Development 121, 2471-2478 (1995)
Printed in Great Britain © The Company of Biologists Limited 1995



# The *Caenorhabditis elegans* heterochronic gene pathway controls stage-specific transcription of collagen genes

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## SUMMARY

**In *Caenorhabditis elegans*, the terminal differentiation of the hypodermal cells occurs at the larval-to-adult molt, and is characterized in part by the formation of a morphologically distinct adult cuticle. The timing of this event is controlled by a pathway of heterochronic genes that includes the relatively direct regulatory gene, *lin-29*, and upstream genes *lin-4*, *lin-14* and *lin-28*. Using northern analysis to detect endogenous collagen mRNA levels and *collagen/lacZ* reporter constructs to monitor collagen transcriptional activity, we show that the stage-specific switch from larval cuticle to adult cuticle correlates with the transcriptional activation of adult-specific collagen genes and repression of larval-specific collagen genes. Heterochronic mutations that cause precocious formation of adult cuticle also cause precocious transcription of the adult-specific collagen genes, *col-7* and *col-19*; heterochronic mutations that prevent the switch to adult cuticle cause continued expression of the larval collagen gene, *col-17*, in adults and prevent adult-specific activation of *col-7* or *col-19*. A 235 bp segment of *col-19* 5′ sequences is sufficient to direct the adult-specific expression of a *col-19/lacZ* reporter gene in hypodermal cells. These findings indicate that the heterochronic gene pathway regulates the timing of hypodermal cell terminal differentiation by regulating larval- and adult-specific gene expression, perhaps by the direct action of *lin-29*.**

Key words: heterochronic, collagen, *Caenorhabditis elegans*, *lin-29*

## INTRODUCTION

A genetic regulatory system controlling the timing of diverse postembryonic developmental events in *C. elegans* has been identified by mutations in the heterochronic genes *lin-4*, *lin-14*, *lin-28* and *lin-29* (Chalfie et al., 1981; Ambros and Horvitz, 1984). The events controlled by this regulatory pathway include the expression of stage-specific patterns of cell division (Ambros and Horvitz, 1984), dauer larva developmental arrest (Liu and Ambros, 1989), progress through the cell cycle (Ambros and Horvitz, 1984; Euling and Ambros, unpublished) and adult-specific terminal differentiation of hypodermal cells (Ambros, 1989). The action of the heterochronic genes in controlling these events must ultimately affect stage-specific gene expression in particular cells. An essential step in analyzing how heterochronic genes specify the timing of specific developmental programs is the identification of target genes whose stage-specific expression is affected by heterochronic mutations.

Previous studies have focused on the effects of heterochronic genes on a stage-specific event named the larva-to-adult switch or 'L/A switch' (Ambros, 1989). In the wild type, lateral hypodermal stem cells (or 'seam cells') divide at least once in each larval stage and synthesize morphologically larval cuticles at the end of the L1, L2 and L3 stages. However, at the end of the fourth larval stage (at the L4-to-adult molt), the seam cells cease cell division, fuse with each other and synthesize a morphologically distinct adult cuticle (Sulston and Horvitz, 1977; Singh and Sulston, 1978). The coordinate morphological and behavioral changes in hypodermal cells that constitute the L/A switch (cell cycle exit, cell fusion, switch in cuticle morphology) signify the terminal differentiation of these cells.

In animals homozygous for loss-of-function (*lf*) mutations of *lin-29*, the L/A switch never occurs, resulting in reiterated larval fates: the animals undergo extra larval molts beyond the L4 stage, characterized by supernumerary seam cell divisions and the formation of morphologically larval cuticle, indicating that *lin-29* is essential for this event. Genetic epistasis has revealed that *lin-29* is a relatively direct regulator of the L/A switch and that *lin-4*, *lin-14* and *lin-28* affect the timing of the L/A switch by regulating *lin-29* (Ambros, 1989). The predicted product of *lin-29* is a transcription factor (Rougvie and Ambros, 1995), which may act directly, or through more specialized regulators, to control the transcription of genes involved in various aspects of hypodermal cell differentiation. Identifying target genes of *lin-29* is essential for understanding the molecular mechanisms by which *lin-29*, and hence the heterochronic gene pathway, regulates the L/A switch.

Candidate target genes for *lin-29* would include genes

involved in cell cycle control, cell fusion and stage-specific cuticle morphology. Collagens are major components of *C. elegans* extracellular cuticles (Cox et al., 1981a,b) and genes encoding collagens are a family of between 50 and 150 members dispersed throughout the genome (Kramer et al., 1982; Cox et al., 1984, 1985, 1989). Collagen genes whose mRNAs accumulate stage-specifically have been identified by high stringency hybridization using stage-specific cDNA probes (Cox et al., 1984). The stage-specific expression of these collagen genes at larval and adult molts may be responsible for some of the morphologically distinct features of larval and adult cuticles.

In work reported here, we show that mutations of heterochronic genes can affect the transcription of stage-specific collagen genes. We show that the precocious and retarded morphological defects exhibited by heterochronic mutants correlate with precocious and retarded transcription of the stage-specific collagen genes *col-7*, *col-17* and *col-19*. These findings provide the first evidence that heterochronic genes function by controlling the stage-specific transcriptional activity of differentiated cell genes.

## MATERIALS AND METHODS

### Strains and cloned DNA

*lin-4*, *lin-14*, *lin-28* and *lin-29* alleles are described in Chalfie et al. (1981), Ambros and Horvitz (1984, 1987), and Ambros (1989). Strains used in this work are N2, (wild-type *C. elegans* var. Bristol), *n1270e187* (containing a *rol-6* putative null mutation which renders the strain more sensitive than wild type to expression of the *su1006* transgene; Mello et al., 1991 and Kramer et al., 1990), MT1397 *lin-14(n179ts)*, VT284 *lin-14(ma135)/sZT1*, MT1176 *lin-29(n546)/mnC1*, MT1155 *lin-4(e912)/mnC1*, MT1388 *lin-14(n355n679ts)*, VT292 *lin-14(ma135)*, MT1524 *lin-28(n719)*, MT1848 *lin-14(n360)*, CB912 *lin-4(e912)*; and VT181 *lin-14(n536) dyp-6(e14)/szT1(X;I)*. Plasmids and lambda clones containing collagen genes were obtained from J. Kramer. β-galactosidase reporter vectors pPD22.04 and pPD35.41 were obtained from A. Fire (Fire et al., 1990).

### Synchronized worm populations

Starvation-arrested newly hatched L1 animals were obtained by treating gravid adult hermaphrodites with 0.5% hypochlorite/0.5 N NaOH solution (Emmons et al., 1979) and allowing the eggs to hatch in sterile M9 solution for 12-24 hours. After feeding with *Escherichia coli* OP50 (Brenner, 1974; Sulston and Brenner, 1974) synchronized L3-to-L4 molt and L4-to-Adult molt animals were harvested after 32-36 hours and 42-46 hours, respectively. Dauer larvae were selected from starved asynchronous cultures with 1% SDS (Cassada and Russell, 1975), and then fed to allow simultaneous resumption of development. Animals at the postdauer L3-to-L4 or postdauer L4-to-adult molt were harvested at about 18-20 and 28-30 hours after feeding, respectively. A sample of each population was monitored for molting behaviour, particularly cessation of pharyngeal pumping. *lin-14(ma135)*, *lin-28(n719)* and *lin-4(e912)* populations were generally less synchronous than wild-type or other mutant strains.

### RNA isolation

5 ml of LETS buffer (100 mM LiCl, 10 mM EDTA, 10 mM Tris-HCl (pH 7.8 adjusted with LiOH), 1% SDS) were added to 1 ml of frozen a worm pellet (kept on ice). An equal volume of phenol/ chloroform and 2 ml of baked glass beads (VWR) were added to the mixture, which was then vortexed to break the worms. RNA was precipitated from the aqueous phase with LiCl and ethanol (Igo and Losick, 1986).

Poly(A)+ RNA was isolated by a modification of Maniatis et al. (1982) (G. Ruvkun, personal communication). 1 mg of total RNA was dissolved in 160 μl of TES (10 mM Tris pH 7.5, 0.1 mM EDTA, 1% SDS), heated to 65°C for 5 minutes and chilled on ice. 40 μl 5× binding buffer (5× is 2 M NaCl, 50 mM HEPES pH 7.5, 1% Sarkosyl, 5 mM EDTA) was added, followed by 0.5 mg oligo(dT)-cellulose (Collaborative Research Inc.) in 150 μl 1× binding buffer. The mixture was shaken at 37°C for 30 minutes, then at room temperature for 30 minutes. The oligo(dT)-cellulose was pelleted at 3000 revs/minute in a microcentrifuge for 2 minutes, washed twice in 15 ml 1× binding buffer, resuspended in 1 ml 1× binding buffer and loaded into a 1 ml pipette tip plugged with glass wool. The loaded pipette tip was washed first with 0.5 ml 1× binding buffer and eluted with 600 μl elution buffer (10 mM Hepes pH 7.5, 0.02% Sarkosyl). The poly(A)$^+$ RNA was precipitated with ethanol and dissolved in DEPC-treated $H_2O$.

### Northern blot hybridization

Northern blots were performed using Gene Screen (DuPont) and kinased oligonucleotide probes (Ausubel et al., 1987). Oligonucleotide sequences were chosen from regions of the collagen transcription units that are not conserved among *C. elegans* collagen genes (Cox et al., 1989). The sequences of the *col-17* and *col-7* 20-base gene-specific oligonucleotide probes are: *col-17*: 5′ AAACTTTTGGAGAAGTTCGC 3′ (antisense strand immediately following the stop codon TAA; GenBank accession number U18384); *col-7*: 5′ TCTACCAGGTGAATGACTGT 3′ (antisense strand immediately preceding the start codon; Cox et al., 1989).

Hybridization conditions (in buffer containing 50% formamide; at 37°C for 16-48 hours using the *col-17* 20-base oligonucleotide probe or 42°C for 16-48 hours using the *col-7* 20-base oligonucleotide probe) and wash conditions (twice for 15 minutes at room temperature with 6× SSPE followed by 30 minutes at a temperature 5°C higher than the hybridization temperature; Sugita et al., 1987) were determined from the base composition of the probe and adjusted empirically so as to result in hybridization to a homogeneous transcript of the collagen gene family. Control probes were labeled using the random primed DNA labeling kit (Boehringer Mannheim) and hybridized to the northern filters at low stringency, followed by a low stringency wash (0.2× SSPE, 0.2% SDS for 1 hour at room temperature).

### Collagen/lacZ reporter constructs

pZC19.61 (*col-19*): A 2.7 kb *Xba*I/*Bam*HI fragment of *col-19* containing 21 bp of coding sequence was fused in frame with the *lacZ* gene at the *Xba*I/*Bam*HI sites of the pPD22.04 vector (Fire et al., 1990; Fig. 2). pZC19Δ846 (*col-19*): A *Hin*dIII fragment (from a site in *col-19* to a site in the vector) was deleted from pZC19.61 to leave 846 bp of 5′ sequences. pZC7.21 (*col-7*): A 2.5 kb *Xba*I/*Bam*HI fragment of *col-7* containing 602 bp of coding sequence and 1900 bp of 5′ non-coding sequences was fused in frame with the *lacZ* gene at the *Xba*I/*Bam*HI sites of the pPD35.41 vector (Fire et al., 1990; Fig. 2). pZC17.6 (*col-17*): A 1.1 kb *Hin*dIII/*Bam*HI fragment of *col-17* containing about 800 bp of 5′ non-coding sequences and 274 bp of coding sequence was fused in frame at the *Hin*dIII/*Bam*HI sites of the pPD35.41 vector (Fire et al., 1990; Fig. 2).

Plasmids were microinjected into *C. elegans* hermaphrodite syncytial gonads at a concentration of 80 μg/ml (Fire, 1986; Mello et al., 1991). Plasmid pRF4 (Kramer et al., 1990) containing a dominant mutant *rol-6(su1006)* gene was co-injected at 80 μg/ml as a behavioral marker for transformation (Mello et al., 1991). β-galactosidase activity was detected in fixed worms as described (Fire et al., 1990).

### *col-19* sequence analysis

pZC19Δ846, and clones derived from pZC19Δ846 by digestion with *Bal*31 (Ausubel et al., 1987), were sequenced by the Sanger method (1977) using M13 reverse primer and primer EGM1 (CCAAGGGTC-

CTCCTGAAA), which is complementary to sequences of pPD22.04 to the right of the *col-19* insert, and the Sequenase kit (United States Biochemicals). Portions of the sequence derived from analysis of pZC19Δ846 were confirmed by sequence analysis of DNA amplified by the Polymerase Chain Reaction (PCR) from phage clone lambdaCG34 (from which the insert in pZC19Δ846 was derived; Cox et al., 1984) and from genomic DNA (see legend to Fig. 4).

## RESULTS

### Stage-specific collagen gene transcripts in wild-type *C. elegans*

Stage-specific collagen transcripts had been identified previously by dot-blot analysis using cDNA probes reverse-transcribed from mRNAs isolated from developmentally synchronized worm populations (Cox and Hirsh, 1985). In those experiments, worms were synchronized by dauer larva arrest (Cassada and Russell, 1975). We repeated the dot-blot analysis of Cox and Hirsh (1985), but used populations of animals that had developed continuously from the L1 stage without dauer larva formation (see Materials and Methods). Our dot-blot results (data not shown) essentially confirm those of Cox and Hirsh (1985) with one exception, *col-17*, described below. Three major classes of collagens were detected based on their expression patterns, (1) stage non-specific, (2) adult-specific (expressed at the L4-to-adult molt only) and (3) larval-specific (expressed at the L3-to-L4 molt but not the L4-to-adult molt).

Collagen genes of the adult-specific class had been previously sequenced by Cox et al. (1989). We sequenced genomic DNA of *col-17*, one of a novel larval-specific class of collagen genes, from 159 bp 5′ of the translation start to 186 bp 3′ of the translational termination signal (sequence not shown; Genbank accession number U18384). The larval-specific expression of *col-17* and the adult-specific expression of *col-7* were confirmed by northern blot analysis (Fig. 1). The adult-specific expression of *col-19* was confirmed by *col-19/lacZ* reporter gene expression (Liu and Ambros, 1991) described below. The expression patterns of *col-7*, *col-17* and *col-19* during postdauer development were the same as during continuous development, as determined by northern blot analysis (data not shown) and *lacZ* reporter studies (Liu and Ambros, 1991).

### *col-17* and *col-7* mRNA accumulation in heterochronic mutants

We used northern blot analysis to examine larva-specific *col-17* and adult-specific *col-7* mRNA accumulation in precocious mutants of *lin-14* and *lin-28* at the L3-to-L4 molt and in retarded mutants of *lin-4*, *lin-14* and *lin-29* at the L4-to-adult molt (Fig. 1). In wild-type animals, *col-17* mRNA was abundant at the L3-to-L4 molt and was not detectable at the L4-to-adult molt (Fig. 1A). The precocious mutants *lin-14(ma135)*, *lin-14(n360)* and *lin-28(n719)* showed reduced *col-17* expression at the L3-to-L4 molt (Fig. 1A). Although none of these precocious mutants exhibited a complete shut-off of *col-17* expression at the L3-to-L4 molt, the *lin-14(ma135)* and *lin-28(n719)* mutations, which cause a more severely precocious defect than *lin-14(n360)* by morphological criteria (Ambros, 1989), caused a more severely reduced *col-17* expression at the L3-to-L4 molt. In contrast, the retarded mutants *lin-4(e912)*, *lin-14(n536/+)* and *lin-29(n546)*, which reiterate larval cuticle synthesis at the L4-to-adult molt, inappropriately expressed the larva-specific *col-17* mRNA at the L4-to-adult molt at levels comparable with the wild type at the L3-to-L4 molt (Fig. 1A).

In the wild type, the adult-specific *col-7* RNA was undetectable at the L3-to-L4 molt and was abundant at the L4-to-adult molt (Fig. 1B). The retarded mutants *lin-4(e912)*, *lin-14(n536/+)* and *lin-29(n546)*, which synthesize morphologically larval cuticles at the L4-to-adult molt, failed to express *col-7* at the L4-to-adult molt (Fig. 1B). Surprisingly, the precocious mutants *lin-14(ma135)*, *lin-14(n360)* and *lin-28(n719)*, which synthesize a morphologically adult-like

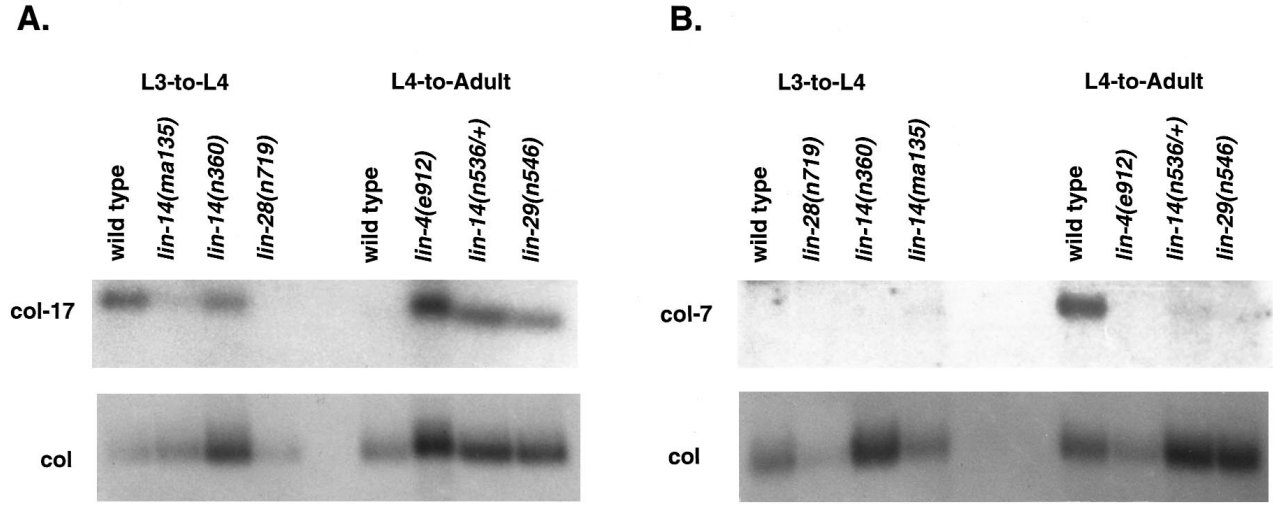


**Fig. 1.** Northern analysis of *col-17* and *col-7* gene expression in heterochronic mutants. Precocious mutants, *lin-14(ma135)*, *lin-28(n719)* and *lin-14(n360)*, were analysed at the L3-to-L4 molt; retarded mutants, *lin-4(e912)*, *lin-29(n546)* and *lin-14(n536) dyp-6(e14)/lin-14(+)szT1(X;I)* were analysed at the L4-to-adult molt. 5 μg total RNA of each mutant was analysed using gene-specific *col-17* or *col-7* oligonucleotide probes (Materials and Methods). (A) *col-17* probe. (B) A separate blot hybridized with *col-7* probe. As a control for the total amount of collagen mRNA in each sample (col), filters were stripped and hybridized with a stage-nonspecific *col-3* probe at low stringency.

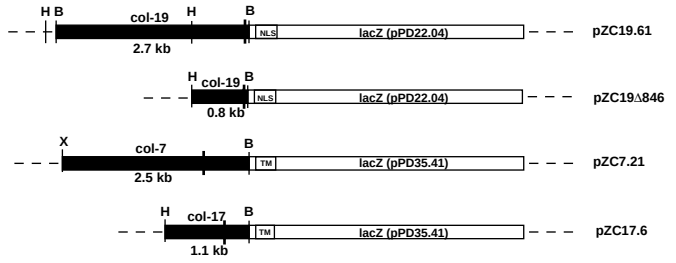


**Fig. 2.** Diagrams of *col-19/lacZ, col-7/lacZ* and *col-17/lacZ* reporter gene fusions (Materials and Methods). The nuclear localization signal (NLS) targets β-galactosidase to nuclei (Fire et al., 1990). The transmembrane domain (TM), which blocks secretion of the fusion protein (Fire et al., 1990), was critical for the detection of *col-7/lacZ* expression. Thick vertical bar marks the position of the translation start site of each fusion gene. X, *Xba*I; B, *BamH*I; H, *Hind*III.

cuticle at the L3-to-L4 molt, did not exhibit detectible levels of *col-7* RNA at the L3-to-L4 molt.

### The expression of *collagen/lacZ* reporter genes in the wild type

We used *lacZ* reporter genes and DNA transformation (Fire et al., 1990) as an independent assay of transcriptional activity that does not depend on detection of stable transcripts. A *col-7/lacZ* translational fusion (pZC7.21) and a *col-19/lacZ* translational fusion (pZC19.61) were constructed (Materials and Methods; Fig. 2). Stably transformed lines were established carrying pZC7.21 or pZC19.61 and transgenic animals were stained with X-gal. Transformed worms containing either the *col-7/lacZ* or the *col-19/lacZ* reporter genes consistently expressed β-galactosidase adult-specifically, beginning at the L4-to-adult molt (Fig. 3; Table 1). The *col-19/lacZ* and *col-7/lacZ* reporter genes were expressed only in hypodermal cells, although not in precisely the same sets of hypodermal cells (Fig. 3). The stage-specific expression of these *col-7/lacZ* and *col-19/lacZ* reporter genes in wild-type animals parallels the results of northern blot and dot-blot analysis. Further, the hypodermal expression of these constructs is consistent with the expected tissue-specificity of cuticle collagen synthesis. Like the *col-19/lacZ* reporter (Liu and Ambros, 1991), the *col-7/lacZ* reporter was expressed adult-specifically whether animals had developed continuously or through a period of dauer larva arrest (data not shown). The expression of a *col-17/lacZ* reporter was not consistent with the northern blot analysis. Worms containing a *col-17/lacZ* translational fusion (pZC17.6; Fig. 2) displayed variable levels of staining at all stages (data not shown), in contrast with the larval-specific expression of the endogenous *col-17* mRNA (Fig. 1). Because the *col-17/lacZ* construct did not express β-galactosidase activity stage-specifically, it was not used in the analysis of heterochronic mutants described below.

### *col-7/lacZ* and *col-19/lacZ* reporter gene expression in heterochronic mutants

*col-19/ lacZ* reporter gene expression was analysed in heterochronic mutant animals to test whether the reporter gene expression correlates with the precocious or retarded execution of the L/A switch. When retarded mutants (*lin-4(e912)* or *lin-29(n546)*) were transformed with a *col-19/lacZ* reporter gene, β-galactosidase expression was not detected in lateral hypodermal cells (Fig. 3; Table 1). The lack of *col-19/lacZ* expression is consistent with the formation of a morphologically larval cuticle at the L4-to-adult stage in these retarded animals. Therefore, *lin-29* and *lin-4* wild-type gene products are required for the activation of *col-19* transcription at the L4-to-adult molt. Interestingly, we observed β-galactosidase staining near the vulva of *lin-29(n546)* and *lin-29(n1368)* adults carrying *col-19/lacZ* constructs (Fig. 3F), indicating that *col-19* expression in those (presumably vulval) cells does not require *lin-29*. The *col-7/lacZ* chimeric gene was not expressed at any stage in *lin-29(n546)* animals (Table 1).

The *col-19/lacZ* reporter was expressed at an abnormally early stage, the L3-to-L4 molt, in precocious mutants of *lin-28(n719)* (Fig. 3E; Table 1), *lin-14(n360)* (Table 1) and *lin-14(n179ts)* (Liu and Ambros, 1991; Table 1) correlating with the abnormally early adult cuticle formation in these mutants. Similarly, the *col-7/lacZ* reporter was expressed precociously in the *lin-14(n360)* mutants (Fig. 3H, Table 1). As described earlier, northern blot analysis failed to reveal precocious *col-7* mRNA in these mutants (Fig. 1). This result suggests that expression of *col-7/lacZ* reporter may be a more sensitive indicator of heterochronic transcriptional activity than northern analysis.

### 5′ sequences of *col-19* required for stage- and tissue-specific expression

A *col-19/lacZ* reporter (pZC19Δ846) containing 846 bp of *col-19* 5′ sequences was constructed from pZC19.61 (Materials and Methods; Figs 2, 4). Worms transformed with pZC19Δ846 expressed the reporter gene adult-specifically (at the L4-to-adult molt), indicating that this 846 bp of *col-19* 5′ sequence contains regulatory sequences sufficient for the stage- and tissue-specific expression of *col-19*. To further delineate essential *col-19* promoter sequences, 5′ deletion derivatives of pZC19Δ846 were generated (Materials and Methods; Fig. 4) and tested for the ability to specify adult-specific *lacZ* expression in transgenic worms. As shown in Table 2, two *col-19* deletion constructs (Δ122 and Δ153) with only 122 or 153 bp of sequences 5′ of the *col-19* ATG, failed to direct the expression of *lacZ* in transgenic worms. In contrast, three *col-19* deletion derivatives (Δ235, Δ315 and Δ448) that retain at least 235 bp of 5′ sequences expressed *lacZ* adult-specifically. One of these constructs, Δ235, expressed *lacZ* relatively weakly, but still adult-specifically. These results indicate that sequences between −153 and −235 may contain elements that control the adult-specificity of *col-19* transcription, and sequences between −235 and −315 contain elements that affect the level of transcription.

In the course of sequencing the above *col-19* deletion constructs, we found that the *col-19* 5′ promoter region contains 296 bp that were not reported in the published *col-19* sequence (Cox et al., 1989). These additional sequences (the underlined nucleotides from positions −337 through −632 in Fig. 4) are present in a *col-19* genomic lambda clone and in wild-type genomic DNA, as revealed by sequence analysis of DNA amplified by Polymerase Chain Reaction (PCR).

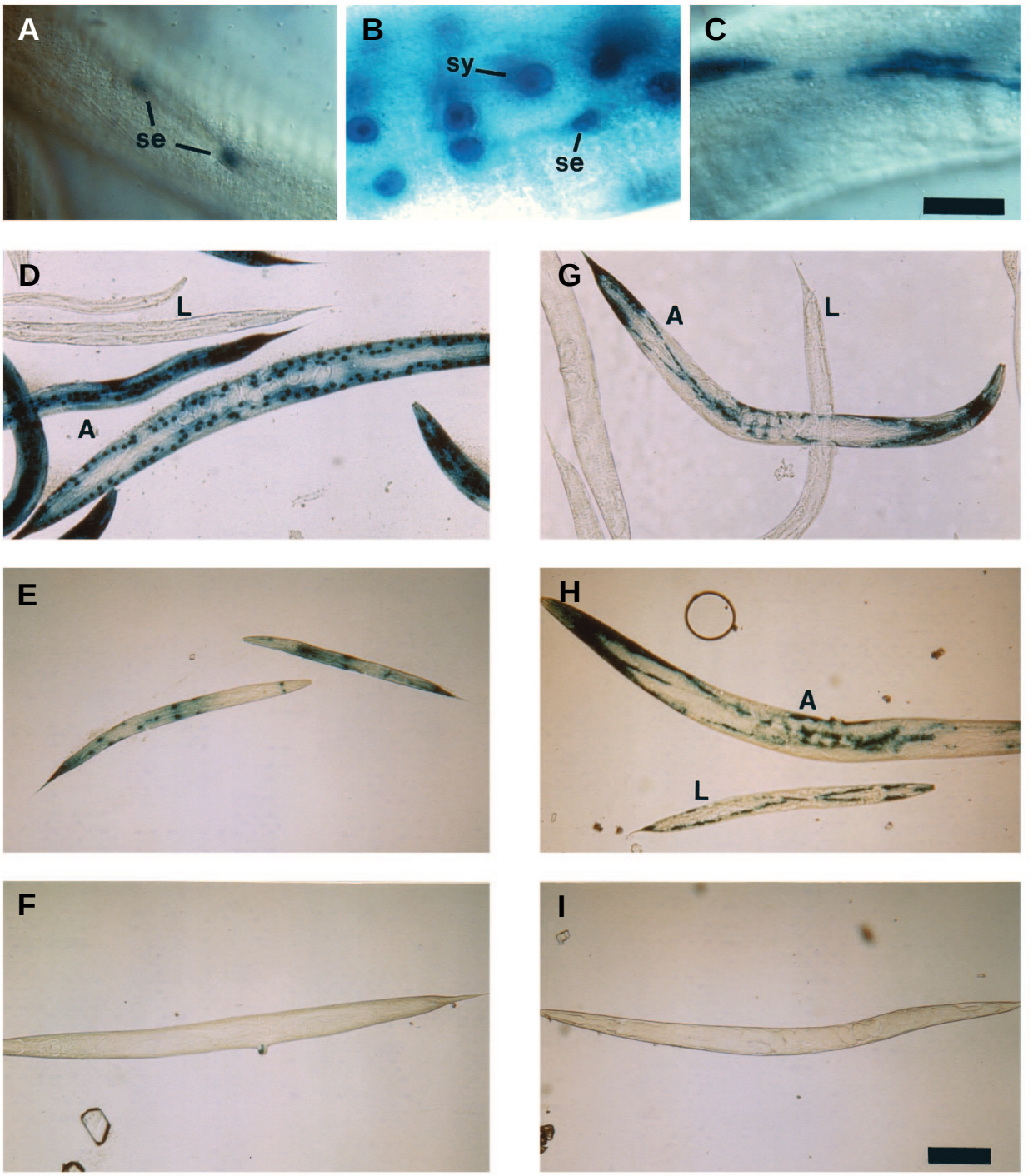


**Fig. 3.** β-galactosidase expression in transgenic wild-type and heterochronic mutant animals transformed with *col-19/lacZ* (pZC19.61) and *col-7/lacZ* (pZC7.21) reporter genes (Materials and Methods; Fig. 2). The rolling phenotype conferred by the *rol-6(su1006)* transformation marker (Mello et al., 1991) is recognizable in fixed animals by a twist in the worm's body. (A,B) Wild-type hermaphrodites at the L4-to-adult molt and adult stages, respectively, transformed with the *col-19/lacZ* reporter. The nuclear localization signal included in the reporter gene product (Fig. 2) directs β-galactosidase to the nuclei of expressing seam (se) cells and syncytial (sy) cells. β-galactosidase staining appeared first in vulva cells at the L4-to-adult molt; then, in seam cells of young adults; finally, in syncytial cells and non-vulval ventral hypodermal cells of older adults. (C) β-galactosidase expression in the hypodermal seam of a wild-type adult hermaphrodite transformed with the *col-7/lacZ* reporter. β-galactosidase staining was adult-specific and restricted to the seam cells. The fusion protein produced from this construct lacks a nuclear localization signal, so much of the β-galactosidase activity is extranuclear. (D) Wild-type larvae (L) and adult (A) hermaphrodites transformed with the *col-19/lacZ* fusion. (E) *lin-28(n719)* larvae transformed with *col-19/lacZ*. (F) *lin-29(n546)* adult transformed with *col-19/lacZ*. Note the staining near the protruding vulva. (G) Wild-type larva (L) and adult (A) hermaphrodites transformed with the *col-7/lacZ*. (The non-expressing adult at left was not a roller). (H) *lin-14(n360)* larva (L) and adult (A) hermaphrodites transformed with *col-7/lacZ*. (I) *lin-4(e912)* adult transformed with *col-19/lacZ*. Bar for A-C, 20 μm; for D-I, 100 μm.

**Table 1. Stage-specificity of collagen/lacZ reporters in transgenic worms**

| Construct | Recipient | Phenotype* | Earliest molt with β-gal staining | Lines |
|---|---|---|---|---|
| pZC19.61 (*col-19*) | wt | WT | L4-to-adult | 5 |
| | *lin-14(n179ts)* 25°C | P | L3-to-L4 | 5 |
| | *lin-14(n360)* | P | L3-to-L4 | 2 |
| | *lin-28(n719)* | P | L3-to-L4 | 3 |
| | *lin-14(n355n679ts)* 15°C | R | L4-to-L5† | 4 |
| | *lin-4(e912)* | R | no expression | 1 |
| | *lin-29(n546)* | R | no expression‡ | 2 |
| | *lin-29(n1368)* | R | no expression‡ | 2 |
| pZC7.21 (*col-7*) | wt | WT | L4-to-adult | 5 |
| | *lin-14(n360)* | P | L3-to-L4 | 3 |
| | *lin-14(n355n679ts)* 15°C | R | L4-to-L5† | 1 |
| | *lin-29(n546)* | R | no expression | 3 |

*P, precocious adult cuticle formation. R, retarded adult cuticle formation.
†A retarded mutant, but less retarded than *lin-29* or *lin-4*, where adults often undergo supernumerary larval stages.
‡These animals often display weak β-galactosidase expression in the vulva area (see Fig. 3F).

## DISCUSSION

### Transcriptional regulation of collagen genes by the heterochronic gene pathway

We employed three methods to monitor the stage-specific expression of the collagen genes *col-17*, *col-7* and *col-19* during development of wild-type and heterochronic mutants. Dot-blot analysis of cloned collagen genes using cDNA probes representing staged mRNA fractions suggested that these genes were expressed stage-specifically during continuous development (this study) and postdauer development (Cox and Hirsh, 1985). Northern analysis with gene-specific oligonucleotide probes detected stage-specific accumulation of endogenous *col-7* and *col-17* transcripts. Finally, extrachromosomal arrays of transgenic DNA containing *col-7/lacZ* or *col-19/lacZ* reporter genes expressed β-galactosidase in a temporal pattern similar to that of the endogenous *col-7* and *col-19* genes.

The adult-specific expression of *col-7* and *col-19* (assayed using *lacZ* reporter constructs) and the larval-specific expression of *col-17* (assayed using northern blots) were altered in accordance with the precocious or retarded expression of cuticle phenotypes in heterochronic mutants. This result indicates that *lin-4*, *lin-14*, *lin-28* and *lin-29* regulate, directly or indirectly, the stage-specific expression of these collagen genes. This regulation is likely to occur on the level of transcriptional initiation and not, for example, mRNA stability, since our *col-19/lacZ* constructs contain only 21 bp of *col-19* protein coding sequence and only approximately 40 bp of 5′ untranslated sequence.

Our results identify *col-17* transcripts as larval-specific by dot-blot (data not shown) and northern blot hybridization (Fig. 1), suggesting that *col-17* represents a novel class of stage-specific collagen gene. Although the *col-17* gene-specific oligonucleotide probe detected a larval-specific mRNA on northern blots, transgenic animals carrying the *col-17/lacZ*

```
-846  AAGCTTCCAAACGTCCCTATTAGGAAATGTAAACTTATTCCCAAAAAATTAAAAATTCCA  -787

-786  GAGAAAGTAGACAAATTTCAGAAAACTTACCGCGCTACATCTAATACTTTCTCATAGGTT  -727

-726  TTTTTATTGGGAAACTGATGAAAATTATTTGAATTCATAATAAAATAAATTCGTATTTAG  -667

-666  CATTTGAAAATTTGCACCAATGTATTATTTAATTTTTTTTTCGAAAATTTAACGCATTT  -607

-606  TCTCTCTAAAAACTCGAAATTTAGTGTGTTCTAAACAACAGTAAGCATACAATACCTTTG  -547

-546  TTCAAAATTGACGTGCTTTCTGAACCAATATGGTTAGTTTCAAAAATTTTTGTATTATAG  -487
                                                  pZC19Δ448
-486  GATAGAAATATTTGGAAATAAATTTTAAAACCAAACTTATGCCTTTCTCTTTTAGTATCC  -427

-426  CAGCTAGGTAATATTTTAGTATTTGCCCAAATCCTTGAAGTAAGGAGTATATAATTTTTG  -367
                                                              pZC19Δ315
-366  AAAAACAATAAAACTCCAGATAATTCATAGTTTTTTCTCGAAAGAAAATTTTTGAGATTA  -307

-306  GTTATTGAACTTCATTTTTGAACATTATTCGTTGAAAAACACTCGCTTTGTCTTTATTTT  -247
                    pZC19Δ235
-246  CAAAAAAATTCCGATTTCCCCAACCAGAAAAAAAAACAGATAGAAGAAATTTCTCCTTAA  -187
                                                  pZC19Δ153
-186  ATTTCATTGTCCATCTCTCTTGGAAACACATTATCTATCAAATGAAAAACGCATTTTTTT  -127
          pZC19Δ122
-126  TCCTGGCAGAAAAATGAAATTGGTTAGATTACACTGGTTAGGTTTGAAGGTGTAACTTTC  -67

 -66  GCTTTCTCAGCAACTTTCAGTATAAAAGGAAACGGTCACCATTTAGAAAGACATCAGTTC  -7

  -6  ATCAACATG
```

**Fig. 4.** 5′ sequences of *col-19* contained in pZC19Δ846 (Materials and Methods). Underlined sequence was not reported in the previously published *col-19* sequence (Cox et al., 1989). ATG (bold), probable initiator methionine codon of *col-19*. The position of the first *col-19* nucleotide of each *Bal*31 derivative (Table 2) is indicated by bold face type. A putative TATA box is positioned at –47 bp from the ATG. The Genbank accession number of this sequence is U18385.

**Table 2. Expression of *col-19* promoter deletions in transgenic worm strains**

| Construct* | bp 5′ sequences | β-gal staining† | Number of lines |
|---|---|---|---|
| pZC19Δ846 | 846 | + | 2 |
| pZC19Δ448 | 448 | + | 2 |
| pZC19Δ315 | 315 | + | 1 |
| pZC19Δ235 | 235 | +/– | 3 |
| pZC19Δ153 | 153 | – | 2 |
| pZC19Δ122 | 122 | – | 5 |

*pZC19Δ846 was derived from pZC19.61 and pZC19Δ122 through pZC19Δ448 were derived from pZC19Δ846 as described in Materials and Methods.
†+, construct expressed β-galactosidase with the same stage-and tissue-specificity, and at approximately the same level as, pZC19.61. –, no β-galactosidase expression was observed in any of at least two independent transformed lines. +/–, β-galactosidase expression was adult- and hypodermal-specific, but at a markedly reduced level compared with pZC19.61.

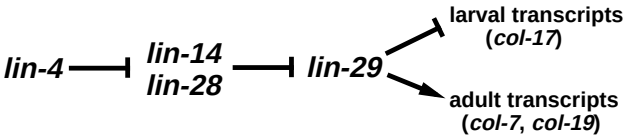


**Fig. 5.** A model for the regulation of stage-specific transcription of hypodermal cell genes by the heterochronic gene pathway. Epistasis relationships (Ambros, 1989) indicate that *lin-29* acts most directly on the adult-specific terminal differentiation program of hypodermal cells. Since *lin-29* encodes a probable transcription factor (Rougvie and Ambros, 1995), the *lin-29* gene product may directly repress and activate, respectively, the transcription of larval- and adult-specific genes. According to this model, *lin-4*, *lin-14* and *lin-28* act to ensure the proper stage-specific activation of *lin-29*.

construct tested here showed β-galactosidase staining at both larval and adult stages. We nevertheless believe that the northern analysis is more likely to reflect accurately activity of the endogenous gene than is reporter gene expression, since the transcriptional regulatory sequences on the transgenic construct may be incomplete. Dot-blot analysis reported by Cox and Hirsh (1985) showed that sequences hybridizing to *col-17* were not expressed larval-specifically, but were detectable in larval and adult RNA samples. It is possible that differences in the dot-blot hybridization conditions detected low levels of *col-17*, or related sequences, in the adult RNA.

In addition to being adult-specific, the expression of these *col-7/lacZ* and *col-19/lacZ* reporter genes is restricted to hypodermal cells, consistent with the presumed role of these collagens in the assembly of the external adult cuticle. There were some anatomical differences between the the adult-specific expression patterns of the *col-7/lacZ* and *col-19/lacZ* reporters (Fig. 3). These differences may either be specific to the transgenes, or reflect anatomical differences in endogenous gene activity between *col-7* and *col-19*.

## Stage-specific collagen gene expression and cuticle morphology

Northern blot analysis indicated that, in some situations, there is not a complete correlation between the level of expression of certain stage-specific collagen genes and the stage-specific cuticle morphology of heterochronic mutants. For example, our northern blots of *lin-14* and *lin-28* precocious mutants (Fig. 1) indicated an apparently incomplete precocious shut-off of larval collagen (*col-17*) and an incomplete precocious activation of adult collagen (*col-7*). This result suggests that the morphological features of adult cuticle expressed precociously in these mutants (lateral alae and specialized ultrastructural layering; Ambros and Horvitz, 1987) are not disturbed by *col-17* expression and also that abundant *col-7* is not essential for their formation.

Collagen/*lacZ* reporter genes may be a particularly sensitive assay for precocious heterochronic phenotypes. For example, in mutants that did not exhibit obvious precocious expression of the endogenous *col-7* gene by northern analysis, precocious *col-7* promoter activity was easily detected using a *col-7/ lacZ* reporter. Further, we have observed that animals displaying a 'leaky' precocious defect, such as *lin-14(n179ts)* at 20°C, can express detectable *col-7/lacZ* expression in hypodermal seam cells not expressing precocious adult lateral alae (data not shown). The precocious reporter gene expression in these mutants may parallel a low level of precocious expression of the endogenous *col-7* gene, or may be a characteristic of the the high copy *col-7* transgenes used here.

## *lin-29* regulates stage-specific collagen gene transcription

Genetic epistasis tests with *lin-4*, *lin-14* and *lin-28* showed *lin-29* to be the most direct regulator of the L/A switch (Ambros, 1989). Exhaustive genetic screening for suppressor mutations of *lin-29* failed to identify any regulatory genes acting between *lin-29* and the L/A switch (Papp, Euling and Ambros, unpublished; Bettinger and Rougvie, personal communication). This suggests that there are few, if any, regulatory steps between *lin-29* and the genes that encode adult and larval-specific proteins of hypodermal cells. Our finding that *lin-29* is required to repress *col-17* and activate *col-7* and *col-19* transcription at the L4-to-adult molt is consistent with the finding that *lin-29* encodes a probable zinc-finger transcription factor (Rougvie and Ambros, 1995). According to one model, *lin-14*, *lin-4* and *lin-28* may control the stage-specific activation of the Lin-29 protein, a transcription factor that triggers the hypodermal cell terminal differentiation process (Fig. 5). Recent findings that *lin-29* protein synthesized in *E. coli* binds in vitro to the promoter sequences of *col-19* and *col-17* supports the view that *lin-29* directly regulates collagen gene transcription and perhaps also the transcription of other hypodermal cell differentiation genes (Rougvie and Ambros, 1995). The roles of *lin-29* protein as either a repressor or activator of stage-specific transcription could reflect the precise nature of its binding to particular promoters. Alternatively, *lin-29* may activate adult-specific transcription in conjunction with certain specific partners and could repress larva-specific transcription when acting together with other partners.

Using the *col-19/lacZ* reporter, we delineated *col-19* promoter sequences sufficient for abundant adult-specific hypodermal expression. This region, extending 315 bp 5′ from the start of translation, likely includes sequences through which *lin-29* acts. However, a simple comparison of *col-7* and *col-19* upstream sequences does not reveal any strikingly conserved sequence motifs that might be Lin-29 binding sites. Detailed analysis of promoter sequences that mediate Lin-29 activity may lead to identification of other direct targets of Lin-29. The activation and repression of stage-specific transcription by the Lin-29 gene product may serve as a model for how developmental regulatory genes control cell differentiation.

We thank Craig Mello for advice on microinjection, Andrew Fire for β-galactosidase reporter vectors and James Kramer for the collagen clones and valuable advice. We also thank Michael Finney, Gary Ruvkun and Rosalind Lee for oligonucleotides, and Rosalind Lee and Ann Rougvie for help with sequencing. This work was supported by US Public Health Service research grant GM34028 and March of Dimes Birth Defects Foundation research grant 5-445.

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(Accepted 20 April 1995)