

## Analysis of RNA associated with P granules in germ cells of *C. elegans* adults

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### SUMMARY

**P granules are cytoplasmic structures of unknown function that are associated with germ nuclei in the *C. elegans* gonad, and are localized exclusively to germ cells, or germ cell precursors, throughout the life cycle. All the known protein components of P granules contain putative RNA-binding motifs, suggesting that RNA is involved in either the structure or function of the granules. However, no specific mRNAs have been identified within P granules in the gonad. We show here that P granules normally contain a low level of RNA, and describe conditions that increase this level. We present evidence that several, diverse mRNAs, including *pos-1*, *mex-1*, *par-3*, *skn-1*, *nos-2* and *gld-1* mRNA, are present at least transiently within P granules.**

**In contrast, *actin* and *tubulin* mRNA and rRNA are either not present in P granules, or are present at relatively low levels. We show that *pgl-1* and the *glh* (Vasa-related) gene family, which encode protein components of P granules, do not appear essential for RNA to concentrate in P granules; these proteins may instead function in events that are a prerequisite for RNAs to be transported efficiently from the nuclear surface.**

Key words: P granules, Germline, Nuclear pore, PGL-1, GLH-1, Oogenesis, *Caenorhabditis elegans*, Germ plasm, Nematode, Germline granules

### INTRODUCTION

In many animal embryos, cells destined to form the germline have distinctive cytoplasmic regions called germ plasm (for general reviews see Beams and Kessel, 1974; Eddy, 1975; Ikenishi, 1998; Wylie, 1999). Germ plasm is widely considered to have a determinative function in germ cell development, as shown by germ plasm transfer experiments in *Drosophila* and *Xenopus* (Ikenishi, 1987; Illmensee and Mahowald, 1974; Illmensee and Mahowald, 1976). While the biochemical functions of germ plasm are not understood, proteins related to *Drosophila* VASA, an ATP-dependent RNA helicase, have been identified in the germlines of vertebrates and invertebrates, including *C. elegans* (Baat et al., 2000; Castrillon et al., 2000; Gruidl et al., 1996; Hay et al., 1988; Knaut et al., 2000; Komiyama et al., 1994; Kuznicki et al., 2000; Olsen et al., 1997; Tsunekawa et al., 2000; Yoon et al., 1997). In addition to containing distinctive proteins, germ plasm stains positively for RNA, contains numerous mitochondria, and has distinctive cytoplasmic granules. These granules are called polar granules in *Drosophila*, dense bodies or germinal granules in *Xenopus*, and P granules in *C. elegans*.

P granules are found in germ cells, or in the embryonic precursors to the germ cells, throughout the *C. elegans* life cycle (Strome and Wood, 1982). In oocytes, the P granules are distributed uniformly in the cytoplasm. After fertilization, P granules move to the posterior pole of the embryo, and at the first cell division are segregated into the posterior daughter cell,

called P1. This pattern of segregation is repeated during the next three cell divisions, resulting in the asymmetric localization of P granules sequentially into the embryonic blastomeres called P2, P3 and P4. In subsequent embryonic and postembryonic development, the descendants of the P4 blastomere divide symmetrically with all cells inheriting P granules; these descendants produce only germ cells.

Several genes have been identified that encode protein components of P granules, most of which are novel. *pgl-1* and the genes *glh-1*, *glh-2*, *glh-3* and *glh-4* encode proteins that are found on P granules at all stages of the *C. elegans* life cycle and are required for postembryonic development of the gonad (Gruidl et al., 1996; Kawasaki et al., 1998; Kuznicki et al., 2000). The genes *gld-1*, *pie-1*, *mex-1*, *pos-1* and *mex-3* encode proteins that are present on P granules only in the early embryo (Draper et al., 1996; Guedes and Priess, 1997; Jones et al., 1996; Mello et al., 1992; Tabara et al., 1999). *gld-1* is required for proper regulation of cell proliferation in the gonad (Francis et al., 1995). The *pie-1*, *mex-1* and *pos-1* genes are required, at least in part, to prevent the germline precursors from producing only somatic tissues (Mello et al., 1992; Mello et al., 1996; Schnabel et al., 1996; Tabara et al., 1999). In contrast, the *mex-3* gene is required in part to prevent certain somatic precursors from adopting germline fates (Draper et al., 1996). Since many of these proteins are present in the cytoplasm or nucleus as well as on P granules, the functional significance of their association with P granules remains to be determined.

Although there are diverse types of proteins in P granules,

each of the known proteins contains a potential RNA-binding motif. For example, PGL-1 has an RGG box and MEX-3 has a KH domain (Draper et al., 1996; Kawasaki et al., 1998). These observations suggest that P granules have functional or structural interactions with RNA; for example, *Drosophila* polar granules contain at least one noncoding RNA (Nakamura et al., 1996). At present, only one specific RNA, the *nos-2* mRNA, has been shown to be associated with P granules in *C. elegans*. *nos-2* and the related genes *nos-1* and *nos-3*, encode proteins that are similar to the *Drosophila* germ plasm component Nanos (Kraemer et al., 1999; Subramaniam and Seydoux, 1999; Wang and Lehmann, 1991). *nos-2* and *nos-1* have redundant functions that are required for germ cell viability (Kraemer et al., 1999; Subramaniam and Seydoux, 1999). *nos-2* mRNA is present throughout the cytoplasm of the early embryonic blastomere P3, but appears to be concentrated in P granules (Subramaniam and Seydoux, 1999). It is not known whether *nos-2* mRNA is associated with P granules in larval and adult gonads, or whether the association with P granules in the early embryonic blastomeres is essential for *nos-2* function.

P granules are localized to the cytoplasm of oocytes and early embryos when the germ lineage appears to be specified. However, P granules are associated closely with germ cell nuclei in older embryos, larvae and adults. Similarly, the germ granules of *Drosophila*, *Xenopus* and *Danio* are associated with germ nuclei during some stage of the life cycle (Eddy and Ito, 1971; Hay et al., 1988; Knaut et al., 2000; Mahowald, 1971; Mahowald and Hennen, 1971). The ultrastructure of the nuclear-associated P granules has been examined in gonads of adult *C. elegans* hermaphrodites (Pitt et al., 2000). In germ nuclei at the pachytene stage of meiosis, each P granule appears to be associated with a cluster of nuclear pores (Pitt et al., 2000). Since about 75% of all nuclear pores are in these clusters, P granules could potentially have functions related to the RNAs that exit from, or enter through, those pores. In previous studies, RNA synthesis has been analyzed in *C. elegans* (Bergerac strain) gonads that were dissected from other body tissues and labeled with [<sup>3</sup>H]uridine (Gibert et al., 1984; Starck, 1977; Starck et al., 1982). These studies showed that the highest level of RNA synthesis occurred in pachytene nuclei, with a large fraction of the label appearing in ribosomal RNA (rRNA). The pachytene nuclei surround a common cytoplasmic region of the gonad called the central core (see Fig. 1); label appeared rapidly and sequentially in germ nuclei, the cytoplasm and then in the central core of the gonad. RNA synthesis appeared to decline markedly as the pachytene nuclei entered diakinesis and differentiated as oocytes. Thus, pachytene germ nuclei, with nuclear-associated P granules, synthesize large amounts of RNA that appears to be transported into the central core of the gonad and incorporated into oocytes.

We have shown previously that P granules on adult germ nuclei show low levels of hybridization with a probe for SL1, a transcribed leader RNA found on about 60% of mRNAs in *C. elegans* (Pitt et al., 2000; Zorio et al., 1994). In this report, we have analyzed whether rRNA and a set of specific mRNAs are associated with P granules in the adult gonad, and describe physiological and genetic conditions that can alter the level of RNA on P granules. Our results provide evidence that the RNA component of P granules is diverse and dynamic.

## MATERIALS AND METHODS

### Strains and culture

The Bristol strain N2 was used as the wild-type strain. *fem-1(hc17)* and *C. remanei(SB146)* were obtained from the *C. elegans* Genetic Stock Center, and *pgl-1(bn101)* was obtained from Susan Strome (University of Indiana).

### Staining and microscopy

SYTO green fluorescent nucleic acid stains were obtained from Molecular Probes. Staged wild-type or *fem-1* hermaphrodites or *C. remanei* females were dissected into 5 µm SYTO 14 in 118 mM NaCl; 48 mM KCl that was prepared just prior to use; extruded gonads were allowed to incubate for 15 minutes before analysis. Gonads incubated for less than 10 minutes showed variable staining on mitochondria, as determined by staining gonads simultaneously with MitoTracker red CMXros (Molecular Probes, Eugene, OR). DNA was visualized simultaneously with Hoechst 33342 (Sigma). In autoradiography experiments, gonads were dissected and labeled following published protocols (Gibert et al., 1984). Labeled gonads were fixed as described (Pitt et al., 2000). Sections were coated with emulsion and processed using published protocols (Kornhauser et al., 1992); sections were exposed to emulsion for 3-4 weeks. Electron microscopy was performed as described by Pitt et al. (Pitt et al., 2000).

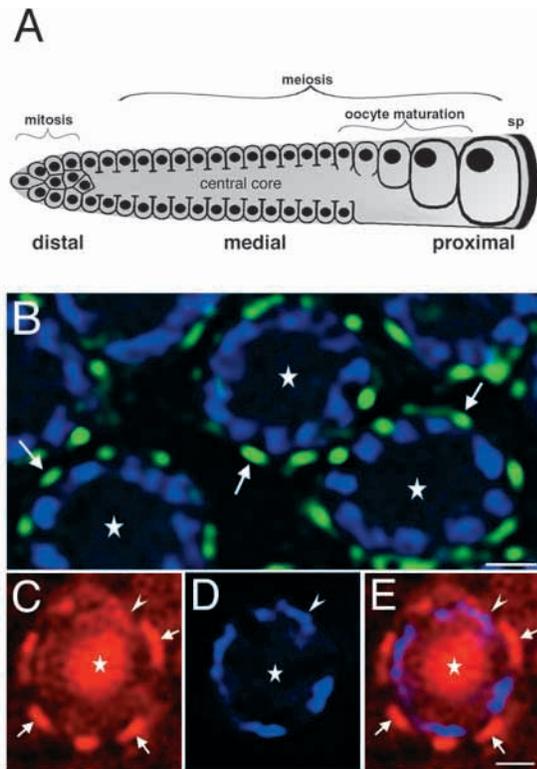
Antibodies, antisera and staining protocols were as described: anti-PGL-1 (Kawasaki et al., 1998; kindly provided by Susan Strome); anti-GLH-1 and anti-GLH-2 (Gruidl et al., 1996; kindly provided by Karen Bennett); anti-MEX-3 (Draper et al., 1996); anti-MEX-1 (Guedes and Priess, 1997); and mAb414 (BABCO; Pitt et al., 2000). Images in Figs 1, 4 and 7 were acquired using a Delta Vision microscope and processed using deconvolution software (Applied Precision); morphometric analyses to analyze intensity and area of P granules was performed using the Delta Vision software. Images in Figs 3, 5 and 6 were acquired using a Zeiss Axioplan microscope.

### In situ hybridization

For all RNAs, both antisense and sense probes were made; for all results reported here, sense probes showed either no, or low uniform levels of hybridization to gonads. Probe sequences were as follows SL1 (Pitt et al., 2000); 5.8S (prepared as in Frank and Roth, 1998); 26S (CCTTAGATGAAGTTTATCACCCACTTTAGG); 18S (TTATCTTTTACTTGAGCATATCGCGC); and 5S (GGACGGGATGGCGTGCATTCAACGTG). Sense and antisense oligo probes were end-labeled with terminal transferase and digoxigenin-dUTP (Roche) and used at a concentration of 1.0 µg/ml in oligo hybridization buffer. cDNA probes were generated by asymmetric PCR with DIG-dUTP (Roche), from either PCR products amplified from genomic DNA (*mtlrRNA*, *par-3*) or the following cDNAs: yk 64a5.5 (*actin*), yk 485d9 (*tubulin*), yk 117h11 (*pos-1*), JPSG9 (*mex-1*), yk 56a9.5 (*nos-2*), yk71d10.5 (*nos-2*), yk 30e10 (*gld-1*) and JP597 (*skn-1*). cDNA probes were used at approximately 5 µg/ml.

Fixation and hybridization procedures were as described by Seydoux and Fire (Seydoux and Fire, 1994) with some modifications. Gonads were dissected in PBS on a glass coverslip. The coverslips were inverted on a 0.1% polylysine-coated slide and frozen on dry ice. After removal of the cover slip, the slide was immersed in 100% methanol at -20°C (5 minutes), 100% methanol at room temperature (5 minutes), 90% methanol (1 minute), 70% methanol (1 minute), 50% methanol (1 minute), and washed twice in PTw (5 minutes; 1× PBS, 0.1% Tween 20). Gonads were treated with protease K (20 µg/ml; 15 minutes) at 37°C, washed in 2 mg/ml glycine in PTw (2 minutes) and PTw (5 minutes). Gonads were fixed for 20 minutes at room temperature in 4% formaldehyde in PBS, then washed in PTw (5 minutes), 2 mg/ml glycine in PTw (5 minutes), PTw (5 minutes) and 2× SSC (5 minutes).

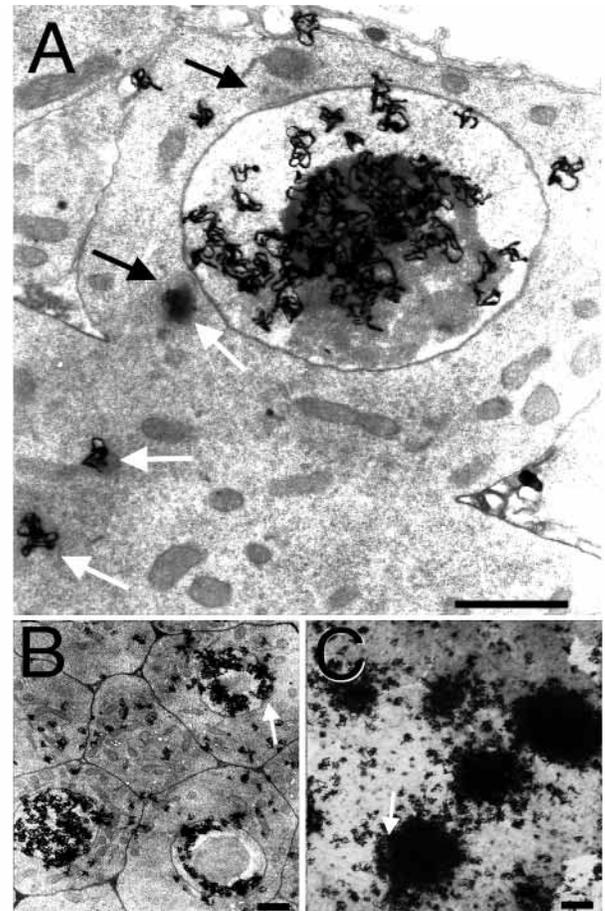
For oligo probes, hybridization buffer consisted of 7.7% formamide, 2× SSC, 100 µg/ml salmon sperm DNA, 50 µg/ml



**Fig. 1.** P granules and germ nuclei. (A) Schematic diagram of longitudinal section through one arm of the adult hermaphrodite gonad showing the germ nuclei (closed black circles) and spermatheca (sp). (B) Fluorescence micrograph of germ nuclei immunostained for the P granule protein PGL-1 (green, arrows) and stained with DAPI (blue) to visualize DNA. The focal plane is a tangential view near the gonad surface and through the center of the three middle nuclei; pachytene chromosomes surround the central, large nucleoli (stars). (C-E) Fluorescence micrograph of a germ nucleus in an old wild-type gonad stained with SYTO 14 (red) to visualize RNA and with Hoechst 33342 (blue) to visualize DNA. Arrows indicate perinuclear RNA foci; note they are concentrated where the chromosomes are not tightly associated with the inner nuclear envelope. Arrowhead indicates chromosomes that are also faintly stained by SYTO 14. Bars, 2  $\mu$ m.

heparin, 0.1% Tween 20. For cDNA probes, hybridization buffer was similar but with 50% formamide and 5 $\times$  SSC. Gonads were prehybridized for 10 minutes at either 37 $^{\circ}$ C (oligo probes) or 48 $^{\circ}$ C (cDNA probes). Probes were boiled in hybridization buffer for 10 minutes and put on ice before being applied to the sample tissue on a microscope slide. The tissue was covered with a glass coverslip, sealed with rubber cement, and incubated at 37 $^{\circ}$ C or 48 $^{\circ}$ C for 12-18 hours. After hybridization, the slide was washed at 37 $^{\circ}$ C or 48 $^{\circ}$ C with hybridization buffer (15 minutes, 30 minutes, respectively). The slide was then washed twice in 2 $\times$  SSC (10 minutes). For alkaline phosphatase detection, the slide was washed in PBT (1 $\times$  PBS, 0.1% BSA, 0.1% Triton X-100) twice for 5 minutes at room temperature.

All RNAs were examined using alkaline phosphatase detection, and all but *tubulin*, *nos-2* and *skn-1* were examined in separate experiments using fluorescence detection. Alkaline phosphatase detection was performed as described by Seydoux and Fire (Seydoux and Fire, 1994). Fluorescence was detected with the fluorescent antibody enhancer set for DIG detection (Roche). In most experiments, anti-PGL-1 antibody (Kawasaki et al., 1998) was applied overnight at 4 $^{\circ}$ C following DIG detection. Gonads were washed twice for 5 minutes in TTBS (100 mM Tris-HCl (pH 7.5), 200 mM NaCl,



**Fig. 2.** RNA synthesis in young wild-type gonads. Electron micrographs of wild-type gonads exposed to [ $^3$ H]uridine for (A) 30 minutes, (B) 45 minutes and (C) 60 minutes. The image in A is from a longitudinal section through the gonad (as in Fig. 1A) showing a single germ nucleus (top) over the central core of the gonad (bottom). Numerous silver grains are visible over the nucleus and nucleolus, and individual grains are indicated in the cytoplasm (white arrows). Two P granules (black arrows) are visible on this nucleus; note silver grain over lower P granule. B and C show tangential sections through the plane of germ nuclei. White arrows indicate the nuclear periphery, and a P granule is present by the arrow in B. Bars, 1  $\mu$ m.

0.1% Tween 20); a rhodamine-conjugated anti-rabbit secondary was applied to detect PGL-1. After two washes in TTBS, gonads were placed in PBS containing 0.08  $\mu$ g/ml DAPI and covered with mounting medium as above.

## RESULTS

### Background

The adult gonad of *C. elegans* consists of two symmetrical arms, and has been described in detail previously (Hirsh et al., 1976; Kimble and Hirsh, 1979). Each arm is an elongated, cylindrical shell of syncytial nuclei that surrounds a common, anucleate cytoplasmic region; this anucleate region is called the central core (Fig. 1A). The distal region of each gonad arm contains nuclei in mitosis and entering meiosis, the medial gonad consists primarily of nuclei at the pachytene stage of

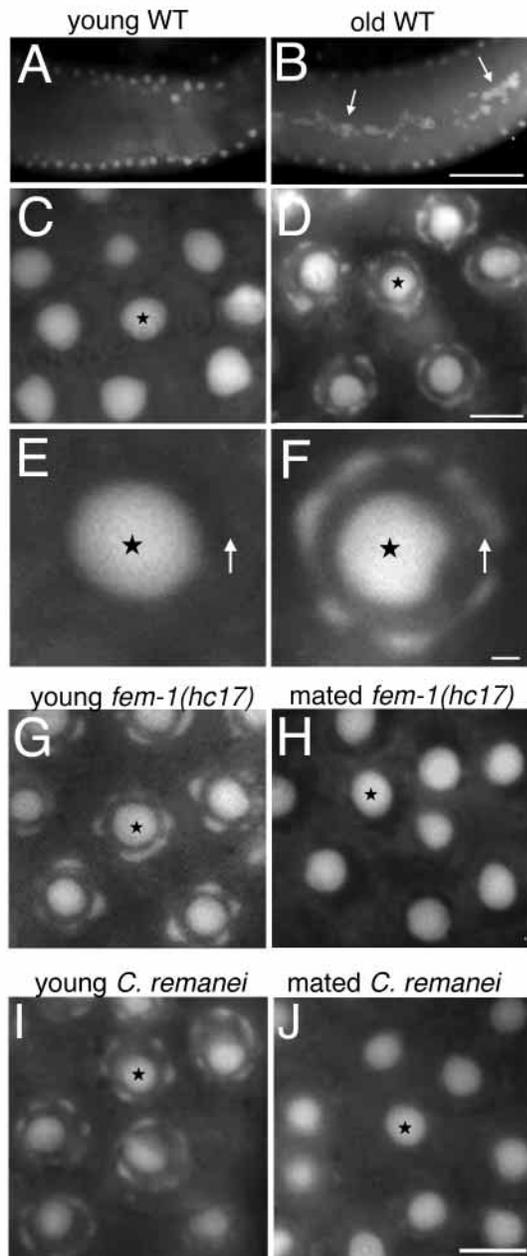
meiosis I, and the proximal gonad contains nuclei that are entering diakinesis and cellularizing to form oocytes (Fig. 1A). *C. elegans* hermaphrodites produce and store sperm during late larval development, prior to producing oocytes as adults. Sperm are stored in a structure, called the spermatheca, at the

proximal end of each gonad arm. As oocytes mature within the proximal gonad, they are ovulated into the spermatheca and self-fertilized. P granules are localized to germ nuclei in the distal and medial gonad, but in the proximal region, P granules detach from the oocyte nuclei and are distributed throughout the cytoplasm (Strome and Wood, 1982). A typical pachytene germ nucleus in the medial gonad contains a very large, central nucleolus (stars, Fig. 1B) that is surrounded by condensed, paired chromosomes. P granules are localized to the outer nuclear envelope, and at this stage appear concentrated on areas where the underlying chromosomes do not appear to be tightly associated with the inner nuclear envelope (Fig. 1B; see also Pitt et al., 2000).

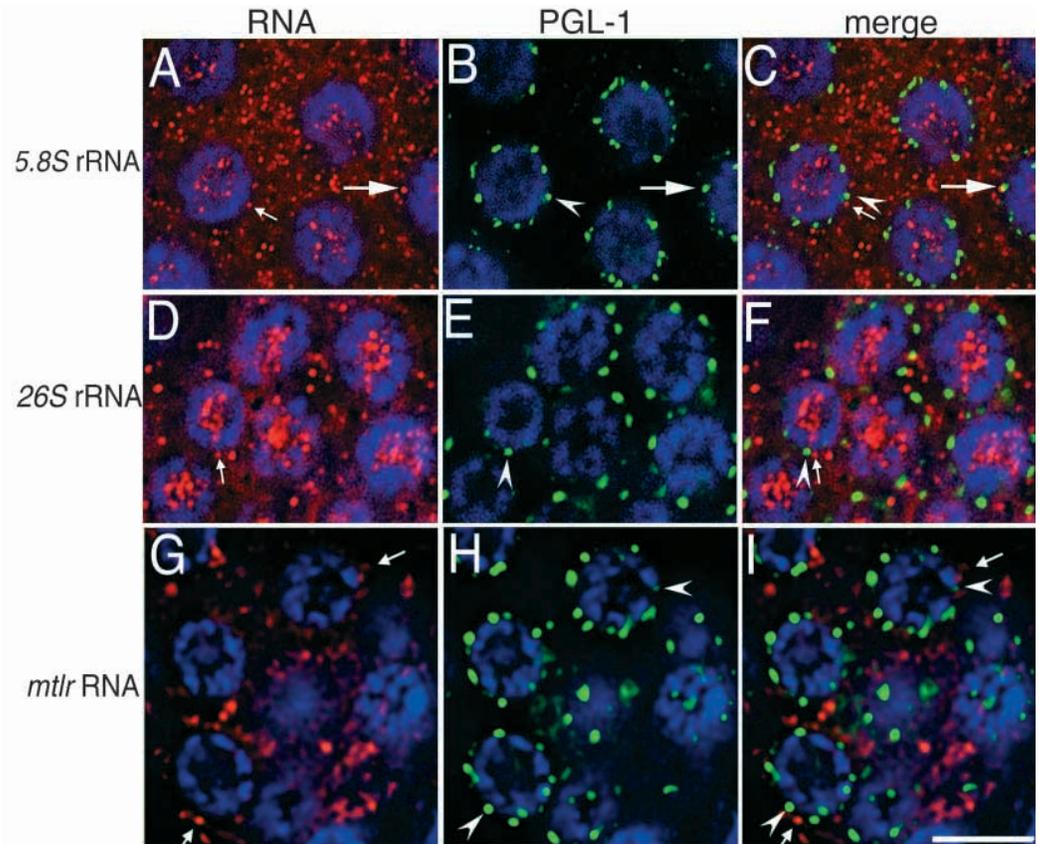
### RNA distribution in wild-type and *fem-1* mutant gonads

Because P granules are associated with many of the nuclear pores on germ nuclei, we determined whether newly synthesized RNA accumulated in P granules. Adult gonads were dissected in culture medium containing [<sup>3</sup>H]uridine for 5, 15, 30, 45, 60 and 120 minutes, then fixed and processed for ultrastructural autoradiography. The general pattern of labeling we observed for *C. elegans* (Bristol strain) was essentially identical to the results reported previously by Gibert et al. (Gibert et al., 1984) for *C. elegans* (Bergerac strain). Germ nuclei were labeled beginning at 5-15 minutes, followed by a progressive increase in labeling in the gonad cytoplasm; in contrast, very little label was detected at any time in either the cytoplasm or nuclei of maturing oocytes. We observed only a few examples of silver grains over P granules on germ nuclei between 15-30 minutes (Fig. 2A), however the entire perinuclear zone, including P granules, showed large amounts of labeling by 60 minutes (Fig. 2C). These results suggest that at least some newly synthesized RNA is associated with P granules.

We dissected live, adult hermaphrodites in the presence of the nucleic acid stain SYTO 14 (Knowles et al., 1996) to visualize the distribution of RNA in the gonad. The highest levels of staining were observed in the nucleolus of each germ nucleus (Fig. 3A; stars in Fig. 3C,E). In almost all of the gonads examined, staining appeared uniform throughout the cytoplasm, including at the perimeter of each germ nucleus (arrow, Fig. 3E) where P granules are located. However, 2 of 40 adults showed slightly elevated levels of staining in perinuclear foci on a few germ nuclei. To determine if this variability was due to differences in the ages of the adults, we stained synchronized populations of animals. None of the adults that were examined up to 4 days post-hatching (hereafter called young adults) showed perinuclear foci (0/80 adults). In contrast, most adults that were examined 6 days post-hatching (hereafter called old adults) showed high levels of staining on perinuclear foci (Fig. 3D,F; 23/31 adults). These old adults also had much higher levels of staining in the central core of their gonads (arrows, Fig. 3B) than did younger adults (Fig. 3A). Staining of the perinuclear foci did not persist through the fixation and permeabilization treatments required to visualize protein components of P granules by immunocytochemistry. However, the perinuclear foci resembled P granules in number, shape and size. Similar to P granules, most of the foci were positioned on the nuclear surface at sites where chromosomes are not juxtaposed to the inner envelope (Fig. 1C-E; see also



**Fig. 3.** RNA in wild-type and *fem-1* mutant gonads. All images are fluorescence micrographs showing SYTO 14 staining in live gonads dissected from adults (ages or genotypes of the adults are indicated). (A,B) Low magnification view of medial region of one gonad arm; nucleoli appear as bright white dots. Note RNA in central core of gonad in B. (C,D) High magnification images of a group of germ nuclei. (E,F) High magnification images of single germ nuclei. Arrows in E and F indicate perimeter of nuclear envelope, as determined by simultaneous imaging with Nomarski optics. (G-J) High magnification images of a group of germ nuclei. Stars in C-J indicate nucleoli. Bars, (A,B) 85  $\mu$ m; (C,D) 10  $\mu$ m; (E,F) 2  $\mu$ m; (G-J) 10  $\mu$ m.



**Fig. 4.** rRNA and P granules in *fem-1* gonads. Each row shows fluorescence images of germ nuclei in *fem-1* gonads after in situ hybridization (left column; probes listed on left) and immunostaining with the PGL-1 antiserum (middle column). The merged image is shown in the right column. Germ nuclei are stained by DAPI (blue). Small arrows indicate the positions of puncta stained by the in situ probes, and arrowheads indicate the positions of individual P granules. The large arrow in A indicates a puncta that partially overlaps with the position of a P granule. Bar, 10  $\mu$ m.

Pitt et al., 2000). For these and additional reasons described below we consider it very likely that the perinuclear foci in old adults are P granules.

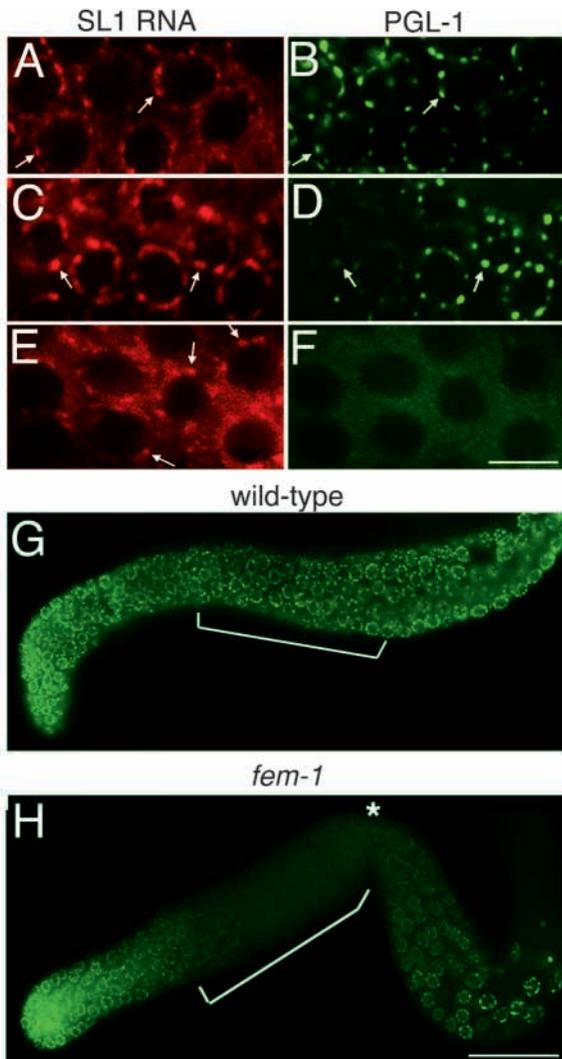
One difference between old and young adult hermaphrodites is that old adults inhibit oogenesis after they deplete their supply of sperm (at about 6 days post-hatching). We analyzed *fem-1* mutant animals to address whether staining of the perinuclear foci resulted from the age of the old adults, or from the inhibition of oogenesis. *fem-1* is a 'feminized' mutant strain of *C. elegans* that is defective in spermatogenesis. Young *fem-1* adults produce several oocytes, however the absence of sperm leads to an inhibition of oogenesis. Similar to old wild-type adults, oogenesis can resume in *fem-1* adults if these animals are mated with males (Nelson et al., 1978). We found that SYTO 14 stained perinuclear foci in non-mated, young *fem-1* adults (Fig. 3G; 30/40 adults 3 days post-hatching). These perinuclear foci were similar to those observed in old wild-type adults, and showed a further increase in staining intensity with age. We mated *fem-1* adults and old wild-type adults with wild-type males, and after 6 hours dissected and stained their gonads. Perinuclear foci were either not visible, or showed only faint staining, in the gonads from each of 30 mated animals (Fig. 3H).

We were interested in whether high levels of P granule-associated RNA, as observed in old wild-type and 'feminized' mutant hermaphrodites of *C. elegans* would be present in naturally occurring female nematodes. We therefore used SYTO 14 to stain gonads from young virgin adult females of *C. remanei*, a non-hermaphroditic, male/female nematode strain. The *C. remanei* gonads showed intense staining in

perinuclear foci (Fig. 3I; 30/36 animals) that appeared identical to the foci in *C. elegans fem-1* and old wild-type adults. We found that oogenesis in *C. remanei* females appeared to be stimulated by mating with males (our unpublished observations), and that within 3 hours after mating the perinuclear foci were not detectable in most animals (Fig. 3J; 19/22 adults). Taken together, these results suggest that the level of RNA in P granules is inversely correlated with the rate of oogenesis (see Discussion).

#### RNA in wild-type and *fem-1* P granules

Because a large fraction of the RNA synthesized in the adult gonad is rRNA (Starck et al., 1982), and P granules can have a level of SYTO 14 staining that is comparable to that of nucleoli, we used in situ hybridization to determine whether P granules might contain rRNA. For these and other in situ hybridization studies reported here, sense and antisense probes were synthesized and probe localization was examined in separate experiments using alkaline phosphatase and fluorescence detection systems (see Material and Methods). Alkaline phosphatase detection generally produced the strongest signal with the least background, but fluorescence detection was necessary for the simultaneous immunolocalization of P granule proteins. Probes for 5S, 5.8S, 18S or 26S rRNA did not show any obvious concentration of rRNA in P granules; these probes hybridized to nucleoli and throughout the cytoplasm of young wild-type gonads and *fem-1* gonads (Fig. 4A-F and data not shown). With fluorescence detection, but not alkaline phosphatase detection, small puncta were visible in nucleoli and in the cytoplasm; these cytoplasmic puncta only rarely



**Fig. 5.** SL1-containing mRNA in wild-type and mutant gonads. (A-F) Fluorescence micrographs of germ nuclei after in situ hybridization with a probe for SL1 (red) and immunostaining for PGL-1 (green). Gonads are from (A,B) a young wild-type adult, (C,D) a *fem-1* adult and (E,F) a *glh-1(RNAi)*; *glh-2(RNAi)* adult. Arrows indicate perinuclear foci or P granules. (G,H) Low magnification fluorescence micrographs of single gonad arms stained with PGL-1 antiserum; genotypes are indicated above panels. The bracketed areas indicate the medial region of the gonad; the distal and proximal regions are left and right of the bracket, respectively. C and D are from a region of a *fem-1* gonad comparable to the transition region in H indicated by a star. Note the low level of PGL-1 immunostaining in the medial region of the *fem-1* gonad (left half of D; bracketed region in H). Bars, (A-F) 10  $\mu$ m; (G,H) 85  $\mu$ m.

overlapped with the locations of P granules (large arrow, Fig. 4C). We conclude that P granules contain, at most, low levels of rRNA, and that the high level of RNA in *fem-1* P granules can not be attributed to an increase in rRNA levels.

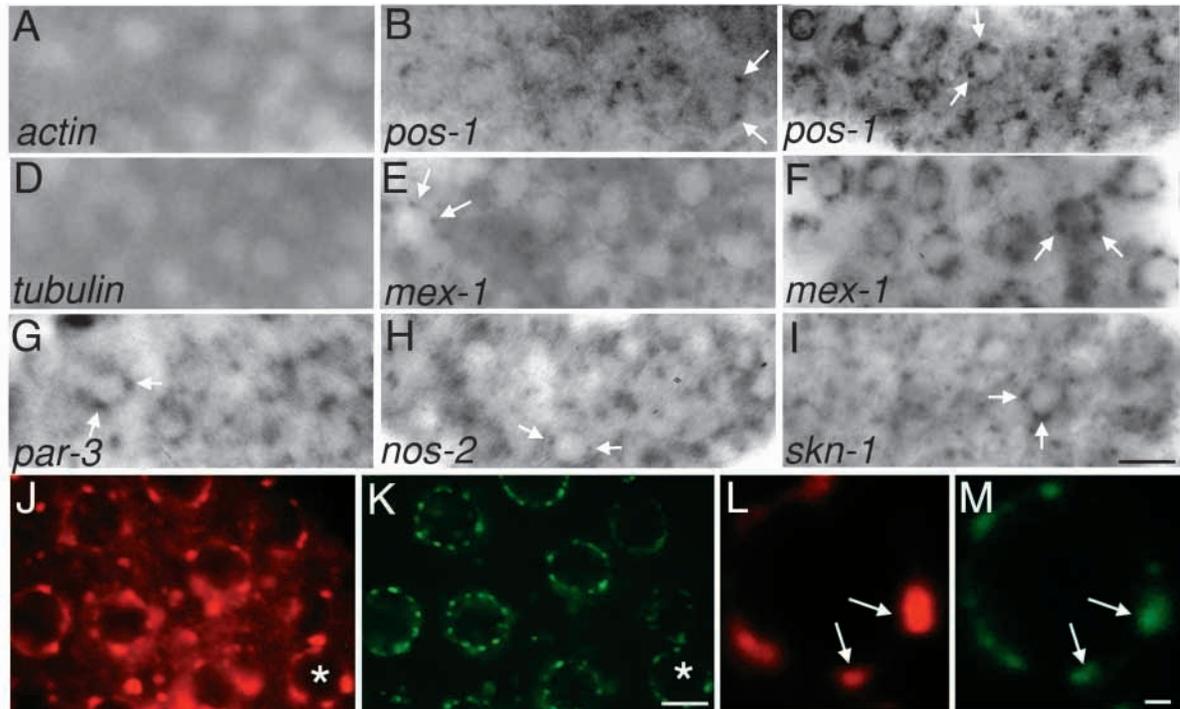
We investigated whether *mitochondrial large rRNA* (*mtlrRNA*) was a component of P granules; *mtlrRNA* has been shown to be present at the periphery of germline granules in both *Xenopus* and *Drosophila* embryos (Kobayashi et al., 1998; Kobayashi et al., 1993). We found that a probe for *C. elegans*

*mtlrRNA* hybridized to irregularly shaped objects throughout the cytoplasm of young wild-type and *fem-1* gonads that appeared similar to the appearance of mitochondria (Fig. 4G-I; see also Pitt et al., 2000). Many of these objects were close to, but not coincident with, P granules, consistent with the localization of mitochondria in the gonad. We thus conclude that *mtlrRNA* is not a prominent component of the RNA in P granules on pachytene-stage nuclei. It remains to be determined whether *mtlrRNA* is associated with P granules at other times in development; for example, *mtlrRNA* is only transiently associated with the germline granules of *Drosophila* and *Xenopus* (Kobayashi et al., 1998; Kobayashi et al., 1993).

SL1 is a transcribed leader RNA that is found on many mRNAs in *C. elegans*, but is not thought to be present on rRNA (Krause and Hirsh, 1987). We found that a probe for SL1 showed a low level of hybridization to P granules in a synchronous population of young wild-type adults (Fig. 5A). In contrast, P granules in *fem-1*, and old wild-type, adults showed a much higher and consistent level of hybridization with the SL1 probe (Fig. 5C). We mated *fem-1* adults with wild-type males, and after 6 hours fixed and hybridized their gonads with the SL1 probe. P granules in the mated animals showed only a low level of hybridization that was comparable to the level observed in young wild-type adults (data not shown). These observations together suggest that the increased levels of RNA in P granules of *fem-1*, and old wild-type, adults result at least in part from an increased level of SL1-containing mRNAs.

As examples of specific, abundant mRNAs, we examined *actin* mRNA and  $\beta$ -*tubulin* mRNA in the gonad (Edwards and Wood, 1983). We found that probes for either mRNA showed hybridization throughout the gonad in *fem-1* adults, but did not show any obvious increase in hybridization at the positions of P granules (Figs 6A,D); we obtained similar results with gonads from young wild-type adults (data not shown). We next examined a set of six developmentally regulated mRNAs that encode proteins that function in either gonadogenesis or embryogenesis; *pos-1* (Tabara et al., 1999), *mex-1* (Guedes et al., 1997); *par-3* (Etemad-Moghadam et al., 1995), *nos-2* (Subramaniam and Seydoux, 1999), *skn-1* (Bowerman et al., 1992), and *gld-1* (Francis et al., 1995). The probe for *gld-1* mRNA hybridized strongly throughout the gonad, while the other probes hybridized predominantly to only the proximal gonad (*nos-2*) or the proximal plus medial gonad (*pos-1*, *mex-1*, *par-3* and *skn-1*). Probes for *pos-1*, *mex-1* and *gld-1* reproducibly showed low levels of hybridization to perinuclear foci in wild-type gonads (Fig. 6B,E and data not shown), but much higher levels of hybridization to perinuclear foci in *fem-1* gonads (Fig. 6C,F). We confirmed that the perinuclear foci stained by the *pos-1* probe (Fig. 6J,L) were P granules by immunostaining for the P granule protein PGL-1 (Fig. 6K,M). We found that probes for *par-3*, *nos-2* and *skn-1* mRNAs showed infrequent, and very weak, hybridization to a few perinuclear foci in young wild-type gonads (data not shown). These same probes showed significant hybridization to perinuclear foci in *fem-1* gonads (Figs 6G-I), though at lower levels than the *pos-1*, *mex-1* and *gld-1* probes. Thus each of these six mRNAs appears to be enriched in P granules relative to the surrounding cytoplasm.

In oocytes from young wild-type adults, where P granules have dissociated from the nuclear envelope, we found that *pos-*



**Fig. 6.** mRNA localization in P granules. (A-I) Light micrographs of gonads after in situ hybridization with probes for (A) *actin* mRNA, (B,C) *pos-1* mRNA, (D) *tubulin* mRNA, (E,F) *mex-1* mRNA, (G) *par-3* mRNA, (H) *nos-2* mRNA and (I) *skn-1* mRNA. Light areas correspond to positions of germ nuclei. Arrows indicate positions of perinuclear foci detected by alkaline phosphatase activity. B and E are of a young wild-type adult; all other panels are *fem-1* adults. (J-M) Fluorescence micrographs of a *fem-1* gonad after in situ hybridization with a probe for *pos-1* mRNA (J,L) and immunostaining for PGL-1 (K,M). L and M are high magnification views of a single germ nucleus; arrows indicate positions of perinuclear foci and P granules. Bars, (A-I) 10  $\mu$ m; (J,K) 5  $\mu$ m; (L,M) 1  $\mu$ m.

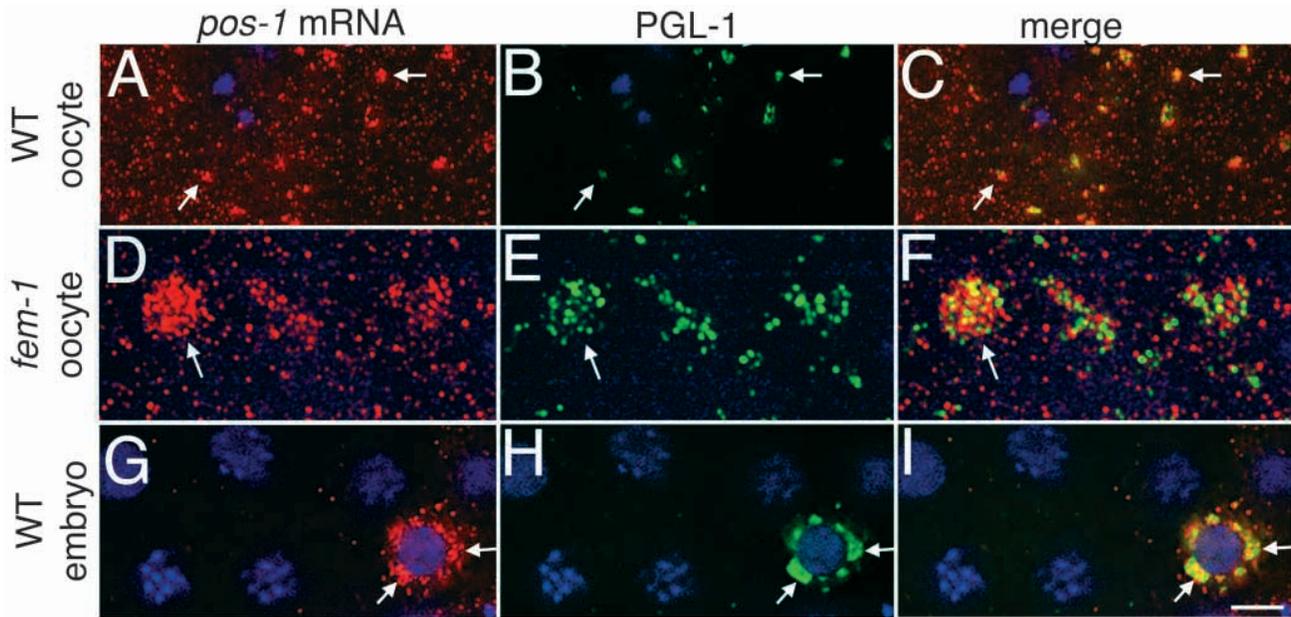
*l* mRNA appeared to be enriched in P granules above the level in the general cytoplasm (arrows Fig. 7A-C). In the arrested oocytes of *fem-1* animals, most of the *pos-1* mRNA appeared to be concentrated in 'giant foci' that could be 20 times larger than typical cytoplasmic P granules in wild-type oocytes (arrow, Fig. 7D). Similarly, probes for all of the other mRNAs except *mex-1* that hybridized to the nuclear-associated P granules of germ cells also hybridized strongly to giant cytoplasmic foci in *fem-1* oocytes. In contrast, probes for *actin* mRNA, *beta-tubulin* mRNA, or rRNA showed only low levels, or no, hybridization to the giant foci (data not shown). The giant foci co-localized with the P granule proteins PGL-1, GLH-1, GLH-2, MEX-3 and MEX-1 (Fig. 7E and data not shown). We note that these giant foci may be related to the large aggregates of MEX-3 protein that have been observed previously in the arrested oocytes of *emo-1* mutants (Iwasaki et al., 1996). Probes for mRNAs like *pos-1* hybridized to the general cytoplasm surrounding the giant foci with much lower intensity than to the general cytoplasm in wild-type oocytes, suggesting that the giant foci originate from an aggregation of mRNA (compare Fig. 7A and D). We mated *fem-1* adults, and old wild-type adults, to wild-type males and after 6 hours immunostained the *fem-1* gonads with antisera against PGL-1, MEX-3 and MEX-1. We found that giant foci were not visible in any of the oocytes in the gonads from the mated animals (data not shown). Before mating, the *fem-1* adults had an average of 30 arrested oocytes, each with giant foci (15 oocytes per gonad arm). Because a maximum of 10 embryos were laid in the 6-hour interval between mating and staining, these

results suggest that the giant foci disperse in all oocytes soon after animals are mated with males. The majority of eggs laid in the 6-hour interval developed into viable larvae, indicating that the giant foci are not deleterious.

We examined *pos-1*, *mex-1* and *gld-1* mRNA localization in the blastomeres in early wild-type embryos that eventually produce the germline. P granules in these blastomeres are initially small and cytoplasmic, as in oocytes, but become larger and associated with nuclei after the first few cell divisions (see Discussion). *pos-1* mRNA appeared to be concentrated at the positions of P granules during each of these divisions (Fig. 7G-I), and *mex-1* and *gld-1* mRNAs showed a similar concentration on P granules beginning at the third embryonic cell cycle (data not shown).

### P granule structure/composition

We were interested in whether the apparent increase in the level of RNA on the nuclear-associated P granules in *fem-1*, and old wild-type, adults was associated with changes in the size or protein composition of P granules. P granules in the distal and proximal regions of *fem-1* gonads appeared, respectively, to be equal to or slightly larger than P granules in the corresponding regions of young wild-type gonads (Fig. 5G,H). Surprisingly, P granules in the medial region of *fem-1* and old wild-type gonads, that contain abnormally large amounts of RNA (see Figs 3G and 5C), appeared smaller than normal and had markedly reduced levels of immunostaining for the P granule proteins PGL-1, GLH-1 and GLH-2 (compare bracketed regions in Fig. 5H with G). In young wild-type adults, P granules increase in size and



**Fig. 7.** *pos-1* mRNA in embryos and oocytes. (A-C) Fluorescence micrograph showing part of a wild-type oocyte after (A) in situ hybridization with the *pos-1* probe (red) and staining with DAPI (blue) to visualize nuclei, and (B) immunostaining for PGL-1; merged image is shown in C. Note numerous, small, cytoplasmic P granules (arrows). Some of the condensed chromosomes in the oocyte nucleus are visible in the top left of these panels. (D-F) Fluorescence micrographs showing part of a *fem-1* oocyte prepared as above. One of the giant cytoplasmic foci is indicated by an arrow. (G-I) Fluorescence micrographs showing part of a wild-type, 24-cell stage embryo, prepared as above. Most of the *pos-1* mRNA is present in perinuclear foci (arrows) on the germ cell precursor P4. Bar, 5  $\mu$ m.

stain with a slight increase in intensity as germ nuclei progress from the distal to medial regions of the gonad (Pitt et al., 2000). In contrast, the P granules in the medial region of the *fem-1* gonads were 56% smaller and stained 65% as intensely as the P granules in the distal region (see Materials and Methods). Because our earlier results indicated that RNA levels in P granules decrease when feminized animals are mated, we immunostained the mated animals to examine P granule proteins. We found that mating either *fem-1* or old wild-type adults with wild-type males resulted in a rapid increase in the level of PGL-1, GLH-1, and GLH-2 on P granules in the medial gonad such that they were comparable to P granules in young wild-type gonads (data not shown).

We used electron microscopy to compare the morphology of P granules in the medial region of the *fem-1* gonads with P granules in young wild-type gonads. A wild-type P granule consists largely of a fine fibrillar/granular matrix, and often contains small, relatively electron dense zones overlying nuclear pores (Fig. 8A; see also Pitt et al., 2000). The matrix of P granules in the medial gonad of *fem-1* adults appeared much smaller, and was more irregular in shape, than the matrix of wild-type P granules (Fig. 8B). In addition, the *fem-1* P granules in both the medial and proximal regions of the gonad appeared to contain more electron-dense material (white arrows, Fig. 8B) than observed in wild-type P granules. Thus conditions that increase the level of RNA in P granules in the medial gonad appear to decrease the level of immunodetectable PGL-1, GLH-1 and GLH-2 associated with P granules, and decrease the size of the P granule matrix.

### P granules and the PGL and GLH proteins

We wanted to determine whether inhibiting the functions of the

PGL or GLH family of proteins would cause changes in P granules that were comparable to those observed in *fem-1* and old wild-type adults, where the levels of PGL-1, GLH-1, and GLH-2 appear to be reduced on P granules. We examined the germ nuclei in the small, sterile gonads of *pgl-1(bn101)* mutant adults by electron microscopy, and found perinuclear foci that we interpret to be abnormal P granules. These perinuclear foci varied considerably in size and shape; many were much smaller than typical wild-type P granules, while some were comparable or even larger in size (Fig. 8C). However, all of the perinuclear foci observed in the *pgl-1(bn101)* sterile gonads appeared to contain more electron-dense material throughout the matrix than is present in wild-type P granules. We found very similar results after using RNA-mediated inhibition (Fire et al., 1998) to simultaneously inhibit the functions of two additional *pgl-1*-related genes (GenBank accession numbers, 1695246 and 3168892) in the *pgl-1(bn101)* mutant (data not shown).

As described previously by others (Gruidl et al., 1996; Kawasaki et al., 1998; Kuznicki et al., 2000), we found that *glh-1 glh-2 (RNAi)* animals and *glh-1, glh-2, glh-3, glh-4 (RNAi)* animals develop small, sterile gonads that lack immunostaining with the GLH-2 antiserum, and that show cytoplasmic, rather than perinuclear, localization of PGL-1 (see Fig. 5F). We examined the germ nuclei in both types of sterile gonads by electron microscopy and found that all of the germ nuclei contained abnormal, small perinuclear foci composed of highly electron dense material that appeared to be associated with nuclear pores (Fig. 8D). These foci appeared to lack completely the fibrillar-granular matrix of wild-type P granules.

We used in situ hybridization with the SL1 probe to determine if RNA was present in the perinuclear foci in *pgl-*

*l(bn101)* gonads, *glh-1-2 (RNAi)* gonads and *glh-1, glh-2, glh-3, glh-4 (RNAi)* gonads. In each case, we observed hybridization to perinuclear foci (Fig. 5E and data not shown). These results indicate that the *pgl* and *glh* genes are required for proper P granule morphology, but these genes do not appear necessary for mRNA to accumulate in perinuclear foci.

## DISCUSSION

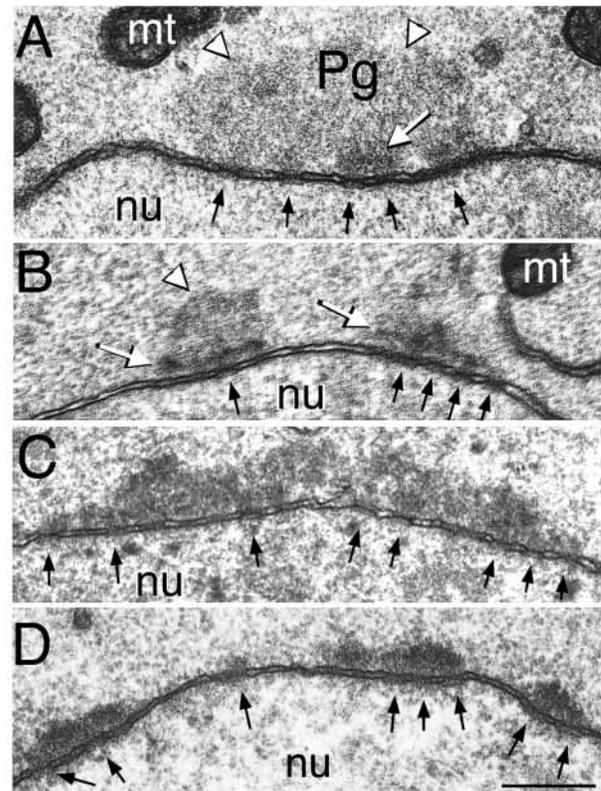
### 'Feminized' strains and P granule analysis

The issues of whether P granules contain RNA, and if so, what type of RNA, have been longstanding questions in the field of germline development in *C. elegans*. Since P granules are associated with many of the nuclear pores in the adult gonad (Pitt et al., 2000), we addressed the possibility that P granules contain several types of RNAs that are synthesized by the germ nuclei. A probe for SL1, the transplliced leader found on the majority of mRNAs, shows a low level of hybridization to P granules (Pitt et al., 2000), and we have shown here that at least some of the newly synthesized RNA in the gonad appears to be associated with P granules. Several previous studies have analyzed the expression of individual mRNAs in the *C. elegans* gonad by in situ hybridization, but have not reported an association of those mRNAs with P granules (for examples, see Draper et al., 1996; Guedes et al., 1997; Gruidl et al., 1996; Jones et al., 1996). However, if the level of hybridization of the SL1 probe to P granules represents the combined signals from many different SL1-containing mRNAs, those mRNAs might be difficult to detect on P granules with specific mRNA probes.

The level of RNA associated with P granules appears to increase markedly in animals that lack sperm; these are old wild-type hermaphrodites and 'feminized' *fem-1* mutants of *C. elegans*, and virgin *C. remanei* females. We propose that sperm indirectly affect the level of RNA in P granules by determining the rate of oogenesis. Young wild-type *C. elegans* adult hermaphrodites produce oocytes in a rapid, assembly-line manner, resulting in the ovulation of an oocyte every 23 minutes (McCarter et al., 1999). In terms of biosynthetic mass, this combined volume of oocytes is equivalent to a doubling in the size of the entire gonad every 6.5 hours (Hirsh et al., 1976). Animals that lack sperm produce several oocytes, but as the unfertilized oocytes accumulate the rate of oogenesis decreases by more than fortyfold (McCarter et al., 1999). If the rate of RNA synthesis does not decrease commensurate with the decreased rate of oogenesis, these animals would be expected to have a higher level of RNA than the level in mated animals with rapid oogenesis. RNA might accumulate in P granules if the transport mechanisms that normally move RNA from P granules into the central core of the gonad were inhibited, or saturated by excess RNA.

### RNAs in P granules

We found that probes for 5S, 5.8S, 18S, and 26S rRNA do not hybridize more intensely to P granules than to the surrounding gonad cytoplasm in either young wild-type adults or *fem-1* mutant adults. The punctae visualized after fluorescence detection in *C. elegans* gonads only rarely coincided with the positions of P granules. These rare punctae may represent new rRNA/ribosomal subunits in transit from the nucleus, or may instead represent cytoplasmic ribosomes that occasionally



**Fig. 8.** Ultrastructure of wild-type and mutant germ nuclei. Panels show electron micrographs of P granules, or perinuclear foci presumed to be abnormal P granules, on germ nuclei. All panels are oriented with the nucleus at bottom and the cytoplasm at top. Small black arrows inside each nucleus (nu) indicate examples of nuclear pores. (A) Wild-type germ nucleus showing a typical, single P granule. White arrowheads indicate the fibrillar or granular matrix of the P granule, and the white arrows indicate electron-dense zones within the P granule. The germ nucleus shown is from the medial region of the gonad. (B) *fem-1* mutant germ nucleus from the medial region of the gonad. Two small, highly abnormal P granules are visible. Labeling as in A. (C) Example of a large, abnormal P granule on a *pgl-1(bn101)* germ nucleus. (D) *glh-1(RNAi);glh-2(RNAi)* germ nucleus. Note that the perinuclear foci appear to consist entirely of electron-dense material. Mt, mitochondria. Bar, 0.2  $\mu$ m.

become trapped in P granules. P granules increase in size as germ cells mature, and P granules infrequently contain microtubules, centrioles, or mitochondria (Pitt et al., 2000). These observations suggest that cytoplasmic components might become engulfed randomly as P granules grow or fuse with neighboring P granules. Our autoradiography results are consistent with the view that newly synthesized rRNA does not accumulate to high levels in P granules. Although a large fraction of the newly synthesized RNA in the adult gonad is rRNA (Starck et al., 1982), we observed only a few examples of label over P granules at early time points when accumulation of label in the cytoplasm was evident. Thus rRNA may not interact with P granule components, or rRNA may be exported from nuclear pores that are not associated with P granules.

In contrast to our results for rRNA, probes for six of the eight mRNAs hybridized to P granules more intensely than to the surrounding cytoplasm. We did not observe hybridization to P granules with the probes for *actin* or *tubulin* mRNAs above

cytoplasmic levels in either wild-type or *fem-1* gonads. Although there may be a significant difference between these two mRNAs and the six positive mRNAs examined, there are several possible explanations for this finding. A potential, though unlikely, explanation is that *actin* or *tubulin* mRNA is not transcribed in the adult gonad, and that high levels of these mRNAs perdure in the gonad from earlier larval stages. Each of the six positive mRNA probes showed a low or moderate hybridization to P granules in young wild-type gonads, and much stronger hybridization to the P granules in *fem-1* gonads. These mRNAs included 'masked messages' that do not appear to be translated until oocyte maturation (*mex-1*) or fertilization (*pos-1*, *skn-1*, *par-3*), and mRNAs that encode proteins required for germline development (*pos-1*, *mex-1*, *nos-2*, *gld-1*), somatic development (*skn-1*), and cell polarity (*par-3*) (Tabara et al., 1999; Bowerman et al., 1992; Etemad-Moghadam et al., 1995; Guedes and Priess, 1997; Mello et al., 1992; Subramaniam and Seydoux, 1999; Kraemer et al., 1999; Francis et al., 1995). The probes for *pos-1*, *mex-1* and *gld-1* mRNA showed the strongest, and most consistent, hybridization to P granules in both wild-type and *fem-1* gonads. Because *skn-1*, *nos-2* and *par-3* mRNA appeared to be present in the cytoplasm at levels comparable to *pos-1*, *mex-1* and *gld-1* mRNA, the latter three may have a higher affinity for P granule components. We hypothesize from our limited survey of individual mRNAs that a large fraction of the mRNAs synthesized in the adult gonad are likely to show an association with P granules. At present, we are aware of only one shared feature of the mRNAs that show localization to P granules; each is a member of a group called class II maternal mRNAs (Seydoux and Fire, 1994). These messages are rapidly degraded during early embryogenesis in blastomeres that generate only somatic cells, but persist in the blastomeres that produce the germline and that contain P granules. *actin* and *tubulin* mRNA do not exhibit this asymmetry and belong to class I maternal RNAs (Seydoux and Fire, 1994). The mechanisms that result in the different patterns of RNA degradation in the embryo are not yet understood.

### P granule mRNA and nuclear export

We have shown that SL1-containing mRNA is present in perinuclear foci after the functions of either the *glh* or *pgl* gene family are inhibited using the technique of RNA-mediated interference (RNAi). If this technique effectively eliminates all *glh* or *pgl* gene function, then their protein products must not be essential for mRNA to accumulate in perinuclear foci. Our results suggest that these proteins are required directly or indirectly for the efficient transport of mRNA away from the germ nuclei. In the medial gonad, the level of immunodetectable GLH-1, GLH-2, and PGL-1 on P granules is inversely correlated with the level of mRNA; wild-type gonads with high levels of the P granule proteins show low levels of mRNA, and feminized gonads with low levels of protein show high levels of mRNA. While inhibiting the functions of the *glh* or *pgl* gene families with RNAi did not result in a higher than wild-type level of mRNA on perinuclear foci, such animals contain only a small number of abnormal germ cells that may not synthesize amounts of mRNA comparable to wild-type pachytene nuclei.

We propose that mRNA enters P granules through the

numerous nuclear pores associated with P granules on germ nuclei. Several of the protein components of nuclear pores, called nucleoporins, contain repeated amino acid sequences such as FXFG, GLFG and FG, and several recent studies in yeast and mammalian cells suggest that proteins involved in RNA export can shuttle across the pore by binding sequentially to these repeats. For example, the nucleoporins RanBP2 and Can/Nup214 on the cytoplasmic fibrils of pores have FG repeats, and the mRNA export protein TAP (yeast Mex67p) binds directly to these repeats (Sträßer et al., 2000). Interestingly, the P granule proteins GLH-1, GLH-2, GLH-3 and GLH-4 each have N-terminal FG repeats. Thus an intriguing possibility is that mRNA export factors in the gonad might interact directly with the GLH proteins. Interactions between export factors and P granule proteins might enable export complexes to move away from the germ nuclei, or allow export factors to interchange with cellular proteins that facilitate mRNA movement. In the *Drosophila* germline, mRNA transport has been hypothesized to involve cytoplasmic structures that are called sponge bodies and that possibly function in the assembly of RNA/protein complexes (Wilsch-Brauninger et al., 1997). The sponge bodies are associated with 'nuage' material that is a likely precursor to polar granules and that contains VASA, the *Drosophila* protein related to the GLH proteins of *C. elegans*.

### Nuclear-localized and cytoplasmically localized P granules

During oogenesis, P granules dissociate from the nuclear surface and become localized to the cytoplasm; the P granule material appears to re-associate with the nuclear surface during embryogenesis in the cells that produce the germline. We have shown that *pos-1* mRNA can be detected on cytoplasmic P granules in oocytes and early embryos, that *mex-1* and *gld-1* mRNAs can be detected on the cytoplasmic/nuclear P granules in embryos, and we have confirmed previous results from Subramaniam and Seydoux (Subramaniam and Seydoux, 1999) that *nos-2* mRNA is associated with P granules in early embryos. Thus we predict that other mRNAs that are found on the nuclear-associated P granules in gonads will show a similar association with the cytoplasmic/nuclear P granules in embryos.

Since the embryonic blastomeres that contain P granules are thought to be transcriptionally quiescent (Seydoux and Fire, 1994; Tenenhaus et al., 1998), we presume that all of the *pos-1*, *mex-1*, *gld-1* and *nos-2* mRNA in the early embryo is transcribed in the gonad. All of these mRNAs show an enrichment in P granules, but are also detected in the cytoplasm surrounding the P granules. At present, we do not know whether P granule-associated mRNA is in dynamic equilibrium with cytoplasmic mRNA, or whether mRNA and P granule proteins remain stably associated after a P granule detaches from the nuclear envelope. *mex-1*, *gld-1* and *nos-2* mRNAs are detectable in the cytoplasmic/nuclear P granules of embryonic blastomeres, but not in the cytoplasmic P granules of oocytes in young wild-type adults. This result is consistent with the view that the RNA component of cytoplasmic P granules is dynamic. However, an alternative explanation is that these mRNAs are more difficult to detect on the P granules of oocytes simply because the oocyte P granules are relatively small. As P granules detach from the nuclear surface in

oocytes, they appear to fragment into small pieces (Strome and Wood, 1982). During the early embryonic cell cycles, the P granules in the germ lineage get progressively larger. For example, typical P granules on germ nuclei in the adult gonad are about 1  $\mu\text{m}$  in diameter, cytoplasmic P granules in oocytes are about 0.5  $\mu\text{m}$ , and P granules in embryonic blastomeres can be 3  $\mu\text{m}$  (see Fig. 7). Since P granules increase in size with a concomitant decrease in number during early embryogenesis, we consider it likely that small P granules aggregate into larger ones. Analogous aggregations of germ plasm have been described in early *Xenopus* embryos (Savage and Danilchik, 1993).

We have described giant cytoplasmic foci in the arrested oocytes of *fem-1* adults and old wild-type adults that resemble P granules in mRNA and protein composition. The amount of mRNA in these giant foci is much larger than the combined amounts of mRNA evident in the P granules in non-arrested oocytes, and formation of the giant foci is correlated with a decrease in cytoplasmic levels of mRNA. Therefore, these foci do not originate simply by the aggregation of oocyte P granules, and instead appear to originate by an aggregation of cytoplasmic mRNAs. One possibility is that certain cytoplasmic mRNAs remain associated with a subset of P granule proteins after they are transported through P granules and dispersed in the cytoplasm; conditions that cause oocytes to arrest may allow large P granule complexes to form analogous to the aggregation of small P granules into large ones during normal embryogenesis. The giant foci in arrested oocytes would be larger than the largest embryonic P granules simply because the oocyte pools of mRNA and P granule proteins are much larger than in any of the embryonic blastomeres. As described above, class II mRNAs such as *pos-1* mRNA are largely degraded in the early blastomeres (except for the germ cell precursors), and some of the maternally supplied P granule proteins are degraded during early cleavage (Hird et al., 1996).

The identification of P granule-associated mRNAs should allow future molecular approaches toward understanding the basis for, and function of, the association. Since germline granules are seen in many animals, it will be interesting to learn if they have conserved functions, and why these functions are unique to the germline.

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