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The *C. elegans* MEX-1 protein is present in germline blastomeres and is a P granule component

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SUMMARY

In the nematode *Caenorhabditis elegans*, germ cells arise from early embryonic cells called germline blastomeres. Cytoplasmic structures called P granules are present in the fertilized egg and are segregated into each of the germline blastomeres during the first few cleavages of the embryo. Mutations in the maternally expressed gene *mex-1* disrupt the segregation of P granules, prevent the formation of germ cells, and cause inappropriate patterns of somatic cell differentiation. We have cloned the *mex-1* gene and determined the distribution pattern of the *mex-1* gene products. The MEX-1 protein contains two copies of an unusual 'finger' domain also found in the PIE-1 protein of *C. elegans*. PIE-1 has been shown to be expressed in germline blastomeres, and is a component of P granules. We show here that MEX-1 also is present in germline blastomeres and is a P granule component, although MEX-1 is a cytoplasmic protein while PIE-1 is present in both the nucleus and cytoplasm. We further show that MEX-1 is required to restrict PIE-1 expression and activity to the germline blastomeres during the early embryonic cleavages.

Key words: *Caenorhabditis elegans*, *mex-1*, *pie-1*, P granules, germ cells

INTRODUCTION

Analysis of the pattern of cell division and differentiation, or lineage, of the *Caenorhabditis elegans* embryo has shown that germ cells have an invariant origin (Sulston et al., 1983). Germ cells arise from only one branch of the embryonic lineage; cells in this branch are called germline blastomeres (see Fig. 1). Cells in all other branches produce only somatic cell types, and are called somatic blastomeres.

How do germline blastomeres differ from somatic blastomeres? Some somatic cell fates in the early embryo appear to be specified in part by two maternally provided transcription factors, SKN-1 and PAL-1 (Bowerman et al., 1992; Hunter and Kenyon, 1996). Germline blastomeres also contain SKN-1 and PAL-1, but do not appear to respond to these factors (Bowerman et al., 1993; Hunter and Kenyon, 1996). Recent studies have shown that embryonic transcription is repressed in germline blastomeres (Seydoux et al., 1996), presumably making these blastomeres refractory to factors that might otherwise direct somatic differentiation. Transcriptional repression in germline blastomeres requires the maternal PIE-1 protein, which is a predominantly nuclear protein that is present in each of the germline blastomeres (Mello et al., 1996; Seydoux et al., 1996). The molecular function of PIE-1 has not yet been determined, nor has the basis for the germline-restricted distribution of the PIE-1 protein.

A second difference between germline and somatic blastomeres is that cytoplasmic structures called P granules are found only in germline blastomeres (Strome and Wood, 1982). P granules are associated with germ cells, or germ cell precursors, at all stages of the *C. elegans* life cycle in embryos, larvae and adults (Strome and Wood, 1982). Thus P granules may function in germ cell development, though this function has not been determined. P granules contain uncharacterized RNA molecules that are capped and polyadenylated (Seydoux and Fire, 1994). Recently, the proteins MEX-3 and PIE-1 have been shown to associate with P granules in the germline blastomeres of early embryos, but not with the P granules found in germ cell precursors in later embryos, larvae or adults (Draper et al., 1996; Mello et al., 1996). MEX-3 is a presumptive RNA-binding protein that is present in the cytoplasms of both somatic and germline blastomeres; the function of MEX-3 in germline blastomeres has not been determined (Draper et al., 1996). PIE-1 is a novel, predominantly nuclear protein with two copies of an unusual 'finger' domain characteristic of the vertebrate TIS11/Nup475/TTP protein (Mello et al., 1996). As described above, PIE-1 is required to repress embryonic transcription in germline blastomeres (Seydoux et al., 1996).

P granules are partitioned to germline blastomeres during each of the early embryonic cleavages (Strome and Wood, 1983). After fertilization there is a general flow of cytoplasm, including P granules, toward the posterior pole of the egg (Hird and White, 1993; Hird et al., 1996). While other cytoplasmic components cycle back anteriorly along the cortical margins of the egg, P granules appear to remain associated with the posterior cortex, and thus are partitioned by the first cleavage

into the posterior cell, a germline blastomere (Hird and White, 1993; Hird et al., 1996). This general pattern of asymmetric localization of P granules happens prior to each of the subsequent cleavages, although additional mechanisms appear to contribute to P granule localization at later cleavages (Hird et al., 1996).

Previous studies have shown that the maternal gene *mex-1* is essential for the development of germ cells, and for the proper segregation of P granules during the first embryonic cleavages (Mello et al., 1992; Schnabel et al., 1996). P granules accumulate posteriorly in newly fertilized *mex-1* mutant eggs, but they do not associate properly with the cortex and are thus spread throughout the posterior half of the egg. In the next and subsequent cleavages, this incomplete localization leads to the mis-partitioning of P granules into somatic blastomeres, and a progressive loss of P granules in cells that would normally be germline blastomeres (Mello et al., 1992; Schnabel et al., 1996).

mex-1 mutants also have complex defects in somatic differentiation (Mello et al., 1992; Schnabel et al., 1996). The SKN-1 transcription factor is required for the proper development of the ventral blastomeres MS and E, and is expressed at high levels in these blastomeres in wild-type embryos. In *mex-1* mutants, SKN-1 is expressed at high levels in anterior blastomeres that inappropriately adopt MS-like (Mello et al., 1992) or E-like (Schnabel et al., 1996) fates. *mex-1* mutants also have a temperature-sensitive defect in the E blastomere, such that at low temperature E has a pattern of development similar to a posterior blastomere called C (Mello et al., 1992). Thus *mex-1* mutations alter the fates of certain somatic blastomeres such that they develop with characteristics of more posterior blastomeres (Mello et al., 1992; Schnabel et al., 1996).

We report here the cloning of *mex-1* and the localization of the *mex-1* gene products. *mex-1* encodes a protein, MEX-1, with two 'finger' domains similar to those found in PIE-1. We show that MEX-1 is a cytoplasmic protein that is distributed unequally between the early blastomeres and that MEX-1, like PIE-1, is a component of P granules. Finally, we demonstrate that MEX-1 is required to restrict PIE-1 localization and activity to germline blastomeres.

MATERIALS AND METHODS

Strains and alleles

Bristol strain N_2 was used as the standard wild-type strain. The *mex-1* alleles used were: *zu120*, *zu121*, and *zu140* (Mello et al., 1992), *it9* (Kemphues et al., 1988), *zu221* (provided by R. Lin), *or4* and *or14* (provided by B. Bowerman), *e2569* (provided by J. Ahringer). *pie-1 (zu154)* was used in in situ hybridization experiments. Nematode strains were cultured as described by Brenner (1974).

Cloning and molecular analysis of *mex-1*

A strain carrying the yeast artificial chromosome Y17G7 (provided by A. Newman and P. Sternberg) was shown by genetic crosses to complement *mex-1(zu120)* (B. Draper, unpublished results). The *mex-1* locus was identified through a series of germline transformation experiments with cosmids and phage spanning Y17G7 (provided by A. Newman and P. Sternberg). Cosmids and phage were injected individually or in groups with the marker *rol-6* (Mello et al., 1991) into the syncytial gonad of *mex-1(zu120)unc-4(e120)/mnCI(II)* animals. DNA was injected at a final concentration between 100 and 200 ng/μl. Four transgenic lines segregating Rol Unc hermaphrodites were isolated after injection of the phage clone VT#YL17 (Papp et al., 1991) containing an approximately 20 kb insert of genomic DNA. The Rol Unc adults were unable to roll, but had the right-handed twist characteristic of Roller animals. Rol Unc hermaphrodites produced either inviable Mex embryos, or viable Unc, or Rol Unc, larvae. Unc larvae became adult hermaphrodites that produced all inviable Mex embryos. We were unable to obtain phenotypic rescue with subclones of VT#YL17.

A 7.2 kb *Bam*HI-*Hin*dIII genomic DNA fragment isolated from VT#YL17 was used to screen a mixed stage *C. elegans* cDNA library (provided by A. Fire). Positive cDNAs were used to construct Bluescript plasmids pJPSG1 (2.7 kb insert) and pJPSG4 (1.1 kb insert). Antisense RNAs corresponding to the full cDNA inserts were produced using an in vitro transcription kit (Promega). RNA injections were carried out as described by Guo and Kemphues (1995). RNAs from pJPSG1 and pJPSG4 were injected at concentrations of 0.5 and 1.8 μg/μl, respectively. Approximately 60% of the resulting embryos resembled *mex-1* mutant embryos. 4 different *mex-1* cDNAs were sequenced on both strands. A potential SL1 trans-spliced acceptor (Krause and Hirsh, 1987) was analyzed by reverse transcription of total mixed stage RNA (provided by M. Morrison and M. Roth) using a random hexamer primer mix (Boehringer Mannheim), followed by polymerase chain reaction (PCR) using a *mex-1*-specific primer and a primer specific for either the trans-spliced leader SL1 or SL2 as described by Spieth et al. (1993). A single product with the predicted size was obtained only when the SL1 primer was included; this product was shown by Southern analysis to hybridize to a *mex-1* cDNA, and coincided with the 5′ end of the *mex-1* cDNA by sequence analysis. *mex-1* alleles were examined for deletions in the *mex-1* gene by single-worm PCR (Barstead and Waterson, 1991) using combinations of *mex-1*-specific primers. The regions of genomic DNA surrounding deletions in the *mex-1(zu140)* and *mex-1(zu221)* alleles were isolated by PCR and sequenced.

In situ hybridization

In situ hybridization using either *pes-10* or *mex-1* probes was done according to the method of Seydoux and Fire (1994). *mex-1* sense and antisense RNA probes labeled with digoxigenin-11-dUTP (Boehringer Mannheim) were made from pJPSG9, a plasmid containing part of the *mex-1* cDNA that includes the entire coding region and 387 bp of the 3′ UTR. The detection reaction with alkaline phosphatase-conjugated anti-digoxigenin (Boehringer Mannheim) was allowed to develop for approximately 45 minutes. Photographs were taken with Ektachrome 160T professional color reversal film (Kodak). Images were digitized using a Nikon Coolscan slide scanner (Nikon). The digitized images were assembled using Adobe Photoshop 3.0 (Adobe).

Production of antibodies and immunostaining

Rabbit polyclonal antisera 8230I and 8325I were generated against branched chain peptides corresponding to amino acids 74-96 and 273-296 of the predicted MEX-1 protein (see Fig. 2A). Peptides were synthesized using a prepared 4-branch MAPs core attached to a Wang resin (Novabiochem) on an Applied Biosystems model 430 peptide synthesizer. Peptide sequences were chosen to exclude the highly conserved finger domains of the MEX-1 protein. Immunizations (Lin et al., 1995) and affinity purification (Robinson et al., 1988) of antibodies were as described previously. Antibodies were eluted from matrix-bound antigen with 0.2 M glycine with 1.0% bovine serum albumin, pH 2.5. Embryos were fixed and stained with antibodies, and with DAPI to visualize DNA, as described previously (Lin et al., 1995). The antisera 8230I and 8325I gave indistinguishable patterns of staining on wild-type embryos. No staining was detected with the 8325I antiserum in embryos from homozygous *mex-1*(zu120) mothers, nor with the 8230I antiserum in embryos from *zu120*, *zu121*, *zu221*, or *e2569* mothers; staining was detected in embryos homozy-

gous for the weak *mex-1* mutation *it9*. P granule/MEX-1 double staining experiments were as described by Draper et al. (1996). Embryos were stained for PIE-1 with a mouse monoclonal antibody, P4G5, that was raised against PIE-1-specific peptides and that will be described elsewhere (C. Schubert unpublished results); we obtained similar embryonic staining patterns with a rabbit polyclonal antibody described by Mello et al. (1996). Embryos were prepared and stained as described previously (Mello et al., 1996). All images were collected on a Deltavision SA3.1 wide-field deconvolution optical sectioning microscope (Applied Precision, Inc.) and assembled using Adobe Photoshop 3.0 (Adobe). The MEX-1 protein and P granule co-localization images were deconvolved using the reiterative constrained method of Sedat and Agard (Hiraoka et al., 1991).

RESULTS

MEX-1 contains a repeated 'finger' motif

Transformation rescue of the mutant strain *mex-1(zu120)* was obtained with a single phage (see Materials and Methods) and probes from this phage were used to isolate candidate *mex-1* cDNAs. Antisense RNA transcribed from the cDNAs SG1 and SG4 resulted in *mex-1*-like embryonic phenotypes when injected into the gonads of wild-type adult hermaphrodites

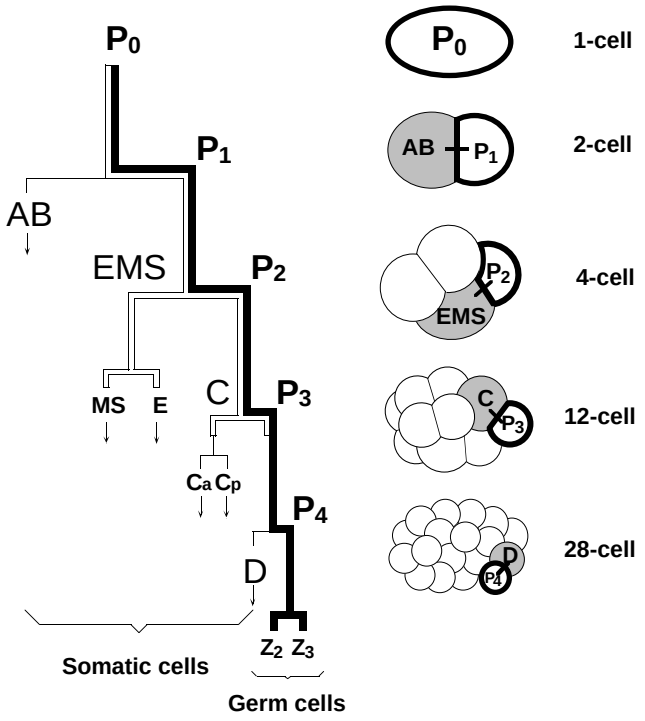

Fig. 1. Schematic diagrams of the early lineage (left) and cleavages (right) of the *C. elegans* embryo. Horizontal lines on the lineage diagram represent cell divisions. The branch of the lineage that produces the germ cells is indicated by the thick black line, with the names of the germline blastomeres (P_0 through P_4) listed for each cleavage stage. Somatic blastomeres mentioned in the text are shown by name. The thick black line also corresponds to the expression pattern of the germline-specific PIE-1 protein. For comparison, the thick white line shows the blastomeres that express high levels of the somatic transcription factor SKN-1. In the cleavage diagram, germline blastomeres are outlined in bold, and the somatic blastomeres that are sisters of germline blastomeres are shown shaded. Embryos are oriented with anterior left and dorsal up.

(data not shown), suggesting that these cDNAs correspond to the *mex-1* gene. The sequence derived from SG1, SG4, and overlapping cDNA clones is shown in Fig. 2A, with the sequence of the predicted protein. We found that two *mex-1* mutants have deletions in the gene defined by these cDNAs. *mex-1(zu221)* has a 437 bp deletion in the 5′ end of this gene that would remove the first 36 amino acids of the predicted protein. *mex-1(zu140)* has an approximately 600 bp deletion toward the 3′ end that would remove the C-terminal 80 amino acids of the predicted protein. These several lines of evidence together indicate that this gene is *mex-1*.

The *mex-1* cDNA can encode a 494 amino acid protein that contains two copies of a predicted Cys/His 'finger' motif with the unusual spacing $CX_8CX_5CX_3H$, initially described in the mouse TIS11/Nup475/TPP protein (Fig. 2B; Varnum et al., 1989; Dubois et al., 1990). This class of 'finger' domain has been identified in proteins from several organisms including *Saccharomyces cerevisiae*, *Drosophila* and *C. elegans* (Ma et al., 1994; Ma and Herschman, 1995; Mello et al., 1996), however the biochemical function of this domain has not been determined. The PIE-1 protein in *C. elegans* has two finger domains similar to the predicted MEX-1 protein (Mello et al., 1996). PIE-1 is a predominantly nuclear protein that is localized to germline blastomeres, and that is required to repress embryonic transcription in these blastomeres (Mello et al, 1996; Seydoux et al., 1996; see Introduction). The vertebrate TIS11/ Nup475/TTP protein can be found in the nucleus (Dubois et al., 1990) and is induced in 3T3 cells in their immediate response to growth factor or serum stimulation, suggesting it may also play a role in regulating gene expression (for review, see Herschman et al., 1994). The predicted MEX-1 protein does not have significant similarity to PIE-1 or TIS11/Nup475/TTP outside of the finger domains, and does not contain any known nuclear localization sequence or other motifs that might suggest its function or distribution.

The *mex-1* gene products localize predominantly to germline blastomeres

We analyzed the distribution of the *mex-1* mRNA by in situ hybridization (see Materials and Methods). *mex-1* mRNA is detected in the syncytial core of the gonad and in oocytes at all stages of maturation (Fig. 3A). In embryos, *mex-1* mRNA has a distribution that is similar to a pattern described previously in *C. elegans* for several unrelated maternally expressed messages, collectively called class II mRNAs (Seydoux and Fire, 1994). There is no genetic evidence for embryonic expression of *mex-1* (Mello et al., 1992), therefore we consider it likely that the expression pattern we describe here represents maternally expressed *mex-1* mRNA, though this has not been tested directly. Like class II maternal messages, *mex-1* mRNA is distributed uniformly in 1-cell and 2-cell stage embryos (Fig. 3B,C) but then appears to be degraded rapidly in somatic blastomeres (Fig. 3D-H). After each of the subsequent cleavages, *mex-1* mRNA persists at high levels in the germline blastomere, but gradually decreases to undetectable levels in the somatic sister of the germline blastomere (compare Fig. 3H with 3G). After the division of the final germline blastomere, P_4, *mex-1* mRNA can no longer be detected in the embryo (data not shown).

To examine MEX-1 protein localization, we used two rabbit polyclonal antisera raised against different regions of the MEX-

1 protein and found identical results. No staining was detected with these antisera in several *mex-1* mutant strains (see Material and Methods). MEX-1 is first detected at low levels in the cytoplasm of mature oocytes (data not shown). The level of MEX-1 increases markedly after fertilization; at the late 1-cell stage, MEX-1 appears most abundant in the posterior half of the embryo (Fig. 4A). During the early cleavage stages, MEX-1 is present in the cytoplasm of each of the germline blastomeres and is not detected in nuclei (Fig. 4C-K). After each division of a germline blastomere, MEX-1 is detected initially in both daughters, but then disappears from the somatic daughter later in that cell cycle (compare the early 4-cell embryo in Fig. 4E with the late 6-cell embryo in Fig. 4G). MEX-1 is present in the germline blastomere P_4, but can not be detected in the daughters of P_4 (see Fig. 5F and data not shown).

MEX-1 is a component of P granules

In addition to the cytoplasmic staining described above, distinct granules are stained prominently by the MEX-1 antisera in the cytoplasm of each germline blastomere (Fig. 4). These granules closely resemble P granules in size, number and distribution (Strome and Wood, 1982). When embryos were stained simultaneously with a MEX-1 antiserum and an anti-P granule antibody, we found that the staining patterns of the granules coincided for each of the germline blastomeres P_0, P_1, P_2, P_3, and P_4 (the P_2 blastomere is shown in Fig. 5A-C). P granules

A

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TTTGAGGAAAGGAGAAGGATGCAATCTTCAAATGGAGAGCATCACCAACAAAAGCAGACGCCGTACTTCATGAATGCTCCATGCGACCCGGCTCTTCTCGCGTTGGCT
                M  Q  S  S  N  G  E  H  H  Q  Q  K  Q  T  P  Y  F  M  N  A  P  C  D  P  A  L  L  A  L  A

CAACAAACGTTTCTAGGCTTCCAACCACAAAAGAATATGTATCAACATGGATTTCCCCCGATGCTTCCCCACAGGAACCTCATTCCGTCGCTGGCGGCCGCGATTCCC
Q  Q  T  F  L  G  F  Q  P  Q  K  N  M  Y  Q  H  G  F  P  P  M  L  P  H  R  N  L  I  P  S  L  A  A  A  I  P

GAAATGGATAAGCTAATGTTGGAGGAGCCAATTGAGGCTGGAAGCATGCCATATCGCAGAGCCACCAACAAGAATATCTCTGTGTCCGATGACTCATTCAACAACATG
E  M  D  K  L  M  L  E  E  P  I  E  A  G  S  M  P  Y  R  R  A  T  N  K  N  I  S  V  S  D  D  S  F  N  N  M

CATCGGTCCTCTTCCTCATCGCAATATCGCCGTCATTCCGCTCAATGGGAGACTATGACTGATGACGAACGAGAGTCGGTTCAGCGTCAGAAGCGCAAGGAAGAAGCA
H  R  S  S  S  S  S  Q  Y  R  R  H  S  A  Q  W  E  T  M  T  D  D  E  R  E  S  V  Q  R  Q  K  R  K  E  E  A

TTCAAGACGGCACTTTGTGACGCTTTTAAGCGCTCAGGCTCCTGCCCGTACGGCGAAGCTTGCCGGTTTGCTCACGGCGAGAACGAGCTGAGGATGCCGTCGCAGCCA
F  K  T  A  L  C  D  A  F  K  R  S  G  S  C  P  Y  G  E  A  C  R  F  A  H  G  E  N  E  L  R  M  P  S  Q  P

CGTGGCAAGGCGCATCCAAAGTACAAGACGCAGCTGTGCGACAAGTTTTCCAACTTTGGACAGTGTCCCTATGGACCACGATGCCAGTTTATTCATAAGCTGAAGAAG
R  G  K  A  H  P  K  Y  K  T  Q  L  C  D  K  F  S  N  F  G  Q  C  P  Y  G  P  R  C  Q  F  I  H  K  L  K  K

GGTCTTCCACTGAGCGAGTACAACCGTGCTCTTCAAGAAGGCGAGATCTCACCGGCTCGCGACGACGAGATCACCAATCCCGACGAATCATCATCCCAAGTGGAGGAT
G  L  P  L  S  E  Y  N  R  A  L  Q  E  G  E  I  S  P  A  R  D  D  E  I  T  N  P  D  E  S  S  S  Q  V  E  D

CTGAGTGAGCTTCACCATCGACAACATCAGCAGCAGCAGCAGTACCAACAACGCTATCGTCGGCCACCATTCAACAATTTCCACGATATGTCGGATAGTGGATACAGT
L  S  E  L  H  H  R  Q  H  Q  Q  Q  Q  Q  Y  Q  Q  R  Y  R  R  P  P  F  N  N  F  H  D  M  S  D  S  G  U  S

GCTCCTCGCCGCCGGCTTCATCATCAATTCGAGCATCTTGGTAGTGAGCAGCAGCAGCAGCAGCAGCAGACACCAGCTCCGAAGATTGTCTATCCATCGATGCAA
A  P  R  R  R  L  H  H  Q  F  E  H  L  G  S  E  Q  Q  Q  Q  Q  Q  Q  T  P  A  P  K  I  V  Y  P  S  M  Q

GTTGTCAATATTGAAATGGCAACTGGTGACGGACCTAAAAGGTCTGACAATCATCATTATCATCATCATCATCATCAACGTCGTCATATTCCGGCGCCACCATCCACC
V  V  N  I  E  M  A  T  G  D  G  P  K  R  S  D  N  H  H  Y  H  H  H  H  H  Q  R  R  H  I  P  A  P  P  S  T

GGTCCACTGAGCTCTCTCATTGATCAGGCCGACTATGAAGCTTCTATGGCAAGCGGGAAAATGTTCGGAAAACCAGAGAACATTCGCACCTATGGAGCCATGATCCGC
G  P  L  S  S  L  I  D  Q  A  D  Y  E  A  S  M  A  S  G  K  M  F  G  K  P  E  N  I  R  T  Y  G  A  M  I  R

GCCAACGAGGAGCACGTCGAGCGTAATCAGACTGGAGTTGTCAAGAAGGAACGGGCGACGGTTCTACCGAAAAGGGATCTTTCACTGATTCGCGAGGAGGATACACTG
A  N  E  E  H  V  R  E  N  Q  T  G  V  V  K  K  E  R  A  T  V  L  P  K  R  D  L  S  L  I  R  E  E  D  T  L

CTCGTCGACGGTTCTCATCTGAATATGACGTCATCGGCGATTCTGCCAGCTCATCAAATCCCTTCCACCACGACCACCAGCACATCATCTCCAGTCCAAGAACAGCCG
L  V  D  G  S  H  L  N  M  T  S  S  A  I  L  P  A  H  Q  I  P  S  T  T  T  T  S  T  S  S  P  V  Q  E  Q  P

TGGTACGAGAAGATTTTCGGGAAAATGACAATGATCCAAGAGGAATCATCGATGGGCGGTGAAGACGACGATGCTCACGAAGATCATTATTCGAGATAATCTGGATTC
W  Y  E  K  I  F  G  K  M  T  M  I  Q  E  E  S  S  M  G  G  E  D  D  D  A  H  E  D  H  Y  S  R  *

CTCCGTCCACCCCCTACCTACTACCTAAAAATCTGCCAAATTCTCACAAATTGTCCCAGTCCAAAATCTCTTACAACAAGAACAAAAAATCCCCTCCAAATTTTCATG

TCATATCATAATTTTCCCCCCACACCACCAAAAAAATGTCCATATTTTGTATTTTTGTGTCCCACCCCCCGGCCAATCTGAGCCATATTTATTTTGATAATGATTGTA

AAAAGTTATTTTGTGATCTCTACCGTTTCCCCCCAAACATTCCCCATTTCTCTATCTCTCTCTCTAGTCTATCTCACTCTCTCTATCGGTGTCTCGCCACGATACCCA

CCGACCCAGAACGCCAAGAACAAATATGAAGAGATTTGAATTTTTATTCAAAAAAAAAAAAAAAAAAAA
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B

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Finger 1

MEX-1 (C. elegans)      140    F K T A L C D A F K R S G S C P Y G E A C R F A H
TIS11 (mouse)            48    Y K T E L C R T Y S E S G R C R Y G A K C Q F A H
PIE-1 (C. elegans)       99    Y K T R L C D A F R R E G Y C P Y N D N C T Y A H


Finger 2

MEX-1 (C. elegans)      182    Y K T Q L C D K F S N F G Q C P Y G P R C Q F I H
TIS11 (mouse)            86    Y K T E L C H K F Y L Q G R C P Y G S R C H F I H
PIE-1 (C. elegans)      185    N R R Q I C H N F E R - G N C R Y G P R C R F I H
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Fig. 2. Cloning and molecular analysis of *mex-1*. (A) Sequence of a *mex-1* cDNA and corresponding protein. The *mex-1* mRNA is trans-spliced to SL1 (Krause and Hirsh, 1987) at the acceptor site shown underlined. The two finger domains of the MEX-1 protein are shown in shaded boxes, with the Cys-His residues in bold. The predicted MEX-1 protein also contains two glutamine-rich, and one-histidine rich, regions of unknown significance. The polyA sequence shown at the 3′ end of the *mex-1* cDNA is present in the *mex-1* genomic sequence, and thus may not represent the true end of the *mex-1* 3′ UTR. Part of the 5′ end of the *mex-1* gene, extending into the second exon, is contained in the cosmid W03C9 and has been sequenced recently by the *C. elegans* Genome Sequencing Project. (B) Alignment of the finger domains, including the 5 preceding amino acids, in MEX-1, TIS11 and PIE-1 proteins. The finger domains are separated by 23, 18, and 66 amino acids in MEX-1, TIS11 and PIE-1, respectively. The GenBank accession number for *mex-1* is U81043.

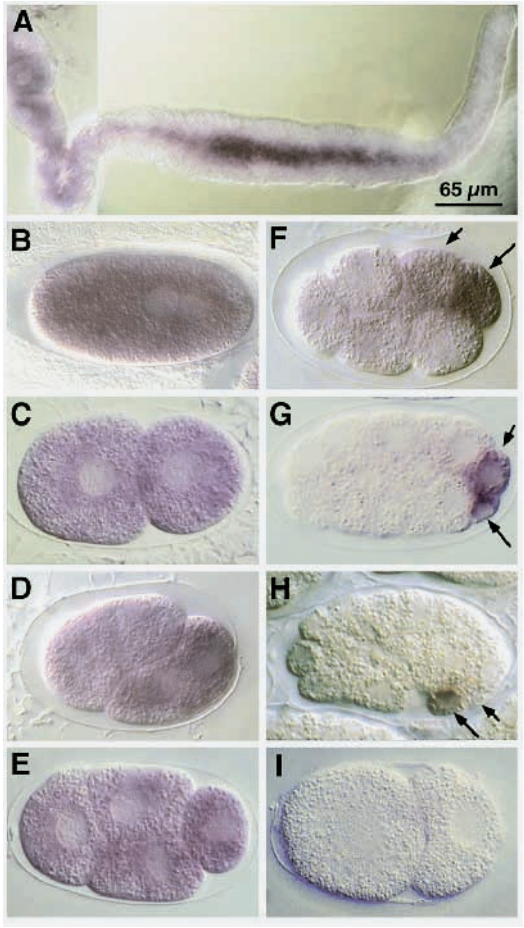

Fig. 3. Localization of *mex-1* mRNA. Photomicrographs of a wild-type hermaphrodite gonad (A) and early embryos at different developmental stages (B-H) after in situ hybridization with a probe for *mex-1* mRNA; positive staining is indicated by purple (see Materials and Methods). In this and all other figures, embryos are oriented as in the schematic diagram of Fig. 1. (A) *mex-1* mRNA is detected in the syncytial core of the gonad beginning in the meiotic region of the distal arm (right), and is distributed uniformly in maturing oocytes (left). *mex-1* mRNA appears to be distributed uniformly in 1-cell (B) and early 2-cell (C) embryos. Beginning in late 2-cell, or 3-cell embryos (D), *mex-1* mRNA appears to be at higher levels in the dividing P_1 blastomere than in AB or the AB daughters. (E) 4-cell embryo: *mex-1* mRNA appears slightly more abundant in the germline blastomere P_2 (posterior) and its sister EMS (ventral) than in the AB daughters (anterior and dorsal). (F) 8-cell embryo: *mex-1* mRNA can be detected at high levels in the germline blastomere P_3 (long arrow) and at lower levels in its somatic sister C (short arrow). (G) Early 28-cell embryo: *mex-1* mRNA is detected only in the germline blastomere P_4 (long arrow) and its somatic sister D (short arrow). (H) Gastrulation stage embryo: *mex-1* mRNA is detected in P_4 (long arrow), but not in D (short arrow). (I) Control 2-cell wild-type embryo stained with a sense probe for *mex-1* mRNA. *C. elegans* embryos are about 45 μm in length.

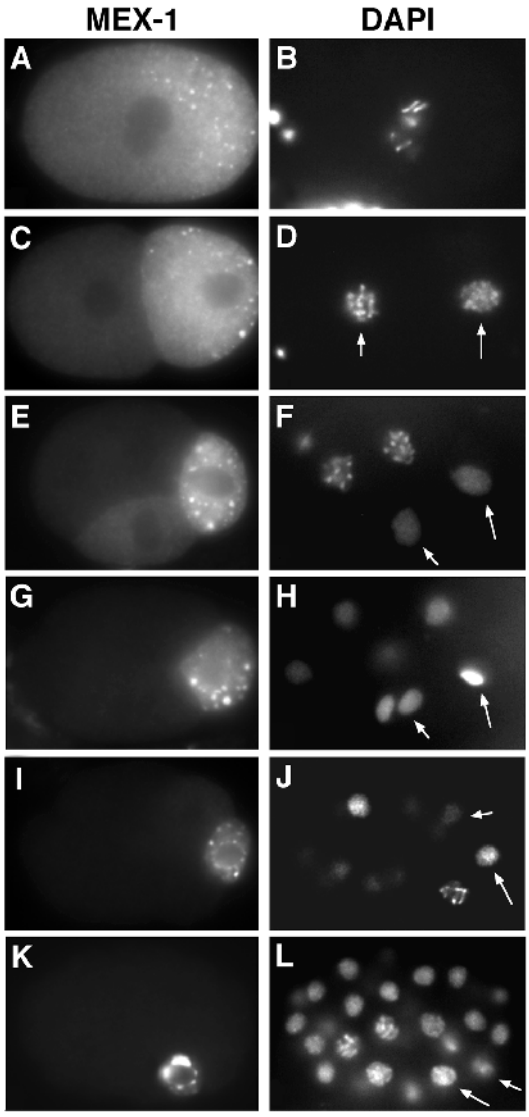

Fig. 4. Localization of the MEX-1 protein. The left column shows immunofluorescence photomicrographs of fixed wild-type embryos after staining with the affinity-purified MEX-1 antiserum 8230I (see Materials and Methods). The right column shows DAPI images of nuclei for each embryo shown on the left; in each of these images the germline blastomere (long arrow) and its sister (short arrow) are indicated. (A,B) 1-cell embryo: MEX-1 is most abundant in the posterior half of the egg, and also is present in distinct granules (for description see Fig. 5). (C,D) 2-cell embryo: MEX-1 is present at high levels in P_1 and at lower levels in AB. (E,F) Early 4-cell embryo: MEX-1 is detected in P_2 and, at lower levels, in EMS. (G,H) Dividing 6-cell embryo with EMS in telophase and P_2 in metaphase: MEX-1 is detected only in P_2. Note that the granules are localized toward the ventral side of P_2; this is the side that becomes the germline blastomere P_3 after division. (I,J) 12-cell embryo: MEX-1 is detected at high levels in P_3 and at lower levels in C. (K,L) Late 28-cell embryo: MEX-1 is detected only in P_4. All images are shown at the same magnification; embryos are 45 μm in length.

are present in both daughters of the P_4 blastomere (Fig. 5D), however MEX-1 is not detectable in either daughter (Fig. 5F). Similarly, P granules are present in the germ cells of larvae and adults, but MEX-1 is not detectable at these later stages (data not shown). Thus MEX-1 may either associate with P granules only transiently during the early embryonic cleavages, or MEX-1 may be masked in P granules at all other stages.

Ectopic repression of *pes-10* in *mex-1* mutants

We have shown that the MEX-1 and PIE-1 proteins share a common 'finger' motif, and that both are present in germline blastomeres (Mello et al., 1996; this study). Because PIE-1 has

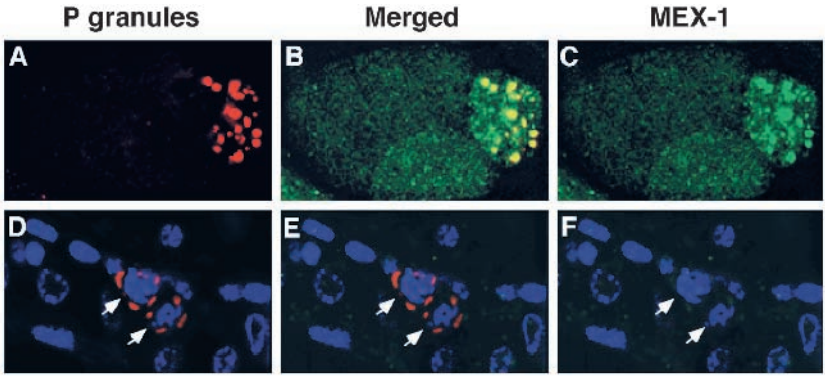

Fig. 5. MEX-1 is a component of P granules. (A-C) Immunofluorescence photomicrographs at the top focal planes of a single 4-cell embryo double-stained for P granules (A) and MEX-1 (C); the merged image is shown in (B). (D-F) Immunofluorescence photomicrograph, at high magnification, of the gonad primordium of a near-hatching embryo stained as above. The gonad primordium contains the two daughters of P_4 (arrows); the nucleus of each daughter is surrounded by P granules (visible in D) but appear to have only background levels of MEX-1 staining (F). Embryo in A-C is about 45 μm in length; Gonad primordium in D-F is about 8 μm in length.

been shown to be required for the proper regulation of embryonic transcription in germline blastomeres (Seydoux et al., 1996), we asked whether MEX-1 might have a similar role. Transcription in the early embryo can be monitored conveniently by analyzing expression of the *pes-10* gene (Seydoux and Fire, 1994). In wild-type embryos, *pes-10* mRNA is present at high levels in all somatic blastomeres from the 8-cell stage until the 15-cell stage, but is not detected in germline blastomeres at any stage (Seydoux and Fire, 1994). For example, in a wild-type 15-cell stage embryo, only one blastomere does not contain detectable levels of *pes-10* mRNA; this blastomere is the germline blastomere P_3 (Fig. 6A; see also Seydoux and Fire, 1994). In this paper we refer to the absence of *pes-10* mRNA in germline blastomeres as *pes-10* repression, though the biochemical mechanism that establishes the soma/germline difference in *pes-10* mRNA levels has not been determined. In *pie-1* mutant embryos, *pes-10* mRNA accumulates in all blastomeres, indicating that *pie-1(+)* is required for the germline-specific repression of *pes-10* (Seydoux et al., 1996).

We examined *pes-10* mRNA levels in *mex-1* mutants by in situ hybridization of 8-cell to 16-cell stage embryos. We found that *pes-10* mRNA was not detectable in multiple blastomeres in all *mex-1* mutant embryos examined (n>100), in contrast to the single blastomere lacking *pes-10* mRNA in wild-type embryos. We identified these blastomeres in the *mex-1* embryos by comparison with the positions of blastomeres in living, lineaged *mex-1* mutants (Mello et al., 1992; Schnabel et al., 1996). In almost all cases, the blastomeres that did not contain *pes-10* mRNA were the descendants of P_2 (see lineage diagram in Fig. 1). For example, *pes-10* mRNA could not be detected in either C or P_3, the daughters of P_2, in all 8-cell stage embryos examined (data not shown). At the 16-cell stage, *pes-10* mRNA could not be detected in any of the four granddaughters of P_2 (Fig. 6C). In addition to the lack of *pes-10* mRNA in the descendants of P_2, about 3% of the embryos also had reduced levels of *pes-10* in the E blastomere. We thus conclude that *mex-1* function is required for the proper pattern of *pes-10* repression, but that mutations in *mex-1* and *pie-1* cause opposite defects in this pattern: *pes-10* is not repressed in any blastomere in *pie-1* mutants, but is repressed in multiple blastomeres in *mex-1* mutants.

To test whether *pes-10* repression in *mex-1* mutants requires *pie-1*(+) activity, we examined *pes-10* mRNA in *mex-1;pie-1* double mutants. We detected *pes-10* mRNA in all blastomeres in *mex-1;pie-1* double mutant embryos (Fig. 6E), as in *pie-1* single mutants (Seydoux et al., 1996). These results suggest that the abnormal pattern of *pes-10* repression in *mex-1* single mutants could result from incorrect localization of PIE-1.

PIE-1 is mislocalized in *mex-1* mutants

We stained *mex-1* mutant embryos with an antibody that recognizes the PIE-1 protein and found that PIE-1 was present in multiple blastomeres. In nearly all *mex-1* embryos, PIE-1 could be detected in the daughters and granddaughters of the P_2 blastomere (90%, n=75; Fig. 6D). In about 46% of the 8-cell stage *mex-1* embryos, low levels of PIE-1 also were detected in the E and MS blastomeres (Fig. 7A; see lineage chart in Fig. 1). In addition to the ectopic localization of PIE-1, we observed that *mex-1* mutants had reduced levels of P granule-associated PIE-1 compared to wild-type embryos (compare P granules in the wild-type P_3 blastomere in Fig. 6B to the P_3 blastomeres in the *mex-1* mutants in Fig. 7A,C).

Because *mex-1* mutants have been shown to have a cold-sensitive defect in intestinal development (Mello et al., 1992), and in some *mex-1* mutant embryos PIE-1 is mislocalized to E, the intestine precursor, we asked if PIE-1 mislocalization was cold-sensitive. We found that at low temperature (15˚C) 74% of the *mex-1* mutant embryos contained relatively high levels of PIE-1 in E and MS (Fig. 7C), while only 46% of the embryos had detectable PIE-1 in E and MS at the standard culture condition of 22˚C (Fig. 7A). We conclude that *mex-1* mutations affect both the embryonic localization and cytoplasmic distribution of the PIE-1 protein, and that the mis-localization of PIE-1 in *mex-1* mutants becomes more severe at low temperature.

DISCUSSION

MEX-1 and PIE-1 have different functions in germ cell development

In this report we have shown that the *C. elegans* MEX-1 protein contains two repeats of a ‘finger’ domain that has been found in several proteins of unknown function from both vertebrates and invertebrates. The only *C. elegans* protein described to date that contains similar domains is the PIE-1 protein (Mello et al., 1996). Both MEX-1 and PIE-1 are required for germ cell development, both are present in the germline blastomeres of early embryos, and both are components of P granules (Mello et al. 1992, 1996, this study). These similarities suggest the possibility that MEX-1 and PIE-1 might have common functions.

However, PIE-1 is a predominantly nuclear protein (Mello et al., 1996), while MEX-1 lacks a recognizable nuclear localization signal and appears to be predominantly, if not exclusively, cytoplasmic.

PIE-1 is required for the repression of embryonic transcription of genes such as *pes-10* in the germline blastomeres (Seydoux et al., 1996). We have shown that MEX-1 is not required to repress *pes-10* transcription. However, MEX-1 is required for the proper spatial pattern of *pes-10* repression, apparently through its role in localizing PIE-1. Ectopic expression of *pie-1* driven by a heat-shock promoter has been shown to reduce the level of mRNA from an embryonically transcribed reporter gene by about 75% in all blastomeres (Seydoux et al., 1996). This result has suggested that PIE-1 may be sufficient to repress embryonic transcription, and is consistent with our finding that ectopic PIE-1 in *mex-1* mutants results in ectopic repression of *pes-10*.

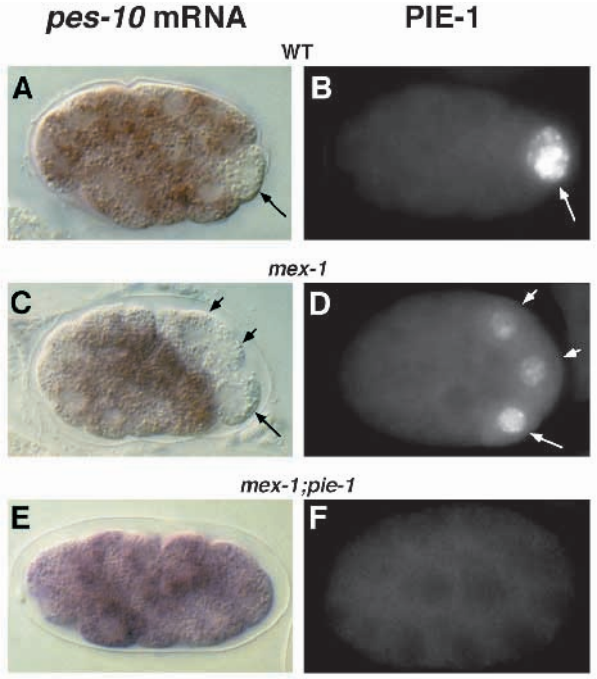

Fig. 6. Embryonic transcription and PIE-1 localization. Each row shows a pair of different embryos that are at the same developmental stage; genotypes are listed above each pair of embryos. Embryos in the left column were cultured at 22˚C and fixed and hybridized with a probe for *pes-10* mRNA. Embryos in the right column were fixed and immunostained for PIE-1 protein (see Materials and Methods). (A,B) In wild-type (WT) embryos, *pes-10* mRNA is not detected in the germline blastomere P_3 (A, arrow), but is present in all other blastomeres. PIE-1 is visible in the nucleus of P_3 (B, arrow), and associated with P granules in the cytoplasm of P_3. (C,D) In *mex-1(zu120)* mutants, *pes-10* mRNA is not detected in the P_2 granddaughters as follows: P_4 (long arrow), the two daughters of C (short arrows), and D (not visible in focal plane); each of these blastomeres contains PIE-1 protein. Note the absence of P granule-associated PIE-1 in the cytoplasm of the P_4 blastomere. (E,F) In *mex-1(zu120);pie-1(zu154)* double mutants, *pes-10* mRNA is present in all blastomeres, and PIE-1 is not detected in any blastomere. All embryos shown were fixed approximately 75 minutes after fertilization (about the 15-cell stage). Because the blastomeres P_2 and P_3 divide slightly faster in *mex-1* mutants than in wild-type embryos (Schnabel et al., 1996), the P_4 blastomere is present in the *mex-1* mutant although P_3 has not yet divided in the wild-type embryo shown. In *mex-1* mutants at earlier cleavage stages, *pes-10* mRNA is not detected in P_3 and C (data not shown). Embryos shown are 45 μm in length.

P granule localization to germline blastomeres

Previous studies have shown that *mex-1(+)* activity is required for the proper cortical association of P granules in germline blastomeres during the early, asymmetrical cleavages (Mello et al., 1992; Schnabel et al., 1996). Our observation that MEX-1 protein is a component of P granules only during these cleavages suggests that P granule-associated MEX-1 might directly bind to proteins at the cortex, such as PAR-1 or PAR-2 (Guo and Kemphues, 1995; Boyd et al., 1996). Alternatively, MEX-1 could be required for the proper structure or assembly of P granules, and thus affect cortical localization indirectly. For example, analysis of *Drosophila* mutants defective in polar granules indicates that the proper assembly of these structures requires the sequential addition of specific components (for review see Lehmann and Ephrussi, 1994).

We have analyzed three P granule components in *mex-1* mutants; an unknown antigen recognized by the K76 antibody (Strome and Wood, 1982), the MEX-3 protein (Draper et al., 1996), and the PIE-1 protein (Mello et al., 1996). In *mex-1* mutants, both the K76 antigen and the MEX-3 protein appear, by immunofluorescence, to be present in P granules at wild-type levels (S. Guedes and J. Priess, unpublished observations). However, we consistently detect abnormally low levels of PIE-1 staining in the P granules of *mex-1* mutants. Because the level of nuclear PIE-1 in the P_3 blastomere of *mex-1* mutants appears only slightly lower than in a wild-type P_3, we do not know whether the diminished PIE-1 staining of P granules can be explained entirely by the reduced level of PIE-1. Alternatively, MEX-1 may have a specific function in the association of PIE-1 with P granules; in future experiments it will be interesting to determine whether there are direct interactions between the MEX-1 and PIE-1 proteins. For example, P granule-associated MEX-1 might recruit PIE-1 into the granules through the common 'finger' domains of MEX-1 and PIE-1.

It is possible that PIE-1 becomes mislocalized in *mex-1*

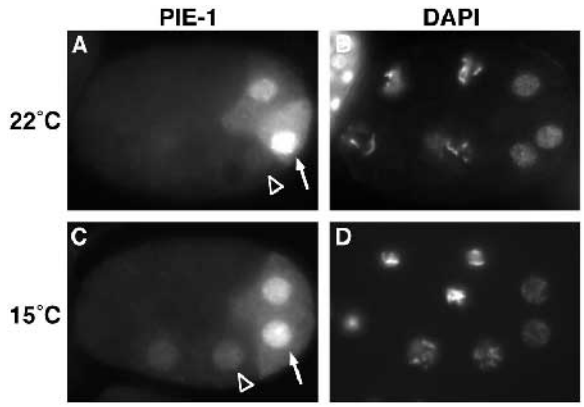

Fig. 7. Temperature-dependent PIE-1 mislocalization. Each row shows a single, late 8-cell stage, *mex-1* mutant embryo that was cultured at either 22˚C or 15˚C prior to fixation. Left column: immunofluorescence photomicrographs of embryos stained for PIE-1 protein. Right column: fluorescence photomicrographs of nuclei stained with DAPI. Arrows point to the P_3 blastomere, and arrowheads point to the E blastomere. Embryos are 45 μm in length.

mutants simply because PIE-1 is a P granule component, and P granules are mislocalized in *mex-1* mutants. However the basis for the wild-type pattern of PIE-1 localization has not been determined, and may not involve the association between PIE-1 and P granules (Mello et al., 1996). For example, PIE-1 appears to be concentrated at the centrosomes of cells prior to division, and is not detected in P granules when cells are dividing (Mello et al., 1996). In dividing cells, PIE-1 is detected at high levels in the centrosome destined for the germline daughter, but is present at much lower levels, or not detected, in the centrosome destined for the somatic daughter. In *mex-1* mutants, we have observed some mitotic spindles with PIE-1 at similar levels in both centrosomes (S. Guedes, unpublished results). Thus *mex-1* mutations may cause defects in the polarity of germline blastomeres that are independent of P granule structure or function.

Germline/soma defects in *mex-1* mutants

If P granules contain germ cell determinants, the lack of germ cells in *mex-1* mutants could result from either the mislocalization of P granules, or from changes in the composition of P granules. We have shown that PIE-1, a component of P granules, is mislocalized in *mex-1* mutants. We do not yet know whether this defect is sufficient to explain the lack of germ cells, or whether other factors that contribute to, or that can interfere with, germ cell development also are mislocalized. In *mex-1* mutants, P_3 and P_4 contain slightly less than wild-type levels of PIE-1. Nevertheless, this level of PIE-1 is sufficient to repress *pes-10* transcription in P_3 and P_4, as PIE-1 does in wild-type embryos. However, the lower levels of PIE-1 could be insufficient to prevent the action of transcription factors, such as SKN-1 and PAL-1, that might promote somatic differentiation in P_3 and P_4 (Bowerman et al., 1992, 1993; Hunter and Kenyon, 1996).

Any model for MEX-1 function must explain the defects in the development of somatic blastomeres that are observed in *mex-1* mutants. Part of the specification of early blastomere fates in *C. elegans* involves the relative activities of PIE-1, that appears to repress embryonic transcription, and the maternally supplied transcription factors SKN-1 and PAL-1 (Bowerman et al., 1992, 1993; Draper et al., 1996; Hunter and Kenyon, 1996; Mello et al. 1996; Seydoux et al., 1996). SKN-1, but not PAL-1, plays a role in the development of the E blastomere in wild-type embryos, although both transcription factors are present in E. This is presumably because SKN-1 appears before PAL-1 in the parent of E, and PIE-1 is not present in that parent. In contrast, PAL-1, but not SKN-1, plays a role in the development of the C blastomere in wild-type embryos, although both factors also are present in C. This appears to be due to the fact that PIE-1 is present in the parent of C, blocking the activities of both PAL-1 and SKN-1. SKN-1 disappears immediately after C is born, allowing C to respond only to PAL-1. In about 50% of *mex-1* mutant embryos cultured at low temperature, the E blastomere is transformed into a C-like blastomere (Mello et al., 1992). This is precisely the transformation in fate that would be predicted from our finding that PIE-1 is mislocalized to the E blastomere and the parent of E; PIE-1 would be expected to prevent SKN-1 from functioning in this lineage, allowing E to respond to PAL-1 once SKN-1 and PIE-1 were no longer present.

PIE-1 mislocalization, however, can not be the sole cause of somatic defects in *mex-1* mutants. Anterior blastomeres in *mex-1* mutants have abnormally high levels of SKN-1 protein and defects that are due, in part, to inappropriate *skn-1(+)* activity (Mello et al., 1992; Bowerman et al., 1993). We have never detected PIE-1 in these anterior blastomeres in *mex-1* mutants, and previous studies have shown that these blastomeres do not return to a wild-type pattern of development in *mex-1;pie-1* double mutants (Mello et al., 1992).

In a wild-type 2-cell stage embryo, SKN-1 is present at much higher levels in the posterior blastomere than in the anterior blastomere (Bowerman et al., 1993). It is not yet understood how this asymmetry is achieved; *skn-1* mRNA is distributed equally between both blastomeres (Seydoux and Fire, 1994), suggesting that there is regulation at the level of *skn-1* mRNA translation, or SKN-1 protein stability. In *mex-1* mutants, SKN-1 is present at nearly equal levels in both anterior and posterior blastomeres. Thus MEX-1 might function as a regulator of *skn-1* mRNA, or be required for the proper localization of such a regulator. For example, a regulator might be associated with P granules, and require MEX-1 for this association.

There are analogies between the somatic/germ cell defects associated with mutations in *mex-1* and mutations in the 'posterior group' genes of *Drosophila*, many of which encode components of polar granules (see Mello et al., 1992). The abdominal determinant *nanos* is associated with polar granules; mutations in genes such as *vasa* that prevent polar granule formation prevent the proper posterior localization of *nanos*, and thus disrupt somatic differentiation indirectly (for review see Lehmann and Ephrussi, 1994). The germline cells, or germline precursors, in many animal embryos contain cytologically distinct structures similar to the polar granules of *Drosophila* or the P granules of *C. elegans*. It will be interesting to determine in future studies whether molecular components of these structures, such as MEX-1, have been conserved in animal evolution, and whether these structures in general serve to localize factors involved in both somatic and germ cell development.

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