

Biology of neglected groups: Annelida /  
Biologie des groupes négligés : Annelida

## An overview of glossiphoniid leech development<sup>1</sup>

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**Abstract:** Dramatic advances in understanding the development of selected “model” organisms, coupled with the realization that genes which regulate development are often conserved between diverse taxa, have renewed interest in comparative development and evolution. Recent molecular phylogenies seem to be converging on a new consensus “tree,” according to which higher bilaterians fall into three major groups, Deuterostoma, Ecdysozoa, and Lophotrochozoa. Commonly studied model systems for development fall almost exclusively within the first two of these groups. Glossiphoniid leeches (phylum Annelida) offer certain advantages for descriptive and experimental embryology per se, and can also serve to represent the lophotrochozoan clade. We present an overview of the development of glossiphoniid leeches, highlighting some current research questions and the potential for comparative cellular and molecular studies.

**Résumé :** Les progrès spectaculaires de la recherche sur le développement d’organismes « modèles » sélectionnés et la constatation que les gènes régulateurs du développement sont souvent conservés d’un taxon à un autre ont ranimé l’intérêt pour leur développement et leur évolution. Les phylogénies moléculaires récentes semblent converger vers un « arbre » consensus nouveau dans lequel les organismes bilatéraux supérieurs appartiennent à l’un ou l’autre de trois groupes principaux, les Deuterostoma, les Ecdysozoa et les Lophotrochozoa. Les systèmes modèles de développement étudiés couramment appartiennent presque exclusivement aux deux premiers de ces groupes. Les sangsues glossiphoniides (phylum Annelida) sont des sujets bien appropriés en embryologie descriptive ou expérimentale et elles peuvent également représenter le groupe des Lophotrochozoa. On trouvera ici une vision globale du développement des sangsues glossiphoniides, dans laquelle sont soulignées les questions courantes en recherche et leur potentiel dans des études comparatives cellulaires et moléculaires.

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### 1. Introduction

Why the leech? In recent years, developmental biologists around the world have converged in applying increasingly powerful genetic and molecular techniques to the embryos of a few vertebrate and invertebrate species, including the mouse *Mus musculus*, the zebrafish *Danio rerio*, the fruit fly *Drosophila melanogaster*, and the nematode *Caenorhabditis elegans* (for reviews see Moody 1999). Given the dramatic advances in understanding the development of these “model” organisms, one may well ask why we should be studying any other species, including annelids.

There are, in fact, several compelling reasons: one is the inherent interest in understanding how all the different kinds of animals develop; another is the intriguing task of prospecting for new developmental phenomena, or for experimentally accessible examples of phenomena that are difficult to study in the model systems.

The last, and perhaps the most important, is the challenge of understanding how changes in developmental mechanisms have contributed to the appearance of new taxa during the evolution of life on earth. Our only hope for obtaining insights into this essentially historical process is to draw inferences as to the nature and timing of developmental changes

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<sup>1</sup>This review is one of a series dealing with aspects of the biology of the phylum Annelida. This series is one of several virtual symposia on the biology of neglected groups that will be published in the Journal from time to time.

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**Table 1.** Stages of glossiphoniid leech embryogenesis.

Stage	Description	Begins with:
1	Uncleaved egg	egg laying
2	Two cells	onset of first cleavage
3	Four cells	onset of second cleavage
4a	Micromere quartet	onset of micromere formation (cleavage of the D to form D' + d')
4b	Macromere quintet	onset of D' macromere cleavage to form DM + DNOPQ
4c	Mesoteloblast formation	onset of cleavage of cell DM'' to form left- and right-hand M teloblasts
5	Ectoteloblast precursor	onset of cleavage of cell DNOPQ''' to form left- and right-hand NOPQ proteloblasts
6a	N teloblast formation	onset of cleavage of cell NOPQ'' to form N and OPQ
6b	Q teloblast formation	onset of cleavage of cell OPQ'' to form Q and OP
7	Germinal-band formation	completion of cleavage of cell OP to form 2 O/P
8	Germinal-band coalescence	onset of coalescence of left- and right-hand germinal bands
9	Segmentation	completion of germinal-band coalescence
10	Body closure	appearance of coelomic space in the 32nd somite
11	Yolk exhaustion	completion of fusion of lateral edges of germinal plate along dorsal midline
Juvenile		exhaustion of yolk in embryonic gut and first feeding

during evolution, by comparing the development of extant species in the context of the phylogenetic tree that relates them. For this purpose we must study the development of embryos representing diverse taxa, and also reach some consensus about their phylogenetic relationships. For example, developmental similarities between *D. melanogaster* and *C. elegans* have been frequently hailed as revealing remarkable conservation of developmental mechanisms because these species represent two disparate phyla (Arthropoda and Nematoda) that were assumed have been separate since before the Cambrian era. But the emerging consensus among molecular phylogeneticists is that the nematodes actually branch within the arthropods into a new protostome clade termed the Ecdysozoa (Aguinaldo et al. 1997). If this is true, the fact that these animals show extensive conservation of developmental mechanisms at the molecular level is less remarkable than the fact that they have evolved such dramatically different body plans and cellular processes of development.

The same consensus now places the mollusks quite firmly with the annelids, flatworms (at least most of them (Ruiz-Trillo et al. 1999)), and a few other groups (McHugh 1997) in a separate protostome clade, the Lophotrochozoa. Operating within this new phylogenetic framework, we hope that our studies of leech development will be useful for making comparative analyses of development and thereby provide insights into the origin(s) of segmentation in protostomes and the remarkable conservation of early cell division patterns among spirally cleaving taxa. Toward that end, our goal here is to recapitulate previous descriptions of the normal development of glossiphoniid leech species, focussing primarily on the small snail-eating species *Helobdella robusta*, and to annotate that description with summaries of progress in areas where cellular and molecular investigations of developmental mechanisms have already been undertaken and with suggestions of interesting areas for further investigation. Glossiphoniid species are useful for studying early leech development because their yolky embryos are relatively large, hardy, and easily cultured. Other leeches, especially the hirudinid *Hirudo medicinalis*, are more widely used for neurobiological and neurodevelopmental studies

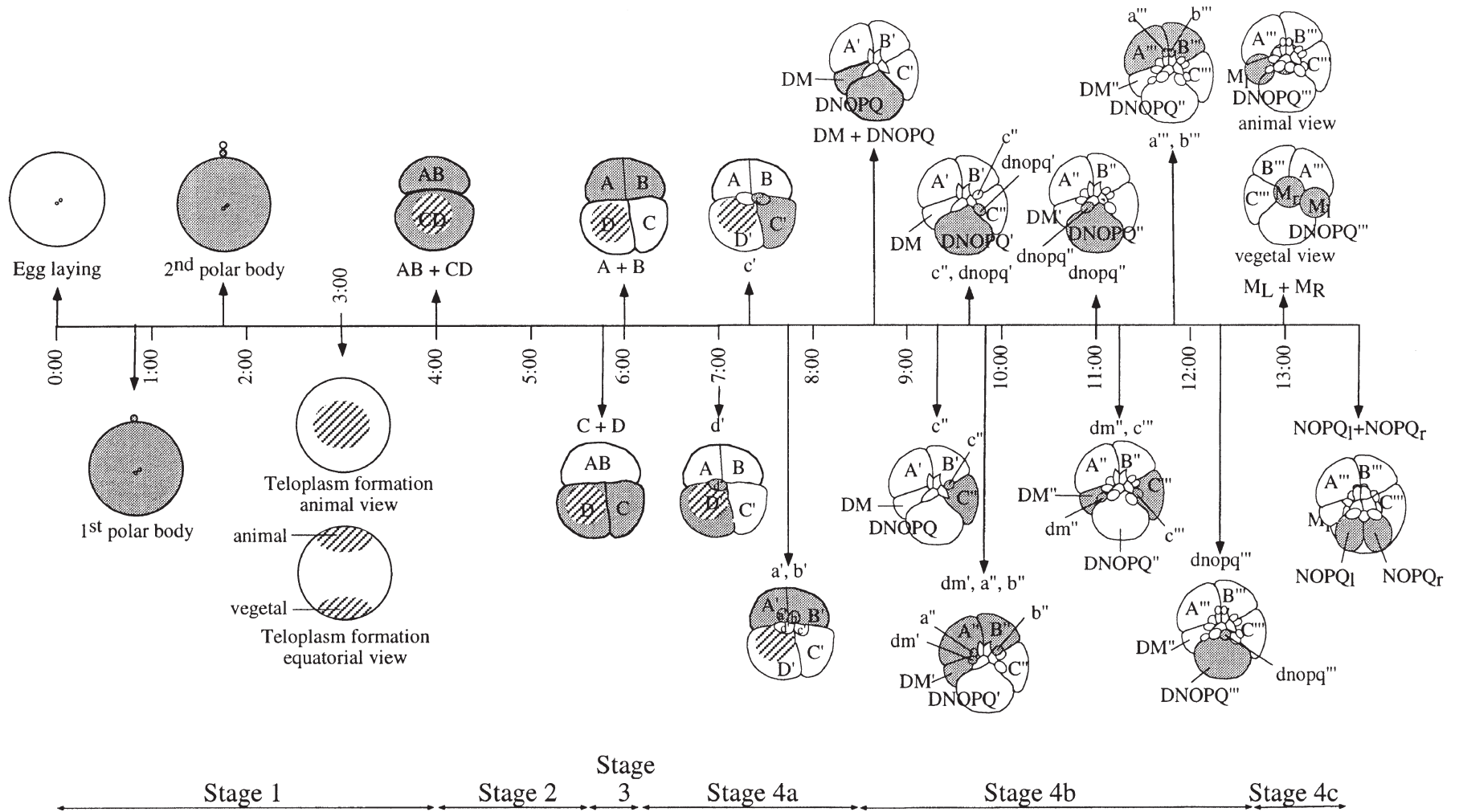
(for review see Stent et al. 1992), but their early embryos are much smaller and more difficult to culture because they are deposited inside an albumen-filled cocoon, the contents of which they must ingest for development to proceed normally. Thus, the extent to which the development of *H. robusta* is characteristic of leeches as a group, not to mention the other classes of annelids, is very much in question.

The system of stages presented here for glossiphoniid leech development and the system of naming embryonic cells are essentially those of Fernandez and Stent (1980; Table 1, Fig. 1). Hermaphroditic like all clitellates, *H. robusta* has also been shown to be capable of self-fertilization (Wedeen et al. 1989) as well as cross-fertilization. The 0.5 mm diameter eggs initiate meiosis upon fertilization, which occurs internally, but meiosis is arrested at metaphase I until the zygotes are deposited in cocoons on the ventral aspect of the parent. Thus, the embryos within each clutch are slightly asynchronous, the stage of development depending on the sequence in which they were deposited. For experiments in which precise knowledge of the timing of cell divisions is required, a large batch or combined batches of embryos can be subdivided into closely synchronized groups by pooling those that pass through a given cell division within a few minutes of each other. When necessary, the timing of developmental events is specified with greater precision than is afforded by the conventional system of stages by indicating them in terms of the number of hours after zygote deposition at a selected temperature, usually 23°C for *H. robusta* (Fig. 1).

## 2. Teloplasm formation

Between the completion of meiosis and the initiation of the first cleavage division, a series of cytoplasmic reorganizations take place, culminating in the formation of yolk-deficient domains of cytoplasm, called teloplasm, at both the animal and vegetal poles of the zygote. The teloplasm is enriched in mitochondria and maternal mRNAs (Fernandez et al. 1987; Holton et al. 1989, 1994). Fernandez et al. (1998b; see also Fernandez et al. 1998a) proposed a three-step model of teloplasm formation, based on ultrastructural and pharmacological studies in *Theromyzon rude*. The first step entails

**Fig. 1.** Time line of *Helobdella robusta* development from egg deposition (stage 1; 0 h after zygote deposition (AZD)) through yolk-depleted juvenile (232 h AZD). At stages 1–4, teloplasm is designated by hatching in quadrant D or its progenitor; embryos in stage 1 are shown in equatorial view, unless labeled otherwise. At stages 1–6, sister cells of the most recent cleavage divisions are shaded. At stage 8, the germinal bands and germinal plate are shaded and the overlying micromere-derived epithelium is depicted as a mosaic of cell outlines. Embryos at stages 2–8 are shown in an obliquely animal pole (prospective dorsal) view, unless labeled otherwise. Embryos at stages 9 and 10 are shown in lateral view unless labeled otherwise. Embryos at stage 11 (juvenile) are shown in dorsal view. Parallel lines indicate breaks in the time line; *gb*, germinal band.



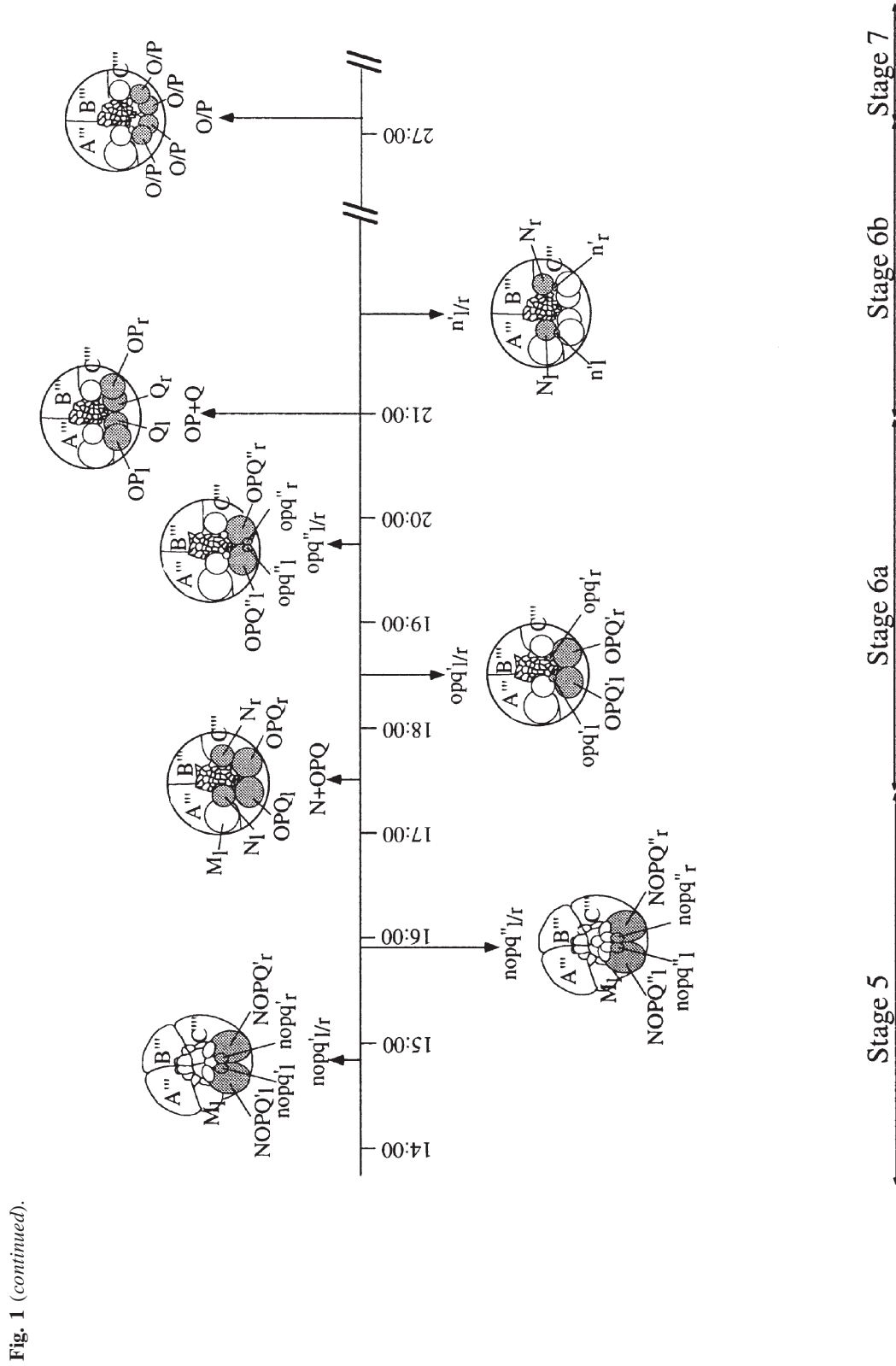


Fig. 1 (continued).

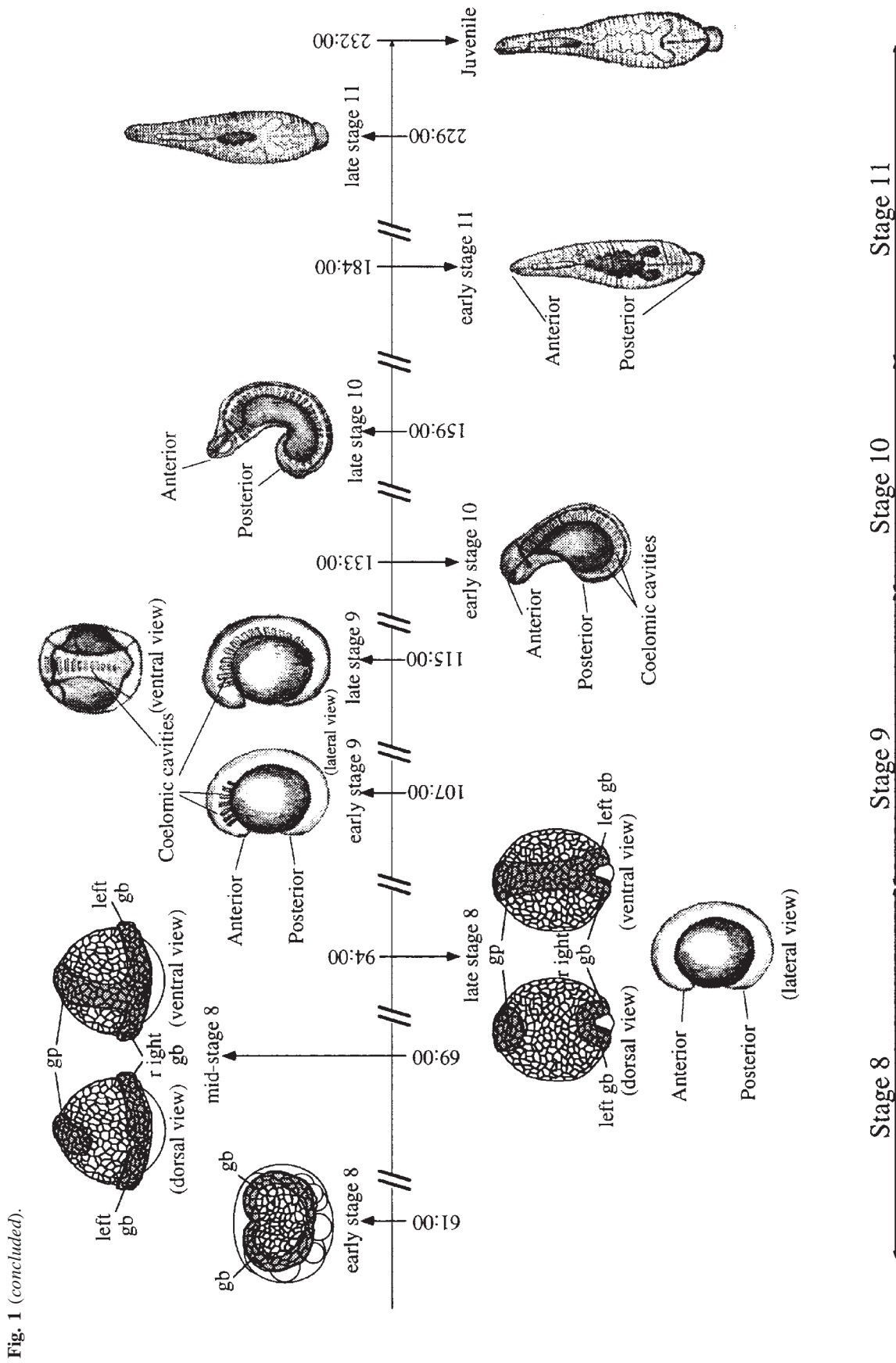


Fig. 1 (concluded).

**Table 2.** Alternative designations of blastomeres.

This paper <sup>a</sup>	Sandig and Dohle 1988
d'	1d
c'	1c
a'	1a
b'	1b
c''	2c
DNOPQ	2d
DM	2D
dnopq'	2d <sup>1</sup>
DNOPQ'	2d <sup>2</sup>
DM'	3D
DM''	3D
a''	2a
b''	2b
dnopq''	2d <sup>21</sup>
DNOPQ''	2d <sup>22</sup>
DM''	4d
DM'''	4d
c'''	3c
a'''	3a
b'''	3b
dnopq'''	2d <sup>221</sup>
DNOPQ'''	2d <sup>222</sup>
NOPQ	T
NOPQ'	T
nopq'	t <sup>I</sup>
NOPQ''	T <sup>II</sup>
NOPQ'''	T <sup>II</sup>
opq'	opq <sup>I</sup>
opq''	opq <sup>II</sup>
n'	n <sup>IV</sup>

<sup>a</sup>After Bissen and Weisblat (1989). Cells are listed in order of birth.

centrifugal movements of mitochondria from deep cytoplasm out to beneath the surface of the zygote, thereby thickening the surface layer of yolk-free cytoplasm. In the second step, this superficial cytoplasmic layer is transported meridionally from equatorial regions to form circumpolar rings of yolk-free cytoplasm. Finally, the constriction of the circumpolar rings to the respective poles results in teloplasm formation. The cytoskeletal basis of these movements is complex in *T. rude* and seems to vary even among the clitellates. In the oligochaete *Tubifex hattai*, teloplasm formation is selectively blocked by cytochalasin B, a microfilament inhibitor (reviewed by Shimizu 1995), while in *H. robusta*, teloplasm formation is blocked by nocodazole, a microtubule inhibitor, but not by cytochalasin (Astrow et al. 1989).

### 3. Cleavage

We define cleavage in glossiphoniid leeches as developmental stages 1–6, which end with the formation of the teloblasts, 10 embryonic stem cells that are the precursors of the segmental mesoderm and ectoderm (Whitman 1878, 1887). As in other annelids, cell divisions in leeches are highly

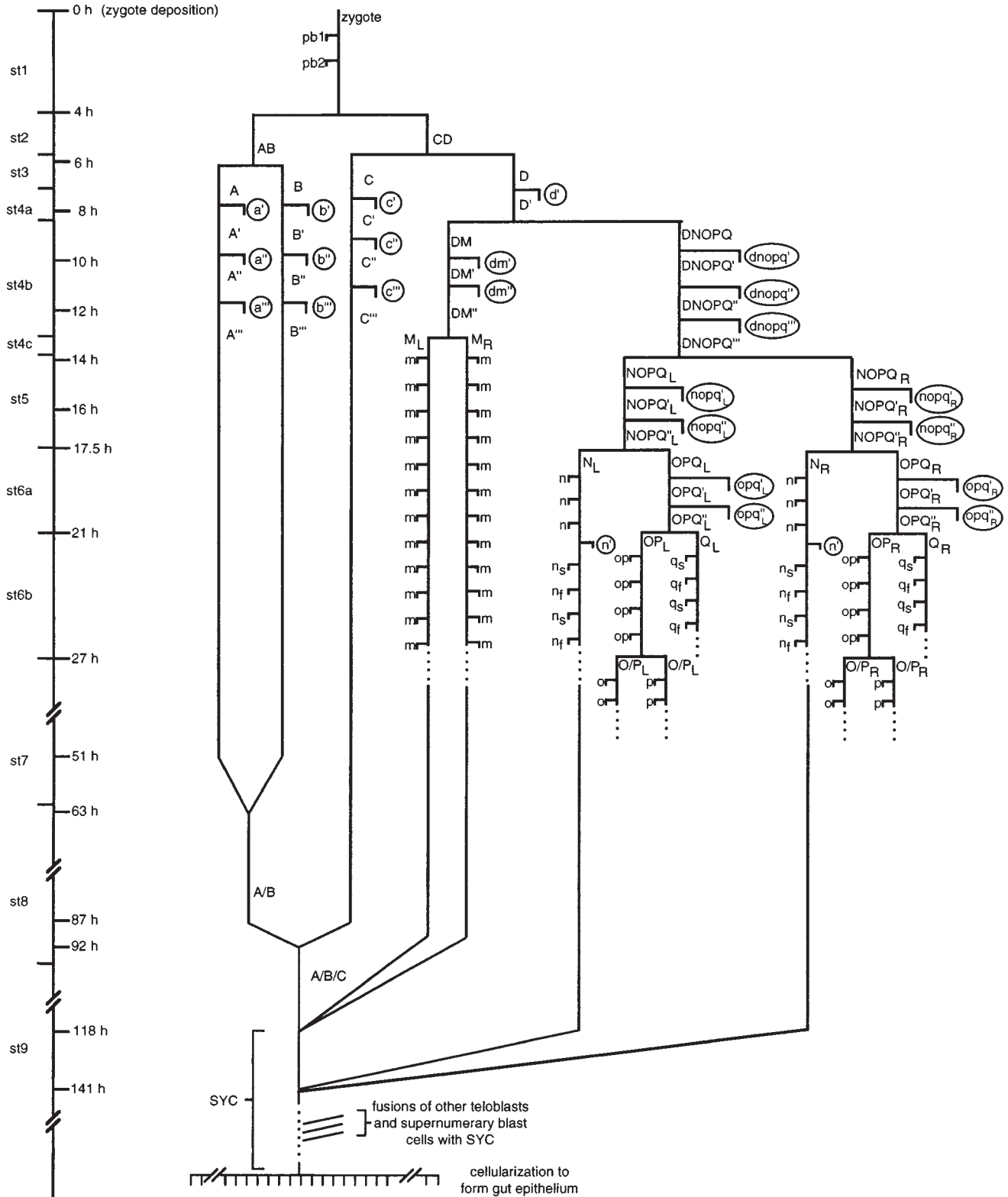
stereotyped by timing, orientation, and the relative size of the sister cells. Thus, early blastomeres can be identified by size, position, birth order, and inheritance of teloplasm. The nomenclature used differs from the standard spiralian nomenclature, as summarized in Table 2.

Three classes of blastomeres arise during cleavage. In addition to the teloblasts there are 3 macromeres, which are the main precursors of the midgut, and 25 micromeres (Sandig and Dohle 1988; Bissen and Weisblat 1989), which contribute to definitive unsegmented tissues and to the epithelium of a provisional integument that undergoes epiboly during gastrulation (stages 7 and 8; Figs. 1, 2). Four macromeres and four micromeres are generated at the highly unequal third cleavage (stage 4a). By the end of stage 6, macromeres A''', B''', and C''' are the largest cells in the embryo, having each produced a total of three micromeres. The teloblasts arise by a unique series of cleavages from macromere D' during stages 4–6; 15 more micromeres also arise from this lineage (Table 2). In this work, micromeres and proteloblasts are designated on the basis of their small size and developmental fates rather than the orientation of the cleavage by which they arise. Thus, for example, the large cell we designate DNOPQ, the precursor of the ectoteloblasts, corresponds to micromere 2d in other spiralian. Moreover, the large cell we designate DM'', the immediate precursor of the mesoteloblasts, is elsewhere referred to as micromere 4d, while the small cell we designate micromere dm'' is elsewhere defined as macromere 4D (Sandig and Dohle 1988; Table 2). Micromeres cluster near the animal pole and are referred to collectively as the micromere cap.

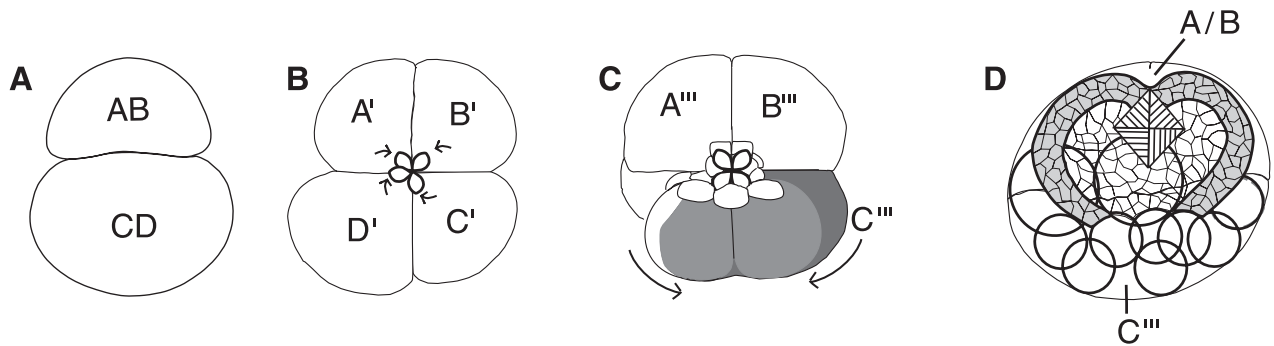
Deviation from the idealized spiral cleavage pattern are apparent beginning at the third cleavage. The blastomeres in quadrants A, C, and D exhibit the normal spiral cleavage pattern, with a dextral third cleavage and then a sinistral fourth cleavage, but quadrant B undergoes sinistral third and dextral fourth cleavages (Sandig and Dohle 1988; F.Z. Huang, unpublished data). Note that this casts the quadrant A and B lineages as mirror-image left–right homologs if the cleavage plane separating A and B is taken as the midline of the embryo (Fig. 3).

This “A–B symmetric” representation of the embryo is in contrast to the usual “D-centric” depiction of spiralian embryos, in which the midline bisects blastomeres B and D at the 4-cell stage. Support for the A–B symmetric representation comes from four further observations. First, it corresponds to the first cleavage plane being transverse to the future anterior–posterior (A–P) axis of the embryo (see Fig. 1), whereas the D-centric representation corresponds to the first cleavage plane being oblique to the A–P axis. Second, micromeres a' and b' give rise to mirror-image symmetric clones of definitive progeny (Nardelli-Haeffliger and Shankland 1993; F.Z. Huang, F. Ramirez-Weber, and D.A. Weisblat, in preparation). Third, as cleavage proceeds, projections from macromere C''' (lying to the right of the midline in the 4-cell embryo) envelop the proteloblasts and teloblasts and fill the spaces between these roughly spherical cells as they form from macromere D' (to the left of the midline in the 4-cell embryo). As a result, the quadrant C and D derivatives both come to straddle the embryonic midline (Fig. 3; see also Weisblat 1999). Fourth, during the stepwise cell fusions that lead to the formation of a syncytial midgut precursor cell

**Fig. 2.** Partial cell-lineage diagram for stages 1–7 of *H. robusta* development. Developmental stages and corresponding development times (at 23°C) are indicated on the time line at the left; breaks in the time line denote changes in scale. Macromeres, protoblasts, and teloblasts are indicated in capital letters, as are the fusion products (A/B, A/B/C, and SYC) leading to gut formation. Lower-case letters denote micromeres (circled) and blast cells (m, n<sub>f</sub>, n<sub>s</sub>, op, q<sub>f</sub>, q<sub>s</sub>). Cell-cell fusions are denoted by the joining of selected lines. Dotted lines indicate the continuing production of blast cells from the teloblasts (M, N, O/P, O/P, Q) and uncertainties in the timing of later SYC fusions (adapted from Weisblat et al. 1999b).



**Fig. 3.** The first cleavage plane in *H. robusta*, which is transverse to the second embryonic axis. Selected stages as viewed from the animal pole are depicted; anterior is up. (A) Stage 2 (~4 h AZD). (B) Stage 4a (~8 h AZD). (C) Stage 5 (~14 h AZD). (D) Early stage 8 (~61 h AZD). Depicting the first cleavage as lying transverse to the future A–P axis (A) at first seems problematic because macromere D', the precursor of the segmental mesoderm and ectoderm, then arises off to one side in the resultant 8-cell embryo (B). But the displacements of the quadrant C and D cells are corrected (large arrows) as macromere C''' (shaded in panel C) envelops the proteloblasts and teloblasts as cleavage proceeds. (In panel C, teloblast precursor NOPQ<sub>L</sub> is shown partially enveloped and precursor NOPQ<sub>R</sub> as fully enveloped.) As a result, by the time cleavage is complete and the germinal bands are forming (shaded in panel D; teloblasts are shown as circles), the progeny of quadrants C and D are effectively superimposed, straddling the midline, by which point macromeres A''' and B''' have fused, forming cell A/B. Moreover, in this representation, since the handedness of quadrant B cleavage is the reverse of that of the other quadrants (Sandig and Dohle 1988), micromeres a' and b' arise as a left–right pair (small arrows in panel B) and only the clones of cells arising from c' and d' must shift to reach their definitive positions (small arrows in panel B). Distributions of the definitive progeny of micromeres a'–d' are indicated schematically in panel D as hatched triangles (a' and b' progeny are denoted by diagonal left-to-right and right-to-left hatching, respectively; d' and c' progeny are denoted by horizontal and vertical hatching, respectively; for a more accurate representation of the positions of these cells see Nardelli-Haeffliger and Shankland 1993; Smith and Weisblat 1994).



(discussed later), macromeres A''' and B''' fuse with one another about 24 h before the resultant A/B cell fuses with macromere C''' (Fig. 2; Liu et al. 1998).

It has been shown that macromere D' inheriting most of the teloplasm during the first three rounds of cell division (Whitman 1878) is the factor that causes the unique series of further divisions leading from it to the formation of the 10 teloblasts and 15 additional micromeres (Astrow et al. 1987; Nelson and Weisblat 1991, 1992; Symes and Weisblat 1992). The obliquely equatorial fourth cleavage of macromere D' separates ectoteloblast from mesoteloblast cell fates (Figs. 1, 2), but contrary to initial expectations, the animal and vegetal domains of teloplasm can each support the production of both meso- and ecto-teloblasts (Astrow et al. 1987; Holton et al. 1989; Nelson and Weisblat 1991, 1992). It appears that the ectodermal fate of the animal daughter (DNOPQ) requires a short-range interaction between teloplasm and the animal cortex of the cell. This fate difference correlates with differences in the expression of a leech *nanos*-class gene (Pilon and Weisblat 1997; D. Kang, M. Pilon, and D.A. Weisblat, in preparation), but no causal relationship has been demonstrated.

The stereotyping of the pattern of cleavage divisions in glossiphoniid leech embryos is accentuated by the fact that they include equal and highly unequal divisions, yielding sister cells that differ manyfold in cell volume. Curiously, while the first round of micromere production proceeds normally even in transcriptionally inhibited embryos, later divisions require zygotic transcription for normal symmetry to be maintained (Bissen and Smith 1996). The links between transcriptional activity, cell-cycle composition, and orientation of cell division have yet to be understood and should prove a productive area of investigation (see Bissen 1999).

As is discussed in detail below, the five pairs of teloblasts normally contribute five distinct segmentally iterated cell lineages of mesodermal (M) and ectodermal (N, O, P, and Q) progeny to the segmented portion of the leech body (Weisblat et al. 1984; Zackson 1984; Weisblat and Shankland 1985). In *H. robusta* and *Helobdella triserialis*, however, much experimental evidence supports the conclusion that the five pairs of teloblasts comprise only four kinds of stem cells, which we designate M, N, O/P, and Q. The O/P teloblasts and their immediate progeny are capable of giving rise to either the O or the P pattern of definitive progeny (Weisblat and Blair 1984; Shankland and Weisblat 1984; Zackson 1984; Huang and Weisblat 1996). Despite a wealth of data concerning phenotypic differences among the different types of teloblasts and their progeny (see following sections), there is as yet no evidence of underlying molecular differences. Given the accessibility of these cells within the embryo, this is a problem that should yield to the application of such techniques as differential-display polymerase chain reaction (PCR) (Zhang et al. 1998) and the construction and comparison of cDNA libraries from single cells (e.g., Korneev et al. 1996).

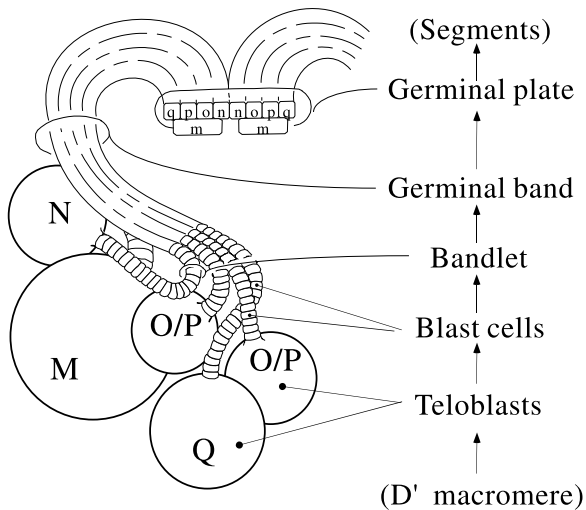
#### 4. Formation of the germinal bands

The preceding description notwithstanding, the embryo comprises more than 38 cells at the end of stage 6. This is because micromeres (such as the primary quartet a'–d') and teloblasts (especially M) that arise early in cleavage initiate further divisions before the final cleavage divisions have occurred.

Leeches possess a fixed number of segments (32), all of which arise during embryogenesis from a posterior growth



**Fig. 4.** Genesis of segmental mesoderm and ectoderm from macromere D' derivatives, showing the arrangement of the bandlets within the germinal bands and germinal plate. Teloblasts and bandlets are shown for the left-hand side only of an early stage 8 embryo; macromeres and micromere derivatives are omitted for clarity (adapted from Weisblat et al. 1984).



zone composed of the 10 teloblasts. (In contrast to many other annelids, leeches cannot regenerate segments or reproduce vegetatively.) The pair of mesoteloblasts (M) generates segmental mesoderm and the four ectoteloblast pairs (N, O/P, O/P, and Q) generate segmental ectoderm. During stages 5–8, each teloblast undergoes several dozen highly unequal divisions, at the rate of about one per hour in *H. robusta*, generating a column of *primary blast cells* called a *bandlet*. We are currently interested in testing the hypothesis that leech homologs of *D. melanogaster* pair-rule genes may be involved in regulating cell division and cell fates within this growth zone (M.H. Song and D.A. Weisblat, in preparation).

Ipsilateral bandlets come together in parallel arrays called *germinal bands*, which contact each other via their distal ends at the future head of the embryo (see Fig. 1, stages 7 and 8; Fig. 4). Within each germinal band, the mesodermal bandlet (m) lies on the surface of the macromeres. The four ectodermal bandlets lie atop and adjacent to it, beneath a micromere-derived squamous epithelium that covers the germinal bands and the territory between them at the animal pole of the embryo. Within the ectodermal layer, the *n* bandlet lies closest to the edge of the epithelium and the *q* bandlet lies farthest from the edge, with the two ipsilateral O/P-derived bandlets between them (Fig. 4). The distinct fates of the O/P-derived blast cells can be reliably assigned on the basis of their position within the germinal band, so their bandlets are now designated *o* and *p* in alphabetic order.

How do the germinal bands form? Only the broadest outline of a descriptive answer to this question is presently available. A key observation is that the distal ends of the *m* bandlets are joined from the time they first form (Fernandez and Stent 1980). This is because the two M teloblasts arise as sister cells from the division of cell DM'' and remain in contact, with their nuclei in apposition across the plasma

membranes. Thus, when the M teloblasts initiate stem-cell divisions, the first blast cells they produce (which constitute the distal ends of the two *m* bandlets) are formed contacting one another between the two teloblasts. Two questions remain: First, how do the conjoined *m* bandlets move from deep inside the embryo to the surface, where the germinal bands form just beneath the micromere-derived epithelium? And second, how do the ectodermal bandlets arrange themselves with respect to one another and to the *m* bandlets?

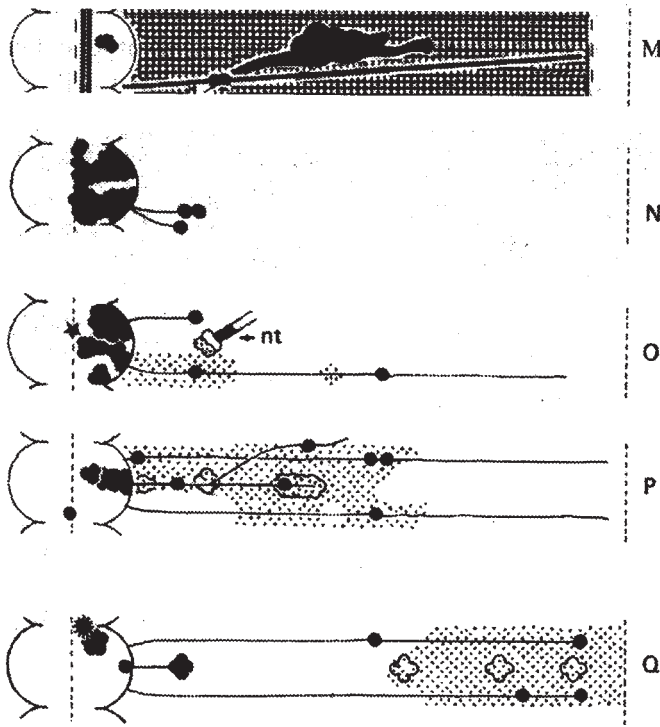
With respect to the first question, it was found that the first blast cells arising from each M teloblast make extensive flattened contact with specific cells, beginning with the left-hand NOPQ proteloblast (M.M. Lee, unpublished data). Thus, we can imagine that these most distal cells in the germinal bands recognize specific cells in sequence and reach the surface by crawling from one to the next, pulling the more proximal blast cells behind them. The first two cells in each *m* bandlet also differ from the standard *m* blast cells in that they later give rise to dispersed clones of progeny in the future head region and not to a set of the segmentally iterated M-lineage descendants (C. Chi, M. Leviten, and D.A. Weisblat, personal communication).

Regarding the ectodermal bandlets, the ectodermal teloblasts on each side of the embryo also arise with close and stereotyped contacts between particular teloblasts, micromeres, and first blast cells. This has been best documented in *Theromyzon tessulatum* (Sandig and Dohle 1988). For example, after each OP proteloblast is born and before it divides to form a pair of O/P teloblasts, it undergoes a few highly unequal divisions (four in *H. robusta*, as shown in Fig. 2; Weisblat and Shankland 1985), yielding a bandlet of 4 blast cells that contribute progeny to the anteriormost segments of the leech. When it then divides equally to form the O/P teloblasts, they are already tethered to the germinal band by the *op* blast cells.

The ectodermal bandlets arise on the surface of embryo, covered only by the micromere-derived epithelium. By this time, the *m* bandlets have reached the surface, and as the ectodermal bandlets on each side arise, they seem to crawl distally along the ipsilateral *m* bandlet. They soon meet at the future head of the embryo, at which time the left- and right-hand germinal bands begin coalescing along the ventral midline to form the germinal plate (Fig. 1, stages 7 and 8).

This process of germinal-band formation means that mesodermal and ectodermal blast cells fated to contribute to the anteriormost segments do not come into register for some time after they are born and do so by moving past *m* blast cells fated to produce more posterior segments. This normal movement of blast cells relative to one another continues throughout the process of germinal-band formation for cells in the *n* and *q* bandlets. This is because, as described in following sections, pairs of sequentially produced blast cells in these bandlets assume distinct identities ( $n_f$  and  $n_s$ ,  $q_f$  and  $q_s$ ) and contribute distinct subsets of definitive progeny to single segments, whereas in the *m*, *o*, and *p* bandlets, each blast cell makes a complete segmental complement of cells (compare Figs. 5 and 6; Zackson 1984; Weisblat and Shankland 1985; Bissen and Weisblat 1987, 1989; Shankland 1999). Because blast cells are produced at about the same rate from each teloblast, there is also a segment-specific age discrepancy between the consegmental

**Fig. 5.** Kinship groups during the formation of segmental mesoderm and ectoderm in five schematic views showing the definitive progeny of the right-hand m, n, o, p, and q bandlets in a typical midbody segment. In each view, the segmental ganglion straddles the ventral midline at the left; the dotted line at the right indicates the dorsal midline. In the mesodermal (M) kinship group, the small solid shape at the left represents M-derived ganglionic neurons and the large solid shape in the center represents the nephridium and its duct; the hatched lines represent circular and longitudinal muscle, the vertical lines represent muscles in the connective nerves, and the diagonal line represents dorsoventral muscle. (Oblique muscles (not depicted) also belong to the M kinship group.) In the ectodermal (N, O, P, Q) kinship groups, dotted domains represent epidermal derivatives, lobed outlines represent epidermal specializations called cell florets, solid shapes represent neurons or clusters of central or peripheral neurons, and stars represent glial cells; *nt*, nephridial-tubule cell (adapted from Weisblat and Shankland 1985).



blast-cell clones in the N and Q lineages relative to the M, O, and P lineages (Lans et al. 1993).

## 5. Epiboly and germinal-plate formation

As more and more blast cells are budded off by the teloblasts, the germinal bands lengthen and move ventrovegetally across the surface of the embryo, coalescing progressively from anterior to posterior along the future ventral midline into a structure called the *germinal plate* (Fig. 1, stage 8). Movements of the germinal bands are accompanied by spreading of the provisional integument. Thus, the micromere-derived squamous epithelium, which covers the germinal bands and the surface of the embryo behind them, comes to cover the entire surface of the embryo through a process of epiboly analogous to that seen in teleost fishes.

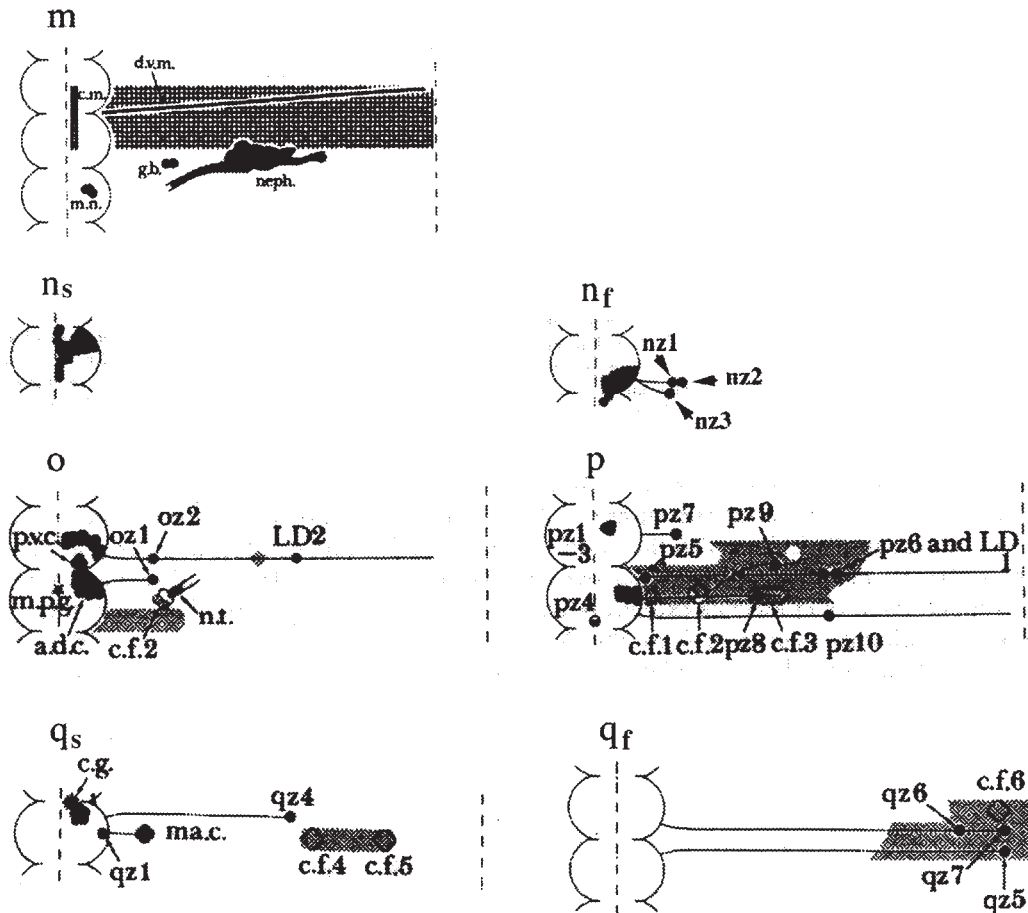
During epiboly, the germinal bands are moving over the surface of the embryo, not spreading, but they do leave a few cells behind them. In addition to the muscle fibers derived from the first 2 blast cells in each m bandlet, 2 or 3 cells migrate out from each m blast-cell clone as it reaches about 50 h of clonal age (in *H. robusta* at 23°C) and contribute additional muscle fibers to the provisional integument (M. Leviten, unpublished observations). These allow the embryo to initiate myogenic peristaltic movements prior to the differentiation of body-wall muscles and nervous system within the germinal plate. During stages 9 and 10, cells proliferate within the germinal plate and it spreads dorsolaterally over the surface of the embryo, displacing the cells of the provisional integument and eventually closing along the dorsal midline of the embryo to form the body tube of the leech.

How do the cell movements associated with germinal-band coalescence and epiboly come about? It was possible to address this question using microinjection of cytotoxic macromolecules such as RNase or the A chain of ricin to poison individual cells without disrupting the embryo as a whole. For example, by poisoning 3 proteloblasts (DM'', OPQ''<sub>R</sub>, and OPQ''<sub>L</sub>) and both N teloblasts after they had produced their n' micromeres, it was possible to block germinal-band formation without disrupting the production of micromeres (Smith et al. 1996). In such embryos the micromere-derived epithelium formed and underwent epiboly, albeit with some delay and with an irregular leading edge relative to control embryos. Thus, the germinal bands are not towing the epithelium vegetally during epiboly.

The converse experiment, preventing the micromere-derived epithelium from forming and looking to see if the germinal bands move and coalesce, is not feasible, partly because it would involve numerous injections of small cells and partly because the micromere may be involved in germinal-band formation. But because each micromere contributes a defined set of cells to the epithelium and because there is little regulation of cell numbers in response to ablation of precursors (Smith and Weisblat 1994), it was possible to create embryos in which the number of cells in the micromere-derived epithelium was reduced by roughly 1/3 (Smith et al. 1996). In these embryos, the germinal bands coalesced and the micromere-derived epithelium underwent epiboly, but in this case the germinal bands led the epithelium during early epiboly, indicating that the epithelium is not responsible for towing the germinal bands vegetally during germinal-band migration.

These observations and others led us to consider a third alternative, that the germinal bands and epithelium are all being towed vegetally by cytoskeletal elements within the underlying macromeres (Weisblat et al. 1999b). We find that epiboly is very sensitive to reagents that interfere with actin microfilaments and actomyosin contractile processes (Cheng and Weisblat 1999), which is consistent with this notion. An important caveat is that the drugs used in that study (cytochalasin D and butanedionemonoxime) cannot be confined to specific cells. Since the drugs were bath-applied, we do not yet know which cells were being affected to block epiboly. Still, the glossiphoniid leech embryo is an interesting system with which to study epiboly, because the relatively small and well-defined populations of cells lend themselves to experimental manipulation.

**Fig. 6.** Spatial distribution of individual blast-cell clones in seven schematic views showing the spatial distribution of the seven types of blast-cell clones (m, n<sub>f</sub>, n<sub>s</sub>, o, p, q<sub>f</sub>, q<sub>s</sub>) with respect to typical midbody segment boundaries. The orientation of the views and the representation of cell types are as in Fig. 5. Identified cells or structures are as follows: a.d.c., anterodorsal neuron cluster; c.f. 1–6, cell florets; c.g., connective glia; c.m., connective muscle; d.v.m., dorsoventral muscle; g.b., gonoblast; LD1 and LD2, lateral dopamine-containing neurons; ma.c., median–anterior nerve neuron cluster; m.n., M-derived neurons; m.p.g., medial packet glia; nz1–nz3, individual neurons; oz1 and oz2, individual neurons; neph., nephridium; p.v.c., posteroventral neuron cluster; pz1 and pz4–pz10, individual neurons; qz1 and qz4–qz7, individual neurons (adapted from Weisblat and Shankland 1985).



## 6. Morphogenesis of segments

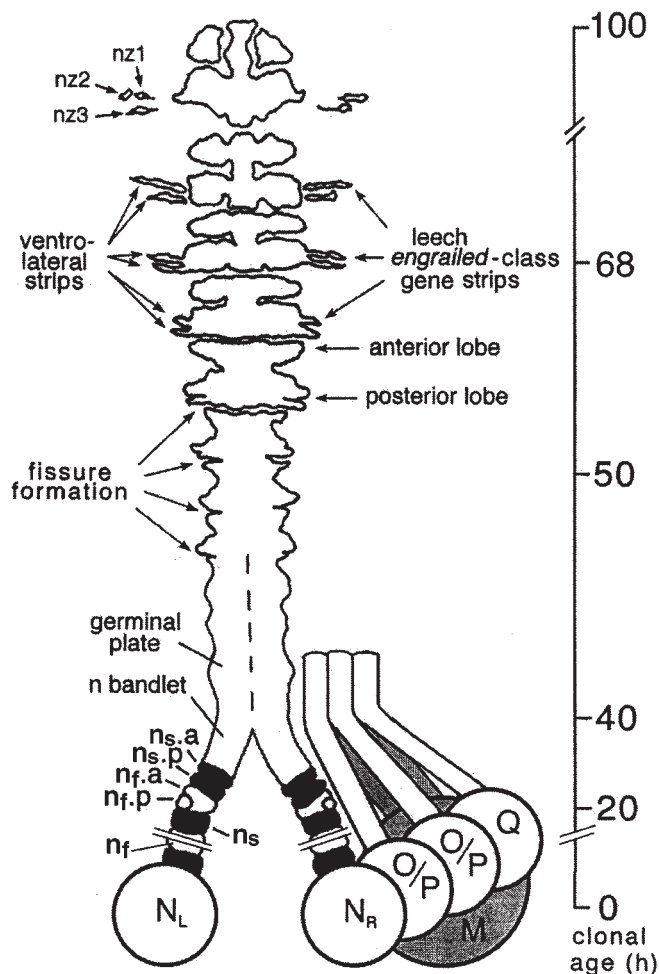
The 32 segments of the leech consist of 4 fused rostral segments (R1–R4), 21 midbody segments (M1–M21), and 7 fused caudal segments (C1–C7). As Stent has pointed out (e.g., Stent 1999), the fundamental problem of segmentation in the leech embryo is “solved” by knowing that the teloblasts undergo repeated divisions and that their blast-cell progeny produce stereotyped clones. Thus, ignoring the gut for now, each morphologically defined segment consists of five bilateral pairs of M, N, O, P, and Q *kinship groups*, a kinship group being defined as all the cells in one segment that arise from a single teloblast (Fig. 5; Stent et al. 1982). Nonetheless, filling out the story of how differentiated, segmentally iterated organs and tissues arise from the blast-cell clones remains a significant challenge for developmental biologists.

Each of the seven classes of blast cells (m, n<sub>f</sub>, n<sub>s</sub>, o, p, q<sub>f</sub>, and q<sub>s</sub>) undergoes a stereotyped series of cell divisions (Zackson 1984; Bissen and Weisblat 1989) to generate a discrete set of roughly 100 definitive progeny that, with certain

exceptions, are identical from one clone to the next (Fig. 6; Weisblat and Shankland 1985; Braun and Stent 1989). Because they are born sequentially, each blast-cell clone of a given type undergoes the same sequence of cellular and molecular events but with a time delay corresponding to its birth order within the bandlet. It is therefore convenient to describe events (cell divisions, gene expression) with respect to the clonal age at which they occur. Although no complete lineages leading from primary blast cell to definitive progeny have been published, this task is feasible (S. Torrence, unpublished analysis of the Q lineage).

Individual blast-cell clones intermingle along all three axes (Weisblat et al. 1984; Weisblat and Shankland 1985; Braun and Stent 1989; Ramirez et al. 1995). Anteroposterior intermingling is most pronounced in the clones of the m, o, and p blast cells; although each of these blast cells makes one segment’s worth of progeny, the individual clones in each lineage interdigitate with anterior and posterior clones of the same type, therefore a kinship group is not a clone (compare Figs. 5 and 6; Weisblat and Shankland 1985; for review see Shankland 1999). Mediolateral intermingling is

**Fig. 7.** Events occurring in the N lineage during gangliogenesis. Mesodermal (M) and other ectodermal (O/P, O/P, and Q) teloblasts and their bandlets are shown only at the right. Bilaterally paired N teloblasts ( $N_L$  and  $N_R$ ) give rise to coherent columns of cells ( $n$  bandlets). Each bandlet comprises two alternating classes of primary blast cells ( $n_f$  and  $n_s$ ), which undergo unequal and approximately equal first mitoses at  $\sim 20$  and  $\sim 28$  h clonal age, respectively, forming secondary blast-cell progeny ( $n_{f,a}$  and  $n_{f,p}$ ,  $n_{s,a}$ , and  $n_{s,p}$ ). Contralateral clones lie next to one another at the ventral midline (broken line) and give rise to  $\sim 2/3$  of the neurons in the segmental ganglia by  $\sim 100$  h clonal age, along with segmentally iterated peripheral neurons (nz1, nz2, and nz3) and a few epidermal cells (not shown). Neurons arising from the other teloblast lineages are not shown. The  $n$  bandlets are divided into ganglionic primordia by transverse fissures that arise at about  $\sim 55$  h clonal age. Two ventrolateral stripes of cells grow out from each posterior ( $n_f$ -derived) lobe later, at  $\sim 68$  h clonal age; the anterior strip in each pair expresses the leech *engrailed*-class gene; anterior is up. Not drawn to scale (adapted from Shain et al. 2000).



most obvious in the Q lineage, which gives rise to mainly dorsal epidermis but also neurons and glia of the central nervous system. Segmental morphogenesis also entails cell movements in the radial direction. For example, the M lineage gives rise to muscle cells that lie between the epidermis

and the ganglion, even though the  $m$  bandlets originate beneath all the ectodermal bandlets.

Within the ectodermal lineages, the most prominent derivatives are the epidermis and the ventral nerve cord, a chain of discrete segmental ganglia linked by intersegmental connective nerves. (A micromere-derived dorsal anterior ganglion, known also as the supraesophageal ganglion, lies in the unsegmented prostomium and is linked to the ganglion in segment R1 by circumesophageal connectives.) Each segmental ganglion contains roughly 200 bilateral pairs of individually identified neurons plus a few unpaired cells (Macagno 1980; Muller et al. 1981). The neural circuitry governing behavior has been extensively studied in leeches, taking advantage of the relatively small numbers of identified neurons and using primarily *H. medicinalis*, in which the ganglia and neuronal cell bodies are large and accessible for making physiological recordings (Nicholls and Baylor 1968; Stent et al. 1979; Friesen 1989).

Each ectodermal lineage contributes both neural and epidermal progeny, but  $\sim 2/3$  of the neurons arise from the N lineage; O-, P-, and Q-derived neuroblasts migrate medially and contribute all but a few of the remaining neurons. How do the discrete ganglia arise from the initially continuous columns of  $n$  blast cells and their progeny in the germinal plate? Working with *T. rude*, Shain et al. (1998) observed the formation of transverse fissures that divide the paired  $n$  bandlets into ganglionic primordia (Fig. 7). In each segment these fissures arise at the junction between the clones of cells arising from secondary blast cells  $n_{f,p}$  and  $n_{s,a}$ . The fissures arise autonomously within the N lineage, i.e., independently of interactions with mesodermal or other ectodermal lineages (Shain et al. 2000). Moreover, differences in cell affinity (adhesivity and (or) motility) between the  $n_f$  and  $n_s$  blast cells have been observed even before these blast cells have gone through their first mitoses (Shain et al. 2000).

It is worth noting that the separation of ganglionic primordia occurs *before* the expression in the N lineage of the leech *engrailed*-class gene, which occurs in transverse, segmentally iterated stripes of cells (Fig. 7; Wedeen and Weisblat 1991). Based on this expression pattern (in the N lineage and others; Lans et al. 1993), by analogy with its function in *D. melanogaster*, and based on the results of blast-cell ablation experiments (Ramirez et al. 1995), we proposed that the leech *engrailed*-class gene is involved in segmentation of the leech nervous system. Instead, it seems more likely that the strips of N-derived cells that express this gene play a role in establishing one of the segmental nerves by which each ganglion connects to the body wall (Shain et al. 1998).

A variety of techniques, including immunostaining for neurons expressing peptide antigens (Shankland and Martindale 1992) and in situ expression to characterize the expression patterns of leech homeobox (Hox) genes (Nardelli-Haeffliger and Shankland 1992; Nardelli-Haeffliger et al. 1994; Kourakis et al. 1997), have been used to reveal segment-specific differences in neuronal phenotypes associated with ectodermal lineages. In conjunction with experimental manipulations that force blast cells to contribute progeny ectopically (Shankland 1984), these results demonstrate conclusively that blast cells within a given lineage can assume segment-specific identities prior to their first mitosis (Nardelli-Haeffliger et al. 1994).

Within the mesoderm (M lineage), segmentation is already overt during stage 7, as each m blast cell gives rise to a discrete cluster of cells, corresponding approximately to hemisomites, within the germinal bands prior to coalescence (Zackson 1982). During stage 9, the coelom arises through the cavitation of mesodermal hemisomites, in an anterior to posterior progression as expected. The septa arise as the juxtaposition of adjacent somite walls. In leeches, in contrast to oligochaetes, the septa are lost during later development. Segmental derivatives of the M lineage include muscles (Torrence and Stuart 1986), the few ganglionic neurons not accounted for by the ectodermal lineages (Kramer and Weisblat 1985), and nephridia (Weisblat and Shankland 1985), which complete differentiation in *H. triserialis* only in segments M2–M5 and M8–M18, thus providing another clear example of segment-specific differences in the fates of individual blast-cell clones. Gleizer and Stent (1993) working with *T. rude* embryos have shown that, as with the ectodermal lineages, these segment-specific differences are to a large extent expressed cell-autonomously in each blast cell.

Little is known about the origins of germ-line precursors in annelids generally. Lineage-tracing experiments with *H. robusta* reveal candidate germ-line cells arising from the M lineage (M. Shankland, personal communication), but uncertainty remains because the gonads of glossiphoniid leeches differentiate so late in development that the lineage tracers are no longer useful. Another approach to this problem springs from the observations that *nanos*-class genes appear to be expressed in and required for developing germ-line precursors in a variety of animals (Kobayashi et al. 1996; Kraemer et al. 1999; MacArthur et al. 1999). The leech *nanos*-class gene, while most heavily expressed during early cleavage, is also expressed in cells that are candidates for testis-sac precursors in midbody segments of *H. robusta* at stages 9 and 10, where lineage-tracing techniques should still be reliable (D. Kang, M. Pilon, and D.A. Weisblat, in preparation). It is hoped that characterizing these cells carefully will permit a definitive conclusion to be drawn as to the origins of the germ-line cells in leeches.

## 7. Gut formation

Beginning at stage 9, the yolk-filled macromeres and remnants of teloblasts become enclosed within the developing midgut and are eventually digested. By the end of stage 11 the yolk has been exhausted and the juvenile leech is ready for its first meal. In leeches, the midgut (crop) and hindgut (intestine and rectum) appear to be secondarily segmented. Both regions feature prominent lobes that are in register with adjacent segments and have been shown to express segmentally iterated patterns of Hox gene expression (*Lox3* and *Lox10*; Nardelli-Haeffliger and Shankland 1993; Wysocka-Diller et al. 1995; Wedeen and Shankland 1997) prior to gut morphogenesis. Gut morphogenesis and *Lox3* expression are both disrupted in regions immediately underlying zones of ablated mesoderm, suggesting that a local signal from mesoderm to endoderm is important in gut morphogenesis (Wedeen and Shankland 1997).

According to the germ-layer theory (Whitman 1887), macromeres A''', B''', and C''' constitute endoderm, but their contributions to the definitive tissues remained unclear until

Nardelli-Haeffliger and Shankland (1993) showed that the gut epithelium is distinct from the (M-derived) visceral mesoderm and arises by cellularization of a multinucleate yolk-filled syncytium, similar to the formation of the *D. melanogaster* blastoderm. The syncytium is derived largely from macromeres A''', B''', and C''', and is termed the syncytial yolk cell (SYC).

The fact that the SYC is a single cell when the midgut epithelium arises by cellularization means that the three macromeres must fuse at some point earlier in development (Fig. 2). Cell–cell fusion is an important aspect of development at various stages in various organisms, but has been little studied apart from the specialized example of sperm–egg fusion. In studying gut formation in *H. robusta* we have found that the three macromeres fuse in a stepwise manner at two different times in development (Fig. 2; Liu et al. 1998; reviewed in Weisblat et al. 1999a). Macromeres A''' and B''' fuse early in stage 8 to form cell A/B. This first fusion step, at least, does not proceed autonomously, but rather has been shown to require signaling from quadrant D and its derivatives (Isaksen et al. 1999). Cell A/B fuses with macromere C''' at about the end of stage 8 to form the SYC, but the process does not end there. It has also been shown that later in development, the meso- and ecto-teloblasts fuse with the SYC, in roughly the order in which they complete blast-cell production (Liu et al. 1998), and that supernumerary blast cells also fuse with the SYC (Desjeux and Price 1999; Shankland 1999).

Thus, as with every other aspect of development, in contemplating gut formation we are left with more questions than answers. In this case, the stage seems set for molecular analyses of signaling from mesoderm to endoderm. The leech embryo may also prove useful for studying the cell biology of cell–cell fusion, and its regulation by “third-party” cells, in this case the quadrant-D derivatives, that do not themselves participate in the fusion event.

## 8. Summary

It should be clear from the preceding overview that studying the development of even a single animal species is a tremendously open-ended endeavor. Even to describe accurately what is happening at the cellular level is a formidable undertaking. And that effort only lays the groundwork for mechanistic analyses, the results of which most typically reveal the need for more detailed description! The wealth of information that has emerged concerning glossiphoniid leech development since Whitman’s work 120 years ago, in fact, merely scratches the surface of what there is to be learned about this one group of annelids. Thus, to gain insights into the evolution of developmental processes by comparing similarly detailed analyses of representatives of all modern taxa will challenge developmental biologists for years to come.

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