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Cell signals allow the expression of a pre-existent neural pattern in *C. elegans*

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Summary

In *C. elegans*, a simple pattern develops within a row of epidermal precursor cells, V1-V6. One cell, V5, gives rise to a neuroblast called the postdeirid neuroblast, while the other V cells produce epidermal cells instead. Here we describe experiments suggesting that in order for V5 to produce the postdeirid it must be in close or direct contact with neighboring V cells. Signaling between V cells is required for the formation of the neuroblast; however, which of the V cells can make a postdeirid is not determined by these signals but rather by the action of the homeotic *lin-22* and *pal-1* genes. These genes prevent V cells in specific body regions from responding to intercellular signals and producing postdeirids. This is a clear example of cell signals playing a permissive rather than an instructive role in neuroblast induction.

Key words: *C. elegans*, pattern formation, cell signaling, homeodomain protein.

Introduction

Intercellular signals play many different roles in pattern formation during development. In some instances, the position of a signal determines where a structure is formed. For example, in *C. elegans* the position of a signaling cell in the gonad determines which epidermal cells initiate vulval development (Kimble, 1981). In *Xenopus*, the position of the organizer, which itself is induced by signals from nearby cells, determines the future positions of structures along the dorso-ventral and anteroposterior axes (reviewed by Gerhart et al., l989). Cell signals can also play a different, more permissive, role in pattern formation (Waring and Kenyon, l991; Sokol and Melton, l991). For example, in the development of a pattern of sensory rays and epidermal structures (called alae) in *C. elegans*, cell-cell signals are required for formation of epidermal structures. However, the signals themselves are not targeted to specific precursor cells. Instead, only certain precursor cells have the potential to respond to the signals (Waring and Kenyon, 1990; Waring and Kenyon, l991).

The same precursor cells that ultimately generate the ray/alae pattern, the V cells, give rise to another neural pattern earlier in development. This is a simple pattern consisting of a single neuroblast, the postdeirid, within a row of epidermal cells (see Fig. 1A; Sulston and Horvitz, 1977). There are several similarities between these two patterning events. Laser ablation experiments have shown that, as in the ray/alae decision, some type of cell-extrinsic signal is required for V5 to make a postdeirid neuroblast instead of behaving like the other V cells and producing only epidermal cells (Sulston and White, 1980). However, the signaling events required for the postdeirid and the ray/alae patterns are distinguishable, since ablation of precursor cells at certain stages can affect one pattern but not the other (Fixsen, l985). In addition, two genes involved in ray pattern formation, the homeobox-containing gene *pal-1* (Waring and Kenyon, 1990; Waring and Kenyon, 1991) and the gene *lin-22* (Horvitz et al., 1983) also affect postdeirid formation.

Here we use genetics and laser microsurgery to investigate the roles of cell-extrinsic signals and the genes *lin-22* and *pal-1* in postdeirid pattern formation. The results indicate that in order to make a postdeirid, a V cell must be in close or direct contact with each of its V cell neighbors, which raises the possibility that it must lie within an intact epithelium. We find that the genes *lin-22* and *pal-1* prevent cells other than V5 from being signaled by their neighbors to produce postdeirids. In these mutants, additional V cells produce postdeirids in a signal-dependent fashion. Mosaic analysis and laser microsurgery indicate that neither *pal-1* nor *lin-22* are likely to affect the production of the signals between V cells required for postdeirid formation. Instead, both genes appear to determine whether V cells are able to produce a neuroblast in response to the signals. Thus, postdeirid pattern formation requires two essential elements: (a) cell signaling to allow neuroblast formation and (b) pattern formation genes to create a prepattern of cells that are competent to respond to the signals.

The postdeirid and ray/alae pattern formation systems differ from one another in many ways. For example, the gene *lin-22* has opposite effects on cell signals in the two cases. Nevertheless, there is a fundamental similarity between them: in both, patterns arise through a mechanism

that creates intrinsic differences in the ability of cells to respond to intercellular signals. This basic strategy can thus be used to produce very different patterns of neurons even within the same tissue.

Materials and methods

General procedures and strains

Methods for routine culturing and genetic analysis were described in Brenner (1974). All experiments were performed at 20°C. The complete genotype of the *pal-1; lin-22* mutant strain was *unc-79(e1068) pal-1(e2091); lin-22(mu2); him-5(e1490).*

Observation of postdeirids

Late L2 or early L3 animals were examined for the presence of postdeirids (wild-type and ectopic). Postdeirids were recognized by their cell morphology and position relative to other cells. By examining animals at this stage, it is possible to determine which cells were derived from each V cell and the type of lineage that had taken place (postdeirid, seam cell, or hybrid lineage). It was sometimes not possible to infer the sequence of cell divisions within a hybrid lineage, but it was always clear that some type of hybrid lineage had occurred. Sodium azide (2 mM) was used in many cases to anesthetize the animals while they were being examined.

Laser microsurgery

Individual cells were killed using a laser microbeam as described in Sulston and White (1980). The laser used was a VSL 334 nitrogen-pumped dye laser using 7-amino 4-methyl coumarin dye, which produces a wavelength of 440 nm. 1-2 mM sodium azide in the agarose pad was used to anesthetize the animals. All ablations were performed on animals within 2 hours of hatching (before the QL cell had migrated above V5). Animals were observed 3-5 hours after surgery to confirm that the ablated cells were in fact dead or dying. Production of postdeirids was scored about 24 hours after ablation when the animals were in the late L2 or early L3 stage.

Mosaic analysis

Mosaic animals arose spontaneously in the strain *sDp3 III; pal-1(e2091) dpy-17(e164) ncl-1(e1865) unc-36(e251) III; him-5(e1490) V. sDp3*, a free duplication carrying wild-type copies of the linked genes, is lost in approximately 1/400 cell divisions (Kenyon, 1986). The mutation *ncl-1(e1865)* causes cells to have unusually large nucleoli. This gene has been shown to act cell-autonomously and thus allows one to determine cell genotypes (E. Hedgecock, personal communication). The Ncl phenotype is especially visible in neuronal cells, which generally have small nucleoli. Class I animals and some of the Class II animals were identified by looking at L2 or L3 animals for the presence of Ncl postdeirids. At this time, all other cells used as markers for the duplication, P(1-10).a descendants, Q-derived neurons, ALM and BDU, could also be scored. Some of the Class II animals were picked as Unc non-Dpy adults and then examined using Nomarski optics for the presence of postdeirids and for the Ncl phenotype.

Isolation of lin-22 *mutations*

The *lin-22(mu2)* and *lin-22(mu5)* alleles were isolated by screening for males that had undergone the alae-to-ray transformation characteristic of *n372* animals (Kenyon, l986). F_2 male progeny from EMS mutagenized *him-5(e1490)* animals were examined under a dissecting microscope using oblique illumination for the absence of adult alae, and then examined with Nomarski optics for the presence of ectopic ray papillae and ray cell groups. (The *him-5* mutation increases the frequency of male self-progeny). From 5402 F_2 male progeny of EMS-mutagenized animals, two independently isolated males were found that displayed an alae-to-ray transformation. One of these males produced progeny when crossed to *unc-17(e245)* hermaphrodites. Complementation tests indicated that this animal contained a new *lin-22* mutation, *mu2.* The second new *lin-22* allele, *mu5*, was isolated in a complementation screen by mating mutagenized *him-5(e1490*) males with *lin-22(n372) unc-17(e245)* hermaphrodites and examining 4267 male F_1 non-Unc cross progeny for the alae-to-ray transformation. In this screen, a single animal with this phenotype was identified. The new mutation was recovered by outcrossing and shown to be a *lin-22* allele by complementation tests with *n372.* In *C. elegans*, the frequency with which null (or severe reduction-of-function) alleles arise in most genes following standard EMS mutagenesis protocols is thought to be approximately 1/2000 haploid genomes. Because of the small sample size, the *lin-22* mutation frequency cannot be determined accurately from these data; however, the results indicate that these alleles do not correspond to rare, highly specific alterations in protein sequence.

Genetic analysis of lin-22

lin-22; him-5(e1490) animals were examined using Nomarski optics for the presence of a postdeirid among the descendants of each V cell. For each allele, heterozygous *lin-22/+* animals were generated by crossing *lin-22; him-5(e1490)* males to *unc-17(e245) dpy-13(e184)* and *dpy-17(e164); him-5(e1490)* hermaphrodites. Cross progeny were then scored for postdeirid formation. In these crosses, the wild-type copy of *lin-22* was always derived from the mother. We also generated *n372/+* animals in which the mutant *lin-22* allele came from the mother. These *n372/+* heterozygotes were produced by crossing *him-5(e1490)* males to *lin-22(n372) unc-17(e245) osm-3(p802); e1490* hermaphrodites (the *osm-3* mutation affects dye uptake in head and tail neurons), *lin-22(n372) unc-17(e245) osm-3(p802) dpy-13(e184); e1490* hermaphrodites, and *lin-22(n372) unc-33(e204); e1490* hermaphrodites. Cross progeny were then examined for postdeirids. When the mutant *n372* allele was provided by the mother, ectopic postdeirid lineages were generated in 4/122 animals (3%) as compared to the 11/250 (4%) animals that made extra postdeirids when the *n372* allele was provided by the father.

lin-22 Gene Dosage Analysis: Animals of the genotype *lin-22(n372)/lin-22(n372)/+* were generated by crossing single hermaphrodites of the genotype *lin-22(n372) unc-17(e245) osm-3(p802); him-5(e1490)* to *n372 e245 p802 dpy13(184); mDp1(lin-22+); e1490* males. Non-Unc F_1 cross progeny, which should carry the duplication *mDp1*, were scored for postdeirids. The *mDp1; n372 e245 p802; e1490* strain is itself *n372/n372/+*; however, we did not want to examine this strain directly because we were unsure whether non-Unc n372/+ recombinants could be generated and could bias the results of a dosage analysis. To ensure that the original male actually carried the free duplication and not a recombinant chromosome, all hermaphrodite F_1 cross progeny examined for postdeirids were subsequently allowed to self-fertilize. The progeny of these hermaphrodites were scored for the presence of wild type, Unc and Unc Dpy phenotypes. This confirmed the presence of *mDp1* and both of the *n372* mutant chromosomes. For the *mu2/mu2/+* genotype, non-Unc non-Dpy F_1 progeny from *mu2 e245 e184; mDp1; e1490* hermaphrodites were scored for the presence or absence of postdeirids. The presence of the free duplication was confirmed whenever possible by scoring the percentage of Lin-22 Unc-17 Dpy-13 progeny produced by the F_1 hermaphrodites. Animals of the genotype *mu2 e245 e184/mu2 e245 e184/+* can be distinguished from *mu2 e245 e184/+* recombinants because loss of the free duplication leads to a higher percentage of Lin Unc Dpy progeny produced than would

be generated from a heterozygote (~35-40% for *mDp1* versus 25%). +/+/+ animals were examined in the same fashion from the strain *e245 e184; mDp1; e1490*.

Results

Background

The V cells are specialized epidermal stem cells called seam cells that divide during each of the four postembryonic larval stages, L1-L4. Each division generates one seam cell and a second cell that becomes either another seam cell, a neuroblast, or a cell that fuses with a large epidermal syncytium that covers much of the animal. The cell V5.pa (the anterior daughter of the posterior daughter of V5), which is generated during L2, becomes the postdeirid neuroblast. This cell goes on to generate the postdeirid sensillum [consisting of a neuron (PDE) and two support cells] and an additional sensory neuron (PVD; Sulston and Horvitz, l977; Way and Chalfie, 1988). For the purposes of this paper we will refer to this group of four cells as a postdeirid. The lineages generated by V cells in wild-type hermaphrodites are shown in Fig. 1A.

The lin-22$^+$ *gene prevents V1-V4 cells from producing postdeirids*

In the wild type, only one V cell, V5, produces a postdeirid. The *lin-22(n372)* mutation, identified by Fixsen and Horvitz (Horvitz et al., 1983; Fixsen, 1985), causes V cells anterior to V5 also to generate postdeirids (Fig. 1B). Therefore *lin-22* is a candidate for a gene that regulates postdeirid pattern formation. The *n372* allele is slightly semidominant; *n372*/+ animals produce ectopic postdeirid lineages at a frequency of about 4%. Either a gain-of-function mutation in *lin-22* or a reduction-of-function (haplo-insufficient or dominant-negative) mutation could produce a semidominant phenotype. In order to infer the wild-type function of this gene, it is necessary to determine whether the *lin-22* phenotype results from elevation or reduction of gene function. We have therefore isolated additional *lin-22* mutations and asked whether these are gain- or reduction-of-function mutations by altering gene dosage.

We have isolated two additional *lin-22* alleles, *mu2* and *mu5* (see Materials and methods). Each of these mutations causes V2-V4, and to a lesser extent V1, to generate a postdeirid. (The postdeirid is always produced by a Vn.pa cell). V6 has never been observed to produce a postdeirid. The frequency with which each V cell generates a postdeirid in homozygous *lin-22* mutants and in heterozygous *lin-22*/+ animals is shown in Table 1. Unlike *n372*, both *mu2* and *mu5* are fully recessive, suggesting that they result in decreased *lin-22* activity.

So far, no attempts to isolate a deficiency of the *lin-22* region have been successful, so in order to better characterize these mutations we have altered gene dosage using the duplication *mDp1*, which carries a copy of the wild-type *lin-22* gene (see Materials and methods). As shown in Table 1, adding a wild-type *lin-22* gene copy to *n372/n372* or *mu2/mu2* homozygotes reduces the severity of the mutant phenotype seen in *lin-22* homozygotes. This means that these *lin-22* mutations do not elevate wild-type gene activity. In that case, adding a wild-type *lin-22* copy should have increased, not decreased, the frequency of ectopic postdeirids. The simplest interpretation of these data is that these alleles lower the level of wild-type *lin-22* activity. While it is still possible that all three mutations create a novel activity that is inhibited by wild-type gene function, this seems unlikely because these alleles are not rare. Therefore, we conclude that wild-type *lin-22(+)* activity prevents V1, V2, V3, and V4 from making postdeirids.

Table 1. *Percentage postdeirid production in lin-22 mutants*

	V1	V2	V3	V4	V5	V6	*n*
+	**0**	**0**	**0**	**0**	**100**	**0**	105
mu5	**21** (5)	**75** (11)	**84** (25)	**90** (15)	**100**	**0**	110
mu2	**39** (5)	**94** (6)	**91** (17)	**95** (19)	**100**	**0**	100
n372	**40** (7)	**88** (10)	**90** (17)	**95** (15)	**100**	**0**	102
mu5/+	**0**	**0**	**0**	**0**	**100**	**0**	168
mu2/+	**0**	**0**	**0**	**0**	**100**	**0**	209
n372/+	**0**	**0.5** (0.3)	**1** (0.3)	**2** (0.5)	**99.7**	**0**	377
n372/n372/+	**0**	**4** (0.9)	**3** (0.9)	**12** (9)	**98**	**0**	114
mu2/mu2/+	**0**	**0.9** (0.9)	**0**	**0**	**99.1**	**0**	106
+/+/+	**0**	**0**	**0**	**0**	**100**	**0**	97
pal-1; mu2	**28** (3)	**56** (7)	**72** (18)	**75** (19)	**100**	**70** (15)	102

The first number in each column represents the percentage of V cells that produced either a hybrid or complete postdeirid.The percentage of V cells that produced hybrid lineages is given in parentheses. *n*: number of animals examined.The frequency of postdeirids in *lin-22/lin-22/+* animals is much lower than in *lin-22/lin-22* animals, indicating that these *lin-22* mutations do not elevate gene activity. The postdeirid frequency in *lin-22/lin-22/+* animals is slightly higher than in *lin-22/+* animals.This could result from several sources, including (i) a slight dominant-negative effect of these mutations (which does not affect the overall conclusion that these mutations lower the level of *lin-22* activity); (ii) genetic mosaicism; and (iii) inefficient expression of the *lin-22(+)* gene when carried on *mDp1*. For complete genotypes, see Materials and methods.

In *lin-22* animals, Vn.pa cells sometimes generate "hybrid" lineages that display elements of both the wild-type V5.pa and wild-type (V1-V4).pa lineages (Fixsen, 1985). An example of the most common hybrid lineage is shown in Fig. 1G. Hybrid lineages were also observed in some wild-type animals following ablations (Tables 2, 3). The fact that Vn.pa cells can undergo hybrid lineages indicates that the choice between the two fates is not simply a single binary decision made by Vn.pa cells between two possible sublineages. Rather it appears that individual descendants of a Vn.pa cell can independently choose between alternative fates.

The pal-1 *protein appears to function cell-autonomously to prevent V6 from making a postdeirid*

So far no single mutation has been identified that is sufficient to cause V6 to generate a postdeirid. However, in

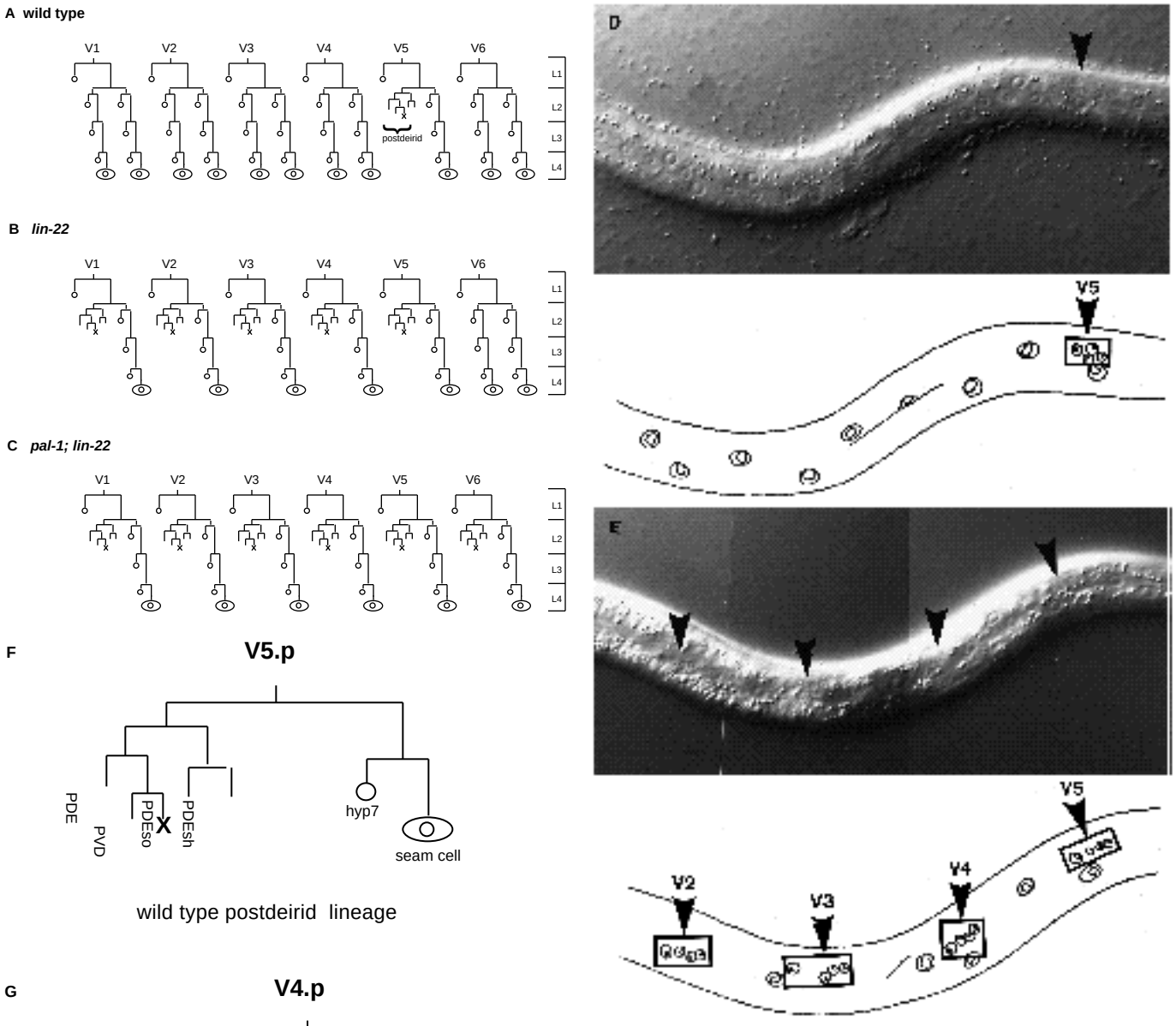

Fig. 1. (A-C) The lineages of V1-V6 in wild-type and mutant hermaphrodites. (Sulston and Horvitz, 1977; Horvitz et al., l983). In the standard *C. elegans* lineage convention anterior daughters of a cell division are drawn to the left and posterior daughters to the right. Symbols: O= cell that joins the hypodermal syncytium, ⓞ = adult seam cell, X= cell death.Archetypal lineages of *lin-22* (Horvitz et al., l983) and *pal-1; lin-22* animals are shown. As shown in Table 1 and discussed in the text, individual V(1-4).pa cells in any given animal may generate a postdeirid, a hybrid lineage, or a seam cell.(D) Nomarski photomicrograph of the lateral epidermis of a *lin-22*$^{+}$*;him-5(e1490)* late L2 animal. The focal plane is subepidermal, so that the postdeirid cells are in focus.(E) Photomicrograph of a *lin-22(mu2); him-5(e1490)* animal. (F) The wild-type L2 lineage of V5.p, showing the production of the postdeirid. The fates of the postdeirid cells are shown. PDE and PVD are neurons, PDEso and PDEsh are the postdeirid support cells (socket and sheath).(G) A typical “hybrid” Vn.pa lineage. This lineage is from a V4 cell in a *pal-1(e2091); lin-22(mu2); him-5(e1490)* animal. In this example (which is by far the most common) the V4.pa cell generated a postdeirid (as judged by lineage and cell morphology) and a seam cell which was smaller than the V4.pp-derived seam cell. The cells of the postdeirid in the hybrid lineages exhibited the typical compact nuclei seen in wild-type postdeirids; however, without EM studies we cannot be certain of the fates of the individual cells.Other hybrid lineages have been observed that yield, for example, two neuron-like cells and an extra seam cell or four cells that are neither seam cells nor neuron-like cells, but appear to be epidermal in character.

Table 2. *Production of postdeirids following ablations*

V1	V2	V3	V4	V5	V6	T
lin-22(mu2); him-5(e1490)						
34/100 (34,5,61)	**88/100** (88,6,6)	**74/100** (74,17,9)	**76/100** (76,19,5)	**100/100** (100,0,0)	**0/100** (0,0,100)	
1/9 (1,1,7)	**4/9** (4,3,2)	**3/9** (3,3,3)	**4/9** (4,3,2)	**0/9** (0,0,9)	X	
7/15 (7,0,8)	**10/15** (9,3,3)	**9/15** (6,6,3)	**1/15** (1,0,14)	X	X	
2/12 (2,1,9)	**8/12** (8,0,4)	**0/12** (0,2,10)	X	X	X	
0/5 (0,0,5)	**0/5** (0,0,5)	X	X	X	X	
X	**1/7** (1,1,5)	**2/7** (2,2,3)	**4/7** (4,1,2)	**7/7** (7,0,0)	**0/7** (0,0,7)	
X	X	**0/4** (0,1,3)	**3/4** (3,0,1)	**4/4** (4,0,0)	**0/4** (0,0,4)	
X	X	X	**0/16** (0,0,16)	**16/16** (16,0,0)	**0/16** (0,0,16)	
pal-1(e2091); lin-22(mu2); him-5(e1490)						
26/102 (26,3,73)	**50/102** (50,7,45)	**55/102** (55,18,29)	**57/102** (57,19,26)	**102/102** (102,0,0)	**56/102** (56,15,31)	
3/9 (3,0,6)	**5/9** (5,0,4)	**6/9** (6,0,3)	**4/9** (5,0,4)	**10/10** (0,0,10)	**0/10** (0,0,10)	X
2/9 (2,0,7)	**0/10** (0,1,9)	X	X	X	**0/10** (0,0,10)	

Table 3. *Production of postdeirids following ablations*

V1	V2	V3	V4	V5	V6
Wild-type					
0	0	0	0	**0/18** (0,0,18)	X
0	X	X	X	**0/9** (0,0,9)	0
0	0	X	X	**5/11** (5,1,5)	0
0	0	0	X	**13/17** (13,2,2)	0
lin-22(mu2)					
34/100 (34,5,61)	**88/100** (88,6,6)	**74/100** (74,17,9)	**76/100** (76,19,5)	**100/100** (100,0,0)	**0/100** (0,0,100)
1/9 (1,1,7)	**4/9** (4,3,2)	**3/9** (3,3,3)	**4/9** (4,3,2)	**0/5** (0,0,5)	X
1/5 (1,0,4)	**4/5** (4,0,1)	**4/5** (4,0,1)	**4/5** (4,0,1)	X	**0/5** (0,0,5)
4/11 (4,1,6)	**9/11** (9,0,2)	**6/11** (6,3,2)	X	**8/11** (8,1,2)	**0/11** (0,0,11)
3/11 (3,0,8)	**5/11** (5,2,4)	X	**4/11** (4,5,2)	**11/11** (11,0,0)	**0/11** (0,0,11)
0/4 (0,1,3)	X	**2/4** (2,1,1)	**2/4** (2,2,0)	**4/4** (4,0,0)	**0/4** (0,0,4)
X	**1/7** (1,1,5)	**2/7** (2,2,3)	**4/7** (4,1,2)	**7/7** (7,0,0)	**0/7** (0,0,7)
X	X	**0/9** (0,2,7)	**4/9** (4,5,0)	**0/9** (0,1,8)	X

The ablated cells are marked with an X.The fraction of animals producing posteirids is shown in large characters.The numbers in parentheses represent postdeirids, hybrid lineages and seam cell lineages observed, in that order.Cases where the ablations have the strongest effects are observed marked with boxes.

double mutants that carry both a *lin-22* mutation as well as a reduction-of-function mutation in the gene *pal-1*, V6 generates a postdeirid (Waring and Kenyon, l990). Thus both *pal-1* and *lin-22* function to prevent V6 from making a postdeirid.

Previously we have shown that *pal-1* acts cell-autonomously to determine whether V6 can produce sensory rays, which arise at a later developmental stage in males. To test whether *pal-1* also functions cell-autonomously to prevent V6 from forming a postdeirid, we generated *pal-1* genetic mosaics in the presence of a *lin-22* mutation (see Materials and methods). We found two classes of mosaic animals (Fig. 2). In the first class, V6 was genotypically mutant for *pal-1*, but all surrounding cells, including V5 and T, were wild type. In these animals, the Pal-1 phenotype of V6 was mutant; that is, V6 produced a postdeirid. In the second class, V6 was genotypically wild type, but the neighboring V5 and T cells were *pal-1*⁻. (T is a seam cell located in the tail. As described below, V5 and T appear to send postdeirid-inducing signals to V6.) In these animals, V6 developed normally, indicating that *pal-1* activity is not required in V5 and T. Thus these data are consistent with the idea that *pal-1* functions cell-autonomously within V6. The data do not rule out the possibility that *pal-1* actually acts in a close relative of V6, and not in V6 itself. However, this seems much less likely because none of these close relatives of V6 are located in the posterior body region, where it appears that the intercellular signaling required for normal V6 development occurs (see below). All other *pal-1*⁻ cells in this class of mosaics were located anterior to V5.

The ectopic postdeirids that form in lin-22 *and* pal-1; lin-22 *mutants require cell-extrinsic signals*

Cell-extrinsic signals are known to be required in order for V5 to produce a postdeirid. When V5's posterior neighbor V6 is ablated, V5 fails to form a postdeirid. In addition, when V5's anterior neighbors are ablated, V5 fails to form a postdeirid (Sulston and White, 1980).

Knowing that cell-extrinsic signals play a role in wild-type postdeirid formation, we can ask whether signals are also required for ectopic postdeirid formation in *lin-22* and *pal-1; lin-22* mutants. One possible explanation for the ectopic postdeirid formation in these mutants is that the mutations render postdeirid formation independent of cell-

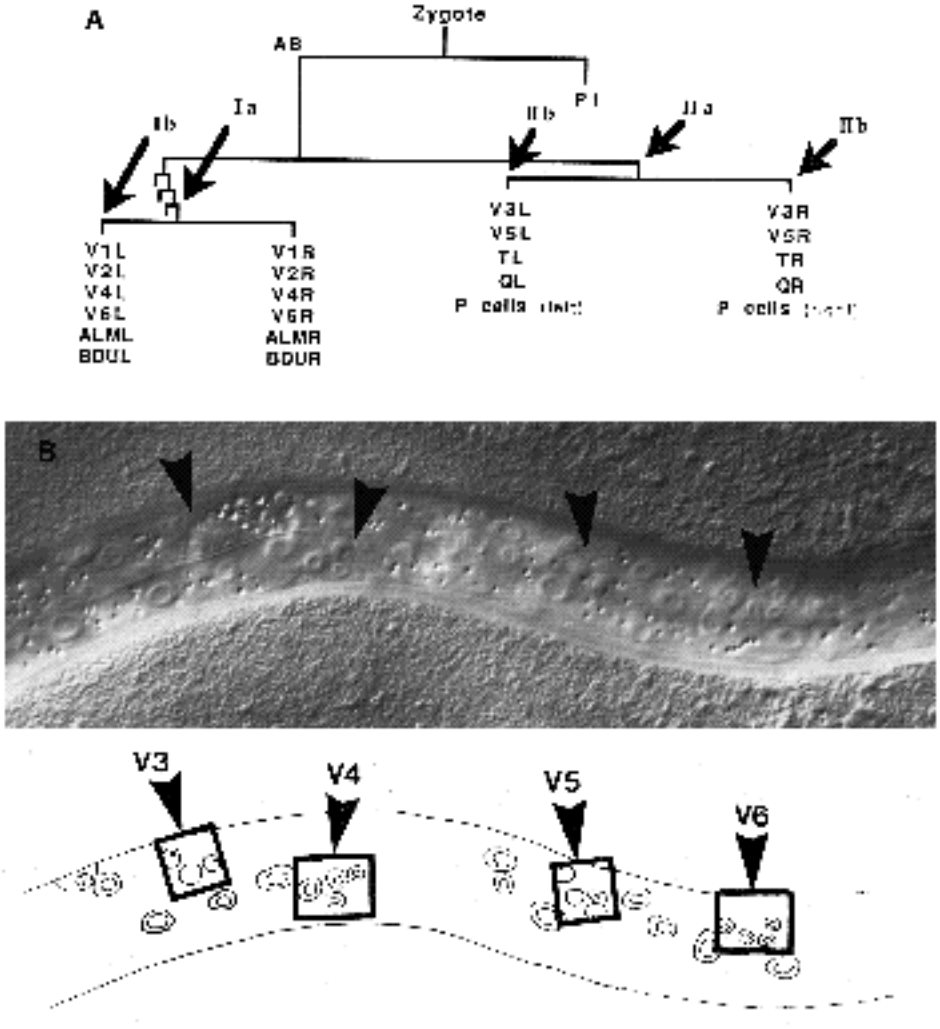

Fig. 2. Mosaic analysis of *pal-1* in a *lin-22* background. (A)The early embryonic lineage showing the origins of the V cells and some of their lineal relatives that were used to score for the loss of the duplication. The arrows indicate the last cell in which the loss could have occurred for each of the two classes of mosaics.Class Ia: (one animal) in this animal the postdeirids derived from V1, V2, V4 and V6 on both sides were Ncl, and the V5- and Pn.a-derived cells were Ncl$^+$. In this animal V6 on one side generated a postdeirid while V6 on the other side did not. Class Ib: (two animals); in these animals ALML, BDUL and those postdeirids derived from V1, V2, V4 and V6 on the left side were Ncl while the Q, V5 and Pn.a derived cells were all wild type (non-Ncl). In both of these animals V6 generated a postdeirid and an extra seam cell (hybrid lineage). Class IIa: (four animals); in these animals, the Q- and Pn.a-derived cells and the V3 and V5 postdeirids on both sides were Ncl, while the ALM cells, and the V1, V2 and V4-derived postdeirids were wild type (non-Ncl). Class IIb: (three animals); in these animals the Q-derived neurons, and the V3 and V5-derived postdeirids on one side and the descendants of one of each pair of Pn.a cells (P1/2-P9/10) were Ncl, while the V1, V2, and V4-derived postdeirids were wild type (non-Ncl). In Class IIa and Class IIb mosaics, V6 did not generate a postdeirid. One additional animal appeared by most criteria to be a Class IIa animal, yet the Q-derived cells appeared non-Ncl.This result cannot be explained as a single or even a double loss of the duplication. Unusual mosaic animals such as this have been reported by others using *sDp3*. It has been suggested that such patterns result from the duplication becoming unstable within a given lineage (E. Hedgecock, personal communication; Austin and Kimble, 1987) (B) Photomicrograph of a mosaic animal (class Ia).Arrowheads point to the postdeirid. The origin of each postdeirid is shown. In this animal the V6 and V4-derived postdeirids have the Ncl phenotype (very large nucleoli) while the V3 and V5 postdeirids do not. This indicates that the free duplication has been lost in a precursor to V4 and V6, and thus that the genotype of V6 is *pal-1*$^-$.

extrinsic signals. For example, if a signal required for wild-type postdeirid formation were normally targeted to V5, signal-independent mutations might allow V cells that normally are not exposed to the signal to produce postdeirids. An example of this situation can be found in vulva development. In wild-type animals a signal from the anchor cell is required to induce vulval development. Many Multivulva mutations cause additional cells to generate ectopic vuval lineages that are anchor cell signal-independent (constitutive) (Ferguson and Horvitz, 1985).

To determine whether ectopic postdeirids require cell-extrinsic signals, we examined postdeirid formation in *lin-22* and in *pal-1; lin-22* mutants following ablation of specific V cells. The results are shown in Table 2. We first asked whether the V5 postdeirid was still signal-dependent in *lin-22* mutants. When V6 is ablated, the V5 postdeirid is not formed, indicating that cell-extrinsic signals are still required. In addition, in *lin-22* mutants, a given V cell almost always failed to produce a postdeirid when we ablated all the V cells posterior to it or all the V cells anterior to it. Likewise, in *pal-1; lin-22* double mutants, V6 did not produce a postdeirid when its posterior neighbor, T, was ablated.

These results indicate that neither *lin-22* nor *pal-1* mutations relieve the requirement for cell-extrinsic signaling in postdeirid formation. Rather, both mutations allow cell-extrinsic signals to signal additional V cells to form postdeirids. This means that the wild-type function of both *lin-22* and *pal-1* is somehow to block signal-dependent postdeirid formation in cells other than V5.

Signals required for postdeirid production are produced by most or all V cells

Two types of signaling models can explain the finding that ablation of the neighbors of V5 abolishes postdeirid formation in the wild type. In the first model a localized inducing signal present in the vicinity of V5 causes V5 to produce a postdeirid. Ablation of neighboring cells could cause V5 to move out of the range of the inducing signal. Alternatively, neighboring V cells could signal V5 to produce a postdeirid. In this case, V5 would change fate after its neighbors are ablated in wild-type animals because it fails to receive intercellular signals from neighboring V cells. The ablation experiments described above provide information about the source of the signals. In *lin-22* and *pal-1*; *lin-22* mutants, postdeirid formation correlates with the presence or absence of a neighboring cell, and not a cell's position along the body axis (Table 2). This is especially apparent when T is ablated in a *pal-1; lin-22* double mutant. In these animals, V6 does not appear to move (as judged by the position of its nucleus) and yet it changes fate when its neighbor is ablated. Other cells, located at many positions all along the body axis, also make postdeirids, but only if their neighbors are present. Thus, signaling from neighboring V cells does appear to be required for postdeirid formation.

Can any V cell provide postdeirid-inducing signals? In the ablation experiments described above, most or all V cells anterior or posterior to the test cell were ablated. We next asked whether the remaining V cells could signal V5 to produce a postdeirid if only one or a few V cells were ablated. We first asked whether ablation of only V5's anterior neighbor, V4, would prevent V5 from making a postdeirid. In this case, there was usually no effect, and V5 produced a postdeirid (Table 3). Thus V3 appears to be capable of signaling V5 to produce a postdeirid. When both V3 and V4 were ablated, V5 still often produced a postdeirid, indicating that V2 is also capable of signaling V5 to produce a postdeirid. However, when three cells, V2, V3 and V4, were ablated, V5 never made a postdeirid. In these types of ablations, we observed that by late L2, the descendants of the remaining V cells had spread across the gap created by V cell ablation, as judged by the positions of their nuclei. Thus it seems likely that if the gap resulting from the ablation is not too large, signaling between the remaining V cells can take place.

In the wild type, ablation of V6 alone prevents V5 from producing a postdeirid. Therefore V6's posterior neighbor T, (a seam cell in the tail), cannot substitute for V6 in these experiments. This could mean that T cannot provide postdeirid-inducing signals. However, the failure of T to signal V5 could also be due to other factors, such as steric constraints preventing V5 and T from moving toward one another following the ablation of V6. The anus, along with many neurons and rectal epithelial cells is positioned between T and V6. In fact, the mosaic analysis of *pal-1* implies that T does produce postdeirid-inducing signals that can act on V6 if V6 is mutant for *pal-1* (see Discussion).

A similar situation was observed when single cells were ablated in a *lin-22(mu2)* mutant (Table 3). Other than V6 ablation, which always prevented V5 postdeirid formation, ablation of single cells generally had no effect. In a previous study, Fixsen and Horvitz ablated single cells in the *lin-22(n372)* mutant and saw no effect on postdeirid formation (Fixsen, 1985).

Together these data suggest that all the V cells are capable of producing signals required for postdeirid formation. They also suggest that a V cell must receive signals from neighboring cells on both sides in order to produce a postdeirid. Are signals from one neighboring V cell on each side of a test cell sufficient for postdeirid formation? If so, in a cluster of three isolated V cells, the central cell should be able to produce a postdeirid. By doing the experiments in a *lin-22* background, it is possible to monitor postdeirid formation in several cells at once. As shown in Table 3, when the central group of V cells, V3-V5, were isolated in *lin-22* animals by ablating all other V cells as well as T, V4, the center cell, produced a postdeirid. V3 and V5 did not.

Discussion

What role do neighboring cells play in postdeirid formation?

In this study we have asked what role extracellular signals play in postdeirid formation, and what types of cell-cell interactions may mediate this signaling. By ablating combinations of V cells in wild-type and mutant animals, we have shown that signaling between V cells or their descendants is likely to be required for postdeirid formation to occur, and that in order to produce a postdeirid, a postdeirid precursor cell must be flanked by neighboring V cells on both sides of itself. In experiments in which all but three V cells are killed, the central cell produces a postdeirid. Thus the two normal neighbors of a V cell are sufficient to allow postdeirid formation.

Two types of experiment suggest that postdeirid-inducing signals are produced by most or all of the V and T cells, and not just by the normal neighbors of V5. First, the results of laser ablation experiments suggest that all V cells produce postdeirid-inducing signals. For example, V2 and V3 are both capable of signaling V5 following ablation of the intervening V cells in wild-type animals. This demonstrates that outlying V cells can provide the postdeirid-inducing signals normally provided by natural neighbors. Second, the mosaic analysis of *pal-1* also indicates that cells other than V5's neighbors (V4 and V6) produce postdeirid-inducing signals in the wild type. In mosaic animals in which V6 lacks *pal-1* activity but neighboring V5 and T cells are *pal-1(+)*, V6 is induced to form a postdeirid. This implies that in the wild type both T and V5 produce postdeirid-inducing signals. The ability of these signals to induce the intervening cell V6 to produce a postdeirid depends upon V6's *pal-1* genotype.

The role played by the presence of neighboring V cells is not at all clear. The data suggest that in order to form a postdeirid, a V cell must be in close or direct contact with V cells on both sides of itself. Why are neighbors on both sides required? It could be that the level of an intercellular signal produced by only one neighbor is insufficient to induce postdeirid formation, and therefore that close neigh-

bors on each side are required. Alternatively, it is possible that a V cell receives different types of signals from its anterior and posterior neighbors.

It is also possible that disruption of the epithelium *per se* somehow changes the development of these cells. For example it could be that signals from only one V cell are sufficient for postdeirid formation, but that disrupting the epithelium on one side of a V cell changes the cell's architecture in such a way that it is no longer capable of receiving signals from V cells on either side. Or, it might be that, rather than requiring active signaling from its neighbor, a V cell may simply have to be part of an intact epithelium in order to produce a postdeirid neuroblast. Several recent findings suggest that molecules involved in forming an epithelial sheet can function in cell fate determination. For example, mutations in plakoglobin, a component of desmosomes, cause specific transformations in cell fate in *Drosophila* (Peifer and Wieschaus, l990). The *Drosophila Notch* protein, which affects the determination of many cell types, also affects cell-cell adhesion (Fehon et al., l990). In these cases the distinction between active signaling and cell adhesion is blurred. It should be informative to identify gene products required for V cell communication.

One feature of this signaling system that sets it apart from certain others is that a V cell sends postdeirid-inducing signals to its neighbor whether or not it produces a postdeirid neuroblast itself. This differs from the phenomenon of lateral inhibition, in which a cell entering a specific pathway of differentiation signals its neighbor to adopt a fate different from its own. In cases of lateral inhibition, a cell's ability to signal its neighbor is correlated with its own developmental fate.

One important question not addressed in this study is exactly which cell in a V lineage sends the signals required for postdeirid formation. During the middle of the first larval stage, each V cell divides, producing one seam cell (the Vn.p cell) and one cell that fuses with the epidermal syncytium that covers much of the animal. In principle, signaling required for the daughter of V5.p, V5.pa, to become a postdeirid could occur between the V cells, their daughters, or even their granddaughters. In a previous study, Fixsen reported that ablation of V6 but not V6.p, its seam cell daughter, prevented postdeirid formation (Fixsen, l985). This suggested that signaling between the V cells themselves is required for postdeirid formation. A more extensive examination of this question is now in progress (Austin and Kenyon, unpublished data).

pal-1 *and* lin-22 *prevent cells other than V5 from producing postdeirids in response to intercellular signals*

The gene dosage analysis of *lin-22* mutations described here, together with previous analysis of *pal-1* (Waring and Kenyon, 1990) indicates that these *lin-22* and *pal-1* mutations decrease the level of gene product, and therefore that in the wild type, both genes prevent V cells other than V5 from producing postdeirids. In postdeirid pattern formation, these genes behave as classical homeotic genes. Mutations in these genes produce discrete transformations in cell fate in which V cells in one body region adopt fates characteristic of V cells in another body region. These two genes function in distinct spatial domains. *lin-22* functions in V1-V4 and, in a *pal-1* mutant, in V6, whereas *pal-1* functions only in V6.

pal-1 and *lin-22* are functionally redundant in V6 postdeirid formation. However, it is not clear whether both genes are active in V6 in the wild type. For example, it is possible that in a wild-type V6 cell, *pal-1* activity represses *lin-22* and also regulates postdeirid development. This possibility is suggested by the finding that *pal-1* behaves as a negative regulator of *lin-22* in V6 during ray development (Waring and Kenyon, 1990). It will be informative to determine directly which genes are expressed in wild-type V6 cells.

Whatever the regulatory relationship between *pal-1* and *lin-22* in V6, it is clear that both genes can function to inhibit postdeirid formation. How do they accomplish this? One possible model, that mutations in these genes allow signal-independent postdeirid formation, has been ruled out. Laser microsurgery indicates that the ectopic postdeirids formed in these mutants are signal-dependent. Thus, in the wild type, these genes prevent V cells from being induced by signals from their neighbors to produce postdeirids.

Genetic mosaic analysis indicates that *pal-1* functions cell-autonomously in postdeirid pattern formation. Thus *pal-1* determines whether or not V6 is competent to respond to postdeirid-inducing signals from its neighbors. Wild-type *pal-1* protein, which is a homeodomain protein, could act in two distinct ways. It could affect cell fate by acting on the signaling pathway directly. For example, it could repress a gene required for signal transduction, or compete with a signal-dependent transcriptional regulator for binding to a target gene. Alternatively, it could bypass the signaling pathway altogether and directly initiate an alternative cell fate.

Because mosaic analysis has not been carried out with *lin-22*, we do not know whether *lin-22* function is also cell autonomous. However, the available data argue against one possibility; namely, that *lin-22* acts by regulating the ability of V cells to produce signals required for postdeirid formation. This is because laser microsurgery experiments show that in wild-type (*lin-22*$^{+}$) animals, V2, V3 and V4 are each capable of providing postdeirid-inducing signals for V5, indicating that *lin-22*$^{+}$ does not prevent these V cells from making signals. It is more likely that *lin-22*$^{+}$ function regulates the ability of V cells to generate a postdeirid in response to signals from their neighbors. *lin-22* could accomplish this by acting cell-autonomously within specific V cells, or possibly by acting in other unidentified cells that, in turn, determine the response properties of the V cells.

The results of this study indicate that together *lin-22* and *pal-1* create a postdeirid prepattern within the lateral epidermis (Fig. 3). The V cells and their daughters appear morphologically similar to one another. However, because of *lin-22* and *pal-1*, they have different developmental potentials: only V5 is competent to be induced by extracellular signals to produce a postdeirid. As long as neighboring V cells are present, V5 will express its potential and produce a postdeirid neuroblast. Otherwise it will produce additional epidermal cells instead. It is not known how *lin-22* and *pal-1* activities are targeted to their respective domains of func-

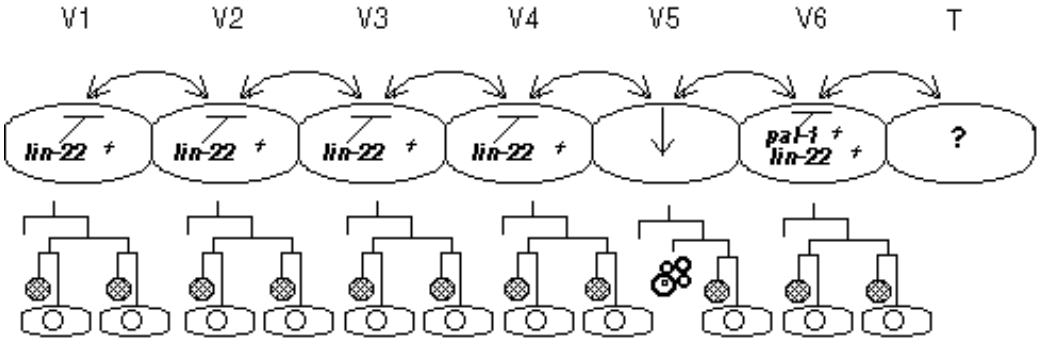

Fig. 3. Genetic model for establishment of the postdeirid pattern. In the model, *lin-22* and *pal-1* activity have the effect of preventing intercellular signals between V cells from inducing postdeirids. The products of these genes could block postdeirid production by acting directly on components of the signal transduction pathway, or they could bypass the effects of signal transduction and act directly on downstream genes. T generates a lineage distinct from any of the V cell lineages, which does not include a postdeirid.

tion, and how V5 is able to escape their effects. Whatever mechanism localizes *lin-22* and *pal-1* ultimately determines the spatial properties of the postdeirid neuroblast pattern.

Similar but distinct regulatory strategies are used in postdeirid and ray pattern formation

The postdeirid pattern is generated during the L2 stage. During the L3 stage in males, certain descendants of the V cells generate another pattern of neurons and epidermal structures called the ray/alae pattern. Rays, which are mating sensilla, are made in posterior body regions by V5 and V6. Alae, which are epidermal structures, are made in anterior body regions by anterior V cells. Intercellular signaling between V cells (or their descendants) is also required for ray pattern formation. Intercellular signals cause certain V cells to produce alae lineages instead of ray lineages. Previously we showed that the gene *pal-1* allows V6 to produce rays by preventing V6 from responding to alae-inducing signals from its neighbor T (Waring and Kenyon, 1991).

Because both the postdeirid and the ray/alae patterning systems involve communication between V cells, one can ask whether a single signal-transduction event initiates one subprogram that generates both the postdeirid and ray patterns. This seems unlikely because, whereas ablation of V cells themselves affects both patterns, ablation of the daughters of the V cells does not affect postdeirid patterning but does affect the ray decision (Fixsen, 1985). Thus, although the same type of signaling mechanisms may well operate in both patterning systems, the developmental decisions required for postdeirid and ray patterning are made independently of one another.

There are several differences between the postdeirid and ray pattern formation systems. One striking difference is that the regulatory relationship between *lin-22* and cell signals is opposite in the two cases. In postdeirid patterning, *lin-22*, like *pal-1*, prevents V cells from responding to signals from their neighbors. However, in ray/alae pattern formation, *lin-22* activity instead promotes the effects of intercellular signals by initiating the signal-dependent pathway, alae lineages. This suggests that *lin-22* may not act directly in a signalling system, but may rather act to bias the regulatory state of particular cells so as to determine the developmental consequences of those signals in particular body areas.

Another difference between the two patterning systems is that ablation experiments give the impression that signals involved in ray/alae pattern formation are unidirectional, as ablation of posterior but not anterior neighbors causes remaining cells to generate extra rays. This apparent unidirectionality may be the result of a strong anteroposterior polarity seen in ray/alae pattern formation. As discussed previously (Waring and Kenyon, 1990; Kenyon 1986), the data suggest the model that some type of graded positional information functions along with intercellular signaling between V cells to determine the pattern of alae and rays. According to this model, a cell must be in the posterior of the animal in order to generate rays. Following ablation of posterior neighbors the remaining cells are able to move into this posterior, ray-permissive region. This is not the case following anterior ablations. To test this model, we ablated anterior neighbors of V6, that is, V2-V5, in *pal-1* animals. In these experiments V6 responded by generating ray instead of alae lineages (6/6 animals; Waring and Kenyon, unpublished results). This indicates that V cells can receive signals from their anterior neighbors, and therefore that signaling is not unidirectional. The most likely explanation for why only V6 can produce ray lineages instead of alae lineages after ablation of anterior neighbors is because V6 is located in the posterior body region.

In spite of the differences between postdeirid and ray pattern formation, the experiments presented here indicate that there is a fundamental similarity in the role that cell signals play in the two processes. In both, intercellular signals are required to specify one pattern element; however, the signals themselves do not provide positional information. Instead a spatial pattern forms because cells differ intrinsically in their ability to respond to intercellular signals. This finding is significant because it indicates that this general patterning strategy - creating a specific arrangement of precursor cells which differ in their ability to respond to intercellular signals - can generate very different types of patterns within a single tissue.

Recent studies on the role of growth factors in *Xenopus* pattern formation indicate that inductive signals may play a permissive rather than instructive role in axial patterning in vertebrates. Dorsal and ventral regions of the ectoderm respond differently to the factor activin in culture, indicating that intercellular signals probably play a permissive role

in allowing the expression of a predetermined pattern in this organism as well (Sokol and Melton, 1991). It may be that the strategy of using intercellular signals to trigger expression of a prepattern is widespread in development.

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