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A Hierarchy of Regulatory Genes Controls a Larva-to-Adult Developmental Switch in C. elegans

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Summary

The heterochronic genes *lin-4, lin-14, lin-28,* and *lin-29* control the timing of specific postembryonic developmental events in C. elegans. The experiments described here examine how these four genes interact to control a particular stage-specific event of the lateral hypodermal cell lineages. This event, termed the "larva-to-adult switch" (L/A switch), involves several coordinate changes in the behavior of hypodermal cells at the fourth molt: cessation of cell division, formation of adult (instead of larval) cuticle, cell fusion, and cessation of the molting cycle. The phenotypes of multiply mutant strains suggest a model wherein the L/A switch is controlled by the stage-specific activity of a regulatory hierarchy: At early stages of wild-type development, *lin-14* and *lin-28* inhibit *lin-29* and thus prevent switching. Later, *lin-4* inhibits *lin-14* and *lin-28,* allowing activation of *lin-29,* which in turn triggers the switch in the L4 stage. *lin-29* may activate the L/A switch by regulating genes that control cell division, differentiation, and stage-specific gene expression in hypodermal cells.

Introduction

Animal development is a complex schedule of cell division, differentiation, and morphogenesis. Understanding how that schedule is specified and executed requires the identification and detailed analysis of genes that control the relative timing of developmental events. In the nematode Caenorhabditis elegans, the rigid temporal control of developmental events is evident from the essentially invariant cell lineages of this animal (Sulston and Horvitz, 1979; Sulston et al., 1983). Patterns of cell division and differentiation always occur in a sequence characteristic of each cell lineage and at specific times relative to events in other lineages.

The proper timing of many postembryonic developmental events in C. elegans requires the normal activity of at least four "heterochronic" genes, *lin-4, lin-14, lin-28,* and *lin-29* (Chalfie et al., 1981; Ambros and Horvitz, 1984). Mutations in these genes result in either precocious or retarded development. In precocious development, certain cells express fates normally specific for cells later in the same lineage; in retarded development, cells reiterate fates normally specific for cells earlier in the same lineage. The products of heterochronic genes may be part of a system by which cells receive temporal information and thereby select and express appropriate stage-specific developmental programs.

An analysis of the stage and lineage specificities of the heterochronic genes revealed a phenotypic hierarchy (Ambros and Horvitz, 1984). *lin-4* and *lin-14* mutations affect the timing of events in diverse cell lineages (including hypodermal, muscle, and intestinal lineages) at all four larval stages. In contrast, *lin-28* mutations affect only a subset of the hypodermal events affected by *lin-4* and *lin-14. lin-29* mutations affect a still smaller subset of those events. One question is whether this phenotypic hierarchy corresponds to a functional genetic hierarchy. For example, are *lin-4* and *lin-14* "general temporal control" genes responsible for the elaboration or reception of temporal information for diverse cell types, and are *lin-28* and *lin-29* more specialized genes involved in the responses of specific cell types to that temporal information?

The genetic properties of *lin-14* mutants have suggested that the level of *lin-14* activity conveys temporal information that directs certain multipotential cells to elect fates appropriate to specific developmental stages. Genetic analysis of *lin-14* alleles that cause either elevation or reduction in the level of gene activity has led to the proposal that *lin-14* activity decreases during development to cause the expression of developmental programs in their proper sequence (Ambros and Horvitz, 1987). Since other heterochronic genes affect at least some of the same events as *lin-14*, the question arises as to whether these other genes temporally regulate *lin-14* or whether they are regulated by *lin-14*.

Epistasis analysis is one approach to answering such questions concerning the relationships among a set of developmental control genes. By comparing the phenotypes of multiply mutant animals to single mutants, one can infer dependent or interdependent functional relationships among genes contributing to the same developmental event. This kind of analysis has been used to assign genes to steps in developmental pathways in such diverse processes as pattern formation in Drosophila (Struhl, 1983; Anderson et al., 1985; Schüpbach, 1987), sex determination in Drosophila (Baker and Ridge, 1980; Steinmann-Zwicky and Nöthiger, 1985), sex determination and dosage compensation in C. elegans (Hodgkin, 1980, 1985; Miller et al., 1988), vulva development in C. elegans (Furguson et al., 1987; Sternberg, 1988), and the specification of cell type in yeast (Rine et al., 1981; Mitchell and Herskowitz, 1986; Kassir et al., 1988). Epistasis analysis provides a genetic formalism to describe the functional nature of interactions among genes without presupposing any particular molecular mechanisms for those interactions. For example, positive or negative regulators of certain genes may be identified by epistasis analysis, even though the regulatory gene products may act indirectly on their genetic targets. Because epistasis analysis identifies the functional nature of gene interactions, it provides a necessary framework for molecular analysis of those interactions.

The experiments described in this paper explore how the activities of *lin-4, lin-14, lin-28,* and *lin-29* interact to

control the timing of a particular stage-specific event in the development of the C. elegans lateral hypodermis—the "larva-to-adult switch" (L/A switch). This switch involves several coordinate changes in the behavior of certain hypodermal cells at the fourth larval molt: cessation of cell division, formation of adult (instead of larval) cuticle, cell fusion, and cessation of the molting cycle. The results of these experiments suggest that the timing of this switch is controlled by a hierarchy of regulatory interactions among *lin-4, lin-14, lin-28,* and *lin-29.* The simplest model from these data is that lateral hypodermal cells at successive stages of development have the potential to switch from larval to adult programs, and the execution of that switch critically depends on the activity of *lin-29. lin-14* and *lin-28* inhibit *lin-29* at early stages of development and thereby prevent early switching. Later, *lin-4* inhibits *lin-14* and *lin-28,* resulting in the activation of *lin-29. lin-29* is proposed to trigger the switch by differentially regulating larval and adult developmental programs in the hypodermal cells.

Results

Experimental Design

The L/A switch in wild-type C. elegans involves several easily detectable changes in the appearance and behavior of certain hypodermal cells and in the morphology of the cuticle. The seam cells (which occupy positions along the left and right lateral midlines of the animal), together with the hypodermal syncytium (which covers most of the length and circumference of the animal), are responsible for synthesis of cuticle prior to each postembryonic molt (Singh and Sulston, 1978; Edwards and Wood, 1983). The seam cells are also stem cells, and divide one or more times at each of the four larval stages (Sulston and Horvitz, 1977). Toward the end of the fourth (L4) stage, the seam cells undergo a marked change in behavior—they fuse with each other and cease cell division. At the L4 molt, the seam cells cause the formation of longitudinal morphological features known as "adult lateral alae" on the newly formed adult cuticle (Sulston

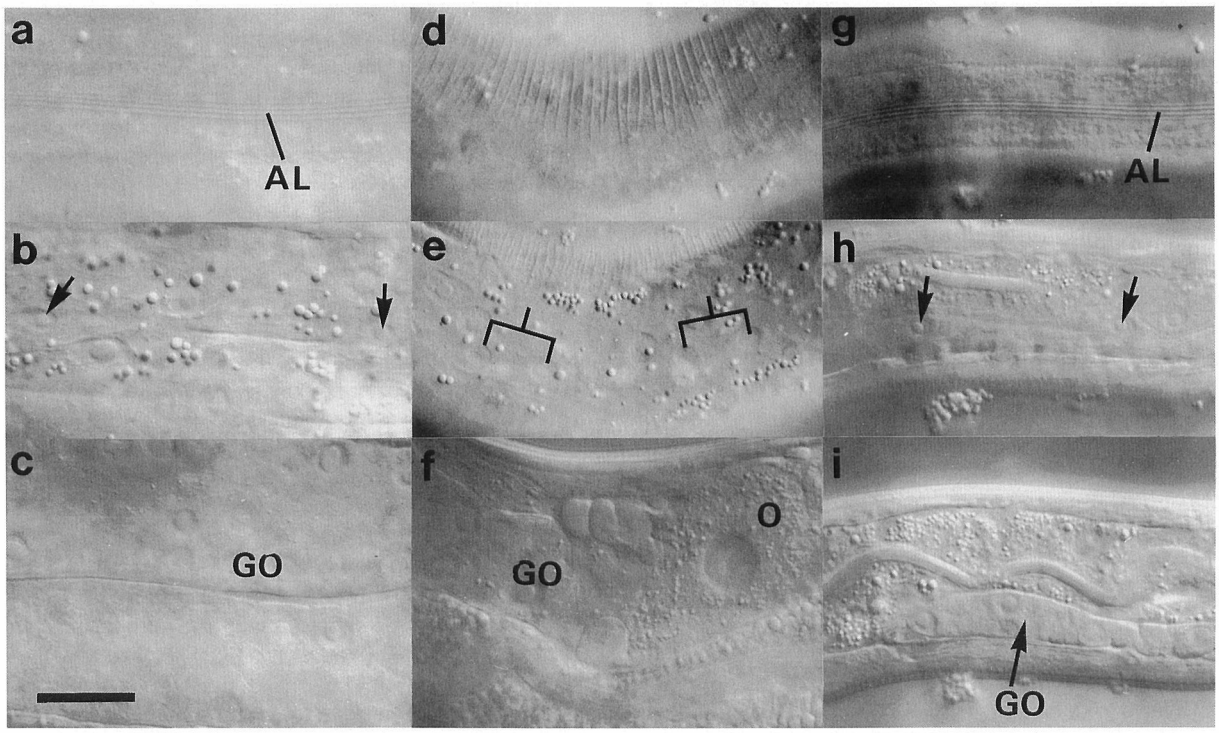


Figure 1. Photomicrographs of Living Wild-Type and Heterochronic Mutant Hermaphrodites at Specific Stages of Development

Nomarski optics; bar = 20 μm. Each vertical series of three photomicrographs is of the same hermaphrodite taken approximately 5–10 sec apart but in different focal planes. Animals were photographed during the L4 lethargus (a–f) or the L2 lethargus (g–i). For each animal, one plane of focus (c, f, and i) shows the gonad (GO) for comparison of developmental stages. Another plane of focus (a, d, and g) shows newly formed cuticles, and a third (b, e, and h) shows lateral hypodermal seam cell nuclei in the corresponding animals. Arrows indicate nuclei of nondividing seam cells; forked lines indicate daughter nuclei of dividing or recently divided seam cells.

(a–c) At the L4 molt of the wild type, lateral alae (AL) are formed on the cuticle (a), and the seam cells that form the alae are nondividing (b).

(d–f) At the L4 molt of the quadruply mutant strain VT364 (*lin-28(n719); lin-4(e912) lin-29(n333); lin-14(ma135)*), seam cells do not express their normal fates; they fail to form adult alae (d) and undergo cell division (e). In (f), a mature oocyte (O) is shown. (The gonad was displaced dorsally in this particular animal.)

(g–i) At the L2 molt of the triply mutant strain VT366 (*lin-28(n719); lin-4(e912); lin-14(ma135)*), lateral alae (g) are formed by the seam cells, and those seam cells are nondividing (h). The L2 molt is the final molt for VT366 animals.

and Horvitz, 1977; Singh and Sulston, 1978). These lateral alae are used in the experiments described here and in previous studies (Ambros and Horvitz, 1984, 1987; DeLong et al., 1987) as an easily detectable adult-specific morphological feature (see Figure 1) to monitor adult cuticle formation in heterochronic mutants.

The constellation of changes exhibited by the hypodermal seam cells at the L4 molt can be considered a "switch" in the developmental state of the seam cells—from the execution of "larval" molts to the execution of an "adult" molt. In wild-type development, larval molts are associated with division of seam cells, larval cuticle formation, and commitment to further molting. In contrast, the adult molt is the final molt and is associated with fusion of seam cells, permanent cessation of seam cell division, and the formation of adult cuticle. In the experiments described here, the presence or absence of seam cell divisions and adult lateral alae formation are the criteria used to characterize the molts at specific stages of mutant development as either "larval" or "adult."

To investigate the functional relationships among the activities of *lin-4, lin-14, lin-28,* and *lin-29,* strains were constructed carrying mutations in one, two, three, or four heterochronic genes. The effects of these single and multiple mutant combinations on the timing of the L/A switch were examined. For these experiments, mutations were used that had previously been determined to cause loss or gain of gene function (Ambros and Horvitz, 1984, 1987; see also Experimental Procedures). Apparent null alleles of *lin-14, lin-28,* and *lin-29* are fully recessive and were identified as mutations that behave equivalently to genetic deficiencies in complementation experiments (Ambros and Horvitz, 1984). Although these alleles likely cause complete loss of function, the actual degree of loss is not known for certain. It is assumed that the one *lin-4* allele, *lin-4(e912),* is a hypomorphic or null allele, since it is fully recessive. However, since the *lin-4* locus is not well studied genetically, this assumption should be considered tentative. In this paper, mutations referred to as "loss of function" include apparent null alleles as well as less well characterized alleles, such as *lin-4(e912),* that may cause incomplete loss of gene activity.

The aims of these experiments were, first, to determine whether L/A switching occurs in the absence of all four genes; second, to identify which of these genes acts most directly to control the switch; and third, to identify which of these genes affect the L/A switch by regulating the activities of certain other genes. Below is a description of the effects of single heterochronic mutations on the L/A switch (Table 1) followed by a description of the effects of multiple

Table 1. Molting Behavior of Heterochronic Mutants

Strain	Genotype	Fraction of Animals Forming Adult Lateral Alae				
		L1 Molt	L2 Molt	L3 Molt	L4 Molt	L5 Molt
Wild type		0%	0%	0%	100%	–
VT292	14^-	0/4*	0/4*	10/10	–/22	
MT1524	28^-	0/5*	(9)/9*	11/11	–/4	
CB912	4^-	0/2[i]	0/5[i]	0/5[i]	0/9[i]	0/4*
MT1149	14^{++}	0%	0%	0%	0%	0%
MT333	29^-	0%	0%	0%	0%	0%
VT364	$4^-14^-28^-29^-$	0/6[i]	0/6[i]	0/6[i]	0/6*	
VT365	$14^-28^-29^-$	0/12[i]	0/12*	0/12*	0/12	
VT366	$4^-14^-28^-$	0/9[i]	9/9	–/9		
MT1537	$14^{++}28^-$	0/9[i]	0/9[i]	4(3)/7*	@/17	
VT291	4^-14^-	0/6*	0/6[i]	6/6	–/6	
MT1538	4^-28^-	0/10[i]	(1)/3[i]	(5)/10[i]	@/28	
VT334	14^-28^-	0/12[i]	12/12	–/7		

These data are summarized in Figures 2 and 3. Genotypes are shown using a genetic shorthand: "+" designates wild-type alleles, "++" designates gain-of-function alleles, and "–" designates loss-of-function alleles. Full genotypes of these strains are listed in Experimental Procedures. Strains were constructed and the fates of seam cells at defined stages of development were assayed as described in Experimental Procedures and in Ambros and Horvitz (1987). Only hermaphrodites were examined. In some cases, but not all, the same animal was observed at successive stages. The denominator of each fraction is the total number of animals assayed, either using the dissecting microscope or using Nomarski optics, at the indicated molt; the numerator is the number of animals that formed adult lateral alae. All animals formed adult alae along their full length except those represented by the numbers in parentheses, which formed partial alae. At stages where no adult alae were formed, at least one animal was examined using Nomarski optics to verify that seam cell divisions occurred either by direct observation of dividing cells (*), or by inference based on an increase in hypodermal cell number between stages (i). (Although all VT365 animals made cuticle at the L4 molt that completely lacked adult lateral alae, seam cell divsions were observed only very occasionally at this stage, suggesting a general inhibition of seam cell divisions after the L4 stage in VT365 animals. This VT365 defect has not been explored further.) A dash indicates that the indicated molt was not observed to occur in animals followed for more than 24 hr after the last molt. The phenotypes of MT1537 and MT1538 are somewhat more variable than these other strains. Occasional MT1538 animals display small patches of adult cuticle amid mostly larval cuticle at the L2 molt, and areas of larval cuticle amid mostly adult cuticle at the L3 molt. The "@" signifies that the molting behavior of MT1537 and MT1538 is unusual after the L4 stage; approximately 20% of animals of both strains undergo a fourth molt, although this L4 molt is abnormal or incomplete, as described in Experimental Procedures. The remaining animals show no molting behavior after the L3 molt. The *lin-4*$^-$ results repeat those of Chalfie et al. (1981). The MT1534 results repeat those of Ambros and Horvitz (1987). The data for wild type are expressed as percentages and are based on numerous observations, including those of Sulston and Horvitz (1977). The phenotypes of MT1149 and MT333 are also expressed as percentages and are based on data from Ambros and Horvitz (1984, 1987). Retarded animals were not followed in detail after the fifth molt, but animals of these genotypes have been observed to undergo additional molts beyond the fifth (Chalfie et al., 1981; Ambros and Horvitz, 1984, 1987). All experiments were performed at 20°C.

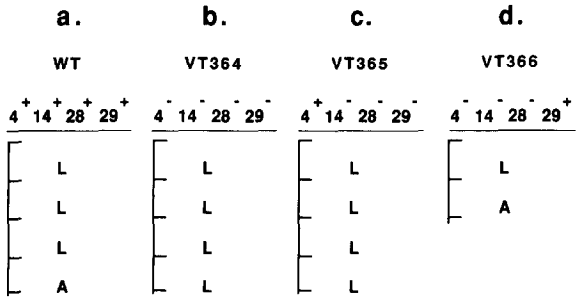


Figure 2. Summary of the the Timing and Sequence of Larval and Adult Molts in Multiply Mutant Strains

Summarized are data from Table 1. To facilitate the comparison of multiply mutant genotypes, wild-type as well as mutant genotypes of these four genes are shown using a genetic shorthand: wild-type alleles are designated by a superscript "+," loss-of-function alleles by a superscript "−." Full genotypes are listed in Experimental Procedures. The time axis at left begins at hatching and is marked with horizontal ticks to delineate postembryonic molts. The vertical letters indicate the type of molt expressed at each stage: larval, "L," or adult, "A." Animals were examined at known developmental stages using Nomarski optics, the number of molts were counted, and the fates of seam cells at each molt were determined as described in Experimental Procedures. The criteria used to interpret molting phenotypes at each developmental stage were the formation of adult lateral alae, the division of seam cells, and the cessation of further molting, as described in Table 1 and Experimental Procedures.

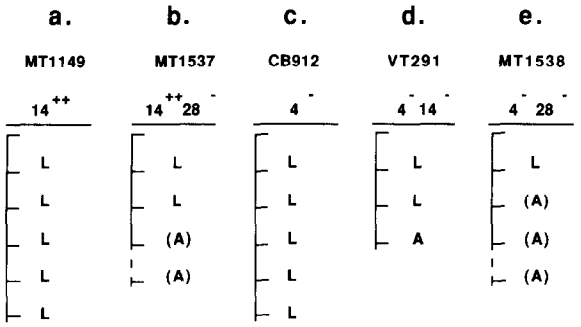


Figure 3. Comparison of the the Timing and Sequence of Molts in Single and Double Heterochronic Mutant Strains

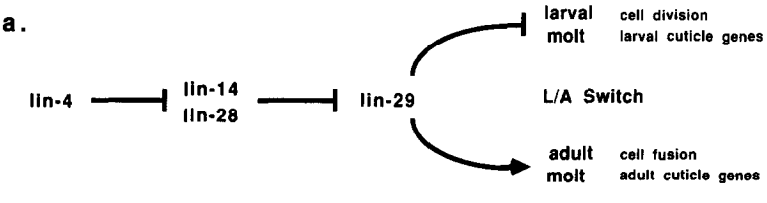
Summarized are data from Table 1. Larval stages and molting phenotypes are shown using the same conventions as in Figure 2. In addition, "(A)" designates a "semiadult" molt wherein aspects of adult molting and larval molting occur in the same animal, as described in Results and Experimental Procedures. For simplicity, in this figure only mutant genotypes are shown using a genetic shorthand: gain-of-function alleles are designated by "++," loss-of-function alleles by "−." Full genotypes are listed in Experimental Procedures. Animals were examined by Nomarski optics, molts were counted, and the fates of seam cells at each molt were determined as described in Table 1 and Experimental Procedures. These strains display essentially complete penetrance of the indicated developmental patterns with the notable exception of MT1537 (b) and MT1538 (e), as described in the legend to Table 1. The infrequency and unusual nature of the L4 molt in MT1537 and MT1538 animals are indicated by the broken time scale lines.

mutations (Table 1, Figures 2 and 3). The chief findings of these experiments are used as a basis for the model shown in Figure 4.

The Wild-Type Activities of *lin-14* and *lin-28* Prevent L/A Switching at the L3 Molt

In animals homozygous for *lin-28* or *lin-14* recessive loss-of-function alleles, the third molt is a precocious adult molt at which seam cells form adult cuticle and permanently cease cell division and then cease molting (Table 1). In previous studies, four molts were observed in *lin-14* precocious mutants (Ambros and Horvitz, 1984, 1987). For the experiments described here, a newly isolated *lin-14* allele, *ma135*, was chosen based on its more extreme precocious phenotype: the occurrence of only three molts. (*ma135* was isolated after ethyl methanesulfonate mutagenesis and a screen for alleles that fail to complement a *lin-14* hypomorphic allele; see Experimental Procedures.) *ma135* seems to represent a class of *lin-14* alleles with complete, or nearly complete, loss of function.

Since loss of either *lin-14* or *lin-28* leads to a precocious



b.

	lin-4	lin-14	lin-28	lin-29	molting
early	off	on	on	off	larval
late	on	off	off	on	adult

Figure 4. Model for the Functional Relationships among *lin-4*, *lin-14*, *lin-28*, and *lin-29*

The phenotypes of multiply mutant strains suggest a hierarchical regulatory relationship among genes controlling the timing of the L/A switch.

(a) *lin-4* is a negative regulator of *lin-14* and *lin-28*; *lin-14* and *lin-28* both negatively regulate *lin-29*, a gene specifically required for execution of the switch. *lin-29* wild-type function is proposed to positively regulate aspects of adult molting, including the expression of adult-specific cuticle genes and functions required for seam cell fusion. *lin-29* is proposed to inhibit aspects of larval molting, including larval-specific cuticle genes and genes controlling seam cell division. These activities of *lin-29* may be direct, as implied here, or via unidentified intermediate regulators. This model is a formal one, and makes no claim to specify the precise molecular nature of the proposed interactions among genes and/or gene products.

(b) The normal timing of the switch is proposed to result from the stage-specific activation of *lin-4*, leading to a corresponding temporal decrease in the combined activities of *lin-14* and *lin-28* and hence a stage-specific activation of *lin-29*. The model does not assume precise times during development that these gene activities would change, only that they would change between early and late stages of postembryonic development such that L4 cells would experience *lin-29* activity and switch molting type.

adult molt, it would appear that both these genes act to inhibit the L/A switch at early stages. The proposal that *lin-14* inhibits switching is further supported by the fact that *lin-14* semidominant gain-of-function alleles, which cause inappropriate expression of *lin-14* at late stages, prevent the L/A switch (Ambros and Horvitz, 1987; see also Table 1). *lin-14* and *lin-28* may inhibit the switch by inhibiting the expression of developmental programs characteristic of the adult molt and/or by activating programs characteristic of the larval molt.

In *lin-28* mutants, precocious adult alae formation can occur at the L2 molt as well as the L3 molt. *lin-28* mutant animals at the L2 molt often contain seam cells expressing larval fates (dividing cells) mixed with seam cells expressing adult fates (nondividing cells forming adult alae) (Ambros and Horvitz, 1984). Despite this expression of some adult characteristics at the L2 molt in *lin-28* animals, the L2 molt has never been observed to be the final one in these particular mutants. Since the L2 molt in *lin-28* mutants shows characteristics of both a larval and an adult molt, it is referred to as a "semiadult" molt.

lin-4 and *lin-29* Are Required for L/A Switching

In animals homozygous for *lin-4* or *lin-29* recessive alleles, the adult molt does not occur and is replaced by a series of supernumerary larval molts (Chalfie et al., 1981; Ambros and Horvitz, 1984, 1987; see also Table 1). Assuming that these mutations cause loss of gene activity, then it would appear that both these genes are required for cells at the L4 molt to execute the L/A switch, perhaps by inhibiting the expression of larval programs and/or by activating adult programs.

Positive and Negative Regulators of the L/A Switch

The phenotypes caused by loss-of-function mutations of *lin-4, lin-14, lin-28,* or *lin-29* suggest two classes of regulatory genes that have opposite effects on the L/A switch: *lin-14* and *lin-28* are required during larval stages to promote larval molting and prevent adult molting, and *lin-4* and *lin-29* are required for cells at the L4 molt to execute the adult molt as opposed to larval molts. The normal temporal control of the L/A switch would appear to require a complementary action and/or interaction of these "adult-inhibiting" and "adult-activating" genes. Without further analysis, various models for such interactions are all plausible.

As a first step toward elucidating the roles of these genes in the control of L/A switching, it is necessary to determine whether this switch occurs in the absence of all four of these genes. The resulting phenotype would reflect the "ground state" of the switch with respect to these four control genes. The second step is to determine whether the wild-type activity of a single gene can toggle the switch in the absence of the other three genes. This latter experiment would identify the gene or genes (among these four) that act most directly on the switch itself.

No Switching Occurs in the Simultaneous Absence of *lin-4, lin-14, lin-28,* and *lin-29*

A strain (VT364) carrying loss-of-function alleles of all four heterochronic genes (*lin-4, lin-14, lin-28,* and *lin-29*) displayed no L/A switching. All animals examined at the L1, L2, L3, and L4 molts executed larval molts. Cuticles formed at all four molts lacked adult lateral alae, and seam cells were observed to divide (Table 1, Figures 1 and 2b). These results suggest that the ground state of the L/A switch with respect to these four genes is the repeated expression of larval molts and that the execution of the L/A switch by hypodermal cells is caused by the imposition over this ground state of a signal to execute an adult molt. This signal may be provided, directly or indirectly, by the activity of one or both of the adult-activating genes, *lin-4* and *lin-29*.

VT364 animals were not observed to undergo additional molts. Every animal that was followed expired approximately 10–20 hr after the L4 molt. This lethality of older adults may be caused in part by nonspecific physiological effects of multiple mutations. Furthermore, VT364 hermaphrodites are fertile, yet they are egg-laying defective due to abnormal vulva morphogenesis and consequently are consumed by their self-progeny. (Whether VT364 animals lacking a gonad would execute further molts has not been tested.)

lin-29 Is Necessary and Sufficient for L/A Switching in the Simultaneous Absence of the Other Three Genes

Since mutation of either *lin-4* or *lin-29* leads to repeated larval molts in place of the adult molt, it was important to test whether one or both of these genes are required to allow L/A switching. To distinguish between these possibilities, two different triply mutant strains were constructed. These strains contained the wild-type alleles of either *lin-4* or *lin-29* and loss-of-function alleles of the other three genes. A strain carrying wild-type function of only *lin-4* displayed no switching after four molts (Table 1, Figure 2c). In contrast, in a strain carrying the wild-type function of only *lin-29*, the L/A switch occurred at the L2 molt (Table 1, Figures 1 and 2d). This result indicates that *lin-29* is necessary and sufficient for activation of the switch in the absence of the other three heterochronic genes. It should be noted that although *lin-29* does not appear to depend on *lin-4, lin-14,* or *lin-28*, it may activate the switch by activating another, unidentified adult activator gene(s) or by inhibiting an unidentified larval inhibitor gene(s). (The L2 molt in *lin-4; lin-14; lin-28* triply mutant animals is the final molt, fully an "adult" molt by all criteria. This apparent enhancement of the effects of *lin-28* mutations when combined with *lin-14* mutations will be discussed further below.)

The wild-type activity of *lin-4* must activate the L/A switch differently than *lin-29*. Specifically, *lin-29* can activate switching in the simultaneous absence of the other three genes, yet *lin-4* cannot. *lin-4* must therefore activate the L/A switch through, or in combination with, the activities of *lin-14, lin-28,* and/or *lin-29*.

Although *lin-29* wild-type activity is necessary and sufficient to allow L/A switching in the absence of *lin-4, lin-14,* and *lin-28*, switching under these conditions is not properly regulated. In animals carrying the wild-type function of

only *lin-29*, the adult molt occurs precociously, at the L2 molt (Figure 2d). This indicates that the roles of *lin-4, lin-14,* and/or *lin-28* in the wild type are to ensure the proper temporal regulation of the L/A switch.

lin-14 and *lin-28* Regulate the Stage Specificity of the L/A Switch by Regulating *lin-29*

The wild-type activities of *lin-14* and *lin-28* appear to prevent early switching by inhibiting *lin-29*. *lin-14* semidominant mutations, which cause inappropriately elevated levels of *lin-14* at late stages of development, block L/A switching (Table 1, Figure 3a; Ambros and Horvitz, 1984). Since these animals possess a wild-type *lin-29*, it would appear that *lin-14* can inhibit or block the action of *lin-29*. This view is further supported by the fact that precocious switching in the absence of *lin-14* or *lin-28* (data not shown) or in the absence of both (compare Figures 2c and 2d) requires *lin-29* function. These results suggest that *lin-14* and *lin-28* act during wild-type larval development to oppose the action of *lin-29* and hence prevent early switching (Figure 4). *lin-14* and *lin-28* may directly or indirectly repress *lin-29* gene expression or inhibit the activity of a *lin-29* gene product.

lin-4 Is A Negative Regulator of *lin-14* and *lin-28*

The retarded defects caused by the *lin-4* loss-of-function mutation *e912* (Chalfie et al., 1981) are nearly identical to those caused by *lin-14* semidominant gain-of-function alleles, *n536* and *n355* (Table 1, Figure 3; Ambros and Horvitz, 1984). Since *lin-14* semidominant alleles cause elevated levels of *lin-14* activity at late stages of development (Ambros and Horvitz, 1987), it is plausible that the *lin-4(e912)* mutation also leads to elevated *lin-14* gene function. This proposal is supported by the fact that the retarded defects of *lin-4(e912)* absolutely require *lin-14* activity; a double mutant carrying *lin-4(e912)* and a *lin-14* loss-of-function allele express only the *lin-14*-associated precocious defects and none of the *lin-4(e912)*-associated retarded defects (Table 1, Figure 3d).

The elevation of *lin-14* activity by a mutation that reduces or eliminates *lin-4* function (*e912*) would suggest that the wild-type *lin-4* product is a negative regulator of *lin-14*. The normal function of *lin-4* may be to cause a temporal decrease in *lin-14* activity during development. *lin-4* may also be a negative regulator of *lin-28*. Either *lin-14* or *lin-28* loss-of-function mutations block the effects of *lin-4(e912)* (Figures 3d and 3e), indicating that *lin-4* acts, directly or indirectly, via both *lin-14* and *lin-28*. Since these data do not discern a hierarchical relationship between *lin-14* and *lin-28*, it is unclear whether *lin-4* acts directly on both *lin-14* and *lin-28* or indirectly on one via the other. Thus, for the sake of this discussion, it will be assumed that *lin-4* negatively regulates both *lin-14* and *lin-28* (Figure 4), with the caveat that these data do not rule out the possibility that *lin-4* may regulate one of these genes via the other.

Two other observations are consistent with the view that *lin-14* and *lin-28* act at the same step or similar steps in a regulatory pathway. First, the simultaneous loss of *lin-14* and *lin-28* leads to a more severe precocious defect than loss of either gene alone (compare the phenotypes of strains VT292, MT1524 and VT334 in Table 1). Although there are various possible explanations for this mutual enhancement of *lin-14* and *lin-28* precocious defects, one possibility is that L/A switching is inhibited in the wild type by the combined activities of these two genes. Second, the effect of a *lin-14* gain-of-function mutation on the L/A switch is blocked in the absence of *lin-28* (Figure 3b), indicating that *lin-14* requires the presence of *lin-28* activity to inhibit L/A switching. Although this suggests that *lin-14* is a positive regulator of *lin-28*, it is also consistent with an activation of *lin-14* by *lin-28*, or an interdependency of the two gene activities.

Discussion

Stage-Specific Regulatory Interactions Control the L/A Switch

In the wild type the L/A switch occurs only at the L4 molt. In *lin-14* and *lin-28* mutants the switch occurs at the L3 molt. In animals homozygous for mutations in both *lin-14* and *lin-28*, the switch can occur at the L2 molt. These results suggest that in the wild type, cells at the L2, L3, and L4 molts are bipotential with respect to the expression of either a larval or an adult molt. The four heterochronic genes examined here seem to be part of a pathway controlling whether or not cells at these stages execute the L/A switch. The model shown in Figure 4 proposes regulatory interactions among *lin-4, lin-14, lin-28,* and *lin-29* that lead to a stage-specific execution of the L/A switch in the lateral hypodermal cell lineages. *lin-14* and *lin-28* prevent switching at the L2 and L3 molts by inhibiting *lin-29* activity. *lin-4* turns *lin-14* and *lin-28* off, resulting in activation of *lin-29* in the L4 stage, which triggers the switch.

Although this model proposes that all four gene activities change during development, the most direct genetic evidence for a temporal change in gene activity applies to *lin-14*. This conclusion is drawn from genetic analysis of temperature-sensitive *lin-14* loss-of-function and gain-of-function alleles (Ambros and Horvitz, 1987) and is supported by the finding that a *lin-14* protein product is concentrated in specific nuclei of L1 larvae but is greatly reduced or absent from later larvae (Ruvkun and Guisto, 1989). Despite the absence of concrete genetic and molecular evidence that *lin-4, lin-28,* and *lin-29* activities also change during development, it is nevertheless plausible to propose that these genes are also temporally regulated.

The phenotypic and genetic properties of the heterochronic genes and the formal nature of their functional interactions may reflect any of a variety of cellular and molecular mechanisms. For example, it is not known whether any of these genes control cell-extrinsic processes, such as the activity of a hormone, or cell-intrinsic processes, such as the regulation of gene expression. Resolution of such issues awaits the analysis of genetically mosaic heterochronic mutants and the further molecular characterization of the heterochronic gene products. Further studies may also determine whether the products of these genes interact directly or whether they participate in processes occurring in distinct cellular compartments or at distinct times during development. For example, tem-

perature-shift experiments have indicated that *lin-14* is required during the L1 stage to prevent adult molting at the L3 molt (Ambros and Horvitz, 1987). *lin-28* could also act in the L1 stage—for example, as a cofactor with *lin-14*. On the other hand, *lin-28* may act subsequently—for example, to maintain during later stages the determinative effects initiated by *lin-14* in the L1 stage.

The Genetic Control of Stage-Specific Cell Division and Differentiation

One aim of the analysis of interactions among developmental mutations is to uncover the genetic circuitry connecting regulatory genes to each other and to the diverse effecter genes involved in the process of cell differentiation per se. The results of these experiments indicate a hierarchical relationship among *lin-4, lin-14, lin-28,* and *lin-29*. These genes seem to function at one or more levels of a pathway by which cells receive temporal information, interpret that information, and choose appropriate developmental programs. For example, *lin-4* and *lin-14,* which show relatively broad tissue specificities, are more likely to convey general temporal information interpretable by a variety of cell types for the execution of diverse developmental choices. In contrast, *lin-29* would seem to represent a gene whose product participates in a cell type–specific response to *lin-14* in the lateral hypodermal cells in particular.

lin-29 may be an interface between, on the one hand, general temporal control genes such as *lin-4* and, on the other hand, specific larval and adult developmental programs. The temporal control of the L/A switch can be interpreted as the control of a set of specific developmental choices for seam cells at the L2, L3, and L4 stages. One choice is to execute molting or to cease the molting cycle permanently. If the choice is made to molt, then additional choices are, one, to form larval cuticle or to form adult cuticle and, two, to divide or to fuse and exit the cell cycle. These developmental choices are controlled most directly by *lin-29*. Thus, *lin-29* may act by regulating genes controlling stage-specific cuticle morphology, molting, and the division and differentiation of seam cells (Figure 4). *lin-29* may regulate these rather diverse cellular processes directly or via more specialized regulators. These matters may be illuminated by further genetic and molecular analysis of *lin-29* and by identification of the molecular targets of *lin-29* activity.

In addition to the formation of adult-specific lateral alae, stage-specific features of cuticle ultrastructure (Cox et al., 1981) and the stage specificity of collagen mRNA accumulation (Cox and Hirsh, 1985) are also altered in heterochronic mutants (Ambros and Horvitz, 1987; Liu and Ambros, unpublished data). Analysis of cuticle ultrastructure and collagen gene transcription in multiple mutants is necessary to determine if the same regulatory relationships apply to stage-specific aspects of cuticle formation other than that assayed in this study, i.e., adult lateral alae formation. Nevertheless, it seems plausible to propose that *lin-29* coordinately controls adult lateral alae formation and other adult-specific cuticle features through the temporal regulation of genes encoding stage-specific cuticle proteins (Figure 4).

lin-4 and *lin-14* affect the timing of certain other developmental events that are not controlled by *lin-29*. These events include the timing of dauer larva formation (Liu and Ambros, unpublished data) and the timing of specific cell divisions in the vulva precursor lineages, in the intestine, and in the sex muscle lineage (Chalfie et al., 1981; Ambros and Horvitz, 1984). Additional epistasis experiments will determine whether *lin-4* and *lin-14* control these other events via regulatory hierarchies analogous to (or perhaps overlapping with) the pathway of L/A switch control. Further genetic studies are also required to identify genes specialized in the regulation of events other than the L/A switch.

Experimental Procedures

General Methods and Genetic Markers

General methods for the culture and handling of C. elegans have been described by Brenner (1974). Methods used for Nomarski differential interference contrast microscopy of living animals and for photography have been described by Sulston and Horvitz (1977) and Sternberg and Horvitz (1981). The fates of seam cells at each postembryonic larval molt were scored using Nomarski microscopy of living heterochronic mutant animals as described elsewhere (Ambros and Horvitz, 1987).

The wild-type parent of all strains used in this work is C. elegans var. Bristol strain N2. The genetic markers listed below are from the Cambridge collection (Brenner, 1974) unless otherwise noted. *lin-14* alleles are described in Ambros and Horvitz (1987) unless otherwise noted. This paper conforms to the standard C. elegans genetic nomenclature (Horvitz et al., 1979). Genetic markers used are: LGI, *lin-28(n719)* (Ambros and Horvitz, 1984); LGII, *lin-4(e912)* (Chalfie et al., 1981), *lin-29(n333)* (Ambros and Horvitz, 1984), *dpy-10(e128), unc-4(e120), unc-52(e444)*; LGV, *him-5(e1490)* (Hodgkin et al., 1979); LGX, *dpy-6(e14), lin-14(n536), lin-14(ma135). lin-14(ma135)* was isolated using a noncomplementation screen: Approximately 10,000 F1 progeny of ethyl methanesulfonate–mutagenized N2 males crossed with *dpy-6(e14) lin-14(n179ts)* hermaphrodites (strain TY172) were examined, and three independent Lin animals were identified. One of these Lin animals, genotype *dpy-6(e14) lin-14(n179ts)/lin-14(ma135)*, was the source of *lin-14(ma135)*. The reciprocal translocation *szT1(X;I)* is a balancer for LGX and LGI that causes recessive lethality in hermaphrodites and carries wild-type alleles of *lin-14* and *lin-28* (Fodor and Deak, 1982). *mnC1* is a chromosomal rearrangement that balances LGII (Herman,1978) and contains the recessive markers *dpy-10(e128)* and *unc-52(e444)* and wild-type alleles of *lin-4* and *lin-29*.

The following strains were used in this work:

CB912	*lin-4(e912)*
MT333	*lin-29(n333)*
MT1149	*lin-14(n536)*
MT1155	*lin-4(e912)/mnC1; him-5(e1490)*
MT1397	*lin-14(n179ts)*
MT1402	*lin-14(n536)/szT1(X;I)*
MT1524	*lin-28(n719)*
MT1537	*lin-28(n719); lin-14(n536)*
MT1538	*lin-28(n719); lin-4(e912)*
TY172	*dpy-6(e14) lin-14(n179)*
VT291	*lin-4(e912); lin-14(ma135)*
VT292	*lin-14(ma135)*
VT329	*lin-4(e912) lin-29(n333)/mnC1*
VT330	*lin-28(n719)/szT1(X;I); lin-14(ma135)/szT1(X;I)*
VT334	*lin-28(n719)/lin-28(n719) szT1(X;I); lin-14(ma135)/szT1(X;I)*
VT359	*lin-28(n719)/szT1(X;I); lin-4(e912) lin-29(n333); lin-14(ma135)/szT1(X;I)*
VT363	*lin-28(n719)/lin-28(n719) szT1(X;I); lin-4(e912) lin-29(n333); lin-14(ma135)/szT1(X;I)*
VT364	*lin-28(n719); lin-4(e912) lin-29(n333); lin-14(ma135)*

VT365 *lin-28(n719); lin-29(n333); lin-14(ma135)*
VT366 *lin-28(n719); lin-4(e912); lin-14(ma135)*

n719, n333, and *ma135* are fully recessive and represent the most extreme phenotype of each gene. These particular alleles are considered null alleles based on the observation that they appear to be equivalent to deficiencies of their respective loci in complementation experiments (Ambros and Horvitz, 1984). It is assumed that the one *lin-4* allele, *lin-4(e912),* is a hypomorphic or null allele, since it is fully recessive (Chalfie et al., 1981).

Strain Constructions

The multiply mutant strains used in this study are homozygous viable and fertile enough for propagation, although, in general, the combined pleiotropic effects of these multiple mutations lead to severe defects in morphology, movement, egg laying, overall growth rate, and fertility. These strains were constructed as outlined below. Care was taken to design and execute strain construction protocols such that genotypes could be unambiguously deduced from phenotypes. Wherever ambiguities existed, genotypes were verified by complementation tests.

MT1537 was constructed by mating *lin-28(n719)/+* males with MT1402 (*lin-14(n536)/szT1(X;I)*) hermaphrodites and identifying among the F2 self-progeny animals of genotype *lin-28(n719)/+; lin-14(n536)* (these are phenotypically retarded; see Ambros and Horvitz, 1987). From among the self-progeny of these latter animals, a strain of the desired genotype was obtained by identifying animals with precocious defects.

MT1538 was constructed by mating *lin-28(n719)/+* males with *lin-4(e912)/mnC1* hermaphrodites, isolating *lin-28(n719)/+; lin-4(e912)* animals among the F2 progeny and then identifying animals of the desired genotype among the F3.

VT291 was constructed by crossing MT1155 (*lin-4(e912)/mnC1; him-5(e1490)*) males with *lin-14(ma135)/szT1(X;I)* hermaphrodites to obtain animals of genotype *lin-4(e912)/+; him-5 (e1490); lin-14(ma135)/+*. From among the F1 self-progeny of these latter animals, animals of the genotype *lin-4(e912); lin-14(ma135)/+* were isolated, and from among their progeny, precocious animals of the desired genotype were obtained.

VT329 was constructed by first identifying a *lin-4–lin-29* recombinant chromosome II in the presence of a temperature-sensitive suppressor of *lin-4, lin-14(n179ts)*: Lin-4, Non-Unc recombinant progeny of *lin-4(e912) lin-29(+) unc-4(e120)/lin-4(+) lin-29(n333) unc-4(+); lin-14(n179ts)* hermaphrodites were isolated at 15°C, and their progeny were raised at 20°C to allow suppression of *lin-4* defects. These progeny (of genotype *lin-4(e912) lin-29(n333)/lin-4(e912) unc-4(e120); lin-14(n179ts)*) were mated with *lin-29(n333)/mnC1; him-5(e1490)* males, and the progeny of this latter cross were isolated and progeny tested for two generations to derive a strain of the desired genotype.

VT330 was constructed by mating *szT1* males with hermaphrodites of genotype *lin-28(n719)/+; lin-14(ma135)/+*. Individual F1 progeny were isolated and allowed to produce self-progeny. Among these F1 animals, a strain containing both the *lin-28* and *lin-14* markers was identified by complementation tests.

VT334 was derived from Lin-28 recombinant progeny of VT330. (*szT1* does not suppress recombination completely in the region near *lin-28,* allowing occasional recombination between *lin-28(n719)* and *szT1*).

VT359 was constructed by mating *lin-4(e912) lin-29(n333)/++; szT1(X;I)* males with *lin-28(n719)/+; lin-14(ma135)/+* hermaphrodites. Cross progeny were isolated and progeny tested to identify animals of genotype *lin-28(n719)/szT1(X;I); lin-4(e912) lin-29(n333)/++; lin-14(ma135)/szT1(X;I)*. From among the progeny of these latter animals, a strain of the desired genotype was obtained.

VT363 was derived from Lin-28 recombinant progeny of VT359.

VT364 was isolated from among the self-progeny of VT363.

VT365 was constructed using a protocol analogous to that for VT364 except that *lin-29(n333)/mnC1; him-5(e1490)* males were used in the initial cross.

VT366 was constructed using a protocol analogous to that for VT364 except that *lin-4(e912)/mnC1; him-5(e1490)* males were used in the initial cross.

Counting Molts

The number of molts expressed by individual animals was determined by periodic observation beginning at known developmental stages. Larvae were obtained that had recently hatched or were in a state of lethargus (Singh and Sulston, 1978). The precise developmental stage was verified using Nomarski microscopy to score for the occurrence of developmental events that serve as markers of developmental stage in heterochronic mutants. These events include the number of gonadal nuclei, extent of gonad morphogenesis, extent of ventral nerve cord development, and extent of lateral hypodermal development (Ambros and Horvitz, 1987). Some individual animals were observed beginning at hatching or at the L1 molt and throughout the remainder of development. Other animals were observed beginning at the L2, L3, or L4 molts. Larvae were placed individually on petri dishes and observed at least once every 4 hr for evidence of molting (reduced feeding behavior, reduced motility, and the occurrence of ecdysis). The occurrence of a molt was scored by the observation of an animal emerging from its old cuticle or by the detection of a recently abandoned empty cuticle on the agar surface. Empty cuticles were easily observable in the dissecting microscope at 25×–50× provided that the bacterial lawn was not appreciably disturbed by the animals' locomotion. So that freshly abandoned cuticles could be readily observed, each animal was transferred to a fresh plate every 3–4 hr and/or after ecdysis. Some of these animals that were followed using the dissecting microscope were also examined from time to time by Nomarski optics to assay for seam cell divisions (Ambros and Horvitz, 1987)

Scoring Larval and Adult Molts

Molts of a "larval" type were distinguished from an "adult" type by examining the behavior of seam cells at the time of the molt using Nomarski differential interference microscopy and by scoring for the presence or absence of the normally adult-specific lateral alae on the surface of the cuticle. Larval molts were identified primarily by the complete absence of adult lateral alae on the newly formed cuticle and also by the occurrence of seam cell divisions. The adult molt was identified by the formation of adult cuticle (adult lateral alae along the full length of the animal), the complete absence of seam cell divisions, and by the fact that the adult molt is the final molt. In certain *lin-28* mutants, some seam cells form alae and do not divide while other seam cells in the same animal at the same molt divide and do not form alae (see Results). Such molts, at which mixtures of larval and adult seam cell fates are expressed, are termed "semiadult."

A novel defect was observed in double mutants carrying mutations that elevate *lin-14* and reduce *lin-28.* In the strains *lin-28(n719); lin-14(n536)* and *lin-28(n719); lin-4(e912),* some animals began the molting process after the fourth stage and then failed to complete the molt. They remained in a state of inactivity, similar to normal L4 lethargus, for abnormally long periods of 10–12 hr or more, eventually being consumed by their progeny because of their inability to lay eggs. A new cuticle was observed to be formed in some of these animals, so the block may be in the initiation of ecdysis. Thus, this somewhat adult-like molt is also designated "semiadult."

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