

# Early events leading to fate decisions during leech embryogenesis



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*This paper reviews leech development up to the 12-cell embryo. Oogenesis proceeds by a system of nurse cells that contribute to oocyte growth via continuous cytoplasmic connections. Development begins when fertilized eggs are deposited: formation of the polar bodies, and centration of the male and female pro-nuclei is accompanied by cytoskeletal contractions, and formation of teloplasm (yolk-free cytoplasm). The first cleavages are asymmetric: cell D', the largest macromere in the eight-cell embryo, contains most of the teloplasm. At fourth cleavage D' divides equally; its animal and vegetal daughters are precursors of segmental ectoderm and mesoderm, respectively. Teloplasm is a determinant of the D' cell fate. The expression pattern of Hro-nos, a leech homolog to the Drosophila gene nanos, suggests that it may be a determinant associated with the animal cortex and inducing the ectodermal fate in the animal daughter cell of the D' macromere.*

**Key words:** cell fate determinant / *Helobdella robusta* / leech / *nanos*

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IN THIS PAPER we review cellular and molecular aspects of the early cleavage stages in glossiphoniid leech embryos. These early cleavages are of special interest for several reasons. On one hand, there is a high degree of conservation in the expression patterns of homologous homeotic genes during the later stages of development of leeches and flies.<sup>1</sup> Indeed, the story of homeotic genes is one of remarkable conservation across many phyla in terms of expression pattern and localization in the genome;<sup>2,3</sup> such homologies have been used to formulate the zootype concept, i.e. that an animal is an organism that displays a particular pattern of gene expression (especially homeotic class genes) during its phylotypic stage.<sup>4</sup> On the other hand, early embryos from different taxa exhibit strikingly dissimilar embryological processes. For example, while the nuclei of segmental founder cells

acquire their fates within a syncytium in *Drosophila*, the leech embryo features holoblastic cleavages and stereotyped cell lineages and cell fates. Do these early embryological differences mask an underlying molecular homology? In other words could the differences in embryological processes at the cellular level between leeches and flies mask underlying molecular homologies that might provide similar foundations for later developmental events?

The early cleavages of the glossiphoniid leech are also interesting in another respect: by the 12-cell stage, the three major cell fates have already been segregated to specific cells. By what mechanisms are these cell fates segregated? And how are the asymmetries of many of these early divisions generated? By virtue of the fact that the leech, an annelid, is closest to the fly, an arthropod, among the well-studied model organisms in developmental biology, we hope to discover how different but related body plans are generated by comparing the developmental mechanisms and the role played by homologous genes in the two organisms. This article concentrates on the establishment of the earliest asymmetries within leech embryos. Later aspects of leech development have recently been reviewed elsewhere.<sup>1,5</sup>

## Oogenesis

What follows is a summary of several descriptive studies that made use of light and electron microscopy to reconstruct the events occurring during leech oogenesis.<sup>6-14</sup>

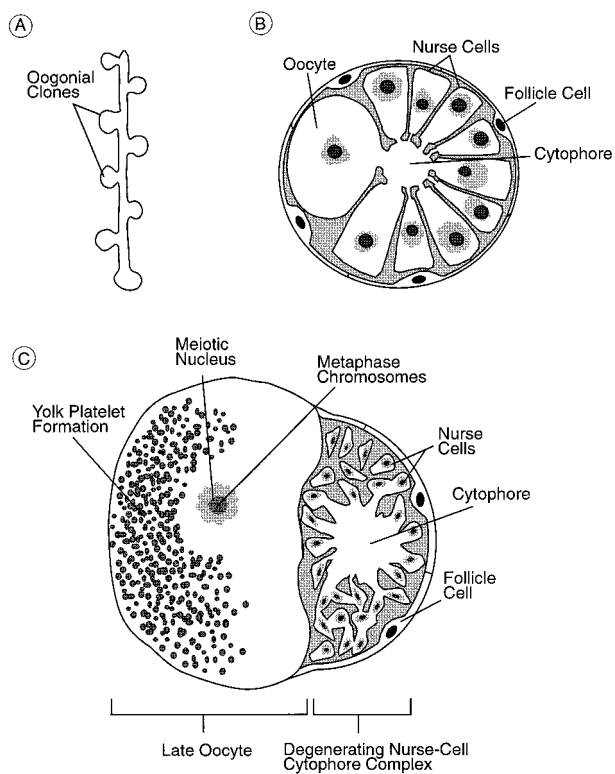
The female germinal epithelium is represented by 'ovary cords' floating freely within the ovisac from which they develop (Figure 1A). At the beginning of a reproductive cycle, the ovary cord becomes thicker and longer due to proliferation of the germinal cells. Division of each germinal cell gives rise to an oogonium and follicle cell. The oogonium proliferates to give rise to a clone of cells, called a polyplast, enclosed within a chamber formed by the descendants of the follicle cell. The early polyplast

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consists of several, apparently identical, pear-shaped cells attached by a stalk of a central anucleate mass, the cytophore (Figure 1B). The exact number of cells in the oogonial clone has not been reported. Eventually one cell of the polyplast differentiates into an oocyte and the remaining cells become nurse cells. The oocyte grows larger than the nurse cells, and microscopy studies indicate that the cytoplasm is, via the cytophore, continuous between nurse cells and the oocyte in early and midclones.<sup>7,8,11,13</sup> This allows transfer of large molecules, and even organelles, from the nurse cell–cytophore complex into the oocyte; oogenesis is therefore remarkably similar to that found in *Drosophila* in that the developing oocyte grows in intimate association with, and at the expense of, nurse cells which transfer material to it. Communication of the oocyte with the cytophore is interrupted when the midclone reaches a certain size.

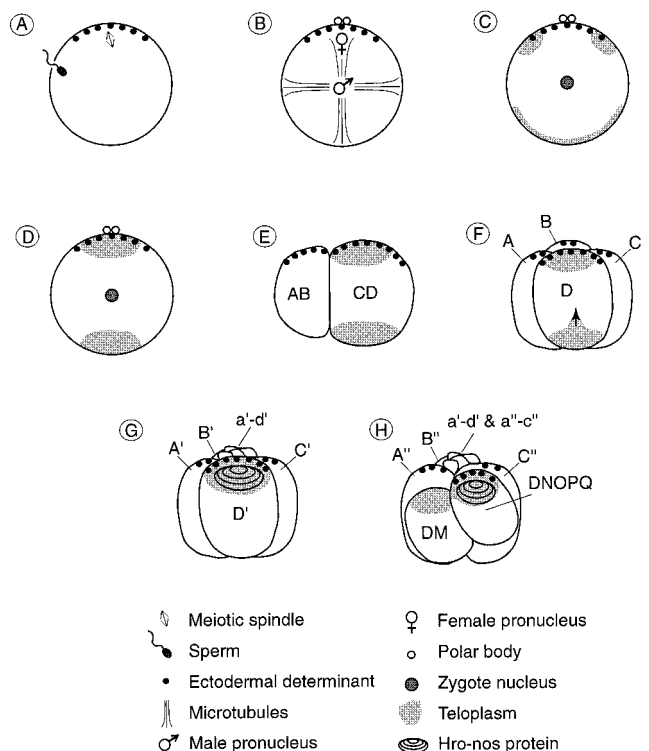


**Figure 1.** Oogenesis in leech. (A) Ovary cords such as the one depicted here float freely within ovisacs in a nutritive fluid also containing some phagocytic cells. Bulges indicate growing oogonial clones. (B) Isolated early oogonial clone. Nurse cells and oocyte share a continuous cytoplasm via a connecting cytophore. Adapted from Sawyer, 1986.<sup>7</sup> (C) Late oogonial clone. The oocyte is enlarged and its cytoplasm is no longer connected to the cytophore; it is soon to be released into the ovisac. Adapted from Fernandez, 1992.<sup>8</sup>

Eventually, the follicle-enclosed clone is released from the ovary cord into the fluid of the ovisac where it continues to enlarge. The assembly of yolk platelets begins in the cytoplasm adjacent to the convex surface of vitellogenic (i.e. yolk-forming) oocytes, which contains stacks of rough endoplasmic reticulum, ribosomes, Golgi complexes and mitochondria. The large meiotic nucleus of vitellogenic oocytes is surrounded by ooplasm rich in organelles and poor in yolk platelets. The first meiotic division progresses to the metaphase stage, at which time the oocyte undergoes meiotic arrest (Figure 1C). At the same time, the nurse cell–cytophore complex degenerates, and cell debris is removed by amebocytes present in the ovisac fluid.

### Precleavage

Leech eggs are fertilized internally but remain arrested at the metaphase of the first meiotic division until after they are laid (ref 15; Figure 2A). Studies of fertilized eggs that were forced to remain within the ovisacs, hours or days beyond the time they were ready



**Figure 2.** Outline of early leech development. See text for details.

to be laid, revealed that after penetration the fertilizing sperm is subjected to migration block: the fertilizing sperm is found along the periphery of the egg animal hemisphere, often close to the meiotic spindle which defines the animal pole.<sup>16</sup> It is not known whether the meiotic spindle is located at the presumptive animal pole before sperm entry or if it migrates near the entry site.

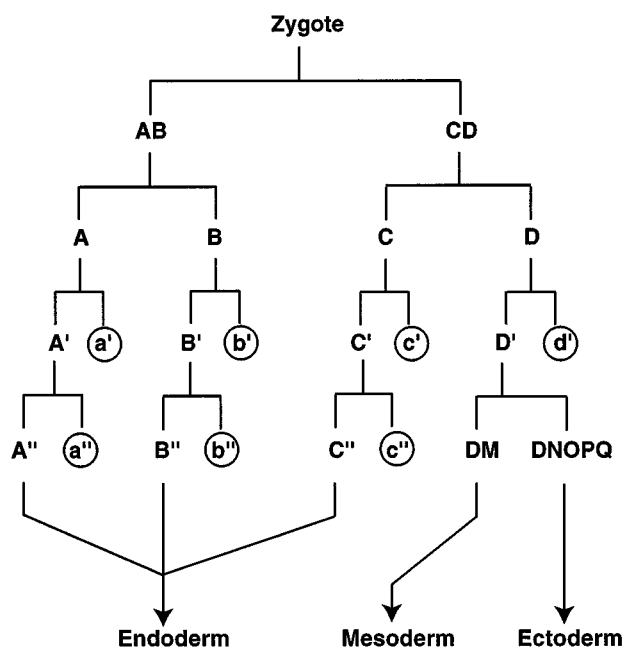
When the eggs are laid, development begins (Figure 2B). Light and electron microscopic observations have made it possible to describe the changes occurring within the uncleaved fertilized egg. The male pronucleus migrates towards the center of the cell as bundles of microtubules grow out of its centrosome, and the meiotic divisions are completed.<sup>16</sup> The production of the two polar bodies is accompanied by actin-based surface contraction waves that likely result in directed cytoplasmic movements.<sup>17,18</sup> Remnants of the disassembled meiotic spindle poles, together with animal ooplasm (a region of cytoplasm rich in organelles that formed during pole cell discharge) and karyomeres (vesicle-like structures containing the female chromosomes) invaginate towards the center of the embryo to fuse with the male pronucleus.<sup>18,19</sup> During the first cell cycle, yolk-deficient domains of cytoplasm, called teloplasm, form at both embryonic poles. At the animal pole, this teloplasm forms as a latitudinal ring centered around the meiotic spindles; in the vegetal hemisphere, teloplasm forms as a continuous disc at the vegetal pole<sup>20</sup> (Figure 2C). Both animal and vegetal teloplasm then condense over their respective poles so that by the end of the first cell cycle there is a pool of teloplasm at each pole (Figure 2D). Teloplasm formation can be inhibited by microtubule destabilizing drugs and is therefore a microtubule-dependent process;<sup>18,20</sup> in the oligochaete *Tubifex hattai* this process is, in contrast, mediated mainly by microfilaments.<sup>20,21</sup> The teloplasm is rich in cytoskeletal elements, membranous vesicles, ribosomes, mitochondria and polyadenylated RNA.<sup>17,22,23</sup> *A priori* any of these materials might function as the determinants associated with inheritance of teloplasm in the experiments described later.

### From zygote to twelve cells

About 4 hours after deposition, the zygote of *H. robusta* elongates along the future dorsal/ventral axis, simultaneously shortening along the animal/vegetal axis. This continues until the first cleavage furrow

appears, segregating most of both teloplasm into the larger cell CD (Figure 2E). At the second cleavage (6.5 hours), cell CD in turn gives rise to a smaller daughter, blastomere C, and a larger daughter, blastomere D, which inherits most of the teloplasm; shortly thereafter, cell AB divides symmetrically to give rise to blastomeres A and B (Figure 2F). Thus, in the four-cell embryo, cells A, B and C are approximately equal in size and contain primarily yolky cytoplasm, whereas cell D is larger and contains both the animal and vegetal teloplasms. At this time, the vegetal teloplasm migrates towards the animal pole of cell D where it forms a single pool with the animal teloplasm.<sup>24</sup> With the third cleavage, quartets of animal micromeres ('primary quartet' micromeres a'-d') and vegetal macromeres (A'-D') arise by highly unequal spiral cleavages (Figure 2C). At fourth cleavage, cells A', B' and C' each divide asymmetrically to produce another set of micromeres (a''-c''). After each producing yet one more micromere, the A'', B'' and C'' macromeres will give rise to the endoderm: they fuse into a syncytium which, upon eventual cellularization, forms the gut epithelium that surrounds the nutritive remnants of the macromeres.<sup>25</sup> Also at fourth cleavage, an obliquely equatorial division of macromere D' separates the segmented ectodermal and mesodermal lineages (Figure 2H). The animal daughter cell of this division, cell DNOPQ, is the precursor to the segmental ectoderm, and the vegetal daughter, cell DM, is the precursor to the segmental mesoderm. According to the standard nomenclature for spiralian embryos, cells DM and DNOPQ would be called macromere 2D and micromere 2d, respectively. But in fact, these cells are approximately equal in size and each inherits a mixture of animal and vegetal teloplasm.<sup>24</sup> Thus, in this article, we use a modified nomenclature for glossiphoniid leech embryos, in which the term 'micromere' has come to mean any of 25 small cells arising from highly unequal cell divisions during cleavage, which do not contribute to definitive segmental tissues.

Cell lineage and cell fates are early and stereotyped decisions in leech embryos. A lineage tree up to the fourth cleavage thus reveals an embryo composed of 12 cells with predictable fates (Figure 3): three yolky macromeres that will form the endoderm, one large cell precursor to the segmented mesoderm, one large cell precursor to the segmented ectoderm, and seven of the eventual 25 micromeres. The micromeres contribute to definitive, non-segmental structures, including the supraesophageal ganglion and proboscis of the prostomium; they also contribute to the



**Figure 3.** Lineage tree of the first four cleavages, leading to the 12-cell embryo. Circles indicate micromeres which contribute non-segmental structures. Cells A'', B'' and C'' give rise to the endoderm of the adult leech. Cell DM contributes most of the segmental mesoderm, and cell DNOPQ contributes most of the segmental ectoderm.

epithelium of the provisional integument, a temporary embryonic structure that is shed at the completion of dorsal closure.<sup>26</sup>

Subsequent to the fourth cleavage, DNOPQ produces four bilateral pairs of ectodermal stem cells (the N, O/P, O/P and Q ectoteloblasts) and 13 micromeres, and DM gives rise to one bilateral pair of mesodermal stem cells (mesoteloblasts) and two micromeres. Teloblasts are embryonic stem cells that constitute a posterior growth zone analogous to the germ band seen in many arthropods, and from which the segments arise.<sup>1,5</sup>

### Early axis and fate specification

In many organisms, egg domains are established during oogenesis in relation to the developing polarized oocyte structure, and are reorganized during oocyte maturation and following fertilization to generate the asymmetries from which embryonic patterning proceeds;<sup>27,28</sup> leeches are no exception to this general rule. The animal-vegetal axis is already evident when the zygote is deposited. This in turn predicts the future position of the teloplasmic pools,

where the quartet micromeres will form, and which of the two large cells born in the D quadrant at the fourth cleavage will become cell DNOPQ, the ectodermal precursor cell. What is the nature of the positional information that correlates with the location of the female pronucleus and on which the subsequent axis specification and cell fate decisions will depend? During oogenesis material is transported from the nurse cells into the oocyte via an opening at one end of the oocyte. By analogy with the situation in *Drosophila*, this is an opportunity for establishing early asymmetries within the oocyte, via the deposition of cortically associated determinants.<sup>29</sup> Whether the animal-vegetal axis is indeed established during oogenesis remains to be tested in leeches, or other annelids. However, that the animal-vegetal axis is fixed prior to first cleavage has been demonstrated by reorientation of this cleavage: micromere production always occurs on that hemisphere containing the site from which the polar bodies were produced.<sup>30</sup>

After egg activation, and as meiosis proceeds, the pre-cleavage surface contraction waves associated with the production of the polar bodies run along the primary animal-vegetal axis of the embryo and likely modify the organization of the egg cytoplasm, possibly bringing together egg components originally localized in different regions. Also, as in many other organisms,<sup>27</sup> centration of male and female pro-nuclei in leech embryos could provide a means of establishing informative cytoskeletal structures along the animal-vegetal axis, since a microtubular network is established by the migrating male centrosome along which the female pronucleus appears to migrate.

The other axis, that running along cells AB and CD following the asymmetrical first cleavage, appears not to be strictly determined during oogenesis nor by the site of sperm entry, as in some other organisms:<sup>27</sup> compression-induced reorientation of the first cleavage does not disrupt development,<sup>30</sup> provided that cleavage occurred roughly parallel to the animal-vegetal axis. The precleavage embryos therefore exhibit apparent radial symmetry along the animal-vegetal axis, up until initiation of the asymmetric first cleavage.

### Evidence of D-fate determinants within the teloplasm

At the four-cell stage, cell D differs from the other three blastomeres in two visible ways: it is larger than the other cells and it contains most of the teloplasm.

The volume occupied by the teloplasm may be sufficient to account for the larger cell volume. It is compelling to imagine that it is the teloplasm which accounts for the unique developmental potential of the D macromere. The idea that the segregation of determinants helps to restrict cell fate has been examined in nematodes, insects, ascidians, amphibians, molluscs and annelids.<sup>31</sup> The notion is particularly substantiated in animals such as ascidians, in which one or several cytoplasmic regions of the egg can be distinguished (e.g. by unique pigmentation) and in which the inheritance of those regions by particular blastomeres correlates perfectly with the fate of their progeny.<sup>32-34</sup> Visually distinct regions of cytoplasm also occur in the early embryos of other annelids such as the oligochaete *Tubifex hattai*: as in leech embryos, a yolk-poor cytoplasm accumulates at the polar regions of the precleavage egg and the cell that inherits it generates the mesodermal and ectodermal precursors.<sup>35</sup>

In leech, several lines of experimental evidence have confirmed that the teloplasm is responsible for the differences in fate that distinguish macromere D from macromeres A, B and C. In one series of experiments, mild centrifugation of two-cell embryos shortly before the second cleavage was used to stratify the teloplasm without killing the embryos.<sup>36</sup> Yolky cytoplasm visibly accumulated at the centrifugal end of the embryo, and yolk-free cytoplasm accumulated at its centripetal end. After centrifugation, teloplasm can be inherited by both cells C and D at the second cleavage. The developmental fates of cells C and D in centrifuged embryos correlate with the amount of teloplasm they receive. In particular, when teloplasm has been distributed roughly equally between the two cells, both cell C and D undergo further cleavages resembling the pattern of divisions normally associated with cell D, thus forming mirror-symmetrical double embryos. Three conclusions may be drawn from these observations: (1) teloplasm is a determinant of D-fate; (2) the C blastomere is fully competent to adopt the fate of the D blastomere, provided that it receives 'enough' teloplasm; and (3) teloplasm does not act strictly quantitatively since both the C and D blastomeres can adopt a 'normal' D-cell fate when each receive roughly half the amount of teloplasm that is normally found in cell D.

These results are consistent with two interpretations: one is that teloplasm contains specific D-lineage instructions (i.e. determinants); the other is that the ability of teloplasm to act as a 'D-fate determinant' resides merely in its richness in translation apparatus,

and other 'house-keeping' cytoplasmic components, which would permit a D-lineage 'program' to unfold in any of the four blastomeres.

### Ectodermal fate determination: teloplasm and cortically associated determinants

The role of the teloplasm in determining the fates of cells DM and DNOPQ has been investigated in experiments where either the animal or vegetal teloplasms were selectively extruded from *H. robusta* zygotes.<sup>37</sup> When animal teloplasm was extruded prior to first cleavage, the ectodermal precursor blastomere, DNOPQ, was converted to a mesodermal fate. Ectodermal fate could be rescued by replacing the extruded animal teloplasm with teloplasm from the vegetal pole, provided that the teloplasm came in direct contact with the animal cortex. The fate of the mesodermal precursor was unaffected by teloplasm extrusions or rearrangements. These results, together with the centrifugation experiments described above, are interpreted to mean that: (1) both cells require teloplasm to undergo the process of teloblast formation; (2) teloplasm is required at or near the animal pole for DNOPQ to produce ectodermal rather than mesodermal teloblasts, i.e. the default fate is mesodermal. This second conclusion led to the proposal that there may be ectodermal determinants localized to the cell cortex in the animal hemisphere that segregate to DNOPQ during early cleavages. This hypothesis was further supported when zygotes were compressed between coverslips in such a way that the animal and vegetal hemispheres were separated by re-orientation of the first cleavage plane from meridional to equatorial: in such embryos the ectodermal fate co-segregates with the animal cortex and the mesodermal fate with the vegetal cortex.<sup>30</sup>

A simple interpretation of these results is that ectodermal determinants are localized to the cell cortex in the animal hemisphere of the zygote; these determinants are activated in the ectodermal precursor by contact with factors in the teloplasm.

### *Hro-nos* a candidate ectodermal determinant

A leech homolog to the *Drosophila* gene *nanos* (*nos*) has been cloned and its expression pattern, studied by northern blots, western blots and immunostaining of embryos, suggest that its mRNA may be a leech ectodermal determinant.<sup>38</sup>

In *Drosophila*, the *nos* and *bicoid* (*bcd*) mRNAs are localized during oogenesis to the posterior and anterior poles of the egg, respectively, and play a role in the formation of the anteroposterior axis within the syncytium.<sup>29,39</sup> The *bcd* protein diffuses from its site of translation at the anterior pole, forming an antero-posterior gradient, while *nos* protein diffuses from the site of its translation at the posterior pole, forming a postero-anterior gradient. These two proteins act antagonistically to regulate the distribution of *hunchback* (*hb*) gene products and hence the differential expression of other interacting regulatory molecules along the length of the embryo.<sup>40-44</sup> In particular, *nos* posteriorly represses translation of the uniformly distributed *hb* RNA through regulatory elements (*nanos* response elements, or NREs) in the *hb* 3' UTR.<sup>45</sup> The ensuing cascade of patterning events, dominated in its early stages by the diffusion of transcription factors within the syncytium, gradually subdivides the embryo into metameric body regions, or segments, which develop synchronously along the length of the embryo.

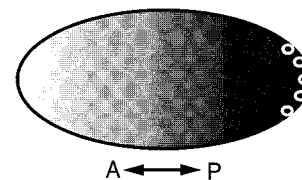
Not surprisingly, the role played by *nos* in establishing embryonic polarity is conserved among other Dipteran species, which, like *Drosophila*, also develop via a syncytial blastoderm.<sup>46</sup> But there is also evidence that *nos*-class genes function in early development of embryos undergoing holoblastic cleavages. A *nos*-class gene designated *Xcat-2* has been cloned from *Xenopus*: its transcript is cortically associated with the vegetal pole of oocytes and, like the *Drosophila nos* mRNA, decays rapidly during early development.<sup>47,48</sup> Furthermore, there are intriguing similarities in the developmental role and the translational regulation of the *hb* gene in *Drosophila* and that of the maternal *g1p-1* gene in *C. elegans*: like *hb*, *g1p-1* contains NRE-like sequences in its 3' UTR and is asymmetrically expressed in the early embryo.<sup>49</sup> These observations have led to the proposal that the non-uniform distribution of a *nos*-class protein, resulting from the cortical association of its mRNA at one embryonic pole, is an ancient mechanism for creating asymmetric patterns of gene expression in early embryos.<sup>50,51</sup>

*Hro-nos*, a leech homolog to the *Drosophila* gene *nos*, encodes a 248-amino-acid protein containing a zinc finger domain characteristic of the *nos*-class proteins. Developmental northern blots showed that the *Hro-nos* mRNA is a maternal transcript that decays during cleavages, with a half-life of approximately 15 hours. A polyclonal antiserum raised against the *Hro-nos* protein was used in developmental western blots and

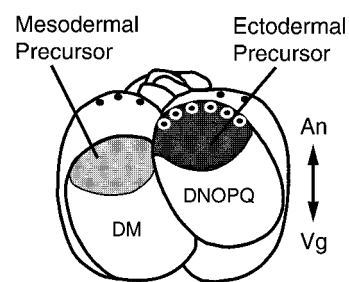
immunostaining of leech embryos of different developmental stages. The *Hro-nos* protein is undetectable in oocytes, first appears in two-cell embryos (4–6 hours of development) and exhibits transient expression peaking in the 12-cell embryo (8–14 hours of development). Finally, suggestive evidence that *Hro-nos* plays a role in establishing early embryonic polarity in leech embryos came from immunostaining experiments (schematized in Figures 2 and 4): the *Hro-nos* protein was asymmetrically distributed between the daughter cells of macromere D': DNOPQ, the animal daughter cell and segmental ectoderm precursor, exhibited much higher levels of *Hro-nos* protein than DM, the mesodermal precursor. The animal to vegetal *Hro-nos* graded distribution is consistent with the following model: translation of the

## Evolutionary Perspective

**Drosophila:** Postero-anterior *nanos* gradient initiates regulatory cascade leading to segmentation.



**Leech:** Animal-vegetal *Hro-nos* graded expression plays a role in ectoderm vs mesoderm specification?



**Figure 4.** Evolutionary perspective. Kimble<sup>50</sup> and Curtis *et al.*<sup>51</sup> have hypothesized that the non-uniform distribution of a *nos*-class protein, resulting from the cortical association of its mRNA at one embryonic pole, is an ancient mechanism for creating asymmetric patterns of gene expression in early embryos (see text for details). While the graded distribution of *Hro-nos* protein in the leech is consistent with this hypothesis, the specific embryological consequences of the *nos* class protein distribution in the ancestor cannot be inferred at present. Shading indicates protein distribution. Black dots indicate localized, translatable *nos*-mRNA in *Drosophila*, or *Hro-nos* (hypothesized) in leech.

*Hro-nos* maternal transcript is initiated at the animal pole when teloplasm comes in contact with the animal cortex. Upon division of D', DM teloplasm is no more in contact with the animal cortex and its *Hro-nos* protein levels declines, while the teloplasm of cell DNOPQ remains in contact with the animal cortex and thus maintains elevated *Hro-nos* protein levels due to sustained translation. Western blots on isolated DNOPQ or DM cells confirm the immunostaining results.

The *Hro-nos* transcript and protein expression patterns are precisely those predicted for the ectodermal determinant as deduced by classical embryological techniques (see above).<sup>30,37</sup> These findings also add to the accumulating evidence that the non-uniform distribution of a *nos*-class protein, resulting from localized translation of its mRNA at one embryonic pole, is an ancient mechanism for creating asymmetric patterns of gene expression in early embryos (Figure 4). This mechanism seems to have been co-opted in the course of evolutionary tinkering to play different roles in different embryos.

## Conclusions

We have reviewed the early events of leech development, from oogenesis to the 12-cell embryo, by which stage the major fates have been segregated to specific cells. These early embryological processes differ dramatically from *Drosophila* development, notably in that leech development begins by holoblastic stereotyped cleavages rather than by a syncytial stage. Nevertheless, some parallels between the early development of leech embryos and that of *Drosophila* have been emphasized.

Our main conclusions, and open questions, are as follows:

1. Leech oogenesis occurs via a system of nurse cells that provides ample opportunity to organize molecular information within the oocyte, but it is not known if the animal-vegetal axis is established prior to fertilization.
2. Zygotes are deposited with an established animal-vegetal axis (meiotic apparatus at animal pole), but the second axis is not fixed until first cleavage.
3. Teloplasm is formed during the first interphase and is a determinant of the 'D-cell' fate.

4. Fourth cleavage segregates the segmental ectoderm and mesoderm lineages among two daughter cells of the D quadrant.
5. Determinants of the segmented ectodermal cell fate are associated with the animal cortex.
6. *Hro-nos* is a leech homolog to the *Drosophila* gene *nanos*, and its high expression in the animal daughter cell of the D' macromere, i.e. cell DNOPQ, suggests that it may play a role in ectodermal fate determination. The maternal mRNA distribution and developmental function of *Hro-nos* remain to be established experimentally.

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