The Maternal Gene skn-1 Encodes a Protein That Is Distributed Unequally in Early C. elegans Embryos

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Summary

The autonomous or cell-intrinsic developmental properties of early embryonic blastomeres in nematodes are thought to result from the action of maternally provided determinants. After the first cleavage of the C. elegans embryo, only the posterior blastomere, P1, has a cell-intrinsic ability to produce pharyngeal cells. The product of the maternal gene skn-1 is required for P1 to produce pharyngeal cells. We show here that the Skn-1 protein is nuclear localized and that P1 appears to accumulate markedly higher levels of Skn-1 protein than its sister, the AB blastomere. We have examined the distribution of Skn-1 protein in embryos from mothers with maternal-effect mutations in the genes mex-1, par-1, and pie-1. These results suggest that mex-1(+) and par-1(+) activities are required for the unequal distribution of the Skn-1 protein and that pie-1(+) activity may function to regulate the activity of Skn-1 protein in the descendants of the posterior blastomere P1.

Introduction

The first cleavage in nematode embryogenesis generates anterior and posterior sister blastomeres that differ in size, cleavage rate, and mitotic spindle orientation. Reproducible differences in the fates of these blastomeres were first described almost 100 years ago. For example, limited cell lineage studies suggested that only the posterior blastomere produces intestinal cells in the nematode Parascaris (Boveri, 1899, 1910). Early experimental studies in which one of the first two blastomeres was killed by ultraviolet irradiation showed that the surviving blastomere still produces at least some of the same cell types it produces in normal development (Stevens, 1909). These results and similar observations in other invertebrate embryos suggested that intrinsic factors, rather than cell–cell interactions, might determine the different fates of some early blastomeres (for a review see Davidson, 1991).

The description of the complete cell lineage of the nematode Caenorhabditis elegans (Sulston and Horvitz, 1977; Kimble and Hirsh, 1979; Sulston et al., 1983) has made it possible to compare in detail the development of an

individual blastomere in an intact embryo with the development of the same blastomere after removing or destroying neighboring blastomeres (Laufer et al., 1980; Schierenberg et al., 1985, 1987; Edgar and McGhee, 1986; Priess and Thomson, 1987; Schnabel, 1991; Bowerman et al., 1992a, 1992b; Goldstein, 1992; Mello et al., 1992). Although these studies have identified multiple cell–cell interactions that contribute to the specification of blastomere fate, they also confirm that some early blastomeres have intrinsic differences in their abilities to produce certain cell types.

One difference between the early blastomeres in C. elegans is their ability to produce pharyngeal cells, which compose a muscular and glandular organ used in feeding (Albertson and Thomson, 1976). Pharyngeal cells can be distinguished easily by morphological and immunochemical criteria from other cell types and thus provide a useful marker for studies of blastomere fate determination. The first two embryonic blastomeres, called AB and P1, both produce pharyngeal cells in normal development. Descendants of the AB blastomere produce pharyngeal cells as a result of cell–cell interactions with P1 descendants (Priess and Thomson, 1987), a process that requires maternal expression of the glp-1 gene (Austin and Kimble, 1987; Priess et al., 1987). In contrast, the P1 blastomere appears to have an intrinsic ability to produce pharyngeal cells; only P1 produces pharyngeal cells when AB and P1 are separated and cultured in isolation (Priess and Thomson, 1987), and only P1 produces pharyngeal cells in embryos from homozygous glp-1 mutant mothers (Priess et al., 1987). After the P1 blastomere divides, only one of its daughters, called EMS, inherits this ability to produce pharyngeal cells. Similarly, after EMS divides, only one of its daughters, called MS, inherits this ability (Priess and Thomson, 1987; Bowerman et al., 1992a; Mello et al., 1992). Thus, some property or factor required for pharyngeal cell development appears to be restricted successively to P1 and specific P1 descendants after each of the first three embryonic cleavages.

To determine the molecular basis for the different abilities of early blastomeres to produce pharyngeal cells in C. elegans, we have isolated and analyzed maternal-effect mutants that produce either too many or no pharyngeal cells. In wild-type embryogenesis the P1 granddaughter that produces pharyngeal cells is called the MS blastomere; MS undergoes an invariant pattern of cleavages, generating pharyngeal cells, body wall muscle cells, and cell deaths (Sulston et al., 1983). In mex-1 mutant embryos both AB and P1 produce MS-like descendants, and in pie-1 mutant embryos both daughters of P1 produce MS-like descendants. Phenotypic and genetic analysis of the mex-1 and pie-1 mutants has suggested that the cell-intrinsic ability of P1 to produce pharyngeal cells results from the activity of an "MS factor" (Mello et al, 1992). mex-1(+) activity would be required to restrict the activity of the MS factor to the posterior blastomere (P1) after the first division, and pie-1(+) activity would be required to restrict

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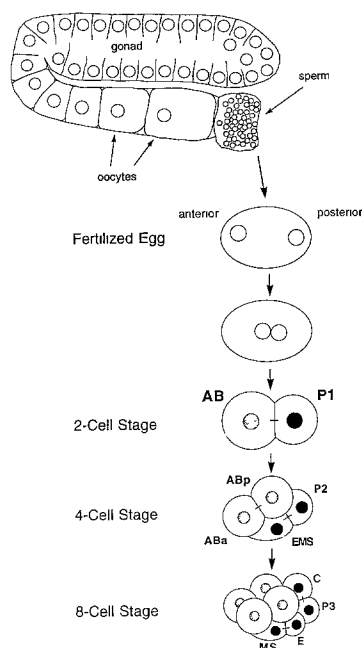
Figure 1. Fertilization and Early Cleavage in C. elegans

Schematic diagrams of a gonad and early embryonic stages. The sperm enters the oocyte at the future posterior pole of the embryo, and the sperm and oocyte pronuclei (open circles) migrate together and fuse. The first mitotic division of embryogenesis results in an anterior blastomere called AB and a posterior blastomere called P1. The nuclei of the AB blastomere and of each of its descendants at the 4-cell and 8-cell stages are stippled, and the nuclei of the P1 descendants are closed. The name of each of the P1 descendants at the 8-cell stage is listed; MS produces pharyngeal cells and body wall muscles, E produces intestinal cells, P3 produces germ cells and body wall muscles, and C produces hypodermal cells and body wall muscles. For further description of embryonic anatomy and blastomere fate, see Sulston et al. (1983).

its activity after the second division to only one daughter of P1; no genes have yet been identified that are likely to perform an analogous role at the third division.

Analyses of *skn-1* mutant embryos and *mex-1;skn-1* and *pie-1;skn-1* double mutant embryos suggest that the product of the *skn-1* gene is a likely component of the presumptive MS factor (Bowerman et al., 1992a; Mello et al., 1992). *skn-1* mutations transform the respective fates of MS and its sister blastomere so that both produce hypodermal cells instead of pharyngeal and intestinal cells (Bowerman et al., 1992a). The *skn-1* gene can encode a novel protein product with a domain similar in sequence to the DNA-binding motif found in a class of transcription factors called bZIP proteins, suggesting that *skn-1*(+) activity may affect blastomere fate in C. elegans by regulating gene expression in the early embryo (Bowerman et al., 1992a).

To examine how the spatial distribution of the *skn-1* gene product corresponds to the different abilities of blastomeres in wild-type and mutant embryos to produce pharyngeal cells, we have generated antisera against the Skn-1 protein. We report here that, in wild-type embryos, the Skn-1 protein appears to accumulate to a much higher level in the posterior blastomere P1 than in the anterior

blastomere AB at the 2-cell stage of embryogenesis. We show that this unequal distribution of the Skn-1 protein is reduced or eliminated in mutant embryos in which both P1 and AB have similar, cell-intrinsic abilities to produce pharyngeal cells. Although in wild-type embryogenesis only one P1 daughter produces pharyngeal cells, we find that both P1 daughters appear to have equivalent levels of Skn-1 protein. Thus, AB and P1 may differ in their cell-intrinsic abilities to produce pharyngeal cells because AB descendants have levels of the Skn-1 protein that are insufficient to promote pharyngeal development. However, additional factors, such as the *pie-1* gene product, must be required to determine which of the descendants of P1 becomes committed to producing pharyngeal cells.

Results

Background

The gonad of the adult C. elegans hermaphrodite contains a distal to proximal sequence of mitotic germ cell precursors, meiotic nuclei joined by a common cytoplasmic core, and individual, maturing oocytes (Hirsh et al., 1976; Kimble and Hirsh, 1979). During maturation the initially symmetrical oocytes increase in volume and elongate, and the female pronucleus becomes positioned at the future anterior pole of the egg (see Figure 1). After fertilization the female and male pronuclei join near the center of the zygote and organize a mitotic spindle aligned parallel to the long, or anterior–posterior, axis. The first cleavage furrow divides the embryo into an anterior blastomere called AB and a posterior blastomere called P1. The 2-cell stage lasts about 20 min; we refer to this interval as the first embryonic cell cycle. The AB blastomere then divides into daughters called ABa and ABp, and P1 divides into daughters called EMS and P2. EMS subsequently divides into 8-cell stage blastomeres called MS and E. The MS blastomere is the only P1 descendant that produces pharyngeal cells in wild-type embryogenesis.

Table 1. Skn-1 Protein in Wild-Type Embryos and *skn-1*(zu67) Mutant Embryos

Stage of Embryogenesis	Wild-Type (%)	*skn-1*(zu67) (%)
Oocytes	0	0
1-Cell	16	0
2-Cell	96	3
4-Cell	99	5
8-Cell	47	2
12-Cell	0	0

Embryos from wild-type mothers or homozygous *skn-1*(zu67) mutant mothers were collected, fixed, and stained for Skn-1 protein as described in Experimental Procedures. The *skn-1*(zu67) mutation corresponds to a premature stop codon in the third *skn-1* exon (C. Schubert, B. B., and J. R. P., unpublished data). Between 31 and 118 individuals were analyzed for each stage listed. The numbers shown represent the percentage of embryos that stained positively for Skn-1 protein at each developmental stage. *mex-1* and *par-1* mutant embryos contain detectable levels of Skn-1 protein at frequencies similar to wild-type embryos (see Experimental Procedures).
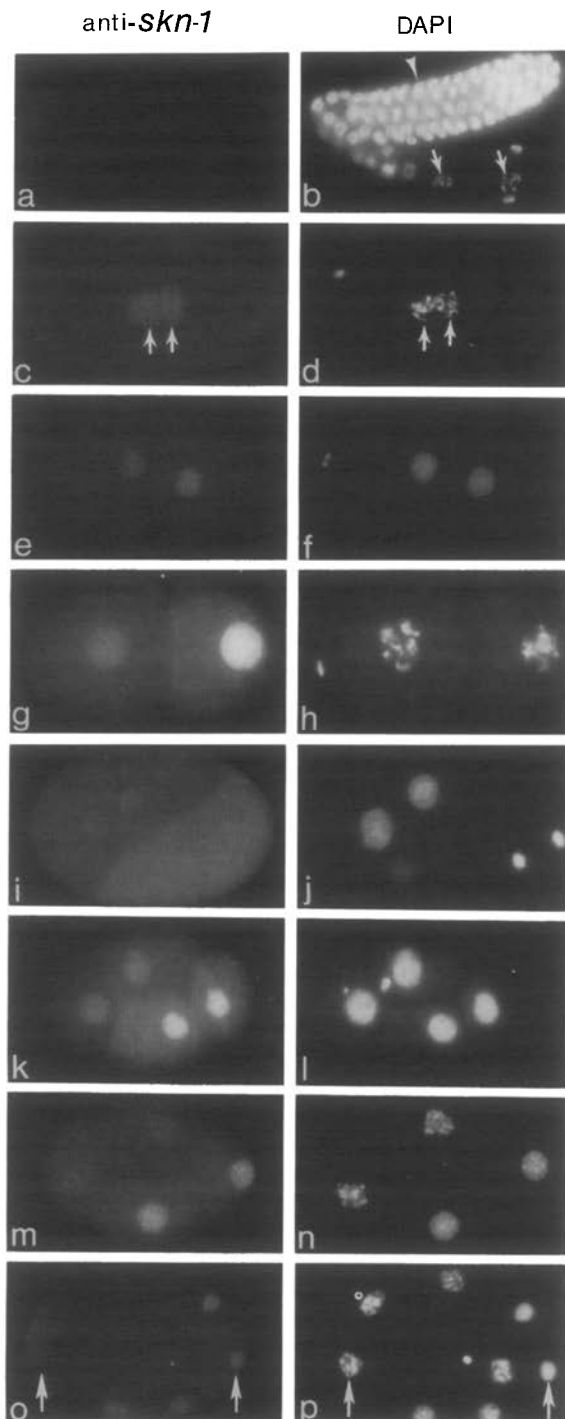
anti-*skn-1* DAPI

Figure 2 Distribution of Skn-1 Protein in Early Embryos.

Fluorescence micrographs of a wild-type adult gonad and early embryos stained for Skn-1 protein (left column) and with DAPI to visualize nuclei (right column). The gonad and embryos are oriented as shown in the schematic diagram in Figure 1.

(a and b) Low magnification pictures of an adult gonad containing germ cell precursors (arrowhead) and maturing oocyte nuclei (arrows).
(c and d) High magnification of an undivided, fertilized egg after the male (left arrow) and female (right arrow) pronuclei have met; the condensed chromosomes of both pronuclei are visible in (d), and the polar body resulting from the second meiotic division of the oocyte is visible on the far left of the panel. Skn-1 protein is detectable in both pronuclei (c).

Production of Skn-1 Antibodies

Both polyclonal antisera and hybridoma cell supernatants that recognize the Skn-1 protein were generated by immunizing rabbits and mice with a bacterially expressed Skn-1–glutathione S-transferase (GST) fusion protein (see Experimental Procedures). The staining patterns reported here were obtained with a mixture of monoclonal cell supernatants FA2 and FC4. Identical staining patterns were observed with monoclonal cell supernatants from four different hybridoma lines and polyclonal antisera from two immunized rabbits (see Experimental Procedures). No staining was seen with preimmune or GST-specific antisera (data not shown), and about 95% of embryos from homozygous *skn-1(zu67)* mutant mothers did not stain with the Skn-1 monoclonal supernatants (Table 1; Figure 3c). Thus, the staining patterns we describe appear to represent expression of the Skn-1 protein.

Skn-1 Protein Is Distributed Unequally in Wild-Type Early Embryos

Skn-1 protein is not detectable in oocytes or sperm prior to fertilization but first becomes visible in both the oocyte and sperm pronuclei shortly before the first mitotic division (Figure 2c; Table 1). After the first cleavage, the nuclei of both the AB and P1 blastomeres appear to contain equivalent levels of Skn-1 protein. During this first embryonic cell cycle, Skn-1 protein appears to accumulate to much higher levels in the nucleus of P1 than in the AB nucleus (compare Figures 2e and 2g). As AB and P1 enter mitosis, the Skn-1 protein is distributed throughout the cytoplasm. After the nuclei of the AB daughters re-form, both appear to contain equivalent levels of Skn-1 protein. Similarly, both P1 daughters appear to contain equivalent levels of Skn-1 protein but much higher levels than the AB daughters. During the second cell cycle the apparent level of Skn-1 protein increases in the nuclei of both P1 daughters

(e and f) Early 2-cell stage embryo showing Skn-1 protein in the nuclei of both the AB (left) and P1 (right) blastomeres.

(g and h) Late 2-cell stage embryo showing an apparent increase in the level of Skn-1 protein in the P1 nucleus. Chromosomes in both the AB and P1 nuclei have begun to condense in preparation for the second cleavage (h).

(i and j) Embryo dividing from 2 to 4 cells, the AB daughter nuclei (left pair) and P2 daughter nuclei (right pair) are reforming, but cytokinesis is incomplete. Skn-1 protein does not appear to be nuclear localized during mitosis and instead appears to be uniformly distributed throughout the cytoplasms of the dividing blastomeres. Note the higher level of cytoplasmic staining in the dividing P1 blastomere than in the dividing AB.

(k and l) Early 4-cell stage embryo.

(m and n) Late 4-cell stage embryo. Skn-1 protein is barely detectable in the AB daughters. In the P1 daughters, Skn-1 protein appears to reach maximum levels in the middle of the cell cycle (compare with Figure 5b) and then decreases as shown.

(o and p) 8-cell stage embryo. Skn-1 protein is detectable in all P1 descendants but not in AB descendants. The arrow to the right of each panel points to the nucleus of the P1 descendant called P3, and the arrow to the left of each panel points to the nucleus of an AB descendant (see also Figure 5c). Fixation and staining procedures are described in Experimental Procedures.

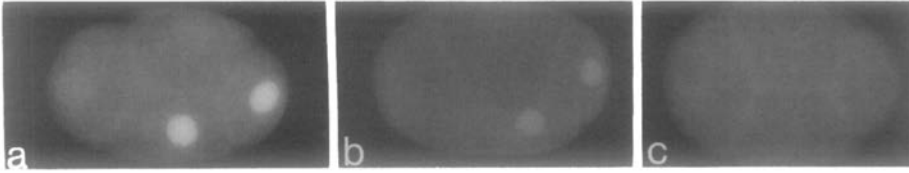(a) and (b), 80× magnification; (c)–(p), 240× magnification.

Figure 3. Comparison of *skn-1* Gene Copy Number and Skn-1 Protein Levels

Immunofluorescence micrographs of 4-cell stage embryos stained for Skn-1 protein.

(a) Embryo from mother with two wild-type copies of the *skn-1* gene (maternal genotype is +/+).

(b) Embryo from mother with one wild-type copy of *skn-1* (maternal genotype is *skn-1(zu67)*/+).

(c) Embryo from mother with no wild-type copies of *skn-1* (maternal genotype is *skn-1(zu67)/skn-1(zu67)*). The staining level in (c) is indistinguishable from background.

(compare the early 4-cell stage embryo in Figure 2k with the intermediate 4-cell stage embryo in Figure 5b). Late in this cell cycle the level of Skn-1 protein begins to decrease in both P1 daughters and in both AB daughters (Figure 2m), and in many embryos the two AB daughters have no detectable Skn-1 protein. After the third cleavage, 8-cell stage embryos do not contain detectable Skn-1 protein in any of the AB granddaughters. Skn-1 protein remains detectable in the P1 granddaughters throughout the 8-cell stage (Figures 2o and 5c), but by the 12-cell stage Skn-1 protein is not visible in any blastomere (Table 1).

Maternal Expression of the *skn-1* Gene

We have shown previously that the *skn-1* gene is expressed both maternally and zygotically during development (Bowerman et al., 1992a; see below). Thus, the different levels of Skn-1 protein detected in early blastomeres could result from either maternal or early zygotic expression of *skn-1* mRNA. Because Skn-1 protein is almost never detected in the progeny of homozygous *skn-1(zu67)* mutant mothers (Table 1; Figure 3c), we were able to test whether the early staining pattern correlates with the maternal or the zygotic number of wild-type *skn-1* genes. One-quarter of the self-progeny of a *skn-1(zu67)*/+ heterozygous mother will have the genotype *skn-1(zu67)/skn-1(zu67)*; if the early Skn-1 staining pattern corresponds to zygotic *skn-1* expression, these embryos should not stain. Instead, we find that nearly all (99.5%, n = 182) of the self-progeny of *skn-1(zu67)*/+ mothers stain positively at the 2- and 4-cell stages. Moreover, all such self-progeny show a uniformly reduced level of staining compared with embryos from wild-type mothers (compare Figures 3a and 3b). This result implies that the +/+ embryos from *skn-1(zu67)*/+ mothers stain less intensely than the +/+ embryos from +/+ mothers. We also have examined the self-progeny of mothers that were heterozygous for a chromosomal deficiency deleting the *skn-1* gene (*nDf41*/+; see Experimental Procedures). Nearly all of these embryos stained positively (96%, n = 39), and there was no variation in staining intensity among embryos (data not shown). Because the protein level correlates with the maternal, rather than embryonic, copy number of the *skn-1* gene, the Skn-1 protein observed in early cleavage stages appears to result from maternal, rather than zygotic, expression.

Maternal-Effect Mutations in the *mex-1* and *par-1* Genes Alter the Distribution of Skn-1 Protein

Because *skn-1(+)* activity is required for the production of pharyngeal cells (Bowerman et al., 1992a; Mello et al., 1992), it is possible that an isolated wild-type AB blastomere cannot produce pharyngeal cells because it contains insufficient levels of Skn-1 protein. To test this possibility, we have examined mutant embryos in which the AB blastomere can produce pharyngeal cells in an apparently autonomous manner. Mothers that are homozygous for maternal-effect mutations in the gene *mex-1* produce embryos in which both P1 and AB have similar, intrinsic abilities to produce pharyngeal cells; both P1 and AB produce MS-like descendants that undergo characteristic patterns of cleavage and generate pharyngeal cells, body wall muscles, and cell deaths (Mello et al., 1992; see Introduction). We also have examined whether the AB blastomere in a *par-1* mutant has the ability to produce pharyngeal cells. Embryos from mothers homozygous for maternal-effect mutations in any of four *par* genes appear to be defective in establishing or maintaining the proper anterior–posterior polarity of the C. elegans embryo and produce highly abnormal patterns of cell differentiation (Kemphues et al., 1988; Kirby et al., 1990). Although the cell fates produced by some *par* mutants are highly variable, *par-1* mutant embryos consistently contain more than twice the normal number of pharyngeal muscle cells and also contain numerous body wall muscles (Kemphues et al., 1988; see also Figures 4a and 4c).

To determine whether the AB blastomere in *par-1* mutant embryos has an intrinsic ability to produce pharyngeal cells and body wall muscles, we asked whether descendants of AB could make these cell types after neighboring blastomeres were killed with a laser microbeam. Because wild-type embryos produce some pharyngeal cells, owing to cell–cell interactions that require maternal expression of the *glp-1* gene (see Introduction), we also examined the ability of early blastomeres to produce pharyngeal cells in *par-1;glp-1* double mutant embryos. We found that both AB daughters can produce pharyngeal muscle cells and body wall muscle cells in *par-1* mutant embryos and that their ability to produce pharyngeal cells does not require *glp-1(+)* activity (Table 2). *skn-1(+)* activity is required for each of the *par-1* mutant blastomeres to produce pharyngeal cells and body wall muscles, because almost no *par-1;skn-1* double mutant embryos contained these cell
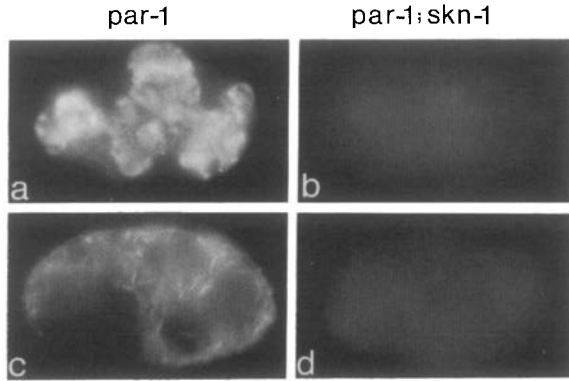
par-1   par-1;skn-1

Figure 4   par-1 Mutant Embryos Require skn-1(+) Activity to Produce Pharyngeal and Body Wall Muscle Cells

Immunofluorescence micrographs of par-1 mutant embryos (left column) and par-1;skn-1 double mutant embryos (right column).

(a and b) Terminal stage embryos stained for pharyngeal muscles with monoclonal antibody 9.2.1 (Miller et al., 1983)

(c and d) Terminal stage embryos stained for body wall muscles with monoclonal antibody 5.6 (Miller et al., 1983).

See Table 3 for quantitation. Although multiple early blastomeres produce body wall muscles in wild-type embryogenesis, previous genetic studies have suggested that skn-1(+) activity is involved only in the MS pattern of body wall muscle production (Mello et al., 1992; Bowerman et al., 1992a). The failure of most skn-1;par-1 double mutant embryos to produce body wall muscles suggests that par-1 mutants produce only skn-1-dependent body-wall muscles.

Table 3. Differentiation of Pharyngeal Muscle and Body Wall Muscle Cells in par-1 and par-1;skn-1 Embryos

	Embryos with Pharyngeal Muscle Cells (%)	Embryos with Body Wall Muscle Cells (%)
par-1(e2012)[a]	100	96
par-1(b274)	100	98
par-1(e2012);skn-1(zu67)[b]	6	20
par-1(e2012);skn-1(zu129)	4	19
par-1(b274);skn-1(zu67)	4	12

Embryos were collected from homozygous mutant mothers with the genotype listed above and allowed to develop at 25°C to terminal stage. The embryos then were fixed and stained for pharyngeal-specific muscle with monoclonal antibody 9.2.1 or for body wall–specific muscle with 5.6 (see Experimental Procedures). Between 400 and 800 embryos were examined in each experiment. Only a few muscle cells (1–10) were observed in each of the par-1;skn-1 embryos that stained positively. Similar results were obtained with embryos allowed to develop at 15°C (data not shown).

[a] For examples of these embryos, see Figures 4a and 4c.
[b] For examples of these embryos, see Figures 4b and 4d.

types (Figure 4; Table 3). par-1;skn-1 double mutants instead appear to produce hypodermal cells, similar to the transformation in blastomere fate observed in skn-1 mutant embryos (data not shown; Bowerman et al., 1992a).

Although the AB blastomeres in par-1 and mex-1 embryos both appear to have a cell-intrinsic ability to produce pharyngeal cells, there are several phenotypic differences between these mutant embryos. The AB descendants in par-1 mutant embryos do not undergo a recognizable MS-like pattern of cleavage; they do not generate an early cell death characteristic of both the wild-type MS blastomere and the ectopic, MS-like blastomeres in mex-1 mutant embryos (data not shown; Mello et al., 1992). Indeed, in par-1 mutant embryos there is variability in whether or not the

AB daughters produce pharyngeal cells (Table 2). Only one P1 daughter in wild-type embryos and in mex-1 mutant embryos produces pharyngeal cells (Mello et al., 1992). However, we find that both P1 daughters can produce pharyngeal cells in par-1 mutant embryos (Table 2). Thus, all early blastomeres in par-1 mutant embryos appear to have similar abilities to produce pharyngeal cells and body wall muscles. We found that the AB blastomere and its descendants had higher levels of Skn-1 protein in mex-1 and par-1 mutant embryos than in wild-type embryos (Figure 5). This difference was most apparent in 4-cell and 8-cell stage embryos, when little or no Skn-1 protein was detectable in the AB descendants of wild-type embryos (Figures 5b and 5c) but Skn-1 protein was easily detected in the AB descendants of both mex-1 and par-1 mutant embryos (Figures 5e, 5f, 5h, and 5i). In mex-1 mutant embryos, each of the 4-cell stage blastomeres appeared to accumulate similar levels of Skn-1 protein, although the AB descendants usually exhibited slightly less intense

Table 2. Muscle Development by Individual Blastomeres in Wild-Type and Mutant Embryos

Blastomere	Wild-Type		par-1		par-1;glp-1
	Pharyngeal Muscle	Body Wall Muscle	Pharyngeal Muscle	Body Wall Muscle	Pharyngeal Muscle
ABa	0/12	0/11	6/8	6/7	7/9
ABp	0/10	0/10	6/9	4/6	8/10
EMS	11/11	11/11	12/12	8/8	15/15
P2	0/9	8/8	11/11	9/9	18/18

Four-cell stage embryos were collected from wild-type or homozygous mutant mothers, and 3 of the 4 embryonic blastomeres present were killed with a laser microbeam. The single remaining blastomere (listed above) was allowed to develop for 12 hr at 25°C, then fixed and stained with either monoclonal antibody 9.2.1, to identify pharyngeal muscles, or monoclonal antibody 5.6, to recognize body wall muscle (see Experimental Procedures). Embryos were scored positively for body wall muscle only if they produced numerous 5.6-positive cells that resembled body wall muscles morphologically. In some experiments wild-type ABp blastomeres produced a few cells that stained positively with 5.6, but the identities of these cells have not been determined. Each of the experiments described in this table also was performed on embryos cultured at 15°C, with similar results (data not shown). The alleles par-1(e2012) and glp-1(e2142ts) were used in all experiments.

staining than the P1 descendants. In *par-1* mutant embryos AB and P1 descendants always have equivalent levels of staining. The staining intensity in each of the blastomeres in *mex-1* and *par-1* mutants at the 4-cell stage appears intermediate between the levels observed in the wild-type P1 daughters and the levels observed in the wild-type AB daughters.

pie-1 Mutant Embryos Have a Wild-Type Distribution of Skn-1 Protein

EMS and P2, the two daughters of the P1 blastomere, have very different fates in wild-type development, although they appear to have equivalent levels of Skn-1 protein. For example, EMS produces pharyngeal cells and P2 does not (Sulston et al., 1983). In embryos from mothers homozygous for maternal-effect mutations in the *pie-1* gene, P2 acquires the ability to produce pharyngeal cells and appears to develop identically to the EMS blastomere by several criteria (Mello et al., 1992). We therefore asked whether *pie-1* mutant embryos had any changes in the distribution of Skn-1 protein. We found that the Skn-1 staining patterns of *pie-1* mutant embryos are indistinguishable from the wild-type patterns shown in Figure 2 (data not shown).

Late Embryonic Expression of the Skn-1 Protein

As described above, Skn-1 protein is not observed after the 8-cell stage in early wild-type embryos. However, nuclear Skn-1 protein can be detected again several hours later in embryogenesis, consistent with previous genetic evidence that zygotic *skn-1* function is required postembryonically (Bowerman et al., 1992a). Embryogenesis in C. elegans involves an early phase of rapid cell proliferation followed by a late phase in which embryos undergo morphogenesis but almost no cell division (Sulston et al., 1983). In postmitotic embryonic cells, Skn-1 protein was present in all of the hypodermal nuclei (Figure 6a) and in all intestinal nuclei (Figure 6b). Zygotic *skn-1*(+) activity is required for the normal postembryonic development of intestinal cells, but *skn-1*(+) activity is not known to be required for development of hypodermal cells, which form the outermost epithelial layer of the worm (Bowerman et al., 1992a). A closely related homolog of the *skn-1* gene (Bowerman et al., 1992a) is expressed postembryonically (B. B. and J. R. P., unpublished data); however, we believe that the staining in intestinal and hypodermal nuclei represents Skn-1 protein, because it is not detected in these cell types in homozygous *skn-1*(*zu67*) mutant embryos (data not shown).
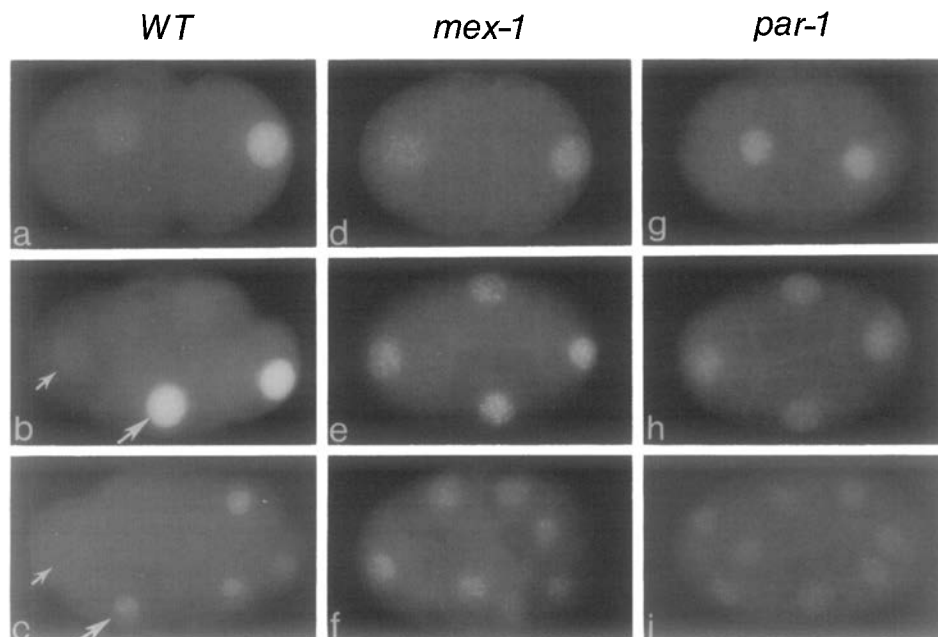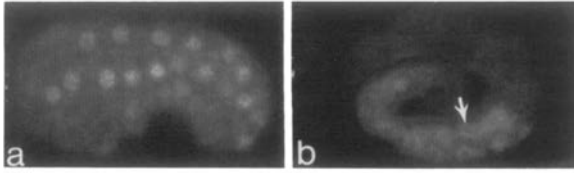

Figure 5. Skn-1 Protein in Wild-Type Embryos and in *mex-1* and *par-1* Mutant Embryos

Immnofluorescence micrographs of wild-type embryos (a–c) and of *mex-1* (d–f) and *par-1* (g–i) mutant embryos stained for Skn-1 protein. The top, middle, and bottom rows show 2-cell, 4-cell, and 8-cell embryos, respectively. Comparisons are between embryos at equivalent times in the cell cycle as determined by DAPI staining (data not shown). In the wild-type 4-cell stage embryo (b) there is a marked difference in Skn-1 protein levels between the AB daughters (left arrow points to the ABa nucleus) and the P1 daughters (right arrow points to the EMS nucleus). In the *mex-1* (e) and *par-1* (h) mutant embryos all blastomeres appear to have nearly equivalent levels of Skn-1 protein; the AB daughters in both mutants appear to have more Skn-1 protein than in the wild-type embryo, and the P1 daughters in both mutants appear to have less Skn-1 protein than in wild-type. At the 8-cell stage, Skn-1 protein is detectable in all nuclei in the *mex-1* (f) and *par-1* (i) mutant embryos, but only in the P1 descendants in the wild-type embryos (c; the left arrow points to the nucleus of the AB descendant called ABa and the right arrow points to the P1 descendant called MS; compare with Figure 1).

Figure 6. Skn-1 Protein in Late-Stage Embryos

Immunofluorescence micrographs of wild-type embryos stained for Skn-1 protein.

(a) Surface view of embryo about 330 min after fertilization, showing positively staining nuclei in the hypodermal cells that form the outermost epithelial layer of the worm.

(b) Internal view of embryo about 420 min after fertilization, showing positively staining intestinal nuclei and some diffuse cytoplasmic staining. The nonstaining disk in the center of each intestinal nucleus is the nucleolus (arrow). Only about 15% of late-stage embryos contained detectable levels of Skn-1 protein, and the staining was often very faint in the embryos that did stain (see Experimental Procedures). Developmental times refer to 25°C; see Sulston et al. (1983) for an anatomical description of late-stage C. elegans embryos.

Discussion

The Skn-1 Protein Is Distributed Unequally in Early Embryos

Maternal expression of the *skn-1* gene is required for the proper specification of certain blastomere fates in the early C. elegans embryo (Bowerman et al., 1992a; Mello et al., 1992). We have shown here that maternally expressed Skn-1 protein appears in the nuclei of early blastomeres during the first three embryonic cell cycles. The nuclear localization of the Skn-1 protein and the occurrence of a DNA-binding motif in the Skn-1 sequence (Bowerman et al., 1992a) suggest that *skn-1*(+) activity may affect blastomere fate by regulating early embryonic transcription in C. elegans. The Skn-1 protein appears to accumulate to peak levels during the 4-cell stage and is not detectable at the 12-cell stage. We do not know when *skn-1*(+) activity is present in the embryo; however, phenotypic defects resulting from *skn-1* mutations are first visible at the 28-cell stage (Bowerman et al., 1992a), about 30 min after Skn-1 protein is no longer visible.

Early blastomeres in the embryo appear to accumulate markedly different levels of Skn-1 protein. During the 2-cell stage the posterior blastomere P1 accumulates a higher level of Skn-1 protein than does the anterior blastomere AB. At the 4-cell and 8-cell stages, the level of Skn-1 protein in the descendants of P1 appears substantially higher than in the descendants of AB. The Skn-1 protein thus provides an example of a nuclear-localized gene product that is distributed unequally in the early C. elegans embryo. At present we do not know why blastomeres differ in their level of Skn-1 protein. Possible mechanisms include differences in the distribution or translation of *skn-1* mRNA or differences in the stability of Skn-1 protein. We have shown that maternal-effect mutations in the genes *par-1* and *mex-1* both lead to approximately equal levels of Skn-1 protein in each of the 4-cell stage blastomeres. Interest-

ingly, these mutations result in levels of nuclear Skn-1 protein that appear to be intermediate between the wild-type levels of the AB and P1 daughters: the mutant AB daughters appear to contain more Skn-1 protein than wild-type AB daughters, and the mutant P1 daughters have less Skn-1 protein than wild-type P1 daughters. Thus, wild-type embryos and *par-1* and *mex-1* mutant embryos may have comparable quantities but different distributions of Skn-1 protein. This result might be expected if *skn-1* mRNA normally is concentrated in the P1 daughters but not localized in *par-1* and *mex-1* mutant embryos. Techniques for in situ RNA hybridization are currently being developed for early C. elegans embryos, but thus far we have been unable to determine the embryonic distribution of *skn-1* mRNA.

Skn-1 Protein Levels and Differences in Blastomere Fates

After the first division of the C. elegans embryo, a cell-intrinsic ability to produce certain cell types appears to be restricted to the posterior blastomere, P1. Only P1 can produce pharyngeal cells, body wall muscle cells, and intestinal cells when AB and P1 are separated at the 2-cell stage (Laufer et al., 1980; Priess and Thomson, 1987). Phenotypic and genetic analyses of *skn-1*, *mex-1*, and *pie-1* mutant embryos suggest that *skn-1*(+) activity is essential for the P1 blastomere to produce pharyngeal cells and a subset of body wall muscle cells (Mello et al., 1992). The P1 blastomere also appears to have some requirement for *skn-1*(+) activity to produce intestinal cells, although this is poorly understood at present (Bowerman et al., 1992a; Mello et al., 1992). Thus, it is possible that an isolated wild-type AB blastomere is unable to produce pharyngeal cells, body wall muscle cells, and intestinal cells simply because it lacks *skn-1*(+) activity.

In *par-1* mutant embryos, as described here, and in *mex-1* mutant embryos, as described previously (Mello et al., 1992), the AB blastomere appears to acquire a *skn-1*-dependent, intrinsic ability to produce pharyngeal cells and body wall muscles. We have shown that, in both *par-1* and *mex-1* mutant embryos, the descendants of AB appear to contain higher levels of Skn-1 protein than do AB descendants in wild-type embryos. Although the level of Skn-1 protein in the *mex-1* and *par-1* mutant AB daughters does not appear to be increased to the level of wild-type P1 daughters, the P1 daughters probably contain more Skn-1 protein than is necessary for normal development. First, all embryos from heterozygous *skn-1(zu67)/+* mothers have normal development (Bowerman et al., 1992a), yet the P1 daughters in these embryos have much less nuclear staining than wild-type P1 daughters. Second, the level of staining in the AB daughters of *mex-1* and *par-1* mutants is comparable both to the level of staining in the EMS blastomere of a *mex-1* mutant (which produces pharyngeal cells and appears to develop normally; Mello et al., 1992) and to the level in both the EMS and P2 blastomeres in *par-1* mutant embryos, which also produce pharyngeal cells. Thus, in descendants of the AB blastomeres of wild-type or mutant embryos, there is a correlation be-

tween genetically defined *skn-1*(+) activity, Skn-1 protein levels, and an intrinsic ability to produce pharyngeal cells and body wall muscle cells. The observation that the AB blastomeres in *mex-1* and *par-1* mutant embryos produce pharyngeal and body wall muscle cells but do not produce intestinal cells suggests that additional factors may act with Skn-1 to determine intestinal cell fates.

Defining the Fates of the P1 Daughters

Although the different levels of Skn-1 protein in AB and P1 descendants may explain why only P1 descendants have an intrinsic ability to produce pharyngeal cells in a wild-type embryo, differences in Skn-1 protein levels cannot account for the different fates of EMS and P2, the daughters of P1. These sister blastomeres appear to have equivalent levels of Skn-1 protein in wild-type embryos, yet only EMS produces pharyngeal cells. *skn-1*(+) activity is essential for the proper development of EMS, but there is no evidence that *skn-1* function is required for the proper development of P2: Skn-1 protein cannot be detected in 95% of the embryos from homozygous *skn-1*(*zu67*) mutant mothers; however, the P2 blastomere in these embryos has a wild-type pattern of development (Bowerman et al., 1992a).

If the Skn-1 protein does not function in the P2 blastomere, factors within P2 may inhibit *skn-1*(+) activity, or P2 may lack factors essential for *skn-1*(+) activity. Regulation of *skn-1*(+) activity in P2 could involve the *pie-1* and *par-1* gene products. In *pie-1* mutants described previously (Mello et al., 1992) and in *par-1* mutant embryos described here, *skn-1*(+) activity results in P2 producing pharyngeal cells; P2 does not produce pharyngeal cells in either *pie-1; skn-1* or *par-1;skn-1* double mutant embryos. *pie-1* mutations cause P2 to adopt a fate very similar or identical to the EMS blastomere but have no effect on other embryonic blastomeres. *pie-1* mutations also appear to have no effect on the distribution of Skn-1 protein. Therefore, the wild-type function of the *pie-1* gene product may play a direct role in specifying the different fates of P2 and EMS. In contrast, *par-1* mutations disrupt most of the anterior–posterior differences observed in wild-type embryos (Kemphues et al., 1988), suggesting that *par-1*(+) activity may play a fairly general role in determining the organization of the early embryo. Indeed, *par-1* mutant embryos resemble *mex-1;pie-1* double mutant embryos in their ability to produce pharyngeal cells from all 4-cell stage blastomeres (Mello et al., 1992). Thus, *par-1*(+) activity may be required for the proper spatial distribution of factors that determine the different fates of P1 descendants, with the *pie-1* gene product perhaps being one of these factors.

In conclusion, understanding how early blastomeres in nematodes and other invertebrates acquire different potentials for producing certain cell types has been a long-standing problem in developmental biology. Our analysis of the Skn-1 protein demonstrates that nematode embryos have an unequal distribution of at least one nuclear factor as early as the 2-cell stage and suggests that other gene products may be distributed unequally at the 4-cell stage. Thus, while cell–cell interactions determine the different fates of AB descendants in the C. elegans embryo (Priess

and Thomson, 1987; Schnabel, 1991; Wood, 1991; Bowerman et al., 1992b), a sequential localization or activation of maternal gene products during the early cleavages may lead to some of the cell-intrinsic properties of posterior blastomeres.

Experimental Procedures

Strains and Alleles

N2 Bristol strain was used as the standard wild-type strain. The marker mutations, duplications, translocations, and deficiencies used in this study are listed by chromosome as follows: LG II: *mnC1*, *mex-1*(*zu120*), *rol-1*(*e91*); LG III: *eDp6*, *glp-1*(*e2142ts*), *pie-1*(*zu127*), *unc-25*(*e156*); LG IV: *DnT1*(*IV;V*), *dpy-13*(*e184sd*), *mDp1*, *nDf41*, *skn-1*(*zu67*), *skn-1*(*zu129*), *unc-5*(*e53*), *unc-8*(*n491sd*); LG V: *par-1*(*b274*), *par-1*(*e2012*), *rol-4*(*sc8*). The translocation *nDf41*, kindly provided by S. A. Kim and H. R. Horvitz, uncovers the markers *unc-5* and *bli-6*, which flank the *skn-1* gene (see Bowerman et al., 1992a). All other mutant alleles were obtained or are available from the C. elegans genetic stock center. The gene name *skn-1* stands for skin excess; *skn-1* mutant embryos make extra hypodermal (or, informally, skin) cells instead of pharyngeal and intestinal cells (see Bowerman et al., 1992a). The basic methods of C. elegans culture, genetics, and nomenclature were as described in Brenner (1974).

Production of Antibodies

An in-frame Skn-1–GST fusion construct was made as follows: The 1 9 kb DraI to EcoRI restriction fragment from the *skn-1* cDNA clone described by Bowerman et al. (1992a) was end filled with Klenow polymerase to generate a blunt end at the EcoRI site, gel purified, and ligated into the SmaI site in the polylinker of the vector pGEX-3 (Smith and Johnson, 1988). This 1.9 kb cDNA fragment contains the entire predicted *skn-1* open reading frame, corresponding to the exon sequences from position 764 to the poly(A) tail at position 5698 in the genomic sequence shown in Bowerman et al. (1992a). An in-frame Skn-1–trpE fusion construct was made by gel purifying the 1.9 kb insert in the Skn-1–GST fusion construct as a BamHI to EcoRI fragment, using the BamHI site in the pGEX-3 vector polylinker. This fragment was ligated into a pATH-1 vector (Spindler et al., 1984; Konopka et al., 1984), using the BamHI and HindIII sites in the pATH-1 polylinker, after end filling the EcoRI site in the *skn-1* fragment and the HindIII site in the vector and gel purifying both the fragment and the vector. Both the Skn-1–GST and the Skn-1–trpE fusion constructs were transformed into the Escherichia coli strain TG-1. Induced and uninduced cultures were run on SDS–polyacrylamide gels that were stained with Coomassie blue to visualize the induced fusion proteins.

The Skn-1–GST fusion protein was purifed from bacterial extracts using glutathione–agarose beads (Sigma) as described by Smith and Johnson (1988) to obtain milligram quantities of the fusion protein. The purified Skn-1–GST fusion protein was run on preparative SDS–polyacrylamide gels that were stained with Coomassie blue. The full-length fusion protein was cut out, ground into a powder using a mortar and pestle on dry ice, and mixed with complete Freund's adjuvant (Difco) for the initial immunization and with incomplete Freund's adjuvant for subsequent immunizations. Preimmune sera were collected from two New Zealand white rabbits and four RBF/Dn mice (The Jackson Laboratory), and the animals were injected subcutaneously with approximately 0.5 mg or 0.1 mg of the purified fusion protein, respectively. Animals were boosted every 3 weeks and bled for serum 1 week after each boost. To test the sera for immunoreactivity, extracts from induced bacteria containing the Skn-1–trpE fusion construct were run out on SDS–polyacrylamide gels and transferred to nitrocellulose. Sera were diluted and incubated with strips of the nitrocellulose transfer. Immunoreactivity was assayed using a Vectastain kit (Vector Laboratories). Animals were boosted until the titer of Skn-1 antisera stopped increasing, as assayed by using serial dilutions of antisera for Western blot analysis. The rabbits were then sacrificed and exsanguinated. The mice were sacrificed, and their spleens were removed, as described by Harlow and Lane (1988). Hybridoma lines producing monoclonal cell supernatants that recognize the Skn-1 protein were made by fusing the spleen cells to FOX-NY myeloma cells as described by Taggert and Samloff (1983). Four hybridoma lines were isolated that recognize

the Skn-1 protein in wild-type C. elegans embryos. Two hybridoma lines, FA2 and FC4, from two different mice were stabilized by subcloning and grown up in RPMI and 20% fetal calf serum (GIBCO) to obtain large quantites of cell supernatants. A 1:1 mixture of these two supernatants gave brighter staining than either supernatant alone, and this mixture was used for the experiments described here. All four cell supernatants and both rabbit immune sera showed the same staining patterns in fixed wild-type and mutant embryos (data not shown). DNA and protein analyses were done using standard techniques as described by Sambrook et al. (1989) and Ausubel et al. (1989).

Microscopy

Light microscopy was performed using a Zeiss Axioplan microscope equipped for Nomarski optics and epifluorescence. Photographs were taken with Kodak Technical Pan film and developed in HC110 developer. To obtain wild-type or mutant embryos, synchronized cultures of animals were raised to adulthood. Several hundred Rolling animals from the strain rol-1(e91) mex-1(zu120)/mnC1 were collected to obtain mex-1 mutant embryos from homozygous rol-1(e91) mex-1(zu120) mothers. Several hundred Uncoordinated early adult animals from the strain unc-25(e156) pie-1(zu127)/qC1 were collected to obtain pie-1 mutant embryos from homozygous unc-25 pie-1 mothers. Several hundred Uncoordinated early adult animals from the strain unc-5(e53) skn-1(zu67)/nT1 were collected to obtain skn-1 mutant embryos from unc-5 skn-1 animals. Alternatively, non-Uncoordinated, non-Dumpy animals were picked from skn-1(zu67)/dpy-13(e184sd) unc-8(e491sd) animals to obtain skn-1 mutant embryos from homozygous skn-1(zu67) mutant mothers. Several hundred unc-5(e53) skn-1(zu67)/nT1, nDf41/ DnT1, and mDp1;unc-5(e53) skn-1(zu67) animals were picked to obtain embryos for correlation of skn-1(+) gene copy number with Skn-1 antibody staining. Several hundred Rolling animals were picked from the strains rol-4(sc8) par-1(e2012)/DnT1, glp-1(e2142ts);rol-4(sc8) par-1(e2012)/DnT1, and skn-1(zu67)/DnT1;rol-4(sc8) par-1(e2012) to obtain par-1/DnT1 single mutant and par-1;glp-1 and par-1;skn-1 double mutant embryos. Analogous strains also were constructed and analyzed with the alleles skn-1(zu129) and par-1(b274). After the adults began to produce fertilized eggs, they were treated with 0.1% sodium hypochlorite and 0.2 N KOH to dissolve the parents, leaving only the early embryos, which were protected by their eggshell. Later embryonic stages were obtained by allowing early embryos harvested as above to develop for 4–6 hr at 25°C (for late-stage Skn-1 staining) or for 12 hr at 25°C (for 9.2.1 and 5.6.1 antibody staining) before fixation.

For fixation and staining with Skn-1 monoclonal supernatants or antisera, 100–500 embryos in distilled water were pipetted onto 0.1% polylysine–coated glass slides. After the embryos adhered to the slide, a pipetman was used to replace the water with 80 μl of a solution of 5% paraformaldehyde dissolved in phosphate-buffered saline (150 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, 1.5 mM NaH_2PO_4, 1 mM $MgCl_2$). The embryos were overlaid with a 22 × 22 mm glass coverslip, and a kimwipe was used to wick out the excess solution until the embryos were flattened visibly as monitored in a dissecting scope. The embryos were allowed to fix for 15 min in a humidified chamber, then were frozen on dry ice. After 5 min, the coverslip was removed, and the embryos were incubated in absolute methanol for 4 min at room temperature, then transferred directly to Tris–Tween (100 mM Tris-HCl [pH 7.5], 200 mM NaCl, 0.1% Tween), also at room temperature. Embryos were incubated with undiluted monoclonal cell supernatant or 1:1000 dilutions of antisera (in Tris–Tween) for 1 hr and rhodamine-conjugated goat anti-mouse immunoglobulin G and immunoglobulin M or anti-rabbit immunoglobulin G secondary antibody (TAGO) for 1 hr. Embryos were washed three times for 4 min each in Tris–Tween after antibody incubations. After the final wash, embryos were transferred to phosphate-buffered saline with 20 ng/ml of 4′,6-diamidino-2-phenylindole (DAPI) to visualize the chromosomal DNA in the nuclei of early blastomeres. The stained embryos were overlaid with a glass coverslip and sealed with fingernail polish for viewing with epifluorescence. Antibody staining (9.2.1 and 5.6) for pharyngeal-specific and body wall muscle–specific myosin staining (Miller et al., 1983) was as described by Bowerman et al. (1992a).

For assessing the levels of Skn-1 staining in embryos from homozygous skn-1(zu67)/skn-1(zu67) or heterozygous skn-1(zu67)/+ mutant mothers, it was necessary to have a standard for comparison on the same slide. Because par-1 mutant embryos are distinguishable from all other mutant embryos used in this study (they have synchronous cleavages and thus distinct patterns of DAPI staining; see Kemphues et al. [1988]), they were mixed with each preparation for comparison. The onset and duration of Skn-1 protein in early mex-1 and par-1 embryos was similar to wild-type. mex-1: 11% (n = 11) of 1-cell stage embryos, 100% (n = 71) of 2-cell stage embryos, 98% (n = 96) of 4-cell stage embryos, and 35% (n = 62) of 8-cell stage embryos; par-1: 14% (n = 14) of 1-cell stage embryos, 100% (n = 68) of 2-cell stage embryos, 99% (n = 109) of 4-cell stage embryos, and 44% (n = 54) of 8-cell stage embryos contained detectable levels of Skn-1 protein.

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