

nos-1 and *nos-2*, two genes related to *Drosophila nanos*, regulate primordial germ cell development and survival in *Caenorhabditis elegans*

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Accepted 19 August; published on WWW 6 October 1999

SUMMARY

In *Drosophila*, the posterior determinant *nanos* is required for embryonic patterning and for primordial germ cell (PGC) development. We have identified three genes in *Caenorhabditis elegans* that contain a putative zinc-binding domain similar to the one found in *nanos*, and show that two of these genes function during PGC development. Like *Drosophila nanos*, *C. elegans nos-1* and *nos-2* are not generally required for PGC fate specification, but instead regulate specific aspects of PGC development. *nos-2* is expressed in PGCs around the time of gastrulation from a maternal RNA associated with P granules, and is required for the efficient incorporation of PGCs into the somatic gonad. *nos-1* is expressed in PGCs after gastrulation, and is required redundantly with *nos-2* to prevent PGCs from dividing in starved animals and to maintain germ cell

viability during larval development. In the absence of *nos-1* and *nos-2*, germ cells cease proliferation at the end of the second larval stage, and die in a manner that is partially dependent on the apoptosis gene *ced-4*. Our results also indicate that putative RNA-binding proteins related to *Drosophila Pumilio* are required for the same PGC processes as *nos-1* and *nos-2*. These studies demonstrate that evolutionarily distant organisms utilize conserved factors to regulate early germ cell development and survival, and that these factors include members of the *nanos* and *pumilio* gene families.

Key words: *nanos*, Primordial germ cells, P granules, Germline, Apoptosis, *Caenorhabditis elegans*

INTRODUCTION

Sexually reproducing organisms use specialized cells, called germ cells, for reproduction. In most animals, these cells are derived from a small group of germline progenitors, or primordial germ cells (PGCs), that are set aside from other lineages early in embryogenesis (Wylie, 1999). During gastrulation, PGCs move inside the embryo, often in association with the developing gut, and eventually migrate out of the gut into the somatic gonad. Once inside the gonad, they undergo extensive proliferation before differentiating into gametes. Another property of germ cells in many organisms is the presence in their cytoplasm of distinctive, electron-dense organelles, often referred to as germ granules. These shared characteristics have suggested that germ cells across phyla may rely on homologous factors to regulate their development and that some of these factors may reside in the germ granules (Eddy, 1975).

In recent years, this hypothesis has received some support from the isolation in several species of factors related to Vasa, a protein component of germ granules first identified in *Drosophila*. *Drosophila* Vasa is a DEAD-box RNA helicase, which is essential for PGC formation in embryos and for oogenesis in adults (Hay et al., 1988; Lasko and Ashburner, 1988). Vasa-related proteins have been identified in many species (*C. elegans*, *Xenopus*, zebrafish, planaria, chicken, mouse and rat), and have begun to be characterized functionally

in *C. elegans*, *Xenopus* and mouse (Fujiwara et al., 1994; Komiyama et al., 1994; Komiyama and Tanigawa, 1995; Gruidl et al., 1996; Olsen et al., 1997; Shibata et al., 1999). So far these analyses have confirmed that Vasa-related factors function in germ cells, but it is not yet known whether these factors regulate similar or different aspects of germline development in different species.

Another component of germ granules first identified in *Drosophila* is *nanos* RNA (Wang and Lehmann, 1991). Maternally loaded *nanos* transcripts are present throughout the early *Drosophila* embryo, but are enriched in the posterior, in a region of the syncytial cytoplasm where the germ granules reside (Wang et al., 1994; Bergsten and Gavis, 1999). *nanos* RNA is translated only in the posterior, and its translation requires germ granule components including Vasa (Gavis et al., 1996). Nanos protein has two functions in early embryos. First, Nanos collaborates with the RNA-binding protein Pumilio to repress the translation of *hunchback* maternal RNA; this function is essential to specify abdominal cell fates in the posterior (Hulskamp et al., 1989; Irish et al., 1989; Murata and Wharton, 1995). Second, Nanos is required for the proper development of primordial germ cells; in the absence of maternal Nanos, primordial germ cells fail to migrate into the somatic gonad and do not become functional germ cells (Kobayashi et al., 1996; Forbes and Lehmann, 1998). Nanos is also expressed zygotically in the germline in adults, where it is required to maintain the

viability of germline stem cells (Forbes and Lehmann, 1998; Bhat, 1999). These studies have raised the question of whether *nanos* homologs in other species function in embryonic patterning, germline development, or both. Genes related to *nanos* have been reported in *Xenopus laevis* [*Xcat-2* (Mosquera et al., 1993)] and in the leech *Helobdella robusta* [*hro-nos* (Pilon and Weisblat, 1997)], but the function of these potential homologs has not yet been reported.

In the present study, we describe three *nanos*-related genes from *C. elegans* and show that two of them, *nos-1* and *nos-2*, function in PGCs. Remarkably, our studies demonstrate that *nos-1* and *nos-2* are required in *C. elegans* for the same aspects of PGC development thought to be regulated by *nanos* in *Drosophila*. We also show that putative RNA-binding proteins related to *Drosophila* Pumilio function in the same PGC processes as *nos-1* and *nos-2*. These results demonstrate that PGCs in evolutionarily distant organisms rely on conserved factors to regulate specific aspects of their development and survival.

MATERIALS AND METHODS

Strains

Caenorhabditis elegans N2 variety Bristol was the wild-type parent of all mutant strains. The following mutations were used: *glp-4(bn2) I*, *tra-2 (q122) II*, *nos-1(gv5) II*, *glp-1(q231) III*, *ced-4 (n1162) III*. Strains were maintained using standard techniques described by Brenner (Brenner, 1974). All experiments were performed at 20°C unless otherwise indicated.

Strain PD4666 carrying the *twist::gfp* transgene (Harfe et al., 1998) was used to determine whether the M blast cell proliferates prematurely in starved *nos-1(RNAi)*; *nos-2(RNAi)* larvae. In this strain, GFP is expressed only in the M blast cell and its descendants. Only one GFP+ cell was observed in each of 196 *nos-1(RNAi)*; *nos-2(RNAi)* L1 larvae cultured in M9, indicating that M does not divide prematurely in the absence of *nos-1* and *nos-2*.

Cloning and sequencing of cDNAs

The full-length coding regions of *nos-1*, *nos-2*, *nos-3*, *fbf-2*, *puf-7* and *puf-8* cDNAs were amplified by reverse transcription – polymerase chain reaction (RT-PCR) from total RNA isolated from wild-type hermaphrodites. Oligonucleotides based on genomic sequences from RO3D7.7 (*nos-1*), ZK1127.1 (*nos-2*), Y53C12B.3 (*nos-3*), F21H12.5 (*fbf-2*), B0273.2 (*puf-7*) and C30G12.7 (*puf-8*) were used as the primers. The RT-PCR products were cloned in the Bluescript KS+ plasmid vector (Stratagene) and sequenced using T3 and T7 primers. The predicted open reading frames of *nos-1* and *nos-2* were confirmed by comparing the cDNA and genomic sequences using the ALIGN program of the DNASTAR sequence analysis software. DNA sequencing was done using an automated DNA sequencer (ABI PRISM 377 XL, Perkin-Elmer).

RNA-mediated interference (RNAi) analysis

Sense and antisense transcripts corresponding to the full-length coding regions were generated from templates cloned in Bluescript KS+ vector using the Megascript kit (Ambion). The two strands were annealed by denaturing at 85°C for 5 minutes and cooling slowly to room temperature. Double-stranded RNA (200 ng/μl) was either microinjected into adult hermaphrodites or used for soaking L4 larvae. For soaking, a synchronous population of about 200 L4 larvae was incubated for 24 hours in 50 μl of M9 solution (22 mM KH₂PO₄ / 42 mM Na₂HPO₄ / 85 mM NaCl / 1 mM MgSO₄) containing 200 ng/μl RNA. We found that microinjection and soaking work with similar efficiency to inactivate *nos-1* and *nos-2*.

The microinjected or soaked worms were allowed to recover overnight on agar plates seeded with *E. coli* (OP50 strain). The worms were transferred to a new plate on the following morning, allowed to lay embryos for 10 hours and then washed off the plate. The larvae that hatched from these embryos were used for phenotypic analysis.

Isolation of *nos-1(gv5)*

A PCR-based deletion screen (Dernburg et al., 1998; G. Moulder and R. Barstead, personal communication) was carried out by Thomas Brodigan and Mike Krause (NIH) to isolate the deletion allele *nos-1(gv5)*. Two sequential rounds of PCR reactions using nested *nos-1* primers were used to screen a library of EMS-mutagenized worms. First round primers were 5'-GGCTCCGGGATATGGTAATT-3' and 5'-CATCTTCCTCCAGCATTTG-3'; second round primers were 5'-AGGCTCAGACTTTGGAGCAA-3' and 5'-CAACTTCTTGAAGGCTTCGG-3'. A single mutant allele, *nos-1(gv5)*, was identified and cloned by sib-selection. *nos-1(gv5)* was subsequently backcrossed to N2 six times to generate the strain JH1270 used in the experiments described here. Sequencing of *nos-1(gv5)* revealed that it contains a 1176 bp deletion starting at amino acid 58 in the *nos-1* ORF and ending 414 bp past the end of the *nos-1* ORF. All experiments with *nos-1(gv5)* were performed in homozygous animals derived from homozygous mothers.

Whole-mount in situ hybridization

In situ hybridization was carried out as described in Seydoux and Fire (1995) using sense and antisense probes of *nos-1* and *nos-2*. No signals were detected using sense probes. Fluorescent in situ hybridization (FISH) was carried out using the Fluorescent Antibody Enhancer Kit (Boehringer Mannheim) following the manufacturer's protocol.

Generation and purification of antibodies

NOS-1 and NOS-2 proteins were expressed in *E. coli* (C600) as TRP-E fusion using the pATH-1 vector. The fusion proteins were partially purified from inclusion bodies (Sambrook et al., 1989), and used as antigens for producing polyclonal antisera either in rats (NOS-1) or rabbits (NOS-2). NOS-1- and NOS-2-specific antibodies were purified by blot affinity purification (Sambrook et al., 1989) against corresponding His-tag fusion created into pRSET vectors (Invitrogen).

Fluorescence microscopy

Immunostaining of *C. elegans* embryos and larvae were carried out essentially as described in Strome and Wood (1983) except that, when using anti-NOS-1 and anti-NOS-2 antibodies, embryos were fixed as described in Seydoux and Dunn (1997). In addition to the NOS-1 and NOS-2 antisera described above, we used the following rabbit polyclonal antisera: anti-LIN-26, a gift from M. Labouesse; anti-MES-2, a gift from L. Xu and S. Strome; anti-GLP-1, a gift from S. Crittenden and J. Kimble; anti-GLD-1, a gift from M. Lee and T. Schedl; anti-CDC25.1, a gift from N. Ashcroft and A. Golden; and anti-GLH-2, a gift from K. Bennett. We also used K76, a mouse monoclonal that recognizes P granules (Strome, 1986), and P4G5, a mouse monoclonal raised against a PIE-1 peptide (a gift from C. Schubert, C. Mello and J. Priess).

Secondary antibodies used were: rhodamine-conjugated goat anti-mouse IgM, rhodamine-conjugated goat anti-mouse IgG, rhodamine-conjugated goat anti-rabbit IgG, Cy3-conjugated chicken anti-rabbit IgG, FITC-conjugated donkey anti-rat IgG and FITC-conjugated goat anti-rabbit IgG (Jackson Immuno Research). The DNA-binding dye, diamidinophenylindole (DAPI), was added at a concentration of 1μg/ml to secondary antibody dilutions to stain DNA. Samples were mounted in Vectashield (Vector laboratories), examined by fluorescence microscopy and Nomarski-optics, and photographed as described in Seydoux and Dunn (1997).

Staining with the vital dye SYTO 12 was performed as described in Gumienny et al. (1999).

35% of *nos-2(RNAi)*, 97% of *nos-1(gv5);nos-2(RNAi)*, and 99% of *nos-1(RNAi);nos-2(RNAi)* animals developed into sterile adults that apparently lacked germ cells (Table 1). No embryonic lethality or gross morphological abnormalities were detected in any combination (data not shown). These results suggest that *nos-1* and *nos-2* are partially redundant and function together during the development of the germline. The phenotypes of *nos-1(RNAi)*, *nos-2(RNAi)* and *nos-1(RNAi);nos-2(RNAi)* animals were not affected significantly by the additional disruption of *nos-3* (Tables 1, 2), suggesting that *nos-3* may not have a unique function distinct from that of *nos-1* and *nos-2*. Consistent with this possibility, recent experiments by Kraemer et al. (1999) have indicated that *nos-3* functions redundantly with *nos-1* and

nos-2 to regulate the sperm/oocyte switch in late larval development.

NOS-1 and NOS-2 are expressed sequentially in the embryonic germ lineage

We first analyzed the embryonic expression pattern of *nos-1* and *nos-2* by in situ hybridization (Methods). As shown in Fig. 2A, both genes encode maternal RNAs that are preferentially maintained in germline blastomeres as is common for many maternal RNAs in *C. elegans* (Seydoux and Fire, 1994). *nos-1* and *nos-2* transcripts are maintained in the germ lineage until approximately the 200-cell stage. *nos-1* transcripts reappear in the primordial germ cells (PGCs) Z2 and Z3 in the 550-cell stage, suggesting that this gene is also transcribed zygotically in PGCs (see below).

In some embryos hybridized to the *nos-2* probe, we noticed that the in situ signal appeared concentrated in small dots around the nuclei of germline blastomeres. This distribution is reminiscent of that observed for P granules. P granules are cytoplasmic organelles unique to the germline (Strome and Wood, 1982). They are known to contain poly(A)⁺ RNAs (Seydoux and Fire, 1994), but a specific RNA associated with P granules has not yet been described. To determine whether *nos-2* RNA is on P granules, we stained embryos with a P granule-specific antibody (K76) following fluorescent in situ hybridization with a *nos-2* probe. As shown in Fig. 2B, we found that the majority of *nos-2* positive foci were also recognized by the K76 antibody. These observations strongly suggest that *nos-2* RNA associates with P granules. Not all *nos-2* transcripts, however, appear to be localized on P granules, since significant levels of *nos-2* RNA could also be detected throughout the cytoplasm of somatic blastomeres that do not contain P granules.

To determine the distribution pattern of the NOS-1 and NOS-2 proteins, we raised polyclonal sera against NOS-1 and NOS-

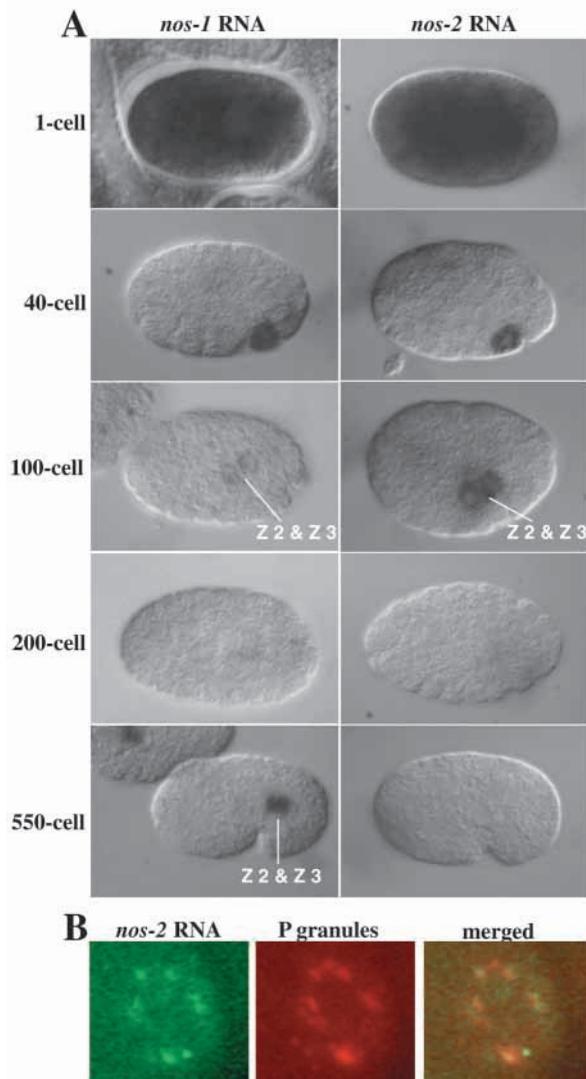


Fig. 2. Distribution of *nos-1* and *nos-2* mRNAs in embryos as revealed by whole-mount in situ hybridization. (A) Like many maternal RNAs, *nos-1* and *nos-2* mRNAs (dark color) are initially present throughout early embryos and are maintained in later stages only in the germline. *nos-1* is also expressed in the PGCs Z2 and Z3 starting in the comma stage (550-cell). (B) Close-up of a P₃ germline blastomere in an 8-cell embryo doubly stained for *nos-2* mRNA (green) and for P granules (red). Many of the *nos-2* foci coincide with P granules (yellow in merged image). In this and Fig. 3, embryos are oriented with anterior to the left.

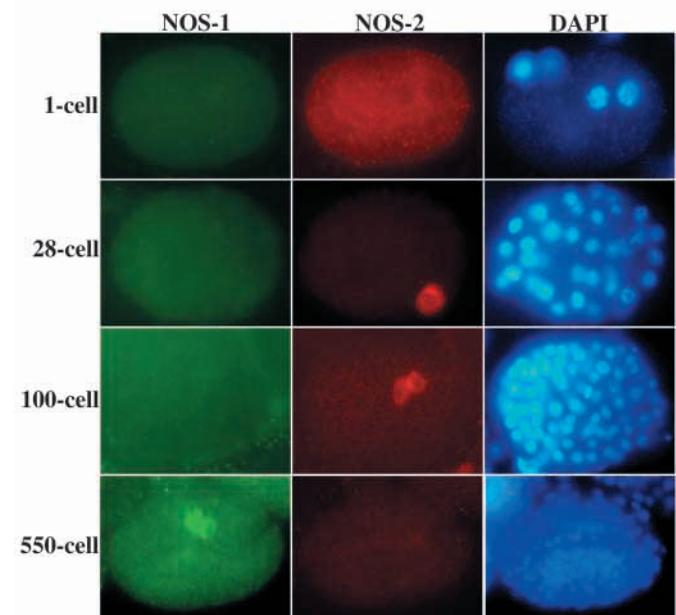
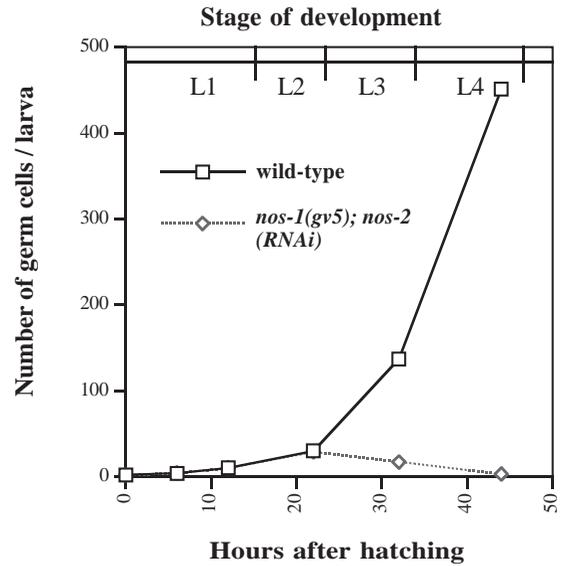


Fig. 3. Distribution of NOS-1 and NOS-2 proteins in *C. elegans* embryos as revealed by whole-mount immunofluorescence. Embryos were triply stained for NOS-1 (red), NOS-2 (green) and DNA (blue). See text for description.

Fig. 4. Germ cell proliferation in wild-type and *nos-1(gv5); nos-2(RNAi)* animals. The number of germ cells per larva presented for the wild-type is an average obtained from 86, 69, 92, 40, 31 and 20 larvae scored, respectively, at 0, 6, 12, 22, 32 and 44 hours after hatching. The number of *nos-1(gv5); nos-2(RNAi)* larvae scored at the same time points were 82, 74, 94, 40, 31 and 21. L1, L2, L3 and L4 are the four larval stages.



2 fusion proteins expressed in *E. coli* (Methods). The specificity of each antibody was confirmed by staining embryos deficient for either NOS-1 or NOS-2. Affinity-purified anti-NOS-1 antibody detected NOS-1 in *nos-2(RNAi)* embryos but not in *nos-1(RNAi)* and *nos-1(gv5)* embryos, whereas affinity-purified anti-NOS-2 antibody detected NOS-2 in *nos-1(RNAi)* and *nos-1(gv5)* embryos, but not in *nos-2(RNAi)* embryos (data not shown). These observations demonstrate the specificity of each antibody, and indicate that NOS-1 and NOS-2 do not depend on each other for expression.

As shown in Fig. 3, we found that NOS-2 and NOS-1 proteins are expressed sequentially during embryogenesis. We first detected expression of NOS-2 in the 1-cell stage; at this stage, NOS-2 was present uniformly throughout the embryo. No NOS-2 expression was detected in 2- to 20-cell embryos. NOS-2 expression reappeared in the 28-cell stage in the cytoplasm of the germline blastomere P₄. In some embryos, NOS-2 staining appeared to be concentrated in a few perinuclear foci suggesting that NOS-2 may associate with P granules in this stage. NOS-2 expression continued in P₄ and its two daughters Z₂ and Z₃ until approximately the 200-cell stage. NOS-2 levels decreased sharply in later stages and were undetectable by the 550-cell stage.

In contrast to NOS-2, no NOS-1 protein was detected in pregastrulation embryos, even though *nos-1* RNA is present in these stages. NOS-1 first appeared in Z₂ and Z₃ at the 550-cell stage and remained expressed in these cells until the end of embryogenesis (Fig. 3). The late onset of NOS-1 expression suggested that NOS-1 might be expressed exclusively from

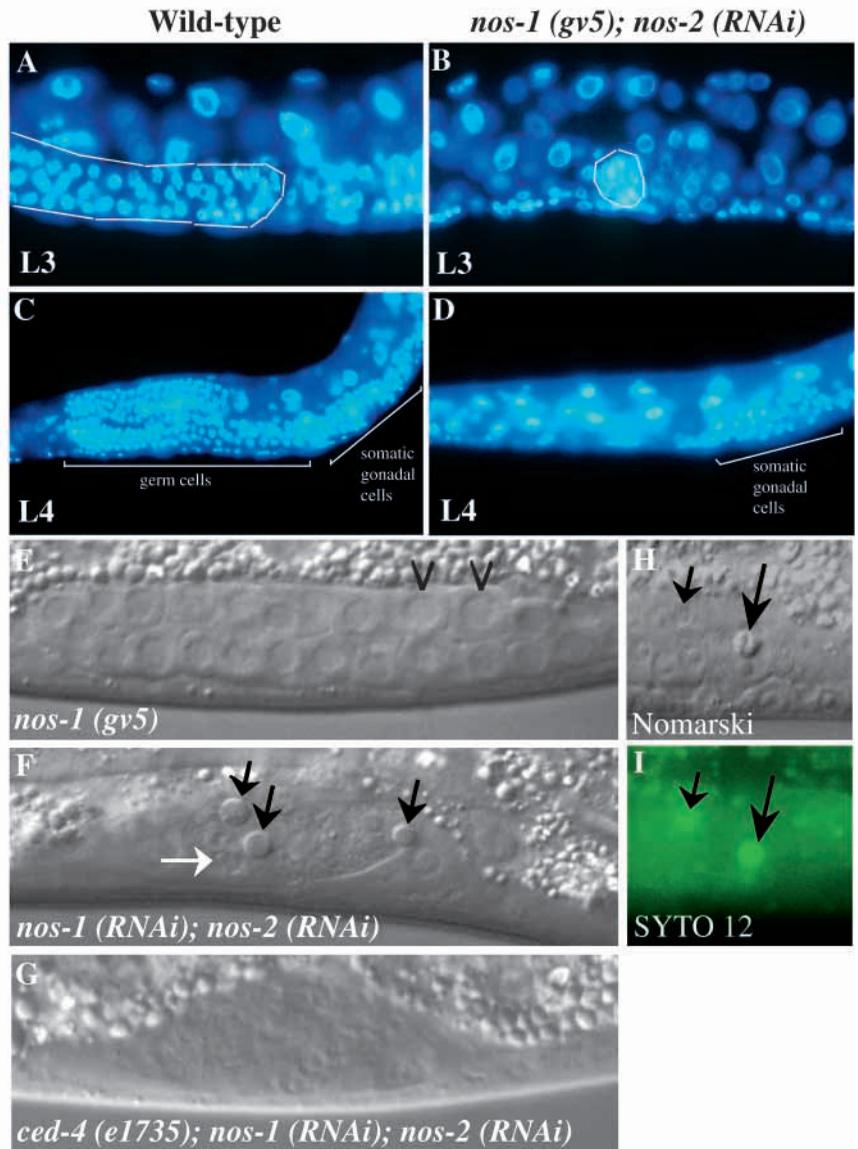


Fig. 5. Germ cell death in *nos-1(gv5); nos-2(RNAi)* animals. (A-D) Wild-type and *nos-1(gv5); nos-2(RNAi)* larvae stained with DAPI. (A,B) Areas containing germ cells are outlined. Note the condensed appearance of the germ cell nuclei in B. (C,D) Germ cells and somatic cells are indicated. No germ cells are detected in D. (E-G) Nomarski photomicrographs of live L3 larvae. Arrowheads point to germ cells with a wild-type phenotype. White arrow points to germ cells with abnormal nuclei. Black arrows point to germ cells undergoing apoptosis ('buttons'). (H,I) *nos-1(RNAi); nos-2(RNAi)* larva stained with SYTO 12, a vital dye that marks cells undergoing apoptosis. Long arrow points to a 'button' that stains with SYTO 12. Short arrow points to another nucleus, which stains more weakly with SYTO 12 and likely is at a more advanced stage of apoptosis (Gumienny et al., 1999). Germ cells in wild-type L3 larvae do not stain with SYTO12 (data not shown).

Table 2. Frequency of mutant PGC phenotypes associated with loss of *nos-1*, *nos-2* and *nos-3*

'Genotype'	% Unfed L1 larvae with			Number of larvae examined
	PGCs outside of somatic gonad	More than 2 PGCs	Both defects	
<i>pes-10(RNAi)</i> – negative control	0	0	0	232
<i>nos-1(gv5)</i>	0	0	0	105
<i>nos-2(RNAi)</i>	38	0	0	140
<i>nos-1(RNAi); nos-2(RNAi)</i>	48	26	14	142
<i>nos-1(gv5); nos-2(RNAi)</i>	32	51	13	84
<i>nos-1(RNAi); nos-2(RNAi); nos-3(RNAi)</i>	33	22	4	95

The F₁ progeny of hermaphrodites injected with dsRNA were hatched in M9 (no food) and fixed 40 hours later for staining with DAPI and the P granule antibody K76. *pes-10* is an embryonically transcribed gene with no known function (Seydoux and Fire, 1994).

embryonically transcribed RNA. To test whether NOS-1 is expressed zygotically, we crossed *nos-1(gv5)* hermaphrodites (which express no NOS-1) to wild-type males and stained their progeny for NOS-1 protein. We detected NOS-1 expression in a pattern identical to that observed in embryos derived from wild-type hermaphrodites (data not shown), confirming that NOS-1 expression is zygotic.

A similar experiment could not be performed for NOS-2, since a *nos-2* mutant is not yet available and since RNAi eliminates both maternal and zygotic expression (Fire et al., 1998). However, the fact that NOS-2 can be detected in the germ lineage as early as the 28-cell stage strongly suggest that NOS-2 is expressed from maternal RNA, since zygotic transcription is not thought to begin in the germ lineage until the 100-cell stage (Seydoux and Dunn, 1997). Whether NOS-1 and NOS-2 are expressed in germ cells during larval and adult stages remains to be determined.

***nos-1* and *nos-2* are required to maintain germ cell viability during postembryonic development**

To determine the nature of the sterility defect resulting from the simultaneous loss of *nos-1* and *nos-2*, we analyzed the development of primordial germ cells in *nos-1(gv5); nos-2(RNAi)* animals. Wild-type larvae hatch with two PGCs (Z2 and Z3), which begin to divide in the late L1 stage and continue to proliferate during the next three larval stages (L2, L3 and L4) to generate approximately 1000 germ nuclei per gonad by the adult stage. *nos-1(gv5); nos-2(RNAi)* larvae also start out with two PGCs but these cells do not proliferate as in wild-type. To quantitate this proliferation defect, we collected *nos-1(gv5); nos-2(RNAi)* larvae at different time points after hatching and stained them with the DNA-binding dye DAPI and the P granule antibody K76 to visualize and count germ cells. The numbers of germ cells present in *nos-1(gv5); nos-2(RNAi)* L1 and L2 larvae were comparable to wild-type (Fig. 4). In contrast, in the L3 stage, at a time where germ cell number increases rapidly in wild-type, we found that *nos-1(gv5); nos-2(RNAi)* germ cells ceased to proliferate and actually declined in number (Fig. 4). In this stage, many germ cells contained few or no P granules (data not shown) and had abnormally condensed nuclei that stained brightly with DAPI (Fig. 5B). These cells were often found proximally surrounded

by somatic gonadal cells, in contrast to wild-type germ cells which by this stage are located in more distal positions in the elongating arms of the gonad (compare Fig. 5A and B). In L4 larvae, individual germ cell nuclei could rarely be recognized and P granules could no longer be detected, suggesting that most germ cells had died by that stage (Fig. 5D). No germ cells, oocytes or sperm were detected in adult animals.

We also examined the appearance of germ cells in live *nos-1(gv5); nos-2(RNAi)* animals by Nomarski DIC microscopy (Fig. 5E-G). Abnormal germ cells were first observed consistently in the early L3 stage. We frequently observed cells with small nuclei filled with tiny particles, as well as clusters of degenerating cells with no recognizable nuclei (Fig. 5F). In addition, in 27% of L3 larvae ($n=47$), we observed nuclei with the flat 'button' morphology typical of programmed or apoptotic cell death (Sulston and Horvitz, 1977; Ellis and Horvitz, 1986; Fig. 5F). These 'buttons' could be stained with SYTO 12, a vital dye taken up by apoptotic cells (Gumienny et al., 1999; Fig. 5H-I). To determine whether these structures indeed represented germ cells undergoing apoptosis, we tested whether their presence required *ced-4* activity, a caspase activator essential for programmed cell death in *C. elegans* (Yuan and Horvitz, 1992; Gumienny et al., 1999). We found that germ cells in *ced-4(n1162); nos-1(RNAi); nos-2(RNAi)* L3 larvae no longer formed button-like structures ($n=82$), but still died and exhibited the other aberrant phenotypes described above (Fig. 5G). These observations suggest that loss of *nos-1* and *nos-2* causes germ cells to activate apoptosis as well as another 'death program' that is independent of *ced-4*.

The experiments described above were all performed in hermaphrodites (XX). To test whether *nos-1* and *nos-2* are also required for germ cell survival in males (XO), we stained *nos-1(RNAi); nos-2(RNAi); tra-2(q122)* L4 larvae [50% female (XX) and 50% male (XO)] with DAPI and the K76 antibody to visualize germ cells. Of 87 larvae examined, 78 (90%) did not contain any germ cells. These results indicate that *nos-1* and *nos-2* are required in both sexes for germ cell survival.

***nos-2* is required for efficient incorporation of primordial germ cells into the somatic gonad**

Next, we wished to determine whether *nos-1* and/or *nos-2* are also required for earlier aspects of PGC development. During embryogenesis, the PGCs Z2 and Z3 associate with the somatic gonad blast cells Z1 and Z4 to form the gonad primordium. In the L1 stage, all four cells appear in a row with Z2 and Z3 in the middle and Z1 and Z4 at each end (Fig. 6A,B) (Kimble and Hirsh, 1979). To determine whether this arrangement is dependent on *nos-1* and/or *nos-2* activity, we co-stained L1 larvae with the P granule antibody K76 and with an antibody against LIN-26, a transcription factor expressed in Z1 and Z4 in this stage (Labouesse et al., 1996). We found that, in 38% of *nos-2(RNAi)* larvae, one PGC was located at an ectopic location anterior and dorsal to the somatic gonad (Fig. 6C,D; Table 2). The other PGC was located in its normal position inside the somatic gonad. The frequency of this phenotype was not increased significantly by the additional disruption of *nos-1*; furthermore, ectopic germ cells were never observed in *nos-1(gv5)* larvae (Table 2). These observations indicate that *nos-2*, independently of *nos-1*, is required to ensure that both primordial germ cells are incorporated into the somatic gonad.

***nos-1* and *nos-2* are required redundantly to block premature proliferation of primordial germ cells in starved larvae**

A second property of Z2 and Z3 is their ability to regulate their cell cycle in response to nutrients. Like other blast cells in the L1 larva, Z2 and Z3 do not begin to divide until the larva has begun to feed. This block to cell proliferation is easily observed by hatching L1 larvae in nutrient-free media. Starved L1 larvae can survive for several days without initiating any cell divisions. To determine whether this cell cycle block requires *nos-1* and/or *nos-2*, we cultured newly hatched *nos-1(gv5); nos-2(RNAi)* larvae in the absence of food for 40 hours and stained these starved L1s with DAPI and the P granule antibody K76 to visualize germ cells. We found that 51% of *nos-1(gv5); nos-2(RNAi)* starved L1 larvae contained more than two germ cells (range 4–12 germ cells; Fig. 6E,F; Table 2). Remarkably, premature proliferation was observed with equal frequency in germ cells located inside or outside of the somatic gonad (Fig. 6G,H; data not shown). This phenotype appears to be specific to germ cells since the somatic gonadal blast cells Z1 and Z4 and the mesodermal blast cell M remained undivided in these animals (Fig. 6D,F; Materials and Methods). Prematurely proliferated germ cells were not observed in *nos-1(gv5)* and *nos-2(RNAi)* single ‘mutants’ (Table 2). These results indicate that *nos-1* and *nos-2* are required redundantly to prevent PGCs from dividing in the absence of food.

***nos-1* and *nos-2* are not generally required for primordial germ cell fate specification**

The finding that two PGC characteristics (association with the somatic gonad and block to cell division under starved conditions) are disrupted in the absence of *nos-1* and *nos-2* led us to ask whether these genes are generally required to specify PGC fate. We began to explore this possibility by examining the expression of several factors specific for PGCs and their descendants in early larval stages, including GLH-2, a RNA helicase component of P granules (Gruidl et al., 1996), MES-2, a nuclear Polycomb-like protein (Holdeman et al., 1998), GLD-1, a cytoplasmic RNA-binding protein (Jones et al., 1996), CDC25.1, a nuclear cell cycle regulator (Ashcroft et al., 1999), and GLP-1, a Notch-like transmembrane receptor required for germ cell proliferation (Yochem and Greenwald, 1989; Crittenden et al., 1997). We also examined the expression of one maternal factor required for the formation of primordial germ cells during embryogenesis [PIE-1 (Mello et al., 1996)]. In all cases, we found that expression of these germline factors was unaffected in *nos-1(RNAi); nos-2(RNAi)* animals (Materials and Methods and data not shown).

In wild-type larvae, mitotic proliferation of germ cells requires the functions of the GLP-1 and GLP-4 proteins. *glp-1* is required to maintain germ cells in mitosis; in the absence of *glp-1*, after a few rounds of mitotic cell divisions, germ cells prematurely enter meiosis and differentiate into sperm (Austin and Kimble, 1987). *glp-4* is required for cell cycle progression; in the absence of *glp-4*, germ cells complete 3–4 rounds of cell divisions before arresting in prophase (Beanan and Strome, 1992). To test whether *glp-1* and *glp-4* are required for the few germ cell divisions that occur in the absence of *nos-1* and *nos-2*, we compared the number of germ cells in *nos-1(RNAi); nos-2(RNAi)* larvae in the presence and absence of *glp-1* or *glp-4*

Table 3. Germ cell proliferation in *nos-1(RNAi); nos-2(RNAi)* animals requires *glp-1* and *glp-4*

‘Genotype’	Number of germ cells per L2 larva	Number of larvae examined
wild-type	14.0	119
<i>nos-1(RNAi); nos-2(RNAi)</i>	14.8	116
<i>glp-1(q231)</i>	6.2	81
<i>glp-1(q231); nos-1(RNAi); nos-2(RNAi)</i>	6.2	90
<i>glp-4(bn2)</i>	5.7	99
<i>glp-4(bn2); nos-1(RNAi); nos-2(RNAi)</i>	5.8	108

The F₁ progeny of hermaphrodites soaked with dsRNA were hatched at 25°C in the presence of food and fixed 11.5 hours later for staining with DAPI and the P granule antibody K76.

function (Table 3). We found that lack of *glp-1* or *glp-4* function significantly reduced the number of germ cells in *nos-1(RNAi); nos-2(RNAi)* larvae. This result indicates that germ cells in *nos-1(RNAi); nos-2(RNAi)* larvae depend on *glp-1* and *glp-4* for their proliferation as in wild type. Germ cells in *nos-1(RNAi); nos-2(RNAi); glp-1(q231)* larvae, however, exhibited the same abnormal nuclear morphologies as in *nos-1(RNAi); nos-2(RNAi)* animals and did not form sperm (data not shown). This observation indicates that *glp-1(q231)* germ cells require *nos-1* and *nos-2* to undergo meiosis. We conclude that *nos-1* and *nos-2* are not generally required to specify PGC fate, but instead are essential for specific aspects of PGC development, differentiation and survival.

RNA-mediated interference of a subset of *pumilio*-related genes causes PGC phenotypes similar to those observed in *nos-1(gv5); nos-2(RNAi)* larvae

During *Drosophila* embryogenesis, Nanos functions with the RNA-binding protein Pumilio to repress the translation of maternal hunchback mRNA (Murata and Wharton, 1995). There are at least eight genes in the *C. elegans* genomic database related to *Drosophila pumilio* (Zhang et al., 1997 and our recent database searches). To determine whether any of these genes function with *nos-1* and *nos-2* during PGC development, we disrupted their expression individually and in combinations using RNA-mediated interference (Table 4, and data not shown). We found that simultaneous disruption of five pumilio-like genes, *fbf-1/fbf-2*, *puf-6/puf-7* and *puf-8*, resulted in phenotypes similar to those observed in *nos-1(gv5); nos-2(RNAi)* larvae, including ectopic PGCs outside of the somatic gonad, premature proliferation of PGCs in starved L1 larvae, and germ cell death (Table 4). These observations indicate that *nos-1* and *nos-2* and a subset of *pumilio*-related genes are required for the same aspects of PGC development.

DISCUSSION

***nos-1* and *nos-2*, two *C. elegans* genes related to *Drosophila nanos*, are expressed in primordial germ cells**

This study reports on the function of two *C. elegans* genes related to *Drosophila nanos* and demonstrates that this family of proteins play essential and evolutionarily conserved roles in the development of the early germline. Like other *nanos*-like genes identified to date, *nos-1* and *nos-2* are related to each other and

Table 4. PGC phenotypes associated with loss of *fbf-2*(*fbf-1*), *puf-7*(*puf-6*) and *puf-8*

RNAi*	% Unfed L1 larvae‡ with		
	PGCs outside of somatic gonad	More than 2 PGCs	Number of larvae examined
<i>fbf-2</i> (<i>fbf-1</i>)	0	0	157
<i>puf-7</i> (<i>puf-6</i>)	0	0	54
<i>puf-8</i>	0	0	51
<i>fbf-2</i> (<i>fbf-1</i>) + <i>puf-7</i> (<i>puf-6</i>)	8.5	0	82
<i>fbf-2</i> (<i>fbf-1</i>) + <i>puf-8</i>	0	0	48
<i>puf-7</i> (<i>puf-6</i>) + <i>puf-8</i>	0	0	55
<i>puf-7</i> (<i>puf-6</i>) + <i>fbf-2</i> (<i>fbf-1</i>) + <i>puf-8</i> §	23	32	108

*dsRNA prepared from *fbf-2*, *puf-7* and *puf-8* cDNAs were injected in the combinations listed. *fbf-1* is highly similar to *fbf-2* (93% base identity) and is inactivated by *fbf-2* dsRNA (Zhang et al., 1997). Similarly, *puf-6* is highly similar to *puf-7* (98% base identity) and is expected to be inactivated by *puf-7* dsRNA (Fire et al., 1999).

‡The F₁ progeny of hermaphrodites injected with dsRNA were hatched in M9 (no food) and fixed 40 hours later for staining with DAPI and the P granule antibody K76.

§Adult F₁ progeny of hermaphrodites injected with this combination were 99.2% sterile (*n*=234). DAPI staining of 15 such animals indicated that they lacked germ cells.

to *Drosophila nanos* only in a small region comprising two putative zinc-binding domains. In *Drosophila nanos*, these motifs are required for high affinity binding to RNA (Curtis et al., 1997), suggesting that *nanos* family members may function as RNA-binding proteins.

Immunolocalization experiments indicate that NOS-1 and NOS-2 are cytoplasmic proteins that are expressed sequentially in the embryonic germ lineage. The first to appear is NOS-2 which is expressed transiently in the germline founder cell P₄ and its two daughters the PGCs Z₂ and Z₃ around the time that these cells begin gastrulation. Like *Drosophila Nanos*, NOS-2 is derived from a maternal RNA that is present throughout the early embryo and is enriched in germ cells on P granules. This similarity suggests that the germline-specific expression of *Nanos* and NOS-2 in early embryos may be regulated by similar mechanisms, possibly involving germ granule components. In contrast to NOS-2, NOS-1 is expressed from embryonically transcribed (zygotic) RNA in mid-embryogenesis after Z₂ and Z₃ have joined the somatic gonad, and continues to be expressed in these cells at least until the first larval stage. Interestingly, *Nanos* expression in *Drosophila* also has a zygotic component: zygotically expressed *Nanos* has been detected in germline stem cells in the adult ovary (Wang et al., 1994). These parallels suggest that NOS-1 and NOS-2 may both be functionally related to *Drosophila Nanos*, and may have diverged from each other only in the timing of their expression during embryogenesis. As described below, the functional redundancy between NOS-1 and NOS-2 is in agreement with this possibility.

NOS-1 and NOS-2 are required redundantly to maintain germ cell viability during postembryonic development

We have investigated the function of *nos-1* and *nos-2* by blocking their expression using RNA-mediated interference (Fire et al., 1998) and, in the case of *nos-1*, also by analyzing a deletion mutant [*nos-1*(*gv5*)] missing most of the *nos-1* open-

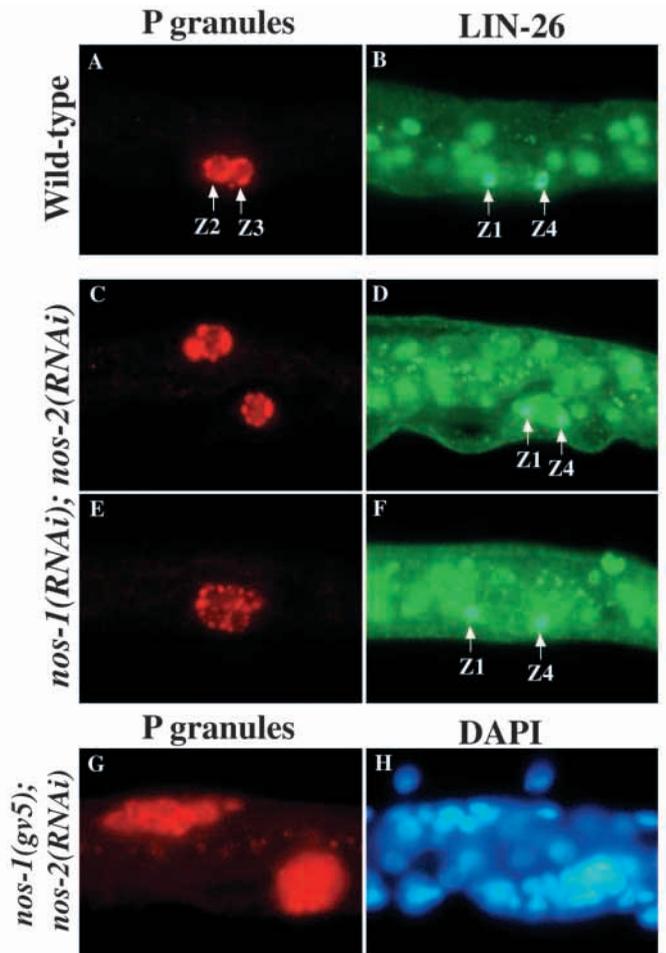


Fig. 6. PGC phenotypes in *nos-1*(*gv5*); *nos-2*(*RNAi*) larvae. The frequencies of these phenotypes are presented in Table 2. (A-F) Unfed L1 larvae doubly stained with the P granule-specific antibody K76 (red) and with an antibody against LIN-26 (green), a transcription factor expressed in the somatic gonadal cells Z₁ and Z₄. (A,B) Wild-type L1 larvae have two PGCs in the somatic gonad. (C,D) *nos-1*(*RNAi*); *nos-2*(*RNAi*) larva with one PGC outside of the somatic gonad. (E,F) *nos-1*(*RNAi*); *nos-2*(*RNAi*) larva with more than two PGCs. (G,H) Unfed *nos-1*(*gv5*); *nos-2*(*RNAi*) L1 larvae doubly stained with the P granule-specific antibody K76 (red) and with the DNA dye DAPI (blue). In this larva, PGCs both inside and outside of the somatic gonad have proliferated prematurely. Larvae are oriented anterior to the left.

reading frame. The most dramatic phenotype associated with the simultaneous loss of *nos-1* and *nos-2* is the death of all germ cells in the third larval stage in 97 to 99% of all double mutants. *nos-1*(*gv5*); *nos-2*(*RNAi*) larvae start out with the normal number (2) of PGCs. These cells proliferate as in wild-type through the first and second larval stages, and then suddenly stop proliferating and die during the third larval stage. In contrast, no detectable phenotype is associated with loss of *nos-1*, and only a partially penetrant sterile phenotype is associated with loss of *nos-2*. These observations indicate that *nos-1* and *nos-2* are required redundantly to maintain germ cell viability in larvae.

Two types of cell deaths have been observed previously in the *C. elegans* germline: programmed cell death (apoptosis)

and degenerative cell death (necrosis). Apoptosis is a natural fate of female germ cells: about half of all germ cells in wild-type adult hermaphrodites undergo programmed cell death as they exit the pachytene stage of meiosis (Gumienny et al., 1999). Apoptosis is characterized by chromatin condensation, formation of button-like, refractile bodies that stain with the vital dye SYTO 12, and a requirement for the caspase CED-3 and the caspase activator CED-4 (Ellis and Horvitz, 1986; Yuan and Horvitz, 1992; Yuan et al., 1993). In contrast, necrosis is characterized by nuclear swelling and cytoplasmic vacuoles and does not require *ced-3* or *ced-4* (Driscoll and Chalfie, 1992). Germ cells have been reported to undergo this second type of cell death in *mes-2*, *mes-3*, *mes-4* and *mes-6* mutants (Garvin et al., 1998). We have found that germ cells in *nos-1(-);nos-2(-)* larvae die in a manner that resembles apoptosis. Like apoptotic cells, *nos-1(-); nos-2(-)* germ cells undergo chromatin condensation and form button-like structures that stain with SYTO12 and require *ced-4* for their formation. These cells, however, also exhibit other phenotypes not reported before for either apoptotic or necrotic cell death, including shrunken nuclei filled with particulate material. These phenotypes cannot be reversed by removing *ced-4*, and germ cells still die in *nos-1(-);nos-2(-);ced-4(-)* animals. These observations indicate that, in the absence of *nos-1* and *nos-2*, germ cells activate the apoptotic machinery as well as another 'death program' that is independent of *ced-4*.

We do not know what triggers the death of germ cells in *nos-1(-);nos-2(-)* animals. However, as described below, these cells exhibit at least two abnormal phenotypes before dying, raising the possibility that death in the L3 stage may be caused by defects accumulated earlier in development.

NOS-1 and NOS-2 regulate specific aspects of PGC behavior, but are not required generally for PGC fate specification

Several observations indicate that *nos-1* and *nos-2* are not generally required for PGC fate specification: *nos-1(-);nos-2(-)* L1 larvae are born with the normal number of PGCs, these cells express several germline-specific markers as in wild type and show normal dependence on the germline mitosis genes *glp-1* and *glp-4* for proliferation. We have identified, however, two aspects of early PGC development that are disrupted in the absence of *nos-1* and/or *nos-2*. First, we found that 35% of *nos-2(-)* L1 larvae hatch with one PGC located outside of its normal location in the somatic gonad. This defect is not seen in *nos-1(-)* animals and is not exacerbated in *nos-1(-);nos-2(-)* double 'mutants', indicating that *nos-2*, independently of *nos-1*, is required for the efficient incorporation of PGCs into the somatic gonad. The second defect that we observed is an apparent disruption of the cell cycle control mechanisms that regulate the onset of PGC divisions in L1 larvae. In wild-type, Z2 and Z3 begin divisions in L1 larvae only after feeding has begun and never divide if larvae are hatched in the absence of food. This block is compromised in *nos-1(-);nos-2(-)* larvae: in these animals, Z2 and Z3 often were observed to divide under starvation conditions. A similar defect was recently reported for somatic blast cells in animals deficient for the cell cycle inhibitor *cki-1* (Hong et al., 1998). Indeed, we have found that loss of *cki-1* also causes Z2 and Z3 to divide in unfed larvae (K. S. and G. S., unpublished results). These observations indicate that *nos-1* and *nos-2* are required to

maintain cell cycle arrest in PGCs in the absence of nutrients, and perhaps do so by regulating the expression or activity of cell cycle inhibitors like *cki-1*.

nanos function in PGCs has been conserved in evolution

The role of *nos-1* and *nos-2* in *C. elegans* resembles that of *nanos* in *Drosophila* in several respects. Like *nos-1* and *nos-2*, *nanos* is not generally required for PGC formation or fate, but is required for specific aspects of PGC development (Kobayashi et al., 1996; Forbes and Lehmann, 1998; Bhat, 1999). First, maternal *nanos* is required in embryos for incorporation of PGCs into the somatic gonad (Kobayashi et al., 1996; Forbes and Lehmann, 1998). As described above, we have shown that *nos-2* performs a similar function in *C. elegans*. This conservation of function is remarkable when one considers that gonad formation in *C. elegans* and *Drosophila* involves strikingly different cell movements. In *Drosophila*, PGCs must migrate through two germ layers to reach the somatic gonad in the mesoderm (Williamson and Lehmann, 1996). In contrast, in *C. elegans*, the PGCs undergo only short-range movements and it is the somatic gonadal precursor cells that migrate to reach the PGCs (Sulston et al., 1983). Perhaps these differences account for the fact that the requirement for *nanos* for germ cell entry into the somatic gonad appears less stringent in *C. elegans* than in *Drosophila*; in *nos-2(-)* animals at least one PGC always makes it into the gonad whereas, in *nanos* mutants, this rarely ever happens (Kobayashi et al., 1996; Forbes and Lehmann, 1998).

Drosophila nanos also functions zygotically later in development to maintain the viability of germline stem cells in the ovary (Forbes and Lehmann, 1998; Bhat, 1999). As described above, this function is shared by *nos-1* and *nos-2* in *C. elegans*. Death of germline stem cells in *Drosophila nanos* mutants has been correlated with the progressive degeneration of their plasma membrane (Bhat, 1999); it is not yet known whether apoptosis might be involved as we report here for *C. elegans*. Additional studies will be required in both systems to identify the primary cause of death and the exact mechanisms that mediate it. It also will be interesting to determine whether *Drosophila nanos* regulates the cell cycle of PGCs as we describe in this study for *nos-1* and *nos-2*.

The functional parallels between *nanos* and *nos-1/nos-2* indicate that *nanos* function in PGCs has been conserved between insects and nematodes. This conservation of function is likely to extend to vertebrates since the *nanos*-related gene *Xcat-2* has been shown to encode an RNA associated with the germline of *Xenopus* (Mosquera et al., 1993; Zhou and King, 1996). Is the function of *nanos* in embryonic patterning similarly conserved? Studies analyzing *nanos* function in several insects suggest that a *nanos*-dependent mechanism to establish embryonic polarity has been conserved throughout Diptera (Curtis et al., 1995). That a similar mechanism operates in nematodes, however, appears unlikely since, as reported here, there is no indication that loss of *nos* function disrupts embryonic patterning in *C. elegans* (although a thorough test of this possibility must await the isolation of null mutations in all three *nanos*-related genes). Furthermore, Hunchback, the target of *nanos* regulation in *Drosophila* embryonic patterning, is not expressed maternally in *C. elegans* and has no function there in early embryos (Fay et al., 1999). Instead, the currently available

data support a hypothesis put forth recently (Forbes and Lehmann, 1998), which proposes that the ancestral function of *nanos* is in primordial germ cell development, and that *nanos* function in embryonic patterning is more recently derived and perhaps specific to insects.

Putative RNA-binding proteins related to *Drosophila* Pumilio are required for the same aspects of PGC development as NOS-1 and NOS-2

How do Nanos and NOS-1/NOS-2 function in PGCs at the molecular level? In early *Drosophila* embryos, Nanos controls embryonic patterning by repressing the translation of *hunchback* mRNA (Lehmann and Nusslein-Volhard, 1987; Barker et al., 1992). This repression requires the activity of Pumilio, a sequence-specific RNA-binding protein and translational regulator that recognizes and binds to 'nanos response elements' in the 3'UTR of *hunchback* mRNA (Murata and Wharton, 1995; Wharton et al., 1998). Whether Nanos also functions with Pumilio in PGCs has not yet been reported. Like Nanos, Pumilio is required for germline stem cell development in adults, but its function there appears distinct from that of Nanos, raising the possibility that Nanos and Pumilio can function independently from one another in the germline (Forbes and Lehmann, 1998).

There are at least eight *pumilio*-like genes in the *C. elegans* genome, including four single-copy genes (*puf-3*, *puf-4*, *puf-5* and *puf-9*), and two highly identical gene pairs *fbf-1/fbf-2* and *puf-6/puf-7* (Zhang et al., 1997; J. Kimble, personal communication; K. S. and G. S., unpublished results). *fbf-1/fbf-2* have been implicated in the translational control of *fem-3*, coding a factor required for the sperm/oocyte switch in hermaphrodites (Zhang et al., 1997). We have found that simultaneous disruption of *fbf-1/fbf-2*, *puf-6/puf-7* and *puf-8* by RNA-mediated interference causes PGC defects identical to those observed in *nos-1(-)*; *nos-2(-)* animals. These results suggest that *nos-1* and *nos-2* function in PGCs much like *nanos* does in embryonic patterning: by regulating the translation of specific mRNAs with the help of RNA-binding proteins related to *Drosophila* Pumilio. We propose that translational control by members of the *nanos* and *pumilio* gene families is a commonly used mechanism to regulate the development and survival of early germ cells.

We are especially grateful to Tommy Brodigan and Mike Krause who isolated the *nos-1(gv5)* allele. We also thank Tim Schedl and Michael Hengartner for insightful suggestions during the course of this work; Sarah Crittenden and Judith Kimble for sharing their unpublished results; Brian Harfe, Catherine Branda and Andy Fire for the PD4666 strain; Michel Labouesse, Lei Xu, Susan Strome, Sarah Crittenden, Judith Kimble, Min-Ho Lee, Tim Schedl, Neville Ashcroft, Andy Golden, Karen Bennett, Charlotte Schubert, Craig Mello and Jim Priess for antibodies; and Denise Montell and Tim Schedl for their comments on the manuscript. This work was supported by funds from the Packard Foundation and the Searle Foundation/ Chicago Community Trust.

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