DOI: 10.1534/genetics.103.023093

The PGL Family Proteins Associate With Germ Granules and Function Redundantly in *Caenorhabditis elegans* Germline Development

Ichiro Kawasaki,*,†,‡,[1] Anahita Amiri,‡,[2] Yuan Fan,‡ Nicole Meyer,‡ Steve Dunkelbarger,‡ Tomoko Motohashi,† Takeshi Karashima,§ Olaf Bossinger‡,[3] and Susan Strome‡

**PRESTO, Japan Science and Technology Agency, Kawaguchi, Saitama 332-0012, Japan, †Genome Biology Laboratory, Center for Genetic Resource Information, National Institute of Genetics, Mishima, Shizuoka 411-8540, Japan, ‡Department of Biology, Indiana University, Bloomington, Indiana 47405 and §Department of Biophysics and Biochemistry, Graduate School of Science, University of Tokyo, Bunkyo-ku, Tokyo 113-0033, Japan*

Manuscript received October 9, 2003
Accepted for publication March 12, 2004

ABSTRACT

PGL-1 is a constitutive protein component of *C. elegans* germ granules, also known as P granules. Maternally supplied PGL-1 is essential for germline development but only at elevated temperature, raising the possibility that redundant factors provide sufficient function at lower temperatures. We have identified two PGL-1-related proteins, PGL-2 and PGL-3, by sequence analysis of the *C. elegans* genome and by a yeast two-hybrid screen for proteins that interact with PGL-1. PGL-3 is associated with P granules at all stages of development, while PGL-2 is associated with P granules only during postembryonic development. All three PGL proteins interact with each other *in vitro*. Furthermore, PGL-1 and PGL-3 are co-immunoprecipitated from embryo extracts, indicating that they are indeed in the same protein complex *in vivo*. Nevertheless, each PGL protein localizes to P granules independently of the other two. *pgl-2* or *pgl-3* single-mutant worms do not show obvious defects in germline development. However, *pgl-1; pgl-3* (but not *pgl-2; pgl-1*) double-mutant hermaphrodites and males show significantly enhanced sterility at all temperatures, compared to *pgl-1* alone. Mutant hermaphrodites show defects in germline proliferation and in production of healthy gametes and viable embryos. Our findings demonstrate that both PGL-2 and PGL-3 are components of P granules, both interact with PGL-1, and at least PGL-3 functions redundantly with PGL-1 to ensure fertility in both sexes of *C. elegans*.

IN multicellular organisms that undergo sexual reproduction, specialized cells, called germ cells, are solely responsible for the generation of offspring and the propagation of species. Germ cells are distinct from somatic cells in several fundamental aspects (reviewed in Marsh and Goode 1994; Saffman and Lasko 1999). Germ cells undergo the specialized cell cycle, meiosis, to reduce the ploidy from diploid to haploid and to generate gametes. The germ lineage is considered to be totipotent and immortal, since united gametes are able to generate the entire somatic body of the organism, as well as more germ cells and thus future generations. In contrast, somatic cells undergo only mitosis, show restricted developmental potential, and senesce and die with each generation. Understanding the molecular mechanisms that underlie the fundamental differences between the germline and soma and confer upon the germline its special characteristics remains a major issue in developmental biology.

In many organisms, primordial germ cells are set apart from the somatic lineages early in embryogenesis (Wylie 1999) and often contain distinctive, electron-dense cytoplasmic organelles, generally called "germ granules" (Eddy 1975; Saffman and Lasko 1999). The presence of germ granules in diverse organisms and the finding that ectopic granules can induce ectopic germ-cell formation in Drosophila and Xenopus (Illmensee and Mahowald 1974 ; Ikenishi 1987; Ephrussi and Lehmann 1992) has led to the widely accepted view that germ granules carry essential factors or "determinants" for germline development.

In the nematode *Caenorhabditis elegans*, germ granules, also called "P granules," are present in germ cells throughout the life cycle (Strome and Wood 1982, 1983; Kawasaki *et al.* 1998). P granules are maternally contributed to the fertilized egg and partitioned to the germline blastomere (P cell) during each of four unequal divisions of the early embryo. This partitioning delivers the majority of granules to the primordial germ cell, P_4. The two daughters of P_4, Z2 and Z3, divide throughout

Sequence data from this article have been deposited with the DDBJ/EMBL/GenBank Data Libraries under accession nos. AB120729 and AB120730.

[1]*Corresponding author:* Molecular Genetics Research Laboratory, University of Tokyo, Science Bldg. No. 7, 7-3-1 Hongo, Bunkyo-ku, Tokyo 113-0033, Japan. E-mail: ikawasak@gen.s.u-tokyo.ac.jp

[2]*Present address:* Department of Biology, University of Ottawa, Ottawa, ON K1H 8M5, Canada.

[3]*Present address:* Institute fuer Genetik, Heinrich-Heine-Universitaet, Düsseldorf 40225, Germany.

larval development, giving rise to the ~1500 germ cells in an adult hermaphrodite. P granules are present in all descendants of P_4 with the exception of mature sperm. At all stages except oogenesis and early embryogenesis, P granules are closely associated with the outer surface of the nuclear envelope. This perinuclear localization is a common feature of germ granules in diverse organisms (Eddy 1975). An ultrastructural analysis of the perinuclearly localized P granules (Pitt *et al.* 2000) revealed their tight association with nuclear pore clusters, suggesting the possible involvement of P granules in nuclear-cytoplasmic transport of RNAs and/or proteins.

P granules are known to contain poly(A)$^+$ RNAs (Seydoux and Fire 1994) and numerous proteins that are predicted to bind RNA. Two maternal mRNAs that associate with P granules in early embryos are *nos-2* (Subramaniam and Seydoux 1999) and *pos-1* (Schisa *et al.* 2001). *nos-2* and the related gene *nos-1* encode proteins similar to the Drosophila germ-granule component Nanos (Wang and Lehmann 1991) and function redundantly to regulate germ-cell proliferation and viability (Subramaniam and Seydoux 1999). *pos-1* encodes a zinc-finger protein that functions at least in part to protect germline fate (Tabara *et al.* 1999). In addition, several class II maternal mRNAs (Seydoux and Fire 1994), which are degraded rapidly in somatic blastomeres but persist in germline blastomeres during early embryogenesis, have been shown to localize to P granules in adult gonads (Schisa *et al.* 2001). All of the identified protein components of P granules contain RNA-binding motifs and thus are predicted to associate with RNA. One class of proteins, including PIE-1 (Mello *et al.* 1992, 1996), GLD-1 (Jones *et al.* 1996), MEX-3 (Draper *et al.* 1996), MEX-1 (Guedes and Priess 1997), and POS-1 (Tabara *et al.* 1999), associates with P granules only during early embryogenesis and disappears in Z2 and Z3. Most proteins in this class function in early embryogenesis to prevent either the germline blastomeres from adopting somatic fates (*pie-1*, *mex-1*, and *pos-1*) or certain somatic blastomeres from adopting a germline fate (*mex-3*).

Proteins that are constitutively associated with P granules are likely to serve crucial roles in the assembly and functions of this organelle. In this class are PGL-1 (Kawasaki *et al.* 1998) and GLH-1, GLH-2, GLH-3, and GLH-4 (Gruidl *et al.* 1996; Kuznicki *et al.* 2000). GLH proteins were identified as *C. elegans* homologs of Drosophila Vasa, a DEAD-box RNA helicase that is a component of Drosophila germ granules (Hay *et al.* 1988; Lasko and Ashburner 1988). Among the four GLH proteins, GLH-1 is essential for germline development at elevated temperature, and GLH-1 and GLH-4 appear to function redundantly in germline development at lower temperatures (Kuznicki *et al.* 2000). Interestingly, the sterile phenotype seen in *glh-1* mutants and in animals depleted of both *glh-1* and *glh-4* by RNAi is very similar to that of *pgl-1* mutants at restrictive temperature (Kawasaki *et al.* 1998; Kuznicki *et al.* 2000). The four GLH proteins localize to P granules independently of each other (Kuznicki *et al.* 2000). GLH-1 is required for PGL-1 to correctly localize to P granules (Kawasaki *et al.* 1998). Correct localization of PGL-1 to P granules in embryos also requires Sm proteins, core components of the spliceosome (Barbee *et al.* 2002). Furthermore, in the adult germline the perinuclear localization of PGL-1 and P granules themselves also requires other core splicing factors, such as U2AF and U170K (Barbee *et al.* 2002). This and the concentration of P granules over nuclear pores (Pitt *et al.* 2000) suggest that P-granule accumulation on germ nuclei requires active mRNA transcription, processing, and/or nuclear export.

This article focuses on the PGL family of proteins. PGL-1 was identified as a protein component of P granules by molecular and genetic analyses of a mutant defective in antibody staining of P granules (Kawasaki *et al.* 1998). PGL-1, a novel protein, is associated with P granules at all stages of development. The presence of an RNA-binding motif, an RGG box, at its C terminus predicts that PGL-1 is an RNA-binding component of P granules. *pgl-1* mutants are sterile, with both a maternal and a zygotic component to the sterility. Sterility is the result of defects in germline proliferation and gametogenesis. Interestingly, the sterility caused by null alleles of *pgl-1* is highly sensitive to temperature, suggesting that either PGL-1 functions as a molecular chaperone whose function is critical primarily at elevated temperature or other partially redundant proteins exist, which function with PGL-1 and are sufficient for fertility at permissive temperature. The latter possibility gained support from our identification and analysis of two *pgl-1*-related genes, *pgl-2* and *pgl-3*. The proteins encoded by both genes are indeed components of P granules, although PGL-2 is notably absent from P granules in embryos. The three PGL proteins interact with each other *in vitro*, and at least PGL-1 and PGL-3 are associated with each other *in vivo* as well. Each PGL protein localizes to P granules independently of the other two. A deletion allele of *pgl-3* does not result in germline defects on its own but significantly enhances the sterility of *pgl-1* at low temperatures, supporting the hypothesis that PGL-1 and PGL-3 function redundantly during germline development. A deletion allele of *pgl-2* does not result in germline defects on its own or significantly enhance the phenotype of *pgl-1* or *pgl-1; pgl-3*, suggesting that PGL-2 is not a major player in germline development.

MATERIALS AND METHODS

Strains and alleles: Maintenance and genetic manipulation of *C. elegans* were carried out as described in Brenner (1974). *C. elegans* strain N2 variety Bristol was used as the wild-type strain. Mutations used in this study were LGI, *glh-1(bn103), glp-4(bn2)*; LGIII, *pgl-2(bn123)*; LGIV, *pgl-1(ct131, bn101), him-*

3(e1147), unc-24(e138); and LGV, *pgl-3(bn104), dpy-11(e224)*. For mutant phenotype analysis of males and hermaphrodites, 25° and 26°, respectively, were used as restrictive temperatures. N2 males and hermaphrodites are mostly fertile at 25° and 26°, respectively.

Yeast two-hybrid screen: *pgl-2* and *pgl-3* cDNAs were isolated in a yeast two-hybrid screen (Fields and Song 1989; Durfee *et al.* 1993) using full-length *pgl-1* cDNA (Kawasaki *et al.* 1998) as "bait" and an oligo(dT)-primed *C. elegans* cDNA library, λACT-RB1, provided by R. Barstead (Barstead and Waterston 1989) as "prey." The positive cDNA clones were sorted by Southern hybridization analysis using some of their cDNA inserts as probes.

Northern and *in situ* hybridization analyses: Northern hybridization analysis was done as in Holdeman *et al.* (1998). Band intensity of transcripts was quantified using a phosphorimager (Molecular Dynamics, Sunnyvale, CA), and the levels of *pgl-2* and *pgl-3* transcripts were normalized to the level of *rpp-1* transcript, which encodes a ribosomal protein (Evans *et al.* 1997). cDNA clones pBS-*pgl-2* and pGEM-*pgl-3*, which contain the longest inserts (1.7 and 2.4 kb for *pgl-2* and *pgl-3*, respectively) among the clones isolated from the yeast two-hybrid screen, were used as probes. *In situ* hybridization was performed as in Tabara *et al.* (1996) with some modifications. For *pgl-1*, an expressed sequence tag (EST) cDNA clone, yk225b8, was used as probe. For *pgl-2*, pBS-*pgl-2* described above, was used as probe. For *pgl-3*, EST cDNA clones, yk518d6 and yk231a5, were used as probes for larval and embryonic *in situ* hybridizations, respectively.

Antibody production: A 1539-bp *pgl-2* cDNA fragment encoding the entire PGL-2 sequence except the first 20 amino acids was cloned into the expression vector pET-30a(+) (Novagen). A portion of *pgl-3* cDNA (279 bp) corresponding to the PGL-3 region from Ser-448 to Ser-540 (see Figure 2), which is relatively unique to PGL-3, was PCR amplified using primers 5′-GAGGGATCCAGTTCGTCGGAACCTTCTGC-3′ and 5′-TCCCAAGCTTAGCTAGAAAATTGAACAGGTG-3′ and cloned into the expression vector pET-28a(+) (Novagen). The 6× His-tagged fusion proteins were expressed in *Escherichia coli* BL21(DE3) by isopropyl thiogalactoside (IPTG) induction and purified using Ni-NTA agarose columns (QIAGEN, Chatsworth, CA) under denaturing conditions. The purified fusion proteins were electrophoresed on SDS-polyacrylamide gels, cut from the gels, and injected with Freund's adjuvant into rabbits for the PGL-2 fusion protein and into rats for the PGL-3 fusion protein. Antibodies against PGL-2 and PGL-3 were purified from crude antisera by blot affinity purification (Olmsted 1986). Monospecific antibodies bound to the 6× His fusion proteins on nitrocellulose-membrane strips were eluted with 0.2 M glycine-HCl (pH 2.8).

Immunofluorescence analysis: DNA staining of intact worms and extruded gonads was carried out as in Kawasaki *et al.* (1998). Immunostaining of extruded gonads and embryos was done as in Strome and Wood (1983). For immunostaining, the following primary antibodies were used: affinity-purified rabbit anti-PGL-1 diluted 1:100, affinity-purified rabbit anti-PGL-2 diluted 1:50, affinity-purified rat anti-PGL-3 diluted 1:10, affinity-purified rabbit anti-GLH-1 diluted 1:100, rabbit anti-HIM-3 (a gift from M. Zetka) diluted 1:500, rabbit anti-phospho-histone H3 (Upstate Biotechnology, Lake Placid, NY) diluted 1:200, mouse monoclonal antichromatin antibody PA3 (a gift from M. Monestier) diluted 1:100, mouse monoclonal anti-PGL-1 antibody K76 (Strome 1986) undiluted supernatant, and mouse monoclonal spermatogenesis-specific antibody SP56 undiluted supernatant. Secondary antibodies used were Alexa Fluor 546 goat anti-rabbit IgG (Molecular Probes, Eugene, OR) diluted 1:400 or 1:100, Alexa Fluor 488 goat anti-rat IgG (Molecular Probes) diluted 1:100, and FITC-conjugated goat anti-mouse IgG (Jackson) diluted 1:100. Samples were examined using a Zeiss Axioskop microscope equipped with Nomarski differential interference contrast and epifluorescence optics. Images were either photographed with Tri-X pan film, scanned, and processed using Adobe Photoshop (Adobe Systems) or acquired with an AxioCam CCD camera (Zeiss) and processed using Openlab 3 (Improvision).

Glutathione S-transferase fusion protein construction, expression, and pull-down experiments: Full-length *pgl-1* cDNA (2190 bp) was amplified by PCR from pBS-*pgl-1* (Kawasaki *et al.* 1998) using primers 5′-CTCGAGATGGAGGCTAACAAGCGAGAA-3′ and 5′-GCGGCCGCTTAGAAACCTCCGCGTCCAC-3′. The PCR product was digested with *Xho*I and *Not*I and ligated to a glutathione-*S*-transferase (GST) gene fusion vector, pGEX-5X-3 (Amersham Pharmacia Biotech), prelinearized by digestion with *Sal*I and *Not*I to form pGEX-*pgl-1*. Full-length *pgl-2* cDNA (1.7 kb) was PCR amplified from pBS-*pgl-2* described in the previous section, using primers 5′-ACGAGATCTATGAAGCCATGTAAAAGGG-3′ and 5′-ACTGAAGCTTAAAGGAATCCTCTACAAT-3′ and cloned into *Bgl*II and *Hin*dIII sites in pGEX-2*, a derivative of pGEX-2T (Amersham Pharmacia Biotech), to form pGEX-*pgl-2*. Full-length *pgl-3* cDNA (2.4 kb) was PCR amplified from pGEM-*pgl-3*, using primers 5′-CCGCTCGACACTAGTATGGAAGCAAACAAACG-3′ and 5′-CCGGAATTCACTAGTTTAGGAACCTCCACGG-3, and cloned into the *Eco*RI and *Sal*I sites in pGEX-2* to form pGEX-*pgl-3*. *E. coli* BL21(DE3) was transformed with pGEX-*pgl-1*, pGEX-*pgl-2*, pGEX-*pgl-3*, or pGEX-2* (as control) and induced with IPTG to express GST-PGL-1, GST-PGL-2, GST-PGL-3, or GST. Purification of the GST fusion proteins was done as in Amiri *et al.* (2001). Radiolabeled PGL-1, PGL-3, and IFE-1 were synthesized by *in vitro* transcription-translation using pBS-*pgl-1*, pGEM-*pgl-3*, and pBS-*ife-1*, respectively, as templates with [^{35}S] methionine in the TNT-coupled reticulocyte lysate system (Promega, Madison, WI). Binding assays were done as in Amiri *et al.* (2001).

Immunoprecipitation and Western blot analyses: Embryo extract was prepared as in Xu *et al.* (2001). A total of 90 μl of N2 embryo extract was incubated with 3 μl of either rabbit anti-PGL-1 (Kawasaki *et al.* 1998) or rat anti-PGL-3 antiserum at 4° for 1 hr after the extract was preabsorbed with 3 μl of either normal rabbit serum or normal rat serum (both from Jackson), respectively. The mixture was then incubated with 20 μl of protein A/G PLUS-agarose (Santa Cruz) at 4° for 2 hr. The immuno-complex was precipitated by centrifugation, washed four times with 500 μl of RIPA buffer (1% NP-40, 0.5% sodium deoxycholate, and 0.1% SDS in PBS) at 4° for 10 min, resuspended in SDS sample buffer, boiled, and analyzed by SDS-PAGE. Western blot analysis was carried out as in Kawasaki *et al.* (1998) with some modifications. For detecting PGL-1 and PGL-3, affinity-purified rabbit anti-PGL-1 diluted 1:100 and affinity-purified rat anti-PGL-3 diluted 1:40, respectively, were used as primary antibodies, and as secondary antibodies, horseradish peroxidase (HRP)-conjugated donkey anti-rabbit IgG and HRP-conjugated donkey anti-rat IgG (both from Jackson), respectively, were used (both at 1:10,000 dilution). Bound antibodies were visualized using enhanced chemiluminescence Western blot detection reagents (Amersham Pharmacia Biotech).

Isolation of *pgl-2* and *pgl-3* deletion mutants: Worm libraries mutagenized with trimethylpsoralen and UV irradiation (Yandell *et al.* 1994) were screened for a deletion mutation in either the *pgl-2* locus (B0523.3) or the *pgl-3* locus (C18G1.4) according to the protocol of G. Molder and R. Barstead (http://pcmc41.ouhsc.edu/Knockout/) with some modifications as described in Karashima *et al.* (2000). PCR primers used to screen for a *pgl-2* deletion mutation were as follows: external primer set, *pgl-2* 367F, 5′-CGTTTTCGTTTAACAGG

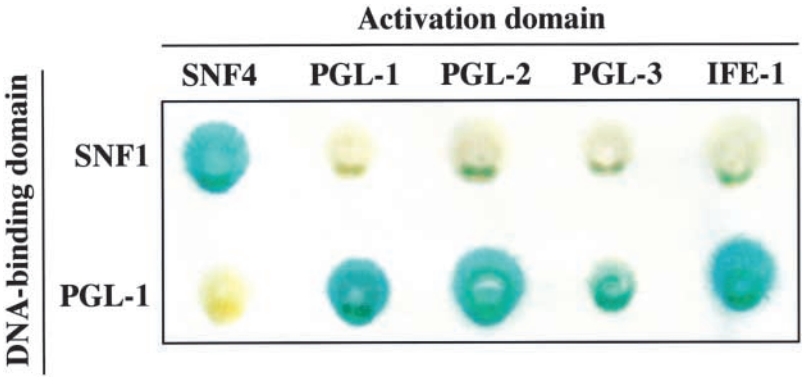

FIGURE 1.—Proteins that interact with PGL-1 in yeast two-hybrid tests. Yeast Y190 cells expressing the indicated proteins as fusions with either the GAL4 DNA-binding domain or the GAL4 activation domain were assayed on filters for β-galactosidase activity. The combination of SNF1 and SNF4 was used as a positive control. As shown, PGL-1 interacts with PGL-1, PGL-2, PGL-3, and IFE-1, but not with SNF4, in yeast cells.

GGA-3′ (9334) and *pgl-2* 1616R, 5′-GATTGCCAAGAAAG AGGCTG-3′ (7352); internal primer set, *pgl-2* -321F, 5′-GAT GCATCAACGAGTGAGGA-3′ (9288) and *pgl-2* 1510R, 5′-CTT CACGACGTAAGGGCAGT-3′ (7458); poison primer, *pgl-2* 731R 5′-TCACTCACAGCAAATGAGCC-3′. PCR primers used to screen for a *pgl-3* deletion mutation were as follows: external primer set, *pgl-3* ExUp, 5′-GTGTTCACAGCGGGAAAAGA TAG-3′ (12588) and *pgl-3* ExLo, 5′-TCCATCGGAAAGTGTA AGTC-3′ (16023); internal primer set, *pgl-3* InUp, 5′-CCC GGAACATCTAGCAGACAGTC-3′ (12618) and *pgl-3* InLo, 5′-CTGCACCATCCTAGAACGGTAGC-3′ (15837). Numbers in parentheses indicate map coordinates of the 5′ ends of the primers on either cosmid B0523 or C18G1. Deletion mutations, *bn123* and *bn104*, were found in the *pgl-2* locus and the *pgl-3* locus, respectively, and homozygous mutant worms were successfully isolated. To eliminate possible additional mutations, the mutant strains were backcrossed eight times before genetic analyses.

RESULTS

Identification of the *pgl-2* and *pgl-3* genes: In a previous study (KAWASAKI *et al.* 1998), we showed that PGL-1, a constitutive component of P granules, is essential for *C. elegans* germline development, but only at elevated temperatures. We reasoned that this temperature sensitivity might be explained by the presence of other proteins that function redundantly with PGL-1. Indeed, our searches of the *C. elegans* genome (*C. ELEGANS* SEQUENCING CONSORTIUM 1998) revealed two open reading frames (B0523.3 and C18G1.4) whose predicted protein products show significant sequence similarity to PGL-1. The encoded proteins were also identified in a yeast two-hybrid screen for *C. elegans* proteins that interact with PGL-1 (Figure 1). In this screen, full-length PGL-1 protein was fused to the GAL4 DNA-binding domain (GAL4DB-PGL-1) and used as bait. Proteins encoded by the library cDNAs were fused to the GAL4 activation domain. Among 2×10^6 independent cDNA transformants screened, 270 clones were found to activate both HIS3 and lacZ reporter genes only in the presence of GAL4DB-PGL-1. Most (260) of the positive clones were found to correspond to one of four genes: *ife-1*, which encodes one of the five *C. elegans* isoforms of eukaryotic translation initiation factor 4E (eIF4E; AMIRI *et al.* 2001); *pgl-1* itself, suggesting that PGL-1 protein can form multimers; and the two *pgl-1*-like genes, B0523.3 and C18G1.4, termed *pgl-2* and *pgl-3*, respectively (Figure 1).

The sequences of the longest cDNA inserts for both *pgl-2* and *pgl-3* were determined, and their splicing patterns were deduced by comparison with their corresponding genomic sequences (*C. ELEGANS* SEQUENCING CONSORTIUM 1998). Their 5′ ends were determined by sequencing RT-PCR products generated using a gene-specific downstream primer and an upstream primer corresponding to the *trans*-spliced leader SL1 or SL2 (SPIETH *et al.* 1993). The *pgl-2* gene contains seven exons (and six introns including a very long fifth intron of 2208 bp). *pgl-2* mRNA is *trans*-spliced to SL1 and encodes a predicted protein of 532 amino acids. PGL-2 protein has 34% identity (140/414) and 67% similarity (279/414) with PGL-1 in its N-terminal 414 amino acids (Figure 2). Its C-terminal portion is not very similar to PGL-1. The *pgl-3* gene contains eight exons. *pgl-3* mRNA is *trans*-spliced to both SL1 and SL2 and encodes a predicted protein of 693 amino acids. PGL-3 protein has 62% identity (431/693) and 77% similarity (535/693) with PGL-1 throughout its length (Figure 2). Also, PGL-3 contains the same RNA-binding motif, an RGG box, as PGL-1 at its C terminus (Figure 2). An RGG box consists of multiple repeats of Arg-Gly-Gly, often interspersed with aromatic amino acids (KILEDJIAN and DREYFUSS 1992). The RGG box of PGL-3 consists of 59 amino acids and contains six Arg-Gly-Gly repeats. As the RGG-box motif is known to act as an RNA-binding domain in some RNA-binding proteins (see BURD and DREYFUSS 1994 for review), PGL-3, like PGL-1, is predicted to be an RNA-binding protein.

***pgl-2* and *pgl-3* transcripts are enriched in the germline:** Densitometric quantification of Northern hybridization blots (Figure 3) revealed that wild-type adult hermaphrodites contain six times more *pgl-2* mRNA and 14 times more *pgl-3* mRNA than *glp-4(bn2ts)* adult hermaphrodites do, which have a severely underproliferated germline at restrictive temperature (BEANAN and STROME 1992). These results indicate that both *pgl-2* and *pgl-3* transcripts are enriched in the germline.

To analyze the temporal and spatial distribution of

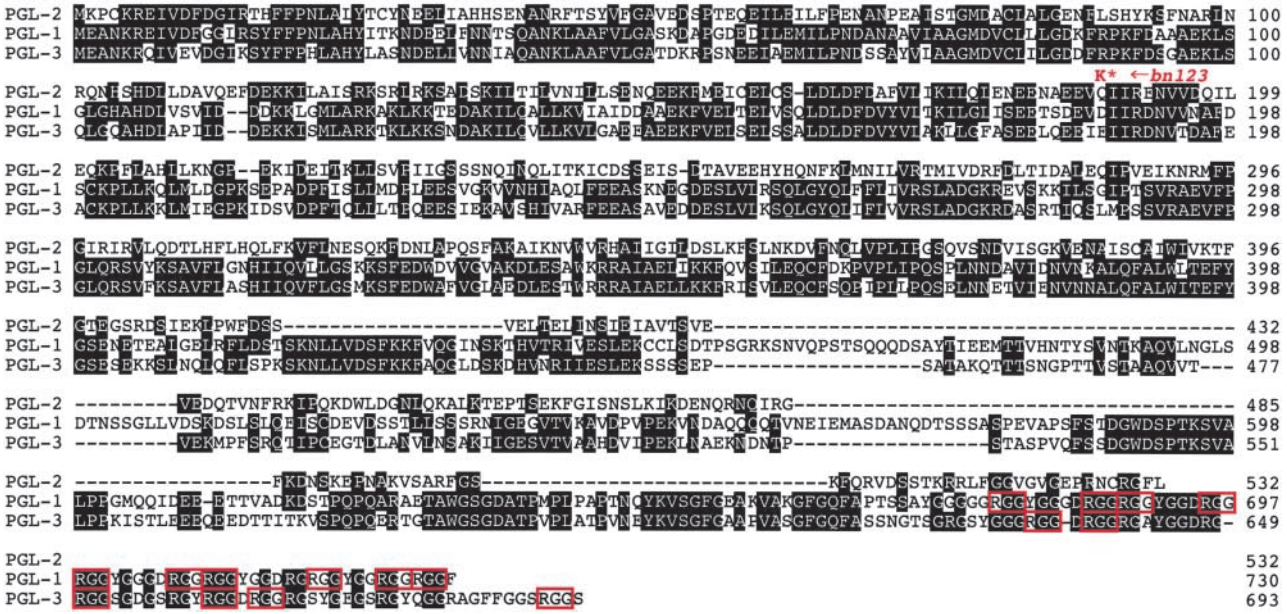

FIGURE 2.—Multiple sequence alignment of PGL-1, PGL-2, and PGL-3. Sequences were aligned using the ClustalW program at DDBJ (http://www.ddbj.nig.ac.jp/). Amino acids that are shared by at least two of the three proteins are marked by solid black boxes. The 10 Arg-Gly-Gly repeats in PGL-1 and the 6 repeats in PGL-3 are marked by red boxes. The red asterisk above the second line of the PGL-2 sequence indicates where PGL-2 is predicted to terminate in *pgl-2(bn123)* mutants.

these transcripts, we performed *in situ* hybridization of *pgl-2* and *pgl-3* probes to whole-mount wild-type embryos and worms at different developmental stages (TABARA *et al.* 1996) and compared their patterns with that of *pgl-1*. Zygotic *pgl-1* transcript first becomes detectable during the twofold stage of embryogenesis in the primordial germ cells, Z2 and Z3 (Figure 4G). *pgl-1* transcript is highly enriched in the germline throughout larval development and in adults (Figure 5, left). *pgl-1* mRNA is maternally loaded into embryos, as the signal is detectable in 1-cell embryos (Figure 4A). Signal appears evenly distributed until the 4-cell stage (Figure 4, B and C), after which it diminishes in somatic blastomeres. Signal persists primarily in P_3 and E at the 8-cell stage (Figure 4D), in P_3 at the 15-cell stage (Figure 4E), and in P_4 at and after the 24-cell stage (Figure 4F). *pgl-1* signal disappears from P_4 before it divides to Z2 and Z3 around the 90-cell stage (data not shown).

Zygotic *pgl-3* transcript first becomes detectable in the germline of L3 larvae (Figure 5, right), much later than the appearance of *pgl-1* transcript. The signal becomes stronger in later stages of larval development and in adults and is highly concentrated in the germline, especially in the pachytene region. Like *pgl-1* mRNA, *pgl-3* mRNA is maternally loaded into embryos (Figure 4O) and is selectively retained by the germline blastomeres (Figure 4, Q–S). *pgl-3* signal becomes undetectable before P_4 is generated at the 24-cell stage (Figure 4T). No signal is detectable throughout the rest of embryogenesis (Figure 4U) or in early larval stages (Figure 5, right).

The significance of the persistence of both *pgl-1* and *pgl-3* maternal mRNAs primarily in the P cells of early embryos is not known, but this pattern is seen for many other class II maternal mRNAs (SEYDOUX and FIRE 1994). Interestingly, the 3′-untranslated region sequences of *pgl-1* and *pgl-3* mRNAs are very similar (data not shown), suggesting that the localization and/or stability of these two mRNAs may be controlled by a similar post-transcriptional mechanism(s).

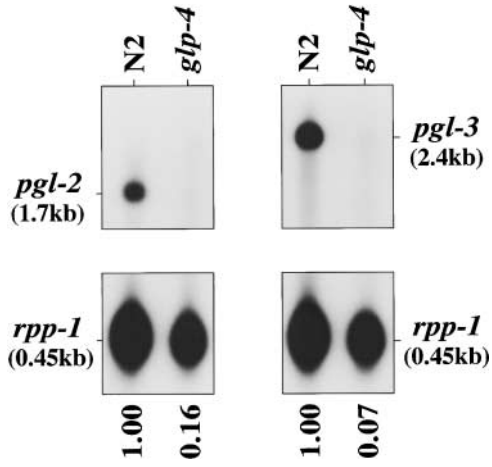

FIGURE 3.—Northern blot analysis of *pgl-2* and *pgl-3*. Poly $(A)^+$ RNA was prepared from synchronous populations of wild-type N2 or *glp-4(bn2ts)* adult hermaphrodites grown at 25° at which temperature *glp-4(bn2ts)* adult hermaphrodites contain a severely underproliferated germline. *pgl-2* and *pgl-3* probes detected 1.7- and 2.4-kb transcripts, respectively. The 0.45-kb transcript of a ribosomal protein gene, *rpp-1*, was used as a loading control. Relative levels of the *pgl-2* and *pgl-3* transcripts are shown at the bottom (see MATERIALS AND METHODS).

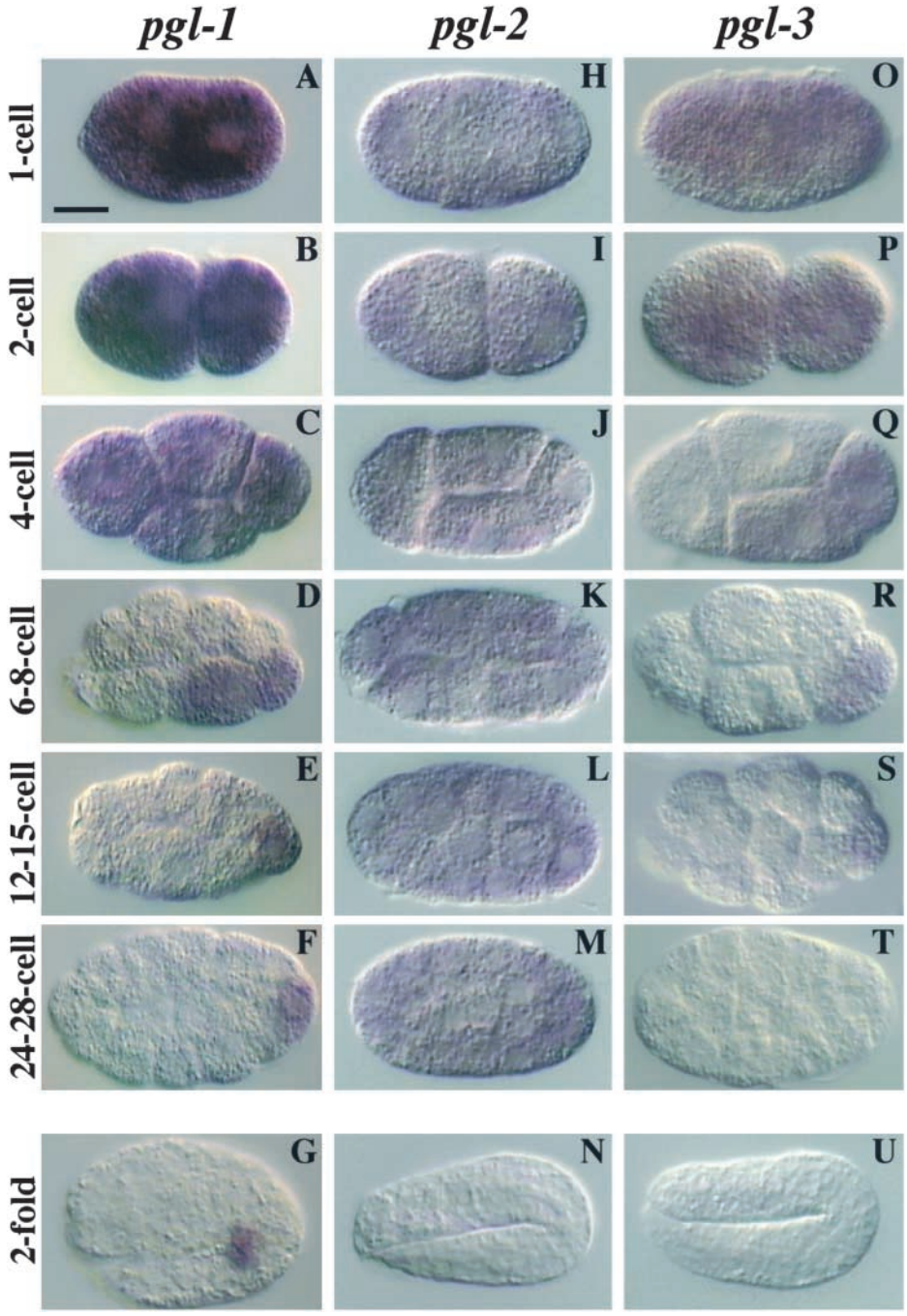

FIGURE 4.—*In situ* hybridization analysis of *pgl-1*, *pgl-2*, and *pgl-3* transcripts in whole-mount wild-type embryos at different developmental stages. Anterior is left, ventral is down, for all but the last row of embryos. Wild-type embryos were hybridized with cDNA probes for *pgl-1* (A–G), *pgl-2* (H–N), and *pgl-3* (O–U), respectively. Developmental stages of embryos are indicated. Bar, 10 μm.

Zygotic *pgl-2* transcript becomes detectable in L1 larvae but is not significantly enriched in the germline (Figure 5, middle). The signal becomes more intense in the germline, with weak signal in somatic tissues, at later stages of larval development and in adults. In embryos, *pgl-2* mRNA shows a uniform distribution in all cells through the early cleavage stages until the 100- to 200-cell stage (Figure 4, H–M), after which it gradually disappears (Figure 4N).

In summary, our *in situ* hybridization results demonstrate the following. First, *pgl-1*, *pgl-2*, and *pgl-3* transcripts accumulate primarily in the germline of L3 and later-stage hermaphrodites and are maternally loaded into embryos. Second, the levels of *pgl-1* and *pgl-3* transcripts in the germline are much higher than the level of *pgl-2*. Third, transcripts of *pgl-1* and *pgl-3*, but not of *pgl-2*, show the class II maternal mRNA behavior of persisting in the germline blastomeres and disappearing from the somatic blastomeres of early embryos. Fourth, *pgl-1* joins *nos-1* in being transcribed in the primordial germ cells, Z2 and Z3, of embryos (SUBRAMANIAM and SEYDOUX 1999).

PGL-2 and PGL-3 are components of P granules: Antibodies were raised against PGL-2 and a central region

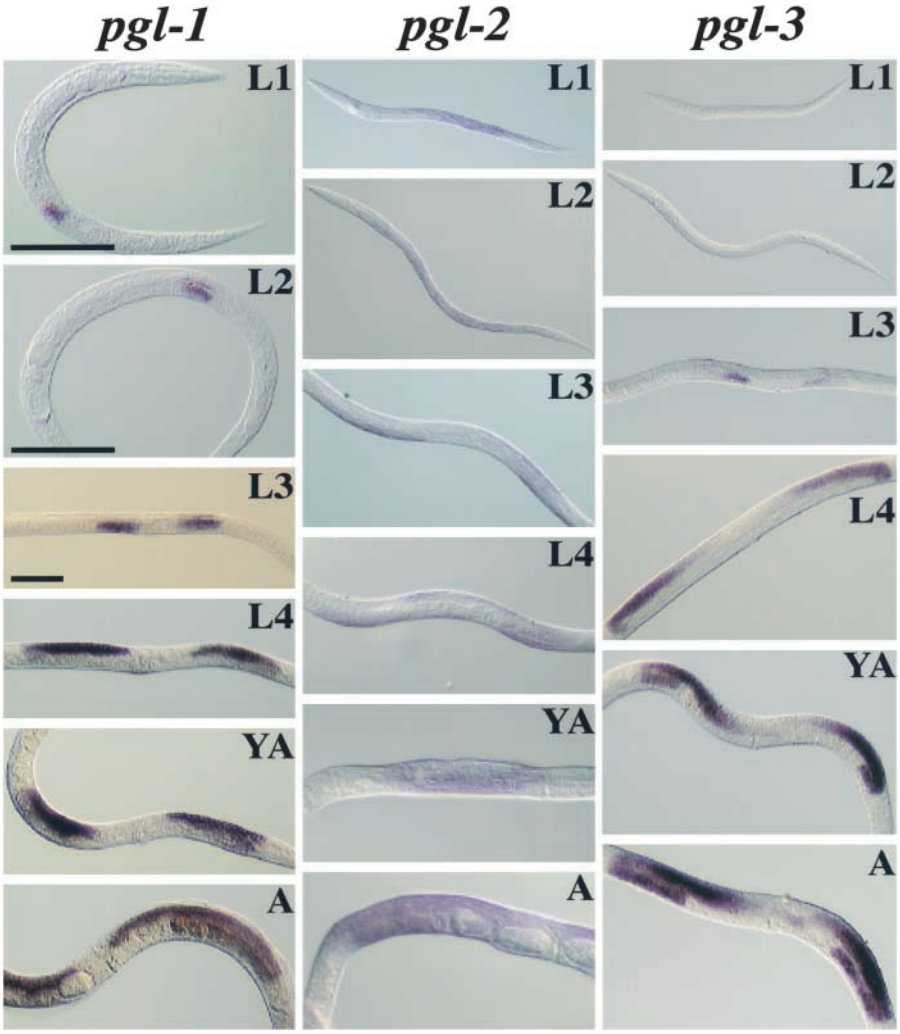

FIGURE 5.—*In situ* hybridization analysis of *pgl-1*, *pgl-2*, and *pgl-3* transcripts in whole-mount wild-type worms at different developmental stages. Anterior is left, ventral is down. Wild-type worms were hybridized with cDNA probes for *pgl-1* (left), *pgl-2* (middle), and *pgl-3* (right). Developmental stages of worms are indicated in each: L1–L4, four larval stages; YA, young adult stage (before egg laying); A, adult stage (containing embryos). Bars, 50 μm. Note that the magnification for *pgl-1* L1 and L2 is twice that used for the rest of the image.

of PGL-3 and then purified by blot affinity purification (OLMSTED 1986; see MATERIALS AND METHODS). Specificity of the affinity-purified antibodies was demonstrated by immunostaining analysis of the *pgl-2* and *pgl-3* deletion mutants (Figure 6). Affinity-purified anti-PGL-2 antibody stained granules in wild-type N2 and *pgl-3(bn104)* mutants but not in *pgl-2(bn123)* mutants, and affinity-purified anti-PGL-3 antibody stained granules in wild-type N2 and *pgl-2(bn123)* mutants but not in *pgl-3(bn104)* mutants.

The affinity-purified antibodies against PGL-2 and PGL-3 stained the same granules as were stained by antibodies to PGL-1 (Figures 7 and 8), demonstrating that both PGL-2 and PGL-3 proteins are components of P granules. However, the distributions of the three PGL proteins show some temporal and spatial differences. In wild-type worms, PGL-1 appears to be associated with P granules evenly in all germ cells in both sexes throughout the life cycle, with one exception: PGL-1 disappears from P granules during spermatogenesis, perhaps to release IFE-1 from P granules (AMIRI *et al.* 2001). Similar to PGL-1, PGL-3 is associated with P granules in both sexes at all stages of development except in spermatogenesis. But unlike PGL-1, PGL-3 is present in a distinct gradient in the adult germline: progressively more enriched in the pachytene and diplotene meiotic regions than in the distal mitotic region (compare Figure 7, E and F). Compared to *pgl-1* mRNA, *pgl-3* mRNA also seems more enriched in the meiotic region of the gonad than in the mitotic region (Figure 5, compare left and right columns).

Notably, PGL-2 is associated with P granules only during postembryonic development (Figure 7B); it is not detectable in embryos (Figure 8, B, D, and F). PGL-2 is first detected in L1 larvae, and like its transcript, it is not significantly enriched in the germline in newly hatched L1's (data not shown). Concomitant with mitotic proliferation of germ cells, PGL-2 appears to accumulate in P granules, with weak signal still remaining in somatic tissues (data not shown). It remains associated with P granules in adult gonads in both sexes until it disappears during spermatogenesis and during the final stages of oogenesis (data not shown).

The three PGL proteins associate with each other *in vitro* and *in vivo*: To verify that the interactions between PGL-1 and itself, PGL-2, PGL-3, and IFE-1 detected by

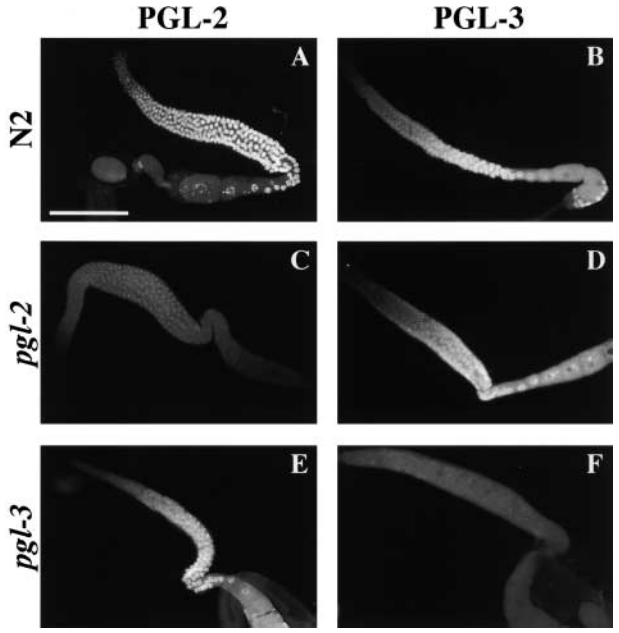

FIGURE 6.—Immunofluorescence analysis of antibody specificity. Adult or L4 hermaphrodite gonads from wild-type N2 (A and B), *pgl-2(bn123)* mutants (C and D), or *pgl-3(bn104)* mutants (E and F) were stained with either affinity-purified rabbit anti-PGL-2 antibody (A, C, and E) or affinity-purified rat anti-PGL-3 antibody (B, D, and F). Anti-PGL-2 stained N2 (A) and *pgl-3* (E) gonads but failed to stain *pgl-2* (C) gonads. Anti-PGL-3 stained N2 (B) and *pgl-2* (D) gonads but failed to stain *pgl-3* (F) gonads. Bar, 100 μm.

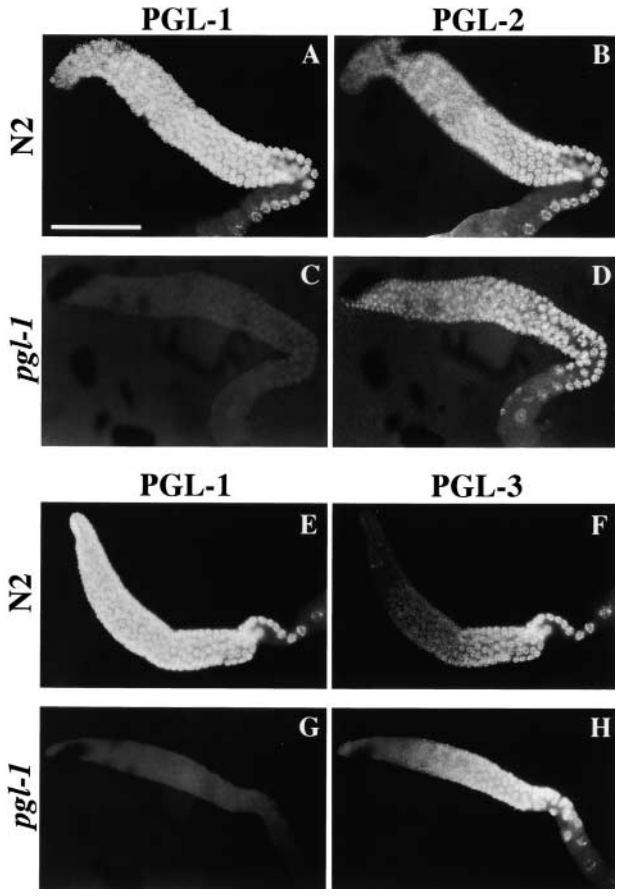

FIGURE 7.—Immunofluorescence analysis of PGL protein distributions in adult hermaphrodite germlines. The distal end of each gonad arm is top left. Adult hermaphrodite gonads from wild-type N2 (A, B, E, and F) or *pgl-1(bn101)* mutants (C, D, G, and H) were costained either with mouse monoclonal antibody K76, which recognizes an epitope on PGL-1 (A and C), and affinity-purified rabbit anti-PGL-2 antibody (B and D), or with affinity-purified rabbit anti-PGL-1 antibody (E and G) and affinity-purified rat anti-PGL-3 antibody (F and H). PGL-1 is associated fairly uniformly with P granules throughout the gonads, while PGL-2 and especially PGL-3 are present in a distinct gradient, most enriched in the pachytene and diplotene regions of adult gonads. PGL-2 and PGL-3 can associate with P granules in the absence of PGL-1 (D and H, respectively). Bar, 100 μm.

the yeast two-hybrid screen are authentic and to test whether PGL-2 and PGL-3 can bind to each other and to IFE-1, we performed *in vitro* binding assays (Figure 9, A and B). ^{35}S-PGL-1 (~100 kD) bound to both GST-PGL-1 and GST-PGL-2, but not to GST alone (Figure 9A, lanes 1–4). Similarly, ^{35}S-PGL-3 (~90 kD) bound to both GST-PGL-1 and GST-PGL-2, but not to GST alone (Figure 9A, lanes 5–8). ^{35}S-IFE-1 (~25 kD) bound to GST-PGL-1, but not to GST-PGL-2 or GST-PGL-3 (Figure 9B). These results indicate that PGL-1, PGL-2, and PGL-3 are all able to bind directly to each other and that at least PGL-1 can bind to itself *in vitro* in the absence of other *C. elegans* proteins. Although the three PGL proteins share significant sequence similarity, only PGL-1 can bind to IFE-1 *in vitro*.

To investigate whether the three PGL proteins are associated with each other *in vivo*, we performed co-immunoprecipitation experiments on embryo extracts (see MATERIALS AND METHODS). After preabsorption with normal serum, wild-type embryo extract was incubated with either rabbit anti-PGL-1 or rat anti-PGL-3 antiserum, followed by precipitation using protein A/G agarose and Western blot analysis of the immunoprecipitated complex (Figure 9C). PGL-1 was co-immunoprecipitated by anti-PGL-3 antiserum (lane 3), and PGL-3 was co-immunoprecipitated by anti-PGL-1 antiserum (lane 7), whereas no PGL protein was immunoprecipitated by normal sera (lanes 4 and 8). PGL-2 was not detected in any of the embryo extract fractions (data not shown), as expected from the immunostaining results described above (see Figure 8). Our results indicate that PGL-1 and PGL-3 are associated with each other in embryos. We expect that PGL-2 is associated with PGL-1 and PGL-3 during postembryonic stages.

PGL proteins localize to P granules independently of each other: GLH proteins, another family of constitutive P-granule components, localize to P granules independently of each other (KUZNICKI *et al.* 2000). To test whether there is a hierarchy among PGL proteins in their recruitment to P granules, the localization of each PGL protein in various *pgl* mutant backgrounds was

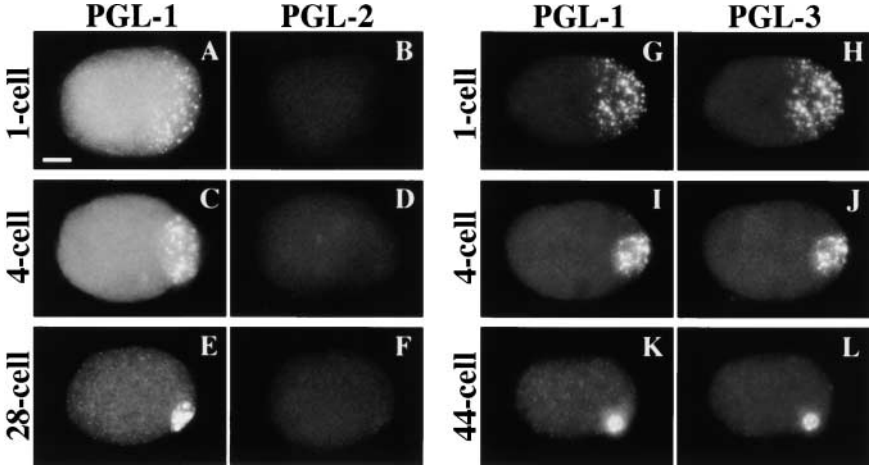

FIGURE 8.—Immunofluorescence analysis of PGL protein distributions in embryos. Anterior is left; ventral is bottom. Wild-type early embryos were costained either with mouse monoclonal antibody K76, which recognizes an epitope on PGL-1 (A, C, and E), and affinity-purified rabbit anti-PGL-2 antibody (B, D, and F), or with affinity-purified rabbit anti-PGL-1 antibody (G, I, and K) and affinity-purified rat anti-PGL-3 antibody (H, J, and L). PGL-1 and PGL-3 are associated with P granules in the germline blastomeres. Shown are P_0 (A, G, and H), P_2 (C, I, and J), and P_4 (E, K, and L) of 1-, 4-, and 28- and 44-cell embryos, respectively. PGL-2 is not detectable in embryos (B, D, and F). Bar, 10 μm.

examined. In *pgl-1* mutant worms, both PGL-2 and PGL-3 localize to P granules (Figure 7, D and H); this is observed even in the defective mutant gonads of *pgl-1* mutant worms raised at the restrictive temperature. Similarly, in *pgl-2* and *pgl-3* mutant worms, the remaining PGL proteins localize to P granules at all temperatures (not shown). Moreover, in *pgl-2; pgl-1*, *pgl-2; pgl-3*, and *pgl-1; pgl-3* double-mutant worms, the single remaining PGL protein localizes to P granules at all temperatures (not shown). These results indicate that, although the PGL proteins associate with each other *in vitro* and *in vivo*, this association is not required for any of them to localize to P granules. All three PGL proteins depend on GLH-1 for proper localization to P granules (KAWASAKI *et al.* 1998; KUZNICKI *et al.* 2000; N. MEYER, A. ORSBORN, K. BENNETT and S. STROME, unpublished results), suggesting that GLH-1 is upstream of all three PGL proteins in a P-granule assembly pathway. Consistent with this, the localization of GLH-1 to P granules in the adult germline does not require the presence of any of the three PGL proteins (Figure 10F). A normal pattern of GLH-1 in embryos may require the PGLs, a possibility that is being investigated (N. MEYER and S. STROME, unpublished results). Regardless of the order of assembly, we have found no evidence from *in vitro* binding tests for a direct interaction between PGL-1 and GLH-1 (data not shown).

PGL-3, but not PGL-2, has a redundant function with PGL-1 in hermaphrodite germline development: To examine the roles of PGL-2 and PGL-3 in *C. elegans* development and to assess their functional redundancy with PGL-1, we isolated deletion alleles of *pgl-2* and *pgl-3* (see MATERIALS AND METHODS). The *pgl-2* allele, *bn123*, has a 472-bp deletion (corresponding to nucleotides 7839–8310 in cosmid B0523) accompanied by a 16-bp insertion of unknown sequence. The deletion/insertion is predicted to cause premature termination after the first 188 amino acids of PGL-2 (asterisk in Figure 2). The *pgl-3* allele, *bn104*, has a 2456-bp deletion (corresponding to nucleotides 12,925–15,380 in cosmid C18G1). The deletion starts 167 bp upstream of the first ATG and removes the N-terminal 654 amino acids of the 693 amino acids of the predicted PGL-3 protein (Figure 2). Thus, both *bn123* and *bn104* are predicted to be either null or strong loss-of-function alleles. Indeed, as described above, *pgl-2(bn123)* and *pgl-3(bn104)* mutant worms fail to stain with anti-PGL-2 (Figure 6C) and anti-PGL-3 antibody (Figure 6F), respectively. Using a null allele of *pgl-1* (*bn101* or *ct131*), *pgl-2(bn123)*, and *pgl-3(bn104)*, we constructed all combinations of double and triple mutants and analyzed their phenotypes as described below.

pgl-1 mutants develop into sterile worms at elevated temperature (KAWASAKI *et al.* 1998). We initially compared *pgl-2*, *pgl-3*, and double and triple mutants with *pgl-1* by shifting homozygous mutant mothers (P_0's) from 20° to 16°, 20°, and 26° as L4's and assessing the fertility/sterility of their F_1 hermaphrodite progeny (Table 1). When grown at permissive temperatures (16° and 20°), 10–14% of *pgl-1* single-mutant hermaphrodites developed into sterile adults that lacked healthy looking embryos in their uterus (their uterus either contained a mass of unfertilized gametes/degenerating embryos or was empty). Growth at restrictive temperature (26°) resulted in 99% sterile adult hermaphrodites. *pgl-2* single mutants did not display significant sterility at any temperature and did not enhance the sterility of *pgl-1* at permissive temperatures. *pgl-3* single mutants also did not display significant sterility at any temperature, but did enhance the sterility of *pgl-1* at permissive temperatures: 71 and 80% of F_1 hermaphrodite progeny from *pgl-1; pgl-3* double-mutant mothers were sterile (38 and 33% of them possessed an empty uterus) at 16° and 20°, respectively. Triple mutants resembled *pgl-1; pgl-3* double mutants. These results suggest that PGL-1 and PGL-3, but not PGL-2, function redundantly and that the low-temperature fertility of most *pgl-1* mutants depends on worms having functional PGL-3 protein.

Sterility in *pgl-1* mutants at 26° has both a maternal and a zygotic component (KAWASAKI *et al.* 1998). The

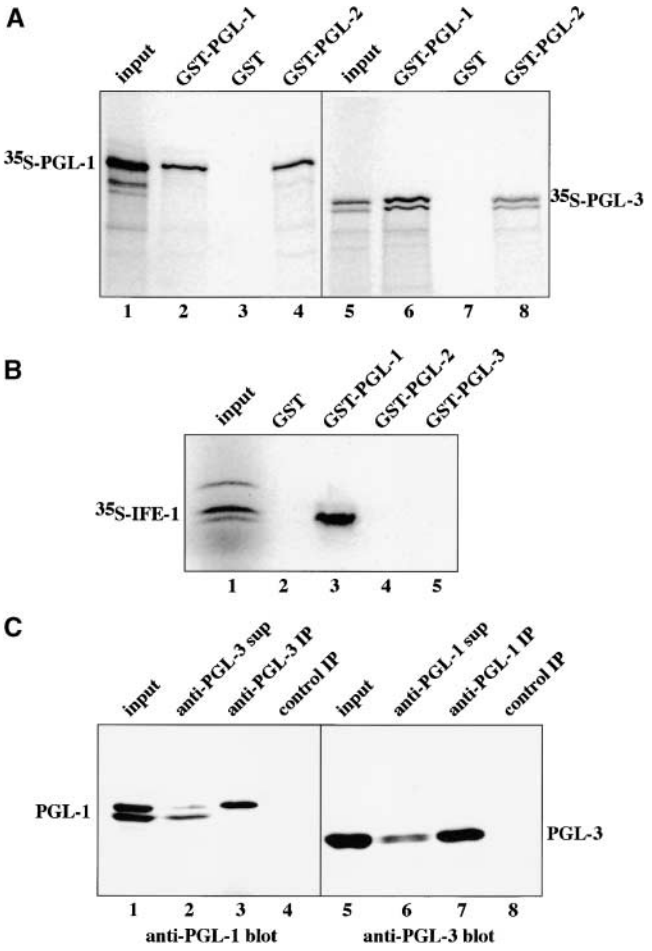

FIGURE 9.—Analysis of PGL protein interactions *in vitro* and *in vivo*. (A and B) Full-length PGL-1, PGL-2, and PGL-3 fused to GST were expressed in *E. coli*, purified, immobilized on glutathione-agarose beads, and tested for binding to radiolabeled full-length PGL-1, PGL-3, and IFE-1 proteins produced by *in vitro* transcription-translation (see MATERIALS AND METHODS). Equal amounts of the ^{35}S-labeled proteins were incubated with GST and each GST fusion protein attached to glutathione beads. After extensive washes, binding of ^{35}S-labeled proteins to GST fusion proteins on the beads was assessed by 10% SDS-PAGE and autoradiography. The input lanes (lanes 1 and 5) contain 10% of the radiolabeled protein used in each binding assay. (A) ^{35}S-PGL-1 and ^{35}S-PGL-3 bind to GST-PGL-1 (lanes 2 and 6) and GST-PGL-2 (lanes 4 and 8), but not to GST control protein (lanes 3 and 7). (B) ^{35}S-IFE-1 binds to GST-PGL-1 (lane 3), but not to GST, GST-PGL-2, or GST-PGL-3 (lanes 2, 4, and 5). (C) Wild-type embryo extract was immunoprecipitated with either rat anti-PGL-3 antiserum (lanes 2 and 3) or rabbit anti-PGL-1 antiserum (lanes 6 and 7) after the extract was preabsorbed with either rat normal serum (lane 4) or rabbit normal serum (lane 8), respectively. Samples of the embryo extract input (lanes 1 and 5) and immunoprecipitated (IP) and unbound (sup) fractions were analyzed by Western blot analysis using either affinity-purified rabbit anti-PGL-1 (lanes 1–4) or affinity-purified rat anti-PGL-3 (lanes 5–8). The input lanes contain 10% of the protein used in each immunoprecipitation.

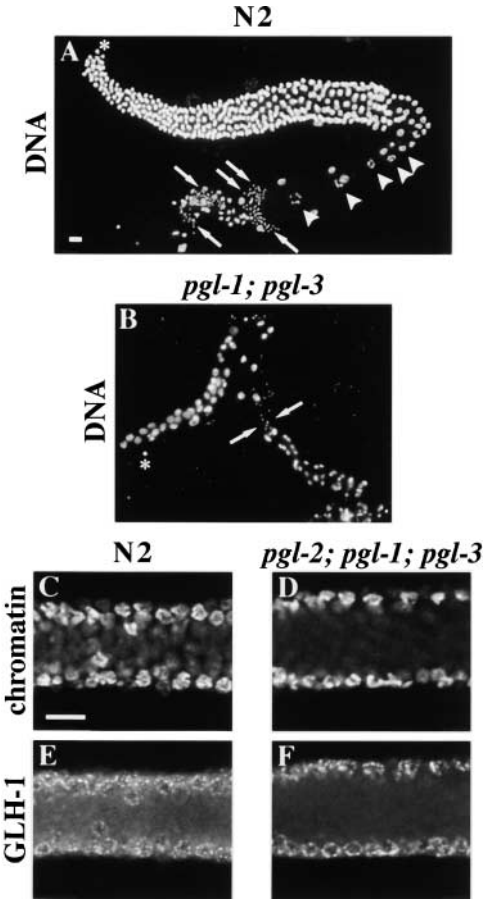

FIGURE 10.—Analysis of germlines in *pgl* double and triple mutants. Gonad arms were extruded from wild-type N2 (A, C, and E), *pgl-1(bn101); pgl-3(bn104)* sterile (B), or *pgl-2(bn123); pgl-1(bn101); pgl-3(bn104)* fertile (D and F) adult hermaphrodites raised at 20°. For A and B, gonads were fixed and stained with DAPI to visualize nuclei; the distal end of each gonad is indicated with an asterisk. For C–F, gonads were fixed and double stained with PA3 mouse monoclonal antibody to chromatin (C and D) and rabbit anti-GLH-1 (E and F); an optical section in the pachytene region of the distal gonad is shown. (A) Gonad arm from a wild-type hermaphrodite. The germline is well proliferated. Arrowheads indicate diakinesis-stage oocytes. Arrows indicate sperm. (B) Gonad arm from a *pgl-1; pgl-3* sterile mutant. The germline lacks detectable PGL-1 and PGL-3 (not shown). It is underproliferated, lacks oocytes, and contains a few sperm. Mutant germlines tend to degenerate and shrink, especially in the proximal region, as worms age. (C and E) Gonad from a wild-type hermaphrodite. (D and F) Gonad from a *pgl-2; pgl-1; pgl-3* fertile hermaphrodite. In both samples, anti-GLH-1 stains perinuclear P granules (E and F). Bars, 10 μm.

zygotic component is seen in the F_1 *pgl-1/pgl-1* progeny from *pgl-1/+* mothers. These F_1 homozygous progeny inherit maternal *pgl-1*(+) product but do not synthesize zygotic product, and presumably as a result, some of the worms are sterile at 26° (KAWASAKI *et al.* 1998). To examine the zygotic component of *pgl-3* enhancement of *pgl-1* sterility, we allowed *pgl-1 unc-24/+ +; pgl-3 dpy-11/+ +* mothers ($n = 8$) to produce self-cross progeny at permissive temperature and compared the sterility of *pgl-1 unc-24; pgl-3 dpy-11* (Unc-24; Dpy-11) progeny and *pgl-1 unc-24* (Unc-24) progeny. Scoring only the "empty uterus" class of sterile worms, we observed higher sterility in *pgl-1; pgl-3* double-mutant progeny (14%, $n = 83$)

TABLE 1

Percentage of sterile hermaphrodite progeny produced by *pgl* mutant mothers at different temperatures

Genotype	% sterile F_1's at 16°	% sterile F_1's at 20°	% sterile F_1's at 26°
unc-24 (IV); dpy-11 (V)	0 (0)	0 (0)	0.5 (0.5)
pgl-1 unc-24 (IV)	14 (3)	10 (1)	99 (99)
pgl-2 (III)	0 (0)	0 (0)	0.2 (0.2)
pgl-3 dpy-11 (V)	0.5 (0.2)	0.1 (0.1)	2.2 (0.6)
pgl-2 (III); pgl-1 unc-24 (IV)	5 (2)	9.2 (1)	99 (99)
pgl-2 (III); pgl-3 dpy-11 (V)	0.4 (0)	0.5 (0.1)	0.6 (0.6)
pgl-1 unc-24 (IV); pgl-3 dpy-11 (V)	71 (38)	80 (33)	100 (100)
pgl-2 (III); pgl-1 unc-24 (IV); pgl-3 dpy-11 (V)	65 (42)	77 (32)	100 (100)

P_0 mothers were transferred to the indicated temperatures as L4's. Their F_1 hermaphrodite progeny were scored as adults on a dissecting microscope for sterility *vs.* fertility. The percentage of sterile F_1's represents the percentage of F_1 hermaphrodite progeny that lacked healthy appearing embryos in their uteruses. Numbers in parentheses indicate the percentage of F_1 hermaphrodite progeny that had an empty uterus. More than 500 F_1 worms were scored for each genotype/temperature, except that 96 *pgl* triple-mutant worms were scored at 26°. The *bn101* allele of *pgl-1* was used.

than in *pgl-1* single-mutant progeny (2%, $n = 248$). Thus, *pgl-3* enhancement of *pgl-1* sterility has a zygotic component. Also, because *pgl-1/+; pgl-3/+* out-cross progeny from *pgl-1; pgl-3* double-mutant mothers show higher "empty uterus" class sterility (17%, $n = 465$) than do *pgl-1/+* out-cross progeny (3%, $n = 645$) from *pgl-1* single-mutant mothers at permissive temperature, *pgl-3* enhancement also has a maternal component.

***pgl-1; pgl-3* hermaphrodites show more severe germline defects than *pgl-1* hermaphrodites do:** To characterize how germline development is compromised in *pgl* multiple mutants, individual gonads of mutant hermaphrodites raised at restrictive temperature were examined and classified (Table 2). In wild-type adult hermaphrodites, each of the two gonad arms contains >300 germ nuclei at 26° (*unc-24; dpy-11* in Table 2). Each gonad arm exhibits a distal-to-proximal pattern of development (Schedl 1997): a distal region in which germ nuclei divide mitotically (this region contains some phospho-histone H3-positive M-phase nuclei); a transition zone in which germ nuclei exit from the mitotic cell cycle and enter meiotic prophase I (indicated by the appearance of HIM-3, a meiotic chromosome core component; Zetka *et al.* 1999); and a pachytene zone in which germ nuclei exhibit a characteristic thread-like chromatin morphology [seen by 4′,6-diamidino-2-phenylindole (DAPI) staining]. In the proximal region of each arm, oocytes develop and arrest at diakinesis of meiotic prophase I (Figure 10A). Sperm made during the L4 larval stage (recognized by the spermatogenesis-specific antibody SP56) are stored in the spermatheca through which oocytes pass and become fertilized.

pgl-1 hermaphrodites raised at 26° are almost 100% sterile (Table 1) and contain a moderately underproliferated germline (Table 2). Each gonad arm contains ~150 germ nuclei on average, and ~25% of the gonad arms lack phospho-histone H3 staining and therefore M-phase nuclei. However, the degree of underproliferation is highly variable among *pgl-1* gonad arms (Kawasaki *et al.* 1998). Seventy percent of the gonad arms contain relatively few germ nuclei, generally lack SP56 sperm staining (~80% of this subpopulation), and do

TABLE 2

Analysis of germline development in *pgl* mutant hermaphrodites raised at 26°

Hermaphrodite genotype	Average no. of germ nuclei per gonad arm[a]	% gonad arms lacking phospho-histone H3 positive germ nuclei	% gonad arms lacking oocytes
unc-24 (IV); dpy-11 (V)	310 ± 52	0[b]	0
pgl-3 dpy-11 (V)	327 ± 68	0	0
pgl-1 unc-24 (IV)	148 ± 185	23	70
pgl-1 unc-24 (IV); pgl-3 dpy-11 (V)	68 ± 50	48	97
pgl-2 (III); pgl-1 unc-24 (IV); pgl-3 dpy-11 (V)	66 ± 42	50	95

P_0 mothers were transferred to 26° as L4's, and the germlines of their young adult F_1 hermaphrodite progeny were analyzed. The *bn101* allele of *pgl-1* was used.

[a] Average number ± standard deviation. A total of 40–92 gonad arms were scored for each genotype.

[b] On average, five phospho-histone H3-positive germ nuclei per gonad arm were observed.

TABLE 3

Production of out-cross progeny by *pgl* mutant males at different temperatures

Male genotype	Average no. of out-cross progeny per male[a] (% out-cross progeny)[b] at 20°	Average no. of out-cross progeny per male[a] (% out-cross progeny)[b] at 25°
him-3 (IV)	149 ± 74 (82)	57 ± 71 (45)
pgl-1 him-3 (IV)	61 ± 83 (33)	1 ± 3 (1)
pgl-2 (III); him-3 (IV)	120 ± 71 (85)	43 ± 71 (33)
him-3 (IV); pgl-3 (V)	120 ± 55 (89)	33 ± 55 (27)
pgl-2 (III); pgl-1 him-3 (IV)	65 ± 87 (35)	4 ± 18 (4)
pgl-1 him-3 (IV); pgl-3 (V)	22 ± 41 (14)	0 ± 0 (0)

P_0 mothers were transferred to 20° or 25° as L4's. Their male F_1 progeny were mated to *unc-24; dpy-11* hermaphrodites and analyzed for production of out-cross progeny. The *ct131* allele of *pgl-1* was used.

[a] Average number ± standard deviation. A total of 18–30 F_1 males were analyzed for each genotype/temperature.

[b] Percentage of out-cross progeny among total (both self-cross and out-cross) F_2 progeny scored.

not contain any oocytes (Table 2). The remaining 30% of the gonad arms contain more germ nuclei and contain oocytes, which are abnormal in appearance and are defective.

pgl-3 hermaphrodites raised at 26° are fertile (Table 1) and contain a well-proliferated and fully differentiated germline (Table 2). However, *pgl-1; pgl-3* double-mutant hermaphrodites raised at 26° display more severe germline defects than do *pgl-1* single-mutant hermaphrodites (Table 2). *pgl-1; pgl-3* gonad arms contain ~70 germ nuclei on average, and ~50% of the gonad arms lack phospho-histone H3 staining. Approximately 70% of the gonad arms lack SP56 sperm staining and 97% of them do not contain any oocytes. Although many of them contain transition zone (HIM-3 positive) nuclei, pachytene nuclei are rarely observed (as judged by nuclear morphology in DAPI-stained samples). *pgl-1; pgl-3* gonads tend to degenerate as adults age, leading to progressively more abnormal morphologies.

At lower temperatures, *pgl-1; pgl-3* double-mutant hermaphrodite germlines are also more underproliferated and more undifferentiated (Figure 10B) than *pgl-1* single-mutant hermaphrodite germlines: *pgl-1; pgl-3* double-mutant hermaphrodites and *pgl-1* single-mutant hermaphrodites contain ~150 and ~500 germ nuclei per gonad arm on average, and ~30 and ~2% of their gonad arms lack oocytes, respectively.

In summary, absence of PGL-3 enhances the Pgl-1 mutant phenotype in hermaphrodite germlines in several respects: increased percentage of animals that are sterile at lower temperatures and more pronounced defects in germ cell proliferation and in production of sperm and oocytes at all temperatures. Absence of PGL-2 does not further enhance any of these phenotypes.

The ability of *pgl-1; pgl-3* males to produce out-cross progeny is greatly reduced: To test whether *pgl* mutants are also defective in male germline development, *pgl* mutations were combined with a *him-3* mutation, *e1147* (Zetka *et al.* 1999), which leads to increased production of self-cross male progeny. Mutant P_0 mothers were shifted to either 20° or 25°, single F_1 self-cross male progeny were mated with single *unc-24; dpy-11* hermaphrodites at 20° or 25°, and the number of non-Unc non-Dpy out-cross F_2 progeny was counted (Table 3). At both temperatures, *pgl-1* males produced substantially fewer out-cross progeny than control *him-3* males did, while *pgl-2* and *pgl-3* mutant males produced close to control numbers of out-cross progeny. Absence of PGL-2 did not enhance the Pgl-1 male phenotype, but absence of PGL-3 did: at 20°, *pgl-1* males, *pgl-2; pgl-1* males, and *pgl-1; pgl-3* males produced 61, 65, and 22 out-cross progeny on average, corresponding to 33, 35, and 14% of total progeny, respectively (Table 3). At 25°, whereas *pgl-1* and *pgl-2; pgl-1* males produced a few out-cross progeny, *pgl-1; pgl-3* males failed to produce any out-cross progeny. Although the ability of *pgl-1* and *pgl-1; pgl-3* males to produce out-cross progeny is greatly reduced at 25°, surprisingly, their gonads have fairly normal morphology. Staining with a DNA dye and the spermatogenesis-specific antibody SP56 revealed that their germlines are well proliferated and contain all of the meiotic stages and mature sperm (data not shown). We noted that *pgl* mutant males appear to be as active as control *him-3* males and show normal mating behavior. We assume that the *pgl* defect in production of out-cross progeny is due to defects in sperm motility and/or fertilization.

Absence of PGL-1 and PGL-3 causes embryonic lethality in addition to germline defects: In addition to their germline defects, *pgl* mutants produce dead embryos, arrested larvae, and more males than usual. To examine those defects, we scored developmental fates of all embryos laid by *pgl* mutant mothers that had been transferred to 26° or 20° as L4's (Table 4, A and B). At 26°, *pgl-2; pgl-1; pgl-3* triple-mutant, *pgl-1; pgl-3* double-mutant, and even *pgl-1* single-mutant mothers laid fewer fertilized embryos (26–50 embryos/mother) than con-

TABLE 4

Developmental fates of *pgl* mutant progeny at different temperatures

Genotype	Average brood size[a]	% embryonic lethal[b]	% larval lethal[c]	% male[d]	% sterile hermaphrodite[e]
		A. At 26°			
unc-24; dpy-11	118 ± 13	2 [1–3] (940)	0 (940)	0.2 (922)	0 (920)
pgl-1 unc-24	50 ± 22	25 [20–43] (397)	9 (397)	5 (262)	100 (250)
pgl-1 unc-24; pgl-3 dpy-11	*31 ± 17*	*37 [14–86] (247)*	*9 (247)*	*2 (134)*	*100 (131)*
pgl-2; pgl-1 unc-24; pgl-3 dpy-11	26 ± 5	58 [33–82] (207)	5 (207)	4 (75)	100 (72)
		B. At 20°			
unc-24; dpy-11	190 ± 14	0.4 [0–1] (1518)	0 (1518)	0.1 (1512)	0 (1511)
pgl-1 unc-24	177 ± 12	5 [1–17] (1412)	0 (1412)	0.8 (1339)	3 (1328)
pgl-1 unc-24; pgl-3 dpy-11	*114 ± 39*	*14 [11–16] (910)*	*0.5 (910)*	*7 (778)*	*30 (720)*
pgl-2; pgl-1 unc-24; pgl-3 dpy-11	156 ± 20	21 [15–29] (1245)	1 (1245)	3 (968)	29 (943)

P_0 mothers were transferred to 26° (A) or 20° (B) as L4's. The broods and fates of F_1 offspring from eight P_0 mothers were analyzed. The *bn101* allele of *pgl-1* was used.

[a] Average number of total embryos laid per mother ± standard deviation.

[b] Percentage of dead embryos. Numbers in brackets indicate ranges of percentage of dead embryos among the eight mothers. Numbers in parentheses indicate total embryos laid.

[c] Percentage of arrested larvae. Numbers in parentheses indicate total embryos laid.

[d] Percentage of male progeny. Numbers in parentheses indicate total adult progeny.

[e] Percentage of sterile hermaphrodite progeny of the "empty uterus" class. Numbers in parentheses indicate total adult hermaphrodite progeny.

trol mothers (~120 embryos/mother) did, indicating reduced numbers or reduced competence of oocytes and/or sperm (Table 4A). Furthermore, a significant fraction of their progeny arrested as late embryos (ranging from 14 to 86% among the mothers) or larvae (5–9% of embryos on average). As a result, they produced very few viable adult progeny: averages of 9, 17, and 33 for the triple, double, and single mutants, respectively, compared to 115 for control worms. Additionally, among the surviving adult progeny, the frequency of males was higher in *pgl-1*-containing mutants (2–5%) than in controls (0.2%). Finally, as discussed above, all of the surviving adult hermaphrodite progeny were sterile. At 20°, although *pgl* mutant mothers laid similar numbers of fertilized embryos as control mothers, *pgl-1; pgl-3* double-mutant and *pgl-2; pgl-1; pgl-3* triple-mutant mothers produced a significant proportion of dead embryos (ranging from 11 to 29% among the mothers) and males (up to 7% on average; Table 4B). As described above, ~30% of the surviving adult hermaphrodite progeny were sterile with an empty uterus.

On the basis of Nomarski observation and DAPI staining, arrest of *pgl-1; pgl-3* double-mutant and *pgl-2; pgl-1; pgl-3* triple-mutant embryos appeared to occur during morphogenesis (ranging from comma to threefold) stages. Because many embryos were fragile and prone to explode, we hypothesized that they had defects in formation of the eggshell/vitelline membrane and in osmoregulation. To test this possibility, *pgl-1; pgl-3* double-mutant embryos and wild-type embryos were released from the uterus of mothers into water containing DAPI. All of the wild-type embryos excluded DAPI and developed well in water. In contrast, many *pgl-1; pgl-3* double-mutant embryos were stained with DAPI, swelled, took on an abnormal appearance, and arrested (data not shown). Thus, at least some *pgl-1; pgl-3* double-mutant embryos appear to be defective in formation of the eggshell/vitelline membrane and in osmoregulation. This phenotype suggests defects during oogenesis or during egg activation upon sperm entry. Such defects probably lead, at least in part, to the high degree of embryonic lethality displayed by *pgl* mutants at 26° (Table 4A). As discussed below, we hypothesize that PGL-1 and PGL-3 are involved in the regulation of many mRNAs in the maternal germline and that the ultimate cause of defects in oogenesis and/or egg activation in *pgl-1; pgl-3* mutants is abnormal localization and/or expression of maternal mRNAs.

DISCUSSION

The PGL and GLH families of P-granule proteins: Two families of proteins, the PGLs and the GLHs, are associated with P granules throughout development and thus are likely to be "core" granule components. Within the PGL family, PGL-1 was discovered through a genetic screen for mutants with altered P-granule staining by a monoclonal antibody (Kawasaki *et al.* 1998), and PGL-2 and PGL-3 were discovered by sequence analysis of the *C. elegans* genome and by a yeast two-hybrid screen for proteins that interact with PGL-1. PGL proteins are novel and do not resemble any of the known components of germ granules in other organisms. PGL-1 and PGL-3 both contain an RGG box and thus are predicted

to bind RNA. PGL-2 lacks an RGG box and also differs from PGL-1 and PGL-3 in being undetectable in embryos. PGL-1 appears to be the most important of the three for germline development, as loss of PGL-1 alone results in sterility at elevated temperature, and, among the three PGL proteins, only PGL-1 can bind to IFE-1, a germline-enriched isoform of eukaryotic initiation factor 4E (eIF4E) that is required for spermatogenesis (Amiri *et al.* 2001). Loss of PGL-2 alone or PGL-3 alone does not cause sterility. However, loss of both PGL-1 and PGL-3 results in more severe germline defects at elevated temperature and in sterility at lower temperatures as well, indicating that PGL-3 functions redundantly with PGL-1. Even though loss of PGL-2 does not cause any obvious germline problems in worms grown in the laboratory, it may contribute to fertility in the wild.

The GLH proteins were identified as *C. elegans* homologs of Drosophila Vasa, a DEAD-box RNA helicase that is a component of Drosophila germ granules (Hay *et al.* 1988; Lasko and Ashburner 1988; Roussell and Bennett 1993; Gruidl *et al.* 1996; Kuznicki *et al.* 2000). Of the four GLH proteins, GLH-1 appears to serve the most important role in the germline; reduction of *glh-1* function results in sterility at elevated temperature, while reduction of *glh-2*, *glh-3*, or *glh-4* function does not result in significant sterility at any temperature (Gruidl *et al.* 1996; Kawasaki *et al.* 1998; Kuznicki *et al.* 2000; N. Meyer, A. Orsborn, K. Bennett and S. Strome, unpublished results). Similar to the PGL-1-PGL-3 relationship, GLH-4 appears to serve a redundant role with GLH-1, as loss of both *glh-1* and *glh-4* functions results in sterility at low temperature (Kuznicki *et al.* 2000). GLH-2 and GLH-3, like PGL-2, may contribute in subtle ways to fertility.

P-granule assembly: Results of molecular epistasis analyses indicate that each GLH protein can assemble into P granules independently of the other GLHs (Kuznicki *et al.* 2000) and that at least GLH-1 can assemble independently of the PGLs. Each PGL protein can assemble into P granules independently of the other PGLs, but all three PGLs depend upon GLH-1 for their efficient recruitment to or retention by P granules (this article; N. Meyer, A. Orsborn, K. Bennett and S. Strome, unpublished results). IFE-1, a germline-enriched isoform of eIF4E that binds mRNA caps, requires PGL-1 to assemble into P granules (Amiri *et al.* 2001). Consistent with this, IFE-1 interacts with PGL-1 but not with PGL-2 or PGL-3 in GST pull-down assays (Amiri *et al.* 2001; this article). These findings generally support a P-granule assembly pathway in which GLH-1 is early in the pathway, the three PGLs are downstream of GLH-1, and IFE-1 is downstream of PGL-1. Interestingly, no mutant has been discovered that prevents P-granule assembly altogether. However, RNAi depletion of core splicing factors, such as the Sm proteins, U2AF, and U170K, disrupts the localization of all three PGLs to granules and may disrupt granule integrity altogether (Barbee *et al.* 2002; I. Kawasaki, unpublished result), suggesting that mRNA processing or export from the nucleus is required to build, stabilize, or localize P granules. The finding that at least some of the Sm proteins associate with P granules raises another possibility: that the physical presence of the proteins in P granules, and not necessarily their splicing functions, is critical to granule integrity.

The protein-protein interactions that have been documented are PGL-1-PGL-1, PGL-1-PGL-2, PGL-1-PGL-3, PGL-2-PGL-3, and PGL-1-IFE-1. PGL-3 appears to associate specifically with the higher-molecular-weight band of the two PGL-1 bands present in embryo extracts. Interestingly, the higher-molecular-weight PGL-1 band is specifically enriched in the nuclear membrane fraction during the course of subcellular fractionation of both embryo extracts and adult worm extracts, whereas the lower PGL-1 band is detected mainly in the cytoplasmic fraction (I. Kawasaki, unpublished result). These results raise the possibility that post-translational modification, such as phosphorylation, affects recruitment of components to perinuclearly localized P granules.

Drosophila polar granules differ from *C. elegans* P granules in several respects. The Drosophila genome lacks an obvious *pgl* homolog, and the *C. elegans* genome lacks an obvious *oskar* homolog. Thus, those germ-granule components are not shared between flies and worms. In flies, germ-granule components are not encoded by multi-gene families, and mutations in numerous polar-granule genes (*e.g.*, *oskar* and *vasa*) prevent the assembly of polar granules during oogenesis (reviewed in Williamson and Lehmann 1996). The "polar-granule null" phenotype appears to be failure to form primordial germ cells and failure to correctly pattern the embryo (Lehmann and Nusslein-Volhard 1986). The latter phenotype is due to a role for Nanos in translational regulation in the somatic embryo as well as in primordial germ cells (Kobayashi *et al.* 1996; Forbes and Lehmann 1998).

The PGL-null phenotype: Since no mutant isolated to date abolishes P granules altogether, the "P-granule null" phenotype is not known at present. This article presents analysis of the phenotype seen in the absence of the entire PGL family. It is useful to consider the defects observed as worms growing at elevated temperature experience progressively declining levels of PGL protein(s) (Kawasaki *et al.* 1998; this article). *pgl-1/pgl-1* F_1 progeny from *pgl-1/+* mothers contain a maternal load of PGL-1 protein, which remains detectable in P granules until at least the L3 larval stage. By adulthood, we expect that P granules in these F_1 worms will be virtually devoid of PGL-1, and probably as a result, ~40% of the F_1's are sterile: their germlines are well proliferated and contain gametes, but they produce no viable progeny. The "fertile" F_1's actually have greatly reduced fertility: they produce reduced numbers of F_2 embryos, ~35% of which die during embryogenesis or

larval development. The few F_2 worms that survive to adulthood are sterile: their germlines are significantly underproliferated and generally lack gametes. The simultaneous loss of PGL-3 enhances all aspects of the phenotype described above for *pgl-1*. Somewhat surprisingly, the PGL-null (*i.e.*, *pgl-2; pgl-1; pgl-3* triple mutant) phenotype remains a mix of zygotic sterility, maternal-effect lethality, and maternal-effect sterility. We think that this reflects the progressive loss of PGL proteins from P granules in F_1 worms and the progressive compromise of P-granule function(s) in F_1 and F_2 worms.

The temperature sensitivity of Pgl phenotypes: We previously speculated that loss of PGL-1 results in sterility predominantly at elevated temperature because redundant factors are sufficient to confer fertility at low temperatures. Indeed, PGL-3 appears to serve just such a redundant role. However, the phenotype of *pgl-1; pgl-3* double (and of *pgl-2; pgl-1; pgl-3* triple) mutants is still more pronounced at high temperature. The temperature sensitivity of Pgl phenotypes may reflect a role for PGL proteins in maintaining the stability and function of multi-protein complexes, most likely P granules. P-granule complexes may be unstable or prone to inactivation, especially at elevated temperature. The presence of PGL-1 in the complex may contribute to maintaining the structure/function of granules at all temperatures. In the absence of PGL-1, PGL-3 may serve this role adequately at low temperatures but not at 26°. In the absence of both PGL-1 and PGL-3, P-granule complexes may tend to dissociate or lose activity, and elevated temperature may further promote granule dissociation/inactivation. An alternative scenario is that the process(es) in which the PGL proteins participate (*e.g.*, export of mRNAs from germ nuclei to the cytoplasm) is inherently sensitive to temperature: in the absence of PGL function, the process(es) can occur with reasonable fidelity or efficiency at lower temperatures but not at elevated temperatures. Whatever the molecular explanation, it is noteworthy that the phenotypes caused by loss of other P-granule components (*e.g.*, *glh-1* and *ife-1*) are also more pronounced at elevated temperature (Kawasaki *et al.* 1998; Amiri *et al.* 2001; N. Meyer, A. Orsborn, K. Bennett and S. Strome, unpublished results). Interestingly, germline expression of transgenes from extrachromosomal arrays is also sensitive to temperature (*e.g.*, Strome *et al.* 2001).

Likely function(s) of P granules and PGL proteins: Schisa *et al.* (2001) observed that perinuclear P granules are rich in RNA and contain even higher levels of RNA under conditions that result in reduced levels of PGL-1, GLH-1, and GLH-2. On the basis of these and other findings, they hypothesized that a primary role of perinuclear P granules in the germline is in export of mRNAs from nuclei to the cytoplasm and that the PGL and GLH proteins promote export. An attractive scenario is that the progressive and variable defects observed in *pgl-2; pgl-1; pgl-3* triple mutants are due to progressive and variable loss of the ability of P granules to export mRNAs. In F_1 *pgl/pgl* worms from *pgl/+* mothers, export may be only mildly impaired, especially early in larval development when some maternal PGL product persists. Some P granules may be more impaired than others, and the export of some mRNAs may be affected more than the export of other mRNAs, leading to variability in defects. By adulthood, those F_1 *pgl/pgl* worms may have lost the ability to export and express at least some mRNAs needed for oogenesis and early embryogenesis, leading to production of defective oocytes and embryos (*i.e.*, sterility and maternal-effect lethality). Certainly any F_2 embryos that survived would be expected to have severely compromised P granules, which might be fully impaired in mRNA export. This could lead to the problems observed in proliferation of the germline in F_2 worms and to their sterility (*i.e.*, maternal-effect sterility).

Another potential role for P granules is delivery of maternal proteins and mRNAs to particular blastomeres during early embryogenesis. Among the proteins that are known to transiently associate with P granules in early embryos are the CCCH-type zinc-finger proteins, PIE-1 (Mello *et al.* 1992, 1996), MEX-1 (Guedes and Priess 1997), and POS-1 (Tabara *et al.* 1999), and the KH-domain proteins, MEX-3 (Draper *et al.* 1996) and GLD-1 (Jones *et al.* 1996). PIE-1, MEX-1, and POS-1 become enriched in the germline blastomeres and participate in preventing the germline blastomeres from adopting somatic fates. Studies of PIE-1 localization illustrate the diversity of mechanisms that can be used to concentrate a protein in the germline blastomere: PIE-1 associates with germline-destined P granules, PIE-1 is selectively retained on the centrosome destined for the germline blastomere, and PIE-1 in the cytoplasm of somatic blastomeres is degraded (Mello *et al.* 1996; Reese *et al.* 2000; DeRenzo *et al.* 2003). It is currently not known whether P granules serve a major or minor role in the delivery of specific proteins to the germline blastomeres and whether the proteins that transiently associate with P granules in the early embryo are important for P-granule functions.

Finally, P granules may recruit translation factors and in that way activate or repress translation. For example, the association of IFE-1, a germline-enriched isoform of eIF4E, with P granules may activate translation of some granule-associated mRNAs. Alternatively, the association of IFE-1 with P granules may inhibit translation: IFE-1 may be sequestered away from the translation machinery, or the binding of IFE-1 to PGL-1 may prevent the binding of IFE-1 to eIF4G, which is essential for initiation of translation (Sonenberg 1996). Indeed, PGL-1 and PGL-3 possess the amino acid motif YXXXXLϕ (where ϕ is a hydrophobic amino acid and X is any amino acid), which is used by eIF4G, eIF4E-binding proteins, and Maskin to bind eIF4E (Mader *et al.* 1995; Amiri *et al.* 2001; I. Kawasaki, unpublished result). However, our

GST pull-down experiments revealed that IFE-1 interacts with PGL-1 but not with PGL-3. Furthermore, a PGL-1 fragment C-terminal of the YXXXXLϕ motif appears to mediate binding of PGL-1 to IFE-1 (N. Meyer and S. Strome, unpublished result).

Future identification of the RNAs to which the PGL proteins bind will help elucidate how the PGLs and P granules function during germline development.

We thank Y. Kohara for allowing I.K. to use his research facilities and for providing the yk clones, R. Barstead for providing the *C. elegans* cDNA library λACT-RB1 for our yeast two-hybrid screens, A. Sugimoto for allowing us to screen her deletion libraries for a *pgl-3* deletion mutant, M. Zetka for providing anti-HIM-3 antibody, M. Monestier for providing the antichromatin antibody PA3, and members of the Strome lab for critical reading of this manuscript. Some nematode strains used in this work were provided by the Caenorhabditis Genetics Center, which is funded by the National Institutes of Health (NIH) National Center for Research Resources. I.K., A.A., Y.F., N.M., and S.D. were supported by NIH grant GM-34059 to S.S. I.K. was also supported by Precursory Research for Embryonic Science and Technology, Japan Science and Technology Agency.

LITERATURE CITED

Amiri, A., B. D. Keiper, I. Kawasaki, Y. Fan, Y. Kohara *et al.*, 2001 An isoform of eIF4E is a component of germ granules and is required for spermatogenesis in *C. elegans*. Development **128:** 3899–3912.

Barbee, S. A., A. L. Lublin and T. C. Evans, 2002 A novel function for the Sm proteins in germ granule localization during *C. elegans* embryogenesis. Curr. Biol. **12:** 1502–1506.

Barstead, R. J., and R. H. Waterston, 1989 The basal component of the nematode dense-body is vinculin. J. Biol. Chem. **264:** 10177–10185.

Beanan, M. J., and S. Strome, 1992 Characterization of a germ-line proliferation mutation in *C. elegans*. Development **116:** 755–766.

Brenner, S., 1974 The genetics of *Caenorhabditis elegans*. Genetics **77:** 71–94.

Burd, C. G., and G. Dreyfuss, 1994 Conserved structures and diversity of functions of RNA-binding proteins. Science **265:** 615–621.

C. elegans Sequencing Consortium, 1998 Genome sequence of the nematode *C. elegans*: a platform for investigating biology. Science **282:** 2012–2018.

DeRenzo, C., K. J. Reese and G. Seydoux, 2003 Exclusion of germ plasm proteins from somatic lineages by cullin-dependent degradation. Nature **424:** 685–689.

Draper, B. W., C. C. Mello, B. Bowerman, J. Hardin and J. R. Priess, 1996 MEX-3 is a KH domain protein that regulates blastomere identity in early *C. elegans* embryos. Cell **87:** 205–216.

Durfee, T., K. Becherer, P.-L. Chen, S.-H. Yeh, Y. Yang *et al.*, 1993 The retinoblastoma protein associates with the protein phosphatase type 1 catalytic subunit. Genes Dev. **7:** 555–569.

Eddy, E. M., 1975 Germ plasm and the differentiation of the germ cell line. Int. Rev. Cytol. **43:** 229–280.

Ephrussi, A., and R. Lehmann, 1992 Induction of germ cell formation by *oskar*. Nature **358:** 387–392.

Evans, D., D. Zorio, M. MacMorris, C. E. Winter, K. Lea *et al.*, 1997 Operons and SL2 trans-splicing exist in nematodes outside the genus *Caenorhabditis*. Proc. Natl. Acad. Sci. USA **94:** 9751–9756.

Fields, S., and O. Song, 1989 A novel genetic system to detect protein-protein interactions. Nature **340:** 245–246.

Forbes, A., and R. Lehmann, 1998 Nanos and Pumilio have critical roles in the development and function of *Drosophila* germline stem cells. Development **125:** 679–690.

Gruidl, M. E., P. A. Smith, K. A. Kuznicki, J. S. McCrone, J. Kirchner *et al.*, 1996 Multiple potential germ-line helicases are components of the germ-line-specific P granules of *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA **93:** 13837–13842.

Guedes, S., and J. R. Priess, 1997 The *C. elegans* MEX-1 protein is present in germline blastomeres and is a P granule component. Development **124:** 731–739.

Hay, B., L. Y. Jan and Y. N. Jan, 1988 A protein component of *Drosophila* polar granules is encoded by *vasa* and has extensive sequence similarity to ATP-dependent helicases. Cell **55:** 577–587.

Holdeman, R., S. Nehrt and S. Strome, 1998 MES-2, a maternal protein essential for viability of the germline in *Caenorhabditis elegans*, is homologous to a *Drosophila* Polycomb group protein. Development **125:** 2457–2467.

Ikenishi, K., 1987 Functional gametes derived from explants of single blastomeres containing the 'germ plasm' in *Xenopus laevis*: a genetic marker study. Dev. Biol. **122:** 35–38.

Illmensee, K., and A. P. Mahowald, 1974 Transplantation of posterior polar plasm in *Drosophila*. Induction of germ cells at the anterior pole of the egg. Proc. Natl. Acad. Sci. USA **71:** 1016–1020.

Jones, A. R., R. Francis and T. Schedl, 1996 GLD-1, a cytoplasmic protein essential for oocyte differentiation shows stage- and sex-specific expression during *Caenorhabditis elegans* germline development. Dev. Biol. **180:** 165–183.

Karashima, T., A. Sugimoto and M. Yamamoto, 2000 *Caenorhabditis elegans* homologue of the human azoospermia factor *DAZ* is required for oogenesis but not for spermatogenesis. Development **127:** 1069–1079.

Kawasaki, I., Y.-H. Shim, J. Kirchner, J. Kaminker, W. B. Wood *et al.*, 1998 PGL-1, a predicted RNA-binding component of germ granules, is essential for fertility in *C. elegans*. Cell **94:** 635–645.

Kiledjian, M., and G. Dreyfuss, 1992 Primary structure and binding activity of the hnRNP U protein: binding RNA through RGG box. EMBO J. **11:** 2655–2664.

Kobayashi, S., M. Yamada, M. Asaoka and T. Kitamura, 1996 Essential role of the posterior morphogen *nanos* for germline development in *Drosophila*. Nature **380:** 708–711.

Kuznicki, K. A., P. A. Smith, W. M. Leung-Chiu, A. O. Estevez, H. C. Scott *et al.*, 2000 Combinatorial RNA interference indicates GLH-4 can compensate for GLH-1; these two P granule components are critical for fertility in *C. elegans*. Development **127:** 2907–2916.

Lasko, P. F., and M. Ashburner, 1988 The product of the *Drosophila* gene *vasa* is very similar to eukaryotic initiation factor-4A. Nature **335:** 611–617.

Lehmann, R., and C. Nusslein-Volhard, 1986 Abdominal segmentation, pole cell formation, and embryonic polarity require the localized activity of *oskar*, a maternal gene in Drosophila. Cell **47:** 141–152.

Mader, S., H. Lee, A. Pause and N. Sonenberg, 1995 The translation initiation factor eIF-4E binds to a common motif shared by the translation factor eIF-4 gamma and the translational repressors 4E-binding proteins. Mol. Cell. Biol. **15:** 4990–4997.

Marsh, J., and J. E. Goode, 1994 *Ciba Foundation Symposium 182: Germline Development*. John Wiley & Sons, Chichester, UK.

Mello, C. C., B. W. Draper, M. Krause, H. Weintraub and J. R. Priess, 1992 The *pie-1* and *mex-1* genes and maternal control of blastomere identity in early *C. elegans* embryos. Cell **70:** 163–176.

Mello, C. C., C. Schubert, B. Draper, W. Zhang, R. Lobel *et al.*, 1996 The PIE-1 protein and germline specification in *C. elegans* embryos. Nature **382:** 710–712.

Olmsted, J. B., 1986 Analysis of cytoskeletal structures using blot-purified monospecific antibodies, pp. 467–472 in *Methods in Enzymology*, edited by R. B. Vallee. Academic Press, Orlando, FL.

Pitt, J. N., J. A. Schisa and J. R. Priess, 2000 P granules in the germ cells of *Caenorhabditis elegans* adults are associated with clusters of nuclear pores and contain RNA. Dev. Biol. **219:** 315–333.

Reese, K. J., M. A. Dunn, J. A. Waddle and G. Seydoux, 2000 Asymmetric segregation of PIE-1 in *C. elegans* is mediated by two complementary mechanisms that act through separate PIE-1 protein domains. Mol. Cell **6:** 445–455.

Roussell, D. L., and K. L. Bennett, 1993 *glh-1*: a germline putative RNA helicase from *Caenorhabditis* has four zinc fingers. Proc. Natl. Acad. Sci. USA **90:** 9300–9304.

Saffman, E. E., and P. Lasko, 1999 Germline development in vertebrates and invertebrates. Cell Mol. Life Sci. **55:** 1141–1163.

Schedl, T., 1997 Developmental genetics of the germ line, pp. 241–269 in *C. elegans II*, edited by D. L. Riddle, T. Blumenthal, B. J. Meyer and J. R. Priess. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Schisa, J. A., J. N. Pitt and J. R. Priess, 2001 Analysis of RNA associated with P granules in germ cells of *C. elegans* adults. Development **128:** 1287–1298.

Seydoux, G., and A. Fire, 1994 Soma-germline asymmetry in the distributions of embryonic RNAs in *Caenorhabditis elegans*. Development **120:** 2823–2834.

Sonenberg, N., 1996 mRNA 5′ cap-binding protein eIF4E and control of cell growth, pp. 245–269 in *Translational Control*, edited by J. W. B. Hershey, M. B. Mathews and N. Sonenberg. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Spieth, J., S. Brooke, S. Kuersten, K. Lea and T. Blumenthal, 1993 Operons in *C. elegans*: polycistronic mRNA precursors are processed by trans-splicing of SL2 to downstream coding regions. Cell **73:** 521–532.

Strome, S., 1986 Establishment of asymmetry in early *Caenorhabditis elegans* embryos: visualization with antibodies to germ cell components, pp. 77–95 in *Gametogenesis and the Early Embryo*, edited by J. G. Gall. Alan R. Liss, New York.

Strome, S., and W. B. Wood, 1982 Immunofluorescence visualization of germ-line-specific cytoplasmic granules in embryos, larvae, and adults of *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA **79:** 1558–1562.

Strome, S., and W. B. Wood, 1983 Generation of asymmetry and segregation of germ-line granules in early *C. elegans* embryos. Cell **35:** 15–25.

Strome, S., J. Powers, M. Dunn, K. Reese, C. J. Malone *et al.*, 2001 Spindle dynamics and the role of gamma-tubulin in early *Caenorhabditis elegans* embryos. Mol. Biol. Cell **12:** 1751–1764.

Subramaniam, K., and G. Seydoux, 1999 *nos-1* and *nos-2*, two genes related to *Drosophila nanos*, regulate primordial germ cell development and survival in *Caenorhabditis elegans*. Development **126:** 4861–4871.

Tabara, H., T. Motohashi and Y. Kohara, 1996 A multi-well version of in situ hybridization on whole mount embryos of *Caenorhabditis elegans*. Nucleic Acids Res. **24:** 2119–2124.

Tabara, H., R. J. Hill, C. C. Mello, J. R. Priess and Y. Kohara, 1999 *pos-1* encodes a cytoplasmic zinc-finger protein essential for germline specification in *C. elegans*. Development **126:** 1–11.

Wang, C., and R. Lehmann, 1991 Nanos is the localized posterior determinant in *Drosophila*. Cell **66:** 637–647.

Williamson, A., and R. Lehmann, 1996 Germ cell development in *Drosophila*. Annu. Rev. Cell Dev. Biol. **12:** 365–391.

Wylie, C., 1999 Germ cells. Cell **96:** 165–174.

Xu, L., Y. Fong and S. Strome, 2001 The *Caenorhabditis elegans* maternal-effect sterile proteins, MES-2, MES-3, and MES-6, are associated in a complex in embryos. Proc. Natl. Acad. Sci. USA **98:** 5061–5066.

Yandell, M. D., L. G. Edgar and W. B. Wood, 1994 Trimethylpsoralen induces small deletion mutations in *Caenorhabditis elegans*. Proc. Natl. Acad. Sci. USA **91:** 1381–1385.

Zetka, M. C., I. Kawasaki, S. Strome and F. Muller, 1999 Synapsis and chiasma formation in *Caenorhabditis elegans* require HIM-3, a meiotic chromosome core component that functions in chromosome segregation. Genes Dev. **13:** 2258–2270.

Communicating editor: R. S. Hawley