MEX-5 and MEX-6 Function to Establish Soma/Germline Asymmetry in Early C. elegans Embryos

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Summary

An asymmetrical network of cortically localized PAR proteins forms shortly after fertilization of the C. elegans egg. This network is required for subsequent asymmetries in the expression patterns of several proteins that are encoded by nonlocalized, maternally expressed mRNAs. We provide evidence that two nearly identical genes, mex-5 and mex-6, link PAR asymmetry to those subsequent protein asymmetries. MEX-5 is a novel, cytoplasmic protein that is localized through PAR activities to the anterior pole of the 1-cell stage embryo. MEX-5 localization is reciprocal to that of a group of posterior-localized proteins called germline proteins. Ectopic expression of MEX-5 is sufficient to inhibit the expression of germline proteins, suggesting that MEX-5 functions to inhibit anterior expression of the germline proteins.

Introduction

Embryonic blastomeres become committed to distinct fates within the first few cell cycles after fertilization of the C. elegans 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 review, see Bowerman and Shelton, 1999).

Little is known about the molecular differences between blastomeres that result in asymmetric protein expression from 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 posterior cortex of the embryo (Guo and Kemphues, 1995; Boyd et al., 1996) and PAR-3, PKC-3, and PAR-6 localize to the anterior cortex (Etemad-Moghadam et al., 1995; Tabuse et al., 1998; Hung and Kemphues, 1999). We refer here to these proteins collectively as PAR proteins. The PAR proteins are 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 and Kemphues, 1999). Mutations in the par genes also cause marked defects in many, or all, of the subsequent asymmetries observed between wild-type blastomeres after cell division. These include differences in blastomere size, 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; J oazeiro et al., 1999; Lorick et al., 1999; Seol et al., 1999; Waterman et al., 1999). However, specific biochemical functions of most PAR proteins have not been determined, nor have any 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 a PAR-dependent distribution. There are three general PAR-dependent patterns of protein distribution that have been described in the early blastomeres (see Figure 1 for diagram). After the first division, anterior proteins are present only in the anterior blastomere; these proteins persist, or continue to be expressed, in all the descendants of the anterior blastomere for 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.,
embryonic lethal mutants, only par-1 mutants were found to completely abolish PIE-1 asymmetry (Tenenhaus et al., 1998). Although there are several possible explanations for these results, they are consistent with the view that there may be only a few factors that link PAR asymmetry to protein localization. In the present study, we provide evidence that two closely related proteins, MEX-5 and MEX-6, are part of this linkage.

Results

The posterior protein SKN-1 is a transcription factor required for the development of muscles in the pharynx, among other cell types. 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 (see Bowerman and Shelton, 1999). 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 novel 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 2C). The mex-5 embryos contain abnormally large numbers of muscles toward their anterior poles (Figure 2D; compare with wild-type embryo in Figure 2B). In cell isolation experiments on 2-cell stage mex-5 embryos, we found that the anterior blastomere produced muscles inappropriately (14/14 cases). 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 2H; compare with wild-type in Figure 2G). 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 (data not shown). In addition, germ cells were visible in almost all mex-5 embryos (15/17 embryos; Figure 2C, white arrows), while par embryos and mex-1 embryos do not produce germ cells (see Bowerman and Shelton, 1999). Thus, the mex-5 gene appears to be a novel component of the pathways that establish asymmetrical patterns of protein expression in the early embryo.

MEX-5 Shares a CCCH Motif with Germline Proteins

We cloned the mex-5 gene as described in Experimental Procedures. In brief, mex-5(zu199) maps to a region on chromosome V near the rescuing cosmid W02A2; RNA-mediated inhibition of the predicted gene W02A2.7 causes wild-type adults to produce inviable embryos that are indistinguishable from mex-5 embryos; the W02A2.7 gene in mex-5(zu199) animals contains a nonsense mutation that would truncate the predicted protein product (Figure 3A); and a monoclonal antibody generated against a W02A2.7 fusion protein stains wild-type embryos (see below), but not mex-5(zu199) embryos. We henceforth name W02A2.7 the gene mex-5,

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**Figure 1. 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 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.

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1998). Therefore, protein asymmetry must result from protein movement, or from differences 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). These proteins persist at high levels in the germline daughter during the cell cycle but rapidly disappear from the somatic daughter (Mello et al., 1996; 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., 1994). 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 suggest that posterior blastomeres contain a trans-acting inhibitor of translation. Thus, it is possible that PAR proteins could modify factors involved in either translation or protein degradation.

A previous genetic screen to identify regulators of GLP-1 localization yielded only alleles of known par genes, and a candidate for a new par gene (Crittenden et al., 1997). Similarly, in a survey of previously identified
The C. elegans TTP finger domains may mediate binding to RNA, mere P2 and its somatic sister (Figure 4A, arrowheads), indicating interactions that involve interactions with RNA, including the In wild-type embryos, the germline protein PIE-1 re-expressed. All of the mex-5; mex-6 that are similar to a CCCH “finger” motif that was first requires SKN-1(+) activity specifically in the germline blastomeres (Mello 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 4A, arrowheads), while PIE-1 is present at high levels only in P2 (Figure 4C). We found that mex-5; mex-6 embryos contained 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 protein TTP/Nup457/Tis-11 (Varmus et al., 1989; Dubois et al., 1990; Lai et al., 1990). The TTP finger motif contains Cys and His residues with CX(8)CX(5)CX(3)H spacing, and additional conserved amino acids; the finger domains of MEX-5 have slightly different CX(9)CX(5)CX(3)H and CX(10)CX(5)CX(3)H spacings (Figure 3B). 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 kDa subunit of cleavage and polyadenylation specificity factor (CPSF) (BARABINO et al., 1997). The finger domains of TTP may mediate binding to RNA (see Discussion; 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 3B).

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 sequence, 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 3A). 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. 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% embryonic 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 in the mex-6 gene by a PCR-based screening method (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; this deletion extends past mex-6 into a neighboring pseudogene (Figure 3A). 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 nonessential 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 present in mex-5 embryos (Figures 2E and 2F). We found that mex-5(zu199);mex-6(RNAi) embryos and mex-5(zu199);mex-6(pk440) embryos had this same, novel phenotype (Figure 2, legend). The two germ cells are indicated by white arrows. (E and F) mex-5(RNAi);mex-6(RNAi) embryos prepared as for (A) and (B). These embryos lack germ cells and pharyngeal muscles (0/24). In a similar analysis, pharyngeal muscles were not detected in any mex-5(mex-6(RNAi) embryos (0/25), or in most mex-5; mex-6 double mutants (1/78).

Figure 2. Comparison of Wild-Type and mex-5/mex-6 Embryos (A and B) Wild-type embryos viewed (A) by light microscopy and (B) after immunostaining for pharyngeal muscles. Arrows point to the two germ cells. (C and D) mex-5 mutant embryos prepared as for (A) and (B). The prominent basement membrane surrounding the pharynx is indicated (black arrow). The two germ cells are indicated by white arrows. (E and F) mex-5(RNAi);mex-6(RNAi) embryos prepared as for (A) and (B). These embryos lack germ cells and pharyngeal muscles (0/24). In a similar analysis, pharyngeal muscles were not detected in any mex-5(mex-6(RNAi) embryos (0/25), or in most mex-5; mex-6 double mutants (1/78).

(G) Two-cell wild-type embryo showing SKN-1 in the posterior blastomere. (H) Two-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 μm in length.
ectopic PIE-1: between the 1-cell stage and the 44-cell stages, PIE-1 was distributed uniformly in all blastomeres (Figure 4D). 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, PIE-1 is mislocalized in mex-5; mex-6 mutants and appears to repress the activity of SKN-1.

We immunostained mex-5; mex-6 embryos to examine other proteins that, like SKN-1 and PIE-1, are asymmetrically localized in wild-type embryos (Figure 4). We found that the germ line proteins MEX-1 and POS-1 were expressed inappropriately in all blastomeres in mex-5; mex-6 mutants (Figures 4F and 4H, 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 require SKN-1 function. For example, while mex-5(RNAi); mex-6(RNAi) embryos lack intestinal cells (0/24 were present in all blastomeres (data not shown), the pie-1(RNAi); mex-5(RNAi); mex-6(RNAi) embryos contained numerous intestinal cells (54/54 embryos). Thus, PIE-1 is mislocalized in mex-5; mex-6 mutants and appears to repress the activity of SKN-1.

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MEX-5, MEX-6, and Soma/Germline Asymmetry

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(pk440) 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 if mex-6(-) activity is removed.

MEX-5 and the Germline Proteins Have Reciprocal Localization Patterns

We generated a mouse monoclonal antibody (mAbCS1) against a full-length MEX-5 fusion protein. Wild-type and mex-6 embryos had identical patterns of mAbCS1 staining, but no staining was 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 detected high levels of MEX-5 in the proximal arm of the gonad and in maturing oocytes; oocytes and newly fertilized eggs have uniform levels of MEX-5 (data not shown). MEX-5 gradually becomes highly asymmetric during and after the 1-cell stage: MEX-5 is present at high levels at the anterior pole of late 1-cell embryos (Figures 5A and 6A), and after cell division MEX-5 remains high in the anterior blastomere (Figure 5B; white arrowhead). After the anterior blastomere divides (Figures 5D and 5E), its daughters inherit high levels of MEX-5 (Figure 5F, doubleheaded arrow). MEX-5 levels subsequently decrease in these daughters during the 4-cell stage (Figure 5G, doubleheaded arrow), and MEX-5 is not detectable in their descendants (Figure 5H).

MEX-5 shows a very different pattern of localization in the posterior of the embryo. During the 1-cell stage, the levels of MEX-5 decrease markedly at the posterior pole (Figures 5A and 6A). At cell division, the posterior daughter inherits low levels of MEX-5 (Figure 5B, black arrowhead); this blastomere is the germ blastomere P1 (see lineage diagram in Figure 1). Toward the end of the 2-cell stage, MEX-5 appears on both centrosomes of the nascent spindle in P1 (Figure 5C, arrows). As the spindle rotates onto the anterior-posterior (a-p) axis of the egg and P1 begins division, MEX-5 disappears from the anterior centrosome (Figure 5D); MEX-5 persists on the posterior centrosome for a brief period before also disappearing (Figure 5E). During these stages, MEX-5 appears to accumulate in the anterior half of P1 (Figures 5D and 5E, asterisks). After cell division, the anterior, somatic daughter has a high level of MEX-5, and the
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Figure 5. MEX-5 Localization in Wild-Type Embryos

Panels show sequential stages of wild-type embryos stained with the MEX-5 antibody mAbCS1, and costained with DAPI to visualize nuclei (blue). Embryos are oriented and blastomeres marked as in Figure 1.

(A) One-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) Two-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) Two-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) Three-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) Four-cell stage.

(G) Late 4-cell stage. Low levels of punctuate staining in the germline blastomere P2 (black arrowhead) are P granules (data not shown).

(H) Twenty-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).

new germline blastomere (P2) has a low level (Figure 5F; white and black arrowheads, respectively). MEX-5 shows a similar, though less marked, asymmetry during the subsequent germline divisions (Figure 5H 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; Tenenhaus et al., 1998; Tabara et al., 1999). 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 6A), there appears to be a synchronous increase in the levels of PIE-1 (Figure 6B) 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 6D) that appears to develop synchronously with a reciprocal, graded distribution of PIE-1 (Figure 6E) 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 6I) 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

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 7C and 7D, respectively). In 1-cell stage embryos, the transition between high and low levels of MEX-5 (Figure 5A, arrowhead) appeared to be coincident with the transition point between the anterior PAR proteins such as PAR-3 (Figure 6G, arrowhead) and PKC-3 (data not shown), and the posterior PAR protein PAR-2 (Figure 6H). In 2-cell stage embryos, where PAR asymmetry is present in the germline blastomere P1, we observed a similar correlation between the distributions of MEX-5 and the PAR proteins. 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 (data not shown).
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 7A and 7B; n = 5–23 embryos). PAR function is required for the normal, asymmetric positioning of the first cleavage furrow in wild-type embryos, 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. Although these results indicate that MEX-5/MEX-6 are not required at the 1-cell stage for either PAR asymmetry or for the PAR-dependent placement of the cleavage furrow, mex-5;mex-6 embryos show defects in PAR asymmetry and cell cleavage in the subsequent divisions of the germline blastomeres. For example, in some 2-cell stage mutant embryos 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).

**Ectopic MEX-5 Can Inhibit the Expression of Germline Proteins**

par-1 and par-3 mutant embryos have low, uniform levels of the germline proteins PIE-1 and MEX-1, and high levels of MEX-5 (Figures 7E and 7F; see also Tenenhaus et al., 1998). We therefore asked whether the low levels of germline protein resulted from mex-5(+) and mex-6(+) activities. par-1(b274);mex-5(RNAi);mex-6(RNAi) and par-3(it71);mex-5(RNAi);mex-6(RNAi) embryos were constructed and immunostained 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 7, legend). For example, while par-3(it71) mutant embryos had low levels of PIE-1 and MEX-1 (Figures 7E and 7F, respectively), the par-3(it71);mex-5(RNAi);mex-6(RNAi) embryos had much higher levels of both proteins (Figures 7G and 7H, respectively).

To test the possibility that high levels of MEX-5 might be sufficient to inhibit the expression of germline proteins, we constructed a transgene to induce ectopic MEX-5 expression from a heat shock promoter. We used mex-5(zu199);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
Table 1. Spindle Orientation in 2-Cell Embryos

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<tr>
<th>Spindle Axis</th>
<th>Wild Type (Ant) (%)</th>
<th>mex-5(−);mex-6(−) (Pos) (%)</th>
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The wild-type pattern is transverse in the anterior blastomere (T) and longitudinal (L) in the posterior blastomere.

embryos were fixed and immunostained for both MEX-5 and MEX-1. Control mex-5(zu199);mex-6(RNAi);pie-1(RNAi) embryos lacking the transgene showed no detectable MEX-5 after heat shock and had high levels of endogenous 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 7I, top embryo) and had high levels of endogenous MEX-1 (Figure 7J, 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 7I, bottom embryo); these embryos had relatively low levels of endogenous MEX-1 (Figure 7J, bottom embryo). The third class of embryos appeared to have mosaic expression of the MEX-5 transgene: blastomeres with high levels of MEX-5 expression showed low levels of MEX-1, while other blastomeres with low levels of MEX-5 had relatively high levels of MEX-1 (Figures 7K and 7L).

Discussion

Through mechanisms that are not yet understood, interactions between the PAR proteins cause 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 nonlocalized, 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

![Figure 7. PAR Proteins and Ectopic Expression of MEX-5](image-url)
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 whether 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 but do not have obvious defects in PAR asymmetry at the 1-cell stage. While mutations in any one of the par genes except par-4 alter the position of the first cleavage furrow (Rose and Kemphues, 1998), mex-5;mex-6 embryos have a normal, asymmetrically positioned furrow. These observations suggest that MEX-5/MEX-6 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, heat shock induction of MEX-5 or after the 2-cell stage can inhibit the expression of the MEX-1 protein in anterior blastomeres that 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 re-forms during each cell cycle of the asymetrically dividing germline blastomeres. 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 (Schiernberg, 1987). Nevertheless, these observations suggest that some factor(s) that regulates PAR asymmetry is restricted to the germline blastomeres 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 function to inhibit 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 embryos, translation of glp-1 mRNA appears to be repressed in posterior blastomeres (see Introduction). 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 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, it correlates remarkably with the absence of the germline proteins MEX-1, PIE-1, and POS-1: during the early cleavages, 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 maternally provided mRNAs (Seydoux and Fire, 1994); these mRNAs are present in the oocyte and persist at high levels in each successive germline blastomere. 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, mex-1 mRNA 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), and 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 the germline 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 expressing the 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 the gradient of MEX-5. We have further 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 were 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 MEX-5/MEX-6 function is required for those low levels. These several results suggest that 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? A mechanism for degrading germline proteins may exist 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 might also be 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 toward the posterior pole. In principle, the gradient could be generated by coupling degradation to nonlocalized translation, or by restricting the 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 predominantly 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 (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; Boweman 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 90° 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 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 (Schrober 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.

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) or unc-30(e191). In addition, the following strains and genetic reagents were used: J, dpy-5(e61); II, rol-1(e187); III, unc-32(e189),par-3(071); Qc1, skn-1(zu67), unc-5(e53), unc-26(e345), dpy-4(e1166), unc-24(e138), unc-22(e7), let-23(e1727), dpy-20(e1282), nDf27, sDf66, nt1; V, dpy-11(e224), par-1(b274), rol-4(e8); and X, lon-2(e678). C. elegans culture, mutagenesis, and genetics were as described in Brenner, 1974).

Isolation of mex-5(zu199) and Mutant Characterization

mex-5(zu199) was isolated in an F2 screen for maternal effect lethal mutants as described by Priess et al. (1987) and mapped near unc-30; map data is available from the C. elegans Genetic Center. All
self-progeny of homozygous mxe-5(zu199) hermaphrodites arrested during embryogenesis (n = 3412). All cross-progeny after male mat- ing were indistinguishable from self-progeny (n = 123). Development of individual early blastomeres was assayed after killing all other blastomeres with a laser microbeam (Bowerman et al., 1992; Mello et al., 1992). Size of the AB blastomere was determined with morpho-metric software in NIH Image on 12 wild-type and 13 mex-5/mex-6 embryos.

Molecular Analysis

For mex-5 rescue, the cosmid W02A2 and marker rol-6 DNA were coinjected into the syncytial gonad of adults following published procedures (Mello et al., 1991). The mex-5(zu199) and mex-6(pk440) alleles were sequenced using standard protocols from PCR-amplified genomic DNA. Mex-6(pk440) DNA is a deletion resulting sequences 12737-15477 within the cosmid AH6 (GenBank).

Predicted structure of MEX-5 from genomic sequence (W02A2.7; GenBank) agreed with sequence analysis of the mex-5 cDNA yk328d9. The predicted MEX-6 protein (AH6.5; GenBank) contains 30 N-terminal amino acids not present in the 5’ end sequences of several mex-6 cDNAs (yk339c12, yk4325a, yt562a8, yk520g6).

dsRNA Inhibition

Regions of the mex-5 and mex-6 genes corresponding, respectively, to amino acids 153-186 and 156-190 in Figure 3 were amplified by polymerase chain reaction (PCR) from phage yk45912 and yk3862, respectively; oligonucleotides contained sites for T3 or T7 polymerase. Pie-1 dsRNA was prepared from the plasmids pJ P660 and pJ P661 (Mello et al., 1996) after PCR amplification. Worms were treated with dsRNA either by injection (Powell-Coffman et al., 1996; Rocheleau et al., 1997) or by soaking (Tabara et al., 1999).

Immunolocalization

Antibodies/antisera against the following proteins were used as described: MEX-3 (Draper et al., 1996), POS-1 (Tabara et al., 1999), MEX-1 (Guedes and Priess, 1997), PIE-1 (Tenenhaus et al., 1998), PAL-1 (Hunter and Kenyon, 1996), SKN-1 (Tabara et al., 1993), PAR-1 (Guo and Kemphues, 1995), PAR-2 (Boyd et al., 1996), PAR-3 (Etemad-Moghadam et al., 1995), PKC-3 (Tabuse et al., 1998), P granules (Strome and Wood, 1982). Pharyngeal muscles were stained with mAb3NB12 (Mello et al., 1992), mAbC51 was generated following published procedures (Wagner and Carter, 1987) in the FHCRC Hybridoma Production Facility using bacterially expressed, full-length, His-tagged MEX-5. Fixation and staining was as in Lin et al. (1995).

PAR and germline protein staining was scored at the 1-cell stage embryos after the pronuclei had decondensed and begun migration. Figure 4 is based on analysis of between 16-40 wild-type embryos and 11-58 mex-5/mex-6 mutant embryos. For the experiments shown in Figures 4 and 7, wild-type and mutant em-}

Heat Shock Experiments

Full-length MEX-5 was cloned into heat shock vectors pPD49.78 and pPD49.83 (Seydoux et al., 1996) and coinjected with rol-6 DNA into unc-30(e199)mex-5(zu199)/nT1 hermaphrodites (Mello et al., 1991). Stage-selected, calibration embryos and gravid hermaphrodites containing embryos at multiple stages were subjected to one D. (1990). A growth factor-inducible nuclear protein with a novel sequence. J. Biol. Chem. Received January 19, 2000; revised March 21, 2000. 

References


