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Heterochronic Genes Control Cell Cycle Progress and Developmental Competence of C. elegans Vulva Precursor Cells

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Summary

Heterochronic genes control the timing of vulval development in the C. elegans hermaphrodite. *lin-14* or *lin-28* loss-of-function mutations cause the vulval precursor cells (VPCs) to enter S phase and to divide one larval stage earlier than in the wild type. A precocious vulva is formed by essentially normal cell lineage patterns, governed by the same intercellular signals as in the wild type. Mutations that prevent the normal developmental down-regulation of *lin-14* activity delay or block VPC division and prevent vulval differentiation. A genetic pathway that includes *lin-4*, *lin-14*, and *lin-28* controls when VPCs complete G1 and also controls when VPCs acquire the competence to respond to the intercellular patterning signals and express vulval fates.

Introduction

Multicellular development requires the temporal coordination of cell division with developmental programs that specify cell fate and differentiation. Production of the correct number of cells and the coordinated differentiation of tissues require the regulation of cell division in conjunction with developmental signals. Where cell fate determination is associated with a phase of the cell division cycle (Pardee, 1989; Thomas et al., 1994) or when cells divide at precise times, progression through cell cycle phases must be developmentally regulated. Although cell cycle regulation has been well characterized at the genetic and molecular levels in cultured cells and yeast (reviewed by Pardee, 1989; Nasmyth, 1993; Sherr, 1994), cell cycle control during multicellular development is not as well understood.

One strategy for coordinating developmental programs with the cell division cycle is to couple components of the cell cycle machinery to developmental regulators (Duronio and O'Farrell, 1994, 1995). Signals that control cell cycle progress include growth factors of mammalian cells (Pardee, 1989), mating pheromones of yeast (Chang and Herskowitz, 1990), and the products of the *roughex* (Thomas et al., 1994) and *anachronism* (Ebens et al., 1993) loci of Drosophila. In different developmental contexts, different cell cycle phases may be regulated. For example, cell cycle progress during Drosophila embryonic cycles 14–16 is controlled at the G2 to M transition by the cyclic transcription of *string* (Edgar and O'Farrell, 1989, 1990; Edgar et al., 1994). After cycle 16, cells acquire a G1 phase when cyclin E and E2F become limiting (Duronio and O'Farrell, 1995).

In the nematode Caenorhabditis elegans, cell cycle regulation during development can be studied at the level of single cells, owing to the animal's relatively simple and invariant cell lineage (Sulston and Horvitz, 1977). Little is known about the developmental control of intrinsic cell cycle regulators in C. elegans, or about the temporal signals specifying when cells divide. Certain cell lineage mutants display defects in cell cycle progression in essentially all cells of the worm (Horvitz and Sulston, 1980; Sulston and Horvitz, 1981; Albertson et al., 1987). These mutants display pleiotropic growth defects, suggesting that they likely identify general cell cycle components and not cell-specific cell cycle regulators.

Heterochronic genes of C. elegans are candidates for developmental regulators that control the cell cycle of specific cells. Mutations in heterochronic genes alter the timing of postembryonic developmental events, including the development of the vulva (Ambros and Horvitz, 1984). Their effects on vulval development include altered timing of vulval precursor cell (VPC) division (Ambros and Horvitz, 1984). This heterochronic cell cycle phenotype, combined with the accessibility of the vulva to developmental and genetic analysis (Sulston and Horvitz, 1977; Ferguson et al., 1987), makes the vulval cell lineage a convenient system to explore mechanisms of developmental cell cycle control.

In the wild type, the vulva is formed by the progeny of three of the six equipotent VPCs (Figure 1A) (Sulston and Horvitz, 1977). VPC cell division and cell fate determination are both developmentally regulated. The VPCs are born in the L1 stage and divide synchronously in the mid-L3 soon after their fates are determined by intercellular signals. These signals include an inductive signal from the gonad, mediated by the LIN-3 epidermal growth factor–like protein and the LET-23 kinase receptor (Aroian et al., 1990; Hill and Sternberg, 1992), an inhibitory signal from the surrounding hypodermis, mediated by the *lin-15* gene product (Herman and Hedgecock, 1990; Clark et al., 1994; Huang et al., 1994), and a lateral signal among the VPCs (Sternberg, 1988; Sternberg and Horvitz, 1989) via the LIN-12 receptor (Yochem et al., 1988). Laser ablation and temperature-shift experiments indicate that these three signals act on the VPCs relatively late during the VPC cell cycle, at approximately the L2 molt or early L3 (Kimble, 1981; Greenwald et al., 1983; Sternberg and Horvitz, 1986; Ferguson et al., 1987; Struhl et al., 1993). Once the VPCs divide, the fates of their progeny are fixed with respect to these signals, indicating that the critical period for vulva cell fate determination corresponds to the last 3–4 hr of the 20 hr VPC cell cycle. Although the signal transduction machinery for VPC fate specification is well characterized (reviewed by Hill and Sternberg, 1993), less is known about how the response to these three distinct signals is temporally coordinated with the VPC cell cycle.

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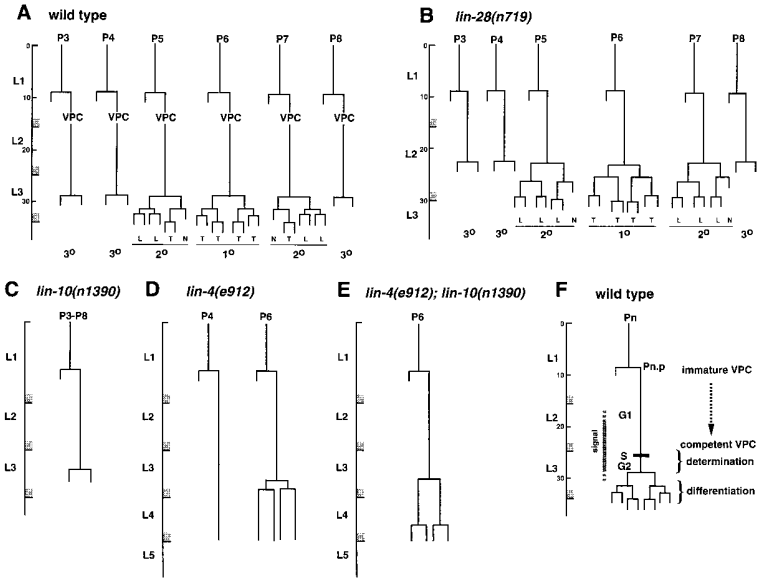

Figure 1. Ventral Hypodermal Cell Lineages in the Wild Type and in Heterochronic Mutants

Each stage ends with a molt (Sulston and Horvitz, 1977; Singh and Sulston, 1978), consisting of lethargus, when the animal is inactive (stippled boxes), and ecdysis, when pumping and locomotion resume and the old cuticle is molted (horizontal lines at the end of lethargus). In (A), (B), and (F), the axis at left is marked in hours after hatching (at 20°C); in (C)–(E) the time axis is normalized to the wild type. (A) Wild-type vulva development; VPCs are "Pn.p" daughters of P3–P8 (Sulston and Horvitz, 1977). Anterior daughters of P3-P8 are neuroblasts whose cell divisions are not shown. Primary and secondary vulval fates are characterized by the division pattern and morphogenetic behavior of the cells produced at the terminal round of cell division: N, no division; L, longitudinal division; T, transverse division (Sternberg and Horvitz, 1986). The 3° fate is characterized by VPC division producing two nondividing progeny. (B) Precocious vulval lineage of a *lin-28(n719)* hermaphrodite. The penetrance of this precocious VPC division defect is 100% for *lin-28(n719)* (n = 50) and *lin-28(n947)* (n = 18). Some minor defects were observed in these precocious vulval cell lineages; the relative timing of VPC divisions was often slightly abnormal, VPC granddaughter cells that would normally divide transversely occasionally divided longitudinally, and the polarity of P5.p or P7.p lineages were sometimes reversed, causing one or two ectopic vulval protrusions (Table 4). The intermolt periods of *lin-14(lf)* and *lin-28(lf)* animals are slightly longer than in the wild type. (C) VPCs of *lin-10(n1390)* animals express only 3° fates (Horvitz and Sulston, 1980; Kim and Horvitz, 1990). (D) Representative retarded vulva cell lineages of *lin-4(e912)* (see also Chalfie et al., 1981). (E) P6 lineage in a representative *lin-4(e912); lin-10(n1390)* animal. (F) A model for the timing of events during wild-type vulval development. DNA quantitation (Table 1) and hydroxyurea sensitivity (Table 2) indicate that VPCs undergo S phase at ~1 hr after the L2 molt. VPCs respond to intercellular signals by becoming determined to express 1°, 2°, or 3° fates (1° fate is shown here) at ~L2 ecdysis or early L3 (Kimble, 1981; Greenwald et al., 1983; Sternberg and Horvitz, 1986; Ferguson et al., 1987). Wild-type VPCs undergo a maturation step whereby they become competent to express one of these fates (see text). The gonadal inductive signal (broad bar at left), and perhaps other intercellular signals that specify VPC fates, are expressed continuously in the L2 and L3 stages, but VPCs cannot implement these signals until they acquire a developmentally regulated competence.

In this paper, we analyze the effects of mutations in heterochronic genes on the timing of VPC cell cycle phases and vulval differentiation. Our results define a pathway of heterochronic genes that, one, controls when the VPCs acquire the competence to respond to intercellular signals and produce differentiated vulval cell progeny and, two, regulates the length of G1 in VPCs, specifying when they divide. By affecting both VPC competence and cell cycle progress, the heterochronic gene pathway provides a mechanism for coordinating the timing of these two critical steps in vulval development.

Results

Time of S Phase in Wild-Type VPCs

To explore the relationship of cell cycle phases to the developmental program of VPCs, we used DNA quantitation to delineate the time of S phase in the VPCs of wild-type animals. Using a DNA stain and quantitative fluorescent confocal microscopy (White et al., 1987), we found that 1 of 11 VPC nuclei of animals in the L2 molt showed >2 N DNA content (Table 1). By contrast, 9 of 11 VPC nuclei analyzed in animals fixed at 0.5–2 hr after the end of the L2 molt showed greater than 2 N DNA content (Table 1). This suggests that wild-type VPC S phase is complete by 2 hr after the L2 molt (Figure 1F).

As an independent measure of the time of S phase, animals at various developmental stages were transferred to plates containing hydroxyurea, an inhibitor of DNA synthesis (Kornberg and Baker, 1992). In the presence of hydroxyurea, cells in G2 complete mitosis, whereas cells in G1 or S arrest in S phase. We found that in wild-type animals placed in hydroxyurea at 20 min into L3 development, 78% of VPC division was inhibited, but in animals placed in hydroxyurea after 50 min into the L3, 94% of VPCs divided (Table 2). Thus, the results of two independent methods, DNA quantitation (Table 1) and hydroxyurea sensitivity (Table 2), concur and suggest that wild-type VPC S phase occurs during the first hour of the L3, ~3 hr before mitosis (Figure 1F).

Precocious VPC S Phase in *lin-14* and *lin-28* Mutants

Previous studies showed that *lin-14(lf)* mutations cause precocious vulval development (Ambros and Horvitz, 1984). We followed the vulval cell lineages in *lin-14(lf)* and *lin-28(lf)* animals and found that both mutants displayed similar precocious defects; the VPCs consistently divided in the mid-L2 stage, ~5–6 hr earlier than in the wild type (Figures 1B, 2C, and 2D). The time that VPCs were born, and the cell cycle lengths of VPC progeny were essentially unaltered in *lin-28(lf)* (Figure 1B) and *lin-14(lf)* animals (Ambros and Horvitz, 1984), indicating that in the ventral hypodermal lineages, *lin-28(lf)* and *lin-14(lf)* mutations specifically affect the length of the VPC cell cycle.

Table 1. Fluorescence Quantitation of the DNA Content of Wild-Type Vulva Precursor Cells

Animal	Stage[a]	Cell[b]	I_p[c]	I_n[d] (n)	SD[d]	t[e]	r[f]	N[g]
1	L2 m	P3.p	372	417 (8)	61	>0.6	1.8	2
		P4.p	476	417 (8)	61	>0.4	2.3	2
		P5.p	791	417 (8)	61	<0.0005	3.8	4
2	L2 m	P8.p	364	412 (7)	88	>0.4	1.8	2
3	L2 m	P5.p	442	499 (7)	100	>0.4	1.8	2
		P6.p	332	499 (7)	100	>0.4	1.3	2
		P7.p	373	499 (7)	100	>0.4	1.5	2
4	L2 m	P1.p	920	1292 (14)	286	>0.4	1.4	2
		P2.p	843	1292 (14)	286	>0.4	1.3	2
		P3.p	948	1292 (14)	286	>0.4	1.5	2
		P4.p	759	1292 (14)	286	>0.4	1.2	2
		P5.p	981	1292 (14)	286	>0.4	1.5	2
		P6.p	771	1292 (14)	286	>0.4	1.2	2
5	L2 m + 0:30	P1.p	313	318 (11)	25	>0.6	2.0	2
		P2.p	374	325 (6)	30	>0.05	2.3	2
		P3.p	423	275 (11)	36	<0.0025	3.1	4
		P5.p	1109	507 (6)	116	<0.0025	4.4	4
		P6.p	941	507 (6)	156	<0.01	3.7	4
		P7.p	532	427 (5)	105	>0.6	2.5	2
		P8.p	806	427 (5)	105	<0.025	3.8	4
6	L2 m + 2:00	P1.p	1864	1693 (7)	157	>0.1	2.2	2
		P2.p	1649	1693 (7)	157	>0.6	2.0	2
		P3.p	3467	1668 (10)	186	<0.0005	4.2	4
		P4.p	3629	1668 (10)	186	<0.0005	4.4	4
		P5.p	1647	989 (11)	192	<0.005	3.3	4
		P6.p	1255	989 (11)	192	>0.1	2.5	2
		P7.p	1357	741 (14)	55	<0.0005	3.7	4
		P8.p	1405	741 (14)	55	<0.0005	3.8	4

[a] See Experimental Procedures. m, molt; times are hr:min after the end of the molt.
[b] P3.p–P8.p are VPCs; P1.p and P2.p are nondividing ventral hypodermal cells (Sulston and Horvitz, 1977).
[c] Above-background fluorescence intensity of the indicated nucleus.
[d] Mean (I_n) and standard deviation (SD) of the above-background fluorescence intensity measurements from a set (n) of nearby ventral cord neuronal nuclei (see Experimental Procedures).
[e] t statistic (see Experimental Procedures).
[f] DNA content $= 2(I_p/I_n)$.
[g] r rounded to 4 for $t < 0.05$; to 2 for $t > 0.05$.

To determine the time of S phase in *lin-14(lf)* and *lin-28(lf)* precocious mutants, we tested for hydroxyurea sensitivity of VPC division. Transfer of *lin-14(lf)* or *lin-28(lf)* animals to hydroxyurea at ~5 hr after the L1 molt permitted VPC division (Table 2), indicating that S phase was complete by ~5 hr into the L2. This result indicates that G1 is shortened from ~17 hr (at 20°C) in the wild type to ~12 hr in *lin-14(lf)* and *lin-28(lf)* mutants. Thus, the approximately 5–6 hr shorter VPC cell cycle in the precocious mutants can be accounted for by a specific shortening of G1.

Signal-Dependent Expression of VPC Fates in Precocious Mutants

The precocious vulval development of *lin-14(lf)* and *lin-28(lf)* animals is otherwise essentially normal in the expression of 1°, 2°, and 3° cell lineage patterns and morphogenesis (Figures 1B, 2C, and 2D). A *lin-11::lacZ* transgene that is expressed in certain progeny of VPCs that adopt 2° fates (Freyd, 1991; G. Freyd and H. R. Horvitz, personal communication) is expressed precociously in *lin-28(lf)* animals, in the proper cells of 2° lineages (Table 3). Precocious vulval lineages can variably express minor abnormalities (Figure 1B, legend) that, combined with the precocious differentiation of vulval cells with respect to interacting tissues, may account for the egg-laying defects of *lin-14(lf)* and *lin-28(lf)* hermaphrodites.

The precociously dividing VPCs of *lin-14(lf)* and *lin-28* mutants express vulval cell fates in response to the usual intercellular signals. In the absence of a gonadal inductive signal, wild-type VPCs adopt the 3° fate, producing two nonvulval progeny (Kimble, 1981). In *lin-28(lf)* hermaphrodites lacking a gonad, VPCs divided in the mid-L2, but expressed only the 3° fate (Table 4). To further test the dependence of precocious vulval cell fates on the anchor cell inductive signal, we used *lin-10(lf)* and *lin-3(lf)* mutations that block vulval induction (Figure 1C) (Ferguson and Horvitz, 1985; Kim and Horvitz, 1990). In most *lin-10(n1390) lin-28(n719)* or *lin-28(n719); lin-3(e1417)* double-mutant animals, the VPCs expressed only 3° fates (Table 4). These genetic and gonad ablation experiments indicate that the timing of VPC division is unaffected by removal of the gonadal signal, but the expression of 1° or 2° fates by these precociously dividing VPCs requires the anchor cell signal, as in the wild type. *lin-28; lin-12(gf)* and *lin-28; lin-*

Table 2. Hydroxyurea Sensitivity of Cell Division in VPCs

Genotype[a]	Stage[b]	Animals #	VPCs divided[b] (%)
N2	L2 m	6	6
N2	L2 m + 0:20[c]	3	22
N2	L2 m + 0:50	3	94
N2	L2 m + 1:20	7	95
N2	L2 m + 1:40	3	78
N2	L2 m + 2:20	3	83
lin-14(n179ts)[d]	L1 m + 0:50	4	4
lin-14(n179ts)	L1 m + 1:45	5	7
lin-14(n179ts)	L1 m + 3:15	5	13
lin-14(n179ts)	L1 m + 4:00	24	37
lin-14(n179ts)	L1 m + 4:45	10	50
lin-14(n179ts)	L1 m + 5:30	24	54
lin-14(n179ts)	L1 m + 7:45	28	63
lin-28(n719)[d]	L1 m	7	0
lin-28(n719)	L1 m + 2:30	7	5
lin-28(n719)	L1 m + 3:30	3	0
lin-28(n719)	L1 m + 4:00	5	13
lin-28(n719)	L1 m + 5:15	11	79

[a] *lin-14(n179ts)* animals were grown at 25° C, and times are converted to 20° C-equivalents; see Experimental Procedures.
[b] See Experimental Procedures. m, molt; times are hr:min after the end of the molt.
[c] One animal in which no VPCs had divided after 8 hr in hydroxyurea was removed from hydroxyurea, and its postdelay vulval development was followed and found to be essentially normal.
[d] *lin-28 (n719)* is 2 null allele (E. Moss, unpublished data), and all VPCs divide in the L2; *lin14(n179ts)* is not a null allele (data not shown), and the precocious VPC division defect is incompletely expressed at 25° C.

15(lf) animals expressed the *lin-28* precocious VPC division phenotype as well as the multivulval phenotype associated with *lin-12* or *lin-15* (Table 4), indicating that the spatial pattern of precocious vulval cell fates also depends on signals mediated by *lin-12* and *lin-15*. These findings indicate that the cell–cell interactions that specify vulval fates, involving a signal from the gonadal anchor cell (Sulston and White, 1980; Kimble, 1981), among the VPCs (Sternberg, 1988), and from the surrounding hypodermis (Herman and Hedgecock, 1990), occur by the mid-L2 in *lin-14* and *lin-28* animals.

We tested whether the gonadal signal mediated by the *lin-3* gene product (Hill and Sternberg, 1992) is normally present in the L2 of the wild type, or if the heterochronic genes might affect its timing. A *lin-3::lacZ* reporter gene, *syIs3*, was observed to be expressed by the L2 molt in the wild type (Hill and Sternberg, 1992). We found that *syIs3* produces β-galactosidase activity in the gonad of wild-type L2 larvae, as early as 2–4 hr after the L1 molt (Table 3). An extrachromosomal transgene, *syEx26*, from which *syIs3* was derived (Hill and Sternberg, 1992), is expressed at a similar stage (data not shown). Animals containing *syIs3* or *syEx26* can display a hyperinduced (multivulva) phenotype, suggesting that these multicopy transgenes produce greater than normal levels of gonadal signal in the form of functional LIN-3::B-GAL protein (Hill and Sternberg, 1992). We found that *lin-14(lf)* does not significantly alter the timing of *syIs3 lin-3::lacZ* expression (Table 3). Assuming that the timing of the transgene expression accurately reflects endogenous *lin-3* activity, these results indicate that gonadal signaling begins in the L2 and that *lin-14* does not affect its timing.

lin-14(n179ts) Temperature-Sensitive Period for the Timing of VPC Division

To test for when *lin-14* acts to control the timing of VPC division, we performed temperature-shift experiments using a temperature-sensitive loss-of-function allele, *lin-14(n179ts)*. *lin-14(n179ts)* animals shifted from the permissive to the restrictive temperature (upshifted) before 2 hr after hatching displayed a precocious VPC division phenotype, whereas animals upshifted after 4 hr of larval development were normal (Figure 3). This result suggests that the *lin-14* gene product can act as early as 2–4 hr after hatching to inhibit precocious VPC division

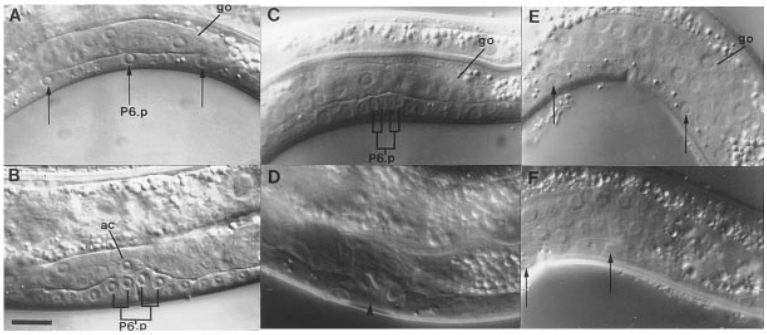

Figure 2. Nomarski Photomicrographs of Heterochronic Vulval Development

(A) N2 at the L2 molt; arrows, undivided VPCs. (B) N2 at the L3 molt; forked lines, four progeny of P6.p. (C) *lin-28(n719)* at the L2 molt; forked lines, four precocious progeny of P6.p. (D) *lin-28(n719)* at the L3 molt; arrow, advanced vulval morphogenesis. (E and F) *lin-4(e912)* at the L2 and L3 molts, respectively; arrows, undivided VPCs. Scale bar = 10 μm; ac, gonadal anchor cell; go, gonad.

Table 3. *lacZ* Reporter Gene Expression in Heterochronic Mutants

Strain[a]	Reporter[a]	Genotype[a]	Stage[b]	Cell(s)	Expression[c] (%)	n[c]
PS1269	*lin-3::lacZ*	wt	L1 m	gonad	5	21
PS1269	*lin-3::lacZ*	wt	L1 m + 2-4	gonad	100	15
VT755	*lin-3::lacZ*	*lin-14(lf)*	L1 m	gonad	3	71
VT755	*lin-3::lacZ*	*lin-14(lf)*	L1 m + 2-4	gonad	78	40
VT756	*lin-3::lacZ*	*lin-4(lf)*	L1 m + 0-1	gonad	0	10
VT756	*lin-3::lacZ*	*lin-4(lf)*	L1 m + 2-4	gonad	70	36
MT5788	*lin-11::lacZ*	wt	L3 m	vulval	100[d]	10
MT5875	*lin-11::lacZ*	*lin-28(lf)*	L2 m	vulval	73[d]	22
MT5790	*lin-11::lacZ*	*lin-4(lf)*	L3 m	vulval	9[d,e]	11
VT720	*lin-12::lacZ*	wt	L3	vulval	100	10
VT719	*lin-12::lacZ*	*lin-4(lf)*	L3	vulval	87	15

[a] Complete genotypes and reporter gene constructs are described in Experimental Procedures, and in the text. *lin-3::lacZ* animals were grown at 25° C.
[b] See Experimental Procedures. m, molt; times are hours after the end of the molt.
[c] For *lin-3::lacZ* and *lin-11::lacZ*, the percent of (n) animals expressing β-galactosidase in at least one nucleus of the indicated cell types is shown; for *lin-12::lacZ*, the percent of animals expressing β-galactosidase in at least four of the six P3.p–P8.p cells is shown.
[d] The animals were examined using Nomarski optics prior to fixation to verify that P5.p and/or P7.p had divided two rounds and, hence, produced cells expected to express *lin-11::lacZ* (Freyd, 1991).
[e] Relatively faint β-galactosidase was detected in one nucleus of a single animal, and that nucleus could not be unambiguously identified as vulval.

in the L2. Animals shifted from the nonpermissive to the permissive temperature (downshifted) at approximately the time of the L1 molt displayed relatively normal development, whereas animals downshifted after the L1 molt displayed precocious VPC division (Figure 3). These results indicate that an interval extending from ~2 hr after hatching to the L1 molt defines a temperature-sensitive period during which the synthesis or function of the *lin-14* gene product can inhibit early VPC division.

Blocks in Vulval Cell Division and Differentiation

If the wild-type *lin-14* activity inhibits VPC division at early larval stages, then *lin-4(lf)* and *lin-14(gf)* mutations, which result in inappropriate *lin-14* activity late in development (Ambros and Horvitz, 1987), would be expected to display delayed or completely blocked VPC divisions. Cell lineage analysis of *lin-4(lf)* (Figures 1D, 2E, and 2F) (Chalfie et al., 1981) and *lin-14(gf)* (data not shown) hermaphrodites revealed that their vulval lineages display variable cell lineage defects, primarily a delay or complete block of Pn.p (VPC) divisions. In cases where a Pn.p does divide, its progeny can also divide, sometimes greater than the normal three rounds of division. Occasionally, Pn.p cells can divide precociously in the L2 of *lin-4(lf)* or *lin-14(gf)* animals (data not shown). Thus, the overall vulval phenotype of *lin-4(lf)* or *lin-14(gf)* animals includes a general block in Pn.p cell division and some temporally unregulated cell divisions.

The phenotype of *lin-4(lf)* and *lin-14(gf)* animals suggests that inappropriate *lin-14* activity affects the cell division of VPCs and the competence of VPCs to generate differentiated vulval cells. When Pn.p progeny are occasionally produced in *lin-4* animals, they are unlike normal vulval cell types by several criteria: first, they

Table 4. Signal Dependence of *lin-28(0)* Vulval Development

Genotype	Gonad	vulva phenotypes[b] (%)			
		vul	wt	muv	n
lin-28(n719)	+	0	88	12[c]	118
lin-28(n719)[a]	−	100	0	0	5
lin-28(n947)	+	0	82	18[c]	84
lin-28(n719); lin-12(n137n460ts)[d]	+	0	1	99	89
lin-28(n947); lin-15(el763)[d]	+	0	0	100	45
lin-10(n1390) lin-28(n719)	+	92	7	1	131
lin-28(n719); lin-3(e1417)[e]	+	95	>4	<1	284

[a] Gonadal cells were ablated using a laser microbeam as described in Experimental Procedures.
[b] Vulval development was assessed by examining adults using the dissecting microscope for the number of ventral vulval protrusions; wt, normal vulval induction, where only one vulva (or, in the case of *lin-28* animals, one ventral protrusion) was observed; vul, no detectable vulval development; muv, two or more ventral protrusions were visible. Some animals were examined using Nomarski optics: 4/4 *lin-28(n719); lin-3(e1417)* animals, 10/10 *lin-10(n1390) lin-28(n719)* animals, 6/6 *lin-28(n947); lin-15(e1763)* animals and 5/5 *lin-28(n719); lin-12(n137n460ts)* animals examined in the L2 showed precocious VPC divisions. Cell lineage analysis of 3 *lin-28(n947); lin-15(e1763)* animals revealed the alternating 1° and 2° cell division patterns typical of *lin-15* animals.
[c] The multiple ventral protrusions sometimes exhibited by *lin-28* animals chiefly result from polarity reversals of P5.p or P7.p lineages, and hence do not represent hyperinduction of vulval fates.
[d] *lin-12(n137n460ts)* (Greenwald et al., 1983) results in 97% (n = 307) muv animals at 15° C. *lin-15(e1763)* also causes a highly penetrant muv phenotype (Ferguson and Horvitz, 1987).
[e] *lin-3(e1417)* and *lin-10(n1390)* result in 89% vul (Ferguson and Horvitz, 1987) and 99.7% vul (Kim and Horvitz, 1990), respectively.

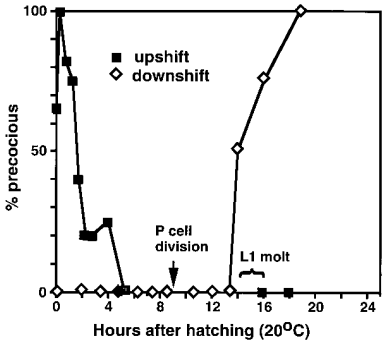

Figure 3. Temperature-Shift Experiments with *lin-14(n179ts)*

Approximately 76% (n = 33) of *lin-14(n179ts)* animals maintained at 25°C executed one or more precocious VPC divisions by the time of the L2 molt. (Small sample sizes probably account for the time points with 100% precocious animals.) In contrast, 0% (n = 26) of *lin-14(n179ts)* animals maintained at 15°C executed precocious divisions. The horizontal axis indicates the time of temperature shift expressed in hours after hatching, converted to hours at 20°C (see Experimental Procedures). The vertical axis shows the percentage of animals shifted at the indicated time that executed one or more VPC divisions by the L2 molt. Solid squares, upshifts from 15°C to 25°C; open diamonds, downshifts from 25°C to 15°C. Approximately 6 animals (range 1–20) were shifted at each time point.

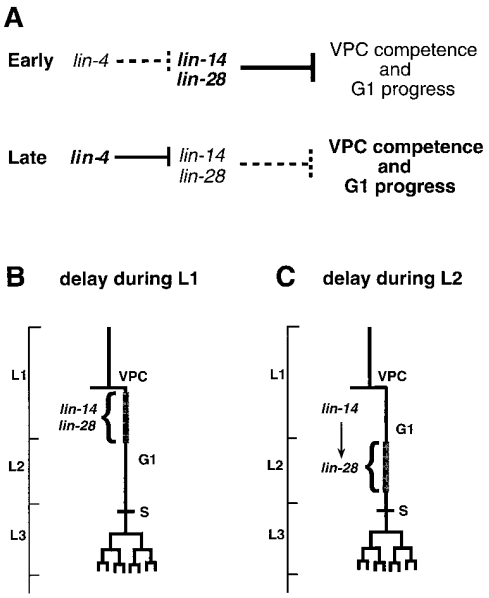

Figure 4. Model for the Temporal Control of VPC Cell Cycle Progression and Competence by *lin-14* and *lin-28*

(A) Epistasis data (see text) are interpreted in terms of proposed negative regulatory interactions. Early in development, *lin-14* and *lin-28* block VPC G1 progress and the competence of VPCs to express 1°, 2°, or 3° fates. Later, *lin-14* and perhaps *lin-28* are inactivated as a result of *lin-4* activity (Ambros, 1989; Lee et al., 1993), permitting VPCs to become competent and to complete G1. *lin-14(lf)* or *lin-28(lf)* mutations result in a shortened G1 and precocious competence. (B) *lin-14* and *lin-28* might both act in the L1 to impose an L1-specific delay in VPC G1 progression and acquisition of competence; or (C) *lin-14* might function in the L1 to activate *lin-28*, which then imposes an L2-specific delay.

never exhibit vulval morphogenesis; second, they display a significantly reduced expression of a 2° lineage-specific transgene, *lin-11::lacZ* (Table 3); and finally, they display no response to the anchor cell inductive signal. Wild-type Pn.p daughter cells (Pn.px cells) do not divide in *lin-10* mutants or in wild-type animals lacking an anchor cell (Kimble, 1981; Ferguson and Horvitz, 1985; Kim and Horvitz, 1990). In *lin-4; lin-10* double-mutant animals (Figure 1E), or in gonad-ablated *lin-4* animals (data not shown; Chalfie et al., 1981), Pn.px cells divided with apparently the same frequency as in unoperated *lin-4* animals. *lin-3::lacZ* is expressed in *lin-4(lf)* animals (Table 3) indicating that the vulval inductive signal is likely produced. These results indicate that the wild-type Pn.px cell identity, as judged by gonad-dependent cell division, is not established in the retarded mutants, even after several rounds of vulval cell division. This could be due to a defect in the Pn.px cells or their Pn.p parents.

To examine further the developmental identity of the vulval cells in *lin-4* animals, we tested for the expression of a *lin-12::lacZ* reporter gene (Wilkinson et al., 1994) that is normally expressed in all six VPCs in the L2 and early L3 stages (Wilkinson and Greenwald, 1995). β-galactosidase expression in *lin-4(e912); lin-12::lacZ* animals was similar to the wild type; at the L2 molt (Table 3), β-galactosidase staining was often detectable in all six P3.p–P8.p cells, although at a somewhat reduced intensity compared to the wild type. These observations indicate that Pn.p cells of *lin-4(e912)* animals express at least one component of the VPC developmental program, the activation of *lin-12* transcription.

Epistasis Analysis

Multiply mutant strains were examined for their precocious or retarded vulval cell division and differentiation defects and compared with the corresponding single heterochronic mutants (data not shown). The precocious defect caused by the *lin-14(ma135)* or *lin-28(n719)* mutations was expressed in the absence of *lin-4* function, and *lin-28(n719)* defect was expressed in *lin-28(n719); lin-14(n536gf)* animals (see Experimental Procedures). *lin-29* mutations that affect the timing of lateral hypodermal development did not affect the timing of VPC development, either alone or in combination with mutations causing precocious VPC division.

Discussion

Developmental Control of VPC G1

Our measurements of the timing of S phase indicate that the wild-type VPC cell cycle contains a relatively long G1 that extends from the mid-L1 until the end of the L2. This suggests that some components of the cell cycle machinery required for completing G1 are limiting in VPCs for much of the L1 and the L2 stages, and that these components are under developmental control, analogous to the situation in the Drosophila blastoderm after cycle 16 (Duronio and O'Farrell, 1994, 1995). The G1 phase of the VPC cell cycle is specifically shortened in *lin-14(lf)* and *lin-28* mutants, indicating that *lin-14* and *lin-28* restrict VPC division to the L3 stage of normal development by controlling some step(s) in G1 (Figure 4A).

The fact that *lin-4(lf)* and *lin-14(gf)* mutations cause

delayed VPC division, and that *lin-14(lf)* is epistatic to *lin-4(lf)*, strongly suggests that the timing of VPC division is governed by a down-regulation of *lin-14* activity by *lin-4* (Figure 4A). In the wild type, the *lin-4* antisense RNA (Lee et al., 1993) causes a down-regulation of LIN-14 protein level after the L1 stage (Ruvkun and Giusto, 1989), and this temporal decrease in LIN-14 specifies the progressive expression of L2- and L3-specific developmental events in various cell lineages (Ambros and Horvitz, 1987). It is not yet known if the *lin-28* gene product is developmentally regulated.

The inhibition of VPC cell cycle progress by *lin-14* and *lin-28* is consistent with the general roles of *lin-14* and *lin-28* in specifying early larval developmental events and delaying later events (Ambros and Horvitz, 1987). However, for other cells affected in *lin-14* or *lin-28* mutants, cell division cycle lengths are not altered; rather, temporal transformations in developmental fates occur, where cells express the developmental fate normally specific for a cell at another stage (Ambros and Horvitz, 1984). Thus, in various cell types, the regulatory targets of the heterochronic genes can include genes specifying stage-specific cell fates (Rougvie and Ambros, 1995; Liu et al., 1995), or as is the case for VPCs, G1 cell cycle regulators.

lin-14 and *lin-28* could affect VPC cell cycle progress at almost any step in G1 between mitosis in the L1 to S phase in the L3. Since the *lin-14(lf); lin-28(lf)* double mutant does not display a significantly shorter G1 than either single mutant alone (data not shown), it appears that these two genes control the same step in G1. Our *lin-14(ts)* temperature-shift experiments, and studies of LIN-14 expression (Ruvkun and Giusto, 1989) support the idea that *lin-14* acts in the L1 stage. *lin-14* and *lin-28* may both act in the L1 stage (Figure 4B) to affect an early step in G1, such as exit from a G0 resting state (Pardee, 1989). However, the fact that *lin-28(lf)* phenotypes are L2 specific in other cell lineages (Ambros and Horvitz, 1984) suggests that the G1 step controlled by *lin-14* and *lin-28* occurs in the L2, and that *lin-14* acts indirectly via *lin-28* (Figure 4C). Analysis of the time of action of *lin-28* will distinguish between these possibilities.

lin-14 and *lin-28* may not be the only genes regulating the length of the VPC cell cycle. Mutations of *lin-31* and *lin-25* cause precocious Pn.p cell divisions (Ferguson et al., 1987; Miller et al., 1993; Tuck and Greenwald, 1995), but at a relatively low frequency compared to the *lin-14(lf)* or *lin-28* mutations. Also, *lin-25* and *lin-31* mutations do not affect the timing of vulva differentiation. These differences suggest that the heterochronic genes affect VPCs independently of *lin-31* or *lin-25*.

Temporal Control of VPC Competence

The heterochronic genes also affect the competence to express vulval differentiation programs. Our results suggest that the Pn.p cells of *lin-4(lf)* and *lin-14(gf)* animals are immature VPCs because they express a marker of VPCs, a *lin-12::lacZ* transgene, but do not generate vulval progeny. Pn.p cells that eventually divide in *lin-4* or *lin-14(gf)* mutants produce cells that do not exhibit the usual characteristics of determined or differentiated vulval cells. *lin-4(lf)* or *lin-14(gf)* mutations do not simply render VPCs insensitive to intercellular signals, since signal-insensitive (but otherwise normal) VPCs would be expected to express 1°, 2°, or 3° fates. Pn.p cells of *lin-4(lf)* and *lin-14(gf)* mutants may correspond to an early developmental state of normal VPCs when they contain the vulval signal transduction machinery, but have not yet acquired the competence to express 1°, 2°, or 3° cell fates (Ferguson and Horvitz, 1985). Thus, the heterochronic genes control two developmental switches for VPCs: one, the acquisition of competence to produce differentiated vulval cells in response to intercellular signals and, two, commitment to the completion of G1 (Figure 4A).

Our results do not directly address whether the heterochronic gene pathway affects VPC cell cycle and competence independently, or whether a causal link between competence and cell cycle phase may facilitate their coordinate regulation. However, in the wild type, a transient delay of VPC S phase by hydroxyurea treatment did not prevent the subsequent expression of normal vulval development (Table 2, legend). Thus, the absence of vulval differentiation by Pn.p progeny in retarded mutants is not likely to be a simple consequence of a lengthened G1 phase.

The competence to generate vulval fates is temporally regulated separately from the vulval patterning signals and signal transduction machinery. In *lin-15(lf)* and *lin-12(sd)* mutants, where vulval fates occur independently of inductive and lateral signals (Ferguson et al., 1987), VPC competence and cell division are nevertheless restricted to the normal time in the L3. Also, we find that the heterochronic genes control when VPCs are competent to respond to intercellular signals but apparently do not affect the time of signal production, or the expression of the signal transduction machinery. Specifically, *lin-14(lf)* and *lin-4(lf)* mutations do not appear to affect the timing of *lin-3::lacZ* expression in the gonad or of *lin-12::lacZ* expression in the VPCs. It remains to be seen if the heterochronic genes affect the timing of other key activities, such as *let-23*, *lin-15*, or the ligand for the LIN-12 receptor.

Our observations suggest that at least some of the signaling mechanisms for vulval determination are present in the wild type earlier than the time that VPCs become competent. A *lin-12::lacZ* reporter gene was expressed in wild-type L2 larvae (Wilkinson and Greenwald, 1995), and similarly, we found that expression of a *lin-3::lacZ* transgene (Hill and Sternberg, 1992) was detectable in the early L2. Although the expression of these reporter genes may not accurately reflect the timing of endogenous gene expression, these results suggest that wild-type L2 animals may produce a gonadal inductive signal, and that VPCs may be able to carry out lateral signaling in the L2. The heterochronic gene pathway may control when VPC cells become competent to respond to these pre-existing signals. Further studies are required to determine if other vulval signaling mechanisms are also active during the L2.

A Genetic Pathway Controlling the VPC Developmental Program

The fact that *lin-28(lf)* is epistatic to *lin-14(gf)* and *lin-4(lf)* for defects in vulval differentiation and VPC cell

cycle progress suggests that both competence and cell division are controlled via *lin-28*. However, this epistasis is interpreted cautiously until it can be determined whether *lin-28* is sufficient to affect VPC division and vulval differentiation in the absence of *lin-14*. Accordingly, *lin-28* and *lin-14* are tentatively assumed to act at the same step of a regulatory pathway (Figure 4A) that controls both the competence and cell cycle progress of VPCs. It is noteworthy that *lin-29*, which acts downstream of *lin-28* for lateral hypodermal terminal differentiation (Ambros, 1989; Rougvie and Ambros, 1995) and for dauer larva differentiation (Liu and Ambros, 1989), is not involved in the timing of vulval development. This suggests that *lin-28* affects vulva timing via targets other than *lin-29*.

Since *lin-14* is a nuclear protein (Ruvkun and Giusto, 1989) and *lin-28* encodes a predicted nucleic acid binding protein (E. Moss, R. Lee, and V. Ambros, unpublished data), it is likely that the LIN-14 and LIN-28 proteins control VPC competence and cell cycle progress by targeting VPC-specific gene expression. Further studies are required to determine whether *lin-14* and *lin-28* function in VPCs, what their regulatory targets are, and how their targets mediate the control of VPC cell cycle and competence. *n300*, associated with the *nT1 IV;V* reciprocal translocation (Ferguson and Horvitz, 1985), may represent a gene activity downstream of *lin-14* and *lin-28* for VPCs. *n300* animals display a vulval phenotype similar to *lin-4(lf)* and *lin-14(gf)* animals; *n300* Pn.p divisions are blocked (Ferguson et al., 1987) or delayed (Euling, 1993; P. Sternberg, personal communication). *n300* is epistatic to *lin-28(lf)* (V. Ambros, unpublished data), suggesting that it acts downstream of *lin-28*. However, it is not known if the *n300* mutation represents a null allele of a single gene (Ferguson et al., 1987).

Developmental Coordination of Signal Transduction and Cell Cycle in VPCs

Vulval development in C. elegans is invariant, both in the timing of VPC division, and in the spatial pattern of vulval fates produced by the VPCs (Sulston and Horvitz, 1977; reviewed by Kenyon, 1995). When and where the vulva forms in the developing larva are critical, since the vulva must connect with the developing uterus and musculature. The normal pattern of vulval fates requires that the VPCs integrate three distinct signaling pathways and perform cell fate choices in concert. By controlling when VPCs become competent to adopt vulval fates in response to intercellular signals and when VPCs execute G1/S, the heterochronic gene pathway may coordinate developmental and cell cycle programs among the VPCs and ensure that they are synchronized in their cell fate determination, cell division, and differentiation.

Experimental Procedures

C. elegans Methods

C. elegans strains were grown and maintained as described (Brenner, 1974; Wood, 1988). All experiments were performed at 20°C unless otherwise noted.

C. elegans Strains

MT873 *lin-4(e912)*, MT1149 *lin-14(n536)*, MT1397 *lin-14(n179ts)*, MT1524 *lin-28(n719)*, MT1537 *lin-28(n719); lin-14(n536)*, MT1538 *lin-28(n719); lin-4(e912)*, MT2015 *lin-28(n947)*, MT3232 *lin-10(n1390)*, MT5788 *nIs2 (lin-11::lacZ)*, MT5790 *lin-4(e912); nIs2*, MT5875 *lin-28(n947); nIs2*, PS1269 *unc-31(e169); syIs3(lin-3::lacZ)*, VT291 *lin-4(e912); lin-14(ma135)*, VT292 *lin-14(ma135)*, VT364 *lin-28(n719); lin-4(e912)lin-29(n333); lin-14(ma135)*, VT377 *lin-4(e912)*, VT517 *lin-28(n719)*, VT518 *lin-4(e912); lin-10(n1390)*, VT594 *lin-10(n1390)lin-28(n719)*, VT719 *smg-1(r861)unc-54(r293); lin-4(e912); arIs11(lin-12::lacZ)*, VT720 *smg-1(r861)unc-54(r293); arIs11(lin-12::lacZ)*, VT721 *lin-28(n947); lin-15(e1763)*, VT723 *lin-28(n719); lin-3(e1417)*, VT757 *lin-28(n719); lin-12(n137n460ts)*, VT755 *syIs3(lin-3::lacZ); lin-14(n179ts)*, VT756 *syIs3(lin-3::lacZ); lin-4(e912)*. The wild-type strain used was C. elegans var. Bristol strain N2 (Brenner, 1974). Multiply mutant strains for epistasis were previously described (Ambros, 1989).

Phenotypic Analysis

Precocious VPC divisions were assayed in animals at the L2 molt using Nomarski optics to score for the presence of multiple hypodermal nuclei in the positions normally occupied by single VPCs. Retarded phenotypes were identified by the presence of undivided VPCs at the L3 molt. Vulval cell lineages were followed continuously (Sulston and Horvitz, 1977) in some animals. Developmental stage was determined using Nomarski optics based on body size, molting behavior (Singh and Sulston, 1978), and stage-specific gonad morphology, which is not appreciably affected by heterochronic mutations (Ambros and Horvitz, 1984).

DNA Quantitation in VPC Nuclei

Animals at defined developmental stages were placed in M9 on a slide and covered with a coverslip. The slides were frozen on dry ice, the coverslip was popped off, and the slides were fixed with 1:3:6 chloroform:acetic acid:ethanol, air dried, treated with RNAse A (20 μg/ml) in 2× SSC for one hr at 37°C, rinsed in 1× phosphate-buffered saline, and stained at room temperature with 0.1–10 μg/ml propidium iodide (Molecular Probes). The specimens were optically sectioned horizontally in 0.7 μm increments by confocal microscopy (White et al., 1987). The above-background fluorescence of VPC nuclei and neighboring neuronal nuclei, which were used as 2 N DNA controls (Hedgecock and White, 1985), were quantified from the digitized image of each section, and total DNA fluorescence was obtained by summing measurements from all sections. Test nuclei (sperm nuclei, 1 N; neuron nuclei, 2 N; oocyte nuclei, 4 N) gave fluorescent intensities consistent with their DNA content. A one-tailed t test (Sokal and Rohlf, 1981) was used to assess whether the DNA fluorescence of individual VPC nuclei was significantly greater than the mean of the fluorescence of surrounding (2 N) neuronal nuclei.

Temperature-Shift Experiments

lin-14(n179ts) animals were shifted between the permissive (15°C) and restrictive (25°C) temperatures at various times in development. The shifted animals were assayed at the L2 molt for evidence of precocious VPC divisions. Rate of development at each temperature was normalized to the rate of development at 20°C: 25°C development = 1.3 × 20°C = 2 × 15°C development (Wood, 1988). The time of shift was determined with respect to hatching or other stage-specific developmental landmarks (Ambros and Horvitz, 1987).

Hydroxyurea Treatment

Animals in L1 or L2 lethargus were identified as described above. They were either shifted immediately to plates containing 25 mM or 30 mM hydroxyurea and a small spot of E. coli, or they were monitored periodically using the dissecting microscope to identify the time of ecdysis (evidenced by resumption of pharyngeal pumping), allowed to grow for a period of time, then transferred to hydroxyurea plates. After 8–10 hr in hydroxyurea, animals were examined using Nomarski optics for VPC division.

lacZ Reporter Analysis

Transgenic DNAs carrying *lacZ* reporters were: *nIs2(lin-11::lacZ)* (Freyd, 1991), *syIs3 (lin-3::lacZ)* (Hill and Sternberg, 1992), and *arIs11(lin-12::lacZ)* (Wilkinson et al., 1994). Transgenic animals were isolated at known developmental stages, placed in a drop of M9 or water on a well slide, dried, fixed in acetone, and stained for 1–16

hr at 37°C in 0.1% X-Gal (5-bromo-4-chloro-3-indolyl-D-galactoside), as described (Fire et al., 1990) and 1 μg/ml DAPI to visualize nuclei (Wood, 1988). *arIs11(lin-12::lacZ)* was maintained in the presence of *smg-1(r861)* at 25°C (Wilkinson et al., 1994).

Laser Microsurgery

Laser microsurgery was performed as described (Sulston and White, 1980; Avery and Horvitz, 1987). Successful destruction of the anchor cell was confirmed using Nomarski optics.

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