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PIE-1, MEX-5, MEX-6 and Soma/Germline Asymmetry in *C. elegans* Embryos

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A thesis submitted in partial fulfillment of the
Requirements for the degree of

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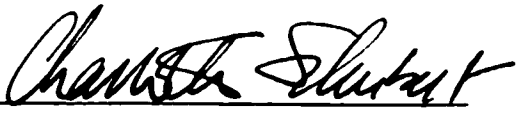
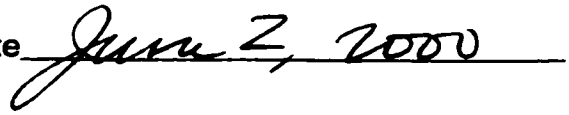
Signature 

Date 

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Abstract

PIE-1, MEX-5, MEX-6 and Soma/Germline Asymmetry in *C. elegans* Embryos

Charlotte M. Schubert

Chairperson of the Supervisory Committee:
Affiliate Professor James R. Priess
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Member, Basic Sciences,
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The separation between soma and germline in *C. elegans* embryos occurs through a series of asymmetric cell divisions initiating with the 1-cell stage. Each of these divisions results in one daughter that will produce only somatic cells, called a somatic precursor. The other daughter will produce germ cells in addition to somatic cells and is called a germline precursor.

Each germline precursor contains, but does not respond to, factors that promote somatic differentiation. This property requires the maternal gene *pie-1;* mutations in *pie-1* result in the germline precursors adopting somatic cell fates. In the first part of this thesis I describe the immunolocalization of PIE-1. I show that PIE-1 is predominantly a nuclear protein, localized exclusively to each germline precursor. The localization of PIE-1 contributes to data that suggest

PIE-1 functions in each germline precursor to prevent the transcription or accumulation of embryonically transcribed mRNAs.

PIE-1 belongs to a group of proteins, called germline proteins, that localize to germline precursors. In the second part of this thesis I describe my work on two highly related genes, *mex-5* and *mex-6*, that have high sequence similarity and appear to function directly in localizing PIE-1 and other germline proteins. Embryos lacking *mex-5/mex-6* show uniform expression of germline proteins and other classes of proteins required for the identities of early blastomeres. I provide evidence that MEX-5/MEX-6 inhibits the expression of germline proteins; MEX-5 is a novel, cytoplasmic protein, localized in a pattern reciprocal to that of the germline proteins, and ectopic expression of MEX-5 is sufficient to inhibit the expression of germline proteins.

I show that MEX-5 expression is dependent on a group of cortical proteins (PAR proteins) that are asymmetrically distributed as early as the 1-cell stage; anterior PAR proteins associate with the anterior cortex, and posterior PAR proteins associate with the posterior cortex of the egg. I show that MEX-5 localization requires both an anterior and a posterior PAR protein, and provide evidence that MEX-5/MEX-6 link asymmetries in the expression of the PAR proteins to asymmetries in proteins, like PIE-1, that specify the fates of somatic and germline precursors.

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This thesis is dedicated to my mother, Ilze Schubert

And to the memory of

my grandmother, Zelma Jirgens

INTRODUCTION

Asymmetric Cell Division and Cortical Polarity Cues

The ability of one cell to produce daughter cells that adopt different fates is a fundamental developmental process and occurs in all kingdoms. In the bacterium *Bacillus subtilis*, and the yeast *Saccharomyces cerevisiae*, two daughter cells with different cell fates are produced during asexual reproduction (reviewed in Chant, 1999; Losick and Dworkin, 1999). Asymmetric cell division gives rise to the two cell types that compose the simple multicellular organism, *volvox* (reviewed in Kirk, 1997). In mammals and other metazoa the asymmetric division of pluripotent stem cells produces differentiated skin, muscle, neural, and other cell types.

All asymmetric divisions must in principle involve the localized activity of a molecular cue that directs the asymmetric localization of determinants in daughter cells. In a variety of organisms, some of the molecules that act as cues and determinants have been identified. In the best-studied eukaryotic systems, described in the next several paragraphs, cortical proteins within dividing cells have been shown to have pivotal functions as localized cues. In *Saccharomyces cerevisiae,* a complex containing the rhoGTPase CDC42 localizes at one pole of the cortex and polarizes the cytoskeleton prior to asymmetric cell division. In *Drosophila* and *C. elegans* a conserved cortical protein (Bazooka in *Drosophila*

and PAR-3 in *C. elegans*) directs asymmetry. Bazooka acts as a cue for asymmetric divisions in dividing neuroblasts of the central nervous system. In *C. elegans*, PAR-3 localizes to one pole within a dividing blastomere and directs the asymmetric localization of molecules that establish cell fate. An understanding of the mechanisms that link these cortical cues to differential gene expression is far from complete. A comparison of these systems yields only a few conserved proteins and highlights the diversity of mechanisms that underlie asymmetric cell division.

In wild-type haploid yeast, the mother and daughter cells each have a distinct potential for mating ("mating type"); (For general reviews of yeast asymmetry see Chant, 1999; Shulman and St Johnston, 1999). The haploid cells of one mating type conjugate only with haploid cells of the opposite type during mating. The difference in mating type occurs through the asymmetric expression of the transcription factor Ash1, which is expressed only in the daughter cell. Ash1 is necessary for the transcription of genes that direct the daughter cell fate, and its ectopic expression is sufficient to transform the fate of a mother cell. Ash1 can thus be viewed as a determinant of daughter cell fate.

The asymmetric distribution of Ash1 is dependent on the previous site of cell separation (the bud scar). Each daughter cell arises adjacent to this bud scar. A group of proteins localize adjacent to the bud scar and appear to polarize the cytoskeleton. As a result of cytoskeletal polarity, the mRNA for Ash1 is

asymmetrically distributed. Ash1 ribonucleoprotein particles have been shown to move along polarized actin cables into the newly forming daughter cell. Consequently, only the daughter cell inherits Ash1 mRNA, and produces Ash1 protein, after cell division. Thus, in *Saccharomyces cerevisiae*, an intrinsic cue establishes polarity in the cytoskeleton. As a consequence, a protein (Ash1p) that regulates cell fate is asymmetrically localized.

The rhoGTPase CDC42 localizes adjacent to the bud scar in a complex required for polarity of the yeast cytoskeleton. In many organisms, proteins in the rhoGTPase family can induce actin-dependent events in processes such as cell migration, axonal pathfinding, and macrophage phagocytosis (St. Johnston, 1999). Thus, molecules involved in asymmetric cell division may have general roles in cell polarity.

Perhaps the best-studied example of asymmetric cell division in higher animals occurs in *Drosophila* during the division of neural precursor cells, called neuroblasts (For general reviews of neuroblast asymmetry see Hawkins and Garriga, 1998; Jan and Jan, 2000). In the central nervous system, neuroblast asymmetry arises through the asymmetric expression of a transcription factor in response to an intrinsic cue. Each division of a neuroblast produces a ganglion mother cell (GMC) and a new neuroblast. The transcription factor Prospero is expressed in the nucleus of the GMC where it appears to act as a determinant for GMC cell fate; daughter cells lacking Prospero become neuroblasts, and

ectopic expression of Prospero is sufficient to transform a neuroblast into a GMC. Prospero and its mRNA are transiently expressed during metaphase in the neuroblast at the cortex of the pole that becomes the GMC at cell division. Thus, asymmetric localization of Prospero mRNA and protein in the mother cell leads to segregation of Prospero mRNA and protein into one daughter cell.

The molecular cue for Prospero asymmetry has recently been shown to involve the protein Bazooka (Schober et al., 1999; Wodarz et al., 1999, reviewed in Jan and Jan, 2000). Bazooka is localized to the apical cortex of a layer of neuroepithelial cells; neuroblasts delaminate from this layer and maintain apical expression of Bazooka during delamination and subsequent division. During division, Bazooka anchors a complex of proteins to the apical neuroblast cortex. This apical complex is required to localize a second complex to the basal side. Within this second, basal complex are "adaptor" proteins that anchor either Prospero protein or Prospero mRNA to the cortex. Surprisingly, neuroblasts that lack expression of the mRNA anchor, Staufen, show proper Prospero protein localization despite the presence of uniform Prospero mRNA. Thus, Prospero mRNA localization does not appear to be the primary mechanism that establishes the asymmetric localization of Prospero protein. In the absence of Bazooka all known components of both apical and basal complexes are delocalized, including Prospero protein and mRNA, which become uniformly expressed throughout the metaphase cortex. Thus, Bazooka serves to transmit

the polarity of the epithelial cell layer to the delaminating neuroblast, and is the earliest acting identified marker of polarity in the neuroblast. Bazooka contains a protein interaction domain called a PDZ domain, but contains no other defined functional domains. The mechanisms by which apical localization of a Bazooka-containing complex results in the basal localization of Prospero are unknown.

In the *C. elegans* embryo, a Bazooka ortholog, PAR-3, likewise has a critical function in generating asymmetry (For general reviews of *C. elegans* embryogenesis see Schnabel and Priess, 1997; Rose and Kemphues, 1998; Bowerman and Shelton, 1999). At the 1-cell stage division, PAR-3 is expressed at the anterior cortex (Figure 2). After this division, two molecularly distinct sister blastomeres are produced; a large anterior blastomere (AB) and a small posterior blastomere (P1) (Figure 1). Each of these blastomeres expresses unique transcription factors and cytoplasmic proteins that establish differences in cell fate. For example, the transcription factor SKN-1 is expressed in P1 and is required for cell fates arising from P1 (Figure 2). In embryos lacking the function of *par-3*, all known aspects of anterior-posterior polarity are disrupted; both the AB and the P1 blastomeres are the same size, and they express the same sets of determinants, including SKN-1.

In *Drosophila* neuroblasts and in *C. elegans* embryos, Bazooka/PAR-3 are required for orientation of the mitotic spindle. In both *Drosophila* neuroblasts and the *C. elegans* blastomere P1, the spindle rotates 90 degrees prior to division

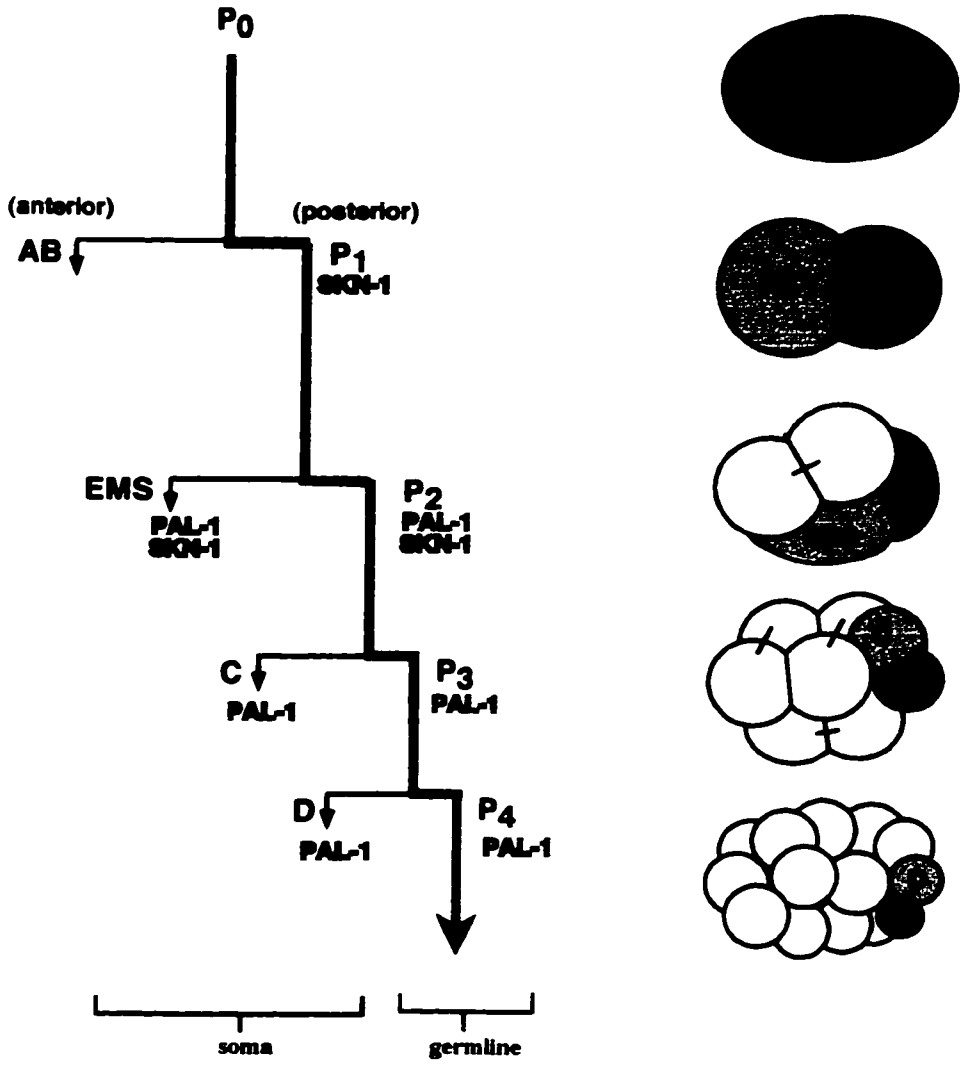

Figure 1: Lineage diagram and cartoon of early embryogenesis. On the left is a lineage diagram of the early cleavage stages. Blastomeres that express PAL-1 and SKN-1 are noted. On the right are cartoons of the early cleavage stages. Germline precursors are shown in dark green. Somatic precursors are shown in light green.

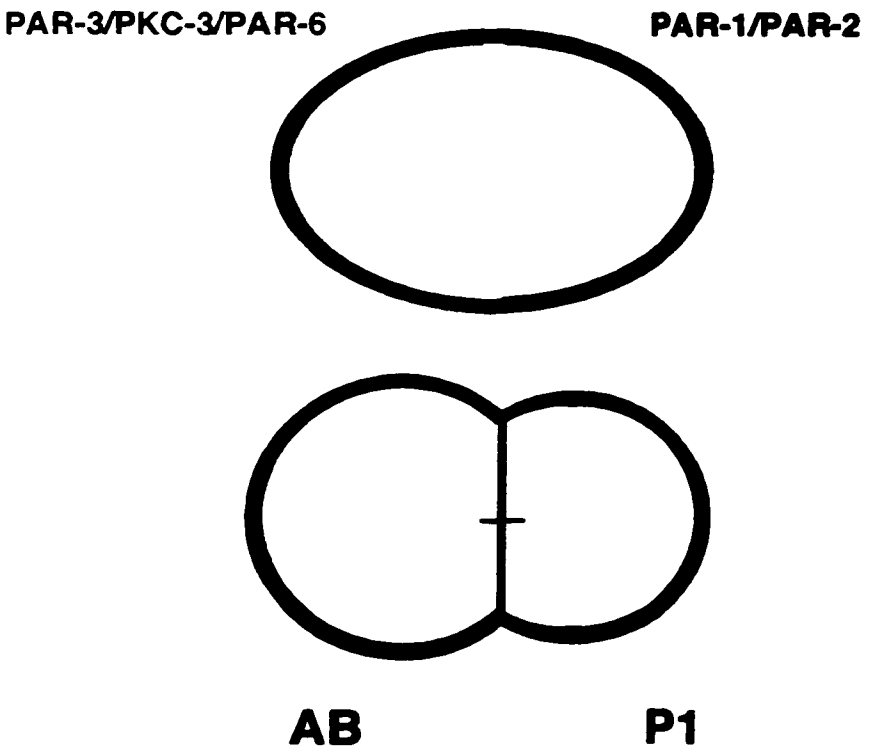

Figure 2: Anterior and posterior PAR proteins show reciprocal patterns of cortical localization.

and determinants are asymmetrically distributed along the resulting axis of division. The correlation of spindle axis and the segregation of determinants has led to speculation that these processes are linked. However, spindle rotation is not a prerequisite for polarization of proteins that regulate cell fate; when spindle orientation is disrupted due to application of cytoskeletal inhibitors (reviewed in Jan, 2000) or through mutation (Rose and Kemphues, 1998; Zwaal et al., 1996), determinants are still asymmetrically distributed. Therefore distinct molecules in both animals could mediate spindle orientation and asymmetric localization of determinants.

In the *Drosophila* central nervous system, proteins localized to the apical cortex (Inscutable and Pins) mediate both Prospero asymmetry and spindle rotation. However, one basally-associated cortical protein is required only for Prospero protein asymmetry. Miranda functions as an "adapter" protein that anchors Prospero to the basal cortex, but mutations in Miranda do not affect spindle orientation. Similarly, neuroblasts lacking Staufen function fail to localize prospero mRNA, but are not defective in spindle orientation. Thus, Miranda and Staufen mediate Prospero asymmetry, but not neuroblast spindle orientation. Whether the molecules that mediate asymmetric distribution of determinants in *C. elegans* are distinct from molecules that mediate spindle orientation remains to be determined.

Several proteins, including Miranda and Staufen, have been characterized that bind to either Bazooka or Prospero. However, database searches reveal that few of these proteins are conserved in *C. elegans*, and none have orthologs shown to be required for early embryogenesis. In *Drosophila*, the determinant Prospero is asymmetrically segregated into one daughter cell through transient cortical association. However, in *C. elegans* none of the transcription factors that appear to function as determinants, including SKN-1, show this type of pattern. Thus, some of the underlying mechanisms that direct asymmetry in *Drosophila* neuroblasts and *C. elegans* embryos may also be different. However, much remains to be learned about how the PAR/Bazooka group of proteins transmits polarity information in both organisms.

At the first cleavage in *C. elegans*, most maternally supplied mRNAs such as *skn-1* mRNA are uniformly distributed (Evans et al., 1994; Seydoux and Fire, 1994; Guedes and Priess, 1997; Tenenhaus et al., 1998). Thus, for most maternal proteins in *C. elegans*, underlying asymmetry in mRNA does not direct asymmetry in respective proteins. Instead, the asymmetric patterns of protein expression must result from protein transport or from differences among blastomeres in either protein stability or mRNA translation.

Several "PAR" proteins have been identified that function with PAR-3 in generating asymmetry (For review see Rose and Kemphues, 1998). PKC-3 and PAR-6 appear to form a complex with PAR-3 and localize to the anterior cortex

(Figure 2). PAR-2 and PAR-1 localize to the posterior cortex (Figure 2). These opposing domains of PAR expression require interdependent interactions among the PAR proteins. For example, mutations in either *par-3* or *par-6* result in uniform localization of all remaining PAR proteins throughout the cortex. Although PAR asymmetry is clearly linked to blastomere differences, the nature of this linkage has remained mysterious, and specific targets of the PAR proteins have not been identified.

PAR-like proteins have been identified in mammals, flies and nematodes. Othologs of PAR-3 and PKC-3 in mammals associate, colocalize, and are distributed in a polarized fashion on the cortex of epithelial cells (Izumi et al., 1998). Mammalian PAR-1 is likewise asymmetrically localized on the cortex of polarized epithelial cells (Bohm et al., 1997). In *Drosophila*, orthologs of PKC-3 and PAR-6 have been identified, but no function in asymmetric cell division has, as yet, been determined (Wodarz et al., 1999; Schober et al., 1999). An understanding of how the PAR proteins in *C. elegans* generate asymmetry could therefore provide general insights into cellular asymmetry.

The *C. elegans* embryo also provides a system for examining how determinants become asymmetrically localized during a series of asymmetric divisions. The P1 blastomere gives rise to, among other cell types, germline, and is therefore called a germline precursor. The anterior blastomere gives rise to only somatic cell fates and is called a somatic precursor (reviewed in Schnabel

and Priess, 1997; Seydoux and Strome, 1999). The first division is followed by a series of similar stem cell-like asymmetric divisions that each produce a large somatic precursor and a small germline precursor (Figure 1). The germline precursors, which repeat the asymmetric divisions, are called P cells, (Po, P1, P2, P3, P4). 4 somatic precursor cells are produced from these divisions: AB, EMS, C and D. P4 is the terminal germline precursor, and gives rise only to germ cells. Intriguingly, the asymmetrical distribution of the PAR proteins is reproduced in germline precursors after the 1-cell stage in *C. elegans* (Figure 2). This has led to speculation that the PAR proteins could function at multiple cell divisions to regenerate asymmetric patterns of cell division (Rose and Kemphues, 1998). However, whether or not the PAR proteins function at these stages is unknown.

In the next section I describe some of the unique properties of germline precursors, and I describe several genes that establish the unique identities of somatic and germline precursors. These descriptions provide background for my molecular studies of a gene, *pie-1*, required for germline precursor fate, and the genes *mex-5/mex-6*, that appear to link PAR-mediated polarity cues to soma/germline asymmetries.

Germline precursors are distinct from somatic precursors

The germline precursors of *C. elegans* and other animals share some characteristics. For example, several studies have shown that *C. elegans* and *Drosophila* germline precursors both appear to silence de novo transcription (Seydoux and Strome, 1999). In the *C. elegans* studies, the in situ expression patterns of 12 zygotically transcribed RNAs were analyzed. These various RNAs were identified by several means, including promotor trapping and molecular screens for genes expressed preferentially in pre-gastrulation embryos. All somatic precursors were found to express these zygotic RNAs, while the germline precursors did not (Figure 3) (Seydoux and Fire, 1994). This result suggested that an inherent property of germline precursors is the inability to transcribe (or stabilize) zygotic RNAs. A different experimental approach led to a similar conclusion in *Drosophila*. In labeling experiments, somatic nuclei but not germline nuclei were shown to incorporate [3H]UTP (Lamb and Laird, 1976; Zalokar, 1976) More recent studies in *C. elegans* and *Drosophila* have shown that the absence of zygotically transcribed RNAs in the germline precursors of both animals correlates with the absence of a specific sub-population of phosphorylated RNA polymerase II. In these studies embryos were stained with two antibodies that recognize phosphorylated epitopes on the C-terminal domain of RNA Polymerase II. While somatic precursors stained brightly with this antibody, germline precursors showed reduced or no staining (Seydoux and

Dunn, 1997). In other systems, phosphorylation of the C-terminal domain of RNA Polymerase II is associated with transcriptional activity (Dahmus, 1996, Zeng at al., 1997, Patturajan et al., 1998). Therefore, the absence of this epitope in *C. elegans* and *Drosophila* germline precursors provides further evidence that the germline precursors do not transcribe zygotic RNAs. As *C. elegans* and *Drosophila* are separated by more than 1,000 million years of evolution (Vanfleteren et al., 1994), it is possible that inhibition of RNA Polymerase II function could represent a general mechanism that establishes some of the unique properties of germline precursors during early embryogenesis.

Distinctive cytoplasmic granules are segregated into the germline precursors of *C. elegans*, as well as the germline precursors of other animals such as *Drosophila* and *Xenopus*. These structures, called polar granules in *Drosophila* and P granules in *C. elegans*, associate with the germline during all developmental stages in *C. elegans* (Reviewed in Seydoux and Strome, 1999). In *C. elegans*, embryos lacking expression of any of several P granule-associated proteins undergo embryogenesis, but do not form functional germ cells. However, the role of P granules during embryogenesis in specifying the differences between each soma/germline pair, if any, is unknown.

PIE-1 prevents SKN-1 and PAL-1 from promoting somatic differentiation in germline precursors

Each of the somatic precursors, AB, EMS, C and D contain unique combinations of transcription factors that initiate distinct patterns of differentiation. For example, the transcription factors SKN-1 and PAL-1 show distinct but overlapping patterns of expression in somatic precursors, and direct distinct differentiation programs. Both SKN-1 and PAL-1 are expressed in germline precursors, but these precursors do not undergo PAL-1 or SKN-1 dependent differentiation programs. Instead, the activity of these transcription factors in germline precursors is prevented by the *pie-1* gene. Below I detail some of the studies that led to these conclusions.

Both the *skn-1* and *pie-1* genes were identified in screens for embryonic lethal maternal effect mutants. These screens focused on mutations that resulted in cell fate transformation, including transformations of the first soma/germline pair, AB and P1. In wild-type embryos the P1 blastomere produces pharynx and intestine when cultured in isolation, while the AB blastomere does not (Priess et al., 1987). Because pharynx and intestine are structures easily identified under the light microscope, alterations in the production of these cell types were used as a basis for genetic screens. Several classes of mutants were identified in these screens. One class, for example,

produces embryos that fail to form pharynx and intestine, and another class produces embryos that generate ectopic pharynx and intestine.

Mutations in the gene *skn-1* fell into the first class; embryos from *skn-1* mutant worms fail to produce pharynx and intestine (Bowerman et al., 1992). *skn-1* was shown to encode a transcription factor that positively regulates the production of pharynx and intestine. At the 2-cell stage SKN-1 is expressed at high levels in the germline precursor P1 and at low levels in the somatic precursor AB (Bowerman et al., 1993). Several mutants have been identified that form pharynx and intestine ectopically from the AB blastomere, and all of these mutants have been shown to express high levels of SKN-1 in the AB blastomere (Bowerman et al., 1993; Bowerman et al., 1997). Furthermore, the production of pharynx and intestine in these mutant embryos has been shown to require *skn-1(+)* activity (Bowerman et al., 1993; Bowerman et al., 1997; Mello et al., 1992). Thus, some of the differences between AB and P1 fate can be explained by the presence of high levels of the transcription factor SKN-1 in the P1 blastomere.

After P1 divides, SKN-1 is expressed in both daughters of P1, but in wild-type embryos only the somatic daughter EMS produces pharynx and intestine (Figure 3). This suggested that *skn-1(+)* activity in the daughter, P2, might be repressed. The *pie-1* (**p**harynx and **i**ntestinal **e**xcess) mutant was recovered because it produced embryos that generated pharynx and intestine from both

EMS and P2 (Figure 3) (Mello et al., 1992). This and other observations suggested that *pie-1(+)* activity normally represses *skn-1(+)* activity in P2 (Mello et al., 1992) (Figure 3).

Genetic analyses demonstrated that *pie-1(+)* activity is also required to repress the activity of the transcription factor PAL-1 in the germline precursors. *pal-1* is required zygotically for multiple developmental events (Waring and Kenyon, 1991), so an analysis of the maternal function for *pal-1* in early embryogenesis was facilitated by the technique of RNA mediated interference (RNAi) (For references on RNAi see Powell-Coffman et al., 1996; Rocheleau et al., 1997). RNAi of *pal-1* showed that *pal-1* was required during the early cleavage stages for proper specification of cell fates from C and D (Hunter and Kenyon, 1996). PAL-1 is expressed in the nucleus of C and D. However, it is also expressed in all descendants of P1, including the germline precursors (Hunter and Kenyon, 1996). The activity of *pal-1* in germline precursors appears to be prevented by *pie-1(+)* activity; in *pie-1;skn-1* mutants the germline precursors produce PAL-1-dependent cell types (Mello et al., 1992; Mello et al., 1996). Thus, *pie-1* is critical for differences between somatic and germline precursors, and prevents the activity of at least two transcription factors (SKN-1 and PAL-1) that would otherwise promote somatic differentiation.

Since I was interested in the processes that establish differences between the somatic and germline precursors I participated in molecular studies of the

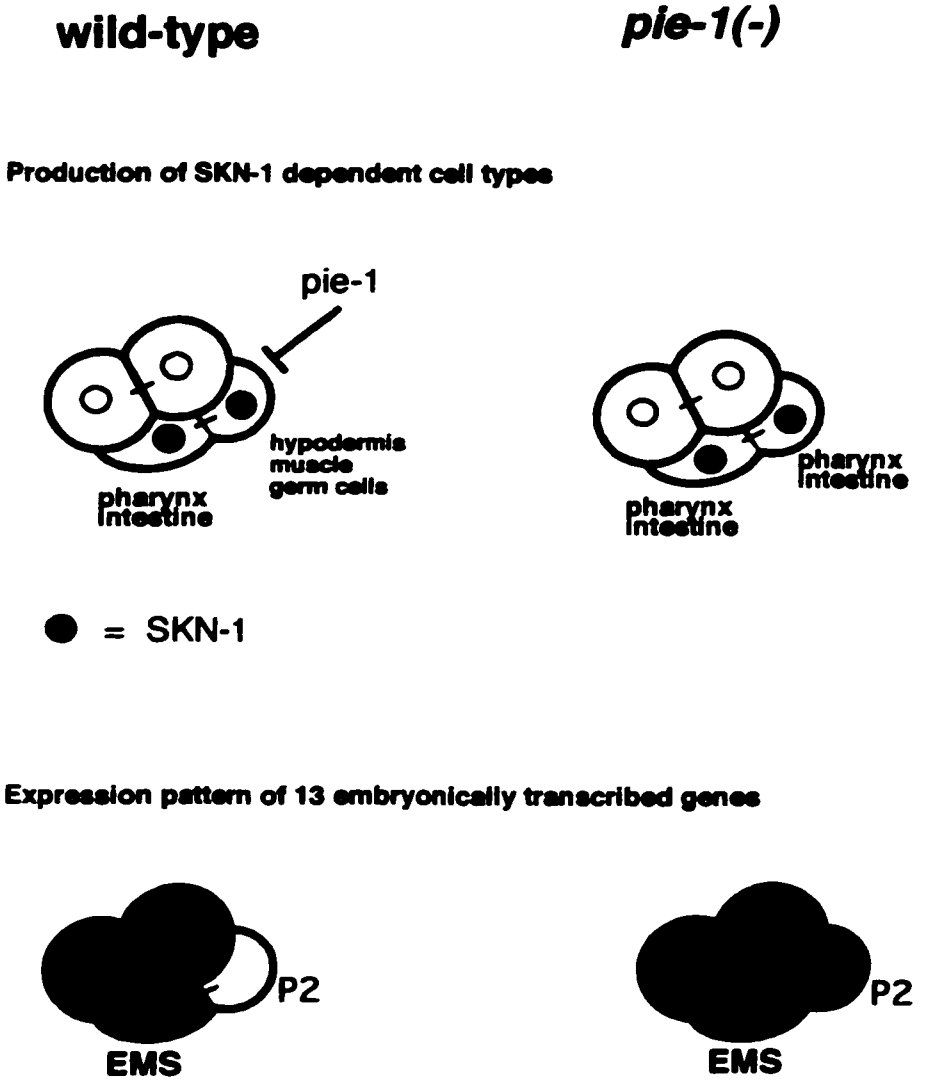

Figure 3: PIE-1 is required to prevent transcriptional activity in germline precursors.

pie-1 gene. These analyses suggested that *pie-1* functioned in P2 to prevent SKN-1 activity. They also provided evidence that *pie-1* had a general function in each germline precursor for establishing a distinct property of germline precursors; the absence of zygotic transcriptional activity. These studies are summarized below and detailed in Chapter 1.

Thesis summary: *pie-1*, *mex-5* and *mex-6* and soma/germline asymmetries

In the next three chapters I describe my work on three genes, *pie-1*, *mex-5* and *mex-6*, that establish the distinct identity of somatic and germline precursors during early embryogenesis. In the first chapter I describe the immunolocalization of the PIE-1 protein. I show that PIE-1 is predominantly a nuclear protein that is expressed at high levels in each germline precursor. This pattern is consistent with a function for *pie-1* in preventing the activity of *skn-1* and *pal-1* in these precursors. The immunolocalization of PIE-1 complements studies undertaken in parallel (Seydoux et al., 1996). These studies provided evidence that *pie-1* functions in each germline precursor to prevent zygotic gene expression.

In the second chapter I describe my work on two genes, *mex-5* and *mex-6*, that are highly similar in sequence. I show that embryos mutant for both *mex-5* and *mex-6* fail to properly localize multiple classes of proteins that are

asymmetrically expressed in wild-type embryos. I show that MEX-5 is a cytoplasmic protein expressed at high levels in each somatic precursor. MEX-5 is also expressed during the division of germline precursors at the pole that gives rise to a somatic precursor at division. I demonstrate that MEX-5/MEX-6 can function to negatively regulate the expression of PIE-1 and other germline proteins; MEX-5 is localized in a pattern reciprocal to that of the germline proteins, and ectopic expression of MEX-5 is sufficient to inhibit the expression of germline proteins.

I show that *par (+)* activity is required for the localization of MEX-5 and provide evidence that MEX-5/MEX-6 link *par (+)* activity to the asymmetric expression of PIE-1 and other proteins that establish the identity of individual blastomeres.

In the last chapter I briefly describe a screen for temperature sensitive embryonic mutants that are defective in asymmetric cell division.

CHAPTER 1: PIE-1 IS ASYMMETRICALLY LOCALIZED TO GERMLINE PRECURSORS

INTRODUCTION

The *pie-1* gene was identified in a screen for general maternal effect mutants by Mello et. al. (1992). This study showed that *pie-1(+)* activity was essential for the germline precursors P2 and P3 to adopt a different fate than their somatic sisters. In a wild-type embryo, the transcription factors SKN-1 and PAL-1 promote somatic fates but have no function in the germline precursors. In a *pie-1* mutant, *skn-1(+)* activity causes P2 and its somatic sister EMS to adopt a fate similar to that of a wild-type EMS blastomere. Similarly, in a *pie-1;skn-1* double mutant, the EMS and P2 blastomeres produce cell types normally dependent on *pal-1(+)* activity (Mello et al. 1992; Mello, et al., 1996). Therefore, *pie-1* appears to prevent germline precursors from responding to factors that determine the identity of the somatic precursors. There were several possible models for how *pie-1* might specify the fate of germline precursors. For example, *pie-1* might have general functions in establishing the identity of germline precursors, or could function as a specific inhibitor of SKN-1 and PAL-1. *pie-1* might function to localize inhibitors of SKN-1 and PAL-1 to germline precursors, or the *pie-1* gene might encode an inhibitor itself.

After C. Mello and coworkers cloned *pie-1*, the predicted protein sequence was used to generate a series of peptides. These peptides were used to immunize rabbits, and the resulting antisera were affinity purified against PIE-1 peptides. I analyzed the immunostaining pattern of these antisera. The studies of Mello et al. (1996) and Seydoux et al. (1996) provide evidence that *pie-1* functions to inhibit the expression of zygotic transcripts in germline precursors.

RESULTS

PIE-1 localizes to Germline Precursors

Results presented in this section are summarized below and detailed in the manuscript in Mello et al., 1996 (Appendix 1).

At the 1-cell stage, PIE-1 is found in the cytoplasm at the posterior pole of the embryo. At the 2-cell stage PIE-1 is present at high levels in the blastomere P1. The somatic precursor AB contains low levels of staining; this staining diminishes as the cell cycle progresses. Similarly, after the division of P1, the germline precursor P2 contains high levels of PIE-1 and the somatic precursor EMS contains low levels; the levels of PIE-1 in EMS subsequently diminish. At subsequent divisions, PIE-1 is detectable at high levels in the germline precursors (P3 and P4), and at low levels in the somatic precursor cells that are their sisters. PIE-1 is detected after the P4 division in the two resulting germ

cells, but does not persist in these cells. PIE-1 is predominantly a nuclear protein, but is also found in the cytoplasm and associated with P granules. I demonstrated that the staining pattern described above is specific for PIE-1 by staining *pie-1* mutant alleles. No staining was observed in each of three *pie-1* alleles (*zu127, zu154, zu213*).

In wild-type embryos each germline precursor fails to express zygotic transcripts (see Introduction). The nuclear localization of PIE-1 in each germline precursor suggested that it could affect transcription in each of the germline precursors. To test this, Seydoux et al. (1996) asked if the germline precursors from *pie-1* embryos expressed zygotic transcripts. In a survey of 10 zygotically expressed mRNAs, Seydoux et al. found that in *pie-1* mutant embryos all germline precursors expressed mRNAs. Seydoux et al. also drove PIE-1 expression ectopically in somatic precursors using a heat shock construct. Seydoux et al. found that zygotic transcripts were not detected in somatic blastomeres ectopically expressing PIE-1. These several experiments showed that PIE-1 expression was both necessary and sufficient to prevent the expression of zygotically transcribed RNAs.

pie-1 mRNA is detected in all blastomeres until the 4-cell stage of embryogenesis (Mello et al. 1996). Thus, the asymmetric distribution of PIE-1 must involve asymmetric translation, or protein stability. My immunolocalization

analysis suggested that a centrosome-mediated mechanism could be involved in generating asymmetric stability of PIE-1 protein.

I demonstrated that PIE-1 shows a cell-cycle dependent association with centrosomes in each germline precursor. In late prophase in germline precursors, the nuclear and P granule staining with PIE-1 antisera are no longer detected. However, both centrosomes show bright PIE-1 staining. The centrosomes rotate 90 degrees prior to division to set up an anterior-posterior axis of division. After this rotation, PIE-1 is observed at high levels associated with the centrosome that will be inherited by the germline daughter after division. The other centrosome shows low levels of staining. As a result of the asymmetry in centrosome association, the germline daughter appears to inherit high levels of centrosome-associated PIE-1 at division. After cell division, PIE-1 is no longer detected on centrosomes. The pattern of centrosome association suggested that cell-cycle dependent centrosome association could be part of a mechanism for assuring that only germline precursors express high levels of PIE-1.

In summary, PIE-1 localizes predominantly to the nucleus of germline precursors. The nuclear localization of PIE-1 provides evidence that PIE-1 can function directly in the germline precursors to prevent the transcriptional activity of SKN-1 and PAL-1. It also contributes to the evidence showing that PIE-1 functions directly in each germline precursor to prevent the expression of zygotic transcripts.

PIE-1 subnuclear localization

I initiated a characterization of the subnuclear localization of PIE-1 subsequent to the studies cited here (Mello et al., 1996; Sedoux et al., 1996). 3D reconstructions of optical thin sections revealed that PIE-1 was found in small speckles that appeared to be associated closely with chromatin (Figure 4). Several classes of proteins have been shown to have a speckled nuclear appearance in mammalian cell types (reviewed in Neugebauer and Roth, 1997). These include components of the basal transcription machinery and proteins involved in RNA processing. These speckled domains have been proposed to be sites of active RNA processing and/or transcription (Neugebauer and Roth, 1997). In the future, double staining experiments with antibodies to proteins involved in transcription or RNA processing could show whether PIE-1 associates with structures analogous to the speckled domains found in mammalian cells.

PIE-1 monoclonal antibody

I generated monoclonal antibodies to PIE-1 peptides in conjunction with Elizabeth Wayner at the Fred Hutchinson Cancer Research Center monoclonal production facility (For methods see Wayner and Carter, 1987). Analysis of one of these antibodies, P4G5, provided additional insight into the PIE-1 immunolocalization pattern.

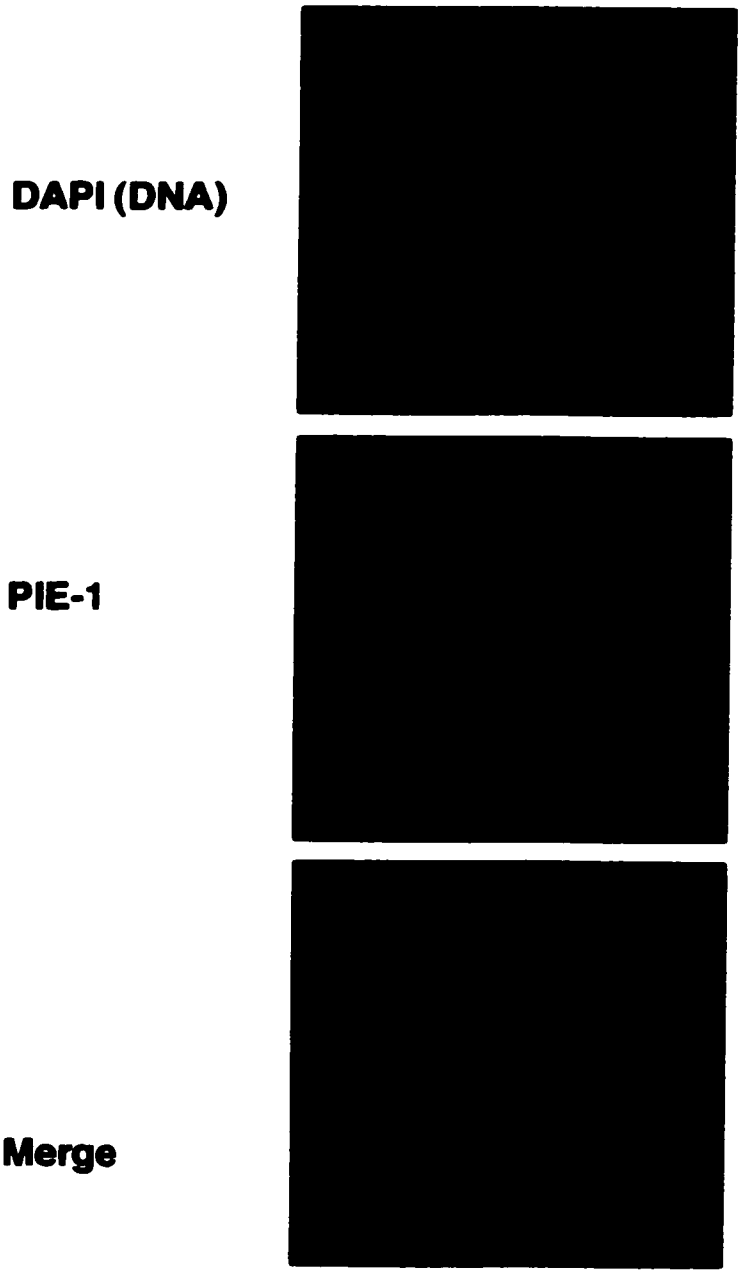

Figure 4: PIE-1 has a subnuclear localization pattern. Shown here is staining of the P3 germline precursor with anti-PIE-1 antibody, and a DNA stain, DAPI

PIE-1 staining with P4G5 was detectable at low levels in the gonad cytoplasm and in the cytoplasm of newly forming oocytes. R. Ciosk (personal communication) asked if PIE-1 might have a transcriptional function in the gonad or in newly forming oocytes. He first asked if the gonad and oocyte nuclei stained with H14, an antibody that recognizes the phosphorylated epitope of the C-terminal domain of RNA polymerase II (Seydoux and Dunn, 1997). In wild-type germline precursors H14 shows reduced or absent staining, consistent with a lack of transcription (Seydoux and Dunn, 1997). Ciosk found that staining with H14 appeared to progressively diminish in the gonad and newly forming oocytes, and does not occur in the most mature oocyte. Ciosk next stained *pie-1* mutant embryos with H14. He found that staining in *pie-1* mutant embyros was wild-type, suggesting that PIE-1 may not have an effect on transcription prior to the 1-cell stage.

Staining with P4G5 revealed that cytoplasmic asymmetry during division appeared to be part of a mechanism to localize PIE-1 into germline precursors. With affinity purified rabbit antisera only very faint cytoplasmic PIE-1 was detectable at cell division. In contrast, cytoplasmic staining with the monoclonal antibody P4G5 was pronounced. During the division of each germline precursor PIE-1 appeared concentrated in the cytoplasm at one pole; this is the pole that gives rise to the germline precursor at division. Thus, the germline daughter appears to inherit higher levels of PIE-1 than the somatic daughter at division.

The centrosome asymmetry observed with the polyclonal sera was still evident with P4G5. Similarly, both P4G5 and the rabbit sera showed low levels of staining in somatic precursors shortly after cell division; this staining diminished as the cell cycle progressed.

DISCUSSION

PIE-1 and germ cell development

A survey by Dixon (1994) showed that separation of germline and somatic cells at early embryonic stages appears to occur commonly throughout the animal kingdom. For example, in *Xenopus*, *C. elegans* and *Drosophila*, germline precursors are segregated from somatic precursors during the first several cleavages. The early separation of germline and somatic cells occurs in many of the less well-studied phyla as well, with several exceptions, such as the Cnidaria. This early separation of somatic and germline precursos has been proposed to serve as a mechanism to maintain the totipotency of the germline (Dixon, 1994). This mechanism can exclude germ cells from the processes of differentiation that occur during specification of somatic cell types.

I have shown that the PIE-1 protein is localized to germline precursors in C. elegans. PIE-1 mediated inhibition of zygotic gene expression appears to maintain the totipotency of the germline precursors; mutations in *pie-1* result in

embryos that express zygotic RNAs in germline precursors, and forced expression of PIE-1 in all early embryonic precursors results in global repression of gene expression.

Molecular mechanisms for PIE-1 function

Two possibilities for a nuclear function of PIE-1 include prevention of RNA processing, or prevention of transcription.. In wild-type embryos zygotic RNA transcripts are detected in the nucleus (Seydoux et al., 1996); these presumably represent newly synthesized transcripts. In germline precursors, nuclear transcripts are not detected, consistent with a complete absence of transcription. However, it is possible that mechanisms exist in *C. elegans*, as in other cell types, to degrade incompletely processed RNAs (Vijayraghaven, et al. 1989). If PIE-1 blocked RNA processing, then it is possible that incompletely processed RNAs would be degraded. An alternative, perhaps simpler, model is that PIE-1 acts directly on the transcriptional apparatus. In support of this view (Batchelder et al., 1999) have shown that the C terminal domain of PIE-1 when fused to the DNA binding domain of GAL-4 is sufficient to strongly inhibit the expression of a promoter containing GAL4-binding sites. Consistent with both models, I have shown that PIE-1 is found in chromatin-associated speckles in the nucleus, in a pattern similar to that observed in mammalian cells in immunostaining

experiments to components of both the transcriptional and mRNA processing machinery.

PIE-1 is detectable in the nucleus, on P granules and in the cytoplasm. Thus, it is possible that PIE-1 has separable functions in different parts of the cell. One curious finding has been that *pie-1* mutant embryos fail to express a protein, APX-1, translated from maternally supplied mRNA (Mickey et al., 1996). APX-1 is normally expressed at high levels in P1 and P2, coincident with the onset of high levels of PIE-1 expression (Mickey, et al., 1996). The absence of APX-1 in *pie-1* mutant embryos is consistent with a function for PIE-1 in positively regulating *apx-1* translation. PIE-1 contains two CCCH domains that are found in proteins that bind RNA, including Cleavage and Polyadenylation Specificity Factor (CPSF) (Barabino et al., 1997), U2AF35 (Zuo and Maniatis, 1996) and Tristetrapolin (TTP) (Varnum et al., 1989; DuBois et al., 1990; Lai et al., 1990). RNA binding by TTP has been shown to require the CCCH domains (Carballo et al., 1998; Lai et al., 1999). Thus, the presence of CCCH domains in PIE-1 is also consistent with a function for PIE-1 in regulating translation. However, the absence of APX-1 in *pie-1* mutant embryos could by due to unknown indirect affects resulting from inappropriate zygotic transcription.

PIE-1 and Totipotency of the Germline Precursors in Other Organisms

Orthologs of PIE-1 in flies or any other organism have not been identified in database searches. However, the *Drosophila* Nanos protein may play a role in the transcriptional inhibition of certain genes. In *Drosophila*, germline precursors lacking Nanos function, several mRNAs normally expressed only in somatic blastomeres are expressed in germline precursors (Deshpande et al., 1999). In *C. elegans* and *Drosophila* embryos, absence of zygotic transcription correlates with the absence of a phosphoepitope of the C terminal domain of RNA Polymerase II. Mutations in *pie-1* result in detection of this epitope in *C. elegans* germline precursors (Seydoux and Dunn, 1997). However, this is not the case for germline precursors in *Drosophila* embryos lacking *Nanos* function (Deshpande et al., 1999). One explanation for this latter result is that *Nanos* may be required to prevent the expression of only a subset of zygotic genes in *Drosophila* germline precursors (Deshpande et al., 1999). In *C. elegans*, three *Nanos* orthologs have been identified (*nos-1-3*) (Subramaniam and Seydoux, 1999). They appear to have functions in germ cell maintenance after the early cleavage stages.

In embryos lacking the function of both *nos-1* and *nos-2*, germ cell maintenance shows defects after the birth of the terminal germ cell precursor, P4 (Subramaniam and Seydoux, 1999). In wild type embryos P4 descendants

proliferate during larval stages; this proliferation does not occur in *nos-1;nos-2* mutant embryos. PIE-1 expression is lost from the daughters of P4 shortly after their birth; the timing of this loss correlates with the resumption of transcription in these cells (Seydoux and Dunn, 1997). The expression of NOS-1 protein (Subramaniam and Seydoux, 1999) and other proteins involved in germline maintenance (for review see Seydoux and Strome, 1999), requires zygotic gene expression. Therefore, PIE-1 mediated transcriptional inhibition appears to be required for germ cell development only during early cleavage stages, and the absence of PIE-1 is required for the transcription of genes involved in germ cell maintenance, including NOS-1.

In mammals, a POU-domain transcription factor, Oct-4 may serve an analogous function to PIE-1 (Reviewed in McLaren, 2000; Pesce and Scholer, 2000). Oct-4 does not appear to inhibit transcription, but like PIE-1, may function during embryonic development in maintaining totipotency in cells that give rise to germline. Oct-4 is present in the early mouse embryo in totipotent cells. As cells differentiate, they lose Oct-4 expression and eventually only the germline precursor cells express Oct-4. Mouse embryos lacking Oct-4 have phenotypes consistent with a role for Oct-4 in maintenance of a totipotent state. In *Oct-4* mutant embryos the inner cell mass, which forms the embryo proper, appears to differentiate as a trophoblast. The tropohoblast normally forms only extra-embryonic structures and is the first differentiated cell type to arise from a

mammalian embryo. Oct-4 has been found to bind to sequences in the promoters of target genes that are coexpressed in the embryo with Oct-4, and whose in vivo expression is dependent on Oct-4. These data are consistent with a function for Oct-4 as a positive regulator of transcription. Thus, Oct-4 could maintain totipotency through the positive transcriptional regulation of specific downstream targets.

POS-1 and MEX-1 are germline proteins with distinct functions from PIE-1

Studies on two proteins related to PIE-1 were undertaken subsequent to my studies on PIE-1 (Guedes and Priess, 1997; Tabara et al., 1999). These two proteins, MEX-1 and POS-1, have two copies of the CCCH domain shared by PIE-1. They also show a similar immunolocalization pattern to PIE-1; they are expressed at high levels in each germline precursor and at low levels in each somatic precursor. Therefore, POS-1, MEX-1 and POS-1 are referred to as germline proteins. However, POS-1 and MEX-1 are not preferentially expressed in the nucleus; POS-1 and MEX-1 are expressed at high levels on P granules and in the cytoplasm. Furthermore, neither POS-1 nor MEX-1 are required for transcriptional silencing. POS-1 and MEX-1 have been proposed to positively regulate the translation of several proteins that are translated from non-localized maternally supplied mRNA (Guedes, 1998, Tabara et al., 1999).

The evidence for this hypothesis is strongest for POS-1 (Tabara et al., 1999). In wild-type embryos the protein APX-1 is expressed in the germline precursors P1 and P2. In *pos-1* mutant embryos, APX-1 protein is not expressed despite the presence of wild-type levels of *apx-1* mRNA. POS-1 has therefore been proposed to act as a positive regulator of APX-1 translation (Tabara et al., 1999). Consistent with this view, the timing of APX-1 protein expression correlates with the onset of POS-1 protein expression in the germline precursors (Mickey et al., 1996, Guedes, 1998, Tabara et al., 1999). In *mex-1* mutant embryos POS-1 protein shows overall reduced levels of expression (Guedes, 1998). This observation has led to the model that *mex-1* could act as a positive regulator of POS-1 translation (Guedes, 1998).

MEX-1 protein is expressed at the posterior pole of the 1-cell stage embryo while POS-1 protein expression is first prominent at the 2-cell stage. This sequential expression has led to the model that cell fate specification in the germline precursors occurs in part through the temporally and spatially distinct translation of proteins in individual germline precursors (Guedes, 1998, Tabara et al., 1999). Such a mechanism could lead to the blastomere-specific expression of proteins such as APX-1.

One prediction of this model is that ectopic expression of POS-1 and MEX-1 should result in the upregulated expression of potential translational targets (POS-1 for MEX-1; APX-1 for POS-1). Some of the effects of ectopic

POS-1 and MEX-1 on the expression of these potential targets can be addressed experimentally in mutant backgrounds that show ectopic POS-1 and MEX-1. In chapter 3 I describe my work on two genes, *mex-5* and *mex-6*, that may serve this purpose. *mex-5;mex-6* mutant embryos show uniform POS-1 and MEX-1 expression and provide a means to address the effect of ectopic POS-1 and MEX-1 on the expression of APX-1 and other potential targets.

The complexity of protein expression in germline precursors underscores the likelihood that several factors in addition to MEX-1 and POS-1 are involved in protein expression specific to germline precursors. NOS-1, for example, has a complex expression pattern; NOS-1 protein is expressed from maternal mRNA; NOS-1 protein is detectable in P0, absent from P1-P3, and re-appears in P4 (Subramaniam and Seydoux, 1999). Another example is APX-1, which is not normally translated in P3 and P4 despite the presence of POS-1 (Mickey et al., 1996). How could proteins such as POS-1, that are expressed in multiple germline precursors, function in regulating the expression of proteins expressed in a subset of these precursors? One possibility is that the germline proteins may cooperate with factors, like SKN-1, that are only transiently expressed in the embryo.

CHAPTER 2: MEX-5 AND MEX-6 FUNCTION TO ESTABLISH SOMA/GERMLINE ASYMMETRY IN EARLY *C. ELEGANS* EMBRYOS

SUMMARY

Shortly after fertilization of the *C. elegans* egg, a group of proteins called PAR proteins localize asymmetrically to either the anterior or posterior cortex. This asymmetrical network of PAR proteins is required for the subsequent, asymmetrical expression patterns of several proteins that are encoded by non-localized, maternally-expressed mRNAs. We provide evidence here that two nearly identical genes, *mex-5* and *mex-6*, function to link PAR asymmetry to subsequent asymmetries in protein expression. Mutations in *mex-5* and *mex-6* together cause the anterior misexpression of several proteins that normally are restricted to the posterior of the embryo. We show that MEX-5 is a novel, cytoplasmic protein that is localized through PAR activities to the anterior pole of the 1-cell stage embryo. The localization of MEX-5 is reciprocal to that of a subclass of posterior-localized proteins called germline proteins. We show that ectopic expression of MEX-5 is sufficient to inhibit the expression of germline proteins, suggesting that MEX-5 functions in the anterior of wild-type embryos to inhibit expression of the germline proteins.

INTRODUCTION

In the nematode *C. elegans*, embryonic blastomeres become committed to distinct fates within the first few cell cycles after fertilization of the egg. This rapid diversification occurs because the egg contains a pool of maternally-provided mRNAs that encode determinative factors, and the early blastomeres have markedly different potentials for expressing these factors. This results in the asymmetric expression patterns of transcriptional factors that appear to determine blastomere fates directly, and components of NOTCH-like and WNT/WINGLESS-like signaling pathways that allow position-specific, cell interactions (for general reviews of *C. elegans* embryogenesis see Schnabel and Priess, 1997; Bowerman and Shelton, 1999).

Very little is known about the molecular differences between blastomeres that result in the asymmetric expression patterns of proteins encoded by these maternal mRNAs. However, a critical first step in this process occurs during the 1-cell stage, shortly after fertilization of the egg. The point of sperm entry appears to define the posterior pole of the *C. elegans* embryo. After fertilization, the proteins PAR-1 and PAR-2 localize to the cortex in the posterior half of the embryo (Guo and Kemphues, 1995; Boyd et al., 1996) and PAR-3, PKC-3 and PAR-6 localize to the cortex in the

anterior half (Etemad-Moghadam et. al.,1995; Tabuse et al., 1998; Hung et al., 1999). We refer here to these proteins collectively as PAR proteins. The localization of the PAR proteins is interdependent, as a mutation in any of several *par* genes can disrupt the localization of all PAR proteins (Etemad-Moghadam et. al.,1995; Boyd et al., 1996; Tabuse et al., 1998; Hung et al., 1999). Mutations in the *par* genes also cause marked defects in many, or all, of the subsequent asymmetries that normally are observed between wild-type blastomeres after cell division. These include differences in blastomere size, in cleavage rate, and in the expression of proteins that specify cell fate (reviewed in Rose and Kemphues, 1998). Although PAR asymmetry clearly is linked to blastomere differences, the nature of this linkage has remained mysterious. Some PAR proteins contain structural domains that are found in diverse cell signaling molecules; PAR-1 and PKC-3, for example, both have putative kinase domains (Guo and Kemphues, 1995; Tabuse et al., 1998). The PAR-2 protein contains a motif called a RING finger (Boyd et al., 1996), and recent studies have shown that RING fingers may function to regulate degradation by adding ubiquitin to proteins (Freed et al., 1999; Joazeiro et al., 1999; Lorick et al., 1999; Seol et al., 1999; Waterman et al., 1999). However, specific biochemical functions of the PAR proteins in *C. elegans* have not been determined, nor have targets of the PAR proteins been identified.

One approach toward elucidating the linkage between the PAR proteins and the differences between early blastomeres is to work upstream from specific proteins that show PAR-dependent, blastomere-specific expression patterns. There are three general, PAR-dependent patterns of protein expression that have been described in the early blastomeres (see Figure 5 for diagram). After the first division, there are proteins that are localized specifically to the anterior blastomere. These anterior proteins persist in, or continue to be expressed in, all the descendants of that anterior blastomere over the next few cell cycles (Evans et al., 1994). Posterior proteins are localized to the posterior blastomere after the first division, and are present in all the early descendants of the posterior blastomere (Bowerman et al., 1993; Hunter and Kenyon, 1996). A third group of proteins is localized to only a single branch of the descendants of the posterior blastomere (Mello et al., 1996; Guedes and Priess, 1997; Tenenhaus et al., 1998; Tabara et al., 1999). Blastomeres in this branch eventually form the germline, and so are called germline blastomeres to distinguish them from all other blastomeres that produce only somatic cell types. We refer here to the proteins that are localized to the germline blastomeres as germline proteins.

Almost all of the mRNAs encoding anterior, posterior, and germline proteins are distributed uniformly throughout the early embryo (Evans et al., 1994; Seydoux and Fire, 1994; Guedes and Priess, 1997; Tenenhaus et al., 1998).

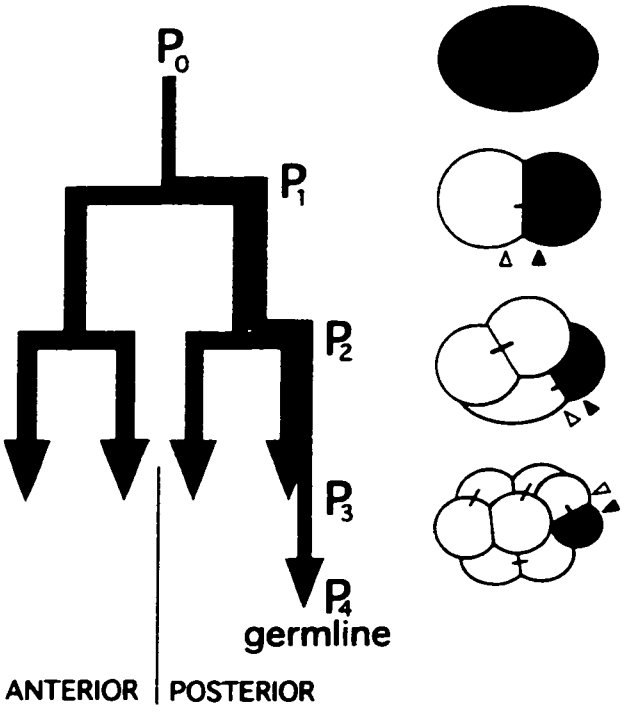

Figure 5: Protein expression patterns in early embryos. A lineage diagram of the first few embryonic divisions is shown on the left. Lineage branches expressing anterior proteins are shown in red, posterior proteins in purple, and germline proteins in green. The successive blastomeres in the germline branch are named P0 (the 1-cell embryo), P1, P2, P3 and P4. Schematic drawings of the early blastomeres are shown on the right; sister blastomeres are indicated by dashes. Each germline blastomere is green, and its somatic sister is light green. For comparison with later figures, the germline and somatic sisters also are indicated by arrowheads. At the beginning of the 4-cell stage, one of the two anterior (white) blastomeres is forced toward the posterior by the surrounding eggshell. This movement results in the two anterior and two posterior blastomeres adopting the rhombohedral configuration shown.

1994; Seydoux and Fire, 1994; Guedes and Priess, 1997; Tenenhaus et al., 1998). Therefore, the asymmetric patterns of protein expression must result from protein movement, or from differences between blastomeres in either protein stability or mRNA translation. There is suggestive evidence that localization of the germline proteins PIE-1, MEX-1 and POS-1 may, at least in part, involve protein degradation. As a germline blastomere divides, high levels of the germline proteins are inherited by the germline daughter, and either low (PIE-1; MEX-1) or high (POS-1) levels are inherited by the somatic daughter (Mello et. al, 1996; Guedes and Priess, 1997; Tenenhaus et al., 1998; Tabara et al., 1999). The levels of the germline proteins in the germline daughter remain stable or increase during the cell cycle. However, the levels in the somatic daughter, as detected by immunostaining, rapidly diminish or disappear (Mello et al., 1996; Tenenhaus et al., 1998; Guedes and Priess, 1997; Tenenhaus et al., 1998, Tabara et al., 1999).

The localization of the anterior protein GLP-1 is due in part to a difference between the anterior and posterior blastomeres in their ability to translate the *glp-1* mRNA (Evans et al, 1995). A reporter construct containing only the 3'untranslated region (UTR) from the *glp-1* mRNA is expressed exclusively in anterior blastomeres. Deletion of a small region within the 3'UTR results in ectopic expression in posterior blastomeres. These results have suggested that posterior blastomeres may contain a trans-acting inhibitor of

EXPERIMENTAL PROCEDURES

Nematode strains and alleles

The Bristol strain N2 was used as the standard wild-type strain. *mex-5(zu199)* was used in cis to *unc-31(e169)* to assay cosmids for transformation rescue; in all other experiments *mex-5(zu199)* was in cis to *unc-30(e191). par-1(b274)* was in cis to *rol-4(sc8)* and *par-3(it71)* was in cis to *unc-32 (e189).* In addition the following strains and genetic reagents were used, arranged by linkage group: I: *dpy-5(e61)* II*: rol-1(e187)*, III: *unc-32(e189)*, Qc1 IV*: skn-1(zu67), unc-5(e53), unc-26(e345), dpy-4(e1166), unc-24(e138), unc-22(s7), let-324(s1727), dpy-20(e1282), nDf27, sDf66, nT1* V*: dpy-11(e224)* X*: lon-2(e678). C. elegans* culture, mutagenesis, and genetics were as described in Brenner (1974).

Isolation of *mex-5(zu199)* and mutant characterization

The *mex-5(zu199)* allele was isolated in an F2 screen for maternal effect lethal mutants as described by Priess et al. (1987), and mapped to the right arm of IV, near *unc-30*, using standard genetic crosses. Map data is available from the *C. elegans* Genetic Center. All progeny produced by self-fertilization of homozygous *mex-5(zu199)* hermaphrodites arrested during embryogenesis (n=3,412). All cross progeny produced after mating to wild-

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development of individual early blastomeres in *mex-5* and wild-type embryos was assayed after killing all other blastomeres with a laser microbeam as described previously (Bowerman et al., 1992; Mello et al., 1992). Production of pharyngeal muscle in *mex-5(zu199);skn-1(zu67)* double mutants was analyzed by examining terminal stage whole embryos. Measurements of the relative size of the AB blastomere were taken with morphometric software in NIH Image; 12 wild-type and 13 *mex-5;mex-6* embryos were examined.

Molecular analysis

The cosmid W02A2 and marker rol-6 DNA were co-injected into the syncytial gonad of *mex-5(zu199)unc-31(e189)/nT1* adults following published procedures (Mello et al., 1991). Three transgenic lines segregating Rol Unc hermaphrodites were isolated. Rol Unc hermaphrodites from these lines containing W02A2 produced either viable embryos that developed into Unc, or Rol Unc larvae, or inviable embryos that appeared identical to those of *mex-5(zu199)* homozygous parents. C. Mello (unpublished) showed that double stranded RNA (dsRNA) corresponding to the W02A2.7 gene could cause wild-type adults to produce inviable embryos in RNAi experiments (see below), and that some of these embryos resembled *mex-5(zu199)* mutant embryos. We found that the penetrance of embryos with a *mex-5*-like phenotype was greatly enhanced using dsRNA from a small region of W02A2.7 that corresponds to

amino acids 153-186 in Figure 6; this region is divergent between *mex-5* and *mex-6*. The *mex-5(zu199)* and *mex-6(pk440)* alleles were sequenced using standard protocols from PCR-amplified genomic DNA. DNA corresponding to bases 12737-15477 on the cosmid AH6 (sequence available from Genbank) are deleted in the *mex-6(pk440)* allele.

The *mex-5* cDNA yk328d9 was sequenced using standard protocols from purified plasmid DNA. Predicted start and stop codons, and exon-intron boundaries agreed with Genefinder predictions from the genomic sequence. The MEX-6 protein predicted by Genefinder contains an extra 30 amino acids N terminal of the MEX-6 protein shown in Figure 6; DNA sequences corresponding to those amino acids were not present in the 5' end sequences of several *mex-6* cDNAs (yk339c12, yk423a1, yk562a8, yk520g6).

dsRNA inhibition (RNAi)

Regions of the *mex-5* and *mex-6* genes corresponding respectively to amino acids 153-186 and 156-190 in Figure 6 were amplified by polymerase chain reaction (PCR) from phage DNA (yk45f12 and yk38b2, respectively); oligonucleotides contained sites for T3 or T7 polymerase. *pie-1* dsRNA was prepared from the clones pJP660 and pJP661 (Mello et al., 1996) after PCR amplification using M13 and M13 reverse primers. The amplified DNAs were gel purified and transcribed using T3 and T7 polymerase (Epicentre Technologies)

and commercially available synthesis kits. Worms were treated with RNAi either by injection (Powell-Coffman et al., 1996; Rocheleau et al., 1997) or by soaking as described, (Tabara et al., 1999); in the latter experiments sucrose and liposomes were omitted.

MEX-5 antibody and immunostaining

DNA corresponding to amino acids 10 to 468 in Figure 6 was cloned from the *mex-5* cDNA phage yk328d9 into the Eag-I and Xho-I sites of pET-28b (Novagen). The resulting MEX-5 fusion protein was expressed in BL21(DE3) (Novagen) cells and purified using Qiagen Ni-NTA agarose. Monoclonal antibody was generated according to published protocols (Wayner and Carter, 1987) in the Hybridoma Production Facility at the Fred Hutchinson Cancer Research Center. The following immunological reagents/protocols were used. Rabbit anti-MEX-3 (Draper et al., 1996), rat anti-POS-1 (Tabara et al., 1999), rabbit anti-MEX-1 (Guedes and Priess, 1997), mouse anti-PIE-1(P4G5); (Tenenhaus et al., 1998), rabbit anti-PAL-1 (Hunter and Kenyon, 1996), mouse anti-SKN-1(F2A and FC4); (Bowerman et al., 1993), rabbit anti-PAR-1 (Guo and Kemphues, 1995), rabbit anti-PAR-2 (Boyd et al., 1996), rabbit anti-PAR-3 (Etemad-Moghadam et al., 1995), and rabbit anti-PKC-3 (Tabuse et al., 1998). P granule staining was determined by staining with the mouse antibody K76 (Strome and Wood, 1982). MEX-5 double staining with either PAR-3, PKC-3 or

PAR-2 was as in Guo and Kemphues (1995). Simultaneous staining with PAR-2, MEX-5 and PIE-1 followed the protocol of Lin et al. (1995), and used the following secondary antibodies: Cy5 anti-rabbit (Jackson Labs), rhodamine anti-mouse IgG1a, and fluorescein anti-mouse IgG2a (Southern Biotechnology Associates) to recognize primary antiserum/antibodies for PAR-2, MEX-5, and PIE-1, respectively. Images were collected on either a Photometrics Sensys digital camera or a Deltavision deconvolution microscope system (Applied Precision).

For experiments analyzing PAR staining in wild-type and mutant strains, 1- cell stage embryos were scored after the pronuclei had decondensed and begun migration. For the experiments presented in Figure 8, between 16-40 wild-type embryos and 11-58 *mex-5;mex-6* mutant embryos were analyzed. For the experiments shown in Figure 8 and Figure 11, wild-type and mutant embryos were placed on the same slide for immunostaining, and were photographed using identical exposure times.

Heat shock experiments

MEX-5 heat shock plasmids were constructed by cloning DNA corresponding to full-length MEX-5 from yk328d9 into Kpn I and BamH I sites in the heat shock vectors pPD49.78 and pPD49.83 (Seydoux et al., 1996) downstream of the hsp16-2 and hsp16-41 promoters, respectively. Correct

insertion of *mex-5* in the resulting clones was confirmed by DNA sequencing. The MEX-5 heat shock constructs were coinjected with *rol-6* DNA into the syncytial gonad of *mex-5(zu199) unc-30(e191)/nT1* hermaphrodites using methods previously described (Mello et al., 1991). Rol Unc larvae carrying the transgene and *mex-5(zu199) unc-30(e191)* control larvae were soaked with dsRNA for *mex-6* and *pie-1* RNA as described (Tabara et al., 1999).

Gravid hermaphrodites containing embryos at multiple stages were subjected to one of two protocols. To calibrate the timing of induction of the transgene, a few, staged embryos also were subjected to these protocols. (#1) A 1 hour heat shock at 34 degrees followed by a 1.5 hour recovery period at room temperature, or (#2) A 3 hour heat shock at 30 degrees with no recovery period. 1-cell stage embryos subjected to protocol #1 had not progressed beyond the 4-cell stage before fixation at the end of the recovery period (6/6 embryos), and most 2-cell stage embryos subjected to heat shock did not progress beyond the 12-cell stage (4/5 embryos). Thus, we infer that embryos that are subjected to protocol #1 and that contain 8-16 cells at the time of fixation must have been past the 1-cell stage before induction of MEX-5. In similar calibration experiments, we found that 1-cell embryos subjected to protocol #2 progressed to about the 24-cell stage at the time of fixation. However no MEX-5 could be detected in these embryos by immunostaining during the first 2 hours of the heat shock period. Thus embryos that are

subjected to protocol #2 and that have 8-16 cells at the time of fixation are unlikely to have had detectable levels of MEX-5 before the 1-2 cell stage. We analyzed 8-16 cell embryos obtained after either protocol and obtained equivalent results as follows: Protocol #1 [MEX-5 levels/MEX-1 levels] high/low (3/17), low/high (7/17), mosaic (7/17). Protocol #2 [MEX-5 levels/MEX-1 levels] high/low (4/18), low/high (10/18), mosaic (4/18). In no case did we observe high/high.

RESULTS

The posterior protein SKN-1 is a transcription factor required for the development of muscles in the pharynx, among other cell types (Bowerman et al., 1992,1993). Mutations in any of several *par* genes, or in a gene called *mex-1*, result in misexpression of SKN-1 in anterior blastomeres; such mutants have abnormally large numbers of muscles (Kemphues et al, 1988; Bowerman et al., 1993, Mello et al., 1992; Bowerman et al., 1997). We performed a genetic screen for maternal-effect lethal mutants with this muscle excess (*mex*) phenotype (see Experimental Procedures). In addition to alleles of the *par* and *mex-1* genes, we identified an allele, *zu199*, of a new gene we call *mex-5*. Embryos from homozygous *mex-5(zu199)* adults (hereafter referred to as *mex-5* embryos) produce approximately the wild-type number of cells, but do not

undergo body morphogenesis and die without hatching (Figure 7C). The *mex-5* embryos contain abnormally large numbers of muscles toward their anterior poles (Figure 7D; compare with wild-type embryo in Figure 7B). In cell isolation experiments on 2-cell stage *mex-5* embryos, we found that the anterior blastomere produced muscles inappropriately (14/14 cases; see Experimental Procedures). In contrast, the anterior blastomere from wild-type embryos, or from *skn-1(zu67);mex-5(zu199)* embryos, did not produce muscles in similar experiments (0/11 and 0/20, respectively). Consistent with these results, we found that 2-cell stage *mex-5* embryos misexpress the posterior transcription factor SKN-1 at high levels in the anterior blastomere (Figure 7H; compare with wild-type in Figure 7G). In this defect, *mex-5* embryos resemble the embryos from *par* mutants or *mex-1* mutants (Bowerman et al., 1993, 1997). However, *mex-5* embryos have normal early cleavage planes and cell cycles, distinguishing them from *par* embryos (reviewed in Guo and Kemphues, 1996). In addition, germ cells were visible in almost all *mex-5* embryos (15/17 embryos; Figure 7C, white arrows), while *par* embryos and *mex-1* embryos do not produce germ cells (Kemphues et al., 1988; Mello et al., 1992). Thus, the *mex-5* gene appears to be a new component of the pathways that establish asymmetrical patterns of protein expression in the early embryo.

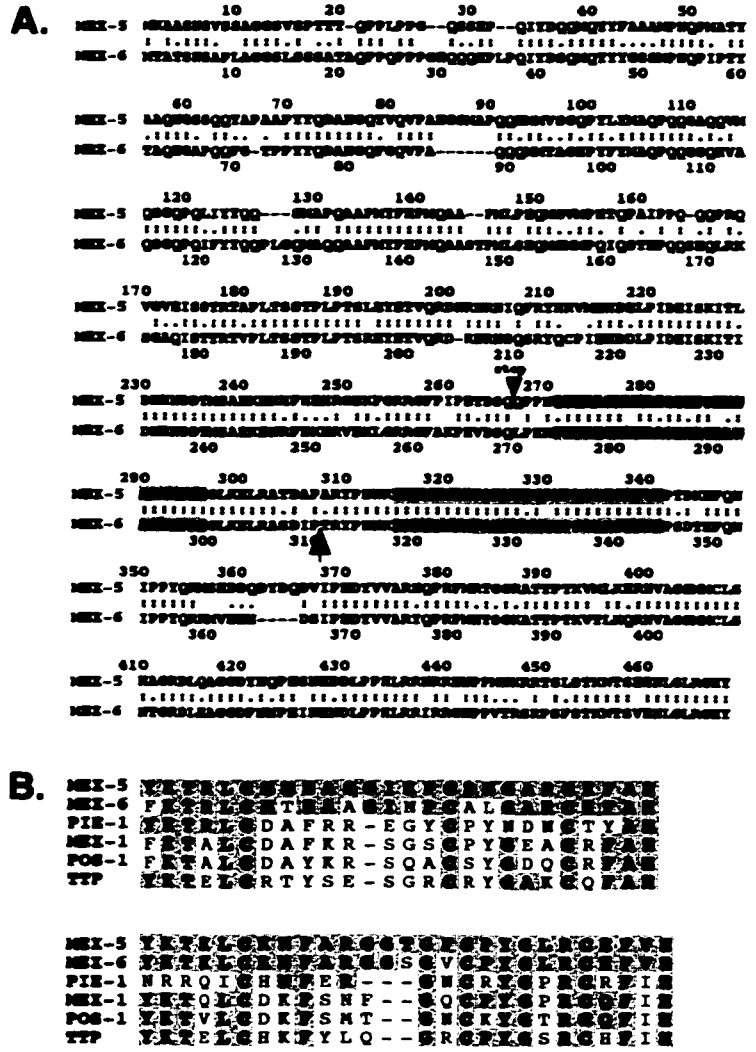

Figure 6: MEX-5 and MEX-6 alignments.
A. Alignment of the predicted MEX-5 and MEX-6 proteins. The CCCH finger domains are highlighted in gray. A point mutation in the *mex-5(zu199)* allele introduces a stop codon (UAA) that is predicted to truncate the protein before the first finger (arrowhead). The *mex-6* allele is a deletion removing the C terminal third of the protein (arrow). Amino acid identities between MEX-5 and MEX-6 are indicated by double dots, similarities with single dots.
B. Alignment of the CCCH fingers. The top and bottom panels compare the first and second CCCH fingers of MEX-5 and MEX-6 with the corresponding fingers in PIE-1, POS-1, MEX-1 (*C. elegans*) and TTP (mouse).

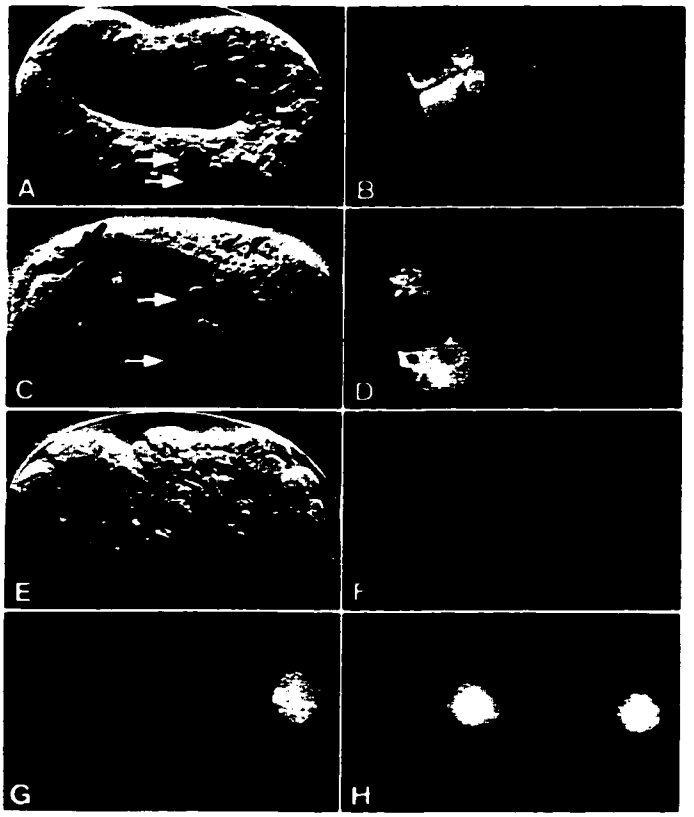

Figure 7: Comparison of wild-type and *mex-5;mex-6* embryos. (A,B) Wild-type embryos viewed (A) by light microscopy and (B) after immunostaining for pharyngeal muscles. Arrows point to the two germ cells. (C,D) *mex-5* mutant embryos prepared as for panels A and B. The prominent basement membrane surrounding the pharynx is indicated (black arrow). The two germ cells are indicated by white arrows. (E,F) *mex-5(RNAi);mex-6(RNAi)* embryos prepared as for panels A and B. These embryos lack germ cells and pharyngeal muscles (0/24). In similar experiments, pharyngeal muscles were not detected in *mex-5;mex-6(RNAi)* embryos (0/25), or in most *mex-5;mex-6* double mutants (1/78). (G) 2-cell wild-type embryo showing SKN-1 in the posterior blastomere. (H) 2-cell embryo *mex-5* embryo with SKN-1 in both blastomeres. In this and all subsequent Figures the size of each embryo is approximately 50 um in length.

MEX-5 shares a CCCH motif with germline proteins in *C. elegans*

We cloned the *mex-5* gene as described in Experimental Procedures. Briefly, we mapped the *mex-5(zu199)* mutation to a region on chromosome V that includes the cosmid W02A2, and showed that this cosmid can rescue the lethality of *mex-5* embryos. RNA-mediated inhibition of a predicted gene, W02A2.7, within this cosmid caused wild-type adults to produce inviable embryos that appeared indistinguishable from *mex-5* embryos. DNA sequence analysis showed that the W02A2.7 gene from *mex-5(zu199)* animals contains a nonsense mutation that would truncate the predicted protein product (Figure 6A). Finally, we found that a monoclonal antibody generated against a W02A2.7 fusion protein stained wild-type embryos (see below), but did not stain *mex-5(zu199)* embryos. We henceforth name W02A2.7 the gene *mex-5*, and refer to the W02A2.7 open reading frame as the MEX-5 protein.

MEX-5 is a novel protein, but contains two regions that are similar to a CCCH "finger" motif that was first described in the vertebrate TTP protein (also called Nup457 and Tis-11; Varnum et al., 1989, DuBois et al., 1990, Lai et al., 1990). The finger motif contains Cys and His residues with a CX(8)CX(5)CX(3)H spacing, and additional conserved amino acids; the finger domains of MEX-5 have CX(9)CX(5)CX(3)H and a CX(10)CX(5)CX(3)H spacing (Figure 6B). Several finger proteins appear to have functions that involve interactions with RNA, including the essential splicing factor U2AF35

(Zuo and Maniatis, 1996), and the 30 kD subunit of Cleavage and Polyadenylation Specificity Factor (CPSF) (Barabino et al., 1997). In TTP, the finger domain may mediate binding to RNA (Carballo et al., 1998; Lai et al., 1999). The *C. elegans* germline proteins PIE-1, MEX-1, and POS-1 each contain two finger domains, but do not appear to have other similarity to MEX-5 (Figure 6B).

MEX-5 and MEX-6 have overlapping functions

Database searches with the *mex-5* sequence showed that the *C. elegans* genome contains a predicted gene, *ah6.5*, that could encode a protein with very high similarity to MEX-5; we have named this protein MEX-6. MEX-6 is about 70% identical and 85% similar to MEX-5 in amino acid sequence (Figure 6A). The CCCH fingers of MEX-6 are more similar to the fingers in MEX-5 than to any other finger protein in the *C. elegans* database (Figure 6B). The *mex-6* and *mex-5* genes contain similarly placed introns, and share extensive nucleic acid identity in 5' and 3' untranslated regions and in intron sequences (data not shown). Thus *mex-5* and *mex-6* are nearly identical genes that are likely to have arisen from a relatively recent gene duplication.

We used the technique of double stranded RNA-mediated inhibition (RNAi) to assay potential functions of MEX-5 and MEX-6 (see Experimental Procedures). When wild-type larvae were treated with *mex-5* dsRNA, the

resulting adults produced embryos (called *mex-5(RNAi)* embryos) that were inviable and appeared indistinguishable from *mex-5(zu199)* embryos by light microscopy (0% viability, n=83). In contrast, adults treated with *mex-6* dsRNA produced viable embryos that hatched and grew into fertile adults (100% embryonic viability, n=277).

We identified a deletion removing part of the *mex-6* gene by a PCR-based screening method (see Experimental Procedures; Jansen et al., 1997). The *mex-6(pk440)* allele has a small deletion predicted to remove the C-terminal third of the predicted MEX-6 protein, including the second finger domain (Figure 6A). This deletion extends past *mex-6* into a neighboring pseudogene (data not shown). We found that embryos from homozygous *mex-6(pk440)* mothers were viable, and grew into fertile, apparently normal adults (99% embryonic viability, n=411). Thus, our RNAi experiments and mutant analysis both suggest that *mex-6* is a non-essential gene.

When mothers were treated with dsRNA from the *mex-5* and *mex-6* genes simultaneously, they produced inviable embryos with a highly penetrant terminal phenotype that differed in several respects from that of *mex-5* embryos. The most striking difference was that *mex-5(RNAi);mex-6(RNAi)* embryos lacked the germ cells and the anterior muscles that are characteristic of *mex-5* embryos (Figures 7E and 7F). We found that *mex-5(zu155);mex-6(RNAi)* embryos and *mex-5(zu199);mex-6(pk440)* embryos had the same,

novel phenotype (Figure 7, legend). Since the formation of the anterior muscles normally requires SKN-1(+) activity, we immunostained the *mex-5;mex-6* embryos to determine if SKN-1 was expressed. All of the *mex-5;mex-6* embryos examined at the 2-cell and 4-cell stages (11/11 and 16/16 embryos, respectively; Figure 8B) appeared to have levels, and localization, of SKN-1 that were identical to *mex-5* embryos. Thus *mex-5;mex-6* embryos express SKN-1, yet lack SKN-1-dependent differentiation.

In wild-type embryogenesis, the germline protein PIE-1 represses SKN-1(+) activity specifically in the germline blastomeres (Mello et al., 1992, 1996; Seydoux et al., 1996). In 4-cell stage embryos, SKN-1 is present at equal levels in the germline blastomere P2 and its somatic sister (Figure 8A, arrowheads), while PIE-1 is present at high levels only in P2 (Figure 8C). We found that *mex-5;mex-6* embryos contained ectopic PIE-1: Between the 1-cell stage and the 44-cell stages, PIE-1 was distributed uniformly in all blastomeres (Figure 8D). We constructed and examined *pie-1(RNAi);mex-5(RNAi);mex-6(RNAi)* embryos, and found that these embryos produced cell types that normally require SKN-1(+) function. For example, while *mex-5(RNAi);mex-6(RNAi)* embryos lack intestinal cells (0/24 embryos), the *pie-1(RNAi);mex-5(RNAi);mex-6(RNAi)* embryos contained numerous intestinal cells (54/54 embryos). Thus the ectopic PIE-1 in *mex-5;mex-6* mutants appears to repress the activity of the ectopic SKN-1.

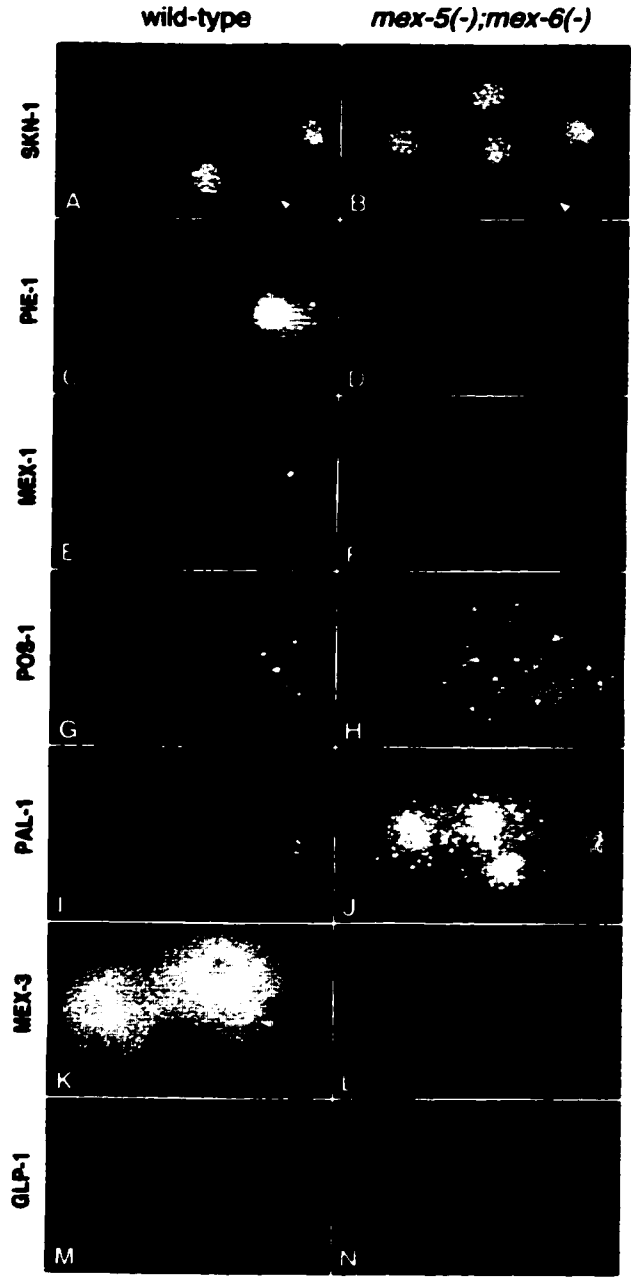

Figure 8: Protein localization in wild-type embryos and in *mex-5;mex-6* embryos. All panels show 4-cell stage embryos stained with antibodies or antisera for the proteins listed at left (see Experimental Procedures for details). Embryos are oriented as in Figure 9, and the germline blastomere (black arrowhead) and its somatic sister (white arrowhead) are indicated in the top row.

We immunostained *mex-5;mex-6* embryos to examine other classes of proteins that, like SKN-1 and PIE-1, are asymmetrically localized in wild-type embryogenesis (Figure 8). We found that the germline proteins MEX-1 and POS-1 were mislocalized to all blastomeres in *mex-5;mex-6* mutants (Figures 8F and 8H, respectively). In wild-type embryos, cytoplasmic granules called P granules are segregated asymmetrically to each germline blastomere; in *mex-5;mex-6* embryos these granules were present in all blastomeres (data not shown). The transcription factor PAL-1 is a posterior, nuclear protein in wild-type embryos (Figure 8I), but was present in all nuclei in *mex-5;mex-6* embryos (Figure 8J). In wild-type embryos, anterior expression of PAL-1 appears to be prevented, at least in part, by high levels of the MEX-3 protein in anterior blastomeres (Figure 8K; Draper et al., 1996; Hunter and Kenyon, 1996). We found that MEX-3 was distributed at low levels uniformly throughout *mex-5;mex-6* embryos, suggesting that the MEX-3 levels may be too low to prevent anterior expression of PAL-1. Finally, we examined the anterior protein GLP-1. In wild-type 4-cell embryos, GLP-1 is present at high levels in the two anterior sister blastomeres (Figure 8M; see also Evans et al., 1994). However, GLP-1 was not detectable in *mex-5;mex-6* embryos (Figure 8N). In summary, germline and posterior proteins are misexpressed in anterior blastomeres in *mex-5;mex-6* mutants, and at least one anterior protein is not expressed.

We immunostained *mex-5* and *mex-6* embryos individually for each of the proteins that were misexpressed in the *mex-5;mex-6* double mutant. Although PIE-1 and MEX-1 appeared to be localized normally in *mex-6* and *mex-6(RNAi)* embryos (n=21 and 32, respectively), we found that about 58% (n= 31) of the *mex-5* embryos had low amounts of PIE-1 and MEX-1 mislocalized to some anterior blastomeres. Thus *mex-5* mutants appear to have a weakly penetrant defect in the localization of the germline proteins PIE-1 and MEX-1 that is greatly exacerbated by the simultaneous removal of *mex-6*(+) activity.

MEX-5 and the germline proteins have asymmetrical and reciprocal localization patterns

We generated a mouse monoclonal antibody (mABCS1) against a full-length MEX-5 fusion protein. Wild-type and *mex-6* embryos show identical patterns of mABCS1 staining, but no staining is detected in *mex-5* embryos. Since our genetic and RNAi experiments demonstrate that *mex-6(+)* activity is present in *mex-5* embryos, this result suggests that either mABCS1 does not cross react with MEX-6, or that the level of MEX-6 is too low to detect with this reagent. We therefore refer here to the staining pattern of mABCS1 as MEX-5 localization. For convenience, we refer to differences in the intensity of

immunostaining as MEX-5 levels, although we do not yet know whether staining levels are determined by protein abundance or antigen accessibility.

We first detect high levels of MEX-5 in the proximal arm of the gonad and in maturing oocytes (data not shown). Mature oocytes show uniform levels of MEX-5 throughout their cytoplasm, and MEX-5 remains uniform in newly fertilized 1-cell stage embryos (data not shown). MEX-5 gradually becomes highly asymmetric during and after the 1-cell stage (Figure 9). MEX-5 is present at high levels at the anterior pole of late 1-cell embryos (Figures 9A and 9A), and after cell division MEX-5 remains high in the anterior blastomere (Figure 9B; white arrowhead). After the anterior blastomere divides (Figures 9D and 9E), both anterior daughters inherit high levels of MEX-5 (Figure 9F, doubleheaded arrow). MEX-5 levels subsequently decrease in these daughters during the 4-cell stage (Figure 9G, doubleheaded arrow), and MEX-5 is not detectable in their descendants at subsequent stages (Figure 9H).

MEX-5 shows a very different pattern of localization in the posterior of the embryo. In the 1-cell embryo, the levels of MEX-5 decrease markedly at the posterior pole (Figures 9A and 9A). At cell division, the posterior daughter inherits low levels of MEX-5 (Figure 9B, black arrowhead); this blastomere is the germline blastomere P1 (see lineage diagram in Figure 5). Toward the end of the 2-cell stage, MEX-5 appears on both centrosomes of the nascent spindle in P1 (Figure 9C, arrows). As the spindle rotates onto the A/P axis of the egg

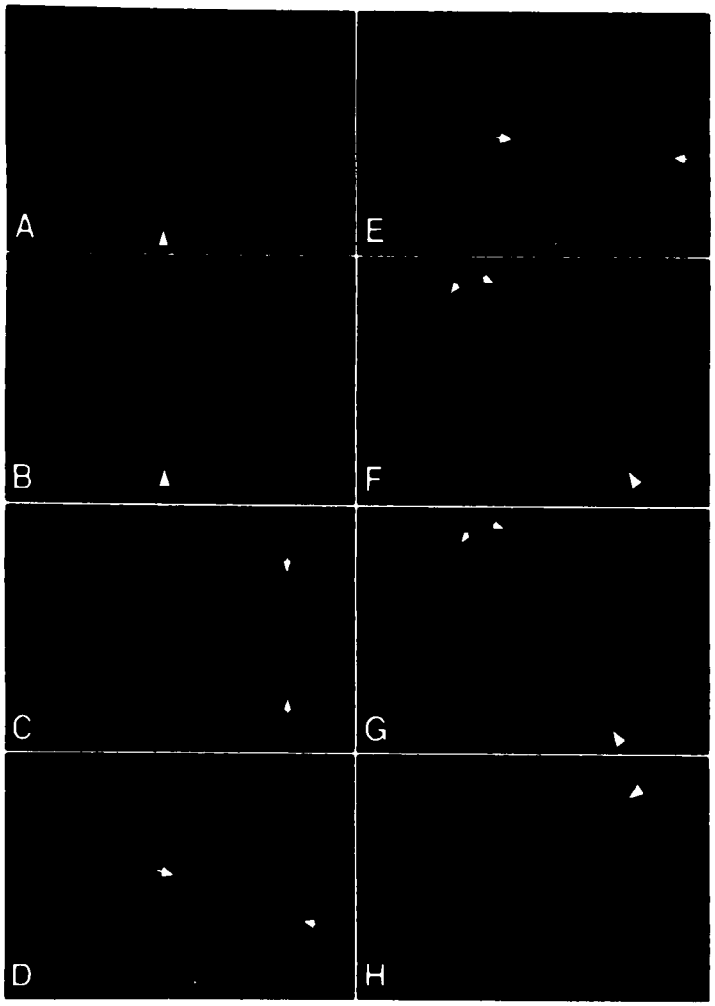

Figure 9: MEX-5 localization in wild-type embryos Panels show sequential stages of wild-type embryos stained with the MEX-5 antibody mABCS1, and co-stained with DAPI to visualize nuclei. Embryos are oriented and blastomeres marked as in Figure 9. (A) 1-cell stage. The male and female pronuclei have not yet fused to form the zygotic nucleus. The arrowhead marks the transition between the high, anterior levels of MEX-5 and the low, posterior, levels. (B) 2-cell stage. (C) Late 2-cell stage. The orientation of the nascent mitotic spindle is indicated by arrows; note MEX-5 on both centrosomes. (D) 2-cell stage at division. The spindle axis in the germline blastomere (right of dotted line) has rotated (arrows). MEX-5 is present at high levels on the posterior centrosome. Note MEX-5 in anterior part of cell (asterisk). (E) 3-cell stage. The anterior blastomere has divided into two cells and the germline blastomere (right of dotted line) is in late anaphase. Note absence of MEX-5 on centrosomes. (F) 4 cell stage. MEX-5 is at high levels in the anterior sister blastomeres (double headed arrow) and in the sister (white arrowhead) of the germline blastomere P2 (black arrowhead). (G) Late 4-cell stage. The level of MEX-5 has decreased markedly in the anterior sister blastomeres (double headed arrow). Low levels of punctuate staining in the germline blastomere P2 (black arrowhead) are P granules (data not shown). (H) 20-cell stage. MEX-5 has disappeared from most of the somatic blastomeres except for the sister (white arrowhead) of the germline blastomere P3 (black arrowhead).

and P1 begins division, MEX-5 disappears from the anterior centrosome (Figure 9D). MEX-5 persists on the posterior centrosome for a brief period before also disappearing (Figure 9E). During these stages, MEX-5 appears to accumulate in the anterior half of P1 (Figures 9D and 9E, asterisks). After cell division, the anterior, somatic daughter has a high level of MEX-5 and the new germline blastomere (P2) has a low level (Figure 9F; white and black arrowheads, respectively). MEX-5 shows asimilar pattern of centrosomal localization, soma/germline asymmetry, and gradual disappearance from somatic lineages, after each of the subsequent asymmetric divisions of the germline blastomeres (Figure 9H and data not shown).

The germline proteins PIE-1, MEX-1, and POS-1 are present at high levels in germline blastomeres and at low levels in the somatic sisters of these blastomeres (Mello et al., 1996; Guedes and Priess, 1997; Tabara et al., 1998; Tenehaus et al., 1998). We therefore immunostained embryos simultaneously for MEX-5 and for the germline proteins to compare their expression patterns. When the level of MEX-5 is high and uniform in a newly fertilized egg, the levels of PIE-1 and MEX-1 are low and uniform (data not shown). As the level of MEX-5 decreases at the posterior pole (Figure 10A), there appears to be a synchronous increase in the levels of PIE-1 (Figure 10B) and MEX-1 (data not shown). In the late 2-cell stage embryo, the germline blastomere (P1) also has a graded distribution of MEX-5 (Figure 10G) that appears to develop

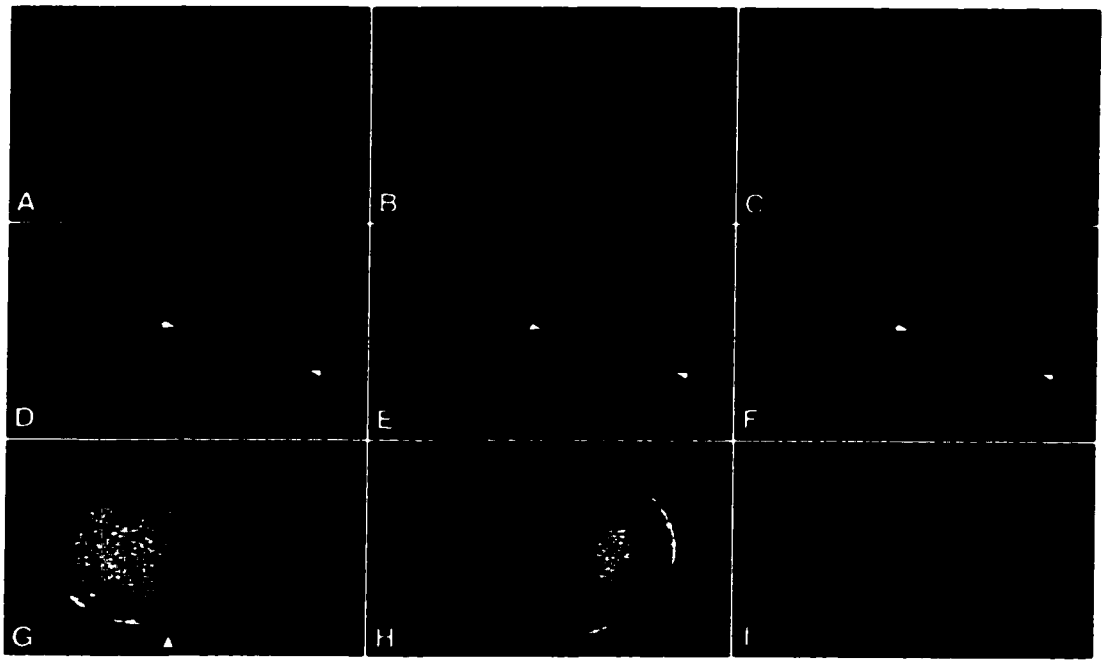

Figure 10: MEX-5, PIE-1, and PAR proteins.
(A-C) A single, wild-type 1-cell embryo immunostained for (A) MEX-5 and (B) PIE-1; the merged image is shown in panel C. (D) Wild-type 1-cell embryo stained for PAR-3. The arrowhead marks the transition from high to low PAR-3 at the cortex. MEX-5 staining in this same embryo is shown in Figure 9A, with the arrowhead in the identical location for comparison. (E) Wild-type 1-cell embryo stained for PAR-2; this is the same embryo shown in panels A-C. (F) 1-cell stage *mex-5;mex-6* embryo stained for PIE-1. (G-H) A single, wild-type 2-cell embryo immunostained for (G) MEX-5, and (H) PIE-1. The merged image is shown in panel I. The germline blastomere (right of dotted line) is in anaphase and arrows mark the axis of the spindle. PIE-1 is visible here in the posterior centrosome (see Mello et al., 1996).

synchronously with a reciprocal, graded distribution of PIE-1 (Figure 10H) and MEX-1 (data not shown). We immunostained 1-cell stage *mex-5;mex-6* embryos for the germline proteins, and found that these embryos did not have graded distributions of either PIE-1 (Figure 10F) or MEX-1 (data not shown); instead, both germline proteins were expressed at high, uniform levels. In contrast, the MEX-5 protein was localized with normal asymmetry in 1-cell stage *pie-1* and *mex-1* mutants (data not shown).

MEX-5 and PAR asymmetry

Wild-type embryos have an asymmetrical network of PAR proteins at the 1-cell stage. During the next few cleavages, the PAR proteins reproduce a similar asymmetry only in the germline blastomeres. We found that MEX-5 asymmetry was strongly dependent on the PAR proteins; mutant embryos lacking either the posterior-localized protein PAR-1, or the anterior-localized PAR-3, had uniform distributions of MEX-5 in all of the early blastomeres (Figures 11C and 11D, respectively). We therefore wanted to compare the asymmetric distribution of MEX-5 in wild-type embryos with the distributions of the PAR proteins. At the 1-cell stage, the transition between high and low levels of MEX-5 (Figure 9A, arrowhead) appeared coincident with the transition point between the anterior PAR proteins such as PAR-3 (Figure 10D, arrowhead) and PKC-3 (data not shown), and the posterior PAR protein PAR-2

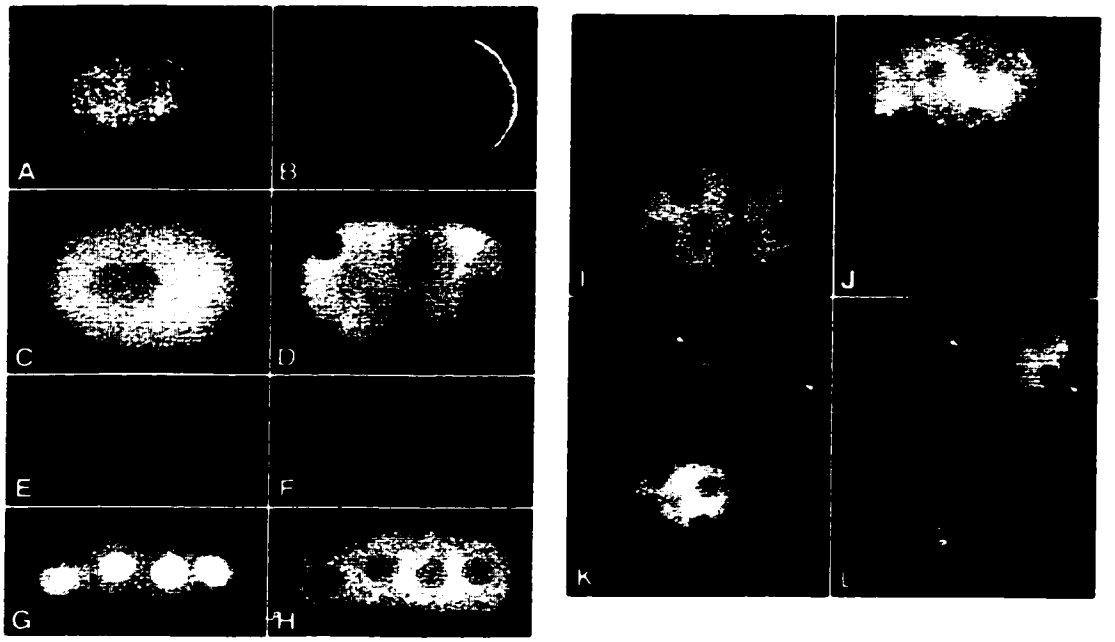

Figure 11: PAR proteins and ectopic expression of MEX-5.
(A and B) 1-cell stage *mex-5;mex-6* embryos showing localization of (A) PAR-3 and (B) PAR-2. Compare with wild-type patterns shown in Figures 10G and 10H, respectively. (C) 1-cell stage *par-1(b274)* embryo stained for MEX-5. (D) 4-cell stage *par-3(it71)* embryo stained for MEX-5. In *par-1* and *par-3* mutants, MEX-5 appeared to be distributed uniformly in 26/27 and 23/24 embryos, respectively, that were scored between the 1-cell and 4-cell stages. Only a slight asymmetry in MEX-5 was apparent in the single, exceptional embryos. (E and F) A single *par-3(it71)* embryo double stained for (E) PIE-1 and (F) MEX-1. Almost all of the *par-3* embryos (61/69) scored from the 1-cell to 12-cell stages had levels of PIE-1 and MEX-1 that appeared lower than wild-type. Similar results were observed in *par-1(b274)* mutants embryos (18/18 embryos; data not shown) (G and H) A single *par-3(it71);mex-5(RNAi);mex-6(RNAi)* embryo double stained for (G) PIE-1 and (H) MEX-1. All of these embryos (25/25) scored from the 1-cell to 12-cell stages contained much higher levels of PIE-1 and MEX-1 than did the *par-3* embryos. Similar results were obtained for *par-1(b274);mex-5(RNAi);mex-6(RNAi)* embryos (11/11 embryos; data not shown). (I -L) Panels show examples of heat-shocked *mex-5(-)mex-6(RNAi);pie-1(RNAi)* embryos carrying an inducible MEX-5 transgene. (I and J) Embryos after double staining for (I) MEX-5 and (J) MEX-1. (K and L) Examples of embryos with mosaic expression of (K) MEX-5 and (L) MEX-1. Long arrows point to a single blastomere with high levels of MEX-5 (K) and low levels of MEX-1 (L). Short arrows point to a single blastomere with low levels of MEX-5 (K) and high levels of MEX-1 (L).

(Figure 10E). In 2-cell stage embryos, we observed a similar correlation between the distributions of MEX-5 and the PAR proteins (data not shown) in the germline blastomere (P1). In both the 1-cell and 2-cell stages, the a/p asymmetry in MEX-5 expression appeared at about the same time as did the a/p asymmetry in the PAR proteins.

The PAR proteins PAR-3, PKC-3, PAR-2 each appeared to be distributed with normal a/p asymmetry in 1-cell stage *mex-5;mex-6* mutants (Figures 11A and 11B; between 5 and 23 wild-type or mutant embryos were scored for each protein). PAR function is required for the normal, asymmetric positioning of the first cleavage furrow in wild-type embryogenesis, creating an anterior blastomere that is larger than the posterior blastomere (reviewed in Rose and Kemphues, 1998). We therefore compared the relative size of the anterior blastomere in 2-cell stage wild-type embryos with the anterior blastomere in *mex-5;mex-6* embryos. The size of the anterior blastomere/total was 58.2 +/- 1.8% for wild-type embryos and 58.2 +/- 1.5% for *mex-5;mex-6* mutant embryos (see Experimental Procedures). Thus, MEX-5/MEX-6 do not appear to be required to establish the asymmetrical network of PAR proteins at the 1-cell stage, nor are MEX-5/MEX-6 required for the PAR-dependent positioning of the first cleavage furrow.

As described above, after the 1-cell stage the PAR proteins repeat their pattern of cortical asymmetry only within the successive germline

TABLE 1: cleavages in *mex-5(-);mex-6(-)* embryos. The wild-type pattern is transverse in the anterior blastomere (T) and longitudinal (L) in the posterior blastomere

Spindle Axis		**wild type**	***mex-5(-);mex-6(-)***
(ant)	(pos)	% (n=50)	% (n=15)
T	**L**	100	26
T	**T**	0	33
L	**L**	0	33
L	**T**	0	7

blastomeres. We detected defects in PAR protein localization in *mex-5;mex-6* embryos beginning late in the 2-cell stage. For example, in some 2-cell stage embryos the posterior PAR protein PAR-2 was localized to an abnormally small zone at the posterior pole (data not shown). By the 4-cell stage, almost all *mex-5;mex-6* embryos (13/14) showed some defects in PAR-2 localization. Furthermore, 2-cell stage and later *mex-5;mex-6* embryos often had cleavage defects that were similar to those observed in some *par* mutants (Table 1). We conclude that MEX-5/MEX-6 do not appear to function in establishing PAR asymmetry at the 1-cell stage, but are required for proper PAR localization at later stages (see Discussion).

Ectopic MEX-5 can inhibit the expression of germline proteins

We have shown that wild-type embryos have an asymmetric distribution of MEX-5, in which high levels of MEX-5 are correlated with low levels of the germline proteins. We found that *par-1* and *par-3* mutant embryos had uniform, high levels of MEX-5, and that these mutants also had low levels of the germline proteins PIE-1 and MEX-1 (Figures 11E,F; see also Tenenhaus et al., 1998). We therefore wondered if the expression of the germline proteins might be inhibited in the *par* mutant embryos by *mex-5(+)* and *mex-6(+)* activities. We constructed *par-1(b274);mex-5(RNAi);mex-6(RNAi)* embryos and *par-3(it71);mex-5(RNAi);mex-6(RNAi)* embryos and immunostained them for

PIE-1 and MEX-1. We observed a large increase in the levels of PIE-1 and MEX-1 in both triple mutants compared to either *par* mutant (Figure 6, legend). For example, while *par-3(it71)* mutant embryos had low levels of PIE-1 and MEX-1 (Figures 11E and 11F, respectively) the *par-3(it71);mex-5(RNAi);mex-6(RNAi)* embryos appeared to have much higher levels of both proteins (Figures 11G and 11H, respectively).

The above results suggest that high levels of MEX-5/MEX-6 might be sufficient to inhibit the expression of germline proteins. To test this possibility further, we constructed a transgene to induce ectopic MEX-5 expression from a heat-shock promoter. We used *mex-5;mex-6(RNAi);pie-1(RNAi)* embryos to assay activity of the transgene; *pie-1(+)* activity was removed to prevent PIE-1 from inhibiting transcription (Seydoux et al., 1996). We analyzed embryos in which induction of the transgene should have occurred at or after the 2-cell stage (see Experimental Procedures); the resulting embryos were fixed and immunostained for both MEX-5 and MEX-1. Control *mex-5;mex-6(RNAi);pie-1(RNAi)* embryos lacking the transgene showed no detectable MEX-5 after heat shock, and had high levels of MEX-1 (data not shown). After heat shock, embryos from the transgene-containing strain showed three patterns of protein expression. Some embryos did not appear to express the MEX-5 transgene (Figure 11I, top embryo); similar to control embryos, these embryos had high levels of endogenous MEX-1 (Figure 11J, top embryo). Since the transgene

was not integrated into a chromosome, embryos or individual cells lacking MEX-5 expression may have lost the MEX-5 transgene during cell division. The second class of embryos appeared to express the MEX-5 transgene at high levels (Figure 11I, bottom embryo); these embryos had relatively low levels of endogenous MEX-1 (Figure 11J, bottom embryo). The third class of embryos appeared to have mosaic expression of the MEX-5 transgene: Blastomeres that showed high levels of MEX-5 expression showed low levels of MEX-1, while other blastomeres in the same embryo that had low levels of MEX-5 had relatively high levels of MEX-1 (Figures 11K and 11L). These results suggest that MEX-5 is sufficient to inhibit expression of the germline protein MEX-1 in *mex-5;mex-6(RNAi);pie-1(RNAi)* embryos.

DISCUSSION

Through mechanisms that are not yet understood, interactions among the PAR proteins causes these proteins to become localized asymmetrically to the cortex of the newly fertilized *C. elegans* egg. This PAR asymmetry is required for subsequent a/p asymmetries in the expression patterns of several cytoplasmic and nuclear proteins that are encoded by non-localized, maternally-expressed mRNAs. In this report, we have shown that the *mex-5* and *mex-6* genes are essential for many of these subsequent asymmetries.

Because the *mex-5* and *mex-6* genes are nearly identical, we consider it likely that the MEX-5 and MEX-6 proteins have the same biochemical function. Although the tissue specification defects in *mex-5;mex-6* embryos appear markedly different from those in *mex-5* embryos, many of these differences can be attributed simply to an increase in the penetrance of a single *mex-5* defect; the mislocalization of the germline protein PIE-1. *mex-6(pk440)* embryos and *mex-6(RNAi)* embryos are viable and develop into fertile adults, suggesting that *mex-6 (+)* function is not essential when *mex-5(+)* function is present. In contrast, *mex-5(zu155)* embryos and *mex-5(RNAi)* embryos are inviable, indicating that *mex-6(+)* activity does not compensate fully for the loss of *mex-5(+)* activity. We do not yet know the expression pattern of the MEX-6 protein, nor do we know if artificially increasing the levels of MEX-6 would be sufficient to rescue the *mex-5* mutant. If the expression pattern of MEX-6 coincides with that of MEX-5 in wild-type embryos, it may be that the levels of MEX-6 in *mex-5* mutants are insufficient to perform all essential functions, such as those required for the proper localization of SKN-1.

MEX-5/MEX-6 are distinct from PAR proteins

Blastomeres in *mex-5;mex-6* embryos have several defects in protein expression that resemble defects described for *par* mutants. However,

mex-5;mex-6 embryos do not have obvious defects in the asymmetrical network of PAR proteins that normally forms during the 1-cell stage. While mutations in any one of the *par* genes except *par-4* alter the position of the first cleavage furrow (see Rose and Kemphues 1998 for review), we have found that *mex-5;mex-6* embryos have a normal, asymmetrically-positioned furrow. These observations suggest that MEX-5/MEX-6 might have functions that are distinct from those of the PAR proteins. In support of this view, we have shown that MEX-5/MEX-6 have effects on the expression of the germline proteins PIE-1 and MEX-1 that are independent of the asymmetrical network of PAR proteins. First, our genetic experiments demonstrate that MEX-5/MEX-6 activities inhibit the expression of both PIE-1 and MEX-1 in mutant backgrounds lacking either anterior PAR proteins (PAR-3), or posterior PAR proteins (PAR-1). Second, we have shown that heat shock-induction of MEX-5 at or after the 2-cell stage in a mutant background can inhibit the expression of the MEX-1 protein in anterior blastomeres; these anterior blastomeres do not contain the asymmetrical network of PAR proteins. Although *mex-5;mex-6* mutant embryos do not show defects in the asymmetry of PAR proteins at the 1-cell stage, *par* mutant embryos show highly penetrant defects in MEX-5 protein localization. Thus, the PAR proteins may not be required for MEX-5/MEX-6 activity, but are required for MEX-5, and possibly MEX-6, localization.

The *mex-5;mex-6* embryos often show PAR-like cleavage defects after the 1-cell stage. It is possible that these later defects result from the misexpression of PAR, or non-PAR, proteins that regulate cell division. In wild-type embryos, the asymmetrical network of PAR proteins is reproduced during each of the early cell cycles only in the germline blastomeres, which divide asymmetrically. PAR asymmetry in the germline lineage has not been studied as extensively as for the 1-cell stage, and is complicated by the fact that the a/p axis of the germline blastomeres appears to invert during the third cell cycle (Schierenberg, 1987). Nevertheless, these observations suggest that some factor(s) that regulate PAR asymmetry may be asymmetrically distributed to the germline blastomeres after the 1-cell stage, and thus may be mislocalized in *mex-5;mex-6* mutants. For example, the germline proteins MEX-1 and POS-1 are required for proper cleavage asymmetry in the germline blastomeres P2 and P3 (Mello et al., 1992; Tabara et al., 1999), and both proteins are mislocalized in *mex-5;mex-6* mutants.

MEX-5/MEX-6 and protein localization in the early embryo

Many of the examples of protein mislocalization we observe in *mex-5;mex-6* mutant embryos involve the anterior misexpression of proteins that normally are expressed in posterior and germline blastomeres. For example, the proteins SKN-1, PAL-1, MEX-1, PIE-1, POS-1, and SKN-1 are each

misexpressed in the anterior of *mex-5;mex-6* mutant embryos. This result suggests that in wild-type embryos MEX-5/MEX-6 functions in inhibiting the anterior expression of these proteins. However, MEX-5/MEX-6 also plays a role in permitting the expression of the anterior protein GLP-1, since this protein is not detectable in *mex-5;mex-6* mutants. In wild-type embryogenesis, translation of the *glp-1* mRNA appears to be repressed in posterior blastomeres (see Introduction; Evans et al., 1994). Thus MEX-5/MEX-6 may prevent a posterior-localized, trans-acting repressor of *glp-1* mRNA from being expressed in the anterior of the embryo, consistent with the effect of MEX-5/MEX-6 on other posterior-localized proteins.

MEX-5/MEX-6 and the germline proteins

In 2-cell stage wild-type embryos, the expression of MEX-5 is reciprocal to that of all posterior proteins. However, this is not the case at the 4-cell stage. For example, SKN-1 is expressed at high, equal levels in both of the posterior sister blastomeres at the 4-cell stage: One sister lacks MEX-5 (the germline blastomere P2), but the other sister contains high levels of MEX-5. Since

MEX-5/MEX-6 functions are required for the proper localization of the posterior proteins, it is possible that MEX-5/MEX-6 functions before the 4-cell stage. It is also possible that MEX-5/MEX-6 affects the localization of some

posterior proteins indirectly. For example, MEX-3 appears to repress the anterior expression of PAL-1 in wild-type embryos (Draper et al., 1996; Hunter and Kenyon, 1996), and we have shown here that MEX-3 is expressed at abnormally low levels in *mex-5;mex-6* mutants.

Although the presence of MEX-5 does not directly correlate with the absence of all posterior proteins, there is a remarkable correlation between the distribution of MEX-5 and that of the germline proteins MEX-1, PIE-1 and POS-1: After fertilization of the egg, MEX-5 is present at high levels in all regions of the embryo that contain the mRNAs for the germline proteins, but that do not accumulate the germline proteins. The mRNAs for the germline proteins are examples of class II mRNAs in *C. elegans* (Seydoux and Fire, 1994). These maternally-provided mRNAs are present in the oocyte, and persist at high levels in each of the successive germline blastomeres. When a germline blastomere divides, its somatic daughter and germline daughter inherit comparable levels of mRNA. However, after one or two additional cell cycles these mRNAs are degraded in the descendants of the somatic daughter. For example, the mRNA for *mex-1* is present at high levels in the anterior, somatic blastomere of a 2-cell embryo, but disappears rapidly from the daughters of the anterior blastomere during the 4-cell stage (Guedes and Priess, 1997). We have shown here that MEX-5 disappears from these same daughters late in the 4-cell stage. Thus MEX-5 may not be necessary to prevent expression of the germline

proteins in anterior blastomeres once their mRNAs are targeted for degradation. In contrast, MEX-5 remains at high levels in the somatic sister of the germline blastomere throughout the 4-cell stage, and this somatic sister is the only blastomere that retains high levels of the germline mRNAs without producing high levels of germline proteins.

The most striking examples of MEX-5 and germline proteins having reciprocal asymmetry are the gradients that form during the 1-cell and 2-cell stages. *mex-5(+)* and *mex-6(+)* activities are required for the gradients of PIE-1 and MEX-1, however neither *pie-1(+)* nor *mex-1(+)* activities are required for gradients of MEX-5. We have shown that MEX-5 expression is reciprocal to that of the germline proteins when MEX-5 is expressed inappropriately in posterior blastomeres. In our heat shock experiments, high levels of induced MEX-5 expression was correlated invariably with low levels of endogenous MEX-1. Similarly, in various *par* mutants, MEX-5 is expressed at high levels in posterior blastomeres that lack, or have low levels, of germline proteins, and we have shown that MEX-5/MEX-6 function is required for those low levels. These several results suggest the possibility that in wild-type embryos MEX-5/MEX-6 may directly inhibit the expression of at least some of the germline proteins.

Control of germline protein levels

Since the mRNAs for proteins like MEX-1 and PIE-1 are distributed uniformly until the 4-cell stage, how might MEX-5/MEX-6 inhibit the expression of these proteins? It is very likely that there is a mechanism for degrading germline proteins in the somatic daughters of germline blastomeres, and therefore MEX-5/MEX-6 could be part of this process (see Introduction). We do not yet know whether degradation also is involved in setting up the gradient of germline proteins that develops in germline blastomeres prior to division. As the gradients of PIE-1 and MEX-1 form during the 1-cell stage, the levels of these proteins remain low at the anterior pole while the levels increase towards the posterior pole. In principle, this gradient could be generated by restricting the de novo translation of the germline mRNAs to the posterior pole.

Finger motifs similar to those found in MEX-5 and MEX-6 have been described in several proteins that appear to interact, directly or indirectly, with RNA. In particular, mouse TTP has been shown to bind tumor necrosis factor (TNF) mRNA, and requires the finger domains to do so (Carballo et al., 1998; Lai et al., 1999). If the finger motifs function in general to bind RNA, an attractive model is that MEX-5 and MEX-6 might bind to, and prevent the translation of, germline messages. Like MEX-5 and MEX-6, the germline proteins PIE-1, MEX-1, and POS-1 each contain two copies of a related finger motif. PIE-1 is a predominately nuclear protein that is markedly different from

MEX-1 and POS-1, and that appears to repress transcription in the germline blastomeres through an unknown mechanism (Seydoux et al., 1996). MEX-1 and POS-1 are cytoplasmic proteins; although their biochemical functions are not known, mutant analysis has suggested that these proteins might positively regulate the translation of maternal mRNAs in the germline blastomere (Guedes and Priess, 1997; Tabara et al., 1999). If so, it will be interesting to determine how the molecular functions of MEX-1 and POS-1 compare with MEX-5 and MEX-6.

PAR asymmetry and MEX-5/MEX-6

Several studies have demonstrated that the asymmetrical network of PAR proteins is required for a/p differences in the fates of the early embryonic blastomeres in *C. elegans* (Kemphues et al., 1988; Bowerman et al., 1997). We propose that MEX-5/MEX-6 is a critical component of the link between PAR proteins and cell fate. We have shown here that the asymmetric localization of MEX-5 closely matches the a/p boundary between anterior and posterior PAR proteins, and that PAR function is required for MEX-5 localization.

One intriguing aspect of MEX-5 localization is the association with centrosomes. MEX-5 becomes associated with centrosomes only in the germline blastomeres, which are the only blastomeres that reproduce the asymmetrical network of PAR proteins during each of the early cell cycles. In

mutants defective for either anterior or posterior PAR proteins, MEX-5 does not associate with centrosomes, though MEX-5 is present at high levels. Thus, in wild-type embryos, PAR functions at the posterior pole could cause MEX-5 to localize initially to both centrosomes. The subsequent ninety degree rotation of the centrosomes/nascent spindle complex toward the a/p axis of the embryo would move the anterior-most centrosome away from the posterior PAR proteins, possibly allowing MEX-5 to disassociate. Additional mechanisms might then degrade the MEX-5 remaining in the posterior centrosome at anaphase.

Recent studies have identified orthologs of PAR-1 and PAR-3 in Drosophila (Kuchinke, et al., 1998) and in mammals (Bohm, et al., 1997; Izumi et al., 1998), where they have asymmetrical, cortical distributions in polarized cells. For example, the PAR-3-related protein in Drosophila (Bazooka) is localized asymmetrically to the cortex of neuroblasts, and is required for the proper, asymmetrical, distribution of other neuroblast proteins (Schober, et al., 1999; Wodarz et al., 1999). In future studies, it will be interesting to determine whether MEX-5-related proteins have general roles in linking cortical asymmetries to cytoplasmic differences in protein expression.

CHAPTER 3: A SCREEN FOR TEMPERATURE SENSITIVE EMBRYONIC MUTANTS

INTRODUCTION

The recovery of temperature sensitive (ts) alleles of embryonic genes has allowed the analysis of requirements for these genes at multiple stages of development. For example, temperature shift experiments using ts alleles of the *C. elegans* Notch-like gene *glp-1* have shown that *glp-1* is required in the embryo for a cell signal at the 4-8 cell stage (Priess et al., 1997), and then later, at the 12-28 cell stage (Mello et al., 1994). In this chapter I report my preliminary data resulting from a screen for temperature sensitive mutants. I focused my screen on embryonic mutants that showed cell fate transformations in the production of pharyngeal and intestinal cell types. Non-conditional screens have successfully identified genes involved in early cell fate decisions by assaying for cell fate transformations in pharyngeal and intestinal cells. These genes include, for example, *skn-1* (Bowerman et al. 1992), *pie-1* (Mello et al. 1992), and *mex-5* (see chapter 2). I describe my preliminary analysis of one mutant, *pie-2*, recovered in the ts screen, and provide a brief description of other mutants.

METHODS

Temperature Sensitive Screen

The screen followed a protocol modified from Priess et al. (1987). *lin-2(e1309)* worms were used for mutagenesis. *lin-2(e1309)* worms have defective vulvas; as a result, hermaphrodites fill up with their own progeny. These progeny are easily scored under Nomarski optics. Mutagenized *lin-2(e1309)* worms were grown until F1 worms filled with F2 progeny. The worms were then treated with hypochlorite (Stiernagle, 1999) to destroy adults and larvae (the unhatched F2 progeny survive this treatment). These F2 progeny were then synchronized according to Stiernagle (1999). The F2 worms were grown at the permissive temperature (15 degrees) until the majority of F2 worms had produced only about 5 embryos. Worms were then upshifted to the non-permissive temperature (26 degrees) for 16 hours. F2 worms were pooled in M9 buffer and were examined under a cover slip by Nomarski optics. As a consequence of the timing of temperature shift, ts embryonic mutants produced older, live progeny and younger, dead progeny. The live embryos were located in a distinct region of the gonad that correlated with their position prior to temperature upshift (Figure 12). Dead embryos were scored by Nomarski optics; ts mutant worms were recovered by removing them from under the cover slip (Figure 12).

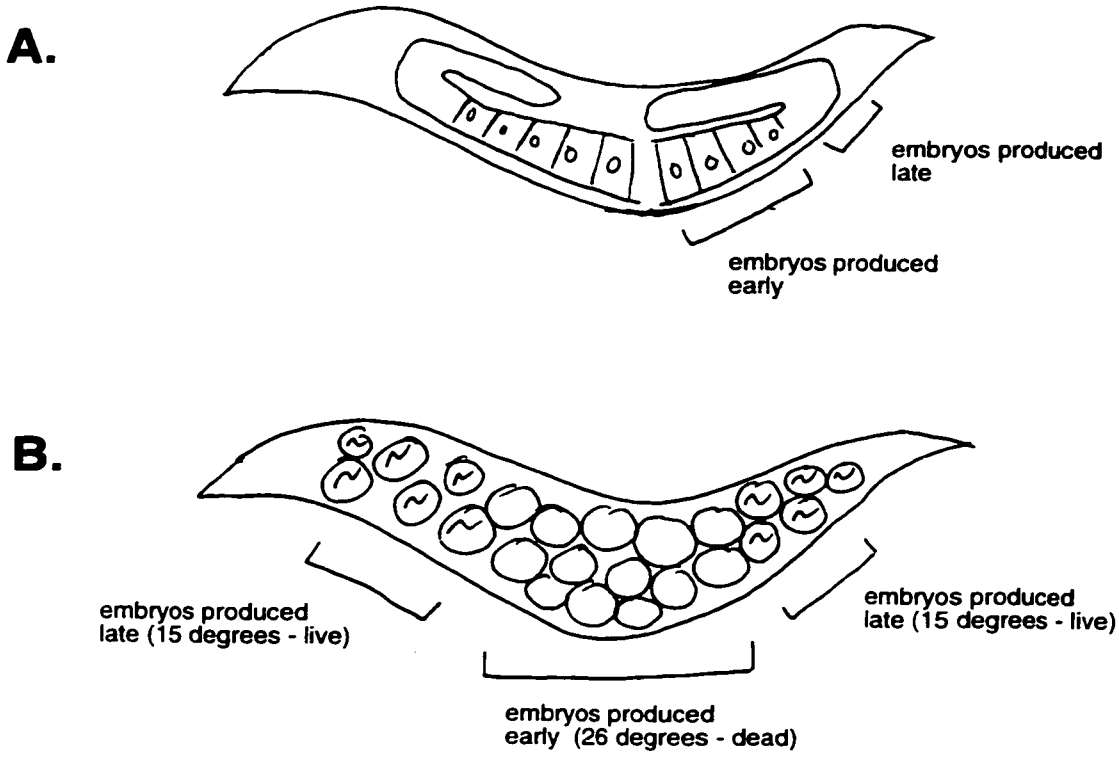

Figure 12: temperature sensitive mutagenesis.

A. A wild type worm at an early stage. The gonad is a symmetric 2-armed structure. Embryos produced first are derived from oocytes at the middle of the animal. Embryos produced later are derived from germ cells at the other arm of the gonad.

B. A typical ts mutant. As a consequence of the anatomy of *lin-2(e1309)*, worms are retained inside the mother. Embryos produced early, at 26 degrees, are visible in the central regions of the worm, and embryos produced later, at 15 degrees, are at the ends.

Genetic analyses

C. elegans culture, mutagenesis and genetics were as described in Brenner (1974). The following strains and genetic reagents were used in complementation analysis and mapping, arranged by linkage group: I: *dpy-5(e61)* II: *rol-6(e187)* III: *unc-32(e189)*, IV: *skn-1(zu67)*, *unc-5(e53)* V: *dpy-11(e224)*, *efl-1(se1)*, *efl-1(n3318)*, *par-1(b274)*,*dpy-4*, *unc-76(0911)*, *sqt-3(sc63)*, *egl-1(n86)*, DnT1 X: *lon-2(e678)*

***pie-2* analysis**

Cell types arising from individual blastomeres were scored by Nomarski optics. Immunolocalization with PIE-1 antibody was as described (Tenenhaus et al., 1998).

RESULTS AND DISCUSSION

Analysis of *pie-2(zu277)*

zu277 was recovered because it produced embryos that did not undergo morphogenesis and appeared to generate extra amounts of pharynx and intestine. *zu277* was therefore named *pie-2* (**p**harynx and **i**ntestinal **e**xcess). 1 and 2-cell stage mutant embryos shifted to the non-permissive temperature died and did not undergo morphogenesis (n=5; pharynx and intestine not scored)

suggesting that *pie-1* can function after the 1-cell stage. L1 larvae upshifted from the non-permissive to the permissive stage grew into viable and fertile adults (20/20).

In wild-type embryos, the autonomous production of pharynx and intestine occurs only in descendants of the blastomere EMS. I asked if blastomeres other than EMS might be producing pharyngeal and intestinal tissue in *pie-2* mutant embryos by using ablation analysis (see Figure 13 for summary of these results). In these analyses, blastomeres are killed using a laser microbeam, and the tissues produced from the remaining blastomere(s) are analyzed. When the P1 blastomere is isolated and the AB blastomere is allowed to develop in isolation, the wild-type AB blastomere never produces pharynx (0/6 in this study, Priess and Thomson, 1987). In contrast, some (3/27) AB blastomeres isolated from *pie-2* mutant embryos produced pharynx. Wild-type P2 blastomeres normally do not produce pharynx or intestine after isolation (Mello et al., 1992). However, when the AB and EMS blastomeres from *pie-2* mutant embryos were killed and the P2 blastomere allowed to develop, I observed that in 6/6 cases, the P2 blastomere produced pharynx and intestine. In this respect *pie-2* mutant embryos resembled *pie-1* mutant embryos. However, *pie-1* mutant embryos fail to make germ cells (Mello et al.,1992), and germ cells were observed in most *pie-2* mutant embryos. I concluded that *pie-2 (+)* activity is required to prevent the production of pharynx in posterior blastomeres and, to a lesser extent, in anterior blastomeres.

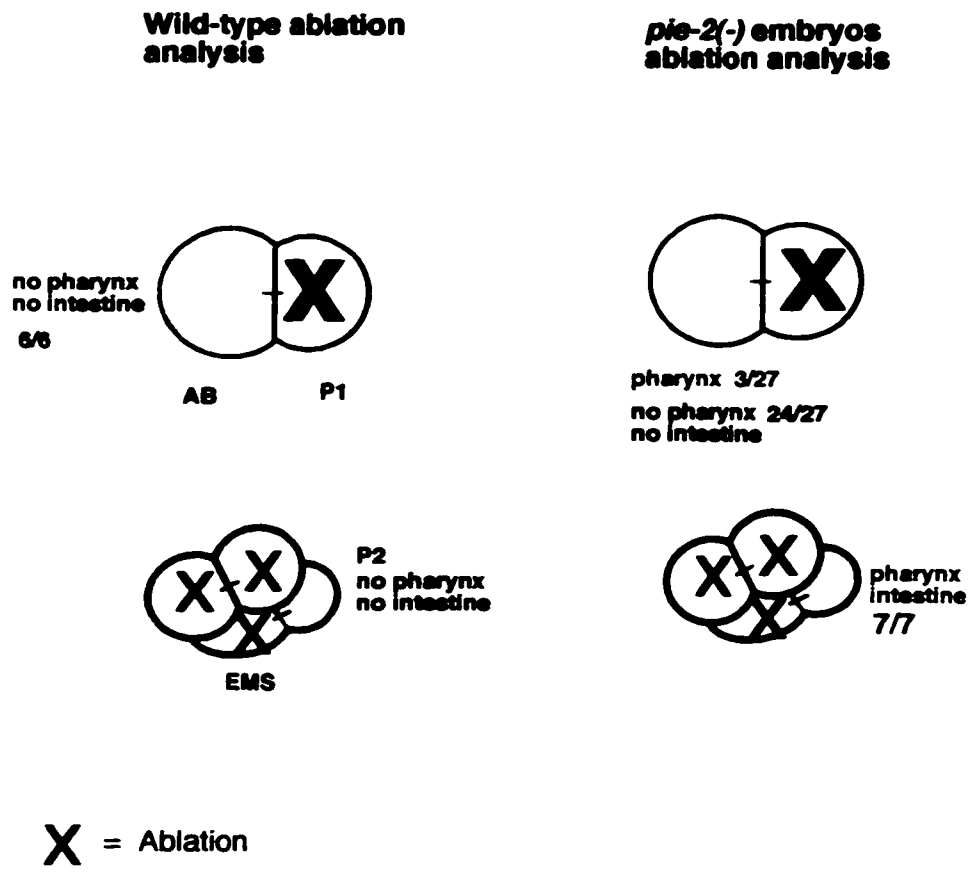

Figure 13: *pie-2* mutant embryos produce ectopic pharynx and intestine from the AB and the P2 precursors.

In wild type 4-cell stage embryos, *pie-1(+)* prevents the formation of pharyngeal and intestinal cell types from P2 (Figure 3) (Mello et al., 1992; Mello et al., 1996). I was interested in whether some of the defects observed in P2 in *pie-2* could be due to defects in PIE-1. In preliminary experiments I stained *pie-2* mutant embryos with the PIE-1 antibody and I observed that PIE-1 expression appeared approximately normal in P2 (3/3 embryos). I concluded that the production of ectopic pharynx and intestine from P2 in *pie-2* mutant embryos appeared, at least upon initial examination, to not be due to aberrant PIE-1 expression.

I considered whether *pie-2* has a role in regulating SKN-1 levels or activity. I first asked if the excess pharyngeal and intestinal tissue in *pie-2* mutant embryos required *skn-1(+)* activity. I constructed *skn-1(zu67);pie-2(zu277)* mutants, and observed that the resulting embryos resembled *skn-1(-)* mutant embryos; no pharynx and no, or very little, intestine was produced (20/20). Therefore, *skn-1(+)* activity is required for the production of pharynx and intestine in *pie-2(-)*. In wild-type embryos SKN-1 is present, although at low levels, in AB and the P2 daughters (Bowerman et al., 1993). These blastomeres do not normally produce pharynx when they are cultured in isolation (Priess et al, 1987; Bowerman et al., 1992), although they do in *pie-2* mutant embryos. Therefore, *pie-2* could normally function to prevent the activity of *skn-1* (See

Figure 14 for a model), or prevent the accumulation of high levels of SKN-1 protein in these blastomeres.

pie-2 mutants also show defects the orientation of the EMS spindle. In wild-type embryos EMS divides along the anterior-posterior axis, but in *pie-2* mutant embryos EMS showed either a left-right or transverse orientation (5/6). It seems unlikely that aberrant EMS orientation could result from defects in SKN-1(+); SKN-1 has not been shown to play a role in spindle orientation (Bowerman et al., 1992, Bowerman et al., 1993).

In order to clone *pie-2*, I initiated genetic crosses to determine its position on the genetic map (data summarized in Figure 15). After determining that *pie-2* mapped to chromosome V, I mapped its position relative to two genetic markers at the right arm of V,*dpy-11* and *unc-76*. These crosses suggested that *pie-2* mapped close to the left of *unc-76*. These data were subsequently shown to be due to a double recombination event. A subsequent cross placed *pie-2* approximately 2.5 map units to the right of *unc-76*. The maternal-effect mutant *efl-1* maps to this same general region. Complementation tests with a putative null *efl-1* allele *(n3318)* showed that *efl-1* and *pie-2* are not allelic (Barbara Page, personal communication). *pie-2* is on a region of chromosome V that contains very few cloned or mapped genes, and work on this locus is difficult to pursue at this time.

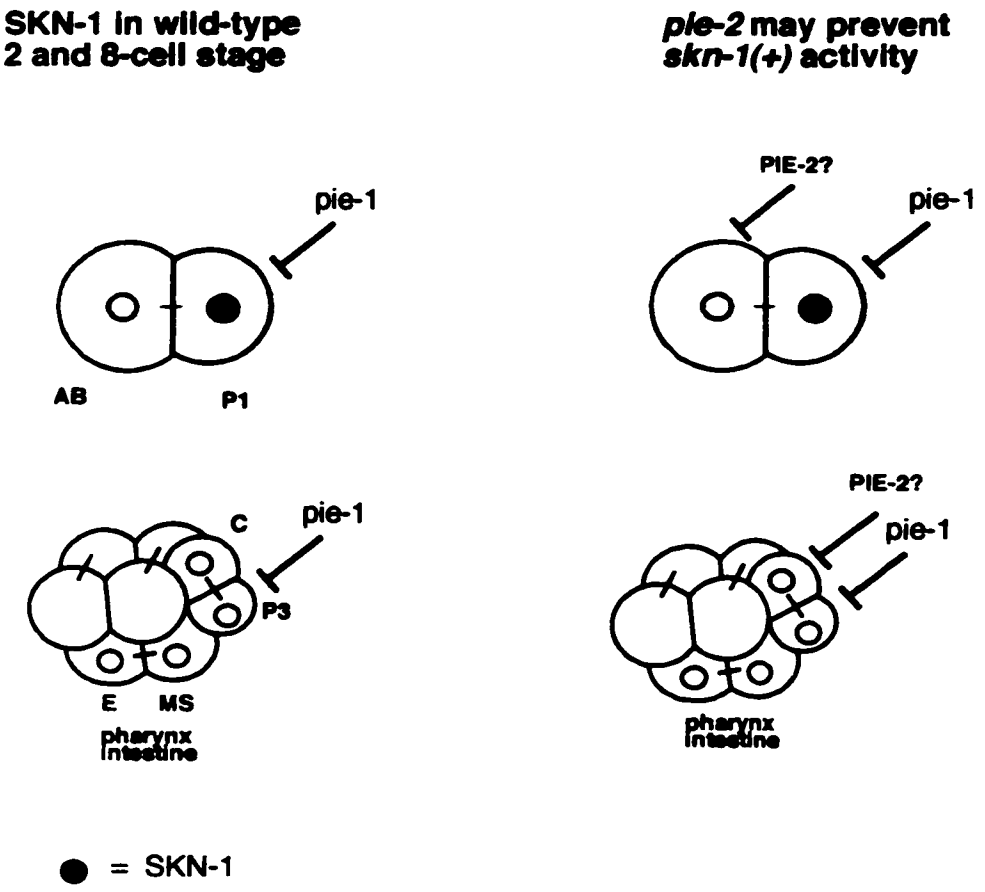

Figure 14: A model for *pie-2* function. In wild-type embryos low levels of SKN-1 are expressed in somatic precursor that normally do not produce pharynx and intestine (shown here are the AB and C precursors). *pie-2* could act to prevent the activity of *skn-1* in these blastomeres.

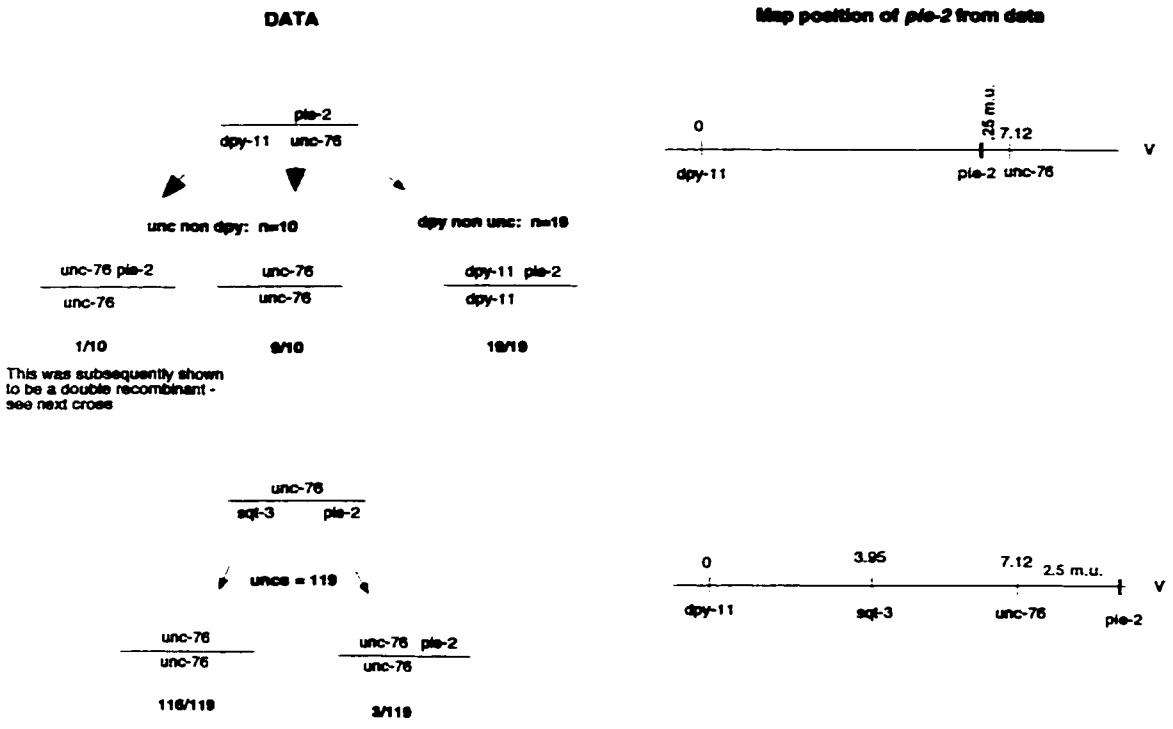

Figure 15: *pie-2* map position.

Temperature sensitive *glp* and *par*-like alleles (Table 2)

10 mutants were recovered because they produced embryos that resemble *glp-1* embryos (Priess et al. 1987). *glp-1* mutant embryos lack anterior pharynx because *glp-1* is required for a signal that induces anterior pharynx from the AB blastomere. I confirmed by complementation tests against *glp-1(e2142)* that 4/10 were new ts alleles of *glp-1 (zu270, zu261, zu259, zu256).* Six of *the* "*glp-1*-like" alleles were tested for post-embryonic phenotypes. Null *glp-1* mutants are sterile because *glp-1* is required for cell signaling in the gonad (Austin and Kimble, 1987). 5/6 of the ts *glp-1*-like alleles also were sterile when upshifted at the L1 larval stage.

Seven mutants were recovered because they produced embryos with excess amounts of pharynx. *par* mutant embryos normally show excess pharynx due to ectopic expression of SKN-1 (Bowerman et al., 1993; Bowerman et al., 1997). I therefore considered whether these new mutations were alleles of *par* genes. I assayed another phenotype characteristic of the *par* mutants; synchronous early cleavages. For each of the ts "*par*" mutants, at least one synchronous early cleavage event was observed. Not all of the ts *"par"* mutants were tested for post-embryonic defects. However, *zu252* (isolated by Jim Priess in the screen) is sterile upon upshift as a larva. The gene defined by the "*par*-like" mutation *zu310* was found by complementation tests to be an allele of *par-1* (Rueyling Lin, personal communication).

Other classes of mutants (Table 2)

Three mutants were recovered because they produced embryos that failed to make pharynx. These mutant embryos superficially resembled *skn-1* mutant embryos. However, they also resembled embryos from the previously identified mutant, *zu130*, that has unusually slow early cleavages (J. Priess personal communication). Two (*zu266* and *zu265*) were shown to have cleavage rates almost twice as long as wild type. Thus this class of mutants was not analyzed further.

zu271 mutant embryos produced small, poorly differentiated pharynx, and no intestinal cell types. These embryos resemble embryos from a previously identified mutant called *pos-1*. Complementation analysis against *pos-1*, however, demonstrated that *zu271* was not a new *pos-1* allele (R. Hill, personal communication).

zu281 mutant embryos had two distinct phenotypes; they appeared to produce excess pharynx and intestine, and, like wild-type embryos, appeared to produce two germ cells. In these respects, *zu281* embryos resembled *mex-5* embryos. However, *zu281* mapped to chromosome IV, while *mex-5* maps to chromosome V. In the future, an analysis of *zu281* may reveal whether the gene identified by *zu281* functions in the same process as *mex-5*.

14 mutations were isolated that had variable phenotypes. Some *zu257* mutant embryos, for example, produced excess pharynx while others produced

decreased amounts of pharynx. As the embryonic phenotypes from these mutants were variable, and thus difficult to analyze, further experiments were not considered.

Table 2: Classes of mutants.

***glp-1* mutants and candidates**

zu256 (confirmed) *zu255*
zu254 (confirmed) *zu260*
zu261 (confirmed) *zu307*
zu270 (confirmed) *zu332*
zu309 *zu335*
zu259

***par* mutant candidates**

zu310 - fails to complement *par-1*
zu273
zu272
zu264
zu258
zu263
zu252 (JP isolated)

slow early cleavages

zu266 - confirmed slow early cleavages
zu338 - three celled embryo
zu265 - confirmed slow early cleavages

variable phenotypes

zu257	*zu268*	*zu276*	*zu337*
zu262	*zu274*	*zu339*	
zu269	*zu350*	*zu308*	
zu267	*zu275*	*zu336*	

***"pos-1"*- like**

zu271 - small, poorly differentiated pharynx

zu 281

pie-2

zu277

CHAPTER 4: CONCLUSIONS AND FUTURE DIRECTIONS

Regulation of germline proteins by MEX-5/MEX-6

The germline proteins appear to be localized by two distinct processes (see Chapter 2). In the first process, PIE-1 and another germline protein, MEX-1 are expressed asymmetrically within each dividing germline precursor. As a consequence of this asymmetry the somatic daughter inherits less MEX-1 and PIE-1 than the germline daughter. In the second process, the low levels of MEX-1 and PIE-1 are further reduced in each somatic precursor as the cell cycle progresses. This reduction also occurs for POS-1. It is possible that MEX-5/MEX-6 control both of these distinct localization processes; in *mex-5;mex-6* mutant embryos, asymmetry in germline proteins is abolished during cell division and no asymmetry is observed after cell division.

How might MEX-5/MEX-6 direct germline protein asymmetry? Each germline precursor expresses uniform levels of *pie-1*, *mex-1* and *pos-1* mRNA. Therefore the first localization process, asymmetry of germline proteins prior to division, must involve either protein transport, or asymmetries in translation or protein stability. The second localization process, reduction of germline protein in each somatic precursor, must involve protein degradation. For example, localized factors could target germline proteins for degradation in somatic precursors. Another possibility is that non-localized factors could degrade MEX-

5, but germline precursors could have a higher rate of MEX-5 translation than somatic precursors. MEX-5 and MEX-6 both contain CCCH domains that implicate them in RNA interactions. Therefore, a reasonable model is that MEX-5 and MEX-6 bind the mRNAs for *pie-1*, *mex-1* and *pos-1* and prevent their translation. However, recent studies by Reese and coworkers (personal communication) provide evidence against this model. Their studies show that PIE-1 localization does not depend on sequences within the *pie-1* mRNA.

Reese and coworkers examined PIE-1 localization in transgenic worms containing coding and non-coding *pie-1* sequences cloned into a GFP reporter construct. Reese and coworkers (personal communication) showed that two domains within the *pie-1* coding region appear to mediate PIE-1 asymmetry; a domain that localizes PIE-1 asymmetrically prior to cell division, and a domain that mediates the reduction of PIE-1 in the somatic precursors. They found that the CCCH domains were critical for both of these processes. The second PIE-1 CCCH domain and sequences 5' to it were necessary and sufficient for PIE-1 asymmetry prior to division. The first PIE-1 CCCH domain was necessary and sufficient for PIE-1 reduction in somatic precursors after division. Similarly, the first CCCH domain of either POS-1 or MEX-1 was also sufficient to cause a reduction in protein in somatic precursors. Cysteine to Serine mutations in each of the PIE-1 CCCH domains abolished or greatly reduced the localization properties of each of the domains.

My immunolocalization analysis with PIE-1 showed that PIE-1 associated transiently with the centrosomes, consistent with the involvement of centrosome-mediated degradation in PIE-1 localization. Reese and coworkers (personal communication) identified a 21 amino acid domain that was sufficient and necessary for PIE-1 centrosome association. Surprisingly, PIE-1 constructs lacking this region still segregated normally to germline precursors. However, reduced PIE-1 accumulation in nuclei was observed. I believe this result is consistent with two possibilities. The first possibility is that the centrosome association contributes to PIE-1 asymmetry and is required for proper levels of PIE-1 in germline precursors. Centrosome association, for example, could stabilize PIE-1 in posterior regions of the germline precursors. The second possibility is that centrosome association facilitates PIE-1 uptake by the nucleus after division.

The experiments of Reese and coworkers do not address whether the CCCH domain-dependent asymmetry is mediated by degradation or transport. In support of a role for actin-based transport, the application of microfilament inhibitors delocalizes PIE-1 in the 2-cell stage embryo (Reese, personal communication). However, this PIE-1 delocalization may be a secondary consequence of delocalized PAR and/or MEX-5 protein; the PAR proteins are likely anchored to the cortex, which shows an accumulation of actin, and application of microfilament inhibitors on PAR localization has yet to be tested. A

more likely model, favored by Reese et al., is that PIE-1 is degraded during division of the germline blastomere. This model is supported by two observations. First, in P1 and later germline precursors, PIE-1 levels appear to decrease on the somatic side of the blastomere before they increase on the germline side (C. Schubert, unpublished observation, and Reese, personal communication). Second, in 1-cell stage embryos, the generation of PIE-1 asymmetry is accompanied by a total increase in PIE-1 protein (C. Schubert, unpublished observation observation, and Reese, personal communication), consistent with a mechanism involving uniform PIE-1 translation coupled with localized degradation.

The finding that the CCCH domains are involved in germline protein asymmetry raises the possibility that MEX-5/MEX-6 interact with the germline proteins via the CCCH domain. MEX-5/MEX-6 do not have any domains that implicate them in protein degradation or transport, but MEX-5/MEX-6 could function in a complex with germline proteins to bring them into proximity with proteins that regulate stability or movement. It also remains possible that MEX-5/MEX-6 affect germline protein localization indirectly, by regulating the expression of unknown proteins that control germline protein asymmetry. The possibility of a direct interaction of MEX-5 and MEX-6 with germline proteins could be tested in future experiments using in vitro binding assays and immunoprecipitation.

MEX-5/MEX-6 and somatic blastomere fates

My experiments demonstrate that MEX-5/MEX-6 function to negatively regulate the expression of germline proteins. However, several classes of proteins that are known to specify cell fate are mislocalized in *mex-5;mex-6* mutant embryos. Perhaps, soma/germline asymmetries in protein expression may be a mechanism that underlies the localization of all cell fate regulators. Asymmetric soma/germline expression could be coupled to the activity of non-localized factors to regulate timing of protein expression. For example, SKN-1 is initially expressed asymmetrically between a somatic and germline precursor (AB and P1). SKN-1 asymmetry is dependent on *mex-5;mex-6* but the timing of onset and disappearance of SKN-1 protein expression is independent of *mex-5;mex-6*. Experiments on GLP-1 provide some insight into how specific patterns in protein expression could arise through a combination of spatial and temporal controls. Experiments using a *glp-1* reporter construct have shown that *glp-1* translation is controlled by two distinct elements in the *glp-1* 3'UTR. One element is required to prevent precocious *glp-1* translation prior to the 2-cell stage. A second element is required to prevent *glp-1* translation in the germline precursor P1 and its descendants. (Evans et al., 1994). In the future it will be interesting to learn how MEX-5/MEX-6 are linked to factors that control the timing of the expression of GLP-1, SKN-1 and other proteins.

MEX-5/MEX-6 and spindle orientation

In wild-type embryos the P1 blastomere divides along the anterior-posterior axis and the AB blastomere divides along the transverse axis. The orientation of these spindles requires MEX-5/MEX-6 and each of the PAR proteins (see Chapter 2). Thus, it is possible that MEX-5/MEX-6 link the PAR proteins to spindle orientation. I have demonstrated that defects in PAR-2 localization occur as early as the 2-cell stage in *mex-5;mex-6* embryos. Therefore spindle orientation defects observed in 2-cell stage *mex-5;mex-6* mutant embryos could be a consequence of the delocalized expression of proteins required for spindle orientation, including the PAR proteins themselves. An analysis of the *par-2* mutant could test this latter possibility. In *par-2* mutant embryos the P1 spindle divides along the transverse axis (Kemphues et al., 1988); this has been show to be due to ectopic *par-3* activity; in *par-2;par-3* mutant embryos the P1 blastomere divides along the a-p axis (Kemphues et al., 1988). If MEX-5/MEX-6 link PAR-3 to spindle orientation then *mex-5;mex-6;par-2* mutant embryos should not show transverse spindle orientation, rather, spindle orientation should appear randomized, as in *mex-5;mex-6* mutant embryos.

Cytoplasmic and Centrosomal Asymmetries in MEX-5 localization

MEX-5 expression in the germline precursors is highly dynamic and localization occurs rapidly. MEX-5 antibodies stain the posterior centrosome

brightly at metaphase, while by late anaphase no centrosome staining is observed. Cytoplasmic asymmetry develops in the P1 blastomere during only part of the 19 minute cell cycle. Due to the rapidity of the changes in MEX-5 localization, it seems unlikely that an underlying asymmetry in MEX-5 mRNA is directing protein asymmetry in germline blastomeres. This possibility cannot be excluded, as the expression pattern of *mex-5* mRNA is not known. However, mechanisms consistent with a rapid localization of MEX-5 include localized translation, protein stability, or transport.

It is possible that centrosomal and cytoplasmic asymmetry are coupled, and that this coupling could involve directed transport of MEX-5. Accumulation of MEX-5 on the centrosomes correlates with the absence of MEX-5 in neighboring cytoplasm. This correlation could reflect a role for the centrosomes as a "sink" to which cytoplasmic MEX-5 is transported. However, cytoplasmic asymmetry appears to precede centrosome association; in some prophase germline precursors, MEX-5 is detected in the anterior cytoplasm in the absence of centrosome association. Thus, some MEX-5 localization must occur independent of the centrosome.

MEX-5 and the PAR proteins

Below I consider how the PAR proteins might transmit positional information to direct the asymmetric distribution of MEX-5.

How might the anterior PAR proteins, PAR-3/PAR-6/PKC-3 promote MEX-5 accumulation in the anterior regions of the germline precursor? One intriguing observation is that in some embryos, MEX-5 staining appears particularly bright at the anterior cortex, in regions that show high levels of PAR expression (C. Schubert, unpublished observation,). MEX-5 could be translated or stabilized at the anterior cortex by proteins anchored or activated in this region by a PAR-3/PKC-3/PAR-6 containing complex; diffusion of MEX-5 from the anterior cortex could contribute to a gradient in MEX-5 asymmetry in the germline blastomere. Alternatively, transport to this cortex could result in a similar graded distribution of MEX-5. If the anterior PAR proteins promote MEX-5 translation or stabilize MEX-5 protein, then their removal might be expected to result in reduced levels of MEX-5. I did not obtain this result when I removed the function of the anterior PAR protein PAR-3; *par-3* mutant embryos showed uniform, high levels of MEX-5 expression. A drawback to this experiment is that I did not remove the activities of the anterior PAR proteins PAR-6 and PKC-3. In *par-3* mutant embryos PAR-6 and PKC-3 are uniformly expressed in the cytoplasm. Thus one or both of these proteins could function to stabilize MEX-5 in *par-3* mutant embryos, resulting in uniform high levels of MEX-5 expression. It would be interesting to ask if the simultaneous removal of the all known anterior PAR proteins resulted in embryos with low levels of MEX-5 expression.

How might the posterior PAR proteins, PAR-1 and PAR-2, downregulate the accumulation, or expression of MEX-5? These proteins could function to prevent MEX-5 translation, destabilize MEX-5 protein, or promote MEX-5 transport to the posterior. The extremely rapid disappearance of MEX-5 from the posterior centrosome at anaphase is suggestive of rapid protein degradation. Also suggestive is the existence of a ring-finger motif in PAR-2 (Boyd et al., 1996), shown in other proteins to be involved in adding ubiquitin to target proteins (reviewed in Freemont, 2000). Intriguingly, PAR-2 and PAR-1 affinity purified antisera recognize the centrosome at anaphase (C. Schubert, S. Guedes (PAR-1), C. Schubert, G. Hermann (PAR-2), unpublished observations). It is possible that this centrosomal staining represents non-specific staining, which could be tested by staining *par-1* and *par-2* mutant embryos. The centrosome-associated staining is not bright compared to the cortical staining but provides suggestive evidence in favor of a role for *par-1* and *par-2* in degrading centrosome-associated MEX-5.

The correlated staining patterns of the cortical PAR proteins and cytoplasmic MEX-5 proteins suggest that cortical asymmetry in the PAR proteins is necessary to define asymmetry; for example, the extent of posterior PAR protein expression could define a region of the cytoplasm that has MEX-5 degradation activity. However, for at least one posterior PAR protein, PAR-1, cortical asymmetry appears to be dispensable for function. *par-1* is normally

required for PIE-1 asymmetry; in *par-1* mutant embryos PIE-1 is expressed uniformly (Tenenhaus et al., 1999; this thesis). However, in *par-2* mutant embryos PAR-1 is found uniformly in the cytoplasm (Figure 16) (Boyd et al., 1996), but PIE-1 shows some asymmetric distribution (Tenenhaus et al., 1999). Thus, in *par-2* mutant embryos, cortical localization of PAR-1 is not required for PIE-1 asymmetry.

I suggest that the anterior PAR proteins provide a positional cue to positively regulate MEX-5 accumulation in the anterior, and that they can do so by inhibiting PAR-1 activity (see Figure 17 for model). Epistasis analysis and immunolocalization studies with the *par* genes could test this model and establish a regulatory hierarchy for PAR and MEX-5 asymmetry. As an example, if removal of all three proteins in the PAR-3/PAR-6/PKC-3 complex results in low levels of MEX-5, then simultaneous removal of PAR-3/PAR-6/PKC-3 and PAR-1 could restore high levels of MEX-5. In *par-2* mutant embryos, anterior PAR proteins show partial extension of cortical staining into the posterior cortex (Boyd et al., 1996) (Figure 16), and PIE-1 is likewise partially delocalized (Tenenhaus et. al., 1998). Co-staining *par-2* mutant embryos with antibodies to MEX-5 and an anterior PAR protein might reveal that the extent of ectopic PAR staining correlates with the extent of ectopic MEX-5 staining. If so, this result would be consistent with a function for the anterior PAR proteins in defining the extent of MEX-5 cytoplasmic expression even in the presence of uniform, cytoplasmic

PAR-1. These several experiments could result in a defined genetic pathway for the establishment of MEX-5 asymmetry.

These experiments, however, would not address possible molecular mechanisms for PAR function; regulation of MEX-5 translation, stability, or transport. One approach that has been particularly successful in the analysis of the PIE-1 protein is the analysis of GFP reporter constructs. Such an analysis with MEX-5 could distinguish among some of the possibilities for the regulation of MEX-5 asymmetry, in particular between translational control and protein-based mechanisms. Analysis of *gfp:mex-5* reporter constructs could be augmented with other methods which would address whether MEX-5 is modified by the PAR proteins. In vitro kinase assays have been developed for PKC-3 (Tabuse et al., 1998); these could be used to assay MEX-5 for phosphorylation. Although in vitro phosphorylation is not strong evidence of in vivo function, functional requirements for phosphorylated residues in MEX-5:GFP reporter constructs would be suggestive. Western blots testing MEX-5 for phosphorylation or ubiquitination in wild-type and *par* mutant embryos would not provide evidence for direct modification by PAR proteins, but could likewise contribute to the existing body of correlative evidence in favor of a function for the PAR proteins in directly regulating MEX-5 asymmetry.

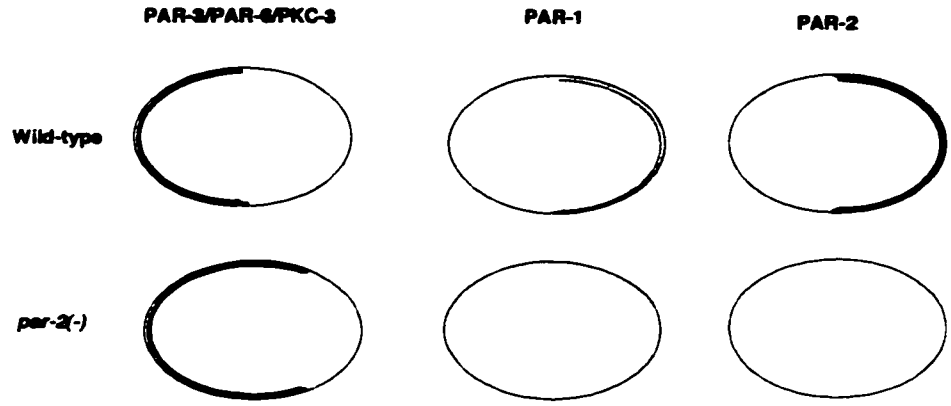

Figure 16: Localization of PAR proteins in *par-2(-)* embryos.

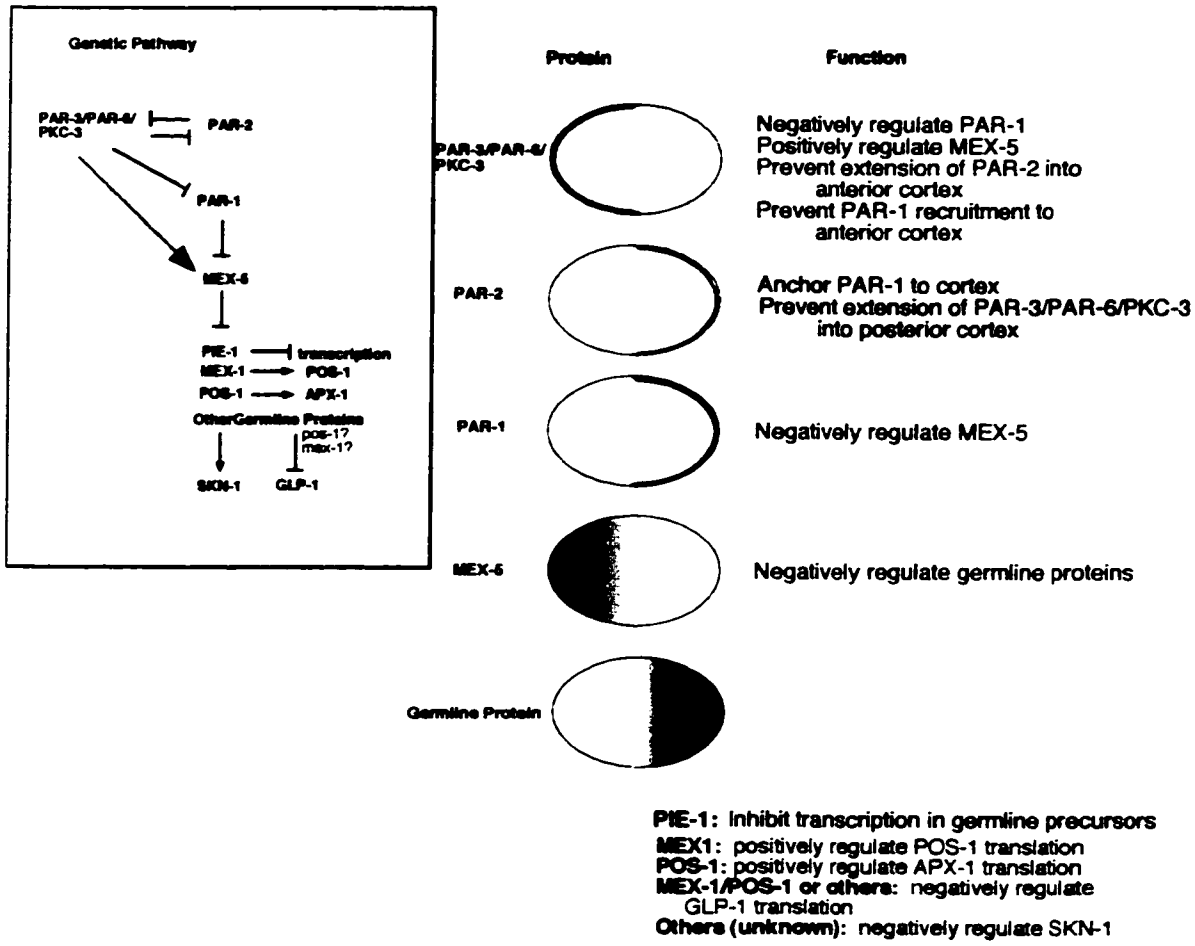

Figure 17: A model for protein asymmetry in *C. elegans*

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Appendix A: The PIE-1 Protein and Germline Specification

The PIE-1 protein and germline specification in *C. elegans* embryos

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TOTIPOTENT germline blastomeres in *Caenorhabditis elegans* contain, but do not respond to, factors that promote somatic differentiation in other embryonic cells[1,2]. Mutations in the maternal gene *pie-1* result in the germline blastomeres adopting somatic cell fates[3]. Here we show that *pie-1* encodes a nuclear protein, PIE-1, that is localized to the germline blastomeres throughout early development. During division of each germline blastomere, PIE-1 initially associates with both centrosomes of the mitotic spindle. However, PIE-1 rapidly disappears from the centrosome destined for the somatic daughter, and persists in the centrosome of the daughter that becomes the next germline blastomere. The PIE-1 protein contains potential zinc-finger motifs also found in the mammalian growth-factor response protein TIS-11/NUP475 (refs 4–7). The localization and genetic properties of *pie-1* provide an example of a repressor-based mechanism for preserving pluripotency within a stem cell lineage.

In the development of all animal embryos, certain cells must remain totipotent to form the reproductive cells, or germ cells, for the next generation. The germ cells of the nematode *C. elegans* arise from a sequence of unequal divisions during early embryogenesis[4] (Fig. 1). After each of these divisions, one daughter will produce only somatic cell types, such as muscle cells or neurons; this daughter can be described as a somatic blastomere. The other daughter will produce germ cells in addition to somatic cells, and thus can be described as a germline blastomere.

Studies on cell-fate determination in *C. elegans* embryos have shown that some maternally expressed factors that function in somatic development also are present in the germline blastomeres[1,2]. For example, development of the somatic blastomere in a four-cell embryo, EMS, but not the sister germline blastomere P_2 requires the maternally expressed transcription factors SKN-1 (refs 9, 10) and POP-1 (ref. 2), although these proteins are present in P_2 at the same levels as in EMS[1,2]. P_2 seems to be prevented from responding to these factors by *pie-1*(+) activity[2,3]. The *pie-1* gene is expressed maternally; in *pie-1* mutant embryos, the P_2 blastomere does not produce germ cells, and instead undergoes a pattern of somatic differentiation similar to a wild-type EMS blastomere[3]. In *pie-1; skn-1* double mutants, the germline blastomere in eight-cell stage embryo, P_4, does not produce germ cells and instead undergoes somatic differentiation similar to a wild-type somatic C blastomere[3]. These results suggest that *pie-1*(+) activity in wild-type development prevents P_2 and P_3 from responding to factors that determine the EMS and C fates, respectively.

In initial experiments to clone the *pie-1* gene, we found that the genetic position of *pie-1* coincided with a region of the physical map of the *C. elegans* genome for which there were no available genomic clones or sequences. We therefore screened for rare, spontaneous *pie-1* alleles in a strain with a high frequency of transposon mutagenesis. A single, transposon-induced allele, *pie-1(zu177::Tc1)*, was obtained from a screen of ~500,000 animals[11], and was used to clone the *pie-1* gene (Fig. 2). A full-length *pie-1* complementary DNA sequence was used to search for related products in the protein and nucleic acids databases (Fig. 2). The *pie-1* gene encodes a novel protein, but has two copies of a motif originally described in the TIS-11/NUP475 family of proteins[4–7] (Fig. 2). This motif consists of a pattern of three cysteine and one histidine residues (Fig. 2*b*) and has been proposed to form a zinc-binding domain, or finger[4]. This motif may have an ancient origin; similar sequences are found in many animal, plant and fungal proteins, and are present in the predicted products of at least 10 genes identified by the *C. elegans* genome sequencing project (C.C.M., unpublished observations). Examples include the mammalian protein U2AF35 (ref. 12) and the *Drosophila* proteins Suppressor of Sable[13] and Unkempt[14]. Although U2AF35 and Suppressor of Sable have been implicated in pre-messenger RNA splicing, a biochemical function has not been established for this motif.

The *pie-1* mRNA is expressed maternally and is detected in gonads, oocytes and in all blastomeres until the four-cell stage of embryogenesis; during later stages the *pie-1* mRNA is degraded in somatic blastomeres but retained in the germline blastomeres (G. Seydoux, personal communication). This distribution has been reported previously for several other unrelated mRNAs in the early *C. elegans* embryo, and may represent the general pattern of degradation for non-localized maternal mRNAs[15].

To examine the distribution of the PIE-1 protein we raised antibodies against three different PIE-1-specific peptides (Fig. 3). Sera against all three peptides reveal a similar embryonic and mitotic distribution of PIE-1 protein. PIE-1 protein first becomes detectable at low levels in the posterior cytoplasm of one-cell stage embryos (data not shown). In two-cell stage embryos, PIE-1 is

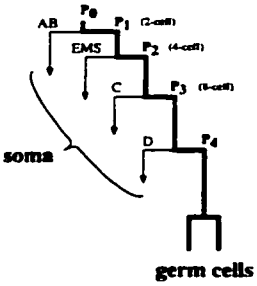

FIG. 1 The origin of germ cells in *C. elegans*, beginning with the fertilized egg (P_0). Each of the early divisions, represented by horizontal bars, produces a somatic blastomere (AB, EMS, C or D) and a germline blastomere (P_1, P_2, P_3 or P_4). Subsequent divisions of the somatic blastomeres are not shown.

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present in the nucleus and cytoplasm of the germline blastomere P_1, but at low or non-detectable levels in the somatic blastomere AB (Fig. 3*a*). In four-cell stage embryos, PIE-1 is present in P_2, but at low or non-detectable levels in its somatic sister EMS (Fig. 3*b*). As described previously[1], the SKN-1 protein is present in P_1, but when P_1 divides SKN-1 is present at equal levels in both of the P_1 daughters, EMS and P_2. Thus the asymmetrical localization of PIE-1 to P_2 nucleus provides a simple explanation of the observation that *pie-1*(+1) activity seems to prevent the activity of the SKN-1 transcription factor in P_2, but not in EMS[1]. PIE-1 protein remains localized to the germline blastomeres at each of the subsequent cleavages: In 8-cell and 16-cell stage embryos PIE-1 is detected only in P_3 and P_4, respectively (Fig. 3*c*, *d*). When P_4 divides, both of its daughters are germ-cell precursors, and PIE-1 is detected in the nuclei of both daughters (Fig. 3*e*).

PIE-1 appears to be associated with punctate structures in the cytoplasm of germline blastomeres (Fig. 3). Double-labelling experiments indicate that these structures are P granules[16], which are germline-associated particles of unknown function and composition (data not shown). P granules are present at all stages of the *C. elegans* life cycle in either mitotic germ cells (larvae), mature germ cells (oocytes in adults), or in germline blastomeres (in embryos)[16]. In contrast, PIE-1 is found in P granules only in germline blastomeres, beginning with the P_1 blastomere in the two-cell stage embryo.

Because the *pie-1* mRNA is present in all blastomeres in two-cell- and four-cell-stage embryos, translational or post-translational mechanisms must control the asymmetrical distribution of PIE-1 protein during these stages. For example, the association of PIE-1 with P granules during the early cell divisions could serve to localize PIE-1 to the germline blastomeres. A second possible contribution to PIE-1 asymmetry is suggested by the finding that PIE-1 antisera specifically stain the centrosomes of dividing germline blastomeres (Fig. 4). We have examined the intracellular distribution of PIE-1 as each germline blastomere divides, and find a common sequence of events. When germline blastomeres begin to divide, the nascent mitotic spindle complex rotates by roughly 90 (ref. 17). Before rotation, PIE-1 accumulates at apparently equal levels around each centrosome of the spindle, and PIE-1 staining diminishes in the nucleus, cytoplasm and P

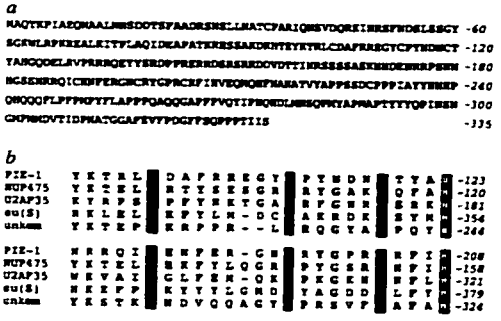

FIG. 2 The *pie-1* locus. *a*, Deduced amino-acid sequence from the nucleotide sequence of a 1,105-bp *pie-1* cDNA clone. Nucleotides 529 to 746 are coding sequences deleted in *pie-1(zu127)* (data not shown). *b*, Alignment of the *pie-1* zinc-finger motifs with sequences from nup475 (ref. 4), U2AF35 (ref. 12) and unkempt[14].

METHODS. The spontaneous mutation *pie-1(zu177::Tc1)* was obtained in a previously described genetic screen[13]. The mutant strain was outcrossed several times and placed over a chromosome marked with *nob-1(ct230)* and *unc-25(e156)* which flank *pie-1*. Recombinant animals yielded a map order of *nob-1*–32–*pie-1*–37–*unc-25*, and in all recombinants a single novel Tc1 transposon co-segregated with the *pie-1* mutation. The *C. elegans* genomic DNA flanking this transposon was isolated as described[24]. Probes were prepared and used to isolate four full-length cDNA clones; each cDNA contained the trans-spliced leader sequence SL1 (ref. 25) at its 5' end and a poly(A) tail of varying length at its 3' end. Three lines of evidence indicate that the open reading frame encodes the PIE-1 protein: The *pie-1(zu177::Tc1)* and *pie-1(zu127)* mutations are a transposon insertion and a deletion, respectively, in the genomic DNA corresponding to this open reading frame; *in vitro* transcribed RNA from these sequences, prepared as described[2,26], can induce a *pie-1* phenocopy when microinjected into wild-type animals; and antibodies raised against peptides deduced from this sequence recognize a protein that is present in wild-type embryos but absent in *pie-1* mutants (Fig. 3 and data not shown).

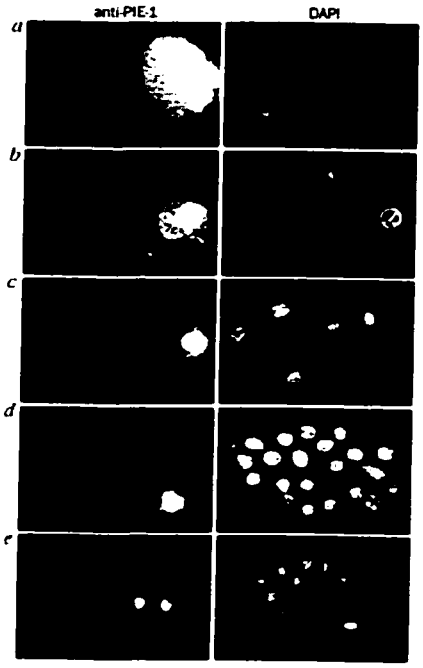

FIG. 3 PIE-1 localization in germline blastomeres. Each row represents a single early embryo stained with an antibody against the PIE-1 protein (left column) or with the dye DAPI to visualize nuclei (right column). *a*, Late two-cell stage embryo showing PIE-1 in the nucleus and cytoplasm of the P_1 blastomere. Faint, punctate staining in the cytoplasm of P_1, and all other germline blastomeres shown corresponds to P granules. *b*, An early four-cell stage embryo with PIE-1 in the nucleus and cytoplasm of the P_2 blastomere (short arrow). Faint PIE-1 staining is detected in the P_2 sister, EMS (long arrow). *c*, 12-cell stage embryo showing PIE-1 in the P_3 blastomere. *d*, 46-cell stage showing PIE-1 in the P_4 blastomere. *e*, 88-cell stage showing PIE-1 in the two P_4 daughters. The P_4 daughters do not divide again during embryogenesis, and in postembryonic development produce only germ cells (sperm and oocytes). PIE-1 is not detected in embryos after roughly 200 min of development. Embryos are 45 μm in length.

METHODS. The PIE-1 protein sequence shown in Fig. 2*a* was used to generate three peptides: A (residues 54–73), B (residues 81–105) and C (residues 134–157). Rabbit polyclonal antisera were generated against the individual peptides. Embryos shown were fixed and stained with antisera generated against peptide C, and with DAPI to visualize nuclei, and examined under the fluorescence microscope[7]. Similar staining results were obtained with each of the peptide antisera. PIE-1 staining with the antiserum against peptide C is not detectable in the nucleus, cytoplasm or P granules of embryos from mothers homozygous for the *pie-1* (zu127) mutation.

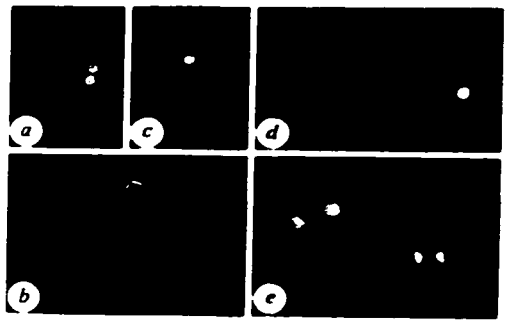

FIG. 4 Centrosomal localization of PIE-1 during division of the germline blastomere P_3. All images except e are of embryos stained with an affinity-purified antibody against PIE-1 peptide A and viewed with either a standard fluorescence microscope (*a*, *c*, *d*, *e*) or an optical sectioning microscope (*b*). *a*, Two-cell stage embryo (only the P_2 blastomere is shown). P_2 is at prophase; PIE-1 is not detected in the nucleus (not visible) but is detected prominently in the two centrosomes, which are shown here just before rotation of the centrosome–spindle complex (compare with the nuclear staining of the interphase P_2 blastomere in Fig. 3a). *b*, High-magnification image of PIE-1 staining at the P_2 centrosomes. *c*, The P_2 blastomere after rotation of the centrosome–spindle complex. The anterior (left) centrosome stains less intensely than the posterior (right) centrosome. *d*, Division of the AB and P_2 blastomeres (compare with DAPI image shown in *e*). The mitotic spindles of AB and P_2 have elongated; both centrosomes associated with the P_2 spindle have detectable PIE-1 staining (arrows), although the posterior centrosome (destined for the germline blastomere P_3) shows a much higher level of staining than the anterior centrosome (destined for the somatic blastomere EMS). Neither of the centrosomes associated with the dividing AB blastomere have detectable PIE-1 staining. *e*, DAPI image of embryo shown in *d*. The two nascent daughter nuclei of the dividing AB blastomere are visible on the left, and the two nascent daughter nuclei of the dividing P_2 blastomere are visible on the right.
METHODS. Identification of the PIE-1-containing structures shown here as centrosomes is based on co-staining experiments with an antibody against beta-tubulin[17]; in such experiments the PIE-1-containing structures are localized at the centre of each spindle aster (data not shown). Embryos were prepared and stained as described in Fig. 3. Image in *b* was collected on a Deltavision SA3.1 wide-field deconvolution optical sectioning microscope (Applied Precision) The image was deconvolved using the reiterative constrained method[27].

granules (Fig. 4*a*, *b*). After spindle rotation, the level of PIE-1 staining in one of the two centrosomes diminishes rapidly and becomes undetectable (Fig. 4*c*, *d*). Thus at the completion of cell division, only one daughter cell contains PIE-1 at its centrosome, and that daughter invariably is the new germline blastomere (P_1, P_2, P_3 or P_4). When the P_4 blastomere divides, PIE-1 staining persists in both centrosomes (data not shown).

Although we do not know the behaviour of individual PIE-1 molecules during the cell cycle, one model is that the PIE-1 protein translocates to centrosomes at cell division. Other proteins, such as NuMA (ref. 18) and CP190 (ref. 19), have been described previously that seem to cycle between the nucleus and centrosomes. We are not aware of proteins other than PIE-1 that disappear from one of the two centrosomes, and thus become distributed asymmetrically after cell division.

What might distinguish the two centrosomes in a dividing germline blastomere? In a study on the control of spindle orientation in *C. elegans*, the relative positions of the two centrosomes of a germline blastomere were interchanged before rotation of the mitotic spindle was complete[20]. These manipulated embryos developed normally, suggesting that both centrosomes initially are equivalent. Thus we propose that rotation of the mitotic into different intracellular environments that affect PIE-1 stability or binding. The idea that asymmetry exists within each germline blastomere is supported by the recent finding that P granules also seem to be degraded or unstable in the pre-somatic 'half' of a germline blastomere before division[21].

The early divisions of the *C. elegans* embryo represent a classic stem-cell-like lineage in which one pluripotent cell gives rise sequentially to daughters with distinct, and more restricted, developmental potentials. In *C. elegans*, the fate of the first somatic blastomere, AB, is determined in part by responses to external cell signals[11,22]. As described above, the fate of the second somatic blastomere, EMS, is determined at least in part by two transcription factors that are present in both P_2 and its sister cell EMS[1,2]. It is likely that differentiation in stem cell lineages in other animals is also determined by several different intercellular or intracellular signals, and that the pluripotent cells must either not contain, or not respond to, these signals.

The PIE-1 protein in *C. elegans* provides an example of how the pluripotent cell in a stem cell lineage might be protected from the signals that promote differentiation in other cells. We have shown here that the PIE-1 protein is localized to the totipotent germline blastomere after each division in the early embryo. This localization correlates with the repression of somatic cell fates[3], and also seems to correlate with a general repression of transcription within the germline[23]. Thus PIE-1 may represent a localized general repressor that serves to antagonize the activity of a more broadly expressed set of transcriptional activators. This may provide an efficient means for generating diversity within a stem cell lineage: if cells in a stem cell lineage express determinative molecules at different times or in response to different signals, there need not be separate mechanisms for segregating these molecules away from the pluripotent stem cell. Instead, a single mechanism for localizing a general repressor at each division could maintain the pluripotency of the stem cell. □

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Curriculum Vitae

EDUCATION

University of Washington Department of Zoology Seattle, Washington	Ph.D.	1/93 - present
Pomona College Claremont, California	B.A. Biology	1987
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RESEARCH EXPERIENCE

1/93 - present Ph.D. thesis Research	Department of Zoology, University of Washington, Fred Hutchinson Cancer Research Center, and Howard Hughes Research Institute, Seattle, Washington Laboratory of James Priess
3/92 - 11/92 Research Technician	Fred Hutchinson Cancer Research Center and Howard Hughes Research Institute, Seattle, Washington Laboratory of James Priess
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HONORS AND AWARDS

3/96	Best Graduate Student Platform Talk, Northwest Regional Developmental Biology Conference, Friday Harbor, WA
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PUBLICATIONS

Craig. C. Mello, **Charlotte Schubert**, Bruce Draper, Wei Zhang, Robert Lobel and James R. Priess, The PIE-1 protein and germline specification in *C. elegans* embryos *Nature*, 382, pp. 710 – 712 (1996)

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