Evolutionary diversification of specification mechanisms within the O/P equivalence group of the leech genus *Helobdella*

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Summary

Developmental fates and cell lineage patterns are highly conserved in the teloblast lineages that give rise to the segmental ectoderm of clitellate annelids. But previous studies have shown that the pathways involved in specification of the ventrolateral O lineage and the dorsolateral P lineage differ to some degree in distantly related clitellate species such as the leeches *Helobdella* and *Theromyzon*, and the sludgeworm *Tubifex*. To examine this developmental variation at a lower taxonomic level, we have explored the specification pathways of the O and P lineages in the leech genus *Helobdella*. In leech, the O and P lineages arise from a developmental equivalence group of O/P teloblasts. In this study, we demonstrate that the cell-cell interactions involved in cell fate specification of the O/P equivalence group differ among three laboratory colonies of closely related species. In two populations, the Q lineage is necessary to specify the P fate in the dorsalmost O/P lineage, but in the third population the P fate can be specified by a redundant pathway involving the M lineage. We also observe interspecific variation in the role played by cell interactions within the O/P equivalence group, and in the apparent significance of extrinsic signals from the micromere cell lineages. Our data suggest that cell fate specification in the O/P equivalence group is a complex process that involves multiple cell-cell interactions, and that the developmental architecture of the O/P equivalence group has undergone evolutionary diversification in closely related species, despite maintaining a conserved morphology.

Key words: Leech, *Helobdella*, O/P equivalence group, Cell-cell interaction, Evolution of a developmental mechanism

Introduction

Recent comparative studies in a variety of animal species have revealed that the developmental pathways underlying homologous morphological patterns can show considerable variation, a phenomenon known as 'developmental system drift' (True and Haag, 2001). For example, variation has been found in nematode vulval patterning mechanisms (reviewed by Sommer, 2001), in which drift has led to a diversity of developmental mechanisms without obvious changes in the final morphology (Sommer, 1997). The vulval precursor cells (VPCs) of the nematode are a classical example of an equivalence group, i.e. a set of equipotent cells that choose between two or more potential fates based on inductive signals from external sources and/or interactions within the group (Greenwald and Rubin, 1992). In the case of the *C. elegans* VPCs, both an inductive signal from the anchor cell and lateral inhibition between VPCs are involved in fate specification of VPCs (Kornfeld, 1997). One might suspect that developmental system drift is a common feature in the evolution of developmental equivalence groups, and to address this question it is necessary to explore the degree of developmental variation manifested by other morphologically conserved equivalence groups.

The O/P lineages in the segmental ectoderm of glossiphoniid leeches also form an equivalence group (Weisblat and Blair, 1984; Zackson, 1984; Huang and Weisblat, 1996; Keleher and Stent, 1990). The segmented ectoderm and mesoderm of the leech are derived from five bilateral pairs of stem cells called teloblasts. There are four pairs of ectodermal teloblasts (N, O/P, O/P and Q) and one pair of mesodermal teloblasts (M), each of which gives rise to a distinct set of differentiated descendants. Through repeated asymmetric cell divisions, each teloblast produces a bandlet, i.e. a string of primary blast cells that are segmental founder cells for their respective teloblast lineage. Bandlets arising from the five ipsilateral teloblasts come together in parallel to form the germinal band, with the four ectodermal bandlets arranged along the dorsoventral axis such that the n bandlet is the ventralmost and the q bandlet is the dorsalmost (Fig. 1). The m (mesodermal) bandlet underlies the four ectodermal bandlets.

The N, Q and M teloblasts can be easily identified based on their cell lineage history and position in the embryo. However, the two remaining ectoteloblasts are named O/P to reflect their developmental equipotency (Weisblat and Blair, 1984). The fate of each o/p bandlet is specified by the position it adopts within the germinal band (Weisblat and Blair, 1984; Shankland and Weisblat, 1984; Huang and Weisblat, 1996). The more ventral o/p bandlet, which is normally in contact with the n bandlet, adopts the O fate; and the more dorsal o/p bandlet,

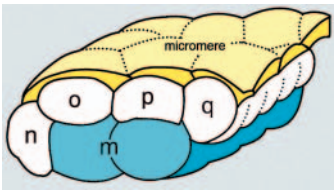

Fig. 1. Cell lineage arrangement in the germinal band of a stage 7/8 leech embryo. Each of the five teloblasts produces a column or 'bandlet' of primary blast cells, shown here in cross-section. The four ectodermal bandlets (white) lie in parallel above a single mesodermal (m) bandlet (cyan), and are overlaid by a provisional integument composed of micromere-derived epithelial cells (yellow). The n and q bandlets are located respectively on the future ventral and dorsal sides of the germinal band. The o and p bandlets are initially equipotent, and are committed to distinct O and P developmental pathways during normal development on the basis of positional cues encountered in the germinal band (Shankland and Weisblat, 1984; Huang and Weisblat, 1996).

which is normally in contact with the q bandlet, adopts a distinct P fate.

Three different cell interactions have been proposed to govern cell fate specification of the O/P lineage. In experiments in which the P lineage is ablated, the presumptive (i.e. positionally defined) O lineage 'transfates' into the P fate. By contrast, the presumptive P lineage does not transfate following the ablation of the O lineage (Weisblat and Blair, 1984; Shankland and Weisblat, 1984; Zackson, 1984). This finding was initially interpreted as implying that the P lineage suppresses the P fate and/or induces the O fate in the O lineage. However, later experiments also revealed an O fate-inducing signal from the micromere-derived provisional integument that covers the germinal band (Ho and Weisblat, 1987), and a P fate-inducing signal from the Q lineage (Huang and Weisblat, 1996). To accommodate these results, Huang and Weisblat (Huang and Weisblat, 1996) have proposed a model in which the 'interaction' between the O/P pair is purely steric, i.e. the presence of the p bandlet prevents the o bandlet from physically contacting, and being induced to the P fate by, the q bandlet.

One potential source of confusion is that these data were obtained from three different *Helobdella* species: *H. triserialis* (Weisblat and Blair, 1984; Shankland and Weisblat, 1984; Zackson, 1984; Ho and Weisblat, 1987), *H. stagnalis* (Zackson, 1984) and *H. robusta* (Huang and Weisblat, 1996). The authors assumed that the developmental mechanisms underlying O/P specification should be very similar in these species given their nearly identical patterns of embryonic cell lineage and cell fate. However, these morphological traits are also conserved in some more distantly related clitellate species in which the O and P lineages behave differently in response to certain experimental paradigms (Goto et al., 1999; Keleher and Stent, 1990). In addition, a variation in developmental cell fates between *Helobdella* species has been reported for the cell lineage arising from one of the embryonic micromeres (Huang et al., 2002). It is possible that the O/P specification mechanism also varies between different *Helobdella* species, and in the present study we provide evidence that this is the case.

In this study, the cell-cell interactions involved in O/P specification are experimentally characterized in three

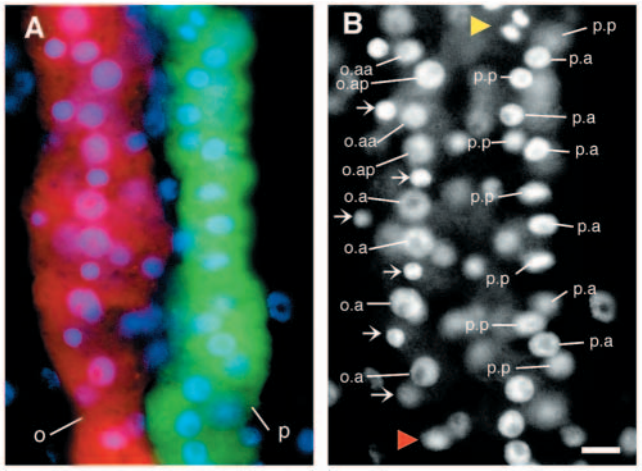

Fig. 2. Normal cleavage pattern of the O/P lineage. (A) Image of the o (red) and p (green) bandlets in the germinal band of a stage 8 embryo. Ventral is towards the left, and anterior towards the top. Nuclei are stained with Hoechst 33258, and appear blue. (B) Hoechst staining shows the shape and location of the nucleus of each individual cell. One o primary blast cell is undergoing mitosis (red arrowhead), and the more anterior (i.e. older) o blast cell clones consist of the larger o.a daughter cell and the smaller o.p daughter cell (arrows). Further anterior, an o.a cell has divided into two cells, o.aa and o.ap. The most posterior cells in the p bandlet are primary blast cells. Anterior to the p primary blast cells, the older p blast cell clones consist of paired daughter cells p.a and p.p. A p.a cell undergoing mitosis is labeled with a yellow arrowhead. Scale bar: 10 μm.

laboratory populations belonging to the leech genus *Helobdella*. As noted above, it was previously shown that the Q lineage plays a central role in O/P specification in a population of *H. robusta* originating from Sacramento, CA (Huang and Weisblat, 1996). Here, we show that there is a significant difference in the response of the O/P lineages to the ablation of the Q lineages in a second leech population from Austin, TX [also described as *H. robusta* (see below)] in which another signaling pathway functions as a redundant component in the O/P specification mechanism. In addition, we find no evidence that the micromere-derived provisional integument influences O/P fate specification in *H. robusta* (Austin), in contrast to a previous report from *H. triserialis* (Ho and Weisblat, 1987). These data suggest that the cell fate specification mechanism of the O/P equivalence group, similar to that of the nematode VPCs, has undergone divergence in closely related populations, while retaining a high degree of evolutionary conservation in cell lineage and terminal morphology.

Materials and methods

Animals

The *Helobdella* leeches used in this study were taken from three separate laboratory breeding colonies with distinct geographical origins. *H. robusta* was originally described (Shankland et al., 1992) with specimens originating from Sacramento, CA. A second population of *H. robusta* was later reported in Austin, TX (Seaver and Shankland, 2000). However, although these two populations are very similar in morphology and development, they have been found to display substantial divergence in partial DNA sequence of the

mitochondrial gene cytochrome c oxidase I (A. Bely, personal communication). The status of *H. robusta* (Austin) and *H. robusta* (Sacramento) as distinct species or geographical variants of the same species requires further evaluation, but in this paper we will refer to the Austin leeches as *H. robusta* (Austin) in order to conform with the published literature (Seaver and Shankland, 2000; Seaver and Shankland, 2001; Kuo and Shankland, 2004).

A morphologically similar but distinguishable leech population originating from Galt, CA, has been previously described as *Helobdella* sp. (Galt) (Huang et al., 2002). Molecular data suggest that these leeches form an outgroup to the two *H. robusta* populations (A. Bely, personal communication).

Embryo culture and staging are as described by Kuo and Shankland (Kuo and Shankland, 2004).

Injection of cell lineage tracer and ablation of teloblasts

Cell lineage tracer labeling was carried out by pressure injecting the OP proteloblast or an O/P teloblast with a 1:1 mixture of 100 mg/ml tetramethylrhodamine dextran (lysine fixable) or fluorescein dextran (lysine fixable) (Molecular Probes, Eugene, OR) and 4% Fast Green (Sigma) in 200 mM KCl. Teloblast ablation was carried out by pressure injection into the target teloblast of a 1:3:4 mixture of 0.83 mg/ml ricin A chain (Sigma), 100 mg/ml fluorescein dextran and 4% Fast Green (Sigma) in 200 mM KCl. Operated embryos were raised separately in 24-well culture plates and were fixed at stage 7/8 or late stage 9 with a 1:1 mixture of 8% formaldehyde (Pella) and HEPES-buffered saline (50 mM HEPES, 150 mM NaCl, pH 7.4) and counterstained with 2.5 μg/ml Hoechst 33258 at 4°C overnight. Stage 7/8 embryos were used for examination of blast cell cleavage patterns. The size and spatial pattern of the blast cells were determined from their nuclei as revealed by Hoechst 33258 staining (Zackson, 1984). To examine the differentiated pattern elements that arose from labeled O/P lineages, stage 9 embryos were dissected and mounted on slides in buffered glycerol and viewed by fluorescence microscopy.

Ablation of the micromere-derived provisional integument

The micromere-derived provisional integument was ablated by a lineage-specific photo-oxidation technique (Shankland, 1984). This procedure was modified from Ho and Weisblat (Ho and Weisblat, 1987) to accommodate differences in developmental timing of the different leech species. First, the target micromeres (opq″ and n′) were injected with fluorescein dextran at stage 6a. At late stage 7/early stage 8, an intense 485 nm light beam was focused on the labeled cells through the 40× water immersion objective of a compound microscope to selectively kill the fluorescein-containing descendants of the injected micromeres. In these same experiments, a 1:1 mixture of 100 mg/ml biotin dextran (lysine fixable) (Molecular Probes, Eugene, OR) and 4% Fast Green (Sigma) in 200 mM KCl was pressure injected into the O/P teloblasts as a lineage tracer. Roughly 24-30 hours after the irradiation procedure, the embryos were fixed, counterstained and washed as described above. To visualize biotin dextran lineage tracer, fixed embryos were incubated in a saline buffer containing 0.8% Triton X-100 and 5 μg/ml avidin-rhodamine complex (Vector) at room temperature for 2 hours and then washed extensively.

Results

Normal development of the O/P equivalence group

The cleavage pattern of the blast cell clone in stage 8 embryos and the set of differentiated pattern elements produced by the blast cell in stage 9 embryos (i.e. its definitive fate) were used here to ascertain whether a given o/p blast cell clone had followed the O or the P developmental pathway. During normal development, all of the primary blast cells in a given o/p bandlet show the same cleavage pattern (Zackson, 1984) and generate very similar clones of differentiated descendants located in different segments (Shankland, 1987b; Shankland, 1987c). The O- or P-type cleavage pattern of a blast cell clone usually predicts its definitive fate (Zackson, 1984); however, this is not true under all experimental conditions (Shankland, 1987a) (see below).

The blast cell clones in a given bandlet exhibit an anteroposterior gradient of clonal age that reflects their order of birth. The older (anterior) blast cell clones exhibit cleavage patterns of more advanced stages, while younger (posterior) blast cell clones exhibit cleavage patterns at earlier stages. The first divisions of the primary o and p blast cells

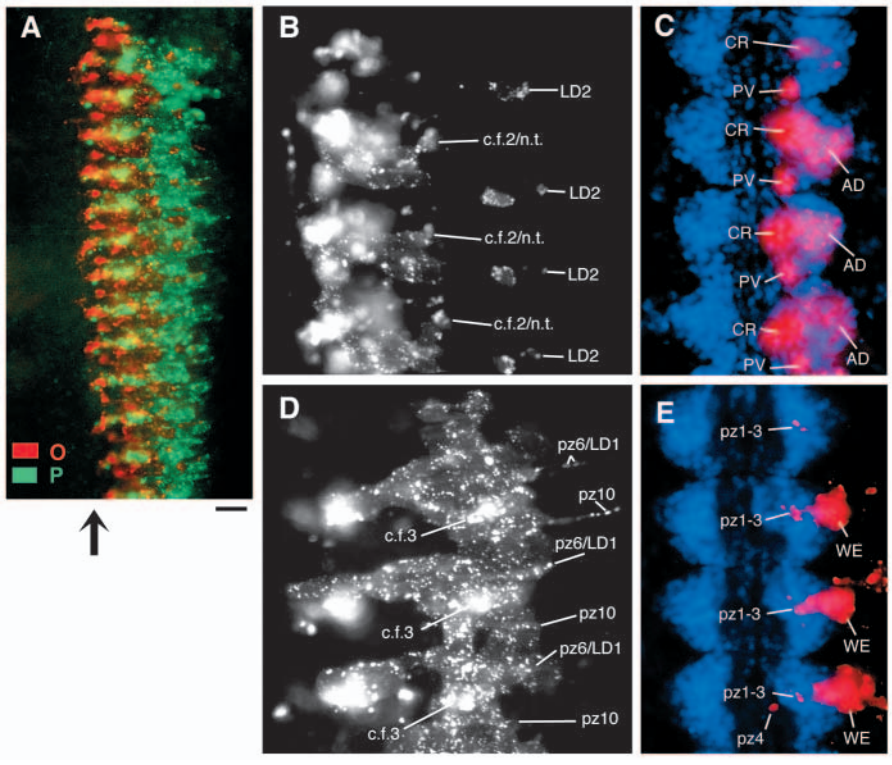

Fig. 3. Differentiated pattern elements arising from the O and P lineages in stage 9 embryos. (A) The descendants of the O lineage (red) are generally located more ventrally than are the descendants of the P lineage (green). The arrow indicates the ventral midline. For labeled tissues, ventral is towards the left and anterior towards the top. (B,C) Pattern elements derived from the O lineage. (D,E) Pattern elements derived from the P lineage. The more superficial pattern elements of the body wall are shown in B and D. Pattern elements in the ganglia of the ventral nerve cord are shown in C and E, in which Hoechst 33258 counterstaining (blue) was used to visualize the segmental ganglia. CR, crescent neuron cluster; AD, anterodorsal neuron cluster; PV, posteroventral neuron cluster; WE, wedge-shaped neuron cluster; c.f.3, cell floret 3; n.t., nephridial tubule. pz6, pz10, LD1 and LD2 are identified peripheral neurons. Scale bar: 30 μm in A; 15 μm in B,E.

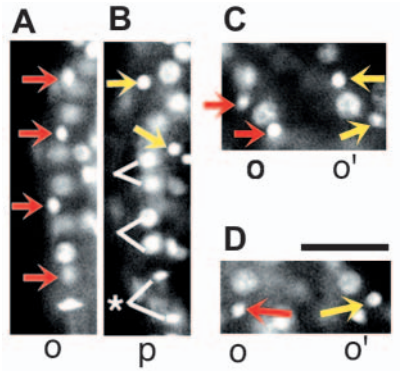

Fig. 4. Effects of the bilateral ablation of the Q lineage on the cleavage patterns of o/p blast cell clones in stage 8 embryos. (A,B) Cleavage pattern of the presumptive o bandlet (A) and the presumptive p bandlet (B) of *H. robusta* (Austin) shown by Hoechst 33258 nuclear staining. Presumptive o blast cell clones express the normal O-type cleavage pattern. The o.p nuclei are marked with red arrows. Most presumptive p blast cell clones express a normal P-type cleavage pattern, and white lines mark the p.a and p.p daughter cell pairs. A few presumptive p blast cell clones express an O-type cleavage pattern (yellow arrows). (C,D) Ablation of bilateral Q lineages causes both o/p bandlets to express the O-type cleavage pattern in *H. robusta* (Sacramento) (C) and *Helobdella* sp. (Galt) (D). The small nuclei of o.p cells in the presumptive o bandlet are labeled with red arrows, and the nuclei of ectopic o.p cells in the presumptive p bandlet are labeled with yellow arrows. See Fig. 2 for orientation. Scale bar: 20 μm.

are both asymmetric, but the degree of asymmetry is significantly greater in the o bandlet (Shankland, 1987b; Shankland, 1987c) (Fig. 2A,B). The subsequent blast cell divisions also exhibit lineage-specific patterns. These cleavage patterns are indistinguishable in the populations examined here.

At stage 9, each O or P lineage gives rise to a definitive set of differentiated pattern elements that consists of parts of the ventral nerve cord, peripheral nervous system and epidermis (Fig. 3). In general, the O lineage contributes primarily to the ventral nerve cord and ventrolateral epidermis and gives rise to a few peripheral neurons, whereas the P lineage contributes more cells to the peripheral nervous system and the dorsolateral epidermis, and fewer cells in the ventral nerve cord. The definitive fates of the O and P lineages appear to be widely conserved among clitellate annelids (see Kramer and Weisblat, 1985; Torrence and Weisblat, 1986; Goto et al., 1999), and are indistinguishable in the three populations examined here.

Variation in response to bilateral ablation of the Q lineage

It has previously been demonstrated that the q bandlet is responsible for inducing P fate in the dorsal O/P lineage of *H. robusta* (Sacramento). When right and left Q teloblasts were ablated, all four O/P lineages (two in the right germinal band and two in the left germinal band) followed the O developmental pathway as evidenced by both cleavage pattern and definitive fate (Huang and Weisblat, 1996). To determine whether O/P specification mechanisms are conserved, we here examined the effect of Q lineage ablation on the development of the O/P lineages in embryos of two other populations.

In a unilateral Q teloblast ablation experiment, transient contact with the q bandlet of the contralateral germinal band is sufficient to specify o/p blast cells to the P fate in *H. robusta* (Sacramento) (Huang and Weisblat, 1996). As described below, we obtained the same result from unilateral Q teloblast ablation experiments in *H. robusta* (Austin). To eliminate completely the influence of the Q lineage, the two Q teloblasts were ablated bilaterally in the following experiments unless otherwise specified.

H. robusta (Austin)

After bilateral ablation of the Q teloblasts, the two ipsilateral O/P teloblasts were injected with fluorescent dextran lineage tracer. In one set of experiments, the cleavage pattern of blast cell clones in 52 pairs of o/p bandlets was scored at stage 8 (Fig. 4A,B; Table 1, row C). In 51 o/p pairs, the blast cell clones in the presumptive o bandlet consistently displayed an O-type cleavage pattern. In 32 out of these 51 pairs, blast cell clones in the presumptive p bandlet displayed a P-type cleavage pattern. But in the other 19 pairs, the majority of individual blast cell clones in the presumptive p bandlet expressed a P-type cleavage pattern, but some presumptive p blast cell clones expressed an O-type cleavage pattern. In one atypical embryo, it appeared that a physical inversion of the o/p bandlets had taken place at the posterior end of the germinal band, and in this case each bandlet maintained the cleavage pattern characteristic of its original position (data not shown). These results are not consistent with the previous observations from *H. robusta* (Sacramento) (Huang and Weisblat, 1996).

The variable cleavage pattern observed in some presumptive p bandlets appeared to correlate with variations in the structure of the germinal band. In those segments in which presumptive p blast cells adopted an O-type cleavage pattern, it was found that the m bandlet was located more superficially, effectively compressing the apicobasal axis of the overlying o/p blast cells and reducing the contact area between the presumptive o and p bandlets (Fig. 5).

In a second set of experiments, we scored the differentiated pattern elements derived from O/P lineages following bilateral ablation of the Q teloblasts. To do so, each of the two ipsilateral O/P teloblasts was injected with a different fluorescent lineage tracer. In contrast to the complex cleavage pattern expressed by the presumptive P lineage in the stage 8 embryos described above, the presumptive o bandlet gave rise to O pattern elements and the p bandlet consistently gave rise to P pattern elements in all stage 9 embryos examined (n=25) (Fig. 6A-D; Table 2, row C). These age-related differences do not appear to be a result of sampling error, as the frequency at which the P fate was manifested in embryos of these two ages differs significantly (χ^2=14.84, P=0.0001). This discrepancy implies that presumptive p blast cells, which exhibit an O-type cleavage pattern following Q lineage ablation can nonetheless go on to produce an essentially normal P-type pattern of differentiated descendants. A comparable result has been previously reported by Shankland (Shankland, 1987a) for *H. triserialis* embryos in which the presumptive o blast cells were deprived of their p blast cell neighbors following a brief period of contact. These findings add support to the idea (Shankland and Weisblat, 1984) that the O/P specification mechanism involves multiple steps.

In sum, the results obtained here for *H. robusta* (Austin)

differ from those reported in *H. robusta* (Sacramento) (Huang and Weisblat, 1996). This suggests that different mechanisms may be involved in O/P specification of these two populations. The results of bilateral Q teloblast ablation in *H. robusta* (Austin) could be taken to imply that the Q lineage has little or no P-inducing capability. However, additional experiments indicate that the Q lineage is able to induce the P fate in the Austin population (see below), suggesting that the persistence of the P fate in Q-ablated embryos is due to a redundant and previously undescribed mechanism of specification.

Table 1. Variation in the effects of combinatorial ablation experiments on the stage 7/8 cleavage pattern of the presumptive O and P lineages in *Helobdella*

			Presumptive O lineage				Presumptive P lineage			
Row	Ablated cell lineage(s)	*n*	O*	P†	O/P‡	?§	O*	P†	O/P‡	?§
	H. robusta (Austin)									
A	No ablation	10	10	0	0	0	0	10	0	0
B	Q_r	12	12	0	0	0	0	12	0	0
C	Q_{l+r} ¶	51	51	0	0	0	0	32	19	0
D	M_{l+r}	40	39	1	0	0	0	40	0	0
E	M_r	14	14	0	0	0	0	14	0	0
F	N_{l+r}	10	10	0	0	0	0	10	0	0
G	N_r	12	12	0	0	0	0	12	0	0
H	Q_{l+r}, N_{l+r}	20	5	2	0	13	3	4	0	13
I	Q_{l+r}, N_r	11	11	0	0	0	0	4	7	0
J	Q_{l+r}, M_{l+r}	12	12	0	0	0	12	0	0	0
K	Q_{l+r}, M_r	12	12	0	0	0	12	0	0	0
L	Micromere	57	57	0	0	0	0	57	0	0
M	Q_{l+r}, micromere	37	37	0	0	0	0	19	18	0
N	$2O/P_l$, $1O/P_r$**	12	0	12	0	0	N/A	N/A	N/A	N/A
O	Q_{l+r}, $2O/P_l$, $1O/P_r$**	57	57	0	0	0	N/A	N/A	N/A	N/A
P	Q_r, $1O/P_r$	15	0	15	0	0	N/A	N/A	N/A	N/A
Q	Q_r, M_r	10	10	0	0	0	0	10	0	0
R	Q_r, M_r, $1O/P_r$**	10	0	10	0	0	N/A	N/A	N/A	N/A
S	Q_r, M_r, N_r, $1O/P_r$**	9	0	9	0	0	N/A	N/A	N/A	N/A
	H. robusta (Sacramento)									
T	Q_{l+r}	10	10	0	0	0	10	0	0	0
	Helobdella sp. (Galt)									
U	Q_{l+r}	24	24	0	0	0	24	0	0	0

The o/p bandlet(s) in the right germinal band were scored in experiments listed above, except for E-H,J, in which the data of left and right O/P lineages were pooled.

*All blast cell clones in the entire bandlet express the O-type cleavage pattern.

†All blast cell clones in the entire bandlet express the P-type cleavage pattern.

‡Some blast cell clones in a bandlet express O-type cleavage pattern, and the other blast cell clones in the same bandlet express P-type bandlet.

§The blast cell clones in a bandlet express unrecognizable cleavage pattern.

¶One morphologically abnormal O/P pair, in which a bandlet inversion takes place, is excluded from this data set (see text).

**For the convenience of presentation here, the surviving O/P lineage is listed under the category of the presumptive O lineage, but in fact the presumptive identity of this surviving O/P lineage cannot be defined here because the presumptive identity is defined by the relative position of the bandlets arising from the two ipsilateral O/P lineages.

Table 2. Variation in the effects of combinatorial ablation experiments on the stage 9 differentiated pattern elements of the presumptive O and P lineages in *Helobdella*

			Presumptive O lineage			Presumptive P lineage		
Row	Ablated cell lineage(s)	*n*	O*	P†	O/P‡	O*	P†	O/P‡
	H. robusta (Austin)							
A	No ablation	4	4	0	0	0	4	0
B	Q_r	18	18	0	0	0	18	0
C	Q_{l+r}	25	25	0	0	0	25	0
D	$2O/P_l$, $1O/P_r$§	15	0	15	0	N/A	N/A	N/A
E	Q_{l+r}, $2O/P_l$, $1O/P_r$§	8	5	0	3	N/A	N/A	N/A
F	Q_r, $1O/P_r$§	10	0	10	0	N/A	N/A	N/A
	H. robusta (Sacramento)							
G	Q_{l+r}	17	17	0	0	17	0	0

O/P lineages on the right side were labeled and scored at stage 9.

*O pattern elements were found in every segment.

†P pattern elements were found in every segment.

‡Segmental mosaicism of O and P pattern elements.

§For the convenience of presentation here, the surviving O/P lineage is listed under the category of the presumptive O lineage, but in fact the presumptive identity of this surviving O/P lineage cannot be defined here because the presumptive identity is defined by the relative position of the bandlets arising from the two ipsilateral O/P lineages.

H. robusta (Sacramento)

To ensure that the apparent difference in the response of the O/P lineages to bilateral ablation of the Q lineages is not an artifact produced by some deviation in the experimental procedure, we likewise performed bilateral ablation of the Q teloblasts in embryos of *H. robusta* (Sacramento). Following bilateral ablation of the Q lineages, the O/P lineages were labeled with fluorescent lineage tracer. Under these conditions, we found that the cleavage pattern of all o/p bandlets was uniformly O-type in the Sacramento population (n=10) (Fig.

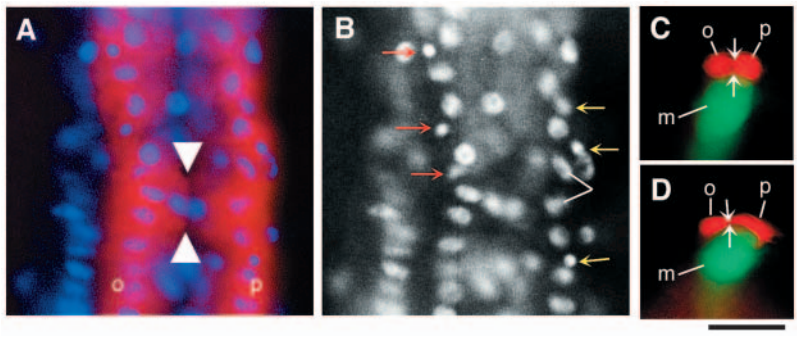

Fig. 5. Following ablation of the Q lineages in *H. robusta* (Austin), the cleavage pattern of presumptive p blast cell clones is correlated to the contact area between the o/p bandlets. (A,B) The presumptive o and p bandlets shown here were labeled with injected rhodamine cell lineage tracer and Hoechst 33258 nuclear counterstaining. The two fluorescent signals are superimposed in A, and nuclear staining alone is shown in B. At the region where the presumptive p bandlets are in better contact with the presumptive o bandlet (between the white arrowheads in A), a presumptive p blast cell expresses the P-type cleavage pattern (white lines). In regions in which the contact area of the presumptive o and p bandlets is reduced, the presumptive p blast cell clones express an O-type cleavage pattern. See Fig. 4 for labeling of cells, and Fig. 2 for orientation. (C,D) Optical cross-sections of the germinal band shows the relative position of the m bandlet (green) and the o/p bandlets (red) in the area where the presumptive p bandlet expresses a P-type cleavage pattern (C) and in the area where the presumptive p bandlet expresses an O-type cleavage pattern (D). The contacting surface between the two o/p bandlets is marked with white arrows. Scale bar: 40 μm in A,B; 80 μm in C,D.

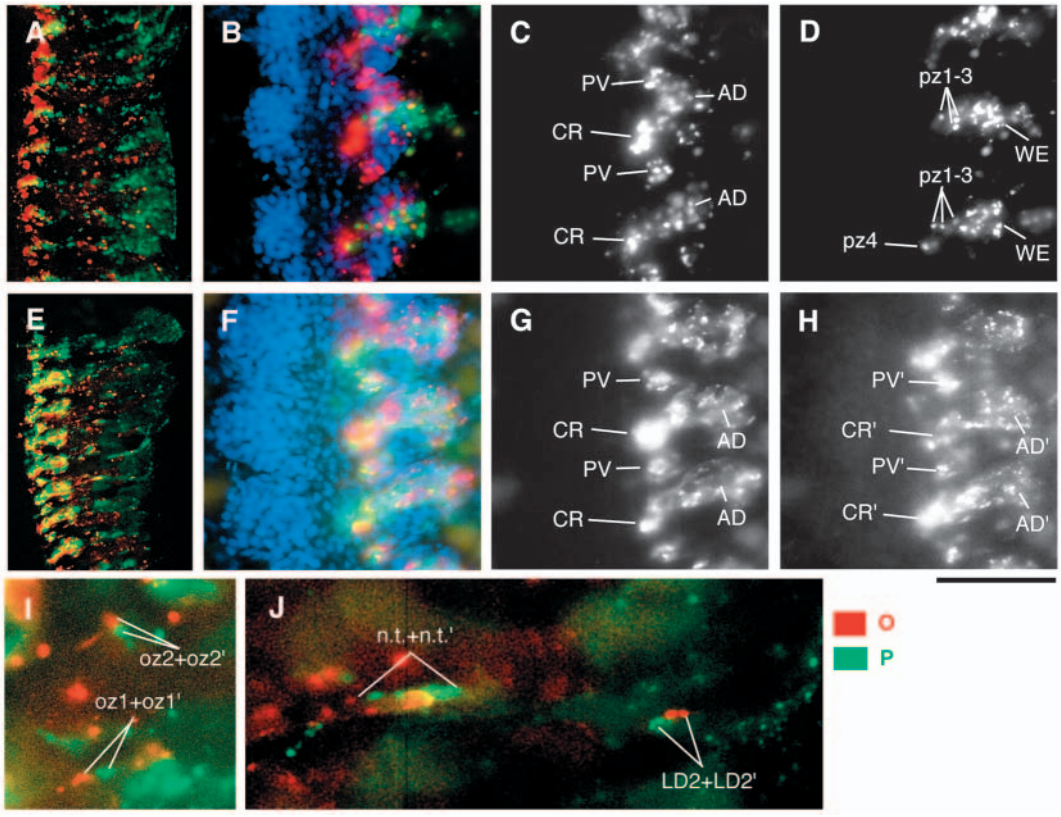

Fig. 6. The differentiated pattern elements arising from the O/P lineages following bilateral ablation of the Q lineage differ in *H. robusta* (Austin) (A-D) and *H. robusta* (Sacramento) (E-J). (A,E) The presumptive O and P lineages give rise to their normally distinct sets of pattern elements in *H. robusta* (Austin) (A), but give rise to nearly identical sets of O pattern elements in *H. robusta* (Sacramento) (E). In both laboratory populations, the presumptive O lineage (red) and the presumptive P lineage (green) are fluorescently labeled. (B,F) Superimposed images of presumptive o bandlet descendants (red), presumptive p bandlet descendants (green), and nuclear staining (blue) in the ventral nerve cord of *H. robusta* (Austin) (B) and *H. robusta*, Sacramento (F). (C,G) The presumptive O lineage gives rise to O pattern elements in both laboratory populations. (D,H) The presumptive P lineage gives rise to normal P pattern elements in *H. robusta* (Austin) (D), but to O pattern elements in *H. robusta* (Sacramento) (H). (I,J) Following Q lineage ablation in *H. robusta* (Sacramento), O pattern elements that are represented by single cells in normal embryos are present as duplicate cells. Rhodamine- and fluorescein-labeling of these duplicate pattern elements reflects their derivation from the two different ipsilateral O/P teloblasts. See Fig. 3 for labeling of pattern elements and orientation. Scale bar: 200 μm in A-E; 50 μm in B-D,F-H; 33 μm I,J.

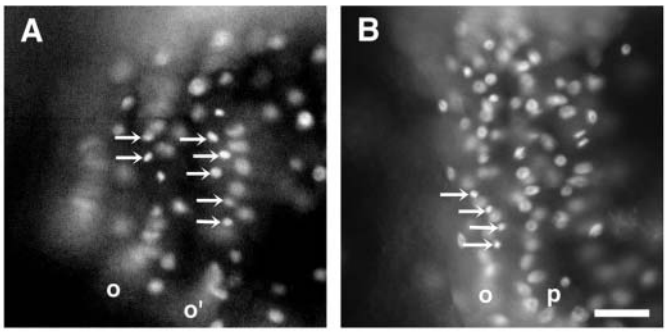

Fig. 7. Mesoderm is required in the Q lineage-independent O/P specification pathway in *H. robusta* (Austin). (A) Ipsilateral o/p bandlets in stage 8 embryo in which both the M and Q lineages were ablated. Both bandlets exhibit the O-type cleavage pattern, with the small o.p nuclei marked by arrows. The presumptive p bandlet has transfated to the O-type cleavage pattern, and is labeled o′.
(B) Ipsilateral o/p bandlets in stage 8 embryo in which only the M lineage was ablated. The presumptive o bandlet expresses the O-type cleavage pattern, while the presumptive p bandlet expresses the P-type cleavage pattern. See Fig. 2 for orientation. Scale bar: 30 μm.

4C; Table 1, row T). In stage 9 embryos, we found that the labeled O/P lineage produced only O pattern elements, and that P pattern elements were completely missing (n=17) (Table 2, row G). In many cases, we could detect duplicated O pattern elements that were labeled with the two different fluorophores and hence arose independently from the two ipsilateral O/P teloblasts (Fig. 6E-J). These findings confirm that both ipsilateral O/P lineages adopt the O fate when the Q lineage is absent in *H. robusta* (Sacramento) (Huang and Weisblat, 1996), and verify that the disparate results obtained with *H. robusta* (Austin) are not a procedural artifact.

Helobdella sp. (Galt)

To explore further the diversity of O/P specification mechanism in the genus *Helobdella*, bilateral ablation of the Q teloblasts was also performed in the more distantly related *Helobdella* sp. (Galt). The O/P lineages were labeled with fluorescent lineage tracer, and the cleavage pattern of the o/p blast cell clones examined in stage 8 embryos. It was found that the o/p blast cell clones in the operated embryos uniformly express an O-type cleavage pattern (n=24) (Fig. 4D; Table 1, row U). The response of the O/P lineage to bilateral ablation of the Q lineages in *Helobdella* sp. (Galt) is therefore largely the same as that of *H. robusta* (Sacramento), which suggests that similar mechanisms may be responsible for O/P specification in these two species.

O/P specification mechanism of *H. robusta* (Austin): mesoderm is involved in O/P specification

The observation that the dorsal o/p bandlet, but not the ventral o/p bandlet, of *H. robusta* (Austin) can adopt a partial or complete P fate in the absence of the Q lineages implies the existence of a redundant inductive signal that may emanate from some other cell population. To identify the source of this redundant signal, we examined the effects of ablating either the M teloblast or the N teloblast, whose descendant lineages are adjacent to the o/p bandlets in a normal germinal band and thus are prime candidates to influence O/P specification.

Ablation of the M or N lineage alone does not produce any consistent alteration in the O/P lineage of *H. robusta* (Austin), consistent with previous observations from *H. robusta* (Sacramento) (Huang and Weisblat, 1996). In experiments in which the M lineage or the N lineage was individually ablated, we saw no visible effect on the O/P cleavage pattern except in one case (Table 1, rows D-G). In this particular embryo, the M lineages of which were bilaterally ablated, both the presumptive o and p bandlets were in contact with the q bandlet at the posterior end of the germinal band, and both displayed a P-type cleavage pattern. The same atypical phenomenon was also reported by Huang and Weisblat (Huang and Weisblat, 1996), and was used to support the notion that an interaction with the Q lineage alone can induce P fate in the O/P lineage.

In order to detect whether the N or M lineages play any role in the Q lineage-independent pathway, either the N or the M teloblast was ablated in combination with the two Q teloblasts. Following ablation of the right N teloblast and two Q teloblasts, the cleavage patterns of the presumptive O and P lineage does not appear to be significantly different from the cleavage pattern seen in embryos whose Q lineages were ablated alone (χ^2=3.28, P=0.070) (Table 1, row H). However, when both N and Q teloblasts were ablated bilaterally, the cleavage pattern of the O/P lineage became highly variable (Table 1, row I). In these embryos, the o/p bandlets frequently failed to enter the germinal band, and were often found in the peripheral region of the micromere cap (data not shown). We believe these variable results are a non-specific effect due to disruption in the relative position of the o/p bandlets with respect to the germinal band as a whole.

By contrast, combinatorial ablation of the M and Q teloblasts had a profound and reproducible effect on the cleavage pattern of the o/p blast cell clones. When either ipsilateral or bilateral M lineage(s) were ablated in combination with bilateral ablation of the Q lineages, blast cell clones in both the presumptive o and presumptive p bandlet exhibited uniformly O-type cleavage patterns on the mesoderm-deficient side (Fig. 7; Table 1, rows J,K). The frequency at which P-type cleavage was expressed in these experiments was significantly different from that seen when the Q lineages were ablated alone (χ^2=24.00, P<0.0001). In *H. robusta* (Sacramento), combinatorial ablation of the Q and M lineages produces the same effect (Huang and Weisblat, 1996).

Thus, while the Q lineage appears to be the sole inducer of the P fate in *H. robusta* (Sacramento), our results indicate that the M lineage and the Q lineage are redundantly involved in the specification of the P fate in *H. robusta* (Austin).

Ablation of the micromere-derived provisional integument does not influence O/P specification of *H. robusta* (Austin)

In addition to the m, n, and q bandlets, the o/p bandlets also contact an overlying provisional integument arising from the micromere lineages. An involvement of this provisional integument in O/P specification was previously demonstrated in *H. triserialis*, in which ablation of the n′ and opq″ micromere clones causes the presumptive O lineage to adopt various aspects of the P fate (Ho and Weisblat, 1987). To determine whether these same micromere lineages are involved in O/P specification of *H. robusta* (Austin), we ablated the provisional integument descending from the n′ and opq″ micromeres at embryonic stage 8 and examined the cleavage

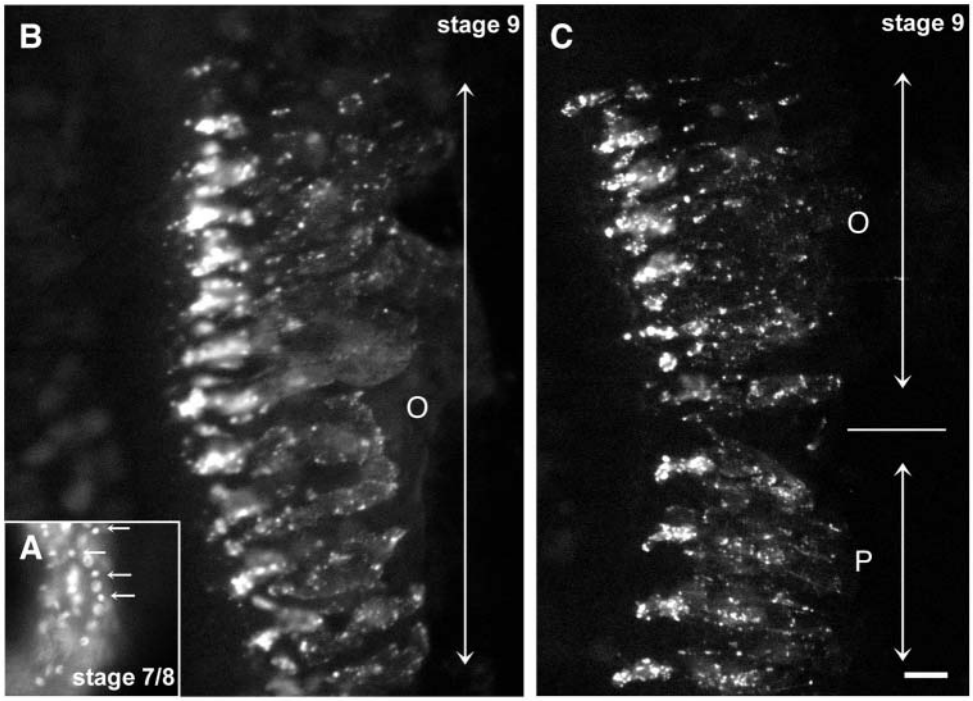

Fig. 8. Development of a 'lone' O/P lineage following ablation of the Q lineages in *H. robusta* (Austin). (A) In early stage 8, the blast cell clones in the 'lone' o/p bandlet express the O-type cleavage pattern. The small o.p nuclei are marked with arrows. Orientation of this panel is the same as Fig. 2. (B,C) Differentiated pattern elements derived from a 'lone' O/P lineage in stage 9 embryos. In some embryos the 'lone' O/P lineage gives rise to O pattern elements in every segment (B); in other embryos it gives rise to a combination of O pattern elements in more anterior segments and P pattern elements in more posterior segments (C). See Fig. 3 for orientation. Scale bar: 30 μm.

pattern of the presumptive o and p bandlets. Unlike *H. triserialis*, the o and p bandlets maintained their normal cleavage pattern following this ablation in *H. robusta* (Austin) (Table 1, row L).

When these two micromere clones were ablated together with the two Q teloblasts in embryos of *H. robusta* (Austin), the presumptive p bandlets exhibited a segmentally mosaic cleavage pattern that did not significantly differ in the rate of P fate specification from embryos in which only the two Q teloblasts were ablated (χ^2=2.05, P=0.1517) (Table 1, row M). These data suggest that ablation of the provisional integument does not have any significant effect on O/P specification in *H. robusta* (Austin).

Q lineage is involved in specification of the P fate in *H. robusta* (Austin)

In the mesoderm ablation experiments described above, the presumptive p bandlet consistently expressed its normal P-type cleavage pattern unless the Q lineages were also ablated. This result suggests that the Q lineage may be able to specify P fate in *H. robusta* (Austin), even though other experiments show that it is not required for P fate specification because of the existence of a redundant Q lineage-independent pathway. To further elucidate the significance of the Q lineage in O/P specification, we performed experiments in which all but one of the four O/P teloblasts were ablated, and the development of the remaining 'lone' O/P lineage was compared in the presence or absence of the Q lineage.

In the presence of the Q lineages, a 'lone' O/P lineage uniformly expressed a P-type cleavage pattern at stage 7/8 (n=12) and gave rise consistently to P pattern elements at stage 9 (n=15) (Table 1, row N; Table 2, row D). This result is similar to that obtained in comparable experiments on *H. triserialis* and *H. stagnalis* (Weisblat and Blair, 1984; Zackson, 1984).

But in the absence of the Q lineages, the cleavage pattern of blast cell clones in a 'lone' o/p bandlet was exclusively O-type (n=57) (Fig. 8A; Table 1, row O). The pattern of differentiated descendants produced at stage 9 by these o/p bandlets was more complicated (Table 2, row E). In five out of eight embryos, the 'lone' o/p bandlet gave rise to O pattern elements exclusively (Fig. 8B). In the remaining three embryos, the 'lone' o/p bandlet gave rise to O pattern elements in some segments and P pattern elements in other segments (Fig. 8C). Here, again, there was a statistically significant inconsistency between cleavage pattern of the blast cell clones examined in stage 8 embryos and the differentiated pattern elements observed in stage 9 embryos (χ^2=34.2, P<0.0001).

Despite this variability both the cleavage pattern and the differentiated pattern elements expressed by the lone surviving O/P lineage are strikingly different in the presence and absence of the Q lineage, and clearly demonstrate that the Q lineage can contribute to the specification of P fate in *H. robusta* (Austin). However, the persistence of some P-type differentiated pattern elements in these experiments suggests that definitive P fate can also be specified by factors other than the interaction between the O/P lineages and the inductive properties of the Q lineage. These additional factors could include signals from the M lineage, as suggested by the experiments above. However, we could not test this hypothesis via cell ablation as it is not feasible to reliably score O and P differentiated fates in stage 9 embryos in which the M teloblast has been ablated.

Transient interaction with the contralateral q bandlet is sufficient for P fate specification in *H. robusta* (Austin)

Huang and Weisblat (Huang and Weisblat, 1996) have shown that a brief contact with the contralateral q bandlet is sufficient to specify P fate in the O/P lineages of *H. robusta* (Sacramento). To determine whether a brief interaction with the contralateral Q lineage is also sufficient to specify P fate in *H. robusta* (Austin), we ablated one of the right O/P teloblasts and the right Q teloblast, and labeled the surviving right O/P teloblast with lineage tracer. Consistent with the results from *H. robusta* (Sacramento), the labeled O/P lineage

consistently expressed the P-type cleavage pattern in stage-7/8 embryos (n=15) and gave rise to P pattern elements in stage 9 embryos (n=10) (Table 1, row P; Table 2, row F).

Given the presence of a second M lineage-dependent pathway for P fate specification in *H. robusta* (Austin), we considered the possibility that the ipsilateral M lineage might be augmenting the P fate-inducing properties of the contralateral Q lineage in this experiment. To test this possibility, we first performed experiments in which the right M and Q teloblasts were ablated (n=10) and found that the cleavage patterns expressed by the two right O/P lineages were normal (Table 1, row Q). Thus, the ipsilateral M lineage is not required for the contralateral Q lineage to specify P fate in the dorsal O/P lineage. Additionally, we performed experiments in which one of the right O/P teloblasts was ablated in combination with the right M and Q teloblasts (n=10) or the right M, N and Q teloblasts (n=9). In each paradigm, the surviving o/p bandlet consistently expressed the P-type cleavage pattern (Table 1, rows R,S).

Similar to what has been reported for *H. robusta* (Sacramento), these data suggest that a brief contact with the contralateral Q lineage can specify P fate in an O/P lineage of *H. robusta* (Austin), and that this specification occurs effectively in the absence of the other ipsilateral teloblast lineages.

Discussion

Studies on the variation of developmental pathways in closely related species are a key to understanding how the evolutionary process generates organismal diversity (Stern, 2000; Simpson, 2002). To explore the evolutionary potential of developmental equivalence groups, we experimentally investigated the variation of cell fate specification mechanisms of the O/P equivalence group in three distinct breeding populations of the leech genus *Helobdella*, two of which were previously described as the same species and one of which has been described as a different species (see Materials and methods). We find that the mesodermal M lineage and the ectodermal Q lineage are redundantly involved in cell fate specification of the O/P equivalence group in *H. robusta* (Austin), whereas in the other two populations – including *H. robusta* (Sacramento) – the Q lineage alone is responsible for specifying the P fate and the M lineage has no detectable effect on O/P specification (Huang and Weisblat, 1996).

This variation in the pattern of cell-cell interactions suggests that the developmental pathway underlying the conserved morphology of the O/P lineages has undergone evolutionary divergence in closely related leech species, and, depending on the taxonomic status of the two *H. robusta* populations, possibly even within a single species. Taken together with the species variation in nematode vulval development (Sommer, 2001), this finding also suggests that a rapid divergence of developmental pathways underlying conserved morphological patterns may be a widespread feature in the evolution of developmental equivalence groups.

A mesoderm-dependent pathway of P fate specification in *H. robusta* (Austin)

H. robusta (Austin) and the other two *Helobdella* populations examined here share the following features: (1) the presumptive O and P lineages both have the developmental potential to adopt either O or P fate, and are thus equipotent; (2) the default fate of the O/P lineage in the absence of cell interactions appears to be the O fate; and (3) the q bandlet alone is able to specify the P fate in an adjacent o/p bandlet. However, *H. robusta* (Austin) differs from the others in that the Q lineage-dependent pathway and a distinct mesoderm-dependent pathway are redundantly involved in P fate specification. The molecular basis of these effects is unknown, but one could imagine that such interspecies variation might arise from either qualitative or quantitative differences in the signals that pattern O and P cell fates.

Teloblast ablation experiments suggest that the P fate can be specified in *H. robusta* (Austin) by a cooperative interaction involving both the two adjacent o/p bandlets and the underlying m bandlet. Removal of either the ipsilateral M lineage or one of the two ipsilateral O/P lineages can, in the absence of the Q lineages, cause the remaining O/P lineage(s) to forsake the P pathway and follow the O developmental pathway with high frequency. Assuming that the two O/P lineages are truly equivalent, it would seem likely that the m bandlet is able to provide extrinsic cues that are sufficient to break that equivalence. The dorsoventral polarity of the m bandlet, which manifests itself in the orientation of the cell division of the primary m blast cell (Zackson, 1984) (D.-H.K., unpublished), may have allowed this structure to assume a novel role in the Austin population as an extrinsic source of dorsoventral asymmetry for the O/P specification.

We envision that the m bandlet and the two o/p bandlets could interact to specify P fate in *H. robusta* (Austin) by either of two distinct mechanisms. In one scenario, the m bandlet may be able to directly induce the P fate in the more dorsal of a pair of overlying o/p bandlets after the Q lineages have been ablated. However, this hypothesis does not readily explain why the m bandlet fails to induce the P fate in a single o/p bandlet (Table 2), although it is formally possible that removal of the q bandlet and one o/p bandlet might prevent the remaining o/p bandlet from adopting a position in which it could receive an inductive signal from the m bandlet.

An alternative scenario is that the m bandlet may potentiate a lateral interaction between the two overlying o/p bandlets, and that it is this lateral interaction which specifies the P fate in the absence of the Q lineages. Huang and Weisblat (Huang and Weisblat, 1996) have proposed that the interaction of the o and p bandlets is purely steric in *H. robusta* (Sacramento), i.e. that the p bandlet merely shields the o bandlet from the P-inducing influence of the dorsally situated Q lineage. However, our findings argue against this interpretation for *H. robusta* (Austin) as the specification of the definitive P fate persists following bilateral ablation of the Q lineages, and is either partially or completely eliminated when one of the two ipsilateral o/p bandlets is also removed (Table 2).

In *H. robusta* (Austin) embryos in which the Q lineages have been ablated and the M lineages are still present, the presumptive p bandlet expresses an ectopic O-type cleavage pattern in some segments. This abnormal pattern of cell division seems to be correlated with a morphological anomaly that reduces the contact area between the two ipsilateral o/p bandlets (Fig. 5). The ectopic O-type cleavages would appear to result from a delayed specification of the P fate, as other data suggest that these same blast cells do go on to produce an

essentially normal pattern of P-type descendants. We do not know what sequence of causal events leads to this anomalous morphology of the germinal band, but one can speculate that a reduction in the contact of the presumptive o and p blast cells may be indicative of an attenuated interaction between them.

Multiple steps in P fate specification

In two of the experiments described here, we observed statistical inconsistencies in the frequency of P fate specification as manifested by O-type blast cell cleavage pattern at stage 7/8 and by the formation of P-type differentiated pattern elements at later stages. This result suggests that even though the o/p blast cell expresses an O-type cleavage at its first division, it can still become committed to the definitive P fate by cell-cell interaction(s) that take place at a later stage of development. In support of this idea, it has previously been shown in *H. triserialis* that presumptive o blast cells that have experienced one or more of their normal cleavages can nonetheless undergo a partial or complete commitment to produce P-type differentiated descendants if the adjoining p bandlet is ablated (Shankland, 1987a; Shankland, 1987b). The reverse has not been observed despite a variety of experimental manipulations, suggesting that commitment of a primary o/p blast cell to the P pathway may be an irreversible event that constrains both cleavage pattern and differentiated cell fate.

Segmental differences have also been reported in the blast cell cleavage patterns that generate O and P cell fates. In the so-called OP lineage of the four most rostral segments in the leech, distinct cleavage programs and cell-cell interaction patterns are used to generate a set of differentiated pattern elements that are segmentally homologous to the combined O and P lineages of the midbody and caudal segments (Shankland, 1987d; Kuo and Shankland, 2004). These findings imply that cleavage programs can be functionally dissociated from differentiated cell fates in the lateral ectoderm of the leech *Helobdella*, raising the possibility that distinct mechanisms may be involved in their specification.

Role of the micromere-derived provisional integument in the O/P specification pathway

It has previously been shown that the micromere-derived provisional integument that covers the o/p bandlets is required for normal specification of the O fate in *H. triserialis* (Ho and Weisblat, 1987). But the developmental fate of the presumptive O lineage was not altered in similar experiments performed here on *H. robusta* (Austin). The most straightforward explanation of these disparate observations is that the role of the provisional integument in O/P specification, like that of the M lineage, varies between species.

However, ablation of micromere clones is inherently transient and variable, apparently because the provisional integument undergoes a wound-healing response (Ho and Weisblat, 1987). Additionally, there are subtle but nonetheless pertinent differences in the positioning of specific micromere clones relative to the time-line of blast cell maturation between *H. triserialis* and *H. robusta* (Austin) (D.-H.K., unpublished). Given these potentially confounding factors, we would not discount the possibility that the provisional integument of *H. robusta* (Austin) could play some role in O/P specification that was not revealed by our experimental procedure.

In any case, the O-fate inducing signal from the micromere-derived provisional integument is unlikely to be a pivotal factor in breaking the equivalence of the O/P lineages in the leech *Helobdella* as this epithelium contacts both the o and p bandlets (Ho and Weisblat, 1987; Smith and Weisblat, 1994). But in some species it may still be involved in promoting O fate specification once the initial equivalence is broken.

Evolution of the O/P equivalence group

The specification of O and P fates has been experimentally studied in several clitellate species. In the sludgeworm *Tubifex*, it has been shown that the P lineage specifies a pluripotent O/P lineage to adopt the O fate, but that the specification of the P lineage does not require any of the other ectodermal lineages (Arai et al., 2001). On the basis of those results, the authors proposed that the O and P lineages of *Tubifex* differ from those of *Helobdella* in that they do not represent an equivalence group (Arai et al., 2001). It should be noted that the role of mesoderm has not yet been experimentally studied in *Tubifex*, and it is possible that, similar to *H. robusta* (Austin), mesoderm may play a role in the specification of P fate. Nonetheless, the fact that the P fate is specified normally in *Tubifex* in the absence of both Q and ipsilateral O lineages strongly suggests that there is a significant difference in the O/P specification

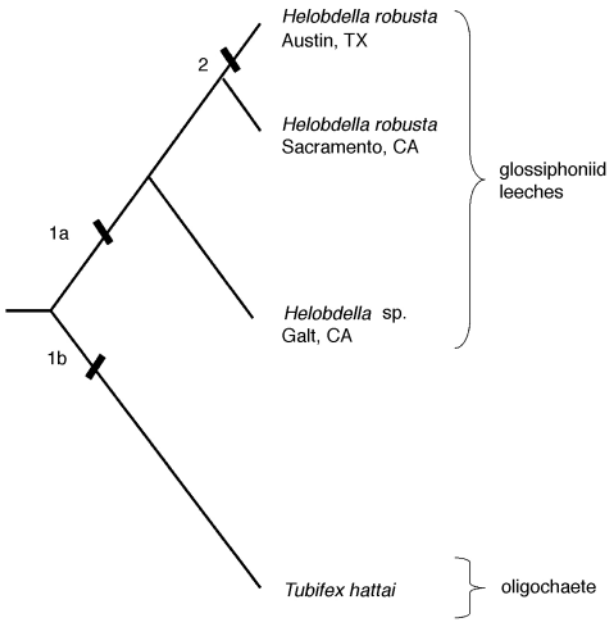

Fig. 9. Evolution of O/P specification mechanisms in clitellate annelids deduced from phylogenetic analysis. The relationship of the three *Helobdella* laboratory populations used here is based on cytochrome oxidase I sequence analysis by A. Bely (personal communication); the oligochaete *Tubifex hattai* is an outgroup. Branch lengths do not reflect the genetic distance between taxa. Evolutionary changes of developmental characters are marked by short bars striking across the tree branches, and are assigned on the basis of parsimony. The role of the Q lineage as a P fate inducer evolved either as an apomorphy (1a) on the branch leading to the *Helobdella* leech species shown here, or is a plesiomorphic trait lost (1b) on the branch leading to *Tubifex*. The role of mesoderm as a redundant P fate inducer appears to have evolved as an apomorphy (2) on the branch leading to *H. robusta* (Austin) following its separation from *H. robusta* (Sacramento).

mechanisms employed by this distantly related clitellate species.

In the leech *Theromyzon*, the O/P lineages do behave as an equivalence group and their cell fates are determined by relative location in the germinal band (Keleher and Stent, 1992). However, simple ablation of the Q lineage does not alter the O/P specification of the *Theromyzon* embryo (F. Z. Huang and D. A. Weisblat, personal communication), as it does in *H. robusta* (Sacramento). Further studies will be required to ascertain whether *Theromyzon* employs an O/P specification mechanism similar to that of *H. robusta* (Austin), or relies on some more divergent mechanism.

As in the leech *Theromyzon*, the O/P lineages of all three *Helobdella* laboratory populations studied here behave as an equivalence group. In *H. robusta* (Sacramento) and *Helobdella* sp. (Galt), the Q lineage is absolutely required for the specification of the P fate. But in *H. robusta* (Austin), the P fate appears to be specified redundantly by the Q lineage and by a set of interactions that involve the two ipsilateral O/P lineages and the mesodermal M lineage. Thus, either one of these pathways can specify the P fate in this particular laboratory population.

Fig. 9 portrays these findings in the context of a phylogenetic tree. Given the data at hand, the most parsimonious interpretation is that the mesoderm-dependent pathway of P fate specification is an apomorphic (derived) trait that arose recently in the branch leading to *H. robusta* (Austin). The role of the Q lineage in O/P specification appears to be evolutionarily conserved among *Helobdella*, and may be a synapomorphy of this group. However, we cannot at this time exclude the possibility that the P fate-inducing ability of the Q lineage in *Helobdella* is a symplesiomorphic (shared ancestral) trait that was lost in the branch leading to *Tubifex*.

Redundancy of specification mechanisms can create opportunities for generating evolutionary novelty through the neutral drift of developmental pathways. For example, redundant pathways of vulval patterning are seen in geographical variants of the nematode *Pristionchus* (Srinivasan et al., 2001), and such variation may account for the evolution of distinct vulval patterning mechanisms that generate the same VPC cell lineage pattern among different diplogastrid nematode species (Sommer, 1997). Similarly, redundancy may have paved the way for the diversification of an ancestral O/P specification pathway into the distinct pathways seen in various clitellate annelids such as *Tubifex*, *Theromyzon* and the various *Helobdella* species.

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