INTRODUCTION

Comparative studies of development are perhaps the most direct means of addressing the fundamental question of how biological diversity arises by modification of developmental processes during evolution. For example, although their segmental body plans are presumed to be homologous, the embryonic development of annelids and arthropods can differ radically at the cellular level. In embryos of the leech *Helobdella triserialis* (an annelid), cleavage divisions are stereotyped and holoblastic. Segmental periodicity is generated by means of an invariant, segmentally iterated sequence of cell divisions and development proceeds via a pronounced rostrocaudal temporal gradient (Stent et al., 1982, 1992). In contrast, the embryos of long germ band insects such as the fruit fly, *Drosophila melanogaster* (an arthropod), undergo multiple rounds of syncytial divisions before cellularization of the blastoderm. Metamerization of the *Drosophila* embryo is achieved by a process in which the blastoderm becomes progressively subdivided into a series of contemporaneously developing compartments (for reviews, see Akam, 1987 and Ingham, 1988). In other arthropods, including short germ band insects and crustaceans, segments are generated sequentially by caudal growth zones (Anderson, 1972; Dohle, 1970, 1976; Dohle and Scholtz, 1988).

One approach to elucidating the similarities and differences in the developmental mechanisms operating in diverse phyla is to compare the function of homologous developmental regulators. Toward this end, we are studying the *Helobdella triserialis* homolog (*ht-en*) of the gene *engrailed* (*en*), a developmental regulatory gene encoding a homeodomain-containing transcription factor (Jaynes and O'Farrell, 1991) expressed during segmentation and neurogenesis in *Drosophila* (Poole et al., 1985). Homologs of *en* have been found in a wide variety of organisms including mouse (Joyner and Martin, 1987), sea urchin (Dolecki and Humphries, 1988), chick (Gardner et al., 1988), zebrafish (Fjose et al., 1988), nematode (Kamb et al., 1989), grasshopper and crayfish (Patel et al., 1989b), honeybee (Walldorf et al., 1989), human (Poole et al., 1989), leech (Wedeen et al., 1991), lamprey and hagfish (Holland and Williams, 1990), frog (Hemmati-Brivanlou et al., 1991), brachiopod (Holland et al., 1991), silkworm (Hui et al., 1992) and flatworm (Webster and Mansour, 1992).

In *Drosophila*, *en* is first expressed in the syncytial blastoderm, as evidenced by western blots (Karr et al., 1989). At the cellular blastoderm stage, *en* is expressed in segmentally iterated circumferential stripes of cells (DiNardo et al., 1985; Fjose et al., 1985; Kornberg et al., 1985; Poole et al., 1985) and is required only in cells of the posterior compartment of the developing segment; in *en* mutants, cells in the anterior compartment develop normally, whereas cells in the posterior compartment adopt other fates and do not respect compartment borders (Garcia-Bellido and Santamaria, 1972; Morata and Lawrence, 1975; Lawrence and Morata, 1976; Kornberg, 1981; Brower, 1984). Thus, it might be expected that the set of cells
expressing en would correspond exactly to the posterior compartment. Recent work, however, indicates that the stable expression of en is not clonally inherited during the blastoderm stage (Vincent and O’Farrell, 1992) and that en expression during larval and pupal development extends into the anterior compartment of the imaginal wing disc (Blair, 1992). Thus, details of the function of en expression during segmentation in Drosophila remain enigmatic. en is expressed again later, during neurogenesis, in a subset of segmentally iterated neurons (Brower, 1986; Patel et al., 1989b). The patterns of en expression in grasshopper and crayfish are similar to those seen in Drosophila, although there are differences in the manner in which these patterns arise, consistent with the different modes by which segments are formed in these organisms (Patel et al., 1989a).

The expression of ht-en during leech embryogenesis occurs in patterns reminiscent of those observed in arthropods; iterated expression patterns are observed during ger­nal plate formation and, later, in the central nervous system and body wall (Wedeen and Weisblat, 1991). We present here a more detailed analysis of the early phase of ht-en expression, in the germinal bands and germinal plate during germinal plate formation. During this time, ht-en is expressed in segmentally iterated patterns in each of the teloblast lineages that contribute definitive progeny to the segments. The exact identities of the earliest cells to express ht-en in several of these lineages have been determined. We observe that after consegmental primary blast cell clones have come into register within the germinal plate, cells that express or have expressed ht-en are confined to the poste­rior portion of the segmental anlage. In addition, we show that the temporal pattern of ht-en expression is correlated with the age of the particular blast cell clone, rather than with any characteristic of the segment taken as a whole.

**MATERIALS AND METHODS**

**Embryos**

*Helobdella triserialis* embryos were obtained from a laboratory breeding colony or from specimens collected from ponds in Golden Gate Park, San Francisco. Embryos were cultured at 23˚C in HL saline (Blair and Weisblat, 1984). The embryonic stages are defined according to Stent et al. (1982), as amended by Stent et al. (1992).

**Lineage tracers and teloblast injections**

Teloblasts were pressure-injected as previously described (Weisblat et al., 1980; Gimlich and Braun, 1985) with a 3:2 mixture of tetramethylrhodamine-dextran amine (FDA) or fluoroscein-dextran amine (FDA) (Molecular Probes, Eugene, OR; catalog nos. D-1817 and D-1820; 50 mg/ml in 0.2 N KCl) and fast green FCF (Sigma; 10 mg/ml in 0.2 N KCl).

The ages of immunoreactive cells within a lineage were assessed relative to the most rostrally positioned primary blast cell clone in a bandlet that contained lineage tracer (i.e. the ‘first labeled clone’). Since only those primary blast cells born after a parent teloblast is injected with lineage tracer inherit the tracer, the age of the first labeled clone is equal to the interval between the time of injection and the time of fixation of the embryo. Because primary blast cells in each lineage are generated at the rate of one per hour and are added to the caudal end of an elon­

gating bandlet, there is a strict correspondence between the birth rank of a primary blast cell clone, itsrostrocaudal location within the bandlet and the identity of the mature segments(s) to which it contributes progeny. This correspondence was used to estimate the ages of blast cell clones in segments rostral and caudal to the first labeled clone, by determining the number of clones separat­ing the clone in question and the first labeled clone in the teloblast lineage. Moreover, the observation that consen segmental clones in the O and P lineages are very close to the same clonal age, as are consen segmental clones in the N and Q lineages (Weisblat and Shankland, 1985), was used to determine the ages of clones in lineages without lineage tracer. Primary blast cells and their progeny are designated according to the system of Zackon (1984) as extended and amended by Shankland (1987a,b) and Bissen and Weisblat (1989).

Due to the rostrocaudal gradient of development, the number of cells in different primary blast cell clones along the length of a bandlet will vary widely. It is useful, therefore, to indicate the developmental state of clones containing ht-en immunoreactive cells by reference to *clonal age*, defined as the time that has elapsed since the birth of the primary blast cell that founded the clone.

To assess the timing of ht-en expression in the N and Q lineages relative to that in the O (or P) lineages, O/P teloblasts were injected either 8 or 20 hours after their birth, generating first labeled o (or p) ‘reference’ clones that contributed progeny to a rostral segment (i.e. M12; Fig. 1, groups 1 and 2) or a caudal segment (i.e. M20; Fig. 1, groups 3 and 4). Embryos were fixed when the o (or p) reference clone was either at clonal age 68 hours (Fig. 1, groups 1 and 3) or 78 hours (Fig. 1, groups 2 and 4). In each group, an N teloblast was injected 6 hours after the O/P teloblast was injected to facilitate the identification of cells expressing ht-en and to provide a means of independently verifying the clonal ages in that lineage. The complete pattern of ht-en expression in each embryo was recorded, and the position of the domains of ht-en expression in the n and q bandlets was compared to the position of the o (or p) reference clone.

**Immunohistochemistry**

At various times after injection of the O/P teloblast, embryos were fixed in 2% formaldehyde (in 50 mM cacodylate buffer, pH 7.4) for 1 hour at room temperature. After fixation, embryos were washed in phosphate-buffered saline (PBS: 130 mM NaCl, 7 mM Na2HPO4, 3 mM KH2PO4, pH 7) for at least 15 minutes, the vitelline membrane was manually removed, and the germinal plate and ger­manal bands were dissected away from the yolk with insect pins. [Some embryos were dissected only after immunohisto­chemical processing.] All washes and antibody incubations were carried out at 4˚C unless otherwise noted. Embryos were blocked in PBT (1× PBS, 2% bovine serum albumen and 1% Triton X­100) for 24-36 hours, then incubated in a 1/25 dilution of anti-h­t-en antibody (Wedeen and Weisblat, 1991) in PBT and gently shaken for 36 hours, washed with PBT for 6 hours (5 changes), incubated in a 1/600 dilution of goat anti-rabbit IgG conjugated to horse­radish peroxidase (GAR-HRP; Calbiochem) and gently shaken overnight. Embryos were washed at room temperature 3× (1 hour each wash) with PBT and then 3× for 1 hour with PBS. The embryos were transferred to a solution of 0.5 mg/ml DAB (3,3’-diaminobenzidine; Sigma) in PBS for 15 minutes, after which H2O2 was added to a final concentration of 0.03% and the reaction was allowed to proceed for 5-10 minutes. Embryos were washed in PBS, stained with DAPI or Hoechst 33258 (1 µg/ml final concentration), and mounted under coverslips ventral side up in 90% glycerol in PBS containing 100 mg/ml DABCO [1,4-diaza­bicyclo [2.2.2]-octane, (Aldrich)] or in 80% glycerol in 0.1 M Tris, pH 7.4 containing 40 mg/ml n-propyl gallate (Giloh and
Sedat, 1982) to retard photobleaching of the fluorophores. Specimens were examined with 40× and 100× objectives on a Zeiss Axiohot microscope using epifluorescence optics alone or in combination with Nomarski optics, and photographed using Ektachrome 400 or Tech Pan film.

RESULTS

Summary of leech development

*Helobdella* embryos undergo highly stereotyped cleavages (stages 1-6) that generate five bilateral pairs of embryonic stem cells, called teloblasts, as well as three macromeres and 25 micromeres. The teloblasts, designated M, N, O/P, O/P and Q, are progenitors of the segmental tissues of the leech (summarized in Fig. 2). Beginning an hour after being born, each teloblast carries out a series of several dozen highly unequal cell divisions (stages 6-8) at the approximate rate of one per hour (Wordeman, 1982), producing a coherent column (bandlet) of segmental founder daughter cells (primary blast cells). The first primary blast cell produced by a teloblast constitutes the distal end of the bandlet and contributes progeny to the most rostral segments in the embryo. Each succeeding primary blast cell is added to the proximal end of the elongating bandlet and contributes progeny to progressively more caudal segments.

On each side of the embryo, the five bandlets merge to form a germinal band (stage 7). There are four ectodermal bandlets on each side, derived from the ipsilateral N, O/P, O/P and Q teloblasts, and one mesodermal bandlet, derived from the ipsilateral M teloblast. Within the germinal band, the ectodermal bandlets (n, o, p, q) are positioned over the mesodermal bandlet (m), relative to the surface of the embryo, in a stereotyped array, with the n and q bandlets at the edges and the two O/P-derived bandlets positioned between them. Once within the germinal bands, the O/P-derived bandlets are designated as distinct o and p bandlets, in alphabetic order relative to the n and q bandlets. A deviation from this general scheme results from the fact that the OP proteloblast itself makes four op blast cells before dividing to produce sister O/P teloblasts. Thus, the rostral end of the germinal band contains only three ectodermal bandlets.

As blast cells are born and added to the caudal ends of the bandlets, the left and right germinal bands elongate and move across the surface of the embryo. They gradually coalesce along the ventral midline of the embryo (stage 8) into a structure known as the germinal plate, from which the segmental tissues arise. The 32 segments are divided into three groups designated R1-R4, M1-M21 and C1-C7, reflecting the inclusion of the four most rostral and seven most caudal segments in specialized terminal structures.

Genesis of segments

There are seven classes of primary blast cells. The M and O/P teloblasts generate bandlets composed of one class of primary blast cell (designated as m and o or p blast cells, respectively). The N and Q teloblasts generate bandlets composed of two classes each of primary blast cells, in exact alternation (designated as nf and ns or qf and qs primary blast cells, respectively). The different classes of primary blast cells can be distinguished on the basis of the stereotyped mitotic patterns within their descendant clones (stages 7-9) and the distinct subset of segmentally iterated definitive progeny to which they give rise in the juvenile leech (stage 10 and beyond; Weisblat et al., 1984; Zаксон, 1984; Shankland, 1987a,b; Bissen and Weisblat, 1989; Weisblat and Shankland, 1985). Moreover, because the primary blast cells and their descendant clones within each bandlet are arranged in a strict birth order, it is possible to reconstruct the temporal progression of developmental events in each class of blast cell clone by examining the status of a series of such clones in a caudal-to-rostral progression (Fernandez, 1980). Although each blast cell (in the

---

**Fig. 1.** Schematic of experiments to assess the relative timing of *ht-en* expression in different teloblast lineages (see Materials and Methods for details; time line is not to scale).
m, o and p bandlets) or pair of blast cells (in the n and q bandlets) makes one segmental complement of definitive progeny, primary blast cell clones interdigitate mediolaterally and longitudinally during development (Weisblat et al., 1984; Weisblat and Shankland, 1985; Torrence and Stuart, 1986), so that most classes of blast cell clones are ultimately distributed across segment boundaries.

Segment-specific age disparity of consegmental primary blast cell clones

The first blast cells born in each ectodermal bandlet (i.e. those fated to contribute progeny to the most rostral segments) are in contact with the consegmental ipsilateral blast cells in other bandlets within the nascent germinal band from the time of their birth (Sandig and Dohle, 1988). However, due to the lineage-specific differences in the number of primary blast cells used to make one segmental complement of progeny, consegmental primary blast cell clones contributing to caudal segments come into register only after the n and q bandlets slide rostrally with respect to the m, o or p bandlets (Weisblat and Shankland, 1985; Shankland, 1991). The details of the timing of this process remain to be determined, but also vary in a segment-specific manner.

Despite the lineage-specific differences in the number of primary blast cells used to make one segmental complement of definitive progeny, each teloblast generates its blast cell progeny at approximately the same rate (i.e., roughly one cell per hour; Wordeman, 1982). A consequence of this type of segmental assembly is that each segmental anlage comprises an aggregate of blast cell clones that have a unique set of age relationships, especially when comparing the N or Q contributions to the M, O or P contributions. While the most rostral segments contain progeny of blast cells born within a few hours of each other, there is a rostrocaudal gradient of increasing age disparity between the m, o or p blast cell clones and the n or q blast cell clones that contribute to the same segment (Fig. 3). Thus, caudal segments comprise progeny of n and q primary blast cells born many hours after the consegmental progeny of m, o and p primary blast cells.

**ht-en is expressed transiently in each teloblast lineage during early development**

During germinal plate formation, *ht-en* is expressed in each of the distinct teloblast lineages that contribute to the mature segments of the leech. Within each teloblast lineage, *ht-en* is expressed in a specific rostrocaudal domain at any one time in early development. αht-en staining is faint at both the rostral and caudal edges of the domains of expression and as development proceeds the domains of *ht-en* expression occupy progressively more caudal portions of the germinal band or germinal plate. These observations indicate that *ht-en* is transiently expressed in each lineage during this period of early development.

To facilitate the characterization of the spatial and temporal pattern of *ht-en* expression in primary blast cell clones, lineage tracers were injected into one or more of the five teloblasts (or OP proteloblast) at various times during stages 6-7 and the patterns of lineage tracer and *ht-en* immunoreactivity were examined in resultant embryos ranging from late stage 7 through late stage 8. Our analy-
The rostrocaudal gradient of increasing age disparity between consegmental n or q and m, o or p blast cell clones. Schematic of one side of the germinal plate. Clones are represented by numbered blocks; numbers denote the hypothetical birth ranks of the parental primary blast cells in each lineage. Each segmental anlage contains the equivalent of one primary blast cell clone from each m, o and p bandlet. Since primary blast cells are born at the rate of approximately one per hour, the birth ranks and clonal ages of consegmental primary blast cell clones are more similar in rostral than in caudal segments.

Engrailed homologue expression in leech embryos

Ectodermal lineages

The earliest expression in the embryo of *ht-en* is in the most rostral segments in the OP lineage during late stage 7, when the embryo is approximately 61 hours old. At this time, the first op blast cell clone is approximately 36 hours of age and contains about 5 cells (data not shown). The pattern of *ht-en* expression is the same in all four op primary blast cell clones. To examine this pattern, an OP proteloblast was injected with lineage tracer immediately prior to the division that generates the third op blast cell and the resultant embryos were fixed at times ranging from 44-74 hours thereafter (n=26). When the third op blast cell clone is at clonal age 44 hours and contains about 8 cells, a single cell, located medially in the most rostral row of cells in each clone expresses *ht-en* (Fig. 4A-C). This cell lies over the transverse cleft that separates the underlying somites in the m bandlet (data not shown). By the time the third op clone is at clonal age 50 hours, a second cell, located just caudal to the first, has become immunoreactive. The lineage of the op blast cell clone has not been analyzed beyond the first few divisions, so that the exact identities of these *ht-en*-expressing cells are unknown. The staining in these immunoreactive cells starts to fade by clonal age 66 hours. One cell still stains faintly at clonal age 74 hours.

In the p bandlet (n=16), *ht-en* expression can first be detected in primary blast cell clones at clonal age 34 hours. At this point the clone contains only five cells; *ht-en* is expressed in cell p.ap (Fig. 4D-F). Cell p.ap divides at clonal age 35 hours, generating daughter cells p.apm and p.apl. Although both cells express *ht-en*, p.apm eventually stains much more darkly than p.apl. The staining in these immunoreactive cells gradually fades and is usually difficult to distinguish from background staining after approximately 60 hours.

In the o bandlet (n=28), *ht-en* expression can first be detected in primary blast cell clones at clonal age 50 hours. At this point the o clone contains six cells; *ht-en* is expressed in cell o.aap (Fig. 4G-I). Cell o.aap divides transversely at clonal age 58 hours. Both daughter cells, o.aapm and o.aapl, continue to express *ht-en* (see also Fig. 5A). By clonal age 70-75 hours, three adjacent *ht-en*-expressing cells in the o bandlet can often be detected along the border between the nf and o clones, but whether or not the third cell is also a descendent of o.aap remains to be determined. Several hours later, *dht-en* staining in the o lineage becomes difficult to distinguish above background.

In the q bandlet (n=20), *ht-en* expression is first detected in a single cell at clonal age 48 hours and soon thereafter in neighboring cells. As early as clonal age 50 hours, *ht-en* is detected in from two to five cells (Fig. 5A-B). All of these immunoreactive cells in the Q lineage are derived from qf primary blast cells: the first cell to stain with *dht-en* is qf.pa or qf.pp, followed later by the mitotic progeny of these cells, namely qf.pam, qf.pal, qf.ppm and qf.ppl. In some specimens, a small, Q-derived cell with a pycnotic nucleus, presumably qf.a, was also stained with *dht-en*. Cell qf.a is difficult to identify by this point in the development of the qf clone (Bissen and Weisblat, 1989), presumably because it is dying (S. A. Torrence, personal communication). Thus, we conclude that, during this period, all of the viable cells in the qf primary blast cell clone express *ht-en*. Immunoreactivity subsequently decreases in some cells before others and by clonal age 62-64 hours no cells stain above background levels (n=13).
In the N lineage, *ht-en* expression begins considerably later than in other ectodermal lineages, at clonal age 63-67 hours \((n=16)\). The first expression of *ht-en* in this lineage is by a descendant of the nf primary blast cell, but the exact identity of this cell remains to be determined, since details of the nf sublineage have not yet been extended to this stage.

---

**Fig. 4.** *ht-en* expression in the OP, O and P lineages. Germinal bands and germinal plates were dissected from stage 8 embryos. Rostral is up and medial is to the right in all panels. Nuclei of cells expressing *ht-en* are stained with the brown HRP reaction product; all nuclei appear blue when illuminated to view Hoechst fluorescence, except when obscured by heavy HRP reaction product (see Materials and Methods). (A) Double exposure (DIC and epifluorescence) showing RDA lineage tracer (red) and immunoreactive nuclei (brown, arrows) in the third and fourth op blast cell clones (clonal ages 51 and 50 hours, respectively) in an early stage 8 embryo. (B) Epifluorescence view of Hoechst-stained nuclei in same field as in A. (C) Tracing of the two op clones. (D) Double exposure (DIC and epifluorescence) showing RDA lineage tracer and immunoreactive nuclei (arrows) in two p blast cell clones in a mid stage 8 embryo. Staining in the medial cell, p.apm, of the caudal clone (clonal age approx. 45 hours) is (uncharacteristically, cf Fig. 5A) lighter than in the lateral cell, p.apl. Immunoreactivity of cells in the more rostral clone (clonal age approx. 46 hours) has started to decline. (E) Epifluorescence view of Hoechst-stained nuclei in same field as in D. (F) Tracing of the p bandlet, with individual blast cells identified. (G) Photomicrograph (DIC optics) showing immunoreactive nuclei (cell o.aap, arrows) in two o blast cell clones (clonal ages approx. 41 and 40 hours for rostral and caudal clones, respectively) in a mid stage 8 embryo. (H) Double exposure showing RDA lineage tracer and Hoechst-stained nuclei in same field as in G. The o bandlet (left) is faintly labeled and the n bandlet (right) is brightly labeled with lineage tracer. Fluorescence of immunoreactive nuclei is partially obscured by the brown precipitate. (I) Tracing of the o bandlet, with individual blast cells identified. In C, F and I, darkly stained *ht-en* immunoreactive nuclei are indicated as shaded circles and more lightly stained nuclei are indicated by circles filled with hatching; dashed lines indicate approximate borders between primary blast cell clones. Scale bars, 5 µm.
Approximately 4 hours later, two cells in an nf clone express *ht-en* and, a few hours later still, three adjacent cells stain with *αht-en*. Additional nf-derived cells stain soon thereafter, so that, by clonal age 78 hours, the previously described stripe of *ht-en* immunoreactive nuclei in the posterior portion of the segmental anlage (Wedeen and Weisblat, 1991) is present and comprises at least five cells. While the stripe is usually composed of a single row of nuclei that extends laterally as more nf-derived cells stain with *αht-en*, occasionally one or two immunoreactive nuclei are seen immediately rostral to the rest. Mitotic cells staining with *αht-en* were frequently observed in the stripe, indicating that at least some of the extension of the stripe reflects clonally inherited expression. At clonal age of about 90–92 hours, gaps in the stripe appear, suggesting that by this time some cells have either stopped expressing the antigen, died or migrated.

**Mesodermal lineage**

The m bandlet is positioned beneath the ectodermal bandlets, relative to the surface of the embryo, and spans almost the entire width of the germinal band (Fig. 2). The expression of *ht-en* in the M lineage can first be detected in a single cell located close to the ventral midline at clonal age

![Figure 5](image_url)
66 hours (n=13). Approximately 2 hours later, a pair of cells in the clone express ht-en (Fig. 5E-G); one cell lies caudal to the other and frequently stains more darkly with αht-en. The two cells appear to be sisters, since mitotic cells expressing ht-en were often observed in segments between those segments with one and those with two stained cells. No additional cells expressing ht-en in the m bandlet were observed, even though mitotic divisions of one or the other of the pair of cells were observed in a few specimens at about clonal age 71-73 hours. By clonal age 78 hours, the expression of ht-en in the m bandlet was difficult to detect above background. The precise identity of the cells expressing ht-en in an m clone remains to be determined because the M lineage has not been analyzed in detail beyond the first few divisions.

**ht-en expression is correlated with clonal age, not segment age**

The notion that all of the clones arising from each class of blast cells exhibit the same pattern of ht-en expression is supported by the observation that equivalent patterns of ht-en expression are present in a range of rostral and caudal segments within each of the five teloblast lineages. Moreover, within each teloblast lineage in a given specimen, ht-en expression is observed in a limited domain of consecutive blast cell clones. As development proceeds, this domain of expression is observed in a progressively more caudal portion of the bandlet, as would be expected if expression is transient and is correlated with the age of the blast cell clone.

If ht-en expression in each teloblast lineage is strictly correlated with clonal age, then the patterns of its expression within any given segment taken as a whole should reflect the segment-specific age disparity of the primary blast cells contributing to that segment. In that case, the timing of ht-en expression in the n or q bandlets relative to that in the m, o or p bandlets should be different in rostral and caudal segments; the delay in the initiation of ht-en expression in the N or Q lineages relative to its initiation in the M, O, or P lineages should be greater in caudal segments than in rostral segments. Alternatively, the initiation of ht-en expression in each teloblast lineage could be determined by some property of the entire segment, in which case the pattern of expression within the segment as a whole should be the same in rostral and caudal segments.

To distinguish between these possibilities, we compared the position of the ht-en expression domains in the N and Q lineages to the position of an o reference clone at two specific clonal ages in a rostral segment and carried out parallel observations using an o reference clone (at the same two clonal ages) in a caudal segment. The design of the experiments is summarized in Fig. 1. In one group of embryos, an O/P teloblast was microinjected with lineage tracer 8 hours after it was born; the reference clone, i.e. the first o (or p) primary blast cell born after the injection of the lineage tracer, contributed definitive progeny to segments M7 and M8. [The definitive progeny of a single o or p primary blast cell are distributed over two adjacent segments (Weisblat and Shankland, 1985).] In another group of embryos, an O/P teloblast was injected 20 hours after it was born; in this group, the o (or p) reference clone contributed definitive progeny to segments M19 and M20. [For simplicity, the positions within the germinal plate of the o (or p) reference clones in these embryos will be...
referred to as M8 and M20, respectively. In both groups of embryos, the ipsilateral N teloblast (and, in several experiments, a Q teloblast as well) was injected with a different lineage tracer 6 hours after the O/P teloblast was injected to facilitate the identification of cells expressing *ht-en* and to verify independently the ages of clones in that lineage. The patterns of *ht-en* expression were examined in 21 such embryos in which the o (or p) reference clones in segment M8 or M20 were at clonal ages of approximately either 68 or 78 hours. After the immunohistochemical procedures, the germinal plates were dissected from the embryos and mounted, and the complete patterns of cells labeled by αht-en and/or with lineage tracer were recorded.

The positions of the domains of *ht-en* expression in the bandlets exhibit a caudal shift in older embryos, as would be expected from the temporal gradient of development within the germinal plate. More importantly, however, a comparison of the positions of the domains of *ht-en* expression in the n and q bandlets relative to the position of the o (or p) reference clones in segment M8 or M20, at both 68 and 78 hours, reveals that *ht-en* expression is correlated with the age of individual blast cell clones (Fig. 6). For example, when the o reference clone in segment M8 is about 78 hours old (with two cells staining darkly and one staining faintly with αht-en), *ht-en* expression in the con- segmental q clone (clonal age 69 hours) is no longer

---

**Fig. 7.** Dynamics of *ht-en* expression: II. Views of an entire germinal plate dissected from an embryo that had been fixed when the o and p clones located in segment M8 were at clonal age 78 hours. (See Fig. 1 and Materials and Methods for details.) Rostral is up and ventral midline is at the center in each panel. Black (in A,B, and D) or brown (in C) spots correspond to *ht-en* immunoreactive nuclei. (A) and (B) Photomontages (DIC optics) at two different focal planes showing primarily mesodermal and primarily ectodermal tissues, respectively. Not all *ht-en* immunoreactive nuclei are in focus in either panel, and some labeled nuclei appear in both panels. Locations of the o reference clone (arrows) and of the most rostral labeled n primary blast cell clone (arrowheads) are indicated. (C) Photomontage (triple exposure, epifluorescence) showing DAPI-stained nuclei (blue dots) and lineage tracers. The o and p bandlets on the right side of the germinal plate are both labeled with RDA lineage tracer (red). The ipsilateral n and contralateral q bandlets are labeled with FDA lineage tracer (green); the most rostral labeled primary blast cell clones (clonal age = 72 hours) in these bandlets are located in segment M7. The brown precipitate corresponding to a few of the *ht-en* immunoreactive nuclei can be seen. (D) Composite drawing showing lineage tracer and *ht-en* immunoreactive nuclei in ectodermal bandlets only. Bracket to left shows the distance between most rostral labeled n and o primary blast cell clones resulting from the six hour gap between the injection of the O/P and N teloblasts (cf. Fig. 8D). Vertical lines to the right of the embryo show domains of *ht-en* expression in the N and Q lineages (cf. Fig. 8D). Arrow and arrowhead point to the same points as in panels A and B. Scale bars, 25 μm.
detectable and expression in the consegmental n clone (clonal age 69 hours) is well under way (Fig. 7). In contrast, when the o clone in M20 has reached clonal age 78 hours (and shows the same three-cell expression pattern; Fig. 8), the consegmental q clone (clonal age 61 hours) still contains an immunoreactive cell. The consegmental n clone (clonal age 61 hours) has just begun to express ht-en.

These results demonstrate that the patterns of ht-en expression reflect the rostrocaudal gradient of increasing age disparity between the N or Q and O lineages. Similar observations have been made regarding the dynamics of the patterns of ht-en expression in the M and P lineages. Thus, we conclude that ht-en expression is correlated with the age of individual blast cell clones and not with the age of the segment taken as a whole. It should be clear, however, that this correlation between clonal age and ht-en expression in unperturbed embryos does not constitute proof of a causal relationship between the two.

Cells expressing ht-en during germinal plate formation lie within a limited posterior region of the segmental anlage

The differences between teloblast lineages in the timing of ht-en expression, combined with the transient nature of its early expression, make it somewhat problematic to compare directly, within one segmental anlage, the spatial rela-

![Fig. 8. Dynamics of ht-en expression: III. Views of an entire germinal plate dissected from an embryo that had been fixed when the o and p clones located in segment M20 were at clonal age 78 hours. (See Fig. 1 and Materials and Methods for details.) Rostral is up and ventral midline is at the center in each panel. Black (in A, B, and D) or brown (in C) spots correspond to ht-en immunoreactive nuclei. (A) and (B) Photomontages (DIC optics) at two different focal planes showing primarily mesodermal and primarily ectodermal tissues, respectively. Not all ht-en immunoreactive nuclei are in focus in either panel, and some labeled nuclei appear in both panels. Locations of the o reference clone (arrows) and of the most rostral labeled n primary blast cell clone (arrowheads) are indicated. (C) Photomontage (triple exposure, epifluorescence) showing DAPI-stained nuclei (blue dots) and lineage tracers. The o bandlet on the left side of the germinal plate is labeled with RDA lineage tracer (red). The ipsilateral n and q bandlets are labeled with FDA lineage tracer (green); the most rostral labeled primary blast cell clones (clonal age = 72 hours) in these bandlets are located in segment M14. (The contralateral n bandlet is faintly labeled with RDA from an injection not relevant to this experiment.) The brown precipitate corresponding to a few of the ht-en immunoreactive nuclei can be seen. (D) Composite drawing showing lineage tracer and ht-en immunoreactive nuclei in ectodermal bandlets only. Bracket to left shows the distance between most rostral labeled n and o primary blast cell clones resulting from the six hour gap between the injection of the O/P and N teloblasts (cf. Fig. 7D). Vertical lines to the right of the embryo show domains of ht-en expression in the N and Q lineages (cf. Fig. 7D). Arrow and arrowhead point to the same points as in panels A and B. Scale bars, 25 µm.
tionship of *ht-en* expression in all of the lineages. The best direct data on this issue may be obtained from examining embryos in which the n and q blast cell clones in segment M20 are at about 61-62 hours clonal age and the conseq-
ential m and o (or p) clones are at about 75 hours and 78 hours clonal age, respectively. Although *ht-en* expression in the P lineage in this segment has long since ceased by this time, expression is seen in the other four lineages (Fig. 9). In the N lineage, in which *ht-en* expression is just begin-
nng, the presence of a single cell expressing *ht-en* may be used as a marker for the position of the nascent transverse band of nf-derived immunoreactive nuclei. Moreover, since the o and p bands do not move relative to one another along the rostrocaudal axis, we can infer the position, within the germinal plate, of the cells in the p bandlet that had expressed *ht-en* from the observation that cells expressing *ht-en* in the o and p bandlets are in close register earlier, when conseqental o and p blast cell clones are 50-60 hours clonal age (Fig. 9D).

Thus, it is possible to form a schematic representation (Fig. 9F) of the distribution of cells within the segmental anlage that express *ht-en* during germinal plate formation (stages 8-9). From this it is clear that the cells expressing *ht-en* lie within a relatively narrow transverse region of the

---

**Fig. 9.** During early *ht-en* expression, conseqential immunoreactive nuclei lie within a relatively narrow region in the segmental anlage. Rostral is up in each panel. Brown precipitate (A and E) and black dots (B-D and F) correspond to immunoreactive nuclei. (A) Photomicrograph (DIC optics) of the caudal portion of a germinal plate dissected from a mid stage 8 embryo. In the most rostral segment shown (B), *ht-en* expression in the N lineage has just begun and domains of *ht-en* expression in the n and m bandlets overlap; in three more caudal segments (C), domains of expression in the m, o and p bandlets overlap and immunoreactive nuclei can be seen in a relatively narrow transverse region of each segment; in the most caudal segments shown (D), domains of expression in the o and p bandlets overlap. (B-D) Drawings of the corresponding sections from panel A, showing immunoreactive nuclei. Bandlets are indicated by lower case letters. Vertical dashed lines in B and C indicate the ventral midline. (E) Double exposure (epifluorescence) of four segments (M19-M22) on the left side of a germinal plate dissected from a mid stage 8 embryo. Ventral midline is to the right; superficial plane of focus, showing the q (left) and n (right) bandlets labeled with FDA lineage tracer (green). The m bandlet is out of the plane of focus. Except where obscured by brown HRP reaction product, all nuclei appear blue. Not all nuclei in the ectodermal bandlets are in the plane of focus. Asterisks in the second segment (M20) indicate the inferred locations of nuclei in the p bandlet that had previously expressed *ht-en* (left) and of *ht-en* immunoreactive nuclei in the underlying m bandlet (right). Scale bar, 50 µm in A; 25 µm in B-D; and 10 µm in E-F.

---

indicated by stippling; circles indicate nuclei in the plane of focus; shaded circles denote immunoreactive nuclei in the plane of focus.
segmental anlage. Moreover, the location of this region relative to the boundaries of the prospective segment can be deduced from the fact that the nf clone contributes progeny primarily to the posterior half of the segmental ganglion. This narrow distribution of immunoreactive cells within a segment is in contrast to the broad rostrocaudal distribution of cells that express ht-en within a segment in the juvenile and adult leech (stage 10 and beyond; Fig. 10).

Cells expressing ht-en during germinal plate formation show no special clonal relationship to those expressing ht-en during terminal differentiation

The spatial and temporal pattern of the segmentally iterated ht-en immunoreactive cells in the ganglion and body wall during late embryonic development has previously been described (Wedeen and Weisblat, 1991). While the specific identity of most of these cells has yet to be determined, the lineage or sublineage has been determined for some of them by using injected lineage tracers in combination with immunohistochemical detection of ht-en expression and is summarized in Fig. 10.

The set of cells that express ht-en after gangliogenesis is clearly not restricted to the descendants of cells that express ht-en during germinal plate formation. Within the N lineage, the peripheral neurons, nz1 and nz2, that express ht-en at stage 10 are definitive progeny of the nf blast cell and arise as part of the segmentally iterated stripe of cells that express ht-en in the posterior portion of the anlage. However, an N-derived definitive neuron located in the anterior portion of the segmental ganglion also expresses ht-en at stage 10; this cell (nz5) is descended from the ns blast cell, whose progeny do not express ht-en during germinal plate formation. Similarly, within the P lineage, a cell in the body wall that either is the peripheral neuron pz8 or is part of the cell floret 3 (cf3) expresses ht-en during stage 10; both pz8 and cf3 are descended from cell p.p (Shankland 1987b), whereas the early expression of ht-en in the P lineage is confined to a subset of the progeny of cell p.a. Finally, within the O lineage, a pair of cells (oz4 and oz5) located in the anterodorsal cluster (AD) of each ganglion expresses ht-en during stage 10, and one of these (oz4) continues to express ht-en at stage 11; cells of the AD cluster are descended from o.apa (Shankland 1987a), whereas progeny of cell o.a express ht-en during the earliest phase of ht-en expression.

DISCUSSION

Early patterns of ht-en expression

In this study, we have used immunostaining with the antibody αht-en (Wedeen and Weisblat, 1991) to analyze the earliest expression of ht-en, an en-specified-class protein, in embryos of the glossiphoniid leech Helobdella triserialis. During germinal plate formation, ht-en is transiently expressed in each of the distinct teloblast lineages that contribute to the mature segments of the leech. In those lineages in which ht-en is expressed at early clonal ages, we have been able to demonstrate unambiguously that ht-en is expressed in a stereotyped subset of identifiable cells in the segmentally iterated primary blast cell clones. The precise timing of ht-en expression within a teloblast lineage is correlated with the age of the primary blast cell clone and not with the age of the segment as a whole. Whether or not this correlation is indicative of a causal relationship between clonal age and protein expression remains to be determined.

During this early period, there is a marked spatial coherence of the subsets of cells that express ht-en in the different teloblast lineages, despite the fact that the timing of ht-en expression differs between lineages. Cells from different teloblast lineages that have expressed, or will express, ht-en are aligned within a relatively narrow transverse region of the segmental anlage. The anterior/posterior position of this region depends on how one defines the segment boundaries in the leech. The commonly accepted definition of segment boundaries places the neural annulus and ganglion centrally within the segment (Sawyer, 1986). Accordingly, the region of early ht-en expression, which is aligned with the posterior portion of the future ganglion, lies in the posterior portion of the segmental anlage.

Functional significance of ht-en expression

Although it is impossible to draw rigorous conclusions of tracer and brown HRP reaction product. Immunoreactive nuclei for which the lineage of origin has been determined are indicated in the second segment (arrowheads), including: a ventral anterior ganglionic neuron derived from an ns primary blast cell (nz5); a dorsal anterior ganglionic neuron derived from the o bandlet (oz4); a lateral ganglionic neuron derived from the p bandlet (pz11); a pair of previously described peripheral neurons derived from an nf primary blast cell (nz1, nz2); and an epidermal or neural cell from the p bandlet (p) that is either a previously identified neuron, pz8, or part of a previously described epidermal specialization, cf3. The most lateral immunoreactive nuclei probably arise from the Q lineage. The vertical dotted line indicates the ventral midline. The dorsal midline is at the right margin. The nephridia (open arrow) stain artifactually. Scale bar, 50 µm.

Fig. 10. Late expression of ht-en. Photomicrograph (DIC optics) showing a ventral view of three midbody segmental ganglia (left) and left body wall (right) of a stage 11 embryo dissected along the dorsal midline and flattened. Rostral is up. The lineage of cells was determined in a different set of embryos (not shown) by injecting individual teloblasts with lineage tracer at stage 6 and looking for colocalization.
regarding the role of a protein merely by observing its expression in normal development, the observed patterns of ht-en expression permit us to speculate about its developmental significance in leech. A priori, this putative transcription factor might have a ‘proximate’ effect, by inducing a phenotypic change in the cells in which it is expressed, or a ‘delayed effect’, by participating in regulatory processes that give observable changes in cell phenotype only after one or more additional rounds of cell division.

ht-en must have a proximate effect, if it has any effect at all, when it is expressed in postmitotic cells, such as the subset of ganglionic neurons in late development. Here, ht-en might participate in controlling genes related to the expression of particular phenotypic characteristics (e.g. branching patterns, excitability, transmitter use) of these individually identifiable neurons in the segmental ganglia.

Another proximate effect of ht-en might be to regulate phenotypic characteristics related to cell adhesion, movement and/or mingling. In Drosophila embryos, en is thought to play a role in establishing or maintaining embryonic compartment boundaries (D'Nardo et al., 1985; Fjose et al., 1985; Kornberg et al., 1985; Poole et al., 1985) and, in zebrafish embryos, the expression of engrailed-like antigens in a subset of cells has been correlated with the subsequent morphogenetic movements of those cells (Hatta et al., 1991). The expression patterns that we observed for ht-en are consistent with the idea that ht-en plays a similar role in the early Helobdella embryo. For example, within the Q lineage, only progeny of qf blast cells migrate medially to contribute cells to the ventral nerve cord (Weisblat et al., 1984; Kramer and Weisblat, 1985; Torrence and Stuart, 1986; Bissen and Weisblat, 1987) and only progeny of qf blast cells show early expression of ht-en. Moreover, expression of ht-en by the qf-derived cells starts to decrease only after cells in adjacent qs clones begin to migrate, consistent with the notion that ht-en serves to restrict migratory propensities that might otherwise be exhibited during this period. In the N lineage, all of the neuronal progeny of an ns primary blast cell are confined to the rostral half of the ganglion; although most of the progeny of an nf primary blast cell lie in the caudal half of the ganglion, some of the progeny come to lie in the rostral part of the next caudal ganglion (D. Stuart, personal communication). The transverse row of cells that express ht-en in the nf clone might serve as a boundary between cells with different adhesion properties or as a barrier to cell movements, thereby defining the rostrocaudal boundary between the subpopulations of the nf clone that will come to occupy different ganglia.

The striking transverse alignment of cells in different teloblast lineages that express ht-en during stages 7-9 might suggest a role for ht-en in the process by which segmental blast cell clones are brought into segmental register. The temporal patterns of ht-en expression, however, argue against any such role. First, from the observation that the first blast cells born in each bandlet are in contact with segmental ipsilateral blast cells from the time of their birth (Sandig and Dohle, 1988) and our results regarding the timing of ht-en expression, it follows that the clones contributing to rostral segments are in register for periods ranging from approx. 36 hours (in the case of op blast cells) to approx. 67 hours (in the case of n blast cells) prior to ht-en expression. Moreover, it is also unlikely that ht-en expression is activated exclusively by positional cues in the posterior region of the segment because, in caudal segments, the interval between segmental registration and the initiation of ht-en expression is much shorter and their order may even be reversed. For example, although the dynamics of segmental alignment have not been measured precisely, it is known that clones contributing to segments M13-M15 do not come into register until the o and p clones are between the ages of 32 and 54 hours (S. Blair, personal communication); ht-en expression in the p lineage begins no later than clonal age 34 hours.

Evidence for the idea that ht-en might act with a ‘delayed’ effect comes from comparing the definitive progeny of cells that express ht-en early in the O and P lineages. Although critical details of these lineages remain to be determined, cells o.aap and p.aap have been shown to give rise to comparable sets of definitive progeny at stage 10, which may include the peripheral dopamine-containing neurons LD2 and LD1, respectively (Shankland, 1987a,b); both o.aap and p.aap express ht-en during stage 8. Similar parallels might exist between cells expressing ht-en in the OP, O and P lineages (Shankland, 1987c; Lans, 1992), since, in normal development, the four primary op blast cells contribute sets of descendants to segments R1-R4 which resemble the combined contributions of primary o and p blast cells in more caudal segments (Weisblat and Blair, 1984).

Comparison of patterns of engrailed-class gene expression in leech and other animals

There is now information available on the expression of engrailed-class genes in representatives of four major animal groups, Annelida, Arthropoda, Echinodermata and Chordata (Wedeen and Weisblat, 1991; this paper; Fjose et al., 1988; Patel et al., 1989a,b; Hui et al., 1992; Dolecki and Humphries, 1988; Joyner and Martin, 1987; Gardner et al., 1988; Hatta et al., 1991; Hemmati-Brivanlou et al., 1991). It is assumed that these groups have been separated for a minimum of 500 million years of evolutionary history and that they arose from an ancestor that already featured bilateral symmetry and a well-defined rostrocaudal axis. However, it seems likely that segmentation arose after the lineage that gave rise to echinoderms and chordates diverged from the lineage that gave rise to annelids and arthropods. Comparing the features of en-class gene expression among these groups is one approach to understanding the nature of the common ancestor and the original function of en-class genes.

The pattern of expression of the en-class protein in leech shows significant similarities to those in arthropods, including early expression in the ectoderm in a transverse region in the posterior of the segmental anlage and later expression in subsets of neuroblasts and/or postmitotic neurons (Wedeen and Weisblat, 1991; this study). The failure to observe iterated transverse bands of expression in the ectoderm of chordates may indicate that this trait arose in the annelid/arthropod line after its separation from the chordate line. Conversely, the presence of neural expression in all three groups (as well as in the echinoderms) is consistent
with the notion that this was a feature of a common ancestor. This latter conclusion is consistent with those of Patel et al. (1989b).

The spatial coherence between the subsets of cells that express ht-en in the different teloblast lineages is reminiscent of the expression of en-class proteins in the posterior compartment of early arthropod embryos. In this regard, it is interesting that prior to cell migration in the germinal plate, the rostral and caudal borders of an o clone align with the rostral border of the nf clone and the caudal border of the ns clone, respectively; thus, a primary o blast cell clone straddles the boundary between prospective segmental ganglia. Although the definitive progeny of a single o primary blast cell are broadly distributed across more than one segment (Weisblat and Shankland, 1985), this early alignment of the o clone astride the prospective segmental borders is reminiscent of the Drosophila parasegment (Martinez-Arias and Lawrence, 1985).

A significant difference between the patterns of en-class gene expression in Helobdella and Drosophila, however, is that mesodermal expression of an en-class protein is seen in leech, but has not been reported in fruit fly. Mesodermal expression of en-class genes has been reported for various vertebrates, including zebrafish (Hatta et al., 1991), chick (Davis et al., 1991) and mouse (Davis et al., 1991). Moreover, the mesodermal expression of the vertebrate en-class proteins is segmentally iterated, whereas the ectodermal expression is not. A parsimonious explanation for these observations is that mesodermal expression of an en-class protein was a feature of the common ancestor of all three groups and was lost after the separation of the annelid and arthropod lineages.

We are grateful to Seth Blair, Richard Kostriken, Marty Shankland, Duncan Stuart and Steve Torrence for helpful discussions. This work was supported by a graduate traineeship to D. L. from NIH Grant GM 07048, fellowship DRG 925 to C. J. W. from NIH First Grant HD 23328 to D. A. W.

REFERENCES


(Accepted 18 December 1992)