Int. J. Dev. Biol. 48: 655-662 (2004)
doi: 10.1387/ijdb.031758ml

Original Article

Differences in maternal supply and early development of closely related nematode species

MAGDALENA LAUGSCH and EINHARD SCHIERENBERG*

Zoologisches Institut, Universität Köln, Köln, Germany

ABSTRACT Comparative analyses revealed considerable differences in embryonic pattern formation and cell-specification between *Caenorhabditis elegans* and *Acrobeloides nanus*, members of two neighboring nematode clades. While *C. elegans* develops very rapidly, *A. nanus* needs 4-5 times as long. To investigate whether differences during early embryogenesis could be related to developmental tempo, we studied three more slowly developing representatives of the genus *Rhabditis*, thus close relatives of *C. elegans*. Besides differences in body size and mode of reproduction, they differ from *C. elegans* in the order of cleavages, germline behavior and requirement for early zygotic transcription, showing evident similarities to *A. nanus*. The distinct variations in cell-cycle rhythms and arrest after inhibition of transcription appear to reflect a species-specific interplay in the timing between exhausting maternal supplies and making available newly transcribed gene products. Looking for the reversal of cleavage polarity in the germline present in *C. elegans* but not in *A. nanus*, two of the studied species express this distinct feature only in a later cell generation. We found that a *C. elegans* mutant in the *mes-1* gene shows a similar deviation. Concerning specification of the gut cell lineage and the potential to compensate for lost cells, the three tested *Rhabditis* species behave less regulatively, like *C. elegans*; in contrast to *A. nanus*, the gut precursor EMS requires an inductive signal from the germline cell P_2 and an experimentally eliminated EMS cell is not replaced by a neighboring blastomere. In conclusion, embryogenesis of the examined *Rhabditis* species includes features of both the fast-developing *C. elegans* and the slow-developing *A. nanus*.

KEY WORDS: *C. elegans, R. dolichura, R terricola, embryogenesis, cell cycle, pattern formation*

Introduction

The embryonic cell lineages of the model organism *Caenorhabditis elegans* have been studied in complete detail (Sulston *et al.*, 1983). Its development is extremely rapid, taking only about 12 hours from fertilization to hatching at 25ºC. Direct manipulation and the analysis of mutants uncovered that, in contrast to the traditional view on nematodes, cell specification involves a combination of cell-autonomous and inductive processes like in other animals (reviews: Schnabel and Priess, 1997; Schierenberg, 1997)

Comparative studies in other nematodes revealed considerable differences in cellular events during early development among them (Skiba and Schierenberg, 1992). An extensive analysis of another soil-dwelling species, *Acrobeloides nanus*, belonging to a neighboring clade, surprisingly uncovered a number of deviations from the pattern observed in *C. elegans* (Wiegner and Schierenberg, 1998; 1999).

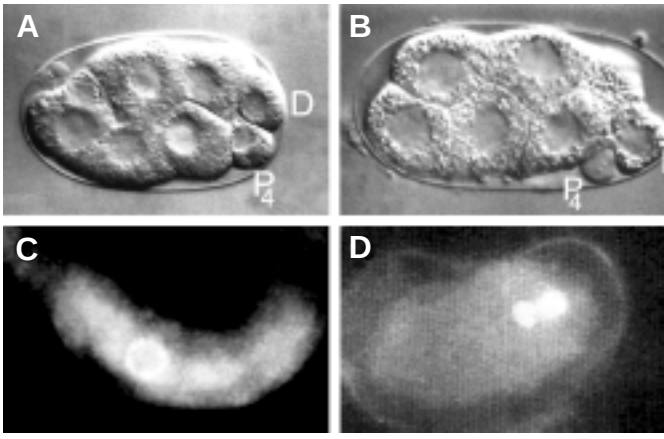
First, embryogenesis of *A. nanus* takes about five times as long. Second, the cleavages in the germline take place prematurely relative to mitoses in somatic cells. Third, a process described as "reversal of cleavage polarity" taking place in the germline cell P_2 and resulting in the new germline cell lying anterior relative to its somatic sister (Schierenberg, 1987), is absent in *A. nanus*. Fourth, in contrast to *C. elegans,* the early *A. nanus* embryo possesses a regulative potential that allows the replacement of eliminated early blastomeres in a sequential and hierarchical manner. Finally, after inhibition of zygotic transcription, development continues to more than 100 cells in *C. elegans*, while in *A. nanus* cleavage arrests very soon under these conditions, indicating a much reduced supply with maternal gene products.

Abbreviations used in this paper: EGM, embryonic growth medium; PR, polarity reversal.

***Address correspondence to:** Dr. E. Schierenberg. Zoological Institute, Universität Köln, Kerpener Str. 15, D-50923 Köln, Germany. Fax: +49-221-470498. e-mail: e.schierenberg@uni-koeln.de

0214-6282/2004/$25.00

Printed in Spain
www.ijdb.ehu.es



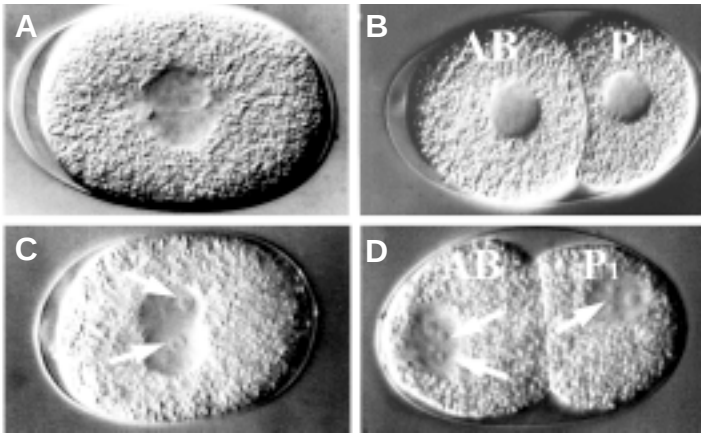


Fig. 1 (Left). Structure and cleavage of the primordial germ cell P_4. (A) R. terricola, *P_4 with yolk granules, nucleus clearly visible;* **(B)** R. belari, *P_4 with essentially no yolk granules, nucleus hardly detectable;* **(C)** R. belari, *immunofluorescence of undivided P_4 in freshly hatched juvenile.* **(D)** R. terricola, *immunofluorescence of P_4 daughters in approx. 150-cell stage embryo. "D" represents the somatic sister cell of P_4. Embryos of all five presented species are about 50-60 µm long.*

Fig. 2 (Right). Presence of nucleoli in early embryos. (A) C. elegans, *1-cell stage just prior to fusion of pronuclei;* **(B)** C. elegans, *2-cell stage;* **(C)** R. terricola, *same stage as (A);* **(D)**R. dolichura, *same stage as (B). Arrows, nucleoli.*

We wanted to know to what extent developmental differences like those found between *C. elegans* and *A. nanus* can be related to their different tempo of development. Therefore, we searched among close relatives of *C. elegans* and *A. nanus* for representatives with considerable differences in developmental timing. So far, all members of the genus *Caenorhabditis* we have looked at develop fast (although somewhat slower than *C. elegans*) and all members of the genus *Acrobeloides* develop slowly. However, we found three *Rhabditis* species (for variations in taxonomic assignment and nomenclature, see Andrassy, 1984; De Ley and Blaxter, 2002), embryogenesis of which is about 2-3x slower than that in *C. elegans*. We examined these with respect to the five aspects laid out above.

Results

Morphology and development: differences and similarities

The three new *Rhabditis* species showed several general differences to each other and in comparison to the two reference systems *C. elegans* and *A. nanus*. These are summarized in Table 1.

Particularly obvious are considerable differences in body size. *A. nanus* has less than half the size of *C. elegans* and *R. dolichura*. In contrast, eggs are quite uniform in size, usually ranging between 50 and 60 µm. However, in all species some eggs of smaller and larger size are found. *R belari* and *A. nanus* share a number of features that are different from the other three species. Both possess only one gonadal arm along with a posterior rather than medial position of the vulva, a correlation typical for nematodes with single-armed (monodelphic) gonads. In both species the division of the germ-cell precursor P_3 is unequal, as found in the other representatives. In addition, P_4 is noticeably more transparent with only few yolk and/or lipid granules making it hard to distinguish the nucleus from cytoplasm (Fig. 1 A,B; for granules, see Schlicht and Schierenberg, 1991; for *A. nanus*, see Skiba and Schierenberg, 1992). This may explain why we found here that P_4 does not divide anymore during embryogenesis (Fig. 1C), while in the other species, including *C. elegans*, it performs one additional equal division generating two germ cells during the first half of embryogenesis (Fig. 1D; Deppe *et al.*, 1978; Sulston *et al.*, 1983). As the applied antibodies directed against *C. elegans* P granules (Strome and Wood, 1983) crossreacted in all analyzed species with germline-specific structures, the staining pattern with one or two germline cells at the end of embryogenesis was used as a confirmation of the lineage studies (Fig. 1 C,D).

An unsual mode of reproduction has been reported for *R. belari* (Belar, 1924; Nigon, 1949), where the sperm is thought to be only required for initiation of embryogenesis as a kind of activating agent without contributing any genetic material to the developing egg (pseudogamy; merospermy). Although we did not study this phenomenon in detail, some of our observations support the original description. These include a low number of males (about 10%), the presence of sperm only in mated females, the inability of isolated, immature females to reproduce, and the presence of only one pronucleus in the egg prior to first cleavage. As pseudogamy can be interpreted as a variant of parthenogenesis,

TABLE 1

MORPHOLOGICAL AND DEVELOPMENTAL FEATURES

feature	*C. elegans* (N2)	*R. dolichura* (ES101)	*R. terricola* (ES102)	*R. belari* (ES103)	*A.nanus* (ES501)
body length (mm)[a]	1.3	0.85	1.4	0.75	0.6
gonadal arms	2	2	2	1	1
vulva position (~%)[b]	50	50	50	80	80
number of pronuclei	2	2	2	1	1
nucleoli present at	~100 cells	pronuclei	pronuclei	2 cells	2 cells
yolk granules in P_4	many	many	many	few	few
germ cells at hatching	2	2	2	1	1
mode of reproduction	hermaph.	hermaph.	dioecious	pseudogamy	partheno.
embryogenesis (h)[c]	12	21	32	26	52
relative duration	1.0	1.7	2.7	2.2	4.3
n =	10	3	6	4	5

a, average of mature, egg-laying adults; b, from anterior; c, approximate absolute duration at 24^0C+/-1°C.

it is another developmental feature connecting *R. belari* with *A. nanus*. While the three *Rhabditis* species need 1.7-2.7 times as long for embryogenesis relative to *C. elegans,* they are by far not as slow as *A. nanus* (Table 1).

There is also one obvious feature found in all analyzed embryos except *C. elegans*. While in the former nucleoli, the organelles of ribosomal biosynthesis (Gerbi *et al.*, 2003), are found already from the pronuclear or 2-cell stages onwards in somatic and in germline cells (Fig. 2 C,D), in *C. elegans* they are absent during this phase (Fig. 2 A,B) and first appear in the gut cell lineage around the 100-cell stage. It remains to be determined whether this indicates differences in early synthetic activity.

Species-specific cleavage patterns

The sequence of the first 16 cell cycles of the three *Rhabditis* species was compared to that of *C. elegans* and *A. nanus* (Table 2).

While each of the five species expresses its own unique and typical sequence of divisions, more detailed examination revealed that the somatic cells initially divide in an identical sequence in all representatives and thereafter also in a very similar order. Essential differences only exist in the timing of germline divisions. While in *C. elegans* the primordial germ cell P_4 is generated only with the 11th cell cycle, in *A. nanus* this takes place already with the 5th cell cycle. The studied *Rhabditis* species uniformly occupy an intermediate position by generating P_4 in the 7th cell cycle. This observation is in accordance with the previously formulated model (Skiba and Schierenberg, 1992) that the germline must be separated from the soma in time, i.e. relatively earlier in slow developing nematodes.

Polarity reversal in the germline

A characteristic developmental event, named reversal of cleavage polarity, takes place in the early *C. elegans* embryo with the cleavage of P_2. This process can most easily be observed in partial embryos from which the anterior AB cell has been removed in order to give ample space for the posterior germline cells. While in the zygote P_0 and in P_1 the new germline cell is always positioned posteriorly to its somatic sister cell, after the division of P_2, P_3 will occupy an anterior and its somatic sister C a posterior position (Schierenberg, 1987; Fig. 3A). However, this is different in *A. nanus*, where the germline cell is always born posterior to its somatic sister but compensating migrations later result in a similar spatial arrangement of cells prior to the onset of gastrulation (Skiba and Schierenberg, 1992).

TABLE 2

SEQUENCE OF EARLY CLEAVAGES

sequence of [a] cell divisions	*C. elegans* (N2)	*R. dolichura* (ES101)	*R. terricola* (ES102)	*R. belari* (ES103)	*A. nanus* (ES501)
1	P_0 [b]	P_0	P_0	P_0	P_0
2	$^1AB^2$ [c]	$^1AB^2$ + [d]	$^1AB^2$	P_1	P_1
3	P_1	P_1	P_1	$^1AB^2$	P_2
4	$^2AB^4$	P_2	$^2AB^4$	P_2	$^1AB^2$
5	EMS	$^2AB^4$	EMS	$^2AB^4$	P_3
6	P_2	EMS	P_2	EMS	$^2AB^4$
7	$^4AB^8$	P_3	P_3	P_3	EMS
8	$^1MS^2$	$^4AB^8$	$^4AB^8$	$^4AB^8$	$^4AB^8$
9	$^1E^2$	$^1MS^2$	$^1MS^2$	$^1MS^2$	$^1MS^2$
10	$^1C^2$	$^1E^2$	$^1E^2$	$^1C^2$	$^1E^2$
11	P_3	$^1C^2$	$^1C^2$	$^8AB^{16}$	$^1C^2$
12	$^8AB^{16}$	$^8AB^{16}$	$^8AB^{16}$	$^1E^2$	$^8AB^{16}$
13	$^2MS^4$	$^2MS^4$	$^2MS^4$	$^2MS^4$	$^2MS^4$
14	$^2C^4$	$^{16}AB^{32}$	$^2E^4$	$^{16}AB^{32}$	$^2C^4$
15	$^{16}AB^{32}$	$^1C^2$	$^1C^2$	$^1C^2$	$^{16}AB^{32}$
16	$^2E^4$	$^2E^4$	$^{16}AB^{32}$	$^2E^4$	$^2E^4$

a, based on the analysis of 3-5 embryos per strain; minor variations may occur among embryos in some species; b, for better visibility germline cells are shown in bold; c, total cell numbers in the lineage before and after cleavage; d, simultaneous division with P_1.

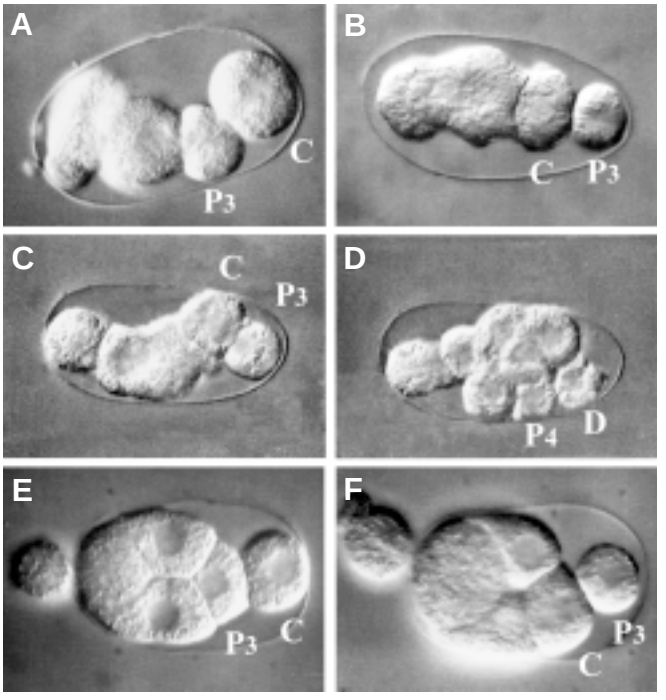


Fig. 3. Reversal of cleavage polarity (PR) in the germline. (A) C. elegans *with PR in P_2;* **(B)** R. belari *without PR;* **(C)** R. dolichura *without PR in P_2;* **(D)** R. dolichura *with PR in P_3;* **(E)** mes-1 *with PR (mothers raised at 16°C);* **(F)** mes-1 *without PR (mothers raised at 25°C).*

In the present study *R. terricola* was found to behave like *C. elegans* (7/7) in this respect. In contrast, in *R. dolichura* (9/11) and *R. belari* (2/4) embryos expressed the polarity reversal only one cell cycle later in P_3 (Fig. 3 B-D).

In an earlier study of the temperature-sensitive maternal-effect *C. elegans* mutant in *mes-1* we had observed (besides other defects) variable abnormalities in the orientation of early germline cleavages (Strome *et al.*, 1995) resembling the pattern found in *R. dolichura* and *R. belari.* This prompted us to examine polarity reversal in embryos of *mes-1* in more detail. We found that in 6/10 cases polarity reversal was absent in P_2 when mothers had been reared at 25ºC (Fig. 3 E,F) but normal when grown at low temperature. However, in four of these six embryos polarity reversal was seen in P_3, similar to our findings in the nematodes described above. Our data indicate that in all cases studied here the control of germline cleavage is less strict than in the *C. elegans* wildtype. Normal development of the two Rhabditis species indicates that modification of polarity reversal alone must not be deleterious.

Establishment of gut cell fate

In *C. elegans* the generation of a gut founder cell requires a receptor-ligand based interaction of P_2 with its sister cell EMS.

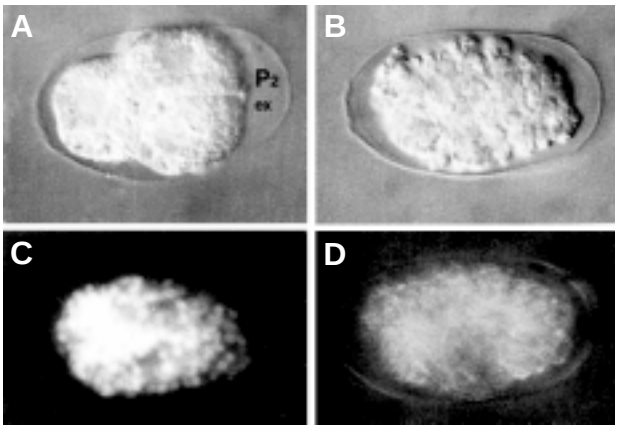


Fig. 4 (Left). No gut induction in *R. dolichura* after removal of P_2 early in the cell cycle. (A) *4-cell stage, P_2 removed; (B-D) terminal phenotype;* **(B)** *DIC optics;* **(C)** *DAPI-labeled, fluorescent nuclei;* **(D)** *polarization optics showing absence of birefringent gut granules. Orientation: anterior, left; dorsal, top.*

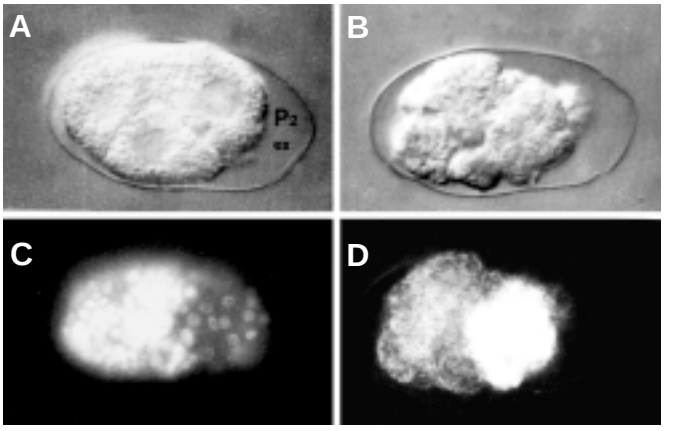


Fig. 5 (Right). Gut induction in *R. dolichura* after removal of P_2 late in cell cycle. (A) *4-cell stage, with P_2 removed; (B-D) terminal phenotype;* **(B)** *DIC optics;* **(C)** *DAPI-labeled, fluorescent nuclei (note large gut nuclei in posterior region);* **(D)** *polarization optics revealing presence of bright birefringent gut granules. Orientation: anterior, left; dorsal, top.*

This interaction takes place in a small time window in the middle of the EMS cell cycle (Goldstein, 1992) and involves genes of the Wnt pathway (Rocheleau *et al.,* 1997; Thorpe *et al*., 1997). In contrast, in *A. nanus* all blastomeres of the 3-cell stage can generate gut autonomously, and inhibiting interactions are required to restrict gut cell fate to EMS (Wiegner and Schierenberg, 1998). To test how the new *Rhabditis* species behave in this respect, we removed the P_2 cell either early or late in the EMS cell cycle. Our results were the same for all three species. When P_2 was removed immediately after its birth, no differentiated gut cells were found in the terminal phenotype (Fig. 4). If, however, P_2 was removed later (just prior to the division of P_2 in *R. dolichura* and *R. belari* or the division of the 2 AB cells in *R. terricola*, see Table 2), the typical differentiation markers for gut cells were formed (Fig. 5). These results demonstrate that concerning this aspect all three *Rhabditis* species behave like *C. elegans*.

TABLE 3

CELL CYCLE LENGTHS (MIN)

Cell(s)	*C. elegans* (N2)	*R. dolichura* (ES101)	*R. terricola* (ES102)	*R. belari* (ES103)	*A. nanus* (ES501)
AB	10[a]	49	39	61	242
2 AB	13	75	37	66	127
4 AB	17	**121**[b]	198	70	121
8 AB	19	85	100	63	93
16 AB	25	65	68	57	83
MS	19	120	243	80	118
2 MS	23	91	123	62	99
E	20	151	286	116	141
2 E	44	119	130	75	185
C	22	195	271	158	376
2 C	25	70	70	74	130
P_1	12	57	46	30	80
P_2	20	62	49	55	110
P_3	27	87	100	73	140
n =	5	3	3	3	5

a, time from generation (cytokinesis of dividing mother cell) to division of a cell at 24ºC (+/-1ºC) is shown. With increasing numbers synchrony within lineages diminishes. Given numbers are values of a single representative specimen per species and mean values for cell cycle durations within individual lineages. In non-*C. elegans* species variations of cell cycle lengths of up to 30% were found between individuals. Nevertheless, the general trends as shown here, including extra-long cell cycles always remained obvious. b, shown in bold, extraordinarily long cell cycle periods in *R. dolichura* and *R. terricola* as reflections of cleavage pauses as described in the text.

Regulative potential

In *C. elegans* eliminated early blastomeres are not replaced, while in the early *A. nanus* embryo cells appear to compete for a higher ranked fate and thus can switch their developmental program in a hierarchical manner if a neighboring cell is lost (Wiegner and Schierenberg, 1999). If for instance EMS is killed, the C cell adopts the EMS fate. When we performed the same experiment (n=3) in each of the 3 *Rhabditis* species, we found in no case that the cleavage program of the C cell changed, which would have indicated a separation into two different lineages (MS and E). In addition, the terminal phenotype never contained differentiated gut cells. From this we conclude that also in this respect the 3 analyzed Rhabditids all behave like *C. elegans.*

Cell cycling and zygotic transcription

We compared the lengths of early cell cycles in each lineage. In the AB lineage 5 cycles were observed, in the germline 3 cycles and in the other lineages 2 cycles (Table 3, Fig. 6). In all five species cell cycles in the germline become longer over time. The behavior of somatic cells is more complex in that each species expresses a different temporal sequence. In *C. elegans* initial somatic cell cycles are short and become longer. In the other species the first cell cycle in MS, E, and C is longer than the second, except *A. nanus* where this is only true for the MS and C lineages. Particularly instructive are the cell cycles in the AB lineage. In the three Rhabditis species studied here, they initially extend and then become shorter.

In *R. dolichura* the third cell cycle and in *R. terricola* in addition the fourth cell cycle in the AB lineage are dramatically prolonged, and as this also occurs in the first cell cycle which the other somatic lineages (MS, E, C; Table 3) pass through at that time, the result is a total absence of any cleavage (all cells remaining in interphase) for more than 90 min prior to the division of P_3 (8-cell

stage). In R. *terricola* an additional phase of no divisions lasting for about one hour was observed after cleavage of P_3.

To test whether this pattern can be correlated to the amount of maternal contribution to the embryonic cleavage program, we blocked zygotic transcription with amanitin in 1-4 cell stage embryos.

It has been shown earlier that under these conditions cleavage in *C. elegans* continues to 120-150 cells (Edgar *et al.*, 1994; Wiegner and Schierenberg, 1998) while *A. nanus* arrests very soon, usually at the 5-cell stage (Wiegner and Schierenberg, 1998). After addition of the drug *R. dolichura* (12/13), *R. terricola* (5/6) and *R. belari* (20/20) stopped with a total of 5-12 cells, independent of when it had been added, i.e. around the same stage when the extended cell cycles appeared (see above). This observation supports the assumption that early transcription is required for continuation of early cleavages in all three *Rhabditis* species, similar to *A. nanus* but unlike *C. elegans*. In addition, the somewhat variable arrest of embryos indicates limited individual differences in maternal supply.

Discussion

The data presented here demonstrate that even closely related nematode species can show considerable differences in anatomy and embryonic development.

Cell cycle control and maternal supply

The species-specific differences in the duration of early cell cycles (Table 3; Fig. 6 a-d) can be best explained with variations in the availability of maternal and zygotic components required for cell division (Fig. 6. A-D).

In *C. elegans* (Fig. 6 a/A) cell cycles increase slowly. According to our interpretation, *C. elegans* possesses a large maternal supply with mitosis-promoting components which diminishes during consecutive cell cycles and thus results in their prolongation. Within the time window we observed, apparently no accelerating zygotic gene products are made available since upon inhibition of transcription early cell cycles remained similar in number and rate compared to controls (data not shown).

A completely different situation is found in *A. nanus* (Fig. 6 d/D). The slow starting zygotic transcription machinery initially causes extremely long cell cycles, but these accelerate increasingly and reach a maximum at a time after the here analyzed phase. This species appears to have a very small maternal supply of cell-cycle relevant components (consistent with the slow cell cycles observed initially), as hardly any divisions take place after transcription is blocked (Wiegner and Schierenberg, 1998).

The cell cycle behavior of *R. belari* (Fig. 6 b/B) with first retarding and then accelerating cell cycles indicates a depleting pool of maternal ressources going along with a simultaneous increase in zygotic components.

R. dolichura (Fig. 6 c/C), and in a similar way *R. terricola* (see Table 3), expressed extremely prolonged cell cycle periods in all somatic lineages in the middle of the observed period, leading to an overall temporary cleavage pause. This behavior indicates a time gap between the depletion of the maternal supply and the availability of zygotic gene products.

The extended cell cycles in the slow-developing species could be due to the presence of a G1 phase, which had been found to be absent during the initial, rapid early cell cycles in embryos of many different species (Zamir *et al.*, 1997; Lefresne *et al.*, 1998). However, our observation that in the analyzed Rhabditis species and in *A. nanus* also mitoses are slower than in *C. elegans* (data not shown) gives more support for a generally low metabolic activity. The changes in cell cycle durations (Table 3) appear to reflect a smooth *(Rh. belari*) or more abrupt (*R. dolichura*, *R. terricola*) transition from maternal to zygotic control of development, resembling some aspects of the midblastula transition in

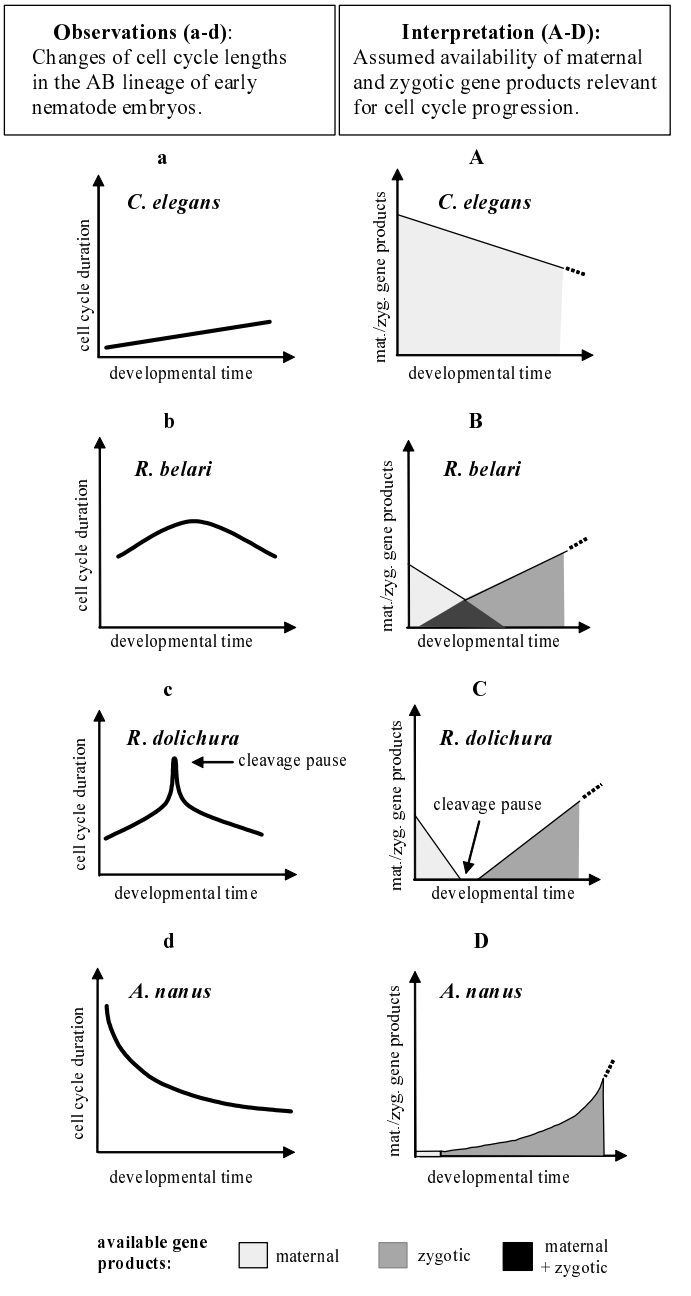


Fig. 6. Different cell cycle patterns and our interpretation in terms of available gene products. (a-d) *Observed changes in cell cycle lengths of the AB cells in four nematode species.* Rh. terricola *behaves similarly to* Rh. dolichura *except for two extra long cell cycles (see Table 3).* **(A-D)** *Proposed decrease of maternal and increase of zygotic components.*

amphibia and other species (Newport and Kirschner, 1982; Großhans *et al.*, 2003).

We do not know the reason for the establishment of different maternal pools, but it may be that the increased storage of maternal products during oogenesis leading to a faster passage through embryogenesis and consequently to more generation cycles per time unit is favorable under specific ecological conditions, while under different circumstances a larger maternal investment does not pay off (Schierenberg, 2001).

In all species studied here the cell cycles in the germline become longer with ongoing divisions (Table 3). A straightforward explanation would be the presence of a transcription block in germ cells as has been reported for *C. elegans* (Seydoux *et al.*, 1996).

Germline and gonad

In contrast to the other representatives considered here (Fig. 1C), *R. belari* and *A. nanus* both possess only one germline cell (P_4) at hatching (Fig. 1D) and the gonad primordium develops only one gonadal arm (Table 1). It has been argued that such monodelphic nematodes constitute a derived form that evolved from didelphic ancestors (Triantaphyllou and Hirschmann, 1980). Cell lineage studies in the monodelphic nematode *Panagrellus redivivus* revealed that in contrast to *C. elegans* an early member of the lineage that generates the somatic gonad (gonadal tube) undergoes programmed cell death (Sternberg and Horvitz, 1981). Cell ablation experiments demonstrated that also in *C. elegans* monodelphic animals can be created if a precursor of the somatic cell that dies in *Panagrellus* is eliminated (Kimble, 1981). However, the presence of two germline cells does obviously not (necessarily) lead to the generation of two gonadal arms, since in *Panagrellus*, like in *C. elegans*, two germline cells are present in the young juvenile.

The asymmetric divisions of the embryonic germline cells in *C. elegans* accompany the unequal distribution of cytoplasmic components, e.g. P granules (Strome and Wood, 1983) and PAR proteins (Kemphues, 2000). In *A. nanus,* and even more dramatically in *R. belari*, an additional polarizing process visible only in the germline cell P_3, results in a grossly unequal segregation of cytoplasmic granules into its descendants (Fig. 1B). Although we can only speculate about the functional significance of this phenomenon, it seems likely that the embryonic cleavage arrest of P_4 is causally related to it. However, our data do not support the notion that the absence of the visible yolk or lipid granules necessarily suppresses cleavage, since granule-free blastomeres (soma and germline) produced by centrifuging embryos of *C. elegans* continue their cleavage program (Schlicht and Schierenberg, 1991; our unpublished results).

The obvious discrepancy between early cleavage arrest in the embryonic germline and extensive divisions after hatching, not only in the examples described here but also in other nematodes, needs to be explained. Based on morphological and experimental data it has been suggested that germ cells of *C. elegans* depend on external nutrition for survival and execution of their postembryonic cleavage program (Sulston *et al.*, 1983; McCarter *et al.*, 1997; Shim *et al.*, 2002).

Reversal of cleavage polarity

We found that two of the examined nematode species show intraspecies variations with respect to polarity reversal in the germline. However, these have no apparent effect on the spatial arrangement of cells produced, suggesting that additional mechanisms are involved to ensure proper localization of blastomeres, in particular the adjacent positioning of germline cells and gut precursors (E cells). This view is supported by the broad contact P_4 makes with Ep in *C. elegans* while loosening contact with its sister D (Schierenberg, 1987) and the extended migration of P_4 to reach the E cells in *A. nanus* (Skiba and Schierenberg, 1992). The analysis of the *mes-1* mutant (Strome *et al.*, 1995) demonstrates that an altered pattern of germline polarity can be induced by mutation of a single gene, suggesting an easy explanation of how variations among closely related species could have been established during evolution. However, in *mes-1* mutants additional defects occur with respect to cell specification. If the genes involved in the expression of germline polarity in the *Rhabditis* species studied here act in the same pathway as *mes-1,* they should do so downstream of it.

Recently, we have started to investigate another nematode of the family Rhabditidae, *Diploscapter coronatus*, where the lack of polarity reversal in P_2 seems to be fully penetrant. On the other hand, as several Plectidae species, phylogenetically much more distant to *C. elegans* (Blaxter *et al.*, 1998), all express a prominent polarity reversal in P_2 (Lahl *et al.*, 2003), this developmental feature must have been modified at least two times independently in different branches of the nematode phylum.

Materials and Methods

Nematode strains

Observations and experiments were carried out with *Caenorhabditis elegans* strain N2 (wild type), the maternal-effect and temperature-sensitive *C. elegans* mutant *mes-1* (Strome *et al.*, 1995)*, Acrobeloides nanus* (strain designation ES501; formerly named *Cephalobus* sp. originally isolated from a soil sample from Peru (Skiba and Schierenberg, 1992), *Rhabditis dolichura* (ES101), *Rhabditis terricola* (ES102) and *Rhabditis belari* (ES103). While the former two Rhabditis species were isolated from soil in the vicinity of the Zoological Institute, Cologne, ES103 was found in a soil sample from the Ligurian coast, Italy.

Nematode culture

Nematode strains were cultured on agar plates with the uracil-requiring strain of *E.coli* OP50 as a food source, essentially as described by Brenner (1974) except that, to keep contamination with other bacteria to a minimum, we used low-salt plates (Lahl *et al.*, 2003). This results in a thinner than normal bacterial lawn.

Microscopical analysis

Adults were picked from the agar plate with a thin insect needle attached to a pasteur pipette and transferred to a drop of autoclaved tap water on a microscope slide. A coverslip sealed on two sides with vaseline was placed on top and water was removed gently from the sides until the specimens were pressed between both slides. Then the remaining sides were sealed. Early stage embryos were either sucked up in distilled water from the agar plate with a drawn-out pasteur pipette (ES103, ES501) or isolated by cutting open gravid hermaphrodites in a drop of distilled water with a scalpel (N2, ES101, ES102) under a dissecting microscope and transferred to a microscope slide carrying a thin pad of 3% agar in H_20. Embryos were covered with a coverslip and sealed with vaseline on the edges. Observations were made at 24 +/- 1^0C, the optimal temperature for rapid development of *C. elegans* embryos.

Visualization of nuclei, germline and gut cells

Cell nuclei of methanol/acetone-fixed embryos were fluorescently marked with diamidinophenylindole (DAPI; 1 µg/ml; Sigma; excitation wavelength, 365 nm). For visualization of germline-specific “P granules” we used a mixture of monoclonal antibodies L416 and K76 (Strome and Wood, 1983)

and as secondary antibody a rabbit-anti-mouse-DTAF conjugate (Dianova; excitation wavelength 450-490 nm). Preparations were embedded with PBS/glycerol 1:1 containing 1mg/ml p-phenylenediamine (Sigma) as an antifading agent (Johnson and Nogueira Araujo, 1981). In advanced-stage embryos differentiated gut cells were identified by their "rhabditin granules" which can first be identified by their autofluorescence (Babu, 1974; excitation wavelength: 340-380 nm) and later in development by their birefringence (Laufer *et al.*, 1980). In some cases, gut differentiation was visualized in laser-penetrated living embryos with the help of accumulating fluorescently-marked transferrin (Bossinger *et al.*, 1996).

Cell culture medium and laser micromanipulation

For laser manipulation we used two types of media, both adapted from Edgar, 1995. "Trypan-blue medium" (TBM; Wiegner and Schierenberg, 1998) was needed for penetration of the eggshell. For this, 600 µl of Leibovitz L-15 medium (Gibco) was freshly mixed with 100 µl inulin (Sigma) stock solution (5 mg/ml), 100 µl 0.25 M HEPES (pH 7.4), 10 µl Base-Mix, 10 µl Penicillin-Streptomycin stock solution (Gibco) and 20% FCS (Gibco). Then 8 mg/ml trypan blue (Sigma) was dissolved in this medium and the resulting solution was centrifuged to remove precipitates.

To better promote embryonic development after manipulation we used "Embryonic growth medium" (EGM). EGM contains over 20 different components (Edgar, 1995). We replaced 1250 µl of "amino acid stock" by 600 µl Grace Amino Acids (Sigma) (Edgar and Wood, 1993).

For cell ablation experiments embryos were fixed to polylysine-coated slides and the distilled water was then replaced by EGM. Cells were ablated by repeated short pulses of an N_2-pumped laser microbeam coupled to a microscope via glassfiber optics (Micropoint Laser; laser dye: Coumarin; absorption maximum 440 nm). For extrusion experiments the distilled water was replaced by TBM (see above) after the embryos had adhered to the polylysine-coated surface, thus allowing absoption of the laser-beam light on the blue-stained eggshell. With repeated short series of laser pulses a prominent hole was burned into the eggshell. After that TBM was replaced by EGM, the slides were incubated at room temperature until embryos had reached the desired cell stages for experimental interference. Single blastomeres adjacent to the laser-induced hole in the eggshell were squeezed out and detached from the remainder of the embryo by gentle pressure on the coverslip (Schierenberg and Wood, 1985). Embryos were cultivated at 25ºC in humid chambers until they had reached their terminal phenotype (*C. elegans*; 12-15 hours; *A nanus*, 72-96 hours).

Inhibition of transcription

Eggshell and underlying vitelline membrane were laser-perforated as described above except that to TBM and EGM was added 150 µg/ml α-amanitin as an inhibitor of mRNA synthesis (Edgar *et al.*, 1994). Trypan blue was removed by replacing TBM with EGM. Embryos were cultivated until they had reached the terminal phenotypes (see above). Control embryos were incubated in inhibitor-free medium.

Acknowledgments

We thank Walter Sudhaus, Berlin for help with classification, Susan Strome for antibodies and cooperation on mes-1, Randy Cassada for valuable comments on the manuscript, Elisabeth Trojtza for expert technical assistance, Jens Schulze for help with cell cycle measurements and the Deutsche Forschungsgemeinschaft for a grant to E.S. through SFB 572.

References

ANDRASSY, I. (1984). Klasse Nematoda. G. Fischer, Stuttgart.

BLAXTER, M.L., DE LEY, P., GAREY, J.R., LIU, L.X., SCHELDEMAN, P., VIERSTRAETE, A., VANFLETEREN, J.R., MACKEY, L.Y., DORRIS, M., FRISSE, L.M., VIDA, J.T. and THOMAS, W. K. (1998). A molecular evolutionary framework for the phylum Nematoda. *Nature* 392: 71-75.

BABU, P. (1974). Biochemical genetics of *C. elegans*. *Molec. Gen. Genet.* 135: 39-44.

BELAR, K. (1924) Die Cytologie der Merospermie bei freilebenden *Rhabditis*-Arten. *Z. Zellforsch.* 1: 1-21.

BOSSINGER, O., WIEGNER, O. and SCHIERENBERG, E. (1996). Embryonic gut differentiation in nematodes: Endocytosis of macromolecules and its experimental inhibition. *Roux's Arch. Dev. Biol.* 205: 494-497.

BRENNER, S. (1974) The genetics of *Caenorhabditis elegans*. *Genetics* 77: 71-94.

DE LEY, P. and BLAXTER, M. (2002). Systematic Position anD Phylogeny. In The Biology of Nematodes (Ed. D.L. Lee). Taylor & Francis, London, pp. 1-30.

DEPPE, U., SCHIERENBERG, E., COLE, T., KRIEG, C., SCHMITT, D.,YODER, B. and VON EHRENSTEIN, G. (1978). Cell lineages of the embryo of the nematode *C. elegans*. *Proc. Nat. Acad. Sci. USA* 75: 376-380.

EDGAR, L.G. and WOOD, W. B. (1993). Nematode Embryos. In *Essential Developmental Biology; A Practical Appproach Series*, (Eds. C.D. Stern and P.W.H. Holland). IRL Press, Oxford, pp. 11-20.

EDGAR, L.G., WOLF, N. and WOOD, W. B. (1994). Early transcription in *Caenorhabditis elegans* embryos. *Development* 120: 443-451.

EDGAR, L.G. (1995). Blastomere culture and analysis. In *Methods in Cell Biology*, Vol. 48. *Caenorhabditis elegans: modern biological analysis of an organism*. (Eds. H.F. Epstein and D.C. Shakes). Academic Press, San Diego, pp. 303-321.

GERBI, S.A., BOROVJAGIN, A.V. and LANGE, T.S. (2003) The nucleolus: a site of ribonucleoprotein maturation. *Curr. Opin. Cell Biol.* 15: 318-25.

GOLDSTEIN, B. (1992). Induction of gut in *Caenorhabditis elegans* embryos. *Nature* 357: 255-257.

GROSSHANS, J., MÜLLER, H.A.J. and WIESCHAUS, E. (2003). Control of cleavage cycles in *Drosophila* embryos by *frühstart*. *Develop. Cell* 5: 285-294.

JOHNSON, G.D. and NOGUEIRA ARAUJO, G.M (1981). A simple method of reducing the fading of immunofluorescence during microscopy. *J. Immunol. Methods* 43: 349-350.

KEMPHUES, K. (2000). PARsing embryonic polarity. *Cell* 101: 345-8.

KIMBLE, J. (1981). Alterations in cell lineage following laser ablation of cells in the somatic gonad of *C. elegans*. *Dev. Biol.* 87: 286-300.

LAHL, V., HALAMA, C. and SCHIERENBERG, E. (2003) Comparative and experimental embryogenesis of Plectidae (Nematoda). *Dev. Genes Evol.* 213: 18-27.

LAUFER, J.S., BAZZICALUPO, P. and WOOD, W.B. (1980). Segregation of developmental potential in early embryos of *C. elegans*. *Cell* 19: 569-577.

LEFRESNE, J., ANDEOL, Y. and SIGNORET, J. (1998). Evidence for introduction of variable G1 phase at the midblastula transition during early development in axolotl. *Dev. Growth Differ.* 40: 497-508.

MCCARTER, J., BARTLETT, B., DANG, T. and SCHEDL, T. (1997). Soma-germ cell interactions in *Caenorhabditis elegans*: multiple events of hermaphrodite germline development require the somatic sheath and spermathecal lineages. *Dev. Biol.* 181: 121-43.

NEWPORT, J. and KIRSCHNER, M. (1982). A major developmental transition in early *Xenopus* embryos: I. Characterization and timing of cellular changes at the midblastula stage. *Cell* 30: 675-86.

NIGON, V. (1949). Modalités de la reproduction et déterminisme du sexe chez quelques nématodes libres. *Ann. Sci. Nat. Zool.* 11: 1-132.

ROCHELEAU, C.E., DOWNS, W.D., LIN, R., WITTMANN, C., BEI, Y., CHA, Y., ALI, M., PRIESS, J.R. and MELLO, C.C. (1997). Wnt signaling and an APC-related gene specify endoderm in early *C. elegans* embryos. *Cell* 90: 707-716.

SCHIERENBERG, E. (1987). Reversal of cellular polarity and early cell-cell interaction in the embryo of *C. elegans*. *Dev. Biol.* 122: 452-463.

SCHIERENBERG, E. (1997). Spezifikation des Zellschicksals im frühen Embryo von *Caenorhabditis elegans*. *Naturwissenschaften* 84: 55-64.

SCHIERENBERG, E. (2001). Three sons of fortune: early embryogenesis, evolution and ecology of nematodes. *BioEssays* 23: 841-847.

SCHIERENBERG, E. and WOOD, W.B. (1985). Control of cell-cycle timing in early embryos of *C. elegans*. *Dev. Biol.* 107: 337-354.

SCHLICHT, P. and SCHIERENBERG, E. (1991). Altered establishment of cell lineages in the *Caenorhabditis elegans* embryo after suppression of the first cleavage supports a concentration-dependent decision mechanism. *Roux's Arch. Dev. Biol.* 199: 437-448.

SCHNABEL, R. and PRIESS, J.R. (1997). Specification of cell fates in the early embryo. In *C. elegans II.* (Eds. D. L. Riddle, T. Blumenthal, B. J. Meyer, and J.

R. Priess). Cold Spring Harbor Laboratory Press, Cold Spring Harbor, pp. 361-382.

SEYDOUX, G., MELLO, C.C., PETTITT, J., WOOD, W.B., PRIESS, J.R. and FIRE, A. (1996). Repression of gene expression in the embryonic germ lineage of *C. elegans*. *Nature* 382: 713-716.

SHIM, Y.H., CHUN, J.H., LEE, E.Y. and PAIK, Y.K.(2002). Role of cholesterol in germ-line development of *Caenorhabditis elegans*. *Mol. Reprod. Dev.* 61:358-66.

SKIBA, F. and SCHIERENBERG, E. (1992). Cell lineages, developmental timing, and spatial pattern formation in embryos of free-living soil nematodes. *Dev. Biol.* 151: 597-610.

STERNBERG, P.W. and HORVITZ, H.R. (1981). Gonadal cell lineages of the nematode *Panagrellus redivivus* and implications for evolution by the modification of cell lineage. *Dev. Biol.* 88:147-66.

STROME, S., MARTIN, P., SCHIERENBERG, E. and PAULSEN, J. (1995). Transformation of the germ line into muscle in *mes-1* mutant embryos of *C. elegans*. *Development* 121: 2961-2972.

STROME, S. and WOOD, W.B. (1983). Generation of asymmetry and segregation of germ-line granules in early *Caenorhabditis elegans* embryos. *Cell* 35, 15-25.

SULSTON, J.E., SCHIERENBERG, E., WHITE, J.G. and THOMSON J.N. (1983) The embryonic cell lineage of the nematode *C. elegans*. *Dev. Biol.* 100: 64-119.

THORPE, C.J., SCHLESINGER, A., CARTER, J.C. and BOWERMAN, B. (1997). Wnt signaling polarizes an early *C. elegans* blastomere to distinguish endoderm from mesoderm. *Cell* 90: 695-706.

TRIANTAPHYLLOU, A.C. and HIRSCHMANN, H. (1980). Cytogenetics and morphology in relation to evolution and speciation of plant-parasitic nematodes. *Ann. Rev. Phytopathol.* 18: 333-359.

WIEGNER, O. and SCHIERENBERG, E. (1998). Specification of gut cell fate differs significantly between the nematodes *Acrobeloides nanus* and *Caenorhabditis elegans*. *Dev. Biol.* 204: 3-14.

WIEGNER, O. and SCHIERENBERG, E. (1999). Regulative development in a nematode embryo: A hierarchy of cell fate transformations. *Dev. Biol.* 215: 1-12.

ZAMIR, E., KAM, Z. and YARDEN, A. (1997). Transcription-dependent induction of G1 phase during zebra fish midblastula transition. *Mol. Cell Biol.* 17: 529-536.

Received: October 2003
Reviewed by Referees: November 2003
Modified by Authors and Accepted for Publication: March 2004