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Two nested gonadal inductions of the vulva in nematodes

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SUMMARY

How do intercellular signals that pattern cell fates vary in evolution? During nematode vulva development, precursor cells acquire one of three fates in a pattern centered around the gonadal anchor cell. Non-vulval fates are at the periphery, outer and inner vulval fates are towards the center. In *Caenorhabditis elegans*, the three fates are specified around the same time by an induction by the anchor cell and lateral signaling between the vulva precursor cells. We find that, in three other nematode species (*Panagrolaimus, Oscheius* and *Rhabditella spp.*) spanning two families, the centered pattern is obtained by two temporally distinct gonadal inductions. The first induction specifies vulval fates; the second induction specifies the inner vulval fates in a subset of the precursors' daughters. This evolutionary change in the spatiotemporal connectivity of cell interactions allows centering of the pattern between two precursors in *Panagrolaimus*.

Key words: nematode, evolution, vulva, patterning, induction, gonad, anchor cell

INTRODUCTION

During development, cell fates are specified by multiple intercellular signals as well as by lineage history. How does this network of cell interactions vary during biological evolution? To address this question, nematode development offers reproducibility at the cellular level and the ability to compare development at a single cell level from species to species (Ambros and Fixsen, 1987; Sternberg and Horvitz, 1981, 1982). Also, cell interactions can be demonstrated after laser ablation of specific cells in different nematode species (Félix and Sternberg, 1996; Sommer and Sternberg, 1994, 1995; Sternberg and Horvitz, 1982). The cell interactions patterning the fates of the precursor cells of the vulva are well known in *Caenorhabditis elegans*. We thus study vulva patterning mechanisms in other nematode species to understand how developmental mechanisms vary in evolution. Such comparison can also shed light on the evolutionary origin of the mechanisms specific to the model organism *C. elegans*.

The precursors of the nematode vulva are the Pn.p cells (posterior descendants of the Pn cells). Of the twelve Pn.p cells aligned in the ventral cord in early *C. elegans* larvae (n=1 to 12 along the anteroposterior axis), six (P3.p to P8.p) are competent to form the vulva. During normal development, three of them (P5.p to P7.p) form the vulva (reviewed by Horvitz and Sternberg, 1991). The central cell, P6.p, acquires a specific, inner fate, characterized in particular by a TTTT lineage (transverse division of its four granddaughters; Fig. 1A), and the ability of its progeny to connect to the anchor cell (AC), a specialized uterine cell that links the gonad to the vulva and is located immediately dorsal to P6.p. The symmetric patterning of the Pn.p fates occurs via multiple cell interactions: a graded inductive signal from the anchor cell of the gonad (AC) to the vulva precursor cells and lateral signaling between the vulva precursor cells. Lateral signaling has been proposed to operate in either an inductive mode (only acting from P6.p to its neighbors P5.p and P7.p), or a comparative mode (acting more strongly from P6.p to its neighbors than vice-versa) (Katz et al., 1995; Koga and Ohshima, 1995; Simske and Kim, 1995; Sternberg and Horvitz, 1986). Induction by the AC and vulval precursor cell fate specification occur in *C. elegans* in early L3, before P(3-8).p divide (Kimble, 1981; M. Wang and P. W. S., unpublished observations).

We find a striking evolutionary change in how the symmetric centered pattern of vulval cell fates is determined. In other nematode species, the centered symmetry of the pattern is achieved by two successive, nested gonadal inductions of the vulva precursor cells and their daughters. This allows a spatial shift of centering of the pattern in the family Panagrolaimidae.

MATERIALS AND METHODS

Nematode strains

Strains are designated by their strain number in the Caltech collection kept by L. Carta. The hermaphroditic *Panagrolaimus sp. cf. hygrophilus* Bassen, 1940 (strain PS1732) was collected by J. DeModena near Iceberg Lake, California, in July 1994 and is kept at 20-25°C. *Oscheius sp.* (PS1131; closely related to *O. tipulae* Lam, 1971 (Sudhaus, 1993); genus is subject to change, L. Carta, K. Thomas, and P. W. S., unpublished data) was collected in Tokyo, Japan, in July 1991 by W. Wood. It is hermaphroditic. *Rhabditella axei* (DF5006) Cobbold, 1884 was isolated by W. Sudhaus and given to us by D. Fitch. It is gonochoristic. Both species are kept at 20°C.

Lineage and cell ablation

For strain culture, cell lineage and laser ablation, we used standard techniques described for *C. elegans* in Wood (1988). The vulva lineages were determined by continuous observation during the L3 molt and early L4.

RESULTS

Centering of the vulva pattern between P6.p and P7.p in the Panagrolaimidae

In all nematodes examined so far, vulval lineages exhibit centered symmetry (Ambros and Fixsen, 1987; Sommer and Sternberg, 1994, 1995, 1996; Sternberg and Horvitz, 1981, 1982). However, a major variation occurs in the family Panagrolaimidae (Sternberg and Horvitz, 1982): the vulva pattern is centered between P6.p and P7.p (not on P6.p like in *C. elegans*) (Figs 1C, 2). The anchor cell is positioned between P6.p and P7.p, and the inner vulval fate is shared between their two central daughters, P6.pp and P7.pa. The progeny of P8.p also participate in the vulva, which is thus formed from the progeny of four (instead of three) Pn.p cells. We find centering of the vulva between two precursors in several other families including the Cephalobidae and the Strongyloidae (R. J. Sommer, M.-A. Félix, P. W. Sternberg, unpublished observations). This centering also appears to be the case in the Tylenchidae (Hirschmann and Triantaphyllou, 1967; Roman and Hirschmann, 1969).

How is this distinct centering of the vulva pattern obtained? By ablating the gonad or the anchor cell in *Panagrolaimus sp.* PS1732 (Family Panagrolaimidae) at different times, we found two temporally distinct signals acting on the Pn.p cells (Table 1B,C). The first signal is produced by the gonad in the early second larval stage (L2) and induces the P(5-8).p cells to divide twice in the late L3 stage and form vulval tissue. The gonad in the early L2 stage in this species comprises the two somatic precursor cells, Z1 and Z4 (before they divide to give rise to the AC), and the two germ-line precursors, Z2 and Z3. The result of the ablation in early L1 is the same whether the two somatic cells are ablated alone or with the two germ-line precursors (data not shown). Either Z1 or Z4 is sufficient to induce the two first rounds of vulva precursor cell divisions (Félix and Sternberg, 1996). The second inductive signal originates from the AC in late L3 and induces the central daughters of the Pn.p cells (P6.pp and P7.pa) to divide a third round ('TT' inner fate vs. 'UU' outer fate). This delayed induction of the vulval inner fate on the daughters of the Pn.p cells allows the change in centering of the pattern in between two Pn.p cells.

Which cells are competent to respond to each inductive signal? In this species (PS1732), as in *Panagrolaimus sp.* PS1159 (Sommer and Sternberg, 1996), P(1-4).p die in the L1 stage (P4.p survives in about 1/3 of the animals in PS1732). After ablation of P(5-8).p, P(9-11).p did not form vulval tissue (12/12 animals) and P4.p did not in the 4 animals in which it survived. Therefore only P(5-8).p are competent to respond to the first induction. Every one of their daughters appears competent to respond to the second induction (Table 1D) (although P8.pp appears less likely to do it). P6.pa and P6.pp can both adopt the 'TT' fate when isolated at the time of, or after, the division of P6.p, indicating that their fate has not been irreversibly determined earlier in P6.p (Table 1C,E). Specification of the inner versus outer fate thus does not occur in the

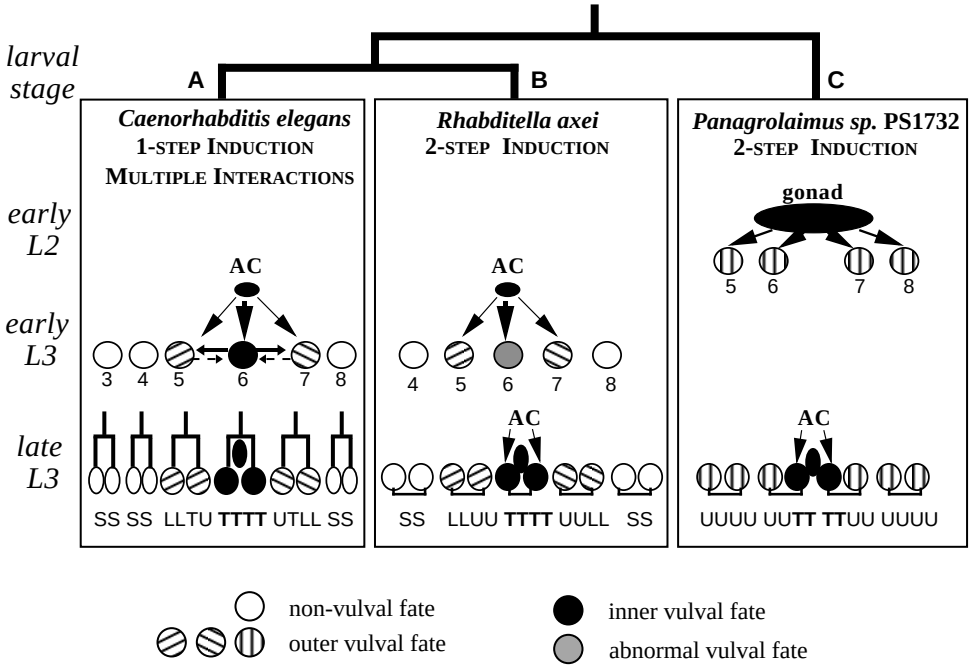

Fig. 1. Evolutionary variations in mechanisms that pattern the vulva precursor cells. The ventral epidermal cells competent to form the vulva are, from anterior to posterior: P(3-8).p in *C. elegans*, P(4-8).p in *R. axei* and P(5-8).p in *Panagrolaimus sp.* PS1732. Their lineages in the late third larval stage (L3) are indicated at the bottom. In *C. elegans* (A), the outermost cells adopt a non-vulval fate (white), P5.p and P7.p adopt an outer vulval fate (hatched) and P6.p adopts an inner vulval fate (black). Patterning of the three fates is accomplished by multiple cell interactions occurring at approximately the same time in the early L3 stage (arrows). Whether P5.p and P7.p normally signal to P6.p in *C. elegans* is not clear. In *R. axei* (B), the first induction is insufficient to completely specify the central fate (grey): the AC is required later to send a signal to the daughters of P6.p (black). We do not know whether lateral interactions between vulval precursor cells participate in specifying their fates in *Rhabditella*. In *Panagrolaimus sp.* PS1732 (C), the pattern is centered between P6.p and P7.p. The gonad sends a first signal in early L2, which induces P(5-8).p to adopt a vulval fate (hatched). P5.p and P8.p could be induced in *Panagrolaimus* directly or through signaling by P6.p and P7.p. In any case, an even number of cells is reached by the inductive signal of the gonad at the time of the first induction. The AC then induces P6.pp and P7.pa in late L3 to adopt the central fate (black). The phylogenetic relationships between these three species are indicated above. Nomenclature follows Sternberg and Horvitz (1986), and Sulston and Horvitz (1977). S, a daughter of a Pn.p cell that fuses with the epidermal syncytium (non-vulval fate); L, longitudinal (anteroposterior) division of a granddaughter; T, transverse (left-right) division; U, no division. We do not distinguish here between N and U. Polarity of the outer vulval lineages is indicated by the direction of hatching. No polarity is visible in the outer vulval lineage in *Panagrolaimus sp.* PS1732 (or in *Oscheius sp.* PS1131).

Table 1. Two-step induction of the vulva by the gonad and the anchor cell in *Panagrolaimus sp.* PS1732

	Cell(s) ablated	Time of ablation		Descendants of P5.p	P6.p	P7.p	P8.p	Invag. in L4	No. of animals
A	–	–	lineage:	UUUU	UU**TT**	**TT**UU	UUUU	+	
			number:	4	6	6	4		
B1	gonad	early L1		1	1	1	1	–	15/15
B2	gonad	L1 lethargus		1	1	1	1	–	8/11
				4	1	1	1	–	1/11
				1	1	4	1	–	1/11
				1	1	1	4	–	1/11
B3	gonad	early L2		4	4	4	4	–	4/10
				4	4	4	3	–	2/10
				4	4	1	4	–	2/10
				3	2	2	1	–	1/10
				1	1	1	1	–	1/10
B4	gonad	late L2		4	4	4	4	–	11/12
				1	4	4	4	–	1/12
C1	AC	mid-L3 (DU and/or VU dividing)		UUUU	UUUU	UUUU	UUUU	–	7/11
				UUUU	UUUU	UUUU	UUUU	+/–	3/11
				UUUU	UUU**O**	UUUU	UUUU	+/–	1/11
C2	AC	Pn.p dividing or early 2-cell stage		UUUU	UUUU	UUUU	UUUU	–	7/9
				UUUU	UUUU	UUUU	UUUU	+/–	1/9
				UUUU	UUUU	**T**UUU	UUUU	+/–	1/9
C3	AC	Pn.p late 2-cell stage		UUUU	UU**TT**	UUUU	UUUU	+/–	3/8
				UUUU	UUU**O**	**T**UUU	UUUU	+	1/8
				UUUU	UUU**T**	**TT**UU	UUUU	+	1/8
				UUUU	UU**TT**	**T**UUU	UUUU	+	1/8
				UUUU	UU**OT**	**TT**UU	UUUU	+	1/8
				UUUU	UU**TT**	**TT**UU	UUUU	+	1/8
C4	AC	Pn.px dividing		UUUU	UU**TT**	**TT**UU	UUUU	+	7/8
				UUUU	UU**TO**	**OT**UU	UUUU	+	1/8
D1	P6.p + P7.p + P8.p (P5.p isolation)	L2		**TTTT**	x	x	x	+	5/7
				UU**D**U	x	x	x	+	2/7
D2	P5.p + P7.p + P8.p (P6.p isolation)	L2		x	**TTTT**	x	x	+	5/9
				x	**DDD**U	x	x	+	2/9
				x	U**DD**U	x	x	+	2/9
D3	P5.p + P6.p + P8.p (P7.p isolation)	L2		x	x	**TTTT**	x	+	5/9
				x	x	U**DDD**	x	+	1/9
				x	x	UU**DD**	x	+	1/9
				x	x	**DDD**U	x	+	1/9
				x	x	**DD**UU	x	+	1/9
D4	P5.p + P6.p + P7.p (P8.p isolation)	L2		x	x	x	**DD**UU	+	8/11
				x	x	x	**DDD**U	+	1/11
				x	x	x	**DDDD**	+	1/11
				x	x	x	**TTTT**	+	1/11
E	P5.px+ P7.px + P8.px (P6.px isolation)	Pn.p dividing or early 2-cell stage		x x	**TTTT**	x x	x x	+	2/9
				x x	**OOTT**	x x	x x	+	1/9
				x x	**DDTO**	x x	x x	+	1/9
				x x	UU**TT**	x x	x x	+	4/9
				x x	UU**TL**	x x	x x	+	1/9

The wild-type vulva lineage of this species is indicated at the top: P(5-8).p each divide twice and their granddaughters either do not divide ('U') or divide once more transversely ('T', bold). To score the two first round of divisions, descendants of P(5-8).p are counted in L4 (numbers). To score the third round of division, lineages are determined by continuous observation at the L3 molt and early L4 (letters) (except for the Pn.p isolations that were scored in early L4). 'Pn.p' designates P5.p, P6.p, P7.p and P8.p; 'Pn.px' their daughters. DU and VU are the dorsal and ventral uterine precursors, respectively (Kimble and Hirsh, 1979). 'Early L2' is before the gonad divisions. 'Late L2' is during the gonad divisions. 'Early' and 'late' 2-cell stage are before or after the AC extends and breaks the basement membrane separating it from the vulva precursor cells, and correspond to Fig. 2C and D, respectively. Pn.p isolations were performed both early and late in L2. Nomenclature follows Sternberg and Horvitz (1986) and Sulston and Horvitz (1977). U, no division; T, transverse division; O, oblique division; L, longitudinal division; D, divided, orientation unknown; 'x', ablated cell. When P(5-8).p divide twice only ('UUUU'), no deep invagination forms: the cells detach slightly from the cuticle in late L4 and small bumps are visible on the adult cuticle. In the Pn.p isolations, the anchor cell contacted the cells that divided a third round. For example, when the isolated Pn.p adopted a UUDD lineage, the invagination formed was asymmetric, with the AC lying dorsally above the DD cells. When the isolated Pn.p adopted a TTTT lineage, the invagination was symmetric, with the AC lying dorsally in the center. Thus there is a correlation between AC contact with vulva precursors and induction in these cells of the inner vulval fate.

Pn.p cells like in *C. elegans*, but in their daughters. When a single Pn.p cell is near the AC, it can generate a lineage like that generated by *C. elegans* P6.p (Table 1D,E).

Two-step vulva induction in the Rhabditidae

In the genus *Oscheius* (Family Rhabditidae, like *C. elegans*), the vulva is centered, as in *C. elegans*, on P6.p. The lineages of P5.p and P7.p are simple: they undergo only two rounds of mitosis and, in contrast to P4.p and P8.p, their progeny participate in the vulval invagination (Sommer and Sternberg, 1995). As in *Panagrolaimus*, we observe two successive inductions in *Oscheius sp.* PS1131. In *Oscheius*, however, both are by the anchor cell. The first induction occurs in early L3 and restricts the vulval fates to P(5-7).p. The second induction occurs in late L3 and induces the inner fate in P6.pa and P6.pp (Table 2A, Fig. 2I). P(4-8).p are each competent to respond to the first induction (Sommer and Sternberg, 1995). All daughters of P5.p and/or P7.p are competent to respond to the second induction after ablation of P6.p in the mid-L3 stage (Table 2H).

In *Rhabditella axei* (Family Rhabditidae, phylogenetically closer to *Oscheius* than to *Caenorhabditis*; Sudhaus, 1976), after AC ablation in mid-L3, P5.p and P7.p adopt their correct fate and lineage, whereas P6.p exhibits abnormal vulval fates (Table 3). We tested whether vulva precursor cells were more sensitive in this species than in *C. elegans* to the ablation conditions by ablating a ventral uterine cell close to the AC. This control ablation had no effect on the vulva lineages (4/4 animals). We interpret these P6.p lineages as outer cell lineages with defective anteroposterior polarity, or as incompletely specified inner fates. Induction of P(5-7).p by the AC is sufficient to specify the outer fates, whereas later induction of P6.pa and P6.pp by the AC is required for the correct specification or execution of the inner fate. Thus, in contrast to *C. elegans*, the AC is still necessary for correct vulval cell lineages after the divisions of the Pn.p cells.

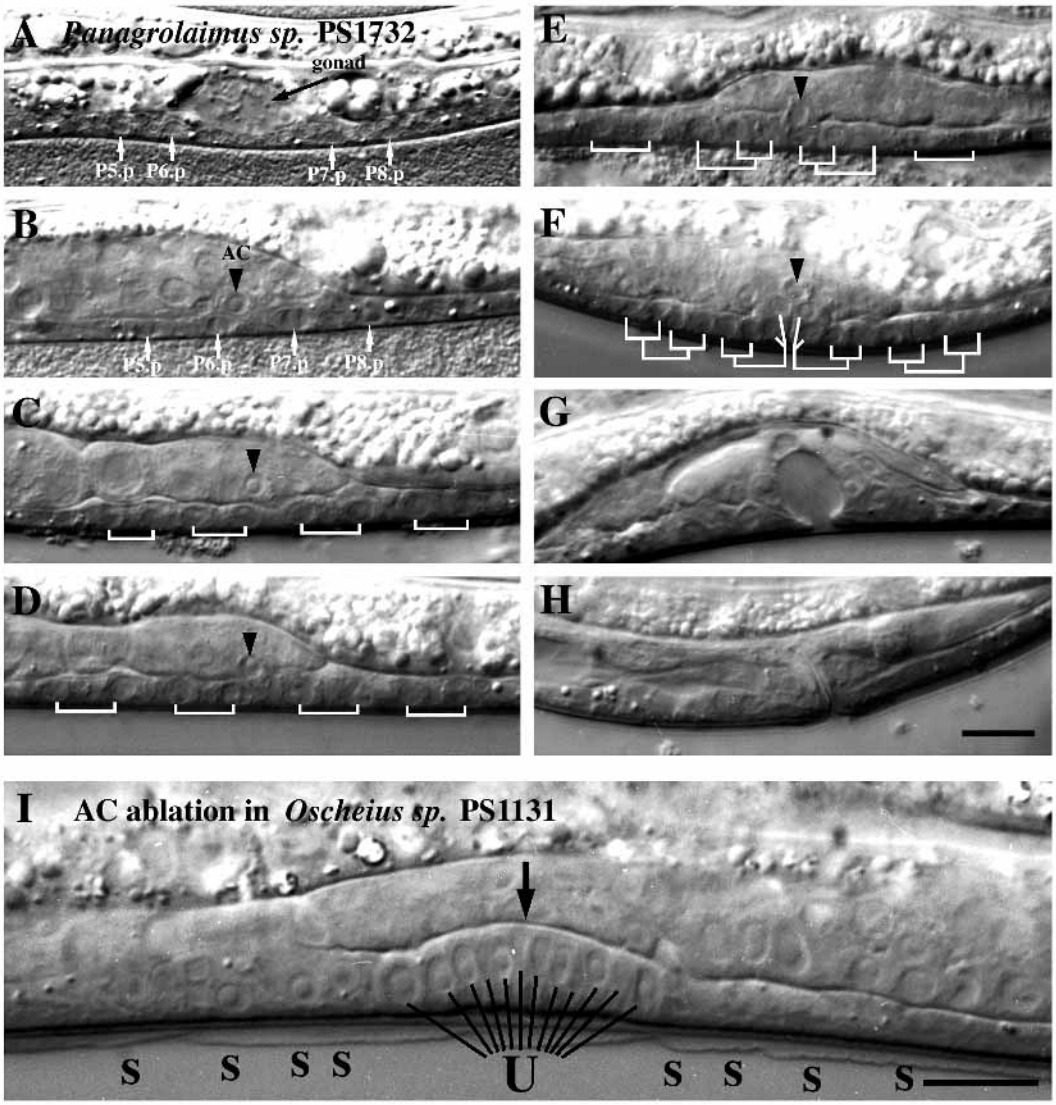

Fig. 2. Vulva development in *Panagrolaimus sp.* PS1732 (A-H) and absence of the inner vulval fate after AC ablation in *Oscheius sp.* PS1131 (I). In *Panagrolaimus sp.* PS1732, in L1 lethargus (A), P(5-8).p are symmetrically positioned ventral to the gonad primordium. The first induction occurs in this configuration. In the early L3 stage (B), the anchor cell (AC, arrowhead) is slightly posterior to P6.p. In mid-late L3 (C), P(5-8).p divided ('early 2-cell stage'). (D) Slightly later, the AC extends between P6.pp and P7.pa and closely apposes them (the basement membrane visible as a black line is discontinuous at this point; 'late 2-cell stage'). The second induction occurs at this time. (E) P6.pp and P7.pa divided (before the outer cells). (F) Their daughters start invaginating. They then divide transversely at the L3 to L4 molt (not shown). (G) In mid-L4, a large vulval invagination forms; the uterus lumen is visible dorsally (the former position of the AC also appears as hollow). (H) In the young adult, the vulva appears as a slit that connects the uterus to the outside. (I) The anchor cell was ablated in *Oscheius sp.* PS1131 in mid-L3, before induction of the central fate in the daughters of P6.p: at the molt to L4, all P(5-7).p granddaughters adopted a vulval fate ('U'), did not fuse with the syncytium and detached from the cuticle. The P6.p granddaughters did not connect to the uterus (the arrow indicates the intact basement membrane, compare with intact animals in Fig. 3B,C in Sommer and Sternberg, 1995) and did not divide further. Nomarski micrographs. Lateral views: dorsal is to the top, anterior to the left (except in E where anterior is to the right). Bar, 10 μm. Same scale in A-H.

Table 2. Two-step induction of the vulva by the anchor cell in *Oscheius sp.* PS1131

	Cell(s) ablated	Time of ablation	Descendants of P4.p	P5.p	P6.p	P7.p	P8.p	No. of animals
A	–	–	ssss	UUUU	**TTTT**	UUUU	ssss	
B	gonad	early L1	ssss	ssss	ssss	ssss	ssss	5/5*
C	AC	early L3	ssss	ssss	ssss	ssss	ssss	7/10
			ssss	UUUU	UUUU	ssss	ssss	1/10
			ssss	ssUU	UUUU	UUUU	ssss	1/10
			ssss	UUUU	UUUU	UUUU	ssss	1/10
D	AC	mid L3	ssss	UUUU	UUUU	UUUU	ssss	8/8
E	AC	P(5-7).p dividing	ssss	UUUU	UUUU	UUUU	ssss	8/9
			ssss	UUUU	UUUU	UUUU	S S	1/9
F	AC	P(5-7).p 2-cell stage	ssss	UUUU	**T**UU**T**	UUUU	ssss	5/9
			ssss	UUUU	**TTTT**	UUUU	ssss	2/9
			ssss	UUUU	UUU**T**	UUUU	ssss	1/9
			ssss	UUUU	**TT**U**T**	UUUU	ssss	1/9
G	AC	P(5-7).px dividing	ssss	UUUU	**TTTT**	UUUU	ssss	7/7
H	P6.p	mid L3	ssss	U**TTT**	x	UUUU	ssss	2/13
			ssUU	**TTTT**	x	UUUU	ssss	2/13
			ssss	UU**DD**	x	**O**UUU	ssss	1/13
			UUUU	UU**TT**	x	**TTTT**	UUss	1/13
			UUUU	**TTTT**	x	UUUU	ssss	1/13
			ssss	**LOLD**	x	UUUU	ssss	1/13
			ssss	U**TTO**	x	UUUU	ssss	1/13
			ssss	U**DTT**	x	UUUU	ssss	1/13
			ssss	UU**TT**	x	UUUU	ssss	1/13
			ssUU	UUUU	x	**TTTT**	UUss	1/13
			ssss	UUUU	x	**TTO**U	ssss	1/13

In *Oscheius sp.* PS1131, P(4-8).p all first divide twice. Their granddaughters either do not divide and fuse to the epidermal syncytium (non-vulval fate: 's'), or do not divide but participate in the vulval ('U'), or divide once more transversely ('T') (Sommer and Sternberg, 1995). This third round of division is indicated in bold. *From Sommer and Sternberg (1995). 'Early L3' is before the dorsal uterine precursors DU divide. 'Mid L3' is during or after the first division of the dorsal or ventral uterine precursors DU and VU, and before the first division of P(4-8).p. In the P6.p ablations, the anchor cell contacted the cells that divided a third round. Nomenclature is as in Table 1. s denotes a granddaughter (S, a daughter) of a Pn.p cell that fuses with the epidermal syncytium (non-vulval fate).

Table 3. Two-step induction of the vulva by the anchor cell in *Rhabditella axei*

	Cell(s) ablated	Time of ablation	Descendants of P4.p	P5.p	P6.p	P7.p	P8.p	No. of animals
A	–	–	S S	LLUU	TTTT	UULL	S S	
B	gonad	early L1	S S	S S	S S	S S	S S	5/5*
C	AC	early L3	S S	ss S	S S	S S	S S	1/5
			S S	ssss	S S	S S	S S	1/5
			S S	LD S	S S	ss S	S S	1/5
			S S	LLUU	ss S	S Ls	S S	1/5
			S S	LLLU	S S	OLLs	S S	1/5
D	AC	mid L3	S S	LLUU	UUUU	UULL	S S	2/7†
			S S	LLLU	UUUU	UULL	S S	1/7‡
			S S	LLOU	UUUL	UDLL	S S	1/7
			S S	LLUU	OUUL	UULL	S S	1/7
			S S	LLUU	DDUU	UULL	S S	1/7
			S S	LLUU	LLUL	UULL	S S	1/7
E	AC	P(5-7).p dividing or 2-cell stage	S S	LLUU	UUUU	UULL	S S	2/6
			S S	LLUU	TUUT	UULL	S S	2/6
			S S	LLUU	UTTT	UULL	S S	1/6
			S S	LLUU	OTUL	UULL	S S	1/6
F	AC	P(5-7).p early 4-cell stage	S S	LLUU	TTTT	UULL	S S	5/6
			LLUU	TTTT	UULL	S S	S S	1/6

In *Rhabditella axei*, P4.p and P8.p divide only once. The outer granddaughters of P5.p and P7.p undergo a third, longitudinal round of division. The four granddaughters of P6.p undergo a transverse division (Sommer and Sternberg, 1995). The cells that remain adherent to the cuticle in mid to late L4 are underlined. *From Sommer and Sternberg (1995); †in one of these animals, P3.p divided as does P4.p (it does not divide in most animals; Sommer and Sternberg, 1995); ‡adherence to the cuticle was not scored in this animal. 'Early L3' and 'mid L3' are as defined in Table 2. Nomenclature is as in Table 1.

DISCUSSION

A major variation in developmental mechanism was previously found in nematode species with a posterior vulva, such as *Mesorhabditis sp.* PS1179, which use mechanisms other than inductive signaling from the anchor cell to specify vulval cell fates (Sommer and Sternberg, 1994). However, the source of patterning information in these species is unknown. Here we demonstrate a unique set of cell interactions used to pattern vulval fates in other nematode species (Fig. 1). Whereas patterning occurs through multiple interactions acting on the Pn.p cells in *C. elegans*, it is achieved in *Panagrolaimus sp.* PS1732 by two successive nested gonadal inductions (i) on the Pn.p cells (specifying vulval versus non-vulval fates) and (ii) on their daughters (specifying inner versus outer vulval fates). In *C. elegans*, after the Pn.p cell is specified, the 1° inner vulval lineage is predominantly specified autonomously. There thus has been a transition between autonomously and non-autonomously specified lineages for the inner vulval cells. In *Oscheius sp.* PS1131 and *Rhabditella axei* (same family as *C. elegans*), the lineage of P6.p is not specified autonomously: it also requires a later induction by the AC.

This 2-step induction mechanism would work without the reinforcement provided by lateral signaling between the vulva precursor cells, especially in species such as *Panagrolaimus sp.* PS1732 in which all the cells competent to respond to the first inductive signal actually do respond: there is no need for spatially precise signal production. Yet we have no evidence against a role for lateral signaling and interactions between the Pn.p cells may contribute patterning information, particularly in *Rhabditella* in which P5.p and P7.p adopt a fate distinct from P6.p when the AC is ablated in mid-L3.

We speculate that the 2-step patterning mechanism is ancestral to the '1-step/many-interactions' mechanism found in *C. elegans*, and that centering of the vulva on P6.p in *Oscheius* and *Rhabditella* is an intermediate. A weak argument in favor of this hypothesis is that this mechanism has been found in distinct nematode families. In this view, the graded action of the anchor cell signal in *C. elegans* could derive from the temporal inductive patterning mechanism that we have described here. Specifically, the first induction might correspond to the intermediate level of inductive signal that promotes 2° fates in *C. elegans*; the second induction would then correspond to the high level of inductive signal that promotes the 1° fate in *C. elegans*.

Irrespective of the direction of evolution, these results demonstrate an extreme variation of developmental mechanism at the level of the spatiotemporal connectivity of cell interactions, that nonetheless results in the same centered pattern of cell fates. This evolutionary variation has however two consequences.

Firstly, heterochrony in the time of induction of vulval (vs. non-vulval) fates allows the change in number of precursor cells participating to vulva formation (four or three). In *Panagrolaimus sp.* PS1732, the first induction occurs when P(5-8).p are positioned symmetrically anterior and posterior to the gonad primordium (Fig. 2A); consequently, an even number of cells are induced (four). In *C. elegans*, mechanisms operate to lock one of the Pn.p cells (normally P6.p) in a position directly ventral to the AC at the time of the induction (K. Tietze and P. W. S., unpublished observations), and an uneven number of cells (three) are induced. The timing and source of the signal can thus specify the number of precursor cells (four or three) forming the vulva.

Secondly, because the inner (vs. outer) vulval fates are specified in the Pn.p daughters, the vulva pattern can be centered in between P6.p and P7.p in the Panagrolaimidae. The sharing of the inner fate between two Pn.p cells in the Panagrolaimidae occurs because the inner 'TT' fate is specified in their daughters, when the AC elongates and closely apposes two of them (normally P6.pp and P7.pa; Fig. 2D). Apparent tight contact of the AC to the vulva precursor cells occurs at the 2-cell stage in the three species considered here, but only later, at the 4-cell stage in *C. elegans* (K. Tietze and P. W. S., unpublished observations). Reproducible centering of the second induction between P6.pp and P7.pa might involve a specific mechanism of alignment of the AC in between two non-sister cells. Altogether, this allows a slight anteroposterior shift of the pattern between the two families Panagrolaimidae and Rhabditidae and contributes to changes in vulval position.

At the molecular level, the two successive inductions might be mediated by distinct signaling pathways, or by two phases of action of one signaling pathway. In *C. elegans* (reviewed in Aroian and Sternberg, 1993; Horvitz and Sternberg, 1991), the AC induction is mediated by the LIN-3 ligand produced by the AC and the LET-23 receptor on the vulva precursor cells. LIN-3 is encoded as a transmembrane protein that is presumably processed and cleaved off the plasma membrane of the anchor cell (Hill and Sternberg, 1992). Lateral signaling between the vulva precursor cells is mediated by the LIN-12 receptor, the known ligands of which are membrane-bound. In species with a 2-step induction mechanism, the first induction presumably can act without cell-cell contact between the anchor cell and the induced vulva precursor cells: the ligand, as LIN-3 in *C. elegans*, presumably traverses the extracellular matrix. This first inductive signal would thus likely be via a non-membrane-bound LIN-12 ligand, or a non-membrane bound form of a LIN-3 homolog. The second induction occurs when the anchor cell is closely apposed to the induced cells; this signal could be membrane-bound, consistent with either a LIN-12 ligand or a membrane-bound form of LIN-3.

This transition between 1-step and 2-step vulval induction now provides a model to analyze at a molecular level how the connectivity and temporal execution of intercellular signaling evolves. Genetics provides one way to begin a mechanistic analysis: as for *Pristionchus pacificus* (Sommer and Sternberg, 1996), we have found that *Oscheius sp.* is amenable to genetic studies (M.-A. F. and P. W. S., unpublished data).

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