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Spatial and Temporal Controls Target *pal-1* Blastomere-Specification Activity to a Single Blastomere Lineage in C. elegans Embryos

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Summary

The early asymmetric cleavages of Caenorhabditis elegans embryos produce blastomeres with distinct developmental potentials. Here, we show that the *caudal*-like homeodomain protein PAL-1 is required to specify the somatic identity of one posterior blastomere in the 4 cell embryo. We find that *pal-1* activity is sequentially restricted to this blastomere. First, at the 4 cell stage, it is translated only in the two posterior blastomeres. Then, its function is restricted to one of these blastomeres. This second targeting step is dependent on the activities of the posteriorly localized SKN-1 and asymmetrically segregated PIE-1 proteins. We propose that the segregation of PIE-1, combined with the temporal decay of SKN-1, targets *pal-1* activity to this posterior lineage, thus coupling the regulation of this conserved posterior patterning gene to asymmetric cell cleavages.

Introduction

The patterns of early embryonic cell cleavages in different species are remarkably diverse, suggesting that the strategies and mechanisms that pattern early embryos have evolved substantially (Davidson, 1990). Studies in Drosophila have identified many genes that function to pattern early Drosophila embryos (for review see St Johnston and Nüsslein-Volhard, 1992). However, few genes important for establishing anteroposterior pattern in Drosophila have homologs in animals with cellular embryos, perhaps because their initial regulatory interactions are dependent on the syncytial nature of the Drosophila embryo (Boring et al., 1993). An interesting exception is the Drosophila gene *caudal* (*cad*); *caudal* homologs, like *cad*, are expressed in posterior regions of both syncytial and cellular early embryos.

Caenorhabditis elegans embryonic development is distinct from both flies and vertebrates, yet a C. elegans *caudal* homolog, *pal-1*, functions in posterior development (Waring and Kenyon, 1990, 1991). Partial loss-of-function *pal-1* alleles cause posterior-to-anterior homeotic transformations in adult males. The observation that a small fraction of these mutant animals hatch with severe posterior defects suggested a more general embryonic function for *pal-1* (Waring and Kenyon, 1990), which was confirmed by the isolation of *pal-1* null alleles (Yandell et al., 1994) that severely disrupt posterior embryonic development (L. G. Edgar and W. B. Wood, personal communication).

We have begun to investigate the function and regulation of *pal-1* in the early embryo. The C. elegans embryo differs from other embryos in which *caudal* function has been studied, in that it generates early blastomeres that have strikingly different developmental potentials (Sulston et al., 1983). For example, the first cleavage generates the anterior blastomere AB, which gives rise to much of the nervous system and anterior ectoderm. The posterior sister of AB, called P_1, divides to produce EMS and P_2. EMS produces primarily endoderm and mesoderm, whereas its posterior sister, P_2, produces mesoderm, posterior ectoderm, and the germline precursor. These blastomere-specific cell types are produced by distinct and invariant cell-division patterns or lineages (Sulston et al., 1983). The potential to execute these blastomere- specific lineages is dependent in some cases on cell–cell interactions and in others on factors that appear to segregate into specific blastomeres (reviewed by Wood and Edgar, 1994).

Genetic screens have identified two classes of early embryonic patterning genes that are required for the development of these blastomeres. Mutations in the *par* genes (for *par*titioning-defective) disrupt the polarity of the early embryo, resulting in symmetric rather than asymmetric early cleavages (Kemphues et al., 1988). Mutations in the second class of genes do not disrupt the asymmetry of the early cleavages but transform the fates of the early blastomeres (Bowerman et al., 1992; Mello et al., 1992, 1994; Hutter and Schnabel, 1994; Mango et al., 1994; Moskowitz et al., 1994; Lin et al., 1995; Draper et al., 1996 [this issue of *Cell*]). Surprisingly, these genes do not appear to have homologs in other species that have similar functions in early embryos.

Here, we report that *pal-1* is a blastomere-specification gene that is required for the normal development of the somatic P_2 descendants. Embryos that lack *pal-1* function lack P_2-derived cell types; conversely, embryos in which *pal-1* is ectopically expressed produce ectopic P_2-like cell types. *pal-1* activity is targeted to the P_2 lineage in two steps. First, *pal-1* translation is inhibited in the anterior by a mechanism that requires the putative RNA-binding protein MEX-3 and *pal-1* 3′ UTR sequences. This localizes PAL-1 protein to both EMS and P_2. Second, *pal-1* activity is confined to the P_2 lineage by a combination of temporal and spatial controls. We find that the putative transcription factor SKN-1 (Bowerman et al., 1992) inhibits *pal-1* activity in EMS and thereby prevents *pal-1* from specifying P_2 cell types in this blastomere. SKN-1 is also present in P_2 (Bowerman et al., 1993); however, the activity of the *pie-1* gene inhibits *skn-1* activity in P_2 (Mello et al., 1992). PIE-1 protein is localized to the P_1–P_4 blastomeres (Mello et al., 1996) and appears to inhibit transcription in these germline precursor blastomeres (Seydoux et al., 1996). Our results suggest that *pie-1* activity in P_2 acts to delay cell fate determination long enough for SKN-1 protein to decay, thus allowing the more stable PAL-1 protein to function in the somatic P_2 descendants. This novel regulatory strategy illustrates how the function of an evolutionarily conserved patterning gene can be adapted to function in different kinds of embryos.

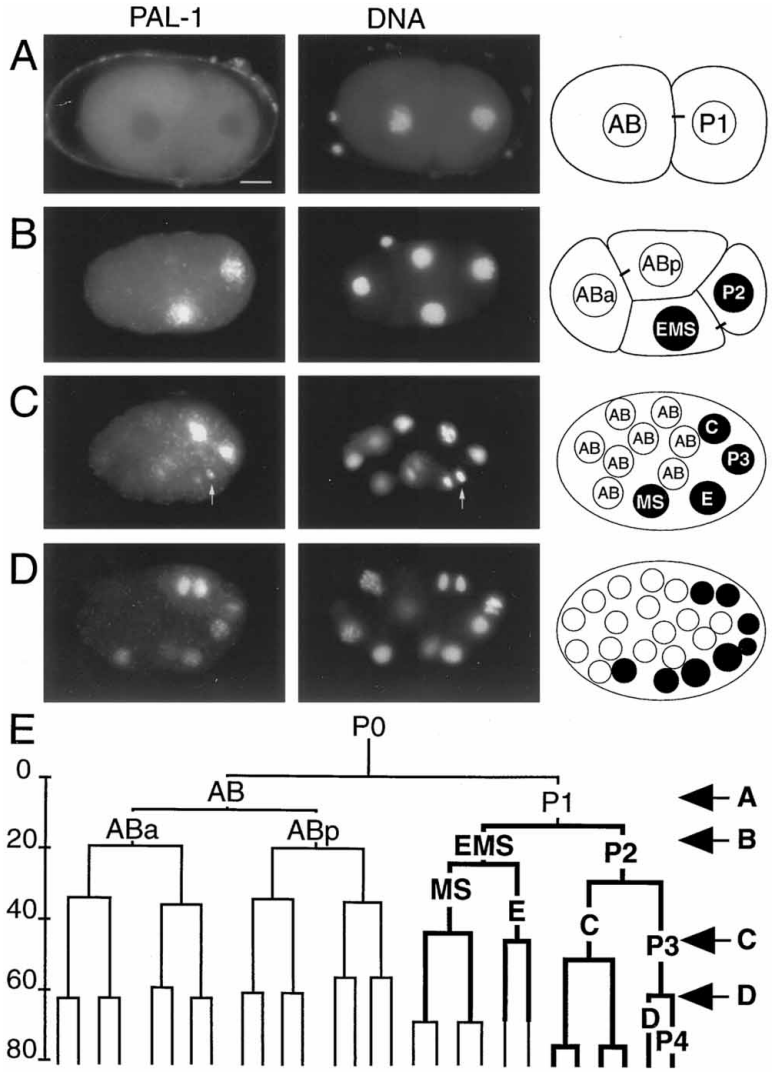

Figure 1. PAL-1 Localization in the Early Embryo

(A–D) Immunofluorescence micrographs of representative wild-type embryos stained for PAL-1 (left column) and DNA (DAPI) (middle column) and a schematic drawing summarizing, in all focal planes, typical protein localization patterns (right column).

(A) Strong PAL-1 staining was never observed in two-cell embryos; very weak staining was occasionally observed (7 of 62 embryos). This image was overexposed to show absence of any nuclear staining.

(B) Strong PAL-1 staining was first detected in 4 cell embryos in the EMS and P_2 nuclei; however, with some fixation methods, very weak staining was occasionally detected in ABa and ABp nuclei (2 of 40 embryos). In this example, ABp is staining weakly.

(C) Strong staining was detected in all four P_1 descendants at the 12 cell stage. In this example, both the E and MS (slightly out of focus) blastomeres were in late mitosis, as indicated in the DNA image. PAL-1 was detected on the condensed chromosomes (arrows). Not all nuclei are visible in this focal plane.

(D) PAL-1 staining continued to be detected in all the P_1 descendants in a 15–24 cell stage embryo (the eight AB descendants and P_3 are in mitosis). The staining intensity increased in Ca and Cp, whereas the signal in the EMS descendants began to decrease; the relative staining levels were quite variable in 24 to 28 cell embryos. Not all nuclei are visible in this focal plane.

(E) Summary of PAL-1 localization through the 28 cell stage. The thick lines and bold lettering represent cells that stain for PAL-1. Approximate developmental time in minutes (at 20°C) from the first cleavage is indicated to the left. The arrows to the right indicate the approximate stage of the representative staining patterns shown in (A)–(D). Scale bar, 10 μM.

Results

PAL-1 Protein Is Asymmetrically Localized in Early Embryos

To learn when and where PAL-1 is expressed, we stained early embryos with antibodies we raised against PAL-1. These antibodies first detected PAL-1 at high levels at the 4 cell stage in the nuclei of the two posterior blastomeres, EMS and P_2 (Figures 1A and 1B). From the 4 cell to the 24 cell stage, PAL-1 was detected in all the descendants of EMS and P_2 (Figures 1C and 1D). The PAL-1 antibody stained the nuclei throughout the cell cycle and was localized to the condensed chromosomes during mitosis.

At the 24 cell stage, the staining intensity appeared to increase in the two C descendants, Ca and Cp (Figure 1D). We found that this increase in PAL-1 levels was dependent on zygotic *pal-1* function. We stained embryos from a strain in which only half the progeny receive a wild-type copy of *pal-1* (see Experimental Procedures) and found that in 4 to 24 cell stage embryos, most (17 of 18) showed wild-type PAL-1 staining patterns. Since half the embryos do not have a wild-type copy of *pal-1*, this shows that maternal RNA is sufficient to generate the EMS and P_2-specific PAL-1 staining pattern. In older (24 to 28 cell stage) embryos, two staining patterns were observed; in some embryos (7 of 20), Ca and Cp stained brightly, whereas in the remaining embryos (13 of 20), the P_2 descendants stained only faintly. Presumably, the strong staining detected in half the embryos represents the initiation of zygotic gene expression from the *pal-1*(+) gene in these animals. In wild-type 28 cell embryos, PAL-1 was detected at a high level in the four C descendants and at a lower level in D and P_4 (Figure 1E). PAL-1 localization patterns from the 28 cell stage will be described elsewhere (L. G. Edgar and W. B. Wood, personal communication).

pal-1 Function Is Required for P_2 Development

At the 4 cell stage, PAL-1 is detected at a high level in P_2 and EMS, suggesting that *pal-1* may function in these blastomeres and their descendants. We showed above that maternal RNA is sufficient to generate the early PAL-1 expression pattern; therefore, to analyze embryos that lack maternal *pal-1* RNA, we devised a screen to

isolate *pal-1* germline mosaic hermaphrodites (see Experimental Procedures). These hermaphrodites have a wild-type allele of the *pal-1* gene in all somatic cells but only mutant alleles in the germline; thus, their self-progeny lack *pal-1* zygotic function and do not inherit any maternally supplied *pal-1* RNA. PAL-1 was never detected in the self-progeny embryos from these hermaphrodites, and the embryos failed to hatch. For simplicity, we refer to these embryos as *pal-1*(−) embryos. The *pal-1*(−) embryos were disorganized but produced all the cell types characteristic of wild-type EMS and P_2 blastomeres (Figure 2A). Injection of anti-sense *pal-1* RNA into the gonad of wild-type hermaphrodites produced embryos with similar phenotypes (data not shown).

Since similar cell types are produced by multiple early blastomeres, we determined what cell types were produced by isolated *pal-1*(−) EMS or P_2 blastomeres. Wild-type isolated EMS blastomeres produce pharyngeal body-wall muscle and intestinal cells. We found that, as in wild type, all these cell types were produced by an isolated *pal-1*(−) EMS blastomere (Table 1; data not shown). Wild-type isolated P_2 blastomeres produce body-wall muscle, epidermal, and germline precursor cells. We found that an isolated *pal-1*(−) P_2 blastomere did not produce the somatic cell types, muscle, and epidermis but instead produced unfamiliar cells (Figures 2B and 2C; Table 1). However, like wild-type P_2 blastomeres, *pal-1*(−) P_2 blastomeres always produced two germline founder cells that stained with antibodies to germ cell–specific P granules (Figure 2C). These results suggest that *pal-1* activity is not required for germline specification but is required for the development of the somatic descendants of the P_2 blastomere.

In early C. elegans embryos, *vab-7* is expressed exclusively in a subset of the epidermal and muscle precursor cells generated by the C blastomere, the anterior daughter of P_2 (Ahringer, 1996). To determine whether *pal-1* function was required for *vab-7* expression, we injected *pal-1* anti-sense RNA into a strain carrying a *vab-7::LacZ* reporter construct. We found that inhibiting *pal-1* function inhibited expression of *vab-7::LacZ* in 12 of 13 embryos examined (Figure 2D). Since *vab-7* function is required for proper patterning of the C descendants (Ahringer, 1996), this is further evidence that *pal-1* function is required for P_2 development.

In summary, PAL-1 protein is localized to both P_2 and EMS, yet its function appears to be restricted to the somatic descendants of the P_2 blastomere. These observations raised two important questions: how is PAL-1 protein localized to P_2 and EMS, and how is *pal-1* function restricted to P_2?

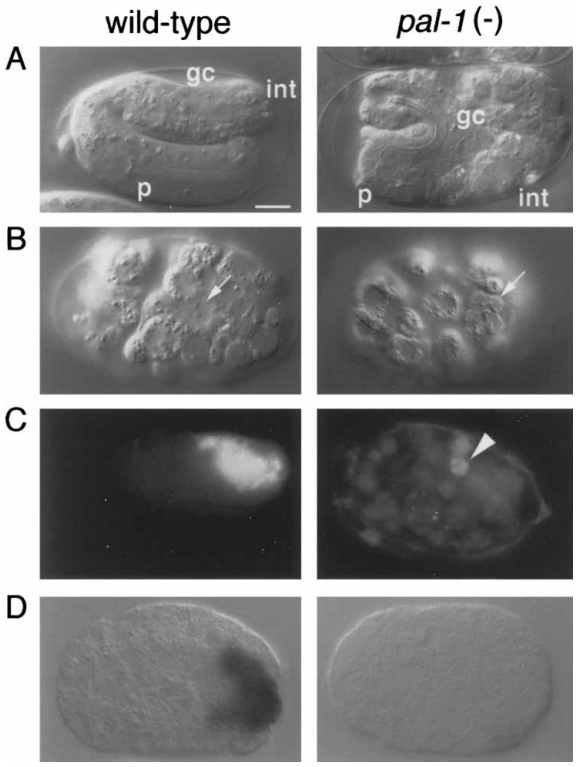

Figure 2. Wild-Type and *pal-1*(−) Embryos and Isolated Blastomeres

(A) Light micrograph of a wild-type embryo and a *pal-1*(−) embryo at 14 hr of development. All differentiated cell types have been made, and morphogenesis is nearly complete. *pal-1*(−) embryos always produced body-wall muscle cells that twitched, pharyngeal cells (p), intestinal cells (int), and two germ cells (gc) that stained with the K76 anti–P granule monoclonal antibody (see [C]). These embryos were never enclosed by epidermal cells, and each differentiated cell type tended to clump together.

(B) Light micrographs showing differentiated epidermal cells in wild-type isolated P_2 blastomeres (left) and unidentified cell types produced by *pal-1*(−) isolated P_2 blastomeres (right). These small cells with smooth nuclei do not resemble any major cell type in the embryo.

(C) Body-wall muscle and P granule–staining of the descendants of isolated wild-type and *pal-1*(−) P_2 blastomeres (both antibodies were detected with the same secondary antibody). The bright body-wall muscle staining in the descendants of isolated wild-type P_2 blastomeres occludes the P granule–staining. In contrast, P granule–staining, which is punctate and perinuclear, is readily detected in the descendants of isolated *pal-1*(−) P_2 blastomeres.

(D) Light micrographs of X-gal–stained embryos containing a *vab-7::lacZ* reporter gene construct (Ahringer, 1996). Inhibition of *pal-1* function by anti-sense *pal-1* RNA injection (right) significantly reduced *vab-7::lacZ* expression. Scale bar, 10 μM.

pal-1 RNA Is Uniformly Distributed in Early Embryos and Posteriorly Localized in Older Embryos

To determine whether *pal-1* RNA, like PAL-1 protein, is localized to the posterior, we examined *pal-1* RNA distributions by in situ hybridization (Seydoux and Fire, 1995). We found that *pal-1* RNA was uniformly distributed in 1 and 2 cell embryos and in about half of the 4 cell embryos (Figures 3A and 3B). In the other half of the 4 cell embryos and in 6 cell and older embryos, *pal-1* RNA was more abundant in the cells that express PAL-1 protein (Figures 3C and 3G). New *pal-1* RNA synthesis, as detected by nuclear localized *pal-1* transcripts, was not detected until the 24 cell stage (Figure 3D); thus, this localization pattern is not likely to be due to differential gene expression. Another explanation for the posterior localization of *pal-1* RNA is differential RNA stability, since nontranslated mRNAs in C. elegans and other animals are often unstable (Pulak and Anderson, 1993; Surdej et al., 1994). To test this idea, we examined the *pal-1* RNA distribution in *smg-3* mutant embryos. The *smg*

Table 1. Production of Body-Wall Muscle Cells from Isolated Blastomeres

Isolated blastomere(s)	Wild-type	*pal-1 (ct224)**	*pal-1 (−)*†	*pal-1 (as)*††
P_2	6/6	22/22	0/23	0/3
EMS	6/6	–	2/2	–
ABa and ABp	0/7	–	–	–

* All the embryos segregating from *pal-1(ct224)*; *sDp3* hermaphrodites inherit maternal *pal-1* (see Experimental Procedures), however, only half inherit a wild-type *pal-1* gene. Since all isolated P_2 blastomeres from this strain produce body-wall muscle cells, we infer that maternally supplied *pal-1* function must be sufficient to specify body-wall muscle cell production.

† Embryos from *pal-1(−)* germline mosaic hermaphrodites that lack both maternal and zygotic *pal-1(+)*.

†† Embryos from wild-type hermaphrodites injected with *pal-1* antisense RNA.

genes function to degrade nontranslated RNAs in C. elegans (Pulak and Anderson, 1993). If the RNA hybridization patterns observed in wild-type embryos reflect the degradation of *pal-1* RNA in the anterior cells, which are not translating *pal-1* mRNA, then in *smg* mutant embryos, *pal-1* RNA should be detected in all cells in early embryos. Indeed, in *smg-3* mutant embryos, *pal-1* RNA was detected in all cells (Figure 3E; PAL-1 protein is still asymmetrically localized). Thus, it is likely that the observed asymmetric *pal-1* RNA localization pattern is a secondary consequence of the asymmetric protein expression.

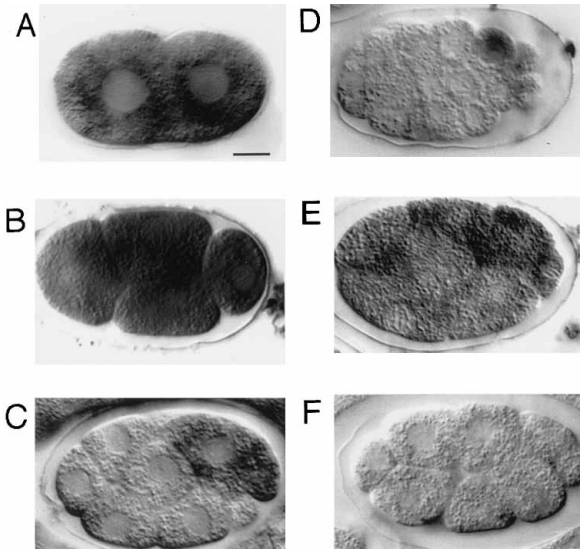

Figure 3. *pal-1* RNA Localization Patterns in Wild-Type and Mutant Embryos

(A) Equal *pal-1* RNA distribution in a wild-type 2 cell embryo. (B) *pal-1* RNA distribution in a wild-type 4 cell embryo. In 4 cell embryos, *pal-1* RNA was either uniformly distributed (approximately 50%) or preferentially localized to the posterior blastomeres. In this example, the staining intensity is slightly decreased in ABa. (C) 12 cell wild-type embryo, at which stage *pal-1* RNA is more abundant in cells that stain for PAL-1. (D) *pal-1* RNA is first detected in the nucleus of wild-type 24 cell embryos. (E) *pal-1* RNA staining in anterior blastomeres in *smg-3* mutant embryo. (F) Wild-type 12 cell embryo hybridized with the sense control probe. Scale bar, 10 μM.

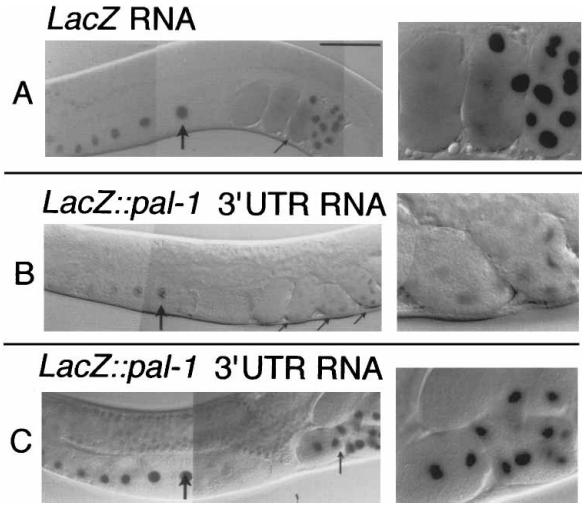

Figure 4. Translation of *lacZ* and *lacZ::pal-1* 3′ UTR RNAs in Wild-Type and *mex-3*(−) Germ Cells and Embryos

(A) Control *lacZ* RNA (Evans et al., 1994) injected into a wild-type hermaphrodite. β-gal activity was detected in oocytes (large arrows) and all cells in young embryos (small arrows). Diffuse cytoplasmic staining was observed in cells during mitosis.

(B) *lacZ::pal-1* 3′ UTR RNA injected into wild-type hermaphrodite. β-gal activity was detected in oocytes (large arrows) and occasionally (Table 2) in posterior cells in young embryos (small arrows). The staining intensity in oocytes was reduced compared with that produced by *lacZ* RNA. This residual oocyte staining was not expected, since PAL-1 is not detected in oocytes in the wild type. This observation may reflect either a requirement for additional control elements or an inadequacy of the assay.

(C) *lacZ::pal-1* 3′ UTR RNA injected into *mex-3* mutant hermaphrodite. β-gal activity was detected in oocytes and early (less than 4 cell) embryos (small arrows). However, the frequency of embryos expressing β-gal was not as high as was observed with *lacZ* RNA (Table 2). Scale bar, 50 μM.

The *pal-1* RNA and protein localization patterns suggest that posterior-specific translation may control the initial PAL-1 protein localization pattern at the 4 cell stage. Control of maternal mRNA translation in C. elegans and other animals is often mediated by sequences located in the 3′ UTR (Ahringer and Kimble, 1991; Goodwin et al., 1993; Evans et al., 1994; Curtis et al., 1995). In C. elegans, these 3′ UTR regions can control the translation of injected *LacZ* reporter RNAs (Evans et al., 1994; Goodwin et al., 1993). To determine whether the *pal-1* 3′ UTR can inhibit translation in anterior cells, we attached the *pal-1* 3′ UTR to a *LacZ* reporter RNA construct (*LacZ::pal-1* 3′ UTR). The control RNA (*LacZ* without a 3′ UTR) was translated efficiently in oocytes and all cells of early embryos (Figure 4A; Table 2). We found that in general, the *LacZ::pal-1* 3′ UTR RNA was translated much less efficiently than *LacZ* RNA (Table 2). However, when this RNA was translated, β-gal activity was primarily detected in the posterior blastomeres and their descendants (Figure 4B), a pattern similar to PAL-1 localization. This result suggests that the *pal-1* 3′ UTR can function to inhibit translation in anterior blastomeres.

mex-3 Functions to Restrict PAL-1 Localization to the P_1 Descendants

To identify genes that regulate PAL-1 localization, we examined PAL-1 staining patterns in embryonic pat-

Table 2. *LacZ* Reporter RNA Expression Patterns

Genotype	RNA injected (ng/μl)*	*LacZ* expression pattern in embryos†		Mex-3–like dead embryos††
		Ant. & Post. Blastomeres	Primarily Post. Blastomeres	
WT	*LacZ* (25-250 ng /μl)	24/30	0/30	0
WT	*LacZ::pal-1* 3′ UTR (25-100)	0/16	5/16	5
WT	*LacZ::pal-1* 3′ UTR (250)	7/11	0/11	17
mex-3(−)	*LacZ::pal-1* 3′ UTR (25-250)	12/26	0/26	NA

* Similar results, except as noted, were obtained with all RNA concentrations (25 ng/μl, 50 ng/μl, 100 ng/μl, and 250 ng/μl).
† Number of hermaphrodites containing embryos with the indicated staining patterns. Only hermaphrodites with oocyte staining and young embryos (less than 24 cell stage) were scored.
†† Number of Mex-3–like embryos produced following reporter RNA injection into wild-type hermaphrodites. Injection of *LacZ* RNA alone produced a few dead embryos but none with MEX-3–like phenotypes. Conversely, a majority of the dead embryos produced by injection of *LacZ::pal-1* 3′ UTR RNA were Mex-3–like. Injection of *LacZ::pal-1* 3′ UTR RNA also produced a few dead embryos resembling those produced by *pal-1* anti-sense RNA injections (about 20% of all dead embryos). The observation that both sense and anti-sense RNAs produce similar phenotypes has been observed for other genes and has not been explained (Guo and Kemphues, 1995; C. C. Mello, B. W. Draper, and J. R. Priess, personal communication).

terning mutants (see Experimental Procedures). We found that PAL-1 was detected in oocytes and all blastomeres in embryos from *mex-3*(−) hermaphrodites (Figures 5A–5D). *mex-3* encodes a putative RNA-binding protein that is present in oocytes and early embryos but at the 4 cell stage is preferentially localized to the anterior blastomeres (Draper et al., 1996). Draper et al. (1996) have also found that MEX-3 is uniformly distributed in *par-1*(−) 4 cell embryos. We found that PAL-1 was never detected in *par-1*(−) embryos (Figure 5E). Thus, the localization of PAL-1 correlates with low MEX-3 levels, suggesting that MEX-3 may inhibit *pal-1* translation.

To determine whether *mex-3* function is required for the *pal-1* 3′ UTR to inhibit translation in anterior blastomeres, we injected the *LacZ::pal-1* 3′ UTR RNA into *mex-3* mutant hermaphrodites. In contrast to the injection of this RNA into wild-type hermaphrodites, β-gal activity was readily detected at a high level in *mex-3*(−) oocytes and embryos (Figure 4C).

In the course of these experiments, we noticed that injection of higher concentration of the *LacZ::pal-1* 3′ UTR RNA into wild-type hermaphrodites resulted in β-gal activity in all blastomeres (Table 2). This suggested that injection of many copies of the *pal-1* 3′ UTR RNA may titrate *mex-3* activity. If so, then these injections should produce embryos with Mex-3(−) phenotypes. Indeed, injection of *LacZ::pal-1* 3′ UTR RNA but not *LacZ* control RNA resulted in dead embryos with Mex phenotypes (Table 2).

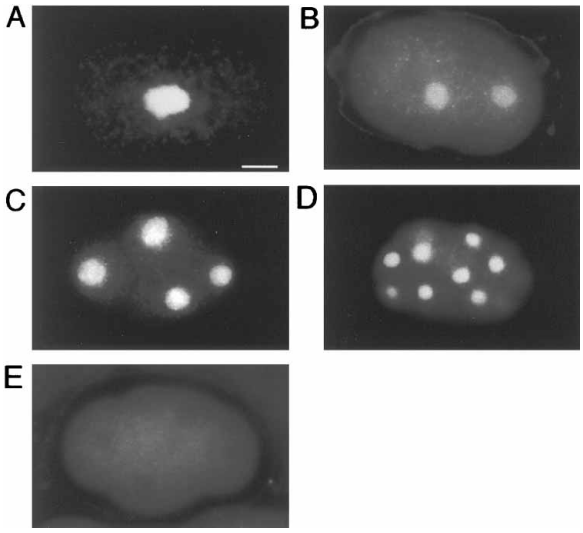

Figure 5. PAL-1 Protein Localization in *mex-3* and *par-1* Mutant Embryos

(A) Nuclear localized PAL-1 in an oocyte from a *mex-3*(−) hermaphrodite.

(B) Both pronuclei stain strongly in *mex-3*(−) 1 cell embryos. The staining intensity was significantly higher in *mex-3*(−) 4 cell (C) and 24 cell (D) embryos than in wild-type embryos (compare with Figures 1B–1D).

(E) PAL-1 was not detected in *par-1*(−) embryos. *pal-1* RNA is normally localized in 4 cell *par-1*(−) embryos and in later embryos is either uniformly distributed or is not detected (data not shown). Scale bar, 10 μM.

pal-1 Function Is Required to Specify Posterior Blastomere Identity

We have found that PAL-1 is normally expressed in and is required for the development of the somatic descendants of the P_2 blastomere (see Figure 2; Table 1). Furthermore, in *mex-3*(−) embryos, PAL-1 is abnormally expressed in the anterior AB descendants (Figure 5). These PAL-1–expressing cells adopt a fate similar to C, the anterior daughter of P_2, and produce body-wall muscle cells instead of anterior pharyngeal cells (Draper et al., 1996). These observations suggested that ectopic *pal-1* activity might provide the AB descendants with a P_2 (or C) blastomere identity, that is, cause anterior-to-posterior cell fate transformations. Alternatively, *pal-1* function may be required simply for the execution of the P_2-like fates; that is, to differentiate certain body-wall muscle and epidermal cell types. To discriminate between these two possibilities, we asked whether inhibiting *pal-1* function in *mex-3* mutant embryos would simply block the execution of the ectopic posterior fates or, rather, would reverse the anterior-to-posterior cell fate transformations.

We first asked whether *pal-1* function was required in *mex-3*(−) embryos for the production of AB–derived muscle cells, a necessary condition for either possibility. We initially determined that maternal *pal-1* function was sufficient for the Mex-3 phenotype (see Experimental

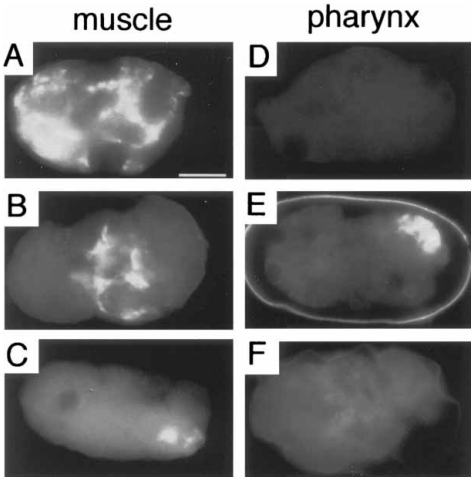

Figure 6. *pal-1*–Dependent Body-Wall Muscle and Pharyngeal Staining in *mex-3*(−) Embryos

Body-wall muscle staining in *mex-3*(−) *pal-1*(+) embryo (A) and *mex-3*(−) *pal-1*(as) embryo (B). EMS–ablated *mex-3*(−) *pal-1*(as) embryo (C) was stained for both body-wall muscle cells and P granules. This embryo contains at least six P granule–staining cells. *mex-3* mutations disrupt the segregation of P granules at the P_3 division, which causes additional P granule–staining cells to be produced (Draper et al., 1996). This Mex-3 phenotype does not appear to be dependent on *pal-1*(+), since multiple P granule–staining cells are often observed in *mex-3*(−) *pal-1*(as) embryos.

(D) Pharyngeal-muscle cell staining in *mex-3*(−) *pal-1*(+) embryo in which MS was ablated after the time of induction. No pharyngeal staining is visible.

(E) *mex-3*(−) *pal-1*(as) embryo in which MS was ablated after the time of induction. Here, *pal-1*(as) restored the pharyngeal staining to AB–derived cells.

(F) *mex-3*(−) *pal-1*(as) embryo in which MS was ablated before the time of induction. This abolished all pharyngeal staining (MS– and AB–derived). Scale bar, 10 μM.

Procedures). To inhibit maternal *pal-1* activity in *mex-3*(−) embryos, we injected *pal-1* anti-sense RNA [*pal-1*(as)] into *mex-3*(−) hermaphrodites. We found that this dramatically reduced but did not eliminate the number of body-wall muscle cells (Figures 6A and 6B). However, the EMS blastomere produced *pal-1*-independent body-wall muscle cells (Table 1). To determine whether the remaining muscle cells in *mex-3*(−) *pal-1*(as) embryos were produced by EMS or AB, we killed EMS. We found that body-wall muscle staining was eliminated in 6 out of 7 embryos (Figure 6C). Thus, *pal-1* function is required in *mex-3*(−) embryos for the production of AB–derived body-wall muscle cells.

We next asked whether inhibiting *pal-1* function in *mex-3*(−) embryos could also restore the production of AB–derived pharyngeal cells. In wild-type embryos, the AB descendants produce half of the pharynx, while descendants of the MS blastomere produce the remaining half. Furthermore, the production of the AB–derived pharyngeal cells is dependent on inductive interactions that occur at the 12 cell stage between MS and descendants of AB (Priess and Thomson, 1987; Hutter and Schnabel, 1994). The production of the MS–derived pharyngeal cells is not affected by *mex-3*(−) mutations. Therefore, to determine whether *mex-3*(−) *pal-1*(as) embryos produce AB–derived pharyngeal cells, we ablated

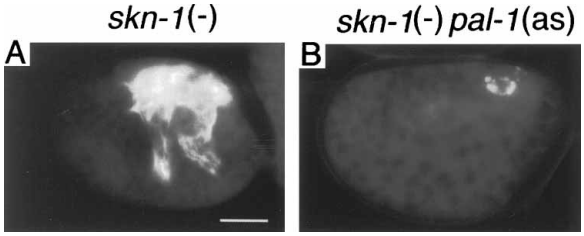

Figure 7. The Body-Wall Muscle Cells Present in *skn-1* (−) Embryos Are *pal-1*–Dependent

Body-wall muscle and P granule–staining in *skn-1*(−) (A) and *skn-1*(−) *pal-1*(as) (B) embryos. The germline-precursor–specific P granule–staining is occluded by the bright body-wall muscle staining in (A). Scale bar, 10 μM.

the MS cell after the time of these inductive interactions and found that six of seven embryos still produced pharyngeal cells (Figures 6D and 6E). To show that these were normal induction-dependent AB–like pharyngeal cells and not ectopic induction-independent MS–like pharyngeal cells, we ablated the MS precursor cell (EMS) and found that no pharyngeal cells were produced (seven embryos; Figure 6F). Therefore, the inhibition of *pal-1* function in *mex-3* mutant embryos restored the production of normal induction-dependent AB–derived pharyngeal cells. These results show that ectopic *pal-1* function is required for the *mex-3*(−) anterior-to-posterior cell fate transformations. Thus, *pal-1* activity is not required simply for the execution of posterior fates but rather is required to specify posterior identity.

skn-1 Activity Prevents *pal-1* from Promoting P_2-Specific Fates in EMS

These findings raised an apparent paradox: the ectopic expression of PAL-1 that occurs in *mex-3* mutants leads to ectopic production of P_2-like cell types, yet PAL-1 is normally present in the EMS cell that does not produce P_2-like fates. Furthermore, the absence of *pal-1* function does not noticeably alter the cell types produced by the EMS blastomere. These observations suggest that in wild-type animals, the PAL-1 protein present in EMS is not active.

Mutations in the bZIP-like putative transcription factor gene *skn-1* do cause the EMS cell to produce P_2-like cell types (Bowerman et al., 1992). This raised the possibility that in wild-type animals, *skn-1*(+) activity might function to inhibit *pal-1* activity in EMS. If this were true, then the P_2 cell types produced by *skn-1*(−) EMS blastomeres should depend on *pal-1* function. To test this, we compared body-wall muscle production in *skn-1*(−) *pal-1*(+) embryos and *skn-1*(−) *pal-1*(as) embryos. We found that the muscle cells produced by *skn-1*(−) embryos were dependent on *pal-1*(+) function (no muscle staining in 10 of 21 embryos and very few muscle-staining cells in 9 of 21 embryos) (Figure 7). Thus, in *skn-1* mutant embryos, *pal-1* activity is required to specify P_2 cell types in EMS. This suggests that in wild type, *skn-1* functions to inhibit *pal-1* activity in EMS. However, SKN-1, like PAL-1, is present in both EMS and P_2 (Bowerman et al., 1993). Therefore, a P_2-specific factor must allow *pal-1* to overcome *skn-1* inhibition in P_2 or its descendants (see Discussion).

Discussion

We have found that *pal-1* activity is required to specify the identity of the somatic descendants of the P_2 blastomere. *pal-1* activity is sequentially restricted to these cells in two steps: first, translation is inhibited in anterior blastomeres; and second, *pal-1* activity is inhibited in one of the two posterior blastomeres. Thus, the domain of *pal-1* activity is restricted first by spatially limiting protein production and second by interactions between embryonic regulatory proteins.

Control of PAL-1 Localization

The spatial and temporal localization of PAL-1 in the early embryo is regulated by *mex-3* activity; in *mex-3* mutants, PAL-1 is detected in oocytes and all cells in embryos (Figure 4). MEX-3 is a putative RNA-binding protein that is present in oocytes, 1 and 2 cell embryos, and preferentially in the anterior AB blastomeres in 4 cell embryos (Draper et al., 1996). Thus, a simple model is that the distribution of MEX-3 determines both the spatial and temporal pattern of PAL-1 localization. Consistent with this are the observations that in *par-1* mutant embryos, MEX-3 is detected at a high level in all four blastomeres in 4 cell embryos (Draper et al., 1996) and PAL-1 is not detected (Figure 4E). We also showed that *mex-3* function is required to inhibit *LacZ::pal-1* 3′ UTR RNA translation in anterior blastomeres and that injection of high concentrations of this RNA into wild-type hermaphrodites leads to β-gal activity in anterior blastomeres and the production of embryos with Mex-3(−) phenotypes. Presumably, this occurs because *pal-1* mRNA is now translated in anterior blastomeres. These results suggest that the *pal-1* 3′ UTR is critical for *mex-3*-dependent regulation of the PAL-1 expression pattern. It will be informative to learn whether MEX-3 directly binds the *pal-1* 3′ UTR.

We also found that once *pal-1* translation begins, the pattern of *pal-1* RNA localization changes; maternal *pal-1* RNA appears to be more stable in cells producing PAL-1. We note that the degradation of *pal-1* RNA precedes the decay of MEX-3 (Draper et al., 1996) and therefore may be functionally relevant to the integrity of the PAL-1 localization pattern. Interestingly, we found that the degradation of *pal-1* mRNA was dependent on *smg-3* function. The *smg* genes function to degrade abnormal RNAs encoding truncated proteins (Pulak and Anderson, 1993). Our observations suggest that the *smg* genes may also have a role in normal development.

Cross-Regulatory Interactions Restrict *pal-1* Activity to P_2 Descendants

The activities of SKN-1 and PAL-1 must be regulated differently in the EMS and P_2 blastomeres. Both proteins are present in both EMS and P_2. However, during normal development, *pal-1* appears to functions only in the P_2 lineage and *skn-1* appears to function only in the EMS lineage (Figure 8A). The explanation for why *skn-1* is active in EMS and *pal-1* is inactive appears to be straightforward. We found that the P_2 cell types produced by *skn-1*(−) EMS blastomeres (Bowerman et al., 1992) are dependent on *pal-1* (Figure 7). Thus, *skn-1*(+) functions directly or indirectly to inhibit *pal-1*(+) activity in EMS (Figure 8B).

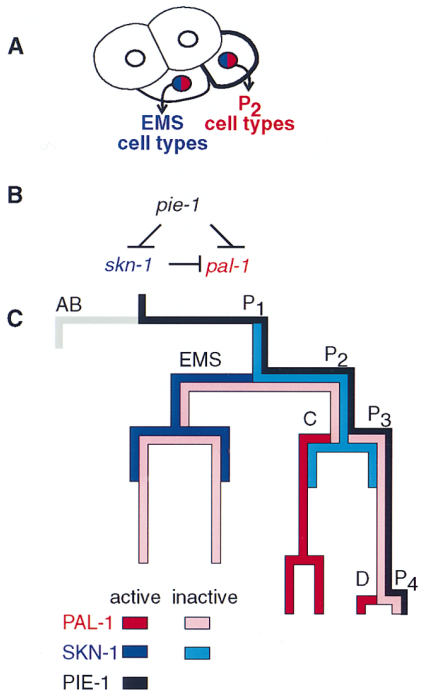

Figure 8. Model for Regulatory Interactions between the *pal-1*, *skn-1*, and *pie-1* Proteins

(A) In 4 cell embryos, both SKN-1 (blue) and PAL-1 (red) are preferentially localized to the posterior blastomeres EMS and P_2, yet SKN-1 functions in EMS to promote the production of EMS fates, and PAL-1 functions in P_2 to promote the production of P_2 somatic fates. PIE-1 (black outline of P_2 blastomere) is localized to P_2 (Mello et al., 1996) and functions to promote the production of P_2 somatic and germline fates (Mello et al., 1992). In this model, regulatory interactions between *pie-1*, *skn-1*, and *pal-1* (B), in conjunction with the protein distributions diagrammed in (C), allow *skn-1* to function in the EMS descendants and *pal-1* to function in the somatic P_2 descendants (see text). In principle, the regulatory interactions could be at the level of the proteins or their targets.

The *skn-1* gene encodes a bZIP-like putative transcription factor (Bowerman et al., 1993; Blackwell et al., 1994) that is detected only in early embryos through the 8 cell stage, yet PAL-1 is present in the EMS descendants through the 24 cell stage (see Figure 8C). This suggests that *skn-1* activity in EMS or its two daughters or both initiates a developmental program that precludes *pal-1* activity from promoting P_2 development later when SKN-1 is gone.

How does *pal-1* overcome *skn-1* inhibition in the P_2 lineage? The simple answer is that *skn-1* function is inhibited in the P_2 lineage. This P_2-specific inhibition of *skn-1* activity is dependent on the *pie-1* gene; in *pie-1* mutants, *skn-1* is active in P_2, where it transforms P_2 into an EMS–like blastomere (Mello et al., 1992). Thus, *pie-1* activity in P_2 allows *pal-1* to function in the P_2 descendants. Interestingly, PIE-1 protein is asymmetrically segregated to the P_1–P_4 blastomeres (Mello et al., 1996), and its activity is associated with the inhibition of zygotic transcription in this germline lineage (Seydoux et al., 1996). This suggests that *pie-1* activity in P_2 inhibits both SKN-1 and PAL-1.

These observations suggest the following interesting regulatory strategy, in which the spatial segregation of PIE-1 combines with the temporal decay of SKN-1 to target *pal-1* activity to the somatic P_2 descendants. In the P_2 blastomere, PIE-1 inhibits the activity of both SKN-1 and PAL-1 (Figures 8B and 8C). When P_2 divides, PIE-1 segregates to the posterior daughter P_3, where it continues to inhibit both SKN-1 and PAL-1. In C, the anterior daughter of P_2, both PAL-1 and SKN-1 protein are initially present, yet *pal-1* functions to promote the production of C cell types. This apparent contradiction is best explained by the decay of SKN-1 at about this time in development (blue lineage in Figure 8C; Bowerman et al., 1993), which would allow PAL-1 to be active in C (red lineage). When P_3 divides, the anterior daughter, D, inherits PAL-1, which, unencumbered by either PIE-1 or SKN-1, leads to the production of *pal-1*-dependent body-wall muscle cells. This model predicts that additional temporally or spatially distributed factors function with *pal-1* to specify the C and D lineages differentially. In summary, in the EMS lineage, *pal-1* activity is inhibited by *skn-1*, and in the P_2 lineage, *pie-1* activity postpones cell fate determination until the inhibitory SKN-1 protein begins to decay.

caudal Genes, Early Embryos, and Analog versus Digital Development

pal-1, like *caudal* homologs in many animals, is expressed in the posterior body region during early embryonic development (Mlodzik et al., 1985; Macdonald and Struhl, 1986; Joly et al., 1992; Frumkin et al., 1991; Gamer and Wright, 1993; Xu et al., 1994); further, *pal-1*, like Drosophila *cad* and mouse *cdx-1* (Macdonald and Struhl, 1986; Subramanian et al., 1995) has a role in posterior patterning. It is even possible that *cad* and *pal-1* regulate a conserved gene, since *vab-7* is an *even-skipped* homolog (Ahringer, 1996), and *cad* mutants have an *evenskipped* phenotype (Macdonald and Struhl, 1986). Our findings have revealed an additional parallel between *caudal* function and regulation in these different early embryos. Both worm and fly *caudal* genes are subject to translational regulation by anteriorly localized RNA-binding proteins. The Drosophila homeodomain protein, *bicoid*, binds to the *cad* 3′ UTR and inhibits initiation of translation (Dubnau and Struhl, 1996; Rivera-Pomar et al., 1996). It is not yet known whether MEX-3 interacts directly with *pal-1* 3' UTR sequences.

These similarities are surprising, since early Drosophila and C. elegans embryos are quite different. In this regard, it is informative to compare the regulation and function of the similar posterior patterning genes, *cad* and *pal-1*, to the regulation and function of the dissimilar anterior patterning genes, *hunchback* and *glp-1*. Both *hunchback* (in flies) and *glp-1* (in worms) are translated in only the anterior of the early embryo by a mechanism that requires similar sequence elements in the 3′ UTR (*nanos* response elements; Wharton and Struhl, 1991; Evans et al., 1994). *Hunchback* protein is found in a gradient in the syncytial embryo and acts as a concentration-dependent transcriptional regulator of the gap genes (Hulskamp et al., 1990; Struhl et al., 1992; Schulz and Tautz, 1994). In contrast, GLP-1 protein, a *Notch*-related transmembrane receptor, is localized to the membranes of anterior blastomeres, where it mediates multiple intercellular interactions with posterior blastomeres (Priess and Thomson, 1987; Evans et al., 1994; Hutter and Schnabel, 1994, 1995; Mango et al., 1994; Moskowitz et al., 1994; Mello et al., 1994). In this case, a similar regulatory mechanism localizes very different types of regulatory molecules, each of which is adapted for the cleavage pattern of the embryo in which it resides.

In contrast, PAL-1 and CAD are similar regulatory proteins. However, following their posterior localization, their regulation and function are each adapted to their different environments. *cad* is a dosage-sensitive regulator of gap genes in Drosophila (Schulz and Tautz, 1995); thus, the posterior-to-anterior gradient of CAD protein (Macdonald and Struhl, 1986; Mlodzik and Gehring, 1987) can provide positional information along the AP body axis, where different protein levels have different developmental consequences (analog information). In contrast, the cellular compartmentalization of PAL-1 in the C. elegans embryo limits its functional range to active versus inactive (on/off) in specific blastomeres (digital information). Combinations of these factors within individual blastomeres then provides the additional information required for further pattern refinement. Thus, this fine-scale regulation of *pal-1* and *cad* activities illustrates how evolutionarily conserved genes can be adapted to function in different kinds of embryos.

Experimental Procedures

Strains and Alleles

Nematodes were cultured using standard conditions (Brenner, 1974). The mutations and chromosomes rearrangements used are derived from wild-type Bristol N2 and are listed by linkage group (LG). LGI: *bli-3(e767), dpy-5(e61), egl-30(ad805), mex-3(zu155), mex-3(zu166::Tc1), hT1(I;IV).* LGII: *rol-1(e91), mex-1(zu121), mex-1(zu120), eIS24(vab-7::LacZ* + pRF4), *mnC1.* LGIII: *pie-1(zu154), unc-25(e156), qC1[dpy-19(e1259) glp-1(q239)], pal-1(ct224) dpy-17(e164) ncl-1(e1865), unc-36(e251); sDp3 (f:III), glp-1(e2141ts), par-3(it71) lon-1(e185) par-2(it5ts). par-2(e2030ts)* LGIV: *skn-1(zu67) DnT1(IV;V), smg-3(ma117).* LGV: *rol-4(sc8), par-1(b247), him-5(e1490).* LGX: *lin-2(e1390).* The *mex-3* mutations were obtained from Bruce Draper and Jim Priess, the *pal-1*(*ct224*) mutation was given to us by Lois Edgar and Bill Wood, and *eIS24*(*vab-7::LacZ*) was provided by Julie Ahringer. Many strains were obtained from the C. elegans genetic stock center.

Antibodies and Immunostaining

Anti-*pal-1* antibodies were purified from the sera of two rabbits immunized with a PAL-1:$(his)_6$ fusion protein by blot affinity purification. For *pal-1* antibody staining, embryos were permeabilized by freeze-cracking and fixed 2–3 min in room temperature methanol (Miller and Shakes, 1995). *pal-1* antibodies (1:50 in phosphate-buffered saline, 0.1% Tween-20, 3% bovine serum albumin) were applied directly to the briefly air-dried sample. Rhodamine-labeled donkey anti-rabbit IGG (Jackson Labs) was used as the secondary antibody (1:200 in phosphate-buffered saline, 0.1% Tween-20). Half the older embryos from *pal-1(ct224); sDp3* hermaphrodites stain; none of the embryos from germline clone hermaphrodites stain; and embryos containing a heat-shock promoter–*pal-1* cDNA construct stain very brightly following heat treatment. *glp-1*, *skn-1*, *pie-1*, and *mex-1* embryos (4 cell) stain similar to wild type. The staining patterns for *par-2* and *par-3* embryos were variable and included embryos with wild-type distributions, no staining, and all four cells staining.

Maternal and Zygotic Requirements for *pal-1* Function

pal-1(ct224) homozygotes inherit maternally contributed *pal-1* RNA. We determined that this RNA was sufficient to specify P_2-like cell

types both from the P_2 blastomere (Table 1) and from the AB blastomeres in *mex-3*(−) embryos: *mex-3(zu155)I; pal-1(ct224)* and *mex-3(zu155) I; pal-1(ct224)/pal-1(+)* embryos die with indistinguishable Mex-3 phenotypes. To eliminate maternal *pal-1* function in embryos, we injected *pal-1* anti-sense RNA (see below) and isolated embryos from *pal-1*(−) germline mosaic hermaphrodites.

Germline mosaic hermaphrodites were isolated from the strain CF418 [*pal-1(ct224) dpy-17(e164) ncl-1(e1865) unc-36(e251); lin-2(e1309); sDp3(III:f)*]. Hermaphrodites homozygous for *pal-1*(*ct224*) and containing the free duplication *sDp3* are wild type. *sDp3* is mitotically unstable and is occasionally not transmitted to both cells at cell division; thus, at some frequency *sDp3* will not be transmitted to P_4, the clonal founder of the germline. The resulting animal will have a *pal-1*(+) gene in somatic cells but not germline cells and will produce only dead [*pal-1*(−)] embryos. The *lin-2* mutation was included in the strain to facilitate the identification of animals that produce only dead embryos. *lin-2* animals cannot lay their eggs; the young hatch internally and eat the mother. Mothers that produce only dead eggs survive. Embryos 4 cell and younger from these hermaphrodites were isolated and used for the experiments described; PAL-1 was never detected in the remaining embryos.

RNA Synthesis and Injection

The *pal-1* 3′ UTR was cloned into the PstI and StuI sites of plasmid pJK370 (pJK370 is essentially the same construct as pJK350 [Evans et al., 1994], except plasmid sequences are from pPD16.43 [Fire et al., 1990; T. Evans, personal communication]). Reporter RNA was synthesized and injected as described in Evans et al. (1994), with minor modifications. The RNA was not polyA-selected. *pal-1* antisense RNA at 100 ng/ml was injected as described in Mello et al. (1991). *pal-1(as) embryos (2 and 4 cell) from eIS24*(*vab-7::LacZ*) hermaphrodites were collected 4–6 hr following injection and incubated with control embryos for 3 hr before fixation and staining for β-gal activity (Fire et al., 1990).

Microscopy and Laser Ablation

For DIC microscopy, embryos were mounted on 2% agarose pads as described in Sulston et al. (1983). Laser ablation of blastomeres in 4 cell embryos was done as described in Mello et al. (1992). Embryos (12–16 hr at 20°C) were scored for cell type–specific phenotypes by DIC (Bowerman et al., 1992) and fixed and stained with cell type–specific monoclonal antibodies, as described in Priess and Thomson (1987). mAb 5.6.1 recognizes body-wall muscle myosin (Miller et al., 1983); 3NB12 recognizes pharyngeal antigens (Priess and Thomson, 1987); and K76 recognizes germline-specific P granules (Strome and Wood, 1982).

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