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Genetic Requirements for PIE-1 Localization and Inhibition of Gene Expression in the Embryonic Germ Lineage of *Caenorhabditis elegans*

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In early *Caenorhabditis elegans* embryos, production of new mRNAs is inhibited in the germ lineage. This inhibition requires the germline factor PIE-1, and correlates with the absence in germline blastomeres of a phosphoepitope on RNA polymerase II (RNAPII-H5). We show that PIE-1 is uniformly distributed in oocytes and newly fertilized eggs, and becomes localized asymmetrically in the late one-cell stage. To begin to dissect the mechanisms required for PIE-1 localization and inhibition of RNAPII-H5 expression, we have examined the distribution of PIE-1 and RNAPII-H5 in maternal-effect mutants that disrupt embryonic development. We find that mutants that disrupt the asymmetric divisions of germline blastomeres mislocalize PIE-1, and activate RNAPII-H5 expression in the germ lineage. In contrast, mutants that alter somatic cell identities do not affect PIE-1 localization or RNAPII-H5 expression. Our observations suggest that PIE-1 represses mRNA transcription in each germline blastomere in a concentration-dependent manner. We also show that in wild-type, and in mutants where PIE-1 is mislocalized, the cellular and subcellular distribution of PIE-1 remarkably parallels that of the P granules, suggesting that the localizations of these two germline components are coordinately regulated.

Key Words: **PIE-1; RNA polymerase II; CTD phosphorylation; transcriptional repression; P granules; germline; *Caenorhabditis elegans*.**

INTRODUCTION

In the nematode *Caenorhabditis elegans*, formation of the germline begins with the asymmetric division of the zygote (P_0) into a larger somatic blastomere (AB) and a smaller germline blastomere (P_1). The AB blastomere divides equally to generate somatic descendants which divide in synchrony (AB lineage). In contrast, the P_1 germline blastomere divides unequally to give rise to the next germline blastomere P_2 and the somatic blastomere EMS. Unequal germline blastomere divisions continue until four somatic blastomeres (AB, EMS, C, and D) and the primordial germ cell P_4 are formed (Fig. 1).

In addition to their asymmetric division pattern and relatively smaller size, two general properties distinguish germline blastomeres from somatic blastomeres: presence of the germline-specific P granules, and absence of embryonically transcribed mRNAs. P granules are cytoplasmic particles that are present in germ cells throughout the life cycle of *C. elegans* (Strome and Wood, 1982). They contain both RNA and protein, and recently several putative RNA-binding proteins have been shown to localize to the P granules (Draper *et al.*, 1996; Jones *et al.*, 1996; Gruidl *et al.*, 1996). P granules are uniformly distributed in oocytes and newly fertilized eggs, but become localized to the posterior region of the one-cell embryo prior to the first cleavage (Strome and Wood, 1982). This localization requires microfilaments (Strome and Wood, 1983), and is correlated with a general flow of cytoplasm toward the posterior (Hird *et al.*, 1996). During this flow, P granules migrate through the cytoplasm toward the posterior pole where they associate with the cortex; P granules that remain in the anterior are disassembled or degraded (Hird *et al.*, 1996). This asymmetric localization ensures that P granules are segregated only to the germline blastomere P_1. During subsequent divi-

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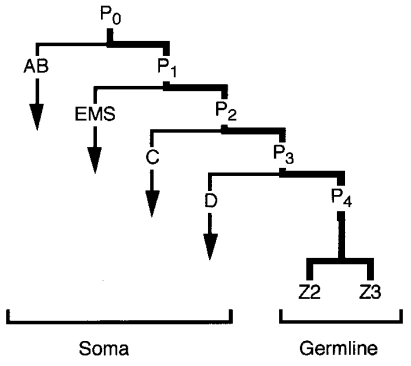


FIG. 1. Origin of the germline in *C. elegans*. The zygote (P_0) undergoes a series of asymmetric divisions (horizontal lines) which give rise to somatic (AB, EMS, C, D), and germline (P_1, P_2, P_3, P_4) blastomeres. In the 100-cell stage, P_4 divides symmetrically to give rise to Z2 and Z3, the two primordial germ cells (Sulston et al., 1983). Thick lines indicate the segregation of the P granules and of PIE-1.

sions, P granules continue to be asymmetrically segregated to germline blastomeres, although a different partitioning mechanism appears to operate in the germline blastomeres P_2 and P_3. In these blastomeres, P granules associate with the nucleus and move with it toward the region of the cytoplasm destined for the germline daughter (Hird *et al.*, 1996). The continuous association of P granules with the germline suggests that they function in some aspects of germline development; the exact function of P granules, however, remains unknown.

A second characteristic which distinguishes germline from soma in early *C. elegans* embryos is the absence of embryonically transcribed mRNAs in germline blastomeres. Embryonically transcribed mRNAs have been detected in somatic blastomeres as early as the 3-cell stage; in contrast, to date no such mRNAs have been detected in germline blastomeres (Seydoux and Fire, 1994; Seydoux *et al.*, 1996). This deficiency correlates with the absence in germline blastomeres of a specific phosphoepitope on the carboxy-terminal domain (CTD) of RNA polymerase II (RNAPII-H5; Seydoux and Dunn, 1997). This phosphoepitope is defined by the monoclonal antibody H5, which recognizes CTD repeats phosphorylated on serine at position 2 (Warren *et al.*, 1992; Bregman *et al.*, 1995; Kim *et al.*, 1997; Patturajan *et al.*, 1998). In mammalian cells, the RNAPII-H5 epitope is present on polymerase subunits engaged in transcriptional elongation (Zeng *et al.*, 1997). In *C. elegans* embryos, RNAPII-H5 appears in somatic blastomeres coincident with the onset of transcription, but is not detected in germline blastomeres until the 100-cell stage (Seydoux and Dunn, 1997). Embryonic germ cells in *Drosophila* also have been shown to lack RNAPII-H5 (Seydoux and Dunn, 1997), and to be deficient in RNAPII-dependent gene expression (Zalokar, 1976; Lamb and Laird, 1976; Kobayashi *et al.*, 1988; Van Doren *et al.*, 1998). These observations have suggested that repression of mRNA transcription may be part of an evolutionarily conserved mechanism that distinguishes germline from soma during early embryogenesis (Seydoux and Dunn, 1997).

Inhibition of RNAPII-dependent gene expression in the embryonic germ lineage of *C. elegans* requires the activity of the *pie-1* gene. In embryos derived from *pie-1* mutant mothers, embryonically transcribed mRNAs and RNAPII-H5 are detected in germline blastomeres (Seydoux *et al.*, 1996; Seydoux and Dunn, 1997). The *pie-1* gene encodes a novel protein, PIE-1, with two CCCH zinc finger-like motifs, also found in the vertebrate TIS11/Nup475/TTP protein (Mello *et al.*, 1996). PIE-1 protein accumulates in the nuclei of germline blastomeres from the late 2-cell stage to the 100-cell stage (Mello *et al.*, 1996). Strikingly, PIE-1's disappearance from the germ lineage in the 100-cell stage coincides with the appearance of mAb H5 immunoreactivity in that lineage (Seydoux and Dunn, 1997). Thus, both the spatial and temporal distributions of PIE-1 are consistent with the possibility that PIE-1 functions to block mRNA transcription in the germ lineage.

To explore this hypothesis further, and to begin to characterize the mechanisms that localize PIE-1 to the germ lineage, we have analyzed the distribution of PIE-1, RNAPII-H5 and P granules in wild-type and in maternal-effect mutants that disrupt early embryogenesis. Our results indicate that PIE-1 and P granule localizations may be coordinately regulated, and support a model in which PIE-1 functions in each germline blastomere as a concentration-dependent inhibitor of mRNA transcription.

MATERIALS AND METHODS

Strains and Maintenance

C. elegans strains were maintained as described by Brenner (1974). Strains used in this study were as follows: LGI, *emb-6(hc65ts), emb-12(g5ts), emb-20(g27ts), zyg-2(b10ts), par-6 (zu222) unc-101 (m1)/hIn1, mes-3(bn35) dpy-5(e61);sDP2, mex-3(zu155)dpy-5/hT1, cib-1(e2300ts), pop-1 (zu189) dpy-5 (e61)/hT1, glp-4(bn2ts);* LGII, *emb-21(g31ts), emb-23(g39ts), emb-27(g48ts), zyg-1(b1ts), zyg-9(b244ts), rol-1(e91) mex-1(zu121/mnC1; rol-1 mes-2(bn11)/ mnC1;* LGIII, *emb-5(hc61ts), emb-8(hc69ts), emb-13(g6ts), emb-16(g19ts), emb-25(g45ts), emb-30(g53ts), emb-33(g60ts), emb-1(hc62ts), emb-7(hc66ts), daf-7(e1372) par-2(it5ts), lon-1 (e185) par-3(e2074); sDp3, pie-1 (zu154) unc-25 (e156)/qCl;* LGIV, *emb-3(hc59ts), emb-11(g1ts), emb-26(g47ts), emb-31(g55ts), mes-6(bn66)DnT1, fem-1(hc17ts), fem-3(g20ts), skn-1(zu67)/nT1; rol-4(sc8) par-1(b274)/nT1;* LGV, *emb-4(hc60ts), emb-18(g21ts), par-4(it47ts), apx-1 (zu183) dpy-11 (e224)/nT1;* LGX, *mes-1(bn7ts).* Temperature-sensitive mutants were maintained at 15°C and shifted to 25°C in the L4 stage in preparation for immunofluorescence experiments; other mutants were maintained at 20°C.

Production of Anti-PIE-1 Monoclonal Antibodies

One hundred micrograms of a peptide corresponding to amino acids 54–73 in PIE-1 (Mello *et al.*, 1996) was injected subcutaneously into RBF/Dn mice (Jackson Laboratory) and reinjected at

2-week intervals. Sera from injected mice were assayed by ELISA, Western blotting, and immunofluorescence. Monoclonal antibodies were generated according to published protocols (Wayner and Carter, 1987) in the Hybridoma Production Facility at the Fred Hutchinson Cancer Research Center. Several monoclonals that recognize PIE-1 in *C. elegans* embryos were isolated, including the IgG1 monoclonal P4G5 used in this study.

Indirect immunofluorescence Microscopy

Gravid hermaphrodites were squashed between slide and coverslip, freeze-cracked on dry ice, and fixed following one of two protocols: 10 to 60 s in −20°C MeOH and 30 min in formaldehyde fix [1× PBS, 0.08 M Hepes (pH 6.9), 1.6 mM $MgSO_4$, 0.8 mM EGTA, 3.7% formaldehyde] for costaining with PIE-1 and RNAPII-H5 antibodies; or 15 min in −20°C MeOH and 15 min in formaldehyde fix for costaining with PIE-1 and P granule antibodies. After fixation, slides were washed five times in PBT (1× PBS, 0.1% Triton, 0.1% BSA), and blocked for 30 min in PBT. Primary antibodies were applied overnight at 4°C, and secondary antibodies were applied for 2 h at room temperature, or overnight at 4°C.

Primary antibodies used were P4G5, an anti-PIE-1 IgG monoclonal antibody (see above); K76, a mouse monoclonal IgM antibody obtained from the Developmental Studies Hybridoma Bank, which recognizes an unknown epitope on P granules (Strome, 1986); and mAb H5, a mouse monoclonal IgM antibody which recognizes phosphorylated serine at position 2 of each CTD repeat in the large subunit of RNAPII (Warren *et al.*, 1992; Bregman *et al.*, 1995; Kim *et al.*, 1997; Patturajan *et al.*, 1998). Secondary antibodies used were: FITC-conjugated goat anti-mouse IgM, and Cy3-conjugated goat anti-mouse IgG (Jackson Immuno Research). DAPI (1 μg/ml) was added to secondary antibody dilutions to visualize DNA. Criteria used to score embryos are described in the legend of Table 1.

To quantitate PIE-1 levels in the one-cell stage, we obtained digitized images of pairs of one-cell embryos using a Dage CCD camera. Each pair consisted of one ''early'' one-cell embryo completing meiosis, and one ''late'' one-cell embryo in mitotic prophase. Embryos in each pair were located in the same field of view, to control for potential variations in staining efficiency within and in between slides. Only digitized images within dynamic range were retained for analysis. Total intensity values were obtained for each embryo using IPLab Spectrum software, and a ratio of late to early values was calculated for each pair. Ratios obtained for each pair were 1.3, 1.4., 1.4, 1.8, 1.5, 1.1, and 1.5, with an average ratio of 1.4.

In Situ Hybridization

In situ hybridization was performed using single-stranded DNA probes labeled with digoxigenin as described in Seydoux and Fire, 1995. *pie-1* probes were derived from JP603 which contains the *pie-1* cDNA (C. Mello, personal communication), and *vet-5* probes were derived from pc101 which contains the *vet-5* cDNA (Seydoux *et al.*, 1996).

Immunoblotting

Total protein extracts were prepared by boiling adult worms twice in denaturing buffer (0.06 M Tris, pH 6.8, 2% SDS, 5% β-mercaptoethanol) and removing the insoluble fraction. Proteins were separated on 10% denaturing gels and transferred to nitrocellulose. Blots were blocked in 5% dry milk for 1 h, incubated with P4G5 antibody (1:200 dilution) for 1 h, followed by HRP-anti mouse IgG (1:2500; Jackson Immuno Research) for 30 min. ECL detection system was used for visualization (Amersham). Anti-tubulin E7 antibody (obtained from the Developmental Studies Hybridoma Bank) was used as a loading control.

Phenotypic Analysis of pie-1 Heterozygotes

pie-1(zu154) unc-25(e156)/dpy-17(e164) unc-32(e189) hermaphrodites were allowed to self for about 6 h at 20 or 25°C, after which adults were removed and the embryos were counted. Sixteen hours later, unhatched embryos were counted to determine the fraction of dead embryos. Live progeny were observed 4 days later for sterility. In two separate trials, no significant lethality or sterility was observed among 2365 progeny of *pie-1(zu154) unc-25(e156)/dpy-17(e164) unc-32(e189).* In parallel experiments *pie-1(zu154) unc-25/dpy-17(e164) unc-32(e189),* and N2 gravid hermaphrodites were immunostained with anti-PIE-1 and mAb H5 antibodies.

RESULTS

pie-1 RNA and Protein Localization in Oocytes and Early Embryos

We have analyzed the distribution of the *pie-1* message by *in situ* hybridization (Fig. 2). The *pie-1* locus encodes a maternal RNA which is distributed uniformly in oocytes and early embryos. After the four-cell stage, *pie-1* RNA was lost from somatic blastomeres and was maintained only in the germ lineage (Fig. 2). This pattern appears to be a common degradation pattern for maternal RNAs (Seydoux and Fire, 1994).

The embryonic distribution of the PIE-1 protein was analyzed previously using three polyclonal antisera (Mello *et al.*, 1996). In this study, we have extended these observations using a monoclonal antibody with increased sensitivity for PIE-1 (Materials and Methods). We used this antibody to describe the distribution of PIE-1 in oocytes and early embryos, and to compare the distribution of PIE-1 to that of the P granules. These analyses were performed by costaining fixed hermaphrodites and embryos with anti-PIE-1 and anti-P granule antibodies as described under Materials and Methods. No PIE-1 was detected in embryos derived from *pie-1(zu154)* mutant mothers (data not shown).

In the adult germline, PIE-1 was detected first in maturing oocytes in the proximal region of each gonadal arm (Fig. 3A; we were first alerted to the presence of PIE-1 in oocytes by L. Timmons and A. Fire, personal communication). PIE-1 was uniformly distributed throughout the cytoplasm of oocytes, as were the P granules. To verify the presence of PIE-1 in oocytes, we performed Western analysis on protein extracts derived from wild-type worms which contain oocytes, sperm, and embryos, *fem-1* worms which contain only oocytes, *fem-3(gf)* worms which contain only sperm, and *glp-4* worms which contain no germ cells. We detected above-background levels of PIE-1 protein only in wild-type

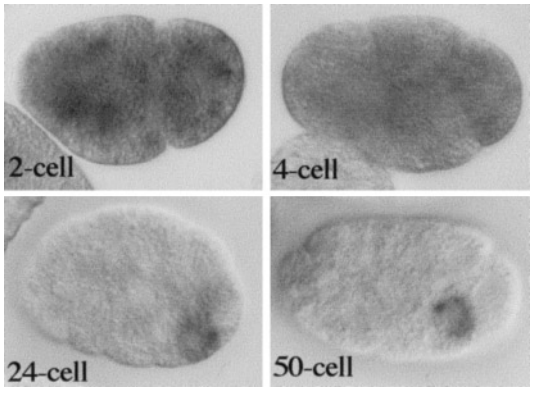


FIG. 2. Localization of *pie-1* mRNA. Wild-type embryos of different stages were hybridized to a *pie-1* antisense probe as described under Materials and Methods. Positive staining is indicated by the dark color. In this and all subsequent figures, embryos are oriented with anterior to the left and posterior to the right. *C. elegans* embryos are approximately 45 μm in length.

and *fem-1* worms, confirming the presence of PIE-1 in oocytes (Fig. 3B).

In newly fertilized eggs, a low level of PIE-1 was uniformly distributed throughout the cytoplasm, as were the P granules (Figs. 4A–4C). During pronuclear migration, when P granules migrate to the posterior, PIE-1 staining decreased in the anterior and increased in the posterior. By pronuclear meeting, both PIE-1 and P granules were detected exclusively in the posterior region of the embryo. In dividing one-cell embryos, PIE-1 was detected throughout the posterior cytoplasm and also on the posterior centrosome (Figs. 4D–4F).

PIE-1 levels appeared to increase during the one-cell stage. We used digitized images to compare PIE-1 levels in one-cell embryos in different stages of the cell cycle. We found that one-cell embryos in mitotic prophase consistently showed higher levels of PIE-1 than newly fertilized embryos (Materials and Methods). These observations suggest that *pie-1* RNA is translated during the one-cell stage. We did not determine whether PIE-1 levels continue to increase after the one-cell stage.

In the germline blastomeres P_1, P_2, and P_3, PIE-1 distribution followed a sequence similar to that observed in the one-cell stage: initially uniform cytoplasmic distribution, followed by asymmetric cytoplasmic localization at the time of P granule migration, and asymmetric cytoplasmic and centrosomal localizations during mitosis (Figs. 4G–4L; and data not shown). In contrast, in the last germline blastomere P_4, which divides symmetrically and segregates P granules to both its descendants, PIE-1 remains uniformly distributed and is segregated equally to both P_4 daughters (Z2 and Z3), where it rapidly becomes undetectable (Mello *et al.*, 1996; Seydoux and Dunn, 1997).

In oocytes, zygotes, and early two-cell embryos, PIE-1 appeared to be excluded from the nucleus (Figs. 3A and 4G). In contrast, starting in the late two-cell stage, PIE-1 was present in both the cytoplasm and the nucleus (Mello *et al.*, 1996; Seydoux and Dunn, 1997). The ratio of nuclear to cytoplasmic PIE-1 appeared to increase after each division, ending with predominantly nuclear PIE-1 in P_4 and in Z2 and Z3 (Mello *et al.*, 1996; Seydoux and Dunn, 1997; C. Tenenhaus and G. Seydoux, unpublished observations).

Although PIE-1 was always detected in areas that also contained P granules, PIE-1 and P granule staining patterns were not identical. PIE-1 staining was predominantly diffuse and cytoplasmic; in contrast, P granule staining was characteristically punctate and was often enriched near the cell cortex (Fig. 4). We occasionally detected higher levels of PIE-1 on P granules (data not shown), as has been reported previously (Mello *et al.*, 1996; Guedes and Priess, 1997). These observations suggest that PIE-1 resides in the cytoplasm surrounding the P granules, and that a subset of PIE-1 molecules may associate directly with the P granules.

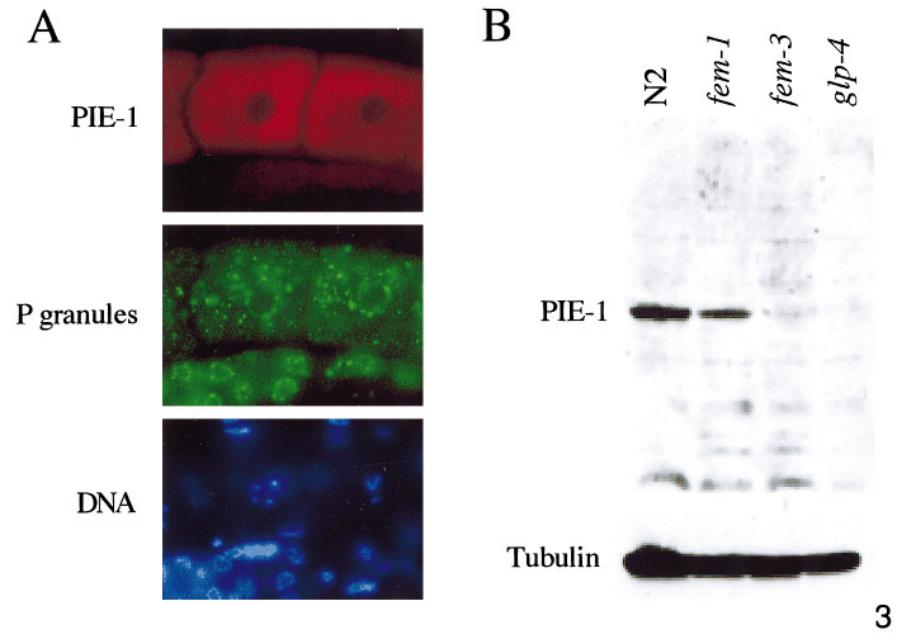
PIE-1 Localization in Mutants That Mislocalize P Granules

We have analyzed the distribution of PIE-1 in eight mutants that mislocalize P granules: *par -1, par-2, par-3, par-4, par-6, mes-1, mex-1, and mex-3.* All are maternal-effect mutants; each exhibit unique cleavage and P granule partitioning defects (Kemphues *et al.*, 1988; Watts *et al.*, 1996; Strome *et al.*, 1995, Draper *et al.*, 1996; Mello *et al.*, 1992; Schnabel *et al.*, 1996). We refer to embryos derived from homozygous mutant mothers as *x* embryos where *x* represents the mutated gene. For example, *par-1* embryos are embryos derived from homozygous mutant *par-1* hermaphrodites (complete genotypes are described under Materials and Methods). We compared the distribution of PIE-1 to that of P granules by staining mutant embryos simultaneously with anti-PIE-1 and anti-P granule monoclonal antibodies.

par-1. In *par-1* embryos, the zygote divides symmetrically and P granules are distributed equally to all blastomeres (Kemphues *et al.*, 1988). In these embryos, we detected low levels of PIE-1 in all blastomeres up to the 4-cell stage (42/45 embryos; Figs. 5A–5C). PIE-1 was rarely observed in later stages, in contrast to P granules which continued to persist in most cells.

par-2. In *par-2* embryos, P granules segregate normally during the first cleavage but are partitioned to both P_1 daughters during the second cleavage (Kemphues *et al.*, 1988). Similarly, PIE-1 was segregated normally during the first cleavage (6/6), but then failed to localize to the posterior in P_1, and was detected in both P_2 and EMS in the 4-cell stage (8/12, Figs. 5D–5F). PIE-1 was typically not detected in later stages, although P granules continued to be present.

par-3. In *par-3* embryos, we observed a variety of PIE-1 and P granule distributions ranging from wild-type to ectopic. When mislocalized in the one- or two-cell stage, PIE-1 was often detected with the P granules in the center of the embryo (7/9, Figs. 5G–5I). In later stages, PIE-1 was



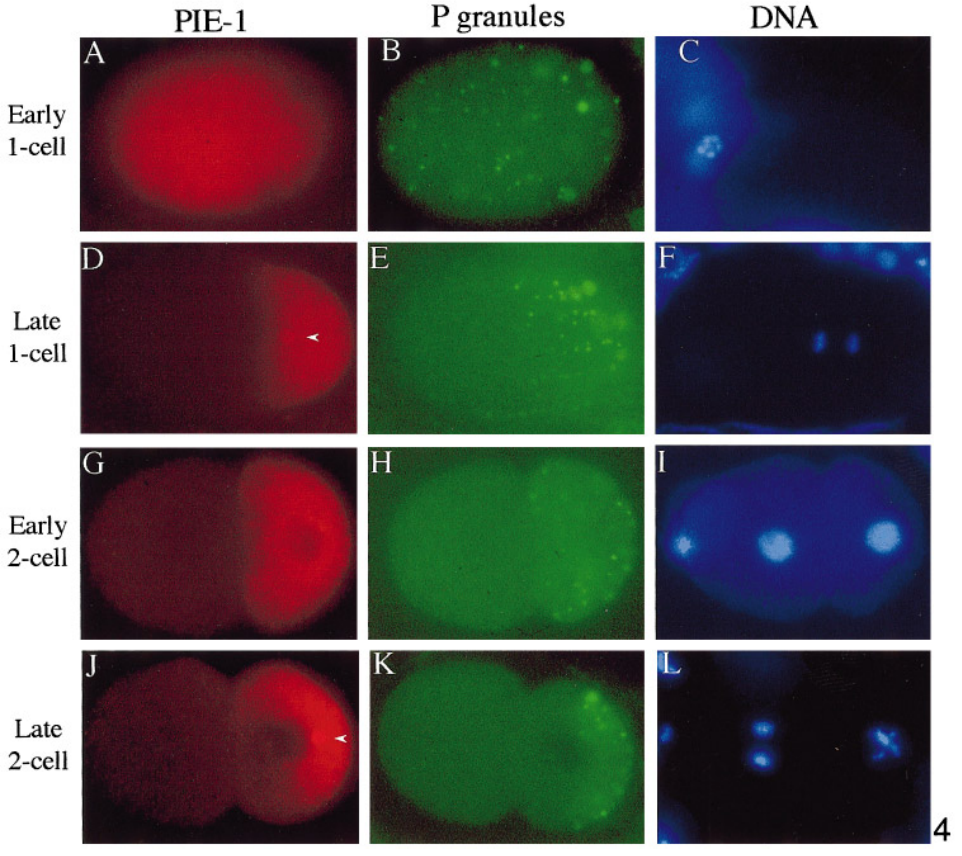


FIG. 3. PIE-1 protein is present in oocytes. (A) Wild-type gonads were triply stained with anti-PIE-1 antibody (red), anti-P granule antibody (green), and the DNA dye DAPI (blue). PIE-1 is detected in the cytoplasm of oocytes (square-shaped cells). (B) Western analysis of PIE-1 expression in hermaphrodites containing different cell types. Wild-type hermaphrodites contain somatic cells, immature germ cells, oocytes, sperm and embryos; *fem-1* hermaphrodites contain somatic cells, immature germ cells and oocytes; *fem-3(gf)* hermaphrodites contain somatic cells, immature germ cells and sperm, and *glp-4* hermaphrodites contain somatic cells only.

sometimes detected in multiple cells (10/25). In all embryos where PIE-1 was present (n = 25), PIE-1 was detected only in areas also containing high levels of P granules.

par-4. In *par-4* embryos, P granules are distributed to all blastomeres (Kemphues *et al.*, 1988). In these embryos, we detected low levels of PIE-1 in all blastomeres up to the 4-cell stage, with occasionally higher levels of PIE-1 in regions containing higher levels of P granules (4/12). We also detected PIE-1 in the second polar body (data not shown). In the 8- to 28-cell stage, PIE-1 was often detected in one blastomere (25/34); most often that blastomere also contained the highest levels of P granules (20/25; Figs. 5J–5L).

par-6. In *par-6* embryos, low levels of PIE-1 were detected in the 1- to 4-cell stage. In most 1- and 2-cell embryos (16/22), PIE-1 and P granules were localized to one pole. In a few embryos (6/22), PIE-1 was detected at both poles. PIE-1 was present at higher levels in the posterior pole where P granules were also present, and at lower levels in the anterior pole where P granules were not detected (5/6) or detected at low levels (1/6). [P granules are thought to disassemble transiently in the anterior pole of *par-6* embryos, making them difficult to detect there (Watts *et al.*, 1996)]. In 4-cell embryos, PIE-1 was often detected in multiple cells; in all cases (7/7), PIE-1 and P granules were detected in the same areas (Figs. 5M–5O). No PIE-1 was detected after the 8-cell stage, whereas P granules continued to be present in most cells.

mex-1. In *mex-1* embryos, P granules are segregated asymmetrically in the first cleavage, but are partitioned equally in the second and subsequent cleavages (Mello *et al.*, 1992; Schnabel *et al.*, 1996). We detected ectopic PIE-1 in 4-cell and older *mex-1* embryos (Figs. 5P–5R), as has been reported previously (Guedes and Priess, 1997). In all cases (n = 45), PIE-1-positive cells also contained P granules.

mex-3 and mes-1. In *mex-3* and *mes-1* mutants, P granules occasionally are segregated to both somatic and germline blastomeres in the P_2 and/or P_3 divisions (Draper *et al.*, 1996; Strome *et al.*, 1995). This missegregation causes P granules to be present ectopically in posterior somatic blastomeres in 8-cell and older embryos. We observed similar ectopic PIE-1 localization in one or more posterior blastomeres (Figs. 5S–5X). In embryos with mislocalized PIE-1, 100% of PIE-1-positive blastomeres were also positive for P granules (n = 25 cells for *mex-3*, and n = 35 cells for *mes-1*). The converse correlation, however, did not always hold; only 62% (n = 40) and 57% (n = 61) of P granule-positive cells, were also positive for PIE-1 in *mex-3* and *mes-1* embryos, respectively. In general, cells containing higher levels of P granules were most likely to also contain detectable levels of PIE-1.

In conclusion, we found that mutants that disrupt P granule segregation also show defects in PIE-1 localization. In the majority of mutant embryos examined, PIE-1 was only found in areas where high levels of P granules were present. In many mutants, however, ectopic P granules were also detected in cells where PIE-1 was not found.

Mutants with Reduced Levels of PIE-1 Activate RNAPII-H5 Expression in the Germ Lineage

We have previously shown that PIE-1 activity is required to repress the appearance in germline blastomeres of a specific phosphoepitope on RNA polymerase II (RNAPII-H5). This phosphoepitope first appears in the 4-cell stage in the interphase nuclei of somatic blastomeres, but remains absent from the germline nuclei until the 100-cell stage (Seydoux and Dunn, 1997). To test the relationship between PIE-1 and RNAPII-H5 further, we analyzed the expression of PIE-1 and RNAPII-H5 in several maternal-effect mutants. These experiments were performed by staining mutant embryos simultaneously with the anti-PIE-1 monoclonal antibody and with mAb H5, an antibody specific for RNAPII-H5 (see Materials and Methods). The frequencies of PIE-1 and RNAPII-H5 patterns observed in each mutant are presented in Table 1.

par mutants. As described above, in most *par* embryos, PIE-1 was rarely detected after the 4-cell stage. In all *par* mutants, we detected RNAPII-H5 in all interphase nuclei after the 4-cell stage (Table 1, section A). These observations are consistent with previous results which demonstrated that transcription is activated in all blastomeres in *par-1*, *par-2*, and *par-4* embryos (Seydoux and Fire, 1994; M. Wallenfang and G. Seydoux, unpublished data).

mex-1, mex-3, and mes-1 mutants. As described above, *mex-1*, *mex-3*, and *mes-1* mutants often mislocalize PIE-1 to one or more posterior somatic blastomeres (Table 1, section B). In embryos where PIE-1 was present in more than one cell, we found that the majority of PIE-1-containing cells were also positive for RNAPII-H5 (92% for *mex-1* (n = 48 cells); 95% for *mex-3* (n = 20 cells), and 97% for *mes-1* (n = 62 cells)). We observed an inverse correlation between PIE-1 and RNAPII-H5 levels; cells with the highest levels of PIE-1 had the lowest levels of RNAPII-H5 and vice versa (Figs. 6A–6F).

To confirm that mRNA transcription is activated in the

FIG. 4. Localization of PIE-1 and P granules in wild-type embryos. Wild-type embryos were triply stained with anti-PIE-1 antibody (red), anti-P granule antibody (green), and the DNA dye DAPI (blue). (A–C) Newly fertilized zygote. PIE-1 (A) and P granules (B) are present throughout the cytoplasm. (D–F) One-cell embryo in mitosis. PIE-1 (D) and P granules (E) are detected only in the posterior of the embryo. PIE-1 is also present on the posterior centrosome (arrowhead in D). (G–I) Two-cell embryo in interphase. PIE-1 (G) and P granules (H) are present in the germline blastomere P_1. (J–L) Two-cell embryo in mitosis. PIE-1 (J) and P granules (K) are detected in the posterior of P_1. PIE-1 is also present on the posterior centrosome in P_1 (arrowhead in J).

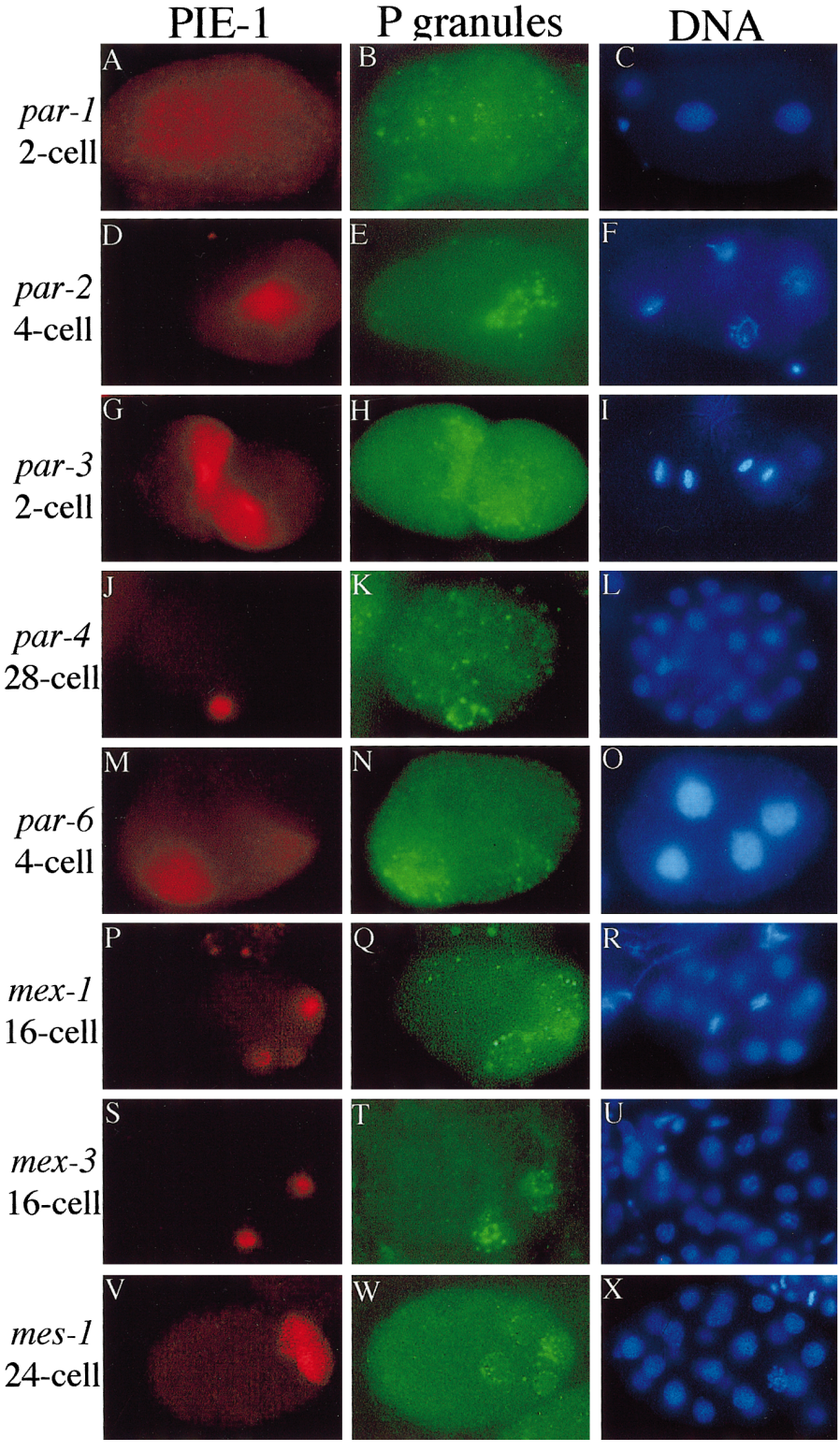


FIG. 5. Localization of PIE-1 and P granules in mutant embryos. Mutant embryos were triply stained with anti-PIE-1 antibody (red), anti-P granule antibody (green), and the DNA dye DAPI (blue). In all cases, PIE-1 is detected in areas where high levels of P granules are also present. PIE-1 and P granules are predominantly cytoplasmic in the 1- and 2-cell stages, and become progressively more nuclear and perinuclear, respectively, in later stages.

TABLE 1

PIE-1 and RNAPII-H5 Expression in Mutants

	1–4 cells					8–18 cells					20–28 cells				
	PIE-1:	WT	Reduced	Absent	Misloc	**PIE-1:**	WT	WT/red[a]	Absent	Misloc	**PIE-1:**	WT	WT/red[a]	Absent	Misloc
	H5:	n/a	n/a	n/a	n/a	**H5:**	H5−	H5+	H5+	H5+	**H5:**	H5−	H5+	H5+	H5+
	n					**n**					**n**				
Wild-type	>50	**100**	0	0	0	>50	**100**	0	0	0	>50	**100**	0	0	0
A															
par-1(*b274*)	45	0	0	7	**93**	7	0	0	**100**	0	5	0	0	**100**	0
par-2(*it5*)[b]	24	**63**	0	0	38	20	5	0	**80**	15	10	10	10	**80**	0
par-3(*e2074*)	23	39	4	0	**57**	15	0	7	**80**	13	6	0	17	**83**	0
par-4((*it47*)	24	8	29	46	17	31	0	42	**52**	6	22	0	18	**68**	14
par-6(*zu222*)	35	40	17	6	37	8	0	0	**100**	0	5	0	0	**100**	0
B															
mex-1(*zu121*)	24	**67**	21	0	13	12	17	8	8	**67**	17	6	24	12	**59**
mex-3(*zu155*)	3	**100**	0	0	0	3	**100**	0	0	0	13	38	15	0	46
mes-1(*bn7*)	15	**100**	0	0	0	12	**58**	0	0	42	12	33	8	0	**58**
mes-2(*bn11*)	10	**100**	0	0	0	0	—	—	—	—	9	**100**	0	0	0
mes-3(*bn35*)	7	**100**	0	0	0	0	—	—	—	—	10	**100**	0	0	0
mes-6(*bn66*)	7	**100**	0	0	0	0	—	—	—	—	6	**100**	0	0	0
cib-1(*e2300*)	30	**60**	0	30	10	25	0	0	**100**	0	2	0	0	**100**	0
C															
emb-1(*hc62*)[c]	25	**56**	0	28	16	n/a	—	—	—	—	n/a	—	—	—	—
emb-5(*hc61*)	5	**60**	20	20	0	15	13	20	**67**	0	15	27	0	**73**	0
emb-6(*hc65*)[c]	16	31	6	**63**	0	6	33	0	**66**	0	n/a	—	—	—	—
emb-7(*hc66*)	12	**75**	25	0	0	34	**62**	38	0	0	16	**69**	31	0	0
emb-11(*g1*)	6	**83**	0	17	0	0	—	—	—	—	7	**100**	0	0	0
emb-12(*g5*)	8	**75**	0	25	0	0	—	—	—	—	7	**100**	0	0	0
emb-13(*g6*)	7	**100**	0	0	0	0	—	—	—	—	9	**78**	0	22	0
emb-18(*g21*)	8	**100**	0	0	0	3	**100**	0	0	0	7	**86**	0	14	0
emb-21(*g31*)[c]	13	**100**	0	0	0	7	14	43	43	0	9	0	0	**100**	0
emb-27(*g48*)[d]	12	**75**	0	25	0	n/a	—	—	—	—	n/a	—	—	—	—
emb-30(*g53*)[d]	18	**78**	0	22	0	4	25	0	**75**	**0**	n/a	—	—	—	—
emb-31(*g55*)	4	**75**	25	0	0	7	**100**	0	0	0	4	**100**	0	0	0
zyg-1(*b1*)[d]	8	**67**	22	0	11	n/a	–	–	–	–	n/a	—	—	—	—
zyg-2(*b10*)[c]	10	**100**	0	0	0	11	**64**	9	18	9	7	0	29	43	29
zyg-9(*b244*)	19	**79**	21	0	0	22	**55**	27	18	0	12	**83**	8	8	0
emb-3(*hc59*)	6	**100**	0	0	0	0	—	—	—	—	5	**100**	0	0	0
emb-4(*hc60*)	7	**100**	0	0	0	0	—	—	—	—	6	**100**	0	0	0
emb-23(*g39*)	8	**100**	0	0	0	0	—	—	—	—	7	**100**	0	0	0
emb-26(*g47*)	13	**100**	0	0	0	0	—	—	—	—	9	**100**	0	0	0
emb-33(*g60*)	9	**100**	0	0	0	0	—	—	—	—	7	**100**	0	0	0
D															
emb-8(*hc69*)	33	**82**	12	6	0	30	23	30	47	0	27	7	11	**81**	0
emb-16(*g19*)[e]	31	**90**	10	0	0	41	**66**	34	0	0	19	47	**53**	0	0
emb-20(*g27*)	5	**100**	0	0	0	9	11	**67**	22	0	12	0	25	**75**	0
emb-25(g45)	14	**100**	0	0	0	41	46	**54**	0	0	24	0	0	**100**	0
E															
apx-1(*zu183*)	10	**100**	0	0	0	0	—	—	—	—	5	**100**	0	0	0
skn-1(*zu67*)	5	**100**	0	0	0	0	—	—	—	—	5	**100**	0	0	0
pop-1(*xu189*)	6	**100**	0	0	0	0	—	—	—	—	5	**100**	0	0	0

Note. Embryos were triply stained with anti-PIE-1 antibody, mAbH5 antibody, and the DNA dye DAPI. Embryos were staged by counting DAPI-stained nuclei and divided into three groups: 1- to 4-cell stage (the germline blastomere P_0, P_1, or P_2 is present), 8- to 18-cell stage (P_3 is present), and 18- to 28-cell stage (P_4 is present). 1- to 4-cell embryos were scored for PIE-1, and 8- to 28-cell embryos were scored for both PIE-1 and RNAPII-H5. Percentages greater than 50% are printed in bold. —, not determined. *n*, number of embryos analyzed. Scoring criteria were as follows. **PIE-1 WT:** Embryos with PIE-1 localized in the posterior (1-cell stage) or in one posterior blastomere (2- to 28-cell stage). This corresponds to the wild-type pattern. **PIE-1 reduced (red):** Embryos with wild-type PIE-1 localization, but with significantly lower levels of PIE-1 compared to wild-type controls. **PIE-1 absent:** Embryos with no detectable PIE-1. **PIE-1 mislocalized (misloc):** 1-cell embryos with PIE-1 in the anterior, and 2-cell and older embryos with PIE-1 in more than one cell. **H5+:** RNAPII-H5 staining in all cells, including PIE-1-containing cell(s) if present. **H5−:** No RNAPII-H5 staining in PIE-1-containing cell(s). (Since RNAPII-H5 is not present in any cell before the 4-cell stage, its expression was scored only in 8- to 28-cell embryos).

[a] Many of these embryos had noticeably reduced PIE-1 levels compared to wild-type controls. In many other embryos, however, changes in PIE-1 levels were small and difficult to identify unambiguously.

[b] Under the conditions tested, some *par-2*(*it5*) embryos were viable.

[c] These mutants arrest between the 4- and the 100-cell stage.

[d] These mutants arrest between the 1- and the 4-cell stage.

[e] *emb-16* embryos lacked detectable PIE-1 between the 28- and 100-cell stage.

germline of embryos that mislocalize PIE-1, we analyzed the distribution of *vet-5* RNA in *mex-1* and *mes-1* embryos by *in situ* hybridization. *vet-5* RNA is a nuclear RNA of unknown function which is transcribed in somatic blastomeres starting in the 4-cell stage (Seydoux *et al.*, 1996). We observed *vet-5* transcripts in germline blastomeres in 43% of *mex-1* embryos (n = 44, 8- to 50-cell embryos) and 43% of *mes-1* embryos (n = 61, 8- to 50-cell embryos) (Figs. 6G–6I). Although this frequency was lower than the frequency of RNAPII-H5-positive germline blastomeres [73% for *mex-1* (n = 26), 62% for *mes-1* (n = 45)], these results confirm that transcription can become activated in the germ lineage of mutants that localize PIE-1 to more than one cell.

In their analysis of *mex-1*, Guedes and Priess (1997) reported that the expression of *pes-10*, another embryonically transcribed gene, was inhibited in multiple cells in *mex-1* embryos. In contrast, in our experiments with *mex-1*, *vet-5* was rarely absent from more than one cell and, as described above, was often detected in all cells including the germline blastomere. This difference most likely reflects the difficulty of detecting low levels of *pes-10*, a mRNA which is quickly exported to the cytoplasm (Seydoux and Fire, 1994). In contrast, *vet-5* RNA remains concentrated in the nucleus, which permits the detection of even low levels of RNA. Nevertheless, the results of Guedes and Priess, in combination with ours, suggest that ectopic PIE-1 in *mex-1* embryos is sufficient to decrease, but not eliminate, transcription in somatic blastomeres.

emb and zyg mutants. The *emb* and *zyg* mutants form a collection of temperature-sensitive embryonic lethal mutations with a variety of terminal phenotypes (Cassada *et al.*, 1981; Isnenghi *et al.*, 1983; Miwa *et al.*, 1980; Wood *et al.*, 1980). The majority of *emb/zyg* mutants examined localized PIE-1 properly, although many occasionally expressed reduced levels of PIE-1. In these mutants, low levels of RNAPII-H5 were often observed in germline blastomeres (Table 1, section C).

We identified a subset of *emb* mutants which exhibited premature loss of PIE-1 at high frequencies (*emb-8*, *emb-16*, *emb-20*, and *emb-25*). In these mutants, PIE-1 levels often were normal in 1- to 4-cell embryos, but decreased significantly in later stages, frequently resulting in a complete lack of PIE-1 by the 28-cell stage (Table 1, section D). We consistently observed premature activation of RNAPII-H5 in the germ lineage of these mutants. The timing of RNAPII-H5 activation closely paralleled the timing of PIE-1 loss. For example, in *emb-25* mutants, P_2 blastomeres had wild-type levels of PIE-1 and showed no RNAPII-H5 expression (Figs. 7A and 7B), P_3 blastomeres often had reduced levels of PIE-1 and showed low levels of RNAPII-H5 expression (Figs. 7D–7F), and P_4 blastomeres had undetectable levels of PIE-1 and showed high levels of RNAPII-H5 (comparable to those detected in somatic blastomeres; Figs. 7G–7I). These results suggest that a certain threshold of PIE-1 is required in each germline blastomere to completely inhibit RNAPII-H5 expression.

Two Copies of pie-1 Are Required to Completely Inhibit RNAPII-H5 Expression in Germline Blastomeres

To test directly the existence of a threshold requirement for PIE-1, we examined embryos derived from *pie-1/+* heterozygotes for RNAPII-H5 expression. We found that 37% (n = 210) of 8- to 28-cell stage embryos derived from *pie-1/+* mothers expressed RNAPII-H5 in the germline blastomeres P_3 or P_4 (Figs. 7J–7L), compared to only 2% (n = 199) of controls. These results confirm that a certain threshold of PIE-1 is required to completely inhibit RNAPII-H5 expression in the germ lineage. We failed, however, to detect any lethality or sterility in the progeny of *pie-1/+* heterozygotes, suggesting that the level of transcription present in the germline blastomeres of these animals was insufficient to negatively affect development under the conditions tested (Materials and Methods).

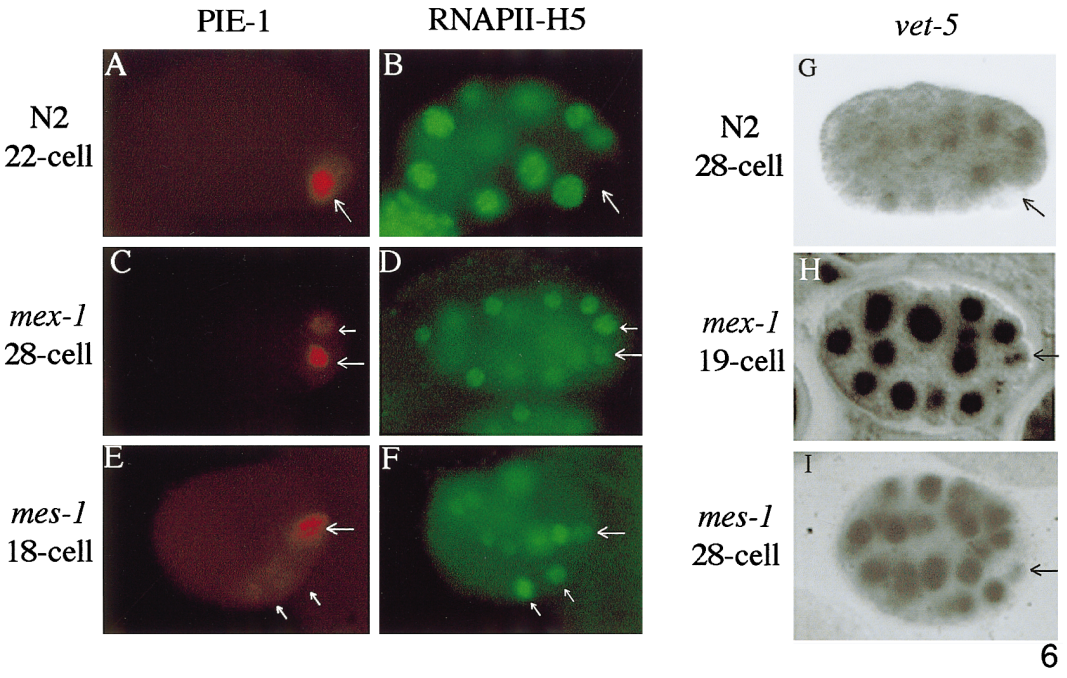
Mutations That Change the Fates of Certain Somatic Blastomeres Do Not Affect PIE-1 Localization

We tested three mutants that affect the fates of somatic blastomeres for PIE-1 and RNAPII-H5 expression. The *skn-1* locus encodes a putative transcription factor, present in the blastomeres EMS and P_2, and required to specify the fate of the somatic blastomere EMS (Bowerman *et al.*, 1992, 1993). *apx-1* encodes a delta-like ligand, present in the germline blastomeres P_1 and P_2, and required to specify the fate of the somatic blastomere ABp (Mango *et al.*, 1994; Mello *et al.*, 1994; Mickey *et al.*, 1996). *pop-1* encodes an HMG-box protein, present in all embryonic nuclei, and required to specify the fate of the somatic blastomere MS (Lin *et al.*, 1994). We observed wild-type patterns of PIE-1 and RNAPII-H5 in all *skn-1*, *apx-1*, and *pop-1* embryos examined (Table 1, section E).

DISCUSSION

PIE-1 Protein Is Distributed Uniformly in Oocytes and Newly Fertilized Embryos and Becomes Localized Asymmetrically in the Late One-Cell Stage

Using a new monoclonal antibody specific for PIE-1, we have shown that PIE-1 is present in oocytes and newly fertilized embryos, and that it is uniformly distributed in the cytoplasm of these cells. In the late one-cell stage, PIE-1 becomes localized to the posterior cytoplasm along with the P granules. PIE-1 protein also accumulates on the posterior centrosome during mitosis. As a consequence of this asymmetric cytoplasmic distribution, PIE-1 is segregated specifically to the posterior blastomere P_1 in the 2-cell stage. This sequence of events, beginning with uniform cytoplasmic distribution followed by asymmetric cytoplasmic and centrosomal localizations, is repeated in



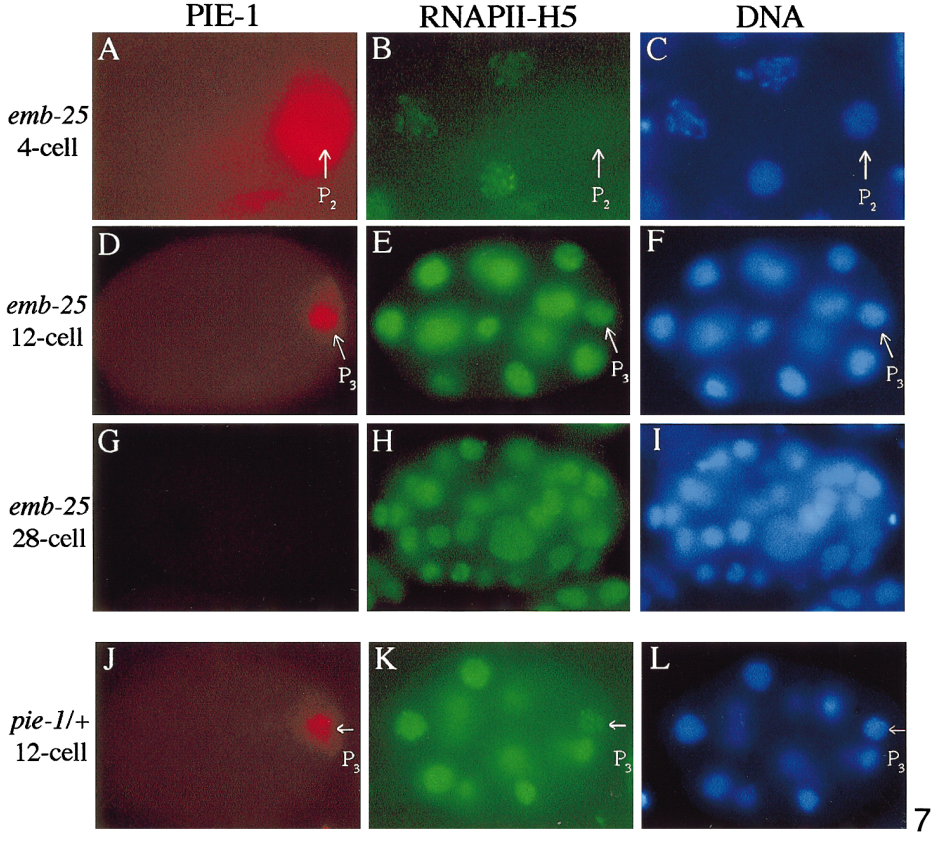


FIG. 6. Mutants that express PIE-1 in more than one cell activate transcription in all cells. (A–F) Embryos were triply stained with anti-PIE-1 antibody (red), mAbH5 (green), and the DNA dye DAPI (not shown). Long arrows point to the cell with the highest level of PIE-1 staining, and short arrows point to cells with lower PIE-1 staining. Note the inverse relationship between PIE-1 and RNAPII-H5 levels. (G–I) Embryos were hybridized to a *vet-5* antisense probe (dark color), and stained with anti-PIE-1 antibody (not shown). Arrows indicate cells with PIE-1 staining. Under these staining conditions, PIE-1 staining is very weak, and was detected in only one cell even in *mex-1* and *mes-1* embryos.

FIG. 7. Cells with reduced PIE-1 levels activate RNAPII-H5 expression. *emb-25* mutants (A–I) and *pie-1/+* heterozygotes (J–L) were triply stained with anti-PIE-1 antibody (red), mAbH5 (green), and the DNA dye DAPI (blue).

each unequally dividing germline blastomere (this study; Mello *et al.*, 1996).

Other proteins have been shown to become localized to the germ lineage; their distribution patterns, however, appear distinct from that of PIE-1. MEX-1 and GLD-1 are two proteins which, like PIE-1, are detected in the cytoplasm of germline blastomeres and on P granules (Guedes and Priess, 1997; Jones *et al.*, 1996). Unlike PIE-1, however, these proteins are distributed to both daughters at each asymmetric cleavage (Guedes and Priess, 1997; Jones *et al.*, 1996). After cleavage, they are lost from the somatic daughter and maintained only in the germline daughter. MEX-3 is another protein associated with P granules (Draper *et al.*, 1996). In early embryos, however, it is also present in the cytoplasm of anterior blastomeres. After the 4-cell stage, it is progressively lost from somatic blastomeres and retained only in germline blastomeres (Draper *et al.*, 1996). Finally, the P granule components GLH-1, GLH-2, and GLH-3 are found predominantly on P granules (Gruidl *et al.*, 1996), in contrast to PIE-1 which is also detected in the cytoplasm surrounding the P granules.

What mechanisms control the asymmetric localization of PIE-1? Like many maternal RNAs (Seydoux and Fire, 1994), *pie-1* RNA is present in all blastomeres up to the four-cell stage, but is later lost from somatic lineages and is maintained only in the germ lineage. Thus, the localization of the *pie-1* RNA may be sufficient to account for the germline-specific localization of the PIE-1 protein after, but not before, the four-cell stage. Total PIE-1 levels appear to increase during the one-cell stage, raising the possibility that localized translation contributes to the posterior localization of PIE-1 during that stage. There are several precedents for translational control as a mechanism to localize maternal proteins. For example, in *Drosophila,* the posterior localizations of the germline proteins Nanos and Oskar are regulated at the level of translation (Gavis and Lehmann, 1994; Kim-Ha *et al.*, 1995; Rongo *et al.*, 1995; Markussen *et al.*, 1995). In *C. elegans,* the localizations of two maternal proteins, GLP-1 and PAL-1, have been shown to be under translational control (Evans *et al.*, 1994; Hunter and Kenyon, 1996). Neither protein, however, is localized in the same pattern as PIE-1: GLP-1 is present in AB-derived somatic blastomeres, and PAL-1 is present in P_1-derived somatic and germline blastomeres.

Another possibility is that PIE-1 localization is achieved through posttranslational mechanisms. For example, the PIE-1 protein may be preferentially degraded in the anterior and/or stabilized in the posterior. Alternatively or additionally, there may exist mechanisms that actively move PIE-1 to the posterior. Both mechanisms (localized degradation and active migration) are used in the localization of P granules to the germ lineage (Hird *et al.*, 1996). Although our observations suggest that PIE-1 and P granule localizations may be coordinately regulated (see below), our results thus far have not determined whether PIE-1 localization is regulated translationally, posttranslationally, or both.

A Link between PIE-1 and P Granules

In wild-type embryos, PIE-1 was detected only in cytoplasmic regions also containing P granules. To test the functional significance of this association, we analyzed the distribution of PIE-1 in eight mutants that mislocalize P granules: *par-1, par-2, par-3, par-4, par-6, mex-1, mex-3,* and *mes-1.* Each of these mutants affects P granule segregation in a unique manner. For example, in *par-1* mutants, P granules remain distributed uniformly throughout the embryo; in *par-3* mutants, P granules are often segregated to the center of the embryo instead of the posterior; and in *mex-1* and *mex-3* mutants, P granules are segregated normally during the first cleavage, but abnormally in later cleavages. In all mutants examined, PIE-1 was mislocalized in the same pattern as the P granules. We also noticed a correlation between PIE-1 levels and local P granule concentration: cells (or subcellular areas) with high numbers of P granules were more likely to contain PIE-1 than cells (or subcellular areas) with low numbers of P granules. For example, in *par* embryos where the P granules are partitioned to all or most blastomeres, PIE-1 was rarely detected after the four-cell stage, and, when present, was only found in cells containing the highest numbers of P granules. These observations suggest the existence of a link between PIE-1 levels and local P granule concentration. One possibility is that the same mechanisms that localize P granules also localize and/or stabilize PIE-1. Another possibility is that the P granules themselves regulate PIE-1 localization, by generating, for example, a cytoplasmic environment that protects PIE-1 from degradation. The reverse possibility (PIE-1 regulating P granule localization) is unlikely since *pie-1* activity is not required for P granule localization or stability in early embryos (Mello *et al.*, 1992).

Of all the mutants analyzed in this study, only *par-1* mutants completely abolish PIE-1 asymmetry. *par-1* is required for the asymmetric localization of several other maternal proteins, including MEX-3, GLP-1, SKN-1, and PAL-1 (Crittenden *et al.*, 1996; Bowerman *et al.*, 1997). These proteins all have localizations distinct from PIE-1, suggesting that PAR-1 may function as a general regulator of cytoplasmic asymmetry (Bowerman *et al.*, 1997). PAR-1 is a putative ser/thr kinase present at low levels throughout the cytoplasm, and at higher levels on the posterior cortex (Guo and Kemphues, 1995). This cortical localization, however, does not appear to be essential for PAR-1 function, since it is lost in *par-2* zygotes where P granules and PIE-1 are still localized posteriorly (Boyd *et al.*, 1996; this study). The mechanisms by which PAR-1 and the other PAR proteins establish polarity are unknown.

Inhibition of mRNA Production in Germline Blastomeres Is Sensitive to PIE-1 Levels

We have shown previously that *pie-1* activity is required to inhibit the appearance in germline blastomeres of RNAPII-H5, a phosphoepitope specific to the CTD of RNA polymerase II (Seydoux and Dunn, 1997). Those studies,

however, did not determine whether PIE-1 is required continuously in the germ lineage to repress RNAPII-H5, or whether PIE-1 activity in the first germline blastomere, for example, is sufficient to repress RNAPII-H5 in all subsequent blastomeres. In our survey of embryonic lethal mutants, we have identified four mutants which lose PIE-1 expression prematurely in P_3 or P_4. In these mutants, RNAPII-H5 expression is absent in P_2, but present in P_3 and/or P_4. These observations suggest that PIE-1 is required in each germline blastomere to repress RNAPII-H5 expression, and are consistent with the hypothesis that PIE-1 itself functions as the repressor.

We also noticed that cells with reduced PIE-1 levels express RNAPII-H5 at low levels. This correlation is particularly striking in mutants where PIE-1 is segregated to more than one cell: in these mutants, cells with the highest levels of PIE-1 have the lowest levels of RNAPII-H5, and cells with the lowest levels of PIE-1 have the highest levels of RNAPII-H5 (comparable to those seen in somatic cells). These observations suggest that a certain threshold of PIE-1 is required to repress RNAPII-H5 expression completely, and that this threshold is rarely met in mutants where PIE-1 is present in more than one cell. We obtained direct evidence for a threshold requirement for PIE-1 by analyzing embryos derived from mothers with only one functional copy of the *pie-1* gene. In these embryos, PIE-1 is localized normally to the germ lineage; yet 37% of these embryos express RNAPII-H5 in the germ lineage. These results indicate that RNAPII-H5 expression is sensitive to PIE-1 dosage.

In mammalian cells, the RNAPII-H5 phosphoepitope is found on polymerase subunits engaged in transcription (Zeng *et al.*, 1997). Therefore, we expected germline blastomeres that express even low levels of RNAPII-H5 to be transcriptionally active. As expected, we found that *vet-5* transcripts are often detected in the germline blastomeres of *mes-1* and *mex-1* embryos which express low levels of RNAPII-H5 in the germ lineage. Together these observations support a model where PIE-1 acts in each germline blastomere as a concentration-dependent, general inhibitor of mRNA transcription. Whether PIE-1 interferes directly with the transcriptional machinery, or functions indirectly by modifying chromatin, for example, remains to be determined.

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