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PGL-1, a Predicted RNA-Binding Component of Germ Granules, Is Essential for Fertility in *C. elegans*

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Summary

Germ cells are distinct from somatic cells in their immortality, totipotency, and ability to undergo meiosis. Candidates for components that guide the unique germline program are the distinctive granules observed in germ cells of many species. We show that a component of germ granules is essential for fertility in *C. elegans* and that its primary function is in germline proliferation. This role has been revealed by molecular and genetic analyses of *pgl-1*. PGL-1 is a predicted RNA-binding protein that is present on germ granules at all stages of development. Elimination of PGL-1 results in defective germ granules and sterility. Interestingly, PGL-1 function is required for fertility only at elevated temperatures, suggesting that germline development is inherently sensitive to temperature.

Introduction

In animals, the generation of offspring and the propagation of species rely on special cells called germ cells. These cells are distinct from somatic cells in several fundamental ways (reviewed in Marsh and Goode, 1994). First, germ cells undergo the specialized cell cycle, meiosis, required for diploid organisms to generate haploid gametes. Second, germ cells are considered to be immortal, since they give rise to the gametes that generate future generations. Third, germ cells are totipotent, since the gametes that unite at fertilization are able to generate the entire somatic body of the organism, as well as more germ cells. In contrast, somatic cells undergo mitosis, but not meiosis, show restricted developmental potential, and senesce and die with each generation. Thus, there is a fundamental dichotomy between the germline and soma. Understanding the molecular mechanisms that underlie this dichotomy and confer upon the germline its special characteristics remains a central issue in developmental biology.

The germ cells of many organisms contain distinctive, electron-dense cytoplasmic organelles, generally referred to as "germ granules" (also called polar granules and pole plasm in *Drosophila*, P granules in *C. elegans*, and germ plasm in *Xenopus*) (Mahowald, 1968; Czolowska, 1972; Eddy, 1975; Wolf et al., 1983). Their presence in diverse organisms and the finding that the cytoplasm containing the granules can induce germ cell formation (Illmensee and Mahowald, 1974; Ephrussi and Lehmann, 1992) have led to the commonly held belief that they carry "determinants" of the germline. However, only in *Drosophila* are roles for germ-granule components known. At least 11 *Drosophila* genes (*cappuccino*, *spire*, *staufen*, *oskar*, *vasa*, *tudor*, *valois*, *mago nashi*, *orb*, *homeless*, and *pipsqueak*) are required for assembly of polar granules at the posterior pole of the oocyte; the products of three of these genes are known to be components of polar granules (reviewed in Rongo and Lehmann, 1996). Maternal-effect mutations in any of the 11 genes result in failure to form polar granules, failure to bud off primordial germ cells or "pole cells" from the syncytial blastoderm embryo, and defective abdomen development (Lehmann and Nusslein-Volhard, 1986). The last defect generally leads to embryonic lethality. Animals that survive to adulthood lack a germline and are sterile. Thus, components of *Drosophila* polar granules serve several roles. One germline role is to induce the formation of pole cells. The granules also must carry instructions for germline development, since induction of ectopic polar granules results in formation of ectopic pole cells capable of generating a functional germline (Ephrussi and Lehmann, 1992; Bardsley et al., 1993). A somatic role of polar granules is to localize mRNA for the abdominal determinant Nanos to the posterior of the embryo (Wang and Lehmann, 1991). Nanos is also essential in the early germline, where it controls gene expression and migration of the germ cells to the gonad (Kobayashi et al., 1996; Forbes and Lehmann, 1998).

Although the role of P granules in *C. elegans* has not been known previously, their behavior during the life cycle has been studied using mouse monoclonal antibodies directed against unidentified P-granule epitopes (Strome and Wood, 1982, 1983; Yamaguchi et al., 1983; Strome, 1986). P granules are maternally contributed to the embryo and progressively partitioned to the germ lineage during each of the unequal divisions that generate a somatic founder cell and a germline blastomere or P cell (Figure 1). This partitioning delivers the granules to the primordial germ cell, P_4. P_4 divides only once during embryogenesis. Its daughters, Z2 and Z3, divide throughout larval development, giving rise to the ~1500 germ cells in an adult hermaphrodite. P granules are present in all of the descendants of P_4 with the exception of mature sperm. At most stages of germline development, P granules are associated with the outer surface of the nuclear envelope. This perinuclear localization is a common feature of germ granules in diverse organisms (Eddy, 1975).

In this paper, we present molecular and genetic analyses of a protein component of P granules and demonstrate that this component serves an essential role in *C.*

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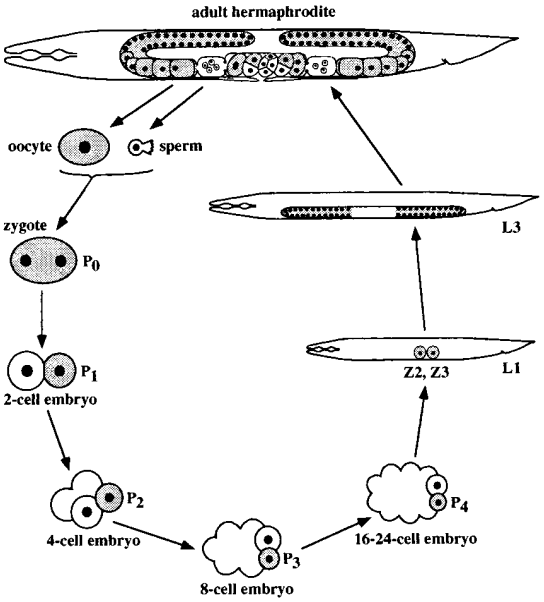

Figure 1. Germline Development and Partitioning of P Granules

Germline cells that contain P granules are shaded. A series of unequal cell divisions generates the germline blastomeres, P_1 to P_4, during early embryogenesis. The two daughters of P_4, Z2 and Z3, divide throughout larval development, giving rise to ~1500 germ cells including sperm and oocytes in an adult hermaphrodite. All germline cells except mature sperm contain P granules. Modified from Strome et al. (1995).

elegans germline development. PGL-1, a novel protein containing a motif (RGG box) commonly found in RNA-binding proteins, is associated with P granules at all stages of development. *pgl-1* mutants have defective P granules, which lack several P-granule epitopes, and display temperature-sensitive sterility. The sterility has both a maternal and a nonmaternal component and is a result of reduced germline proliferation and defects in gametogenesis. The role served by PGL-1 is distinct from the known roles of germ-granule components in *Drosophila*.

Results

Cloning the *pgl-1* Gene

The original allele of *pgl-1*, *ct131*, was isolated in screens for mutants that fail to stain with the monoclonal anti-P-granule antibody, K76 (Wood et al., 1984). The absence of a P-granule epitope suggested that *pgl-1* might encode a component of P granules. We demonstrated that this is the case by cloning the *pgl-1* gene and immunolocalizing the encoded protein product.

The *pgl-1* gene was mapped (see Experimental Procedures) by three-factor crosses to the *lin-45–deb-1* interval on LGIV and then by RFLP mapping to the overlapping cosmids, C02F1 and B0318. In DNA transformation rescue tests, the Pgl-1 phenotype was rescued by cosmid B0318 and by a 20.2 kb KpnI fragment of B0318. The 20.2 kb KpnI fragment recognizes two transcripts (2.6 and 1.3 kb) on Northern blots. RNA from numerous genes, injected into the gonads of wild-type hermaphrodites, produces a gene-specific phenocopy (Guo and Kemphues, 1995; Rocheleau et al., 1997; Fire et al., 1998). Injection of wild-type worms with RNA produced from the 2.6 kb transcript cDNA caused the production of sterile progeny (at elevated temperature, see below) that lacked staining by K76 antibody, suggesting that the 2.6 kb transcript corresponds to *pgl-1*.

The *pgl-1* Transcript Is Germline-Specific

Northern hybridization analysis demonstrated that the *pgl-1* transcript is specifically expressed in both the female and male germlines (Figure 2A). *glp-4(bn2)* adult hermaphrodites, which have a severely underproliferated germline (Beanan and Strome, 1992), contain <1% the level of *pgl-1* transcript present in wild-type adult hermaphrodites. *pgl-1* transcript is present at high levels (~70% of wild type) in *fem-2* hermaphrodites, which produce only oocytes (Kimble et al., 1984), and at lower levels (~30% of wild type) in *fem-3* gain-of-function hermaphrodites, which produce only sperm (Barton et al., 1987).

pgl-1 transcript levels are low in young larvae, higher at later stages of larval development, and highest in

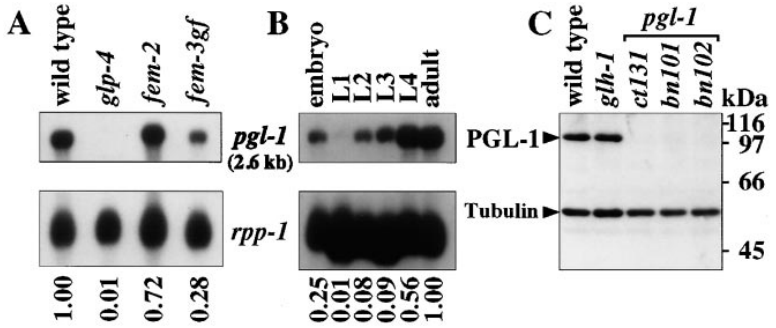

Figure 2. Northern and Western Analyses of *pgl-1*

(A) *pgl-1* mRNA accumulation in germline mutants. Poly(A)$^+$ RNA was prepared from synchronous populations of wild-type or mutant adult hermaphrodites. *glp - 4 (bn2ts)* adult hermaphrodites contain a severely underproliferated germline. *fem-2(b245ts)* and *fem-3(q20ts)* gain-of-function adult hermaphrodites contain only oocytes and only sperm, respectively. All mutant populations were grown at restrictive temperature.

(B) *pgl-1* mRNA accumulation during development. Poly(A)$^+$ RNA was prepared from wild-type hermaphrodite populations synchronized at each of the six different developmental stages (embryonic, four larval, and adult stages). In (A) and (B), cloned *pgl-1* cDNA was used as a probe to detect the 2.6 kb transcript. Transcript of a ribosomal protein gene, *rpp-1*, was used as a loading control. Relative levels of the *pgl-1* transcript are shown at the bottom.

(C) Western analysis of *pgl-1* mutant alleles and of *glh-1*. Whole-worm protein extracts were prepared from wild type, a *glh-1* mutant, and three homozygous *pgl-1* mutants (*ct131*, *bn101*, and *bn102*) grown at 20°C. The protein blot was probed with rabbit anti-PGL-1 and mouse anti-tubulin antibodies. Tubulin served as a loading control. Size markers are shown on the right.

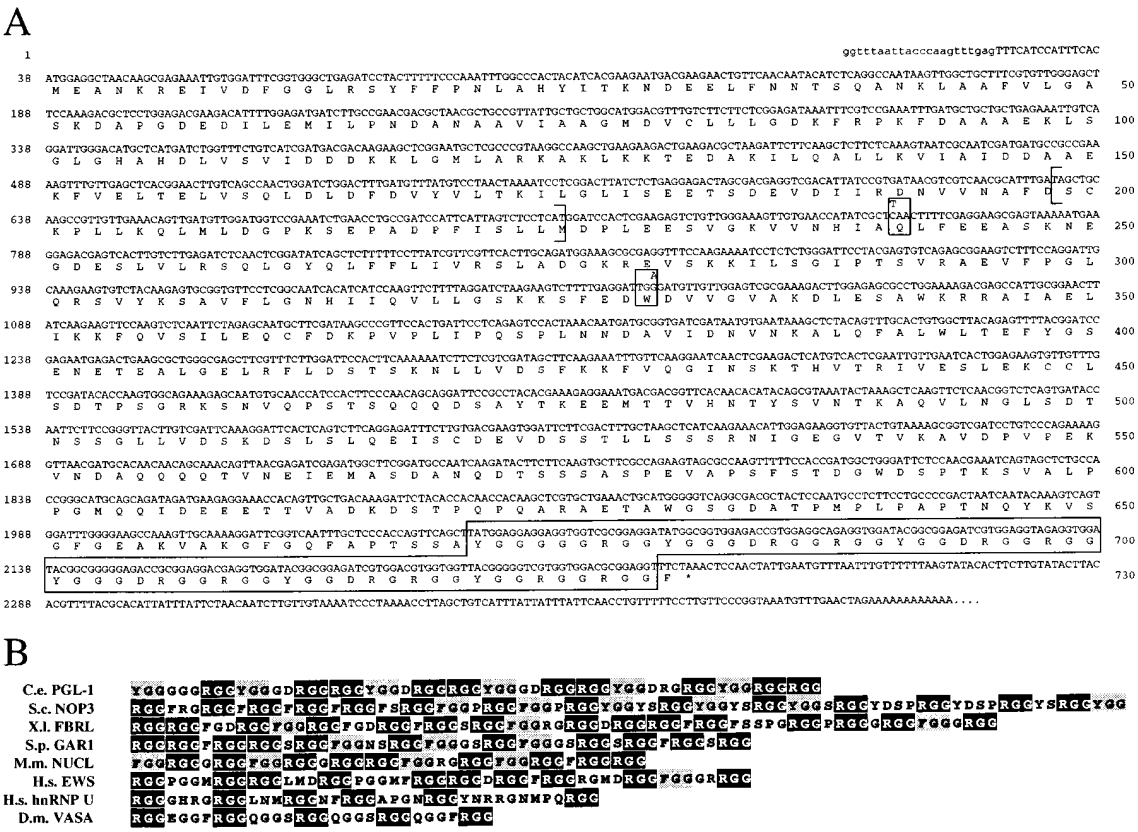

Figure 3. Sequences of *pgl-1* cDNA and PGL-1 Protein

(A) The nucleotide and predicted amino acid sequences from the *pgl-1* cDNA. The 5′-end sequence corresponding to SL1 is shown in lower case letters. A bracket indicates the region deleted in *ct131* (129 nt including an intron in the genomic sequence, and 81 nt [nt 631–711] in the cDNA sequence). The deletion is accompanied by a 1 nt insertion and causes a reading-frame shift, resulting in premature termination of translation. Two small boxes show the positions of other mutations: CAA to TAA at Gln-241 in *bn101*, and TGG to TGA at Trp-329 in *bn102*, respectively. A large box at the C terminus encloses the RGG box (aa 671–729).

(B) Comparison of RGG-box sequences. Arg-Gly-Gly repeats are boxed in black. Tyr-Gly-Gly repeats and Phe-Gly-Gly repeats are boxed in gray. The RGG-box sequences shown are *C. elegans* PGL-1, *S. cerevisiae* NOP3 (Russell and Tollervey, 1992; aa 284–368), *X. laevis* fibrillarin (Lapeyre et al., 1990; 8–81), *S. pombe* GAR1 (Girard et al., 1993; 137–189), *M. musculus* nucleolin (Bourbon et al., 1988; 650–693), *H. sapien* EWS (Plougastel et al., 1993; 565–617), *H. sapien* hnRNP U (Kiledjian and Dreyfuss, 1992; 683–722), and *D. melanogaster* Vasa (Hay et al., 1988; Lasko and Ashburner, 1988; 94–124).

adults (Figure 2B). This increase in RNA accumulation is presumably due to transcription by a growing population of germ cells. The presence of *pgl-1* transcript in embryos probably reflects both a maternal load and new synthesis (see below).

PGL-1 Protein Has an RGG Box and Is Likely to Be an RNA-Binding Protein

The nucleotide sequence of the *pgl-1* cDNA (Figure 3A) predicts that *pgl-1* encodes a novel protein of 730 amino acids, containing at its C terminus an RGG box (Kiledjian and Dreyfuss, 1992), a motif found in certain RNA-binding proteins, such as hnRNPs, fibrillarins, and nucleolins (for review, see Burd and Dreyfuss, 1994) (Figure 3B), and shown in the cases of hnRNP U and nucleolin to have RNA binding activity (Ghisolfi et al., 1992a, 1992b; Kiledjian and Dreyfuss, 1992). Therefore, PGL-1 is predicted to be an RNA-binding protein.

Three *pgl-1* Mutant Alleles Are Likely to Be Null Alleles

Sequence analysis of the *pgl-1* gene from worms bearing the three mutant alleles was performed (Figure 3A). *ct131* has a 129 bp deletion accompanied by a 1 bp insertion, which removes parts of exons 3 and 4 and the intervening intron and causes a frame shift after that, resulting in premature termination of translation. *bn101* and *bn102* have single base-pair changes that introduce premature stop codons at positions Gln-241 and Trp-329 of PGL-1, respectively.

Rabbit antisera were raised against a 6×His-tagged fusion protein containing the central 357 amino acids of PGL-1. Western analysis revealed that the rabbit anti-PGL-1 antisera recognize exclusively a ~100 kDa protein in wild-type worm extracts, whereas no protein is detected by the anti-PGL-1 antisera in any of the three *pgl-1* mutant worm extracts (Figure 2C). From the results

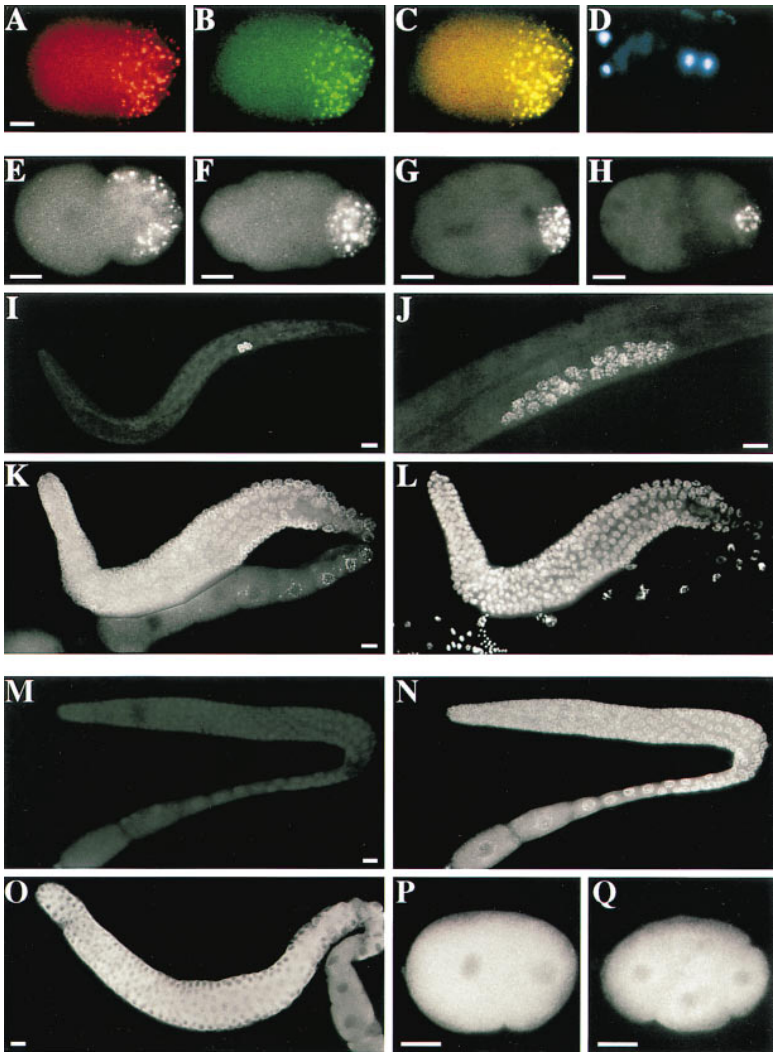

Figure 4. PGL-1 Is a Constitutive Component of P Granules

Immunofluorescence micrographs of embryos, larvae, and extruded adult hermaphrodite gonads. Anterior is left; ventral is down.

(A–D) Wild-type late 1-cell embryo costained with rabbit anti-PGL-1 antibody (A), mouse monoclonal antibody K76 (B), and the DNA dye, DAPI (D). The merged image (C) demonstrates double staining of granules by anti-PGL-1 and the original anti-P-granule monoclonal antibody, K76.

(E–H) Wild-type early embryos stained with anti-PGL-1. P granules in the germline blastomere, P_1 (E), P_2 (F), P_3 (G), and P_4 (H) of a 2-, 4-, 8-, and 24-cell embryo, respectively, are stained.

(I and J) Wild-type L1 (I) and L2 (J) stained with anti-PGL-1. P granules surround the germ nuclei.

(K and L) Wild-type adult hermaphrodite gonad stained with anti-PGL-1 (K) and DAPI (L). P granules are perinuclear in the distal arm (upper left) and dissociate from the nuclear membrane during oogenesis (lower right).

(M and N) *pgl-1(bn101)* adult hermaphrodite gonad costained with rabbit anti-PGL-1 (M) and mouse anti-GLH-1 (N). P granules fail to stain with anti-PGL-1 but stain with anti-GLH-1.

(O–Q) *glh-1* adult hermaphrodite gonad (O) and early embryos (P and Q) stained with anti-PGL-1. PGL-1 is present but not associated with P granules. Bars, 10 μm.

of genomic sequencing, Western analysis, and immunostaining (see below), and from the finding that hemizygous mutants (*pgl-1/nDf41*) display the same phenotype as homozygous *pgl-1* mutants, we judge that the three *pgl-1* mutant alleles are null alleles.

PGL-1 Is a Constitutive Component of P Granules

Affinity-purified anti-PGL-1 antibody stains the same granules that are stained by our collection of monoclonal anti-P-granule antibodies (Figures 4A–4C). PGL-1 is associated with the P granules of wild-type worms throughout the life cycle (Figures 4A and 4E–4K) and shows the same stage- and cell cycle–specific changes in localization as previously visualized using the monoclonal antibodies to P granules: P granules are small and numerous and dispersed throughout the cytoplasm of newly fertilized embryos, become segregated to the posterior pole of the zygote (Figures 4A–4D), and are progressively partitioned to the germline blastomeres, P_1, P_2, P_3, and P_4, during the early unequal divisions (Figures 4E–4H). P granules are perinuclear in P_4 and all of the descendant germline cells in larvae and adults (Figures 4I–4L). During spermatogenesis, the granules are partitioned to the residual body (data not shown) and thus are not visible in mature sperm. During oogenesis, they dissociate from the nuclear membrane and become dispersed in the cytoplasm (Figures 4K and 4L), presumably in preparation for cytoplasmic localization to the germ lineage during embryogenesis. In worms homozygous for any of the three *pgl-1* mutant alleles, P granules fail to stain with anti-PGL-1 antibody at all stages of development (Figure 4M). Our immunostaining results demonstrate that PGL-1 is a constitutive component of P granules.

To determine how long maternal PGL-1 persists, we immunostained *pgl-1/pgl-1* offspring from *pgl-1/+* mothers with anti-PGL-1 antibody. PGL-1 expressed from the wild-type maternal allele persists at easily detectable levels in the progeny's germline until the L3 stage, when the germline contains ~70 germ nuclei (data not shown). Faint PGL-1 staining can be seen in some L4-stage progeny as well. To determine when newly synthesized PGL-1 becomes detectable, we immunostained *pgl-1/+* offspring produced from *pgl-1/pgl-1* mothers mated to +/+ males. PGL-1 staining becomes detectable in the two primordial germ cells, Z2 and Z3, around the comma

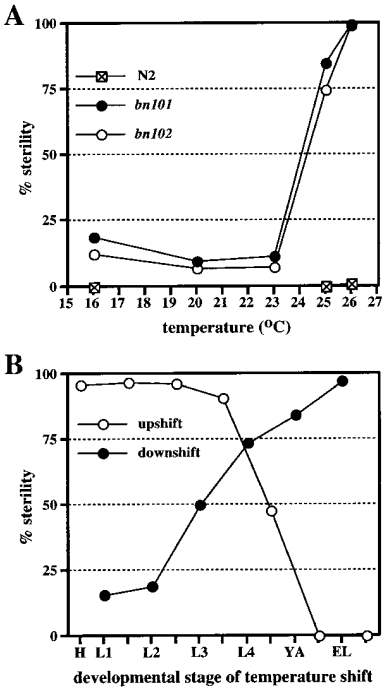

Figure 5. *pgl-1* Mutants Display Temperature-Sensitive Sterility and a TSP from Midlarval to Young Adult Stages

(A) Wild-type (N2), *pgl-1(bn101)*, and *pgl-1(bn102)* mothers were shifted from 16°C to the indicated temperatures as L4s, and their hermaphrodite offspring were scored for fertility/sterility. Number of animals scored at each point ranged from 558 to 3450. (B) *pgl-1(bn101) dpy-20* worms were upshifted from 16°C to 26°C or downshifted from 26°C to 16°C at different developmental stages, grown to adulthood, and scored for fertility/sterility. Number of animals scored at each point ranged from 33 to 95. H, newly hatched; L1–L4, four larval stages; YA, young adult (egg laying has not commenced); EL, egg-laying adult.

stage of embryogenesis and becomes brighter by the 1.5- to 2-fold stage (data not shown). Thus, both maternally contributed and embryonically and larvally synthesized PGL-1 coexist in a wild-type germline from late embryogenesis to late larval stages.

In *pgl-1* Mutants, P Granules Lack Some Epitopes

In wild-type worms, P granules are stained at all stages of development by several monoclonal antibodies, K76, OIC1D4, L416, N123, and PIF4, directed against unidentified P-granule epitopes (Strome and Wood, 1983; Strome, 1986; Hird et al., 1996), and by antibodies directed against the putative germline RNA helicases, GLH-1 and GLH-2 (Gruidl et al., 1996). Certain proteins present in early embryos, such as MEX-1 (Guedes and Priess, 1997) and MEX-3 (Draper et al., 1996), also transiently associate with P granules. In homozygous *pgl-1* worms, P granules are not stained by K76 or OIC1D4. However, P granules are present in the mutant worms, since all of the other antibodies listed above stain them (Figure 4N).

On Western blots, K76 was found to bind to a bacterially expressed fusion protein containing the central half of PGL-1; OIC1D4 did not (data not shown). Thus, absence of K76 staining of P granules in *pgl-1* mutants is due to absence of PGL-1. We do not know whether absence of OIC1D4 staining reflects absence of a P-granule protein distinct from PGL-1.

Table 1. Sterility in *pgl-1* Mutants Has Both a Maternal and a Zygotic Component

Generation	Genotype	Sterile Adult Hermaphrodites (%)
P0[a]	*dpy-20*/+	
F1	*dpy-20*	10.5[b]
F2	*dpy-20*	19.0[b]
P0[a]	*bn101 dpy-20*/++	
F1	*bn101 dpy-20*	40.8[c]
F2	*bn101 dpy-20*	100
P0[a]	*bn102 dpy-20*/++	
F1	*bn102 dpy-20*	37.8[c]
F2	*bn102 dpy-20*	100

[a] Heterozygous P0 mothers were generated by mating *dpy-20*/+ or *pgl-1 dpy-20*/++ males to wild-type hermaphrodites at 26°C. All subsequent generations were maintained at 26°C. Number of animals scored at each point ranged from 70 to 779.

[b] *dpy-20(e1282)* itself displays some sterility at 26°C. Under the same conditions, wild type (N2) showed 0.9% and 1.0% sterility in the F1 and F2 generations, respectively (also see Figure 5A).

[c] <1% of the non-Dpy F1 worms, of the genotypes *pgl-1 dpy-20*/++ and ++/++, were sterile. This indicates that the sterility of *pgl-1 dpy-20* F1 worms is due to lack of embryonic and larval PGL-1 synthesis and not to maternal-effect haploinsufficiency of *pgl-1*.

pgl-1 Mutants Display Sterility that Is Temperature-Sensitive and Has Both a Maternal and a Nonmaternal Component

pgl-1 mutants display sterility that has two unique features: the sterility is sensitive to temperature and has both a maternal and a nonmaternal (referred to as "zygotic" in the remainder of the paper) component. *pgl-1* homozygous strains can be maintained indefinitely at low temperature (16°C–23°C). When such low-temperature-grown homozygotes are allowed to produce progeny at different temperatures, the percentage of sterile offspring is 7%–19% at 16°C–23°C, 75%–85% at 25°C, and 100% at 26°C (Figure 5A). It is noteworthy that the P-granule staining defect described in the previous section is not sensitive to temperature. Thus, the absence of PGL-1 does not severely compromise fertility at low temperature but leads to 100% sterility at high temperature.

Sterility in *pgl-1* mutants at 26°C has both a maternal and a zygotic component (Table 1). The zygotic component is seen in the F1 *pgl-1/pgl-1* progeny from *pgl-1*/+ mothers. These F1 homozygous progeny inherit maternal *pgl-1*(+) product but do not synthesize zygotic product (abbreviated M^+Z^-), and presumably as a result, ~40% of the worms are sterile. These sterile worms contain fairly well-proliferated germlines but produce defective oocytes and dead embryos (see below). The remaining ~60% of the F1 *pgl-1/pgl-1* worms are fertile and produce viable F2 progeny. These F2 progeny lack both maternal and zygotic *pgl-1*(+) products (i.e., M^-Z^-), and 100% of them develop into sterile adults. As described in the following section, these M^-Z^- sterile worms display more severe germline defects than the

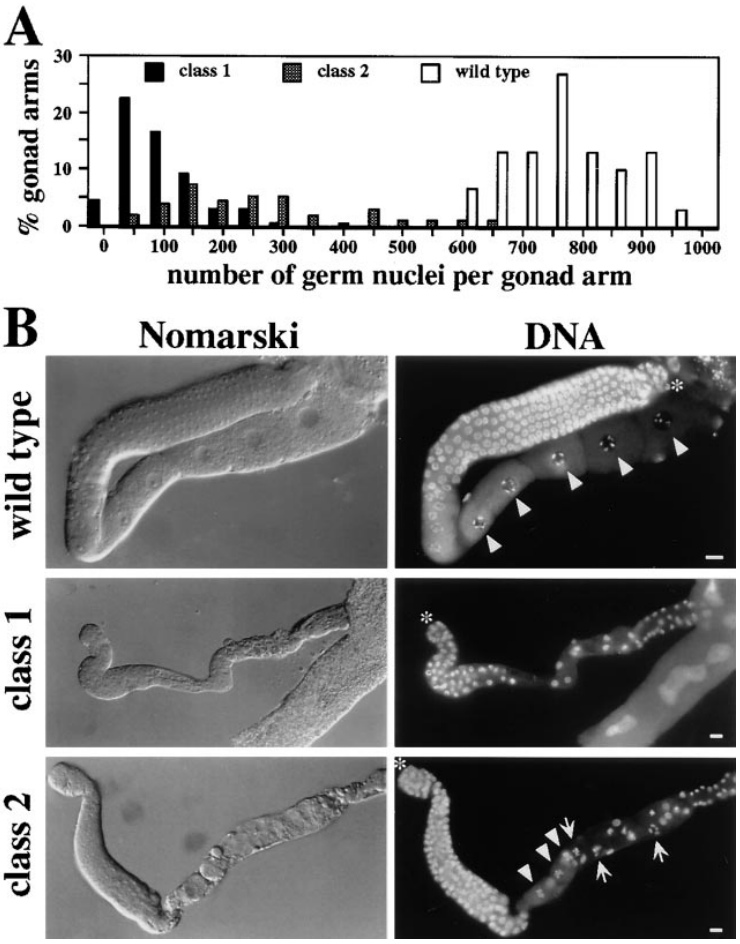

Figure 6. Germline Phenotypes of *pgl-1* Sterile Worms

(A) Frequency distribution of gonad arms containing different numbers of germ nuclei. Wild-type (*pgl-1*[+] *dpy-20*) and *pgl-1(bn101) dpy-20* F2 adult hermaphrodites raised at 26°C (see Table 1) were stained with Hoechst 33342 to visualize and count germ nuclei. Germ nuclei were counted in 30 and 151 gonad arms of *dpy-20* and *pgl-1 dpy-20* worms, respectively. Gonad arms of the *pgl-1* worms were further classified into two classes, those with no gametes (class 1, n = 91) and those with defective gametes (class 2, n = 60). Each bar represents the percentage of gonad arms containing a similar number of germ nuclei, within a range of 50 germ nuclei. *pgl-1* sterile gonads contain underproliferated germlines. (B) Morphology of *pgl-1* sterile gonad arms. Wild-type and sterile *pgl-1(bn101)* adult hermaphrodites raised at 26°C were dissected, stained with Hoechst 33342, and fixed with glutaraldehyde, and their gonads were viewed with Nomarski DIC (left) and fluorescence (right). Representative gonad arms are shown for wild type and *pgl-1* class 1 and class 2. The distal end of each gonad is indicated with an asterisk in the right panel. At the proximal end, the pairs of nuclei (obvious in the class 1 and 2 gonads) are somatic nuclei of the spermatheca, and certain of the other proximal nuclei are somatic oviduct nuclei. Arrowheads indicate germ nuclei in diakinesis. Class 1 gonads lack diakinesis-stage germ nuclei. In class 2 gonads, diakinesis-stage germ nuclei are observed either in a few small oocytes or in noncellularized regions (arrows). Bars, 10 μm.

M^+Z^- sterile worms. Mating of F1 fertile *pgl-1/pgl-1* hermaphrodites to wild-type males results in the production of 100% sterile outcross (*pgl-1*/+ and M^-Z^+) progeny, demonstrating that neither a paternal supply of *pgl-1*(+) product nor zygotic expression of *pgl-1*(+) is sufficient to restore fertility to the progeny. Thus, both maternally supplied and zygotically expressed *pgl-1*(+) products are required to ensure fertility at high temperature.

pgl-1 Worms that Lack Both Maternal and Zygotic *pgl-1*(+) Products Have Underproliferated Germlines and Generally Lack Gametes

In wild-type adult hermaphrodites, each of the two gonad arms contains 500–1000 germ nuclei (Figure 6A). Each gonad arm contains a distal region in which germline stem cells divide mitotically, a transition zone in which germ nuclei exit from the mitotic cell cycle and enter meiotic prophase I, and a pachytene zone in which germ nuclei exhibit a characteristic thready chromatin morphology (Francis et al., 1995). Oocytes form and enlarge in the proximal region of each gonad arm and arrest at diakinesis of meiotic prophase I (Figure 6B). Sperm made during the L4 stage are stored in the spermatheca, through which oocytes pass and become fertilized.

All of M^-Z^- *pgl-1* worms that have been raised at 26°C are sterile and contain an underproliferated germline. We classified worms into two classes, those with no gametes (class 1) and those with defective gametes (class 2) (Figure 6). Class 1 gonads contain severely underproliferated germlines; a distal stem cell region and sometimes a transition zone are present, but a pachytene zone and diakinesis nuclei are not seen. Therefore, germ nuclei in class 1 worms are defective in proliferation and may also be defective in entry or progression through meiotic prophase I. This severe phenotype is displayed by the majority (~60%) of *pgl-1* sterile worms. Class 2 gonads contain more proliferated germlines, which have relatively normal appearing distal arms but fewer pachytene and diakinesis nuclei than in wild type. Any cellularized oocytes are abnormally small.

Our finding that all M^-Z^- mutant worms display defects in germline proliferation suggests that PGL-1's primary role is in maintaining a population of proliferating germ cells. Defects observed in other germline-proliferation mutants include degeneration of the germline (e.g., *mes-3*, Paulsen et al., 1995), arrest of germ nuclei in a particular stage of the mitotic cell cycle (e.g., *glp-4*, Beanan and Strome, 1992), and premature and global entry of germ cells into meiosis (e.g., *glp-1*, Austin and Kimble, 1987). *pgl-1* mutant germ cells do not display these phenotypes, suggesting that PGL-1 is not required for germline survival, to promote a particular step of

mitosis, or to control the switch from mitosis to meiosis. Instead PGL-1 may be required to maintain germline stem cells in a mitotic state or to activate the mitotic cell cycle. The production of defective oocytes in some M^+Z^- and some M^-Z^- worms suggests that PGL-1 is also required for normal oogenesis.

The issue of whether PGL-1 must be associated with P granules in order to function correctly is addressed by analysis of a *glh* mutant. Multiple *glh* genes encode P-granule-associated GLHs (predicted germline helicases) (Gruidl et al., 1996). Mutation of *glh-1* causes PGL-1 to lose its association with P granules: PGL-1 is present in *glh-1* worm extracts (Figure 2C) and in the germline cytoplasm but is not associated with P granules (Figures 4O–4Q). This *glh-1* mutation results in sterile phenotypes identical to those of *pgl-1*: 40%–50% zygotic sterility at high temperature, 10%–15% maternal-effect sterility at low temperature, and 100% maternal-effect sterility at high temperature (J. K. et al., unpublished results). Since *glh-1* worms still contain other GLH protein on P granules, we think that the sterility is a consequence of the dissociation of PGL-1 from P granules, which would support the notion that PGL-1 must be associated with P granules to perform its normal role in the germline.

The TSP for *pgl-1* Sterility Extends from Midlarval to Young Adult Stages

The temperature sensitivity of the sterile phenotype caused by null alleles of *pgl-1* suggests that the gene participates in an inherently temperature-sensitive process. We expect the temperature-sensitive period (TSP) for *pgl-1* to indicate when that process is occurring. To determine the TSP, *pgl-1* hermaphrodites were shifted from 16°C to 26°C or vice versa at different developmental stages, grown to adulthood, and scored for their fertility/sterility (Figure 5B). Upshifting *pgl-1* worms at any stage before L4 caused most of the shifted worms to develop into sterile adults. When worms were upshifted as L4s or young adults (before egg laying had commenced), about 50% of them developed into sterile adults, and the remainder of them produced a small number (average = 4) of viable progeny and many inviable embryos. (The results of temperature shifts of embryos suggest that embryonic lethality is due to defects in oogenesis at elevated temperature; data not shown.) Downshifting *pgl-1* worms before the L3 stage restored fertility to the majority of worms. In contrast, mutant worms downshifted at or after the L3 stage displayed a progressive increase in frequency of sterility. These results indicate that the TSP for *pgl-1* sterility extends from midlarval to young adult stages. During these stages germ cells are proliferating, entering meiosis, and forming gametes. Apparently *pgl-1*(+) product is required to protect one or more of these processes from the deleterious effects of elevated temperature.

Discussion

We have demonstrated that the protein product of the *pgl-1* gene is a constitutive component of P granules. Analysis of the mutant phenotype revealed that PGL-1 is not required for establishment of the germline but is required for normal postembryonic germline development. Our findings demonstrate that a component of P granules is essential for fertility in *C. elegans* and reveal a role for a germ-granule component that is distinct from what has emerged from analysis in *Drosophila*. The presence of an RGG box in PGL-1 predicts that it serves as an RNA-binding component of P granules, and the temperature sensitivity of the Pgl-1 sterile phenotype indicates that PGL-1 function is required only at elevated temperature.

Identifying Components of P Granules

Germ granules were first visualized in *C. elegans* by electron microscopy (Wolf et al., 1983) and by antibody staining (Strome and Wood, 1982, 1983; Yamaguchi et al., 1983) over a decade ago, and yet their composition and function have remained elusive. The existing collection of mouse monoclonal anti-P-granule antibodies was raised against *C. elegans* crude early embryo homogenates (Strome and Wood, 1983; Strome, 1986). K76 is now known to recognize PGL-1, but the antigens recognized by the other monoclonal antibodies remain unidentified. GLH-1 and GLH-2, putative germline RNA helicases identified on the basis of their similarity to *Drosophila* Vasa, were recently shown to be components of P granules (Gruidl et al., 1996). RNA interference with expression of *glh-1* and *glh-2* results in the production of ~10% sterile progeny, suggesting that the GLHs are required to ensure fertility (Gruidl et al., 1996). At present, four *glh* genes have been identified in the *C. elegans* genome (K. Bennett, personal communication), raising the likelihood of significant functional redundancy in the germline. Future analysis of *glh* mutants will clarify whether the GLH proteins serve overlapping roles and in which germline processes these proteins participate.

Evidence that P granules contain RNA initially came from the finding that P granules stain with Bernhard's reagent, which reacts with ribonucleoproteins (D. Albertson and N. Thomson, personal communication). Subsequently, Seydoux and Fire (1994) found that oligo(dT) and an antisense probe to the *trans*-splice leader SL1 hybridize to P granules, indicating the presence of RNAs that contain poly(A) and SL1. The identities of P-granule RNAs are not yet known.

Recently, several proteins encoded by maternal-effect lethal genes have been shown to transiently associate with P granules during early stages of embryogenesis. PIE-1, a Cys-His finger protein that represses transcription in the germline blastomeres, is predominantly nuclear but also associates with P granules (Mello et al., 1996). MEX-1, a related Cys-His finger protein required for correct localization of PIE-1, is both dispersed in the cytoplasm and associated with P granules in the germline blastomeres (Guedes and Priess, 1997). MEX-3, a probable RNA-binding protein required for normal development of multiple blastomeres, is cytoplasmic in the AB descendants and associated with P granules in the germline blastomeres (Draper et al., 1996). The three proteins are not detectably associated with P granules in late-stage embryos or in larval or adult germlines.

Whether the transient association of these proteins with P granules is required for the functions of the proteins or of P granules is not known.

pgl-1 was identified on the basis of its P-granule staining phenotype: *pgl-1* mutant embryos and worms lack the P-granule epitopes recognized by the monoclonal antibodies K76 and OIC1D4. However, P granules are present and contain GLH-1, GLH-2, MEX-1, MEX-3, and the epitopes recognized by the other anti-P-granule monoclonal antibodies. Thus, absence of PGL-1 does not prevent assembly of P granules. This phenotype is distinct from that seen in *Drosophila*, in which absence of any of the known polar-granule components results in failure to assemble polar granules (for review, see Rongo and Lehmann, 1996). The consequence of absence of polar granules is failure to form primordial germ cells, which reveals that the earliest-acting granule components function in establishment of the germline during embryogenesis. Our studies of *C. elegans* mutants lacking the PGL-1 component (and perhaps a different protein recognized by OIC1D4) but not lacking granules altogether have revealed a distinct, presumably later, role for a germ-granule component. Ultimately, dissecting the functions of the various proteins and RNAs assembled into germ granules will reveal how many different germline processes are controlled by germ granules and which are evolutionarily conserved across species.

Functions of PGL-1 in Germline Development

Genetic analysis of *pgl-1* mutants has demonstrated that the gene product is required for postembryonic germline development. The Pgl-1 phenotype is unusual in several respects: *pgl-1* mutants display both maternal-effect and zygotic sterility, the sterility is sensitive to temperature, and mutants display a variety of germline defects. These properties may provide clues to PGL-1's function.

Both a maternal load and zygotic synthesis of PGL-1 are required to ensure normal germline development. In worms lacking both maternal and zygotic PGL-1 (M^-Z^-), germlines are underproliferated. Thus, the earliest role of PGL-1 is to maintain a population of proliferating germ cells; PGL-1 may affect the identity or health of the germline stem cells or may directly promote mitosis in the germline. In worms that inherit maternal PGL-1 (which persists until at least the L3 stage) but fail to synthesize new *pgl-1*(+) product (M^+Z^-), germline proliferation is extensive and germ cells enter meiosis, indicating that maternal PGL-1 is sufficient to promote these events. However, about 40% of such M^+Z^- worms develop into sterile adults, which produce defective oocytes and dead embryos. This demonstrates that zygotically produced PGL-1 is required to ensure correct oogenesis. Because zygotic production of PGL-1 begins during embryogenesis, well in advance of germline proliferation and gametogenesis, we expected that zygotically produced PGL-1 would restore fertility in worms that did not inherit maternal PGL-1. However, supplying one copy of *pgl-1*(+) to embryos that lacked maternal PGL-1 (M^-Z^+) did not enable them to develop into fertile adults. This finding suggests either that the germline requires a high threshold level of PGL-1 protein, which cannot be supplied by zygotic synthesis alone, or that having maternal PGL-1 protein in P granules is essential for zygotically expressed PGL-1 to correctly associate or function.

Mutant mothers that lack PGL-1 protein produce predominantly fertile progeny at low temperature (16°C–23°C) and 100% sterile progeny at high temperature (26°C). This suggests that the process in which PGL-1 functions is intrinsically sensitive to temperature and that PGL-1 functions to render the process more heat resistant. Two possible explanations for this sensitivity to temperature are as follows: (1) Other genes may provide functions similar to that of PGL-1. In the absence of PGL-1, the redundant factors may be sufficient to support normal germline development at low temperature but insufficient at high temperature. *C. elegans* development proceeds faster at higher temperature (e.g., about 2-fold faster at 26°C than at 16°C), which may create a greater demand for both PGL-1 and the redundant factors at high temperature. (2) PGL-1 may function as a molecular chaperone. For example, some germline proteins or RNAs may be stably folded and active at low temperature but prone to be unfolded and inactivated at high temperature. PGL-1 may interact with such proteins or RNAs to guarantee that they remain correctly folded and functional even at elevated temperature.

The TSP for *pgl-1* reflects the period during which germline development is dependent upon PGL-1's function. The TSP extends from midlarval to young adult stages and thus encompasses germline proliferation, meiosis, and gametogenesis. In fact, we see defects in all of these germline events, as described above.

The presence of an RGG box in PGL-1 predicts that PGL-1 binds RNA. The RGG-box motif is widespread among RNA-binding proteins involved in diverse aspects of RNA metabolism (Burd and Dreyfuss, 1994). Most of these proteins also contain additional RNA-binding motifs, such as an RNP motif or a KH motif. However, an RGG box is the only recognizable RNA-binding motif in hnRNP U protein, and the RGG box of this protein is necessary and sufficient for RNA binding (Kiledjian and Dreyfuss, 1992). Additionally, it has been shown that the RGG box of nucleolin is capable of binding RNA and is essential for efficient binding of nucleolin to RNA (Ghisolfi et al., 1992a, 1992b).

An attractive scenario is that PGL-1 regulates multiple aspects of germline development by binding and controlling the translation of multiple germline RNAs. Interestingly, the *Drosophila* polar-granule component, Vasa, also contains an RGG box (see Figure 3B) and is thought to regulate translation (Styhler et al., 1998). Vasa is best known for its maternal-effect role in assembly of polar granules and in formation of pole cells in the embryo (see above). PGL-1 clearly has a different maternal-effect role than Vasa, since PGL-1 is not required for either assembly of germ granules or formation of the germline in *C. elegans*. Vasa is also required in the female germline for several aspects of oogenesis that precede polar-granule formation: growth of germline cysts, oocyte determination and differentiation, and patterning of the egg (Lasko and Ashburner, 1988, 1990; Styhler et al., 1998). Vasa's primary role during oogenesis may be in

control of translation (Styhler et al., 1998). PGL-1 also participates in oogenesis, since some M^+Z^- hermaphrodites produce only defective oocytes and dead embryos that likely die due to defects in oogenesis. It remains to be determined whether PGL-1's roles in oogenesis are similar to those of Vasa. Regardless of the precise roles served, both proteins may function through a similar mechanism, control of translation.

Experimental Procedures

Strains and Alleles

Maintenance and genetic manipulation of *C. elegans* were carried out as described in Brenner (1974). *C. elegans* variety Bristol, strain N2 was used as the wild-type strain. The restrictive temperature used in this study was 26°C because the *pgl-1* sterile phenotype is not 100% penetrant at 25°C. N2 can be maintained at 26°C without compromising its fertility (Figure 5A). The following mutations, polymorphisms, balancers, and deficiencies were used in this study. LGI: *dpy-5(e61)*, *glh-1(bn103)*, *glp-4(bn2ts)*. LGII: *bli-2(e768)*, *dpy-10(e128)*, *unc-4(e120)*, *unc-52(e444)*, *mnC1*. LGIII: *fem-2(b245ts)*, *unc-32(e189)*. LGIV: *unc-17(e245)*, *dpy-13(e184)*, *unc-5(e53)*, *unc-44(e362)*, *bli-6(sc16)*, *lin-45(sy96)*, *bnP12*, *pgl-1(ct131, bn101, bn102)*, *him-3(e1147)*, *bnP13*, *stP44*, *deb-1(st555)*, *unc-24(e138)*, *fem-3(q20ts)gf*, *dpy-20(e1282ts)*, *unc-26(e205)*, *dpy-4(e1166)*, *mDf9*, *nDf41*, *eDf18*, *eDf19*, *sDf2*. LGV: *dpy-11(e224)*. LGX: *lon-2(e678)*, *lin-2(e1309)*. *C. elegans* variety Bergerac, strain RW7000 was used to generate Bristol-Bergerac hybrid recombinants for RFLP mapping. All strains were provided by the *Caenorhabditis* Genetics Center, except that a strain containing *nDf41* was a gift from Bruce Bowerman, a strain containing *bli-6(sc16)* was a gift from James Kramer, and strains containing *lin-45(sy96)* were gifts from Min Han.

Isolation of *pgl-1* Alleles

The original allele of *pgl-1*, *ct131* was isolated by EMS-mutagenizing *unc-4/mnC1; him-3; lin-2* worms (Kemphues et al., 1988). *ct131* homozygotes were detected in the F2 generation by their failure to stain with the anti-P-granule monoclonal antibody, K76 (Wood et al., 1984). Subsequent analysis revealed that, although *ct131* homozygous worms are mostly fertile at permissive temperature, they become sterile at restrictive temperature. The other two *pgl-1* alleles, *bn101* and *bn102*, were isolated by noncomplementation screens using the temperature-sensitive sterile phenotype. *dpy-20* males were mutagenized with EMS and mated with *unc-44 pgl-1(ct131) unc-24* hermaphrodites. F1 outcross hermaphrodite progeny (n = 3620) were scored for the production of all sterile progeny at restrictive temperature and fertile progeny at permissive temperature.

Mapping *pgl-1*

pgl-1(ct131) was positioned between *lin-45* and *deb-1* on LGIV by performing a series of three-factor crosses. The *pgl-1* locus was further narrowed down to two overlapping cosmids, C02F1 and B0318, by mapping *pgl-1* relative to Bristol-Bergerac RFLPs in the *lin-45–deb-1* interval. C02F1 detects an EcoRI RFLP, *bnP12*, and B0318 detects another EcoRI RFLP, *bnP13*. From Bristol *unc-44 pgl-1 unc-24*/ Bergerac +++ hybrid heterozygous worms, 157 non-Unc-44 Unc-24 recombinants were isolated and made homozygous for the recombinant chromosome. By analyzing the recombinants by a combination of staining with the anti-P-granule monoclonal antibody, OIC1D4 (the Bristol *pgl-1* locus fails to stain; the Bergerac *pgl-1*[+] locus stains), and genomic Southern hybridization with cosmids C02F1 and B0318, we determined that *pgl-1* lies between *bnP12* and *bnP13* and therefore within cosmids C02F1 and B0318.

Cloning *pgl-1*

Cosmids C02F1 and B0318 were tested for transformation rescue of the Pgl-1 mutant phenotype. Each DNA was coinjected with pRF4, a plasmid carrying a dominant marker, *rol-6(su1006)*, into the gonad arms of *pgl-1(ct131)* homozygous hermaphrodites, using the procedure of Mello et al. (1991). Heritable lines of Rol transformants were obtained and examined for rescue of the Pgl-1 phenotype. B0318 and a 20.2 kb KpnI fragment of B0318 rescued. The rescuing 20.2 kb KpnI fragment, which detects two transcripts (2.6 and 1.3 kb) on Northern blots, was used as a probe to screen a λZAP mixed-stage *C. elegans* cDNA library (Barstead and Waterston, 1989) to obtain cDNA clones corresponding to each transcript. Antisense and sense RNA from the cDNAs corresponding to the 2.6 or 1.3 kb transcript were prepared essentially as described by Guo and Kemphues (1995). Antisense or sense RNA was injected into the gonad arms of wild-type hermaphrodites at a concentration of ~1 mg/ml, and the progeny of injected worms were examined. Injection of RNA from cDNAs corresponding to the 2.6 kb transcript produced an exact phenocopy of the Pgl-1 mutant phenotype: absence of some P-granule epitopes and temperature-sensitive sterility. Injection of RNA from the 1.3 kb transcript cDNA did not produce a Pgl-1 phenocopy.

Northern Analysis

Northern hybridization analysis was done as in Holdeman et al. (1998), using the *rpp-1* transcript, which encodes a ribosomal protein (Evans et al., 1997), as a loading control.

Sequencing

All DNA templates were sequenced using SequiTherm Long-Read Cycle Sequencing Kit (Epicentre Technologies) and a LI-COR model 4000 automated DNA sequencer. To sequence the full length of *pgl-1* cDNA, five cDNA clones of different insert sizes were sequenced on both strands. The 5′ end of the *pgl-1* transcript was determined by sequencing an RT-PCR product, generated using a *pgl-1*–specific downstream primer and an upstream primer specific to the SL1 *trans*-splice leader (Spieth et al., 1993). For sequencing *pgl-1* mutant alleles, genomic DNA was prepared from homozygous mutant worms carrying each of the three *pgl-1* alleles. The *pgl-1* gene was PCR-amplified as five overlapping segments, using primers with 5′ extensions consisting of either T3 or T7 promoter sequence, and each segment was cycle sequenced. The wild-type *pgl-1* genomic sequence was determined by the *C. elegans* Genome Sequencing Consortium. Databases were searched using the BLAST program (Altschul et al., 1990) at NCBI.

Antibody Production

The PGL-1 expression vector was constructed by cloning a 1071 bp HincII fragment of *pgl-1* cDNA, which encodes the central 357 amino acids of PGL-1 (Asn-195 to Val-551), into the expression vector pQE-32 (Qiagen). A 6×His-tagged fusion protein of the expected size (~45 kDa) was expressed in *E. coli* and purified using a Ni-NTA agarose column (Qiagen). The purified fusion protein was injected into rabbits. Antibodies against PGL-1 were purified from crude antisera by blot affinity purification (Olmsted, 1986), using 0.2 M glycine-HCl (pH 2.8) for elution.

Western Analysis

Western blotting was performed as described by Towbin et al. (1979), using protein extract from ~100 gravid hermaphrodites of each genotype per gel well. Primary antibody was rabbit anti-PGL-1 antiserum diluted 1:2000 and mouse anti-tubulin antibody (from Margaret Fuller) diluted 1:500. Secondary antibody was HRP-conjugated goat anti-rabbit IgG (Miles) diluted 1:2000 and HRP-conjugated goat anti-mouse IgG (Jackson) diluted 1:10,000. Bound antibodies were visualized using ECL Western blotting detection kit (Amersham).

Immunofluorescence Analysis

To examine germlines in intact worms, single worms were placed in 1 μl of H_2O on a polylysine-treated slide. The slide was flamed briefly to evaporate the H_2O. The dried worms were mounted in Elvanol (Dupont) mounting fluid containing 5 μg/ml Hoechst 33342 to visualize DNA. To examine extruded germlines, worms were cut open in 10 μl of 100 μg/ml Hoechst 33342 in 0.5× egg buffer (Edgar, 1995) on a polylysine-treated slide. Ten microliters of 5% glutaraldehyde in 0.5× egg buffer was added to fix the sample, which was covered with a coverslip and observed.

To visualize PGL-1 localization in worms and embryos, L4 or adult

hermaphrodites were cut open, fixed in cold methanol followed by cold acetone, and stained with affinity-purified rabbit anti-PGL-1 antibody as described by Strome and Wood (1983). Secondary antibody was rhodamine-conjugated goat anti-rabbit IgG (Jackson) diluted 1:200. L1, L2, and L3 larvae were not cut open but were otherwise fixed and stained as described above. Mouse monoclonal anti-P-granule antibodies, K76 and OIC1D4, and mouse anti-GLH-1 antibody (Gruidl et al., 1996) were also used for staining, followed by fluorescein-conjugated goat anti-mouse IgG (Jackson) diluted 1:100.

Samples were observed and photographed using a Zeiss Axioskop equipped with Nomarski DIC and epifluorescence optics and using Tri-X pan film. Developed negatives were scanned with SprintScan 35 (Polaroid), and image files were processed in Adobe Photoshop 3.0 (Adobe Systems).

Temperature Shift Experiments

For upshift experiments, *pgl-1(bn101) dpy-20* worms that had been maintained at 16°C were upshifted from 16°C to 26°C at different developmental stages, grown to adulthood, and scored for their fertility/sterility. For downshift experiments, *pgl-1(bn101) dpy-20* homozygous mothers were generated from *pgl-1(bn101) dpy-20*/++ heterozygous grandmothers and grown at 26°C; the progeny of the *pgl-1 dpy-20* mothers were downshifted from 26°C to 16°C at different developmental stages, grown to adulthood, and scored for their fertility/sterility. Developmental stages were assessed by time after hatching and by Nomarski DIC analysis of the ventral hypodermis and vulva (Sulston and Horvitz, 1977).

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