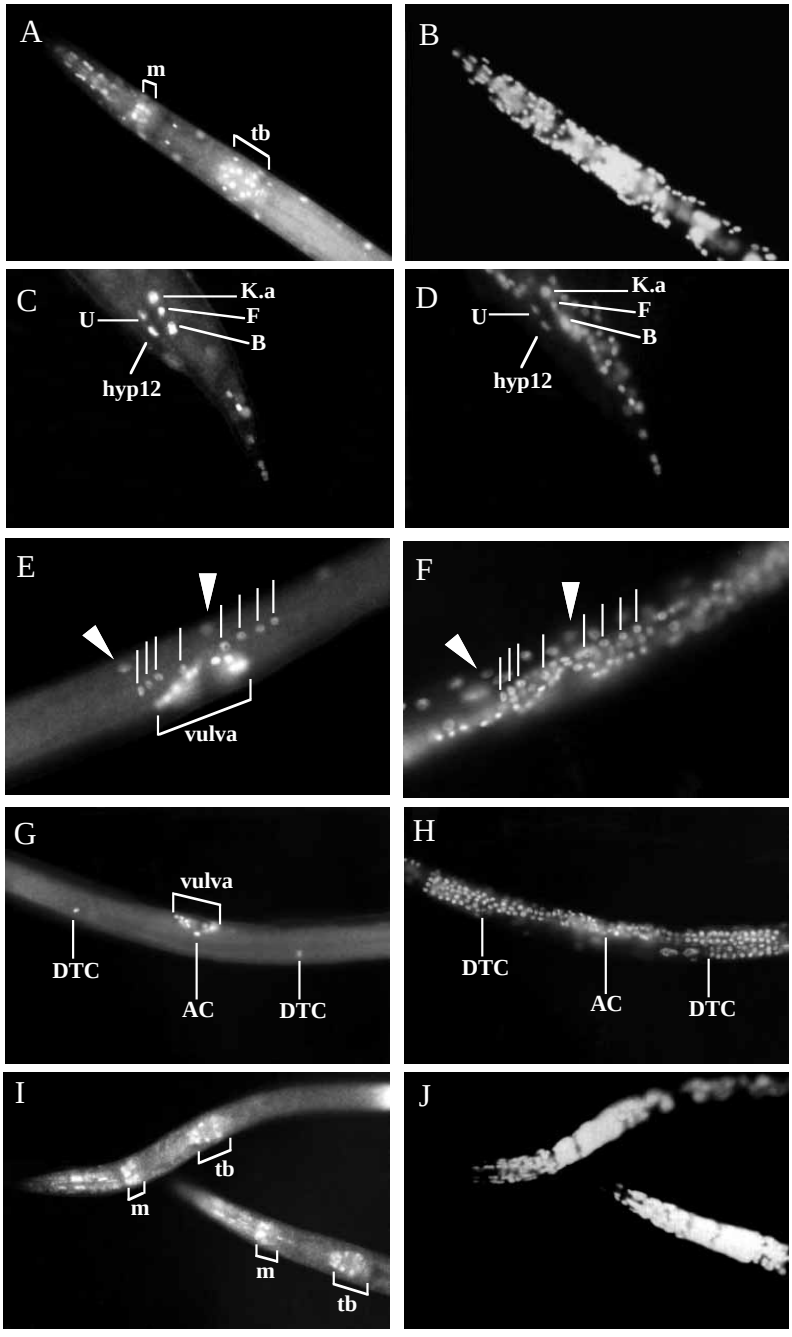

Fig. 6. Accumulation of LIN-29 in non-hypodermal cells. Left panels, wild-type worms stained with anti-LIN-29 antibodies. Right panels, corresponding focal plane showing Hoechst-staining nuclei. (A-B) Mid-section focal plane through the head of a wild-type L4 molt animal. Many non-hypodermal nuclei in the head accumulate LIN-29, particularly in the metacorpus (m) and the terminal bulb (tb) of the pharynx. (C-D) Tail of a wild-type animal during the L4-molt. The rectal cells B, F and U accumulate LIN-29. Several tail hypodermal nuclei, including hyp12 and K.a, have accumulated LIN-29 and are also visible in this focal plane. (E-F) The mid-region of a wild-type early L4-stage animal. Ventral is down. The eight left sex muscle cell nuclei accumulate LIN-29 and are indicated by vertical lines. The eight sex muscle cells on the right side of the worm also accumulate LIN-29. LIN-29 first appears in this lineage during the L3 stage. Two seam cell nuclei that are present in this focal plane are indicated by arrowheads. Vulval cell nuclei present on the ventral side of the worm also accumulate LIN-29 and are indicated by a bracket. (G-H) The mid-portion of a wild-type L3 molt-stage worm. Early during the L3 stage, the anchor cell (AC) nucleus accumulates LIN-29. A short time later the two distal tip cell nuclei (DTC), present at the tips of the developing gonad, accumulate LIN-29. The cell nuclei of the developing vulva also accumulate LIN-29 and are visible in this focal plane (bracket). (I-J) Heads of two wild-type L2-stage animals. LIN-29 accumulation is mainly in pharyngeal cell nuclei. LIN-29 is present in these nuclei at low, but detectable, levels beginning during the L1 stage, and appears to increase in abundance as development proceeds to the L4 stage. Hypodermal accumulation of LIN-29 is not observed in the head prior to the L4 stage.

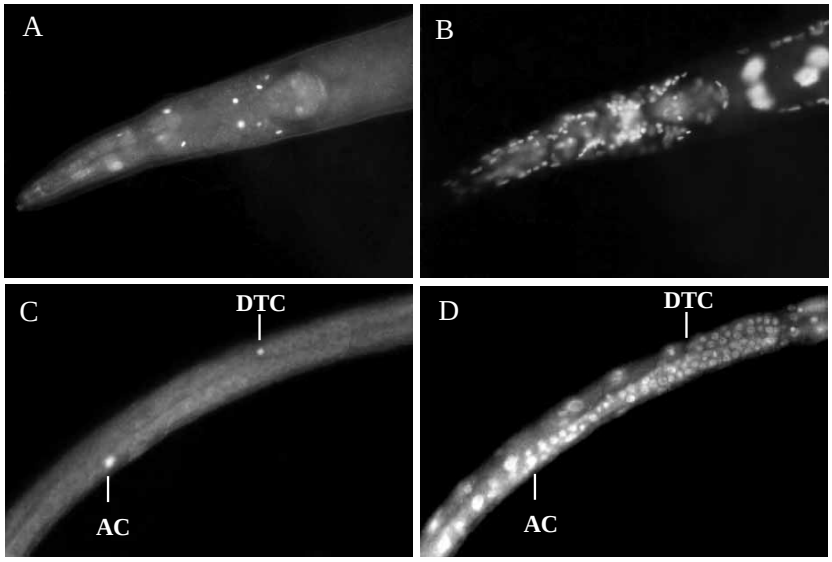

Fig. 7. *lin-4*-independent accumulation of LIN-29. Left panels, middle focal plane of worms stained with anti-LIN-29 antibodies. Right panels, corresponding focal plane showing Hoechst-staining nuclei. (A,B) Head of a *lin-4(e912)* mutant. LIN-29 accumulates in approximately 14 neurons in the head. Weak accumulation of LIN-29 is also detected in the terminal bulb of the pharynx. (C,D) Mid-body view of a *lin-4(e912)* mutant during the L3 molt. LIN-29 accumulates in the anchor cell nucleus (AC) and in the distal tip cell nuclei (DTC), the posterior one of which is shown in this view. Vulval cell types that would normally have accumulated LIN-29 by this stage are not properly specified in *lin-4* mutants (Chalfie et al., 1981).

analysis of the signals from *lin-29* relative to *act-1* shows that the steady-state levels of the lin-29A and lin-29B transcripts are somewhat reduced in the mutant backgrounds compared to wild type. Steady-state lin-29A and lin-29B levels were lower by factors of 2.0 and 4.3, respectively, in *lin-4(e912)* mutants and by factors of 2.8 and 3.0 in *lin-14(n536)* mutants. We note that the lin-29B transcript reproducibly shows a greater decrease in abundance in *lin-4* mutants with respect to wild type than does lin-29A. This alteration in relative transcript abundance may reflect specificity of lin-29B transcription for a cell type that is missing or underrepresented in *lin-4* mutants. The northern analysis shows that steady state *lin-29* mRNA levels are not dramatically altered in the upstream retarded mutants. However, this analysis does not allow us to determine the cell-type specificity of *lin-29* transcription in the mutants.

We wished to learn if the observed accumulation of *lin-29* transcripts prior to the L4 stage in wild-type animals could be due, in part, to transcription of *lin-29* in the hypodermis. The analysis of transgenic lines bearing *lin-29::lacZ* reporter gene fusions is presently the best method for assaying *lin-29* gene expression in larvae. We analyzed the expression of two *lin-29::lacZ* reporter gene fusions, one containing genomic DNA from the lin-29A promoter region and the other containing sequences from the lin-29B promoter (see Materials and Methods) in transgenic worms. Both constructs program hypodermal transcription prior to the L4 stage (Fig. 9). An integrated *lin-29A::lacZ* fusion construct containing 3.9 kb upstream from the start of exon 1 of lin-29A programs β-galactosidase accumulation in the hypodermis, beginning during the L2 stage. β-galactosidase was detected in hypodermal nuclei of hyp7 (Fig. 9B) and in the hypodermal syncytia of the head and tail, two stages prior to the time when LIN-29 is detected in those nuclei. The pattern of β-galactosidase accumulation programmed by this construct is indistinguishable from a similar construct containing 12.3 kb of upstream sequences (not shown). A *lacZ* fusion containing 4.8 kb upstream of the lin-29B ATG programs hypodermal expression of β-galactosidase beginning during the L2 stage in lateral seam cell nuclei (Fig. 9D). In addition, hypodermal expression of both *lin-29::lacZ* fusions is unaltered in a *lin-4* mutant background (not shown). Thus, sequences from the lin-29A and lin-29B promoters can program hypodermal transcription prior to the L4 stage, and this transcription is *lin-4*-independent.

Taken together, these experiments suggest that hypodermal control of *lin-29* activity is not strictly transcriptional.

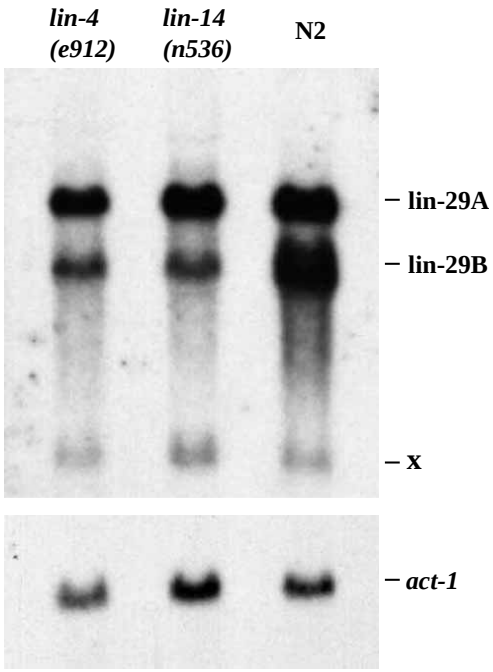

Fig. 8. Accumulation of *lin-29* mRNAs in retarded heterochronic mutants. Poly(A)+ RNA was isolated from synchronized wild-type or heterochronic mutant worm populations during the L4 stage and analyzed by northern hybridization. The upper panel is an autoradiograph of a northern blot probed with a 1.1 kb *Eco*RI fragment (Rougvie and Ambros, 1995) that hybridizes to both the lin-29A and lin-29B transcripts. A smaller third transcript is detected in this blot (X). Its presence on northern blots using *lin-29* probes is variable and its precise origin is unknown. It is unaffected by the *lin-4* and *lin-14(gf)* mutations. The lower panel shows an autoradiograph of the same blot stripped and reprobed with the *act-1* gene (Files et al., 1983) as a control for equal loading of RNA.

However, we cannot be certain if the early accumulation of β-galactosidase from the *lin-29::lacZ* fusions mimics normal *lin-29* transcriptional activity, or if cis-acting regulatory sequences are missing from these constructs. The ultimate resolution of this issue will require direct examination of *lin-29* mRNA accumulation once reliable techniques for in situ hybridization are developed for larval stages.

DISCUSSION

LIN-29 accumulates stage-specifically in hypodermal cells

In this study we have shown that *lin-29* protein accumulation in wild-type hypodermal seam cell nuclei is temporally restricted to the L4 and adult stages. LIN-29 is thus present in wild-type seam cells during the time that defects in seam cell terminal differentiation are observed in *lin-29* mutants. However, the hypodermal defects in *lin-29* mutants are not restricted to the lateral seam. Electron microscopic sections of *lin-29* adults reveal that the entire adult stage cuticle, not just the cuticle secreted by the seam cells, has a larval-type appearance at the ultrastructural level (Ambros and Horvitz, 1984). In addition, the expression of certain collagen gene-*lacZ* fusions in the head, tail and main body hypodermal syncytia is dependent on *lin-29* activity (Liu et al., 1995). These hypodermal defects could be an indirect result of *lin-29* expression in the lateral seam syncytia; for instance, the *lin-29*-controlled terminal differentiation of the lateral seam could signal changes in the surrounding hyp7. Instead, our finding that LIN-29 accumulates in hyp7, and in the hypodermal syncytia of the head and tail beginning during the L4 stage, suggests a direct role for LIN-29 in these cells. Thus, LIN-29 appears to function directly throughout the hypodermis. One role for *lin-29* in these cells is to regulate stage-specifically the expression of collagen genes such as *col-19* (Liu et al., 1995; Rougvie and Ambros, 1995).

Heterochronic gene control of *lin-29* activity

To address the question of how the upstream heterochronic genes control the timing of *lin-29* activity, we examined LIN-29 accumulation in heterochronic gene mutant backgrounds. We found that the accumulation of *lin-29* protein in the lateral seam occurred abnormally early, during the third larval stage, in precocious *lin-14*, *lin-28* and *lin-42* mutants. LIN-29 accumulated late or failed to accumulate in retarded *lin-4* and *lin-14(gf)* mutants (Figs 4, 5). Thus, one consequence of mutations in the upstream heterochronic genes is to alter, directly or indirectly, the timing of *lin-29* protein accumulation.

If the upstream heterochronic genes control LIN-29 accumulation by altering the time of *lin-29* transcription, then the abundance of *lin-29* transcripts should mirror the dramatic difference in the number of cells that accumulate LIN-29 in wild-type L4-stage animals versus retarded mutants (greater than 150 versus approximately 15), and be severely reduced. Instead, we find that both the lin-29A and lin-29B transcripts are only two- to fourfold reduced in *lin-4* and *lin-14(gf)* mutant animals during the L4 stage (Fig. 8). Thus, there does not appear to be a strict correlation between *lin-29* mRNA and protein accumulation. If LIN-29 accumulation is controlled post-transcriptionally, then the observed small reduction in steady state transcript levels in the mutants may reflect a decrease in stability of *lin-29* message, due to its lack of translation. An additional possibility is that the reiterative cell divisions that occur in *lin-4* mutants (Chalfie et al., 1981), some of which are in lineages that do not normally accumulate LIN-29, may alter the relative number of cells that express *lin-29*, thereby affecting the transcript abundance of *lin-29* relative to the *act-1* control.

The expression patterns of *lin-29A*- and *lin-29B*-

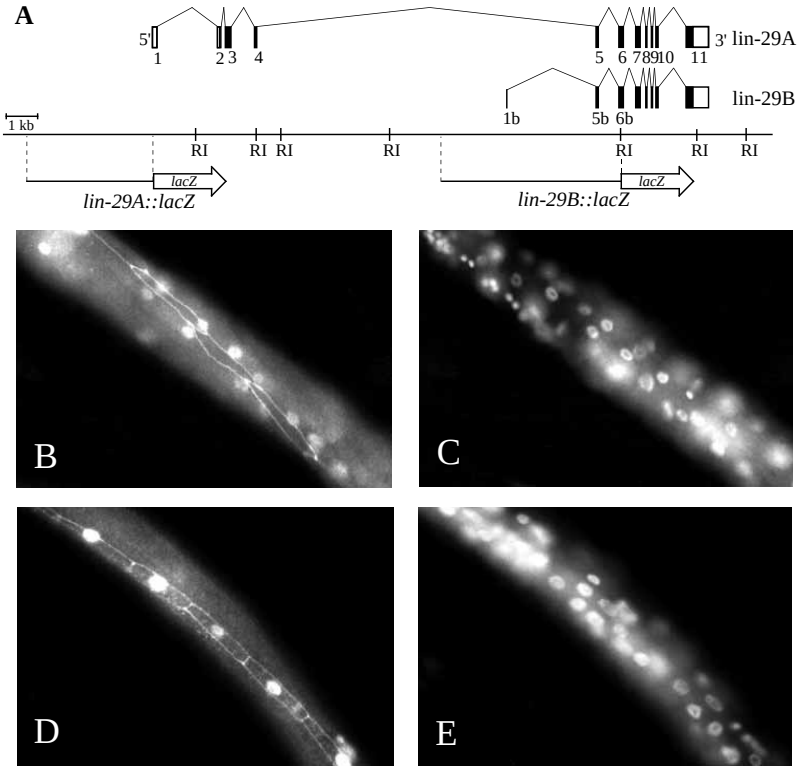

Fig. 9. Hypodermal expression of *lin-29::lacZ* reporter fusions. Strains transgenic for *lin-29A::lacZ* and *lin-29B::lacZ* were assayed for β-galactosidase accumulation by indirect immunofluorescence, as described in Materials and Methods. Both constructs program hypodermal expression beginning during the L2 stage. (A) *lin-29* gene structure. The horizontal line depicts the *lin-29* locus with respect to *Eco*RI restriction sites (RI). The lin-29A and lin-29B transcripts are shown above this line. The boxes represent exons. Filled boxes are protein coding regions. lin-29A exons are numbered 1-11. Exon 1 of lin-29A is trans-spliced to SL1. The lin-29B-specific exon is referred to as exon 1b. The remaining exons are shared with lin-29A and are referred to as exons 5b-11b. cDNA analysis has revealed that lin-29B can also be trans-spliced to SL1 at the start of exon 5b (Rougvie and Ambros, 1995). *lin-29* gene fragments used in *lacZ* reporter gene constructs are shown below the restriction map (see Materials and Methods). (B) A L3 larva transgenic for *lin-29A::lacZ* and stained for β-galactosidase and MH27. β-galactosidase has accumulated in hyp7 nuclei surrounding the unfused lateral seam. (C) Hoechst staining of same focal plane. (D) A L3 larva transgenic for *lin-29B::lacZ* and stained for β-galactosidase and MH27. β-galactosidase has accumulated in hypodermal seam cell nuclei. (E) Hoechst staining of same focal plane.

promoter::lacZ fusions are consistent with a post-transcriptional mechanism for control of LIN-29 accumulation. Hypodermal expression of *lin-29::lacZ* reporter fusions begins during the L2 stage in transgenic wild-type worms (Fig. 9). Because the *lin-29* locus is complex and encodes two transcripts, one with a primary transcription unit containing 11 exons spanning 17 kb, we cannot be sure if all *cis*-acting regulatory sequences are present in each construct. Nevertheless, the L2- and L3-stage hypodermal expression of these *lin-29::lacZ* fusions demonstrates that the *lin-29* genomic DNA fragments are inherently capable of programming transcription prior to the L4 stage. The hypodermal expression of these reporter fusions is unchanged in a *lin-4* mutant background (not shown), further supporting the idea that *lin-29* transcription in the hypodermis does not require *lin-4* activity. Since the main role of *lin-4* is to negatively regulate *lin-14* (Ambros, 1989), *lin-29* transcription must also be independent of LIN-14. Further analysis of how LIN-29 accumulation is controlled will require the development of in situ hybridization techniques with sufficient sensitivity to detect endogenous *lin-29* message accumulation in wild type and heterochronic mutant animals.

Are any of the identified upstream heterochronic genes good candidates for participating directly in control of *lin-29* protein accumulation? The answer appears to be no, since these genes probably act earlier in development than the time at which *lin-29* must be regulated. Temperature-shift experiments reveal a requirement for *lin-14* activity during the L1 stage in order to achieve correct temporal execution of seam cell terminal differentiation during the L4 molt (Ambros and Horvitz, 1987). *lin-14* encodes a nuclear protein that decreases in abundance during the L1 stage and is not detectable after the beginning of the L2 stage (Ruvkun and Giusto, 1989). It is thus difficult to imagine a direct role for LIN-14 in the control of *lin-29* protein accumulation during the L4 stage. Whether *lin-28* functions to control *lin-29* directly is, as yet, unknown, but evidence suggests that it does act during early post-embryonic development. Cell lineage analysis of *lin-28* mutants reveals that certain L2-stage seam cell divisions are deleted (Ambros and Horvitz, 1984). In addition, LIN-14 levels in late L1-stage larvae appear to be reduced in *lin-28* mutants relative to wild type, implying that *lin-28* is required for the appropriate accumulation of LIN-14 during early larval stages (Arasu et al., 1991). Less is known about the time of action of *lin-42*, since much of its analysis has been based on one allele. Cell lineage analysis indicates that the L1- and L2-stage-specific seam cell divisions (Fig. 4C) occur normally in *lin-42* animals (Liu, 1990). On the contrary, *lin-42* enhances certain L2-stage cell lineage defects of a weak *lin-14* allele (Liu, 1990), suggesting an early role for *lin-42*. Whether the time of *lin-42* action is restricted to early larval stages remains to be determined. In addition to the heterochronic genes described here, there are likely to be other genes involved in the timing of seam cell terminal differentiation. These genes may function in other developmental processes that have obscured detection of their roles in the heterochronic gene pathway. Among these may be genes that act primarily during late post-embryonic development and regulate *lin-29* activity more directly.

The role of LIN-29 in non-hypodermal cell types

LIN-29 is also found in some non-hypodermal cell nuclei, including the nuclei of cells where it was not expected on the basis of previous characterization of the *lin-29* mutant phenotype (Ambros and Horvitz, 1984). These cells include the distal tip cells, the sex myoblasts, many pharyngeal cells, the anchor cell, vulval cells and a subset of neurons in the head. The accumulation of LIN-29 in cells of the vulva and the somatic gonad, together with the egg-laying defect associated with *lin-29* mutants (Ambros and Horvitz, 1984), may reflect a functional requirement for LIN-29 in these cells. However, the precise role of LIN-29 in non-hypodermal cells remains to be determined. *lin-29* may be inactive in these cells or play a redundant role such that loss of LIN-29 has little or no phenotypic consequence. Alternatively, there may be defects in these cells in *lin-29* mutants that have not been detected.

LIN-29 accumulation in many of these non hypodermal cells is independent of the heterochronic gene pathway. For example, LIN-29 accumulates in a *lin-4*-independent fashion in the distal tip cells, the anchor cell and in approximately 14 neurons in the head. At least two regulatory pathways must therefore exist to control *lin-29* expression: one that acts largely in the hypodermis and depends on *lin-4* activity, and a second *lin-4*-independent pathway that controls LIN-29 accumulation in the anchor cell, distal tip cells and neurons. A central issue now is to identify the genes directly responsible for specifying the times and places of LIN-29 accumulation.

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