Cell lineage analysis of pattern formation in the *Tubifex* embryo. I. Segmentation in the mesoderm

ASAKO GOTO, KAORU KITAMURA and TAKASHI SHIMIZU*

Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo, Japan

ABSTRACT   Annelids are strongly segmented animals that display a high degree of metamerism in their body plan. The embryonic origin of metameric segmentation was examined in an oligochaete annelid *Tubifex* using lineage tracers. Segmental organization arises sequentially in the anterior-to-posterior direction along the longitudinal axis of the mesodermal germ band, a coherent column of primary blast cells that are produced from the mesodermal teloblast. Shortly after its birth, each primary 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. Each cluster is composed of descendants of a single primary blast cell; there is no intermingling of cells between adjacent clusters. Relatively small-sized cells in each cluster become localized at its periphery, and they form coelomic walls including an intersegmental septum to establish individuality of segments. A set of cell ablation experiments showed that these features of mesodermal segmentation are not affected by the absence of the overlying ectodermal germ band. These results suggest that each primary 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.

KEY WORDS: *cell lineage, annelid metameric body plan, Tubifex, germ band, mesoderm*

Introduction

Annelids are strongly segmented animals that display a high degree of metamerism in their body plan. The segmented part of the body is limited to the trunk; the head, represented by the prostomium and containing the brain, is not a segment, nor is the pygidium, the terminal part of the body in which the anus is located. 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 (Brusca and Brusca, 1990).

The mechanisms that lead to metameric segmentation during annelid embryogenesis have mainly been studied in leeches. In embryos of this animal, five bilateral pairs of embryonic stem cells called teloblasts are formed early in development; each teloblast divides repeatedly to give rise to primary blast cells, which are arranged into a coherent column (bandlet; see Fig. 1D). Four 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 that underlies the ectodermal GB (Fernandez and Stent, 1980). Extensive cell lineage studies with reliable lineage tracers have shown that segmental tissues and organs are exclusively derived from the GBs (for reviews see Weisblat *et al.*, 1994; Irvine and Martindale, 1996). Blair (1982) and Torrence *et al.* (1989) have shown that the mesodermal GB plays an essential role in generating segmental organization in the overlying ectodermal GB. Furthermore, Zackson (1982) has demonstrated that each mesodermal segment is comprised of descendants of a single primary blast cell, suggesting that the boundary between segments is determined autonomously. Thus, it appears that mesodermal blast cells (or mesodermal GB collectively) play a key role in segmentation in the leech embryo.

Oligochaetes, another annelid class, exhibit many developmental features that appear to be homologous to those of leeches: e.g., generation of five bilateral pairs of teloblasts and ensuing formation of GBs (Fig. 1D; Penners, 1924; Anderson, 1973; Devries, 1973).

Address for reprints: Division of Biological Sciences, Graduate School of Science, Hokkaido University, Sapporo 060-0810, Japan. FAX: +81-11-706-4851. e-mail: stak@sci.hokudai.ac.jp

Fig. 1. Diagrammatic summary of telo-blastogenesis in *Tubifex*. (A-C) *Posterior views. Dorsal is to the top.* **(A)** *Twenty two-cell stage embryo. The second (2d) and fourth (4d) micromeres of the D cell line and a macromere 4D all come to lie in the future midline of the embryo.* **(B)** *Among these three blastomeres, 4d first divides equally into a bilateral pair of teloblasts (Ml and Mr).* **(C)** *About 2.5 h later, 2d divides equally into a pair of ectodermal teloblast precursors NOPQ. 4D also divides into a pair of endodermal precursor cells ED before 2d division.* **(D)** *Embryo at about 36 h following the bilateral division of 2d. Dorsal view. Only*

(A)   **(B)**   **(C)**   **(D)**

teloblasts and associated structures are depicted. NOPQ on each side of the embryo has produced ectodermal teloblasts N, O, P and Q. At this stage, a short germ band (GB) extending from these teloblasts is seen on either side of the embryo. Mesodermal teloblasts (Ml and Mr) located at the posterior end of the embryo are separated from the ectodermal teloblasts.
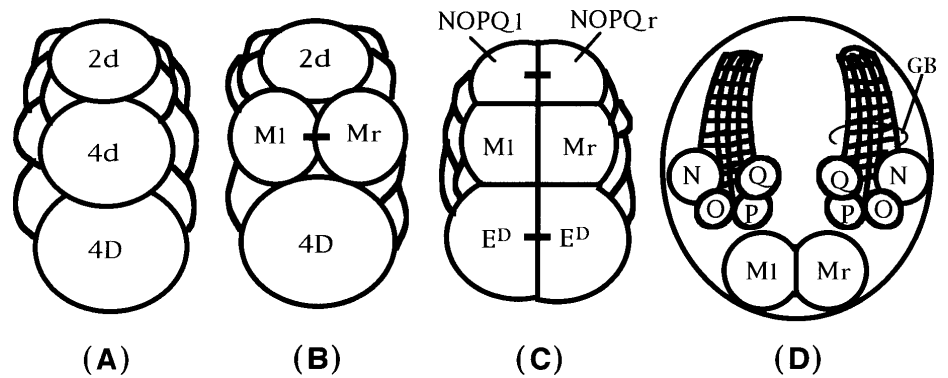
In addition, it has also been demonstrated that, as in leeches, segmental tissues and organs originate exclusively from the GBs (Goto *et al.*, unpublished). However, it is still unclear whether or not oligochaetes and leeches share the same developmental mechanisms for metameric segmentation. So far, only a few studies have been undertaken to examine segmentation in oligochaetes (Penners, 1924; Devries, 1974, 1983). Furthermore, it should be noted that these studies were all based on observations on intact or operated embryos in which cell lineages were not determined with reliable methods. Thus, presently available information about metameric segmentation in oligochaetes is fragmentary and not certain.

Because of our great interest in learning the extent to which the developmental mechanisms for metameric segmentation have been conserved in annelids, and because of the apparent lack of the knowledge about cellular events leading to segmen-

tation in the mesodermal GB in either of the annelid classes, we decided to investigate the process by which the mesodermal GBs acquire segmental organization in a freshwater oligochaete *Tubifex*.

In this study, we examine the process of mesodermal segmentation in *Tubifex* embryos using lineage tracers. We also present experiments in which single blast cells are labeled, extending, to *Tubifex*, the labeling procedure devised originally by Zackson (1982) for a leech *Helobdella*. Finally, effects of ectodermal GB removal on mesodermal segmentation are examined.

Results

General features of tracer-injected Tubifex embryos

Blastomeres that had been injected with 5% horseradish peroxidase (HRP) or fluorescently labeled dextrans continued cell divi-
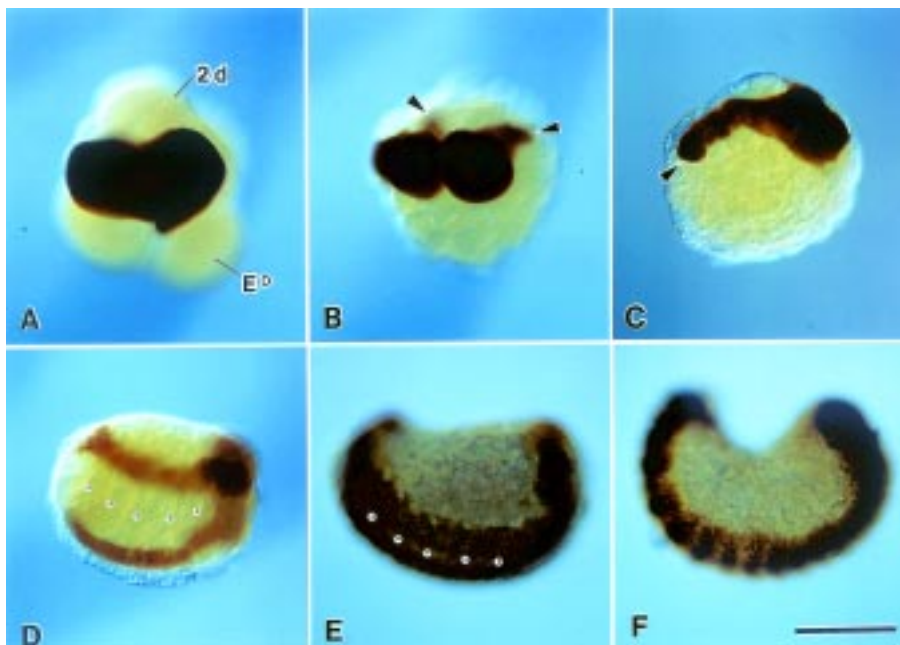

Fig. 2. Formation of mesodermal GBs in the *Tubifex* embryo. *4d-cells of 22-cell embryos were injected with HRP and allowed to develop, before fixation, for 2 (A), 24 (B), 48 (C), 72 (D), 96 (E) and 110 (F) h. HRP-containing cells were visualized as described in Materials and Methods. All panels show whole-mount preparations at the same magnification. Bar, 200 μm.* **(A)** *Posterior view of a 2-h embryo. Dorsal is to the top. The 4d cell has divided into a bilateral pair of M cells. Both cells have inherited injected HRP equally. Note that no trace of HRP is seen in adjacent cells such as 2d and E^D.* **(B)** *Posterior view of a 24-h embryo. Dorsal is to the top. M cells have extended short columns of cells (i.e., GBs) anteriorly at the dorsal side of the embryo. Arrowheads indicate the anterior ends of the GBs (these ends are out of focus here).* **(C)** *Side view of a 48-h embryo. Anterior is to the left; dorsal is to the top. An elongating GB is seen at the dorsal side of the embryo. M cells are located at the posterior end of the embryo, but their contour is not depicted here due to overlapping of labeled cells.* **(D)** *Ventral view of a 72-h embryo. Anterior is to the left. GBs are curving round toward the ventral midline (as indicated by dots) of the embryo. Note that the GBs exhibit*

uniform thickness along their length at this stage. **(E)** *Ventrolateral view of a 96-h embryo. Anterior is to the left. GBs on both sides have coalesced along the ventral midline (indicated by dots). Note that dorsal edges of the GB on the left side have begun expanding in the anterior half of the embryo.* **(F)** *Side view of a 110-h embryo. Anterior is to the left; dorsal is to the top. Segmental organization is evident in the anterior half of the GB at this stage.*

sions in a normal pattern except when a single cell was doubly injected within a short time (see below). In some of the embryos injected, however, gastrulation halted and tracer-containing cells were found to disperse throughout the body. This is probably due to abnormal division of descendants of HRP-injected cells, which might be brought about accidentally during handling of embryos. Such abnormal embryos were not included in the results described in this paper.

In histochemical observation, HRP-containing cells were brown-colored. When HRP-injected embryos were fixed within 6 h following HRP-injection, the brown color was found to be confined to the injected cell itself or its descendants, not to their adjacent cells. Similarly, both TRD and FLD were confined to the injected cells and their descendants. This preliminary experiment suggests that either HRP, TRD or FLD does not pass through gap junctions between cells of the *Tubifex* embryo (see Shimizu, 1995). In all stages examined cell nuclei were found to be labeled more intensely than any other portion of cells; nevertheless, as described above, development proceeded normally in tracer-injected embryos, suggesting that nuclear localization of injected tracers had no influence upon cleavages. Similar intense nuclear staining of HRP-containing embryonic cells has also been reported in other animals (Nishida and Satoh, 1983; Weisblat *et al.*, 1984).

Judging from the intensity of nuclear staining, injected HRP appeared to remain active within embryonic cells for at least 7 days following injection; color development of HRP activity was sufficiently detectable in 7-day embryos. It is unlikely that injected HRP is digested, inactivated or diluted so much in developing *Tubifex* embryos.

Finally, we should mention that in embryos at 8 days or later, endogenous peroxidase activity becomes detectable in developing blood vessels and setae. Unlike injected HRP, however, this endogenous activity never exhibits nuclear localization, but appears as diffuse staining.

M cells derived from 4d micromere behave as embryonic stem cells

The 4d cell, the fourth micromere of D quadrant, divides bilaterally and equally, and produces a pair of cells designated as M cells (Figs. 1B and 2A; Shimizu, 1982). M cells are identified by their large size and position in living embryos. In fact they are the largest cells in the embryo after the 4D cell, the sister of 4d, divides twice (see Fig. 2B). Since the cleavage furrows of dividing M cells are formed at their interior side and the surrounding cells are largely opaque, it is impossible to directly see the division itself of M cells in living intact embryos. On the other hand, it is possible to distinguish M cells in mitosis from those in interphase through their shape change, since M cells round up at mitosis and relax at interphase. Time-lapse video microscopy shows that M cells of the *Tubifex* embryo repeat rounding-up and relaxation, at least 35 times, at about 2.5-h intervals at 22°C. This suggests that M cells divide ~35 times at 2.5-h intervals (at 22°C).

At each division, M cells produce smaller cells designated as *m blast cells* at their anterior side and add them to the posterior side of the previously generated m blast cells, giving rise to a column of cells (designated as *germ band;* GB) running along the anteroposterior axis of the embryo (Fig. 2B-D; also see Fig. 6A). M cells become smaller as they repeat divisions. At the 35th division, for example, they are almost the same in size to m blast cells; they are
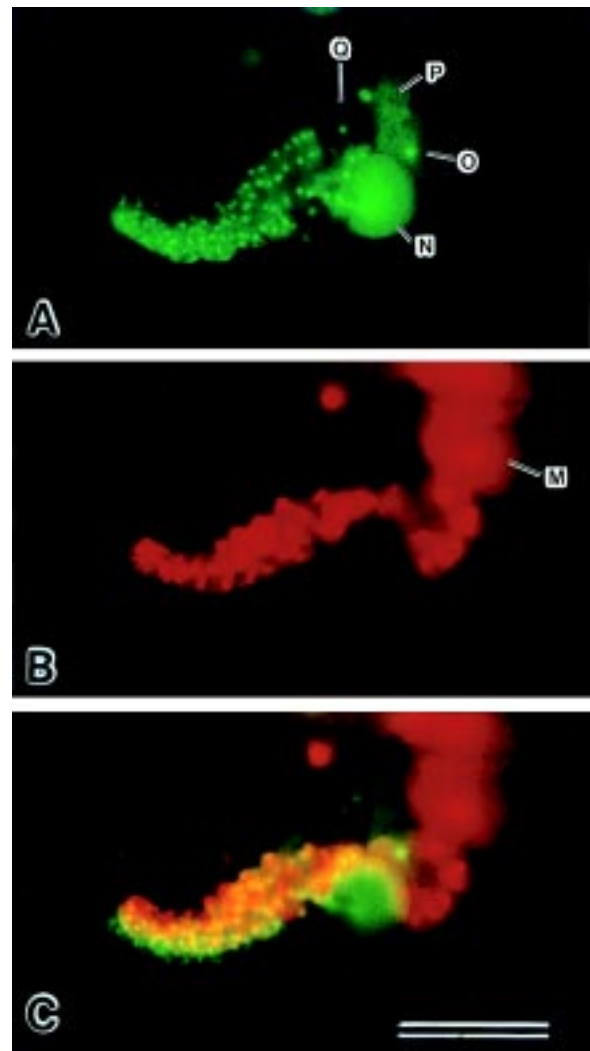

Fig. 3. Fluorescence micrographs showing overlap of mesodermal and ectodermal GBs. *A 4d cell of a 22-cell embryo was injected with TRD and, 3 h later, the left NOPQ of the same embryo was injected with FLD. After a 36-h culture in the darkness, the embryo was fixed and photographed by epifluorescence microscopy using filter cassettes for fluorescein (A) and rhodamine (B). All panels show dorsal views of the same field at the same magnification; (C) shows a merged image of (A) and (B). Anterior is to the left.* **(A)** *Distribution of FLD. The ectodermal GB generated by the posteriorly located teloblasts (N,O,P and Q) inherits FLD. Bright dots are nuclei in blast cells and teloblasts.* **(B)** *Distribution of TRD in the teloblasts (M) and the GBs extending therefrom.* **(C)** *The ectodermal GB (green) is superimposed on the mesodermal GB (red). Regions of overlap are yellow. Note that mesodermal blast cells (red) located in the vicinity of the M cell are not overlain by the ectodermal GB. Bar, 200 μm.*

only recognizable by their posteriormost position in the GB. In this study, we were unable to follow the subsequent fate of the M cells. Nevertheless, there is no doubt that M cells behave as embryonic stem cells during *Tubifex* embryogenesis. As described below, unlike M cells, their daughter cells, m blast cells, undergo a stereotyped sequence of cell divisions and cell differentiation.

In intact embryos, the GBs derived from M cells are overlain by an array of cell columns generated by ectodermal teloblasts N-Q
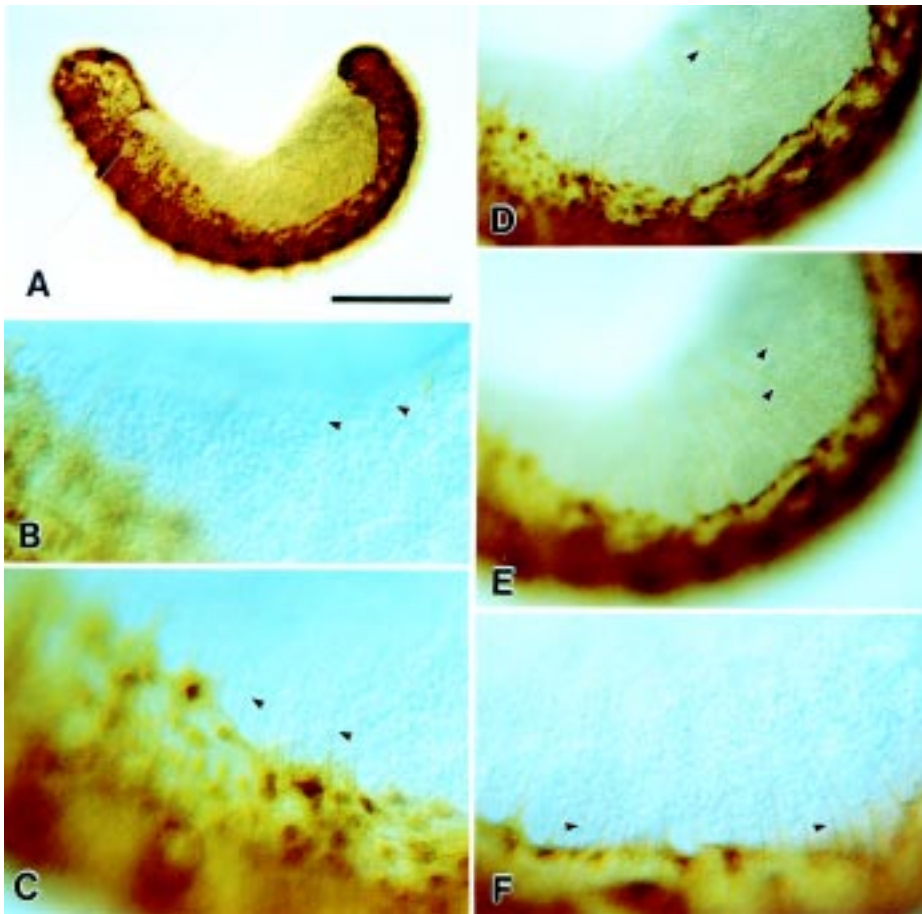
Fig. 4. Dorsal expansion of the mesodermal GB is preceded by elongation of circumferential fibers. *A 4d cell of a 22-cell embryo was injected with HRP and allowed to develop for 120 h before fixation. Whole-mount preparations are shown.* **(A)** *Survey of the embryo at a lower magnification. Side view. Anterior is to the left; dorsal is to the top. In this embryo, three portions are discernible with respect to the extent to which the dorsal expansion proceeds. The dorsal expansion is completed in the anteriormost 4 segments and is underway in the next 6 segments. In the remaining posterior half of the embryo, the dorsal expansion has not begun yet.* **(B-F)** *Higher magnification of the embryo shown in (A).* **(B,C)** *In the region where the dorsal expansion is underway, circumferential fibers (arrowheads) extend from the dorsal edges of the GB (C) and cross the dorsal midline (B).* **(D-F)** *In the posterior region where the dorsal expansion has not begun yet, circumferential fibers (arrowheads) extend from the dorsal edges of the GB (E,F) and reach close to the dorsal midline (D). Bar, 50 μm (A); 100 μm (D,E); 200 μm (B,C,F).*

about 3.5 days after BMC, and it progresses in an anterior-to-posterior fashion. Around this stage, segment-like organization of cells becomes recognizable along the GBs even at a lower magnification (Fig. 2F).

The coalescence of the GBs is followed by the dorsalward expansion of their edges. This expansion begins first at the anteriormost part of the embryo at about 3.75 days after BMC and progresses in anterior-to-posterior succession (Figs. 2E and 4A). As Figure 5A and B show, the expansion is a process by which dorsal edges of coelomic walls migrate between the yolky endoderm and a thin layer of squamous epithelium. 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. 5C).

It is interesting to note that the dorsal expansion of the GBs is accompanied by change in the shape of the embryo's body. As Figure 4A shows, the anterior portion of the embryo, where dorsalward expansion has been completed or is underway, is more slender than the posterior portion that does not exhibit GB expansion. These two portions also differ from each other in that circumferential fine fibers extending from the GB have crossed the dorsal midline to the opposite side of the embryo in the anterior slender portion (Fig. 4B,C) but not yet in the remaining posterior portion. It should be noted that in the posterior portion, these circumferential fibers have elongated, almost reaching the dorsal midline of the embryo (Fig. 4D-F).

Cellular events leading to segmentation in the mesodermal GB

To investigate the processes by which the mesodermal GBs become organized into segments, M cells shortly after or at 15 h after their birth (see Fig. 1B) were micro-injected with HRP and allowed to develop

(Fig. 3) and are underlain by the endoderm (not shown). Furthermore, M cells contribute exclusively to mesodermal tissues in *Tubifex* (Goto *et al.*, unpublished). Therefore, we will hereafter refer to M cell-derived GBs as mesodermal GBs.

Behavior of mesodermal GBs

During the first 48 h following the birth of the M cells (BMC), the GBs extending anteriorly from the M cells are located at the dorsal side of the embryo (Fig. 2B,C). During the next 48 h, they further elongate and gradually curve round, first toward the embryo's side and then toward the ventral midline (Fig. 2D). Thereafter, the GBs on both sides of the embryo move more ventrally and finally coalesce with each other along the ventral midline (Fig. 2E). The coalescence first occurs at the anteriormost part of the embryo at

for 12, 24, 48 or 72 h before fixation. HRP-labeled GBs (together with overlying ectoderm and a part of the underlying endoderm) were dissected out and examined for distribution of cells and mitotic figures along the GBs. We found that blast cells are organized in clusters (or blocks), which are of about one-cell width (Fig. 6A). We also noticed that the number of clusters (including primary m blast cells, each of which was counted as one cluster) was equal to the number of primary blast cells that were expected to be produced from the M cell following HRP-injection. For the convenience of description, the position of a given cluster along the GB is expressed as its position (Cn) relative to the M cell. The cluster located at C10, for example, is separated from the M cell by 9 clusters (see Fig. 7). Cn also indicates the approximate time (nx2.5 h) that lapses after the birth of a given cluster.

Fig. 5. Cross sections of mid regions of embryos showing dorsal expansion of the mesodermal GB. *4d cells of 22-cell embryos were injected with HRP and allowed to develop for 96 (A), 120 (B) and 150 (C) h before fixation. Dorsal is to the top. All panels are at the same magnification. Bar, 50 µm.* **(A,B)** *Right GBs are shown here. The dorsal edges (arrowheads) of the GBs extend dorsally between the yolky endoderm and overlying thin layer of epidermis.* **(C)** *The dorsal expansion of the mesodermal GBs results in the dorsal closure of the endoderm. g, ganglion.*
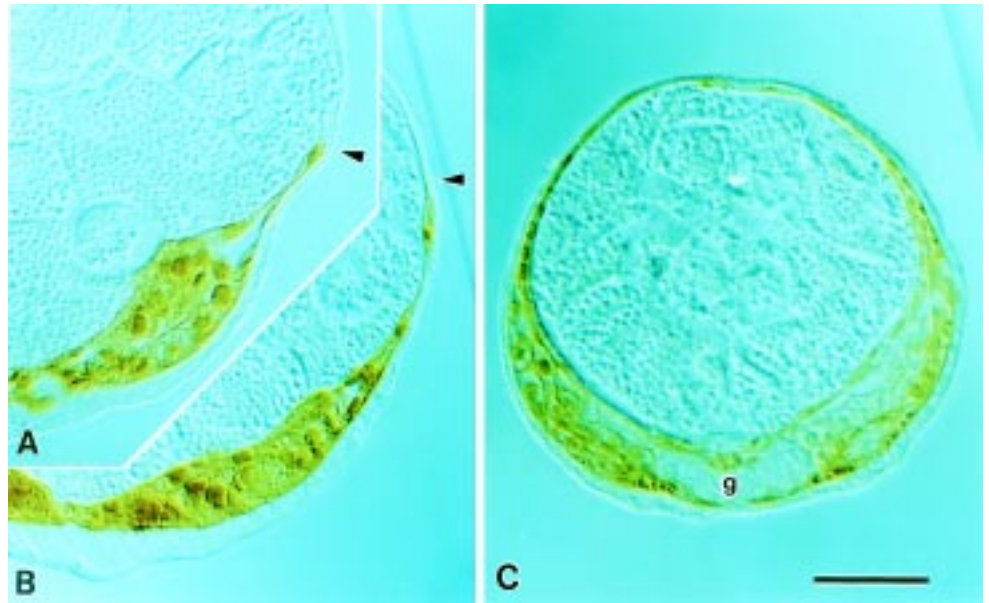
Proliferation of m blast cells

The first division of a primary m blast cell occurs at a distance of 2 clusters (C2) from the M cell. The cell divides quasi-equally in the direction perpendicular to the longitudinal axis of the GB (Fig. 6B); the resulting two cells are arranged along the dorsoventral axis. The second division is also quasi-equal and occurs in the cluster located at C4, and the division axis is parallel to the axis of the GB (Fig. 6C). The resulting four cells, however, are packed in a cluster of one-cell width in most embryos observed.

The third division is extremely unequal and occurs asynchronously in the four cells resulting from the second division. Two of the four cells divide at C6 to produce tiny cells posteriorly (Fig. 6D). The remaining two cells divide unequally at C7 to produce tiny cells at their anterior side (Fig. 6E). The fourth division also occurs asynchronously. Two of the four larger cells resulting from the third division divide unequally at C9-10 to form tiny cells anteriorly (Fig. 6F). Division of the remaining two larger cells, which occurs in the direction of the GB axis at C10, produces two cells of similar size at the ventral side of the cluster (Fig. 6G). Thus, each cluster located at C11-12 is comprised of two dorsally located larger cells and four ventrally located smaller cells (Fig. 6F-H). The sequence of these early divisions is summarized schematically in Figure 7.

Subsequent divisions were difficult to trace precisely because the position of the occurrence of dividing cells in clusters beyond C12 varied among embryos. However, we noticed that both the dorsal and ventral cells resulting from the fifth division undergo unequal divisions and that the clusters located at C18 or more anteriorly are comprised of many small cells and two relatively large cells (see Fig. 8C). These larger cells are comparable in size to the dorsal (larger) cells found in younger (posterior) clusters.

Development of coelomic walls

In clusters located at C1-12, cells are tightly packed, and no space is seen between cells except when cells undergo divisions. In contrast, clusters located more anteriorly exhibit a distinct internal space (i.e., coelomic cavity) surrounded by a coelomic wall, a thin layer of cells. As the coelomic cavity develops with time, cells located interiorly tend to localize at the posterior side of the cluster.

The first sign of coelomic cavity appears at the antero-ventral side of the cluster located at C13-14 (Fig. 8A). A small, but distinct, space is formed between the clump of ventral smaller cells and the anterior layer of tiny cells. This space expands posteriorly in the clusters at C15-17 as a thin layer of cells emerging on the ventral edge forms a coelomic wall (Fig. 8D).

The coelomic cavity at the dorsal side is first detected in the cluster at C15-16. It is formed between an aggregate of dorsal larger cells and overlying flattened cells (Fig. 8C). These flattened cells are apparently derived from small cells that emerge at the dorsal side of clusters at C12-14 (see Fig. 8A,B). As development proceeds, cells comprising the dorsal coelomic wall become much thinner (Fig. 8G); at the same time, the dorsal coelomic space becomes larger in the clusters located at C20 or more anteriorly.

The lateral coelomic wall appears to be formed in clusters located at C17-18. A sheet of three or four flattened cells emerges on the lateral surface of the cluster (see Fig. 8C). This sheet is continuous structurally to dorsal and ventral coelomic walls (Fig. 5A).

Development of fibrous structures

Cell clusters located at C18 or more anteriorly are characterized by the presence of longitudinally and circumferentially running fine fibers on their surface. Longitudinally running bundles of fibers are located at both the mid region and near the ventral margin of the cluster; these bundles are often found to be continuous to those on more anteriorly located clusters (Fig. 8F). Circumferentially running fibers originate from the dorsal edge of the coelomic wall; as development proceeds, these circumferential fibers become longer and extend into dorsal territory (Figs. 4D,E and 8E). It should be noted that during the dorsalward elongation of circumferential fibers, neither the size of the dorsal coelomic cavity nor the position of the dorsal edge of the coelomic wall relative to the ventral midline of the embryo changes significantly in clusters at least up to C25 (Fig. 8E-G).
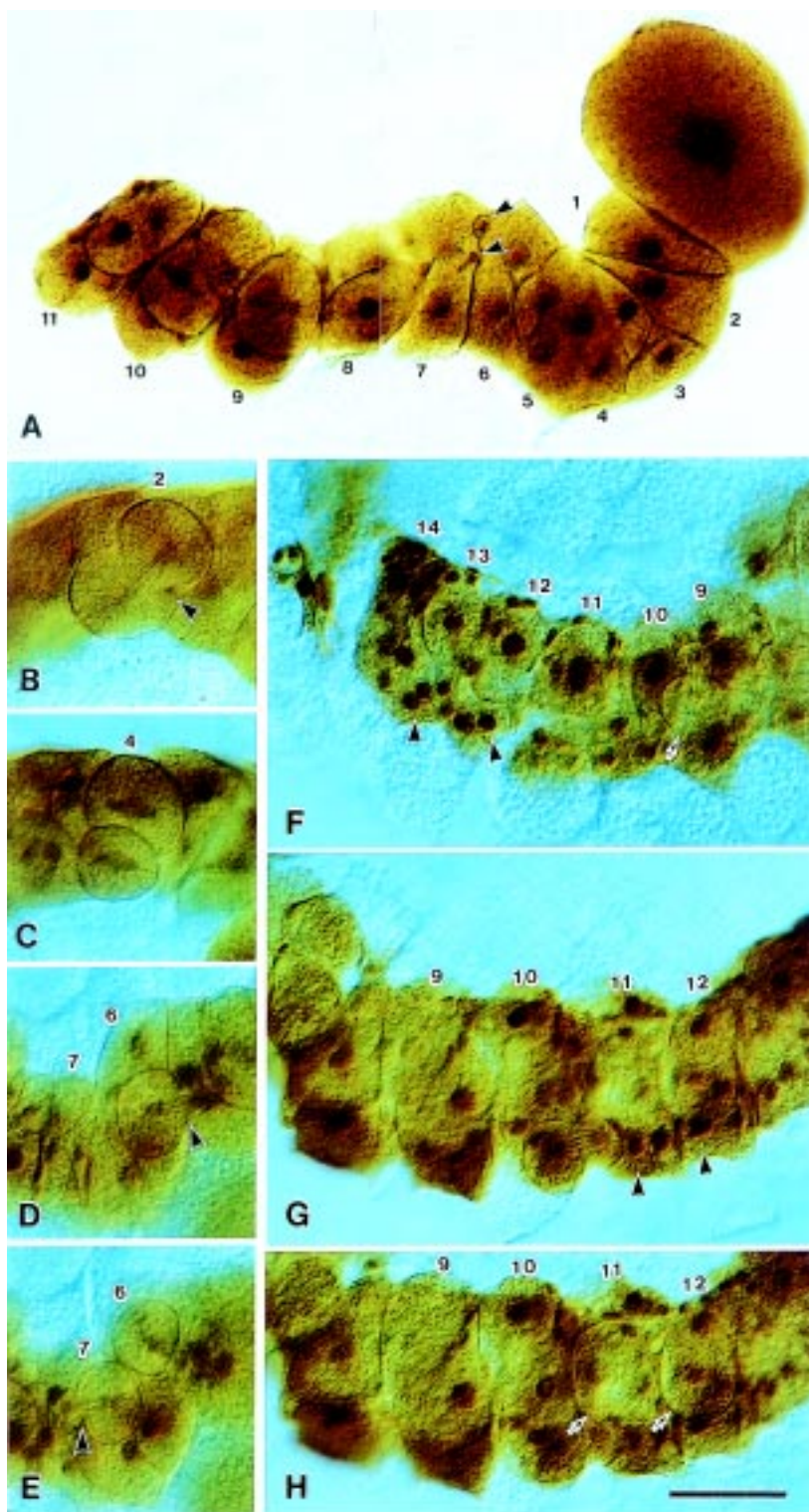
Both the circumferential and longitudinal fibers are specifically stained with rhodamine-phalloidin (data not shown). This suggests that those fibers contain the actin cytoskeleton.

Dorsalward expansion of coelomic walls

As described earlier, dorsalward expansion of the coelomic wall is first detected at the anteriormost clusters (i.e., segments) in embryos at 3.75 days after BMC. The present observation suggests that the coelomic wall expansion is unlikely to begin soon after formation of coelomic walls or circumferential fibers. Given that the dorsal coelomic wall is established at 50 h after BMC (i.e., in the cluster located at C20), it is assumed that the dorsal expansion begins at about 30 h following the coelomic wall establishment. This period of time is equivalent to 12 clusters. (In this study, the position of the first cluster that exhibits dorsal expansion cannot be expressed relative to the M cell, since we were unable to determine the number of clusters in embryos at 3.75 days or more after BMC due to the failure to identify M cells and to the complex organization of the posterior portion of the GB.)

Coelomic walls that are undergoing dorsal expansion are characterized by a jagged contour of their dorsal edges (compare Fig. 4C with Fig. 4F). It appears as if the dorsal edges of coelomic walls are pulled toward the dorsal side via circumferential fibers that are structurally continuous to these edges (Fig. 4C).

Fig. 6. Organization of blast cells and occurrence of mitotic cells in the mesodermal GBs. *M cells were injected with HRP shortly after (F-H) or 15 h (A-E) after their birth and allowed to develop for 24 (A-E) or 36 (F-H) h before fixation. GBs shown here were all dissected out and cleared as described in Materials and Methods. Numerals (1-14) indicate the positions of cell clusters (Cn) relative to the M cell. All panels are at the same magnification. Bar, 50 μm.* **(A-F)** *GBs generated from M cells located on the left side of the embryo. Anterior is to the left; 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 C1 and 2. In this preparation, unlabeled clusters are present in front of this labeled portion of the GB, though they are invisible here. Arrowheads indicate tiny cells that have been produced by cells located in the cluster at position C7. Note that tiny cells form a thin layer at the boundary between the clusters at C10 and 11. Clusters at positions C4 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-E)** *Representative mitotic figures seen along the GBs.* **(B)** *First division of a primary blast cell occurring at position C2.* **(C)** *Metaphase cells located at position C4. Judging from the position of mitotic spindles, these cells would divide equally along the axis of the GBs.* **(D,E)** *Two focal planes of the same field showing unequal divisions of cells located at positions C6 and 7. Cells in position C6 are forming smaller cells on the right (i.e., posteriorly), while a cell in position C7 is producing a smaller daughter cell on the left (i.e., anteriorly). Arrowheads indicate cleavage furrows.* **(F)** *Anteriormost portion of the left GB. Clusters located at positions C11-13 are comprised of larger dorsal cells and smaller ventral cells (arrowheads). In this preparation, the first cluster is seen at position C14. In addition, a clump of labeled cells is also seen on the left of this cluster. Although these two groups of cells are separated from each other at this stage, they both originate from the first primary blast cell. Note that the first cluster at C14 is distinct from the ensuing clusters in that it is composed of many small cells. The arrow indicates an unequally dividing cell located at position C10.* **(G,H)** *Two focal planes of a GB generated from an M cell located on the right side of the embryo. Anterior is to the right; dorsal is to the top. Clusters at positions C11 and 12 are comprised of larger dorsal (arrows) and smaller ventral (arrowheads) cells. Note that ventral cells at C10 are dividing equally (G).*

Segmental contribution of primary m blast cells

The aforementioned results suggest that each primary blast cell undergoes a stereotyped sequence of divisions to form a cell cluster. This suggests that each cluster is derived from a single primary m blast cell. To test this possibility, left M cells of embryos at about 15 h after BMC were doubly injected first with TRD and then with FLD 2.5 or 5 h later. Since M cells divide repeatedly at 2.5-h intervals, it was expected that the first one (in the case of 2.5-hr intervals) or two (in the case of 5-h intervals) primary m blast cells produced from these M cells would be labeled singly with TRD, whereas subsequent primary blast cells would inherit both of TRD and FLD. Injected embryos were allowed to develop for 72 h before fixation and were examined for distribution of TRD and FLD along the GBs. Of a total of 20 embryos that had been doubly injected at 5-h intervals, 13 exhibited fluorescently labeled GBs that were normal in appearance. In the case of 2.5-h intervals, however, only four (out of a total of 35 embryos injected) underwent normal GB formation. In most of the remaining embryos, doubly labeled cells were found to be dispersed throughout the embryo, suggesting abnormal divisions of injected M cells. It is conceivable that a 2.5-h interval between two successive injections was too short to cure trauma resulting from the first injection.

Figure 9 shows GBs in representative embryos that were doubly injected with TRD and FLD. In embryos that had been injected doubly at 2.5-h intervals, an anteriormost cluster of the fluorescently labeled portion of the GB exhibited TR fluorescence only, and ensuing clusters inherited both TRD and FLD labels (Fig. 9A). In the case of 5-h-interval double labeling, two anteriormost clusters were labeled with TRD only, followed by doubly labeled clusters (Fig. 9B). In both cases, the boundary of the adjacent clusters that were differently labeled was sharp. We did not find any cases where clusters were 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.
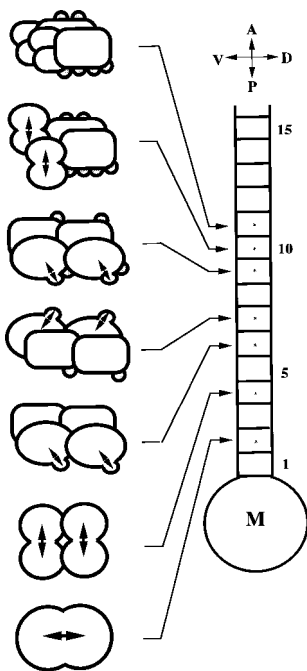

Fig. 7. Schematic summary of pattern and sequence of divisions in mesodermal blast cells. *Inequality and direction of divisions are reflected by position and orientation of mitotic spindles in dividing cells. The mesodermal GB extending from the M cell is illustrated to the right of the figure; each block in the GB represents a cell cluster. Arrows indicate the approximate position, along the GB, where each division occurs. A, anterior; D, dorsal; P, posterior; V, ventral.*
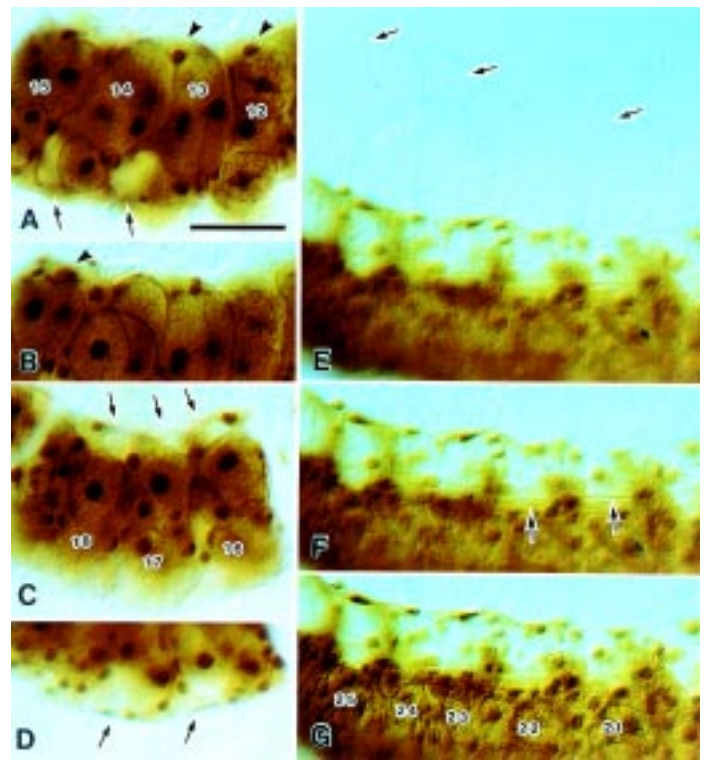

Fig. 8. Morphogenesis of the mesodermal GB. *M cells were injected with HRP at 15 h after their birth and allowed to develop for 48 (A-D) or 72 (E-G) h before fixation. GBs shown here were all dissected out and cleared as described in Materials and Methods. Numerals indicate the positions of cell clusters relative to the M cell. All panels are at the same magnification. Bar, 50 μm.* **(A,B)** *Two focal planes of the same field showing the emergence of spaces (arrows) in the anteroventral region of the clusters at positions C13 and 14. Arrowheads indicate smaller cells located on the dorsal surface of the clusters at positions C12-15.* **(C,D)** *Two focal planes of the same field showing emergence of rudiments of dorsal coelomic walls (arrows) in clusters at positions C16-18. Arrows in D indicate coelomic walls bounding the ventral coelomic cavity. In the cluster at position C17 in (C), a sheet of a few flattened cells is seen on its lateral surface.* **(E-G)** *Three focal planes of the same field showing development of circumferential (arrows in E) and longitudinal fibers (arrows in F) and flattened cells in the dorsal coelomic wall (G).*

Segmentation in the mesoderm does not require overlying ectodermal GBs

As described earlier, m blast cells come to be overlain by the ectodermal GBs soon after primary m blast cells undergo the first two cell divisions. 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. There is a possibility that segmentation in the mesodermal GB is regulated by the overlying cell layers. To test this possibility, we ablated 2d cells from embryos, whose 4d cells had been injected with HRP (see Fig. 1A), and observed the behavior of HRP-labeled cells in these operated embryos.

Even after deletion of 2d cells, M cells repeat cell divisions to form GBs (Fig. 10A-C). As development proceeds, the GBs lengthen and curve round toward the ventral side of the embryo, in a very similar manner to that in intact embryos (compare Fig. 10A with Fig.
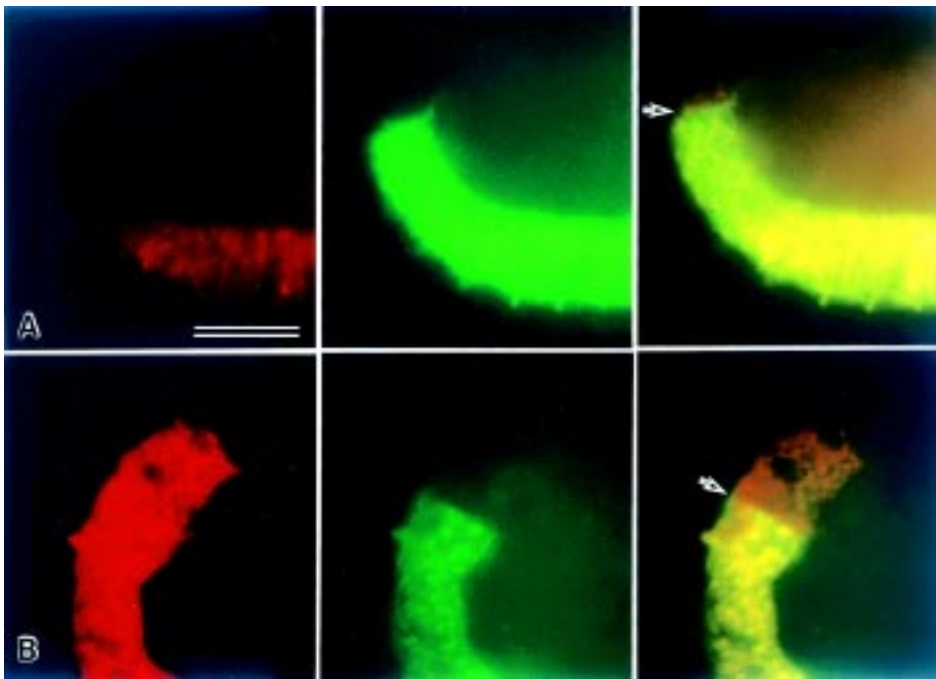
Fig. 9. Each cell cluster is composed of descendants of a single primary blast cell. *Left M cells (at 15 h after their birth) were injected with TRD, and 2.5 (A) or 5 (B) h later, they were injected again with FLD. After a 72-h incubation in the darkness, these embryos were fixed and photographed by epifluorescence microscopy using filter cassettes for rhodamine (left; red) and fluorescein (middle; green). Right panels are double-exposure micrographs; regions of overlap are yellow. Arrows indicate the boundary between the singly labeled cluster and the doubly labeled cluster. Bar, 100 μm.*

2D). However, coalescence of the GBs along the ventral midline, which normally follows their ventral migration, does not occur in operated embryos. The GBs on either side are located at a distance from the ventral midline; they are separated from each other furthest at their anterior ends (Fig. 10B).

In spite of this incomplete translocation of the GBs, cellular events that lead to segmentation appear to occur in a manner comparable to that in intact embryos. Blast cells are organized in clusters surrounded by tiny cells. Each cluster is transiently polarized in that it is comprised of dorsal larger cells and ventral smaller cells (Fig. 10D). This polarizaton is followed by development of coelomic walls and cavities and appearance of fine fibrous structures (Fig. 10E). Furthermore, as demonstrated in Figure 11, each cluster is comprised of progeny cells of a single primary m blast cell.

Figure 10F shows a representative embryo that developed for 6 days after HRP injection of a 4d cell and deletion of a 2d cell. Apparently this embryo failed to transform into a vermiform shape (compare Fig. 10F with Fig. 4A). Nevertheless, circumferential expansion of the coelomic cavity appears to have occurred to some extent.

Discussion

In this study, we have traced the process by which the mesodermal GBs acquire segmental organization during early *Tubifex* embryogenesis. Our major findings are summarized as follows: (a) segmental organization arises sequentially in the anterior-to-posterior direction along the longitudinal axis of the GB; (b) shortly after its birth, each primary m blast cell undergoes a spatiotemporally stereotyped sequence of cell divisions to give rise to a distinct cluster of cells, which becomes a segment; (c) each segment is comprised of descendants of a single primary m blast cell; (d) segmentation in the mesodermal GB proceeds normally in the absence of the ectodermal GB.

Segmental founder cells

The present study strongly suggests that each primary m blast cell serves as a founder cell of each mesodermal segment. If so, the mesodermal GB in the *Tubifex* embryo could be regarded as a linear series of segmental founder cells, which runs along the anteroposterior axis. We consider that the metameric body plan of *Tubifex* arises from this initially simple organization of segmental founder cells.

During normal *Tubifex* embryogenesis, more than 30 primary m blast cells are produced on either side of the embryo. We did not find any evidence that these cells are different from one another in either spatial or temporal aspects of their proliferation. It appears that primary m blast cells are all identical in their ability to execute mitotic events that lead to segmentation. We think that primary m blast cells are endowed with identical developmental properties that render them able to found segments, probably at the time when they are produced from their parent teloblasts. At present, it is unclear whether segmental founder cells are specified autonomously or via inductive processes, although it is known that primary m blast cells are specified as segmental founder cells in the absence of overlying ectodermal GBs.

Coelomic wall formation

In oligochaetes, each segment contains a coelomic cavity bounded by a parietal layer (or somatopleura), a visceral layer (or splanchnopleura) and transverse septa (Dixon, 1915; Jamieson, 1981). Apparently, individuality of segments is established through coelomic wall formation. It is thought that coelomic wall formation is an essential part of mesodermal segmentation in oligochaetes.

The present observations suggest that relatively small cells resulting from unequal divisions of m blast cells are the source of the coelomic walls. Judging from the sizes of cells involved, two steps are discernible in the process of coelomic wall formation. First, tiny cells, which emerge during the third and fourth divisions, form a thin layer at the boundary between adjacent cell clusters.

This is soon followed by formation of dorsal, ventral and lateral coelomic walls, all of which are made of smaller cells that are produced later than the fifth division. Around this time, cells of a similar small size spread along the thin layer of tiny cells at the cluster boundary, and they establish the septum; apparently the thin layer of tiny cells serves as a template for the septum. Furthermore, this layer may also play a role in preventing cells of adjacent clusters to intermingle.

As development proceeds, cells comprising coelomic walls individually become flattened and the coelomic cavity becomes more distinct through cumulation of spaces between inner cells. At this stage, each mesodermal segment appears as a vesicle containing inner cells at one end. This configuration is reminiscent of mouse blastocysts, which contain a fluid-filled cavity (i.e., blastocoel) bounded by an epithelium (trophoectoderm) and an inner cell mass at one end (Cruz, 1997). It is known that fluid is pumped by the trophoectoderm into the interior of the blastocyst, which gives rise to expansion of the trophoectoderm and formation of the blastocoel (Fleming, 1992). Similar mechanisms may operate in flattening of coelomic walls and the formation of the coelomic cavity in *Tubifex* embryos.

Polarized expansion of segments

Developing segments are characterized by their expansion toward the dorsal midline, which results in enclosure of the endo-dermal tube. In normal embryos, the mesodermal GBs on either side are in contact with each other along the ventral midline. This raises the possibility that, due to this contact, the GBs are unable to expand their ventral edges and they are forced to expand in the opposite direction, viz. toward the dorsal side. This is unlikely, however, because segments expand only to the dorsal side even in embryos where GBs are prevented from coalescing. Rather, dorsalward expansion may reflect the regional differences in motility of the coelomic wall. That is, dorsal edges of the coelomic wall acquire higher motile properties than ventral edges do. In support of this notion, we have found that circumferential fibers, which contain actin cytoskeleton, are localized at the dorsal side of each segment. At present, the mechanisms for dorsal expansion of segments are not known, but it seems likely that circumferential fibers act as contractile fibers, which mediate dorsalward expansion of the coelomic wall.

It remains to be explored how the regional differences in the coelomic wall are generated. One possibility is that the dorsal and ventral edges are differentiated according to positional cues lying in the GB itself or other parts of the embryo. Alternatively, cells comprising the coelomic wall may be specified according to their
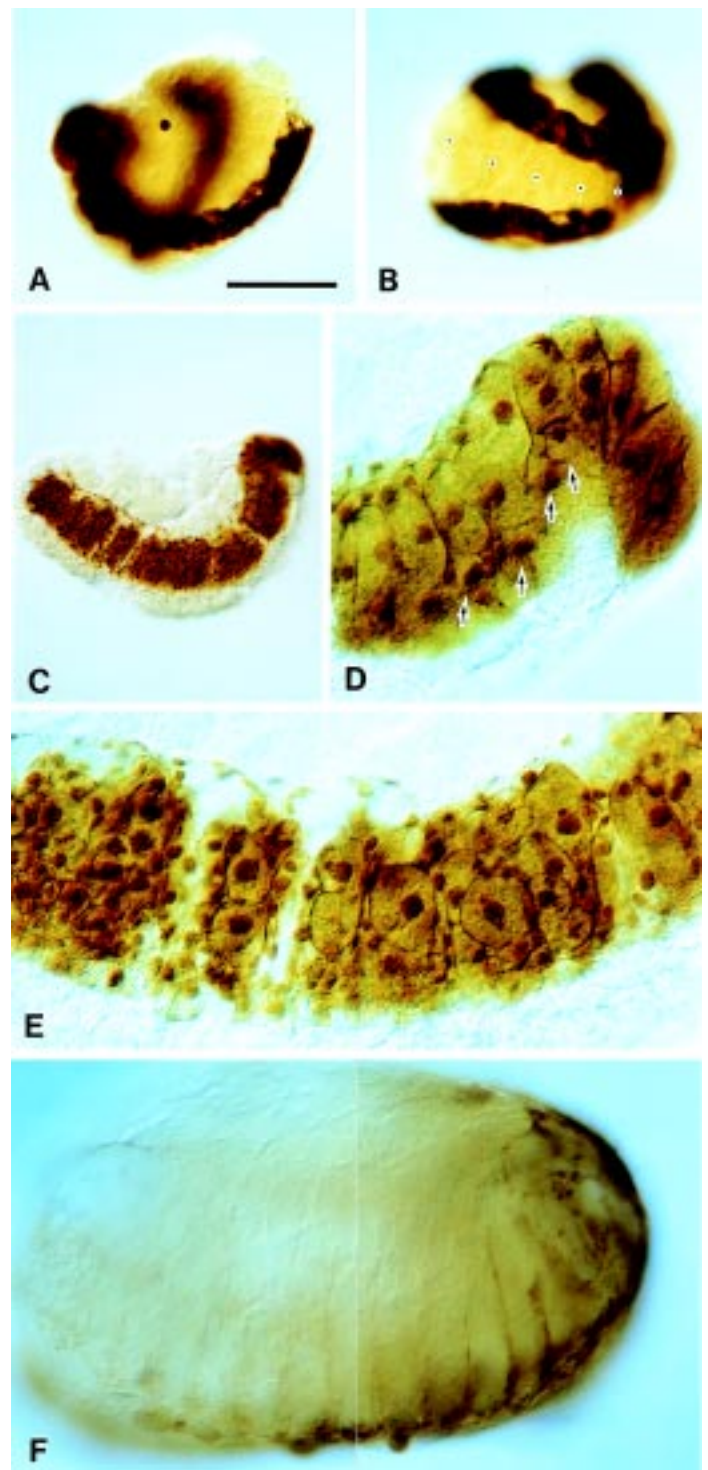

Fig. 10. Formation and segmentation of the mesodermal GB in the absence of the ectodermal GB. *4d cells of 22-cell embryos were injected with HRP, and 2d cells of the same embryos were ablated shortly after division of 4d cells. The operated embryos were allowed to develop for 72 (A-E) or 144 h (F) before fixation.* **(A,B)** *Right side (A) and ventral (B) views of an operated embryo. Whole-mount preparation. HRP-labeled GBs have elongated and curved round toward the ventral side. Note that these GBs are located at a distance from the ventral midline (indicated by dots).* **(C-E)** *A GB dissected out from an operated embryo. Anterior is to the left; dorsal is to the top. (D) and (C) are higher magnifications of the posterior and middle parts of the GB shown in (C).* **(D)** *In the posterior portion, each cluster is comprised of larger dorsal cells and smaller ventral cells (arrows). There are also tiny cells at the boundary between clusters (though these cells are out of focus here).* **(E)** *As development of clusters proceeds, each cluster exhibits coelomic cavities and walls composed of flattened cells. Note longitudinal fibers seen at the ventral edges.* **(F)** *A representative embryo that has developed for 6 days after 2d cell ablation. Whole-mount preparation. Anterior is to the left; dorsal is to the top. HRP-containing cells have formed septum-like structures, which divide the embryo body into segments. Bar, 50 μm (A-C); 100 μm (F); 200 μm (D, E).*
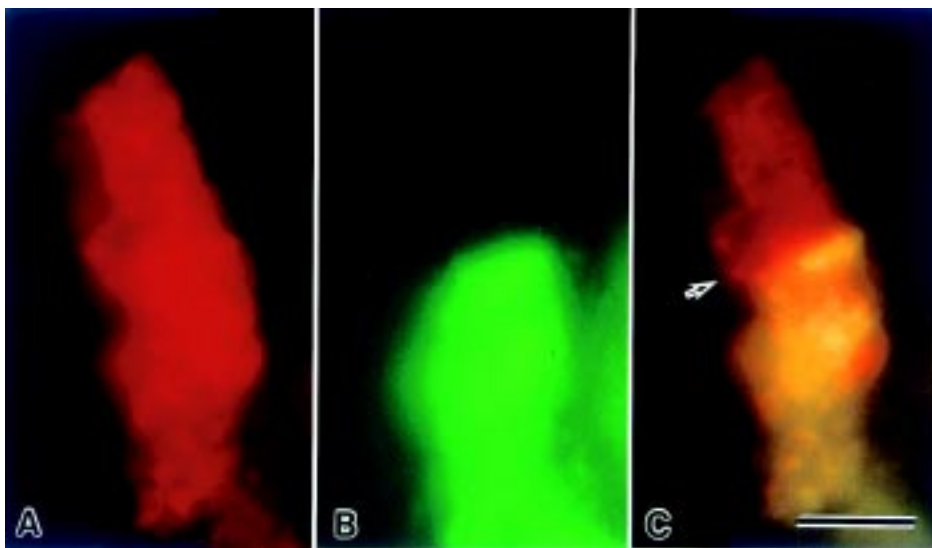
Fig. 11. Evidence for clonal origin of mesodermal segments in the embryo without ectodermal GBs. *2d cells were ablated from 22-cell embryos, and 15 h later, right M cells of the operated embryos were doubly injected with TRD and FLD at a 5-h interval in this order. After a 72-h culture in the darkness, these embryos were fixed and photographed as indicated in Figure 9. Anterior is to the top; dorsal is to the left. All panels show the same field.* **(A)** *TRD distribution.* **(B)** *FLD distribution.* **(C)** *Merged image of TRD and FLD fluorescence. The arrow indicates the boundary between the singly labeled portion (red) and the doubly labeled portion (yellow). Bar, 100 μm.*

lineages; that is, cells in different portions may be distinct from each other as early as their birth. Although we are unable to differentiate between these possibilities at present, it is interesting to note that cell clusters are polarized along the dorso-ventral axis as early as the 5th cell division (C11 in Fig. 6). This suggests that each cluster acquires positional information at an early stage of its development.

Comparison with other annelids

Mesodermal segmentation has been examined in a leech *Helobdella* (Blair, 1982; Zackson, 1982) and oligochaetes *Eisenia* and *Tubifex* (Devries, 1974, 1983; this paper). These annelids exhibit very similar patterns of early development: a pair of M teloblasts bud off small blast cells to generate mesodermal GBs, which acquire segmental organization sequentially in the anterior-to-posterior direction (Anderson, 1973). In both *Helobdella* and *Tubifex*, it has been demonstrated that each mesodermal segment (viz. hemisegment) is comprised of descendants of a single primary blast cell produced from the M teloblast (Zackson, 1982). A similar one-to-one relationship between primary m blast cells and segments has also been suggested in *Eisenia* (Vandebroek, 1934; Devries, 1983; Storey, 1989). Thus, it appears that key elements of the mechanisms underlying mesodermal segmentation have been conserved among oligochaetes and leeches.

In contrast to the aforementioned common framework of mesodermal segmentation, the behavior of individual blast cells during the process leading to segmentation appears to be diverse among annelid species. Although the first blast cell division is oriented perpendicularly to the longitudinal axis of the GB in either annelid species, it occurs at a distance, from the parent teloblast, of ~10 blast cells in *Helobdella* (Zackson, 1984; Nelson and Weisblat, 1992) and ~14 cells in *Eisenia* (Devries, 1983; Storey, 1989), whereas in *Tubifex*, only one cell separates the first dividing cell from the parent teloblast. At present, we cannot make a direct comparison of the division patterns of blast cells due to the paucity of information about this issue in *Eisenia* and *Helobdella*. However, there is some evidence that suggests a difference in the properties of blast cell organization. In *Helobdella*, Blair (1982) has shown that

ablation of ectodermal GBs results in the loss of mesodermal segmental organization. This is in sharp contrast to the situation in oligochaetes, where, as demonstrated in the present study, mesodermal segmentation proceeds normally in the absence of ectodermal GBs (Devries, 1974). It seems likely that, compared with those in leech embryos, the boundaries between adjacent developing segments are stable in oligochaete embryos.

Materials and Methods

Embryos

Embryos of the freshwater oligochaete *Tubifex hattai* were obtained according to Shimizu (1982). For the experiments, embryos were all freed from cocoons in the culture medium. Unless otherwise stated, all experiments were carried out at room temperature (20-22°C).

Microinjection of tracer enzyme HRP

Injection micropipettes were prepared by pulling thin-walled capillaries using a microelectrode puller. HRP (Sigma, type VI-A) was dissolved at 5% in 0.2 M KCl containing 0.5% fast green, and stored at -20°C (Weisblat *et al.*, 1984). This dye inclusion allowed us to monitor the progress of the injection. To sterilize their surface, cocoons were treated with 0.02% chloramine T (Wako Pure Chemical, Osaka, Japan) for 3 min, and washed thoroughly in three changes of the culture medium.

Embryos were freed from the cocoon and removed from vitelline membranes on 2% agar bed. For injection, the embryos were placed in shallow holes made in the agar layer and carefully oriented with target cells upward. Target cells were impaled with micropipettes that had been backfilled with HRP solution, and a small volume (~4% of the cell volume) of the solution was forced into the cells by pressure.

HRP-injected embryos were transferred to petri dishes (covered with 2% agar) with the culture medium containing antibiotics (penicillin G and streptomycin, 20 units/ml each) and allowed to develop at 22°C. The culture medium containing antibiotics in the petri dishes was renewed daily.

Detection of HRP-containing cells

Embryos were fixed with 1% glutaraldehyde in phosphate buffer (40.5 mM Na_2HPO_4, 9.5 mM NaH_2PO_4·$_2H_2O$) for 1 h and washed with phosphate buffer containing 0.5% Triton X-100. The embryos were then incubated for 30 min in phosphate buffer containing 0.025% diaminobenzidine. Color development was carried out in phosphate buffer containing 0.025%

diaminobenzidine and 0.01% hydrogen peroxide for 5-10 min. HRP-containing cells became brown-colored by this treatment.

Stained embryos were dehydrated in ethanol and cleared in a mixture of one part benzyl alcohol and two parts benzyl benzoate, and mounted in this mixture for observation. In some experiments, stained portions were dissected out with a sharpened razor blade before dehydration. In order to closely examine the distribution of the progeny of HRP-injected cells, some embryos were embedded in epoxy resin and serially sectioned with glass knives. Both whole-mount preparations and epoxy-resin thick sections were observed with Nomarski differential interference contrast optics.

Microinjection of FLD and TRD

Both fluorescein dextran (10,000 MW, anionic, lysine fixable; FLD) and Texas Red dextran (10,000 MW, lysine fixable; TRD) were obtained from Molecular Probes, Inc. (Eugene, USA). FLD and TRD were each dissolved at 100 mg/ml in 0.2 M KCl plus 5 mM HEPES (pH 7.2) and 0.5% fast green, and stored at -20°C in the dark. Microinjection of these solutions was performed as described above for HRP. Injected embryos were cultured at 22°C in the dark, and fixed with 3.5% formaldehyde in phosphate buffer for 1 h before observation. Fixed specimens were immersed in 50% glycerol in phosphate buffer containing 2.5% n-propyl gallate and examined by epifluorescence microscopy.

Ablation of precursor cells of ectodermal teloblasts

Embryos without vitelline membranes were placed on 2% agar in the culture medium containing antibiotics. A wound was made with fine forceps on the surface of 2d cells, and within a minute the yolk mass of these cells began to coagulate. The coagulating blastomeres were removed by pulling them away from the remaining living blastomeres. The operated embryos were allowed to develop in the culture medium containing antibiotics at 22°C.

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