

## Segmentation of the central nervous system in leech

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### SUMMARY

Central nervous system (CNS) in leech comprises segmentally iterated progeny derived from five embryonic lineages (M, N, O, P and Q). Segmentation of the leech CNS is characterized by the formation of a series of transverse fissures that subdivide initially continuous columns of segmental founder cells in the N lineage into distinct ganglionic primordia. We have examined the relationship between the N lineage cells that separate to form the fissures and lateral ectodermal and mesodermal derivatives by differentially labeling cells with intracellular lineage tracers and antibodies. Although subsets of both lateral ectoderm and muscle fibers contact N lineage cells at or near the time of fissure formation, ablation experiments suggest that these contacts are not required for initiating fissure formation. It appears, therefore, that this aspect of segmentation occurs autonomously within the N lineage. To support this idea, we present evidence that fundamental

differences exist between alternating ganglionic precursor cells (nf and ns primary blast cells) within the N lineage. Specifically, ablation of an nf primary blast cell sometimes resulted in the fusion of ipsilateral hemi-ganglia, while ablation of an ns primary blast cell often caused a 'slippage' of blast cells posterior to the lesion. Also, differences in cell behavior were observed in biochemically arrested nf and ns primary blast cells. Collectively, these results lead to a model of segmentation in the leech CNS that is based upon differences in cell adhesion and/or cell motility between the alternating nf and ns primary blast cells. We note that the segmentation processes described here occur well prior to the expression of the leech *engrailed*-class gene in the N lineage.

Key words: *Theromyzon rude*, Leech, Gangliogenesis, Pattern formation, Cell lineage, *engrailed*

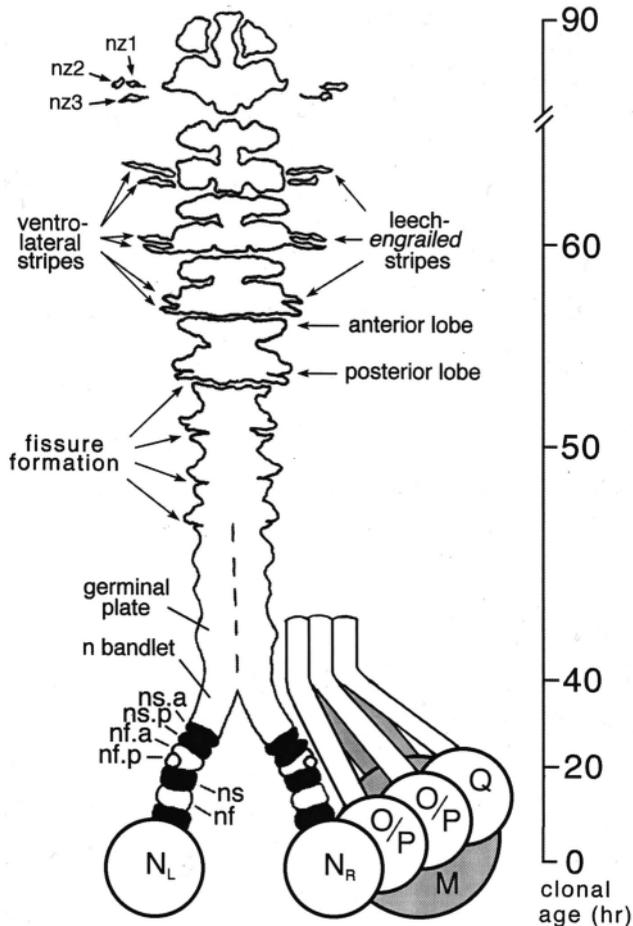
### INTRODUCTION

Segmented mesoderm and ectoderm in leech embryos arise from five bilateral pairs of stem cells, the M, N, O/P, O/P and Q teloblasts. The two M teloblasts give rise to mesoderm while the remaining four pairs of teloblasts give rise to separate ectodermal sublineages, designated N, O, P and Q. Each teloblast divides repeatedly, giving rise to segmental founder cells in a coherent column called a bandlet. In the n and q bandlets, alternating blast cells follow two different fates, as defined by the patterns of their subsequent divisions (Zackson, 1984; Bissen and Weisblat, 1989) and of their definitive, segmentally iterated progeny (Weisblat et al., 1984; Weisblat and Shankland, 1985; Bissen and Weisblat, 1987). Thus, the n bandlets contain alternating nf and ns primary blast cells (and q bandlets contain alternating qf and qs primary blast cells). The five bandlets on each side of the embryo join together in parallel to form the germinal bands, which then coalesce from anterior to posterior along the ventral midline into the germinal plate, forming a superficial ectodermal layer of N, O, P and Q lineage progeny, and an underlying mesodermal layer of M-derived progeny.

Definitive tissues arise from the five distinct teloblast lineages present in the germinal plate via stereotyped lineages from the seven classes of primary blast cells (m, nf, ns, o, p, qf and qs). Because the blast cells in each lineage are born

sequentially, blast cells contributing to more anterior segments are born earlier than those contributing to more posterior segments, and their clones are correspondingly more advanced in development. Thus, it is possible to infer the sequence of developmental processes within each lineage by observing the state of its constituent clones from posterior to anterior within the germinal plate (Zackson, 1982, 1984; Torrence and Stuart, 1986; Bissen and Weisblat, 1989; Lans et al., 1993). The developmental state of a given clone is specified in terms of its 'clonal age', i.e. the time since the birth of the primary blast cell that founded the clone (Lans et al., 1993), and progeny of the primary blast cells are designated by a nomenclature similar to that used for *Caenorhabditis elegans* (Zackson, 1984; Bissen and Weisblat, 1989; Fig. 1).

Except for fused ganglia in segments at the anterior and posterior ends of the leech, each midbody segment of an adult animal contains a discrete ganglion, linked to ganglia in adjoining segments by connective nerves as part of the ventral nerve cord. The work presented here addresses the question of how the segmented nerve cord forms from the initially continuous array of cells within the germinal plate. Within the germinal plate, the two n bandlets lie in direct apposition at the ventral midline, site of the future ventral nerve cord (Whitman, 1887). Of the ~400 neurons in each ganglion (Macagno, 1980), more than two thirds are descendants of the N teloblasts (Kramer and Weisblat, 1985). Thus, studies of gangliogenesis



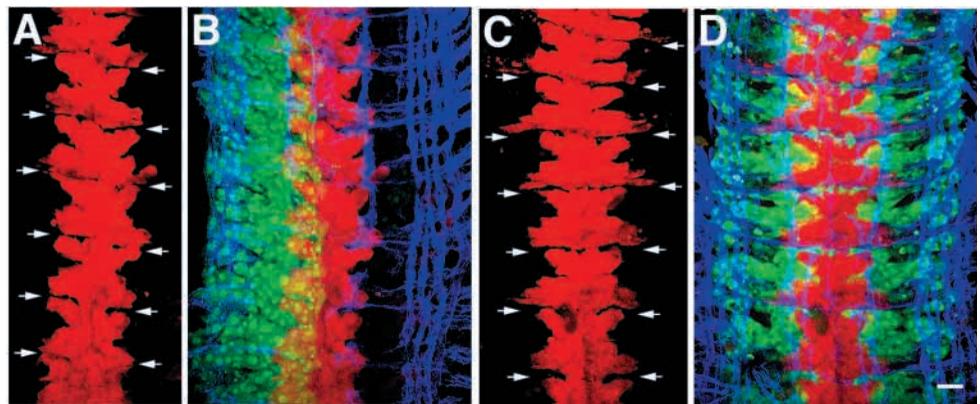
**Fig. 1.** Schematic showing the progression of events in the N lineage during gangliogenesis. The relative positions of the mesodermal (M) and other ectodermal (O/P, O/P and Q) teloblasts and their progeny are indicated on the right side only. Bilaterally paired N teloblasts ( $N_L$  and  $N_R$ ) give rise to coherent columns of cells (n bandlets). Each bandlet comprises two alternating classes of primary blast cells (ns and nf), whose distinct fates are first indicated by the differences in size of their respective progeny (ns.a and ns.p, nf.a and nf.p). Within the germinal plate, contralateral n blast cell clones lie in apposition across the ventral midline (dashed line) and subsequently give rise to the bulk of the segmental ganglia of the ventral nerve cord, along with segmentally iterated peripheral neurons (nz1, nz2 and nz3) and a few epidermal cells (not shown). The O/P, O/P and Q teloblasts on each side give rise to distinct O, P and Q lineages that generate progressively more lateral and dorsal ectoderm (Weisblat et al., 1984). Ganglionic primordia arise by the formation of transverse fissures that arise when the parent blast cell clones are ~50 hours old. The outgrowth of two ventrolateral stripes of cells from each posterior lobe (the anterior of which expresses the leech *engrailed*-class gene) occurs later at ~65 hours clonal age. Approximate clonal ages for n blast cells and their derivatives are indicated at right. Anterior is up. Not drawn to scale.

have focussed on events in the N lineage and on the differences between nf and ns blast cells (Bissen and Weisblat, 1987; Wedeen and Weisblat, 1991). It had previously been shown that, within the N lineage, the leech *engrailed*-class gene was

expressed near the posterior edge of prospective ganglia in transverse rows of cells derived from the nf clone (clonal age 63–78 hours; Lans et al., 1993), and that early ablation of the nf clone (2-cell stage; clonal age 28–30 hours) results in a ‘fused hemi-ganglia’ phenotype, apparently due to the failure of two ipsilateral hemi-ganglionic primordia to separate (Ramirez et al., 1995). These results were interpreted as suggesting that the nf-derived stripes of cells expressing the leech *engrailed* gene are instrumental in separating the N lineage into discrete ganglionic primordia (Ramirez et al., 1995).

Recent observations have called this interpretation into question. We have observed a series of transverse fissures between cells in the N lineage that subdivide the n bandlets into distinct ganglionic primordia (Shain et al., 1998). Fissures form at approximately 50 hours clonal age, and invariably

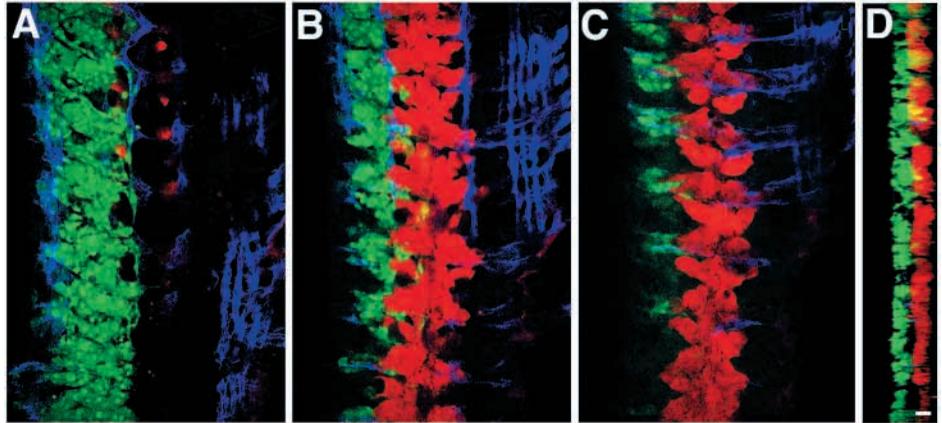
**Fig. 2.** Cell neighbor relations during fissure formation. Confocal image projections showing the positions of N lineage cells relative to cells in the mesodermal and other ectodermal lineages. In this and all subsequent figures, anterior is up, ventral midline is in the center of each panel and progeny of the N teloblasts are shown in red, unless otherwise indicated. (A) A view of five complete ganglionic primordia, plus parts of two others, from an embryo in which both N teloblasts were labeled with RDA. Arrows indicate the fissures between ganglionic primordia. In posterior segments (clonal age ~45



hours), fissures are beginning to form, while in the anterior segments (clonal age ~52 hours) fissures extend to the ventral midline. (B) The same preparation as in A, but showing the left M lineage (green), which had been labeled by FDA injection of the M teloblast, and differentiated muscle fibers (blue), which were labeled using the monoclonal antibody Lan 3-14 and a Cy5-labeled secondary antibody. The mesoderm lies dorsal to the neuroectoderm and muscle fibers appear to make contact with N-derived cells proximal to the boundary of the fissure. (C) A view of six complete ganglionic primordia, plus parts of two others, from an embryo in which both N teloblasts were labeled with RDA. Arrows indicate the fissures separating ganglionic primordia. (D) The same preparation as in C, but showing bilateral O, P and Q lineages (green) and differentiated muscle fibers (blue). Collectively, the ectoderm forms a continuous layer of cells on the ventral aspect of the embryo. Ganglionic primordia are essentially surrounded by the remaining ectoderm during fissure formation. In this and other figures, yellow color occurs where mesodermal or ectodermal cells (green) overlap with N-derived cells (red). Scale bar, 25  $\mu$ m.

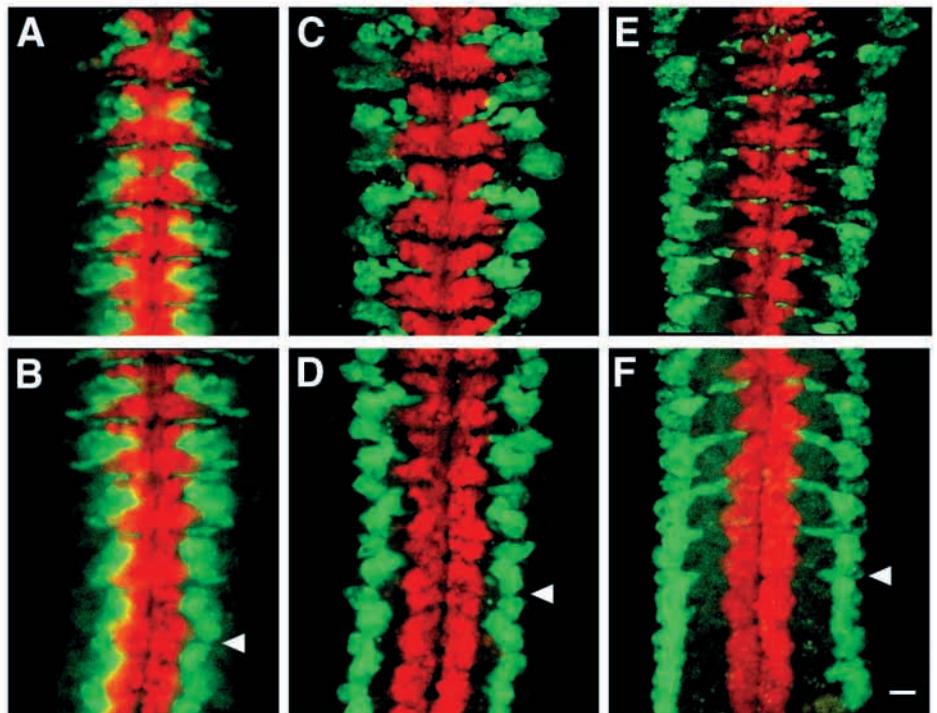
**Fig. 3.** Relative movements of mesodermal and neuroectodermal progeny. Differentially labeled cells are as described in Fig. 2B.

Anterior is up. (A-C) Selected optical sections of six intact segments and parts of two others, taken with the confocal microscope at  $\sim 20 \mu\text{m}$  intervals beginning from the dorsal aspect of the dissected germinal plate. Note that the mesoderm (green) and the muscle derived from it (blue) occupies more ventral positions in anterior segments than in posterior segments, and that the nerve cord in posterior segments lies ventral to virtually all the mesodermal derivatives. (D) Digital rotation of the preparation shown in A-C to a sagittal position. A medial section through the nerve cord (red) was merged with a lateral section of the mesoderm (green) to demonstrate the relative movements of these two lineages (dorsal is left). Scale bar,  $25 \mu\text{m}$ , except that in F, the dorsoventral axis has been expanded two-fold to emphasize the changes in relative positions of the M and N lineages.



appear as a separation between two sublineages derived from the nf and ns blast cells (the nf.p clone and ns.a clone). Fissure formation seems to be a pivotal event during leech gangliogenesis, because fissures are the first overt indication of the separations between ganglionic primordia (Shain et al., 1998). A key observation was that fissures form prior to the appearance of leech *engrailed*-class gene expression in the N lineage, suggesting that the expression of leech-*engrailed* is not required for this aspect of segmentation. Leech-*engrailed* is expressed earlier in other lineages, however (Lans et al., 1993). Therefore, in this study, we sought to determine whether fissure formation occurs autonomously within the N lineage, or in response to signals from the mesodermal or ectodermal lineages that lie next to the n bandlets. We found that both ectodermal and mesodermal progeny are closely associated with N-derived cells that form the fissure. There is no absolute requirement for either the mesodermal or ectodermal progeny in initiating the formation of the fissure, although, as reported previously (Blair, 1982; Torrence et al., 1989; Torrence, 1991), interactions with mesoderm are required to maintain the separation and organization of ganglionic primordia. Rather, we present evidence that fissures form autonomously within the n bandlet as the result of differences between alternating nf and ns primary blast cells.

**Fig. 4.** Distribution of lateral and dorsal ectodermal lineages during fissure formation. View of 4-5 segments in preparations at 60-70 hours clonal age (A,C,E) or 6 segments in preparations at 45-60 hours clonal age (B,D,F), with the O (A,B), P (C,D) and Q (E,F) lineages labeled with FDA (green). Note that the O and Q lineages project into the fissures separating ganglionic primordia, while P does not. The arrowhead in each of the lower panels marks the first segment in which fissure formation can be detected. Scale bar,  $25 \mu\text{m}$ .



## MATERIALS AND METHODS

### Embryos

*Theromzyon rude* embryos were obtained from specimens collected in the ponds of Golden Gate Park, San Francisco, and were cultured as previously described (Torrence and Stuart, 1986), except that they were maintained at  $12^\circ\text{C}$  or  $23^\circ\text{C}$ .

### Lineage tracer injections and cell ablations

Fluorescent lineage tracer [either fluorescein-dextran amine (FDA, Molecular Probes) or tetramethylrhodamine-dextran amine (RDA, Molecular Probes) was injected into M, N, O, P or Q teloblasts after their birth, using fast green dye (1% final concentration) to monitor the course of the injections, as previously described (Weisblat et al., 1980). All injections were performed under a dissecting microscope. Cell ablations were achieved by irradiating FDA-labeled cells with the focused beam of a 488 nm laser (Lexel, Model 65; Braun and Stent,

1989b) or by 'over-injecting' the cell of interest (i.e., to the point at which fast green could be visualized by the unaided eye several hours after injection) with either DNase (Blair, 1982) or ricin A chain (Nelson and Weisblat, 1992).

### Histochemistry

Embryos were fixed overnight at 4°C in 4% formaldehyde (in 1× PBS, pH 7.4) containing Hoechst 33258 (1 µg/ml final concentration). The vitelline membrane was removed manually and the germinal plate was dissected from the yolk with fine pins (Fine Science Tools, Cat. No. 10130-05). Germinal plates were mounted in 80% glycerol containing 4% n-propylgallate; for viewing, embryos were placed on a slide, drawn out of the glycerol solution with pins and covered with a glass cover slip.

### Antibody staining of embryos

Fixed, dissected germinal plates were rinsed for 30 minutes at room temperature in 1× PBS, then blocked for 1 hour in a solution containing 1× PBS, 10% goat serum, 1% BSA, 2% Triton X-100 and 0.001% sodium azide. A monoclonal antibody specific for leech muscle (Lan 3-14; Zipser and McKay, 1981) was added at a dilution of 1:1000 and incubated overnight at room temperature with agitation. Following five 1 hour washes in 1× PBS, Cy5-conjugated secondary antibody (Jackson Lab) was added at a 1:400 dilution in blocking solution and incubated overnight at room temperature. Following five additional washes in 1× PBS, germinal plates were mounted in 80% glycerol containing 4% n-propylgallate, as described above.

### Microscopy

Live embryos were viewed by a CCD camera (DC 330, MTI) mounted on a compound microscope (Zeiss) and images were captured using Scion Image software. Stained germinal plates were viewed and photographed using either a Zeiss Axiophot microscope or a confocal microscope (BioRad model MRC-1000/1024). Slides were taken using Ektachrome 400 film (Kodak) and scanned with a SprintScan 35 Plus (Polaroid) slide scanner. Adjustment of color levels and merging of images was performed with Adobe Photoshop (version 4.0).

## RESULTS

### Cell neighbor relations during fissure formation

To identify cells that interacted with N-derived progeny during fissure formation, both N teloblasts were injected with RDA and other teloblasts of interest were injected with FDA. In addition, the Lan 3-14 antibody was employed to identify muscle fibers, all of which arise from the M lineage in the segmental tissues. Most mesodermal progeny lie dorsal to ectodermal lineages from the time germinal bands first form until approximately the onset of fissure formation. As fissures formed within the N lineage and ganglionic primordia separated from each other (~45 hours clonal age; Fig. 2A), prominent circular muscle fibers appeared within the gap between *nf.p* and *ns.a* clones at the dorsal aspect of prospective ganglia (Figs 2B, 3A-C; Torrence and Stuart, 1986). In midbody segments, circular muscle fibers appeared within the fissure as it became evident, raising the possibility that these muscle fibers played an active role in fissure formation. In older segments, the circular muscle fibers occupied progressively more ventral positions and eventually came to lie along the ventral body wall. The ventral movement of a portion of the mesodermal progeny (e.g. prospective body wall muscles) with respect to the neuroectoderm was monitored by observing

consecutive confocal slices through the embryo between clonal ages ~50-65 hours (Fig. 3A-C). Older segments (anterior) contained mesoderm that was shifted ventrally with respect to the neuroectoderm, while in younger (posterior) segments, neuroectoderm was positioned ventral with respect to mesoderm. Longitudinal muscle fibers that contacted the lateral edge of each ganglionic primordia also appeared to move from dorsal to ventral positions relative to the ganglia (Fig. 3A-C). The movement of N lineage cells relative to lateral M lineage derivatives is revealed by a computer-generated sagittal view of the same region of the germinal plate (Fig. 3D).

Similar experiments were carried out to examine cell neighbor relations between the N lineage and the lateral and dorsal ectodermal lineages, O, P and Q. Muscle fibers were also immunostained in these experiments to provide reference markers within the germinal plate. As fissures formed within the N lineage (Fig. 2C), lateral ectodermal progeny appeared to contact N-derived progeny that lined the fissure (Fig. 2D). As the fissures progressed medially, lateral ectoderm extended finger-like projections into the gaps and eventually formed continuous stripes that lay between each ganglionic primordia. At this point (~50 hours clonal age – see Fig. 1), ectodermal cells from O, P and Q surrounded the periphery of each ganglionic primordia but did not appear to intermingle with N-derived cells. After 70 hours clonal age, neural cells from O, P and Q lineages were observed in their previously described positions within the ganglion (data not shown; Braun and Stent, 1989a).

To distinguish the separate contributions of each ectodermal lineage (O, P and Q) during the process of fissure formation, each of these teloblasts was microinjected with FDA (Fig. 4). Results from this analysis demonstrated that progeny from the O and Q lineages, but not the P lineage, entered the fissure, and extended toward the midline.

### Fissure formation and gangliogenesis in the absence of lateral and dorsal ectoderm

The close association of N-derived cells that form the fissure with M-derived circular muscle fibers and with O- and Q-derived progeny raised the possibility that cells from M, O and/or Q lineages may be required for fissure formation, and for the subsequent separation of ganglionic primordia. It has previously been shown that ganglia still form in *Helobdella* embryos from which the O, P and Q lineages have been ablated, but these ganglia are often malformed (Blair and Weisblat, 1982). To determine if the O, P and Q lineages were required for fissure formation, the Q and both O/P teloblasts were ablated unilaterally. The effects of this manipulation on fissure formation and the later separation of definitive ganglia was monitored at stages 8 and 10, respectively. Fig. 5 demonstrates that fissures form, albeit larger than normal and often at abnormal angles as opposed to their normal transverse orientation. Aside from a small decrease in the size of each ganglion, due to the absence of the normal complement of O-, P- and Q-derived neurons, ganglion formation and morphology did not differ significantly from control embryos, except that N-derived neurons from the anterior and posterior parts of the ganglion (corresponding to progeny of *ns.a* and *nf.a*, respectively) appeared as a single cluster of cells (Fig. 5B) in the absence of the O-, P- and Q-derived ventromedial neurons (Weisblat and Shankland, 1985; Torrence and Stuart,

1986). To test for signalling across the ventral midline from the contralateral O-, P- or Q-derived lineages, embryos were also prepared in which O/P and Q teloblasts were ablated bilaterally. Of approximately 40 such embryos, 15 survived to stage 10 and were successfully dissected for analysis. In these embryos, ganglia had separated but, as expected, were smaller than normal (data not shown).

### Independence of fissure initiation from mesodermal signaling

Previous studies have demonstrated that, in the absence of underlying mesoderm, leech ectoderm is disorganized and discrete ganglia fail to form; when mesoderm is ablated unilaterally, cells in the N lineage from the ablated side cross the ventral midline in numbers that vary from segment to segment, resulting in asymmetric and irregular ganglia (Blair, 1982; Torrence et al., 1989; Torrence, 1991). But these previous observations, made at later stages of development, do not distinguish between the possible roles of mesoderm in initiating the separation of ganglionic primordia as opposed to maintaining distinct ganglia once gangliogenesis has begun. We therefore carried out ablation experiments to determine if the initial steps of ganglionic separation can occur in the absence of mesoderm. Germinal plate formation often fails even after unilateral mesodermal ablation, and the resultant embryos are more fragile and difficult to dissect than unmanipulated controls. Thus, in these experiments, M teloblasts were ablated only after the birth of about 10 m blast cells, so that a small region of anterior germinal plate could form normally, increasing the probability that ectodermal bandlets would coalesce into the germinal plate in more posterior regions where the m bandlet was missing. Out of more than 30 experimental embryos, 10 survived for subsequent analysis. In these embryos, the earliest stages of ganglion formation, namely formation of the fissure, appeared to proceed in hemi-ganglia that lacked mesodermal contributions (Fig. 6A,B). To test for the possibility of mesodermal signals crossing the midline from the unablated side, bilateral mesodermal ablations were attempted, but the resultant embryos did not survive to the point where observations of fissure formation could be made.

Further evidence for the idea that primary circular muscle fibers are not required for fissure formation was obtained using the segment-specific disparity in the age of consegmental m and n blast cell clones. As described previously (Lans et al., 1993), the m and n blast cell clones contributing to anterior segments are of approximately the same age, whereas in each more posterior segment, the m blast cell clone is progressively older, and correspondingly more advanced in development, than the consegmental n blast cell clones. Thus, in posterior segments, primary circular muscle fibers often extended to the midline prior to fissure formation (Fig. 6C) while, in anterior segments, primary circular muscle fibers were often incomplete although the process of fissure formation was well under way (Fig. 6D,E). Moreover, in several segments, primary circular muscle fibers were clearly out of register with the fissure, suggesting that this component of the M lineage is not required for fissure formation (Fig. 6E).

We also determined the clonal age (in the N lineage) at which fissures formed along the anteroposterior axis, by fixing embryos from the same clutch at various times during

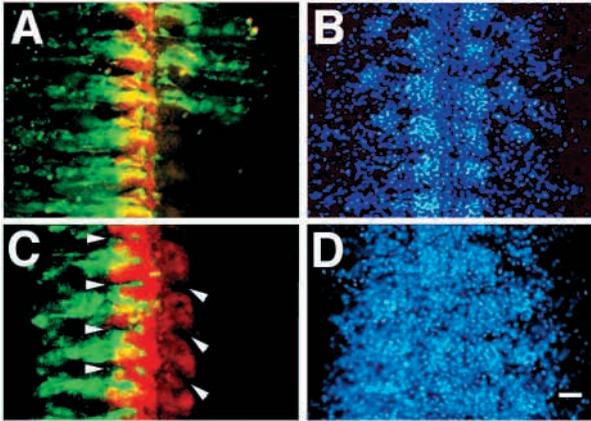
development (to observe fissure formation in anterior and posterior segments). We found that fissure formation occurred at ~50 hours clonal age in both anterior and posterior midbody segments and was therefore independent of the age of the con-segmental m blast cell clone (data not shown). These observations suggest that, even if fissure formation is induced by an inductive signal from the M lineage, the exact location of the fissure and the timing of its formation are determined within the N lineage.

### Blast cell ablations

It is not feasible to test for fissure formation after having ablated both ectoderm and mesoderm, because the development of the resultant embryos is too severely disrupted. Thus, we cannot rule out the possibility that ectoderm and mesoderm signal redundantly to induce fissure formation in the N lineage. That possibility notwithstanding, the preceding results suggest that fissure formation can occur autonomously within the N lineage, i.e. that clones within the N bandlet may be programmed to separate from each other at a designated time during development (at ~50 hours clonal age). Previous studies showed that fissures separating ganglionic primordia form between the posterior edge of the nf.p clone and the anterior edge of the ns.a clone (Shain et al., 1998). To begin to distinguish the separate roles of nf.p and ns.a clones in the process of gangliogenesis, we observed the effects of ablating these and other secondary blast cells derived from the nf and ns primary blast cells. As a first step in this process, we repeated the ablations of primary nf and ns blast cells (Ramirez et al., 1995) but, in the course of these experiments, we made new observations, as described below.

In the majority of cases (Table 1), an ablation of either nf or ns resulted in a deficiency of cells in the positions that would have been occupied by the ablated clone. For example, nf-derived progeny constitute the posterior lobe of each ganglion plus peripheral neurons nz1, nz2 and nz3 (Shain et al., 1998), and the ablation of this clone usually resulted in the expected deficiency of neurons (Fig. 7A,B). Similarly, ablation of the ns primary blast cell usually resulted in a deficiency in the ipsilateral anterior lobe of the ganglion (Fig. 7E,F).

In some embryos, however, either of two other phenotypes were obtained, one of which was unique to nf blast cell ablations and the other to ns blast cell ablations. Roughly 20% of nf ablations (4 of 22 embryos) resulted in a 'fused hemi-ganglia' phenotype (Ramirez et al., 1995) in which two adjacent hemi-ganglia on the side of the ablation fused, while those on the contralateral side separated normally (Fig. 7C,D). By contrast, in ~50% of the cases in which an ns blast cell was ablated (31 of 64 embryos), the bandlet posterior to the ablated cell slipped backwards by one or more segments relative to the other bandlets, as evidenced by the smaller size of the intervening ganglia and the absence of labeled progeny, causing a deficiency of neurons often several segments in length (Fig. 7G,H). The phenomenon, referred to as 'slippage' (Shankland, 1984), has been observed in the m, n, o and p bandlets, following the ablation of one or more of the respective primary blast cells (Shankland, 1984; Gleizer and Stent, 1993; Ramirez et al., 1995). The important finding here is that hemi-ganglionic fusion was observed only after nf ablations and slippage only after ns ablations, suggesting that a difference in cell motility and/or adhesivity exists between nf

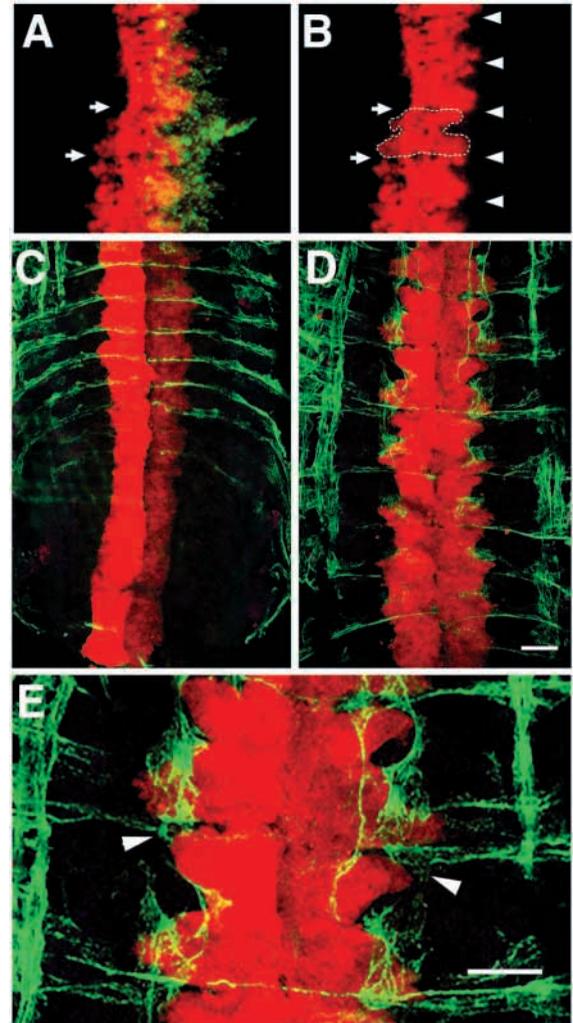


**Fig. 5.** Ganglia separate in the absence of the O, P and Q lineages. The O, P and Q lineages on both sides of the embryo were labeled with FDA (green) and the N teloblasts with RDA (red). O/P and Q teloblasts on the right side of the embryo were biochemically arrested by micro-injecting ricin A chain ~10 hours after the birth of the O/P teloblasts. (A) Segmental ganglia ranging from ~65–75 hours clonal age; posterior segments lack O, P and Q progeny on the right side of the embryo, but ganglia have separated. (B) Hoechst 33258 staining of nuclei in the same preparation as A. (C) Segmental ganglia ranging from ~45–55 hours clonal age; all segments lack O, P and Q progeny on the right side of the embryo. Arrowheads indicate the formation of fissures on both sides; those on the left have formed normally, while those on the right are larger than normal and at an oblique angle to the A-P axis. (D) Hoechst 33258 staining of nuclei in the same preparation as C. Scale bar, 25  $\mu$ m.

and ns primary blast cells (and their respective derivatives). We also noted that in the large majority of cases, bandlets that had slipped posteriorly came to lie so that the nf and ns clones were in register with contralateral n blast cell clones of the same type.

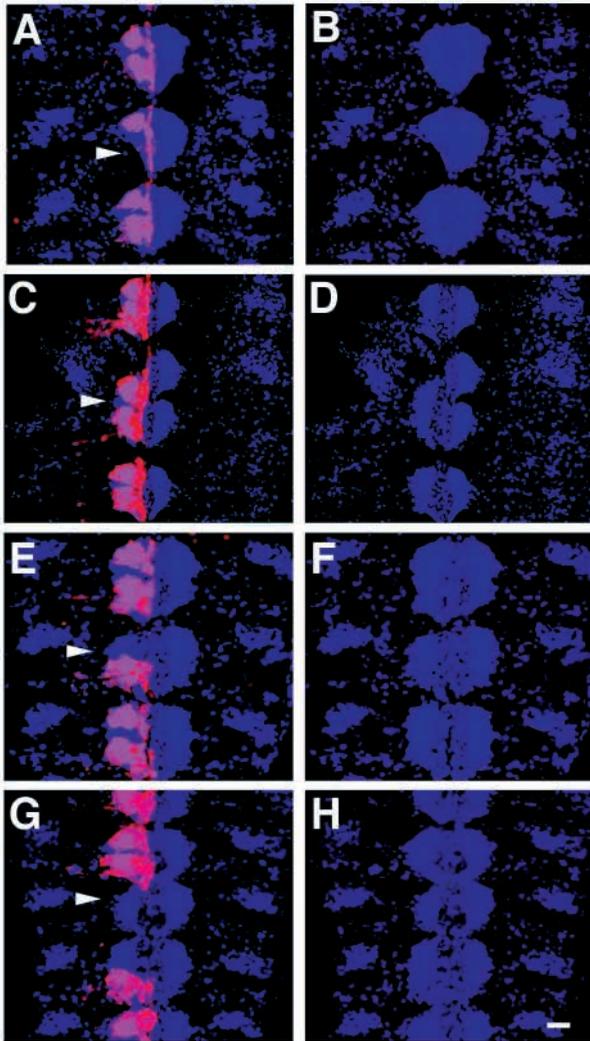
To determine when differences could first be detected following nf versus ns blast cell ablations, we monitored a group of 24 embryos at selected times after microinjecting primary blast cells with DNase. Of these embryos, nine survived handling and UV exposure to a stage where the distribution of definitive blast cell progeny could be examined. Three embryos were identified as having undergone nf ablation and displayed the ‘deficiency’ phenotype – no fused hemiganglia were observed (Fig. 8A–D). In these ablations, a small gap was observed between apposing ns cells shortly after the ablation, but in all three cases the gap had closed within the next ~14 hours. Six ablations were identified as ns, but only one of these displayed a simple ‘deficiency’ phenotype. The remaining five displayed a ‘slippage’ phenotype (Fig. 8E–H). In all cases, a large gap (greater than the diameter of one blast cell clone) was observed between apposing nf cells by 9 hours following the lesion. This gap remained approximately the same size (i.e., there was no further slippage) during subsequent development. These results indicate that nf and ns blast cells differ in adhesivity and/or motility by the time they have entered the germinal bands; apposing ns blast cells maintained their normal positions following an nf ablation, whereas apposing nf blast cells typically underwent ‘slippage’ shortly after a ns ablation.

The ablation of secondary blast cells, nf.a, nf.p, ns.a and ns.p was technically difficult because of the small size of these cells.



**Fig. 6.** Fissure formation in the absence of the M lineage. (A) Unilateral  $M_L$  ablation; progeny from  $M_R$  are shown in green. Arrows indicate the boundaries of a putative ganglionic primordium. At the lower arrow, a fissure extends to the ventral midline. Most N-derived cells anterior to the upper arrow have crossed over the ventral midline, so no fissure is evident on the ablated side; in the more posterior segments, ganglionic primordia are still asymmetric due to some N-derived cells having crossed the midline. (B) Same as A, except the N lineage is shown independently of the mesoderm. A dotted line surrounds one ganglionic primordia, indicating the formation of two independent fissures (arrows). Arrowheads indicate the position of fissures formed (anterior) or forming (posterior) on the right side of the embryo. (C) Circular muscle fibers (green) extend to the midline prior to fissure formation in posterior regions of the embryo where M is developmentally more advanced than N (see text). (D) Circular muscle fibers in the anterior region of the midbody are variable: fissure formation is well under way before circular muscle fibers are continuous across the midline, and muscle fibers often form out of register with the fissures. (E) A higher magnification of the fissure separating the two most anterior ganglionic primordia in D. Arrowheads indicate the position of the fissure. Scale bar, 25  $\mu$ m.

This was especially true in the case of nf.p, which is of particular interest because its clone comprises a thin stripe of ~4–6 cells that forms the anterior margin of the fissure (Shain et al., 1998), and because hemi-ganglionic fusions sometimes



**Fig. 7.** Ablation of primary n blast cells results in distinct phenotypes in the differentiated nerve cord (clonal age >90 hours). Only the left N teloblast was labeled (red) and ablations were performed either by laser illumination or by microinjecting a cellular toxin (ricin A chain or DNase). (A) Most nf ablations resulted in a deficiency of neurons corresponding to the usual distribution of the missing clone, but (C) a small percentage (~20%) resulted in the fusion of adjacent, ipsilateral hemi-ganglia (Ramirez et al., 1995). (E) Ablations of ns resulted in either a deficiency (~50%) or (G) slippage of the bandlet posterior to the ablated cell (~50%). (B,D,F,H) The nuclear stain (Hoechst 33258; blue) only of A,C,E,G. Arrowheads indicate the site of ablation. Scale bar, 25  $\mu$ m.

processes into different segments (Fig. 10A). The morphology of ricin-arrested nf cells was even more dramatic, as these cells extended a broad process resembling the lateral stripes of 5-7 nf-derived cells that give rise to the peripheral nz neurons (Fig. 10B; Braun and Stent, 1989a; Wedeen and Weisblat, 1991; Shain et al., 1998). In all the cases in which ricin-injected cells survived, ganglia always separated normally. The observation that arrested nf and ns primary blast cells exhibited clone-specific features despite severe biochemical insult further support the notion that differences exist between these primary blast cells before their first mitoses.

## DISCUSSION

### Autonomy of fissure formation

The work presented here suggests that in the leech *Theromzyon rude*, segmentation of the nerve cord is initiated autonomously within the primary neurogenic (N) lineage. Fissures that divide initially continuous columns of cells (derived from the primary nf and ns blast cells) into discrete ganglionic primordia arise between the nf.p and ns.a subclones about 50 hours after the birth of the corresponding primary blast cells. The fissures arise in close association with cells arising from other ectodermal lineages, especially the O and Q lineages, and with primary circular muscle fibers derived from the M lineage. But ablation experiments show that the fissures can form in the absence of either the normal ectodermal or mesodermal neighbors. It is also important to note that the clonal age of fissure formation remains constant along the anteroposterior axis, despite the age disparity between the N-lineage and mesoderm (and the O and P ectodermal lineages). Therefore, even if some secreted signal from another lineage is required to trigger fissure formation, the temporal and spatial details of the process seem to be established autonomously within the N lineage.

While the results presented here suggest that the initiation of fissure formation occurs autonomously, there are caveats to this conclusion. In particular, we have not been able to score embryos in which the mesoderm was removed bilaterally or in which both mesoderm and other ectodermal lineages were removed simultaneously. Thus, we cannot exclude the possibility that these tissues signal redundantly to induce fissure formation, or that the contralateral mesoderm signals across the ventral midline. And, as has been shown previously (Blair, 1982; Torrence et al., 1989), interactions with mesodermal derivatives are required for maintaining ganglionic organization.

occur when the parent nf cell is ablated (Ramirez et al., 1995; this paper). Of more than 200 embryos, only 34 successful secondary blast cell ablations were achieved (Table 1). On the basis of these limited observations, ablation of any of the secondary n blast cells, including nf.p, resulted in the simple deficiency phenotype [i.e., the absence of labeled neurons in the position that would normally be occupied by the ablated clone (Fig. 9)].

### Behavior of biochemically arrested primary blast cells

The ricin A chain is an inhibitor of protein synthesis in eukaryotic cells (Endo and Tsurugi, 1988). Leech blastomeres injected with ricin A chain cease dividing after at most one further division, but typically do not immediately die (Nelson and Weisblat, 1992; Liu et al., 1998). In ~10 of the more than 200 embryos studied in the present experiments, ricin injections failed to lyse the primary n blast cell, but merely prevented further cyto- and karyokineses. In such cases, we observed that the injected cell occupied the position that would normally be occupied by its descendant clone and even took on a similar shape, despite the fact that it remained only as a single cell. For example, a ricin-injected ns cell formed the anterior lobe of the ganglion and, in some instances, extended

### Differences between nf and ns blast cells

That distinct nf and ns primary blast cells arise in exact alternation in the n bandlet (Zackson, 1984; Weisblat et al., 1984; Bissen and Weisblat, 1987, 1989) seems an obvious key to understanding gangliogenesis in leech. The results obtained here with ablation of primary nf and ns blast cells reveal significant differences between the two cell types prior to their first mitoses. In ~20% of nf ablations, adjacent hemi-ganglia fused, while in ~50% of ns ablations, the fragment of the n bandlet proximal to the teloblast slipped posteriorly. Fusion was never seen upon ns ablation and slippage never occurred upon nf ablation. We attribute these results to inherent differences in cell adhesivity and/or motility between nf and ns blast cells. Differences also exist in lengths of the G<sub>2</sub> phase of their cell cycles and the symmetry of their first mitoses (Zackson, 1984; Bissen and Weisblat, 1987, 1989).

When an nf cell is ablated, the severed anterior and posterior ends of the lesioned n bandlet terminate in ns blast cells, and vice versa for an ns ablation. If ns cells are more adhesive and/or more motile than nf cells, then the apposing anterior and posterior ends would be more likely to retain their relative positions, and may even become contiguous with one another, which we speculate could result in the fused hemi-ganglia phenotype that is sometimes observed after an nf ablation. By this same logic, when an ns cell is ablated, the apposing ends of the severed bandlet would terminate in the less adhesive and/or motile nf blast cells, which could account for the frequent observations of 'slippage' upon ablation of ns primary blast cells (see Fig. 8). We observed that the posterior sectors of slipped n bandlets usually came to lie with their nf and ns clones in register with the contralateral n bandlet. This could represent homotypic adhesive interactions between contralateral n blast cell clones within the germinal plate, or recognition by n blast cell clones of iterated domains in any of the other bandlets within the germinal band or germinal plate.

Comparing the present results of blast cell ablation with those obtained previously (Ramirez et al., 1995), we find two significant differences. First, the frequency of hemi-ganglionic fusions obtained here is lower, which may result from differences in the experimental techniques used to perform the ablations. In the previous work, ablations were carried out by photoablating 2-cell clones of nf or ns blast cells, whereas in the present experiments, microinjection of ricin was used for most ablations. Second, in previous experiments, slippage was ascribed to inadvertent killing of primary blast cells adjacent to the target clone, which is a technical problem associated with dye-mediated laser ablations. In the present experiments, using direct microinjection of the target cell, we were able to circumvent this problem and thereby discover that slippage occurs only when an ns cell is ablated.

Previous analyses showing the positions of secondary blast cell clones indicated that the separation between ganglionic primordia occurs between the nf.p and ns.a clones (Shain et al., 1998). Based upon the thin stripe of ~4-6 nf.p-derived cells at this boundary, it was proposed that nf.p may provide a 'spacer' between ganglia, and we therefore anticipated that selectively ablating the nf.p clone would result in ganglion fusion. Contrary to this prediction, we find that no hemi-ganglionic fusions were observed with any of the secondary blast cell ablations. Because of the difficulty of the experiments,

**Table 1. Distribution of phenotype for blast cell ablation**

| Cell ablated (total) | Phenotype  |        |          |
|----------------------|------------|--------|----------|
|                      | Deficiency | Fusion | Slippage |
| nf (22)              | 18         | 4      | 0        |
| ns (64)              | 33         | 0      | 31       |
| nf.a (17)            | 17         | 0      | 0        |
| nf.p (6)             | 6          | 0      | 0        |
| ns.a (10)            | 10         | 0      | 0        |
| ns.p (1)             | 1          | 0      | 0        |

however, we were unable to achieve more than a few nf.p ablations. Therefore, if ganglion fusion is occurring at the same low frequency for nf.p secondary blast cell ablations as for nf primary blast cell ablations, it is not surprising that we observed no hemi-ganglionic fusions. Alternatively, it may be that when the nf.p clone is selectively removed, leading to apposition of ns.a and nf.a clones (see Fig. 1), these clones exhibit no proclivity to adhere or merge with one another.

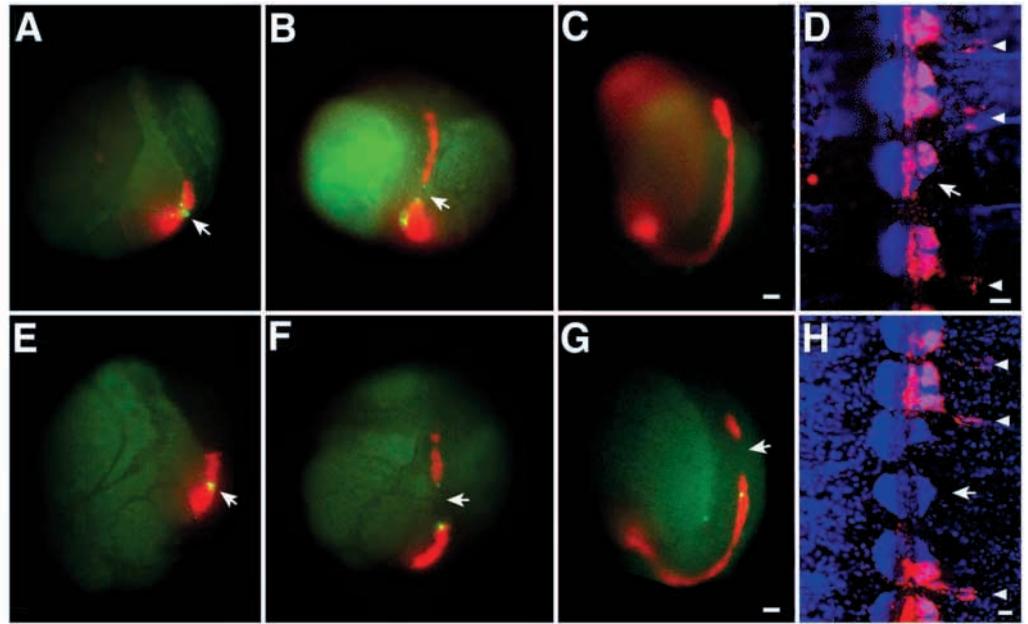
### *Engrailed*-class genes and segmentation

The conserved patterns of *engrailed*-class gene expression have been interpreted as reinforcing the notion that the ancestor to arthropods, annelids and chordates was already overtly segmented (Wedeen and Weisblat, 1991; Holland et al., 1997). In *Drosophila*, the only organism for which a detailed molecular genetic analysis is available, *engrailed* is expressed in stripes that demarcate the posterior compartment of each segment; *engrailed*-expressing cells appear prior to overt segmentation and are required for normal segmentation (Kornberg, 1981a,b).

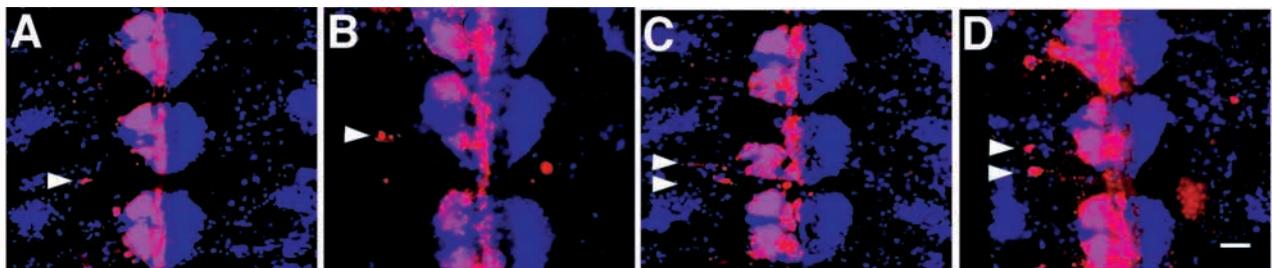
*Engrailed* protein specifies the posterior compartment cells (Lawrence et al., 1999a), which then secrete *hedgehog* protein (Lee et al., 1992). *hedgehog* protein diffuses into the anterior compartment across the A/P and P/A boundaries and directly affects cell 'affinities', thereby influencing the normal positioning and differentiation of cells in the anterior compartment (Lawrence et al., 1999b). Thus, it seems that *engrailed* acts upstream to regulate cell affinities in anterior compartment cells in *Drosophila*. While the ablation of the *engrailed*-expressing cells in *Drosophila* has not been carried out, one is lead to conclude that ablating these cells would remove the source of *hedgehog* protein and thus have a significant effect on the fates of remaining cells, thereby disrupting segmentation.

In leech, *engrailed*-class gene expression in the N lineage occurs in discontinuous, iterated stripes of cells that lie close to the ganglionic margin (Wedeen and Weisblat, 1991; Lans et al., 1993). But a detailed lineage analysis of these cells revealed that the stripes of N-derived cells appear only after ganglionic primordia have already separated (Shain et al., 1998). Leech-*engrailed* is expressed transiently by individually identified cells in primary blast cell clones within all five of the lineages contributing to segmental tissues (Lans et al., 1993). In each prospective segment, expression in the O, P and Q lineages occurs prior to the separation of ganglionic primordia in the N lineage, and could therefore play an inducing role in segmenting the N lineage. But here we have shown that even bilateral ablation of the O, P and Q lineages did not prevent ganglion separation. Evidence presented here further supports the notion that ganglia in leech separate in an N lineage-autonomous process, prior to the birth of the cells that will

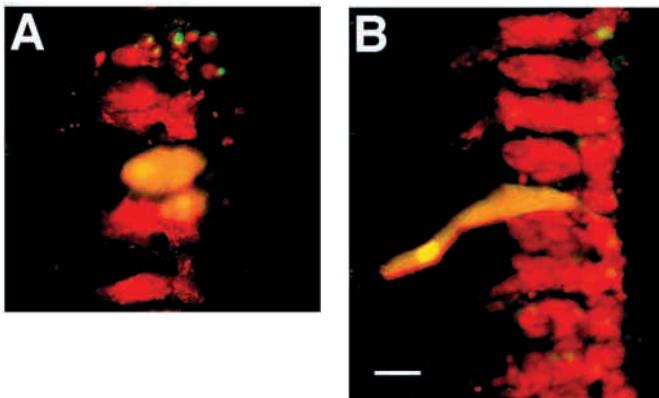
**Fig. 8.** Time course analysis of primary n blast cell ablations. In each embryo, the left N teloblast was labeled with RDA (red). About 12 hours later, a single primary n blast cell in each labeled bandlet was microinjected with a mixture of DNase and FDA (green). Embryos were maintained individually at 15°C and images of living embryos were taken ~10 minutes (A,E), ~9 hours (B,F) and ~23 hours (C,G) after the DNase injection. Embryos were then grown at room temperature (23°C) until ~140 hours after the DNase injection, at which point they were fixed, counterstained with Hoechst 33258 and dissected to observe the definitive progeny of the cells adjacent to the ablated blast cell (D,H). Arrows indicate the site of the lesion; arrowheads indicate nf-derived peripheral neurons nz1-nz3. (A-D) A representative embryo in which an nf blast cell was lesioned,



as judged by the missing nz neurons and the reduced numbers of N-derived progeny in the posterior hemi-ganglion in the affected segment in D. Note that the cell labeled with green in A has vanished, except for bits of cellular debris, by ~9 hours later (B); the gap corresponding to the missing cell in B is larger than in A because the blast cells are compressed in the proximal regions of the bandlet and then expand as they move distally (compare the labeled segment of bandlet anterior to the lesion site in A and B. By ~23 hours after the ablation (C), no obvious break in the bandlet can be detected, and an nf deficiency with no slippage or fusion was observed in the germinal plate (D). (E-H) A representative embryo in which an ns blast cell was lesioned, as judged by the observation that, in the dissected embryo (H), the lesion site is flanked by two nf blast cell clones, with progeny in the posterior part of each ganglion and peripheral nz neurons. Here, too, the initially injected blast cell (E) has vanished by ~9 hours after the injection except for fluorescent debris (F), but in this case the gap between the flanking cells is somewhat larger in F than in B. The gap persists at the ~23 hour time point (G) and in the nerve cord of the dissected embryo (H), in which it can be seen that the nf clone posterior to the ablated cell has slipped two segments posterior. Scale bar, 100 μm in A-C, E, F; 25 μm in D, H.



**Fig. 9.** Ablation of secondary n blast cells results in a simple deficiency of the corresponding clone in the differentiated nerve cord. In each panel, the middle ganglion is affected. Cells derived from the left N teloblast are labeled (red). (A) nf.a, (B) nf.p, (C) ns.a, (D) ns.p. Arrowheads indicate neurons nz1 and nz2 (B; upper arrowheads in C and D) and nz3 (A; lower arrowheads in C and D) Scale bar, 25 μm.



**Fig. 10.** Behavior of biochemically arrested blast cells. Examples of primary blast cells in an RDA-labeled N lineage (red) that were injected with ricin and FDA (appear as yellow cells). Neither cell died, but remained as 1- or 2-cell clones even when segmental ganglia were well differentiated. (A) ns, (B) nf. Note the prominent lateral projection, which recapitulates the parallel stripes that normally arise from the nf.a and nf.p clones (cf. Fig. 2A,C). Scale bar, 25 μm.

ultimately express the *engrailed*-class gene in the N lineage. We note also that ablation of cells expressing the *engrailed*-class gene in the O lineage has no effect on the fates of the remaining O-derived cells (Seaver and Shankland, 1999).

Thus, it appears that in the leech *Theromyzon*, critical differences in cell adhesivity and/or motility (i.e., cell affinity) occur prior to the expression of the leech *engrailed*-class gene, in apparent contrast to the situation in *Drosophila*. Moreover, both our ablation experiments and those of Seaver and Shankland suggest that other cell fates are not affected by the removal of cells expressing the *engrailed*-class gene. These results imply that the mechanism of segmentation in leech is different from that in fly, at least with respect to *engrailed*-class gene function. Clearly, this calls into question what aspects of segmentation are conserved among ecydsozoan and lophotrochozoan protostomes, not to mention deuterostomes.

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