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[REVIEW]

Segmentation in Annelids: Cellular and Molecular Basis for Metameric Body Plan

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ABSTRACT—Annelids are segmented animals that display a high degree of metamerism in their body plan. This review describes the segmentation of clitellate annelids (i.e., oligochaetes and leeches) and polychaetes with special reference to cellular and molecular mechanisms elaborating the metameric body plan. In clitellate embryos, segments arise from five bilateral pairs of longitudinal coherent columns (bandlets) of primary blast cells that are generated by five bilateral pairs of embryonic stem cells called teloblasts (M, N, O, P and Q). Recent cell-ablation experiments have suggested that ectodermal segmentation in clitellates consists of two stages, autonomous morphogenesis of each bandlet leading to generation of distinct cell clumps (i.e., segmental elements; SEs) and the ensuing mesoderm-dependent alignment of separated SEs. In the N and Q lineages, SEs are each comprised of clones of two consecutive primary blast cells. In contrast, in the O and P lineages, individual blast cell clones are distributed across SE boundaries; each SE is a mixture of a part of a more-anterior clone and a part of the next more-posterior clone. In contrast, the metameric segmentation in the mesoderm (M lineage) is a one-step process in that it arises from an initially simple organization (i.e., a linear series) of primary blast cells, which individually serve as a founder cell of each segment; the boundary between mesodermal segments is determined autonomously. Cell-autonomous properties of primary blast cells have also been suggested in two fundamental aspects of segmentation, viz., specification of segment polarity and determination of segmental identities. Recent cell-ablation and -transplantation studies have suggested that segmental identities in primary blast cells derived from M teloblasts are determined according to the genealogical position in the M lineage and that the M teloblast possesses a developmental program through which the sequence of blast cell identities is determined. It is unlikely that either a segment polarity gene *engrailed* or *Hox* genes are involved in specifying polarity or identities of segments of clitellates, since these genes (in leeches) are reportedly expressed long after the establishment of these segmental properties. As in clitellates, segments in polychaetes arise from descendants of teloblasts located in a posterior growth zone, but it is only during trochophore larval stages that overt segmental organization becomes recognizable. At present, it is not known how the posterior growth zone generates trunk segments either during larval stages or after metamorphosis. However, the recent finding that *Hox* genes are expressed in the growth zone (probably in teloblasts) suggests that segmentation mechanisms in polychaetes are distinct from those in clitellates.

INTRODUCTION

Metameric body plans are found in several metazoan phyla, including vertebrates, annelids and arthropods. It remains unclear, however, whether the segments of these animals evolved independently or whether they were derived from a common ancestor (Davis and Patel, 1999). To address this question, it is prerequisite to identify the ancestral condition within each phylum. It is therefore essential to examine not only the similarities and differences in the process of segmentation between these phyla but also how this process varies within phyla.

In the present article, we review what is known about the segmentation process in annelids. Annelid segmentation was long held to be homologous to that of arthropods, and segmentation was often used to unite these segmented phyla within a single clade “Articulata” (Willmer, 1990). However, recent analyses of molecular phylogenetic data, as well as evaluation of morphological data, have suggested that these two segmented phyla are actually more closely related to sev-

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eral unsegmented phyla than they are to each other (Eernisse *et al*., 1992; Aguinaldo *et al*., 1997; Moore and Willmer, 1997; Giribet and Ribera, 1998; Adoutte *et al*., 1999). Thus, the homology of annelid and arthropod segmentation has now been called into question. Embryological data obtained from recent analyses of annelid segmentation do not appear to favor the idea the segments of annelids and arthropods were derived from a common segmented ancester.

BASIC ANNELID ORGANIZATION

The phylum Annelida is subdivided into thee major groups (classes): Polychaeta, Oligochaeta and Hirudinida. Oligochaetes and hirudinidans (leeches) are sometimes united as a single class, Clitellata, due to their common possession of a clitellum, an organ that secretes a cocoon protecting the early embryo (Brusca and Brusca, 1990). Although annelids display great diversity in morphology and ecology, they share a remarkable degree of conservation of basic body plans as

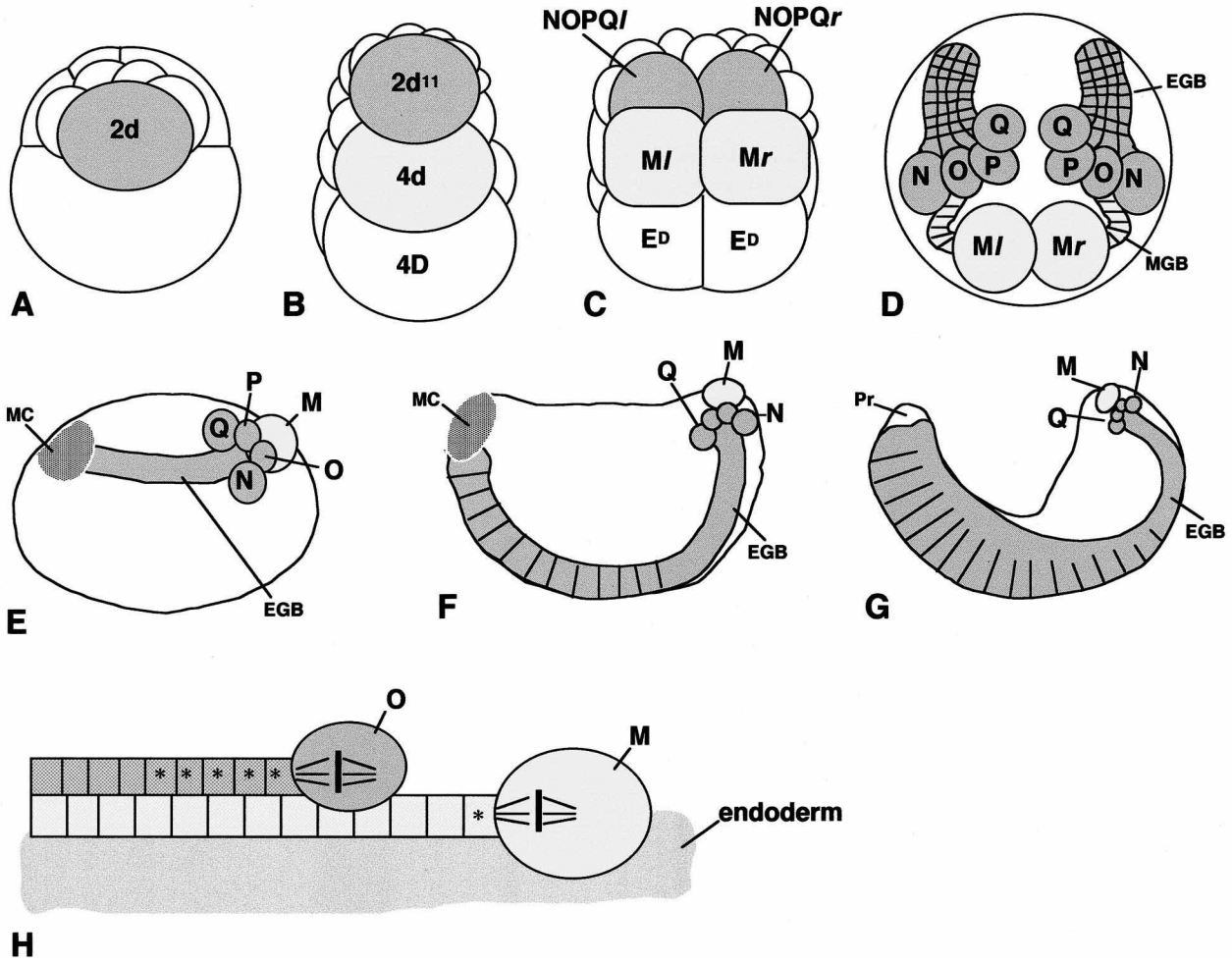

Fig. 1. Summary of *Tubifex* development. (A-C) Posterior view with dorsal to the top; (D) dorsal view with anterior to the top; (E–G) side view with anterior to the left and dorsal to the top. (A) A 9-cell-stage embryo shortly after the formation of cell 2d. (B) A 22-cell-stage embryo. Cells $2d^{11}$, 4d and 4D all come to lie in the future midline. (C) 4d divides bilaterally into left and right mesoteloblasts, M*l* and M*r*, $2d^{111}$ derived from $2d^{11}$ divides into a bilateral pair of ectoteloblast precursors (NOPQ*l* and NOPQ*r*), and 4D divides into a pair of endodermal precursor cells E^{D}. (D) A two-day-old embryo following the bilateral division of 4d. Only teloblasts and associated structures are depicted. NOPQ on each side of the embryo has produced ectoteloblasts N, O, P and Q. A short ectodermal germ band (EGB) extending from the ectoteloblasts N, O, P and Q is seen on either side of the embryo. A mesodermal germ band (MGB) extending from the M teloblast is located under the ectodermal germ band. (E-G) Morphogenesis of the ectodermal germ band. Embryos are shown at 2.5 (E), 4 (F) and 6 (G) days after the 4d cell division. The germ band (EGB) is associated, at its anterior end, with an anteriorly located cluster of micromeres (called a micromere cap; MC), and it is initially located at the dorsal side of the embryo (E). Along with their elongation, the germ bands (EGB) on both sides of the embryo gradually curve round toward the ventral midline and finally coalesce with each other along the ventral midline (F). The coalescence is soon followed by dorsalward expansion of the edge of the germ band (G). Pr, prostomium. (H) Longitudinal section showing the relative positions of the endoderm and bandlets extending from teloblasts M and O. Anterior is to the left and posterior is to the right. The bandlet (germ band) derived from the M teloblast is overlain by the o-bandlet and is underlain by the endoderm. Asterisks indicate the presence of a single primary blast cell in each block of the bandlet; the remaining blocks individually represent a cell cluster, which is derived from a single primary blast cell.

well as developmental patterns (for early embryonic development, see Anderson, 1966; Devries, 1968, 1973a; Fernandez and Olea, 1982; Shimizu, 1982). The distinguishing characteristic of the annelids is the division of the body into similar parts, or segments, which are arranged in a linear series along the anteroposterior axis. The youngest segments occur at the posterior end of the series. The segmented part of the body is limited to the trunk; the head, representated by the prostomium, is not a segment, nor is the pygidium, the terminal part of the body in which the anus is located (see Fig. 1G). The trunk segmentation is visible externally as rings (or annuli) and is reflected internally not only by the serial arrangement of coelomic compartments separated from one another by intersegmental septa but also by the metameric arrangement of organs and system components. It should be noted that unlike those in other segmented animals, the endodermal tissues of the midgut are also segmented in annelids (see Wedeen and Shankland, 1997).

The number of adult segments varies considerably among annelid species. In this connection, it is noteworthy that the number of "true" segments in the leech is fixed at 32 (Stent *et al*., 1982), while other annelids continue to elongate the body trunk during postembryonic development by the addition of segments to the caudal end of the body. At present, nothing is known about the mechanisms controlling the number of segments other than the reported findings in leeches that more than the required number of segmental founder cells are produced and that supernumerary cells are subsequently eliminated (see Shankland, 1984; Desjeux and Price, 1999).

EMBRYONIC ORIGIN OF SEGMENTAL BODY PLAN: TELOBLASTS

A long-held view as to the mode of spiralian development is that developmentally important cells, which are few in number, are set aside or segregated from the rest of the embryo early in development (Wilson, 1925; Freeman and Lundelius,1992; van den Biggelaar *et al*., 1997). Classic cell-ablation studies on clitellate embryos clearly showed that morphogenetic events such as body elongation and segmentation depend solely on the presence of the second (2d) and fourth (4d) micromeres of the D quadrant (Penners, 1924, 1926; Mori, 1932; Devries, 1973b). In fact, these micromeres are the main source of ectodermal and mesodermal segmental tissues (Weisblat *et al*., 1984; Goto *et al*., 1999b); none of the remaining cells can replace missing 2d and 4d micromeres in this respect. Consequently, oligochaete embryos from which both 2d and 4d micromeres have been ablated develop only into a ball of endoderm covered with an epithelial sheet of ectoderm (Penners, 1926). Recently, we have shown that D-cell line micromeres that have been transplanted to ectopic positions can induce segmental organization in embryos of the oligochaete *Tubifex* (A. Nakamoto, unpublished observation), confirming the developmental importance of the second and fourth micromeres of the D-cell line in generating segmental organization in annelid body plans.

The 2d and 4d micromeres of the D quadrant, which have been differently designated in different annelids (see Table 1), are precursors of embryonic stem cells called *teloblasts*. In clitellate annelids, the 2d micromere divides into a bilateral pair of proteloblast NOPQ, each of which generates, through an invariable sequence of cell division, four ectodermal teloblasts (ectoteloblasts N, O, P and Q) on either side of the embryo (Fig. 1C, D); the 4d micromere divides directly into a bilateral pair of mesodermal teloblasts (mesoteloblasts M; Fig. 1B, C). These teloblasts assume distinct fates; mechanisms for specification of teloblast fates have been discussed elsewhere (Huang and Weisblat, 1996; Arai *et al*., 2001).

Teloblasts in clitellate embryos are large enough to be identified under a dissecting microscope (Fig. 1A–D) and, as their name indicates, they are located at the posterior end of the embryo (Fig. 1E). Soon after their birth, teloblasts undergo extremely unequal divisions repeatedly to produce a coherent column (bandlet) of smaller daughter cells referred to as primary blast cells (Each bandlet and each blast cell are designated by the lower case letter corresponding to the teloblast of origin.). Four (n, o, p and q) of the five bandlets on each side of the embryo join together to form an ectodermal germ band (GB), while the remaining bandlet becomes a mesodermal GB, which underlies the ectodermal GB (Weisblat *et al*., 1980, 1984; Goto *et al*., 1999a, b). The GBs are initially

Table 1. Designations of D-cell line micromeres of annelids

	2^{nd} cell (ETB precursors)	~~4th~~ cell (MTBs)	Reference
Polychaeta			
Nereis	d^2 (X/X)	d^4 (M/M)	a
Platynereis	2d ($2d^{1121}$/$2d^{1122}$)	4d ($4d^1$/$4d^2$)	b
Oligochaeta			
Eisenia	2d (Ed/Eg)	4d (Md/Mg)	c
Tubifex	2d (NOPQl/NOPQr)	4d (Ml/Mr)	d
Hirudinida			
Helobdella	DNOPQ (NOPQl/NOPQr)	DM (Ml/Mr)	e
Theromyzon rude	SNOPQ (NOPQl/NOPQr)	SM (Ml/Mr)	f
T. tessulatum	2d (Tl/Tr)	4d (Ml/Mr)	g

ETB, ectoteloblast; MTBs, mesoteloblasts.
a, Wilson (1892); b, Dorresteijn (1990); c, Devries (1968); d, Shimizu (1982); e, Weisblat *et al.* (1980); f, Fernandez and Olea (1982); g, Sandig and Dohle (1988).

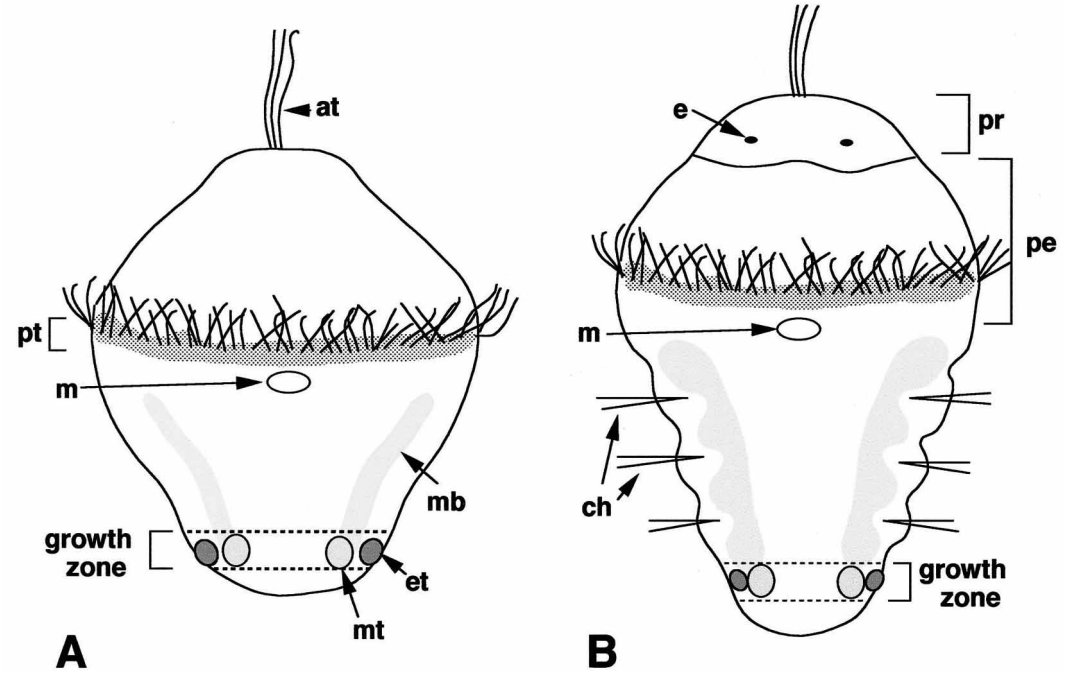

Fig. 2. Growth of a generalized polychaete trochophore larva. Frontal views. (A) Early larva with apical tuft (at), prototroch (pt), and a growth zone comprised of ectoteloblasts (et) and mesoteloblasts (mt). Note a bilateral pair of mesodermal bands (mb) extending from the mesoteloblasts. m, mouth. (B) Later larva with three segments. Each segment exhibits provisional chaetae (ch). e, eye; m, mouth; pe, peristomium; pr, prostomium.

located at the dorsal side of the embryo (Fig. 1D, E). Along with their elongation (which is brought about by addition of primary blast cells as well as proliferation of preexisting blast cells), they gradually curve round toward the ventral midline and finally coalesce with each other along the ventral midline (Fig. 1F). The coalescence is soon followed by dorsalward expansion of GBs. The edges of the expanding GBs on both sides of the embryo finally meet along the dorsal midline to enclose the yolky endodermal tube (Fig. 1G). As described below, segmentation in clitellate annelids is the process by which an initially continuous GB is divided into repeating units. In both oligochaetes and leeches, the formation of repeating units proceeds in an anterior-to-posterior succession in each GB. It should be noted, however, that the timing when each GB exhibits the first indication of this process is different between these annelid classes. While GBs in *Tubifex* embryos undergo division prior to ventral coalescence of GBs (Nakamoto *et al.*, 2000), those in the leech *Theromyzon* show indication of segmentation only after completion of GB coalescence (Shain *et al.*, 1998). However, in both classes, repeating units are established in each GB well before the onset of dorsalward expansion. We hereafter focus on segmentation events occurring prior to dorsal expansion of GBs.

Trunk body segments of polychaetes are also traced back to teloblasts derived from the D-cell line micromeres (Anderson, 1966). As is well known, polychaete embryos develop into trochophore larvae after gastrulation. Mesoteloblasts derived from the 4d micromere are initially located at the posterior surface of the embryo and they move into the interior, to a position on either side of the midline behind the midgut rudiment, during gastrulation. Ectoteloblasts derived from the 2d micromere are also located in the posterior region of the embryo; as expected, they remain at the embryo's surface during gastrulation. Thus, polychaete trochophore larvae possess a bilateral pair of mesoteloblasts and a superficial ring of ectoteloblasts just anterior to the pygidium (Fig. 2A); a posterior narrow zone comprised of the teloblasts has been called a (posterior) *growth zone* (Anderson, 1966).

As in clitellates, mesoteloblasts in polychaetes bud off smaller cells and form a pair of mesodermal bands, which later contribute mesoderm to trunk segments (Fig. 2). Although less characterized, ectoteloblasts in the growth zone have been thought to proliferate to supply segmental ectoderm. As described later, in many polychaete species, segmentation has a biphasic character; some anterior segments are formed during the larval stage and formation of subsequent adult segments occurs after metamorphosis. Both mesoteloblasts and ectoteloblasts are involved in segmentation of both phases.

SEGMENTATION IN OLIGOCHAETES

Segmentation of the ectoderm

Ectodermal segmentation in *Tubifex* is a process of separation of 50-μm-wide blocks of cells from the initially continuous ectodermal GB (Fig. 3). The formation of ectodermal segments begins with formation of fissures, first on the ventral side and then on the dorsal side of the GB; the unification of these fissures gives rise to separation of a 50-μm-wide block of cells from the ectodermal GB (Fig. 3A, B; Nakamoto *et al.*, 2000). As development proceeds, an initially linear array of

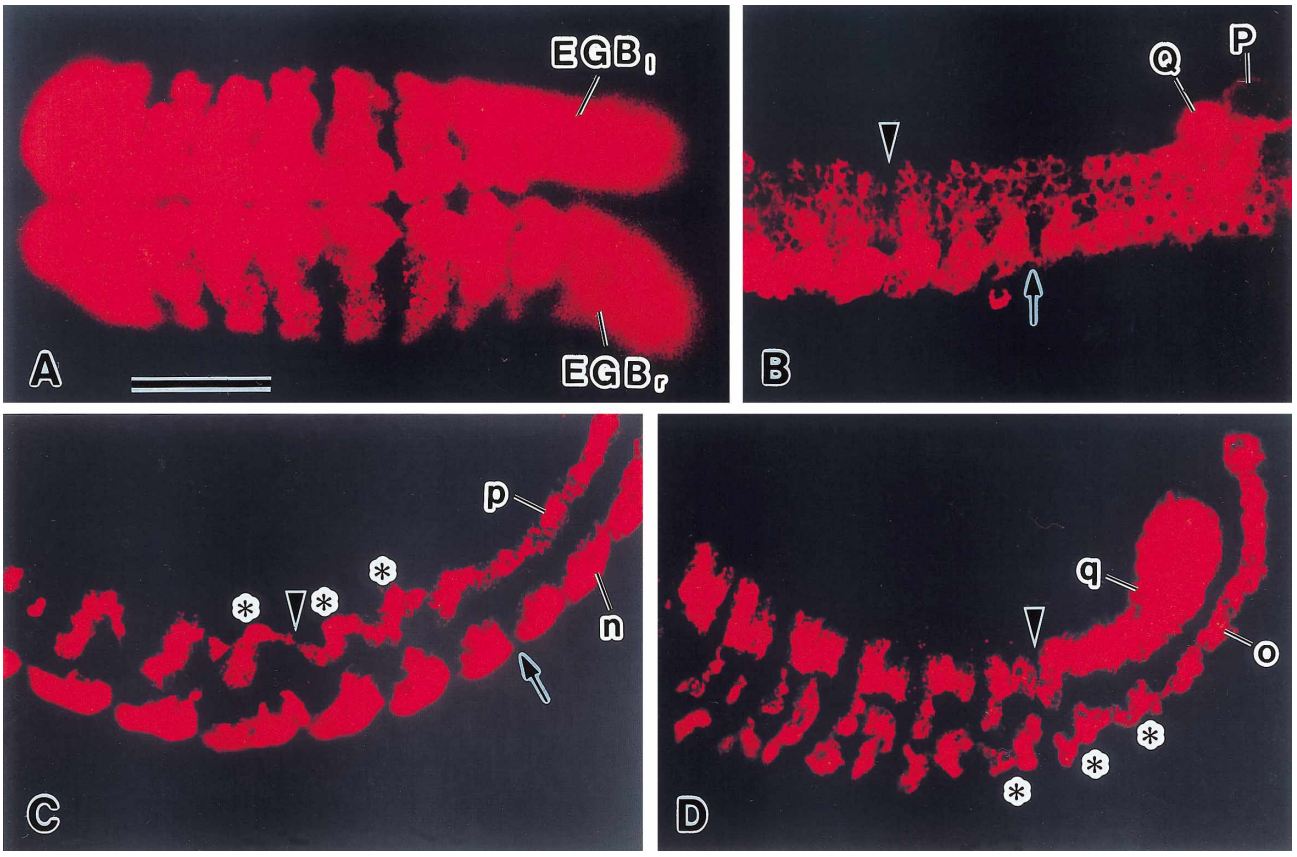

Fig. 3. Fluorescence micrographs showing segmentation of ectodermal germ bands (GBs) in *Tubifex* embryos. $2d^{111}$ cell (A), left NOPQ (B) or individual teloblasts (C, D) were injected with DiI and allowed to develop for 3 days before fixation. Wholemount preparations were viewed from the ventral side (A) or left side (B–D). In all panels, anterior is to the left; in B–D, dorsal is to the top. (A) Both the left and right germ bands (EGBl and EGBr, respectively) are labeled with DiI. Both GBs have coalesced with each other along the ventral midline in the anterior and mid regions of the embryo. Only the mid region of the embryo is in focus here. Note that GBs are divided into 50-µm-wide blocks of labeled cells by intersegmental furrows, which are recognized as non-fluorescent transverse stripes. (B) The posterior portion of the left GB is shown. P and Q teloblasts are seen, but N and O teloblasts are out of the field. The arrow indicates the site where a fissure becomes evident in the ventralmost bandlet (i.e., n-bandlet). The arrowhead indicates fissures at the dorsal side of the GB. (C) Fluorescent n- and p-bandlets in the left GB. These bandlets were derived from left N and P teloblast that had been injected simultaneously with DiI shortly after the birth of the P teloblast. Asterisks indicate S-shaped segmental elements (SEs) in the P lineage. The arrow and arrowhead indicate the sites where separation of an SE from the bandlet has taken place. Note that the separation of an SE in the P lineage lags behind that in the N lineage by three segments. (D) Fluorescent o- and q-bandlets in the left GB. These bandlets were derived from O and Q teloblasts that had been injected simultaneously with DiI shortly after the birth of the O teloblast. Asterisks indicate W-shaped SEs of the O lineage. The arrowhead indicates the boundary between two consecutive SEs. Bar: 100 µm (A, B); 80 µm (C, D). From Nakamoto *et al.* (2000).

blast cells in each ectodermal bandlet gradually changes its shape and its contour becomes indented in a lineage-specific manner (Fig. 3C, D). These morphogenetic changes result in the formation of distinct cell clumps, which are separated from the bandlet to serve as segmental elements (SEs; Fig. 4A). SEs in the N and Q lineages are each comprised of clones of two consecutive primary blast cells. In contrast, in the O and P lineages, individual blast cell clones are distributed across SE boundaries; each SE is a mixture of a part of a more-anterior clone and a part of the next more-posterior clone (Fig. 4B).

Autonomy of bandlet morphogenesis

When an ectoteloblast is forced to be isolated or "solitary" by removal of all of its (ipsilateral) sister teloblasts, it continues dividing at a rate comparable to that in intact embryos. All but o-bandlets are very similar to the respective bandlets in intact embryos, not only in shape but also in periodicity of separated SEs. This suggests that lineage-specific bandlet transformation in the N, P and Q lineages occurs independently of adjacent bandlets (Nakamoto *et al.*, 2000).

In contrast, "solitary" o-bandlets exhibit features characteristic to the P lineage rather than the O lineage. This finding suggests that "solitary" o-bandlets adopt the P fate rather than the O fate. The O-to-P fate conversion of "solitary" o-bandlets has been shown to be maintained in the terminally differentiated progeny of the primary blast cells. Recently, it has also been shown that o-bandlets are induced to assume the O fate by signals from the p-bandlets (Arai *et al.*, 2001).

Mesodermal control

During *Tubifex* embryogenesis, the ectodermal GB is

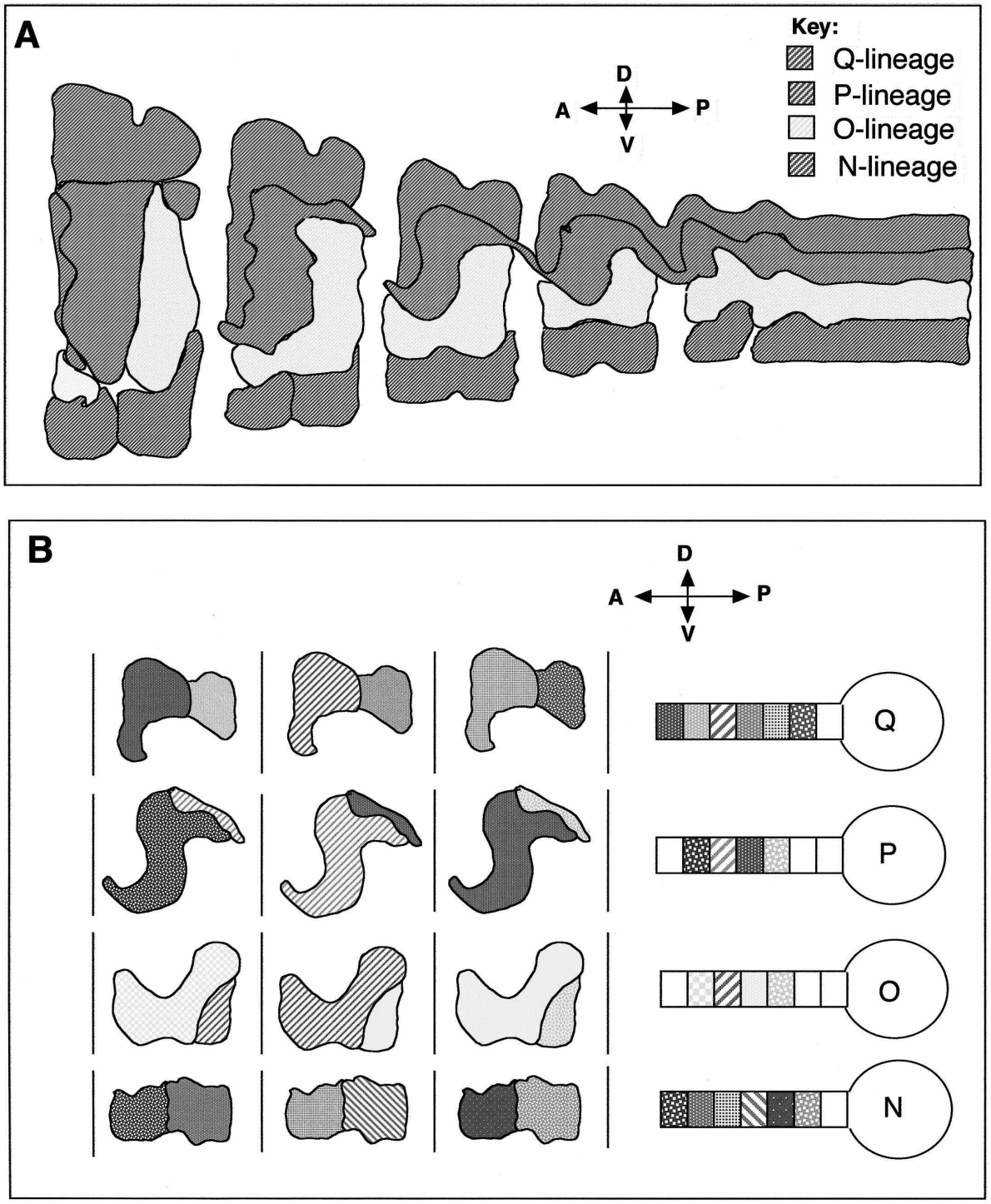

Fig. 4. Schematic summary of morphogenetic events leading to ectodermal segmentation in the *Tubifex* embryo. (A) Formation of segmental elements (SEs) is followed by their separation from bandlets. This separation occurs first in the N lineage and then in the O, Q and P lineages in this order. Upon their integration into a discrete segment, SEs further change their shape to intermingle with each other within the segment. (B) Segmental contribution of clones of primary blast cells. Three segments are shown; each pattern represents an individual clone. In each lineage, the order of primary blast cells is shown to the right of the figure, together with their parent teloblasts. In the N and Q lineages, two consecutive primary blast cells give rise to one segmental complement of progeny. In the O and P lineages, each of the serially homologous primary blast cell clones is divided into two parts, which are inherited separately by two consecutive segments. A, anterior; D, dorsal; P, posterior; V, ventral. Adapted from Nakamoto *et al.* (2000).

normally underlain by the mesodermal GB (see Fig. 1H; Goto *et al.*, 1999a). There is a possibility that the mesodermal GB plays a role in ectodermal segmentation. In fact, the results of recent cell-ablation and -transplantation experiments have suggested that the mesodermal GB plays an important role in two aspects of ectodermal morphogenesis, viz., spatial

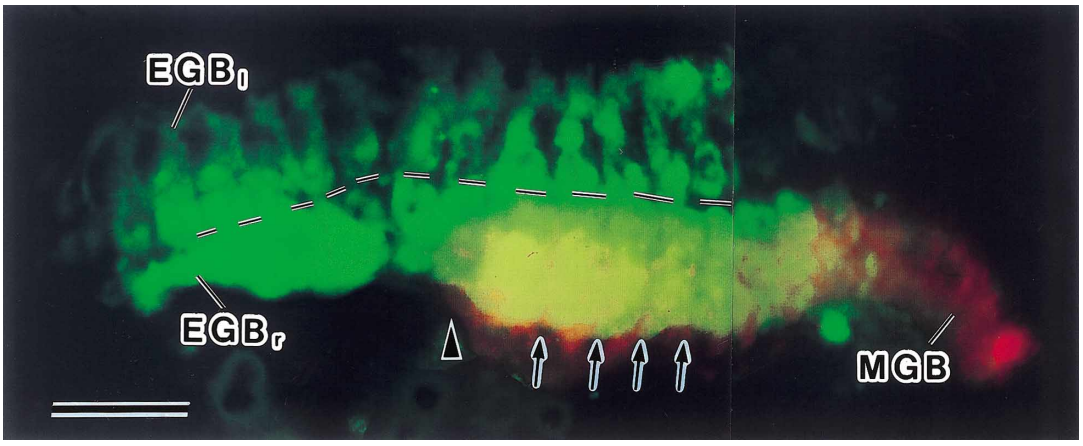

Fig. 5. Segmentation and dorsalward expansion of ectodermal GB depends on the underlying mesoderm. $2d^{11}$ cell of a 22-cell *Tubifex* embryo (see Fig. 1B) was injected with a lineage tracer Oregon-Green dextran, and the right M teloblast of the same embryo was ablated shortly after its birth; this embryo served as a host embryo. About 24 hr later, a right M teloblast, which was isolated from another embryo and injected with DiI, was transplanted to the right side (just behind right ectoteloblasts) of the host embryo. This reconstituted embryo was allowed to develop for 5 days before fixation and was photographed by epifluorescence microscopy. Double-exposure micrograph of a ventral view is shown. Anterior is to the left. The Oregon-Green dextran-labeled ectoderm appears green, and the DiI-labeled mesoderm appears red. The anterior portion of the right ectodermal GB (EGBr) is mesoderm-deficient and does not show any sign of segmentation or dorsal expansion. Note that labeled cells (green) are confined near the ventral midline (dashed line). In contrast, the remaining posterior portion of this GB, which is underlain by descendants (MGB) derived from the transplanted M teloblast, exhibits normal-appearing segmental organization. Note that intersegmental furrows (arrows) are arranged with the same periodicity as those seen in the contralateral GB (EGBl), which is underlain by mesodermal GB derived from the host embryo. The arrowhead indicates the anterior margin of the DiI-labeled GB. Bar: 100 μm.

arrangement of SEs along the anteroposterior axis and dorsalward expansion of the ectodermal GB (Nakamoto *et al.*, 2000).

In embryos from which left M teloblasts have been ablated, ectodermal GBs on the mesoderm-deficient (left) side fail to generate any segmental organization. These GBs do not undergo the dorsalward expansion that normally accompanies ectodermal segmentation; as a result, they appear as a long rod running along the ventral midline. If a cell layer derived from M teloblasts is “transplanted” to the space between the mesoderm-deficient ectodermal GB and the endoderm, the ectodermal GB undergoes segmentation and dorsalward expansion only in the portion underlain by the transplanted (mesodermal) cell layer (Fig. 5; A. Nakamoto, unpublished data).

Two-step process

Ectodermal segmentation in *Tubifex* involves three key events: (a) generation of SEs within each bandlet, (b) separation of SEs from bandlets, and (c) arrangement of separated SEs at 50-μm intervals along the anteroposterior axis. The first two events occur normally in each ectodermal bandlet in the absence of its neighboring bandlets. This suggests that morphogenetic processes leading to generation and separation of SEs are initiated autonomously in each bandlet. In contrast, the distribution pattern of separated SEs along the anteroposterior axis apparently depends on the germ layers underlying ectodermal bandlets. Thus, we suggest that the ectodermal segmentation in *Tubifex* is divided into two stages, autonomous morphogenesis of each bandlet leading to generation of SEs and the ensuing non-autonomous alignment of separated SEs (Nakamoto *et al.*, 2000).

Segmentation of the mesoderm

Mesodermal segmentation is a process of reorganization of the mesodermal GB into a linear array of 50-μm-wide clusters of cells. Careful stage-by-stage observations of GBs labeled with lineage tracers have shown that the number of clusters (including primary m-blast cells, each of which is counted as one cluster) is equal to the number of primary blast cells that are expected to be produced from the M teloblast following lineage tracer injection. After birth from M teloblasts, each primary m-blast cell undergoes a spatiotemporally stereotyped sequence of cell divisions to generate three classes of cells (in terms of cell size), which together give rise to a distinct cell cluster (i.e., a mesodermal compartment; Fig. 6A). As development proceeds and blast cells proliferate, each compartment becomes a mesodermal segment consisting of a surrounding thin layer of tiny cells (i.e., a coelomic wall), inner cells at one end, and the coelomic cavity (Fig. 6B, C; Goto *et al.*, 1999a).

Segmental founder cells

Is each cluster derived from a single primary m-blast cell or a mixture of progeny cells of two or more consecutive primary blast cells? To answer this question, we have performed double-labeling experiments in which M teloblasts were doubly injected first with Texas Red dextran (TRD) and then with fluorescein dextran (FLD) 2.5 or 5 hr later (Goto *et al.*, 1999a). Since M teloblasts divide repeatedly at 2.5-hr intervals (at 22°C), it is expected that the first one (in the case of 2.5-hr intervals) or two (in the case of 5-hr intervals) primary m-blast

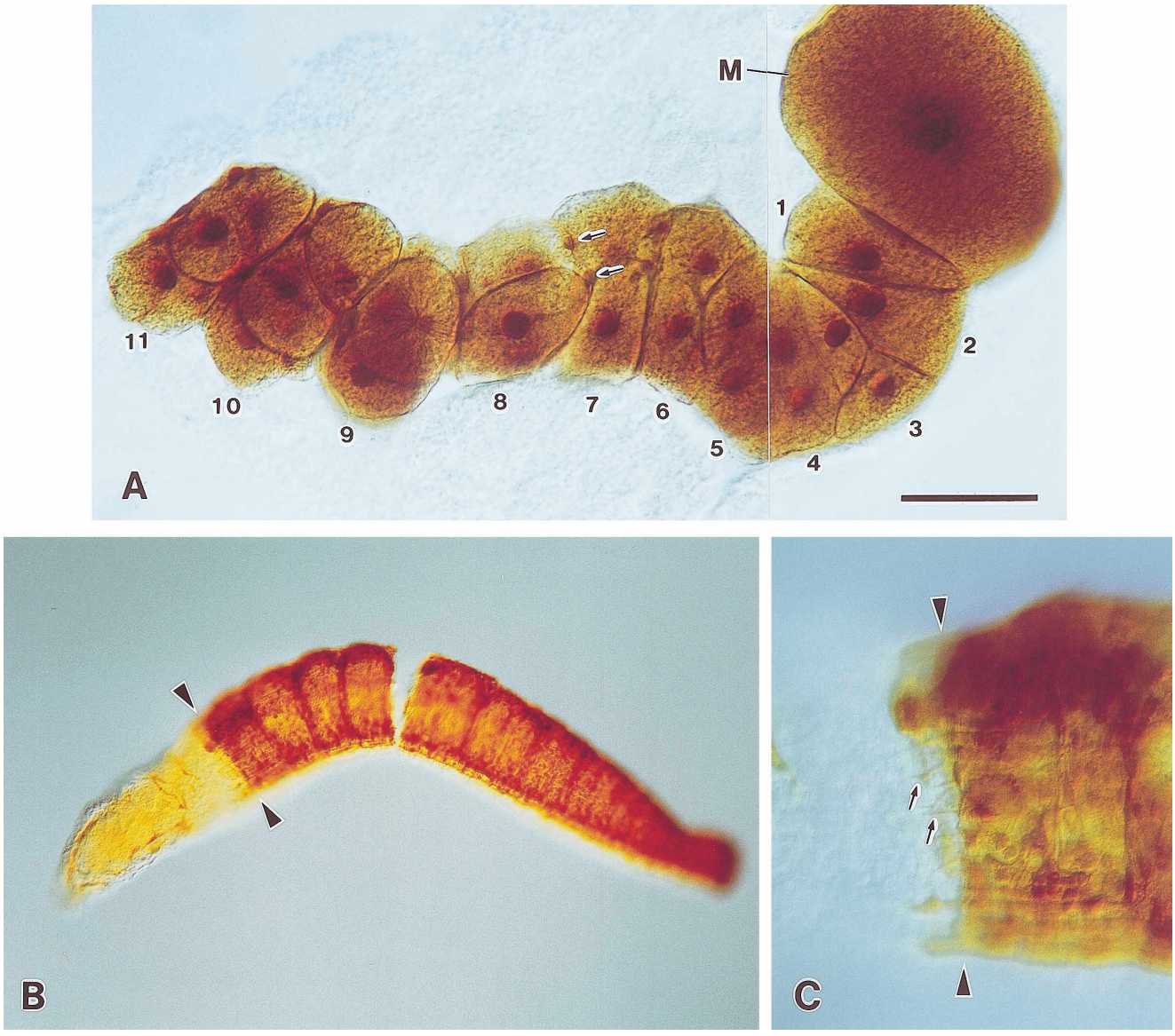

Fig. 6. Organization of blast cells in the mesodermal GB. Left M teloblasts of *Tubifex* embryos were injected with horseradish peroxidase (HRP) 15 hr after their birth and allowed to develop for 24 hr (A) or 7 days (B, C) before fixation. HRP-containing cells were visualized histochemically according to the method described in Goto *et al.* (1999b). Anterior is to the left and dorsal is to the top. (A) The HRP-labeled portion of the GB is comprised of 11 cell clusters, including primary blast cells located at positions 1 and 2. In this preparation, unlabeled clusters are present in front of this labeled portion of the GB, though they are invisible here. Arrows indicate tiny cells that have been produced by cells located in the cluster at position 8. Note that tiny cells form a thin layer at the boundary between the clusters at positions 10 and 11. Clusters at positions 4 and 5 are out of focus here due to the presence of overlying ectodermal teloblasts (O and P), which are invisible in this preparation. (B, C) This embryo shows that during 7 days of development, mesodermal cells in a segment do not migrate into a more-anterior segment. The boundary (arrowhead) between the unlabeled anterior segment and the HRP-labeled segment is sharp. As shown in C, however, longitudinal muscle fibers (arrows) are seen to extend into the adjacent segment. Bar: 50 μm (A, C); 200 μm (B).

cells produced from these M teloblasts would be labeled singly with TRD, whereas subsequent primary blast cells would inherit both TRD and FLD labels.

In embryos that have been injected doubly at 2.5-hr intervals, an anteriormost cluster of the fluorescently labeled portion of the GB exhibits TR fluorescence only, and ensuing clusters inherit both TRD and FLD labels. In the case of 5-hr-interval double labeling, two anteriormost clusters are labeled with TRD only, followed by doubly labeled clusters. In both cases, the boundary of the adjacent clusters that are differently labeled is sharp. There is no case in which clusters are a mosaic of singly labeled cells and doubly labeled cells. These results suggest that each cluster is a clone of a single primary m-blast cell (Goto *et al.*, 1999a). A similar one-to-one relationship between primary m-blast cells and compartments (i.e., segments) has also been suggested in another oligochaete *Eisenia* (Vandenbroek, 1934; Devries, 1983; Storey, 1989).

Absence of ectodermal control

Soon after primary m-blast cells undergo the first two cell divisions, m-blast cells come to be overlain by the ectodermal GBs. Thereafter, mesodermal GBs are always sandwiched between the overlying ectodermal GB and the underlying endoderm, at least up to the completion of their coalescence along the ventral midline (Fig. 1H). There is a possibility that segmentation in the mesodermal GB is regulated by the overlying cell layers. This is unlikely, however, since even after deletion of 2d cells (precursors of ectoteloblasts), cellular events that lead to segmentation occur in a manner comparable to that in intact embryos (Devries, 1974; Goto *et al*., 1999a). Blast cells are organized in clusters surrounded by tiny cells; furthermore, as demonstrated by double-labeling experiments, each cluster in ectoderm-deficient embryos is comprised of progeny cells of a single primary m-blast cell. These results suggest that segmentation in the mesodermal GB proceeds normally in the absence of the ectodermal GB.

Taken together, it is reasonable to assume that each primary m-blast cell serves as a founder cell of each mesodermal segment and that the boundary between segments is determined autonomously. It is concluded that the metameric body plan of *Tubifex* arises from an initially simple organization (i.e., a linear series) of segmental founder cells (Goto *et al*., 1999a).

SEGMENTATION IN LEECHES

Segmentation of the ectoderm

The process of segmentation during leech embryogenesis is strikingly similar to that in oligochaetes. First, the formation of ectodermal segments begins with formation of fissures in an initially linear array of blast cells (i.e., a bandlet), which results in generation of segmental elements (SEs; Shain *et al*., 1998). As in *Tubifex*, there is not a one-to-one relationship between primary blast cells and SEs in any of the leech ectoteloblast lineages (Weisblat and Shankland, 1985). In the N and Q lineages, two classes of primary blast cell exist alternately in the bandlets, and two consecutive primary blast cells give rise to one segmental complement of progeny (Bissen and Weisblat, 1987). In contrast, there is only one type of primary blast cell clone in the O and P lineages. In these lineages, one primary blast cell generates one segmental complement of tissue, but during segmentation, each of the serially homologous primary blast cell clones is divided into two parts, which are inherited by two consecutive SEs (i.e., segments).

Second, morphogenetic events leading to SE formation in each ectodermal bandlet are initiated autonomously. Shain and others (2000) have recently shown that in the leech *Theromyzon*, the separation of ganglionic primordia (which correspond to SEs) from the N lineage bandlet occurs independently of neighboring bandlets (including mesodermal GB), suggesting autonomy of early events of ectodermal bandlet morphogenesis. These authors have also suggested that segmentation in the n-bandlet might be achieved through differences in cell adhesion and/or cell motility between two alternating classes of primary blast cells (designated as nf and ns; also see Bissen and Weisblat, 1987).

Third, segmentation of the leech ectodermal GB appears to depend on the presence of the underlying mesoderm. Blair (1982) and Torrence (1991) reported that when M teloblasts (hence mesodermal GB) in leech embryos were ablated, no segmentally iterated structures formed in the ectodermal GB on the mesoderm-deficient side, suggesting mesodermal control of ectodermal morphogenesis. Given that, as mentioned above, ectodermal bandlet morphogenesis leading to SE formation occurs autonomously (Shain *et al*., 2000), it is considered that as in *Tubifex*, the ectodermal segmentation in leech embryos consists of an early autonomous morphogenetic process (including SE formation) followed by a mesoderm-dependent process. Even if this is the case for leech embryos, it is still not known when the mesoderm is required to function in the ectodermal segmentation. It is not clear when discrete segmental organization emerges in the ectodermal GB as a whole.

Segmentation of the mesoderm

The first overt sign of segmentation of the leech mesoderm is subdivision of the m-bandlet (GB) into iterated clusters of cells (Stent *et al*., 1982). These clusters (~25 μm in width) emerge in an anterior-to-posterior succession during development. In his elegant, double-labeling experiments, Zackson (1982) clearly showed that each cluster is comprised of descendants of a single primary blast cell produced from the M teloblast. Thus, as in oligochaetes, there is a one-to-one relationship between primary m-blast cells and mesodermal segments in leeches.

Adjacent clusters are isomorphic in that their cells are in topographical and morphological correspondence (Stent *et al*., 1982). This suggests that each cluster may be generated by a sequence of stereotyped cell divisions of primary m-blast cells. At present, however, nothing is known about cell divisions in m-blast cells other than the first division occurs perpendicularly to the long axis of the bandlet (Zackson, 1984; Nelson and Weisblat 1992). Thus, little is known about the process by which clusters develop into individual mesodermal segments or how segmental boundaries are established. In this connection, it is noteworthy that ablation of ectodermal GBs results in the loss of segmental organization in the mesoderm (Blair, 1982). This is in sharp contrast to the situation in oligochaetes, where, as described earlier, mesodermal segmentation proceeds normally in the absence of ectodermal GBs (Devries, 1974; Goto *et al*., 1999a). It seems likely that, compared with those in oligochaetes, the boundaries between adjacent developing segments are labile in leech embryos. This property of the segmental boundaries in leech embryos may be related to the naturally occurring breakdown of septa (Mann, 1962). Recently, septal breakdown in *Helobdella* has been suggested to be mediated by apoptosis (Tsubokawa and Wedeen, 1999).

Segmental expression of an *engrailed*-class gene

In *Helobdella*, Wedeen and Weisblat (1991) showed that during the early development, an *engrailed*-class gene, *ht-en*, is expressed in segmentally iterated stripes of cells within the posterior portion of the segment. This spatial pattern of *ht-en* expression was sufficient to allow us to envisage the involvement of this gene in various aspects of segmentation. However, subsequent detailed cell lineage analyses of *ht-en* expression cast doubt on its functional role in the segmentation process. First, in the N lineage, *ht-en* expression begins after the onset of segmentation (i.e., fissure formation; see below) in the n-bandlet (Lans *et al*., 1993; Shain *et al*., 1998). Second, although the O, P and Q lineages begin to express *ht-en* prior to the onset of segmentation of the n-bandlet (Lans *et al*., 1993), the *ht-en* expression in these lineages (bandlets) is unlikely to be essential for the n-bandlet segmentation, since the n-bandlet undergoes segmentation in a normal fashion without any of the other bandlets (Shain *et al*., 2000). Furthermore, as described later, segment polarity develops normally regardless of whether or not *ht-en* expression occurs (see below).

Thus, in leeches, it is unlikely that *ht-en* expression is associated with the morphogenetic processes leading to segmentation. These results also support the notion that segmentation is an autonomous process. Nevertheless, *ht-en* is expressed in a strictly reproducible manner in a stereotyped subset of identifiable cells in the segmentally iterated primary blast cell clones (Wedeen and Weisblat, 1991; Lans *et al*., 1993), suggesting its role in specification of cells occurring after segmentation.

SEGMENTATION IN POLYCHAETES

Unlike the aforementioned clitellate annelids, which develop directly into juveniles, polychaetes are indirect developing annelids. Their embryogenesis results in the production of trochophore larvae, which are initially devoid of any segmental organization. As the larva grows, it elongates by proliferation of tissue in a growth zone (ectoteloblasts surrounding mesoteloblasts) located immediately in front of the pygidium and simultaneously undergoes subdivision of its preexisting body into presegmental prostomium and peristomium and succeeding anterior trunk segments (which are referred to as larval segments; Fig. 2B). This is the first phase of polychaete segmentation. The number of larval segments is species-specific but varies considerably among polychaete species (Schroeder and Hermans, 1975). In many species of polychaetes, three segments are formed during this phase; in *Chaetopterus*, exceptionally, 15 segments are generated during the larval stage (Irvine *et al*., 1999). The second phase of polychaete segmentation is characterized by the addition of segments to the pre-existing trunk region, which is achieved through interpolation of segments between the pre-existing segments and the pygidium.

As described earlier, the mesoderm and ectoderm of the trunk segments of polychaetes have long been thought to arise from mesoteloblasts (derived from the 4d micromere) and ectoteloblasts (derived from the 2d micromere) in the posterior growth zone (Fig. 2A; Anderson, 1966, 1973). Mesoteloblasts, which are located on either side of the pygidium, proliferate a pair of mesodermal bands anteriorly. Similarly, ectoteloblasts in the growth zone have been believed to serve as stem cells that contribute ectodermal cells to the trunk segments. So far, however, lineal relations between the teloblastic growth zone and trunk segments have not been clearly demonstrated. Furthermore, almost nothing is known about the dynamics of cell division in the growth zone. As such, no information is available on either the mode of cell arrangement in mesodermal bands and ectodermal sheets or the processes by which the germ layers acquire segmental organization.

Due to the scarcity of information on polychaete segmentation, we cannot make a direct comparison of cellular mechanisms for clitellate and polychaete segmentation. However, a recent finding that *Hox* genes are expressed in the growth zone of *Chaetopterus* larvae prior to the emergence of overt segmental organization (Irvine and Martindale, 2000; Petersen *et al*., 2000) is suggestive of differences in segmentation between clitellates and polychaetes (Leech *Hox* genes begin to be expressed long after completion of the segmentation process; see below.). On the other hand, it should be noted that mechanisms for segmentation appear to be diverse among polychaete species. For instance, in the orbiniid *Scoloplos*, mesodermal segmentation precedes ectodermal (external) segment delineation (Anderson, 1959); in contrast, in the serpulid *Eupomatus*, ectodermal segment delineation occurs before mesodermal segmentation (Ivanov, 1928).

SEGMENT POLARITY

Trunk segments of annelids exhibit similar composition and distribution of differentiated cells except for segment-specific organs such as nephridia and genital primordia (see below). In each segment, however, these cells are distributed not evenly but differentially along the anteroposterior (AP) and the dorsoventral (DV) axes. For instance, central neurons are localized in the ventral region, while peripheral neurons are present in the rest of each segment. Such a differential distribution of blast cell progeny is apparently a consequence of asymmetric divisions of primary blast cells. Careful observations on clitellate teloblast lineages have shown that blast cell divisions are polarized, in terms of their direction and inequality, as early as the time of first division (Zackson, 1984; Shankland, 1987a, b, c; Goto *et al*., 1999a; Arai *et al*., 2001). This suggests that primary blast cells are each differentiated along its AP and DV axes. How do these cells acquire polarized properties? Recent cell ablation studies on leech and oligochaete embryos suggest that primary blast cells are polarized independently of neighboring cells.

In leech embryos, Seaver and Shankland (2000) have shown that in the absence of both its anterior and its posterior neighbor, an “isolated” primary blast cell in the O and P lin-

eages develops normally to generate an appropriate complement of descendant cells, which are specified and positioned in a normal fashion along the AP axis. Although these authors did not test the possibility of blast cell clones being polarized by signals from other sources, e.g., ipsilateral ectodermal bandlets and the underlying mesoderm, this possibility seems unlikely, because, as demonstrated both in leeches and oligochaetes, ablation of neighboring bandlets does not influence AP polarity of primary blast cells in the O and P lineages (Huang and Weisblat, 1996; Nakamoto *et al*., 2000; Arai *et al*., 2001).

As to DV polarity, it is unlikely that primary blast cell clones acquire DV polarity under the influence of neighboring bandlets. In *Tubifex* embryos, ectodermal bandlets that have been forced to be isolated or "solitary" by ablation of ipsilateral and contralateral bandlets exhibit a pattern of descendant cells, which is identical to that of intact bandlets (Nakamoto *et al*., 2000; Arai *et al*., 2001). Similarly, the mesodermal GB in *Tubifex* embryos exhibits normal DV polarity in the absence of an overlying ectodermal GB (Goto *et al*., 1999a).

Taken together, it is unlikely that intercellular signaling in the transverse plane plays a role in specification of segment polarity, i.e., the differential specification of anterior/posterior and dorsal/ventral cell fates within a blast cell clone. Rather, it is more likely that primary blast cells are polarized as early as their birth. At present it is unknown how these cells acquire AP and DV polarities. In this connection, it is noteworthy that in *Tubifex*, parental teloblasts have intrinsic DV polarity, which is maintained if the teloblasts are transplanted to ectopic positions (A. Arai, unpublished data). Polarities in primary blast cells might be properties inherited from their parent teloblasts.

SEGMENTAL IDENTITY

Each segment contains a similar complement of ectodermal and mesodermal tissues. Although segments are homologous, they are regionally differentiated along the longitudinal body axis. Among mesodermal organs, for instance, nephridia and genital primordia are localized in a subset of segments, in a species-specific manner (Brusca and Brusca, 1990). These are morphological features that give each of the segments their own identity. There is no doubt that individual segments are assigned specific identities during embryonic development of annelids.

As to the developmental origin of segmental identity, two types of mechanisms have been suggested from recent cell-ablation and -transplantation studies (Martindale and Shankland, 1988, 1990; Gleizer and Stent, 1993; Nardelli-Haefliger *et al*., 1994; Kitamura and Shimizu, 2000b). One is a cell-intrinsic mechanism in which the commitment of each cell line to a particular fate is governed by its genealogical position in the cell lineage, and the other is a cell-extrinsic mechanism, which is based on the interactions of cells with their environment.

Cell-intrinsic mechanisms

Using a photolesioning technique that causes a shift of a blast cell bandlet out of its normal segmental register, Shankland and his collaborators showed that in *Helobdella*, blast cells of both the N and O teloblast lineages give rise to neurons with specialized properties to express a specific neuropeptide or LOX2 protein when shifted to segments that normally lack such neurons and that, conversely, N-derived neurons normally destined for a domain lacking LOX2 protein expression do not undertake LOX2 expression when transplanted into the normal expression domain (Martindale and Shankland, 1990; Nardelli-Haefliger *et al*., 1994). Applying a similar "cell-transplantation" technique to another leech, *Theromyzon rude*, Gleizer and Stent (1993) demonstrated that m-blast cells give rise to segment-specific mesodermal structures such as nephridia and genital primordia, according to their birth rank rather than to their actual segmental position. These results suggest that primary blast cells of M, N and O lineages in leech embryos have an intrinsic segmental identity. It has also been indicated that primary blast cells could autonomously be committed to segment-specific fates before or shortly after their birth.

Similar cell-intrinsic development of segmental identity during embryogenesis has also been suggested for mesodermal segments of the *Tubifex* embryo (Kitamura and Shimizu, 2000b). In the embryos of *Tubifex*, segments VII and VIII specifically express mesodermal alkaline phosphatase (ALP) activity in the ventrolateral region (Kitamura and Shimizu, 2000a). Cell lineage analyses show that the ALP-expressing cells (which are to develop into a nephridium) originate from M teloblasts. Furthermore, a set of teloblast-ablation experiments demonstrated that the 7th and 8th primary m-blast cells (m7 and m8) produced from M teloblasts give rise to ALP-expressing cells in segments VII and VIII, respectively, and that primary m-blast cells other than m7 and m8 lack the ability to generate ALP-expressing progeny cells. The results of another set of blastomere-ablation experiments suggest that ALP-expressing cells emerge independently of interactions with surrounding tissues. Teloblast-transplantation experiments demonstrated that m8 can generate ALP-expressing cells in an ectopical position, suggesting that it is unlikely that ALP activity emerges in response to the positional cues residing in the embryo. These results suggest that m7 and m8 are exclusively specified as precursors of ALP-expressing cells at the time of their birth from M teloblasts and that segmental identities in primary m-blast cells of the *Tubifex* embryo are determined according to the genealogical position in the M lineage. It is conceivable that the M teloblast possesses a developmental program through which the sequence of blast cell identities is determined.

Apparently, cell-intrinsic development of segmental identity during embryogenesis is widespread in annelids. It appears that the acquisition of specific identities by individual blast cells according to their birth rank, especially in the mesoderm, may have been conserved among oligochaetes and leeches.

Cell-extrinsic mechanisms

It should be noted, however, that there are some features of segmental identity that are specified extrinsically. For instance, in the leech *Hirudo medicinalis*, distinct patterns of cell morphology and synaptic contacts of the serotonergic Ratzius neurons in the genital segments devolve from interactions of the immature Retzius cell with the genital primordia (Loer *et al*., 1987; Loer and Kristan, 1989a, b). Similarly, segment-specific survival of distal tubule cells (which form the distal end of the nephridial tubule) in an ectodermal lineage in *Helobdella* is achieved via interactions with surrounding tissues, probably nephridial primordia (Martindale and Shankland, 1988). Given that both genital and nephridial primordia are intrinsically specified mesodermal features (Gleiter and Stent, 1993), these results suggest that at least some ectodermal features owe their segmental identity indirectly to early segmental blast cell clones.

Hox genes

In view of the fact that *Hox* genes play a critical role in the patterning of the AP body axis in fruitflies, mice and nematodes (Kenyon *et al*., 1999; McGinnis and Krumlauf, 1992; Gellon and McGinnis, 1998), it is reasonable to postulate that *Hox* genes are also involved in the assignment of different identities to annelid body segments. In fact, the presence of HOM/*Hox*-type homeobox genes has been confirmed in all of the three annelid classes (Snow and Buss, 1994; Dick and Buss, 1994). In leeches (*Helobdella* and *Hirudo*), seven *Hox* genes have been characterized to date and shown to be expressed in segmentally restricted domains along the AP axis; it has also been revealed that their expression domains exhibit the same AP order as that seen for the orthologous genes from other animals (Wysocka-Diller *et al*., 1989; Nardelli-Haefliger and Shankland, 1992; Aisemberg and Macagno, 1994; Kourakis *et al*., 1997; Wong and Macagno, 1998). If these leech *Hox* genes are involved in specifying segmental identity, it is expected that they would be expressed around the time of birth of primary blast cells from teloblasts. However, temporal and spatial patterns of their expression revealed to date suggest that this possibility is unlikely. Most of the leech *Hox* genes are expressed at detectable levels in terminally differentiated cells during late stages of development.

Thus, *Hox* genes in leeches are not responsible for providing the repeat units of the segmental body plan (blast cell clones) with an integral segment identity. At present, however, it is unclear whether this is the case for other annelids. It has recently been shown that five *Hox* genes of the polychaete *Chaetopterus* are expressed in the posterior growth zone of the trochophore larva (Irvine and Martindale, 2000). Apparently, in this polychaete species, *Hox* genes are expressed in undifferentiated cells at very early stages of segmentation. These results suggest that polychaete *Hox* genes might be involved in specifying early events of segmentation, including assignment of segmental identities.

COMPARISON WITH ARTHORPODS

As mentioned earlier, there is an ongoing discussion on whether the segments of annelids and arthropods were derived from a common ancestor. Here we make a brief comparison of the segmentation processes in these two phyla. As has been well documented, the segmentation process is diverse among arthropod groups (Tautz *et al*., 1994; Gilbert, 1997). In long-germ insects, such as *Drosophila*, all segments are patterned simultaneously within the blastoderm. In contrast, in short-germ insects, such as *Tribolium*, and in many species of crustaceans, only segments of the head are patterned in the initial blastoderm, whereas the remaining segments of the thorax and abdomen form progressively from a posterior growth zone. In spite of such a diversity in the mode of segmentation, the expression patterns and functions of segmentation genes, including segment-polarity and pair-rule classes, are conserved in arthropod groups, such as insects, crustaceans and chelicerates (Scholtz *et al*., 1993; Patel, 1994; Damen *et al*., 2000).

Morphologically, the segmentation process of annelids, in which segments form progressively from the posteriorly located teloblasts or growth zone, resembles that in short-germ insects and crustaceans. Furthermore, the expression patterns of a segment-polarity gene *engrailed* as well as *Hox* genes, revealed in leeches, are similar to those seen in arthropods. However, as described already, these genes are unlikely to be involved in the process that specifies polarity and identity of segments. At present, it is not known to what extent such spatiotemporal patterns of gene expression as those seen in leeches are general in annelids, since leeches are not basal annelids but derived clitellates (Brusca and Brusca, 1990).

CONCLUDING REMARKS

Segmentation in annelids is a relatively simple process and consists of three stages. First, embryonic stem cells called teloblasts, which serve as the main source of segmental tissues, are produced (or set aside) early in development. Second, segmental founder cells produced from the teloblasts are arranged in a linear series along the anteroposterior axis. Third, each segmental founder cell proliferates according to its intrinsic temporal and spatial program, and the resulting clone is rearranged into a definitive segmental unit. These three steps are basically conserved in all of the three annelid classes (Polychaeta, Oligochaeta and Hirudinida) but have been modified to various degrees in each class. This three-step mode of segmentation in annelids is totally different from that in arthropods, even crustaceans that employ teloblasts in their segmentation.

Embryological studies so far done on annelids have uncovered many similarities and differences in the segmentation processes in the three annelid groups. However, it is clear that further studies are needed to gain a full understanding of annelid segmentation. Apparently, unique aspects of

annelid segmentation include the involvement of teloblasts that serve as embryonic stem cells and the autonomous properties of segmental founder cells (i.e., primary blast cells). If molecular mechanisms for specification of primary blast cells as well as teloblasts are elucidated in each of the three annelid groups, we would be able to gain an insight into not only the diversity of segmentation mechanisms but also their origins during animal evolution.

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