Organization and Expression of Globin Genes, G. Stamatoyannopoulos and A. W. Nienhuis, Eds. (Liss, New York, 1981), pp. 313–334; M. Bar-Eli, H. D. Stang, K. E. Mercola, M. J. Cline, *Somatic Cell Genet.* **9**, 55 (1983).
30. W. F. Anderson, L. Killos, L. Sanders-Haigh, P. J. Kretschmer, E. G. Diacumakos, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 5399 (1980); M. R. Capecchi, *Cell* **22**, 479 (1980); A. Graessman, M. Graessman, W. C. Topp, M. Botchan, *J. Virol.* **32**, 989 (1979); W. F. Anderson and E. G. Diacumakos, *Sci. Am.* **245**, 106 (July 1981); C. W. Lo, *Mol. Cell. Biol.* **3**, 1803 (1983).
31. E. Neumann, M. Schaefer-Ridder, Y. Wang, P. H. Hofschneider, *EMBO J.* **1**, 841 (1982); H. Potter, L. Weir, P. Leder, *Proc. Natl. Acad. Sci. U.S.A.*, in press.
32. E. G. Diacumakos, L. Killos, L. Lee, W. F. Anderson, *Exp. Cell Res.* **131**, 73 (1981).
33. J. W. Gordon and F. H. Ruddle, *Science* **214**, 1244 (1981); E. F. Wagner, T. A. Stewart, B. Mintz, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 5016 (1981); T. E. Wagner *et al.*, *ibid.*, p. 6376; F. Costantini and E. Lacy, *Nature (London)* **294**, 92 (1981); R. L. Brinster *et al.*, *Cell* **27**, 223 (1981); R. D. Palmiter *et al.*, *Nature (London)* **300**, 611 (1982); G. S. McKnight, R. E. Hammer, E. A. Kuenzel, R. L. Brinster, *Cell* **34**, 335 (1983); R. D. Palmiter, G. Norstedt, R. E. Gelinas, R. E. Hammer, R. L. Brinster, *Science* **222**, 809 (1983).
34. J. W. Gordon, G. A. Scangos, D. J. Plotkin, J. A. Barbosa, F. H. Ruddle, *Proc. Natl. Acad. Sci. U.S.A.* **77**, 7380 (1980).
35. R. L. Brinster, K. A. Ritchie, R. E. Hammer, R. L. O'Brien, B. Arp, U. Storb, *Nature (London)* **306**, 332 (1983).
36. T. E. Wagner, personal communication.
37. E. Lacy, S. Roberts, E. P. Evans, M. D. Burtenshaw, F. D. Costantini, *Cell* **34**, 343 (1983).
38. E. F. Wagner, L. Covarrubias, T. A. Stewart, B. Mintz, *ibid.* **35**, 647 (1983); R. D. Palmiter, T. M. Wilkie, H. Y. Chen, R. L. Brinster, *ibid.* **36**, 869 (1984); F. H. Ruddle, personal communication.
39. This is true for recessive genetic diseases. Dominant diseases will probably not be treated by gene therapy in the near future because the defective gene appears to make a damaging product. It is not now possible to engineer the shut-off of an endogenous gene, nor to replace a gene. All that appears likely in the foreseeable future is to be able to add a normal gene to a cell, not to repair or replace a defective gene already present.
40. G. Swift, R. Hammer, R. MacDonald, R. Brinster, *Cell*, in press.
41. R. K. Humphries *et al.*, in preparation.
42. R. D. Palmiter, H. Y. Chen, R. L. Brinster, *Cell* **29**, 701 (1982).
43. R. C. Willis *et al.*, *J. Biol. Chem.* **259**, 7842 (1984).
44. R. C. Willis, A. H. Kaufman, J. E. Seegmiller, *ibid.*, p. 4157.
45. G. Khoury and P. Gruss, *Cell* **33**, 313 (1983); P. Gruss, *DNA* **3**, 1 (1984).
46. L. A. Laimins, G. Khoury, C. Gorman, B. Howard, P. Gruss, *Proc. Natl. Acad. Sci. U.S.A.* **79**, 6453 (1982); J. de Villiers, L. Olson, C. Tyndall, W. Schaffner, *Nucleic Acids Res.* **10**, 7965 (1982); P. E. Berg *et al.*, *Mol. Cell. Biol.* **3**, 1246 (1983).
47. P. E. Berg, Z. Popovic, W. F. Anderson, *Mol. Cell. Biol.* **4**, 1664 (1984).
48. S. D. Gillies, S. L. Morrison, V. T. Oi, S. Tonegawa, *Cell* **33**, 717 (1983); J. Banerji, L. Olson, W. Schaffner, *ibid.*, p. 729; C. Queen and D. Baltimore, *ibid.*, p. 741; U. Storb, R. L. O'Brien, M. D. McMullen, K. A. Gollahon, R. L. Brinster, *Nature (London)* **310**, 238 (1984); F. K. Fujimura, P. L. Deininger, T. Friedmann, E. Linney, *Cell* **23**, 809 (1981); M. Katinka, M. Vasseur, N. Montreau, M. Yaniv, D. Blangy, *Nature (London)* **290**, 720 (1981); M. D. Walker, T. Edlund, A. M. Boulet, W. J. Rutter, *ibid.* **306**, 557 (1983); I. S. Y. Chen, J. McLaughlin, D. W. Golde, *ibid.* **309**, 276 (1984); M.-J. Chen and A. W. Nienhuis, *J. Biol. Chem.* **256**, 9680 (1981); P. Mellon, V. Parker, Y. Gluzman, T. Maniatis, *Cell* **27**, 279 (1981); R. K. Humphries, T. Ley, P. Turner, A. D. Moulton, A. W. Nienhuis, *ibid.* **30**, 173 (1982).
49. M. Perucho, D. Hanahan, M. Wigler, *Cell* **22**, 309 (1980); D. M. Robins, S. Ripley, A. S. Henderson, R. Axel, *ibid.* **23**, 29 (1981); P. J. Kretschmer, A. H. Bowman, M. H. Huberman, L. Sanders-Haigh, L. Killos, W. F. Anderson, *Nucleic Acids Res.* **9**, 6199 (1981).
50. R. L. Brinster, H. Y. Chen, R. Warren, A. Sarthy, R. D. Palmiter, *Nature (London)* **296**, 39 (1982); K. E. Mayo, R. Warren, R. D. Palmiter, *Cell* **29**, 99 (1982).
51. K. Chada *et al.*, in preparation.
52. M. V. Chao, P. Mellon, P. Charnay, T. Maniatis, R. Axel, *Cell* **32**, 483 (1983).
53. S. Wright, E. deBoer, F. G. Grosveld, R. A. Flavell, *Nature (London)* **305**, 333 (1983).
54. A. D. Miller, E. S. Ong, M. G. Rosenfeld, I. M. Verma, R. M. Evans, *Science* **225**, 993 (1984).
55. K. Shimotohno and H. M. Temin, *Nature (London)* **299**, 265 (1982); J. Sorge and S. H. Hughes, *J. Mol. Appl. Genet.* **1**, 547 (1982).
56. M. A. Martin, T. Bryan, S. Rasheed, A. S. Khan, *Proc. Natl. Acad. Sci. U.S.A.* **78**, 4892 (1981); T. I. Bonner, C. O'Connell, M. Cohen, *ibid.* **79**, 4709 (1982).
57. M. Martin, personal communication.
58. *Fed. Regist.* **49** (No. 81), 17846 (25 April 1984).
59. Report of the RAC Working Group on Social and Ethical Issues discussed at RAC meeting on 6 February 1984.
60. H. I. Miller *Gene* **27**, 1 (1984) (editorial).
61. F. Young, personal communication.
62. The creation of a President's Commission on Human Applications of Genetic Engineering is one part of bill H.R. 2350.
63. J. Jensen, personal communication.
64. This position is, in fact, consistent with the existing regulation which states: "The IRB should not consider possible long-range effects of applying knowledge gained in the research . . . as among those research risks that fall within the purview of its responsibility" [45 *CFR* 46 (March 8, 1983) 46.111(a)(2)].

RESEARCH ARTICLE

Heterochronic Mutants of the Nematode *Caenorhabditis elegans*

Victor Ambros and H. Robert Horvitz

Significant evolutionary change in multicellular organisms may arise from mutations in genes that control temporal or spatial patterns of development. Much of the evolutionary variation in morphology and life history among related species has been proposed to result from "heterochrony," that is, from differences in the relative timing of developmental events (*1–3*). In some cases, radical morphological differences between species appear to result from simple differences in developmental timing. The isolation of mutations that lead to heterochrony may identify genes that control temporal patterns of development and also that could mutate to introduce heterochronic variation between species.

We have sought heterochronic mutants of the nematode *Caenorhabditis elegans*. The relatively simple anatomy and virtually invariant cell lineage of *C. elegans* (*4–6*) facilitate the detailed comparison of mutant and wild-type developmental patterns. Observation of living worms in the light microscope with the use of Nomarski differential interference contrast optics allows individual cells to be studied. Changes in temporal patterns of development can be characterized by examining the fates of individual cells at specific times during development. Each cell can be recognized by its lineage history and position, and its developmental fate can be defined by its morphology (an indicator of cell type), and in the case of blast cells, by the number and types of its progeny cells (*4*). The four larval stages of *C. elegans* are characterized by stage-specific patterns of cell division and differentiation (*4, 5*) and stage-specific cuticle formation (*7*).

Many mutants of *C. elegans* have been identified that are abnormal in cell lineage (*8*). The mutant *lin-4(e912)*, isolated because of its inability to lay eggs, displays multiple and complex cell lineage defects, with some patterns of cell division occurring repeatedly and others not occurring at all (*8, 9*). These mutant cell lineages, as well as other defects of *lin-4(e912)* can be interpreted as heterochronic; for example, the times of expression of certain cell division patterns are altered, while other cell division patterns occur at their normal times. Because a defect in egg-laying appeared to be one consequence of the heterochronic development of *lin-4(e912)*, we have screened other egg-laying defective mutants (*10*) for similar alterations in the stage specificity of developmental events. In this article, we describe the heterochronic developmental defects

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Abstract. *Mutations in the* Caenorhabditis elegans *genes* lin-14, lin-28, *and* lin-29 *cause heterochronic developmental defects: the timing of specific developmental events in several tissues is altered relative to the timing of events in other tissues. These defects result from temporal transformations in the fates of specific cells, that is, certain cells express fates normally expressed by cells generated at other developmental stages. The identification and characterization of genes that can be mutated to cause heterochrony support the proposal that heterochrony is a mechanism for phylogenetic change and suggest cellular and genetic bases for heterochronic variation.*

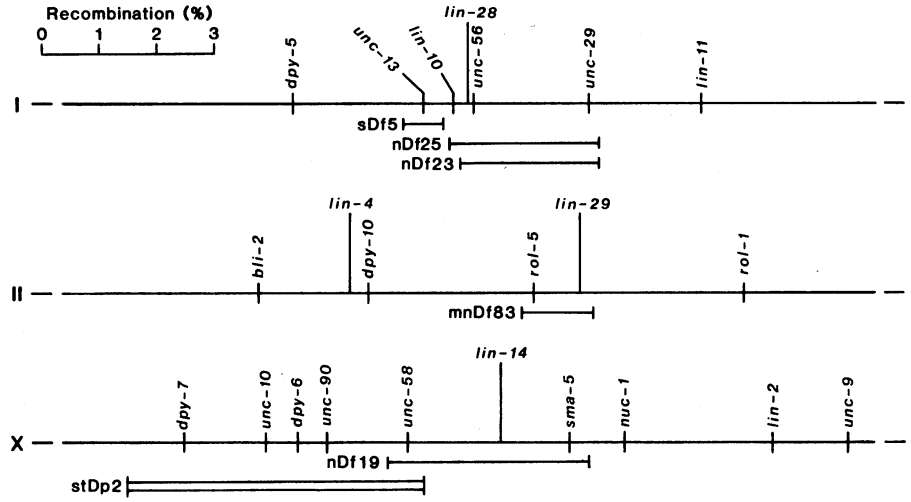


Fig. 1. Partial genetic map showing positions of genes defined by heterochronic mutants. This map is based on our mapping experiments (*16*) and the 1984 *Caenorhabditis elegans* genetic map (*34, 35*). Heterochronic genes are shown further above the line of each linkage group.

caused by mutations in three new genes, *lin-14*, *lin-28*, and *lin-29*. In these heterochronic mutants, specific cells adopt fates normally expressed by cells at other stages. As a result of these temporal transformations in cell fates, certain developmental events occur either earlier than normal ("precocious" events) or later than normal ("retarded" events) with respect to other, unaffected events.

Retarded lin-14 *mutants*. The X-linked semidominant *lin-14* alleles *n536* and *n355* (Fig. 1) (*11*) result in supernumerary molts and cell lineage defects similar to those caused by *lin-4(e912)*, a recessive mutation on linkage group II (*9*). These mutants display "retarded" development. Certain stage-specific events—such as molting and larval cuticle synthesis—are repeated ("reiterated") at abnormally late stages of development; this reiteration of early stage events leads to retardation of succeeding events, such as adult cuticle synthesis, by displacing them to still later stages.

The most apparent visible defects of *lin-14(n355)* and *lin-14(n536)* hermaphrodites are their inability to lay eggs and the occurrence of supernumerary molts beyond the normal four molts of the wild type. Sexual maturation of *lin-14(n355)* and *lin-14(n536)* hermaphrodites occurs at the normal stage, that is, after the L4

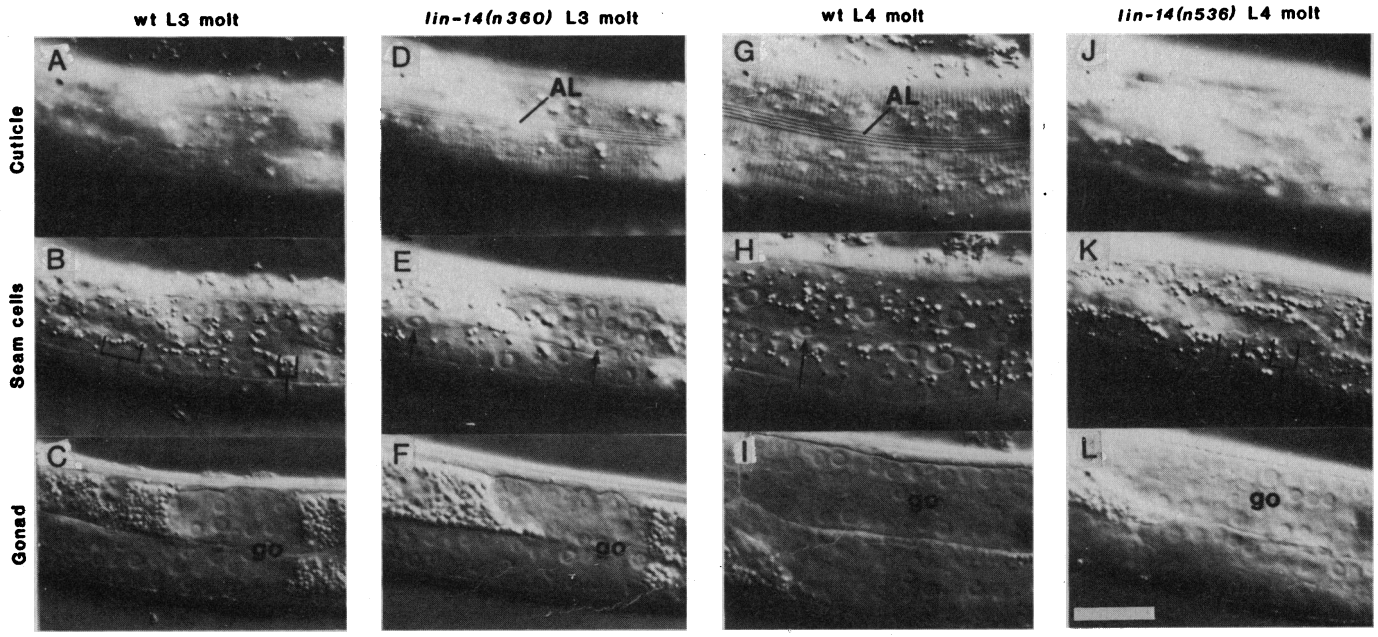


Fig. 2. Photomicrographs of living wild-type and heterochronic mutant hermaphrodites at specific stages of development (Nomarski optics; bar, 20 μm). Each vertical series of three photomicrographs is of the same hermaphrodite taken approximately 5 to 10 seconds apart but in different focal planes. The set A to L shows the expression of heterochronic seam cell fates at the L3 and L4 molts. For each animal, one plane of focus (panels C, F, I, and L) shows the gonad (go) for comparison of developmental stages. Panels A, D, G, and J show the plane of focus of the newly formed cuticles and panels B, E, H, and K show the plane of focus of the lateral hypodermal seam cell nuclei in the corresponding animals. AL, lateral alae; arrows indicate nuclei of nondividing seam cells; forked lines indicate daughter nuclei of dividing seam cells. (A, B, and C) At the L3 molt of the wild type, lateral alae are absent from the new cuticle (circumferential ridges, or annulae, are visible); seam cells at this stage undergo cell division. (D, E, and F) At the L3 molt of the precocious mutant *lin-14(n360)*, lateral alae (D) are formed by the seam cells and those seam cells are nondividing (E). (G, H, and I) In the wild type, these seam cell fates (adult alae formation and failure to divide) do not occur until one stage later, at the L4 molt. (J, K, and L) At the L4 molt of the retarded mutant *lin-14(n536)*, seam cells do not express their normal fates; they fail to form adult alae (J) and undergo cell division (K) (these are the same fates normally expressed earlier in the wild type (A and H).

molt; yet the cuticle of sexually mature fifth- and sixth-stage animals is unlike that of wild-type adults and instead appears similar to that of L2, L3, and L4 larvae. We have identified this cuticle as larval on the basis of (i) the absence of adult-specific external longitudinal ridges or "lateral alae," which extend the entire length of wild-type adults (Fig. 2J); and (ii) the presence of an ultrastructural layering pattern like that of wild-type larval cuticle (Fig. 3E). The expression of adult cuticle appears to be delayed to a later stage rather than blocked absolutely; the cuticles of *lin-14(n536)* and *lin-14(n355)* L6 animals can contain patches of alae in regions that lacked alae at the L5 stage, and always contain alae in those few regions that had alae at the L5 stage.

Other retarded developmental events include stage-specific patterns of cell division and differentiation. For example, certain cell lineage patterns specific for early larval stages in the wild type are reiterated at later stages in *lin-14(n355)* and *lin-14(n536)* animals. This defect is illustrated in Fig. 4 for the lateral hypodermal T lineage. In the T lineage of *lin-14(n536)* animals, a distinct cell division pattern occurs during the first stage and then is repeated during the second stage (Fig. 4B). In the wild-type T lineage, this pattern occurs only during the first stage (Fig. 4A). Other lateral hypodermal cell lineages of *lin-14(n536)* and *lin-14(n355)* reiterate first stage cell lineage patterns (S1) at later stages (Fig. 5, C and D). In *lin-14(n536)* animals (Fig. 5D), lineage patterns normally specific for the second stage (that is, "S2" patterns) do occur, but are often delayed by one or two larval stages (as a result of prior reiteration of S1 patterns) and are often then reiterated.

These cell lineage reiterations in *lin-14* retarded mutants seem to reflect transformations in cell fates. These transformations can be exemplified by the fates expressed by the lateral hypodermal "seam cells"—morphologically distinct cells that are blast cells during wild-type larval development and that participate in cuticle synthesis at each stage (*12*). For example, in *lin-14(n355)* and *lin-14(n536)* animals, the lateral hypodermal blast cell T.ap, which is the posterior daughter of the anterior daughter of the blast cell T [this cell nomenclature is described in (*4*)], fails to generate its normal pattern of progeny during the L2 and instead generates a lineage identical to that normally generated one larval stage earlier by its grandparental precursor, T (Fig. 4 and Fig. 5, C and D). Similarly, seam cells at the L4 molt do not express their normal fate, that is, they do not form adult lateral alae, but instead they express the fate normally specific for seam cells at earlier stages; that is, they divide (Fig. 2K and Fig. 5, C and D). These temporal transformations in cell fates, in which cells express fates normally expressed at an earlier stage of development, are analogous to the spatial transformations in cell fates seen in homeotic mutants of insects (*13*) and nematodes (*14*), in which cells express fates normally expressed at another position in the animal.

Other postembryonic cell lineages in *lin-14(n355)* and *lin-14(n536)* animals display abnormalities that can be interpreted as retarded development. For example, as in *lin-4(e912)* animals (*9*), the lineages of the vulval precursor cells, the sex mesoblasts and the intestinal nuclei of *lin-14(n536)* and *lin-14(n355)* animals often contain supernumerary divisions (data not shown). These supernumerary divisions may reflect the repeated expression of programs normally expressed at earlier times during development. Gonadal cell lineages have not been followed in *lin-14(n355)* or *lin-14(n536)* animals, but hermaphrodites are fertile and, aside from reduced brood size, these mutants appear to undergo

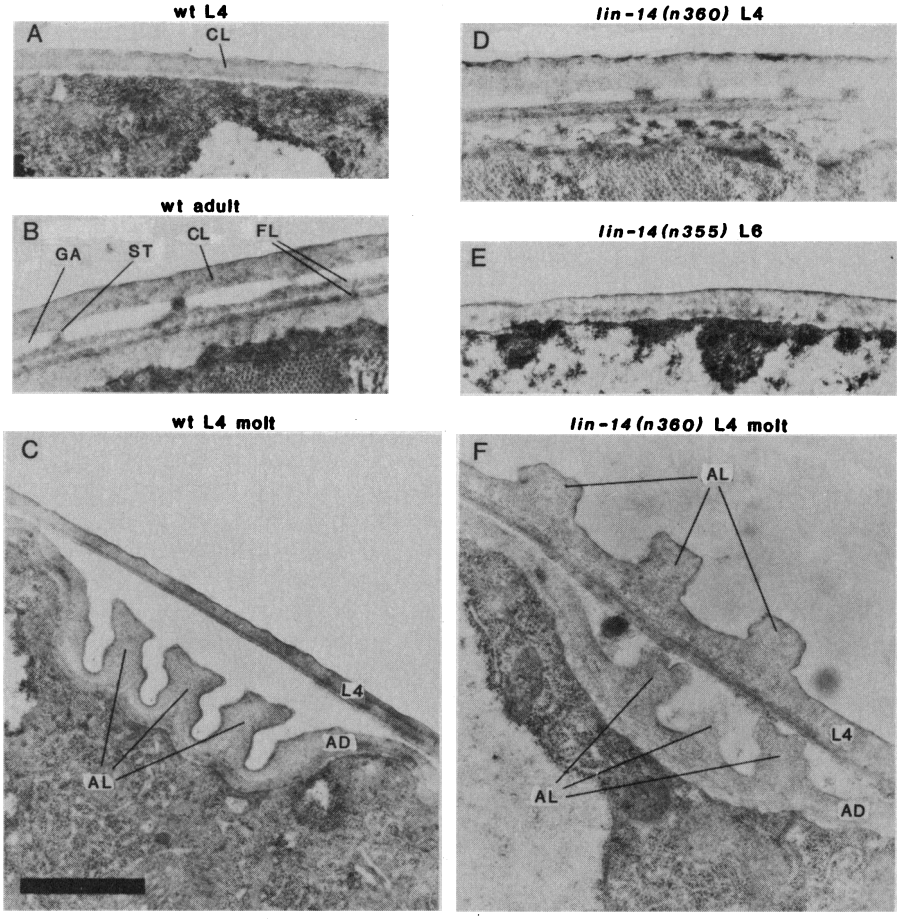


Fig. 3. Electron micrographs of thin sections of wild-type and heterochronic mutant cuticles. Stage-specific cuticle morphology of wild-type *C. elegans* has been described by Cox *et al.* (*7*). L4 larvae or adult animals are fixed and embedded according to Ward *et al.* (*36*). Animals were sectioned approximately perpendicular to their longitudinal axes. Sections were placed on slotted grids that had been coated with Parlodion and carbon. Sections were stained for 10 minutes at 70°C with uranyl acetate and at room temperature for 3 minutes with lead citrate (bar, 1 μm). (A) Wild-type L4 cuticle consists of a cortical layer (CL) apparently in direct contact with underlying layers. L2, L3, and L4 cuticles are morphologically similar (*7*). (B) Wild-type adult cuticle is distinguishable from larval cuticle on the basis of an electron transparent gap (GA) and opaque struts (ST) separating the outer cortical layer (CL) from the underlying fiber layers (FL). (C) Cross section of a wild-type L4 hermaphrodite fixed during L4 lethargus, prior to molting of the L4 cuticle. The alae (AL) of the newly formed adult cuticle (AD) are visible beneath the larval cuticle (L4), which lacks alae. (D) Cuticle of an L4 larva of the precocious mutant *lin-14(n360)* (strain MT1848) displays layering features, including gap and struts normally specific for adult cuticle (B). (E) Cuticle of a sixth stage ("L6") animal (sexually mature) of the retarded mutant *lin-14(n355)* (strain MT355) is structurally similar to wild-type L4 cuticle (A). Cuticle of L5 animals of this strain (not shown) also appear similar to wild-type L4 cuticle. (F) Cross section of a *lin-14(n360)* L4 hermaphrodite fixed at similar stage of development as the animal in (C). Alae, normally specific for adult cuticle (C), are visible on both the L4 cuticle and adult cuticle of this precocious mutant.

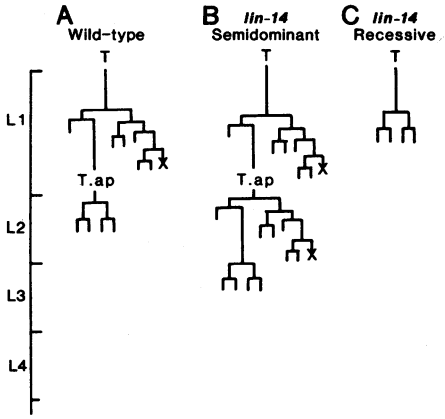


Fig. 4. Lateral hypodermal T cell lineages of the wild type and two opposite classes of *lin-14* mutants. Cell divisions were followed in living nematodes (*4*). Cell lineages are diagrammed and cells are named according to (*4*). Vertical scales to the left of each panel indicate developmental stage with the time of each postembryonic molt marked by a horizontal line. L1, L2, L3, and L4 are the four larval stages; supernumerary stages of *lin-14(n536)* (L5, and so on; see Fig. 5) are not shown. Unless otherwise noted in this and other figures, all cell divisions were along an anterior-posterior axis in the animal. Anterior is to the left in the diagram. The semidominant mutant *lin-14(n536)* (B) displays retarded development in the T lineage. In the L2 stage, T.ap generates a cell division pattern and descendant cell types very similar to those normally generated only during the L1 by the T cell (A). Conversely, in the recessive mutant *lin-14(n536 n540)* (C), the T cell precociously generates a cell division pattern normally generated during the L2 by T.ap. The four progeny of T in *lin-14(n536 n540)* appear by Nomarski microscopy (*4*) morphologically similar to the corresponding progeny of T.ap in the wild type.

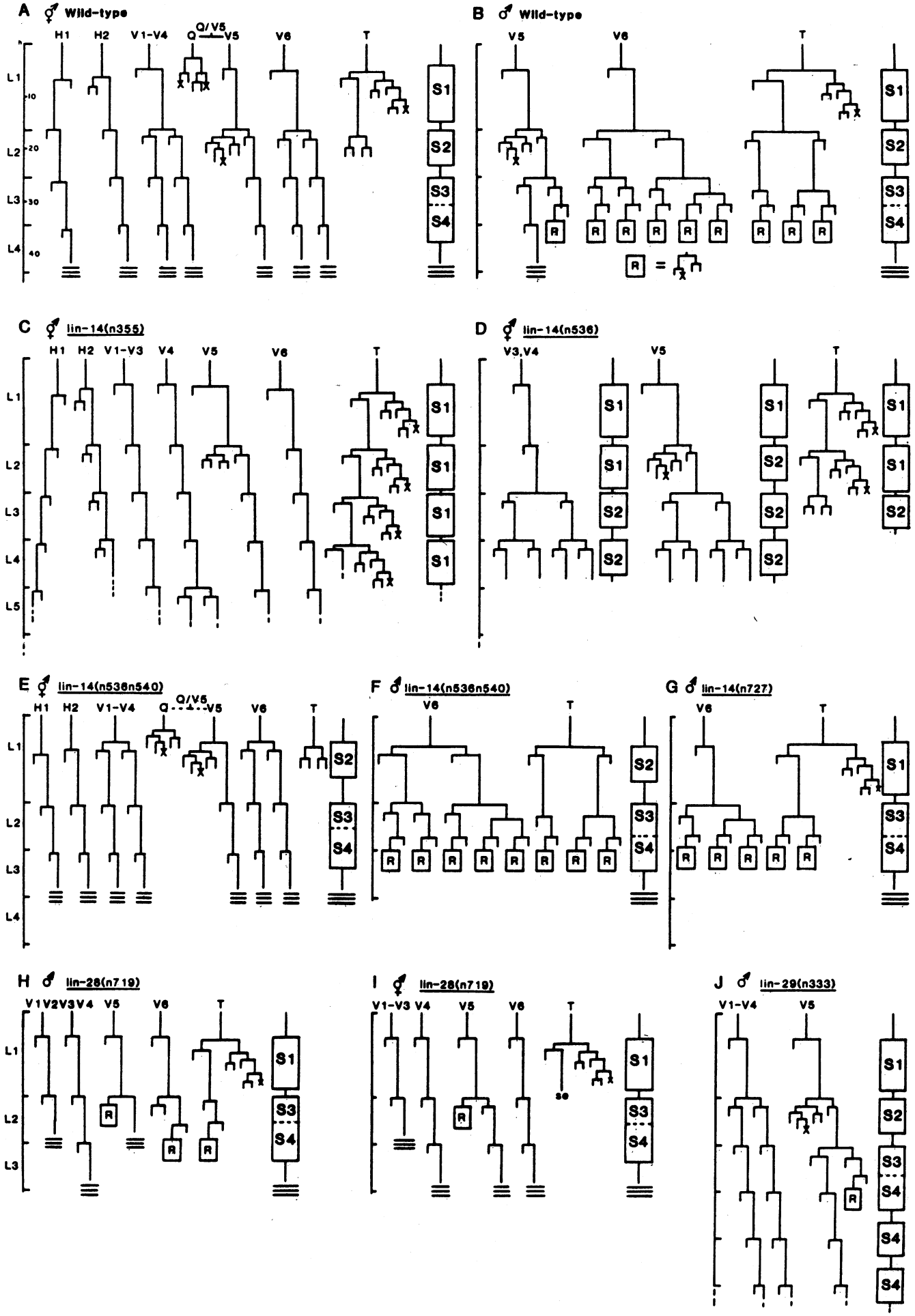
normal gonadal development. Sexual maturation, marked by the first appearance of fertilized oocytes, occurs after the fourth molt, as in the wild type. The vulval and mesodermal cell lineage abnormalities are sufficient to account for the egg-laying defects of these mutants.

Precocious lin-14 *mutants*. Recessive mutations of *lin-14* (*15*) cause developmental defects opposite to those caused by *lin-4(e912)* or semidominant *lin-14* mutations; that is, they cause precocious development, in which certain developmental events occur earlier than normal during development. The most frequent class of *lin-14* recessive mutations, typified by *lin-14(n536 n540)*, causes the precocious expression of specific developmental events in the same tissues affected by *lin-14* semidominant mutations.

The precocious development of *lin-14(n536 n540)* animals seems to reflect transformations in cell fates such that certain cells express fates normally expressed by cells at later stages of the same lineages. For example, in the L1 stage of *lin-14(n536 n540)* animals, the blast cell T does not generate its normal cell lineage pattern. Instead, the T cell generates during the L1 a pattern normally generated during the L2 by T.ap (Fig. 4C). Other lateral hypodermal blast cells are similarly affected; cells fail to generate their normal L1-specific patterns (S1 lineage patterns) but instead generate S2 lineage patterns (Fig. 5, E and F). The third and fourth stage lineage patterns (S3 and S4) similarly occur one larval stage early. Seam cells cease division one stage early, in the third larval (L3) stage, and produce adult lateral alae at the L3 molt, which is one stage earlier than normal (Fig. 5; see also Figs. 2 and 3, which indicate this phenotype for another *lin-14* recessive mutant). The cuticle formed at the L3 molt in these mutants is unlike wild-type L4 cuticle and is similar to wild-type adult cuticle, on the basis of both the presence of adult lateral alae overlying the seam cells (Fig. 2, D and E) and an adult-specific layered structure in regions distinct from the seam cells (Fig. 3D). Hermaphrodites of *lin-14* recessive mutants synthesize a second adultlike cuticle at the L4 molt (Fig. 3F).

Recessive mutations of *lin-14* also cause precocious expression of male-specific ectodermal lineages and hermaphrodite-specific ventral hypodermal (vulval) lineages (Fig. 6). In *lin-14(n536 n540)* hermaphrodites, the vulval precursor cells divide during the L2 stage, whereas in the wild type, the vulval precursor cells do not divide until the middle of the L3 stage. The *lin-14(n536 n540)* L4 hermaphrodites have one or two ventral protrusions resulting from precocious (and often abnormal) vulval morphogenesis. Abnormal vulval development is the source of the egg-laying

Fig. 5. Lateral hypodermal cell lineage patterns of wild-type and heterochronic mutant animals. Wild-type lineages (A and B) are from (*4*). Lineages are diagrammed as in Fig. 4, except that time scales are calibrated in hours after hatching (h). The embryonically derived lateral hypodermal blast cells (H1, H2, V1 to V6, and T) and their blast cell progeny divide at approximately the times of the first three larval molts. These nine cells, and their lateral hypodermal blast cell descendants, are known as "seam" cells and are arranged along each lateral line of the animal (*4*, *12*). In the wild type during the late L4 stage, seam cells generate the adult lateral alae (cuticular ridges); the approximate time of adult alae formation by a seam cell is indicated by three horizontal lines. The Q neuroblast lineage is shown only in the one mutant in which it is abnormal (E; compare A). The boxes (S1, S2, S3, and S4) to the right of each lineage or set of lineages refer to cell lineage patterns that are normally stage-specific in the wild type and that are coordinately affected in the mutants described (*37*). Each of these patterns consists of the specific cell divisions and the associated production of differentiated cell types that occur within the time interval defined by the vertical span of each box. At a given stage, these patterns differ in certain lineages from the corresponding patterns in certain other lineages; for example, the S1 pattern of the T lineage is different from the S1 pattern of the V6 lineage (A). Each of the nine sensory rays on each side of the male tail is formed from the same "sublineage" (*9*, *38*), designated R and indicated in panel (B). The time scales for all mutants have been normalized to that for the wild type (*39*). The mutant cell lineages shown in each panel (C to J) is from a single animal in which every cell division was followed (*40*). (C) *lin-14(n355)* hermaphrodite; strain MT355. Dotted lines extending down from the seam cells indicate that further cell divisions occurred but were not followed in detail. V5.pa generated four compact neural-like nuclei in this animal. We found the lineage generated by V5.pa to be highly variable. (D) *lin-14(n536)* hermaphrodite; strain MT1149. Selected lateral hypodermal lineages are shown to demonstrate the different lineage pattern reiterations typical of this mutant. This animal was not followed after the L4, but extra molts characteristic of this strain are indicated. (E) *lin-14(n536 n540)* hermaphrodite; strain MT1153. Both Q and V5.a generated during the L1 a set of compact nuclei and a cell death that by lineage and morphology are identical to those normally generated by V5.pa of the wild type (*4*). The sensory structure formed by these progeny, called a postdeirid, normally has one dopaminergic neuron, which can be visualized with formaldehyde-induced fluorescence (*41*). Each of the two precocious postdeirid lineages may generate a dopaminergic neuron, for *lin-14(n536 n540)* animals often have two dopaminergic neurons on one side (*42*). (F) *lin-14(n536 n540)* male. These lineages appear to be variable during the L2 resulting in variation in the number of rays generated. These males were obtained among the non-Dpy progeny of wild-type males crossed with hermaphrodites of the strain MT1394 *dpy-17(e164); szT1/lin-14(n536 n540)*. *szT1* is a balancer for linkage group X (*43*). (G) *lin-14(n727)* male. Obtained from a cross between N2 males and MT1851 *lin-14(n727) X* hermaphrodites. (H) *lin-28(n719); him-5(e1467)* male; strain MT2005. There is some uncertainty in the assignment of pattern identity to the lineages that occurred during the L2 and L3 stages. However, the T lineage of this animal generated, beginning in the L2 stage, a pattern identical to the anterior branch of the wild-type T lineage S3 and S4 patterns. The assignment of S3 to the patterns occurring during the L2 is based on this T lineage pattern. The *him-5* mutation causes a high incidence of males among the self progeny of hermaphrodites as a consequence of the nondisjunction of the X chromosome (*44*). (I) *lin-28(n719)* hermaphrodite; strain MT1524. (J) *lin-29(n333); him-5(e1467)* male. Strain MT2006. This animal was followed continuously only from the L3 molt to shortly after the L5 molt. Anatomical screens and partial lineages of animals at earlier stages indicated that all lateral hypodermal lineages are normal prior to the L4 stage.



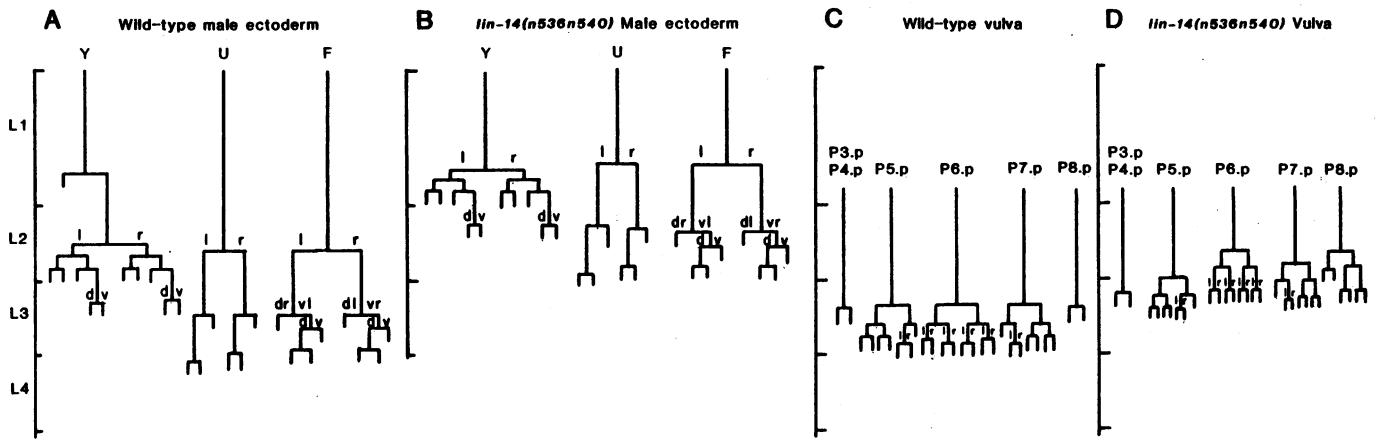


Fig. 6. (A) Cell lineages of the male ectoblasts Y, U, and F in the wild type (*4*). (B) Y, U, and F lineages of *lin-14(n536 n540)*; strain MT1153. (C) Ventral hypodermal (vulva) development in the wild type (*4*). (D) Vulva development of *lin-14(n536 n540)*. In this animal, the extra cells generated by P8.p formed an ectopic ventral protrusion (pseudovulva) posterior to the pseudovulva formed by the progeny of P5.p, P6.p, and P7.p. Vulva morphogenesis began at the L2 molt, approximately one larval stage earlier than in the wild type. The relative timing of the divisions of P3.p–P8.p and the number of progeny generated from each was somewhat variable.

defect of *lin-14* recessive mutants (*16–26*).

The major class of *lin-14* recessive mutations cause precocious expression of events at all larval stages and in several different lineages and tissues. Mutations of a second class of *lin-14* recessive alleles, typified by *n360* and *n727*, cause a subset of these same defects, that is, precocious adult cuticle formation (Fig. 2D and Fig. 3, D and F), precocious vulval development, and precocious S3 and S4 lineage patterns (Fig. 5G). However, unlike mutants of the major class, these mutants do not express precocious S2 lineage patterns. S1 lineage patterns occur at the normal stage (L1), and S2 lineage patterns are deleted (compare Fig. 5, G to F).

In *lin-14* precocious mutant hermaphrodites, the normal number of molts (four) occurs and gonadal development appears normal in that sexual maturation is reached shortly after the L4 molt. However, males of genotypes *lin-14(n179ts)* at 25°C, *lin-14(n536 n540)*, or *lin-14(n360)* appear to cease the molting cycle after three molts (Fig. 5F), suggesting that these males are more severely affected than hermaphrodites. Furthermore, the timing of gonadal development at later stages in these males appears to be abnormal, although we have not characterized this defect in detail.

Precocious lin-28 *mutants*. Mutations of *lin-28* cause precocious development and a partial transformation in sexual phenotype. The four alleles of *lin-28 I (n719, n947, n1119, n1120)* are recessive and cause precocious defects similar to those caused by the second class of *lin-14* recessive alleles (such as *n360* and *n727*) (Fig. 5). For example, in *lin-28(n719)* animals, S2 lineage patterns are deleted from each lateral hypodermal lineage, and S3 and S4 patterns occur earlier than in the wild type (Fig. 5, H and I). Seam cells cease cell division and form adult alae precociously (Fig. 5), and the cuticle of L4 larvae is also similar to normal adult cuticle in regions distinct from the seam cells (data not shown). The ventral hypodermal vulval precursors (P3.p to P8.p) divide earlier than in the wild type, and their progeny undergo vulval morphogenesis precociously (data not shown).

Although these defects of *lin-28* mutants are similar to those of *lin-14* recessive mutants, *lin-28* mutant development seems to be more severely affected. The *lin-28(n719)* seam cells occasionally form adult alae at the L2 molt, which is two stages earlier than in the wild type. Furthermore, *lin-28(n719)* hermaphrodites as well as males cease the molting cycle after the L3 molt (Fig. 5, H and I). Despite the precocious cessation of molting, gonadal development of *lin-28(n719)* hermaphrodites seems to follow approximately the wild-type schedule, based on the number of germ line and somatic gonad nuclei, the time of gonad reflection, and the time of appearance of fertilized oocytes with respect to the series of molts.

Another difference between the phenotypes of *lin-28* and *lin-14* recessive mutants is that *lin-28* hermaphrodites appear to be partially transformed into males. Specifically, in *lin-28* hermaphrodites, a normally male-specific sensory structure known as a "ray," which is recognizable by lineage and morphology (*4*), is formed (Fig. 5I). In wild-type males (Fig. 5B), ray formation is an L4-specific event. In both sexes of *lin-28* mutants, ray cells are generated during the L2 stage by a descendant of the lateral hypodermal blast cell V5 (V5.pa). This precocious ray formation supplants the normal fate of V5.pa, which is to form a different sensory structure called the "postdeirid" (*4*) (Fig. 5A). Ray formation in the L2 stage suggests that two lineage patterns (presumably S2 and S3) are deleted from the V5 lineage in *lin-28(n719)* animals.

One possible explanation for this partial transformation in the sexual phenotype of *lin-28(n719)* hermaphrodites is that *lin-28* activity directly controls the expression of both stage-specific and sex-specific cell fates in the V5 lineage. Alternatively, the sexual transformation may result indirectly from the severe precocious defects of *lin-28(n719)*. For example, *lin-28(n719)* may cause the deletion of a critical developmental period during which sexual determination normally occurs in the V5 lineage. As a result of the deletion of that critical period, perhaps the determination of the hermaphrodite fate cannot be made, and the male fate is expressed by default.

Retarded lin-29 *mutants*. The three alleles of *lin-29 II (n333, n546,* and *n836)* are recessive. Like *lin-14* retarded mutants, *lin-29* mutants are egg-laying defective and undergo supernumerary molts. However, *lin-29* mutations are more tissue- and stage-specific in their effects than are *lin-14* and *lin-28* mutations. In *lin-29* mutants the only cell lineages we have observed to be altered are those of the lateral hypodermis at the L4 stage. In each lineage, seam cells at the L4 molt do not form adult lateral alae (as they do in the wild type) but instead

divide (Fig. 5J); they also divide again at later stages. The progeny of these supernumerary divisions are like those normally generated at earlier stages of wild-type development, including the L4 stage. For the sake of discussion and by analogy with other heterochronic mutations, we postulate that *lin-29* mutations cause a reiteration, in this case of L4-specific divisions. Therefore, *lin-29* mutations seem to cause the expression of an early L4 seam cell fate (cell division) instead of the normal adult seam cell fate (cessation of cell division and formation of alae). In addition to lacking lateral alae, the cuticle of *lin-29* adults is also similar in its ultrastructure to normal L4 cuticle in regions not overlying the seam cells (data not shown). These overall effects of *lin-29* mutations can be interpreted as a failure of hypodermal cells to switch from a larval (L4) state to the adult state. Why *lin-29* mutants are defective in egg-laying is not clear; perhaps some characteristic of adult cuticle is required for proper vulval morphogenesis.

Possible phylogenetic role for mutations that cause heterochrony. The mutants described in this article display the two major complementary classes of heterochrony defined by evolutionary biologists (*2, 3*): in retarded mutants, mature individuals express normally juvenile characteristics (such as larval cuticle at adult stages; Fig. 3E); conversely, in precocious mutants, normally adult characteristics appear in immature individuals (such as adult cuticle on larvae; Fig. 3F). Such heterochrony may be a significant component of evolutionary change among a wide spectrum of animal species (*2*). The existence of the mutants we describe in this article indicates that mutations in single genes could cause such heterochronic developmental change during phylogeny.

We have discovered that the cell lineages of certain nematode species isolated from the wild differ from the corresponding cell lineages of other nematode species in a manner consistent with heterochrony. For example, the lateral hypodermal cell lineages of one *Rhabditida* species have characteristics similar to lineages of the *C. elegans* mutant *lin-14(n536)* (*27*). Perhaps mutations in heterochronic genes such as those we have described in this article could have led to heterochronic differences between the cell lineages of different nematode species. Such changes in the times of expression of various cell fates could lead to evolutionarily significant changes in nematode anatomy and life history. For example, the alterations in developmental timing caused by the heterochronic mutations described here result in the absence of specific structures, the duplication of other structures, partial sexual transformation, and changes in the number of larval stages.

Genes with properties similar to those of *lin-4, lin-14, lin-28*, and *lin-29* could be responsible for interspecific heterochrony in organisms other than nematodes. If so, heterochrony may often be caused by temporal transformations in cell fates, so that cells or groups of cells adopt fates specific for cells at other developmental stages. One consequence of such a mechanism for heterochrony would be that certain developmental events (the generation of specific differentiated cells or structures) of an ancestral species could be deleted or reiterated in a descendant. This mechanism is distinct from one involving changes in the relative rates of cell division within different lineages (*28*).

Heterochronic genes may control temporal patterns of development. The temporal transformations in developmental fates caused by heterochronic mutations suggest that heterochronic genes may control the times during *C. elegans* development at which specific cell fates are expressed. These genes may encode elements of a system by which cells receive and interpret temporal information and, on the basis of that information, express appropriate stage-specific characteristics. Detailed genetic analysis of each of these loci is required to determine its role in wild-type development. The genetic properties of *lin-14* (*29*) suggest that the dosage of *lin-14* gene activity specifies the fates expressed by cells at several stages of development, that is, high levels specify earlier fates and low levels specify later fates. The activity of *lin-14* may diminish during development and cause various cell fates to be expressed in defined temporal sequences. Perhaps genes such as *lin-14*, which has highly pleiotropic effects, are involved in conveying temporal information to diverse cell types. Genes with more tissue- or stage-specific effects (*lin-29*) may be required for the reception or utilization of that temporal information for cell type-specific or developmental stage-specific determinative choices.

By what molecular mechanisms might heterochronic genes act? One possible mechanism is illustrated by pharmacologically or genetically induced heterochrony in other organisms. Retarded expression of larval characteristics in the Mexican axolotl, *Ambystoma mexicanum*, seems to be caused by homozygosity for a single recessive allele (*30*). Aspects of this retarded development can be overcome by exogenous application of the thyroid hormone, thyroxine, indicating that axolotl heterochrony may have arisen from mutation of a gene or genes affecting thyroxine levels (*30*). Similarly, manipulation of the level of juvenile hormone at specific stages of insect development can cause heterochrony (*31, 32*). These effects include extra larval stages or, alternatively, reduction of the number of larval stages and precocious differentiation of adult features. The analogies between these examples of apparent hormonally mediated heterochrony and the defects of *C. elegans* heterochronic mutants suggest a possible role for hormones in the temporal regulation of cell fates during *C. elegans* development. Alternatively, *C. elegans* heterochronic genes could affect the fates of cells intrinsically, in a manner analogous to that proposed for insect homeotic genes (*33*).

References and Notes

1. G. R. DeBeer, *Embryos and Ancestors* (Clarendon, Oxford, 1958).
2. S. J. Gould, *Ontogeny and Phylogeny* (Harvard Univ. Press, Cambridge, Mass., 1977).
3. P. Alberch, S. J. Gould, G. F. Oster, D. B. Wake, *Paleobiology* **5** (No. 3), 296 (1979).
4. J. Sulston and H. R. Horvitz, *Dev. Biol.* **56**, 110 (1977).
5. J. Kimble and D. Hirsh, *ibid.* **70**, 396 (1979).
6. J. E. Sulston, E. Schierenberg, J. G. White, J. N. Thomson, *ibid.* **100**, 64 (1983).
7. G. N. Cox, S. Staprans, R. S. Edgar, *ibid.* **86**, 456 (1981).
8. J. E. Sulston and H. R. Horvitz, *ibid.* **82**, 41 (1981).
9. M. Chalfie, H. R. Horvitz, J. E. Sulston, *Cell* **24**, 59 (1981).
10. C. Trent, N. Tsung, H. R. Horvitz, *Genetics* **104**, 619 (1983).
11. The mutations *n536* and *n355* are defined as semidominant because heterozygotes show the same general defects as homozygotes, but to a lesser degree. The *lin-14* locus has been defined by a series of recessive mutations described in the text and below (*16, 29*). Both *n355* and *n536* appear to be *lin-14* alleles based on their close linkage to *lin-14* (Fig. 1) (*16*), and the nature of their interactions with *lin-14* recessive alleles (*29*).
12. R. Singh and J. E. Sulston, *Nematologica* **24**, 63 (1978).
13. G. Morata and P. A. Lawrence, *Nature (London)* **265**, 211 (1977).
14. I. S. Greenwald, P. W. Sternberg, H. R. Horvitz, *Cell* **34**, 435 (1983).
15. The *lin-14* recessive alleles were obtained (i) from a collection of mutants defective in egg-laying (*10*) and (ii) as revertants of *lin-4(e912)* and *lin-14* retarded mutants (*16*).
16. Mapping was performed by standard two- and three-factor crosses (*17*) (at 25°C unless otherwise indicated) and by complementation experiments between recessive mutations of each gene and the duplication *stDp2* (*18*) or the deficiencies *nDf23* and *nDf25* (*19*), *mnDf83* (*20*), *sDf5* (*21*), and *nDf19*. We generated *nDf19* by γ-ray induced reversion of the dominant allele *e665* of the gene *unc-58*, which has a recessive null phenotype (*22*). The phenotypes of animals heterozygous for the relevant deficiency and recessive alleles of *lin-14*, *lin-28*, or *lin-29* appear similar or identical to the phenotypes of animals homozygous for the corresponding recessive mutation. These observations, together with the fact that these deficiencies are themselves recessive, suggest that recessive alleles of *lin-14*, *lin-28*, and *lin-29* cause loss of gene activity. *lin-28(n719, n947, n1119*, and *n1120)* were isolated

after ethyl methanesulfonate (EMS) mutagenesis (*17*). Recombination data: Among 31 Unc recombinant progeny of animals of genotype + *lin-28(n719)* + / *dpy-5(e61)* + *unc-56(e403)*, one carried the recombinant chromosome + *lin unc*, and 30 carried recombinant chromosomes + + *unc*. Among three Lin recombinant progeny of animals of the genotype + + *lin-28(n719) unc-56(e403) / dpy-5(e61) unc-13(e51)* + +, all carried the recombinant chromosome + + *lin* +. The isolation and mapping of *lin-4(e912)* have been described (*23*, *24*). *lin-29(n333, n546,* and *n836)* were isolated as egg-laying defective mutants after EMS mutagenesis. Recombination data: Among 12 Rol recombinant progeny (picked at 20°C) of + *lin-29(n546)* + / *dpy-10(e128)* + *rol-1(e91)*, five carried the recombinant chromosome + *lin rol* and seven carried + + *rol*. Eight of 71 Lin-29 progeny of *lin-4(e912)* + / + *lin-29(n333)* carried the recombinant chromosome *lin-4 lin-29*. Among 35 Rol recombinant progeny of *dpy-10(e128)* + *lin - 29(n333)* + / + *rol - 5(sc13)* + *unc-52(e444)*, two carried the recombinant chromosome + *rol lin* +.

Of the *lin-14* semidominant mutations, *n355* was isolated on the basis of defects in egg-laying and general morphology after γ-irradiation of the wild type. *n536* was isolated as an egg-laying defective mutant after EMS mutagenesis of the wild type. *lin-14* recessive alleles cause heterochronic defects opposite to those of *lin-14(n355), lin-14(n536),* and *lin-4(e912)* and lead to incompletely penetrant defects in egg-laying. One *lin-14* recessive allele (*n530*) was isolated after EMS mutagenesis of the wild type on the basis of defects in egg-laying (*10*). This incompletely penetrant egg-laying defect of *lin-14* recessive mutants is epistatic to the extremely penetrant egg-laying defects of *lin-14(n355), lin-14(n536),* and *lin-4(e912)*. Thus animals carrying a *lin-14* recessive allele and *n355, n536,* or *e912* display a low penetrance egg-laying defect and can be selected as revertants of *lin-14(n355), lin-14(n536),* or *lin-4(e912)* with partially restored egg-laying ability. For this reason, *lin-14* recessive alleles also have been isolated both as suppressors of *lin-14* semidominant mutations and as suppressors of *lin-4(e912)*. Fifteen recessive *lin-14* alleles, for example, *lin-14(n536 n540),* were isolated by reversion of the egg-laying defective phenotype or abnormal morphology of *n536* or *n355* after mutagenesis with γ-rays or EMS. These revertants carry *cis* dominant, closely linked suppressor mutations (at 20°C *n536* maps less than 0.03 percent from the presumably intragenic suppressor mutation *n540*). Mutations *n179, n360,* and *n727* were isolated as partial suppressors of the egg-laying defects of *lin-4(e912)*. Animals carrying the *lin-4(e912)* mutation alone lack vulvae and almost never lay eggs, while *lin-4(e912); lin-14(suppressor)* animals form vulvae and often are capable of egg-laying. The *n179* mutation arose spontaneously in a *lin-4(e912)* stock; *n360* was isolated after γ-irradiation; *n727* was isolated after EMS mutagenesis. Recombination data: Among three Lin recombinant progeny of + *lin-14(n179) sma-5(n678) / unc-10(e102)* + +, all carried a + *lin* + recombinant chromosome. Among three Sma recombinant progeny of + *lin-14(n179) sma-5(n678) / unc-10(e102)* + +, all carried an *unc* + *sma* recombinant chromosome. Four phenotypically wild-type recombinant progeny were obtained from 677 total progeny of *lin-14(n536)* + /+ *sma-5(n678)*. Among 11 phenotypically wild-type recombinant progeny of *dpy-7(e1324) unc-58(e665)* + / + + *sma-5(n678)*, ten carried + + + recombinant chromosomes and one carried a *dpy* + + double recombinant chromosome (*e665* is dominant). Among nine Unc recombinant progeny of *dpy-7(e1324)* + *unc-9(e101)* / + *nuc-1(e1392)* +, two carried the recombinant chromosome + *nuc unc*. Among six Lin recombinant progeny of *sma-5(n678)* + *lin-2(e1309)* / + *nuc-1(e1392)* +, two carried the recombinant chromosome + *nuc lin*. Among five *lin-2* recombinants from + *lin-14(n179) lin-2(e1309)* + / *dpy-7(e1324)* + + *unc-9(e101)*, all carried the recombinant chromosome *dpy* + *lin-2* +.

In this article, we conform to the standard system of *C. elegans* genetic nomenclature (*25*). The wild-type parent of all strains used in this work is *C. elegans* var. Bristol strain N2 (*17*). Most genetic markers used are described in (*34*). *nuc-1* is described in (*26*). We isolated and characterized *sma-5(n678)*.

17. S. Brenner, *Genetics* **77**, 71 (1974).
18. R. Waterston, personal communication.
19. E. Ferguson, personal communication.
20. C. Sigurdson, G. Spanier, R. Herman, *Genetics*, in press.
21. A. M. Rose and D. L. Baillie, *ibid.* **96**, 639 (1980).
22. I. S. Greenwald and H. R. Horvitz, *ibid.*, p. 147.
23. J. Hodgkin, thesis, Darwin College, University of Cambridge (1974).
24. P. Babu and S. Brenner, *Mutat. Res.* **82**, 269 (1981).
25. H. R. Horvitz, S. Brenner, J. Hodgkin, R. Herman, *Mol. Gen. Genet.* **175**, 129 (1979).
26. J. E. Sulston, *Philos. Trans. R. Soc. London Ser. B* **275**, 287 (1976).
27. W. Fixsen, V. Ambros, P. W. Sternberg, H. R. Horvitz, unpublished observations.
28. F. R. Lillie (1898), reviewed in R. A. Raff and T. E. Kauffman, *Embryos, Genes and Evolution* (Macmillan, New York, 1983).
29. V. Ambros and H. R. Horvitz, in preparation.
30. R. Tompkins, *Am. Zool.* **18**, 313 (1978).
31. V. B. Wigglesworth, *Q. J. Microsc. Sci.* **77**, 191 (1934).
32. ———, *J. Exp. Biol.* **17**, 201 (1940).
33. A. Garcia-Bellido, in *Cell Patterning*, S. Brenner, Ed. (Elsevier/North Holland, Amsterdam, 1975), p. 161.
34. M. Swanson, M. Edgley, D. Riddle, in *Genetic Maps 1984*, S. J. O'Brien, Ed. (Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y., 1984), p. 286.
35. D. Riddle and M. Swanson, personal communication.
36. S. Ward, N. Thomson, J. G. White, S. Brenner, *J. Comp. Neurol.* **160**, 313 (1975).

37. In wild-type males (Fig. 5B), based upon the lineages derived from the blast cells V5, V6, and T, each of the four patterns (S1 to S4) is distinct. In wild-type hermaphrodites, S1 is distinct from S2, and S2 is distinct from S3, but the S3 and S4 patterns appear identical. The precise demarcation between the S3 and S4 patterns is uncertain as judged by the mutants described in this article. On the basis of the phenotype of *lin-29*, we have defined the S4 lineage pattern as being composed of at least the division of V5 · pppap that occurs at the L3 molt in wild-type males.

38. P. W. Sternberg and H. R. Horvitz, *Dev. Biol.* **93**, 181 (1982).

39. The intermolt periods of these mutants are approximately the same length as those of the wild type, except in two cases: *lin-14(n536 n540)* animals develop more slowly [66 hours from hatching to L4 molt at 20°C, compared to 45 hours for the wild type (Fig. 5A)]. Also, like *lin-4(e912)* (*9*), the overall development of *lin-14(n355)* is slowed slightly with respect to the wild type (for example, 18 hours L1 for *n355* compared to 15 hours for the wild type at 20°C). This slowing of overall development does not contribute to the heterochronic phenotype, since all lineages appear coordinately affected.
40. These mutants are not invariant in cell lineage. We have observed variability in the specific lineages and stages affected and in the precise pattern of cell divisions at particular stages. On the basis of lineages followed in several individuals of each genotype, we have chosen the individual shown here as typical of the strain. In these other animals, lineages were followed continuously beginning at hatching, or partial lineages were followed for defined intervals beginning at specific developmental stages. We have also screened larger numbers of hermaphrodites at defined developmental stages for anatomical abnormalities indicative of heterochronic expression of lateral hypodermal cell lineage patterns.

41. J. Sulston, M. Dew, S. Brenner, *J. Comp. Neurol.* **163**, 215 (1975).
42. V. Ambros and N. Tsung, unpublished observations.
43. A. Fodor and P. Deak, *Acta Biol. Acad. Sci. Hung.* **32**, 229 (1982).
44. J. Hodgkin, H. R. Horvitz, S. Brenner, *Genetics* **91**, 67 (1979).

45. We thank C. Desai, H. Ellis, K. Edwards, E. Ferguson, M. Kusch, and N. Tsung for providing mutants; R. Edgar, W. Fixsen, I. Greenwald, C. Kenyon, and P. Sternberg for stimulating discussions, and E. Hartweig for help with the electron microscopy; R. Lee, G. Struhl and members of our laboratory for comments on the manuscript; E. Ferguson, who initially discovered and mapped *lin-14*, and P. Sternberg, who suggested to us that the phenotype of *lin-4(e912)* might be heterochronic. Supported by PHS research grants GM24663 and GM24943, PHS Research Career Development Award HD00369 (H.R.H.), NIH postdoctoral fellowship F32 GM08642-01 (V.A.), and American Cancer Society fellowship NY PF-1726 (V.A.).

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Heterochronic mutants of the nematode Caenorhabditis elegans

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