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Cellular pattern formation, establishment of polarity and segregation of colored cytoplasm in embryos of the nematode *Romanomermis culicivorax*

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Abstract

We have begun to analyze the early embryogenesis of *Romanomermis culicivorax*, an insect-parasitic nematode phylogenetically distant to *Caenorhabditis elegans*. Development of *R. culicivorax* differs from *C. elegans* in many aspects including establishment of polarity, formation of embryonic axes and the pattern of asymmetric cleavages. Here, a polarity reversal in the germline takes place already in P_1 rather than P_2, the dorsal–ventral axis appears to be inverted and gut fate is derived from the AB rather than from the EMS blastomere. So far unique for nematodes is the presence of colored cytoplasm and its segregation into one specific founder cell. Normal development observed after experimentally induced abnormal partitioning of pigment indicates that it is not involved in cell specification. Another typical feature is prominent midbodies (MB). We investigated the role of the MB region in the establishment of asymmetry. After its irradiation the potential for unequal cleavage in somatic and germline cells as well as differential distribution of pigment are lost. This indicates a crucial involvement of this region for spindle orientation, positioning, and cytoplasmic segregation. A scenario is sketched suggesting why and how during evolution the observed differences between *R. culicivorax* and *C. elegans* may have evolved.

Keywords: Embryogenesis; Nematode; Polarity; Asymmetry; Midbody; Germline; Axis formation; Cytoplasmic segregation; Evolution; *C. elegans*

Introduction

Establishment of cellular polarity, cell lineages, and cell specification have been analyzed in detail in the embryo of *C. elegans* (clade 9; Fig. 1). However, comparative studies in other nematodes have now revealed remarkable differences between species. For instance, in *Acrobeloides nanus* (clade 11, Fig. 1) early blastomeres are pluripotent and seem to compete for a primary fate. Here, eliminated cells can be replaced by neighbors, which adopt the developmental program of the missing blastomere (Wiegner and Schierenberg, 1998, 1999). This and other findings indicate that different paths can lead to essentially the same result.

As nematodes belong to an ancient and diverse phylum (De Ley and Blaxter, 2002; Meldal et al., 2007) and many species can be cultured in the laboratory, they appear to be excellent candidates to study the evolution of developmental processes.

Over the last decades various attempts have been made to better understand phylogenetic relationships among nematodes. Based mainly on DNA sequence data, a modern nematode phylogeny was suggested by Blaxter et al. (1998), extended and modified by De Ley and Blaxter (2002). Recently, drawing on a larger set of species, more than 300 nearly full-length SSU rDNA sequences were analyzed, revealing a backbone of twelve consecutive dichotomies that subdivide the phylum Nematoda into twelve clades (Holterman et al., 2006; Fig. 1). In the following we will rely on and refer to this work. A further extended phylogenetic tree with emphasis on marine taxa was presented by Meldal et al. (2007).

Embryogenesis of representatives from clades 6 to 12 has been studied at least to some extent (Fig. 1; Sulston et al., 1983; Skiba and Schierenberg, 1992; Malakhov, 1994; Lahl et al., 2003; Houthoofd et al., 2003, 2006; Zhao et al., in press), including the classical model organism *Ascaris* (clade 8, Fig. 1; Boveri, 1899; Müller, 1903). They all show essentially the

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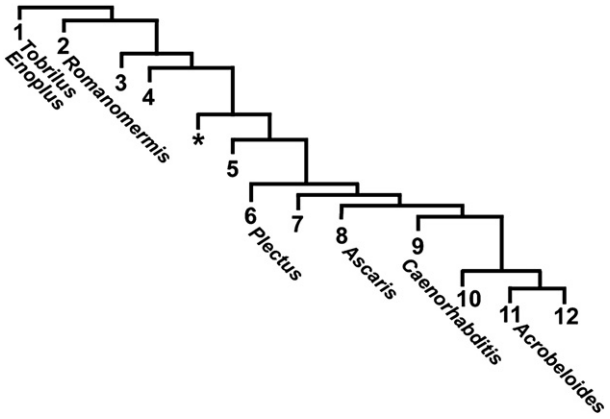

Fig. 1. Phylogenetic tree of nematodes. Simplified phylogenetic tree of nematodes based primarily on rDNA sequence data (Holterman et al., 2006). The tree is subdivided into twelve clades (1–12) and one unresolved branch (*). Branch lengths reflect substitution rates. Affiliations of selected representatives whose early embryogenesis has been studied are shown. For references, see Introduction.

same early cleavage pattern with a germline generating a small number of somatic founder cells. Also fate distribution seems to be similar among these taxa. We know much less in this respect about species belonging to clades 1–5 (Malakhov, 1994) positioned closer to the root of the nematode phylum. It was found that representatives of Enoplida (clade 1; Fig. 1) pass through early equal cleavages without a detectable germline (Malakhov, 1994), and the fate of most blastomeres appears not to be fixed during early development (Voronov and Panchin, 1998; Voronov, 1999). Gastrulation in the genus *Tobrilus* (clade 1, Fig. 1) differs from all other nematodes studied so far in that a large blastocoel forms, as is typical for most other animals (Schierenberg, 2005; our unpublished results).

We chose to study *Romanomermis culicivorax* (clade 2) because its embryos are more transparent and develop faster than other members of this clade we have looked at (e.g., Dorylaimida and Mononchida) and because large numbers of fertilized eggs laid at the 1-cell stage are available. Another advantage of this species is that it can be cultured in large quantities in the laboratory. However, because of its partial parasitic life cycle in insects, refined procedures are required to pass the cultures from one generation to the next (Petersen, 1985; Platzer et al., 2005). Once specimens have emerged from the host, they develop to egg-laying adults without feeding. During recent decades *Romanomermis* and other mermithids have been used as biological control agents to fight insect-borne pests like malaria and dengue fever (Platzer, 1981; Petersen, 1985; Perez-Pacheco et al., 2005).

In this paper we investigate to what extent major developmental differences to *C. elegans* are found during early embryogenesis of *R. culicivorax*, in particular with respect to the establishment of cellular polarity, asymmetric divisions, soma/germline separation, and cytoplasmic segregation, and gain some understanding of how these differences are generated.

Materials and methods

Nematode strains and culture

Caenorhabditis elegans (N2) was cultured on agar plates with the uracil-requiring strain of *Escherichia coli* OP50 as a food source, essentially as described by Brenner (1974) except that, to reduce contamination with other bacteria, we used minimal medium plates (Lahl et al., 2003).

Romanomermis culicivorax (Petersen et al., 1978), *Romanomermis iyengari* (Perez-Pacheco et al., 2005) and *Strelkovimermis spiculatus* (Poinar and Camino, 1986) were kindly provided by Dr. Edward Platzer, University of California, Riverside. Mermithids can be cultured in the laboratory using a variety of different mosquito genera as hosts (Perez-Pacheco et al., 2004). Insect larvae were hatched from eggs and infected as L3 stages by adding infective juvenile nematode worms to the liquid culture. Post-infective nematode larvae kill the host when they emerge and afterward can be cultured in distilled water without any food. More details are found in Petersen and Willis (1972) and Platzer and Stirling (1978). While nematodes which we had received from Dr. Platzer had been passaged through *Culex pipiens* as a host, we successfully initiated a culture using *Aedes aegypti*. Embryos of *R. culicivorax* generally develop inside the eggshell to the L2 stage, where they arrest. Hatching can be induced by osmotic shock (Perez-Pacheco et al., 2004).

Cell nomenclature

For better comparison between *R. culicivorax* and *C. elegans* standard names for germline and somatic founder cells (Sulston et al., 1983) were given based on cell behavior (germline) and sequence of birth (somatic founder cells) as outlined in Results. This can lead to confusion if cells with the same name and origin make different contributions to the embryo. Depending on the outcome of our lineage studies, a different nomenclature may be considered at a later date.

Microscopy

Development was studied with Nomarski optics and pigmented cytoplasm with brightfield illumination, both using a 100× objective. For cell lineage studies early stage embryos were collected from culture dishes with a drawn-out pasteur pipette. Specimens were mounted on slides carrying a thin 3% agarose layer as a mechanical cushion. The coverslip was sealed with petroleum jelly (Vaseline). Embryos were recorded at 23 °C, either on video tape (Lahl et al., 2003) or with a 4-D microscope (Schnabel et al., 1997). A Zeiss Axioskop 2 microscope with an internal focus drive was used to record Z-series (45 focal levels, increment 1.5 μm) through the embryo. Images from an analog camera (VC 45, PCO, Kelheim, Germany) were digitized with an Inspecta-2 frame grabber (Mikroton, Eching, Germany) and compressed 10x with a wavelet function (Lurawave, LuraTech, Berlin, Germany). 3-D tracing of cell behavior was software-supported (Biocell, Simi, Unterschleissheim, Germany). Laser scan microscopy was done with a Zeiss Axiovert 100M microscope coupled to an LSM 510 Meta laser unit.

Laser micromanipulation

For irradiation an N_2-pumped laser microbeam (Photonic Instruments, St. Charles, Ill.; coumarin dye, absorption maximum 440 nm) coupled to a Leica DMLB microscope via glassfiber optics was used. Each embryo was irradiated 5×1 min (at 5–10 Hz) with 2-min intervals in between.

Antibody staining

To decrease the thickness of the eggshell, eggs were incubated for about 4 min in a NaOCl solution (0.75% NaOCl, 25 mM KOH) and afterward washed 3× in distilled water. Treated eggs were transferred to polylysine-coated microscope slides and covered with a coverslip. After freezing in liquid nitrogen, the coverslip was quickly pried off and slides were incubated in methanol followed by acetone (20 min each at −20 °C). Slides were washed 30 min in PBST (PBS, pH 7.4, 0.1% Tween 20) followed by 30 min in blocking solution (PBS, pH 7.4,