Identification of a neurogenic sublineage required for CNS segmentation in an Annelid

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SUMMARY

In embryos of leeches (phylum Annelida), metameric structures arise sequentially from a germinal plate comprising the descendants of five pairs of embryonic stem cells called teloblasts. It has been shown that transverse stripes of cells expressing *ht-en* (a homolog of *engrailed*, a *Drosophila* segment polarity gene), arise in the germinal plate prior to the appearance of segmental ganglia and that, in the main neurogenic lineage (derived from the *N* teloblasts), the stripe of cells expressing *ht-en* demarcates the boundary between prospective segmental ganglia. Previous lineage-tracing experiments had suggested that the clones of *nf* and *ns* primary blast cells in the *N* lineage are confined to within segmental borders. This conclusion was called into question by the observation that the cells expressing *ht-en* do not appear to be at the very posterior edge of the *nf* clone, from which they arise. To resolve this issue, we have injected individual primary blast cells with fluorescent lineage tracers; we find that cells in the *nf* clone actually straddle two adjacent ganglia. Moreover, using photoablation techniques, we find that the *nf* clone is required for proper morphogenesis of the segmentally iterated central nervous system (CNS).

Key words: *engrailed*, segmentation, gangliogenesis, leech, annelid

INTRODUCTION

Questions as to how homologous developmental processes are realized at the cellular and molecular level in various organisms have reinvigorated studies in the area of comparative development, especially through the analysis of evolutionarily conserved developmental regulatory genes. One class of genes that has been used for such comparisons is the *Drosophila* gene *engrailed* (*en*) and its homologs in other animals, including annelids (Wedeen et al., 1991), other arthropods (Patel et al., 1989a; Walldorf et al., 1989; Hui et al., 1992), brachiopods (Holland et al., 1990), chordates (Joyner et al., 1985; Fjose et al., 1988; Gardner et al., 1988; Poole et al., 1989; Holland and Williams, 1990; Hemmati-Brivanlou et al., 1991), echinoderms (Dolecki and Humphries, 1988), nematodes (Kamb et al., 1989) and platyhelminthes (Webster and Mansour, 1992). Expression of *en*-class genes has been seen in most of these groups during neurogenesis, but to date, only arthropods and annelids have been shown to express *en*-class genes during segmentation (Patel et al., 1989a; Wedeen and Weisblat, 1991).

Comparing segmentation in annelids and arthropods is of particular interest because these phyla are generally assumed to have had a common segmented ancestor (Clark, 1964; Valentine, 1973), even though the mechanisms of segmentation can differ widely between them at the cellular level. For example, long germ band insects such as *Drosophila* (phylum Arthropoda), generate segments simultaneously from a syncytial blastula. The *Drosophila* blastoderm becomes organized into parasegmental units demarcated by the expression pattern of pair-rule genes (Ingham and Martinez Arias, 1992). Parasegmental units in the blastoderm are defined simultaneously along the length of the *Drosophila* embryo (Ingham and Martinez Arias, 1992). Parasegments are further divided into compartments, the borders of which restrict cell mingling (Martinez Arias and Lawrence, 1985), by the expression patterns of segment polarity gene(s), including *en*, a homeodomain-containing transcription factor (Kornberg, 1981; Poole et al., 1985). The anterior limit of *en* expression defines the parasegmental borders (Kornberg et al., 1985; DiNardo et al., 1985).

In contrast to the situation in *Drosophila*, other arthropods, such as short germ band insects and crustaceans, generate caudal segments sequentially from a posterior growth zone (for reviews see Patel, 1994; Tautz et al., 1994). Examples of purely sequential segmentation are found in the annelid phylum. In leeches, such as the glossiphoniid species *Helobdella robusta* and *Theromzyon rude*, all the segments arise in a strict rostrocaudal progression from longitudinally arrayed columns of primary blast cells that are produced sequentially from five bilateral pairs of embryonic stem cells (M, N, O/P, O/P and Q teloblasts) (Weisblat and Shankland, 1985) (Fig. 1). Although the O/P teloblasts are equipotent, each teloblast normally gives rise to a unique pattern of segmentally iterated progeny, so we can treat the segmental ectoderm and mesoderm as arising from distinct M, N, O, P and Q cell lines.
In the M, O and P cell lines, each primary blast cell gives rise to one segment’s worth of definitive progeny; m, o and p blast cell clones interdigitate extensively along the rostrocaudal axis, however, indicating that blast cell clones are not compartments as defined in *Drosophila* (Weisblat and Shankland, 1985). Metameric structures form nonetheless, because the iterated clones in each cell line are stereotyped in terms of division pattern, definitive cell phenotype, and spatial distribution.

The N (and Q) cell lines each consist of two different classes of blast cells (nf and ns, qf and qs) in exact alternation (Fig. 1), and two consecutive blast cell clones are required to form one segment’s worth of definitive progeny for these cell lines (Weisblat and Shankland, 1985). The columns of n and q blast cells compress themselves relative to the columns of m, o and p blast cells to achieve their proper segmental register, and the age of n and q clones relative to consegmental m, o and p clones changes markedly along the rostrocaudal axis of the embryo. For this reason, and because the sequential nature of blast cell production dictates that blast cell clones in anterior segments are developmentally advanced relative to those in posterior segments of the same embryo, it is more precise to refer to events (such as cell division or gene expression) within the five segmental founder cell lines by the *clonal age* at which they occur, meaning the time elapsed since the birth of the primary blast cell that founded the clone in question, rather than simply referring to the embryonic stage (Lans et al., 1993).

In early development, the leech *en*-class gene (*ht-en*) is expressed transiently by a subset of cells in each of the five cell lines; within the N cell line in particular, the early expression of *ht-en* occurs during stages 8-9 as transverse stripes of up to 7 immunoreactive nuclei arising within the nf clones of clonal age 63-78 hours (Fig. 1) (Wedeen and Weisblat, 1991; Lans et al., 1993). Previous experiments had led to the conclusion that nf and ns blast cell clones are confined to a single segment (Weisblat and Shankland, 1985; Bissen and Weisblat, 1987), but in those experiments only the anterior border of individual blast cell clones was observed directly. The validity of this conclusion for the posterior border of the clone was called into question when the expression of *ht-en* was analyzed in detail (Lans et al., 1993; Wedeen and Weisblat, 1991). In particular, the transverse stripe of cells expressing *ht-en* in the N cell line was seen to lie at the prospective boundary between segmental ganglia, prior to the appearance of overt ganglionic borders (Wedeen and Weisblat, 1991).
Clonal ablations were performed by injecting a photosensitizing Ektachrome 400 film. At early stage 8, primary nf and ns blast cells were pressure injected with tetramethylrhodamine dextran amine (RDA, Molecular Probes Inc.) along with RDA into an N teloblast. After culturing injected embryos to stage 8, secondary blast cells were visualized by rhodamine epifluorescence and identified by their characteristic sizes and shapes using a Zeiss standard microscope equipped with a 50x or 100x NPL Fluor Leitz water immersion objective. Cells were photobleached by intracellular excitation of fluorescein (Shankland, 1984) using a 485 nm laser beam (Lexel, Model 65) focused through the objective using fluorescein optics (Braun and Stent, 1989b). Death of the irradiated cell is presumably the result of singlet oxygen produced by energy transfer from the excited fluorescein (Braun, 1985). That both of the intended secondary blast cells and none of the adjacent cells had been photobleached was determined by examining the patterns of definitive nf or ns progeny adjacent to the lesioned zone at stage 10. The RDA lineage tracer (which persists after the 485 nm irradiation) reveals the N lineage progeny remaining in the hemiganglion. Correct ablation of an ns clone reveals a gap of tracer in the anterior of only one hemiganglion with the nf clones just anterior and posterior to the ablated ns clone still present and unaffected. Correct ablation of an nf clone reveals that stereotyped peripheral neurons, nz neurons, (Braun and Stent, 1989a) are missing in only one hemiganglion and ns clones anterior and posterior to ablated nf clone are still present.

By these criteria, 6 embryos with successful nf ablations and 7 with successful ns ablations were obtained from a total of 59 experimental embryos. The remaining embryos either exhibited slippage of the n bandlet (indicating that a primary blast cell next to the 2-cell clone had also been killed; Shankland, 1984; n=25), incomplete ablation (n=17), or were destroyed during dissection (n=4).

N lineage ablation

The N lineage was ablated on one side of an embryo by injecting one N teloblast with both the toxin A chain of ricin and the RDA lineage tracer shortly after its birth as described by Nelson and Weisblat (1992).

ht-en antibody staining

The N teloblast was injected with RDA lineage tracer shortly after its birth as described above and allowed to develop to stage 9. Embryos were fixed and processed with the ht-en antibody as described previously (Wedeen and Weisblat, 1991). Confocal images were obtained using a BioRad 600 confocal microscope.

RESULTS

The nf clone is not confined to a single ganglia

The distribution of the nf and ns clones was examined directly by injecting individual blast cells with fluorescent dextran lineage tracers at stage 7 (Fig. 3A,B) and examining the distribution of their definitive neuronal progeny at stage 10 (clonal ages 100 hours onwards). The results show that the nf clone is not confined to the posterior of the segmental hemiganglion, as previously supposed (Weisblat and Shankland, 1985; Bissen and Weisblat, 1987), but rather that it includes three cells in the anterior margin of the adjacent ganglion (n=31) (Fig. 3B). In contrast, ns clones are restricted to a single ganglion (n=20). This is despite the fact that the labeled clone extends throughout the anteroposterior extent of the ganglion, with many cells in the anterolateral and posterior medial regions of the hemiganglion (Fig. 3A).

The nf clone is essential for ganglionic separation

The finding that the nf clone contributes progeny to two
adjacent ganglia indicates that, contrary to prior belief, the clonal boundary is not coincident with ganglionic borders. Rather, the observation that the interganglionic boundary is better predicted by the stripe of *ht-en* expression (Wedeen and Weisblat, 1991), suggests that the subset of nf-derived cells expressing this gene might be involved in the process of defining ganglionic borders.

This issue was investigated further by comparing the roles of nf- and ns-derived cells in segmenting the ventral nerve cord. For this purpose, laser ablation was used to selectively ablate nascent nf or ns clones (clonal ages 28-30 hours). Previous work has shown that there is no regulative replacement of N lineage cells by other teloblast lineages when the N lineage is ablated (Blair and Weisblat, 1982; Stuart et al., 1987). Moreover, nf and ns blast cells retain their distinctive identities when adjacent cells within the bandlet are ablated in a manner similar to that described here (Bissen and Weisblat, 1987). Accordingly, we find no regulative replacement of the nf-derived stripe of *ht-en*-expressing cells when the N teloblast is ablated (Fig. 4). The time point chosen for ablation of individual blast cell clones is approximately 30 hours prior to *ht-en* expression in the N lineage and thus well prior to gangliogenesis. In addition, at clonal age 28-30 hours, the nf and ns primary blast cells have divided into secondary blast cells (Fig. 1). This simplifies identification of nf and ns clones, and eliminates the longitudinal displacement of blast cells posterior to
showing that In this study, we have followed up on previous experiments system. established ganglionic borders in the leech central nervous the clone, fail to separate on that side (adjacent hemiganglia, which would otherwise be spanned by affec
ted ganglion is reduced in size but forms normal bound-
aries (Kramer and Shankland, 1984; see Materials and Methods).
The resultant embryos were examined at stage 10 as before. Normally, nf and ns clones together contribute roughly two thirds of the neurons in the hemiganglion (Kramer and Weisblat, 1985). When a nascent ns clone is ablated, the affected ganglion is reduced in size but forms normal bound-
aries (n=7) (Fig. 3C). In contrast, when an nf clone is deleted, adjacent hemiganglia, which would otherwise be spanned by the clone, fail to separate on that side (n=6) (Fig. 3D). Therefore, one or more nf-derived cells are required to establish ganglionic borders in the leech central nervous system.

DISCUSSION

In this study, we have followed up on previous experiments showing that ht-en in leech is expressed in transverse stripes that demarcate ganglionic boundaries. Between clonal ages 63 and 78 hours, ht-en-expressing cells are located in the mid-
posterior portion of the nf clone, leading to the prediction that this clone should straddle the mature ganglionic segment boundary. Using an intracellular cell lineage tracer, we have verified this prediction by demonstrating that a single nf clone contributes descendant neurons to two successive ganglia. Specifically, three cells are found in the posterior ganglia.

These cells come from nf,p and are posterior to the ht-en positive cells (Ramirez et al., unpublished data).

The cellular mechanism(s) by which one or more cells in the nf clone contribute to sculpting discrete ganglia from the continuous sheet of cells in the germinal plate remains to be determined. Ablating the entire nf clone eliminates both ht-en-expressing and non-expressing cells, but the congruence between the ht-en expressers and the prospective border leads us to consider the possibility that it is the cells expressing ht-en that are required to form the interganglionic border.

One possibility is that cells expressing ht-en fail to con-
tribute to the ganglion, thereby creating gaps between adjacent ganglionic masses. Such a failure could be explained if the cells in question die or migrate, or if they fail to migrate relative to their neighbors. Consistent with this latter possibility is the observation that cells expressing ht-en in the Q cell line maintain their lateral positions while other cells in the same line migrate medially (Lans et al., 1993). Another correlation between ht-en expression and cell movements comes from the observation that the subsets of cells that express ht-en transiently in each of the five cell lines form a narrow trans-
verse array within the posterior portion of the segmental anlage, prior to the interdigitation of the m, o and p clones (Lans et al., 1993). These transient alignments of ht-en-
expressing cells in leech resemble the parasegmental organiza-
tion of cells within the germ bands of arthropods, and may therefore represent a primordial stage of development common to both simultaneously and sequentially segmenting proto-
 stomes.

As is to be expected, comparing the patterns of expression of en-class genes in leech and sequentially segmenting arthro-
pods reveals both similarities and differences (Fig. 5). In some malacostracan crustacean embryos, for example, the postnau-
pilar ectoderm typically arises from multiple bilateral pairs of ectoteloblasts that generate columns of segmental founder cells via stem cell divisions, as in leech (Dohle, 1970, 1976; Dohle and Scholtz, 1988; Patel et al., 1989b). Each malacostracan founder cell generates one segment’s worth of definitive progeny, in contrast with the two cells required to generate one segmental complement in the N (and Q) cell lineages of leech. In addition, the expression of en-class genes in the 2- to 4-cell clones of the malacostracans is much earlier than in the N lineage of leech. But parallels can be drawn between the 2-cell (ab and cd) clone in malacostracans, and the nf and ns blast cells of the leech embryo.

Typically, the ab row of blastomeres divides to produce an anterior row of a cells and a posterior row of b cells; the a cells either initiate expression of en-class genes or maintain expression that they inherited from the ab progenitor. Within the clones descended from the row of b cells, anterior progeny express en-class genes and posterior progeny do not; the segmental boundary falls within the b clones, just posterior to the cells expressing en-class genes (Patel et al., 1989b; Scholtz et al., 1994). Thus, both anterior and posterior margins of the segment boundary are produced by progeny of the ab cells, and we suggest that they may play a role in segment formation analogous to that of the nf clone in the leech. More detailed comparisons await further investigations in both groups, but the parallels seen are consistent with the notion that sequential segmentation in arthropods and annelids is a shared, primitive trait.
It is also interesting to compare the role proposed here for *ht-en* in the morphogenesis of leech CNS with that of *En-2*, an *en*-class gene that is expressed in the region of the presumptive midbrain-hindbrain border of mouse (Millen et al., 1994). While the phenotype of mice homozygous for *En-2* mutations is complex, cerebellar morphogenesis is clearly affected. Apart from localization of the defects to the posterior region of the cerebellum, the developmental role of this *en*-class gene in the morphogenesis of nonsegmental CNS in mouse (phylum Chordata) bears little semblance to its role in the formation of segmental boundaries in arthropod ectoderm. Thus, if, as suggested here, *ht-en* plays a role in the morphogenesis of segmentally iterated CNS in leech, this would provide overlap with the otherwise disparate functions of *en*-class genes in arthropods and chordates.

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**REFERENCES**


